The regulation of mesangial cell apoptosis by inflammatory macrophages

Jeremy Stuart Duffield
M.A. oxon, B.M. B. Ch. oxon, M.R.C.P. UK

Thesis presented for the degree of Doctor of Philosophy
University of Edinburgh

M.R.C. Centre for Inflammation Research
Medical School
Teviot Place
University of Edinburgh
July 2001
Abstract

Inflammation of any organ frequently resolves by remodelling of the tissue. Normal tissue function is restored. However, inflammation can lead to scarring, dysfunction and even failure of function of the tissues. The kidney, in particular the glomerulus, is a focus for inflammation by many, currently obscure mechanisms. Inflammation here is not exceptional, and failed resolution leads to organ failure and death. However, in humans and animals complete resolution of apparently severe inflammatory injury of the glomerulus can occur. Mesangial cells (MC) are a major cell-type in the healthy glomerulus. They respond to inflammation by proliferation and laying-down scar tissue. In animal models, deletion of excess MC by a cell death process called apoptosis is crucial for resolution and restoration of the normal tissue. Coincident with the occurrence of MC apoptosis is the presence of inflammatory macrophages (MΦ), in the mesangial areas of the glomerulus. Motivated by a clear role for MΦ in deleting excess cells in developmental remodelling, I sought to determine whether inflammatory MΦ as found in the diseased glomerulus could induce apoptotic death in of cultured MC.

By developing an in vitro assay of proliferating MC with MΦ as a model of glomerular inflammation, MΦ, either activated by cytokines, or taken directly from diseased glomeruli, induced apoptotic death of MC. Thus, MΦ were able to control MC populations. Roles for MΦ derived nitric oxide and tumour necrosis factor (TNF) in mediating these effects on MC were clearly shown. Further, when MΦ ingested opsonised particles (to model immune complex activation), hydrogen peroxide release was also found to induce MC apoptosis.

Excessive MΦ killing of MC is also deleterious to healing of the glomerulus, since excessive MC loss also leads to scarring. Negative feedback in MΦ killing was sought. Since MΦ ingestion of apoptotic cells leads to transforming growth factor beta release, I hypothesised that once MΦ had induced MC apoptosis and ingested the dead cells, they might deactivate. Co-culture was modified whereby activated MΦ ingested pre-formed apoptotic cells whilst in the process of killing healthy MC. These MΦ were rendered incapable of inducing TNF dependent MC apoptosis, indicating a novel negative feedback loop in the inflammatory response. TNF receptor 1 signalling pathways of apoptosis in MC were investigated. Following stable transfection, an important role for interferon regulatory factor-1 was shown.

This thesis has contributed to the understanding of the inflammatory process both in the glomerulus and in a wider context. It has demonstrated that inflammatory MΦ induce apoptosis of MC, and indicated that normal ingestion of apoptotic cells brings about a form of deactivation of the inflammatory MΦ phenotype.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Jeremy Stuart Duffield

Dated
PUBLICATIONS, PRESENTATIONS, SCHOLARSHIPS AND AWARDS

Publications arising from this thesis
Activated macrophages direct apoptosis and suppress mitosis of mesangial cells
**Duffield J S, Erwig L-P, Wei X-Q, Liew F Y, Rees A J, Savill J S.**
Journal of Immunology (2000);164:2110-2119

Suppression by apoptotic cells defines tumour necrosis factor-mediated induction of glomerular mesangial cell apoptosis by activated macrophages
**Duffield J S, Ware C F, Ryffel B, Savill J S**
American Journal of Pathology (2001) accepted for publication

Other work published but not reported in this thesis
Matrix metalloprotease inhibitors induce apoptosis in mesangial cells in resolving glomerular inflammation
J. Pharmacol. Exp. Ther. (2001);297:57-68

Presentations of work from this thesis to learned societies

Cold Spring Harbor: apoptosis 1999
Keystone Symposia: macrophage activation and deactivation 2001

Scholarships and awards

Medical Research Council Clinical Training Fellowship 1997-2000
Table of contents

Abstract ii
Declaration iii
Publications, presentations, scholarships and awards iv
Table of contents v
Index of figures xvi
Index of tables xxii
Acknowledgements xxv
Abbreviations xxvi
Dedication xxviii

Chapter 1 Introduction 1
1.1 INFLAMMATION 2
1.2 APOPTOSIS – A FORM OF CELL DEATH 4
1.3 EFFECTOR MECHANISMS IN THE APOPTOTIC PROCESS 6
1.4 MESANGIAL CELL AS A MYOFIBROBLAST; ITS ROLE IN REMODELLING 10
1.5 MACROPHAGE EFFECTORS AND RESIDENT CELL APOPTOSIS IN TISSUE REMODELLING 14
1.6 MACROPHAGES IN GLOMERULAR INFLAMMATION 17
1.7 DIFFERENT PATTERNS OF GLOMERULAR INFLAMMATION 18
1.8 DEATH RECEPTORS AND SIGNALLING PATHWAYS IN GLOMERULAR INFLAMMATION 20
1.9 OXYGEN RADICALS IN CELL DEATH 24
1.10 NITRIC OXIDE IN CELL DEATH 25
1.11 CELL CYCLE AND APOPTOSIS 28
1.12 DEACTIVATION OF MACROPHAGES 29
1.13 IN VITRO CELL CULTURES – A MODEL FOR IN VIVO CELLULAR INTERACTIONS 30
1.14 AIMS OF THIS STUDY 31
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.1</td>
<td>Purification of Human Leucocytes from Whole Blood</td>
</tr>
<tr>
<td></td>
<td>2.1.1</td>
<td>Separation of leucocytes from whole blood</td>
</tr>
<tr>
<td></td>
<td>2.1.2</td>
<td>Separation of granulocytes from mononuclear cells (PBMC) by Percoll discontinuous gradient</td>
</tr>
<tr>
<td></td>
<td>2.1.3</td>
<td>Preparation of Platelet rich plasma-derived serum and platelet poor plasma</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>Generation of Mesangial Cells</td>
</tr>
<tr>
<td></td>
<td>2.2.1</td>
<td>Preparation of human mesangial cells (HMC)</td>
</tr>
<tr>
<td></td>
<td>2.2.2</td>
<td>Collection of human glomeruli</td>
</tr>
<tr>
<td></td>
<td>2.2.3</td>
<td>Growth of mesangial cells from glomeruli</td>
</tr>
<tr>
<td></td>
<td>2.2.4</td>
<td>Preparation of rat mesangial cells.</td>
</tr>
<tr>
<td></td>
<td>2.2.5</td>
<td>Purification of rat glomeruli</td>
</tr>
<tr>
<td></td>
<td>2.2.6</td>
<td>Growth of mesangial cells from whole glomeruli</td>
</tr>
<tr>
<td></td>
<td>2.2.7</td>
<td>Characterisation of mesangial cells</td>
</tr>
<tr>
<td></td>
<td>2.2.8</td>
<td>Passaging of cells</td>
</tr>
<tr>
<td></td>
<td>2.2.9</td>
<td>Preparation of steroid (oestrogen) depleted FCS</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>Generation of Macrophages</td>
</tr>
<tr>
<td></td>
<td>2.3.1</td>
<td>Collection of bone marrow from rats.</td>
</tr>
<tr>
<td></td>
<td>2.3.2</td>
<td>Collection of bone marrow from mice.</td>
</tr>
<tr>
<td></td>
<td>2.3.3</td>
<td>Differentiation of bone marrow (rat and mouse) to macrophages</td>
</tr>
<tr>
<td></td>
<td>2.3.4</td>
<td>Isolation of rat glomerular macrophages</td>
</tr>
<tr>
<td></td>
<td>2.3.5</td>
<td>Isolation of monocytes from peripheral blood mononuclear cells (PBMC) by lymphocyte depletion</td>
</tr>
<tr>
<td></td>
<td>2.3.6</td>
<td>Isolation of monocytes from peripheral blood mononuclear cells (PBMC) by elutriation</td>
</tr>
<tr>
<td></td>
<td>2.3.7</td>
<td>Differentiation of human macrophages from peripheral blood monocytes</td>
</tr>
<tr>
<td></td>
<td>2.3.8</td>
<td>Preparation of Teflon wells</td>
</tr>
</tbody>
</table>
2.3.9 Red cell lysis 47
2.4 PREPARATION OF APOPTOTIC CELLS FOR INGESTION BY Mφ 48
2.4.1 Induction of apoptosis of rat mesangial cells 48
2.4.2 Percoll density gradient purification of human neutrophils 48
2.4.3 CD16 enrichment of apoptotic neutrophils 48
2.4.4 Preparation of murine peritoneal neutrophils 48
2.4.5 Induction of apoptosis of neutrophils 50
2.4.6 Preparation of murine thymocytes 50
2.4.7 Induction of apoptosis of thymocytes 51
2.5. HYBRIDOMA/CELL SUPERNATANT HARVESTING 52
2.5.1 Production of supernatant containing murine M-CSF (CSF-1) 52
2.5.2 Production of supernatant containing mouse anti-human CD16 antibody 52
2.6. CO-CULTURE 52
2.6.1 Rodent co-culture with bone-marrow-derived macrophages 52
2.6.2 Distinguishing rat mesangial cells from macrophages 53
2.6.3 Rat co-culture with glomerular macrophages 54
2.6.4 Human co-culture 54
2.6.5 Distinguishing human mesangial cells from macrophages 55
2.6.6 Activation of the co-culture with cytokines 55
2.6.7 Activation of the co-culture with opsonised particles 55
2.6.8 Termination of co-culture 56
2.6.9 Tri-culture 57
2.7. ASSESSMENTS OF MESANGIAL CELL APOPTOSIS 58
2.7.1 Fluorescence microscopy 58
2.7.2 Blinded microscopical counting 60
2.7.3 Hoechst and PI staining of live cells 61
2.7.4 Trypan blue staining 62
2.7.5 Flow cytometric analysis of MC apoptosis in co-culture 62
2.7.6 Terminal deoxy-uridine nick end labelling (T.U.N.E.L.) and DNA fragmentation assays 63
2.8. SEMIQUANTITATIVE COLORIMETRIC ASSAYS 63
2.8.1 Griess assay 63
2.8.2 Nitrite plus nitrate 64
2.8.3 TNF elisa 64
2.8.4 Assays for estimating protein concentration 65
2.8.5 Hydrogen peroxide assay 65
2.9. WESTERN BLOTTING 65
2.9.1 Preparation of cellular proteins 65
2.9.2 Preparation of soluble supernatant proteins 66
2.9.3 Preparation of SDS-PAGE 66
2.9.4 Wet transfer of proteins 67
2.9.5 Incubation with antibodies 68
2.9.6 Detection of antibody binding 68
2.9.7 Immunoprecipitation 69
2.9.8 Estimating protein concentration 69
2.9.9 Staining of membranes and gels 70
2.10. ELECTROMOBILITY SHIFT ASSAY 70
2.10.1 Preparation of nuclear proteins 70
2.10.2 Generating labelled oligonucleotides 71
2.10.3 Binding reaction 71
2.10.4 Gel electrophoresis 71
2.10.5 Visualisation of radioactive gel 72
2.10.6 Super shift and cold-competition assays 72
2.11. MOLECULAR BIOLOGICAL TECHNIQUES 72
2.11.1 Preparation of agar plates 72
2.11.2 Preparation of broth 73
2.11.3 Preparation of agarose gels 73
2.11.4 Generation of competent bacteria 73
2.11.5 Transformation of competent bacteria 73
2.11.6 Selection of resistant clones 74
2.11.7 Screening of colonies by STET preparation 74
2.11.8 Purification of DNA for transfection 75
2.11.9 Precipitation of DNA in solution 76
2.11.10 Gel purification of DNA 77
2.11.11 Ligation of insert DNA and plasmid vector 77
2.11.12 Generation of a vector for stable transfection of HERIRF-1 under the CMV promoter 77
2.11.13 Generation of stably transfected rat mesangial cells 81
2.11.14 Polymerase chain reaction methods for genomic DNA 83
2.11.15 Purification of mRNA from eukaryotic cells 84
2.11.16 Polymerase chain reaction methods for mRNA 85
2.12. IMMUNOHISTOCHEMISTRY 86
2.12.1 Proliferating cell nuclear antigen 86
2.12.2 Anti-α-smooth muscle actin 87
2.12.3 Anti-ED-1 87
2.12.4 Anti-Thy1.1 87
2.12.5 Anti-cytokeratin 88
2.12.6 Anti iNOS-haem 88
2.13. FLOW CYTOMETRIC IMMUNOFLOUORESCENCE 88
2.13.1 Anti-Fas 88
2.13.2 Anti TNFR1 89
2.13.3 Anti F480 89
2.14. ACCELERATED NEPHROTOXIC NEPHRITIS IN THE RAT 90
2.14.1 Induction of accelerated nephrotoxic nephritis 90
2.14.2 Assessment of proteinuria and renal function 90
2.14.3 Histology 90
2.16. ANALYSIS OF DATA 90

Chapter 3 Results 1: Inflammatory Mϕ induce apoptosis of mesangial cells 92
3.1 QUIESCENT OR UNACTIVATED RODENT Mϕ DO NOT INFLUENCE RAT MC APOPTOSIS 93
3.2 CYTOKINE ACTIVATED RODENT Mϕ INDUCE APOPTOSIS AND CELL CYCLE ARREST OF RAT MC 96
3.3 SUPERNATANTS FROM ACTIVATED RODENT Mϕ HAVE NO EFFECT ON RAT MC CYCLE OR APOPTOSIS 105
3.4 Mφ INDUCTION OF BOTH CELL CYCLE ARREST AND APOPTOSIS IS BLOCKED BY INHIBITORS OF NO GENERATION

3.5 CYTOKINE-ACTIVATED RODENT Mφ INDUCE NITRIC OXIDE SYNTHASE 2 AND ABUNDANTLY PRODUCE NO

3.6 CYTOKINE-ACTIVATED RODENT MC INDUCE NOS 2 AND PRODUCE LOW LEVELS OF NITRIC OXIDE

3.7 RODENT MC UNDERGO CELL CYCLE ARREST AND APOPTOSIS IN RESPONSE TO HIGH LEVELS OF NO DONORS

3.8 NOS 2 KNOCKOUT MURINE Mφ CAN NOT INDUCE CELL CYCLE ARREST OR APOPTOSIS OF RAT MC

3.9 Mφ FROM INFLAMED RAT GLOMERULI SPONTANEOUSLY PRODUCE COMMENSURATE AMOUNTS OF NO

3.10 Mφ FROM INFLAMED BUT NOT NORMAL RAT GLOMERULI INDUCE CELL CYCLE ARREST AND APOPTOSIS OF RAT MC

3.11 GLOMERULAR Mφ INDUCED DEATH OF RAT MC IS BLOCKED BY INHIBITORS OF NO GENERATION

3.12 L-ARGININE, THE PRINCIPAL NOS 2 SUBSTRATE IS NECESSARY FOR Mφ-DEPENDENT EFFECTS ON RAT MC

3.13 THE RATIO OF RAT Mφ TO RAT MC INFLUENCES Mφ-DEPENDENT KILLING

3.14 QUIESCENT HUMAN MONOCYTE DERIVED Mφ DO NOT INFLUENCE HUMAN MC APOPTOSIS

3.15 CYTOKINE ACTIVATED HUMAN Mφ INDUCE HUMAN MC APOPTOSIS

3.16 HUMAN Mφ KILLING OF HUMAN MC IS NOT BLOCKED BY INHIBITORS OF NO GENERATION

3.17 HUMAN Mφ DO NOT PRODUCE NITRIC OXIDE

3.18 HUMAN MC DO NOT PRODUCE NITRIC OXIDE

3.19 HUMAN MC UNDERGO CELL CYCLE ARREST AND APOPTOSIS IN RESPONSE TO HIGH LEVELS OF NO DONORS

3.20 SUMMARY
Chapter 4 Results 2: Regulation of mesangial cell apoptosis in co-culture by interferon-γ

4.1 IN CYTOKINE-ACTIVATED CO-CULTURE WITH RODENT Mφ, IFN-γ PRIMED RAT MC ARE MORE SUSCEPTIBLE TO APOPTOSIS THAN UNPRIMED CELLS

4.2 L-NMMA PARTIALLY INHIBITS Mφ MEDIATED APOPTOSIS OF PRIMED RAT MC

4.3 BMD Mφ FROM NOS 2 KNOCKOUT MICE CAN INDUCE APOPTOSIS OF PRIMED MC

4.4 SUPERNATANT TRANSFER FROM ACTIVATED Mφ TO PRIMED MC INDICATED CLOSE APPosition OF CELLS IS REQUIRED FOR KILLING

4.5 RAT MC PRIMED WITH IFN-γ PLUS TNF-α ARE SUSCEPTIBLE TO APOPTOSIS BY SOLUBLE RECOMBINANT FAS LIGAND

4.6 PRIMED RAT MC INCREASE EXPRESSION OF THE DEATH RECEPTOR FAS

4.7 RED-OX POTENTIAL AND FAS-MEDIATED KILLING

4.8 IFN-γ PLUS TNF-α TREATED HUMAN MC ARE ALSO SUSCEPTIBLE TO FAS-MEDIATED APOPTOSIS

4.9 PRIMED HUMAN MC HAVE INCREASED EXPRESSION OF CELL SURFACE FAS

4.10 HUMAN AND RAT Mφ HAVE FAS LIGAND, BUT ONLY PARTICULATE PHAGOCYTOSIS RESULTS IN RELEASE OF SOLUBLE FAS LIGAND

4.11 RAT MC EXPRESS FAS LIGAND

4.12 FAS LIGATION IS NOT RESPONSIBLE FOR Mφ MEDIATED KILLING OF PRIMED RAT MC

4.13 IN CYTOKINE ACTIVATED RODENT CO-CULTURE WITH IFN-γ PRIMED MC, BLOCKADE OF TUMOUR NECROSIS FACTOR RECEPTOR 1 INHIBITS MC APOPTOSIS
4.14 TNF-α/TNF-β DOUBLE KNOCKOUT MOUSE MΦ
SHOW IMPAIRED ABILITY TO INDUCE APOPTOSIS
OF IFN-γ PRIMED MC 167
4.15 IN LOWER SERUM CONDITIONS, TNF-DIRECTED
KILLING OF RAT MC PREDOMINATES OVER NITRIC OXIDE 168
4.16 IN CYTOKINE-ACTIVATED CO-CULTURE WITH
HUMAN MΦ, IFN-γ PRIMED HUMAN MC ARE MORE
SUSCEPTIBLE TO APOPTOSIS THAN UNPRIMED CELLS 169
4.17 TNFR1 BLOCKADE RATHER THAN FAS BLOCKADE
INHIBITS HUMAN MΦ-DERIVED KILLING OF BOTH
PRIMED AND UNPRIMED HUMAN MC 170
4.18 LOW SERUM CONDITIONS DO NOT AUGMENT
HUMAN MΦ-DERIVED KILLING OF HUMAN MC 172
4.19 TNFR1 CELL SURFACE EXPRESSION IS NOT
INCREASED BY IFN-γ PRIMING OF HUMAN MC 173
4.20 SUMMARY

Chapter 5: Results 3 Mechanisms of mesangial cell death by immune
complex-activated macrophages 176
5.1 OPSONISED ZYMOSAN-ACTIVATED RODENT
MΦ INDUCE APOPTOSIS BUT NOT CELL CYCLE
ARREST OF RAT MC 177
5.2. OZ ACTIVATED RODENT MΦ RELEASE
LITTLE NO BUT ABUNDANTLY RELEASE H₂O₂ 177
5.3. INHIBITORS OF H₂O₂ RELEASE, BLOCK
OZ-ACTIVATED MΦ-DEPENDENT KILLING OF RAT MC 180
5.4. RAT MC, PRIMED WITH IFN-γ PLUS TNF-α ARE
RESISTANT TO H₂O₂ MEDIATED INDUCTION OF APOPTOSIS 182
5.5 OZ ACTIVATED MΦ FROM GLD/GLD AND
WILD TYPE MICE INDUCE CELL DEATH OF
PRIMED RAT MC EQUALLY 184
5.6 KILLING OF PRIMED RAT MC BY OZ-ACTIVATED
Mφ derived from TNF-α/β deficient mice is attenuated.

5.7 OZ-activated human Mφ induce apoptosis of human MC through release of H₂O₂ but not Fas ligand

5.8 IFN-γ primed human MC undergo apoptosis in co-culture with OZ-activated human Mφ by a TNF-inhibitable mechanism

5.9 Summary

Chapter 6: Results 4 The regulation of TNF-mediated apoptosis in mesangial cells

6.1 Introduction

6.2 IFN-γ enables TNF induction of apoptosis in rat MC

6.3 Inhibitors of nitric oxide generation do not block IFN-γ/TNF-α induction of MC apoptosis

6.4 Inhibitors of intracellular oxygen radical generation do not inhibit TNF-mediated killing of IFN-γ primed rat MC

6.5 IFN-γ primed MC translocate more NFκB to the nucleus than unprimed cells upon exposure to TNF-α

6.6 Interferon regulatory factor 1 is translocated to the nucleus under the influence of IFN-γ with TNF-α

6.7 Wild type and IRF-1 knockout cultured mouse mesangial cells do not undergo apoptosis in response to IFN-γ and TNF-α

6.8 Transient transfection of rat MC with IRF-1 under the CMV promoter results in expression of IRF-1 mRNA

6.9 Over-expression of IRF-1 in rat MC renders
THEM SUSCEPTIBLE TO TNF-MEDIATED APOPTOSIS WITHOUT THE REQUIREMENT FOR IFN-γ

6.10 CASPASE INHIBITORS DO NOT BLOCK TNF-MEDIATED KILLING OF IFN-γ PRIMED RAT MC

6.11 SUMMARY

Chapter 7: Results

7.1 IN CO-CULTURE, ACTIVATED MΦ PHAGOCYTOSE MC THAT HAVE BEEN INDUCED INTO APOPTOSIS

7.2 INGESTION OF APOPTOTIC MC BY MΦ IN CO-CULTURE INHIBITS FURTHER INDUCTION OF MC APOPTOSIS

7.3 INGESTION OF APOPTOTIC LEUCOCYTES BY MΦ IN ACTIVATED CO-CULTURE INHIBITS THE INDUCTION OF MC APOPTOSIS BY MΦ.

7.4 NITRIC OXIDE RELEASE BY ACTIVATED MΦ IS AUGMENTED BY INGESTION OF APOPTOTIC CELLS BUT NOT LATEX BEADS

7.5 TNF-α RELEASE BY ACTIVATED MΦ IS INHIBITED BY INGESTION OF APOPTOTIC CELLS, BUT NOT LATEX BEADS

7.6 TRANSFORMING GROWTH FACTOR-β RELEASE IS AUGMENTED BY MΦ AFTER INGESTION OF APOPTOTIC CELLS

7.8 SUMMARY

Chapter 8: Discussion

8.1 SUMMARY

8.2 MACROPHAGES KILL RESIDENT CELLS; A SPECIFIC OR GENERAL PHENOMENON?

8.3 THE INTERPLAY OF CELL CYCLE AND APOPTOSIS
Index of Figures

Figure 1-1  Micrograph of purified human monocytes differentiating into macrophages in vitro 2
Figure 1-2  Flow diagram of cellular generation of reactive oxygen and nitrogen intermediates 4
Figure 1-3  Fluorescent micrograph of apoptotic neutrophil labelled with annexin-V-FITC 6
Figure 1-4  Diagram of the caspase cascade and the role of mitochondria as a regulator 8
Figure 1-5  Diagram of nuclear factors which have important roles in regulating apoptosis 10
Figure 1-6  Diagram of the nephron 11
Figure 1-7  Fluorescent micrograph of human mesangial cells labelled with anti- α.SMA 12
Figure 1-8  Resident cell apoptosis, resident cell and macrophage number, in nephrotoxic nephritis 14
Figure 1-9  Resident cell apoptosis, resident cell and macrophage number, in Thy 1.1 nephritis 16
Figure 1-10  ED-1 positive macrophages in both early and late glomerular inflammation 18
Figure 1-11  Adapter proteins and their role in regulating the caspase cascade 22
Figure 1-12  Death receptors and their ligands 24
Figure 1-13  Cartoon of inducible nitric oxide synthase with cofactors 26
Figure 2-1  Phenotyping of cultured rat mesangial cells 39
Figure 2-2  Flow cytometric histograms showing selective depletion of lymphocytes from PBMCs 44
Figure 2-3  Separation of leucocytes by elutriation 45
Figure 2-4  Purification of apoptotic human neutrophils 49
Figure 2-5  Flow cytometric histograms showing induction of apoptosis of thymocytes 51
Figure 2-6  Diagram showing co-culture experimental protocol 58
Figure 2-7  Fluorescent micrographs defining mesangial cell
apoptosis in co-culture

Figure 2-8 Fluorescent micrographs defining mesangial cell mitosis in co-culture

Figure 2-9 Dose response curve used in the Griess assay

Figure 2-10 Restriction map for the 767 vector containing the IRFHER gene

Figure 2-11 Plasmid map showing insertion of the IRFHER gene into the eGFP-N1 vector

Figure 2-12 Agarose gels confirming excision and purification of the IRFHER gene

Figure 2-13 Agarose gels confirming successful insertion of IRFHER into eGFP-N1

Figure 2-14 Flow cytometric histograms confirming superiority of electroporation in transfection

Figure 2-15 Agarose gel confirming isolation of RNA from rat mesangial cells

Figure 3-1 Fluorescent photomicrograph (X 200) showing fixed co-culture of CMFDA green-labelled rat MC co-cultured with rat BMD Mφ

Figure 3-2 Mφ do not influence apoptosis or mitosis of proliferating MC in co-culture

Figure 3-3 Assessment of co-culture by flow cytometry

Figure 3-4 Activated co-culture leads to MC apoptosis and suppression of mitosis

Figure 3-5 Fluorescent micrograph of activated rat co-culture of BMD Mφ with CMFDA-labelled MC

Figure 3-6 Confirmation of MC apoptosis in live co-culture at 24h

Figure 3-7 Fluorescence intensity histograms showing G0/G1, G2/M and hypodiploid cell DNA content

Figure 3-8 Dose response curve showing the dose of L-NMMA required in co-culture

Figure 3-9 The effect of activated Mφ on rat MC apoptosis and mitosis is largely abrogated by addition of L-NIL

Figure 3-10 Nitrite generation in the supernatants of activated rat BMD Mφ

Figure 3-11 Accumulation of nitrite in supernatants of cultures of activated rat BMD Mφ with time

Figure 3-12 Immunofluorescent micrograph (X 200) of iNOS in activated rat BMD Mφ

Figure 3-13 Induction of iNOS in rat MC

Figure 3-14 Nitrite accumulation in supernatants of rat MC
Figure 3-15  Fluorescent micrographs (X200) early passage rat MC cultures showing induction of iNOS by LPS 115
Figure 3-16  Phase contrast photomicrographs of rat MC 116
Figure 3-17  Representative study of nitrite generation in the supernatant of MZ mesangial cells 117
Figure 3-18  Induction of rat MC apoptosis by donors of NO 119
Figure 3-19  Representative study of induction of apoptosis of unprimed or IFN-γ/TNF-α primed rat MC by the NO donor GSNO 121
Figure 3-20  Photomicrograph (X 400) of Diff-Quik stained cytopsins of murine BMD Mφ 122
Figure 3-21  The effect of Mφ from iNOS knockout mice on rat MC apoptosis and mitosis 123
Figure 3-22  Spontaneous nitrite generation by glomerular Mφ from telescoped nephrotoxic nephritis compared with cytokine-activated resident glomerular and BMD Mφ 125
Figure 3-23  The effect of Mφ from glomeruli of rats with day 2 telescoped NTN on MC apoptosis and mitosis 127
Figure 3-24  Fluorescent micrographs (X200) from co-culture using glomerular Mφ from NTN 129
Figure 3-25  Induction of rat MC apoptosis by pre-activated rat BMD Mφ that had been pre-treated with a collagenase digest 129
Figure 3-26  The effect of Mφ from glomeruli of rats with day 4 telescoped NTN on MC apoptosis and mitosis in the presence of L-NMMA 131
Figure 3-27  Cartoon showing inducible NOS with its cofactors and endothelial NOS 132
Figure 3-28  The effect of low levels of L-arginine on Mφ killing of MC in co-culture 134
Figure 3-29  L-NMMA, but not catalase or SOD inhibits Mφ killing MC in L-arginine depleted conditions 134
Figure 3-30  The effect of increasing Mφ to MC ratio on induction of apoptosis of MC in activated co-culture 135
Figure 3-31  The effect of co-culture on human MC mitosis and apoptosis 137
Figure 3-32  Fluorescent micrograph (X 320) of CMFDA green-labelled MC in co-culture with Mφ 138
Figure 3-33  The effect of activation of human co-culture with IFN-γ/LPS on MC mitosis and apoptosis 140
Figure 3-34  Fluorescent micrograph of activated human co-culture 141
Figure 3-35  Diagram indicating the factors influencing the ability of human iNOS to generate NO 142
Figure 4-1  The effect of priming with γIFN plus TNFα of rat MC on Mϕ induced apoptosis in activated co-culture 147
Figure 4-2  The effect of priming rat MC with γIFN plus TNFα on susceptibility to apoptosis by activated rat BMD Mϕ in the presence of iNOS inhibitors 148
Figure 4-3  The effect of activated Mϕ from NOS 2 knockout mice on unprimed and primed MC apoptosis 149
Figure 4-4  The effect of priming rat MC with γIFN plus TNFα for 24h on susceptibility to Fas ligand 152
Figure 4-5  Dose response curve for induction of apoptosis by sFasL on IFN-γ/TNF-α primed rat MC 153
Figure 4-6  Immunoblot showing increased expression of the Fas protein with IFN-γ/TNF-α priming of rat MC 155
Figure 4-7  Induction of apoptosis by CH11 anti-Fas antibody after priming of human MC 157
Figure 4-8  Immunofluorescence flow cytometry histograms showing induction of Fas on the cell surface of human MC 158
Figure 4-9  Immunoblot for Fas ligand from Triton-X soluble extracts from human and rat Mϕ 160
Figure 4-10  Immunoblots of soluble FasL in protein extracts from supernatants of Mϕ 161
Figure 4-11  Immunoblot for Fas ligand in rat MC 162
Figure 4-12  The effect of murine BMD Mϕ with mutated, non-functional Fas ligand (gld/gld) in co-culture with rat MC 164
Figure 4-13  Fluorescent dideoxy sequencing of PCR products from genomic DNA of gld/gld and WT Mϕ 164
Figure 4-14  Soluble tumour necrosis factor receptor 1 inhibits Mϕ killing of primed rat MC 166
Figure 4-15  The comparative effect of activated WT and TNFα/TNFβ 19
knockout murine Mφ on apoptosis of primed rat MC

Figure 4-16 Mφ directed killing in the absence of NO is TNF restricted

Figure 4-17 The effect of WT and TNF-α/TNF-β knockout Mφ on unprimed MC in low-serum conditions

Figure 4-18 Interferon-γ augments killing of MC by apoptosis in activated human co-culture

Figure 4-19 Induction of apoptosis of human MC by human Mφ is inhibitable by TNF receptor 1 blockade

Figure 4-20 Fluorescence histogram of human MC showing TNF receptor 1 expression

Figure 5-1 Co-culture at 24h of CMFDA- labelled rat MC with rat BMD Mφ treated with OZ

Figure 5-2 The effect of OZ activated rat Mφ on MC apoptosis and mitosis

Figure 5-3 Induction of MC apoptosis by Mφ in OZ activated co-culture is inhibitable by catalase

Figure 5-4 The effect of priming rat MC with IFN-γ plus TNF-α on the susceptibility of MC to killing by OZ activated Mφ.

Figure 5-5 The effect of TNF-α/β knockout or wild type mouse BMD-Mφ activated by OZ on killing of IFN-γ/TNF-α primed MC in co-culture

Figure 5-6 The effect of inhibitors of Fas ligation and ROI on OZ-activated human Mφ killing of human MC

Figure 6-1 Fluorescent photomicrograph (X200) showing apoptotic rat MC

Figure 6-2 Fluorescent histograms of PI stained RNAse treated rat MC indicating DNA content

Figure 6-3 Induction of apoptosis by IFN-γ/TNF-α in rat MC is not suppressed by iNOS inhibitors

Figure 6-4 Induction of apoptosis and suppression of mitosis by IFN-γ/TNF-α in rat MC is not suppressed by intracellular oxygen radical scavengers

Figure 6-5 Representative band shift assay for nuclear NFκB species

Figure 6-6 Supershift EMSA for NFκB using nuclear extract from TNF-α stimulated rat MC

Figure 6-7 Representative band shift using the ISG15 promoter IRF-1 binding sequence

xx
Figure 6-8  Supershift EMSA using the ISG15 promoter sequence for IRF-1 binding
Figure 6-9  Agarose gel showing PCR products from transiently 
transfected rat MC using IRF-1 primers
Figure 6-10  Polyclonal HERIF-1 stably transfected rat MC undergo 
increased apoptosis
Figure 6-11  Rat MC clones over-expressing IRF-1 have abnormalities 
of cell cycle progression
Figure 6-12  Rat MC do not obviously have Caspase 3 in the cytosol
Figure 7-1  Ingestion of apoptotic MC by activated Mϕ does not prevent 
 Mϕ induced killing of live primed MC in co-culture
Figure 7-2  Ingestion of apoptotic MC by activated Mϕ blocks TNF-dependent 
killing of live MC in co-culture
Figure 7-3  Ingestion of apoptotic neutrophils by quiescent BMD Mϕ viewed 
by phase contrast microscopy
Figure 7-4  In activated co-culture, prior ingestion of apoptotic leucocytes 
by Mϕ does not inhibit Mϕ induction of MC apoptosis
Figure 7-5  In activated co-culture, prior ingestion of apoptotic leucocytes 
by Mϕ inhibits TNF-restricted Mϕ induction of MC apoptosis
Figure 7-6  Mϕ that have ingested apoptotic cell produce more NO when 
activated by IFN-γ/LPS than Mϕ which have ingested inert particles
Figure 7-7  Mϕ that have ingested apoptotic cell release less TNF-α when 
activated by IFN-γ/LPS than Mϕ which have ingested inert particles
Figure 8-1  Schematic of the mechanisms by which Mϕ induce apoptosis of MC
Figure 8-2  Simplified schematic showing cell cycle proteins
Figure 8-3  Schematic showing predicted roles of Mϕ in NTN
Figure 8-4  Differentiation of monocytes to classically activated or alternatively 
activated Mϕ
Figure 8-5  Negative feedback in killing of MC by Mϕ by ingestion of apoptotic MC
Index of Tables

Table 3-1  Flow cytometric analysis of cellular DNA content in unactivated rodent co-culture  
Table 3-2  Microscopical assessment of rat MC apoptosis and mitosis in medium with 1% FCS  
Table 3-3  Percentage apoptotic rat MC in live co-culture by exclusion of propidium iodide but not Hoechst 33342  
Table 3-4  The effect of activated rodent co-culture on percentage G2M and hypodiploid MC  
Table 3-5  The effect of co-culture on PCNA-positivity of rat MC at 8h and 24h  
Table 3-6  The effect of activated co-culture on apoptosis of primary polyclonal rat MC at 24h  
Table 3-7  The effect of supernatant transfer from IFN-γ/LPS activated rat Mφ to rat MC, on MC apoptosis and mitosis  
Table 3-8  The effect of nitric oxide and super-oxide radical inhibitors on induction of rat MC apoptosis in co-culture with Mφ at 24h  
Table 3-9  The effect of L-NMMA and D-NMMA on activated Mφ-induced induction of rat MC apoptosis and suppression of mitosis  
Table 3-10  Generation of nitrite in supernatant of murine Mφ  
Table 3-11  Induction of apoptosis of MZ rat mesangial cells by rat BMD Mφ in activated co-culture  
Table 3-12  Induction of apoptosis and cell cycle arrest of rat MC by 10mM GSNO with time  
Table 3-13  Induction of polyclonal rat MC apoptosis by the NO-donor, GSNO at 24h  
Table 3-14  Nitrite generation by wild type and iNOS knockout murine BMD Mφ  
Table 3-15  The effect of resident glomerular Mφ and diseased glomerular Mφ from rats with NTN at upon MC apoptosis, mitosis and cell number  
Table 3-16  The effect of a confluent monolayer of Mφ on human MC proliferation  
Table 3-17  The effect of L-NMMA in cytokine-activated human co-culture  
Table 3-18  Nitrite generation by human monocyte derived Mφ over 24h  
Table 3-19  Nitrite generation by human MC over 48h  

xxii
Table 3-20: Induction of apoptosis of human MC in response to the NO donor, GSNO

Table 4-1: Augmented induction of rat MC apoptosis by activated Mφ following MC priming with IFN-γ alone

Table 4-2: The effect of conditioned medium from activated Mφ on MC apoptosis and mitosis in the absence of FCS

Table 4-3: Time course for induction of apoptosis of primed rat MC by soluble Fas ligand

Table 4-4: Inhibition of primed human MC apoptosis induced by activated human Mφ at 48h

Table 4-5: Inhibition of unprimed human MC apoptosis by human Mφ using TNFR1-Fc

Table 4-6: Induction of human MC apoptosis in the presence of 10% FCS or 1% FCS

Table 5-1: Nitrite generation by rat BMD Mφ following phagocytosis of OZ

Table 5-2: Peroxide generation by activated macrophages

Table 5-3: The effect, in rodent co-culture of oxygen radical scavengers on OZ-activated killing of IFN-γ/TNF-α primed rat MC

Table 5-4: Catalase inhibitable induction of MC apoptosis by activated neutrophils

Table 5-5: The effect of OZ-activated wild-type and gld murine Mφ on killing of IFN-γ/TNF-α primed rat MC

Table 5-6: Inhibition of OZ killing of primed rat MC by soluble TNF receptor 1

Table 5-7: The effect of candidate inhibitors on OZ-activated Mφ-mediated killing of IFN-γ primed human MC

Table 6-1: The synergistic effect of IFN-γ on recombinant TNF-α-induced apoptosis of rat MC

Table 6-2: The effect of recombinant IFN-γ plus TNF-α on IFN-γ primed and unprimed rat and human MC

Table 6-3: Polyclonal IRFHER expressing MC are susceptible to TNF-α mediated apoptosis

Table 6-4: IRFHER transfected MC clones are susceptible to TNF-α mediated apoptosis

Table 7-1: The effect of ingestion of apoptotic MC by Mφ in co-culture with IFN-γ primed MC on Mφ-induction of MC apoptosis
Table 7-2  Release of TGFβ (pg/ml) by Mφ following ingestion of apoptotic cells

<table>
<thead>
<tr>
<th>Mφ following ingestion of apoptotic cells</th>
<th>221</th>
</tr>
</thead>
<tbody>
<tr>
<td>Release of TGFβ (pg/ml) by Mφ</td>
<td>221</td>
</tr>
</tbody>
</table>

xxiv
Acknowledgements

Prof. John Savill. For excellent judgement in his supervision of me, allowing great freedom to diverge, but redirecting the ‘tiller’ at the appropriate time, for expertly tutoring me in the art of presentation and directing me to the right people at the right time.

Dr. Simone Brown. For excellent, expert advice about basic scientific principles, assistance with setting up the in vitro assays, and many fruitful and educational discussions.

Dr. Hitoshi Sugiyama. For assistance with EMSA and assays of human MC apoptosis.

Dr. Eleni Stylianou. For advice and direction about TNF signalling, basics of cell culture and helpful discussions.

Dr. Adam Lacy-Hulbert. For setting me straight with molecular biology, and many useful discussions about macrophages.

Ms. Amanda Watson and Dr. Tim Jobson. For help and support during my time in Nottingham.

Department of Renal Medicine. To all members of staff, for their support in undertaking the thesis and assisting with facilities and time to complete the writing of the thesis.

Prof. Shigekazu Nagata. For advice about Fas/FasL interactions and choice of antibodies.

Prof. Andy Rees and Dr. Lars Erwig. For enthusiastic support of ex vivo co-culture experiments.

Dr. Vicky Cattell and Dr. Simon Waddington. For open support and interest in pursuit of understanding nitric oxide in the kidney, and supply of materials.

Prof. Josef Pfeilschifter. For helpful advice and supply of materials for investigation of iNOS in mesangial cells.

Prof Brice Weiberg. For useful conversations about nitric oxide in glomerulonephritis.

Dr. Jeremy Hughes. For helpful advice during the thesis and assistance with acquisition of gld mice.

Prof. Bernhardt Ryfell. For supply of tnf-α-/- and tnf-β-/- double knockout mice.

Prof. Carl Ware. For advice, interest and assistance with investigations of death receptors in macrophage killing of mesangial cells, and for the supply of soluble receptors.

Prof. Hansorg Hauser. For supply of the original IRFHER vector.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAF-1</td>
<td>apoptotic protease activating factor</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>(Bcl-2 family protein)-x-long</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell leukaemia-2</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>BMD</td>
<td>bone marrow derived</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CMFDA</td>
<td>Chloromethyl fluorodiamine (cell tracker Green)</td>
</tr>
<tr>
<td>CR1,CR3</td>
<td>complement receptor 1, or 3</td>
</tr>
<tr>
<td>CSF-1/M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>DAXX</td>
<td>Fas death domain associated protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>eGFP</td>
<td>extranuclear green fluorescent protein</td>
</tr>
<tr>
<td>F12</td>
<td>Ham’s F12 medium</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
</tr>
<tr>
<td>Fas</td>
<td>Fas/Apo-1/CD95</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>-Fc</td>
<td>The constant region of immunoglobulin</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HVEM</td>
<td>herpes virus entry mediator</td>
</tr>
<tr>
<td>IL-1$\beta$</td>
<td>interleukin-1 beta</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin-</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IRF-1</td>
<td>interferon regulatory factor-1</td>
</tr>
<tr>
<td>IRF-2</td>
<td>interferon regulatory factor-1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT-(\beta)</td>
<td>lymphotoxin-beta</td>
</tr>
<tr>
<td>LT(\beta)-R</td>
<td>lymphotoxin-beta receptor</td>
</tr>
<tr>
<td>M(\phi)</td>
<td>macrophage</td>
</tr>
<tr>
<td>MCP-1</td>
<td>M(\phi) chemotactic protein 1</td>
</tr>
<tr>
<td>MC</td>
<td>mesangial cell</td>
</tr>
<tr>
<td>MDM</td>
<td>monocyte-derived macrophage</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>iNOS/NOS 2</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>NOS 1/nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS 3/eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NTN</td>
<td>nephrotoxic nephritis</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>reactive oxygen</td>
</tr>
<tr>
<td>OZ</td>
<td>Opsonised zymosan</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>neutrophil</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediates</td>
</tr>
<tr>
<td>RNA</td>
<td>ribose nucleic acid</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Rosewell Park Memorial Institute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl (lauryl) sulphate</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>Thy1.1</td>
<td>Thymus antigen 1.1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TNF-β</td>
<td>tumour necrosis factor-beta</td>
</tr>
<tr>
<td></td>
<td><strong>Buffered Culture Medium</strong></td>
</tr>
<tr>
<td>TGF-β</td>
<td><strong>Transforming Growth Factor-Beta</strong></td>
</tr>
<tr>
<td>Thy1.1</td>
<td><strong>Thymus Antigen 1.1</strong></td>
</tr>
<tr>
<td>TNF</td>
<td><strong>Tumour Necrosis Factor</strong></td>
</tr>
<tr>
<td>TNF-α</td>
<td><strong>Tumour Necrosis Factor-Applha</strong></td>
</tr>
<tr>
<td>TNF-β</td>
<td><strong>Tumour Necrosis Factor-Beta</strong></td>
</tr>
</tbody>
</table>
Dedication

This thesis is for my daughter Madeleine Alice Calder Duffield whose conception, birth, early development and now flourishing life, coincided with the major events in the life of this work. I hope that this thesis might be the building block of future learning knowledge and research, much as with her basic life-skills established, she will go on to great things.
Chapter 1

Introduction
1.1 INFLAMMATION

Inflammation is a tissue response to injury characterised by rubor, calor, tumor, dolor (redness, heat, swelling and pain). The injured tissue has responded by increased vascular flow, increased vascular permeability, and has attracted inflammatory leucocytes to leave the capillaries and enter the tissue. Many types of injury allow (particularly to epithelial surfaces) pathogenic organisms to enter, therefore requiring a swift response by the innate immune system. The most efficient cell types at clearing invading organisms are the neutrophil and the inflammatory macrophage. These two cell types are the first to appear at a site of local injury; usually the neutrophil precedes the macrophage. Diapedesis, the movement of leucocytes across the endothelial barrier, and the extravascular, cytokine-rich environment trigger differentiation. The monocyte differentiates into a macrophage phenotype (Figure 1-1). Neutrophils entering the injured tissue, upregulate adhesion molecules and may produce oxygen radicals or proteases, thereby becoming activated.

Figure 1-1: Human monocytes (X400) purified from fresh buffy coat show characteristic morphology by haematoxylin and eosin stain (Panel A). In the presence of autologous serum they differentiate into macrophages (X400) (panel B). Note characteristic ‘crystals’ in the nucleus of monocytes, and the characteristic ‘fried egg’ morphology of macrophages with greater diameter and cytoplasm.

Increased blood flow and permeability allow increased transfer of plasma borne proteins of the innate immune system to enter the injured tissue (such as C-reactive protein). Vascular damage results in activation of the coagulation cascade and release of α granules from platelets. Amongst
other mediators, a multitude of growth factors (found in serum) are released locally by platelet
degranulation and initiate de-differentiation and proliferation of local resident tissue cells. In all
tissues, are mesenchymal cells that de-differentiate into myofibroblasts. In health, these cells are
major contributors to the normal extracellular matrix comprising laminins and collagens
(principally type IV). In response to injury and growth factors, myofibroblasts proliferate and are
responsible for laying down abnormal matrix or scar tissue. Once proliferating, these cells generate
their own growth factors which, by autocrine mechanisms, perpetuate proliferation and the
myofibroblast phenotype. The matrix generated now contains an excess of collagen I, which is not
normally synthesised by these cells in health. The generation and degradation of matrix is dynamic
during the inflammatory response as the very cells that lay down matrix also break it down using
enzymes called metalloproteinases. This family of enzymes is also active during inflammation. It is
not true to say therefore that any scar tissue laid down will be permanent.

Neutrophils that are activated at the site of inflammation produce many proteases that can
breakdown extracellular matrix. They also undergo a 'respiratory burst', swiftly generating
abundant oxygen radicals (Figure 1-2) and hydrogen peroxide. Lipopolysaccharide (LPS) and
cytokines such as tumour necrosis factor alpha (TNF-α) can result in induction of the oxygen
radical burst of neutrophils, but many stimuli can activate them in this stereotyped fashion. There
is much evidence in models of inflammation that neutrophils can inflict 'bystander' or 'friendly
fire' damage on the tissue concerned (Johnson, Guggenheim et al., 1988; Johnson, Alpers et al.,
1988; Albina et al., 1990; Mateo et al., 1994; Worthen et al., 1987; Savill et al., 1989). Indeed the
induction of many sterile inflammatory responses does not occur without neutrophil mediated local
damage (Santos & Tipping 1994; Henson & Johnson 1987; Sandoval et al., 1996). Excessive local
damage mediated by neutrophils would cause perpetuation of the inflammatory response leading to
further influx of leucocytes. Monocyte-derived MΦ entering the site of inflammation appear to take
on an activated phenotype (Albina et al., 1995; Nessel et al., 1999; Meszaros et al., 2000; Erwig
et al., 1999). These cells when activated can produce a variety of free radicals and cytokines. In
addition, they are another potent source of growth factors. Macrophages, so called because of their
appetite for phagocytosing particles have long been thought of as the 'hoover' in the inflammatory
response. Their role however is less clear and is the focus of this study.
Figure 1-2 Flow diagram indicating the key steps in the generation of reactive oxygen intermediates. Key enzymes and co-factors are marked. Also, key regulatory enzymes such as catalase are also shown. Generation of O$_2^-$ is essential for nitric oxide generation by the NOS family of enzymes from L-arginine. The pathway of NO metabolism is also shown.

1.2 APOPTOSIS – A FORM OF CELL DEATH

Apoptosis is a term used to describe programmed or physiological cell death. It appears that all cells can undergo apoptotic cell death. This form of death is sometimes induced by noxious stimuli, sometimes induced by specific ligand receptor interactions either at the cell surface or intracellularly, sometimes by deprivation of vital survival signals initiated at the plasma membrane, and sometimes occurs as a default process, when the cell becomes damaged, unable to repair itself, or if it becomes effete (Duke & Cohen 1986; Williams et al., 1990; Clarke et al., 1993; Soengas et al., 1999; Talhouk et al., 1992; Itoh et al., 1991; Smith et al., 1994). Many non-haematopoetic cells require survival signals from adhesion to extracellular matrix. In the event that these cells become non-adherent (such as columnar epithelial cells at the apex of villi in the gut), the cell defaults into apoptotic cell death (Frisch & Francis 1994; Bates et al., 1994; Ruoslati & Reed 1994).

It is important to distinguish apoptosis from non-programmed cell death where cells are so abruptly damaged that they 'fall apart' without being able to initiate the cellular program or organised death (Duvall & Wyllie 1986; Kerr et al., 1972). In the case of non-programmed cell death, intracellular contents are randomly released into the extracellular space. One can envisage
that necrotic death of inflammatory cells such as eosinophils and neutrophils will lead to uncontrolled release of granules spilling proteases that degrade normal tissue architecture. By contrast programmed cell death leads to maintenance of the plasma membrane and intracellular organelles. The Golgi is disrupted but compacted into vesicles and importantly, DNA is characteristically cleaved into small sections by apoptosis-specific endonucleases. This cleavage can be seen as a characteristic ladder if DNA from apoptotic cells is electrophoresed (Wyllie et al., 1980). Significantly, apoptotic cells are specifically phagocytosed by phagocytes of the monocyte lineage and also semi-professional phagocytes such as fibroblasts (Savill et al., 1989; Savill et al., 1992). Most cells when de-differentiated can phagocytose apoptotic cells. The mechanisms of phagocytic clearance of apoptotic cells are distinct from phagocytosis of other particles including necrotic debris and unlike phagocytosis of many particles do not induce an inflammatory response in the phagocytosing cell (Meagher et al., 1992).

Apoptosis as opposed to necrosis is defined both morphologically and functionally. The distinction between these forms of cell death for inflammatory cells such as neutrophils is obvious since necrotic death might lead to enhancement of tissue destruction through release of intracellular contents and by further exciting local inflammatory responses (Stern et al., 1996). However, in the case of resident tissue cells the functional distinction is less clear cut since most cells do not contain vesicular enzymes capable of tissue destruction. More importantly, possibly, is the effect uptake of apoptotic cells as opposed to necrotic cell debris has on the phagocytosing cell. This issue is pursued in this thesis.

Apoptosis was defined morphologically in 1972 (Kerr et al., 1972). This remains the gold standard, though there are now biochemical markers that are variably reliable depending on the cell type under investigation. Morphologically, the features are highly stereotyped across species and cell type. Indeed there is evidence that plant eukaryotic cells undergo apoptosis! Features include cell shrinkage, cytoplasmic condensation and vacuolation, condensation of nuclear heterochromatin, abutting the nuclear membrane, then distinction of nuclear material into homogeneous spheres. In some cell types there is dramatic cytoplasmic blebbing. When viewed in real time the blebbing looks like the cell is 'boiling away'. Some of these buds contain nuclear material and are termed apoptotic bodies. These bodies are also swiftly phagocytosed, non-phlogistically, by macrophages.

Functionally, apoptotic death can be distinguished from necrotic cell death by maintenance of function of the plasma membrane. The necrotic cell membrane can be breached by all manner of molecules and is unable to maintain ion gradients. This is manifested by leakage of dyes into necrotic cells that are excluded from apoptotic and live cells. Commonly, trypan blue and the fluorescent nucleic acid-binding dye, propidium iodide (PI), can assist in assessing the integrity of
the plasma membrane. Another fluorescent molecule that binds avidly to DNA only, Hoescht 33342, is freely permeable to the plasma membrane of healthy cells but is pumped out actively (Sun et al., 1992) (see Figure 3-5). In apoptotic cells, this pump does not function, permitting accumulation and nuclear labelling of DNA in the apoptotic cells. The cell membrane, though intact is manifestly different from the healthy plasma membrane in many ways. For example, many cell surface receptors and ligands are shed, the actin cytoskeleton immediately below the plasma membrane is disrupted and the composition of the external lipid bilayer changes due to down regulation of scramblases which exchange phosphatidylserine in the inner leaflet for phosphatidylcholine in the outer leaflet (Fadok et al., 1992). Furthermore, as a part of the apoptotic process, certain intracellular contents such as nuclear proteins that are not normally displayed, become localised to the external plasma membrane (Casciola-Rosen et al., 1996). Many of these biochemical changes have been exploited as biological tools to define apoptosis. One commonly used tool is the appearance of phosphatidylserine in the external leaflet of the lipid bilayer which can be specifically recognised by Annexin V (Figure 1-3).

![Figure 1-3: Apoptotic neutrophils labelled with Annexin V-FITC. Note bright green cell surface fluorescence.](image)

1.3 **EFFECTOR MECHANISMS IN THE APOPTOTIC PROCESS.**

Whilst apoptosis can be defined by morphological and biochemical processes it is clear that many stimuli can result in a final common outcome for the cell. Much effort has been devoted to unravelling the effector mechanisms in this final common pathway. What has become clear is that for many types of apoptotic death an intracellular cascade of aspartic acid proteases (the caspases)
Chapter 1 Introduction

is important in directing the manifestations of cell death. The key pro-enzymes were first delineated from mutant studies of the developing worm C. elegans. This worm characteristically loses 131 of the original 1090 somatic cells by apoptosis during development. CED-3, a caspase was discovered from these mutant studies and shares great homology with enzymes in mammalian cells and in many cases are functionally intact when transferred to mammalian cells (Yuan, & Horvitz 1990; Yuan et al., 1993). In mammalian cells this caspase pathway, which is analogous to the coagulation and complement cascades, has many amplification steps. Whilst the nucleus is dispensable (Jacobson et al., 1994), for many types of apoptotic cell death the mitochondria have proved vital in the amplification process of the cascade (Mignotte & Vayssiere 1998; Yang et al., 1997; Susin et al., 1999). It is worth emphasising that whilst this pathway is undoubtedly very important, there do appear to be examples of apoptotic death where caspases are not necessary. Indeed in vivo some cells do not express key members of this pathway (Horvitz personal communication; Bennett personal communication; Doerfler et al., 200; Carmody & Cotter 2000; Kuida et al., 1998; Kuida et al., 1996; Woo et al., 1999). It is perhaps not surprising that such an evolutionarily conserved event (apoptosis) has many levels of mechanistic redundancy. However, knockout studies in worms and mice have emphasised a key role for the caspase cascade (Figure 1.4) (Kuida et al., 1998; Kuida et al., 1996; Woo et al., 1999).

Cytolysis of tumour cells by soluble cytokine tumour necrosis factor was recognised in 1983 (Drysdale et al., 1983). However, confirmation that this ligand could swiftly induce apoptotic death of certain tumour cells through specific ligand-receptor interactions was not described until 1991 (Tartaglia et al., 1991). This indicated that signalling events at the cell surface could link into effector mechanisms operating intracellularly. A receptor named Fas (Apo-1), homologous to the TNF receptor 1, whose activity was shown to also result in cell death (Itoh et al., 1991), was co-precipitated with one such caspase named FLICE (subsequently caspase 8) (Muzio et al., 1996). This provided a direct link between cell surface receptor ligation and the death-signalling cascade. Subsequently, TNFR1 and more recently many other homologous 'death' receptors have been shown to activate the caspase pathway through cleavage (hence activation) of caspase 8 (Hsu et al., 1996). It should be noted that these death receptors also cleave other caspases (eg caspase 2) in certain cell types (Cohen 1997). Furthermore activation of other signalling pathways such as DAXX (Yang, Khosravi-Far, et al., 1997) and Jun kinases (Latinis & Koretzky 1996), extracellular signal-regulated kinases (Goillot et al., 1997) and acid sphingomyelinase-ceramide (Cifone et al., 1995) pathways are likely to be important in induction of apoptosis in some cell types following death receptor ligation (Figure 1.4). At the effector end of the caspase cascade, caspase-specific final effectors such as the caspase activated DNase (CAD) have been clearly demonstrated. Other targets for proteolysis include kinases such as FAK, MEKK1, structural proteins such as laminins,
fodrin, gelsolin. Many factors involved in transcription, translation, DNA repair and replication are also specifically cleaved.

Recent attention has been paid to the role of cytochrome C released from mitochondria in augmenting the caspase cascade. Cytochrome C (cyt. C) has traditionally been associated with the electron transport chain in the inner lipid bilayer of mitochondria, crucial in ATP generation. There is good evidence that caspase 9 binds to, and is cleaved to the active form by a factor called APAF-1 when cyt. C is released from mitochondria. This 'apoptosome' is able to cleave caspase 3 which is thought to be the final common effector in caspase dependent apoptotic death (or at least a component of the final common pathway) (Susin et al., 1999; Susin et al., 1999; Mignotte et al., 1998; Li et al., 1997). Release of cyt. C from mitochondria is modulated by numerous factors including members of the B-Cell Lymphoma-2 (Bcl-2) family of pore-forming proteins (Yang, Liu, et al., 1997). It appears that many survival signals derived from cell-surface integrins and

Figure 1-4: Diagram of a cell giving an interpretation of current data in the literature regarding pathways involved in apoptosis following death receptor trimerization at the cell surface. The key players and their interactions are indicated though aspects of this pathway might be refuted by some and it is by no means comprehensive. For a more detailed overview refer to Schulze-Osthoff et al., 1998; Mignotte et al., 1998.
growth factors such as insulin-like growth factors impinge on mitochondrial release of cyt C, thereby 'plugging in' to the caspase cascade, possibly by regulating phosphorylation and therefore activity of Bcl-2 family members such as Bid.

This model of regulation of the apoptotic process cannot be the whole story since attenuation of the function of caspase 3 either in knockout mice or in antisense/dominant negative studies does not necessarily block induction of apoptosis (Kuida et al., 1998; Kuida et al., 1996; Honarpour et al., 2000). Indeed in caspase 3, caspase 9 and APAF knockout mice the principal defect is impaired neuron deletion. Other organs developed normally. However, when such caspase 3 knockout mice were challenged with agonistic anti Fas antibodies, the hepatocytes were protected from apoptosis for 72h. Notably in these mice, lymphocyte development appeared normal, though when thymocytes were challenged with apoptotic stimuli, knockout thymocytes were variably resistant (Doerfler et al., 2000). These knockout animals indicate that there are either caspase-independent mechanisms of apoptosis or that other caspases can take over the function of deleted family members.

It is evident that many cells exposed to noxious stimuli phosphorylate and thereby activate a nuclear factor p53 (Ko et al., 1997). This family of nuclear factors is induced both functionally and quantitatively prior to morphologically detectable apoptosis. Over-expression of some of the p53 isotypes results in induction of apoptosis. p53-mediated apoptosis has been closely associated with DNA damage due to radiation (UV, gamma) and noxious stimuli such as nitric oxide and oxygen radicals (Messma et al., 1994; Albina et al., 1993; Clarke et al., 1993). Induction of p53 is associated with cell cycle arrest, frequently at the G1/S border (Baker et al., 1990; Harper et al., 1993). However, it has become apparent that other forms of apoptotic death such as developmentally regulated cell deletion may involve p53 (Almog & Rotter 1997). Indeed, many stimuli that activate p53 also seem to induce caspase 3 cleavage (Zhao et al., 2000; Soengas et al., 1999) and others have indicated a p53-dependent activation of caspase 8 via Fas at the plasma membrane (Bennett et al., 1998) (Figure 1-5). For example, nitric oxide induces p53 activity, but also results in caspase 8 and caspase 1 cleavage (Bennett et al., 1998, Zhou et al., 2000). The mechanisms by which p53 enables apoptosis have remained obscure, but recently it has become clear that p53 controls Bax activity, which in turn regulates caspase 3 activity (Miyashita et al., 1994; Cregan et al., 1999). One mechanism, therefore, by which p53 can induce apoptosis is by cleaving key caspase family members. Investigations of toxic stress-induced apoptosis have shown that not all cells require p53 to undergo apoptosis. Many of the cells that die in response to DNA-damaging stimuli undergo apoptosis independently of p53 require induction and translocation to the nucleus of another nuclear protein, interferon regulatory factor-1 (Tamura, Ishihara, et al.,
First noted as an interferon induced gene (Tanaka & Taniguchi 1992), this nuclear factor is also induced synergistically by interferon-γ in association with TNF-α in addition to other factors including DNA damage. Transgenic overexpression in B-lymphocytes lead to developmental deletion, of those cells (Yamada et al., 1991). In murine knockout studies lymph node architecture is disrupted with abnormal CD8 T cell populations (Matsuyama et al., 1993).

Figure 1-5: Diagram of a cell with nucleus indicating mechanisms potentially responsible for mediating apoptosis of cells following removal of survival signals from the plasma membrane or following DNA damaging insults such as gamma or UV irradiation.

1.4 MESANGIAL CELL AS A MYOFIBROBLAST; ITS ROLE IN REMODELLING

The principal function of the kidney is concerned with extracellular homeostasis, that is fluid and electrolyte control in the extracellular space. The kidneys achieve this by relatively non-specific ultrafiltration of protein deplete fluid from the plasma component of blood through many sieve-like structures, called glomeruli. There are between $2 \times 10^4$ and $5 \times 10^5$ glomeruli per kidney depending
on the species. This functional unit comprises capillaries with specialised endothelium, a specialised basement membrane upon which lie the endothelial cells on one side and specialised epithelial cells, the podocytes. Holding this structure of many capillaries surrounded by podocytes together is the mesangium, containing mesenchymal cells that produce extracellular matrix and function in addition to structural support to regulate capillary blood flow. Surrounding this central structure (looking like the surface of the brain) is a smooth spherical skin of parietal epithelium which at one pole becomes the proximal tubule or the 'drain' of the glomerulus. The normal glomerulus is a tightly regulated structure with a fixed number of mesangial cells and little cell turnover. Filtered fluid is enriched for waste and has useful, beneficial compounds selectively re-absorbed by tubular cells that constitute the tubular structure carrying away the filtered fluid. The final concentrated, enriched waste fluid is urine.

![Diagram of a nephron in the kidney, indicating the glomerulus](image)

Figure 1-6: Diagram of a nephron in the kidney, indicating the glomerulus

Inflammation involving either the glomerular or tubular structures is very common and is a manifestation of many poorly understood diseases. Glomerulonephritis, inflammation of the kidney specifically directed at either some or all glomeruli (Figure 1-6). During inflammation of the glomerulus there is a stereotyped response to many different stimuli. The responses include increased and altered matrix production by mesangial cells, induction of proliferation of mesangial cells (one of the most common findings in human biopsies from kidneys with disease is an increase in mesangial cellularity and matrix expansion). Other responses include changes (ultrastructural) to the basement membrane that render it permeable to albumin and other plasma proteins and loss of,
or damage to, endothelial cells. When MC respond to inflammatory stimuli they produce α-smooth muscle actin (and other proteins of the contractile apparatus), and in models of glomerular disease (Thy1.1 nephritis) they show distinct migratory and contractile tendencies (Johnson et al., 1991; Floege et al., 1991; Haseley et al., 1999).

This differentiation of MC to myofibroblast like cells and the consequences of this differentiation on the tissue, however, is reiterated in many types of inflammation throughout the body (Figure 1-7).

Myofibroblasts are de-differentiated cells that express α-smooth muscle actin and are both migratory and contractile (smooth muscle cell phenotype) and they lay down extracellular matrix containing Collagen I (fibroblast phenotype). Whilst the skin fibroblast during injury might be thought of as the archetypal myofibroblast (Schurch et al., 1992), many other tissues have cells in the interstitium that, when provoked, de-differentiate into this phenotype. Such sites include atherosclerotic plaques (Bennett 1999), the septae of liver lobules during inflammation (Iredale et al., 1998), lungs during the inflammatory response (Brewster et al., 1990; Polunovsky et al., 1993), gut (Jobson, et al., 1998; Shuppan et al., 2000) and the kidney (interstitial fibroblasts and mesangial cells). Further, there is evidence that epithelial cells can trans-differentiate into
myofibroblasts. As inflammation in diverse sites is characterised by infiltrating bone-marrow-derived cells, proliferating resident cells, myofibroblasts, and laying down of abnormal matrix it seems reasonable to consider glomerular inflammation in that light.

In self-resolving models of glomerulonephritis, Baker and colleagues (Baker et al., 1994), and Shimizu and co-workers (Shimizu et al., 1995) have both demonstrated that resolution of glomerular hypercellularity (and therefore disease) is brought about by loss of excessive mesangial cells by apoptosis. Return to the normal cell complement is therefore brought about by apoptosis. Concomitantly, there is loss of excessive matrix. Therefore, like skin wounding (Desmouliere et al., 1995), and resolving hepatic inflammatory scarring (Iredale et al., 1998), co-ordinate loss of myofibroblasts brings about resolution of disease.

In models of progressive disease, there is good evidence that excessive mesangial cell loss is also brought about largely by apoptosis. Firstly, in a study of progressive anti-GBM nephritis in the WKY (Wistar-Kyoto) rat, apoptosis occurs in proliferating (PCNA +ve) MC. The incidence of apoptotic cells is high compared with the resolving Thy 1.1 nephritis and correlated with infiltration of leucocytes. However, the rate of apoptosis increases further in the fibrotic later stages of the disease (Shimizu et al., 1996). Similar findings were reported using the same nephrotoxic nephritis model, but in the Sprague Dawley rat (Sugiyama et al., 1997). However, NTN induced by El Nahas’ group using the WKY rat, found that maximum apoptosis of resident cells occurred earlier in the disease and correlated more closely with leucocyte infiltration (Yang et al., 2000) (Figure 1-8). In the latter study, all the cell loss could be accounted for if clearance time of apoptotic cells was 3.5h. In human glomerulonephritis there are many reports of increased MC apoptosis in both progressive and resolving diseases (Harrison 1988; Baker et al., 1994; Shimizu et al., 1995; Sugiyama et al., 1997).
It appears therefore that in glomerular disease the myofibroblast-like mesangial cell regulates scar formation and healing in the wounded tissue. Factors that alter that process appear to impinge on resolution or progression of the disease process.

1.5 MACROPHAGE EFFECTORS AND RESIDENT CELL APOPTOSIS IN TISSUE REMODELLING

Tissue remodelling is the ability of a tissue to change co-ordinately form or shape. This is obvious during embryogenesis when many tissues change form dramatically. During this remodelling there are co-ordinated waves of apoptosis and cell division as well as differentiation of cells (Johnson 1994; Sorensen et al., 1998; Gassler et al., 1998; Araki et al., 1999). Developmental remodelling occurs no more obviously than during development of digits from limb buds. In this instance as the digits develop co-ordinated apoptosis results in the progressive loss of inter-digital webbing. In fact, involution of tissue is characterised by apoptosis. For example, involution of ductules in the breast after lactation (Kerr et al., 1972; Maclaren et al., 2001). In seminal work during the mid-1990s, Lang and colleagues discovered that during remodelling of the neonatal eye in rodents, a leash of vessels in the vitreous humor disappears due to apoptosis of endothelial cells and the other cells of the vascular structure. Curiously, for this phenomenon to occur, macrophages were required. It seemed that the Mφ dock onto the vascular cells and induce cell death prior to
phagocytosis. This observation was reproduced using a variety of interventions (Lang & Bishop 1993; Lang 1997; DiezRoux et al., 1997). In animals without Mφ, the deletion of the vascular structures can be induced by reconstituting the eye with Mφ.

Tissue remodelling also occurs in inflammation. The inflammatory process, characterised by infiltrating leucocytes and increased capillary permeability, is also characterised by proliferation of local resident cells, which lay down Collagen I rich extracellular matrix. One such cell type which predominates in this setting is the myofibroblast (see above). Considering skin wounding, during the healing process, the transition from granulation tissue (containing fibroblasts, endothelial cells, vascular structures and pericytes) to scar is mediated by apoptosis of wound (myo)fibroblasts and other cell types (Desmouliere et al., 1995). This process appears to be co-ordinated. Histological examination of healing wounds reveals that up to 10% of all myofibroblasts can be apoptotic in tissue sections. Curiously, in these studies it was noted that Mφ containing phagolysosomes were always seen in close apposition to the apoptotic cells, indicating Mφ to be the major cell in clearance of apoptotic cells. Indeed, observations of resolving inflammation in the lung, liver and kidney indicate that myofibroblast apoptosis is a characteristic of the resolution phase (Polunovsky et al., 1993; Iredale et al., 1998; Baker et al., 1994). This wave of apoptosis results in deletion of excessive cells that are producing abnormal (Collagen I-containing) matrix that ultimately if not resorbed will become scar tissue. Thus deletion of these myofibroblast cells is crucial to the restoration of normal tissue architecture. There is evidence in models of inflammation that disturbances in the balance of cell loss results in persistent inflammation or inappropriate scarring which may ultimately lead to loss of function. For example in skin wounding the hypertrophic and keloid scars show a persistence of granulation tissue which remains hypercellular i.e. there appears to be a failure of apoptosis of myofibroblasts (Rockwell et al., 1994; Erlich et al., 1989). In studies where proliferation is promoted and apoptosis decreased (Shankland 2000; Guervara et al., 1999) the disease process and outcome is worse. Arguably in leukocytoclastic vasculitis there is an excess of apoptotic neutrophils that are not cleared from the tissue. Perhaps this is the cause of persistent inflammation. Recently, in models of multisystem inflammation as seen in Systemic Lupus Erythematosus there is evidence of failed clearance of apoptotic cells triggering further inflammation (Taylor et al., 2000).
Chapter 1 Introduction

Figure 1-9: Time course of total cell number per glomerular cross section compared with macrophage number and apoptotic cells seen per 50 glomerular cross sections in resolving Thy 1.1 mesangioproliferative glomerulonephritis. Note presence of macrophages mirrors appearance of apoptotic mesangial cells. Data derived from experiments by Baker and colleagues (Baker et al., 1994).

At inflamed sites there are abundant macrophages, lymphocytes and neutrophils. Mφ are described in apposition to apoptotic resident cells, often containing phagocytosed apoptotic cells (Desmouliere et al., 1997; Grigg et al., 1991). However, a link between the presence of Mφ and mechanisms of induction of apoptosis of resident cells during inflammatory remodelling has not been made. The work of Baker and others (Baker et al., 1994) showed a striking correlation between the presence of Mφ and apoptosis of mesangial cells (glomerular myofibroblasts) during a self resolving (healing) model of inflammation in the kidney (Figure 1-9). Further, it has long been reported that Mφ possess the 'machinery' to limit tumour growth by induction of apoptosis of tumour cells utilising a variety of mechanisms. Albina's group reported roles for nitric oxide, and TNFα in deletion of tumour cells in co-culture in vitro, and a role for macrophages in tumour immune defence has been shown in various models of tumour growth and regression (Hagari et al., 1995).

It is conceptually plausible that since Mφ are able to regulate tumour growth and regulate deletion of unwanted structures during development. This function might be re-iterated during the inflammatory process; that is Mφ actually induce apoptosis of resident cells (in particular
myofibroblasts).

An extrapolation of this idea would be that too many Mφ or rather too many activated 'killer' Mφ at an inflammatory site would disrupt the balance between the tendency of myofibroblasts to proliferate, deposit matrix or to be induced to die by apoptosis. Thus if it were true that Mφ in inflammation were regulators of resident populations the outcome of tissue repair would lie with processes regulating Mφ activation (and deactivation) and localisation.

Further evidence in the kidney for a role of Mφ in regulating resident cell populations comes from studies contemporaneous with this Thesis. Firstly, Hughes and Johnson (Hughes et al., 1999), in a model of ureteric obstruction confirmed an accumulation of cortical Mφ adjacent to proliferating interstitial myofibroblasts and proliferating tubular cells, as reported by Diamond (Diamond et al., 1995). Using gld/gld (FasL deficient) mice, they showed that Mφ may play a role in inducing distal tubular cell apoptosis. Also, in a model of anti glomerular basement membrane glomerulonephritis in mice (with concomitant interstitial nephritis), tubular epithelial cell apoptosis occurred with Mφ in close apposition. However, in MCP-1 -/- mice (monocyte chemoattractant protein-1), fewer interstitial Mφ were recruited and a corresponding reduction in tubular cell apoptosis was seen, suggesting Mφ were responsible for tubular cell apoptosis (Tesch et al., 1999). In vitro they demonstrated that supernatants from activated Mφ resulted in apoptosis of proliferating tubular cells.

1.6 MACROPHAGES IN GLOMERULAR INFLAMMATION

So far, I have referred to the presence of Mφ in the Thy1.1 model of resolving nephritis in the rat, but a striking feature of glomerular inflammation is the preponderance of Mφ in many types of disease (Figure 1-10). There is an equally striking paucity of lymphocytes to be found in the glomerulus, compared with the tubulo-interstitial compartment of the kidney and many other sites of inflammation. A well-established model of nephritis is the nephrotoxic nephritis model. This disease is initiated by planted antigen in the form of foreign antibody against glomerular basement membrane. However, priming animals with the foreign immunoglobulin results in a host antibody and cell-mediated response to the anti-GBM antibody and augmentation of glomerular disease, once the anti-GBM is planted in the glomerulus. In many ways this model mirrors events in human diseases such as anti-GBM disease, and other forms of rapidly progressive glomerulonephritis. In rabbit, sheep rat and mouse the model is characterised by abundant intra-gglomerular, mesangial-
infiltrating Mφ (Holdsworth et al., 1985; Tesch et al., 1999; Erwig et al., 2000). Interestingly, rapidly progressive GN in humans is also characterised by infiltration with Mφ (Nolasco et al., 1987; Cockwell et al., 1998; Cunningham et al., 1999). Indeed, in crescentic GN in humans and animals, intraglomerular Mφ can contribute 50% of all cells. In other disease models such as active Heymann nephritis, a model of human membranous GN, there are also abundant Mφ (Cattell et al., 1991). It seems that many forms of inflammation in the glomerulus are characterised by predominantly Mφ infiltration. Interestingly, Tipping irradiated rabbits with the telescoped form of nephrotoxic nephritis and was able to reduce Mφ infiltration, TNF-α production and ameliorate disease, implicating Mφ as being important in disease progression (Tipping et al., 1991). Similar experiments in the active Heymann nephritis did not ameliorate disease. These bone marrow depletion experiments are open to interpretation due to the crude techniques employed in Mφ depletion, but they do point to Mφ as key players in glomerular disease.

Figure 1-10: Photomicrograph (x1000) of an acutely inflamed glomerulus showing ED-1 positive Mφ (black) in immune complex glomerulonephritis at 4h after onset of disease (left hand panel). Telescoped rat nephrotoxic nephritis at day 7 stained for the macrophage marker ED-1 (brown stain) counterstained with haematoxylin. Intense mesangial infiltration of macrophages can be seen in both diseases (provided by J. Hughes and G.L. Thomas).

1.7 DIFFERENT PATTERNS OF GLOMERULAR INFLAMMATION

From studies of the inflammatory response, two distinct patterns of inflammation emerge. Firstly there is a rapid inflammatory response to a local insult such as skin wounding or mesangiolysis in the kidney, or possibly to immune complex deposition in the mesangium (as characterised by post infectious glomerulonephritis in humans. This inflammatory response is innate in that the immune system was already poised to rapidly respond in this way and is characterised by initial neutrophil influx (within minutes), which is followed some hours later by Mφ influx. It is undoubtedly true that during localisation to the site of inflammation neutrophils are activated and undergo a
respiratory burst (Johnson et al., 1987; Johnson et al., 1988; Rehan et al., 1984). Studies would suggest that inhibition of this burst prevents local tissue injury (Rehan et al; 1984; Mosely et al., 1999). In diseases such as post-infectious nephritis, the inflamed mesangium has similar numbers of Mφ and neutrophils. Studies by Cattell (Cattell et al., 1993), Rees (Rees, personal communication), and Johnson (Johnson et al., 1995) on Mφ in the Thy1.1 model of GN indicate that the macrophages are activated, that they produce nitric oxide abundantly and that a proportion are positive for staining for myeloperoxidase indicating that they undergo a respiratory burst. Further, the heterologous (non-telescoped) nephrotoxic nephritis model, which usually resolves spontaneously, again, has neutrophils and Mφ co-existing in the glomerulus. Detailed studies (Waddington et al., 1996) indicate that Mφ and neutrophils in this setting are activated, since both produce nitric oxide. Many diseases of the glomerulus feature immune complexes in the glomerular basement membrane and/or mesangium. It is evident from the Con A and anti-GBM models of nephritis where immune complexes bind to the glomerular basement membrane and fix complement, that the inflammatory response is potently activated and typified by a neutrophil/macrophage inflammatory influx with renal damage. This inflammation is dependent on FcγR and complement receptor (CR1, CR3) signalling (Couser et al., 1995; Hughes et al., 1999; Wakayama et al., 2000; Gomez-Guerrero et al., 2000; Launay et al., 2000).

A yeast cell wall extract, zymosan, is a potent activator of immune cells cultured in vitro and is believed to act through ligation of mannose receptors. Macrophages secrete complement factors in vitro and rapidly opsonize the particles enabling a CR3 dependent phagocytic pathway subsequently (Bodmer & Dean 1983; Bodmer & Dean 1983a; Ezekowitz et al., 1984; Ezekowitz et al., 1985). However, when zymosan is opsonised with either IgG or serum, it binds immunoglobulins and complement forming immune complexes on the cell surface. Its activation of Mφ upon phagocytosis has been shown to be dependent on Fcγ polymericisation, CR1 and CR3 ligation (Klebanoff et al., 1985; Porteu et al., 1986; Gresham et al., 1988; Klegeris & McGeer 1994). This opsonised particle can therefore be used as a model for immune complex activation.

In other models of nephritis there appears to be a switch in the inflammatory response, i.e. a different pattern of inflammation. This is demonstrated no better than in the telescoped model of nephrotoxic nephritis. After several days of injury mediated by the innate immune response, there is a much greater influx of Mφ, a notable absence of neutrophils and the presence of T-lymphocytes with an activated phenotype suggesting cognate activation (Diaz Gallo et al., 1992; Cunningham et al., 1999). Much evidence exists to suggest that these Mφ are activated under the influence of lymphocytes, rather than local stimuli such as immune complexes hypoxia or other chemokines. In these circumstances there is little evidence of respiratory burst in the Mφ. Early
studies (1980's) on rabbits and more recent work with mouse models of immune complex nephrotoxic nephritis have shown unequivocally that T cell transfer experiments could recapitulate the Mφ-rich glomerular disease purely by adoptive transfer (Chandra 1988). This work and the idea of T-cell dependent Mφ activation has more recently been supported by experiments using animals knocked-out for different T cell sub-sets (Rosenkranz et al., 2000).

1.8 DEATH RECEPTORS AND SIGNALLING PATHWAYS IN GLOMERULAR INFLAMMATION

Fas and its natural ligand were found to be important regulators of lymphocyte development since naturally occurring mutations of these genes in mice (and humans) result in a syndrome comprising striking similarities to Systemic Lupus Erythematosis (SLE). These animals and humans have lymphadenopathy and a preponderance of CD4-ve, CD8-ve lymphocytes that are believed to be deleted during development in normal animals. This observation lead to the demonstration that ligation and activation of the Fas receptor was indeed important in lymphocyte deletion. These animals have anti dsDNA antibodies, a hallmark of SLE and many develop glomerulonephritis with characteristic immune complex deposition in the basement membrane.

More widespread interest in Fas as a molecule signalling cell death came when it was realised that agonistic anti-Fas antibodies could induce rapid apoptosis in many cells in culture (particularly tumour cells). Intra-peritoneal administration of moderate doses of this antibody resulted in massive hepatocyte apoptosis and secondary necrosis, and ultimately death of the animal (Ogasawara et al., 1993).

Spanish researchers showed weak induction of apoptosis of cultured human MC that had been primed with IFN-γ, upon application of large doses of agonistic antibody (Gonzalez-Cuadrado et al., 1996). They supported this work with in vivo induction of massive mesangiolysis of glomeruli after administration of agonistic anti-Fas antibodies (Gonzalez-Cuadrado et al., 1997). Whilst this renal pathology was not reported in the earlier administrations of antibody (hepatic cell death only), this study implicated Fas/FasL interactions in glomerular injury or glomerular remodelling; it was possible that glomerular scarring in the Fas/FasL deficient mice was due to failed (or excessive) deletion of mesangial cells. Importantly, it was noted that whilst the T-lymphocyte had been described as the archetypal bearer of FasL, Mφ also expressed cell surface FasL. Indeed evidence amassed for a potential role for Mφ-FasL in induction of apoptosis of other cell types, including neutrophils, lymphocytes and pancreatic islets of Langerhans β cells (Badley 1997;
Brown & Savill 1999; Kiener et al., 1997; Signore et al., 1998). These observations could lead one to speculate about the role of MΦ-FasL in regulating mesangial cells in vivo.

The role of FasL became more complicated following observations in vivo and in vitro. Firstly, when FasL was injected into, or over-expressed in tissues in animals, rather that induce local cell death, the area became infiltrated by neutrophils, suggesting that soluble FasL was acting as a chemokine (Ottonello et al., 1999; O'Connell 2000). Molecular biological studies deduced that in addition to the caspase-dependent apoptotic signalling pathway, other signalling pathways existed (Cifone et al., 1995; Um et al., 1996; Yin et al., 1999). Furthermore, there appeared to be a multitude of ‘adaptor proteins’ able to link into the death inducing signalling complex (DISC) (Figure 1-11), and modulate it or regulate Fas directly. The biological implication from these studies was that regulation of apoptosis by Fas was complicated. It did not follow that activation of Fas necessarily resulted in death of the cell. Indeed, the in vivo data have confirmed that suspicion, and there are studies indicating other cellular consequences upon Fas activation (trimerisation) including proliferation (Alderson et al., 1994). The role of Fas in the kidney has become more controversial since tubular cell biologist have demonstrated both Fas and FasL on tubular cells in vitro and implicated these molecules in cell fratricide though little data exists to confirm this hypothesis in vitro or in vivo (Schelling et al., 1998; Koide et al., 1999). However, the idea that only inflammatory cells exhibit FasL has had to be re-addressed in the light of many cell types including endothelia being shown to express membrane bound FasL.

Tumour necrosis factor receptor 1 (TNFR1), the archetypal death receptor, upon trimerization activates pro-inflammatory genes by signalling through the (nuclear factor kappa B) NFkB pathway. Paradoxically the same receptor can induce cell death upon signalling through the caspase 8 pathway. This receptor, like Fas, also seemed to be regulated by many adapter proteins (Baker & Reddy 1996; Chan & Lenardo 2000), including concomitant intracellular signalling through TNFR2 trimerization (which lacks a death domain). For many cell types, studies of activation of the NFkB signalling pathway upon TNFR1 trimerization protected the cell from undergoing apoptosis (Figure 1-4). This observation was made apparent in studies where translocation of NFkB species to the nucleus (where they act) was blocked using IkB (inhibitor of κB proteins) over-expression or dominant negative transfection of cells. In these cells, trimerization of TNFR1 lead to rapid apoptosis in cells that were otherwise resistant (Sugiyama et al., 1999). Thus, regulation of the NFkB signalling pathway is implicated in modulation of susceptibility to apoptosis.
Proteolysis of death substrates: Structural proteins, kinases, DNA cycle repair, CAD, transcription factors

Figure 1-11: Diagram indicating different factors and pathways responsible for preventing apoptosis following ligation of death receptors. Note, plasma membrane density, plasma membrane phosphatases such as FAP-1, and the proportion of splice variants of FADD or TRADD regulate the ability of the death receptor to recruit caspase 8. Cleavage of the latter is blocked by inhibitors such as FLIP family and IAP family members. IAPs also inhibit cleavage of other caspases. Importantly regulation of the mitochondrial amplification step by survival factors such as BCL-2 plays a key role. Finally pathways such as NFκB pathway promote survival in the presence of pro-apoptotic signalling.

In addition to NFκB, TNFR1 activates another nuclear factor Interferon Regulatory Factor 1 (IRF-1). This factor, originally described as one of the genes up-regulated by interferons, is also modulated by TNFR1 ligation. Interferon gamma (IFN-γ) and TNF-α synergistically activate this protein in a variety of cell types, where it appears essential in the pro-inflammatory response (eg iNOS induction requires IRF-1) (Martin et al., 1994; Kamijo et al., 1994). Importantly, IRF-1 induction is NFκB dependent (Ohmori et al., 1997; Pine 1997). Studies both in vivo and in vitro, suggest this factor has a role in cell cycle arrest and possibly apoptosis in a similar fashion to p53 (Burke et al., 1999; Kano et al., 1999; Kirchoff et al., 1999). However, its precise relationship to the caspase cascade is unclear. IRF-1 knockout mice show reduced CD8 T cell populations, yet transgenic over-expression of IRF-1 in B lymphocytes leads to B cell deletion (Yamada et al., 1991). Indeed, ex vivo studies of activated splenocytes and thymocytes implicated IRF-1 to be a key player in induction of apoptosis of splenocytes after exposure to agents that induce DNA
damage (γ irradiation and chemotherapeutic agents) (Tamura et al., 1995). Thus IRF-1 which is in part regulated by TNFR1 signalling through NFκB is responsible for controlling DNA repair (Prost et al., 1998) and apoptosis in cells following noxious stimuli such as irradiation, in some cell types. It appears that both in vitro and in vivo, the synergistic effect of IFN-γ and TNF-α in bringing about apoptosis is being recognised in tissue inflammation (Kirchhoff et al., 1993; Burke et al., 1999; Kano et al., 1999; Ohta et al., 2000; Suk et al., 2001). One can speculate that IRF-1 might be the common denominator in the intracellular signalling events required to bring about cell death.

An ever increasing family of death receptors is developing. Macrophages possess on their cell surface ligands for many of these receptors. Recently work from two groups (Browning et al., 1996; Zhai et al., 1998) on the role lymphotoxin β receptor and another death receptor HVEM (herpes virus entry mediator; its sole function when it was originally discovered) in tumour cell killing by apoptosis suggested a possible role for macrophages in such killing since they express on their cell surface the ligands LIGHT (homology with lymphotoxin) (Zhai et al., 1998), and lymphotoxin LTα1β2. These trimeric ligands can bind to trimeric death receptors, which then either activate the NFκB pathway or alternatively induce apoptosis. LT-α1β2 is an exclusively membrane bound ligand is trimeric and binds to the LTβR. Further, they produce a membrane bound and soluble ligand called LIGHT which activates the LTβR. The receptor-ligand interactions are more complicated since LIGHT can ligate and activate HVEM. The cartoon shown (Figure 1-12) illustrates the death receptors that might potentially be important in macrophage mesangial cell interactions.

Interestingly the work on tumour cells indicated that IFN-γ was important in mediating the switch in LTβR and HVEM receptor function from pro-inflammatory to death inducing (Zhai et al., 1998; Browning et al., 1996). Indeed there is evidence that IFN-γ may play a general role in switching cells to susceptibility to apoptosis from many death ligands and other stimuli (Ossina et al., 1997).
Chapter 1 Introduction

1.9 OXYGEN RADICALS IN CELL DEATH

Much work has focussed on bystander damage by oxygen radicals in the inflammatory response, particularly in the context of neutrophil rich acute inflammation such as pneumonia, and vasculitis. There is good evidence that re-perfusion injury to organs is largely mediated by reactive oxygen intermediate (ROI) release. Indeed studies of the early events (neutrophil dependent) in different forms of glomerulonephritis have clearly implicated ROI (including hydrogen peroxide) in the early endothelial damage that is seen. Further work on the role of neutrophils as disease-mediators in vasculitis have again implicated neutrophil-derived ROI in local small vessel damage (Harper et al., 2001).

Relatively little attention has been paid to oxygen radicals as a potential disease mediator when released from MΦ. Albina’s group suggested that wound MΦ in rats have only a vestigial respiratory burst (Nessel et al., 1999). However, Cook and co-workers (Cook et al., 1989), and
Holdsworth’s group (Boyce et al., 1986) indicated that Mφ in rabbit models of nephrotoxic nephritis appeared to express key enzymes of the respiratory burst. Johnson, confirmed similar findings in Mφ present in inflamed glomeruli of Thy1.1 nephritis (Johnson et al., 1995). Cultured human MC are susceptible to apoptosis in the face of exogenous hydrogen peroxide (Sugiyama et al., 1996), though it is widely accepted that high levels of peroxide can induce necrosis in cells rather than apoptosis.

There is another, more subtle, role of the oxidative burst. Whilst some cell-types release large amounts of toxic radicals that induce peroxidation of lipids and proteins, in other cell-types, ligation of death receptors leads to a swift low level oxidative burst. This phenomenon has received relatively little attention compared with the caspase cascade. However, in some cells, inhibition of this respiratory burst with reducing agents such as glutathione effectively blocks TNFR1 and Fas mediated apoptosis (Um et al., 1996; Spanaus et al., 1998; Sidoti-de Fraisse et al., 1998). In the latter study of TNFR1-mediated apoptosis of HELA (uterine malignant epithelial) cells, inhibition of the oxidative burst after TNFR1 ligation prevented about 50% of induction of apoptosis of the cell population. Application of ZVAD (peptide), the pan-caspase inhibitor, blocked the other 50%. It has been postulated that the oxidative burst merely reflects cytochrome C release from mitochondrial stores. However, if this were simply a side effect of cyt. C release, inhibition by reducing agents would not be expected to block the apoptotic process. Thus, oxidative burst has a potential role in induction of bystander leucocyte-mediated apoptosis and as an intracellular regulator of death receptor signalling.

1.10 NITRIC OXIDE IN CELL DEATH

Nitric oxide is generated by nitric oxide synthases. There are three forms: nNOS (NOS-1) is constitutively present in neuronal tissue and its low level activity is dependent on intracellular calcium fluxes. iNOS (NOS-2) is inducible in response to a variety of pro-inflammatory signals, and was first described in macrophages. It is rapidly induced and when present usually results in high concentrations of NO generation. It avidly binds calcium, therefore functions independently of intracellular calcium fluxes (Figure 1-13). eNOS (NOS-3) is found in the endothelium constitutively and its generation of low levels of NO is again calcium dependent. All three enzymes share homology with cytochrome P450 family since one subunit contains binding sites for NADPH, FAD and FMN, giving them a functional electron transport chain as seen in mitochondria. The other subunit contains a haem-group and requires calcium binding for stabilisation. Tetrahydrobiopterin is also required to stabilise the haem-moeyity (Figure 1-13).
NO production is a stress response and can lead to either tissue injury because of its radical chemistry, or be cyto-protective, protecting cells from damage by destroying pathogenic microorganisms first (e.g. neutrophils and macrophages). Nitric oxide and particularly its superoxide derivative peroxynitrite have been reported to cause DNA damage in bacteria for example in the context of Helicobacter pylori infection (Nagata et al., 1998).

Figure 1.13: Cartoon of iNOS showing the binding sites for the cofactors NADPH, FAD, FMN. The electron transport chain is shown. The haem-group is denoted by Fe$^{2+}$ and both BH4 and calcium are shown binding to this part of the molecule. L-arginine plus molecular oxygen are required. The intermediate, hydroxy-arginine is shown prior to generation of L-citrulline plus nitric oxide.

One can consider NO generation as part of the defence arsenal, since, in addition, it is required in host defence to certain tumours such as gastric epithelia, breast tissue, brain, to suppress growth (Edwards et al., 1996; Gal et al., 1997; Hajri et al., 1998; Nishikawa et al., 1998). Such a defence mechanism, however, has limitations since it is also linked to septic shock. Bacterial endotoxins
Chapter 1 Introduction

induce the iNOS, which in turn produces high levels of NO, damaging pathogenic DNA and inhibiting respiration (inhibits metabolic energy production needed for cell division). The free radicals, however, do not discriminate pathogenic DNA from host DNA and indiscriminate induction of iNOS in the body therefore induces cell and tissue damage, sometimes leading to a fatal development (septic shock) in the course of bacterial infections (Parratt 1997).

Nitric oxide is a free radical molecule and one of its major effects is the activation of cytoplasmic, soluble guanyl cyclase (Callsen et al., 1998). This enzyme catalyses the conversion of GTP to cyclic GMP. Cyclic GMP is a signalling molecule (similar to cAMP) by virtue of activating protein kinases. Nitric oxide binds to the haem group of the cyclase. Other protein targets are metallo-enzymes, where NO binds to Fe-S clusters. Aconitase is inactivated by NO, as is complex IV, the cytochrome oxidase in the inner membrane of mitochondria. Thus NO has an inhibitory effect on oxidative phosphorylation by blocking the electron transport chain and controlling the levels of citrate in the Krebs cycle essentially blocking the oxidative degradation of acetyl-CoA (Drapier 1997).

NO in lower concentrations is a short-lived chemical transmitter, which is freely diffusible across membranes. The molecule possesses a small dipole moment because of the similar electronegativity of oxygen and nitrogen, making it essentially hydrophobic. Its reactivity is due to the unpaired electron in the outer valence orbital of its oxygen constituent. NO is almost unreactive compared to other oxygen radicals. Indeed, NO decays within seconds after its synthesis if left unbound in solution because it reacts with either molecular oxygen or superoxide.

NO strongly interacts with molecular oxygen to form dinitrotetroxide (N₂O₄), or with superoxide O₂⁻ to form peroxynitrite (ONOO⁻). NO also binds to sulphydryl groups (SH) and unsaturated fatty acids. The reaction with superoxide can be diminished by superoxide dismutase (SOD) which removes O₂⁻ to form hydrogen peroxide (H₂O₂). NO can be 'stored' by covalent interaction to glutathione to form S-nitroso-glutathione. Both H₂O₂ and S-nitroso glutathione can have a stimulatory effect on guanine cyclase. Superoxide dismutase thereby prevents the loss of nitric oxide to peroxynitrite forming hydrogen peroxide instead and increasing the cyclase stimulatory capacity of the cell.

NO can be regenerated from ONOO⁻ by the enzyme nitrate reductase. The enzyme exists in two isoforms, a mitochondrial type and an endoplasmic reticulum resident protein. Both receive their electrons needed for nitrite reduction to NO from either NADH or NADPH, and interact with flavoproteins (FAD prosthetic groups) and cytochromes (cyt. C oxidase in mitochondrial membrane; cytochrome P450 in ER membrane).
Nitrite reductase therefore not only prolongs the effective 'life time' of NO, but also reduces the concentration of highly reactive, secondary metabolites. Peroxynitrite, hydrogen peroxide, and dinitrotetroxide all have been linked to apoptotic cell death through protein nitration and DNA damage. The latter is a consequence of DNA stand breakage and guanine nitration. Inhibitors of nitric oxide synthase and antioxidants are known to have cell-protective properties because they limit the formation of highly reactive nitrogen containing radicals (Sandau et al., 1997).

1.11 CELL CYCLE AND APOPTOSIS

Shankland has drawn attention to the role of cell cycle and cell cycle regulatory proteins in disease regulation in the kidney (Shankland 2000). It was notable in the earlier studies of Thy 1.1 nephritis (Baker et al., 1994) that the presence of apoptotic bodies in glomerular cross sections also correlated with the presence of mitotic cells in the same glomerulus, i.e. the more proliferation, the more apoptosis. This might suggest that cells that are proliferating are more susceptible to apoptosis. This hypothesis appears to be borne out in several studies of cell cycle. Indeed, when p53 is induced in cells it appears to induce cell cycle arrest at the G1/S border most frequently, though there are reports of cell-cycle arrest at the G2/M checkpoint (Allday et al., 1995; Aloni-Grinstein et al., 1995; Enoch et al., 1995; O'Connor et al., 1997; Deptala et al., 1999). It is thought that these checkpoints allow time for DNA repair, to prevent the possibility of mutated daughter cells. If repair is unsuccessful, apoptosis ensues. Thus, cellular decisions about cycle also influence apoptosis. Whilst it is evident that cells do not need to be cycling for apoptosis to be induced (Ogasawara et al., 1993) it is plausible that cycling cells are more susceptible to apoptosis than cells that are not cycling. Indeed, this appears to be the case. (Duttaroy et al., 1997; Kato et al., 1997). IRF-1 induction also appears to induce cell cycle arrest (Tanaka et al., 1996; Kano et al., 1999; Lieberman & Hoffman 1998; Tada et al., 1998; Prost et al., 1998). Following arrest of cell cycle, again apoptosis may ensue. However, there has been little attention to this nuclear factor as a regulator of cell cycle and apoptosis. Understanding some of the molecular processes controlling the cell cycle is providing links with factors controlling apoptosis. For example, at the G2/M point in the cycle, one of the anti-apoptotic, survival proteins Bcl-2 becomes phosphorylated. This protein is involved in pore formation in the mitochondrial outer membrane. This step results in deactivation. The cell no longer has the protective function of Bcl-2 during any cellular insults or pro-apoptotic stimuli. The stress induced kinase pathway involving ASK1 and JNK1 was found to be active and responsible for BCL-2 phosphorylation in lymphocytes. Following successful mitosis Bcl-2 is re-activated by de-phosphorylation (Yamamoto et al., 2000).
1.12 DEACTIVATION OF MACROPHAGES

Macrophages can express many cytokines and toxic radicals that can inflict damage on neighbouring tissue or cells. These cytokines and radicals can induce apoptosis of neighbouring cells in models of tumour growth. However, not all Mφ have the killer phenotype. Indeed many cells of the Mφ lineage do not exhibit pro-inflammatory cytokines, such as Kupffer cells and oligodendrocytes. Moreover, it appears that even in an inflammatory tissue, macrophages may have more than one phenotype. Macrophages can be characterised by TGFβ production, hyaluronidase or class II MHC expression. It is likely that Mφ expressing high levels of these cytokines or matrix proteoglycans might be involved in tissue repair. Mφ expressing high levels of MHC molecules may be involved in antigen presentation. In immunohistochemical studies of glomerulonephritis, it is apparent that only a proportion of the Mφ in the inflamed glomerulus express in iNOS in human IgA nephritis (Furusu et al., 1998), in keeping with these hypotheses. Why however, macrophages exposed to a similar inflammatory milieu, should have dissimilar phenotypes is unclear since it is generally accepted that macrophages in inflammatory sites derived from blood borne monocytes which differentiate following initial cytokine exposure (Riches 1995; Fournier et al., 1995; Erwig et al., 1998). It is plausible that this reflects slightly different chemokine and cytokine exposure. However, it might suggest that with time, macrophage phenotype in the inflammatory milieu is changing. Kluth and colleagues (Kluth & Rees 1999), have demonstrated that the disease progression of a rat model of telescoped nephrotoxic nephritis can be modulated by infusing gene targeted macrophages into the inflamed kidneys. For example, macrophages over-expressing TGF-β that enter the mesangium, result in less protein leakage from the glomeruli, than normal monocyte derived macrophages entering the mesangium in vivo. Thus, in this instance, Mφ over-expression of TGF-β is beneficial. There is good evidence that the cytokine TGF-β in some diseases can result in excessive scarring of remodelling tissues, whereas in others it is important to normal healing and tissue resolution (Kitamura et al., 1995; Sime et al., 1997). It may be that the balance of activated killer Mφ and the healing, matrix-generating Mφ is important in successful remodelling of inflamed tissues.

Reiter (Reiter et al., 1999) made the interesting observation that activated mouse bone marrow-derived macrophages, following ingestion of apoptotic tumour cells were less able to kill other tumour cells. Voll had previously (Voll et al., 1997) suggested that human Mφ produce less TNF-α and more IL-10 following ingestion of apoptotic lymphocytes. It is possible therefore that activated macrophages can alter their phenotype by phagocytosis of apoptotic cells. This work has been more thoroughly investigated by Fadok and colleagues (Fadok et al., 1998; McDonald et al., 1999).
They implicate TGF-β as one candidate autocrine signal to deactivate the Mφ. It is released by Mφ that ingest apoptotic cells and is able to down-regulate TNF-α release from those Mφ.

1.13 IN VITRO CELL CULTURES – A MODEL FOR IN VIVO CELLULAR INTERACTIONS?

In order to tease out precise cellular interactions, their regulation and cell signalling pathways, it is necessary to turn to cell cultures, since it has become clear that cells interact with many cell types in vivo, and cytokines act at many different levels of the inflammatory process. For example, regulation of local blood flow might obscure an important biological event if a cellular interaction of interest also modulates vascular wall tone. To dissect out cellular mechanisms in vitro assays offer a simple method to investigate specific interactions. Mesangial cells grow readily in culture in a de-differentiated, myofibroblast phenotype as do many specialised mesenchymal cells such as vascular smooth muscle cells from blood vessels, fibroblasts in skin and interstitia, and stellate cells in the liver. Fortunately, in disease mesangial cells adopt this phenotype. The validity of using primary mesangial cells in culture has been reviewed comprehensively by Floege (Floege et al., 1994). For many years Mφ have been obtained for in vitro studies from bone marrow precursors, inflamed peritoneum and blood monocytes. These cells have been well validated, and compared in terms of cell surface receptor profile, cytokine production and in functional studies. Work of Riches amongst others has indicated that Mφ both in vitro and in vivo may be ‘relatively undetermined’ and their local environment induces phenotypic changes that are may be irreversible (Riches 1995; Erwig et al., 1999). It appears that bone marrow Mφ are partially undetermined whereas elicited peritoneal Mφ are different and are more determined. Cytokine exposure can induce in bone marrow cells a phenotype similar to elicited peritoneal cells. However, work from Albina’s group on wound Mφ indicates that they might have a distinct phenotype from elicited peritoneal cells. Thus whilst in vitro Mφ cultures can be induced to mimic in vivo Mφ populations it is clear that the in vivo environment is important to Mφ phenotype and function. It is clear that Mφ populations exist at the inflamed site and that in vitro observations of Mφ function require supplementation with carefully planned in vivo or ex vivo studies.
1.14 AIMS OF THIS STUDY

- To determine whether macrophages are able to regulate populations of mesangial cells in an in vitro model of glomerular inflammation.

- To determine the phenotype of macrophages that are able to perform such functions.

- To delineate mechanisms employed by both macrophages and mesangial cells to bring about the macrophage-mediated regulation of mesangial cells.

- To investigate factors involved in susceptibility of mesangial cells to macrophage killing.

- To determine whether macrophage killing of mesangial cells is regulated by phagocytosis of killed cells.
Chapter 2

Methods
Chapter 2  Methods

2.1 PURIFICATION OF HUMAN LEUCOCYTES FROM WHOLE BLOOD

2.1.1 Separation of leucocytes from whole blood
Blood was drawn from a peripheral arm vein of a healthy donor, on no medication, using an 18 gauge butterfly needle. 40ml of blood was mixed by gentle inversion with 4ml of sodium citrate (3.8%). Care was taken not to aerate the blood, during collection and to avoid red cell lysis during letting lest platelets or neutrophils should become activated. 160 ml of blood typically yielded 250 x 10⁶ granulocytes which were usually more than 95% neutrophils (PMN) and 150 x 10⁶ PBMC which were often 75% lymphocytes, 25% monocytes.

Aliquots of 44ml of citrated blood were centrifuged at 350g for 20min (no brake) in 50ml tubes (Falcon). Care was taken to avoid an aerosol of blood being generated at this stage. Plasma was gently removed using a transfer pipette, to avoid disturbing the buffy coat. The plasma was used to generate platelet rich plasma derived serum (PRPDS) or platelet poor plasma (PPP) (Chapter 2.1.3). The remaining cells including buffy coat were resuspended, by inversion, in warmed (37°C) normal saline with dextran MW500,000 (6% stock solution made up in normal saline) to give a final concentration of 0.6% if the haematocrit was < 0.55 and 0.72% if the haematocrit was > 0.55. The final volume was 50mls. Dextran induces aggregation of red cells thereby inducing rapid red cell precipitation. The tubes were left without disturbing for 20-30min whilst the red cell aggregates settled. The upper layer of saline was now depleted of red cells and was harvested into fresh 50ml tubes using a transfer pipette. Care was taken not to disturb the sedimented red cells whilst collecting all the supernatant. The supernatant was now leucocyte enriched and was ready for further purification to yield pure cell types. It was pelleted at 350g for 6min (low brake).

2.1.2 Separation of granulocytes from mononuclear cells (PBMC) by Percoll discontinuous gradient
The Percoll discontinuous gradient separates granulocytes from PBMC relying on buoyancy of the different cell types, and further purifies by separation of remaining erythrocytes.

To generate a gradient, Percoll at 4°C and diluted with 10x PBS at 4°C to generate a 90% solution in PBS (not DPBS). After thorough mixing, three solutions were prepared in PBS (expressed as percentages of the 90% solution); 81% (recently modified to 79%), 70% (recently modified to 68%), and 55%. Each solution was thoroughly mixed. To a 15ml polystyrene tube (Falcon) 2.5ml of the 81% solution was added, followed by 2.5ml of the 70% solution which was carefully layered on top, by very
slow, steady pipetting (either transfer pipette or plastic Pasteur pipette). The leucocytes from one 44ml of citrated blood were resuspended in 2.5ml of the 55% Percoll solution. One gradient is sufficient for separation of leucocytes from 2 tubes or 88ml of citrated blood. Very gently (one drop at a time initially), 2.5mls of leucocytes in 55% Percoll were layered on the top of the gradient. When completed, the gradient was centrifuged at 720g for 20min (no brake).

The gradient now contained three layers: at the apex of the tube, a pellet of red cells. In the interface between the 81% and 70% Percoll were the granulocytes and between the 55% and 70% were the PBMC. To collect these, the first 0.5-1.5ml of gradient was discarded. The PBMC layer was aspirated into a clean 50ml tube. The gradient between the two layers was discarded and, using a fresh pipette, the granulocyte layer was collected into a different 50ml tube. Cells were washed x2 in 50ml of PBS, with intervening centrifugation at 220g for 6min (low brake).

2.1.3 Preparation of Platelet rich plasma-derived serum and platelet poor plasma

10ml of citrated plasma was incubated with 200μl of 13% CaCl₂ solution in a glass vial, for 15min at 37°C to generate PRPDS. For PPP (platelet-poor plasma), platelets were pelleted in citrated plasma by centrifugation at 350g for 20min. The supernatant was then decanted and stored.

2.2 GENERATION OF MESANGIAL CELLS

2.2.1 Preparation of human mesangial cells (HMC)

MC were prepared as outgrowths from purified glomeruli. After several passages in culture, (usually three) other cell types such as epithelial, endothelial cells and macrophages were lost leaving pure mesangial cells as assessed by immunofluorescence (Methods 2.12, Methods 2.2.7, Figure 1-7). Of note, the human kidneys utilised were from 50+ year old patients. The glomerular outgrowths often proliferated rapidly in culture but cells divisions became limited often by the tenth passage. It is interesting to note that a group in Germany has a human MC clone from a foetal kidney that is immortalised and has grown for more than 100 passages (personal communication Dr. B. Banas).
2.2.2 Collection of human glomeruli

Human kidney cortex was collected from patients after nephrectomy for renal cell carcinoma (epithelial). Every care was taken to take pieces of cortex from the unaffected area of the kidney. The cortex was transported on ice in Hanks buffer (HBSS w/o).

In a tissue culture hood, the renal capsule was removed and the cortex chopped into small pieces using a scalpel. Three Endocotts sieves were assembled in series, with 150μm sieve on top, followed by a 210μm, followed, at the bottom by a 106μm sieve. The sieve system was placed in a sterile bacterial grade culture dish to collect effluent. Hanks solution (HBSS w/o) was passed through the system to wet the meshes. The kidney cortex was then placed in the top sieve and gently pressed through using the inverted end of a 50ml syringe plunger. Hanks solution was poured into the top sieve to wash through the mashed tissue; the process being repeated until the entire cortex has passed through the first sieve. Repeated flushing with Hanks solution was continued with aspiration of the petri dish until the effluent ran clear.

The glomeruli now rested in the 106μm sieve, and were collected by gently washing the mesh at an angle with Hanks solution, so that the glomeruli collected in the lip of the sieve. Aspiration around the lip of the mesh with the same pipette, where the glomeruli had come to rest ensured complete collection. The glomeruli were then washed by centrifugation at 150g for 2min. x 2.

2.2.3 Growth of human mesangial cells from glomeruli

The purified glomeruli were subjected to a gentle collagenase digest to remove the collagenous capsule. Collagenase was prepared in 0.1mg/ml (Sigma) in Hanks with Ca²⁺ and Mg²⁺. The glomeruli were resuspended in 10mls of the collagenase and incubated for 1-5min at 37°C. They were regularly viewed by microscopy (x100) to look for loss of the glomerular capsule (seen as increased fluffiness of the surface of the glomerulus). Medium containing 10% FCS was added to inactivate the collagenase and the glomeruli were then centrifuged at 150g for 2min. After aspiration of medium, they were resuspended in RPMI medium containing L-glutamine, penicillin, streptomycin (500U/ml) and 16% FCS. The density was 1000 glomeruli/ml. This suspension was placed in T25 or T75 flasks and incubated without disturbance for one week. Additional medium was gently added after 4 days if necessary.

After one-two weeks the glomeruli became adherent to the plastic and cells were seen growing out. Regular replacement with fresh medium ensured proliferation of these cells. Once the plastic was
confluent with cells they were passaged. Medium was aspirated away and the cells washed with PBS. 1x trypsin-EDTA (TE) was added (here 1ml per T75), and incubated at 37°C for 5min. Vigorous agitation was sometimes required to loosen these cells. Floating cells were collected into fresh medium (minimum 10ml) and cells and re-plated after a 1:2 split. Cells were passaged in the same fashion for two further passages.

At this stage the medium was changed to the less nutrient rich DMEM/F12 (containing glutamax) with penicillin/streptomycin and now 10% FCS. This change was made whilst the cells are adherent and growing well.

The proliferative burst of these cells was limited to approximately 10 passages. In addition they were very sensitive to a variety of factors and stopped dividing if conditions were not optimal.

To maintain optimal conditions, the cells were fed with fresh medium every 3-4 days. At passage, cells were split on a 1:2 basis. Often 1:3 splits were not deleterious but this was not uniformly so. Greater splits inevitably resulted in failure of the cells to grow. In addition, if the cells were allowed to become confluent they appeared to stop dividing (contact inhibition). Further passaging of cells would not result in further proliferation. It was imperative therefore that human mesangial cells were closely monitored and treated.

2.2.4 Preparation of rat mesangial cells.

The advantage of using rat mesangial cells over human cells was that, after 3-5 passages, they grew clonally in culture and were much more hardy than human mesangial cells. These clonal cells were not limited by passaging more than 20 times but, for the purpose of these studies all experiments were been performed on cells no greater than 16 passages. Indeed the rat cells were all derived from young animals (8-12wks). The rat cells can grow for more than 100 passages (Personal communication Dr. M Kitamura, Imperial College).

To demonstrate the validity of some of the findings with the clonal cells, polyclonal, early passage, cells were also tested and appeared to corroborate observations using the clonal cells.

The principal clone used in this study was a gift from Dr. M. Kitamura, and its phenotypic characteristics have been verified (Kitamura et al., 1995). This clone was derived from a Sprague-Dawley rat, in a similar fashion to that described below (i.e. from the outgrowths of whole glomeruli).
2.2.5 Purification of glomeruli

The method for the purification of glomeruli from rat kidneys was similar to that for human glomeruli.

Whole rat kidneys were isolated from an 8-12 week Wistar rat. The capsule was removed and the whole kidneys chopped with a scalpel.

The kidneys were then pressed through a sieve system as described for human mesangial cells. However, due to the glomerular size differences the sieves were 106μm, 150μm followed by 53μm. Pure glomeruli were collected from the 53μm mesh after flushing with large volumes of Hanks solution.

2.2.6 Growth of rat mesangial cells from whole glomeruli

Glomeruli from rats did not require collagenase digestion to allow cellular outgrowth. Washed, purified glomeruli were resuspended in RPMI with L-glutamine, penicillin/streptomycin and 16% FCS and cultured in T25 flasks (usually one flask per kidney) in 5mls of medium. Medium was carefully replaced after 4 days, and after one week the glomeruli were adherent with obvious cellular outgrowths. After 3-4 weeks, flasks were near-confluent and passaged as described for HMC. After 3 passages (50% splits), the medium was changed to DMEM/F12 with 10% FCS.

2.2.7 Characterisation of mesangial cells

Glomeruli contain mesangial epithelial and endothelial cells. In addition, they contain resident macrophages and podocytes. There is potential for all of these cells to grow in the in vitro environment, though the conditions used favour the hardier cell-type, the mesangial cell.

For the purposes of these studies, previous investigators have thoroughly characterised the HMC outgrowths (Savill et al., 1992). By the third passage, the cells do not express cytokeratin (epithelial cell marker), nor do they express VWF (endothelial cell marker) or leucocyte common antigen (CD45). The cells however are positive for α-smooth muscle actin, and desmin. For my own satisfaction I characterised one of the cell preparations looking for cytokeratin and α-SMA (Figure 1-7)

Since the rat mesangial cells had not been previously grown in the department, rat cell outgrowths were characterised using anti-α-SMA (Sigma), anti-CD90 (Thy1.1) (Serotec), anti-cytokeratin (Sigma), and anti-ED-1, a macrophage marker (Serotec).
Cells were grown (P4) to 70% confluence. They were washed and fixed with ice-cold methanol for 5 min, before air-drying. Once dry, non-specific binding was blocked using PBS containing 10% new born calf serum (NBCS) with 0.01% NaNO (preservative). After 30 min at room temperature specific antibody or isotype matched negative control was added (diluted in PBS/10%NBCS blocking solution). Overnight incubation at 4°C achieved most favourable results. After, washing x3 with PBS (containing 0.05% Tween 20) and a further washing x3 with PBS alone, the appropriate FITC-conjugated anti-immunoglobulin was applied. After 1h at room temperature, the secondary antibody was removed by washing x 3 with PBS and the well covered with a fluorescent (glycerol based) mountant (Chapter 2.7.1). Characterisation of cells by immunofluorescence can be seen (Figure 2-1).

2.2.8 Passaging of cells

Once established in culture all primary proliferating cells (principally mesangial cells) were grown in a combined medium: Dulbecco’s modified Eagle medium with F12. (DMEM/12) This contains glutamax (a stable replacement for L-glutamine: Life Technologies). To this medium was added Penicillin (500U/ml), Streptomycin (500U/ml), and 10% FBS (Life Technologies). The added ingredients were routinely filtered through a 0.45μm filter. Medium was stored at 4°C when not in use.

Cells were grown routinely in Costar flasks (T25, T75, or T162). They were maintained at 37°C with 5% carbon dioxide atmosphere (Binder). They were routinely screened for Mycoplasma infection by Hoechst 33342 fluorescent microscopy.

The exact timing of passage of cells was specific to the cell type cultured. Typically, however cells were split when they were 80-95% confluent. In a pre-cleaned tissue culture hood (Neutrocon followed by 70% ethanol with water), medium was aspirated using an autoclaved glass pasteur pipette. The cell monolayer was washed with 2-10mls of sterile, autoclaved PBS to remove calcium and serum. An aliquot of sterile 1x trypsin-EDTA (trypsin 50 units/ml and EDTA 5mM) solution (0.5ml for T25, 1ml for T75, 2ml for T162) was placed into the flask using a transfer pipette. Cells were covered with the TE and replaced in the 37°C incubator for 2-5 min, until cells were evenly detached. Following a gentle tap of the flask to loosen all cells, an appropriate volume of fresh medium containing 10% FCS was added and the cells suspended by gentle pipetting with a transfer pipette. The resuspension volume was ≥ 10x the volume of TE applied to ensure full inactivation of trypsin. An aliquot of the suspension was placed into a new flask containing fresh medium (typically mesangial cells were split 1:2 or 1:3 apart from clonal immortalised rat mesangial cells which were split 1:10). The flask was immediately placed
in the 37°C incubator in order that the cells could adhere. All waste medium was treated with bleach for >24h prior to disposal. Used flasks and transfer pipettes were autoclaved.

Figure 2-1: Fluorescent photomicrograph (X 400) of rat mesangial cells P4 fixed in wells with ice-cold methanol visualised by phase contrast microscopy (A), or labelled with (B) anti-α-smooth muscle antibody or (C) anti-Thy1.1 antibody, followed by secondary FITC-conjugated anti IgG, IgG2a and IgG1 control antibody gave no specific binding (not shown). Cells were prepared and labelled according to Chapter 2.12
2.2.9 Preparation of steroid (oestrogen) depleted FCS

Mesangial cells transfected with the interferon regulatory factor 1 producing vector were grown in medium containing oestrogen-depleted FCS. This was because the IRF-1 protein was fused to part of the oestrogen receptor that is responsible for translocation to the nucleus. In the presence of oestrogens, transfected cells would translocated the IRF1 to the nucleus where it is active. To control this cells were grown in medium containing depleted FCS. This was achieved as follows (ref. Green & Leake Steroid Hormones- a practical approach. Dextran coated charcoal was prepared by incubating for 16h at 4°C, 0.25% Charcoal (Norit A-Sigma) with 0.0025% dextran T-70 (Pharmacia) in a buffer of 0.25M sucrose, 1.5mM MgCl₂, 10mM Hepes pH 7.4.

To an aliquot of FCS and equal volume of the dextran coated charcoal suspension was taken and the charcoal pelleted by centrifugation (500g, 10min). The supernatant was decanted and replaced by an equal volume of FCS and agitated for 12h at 4°C. FCS was then separated by centrifugation, followed by 50µm filtration.

2.3 GENERATION OF MACROPHAGES

2.3.1 Collection of bone marrow from rats.

A 200g Wistar rat (male) was killed by cervical dislocation. The animal’s coat was cleaned with 0.5% chlorhexidine. Using autoclaved surgical instruments, skin was removed from the lower abdomen bilaterally without disturbing the peritoneal cavity. This involved lateral incisions between the skin and first muscle layer. The lower limb muscle was then exposed by cutting the skin medially along the length of each limb. By blunt dissection, skin covering the legs was removed. Next the lower limbs were separated from the animal by cutting through the pelvic muscles and the pelvic bones. Sometimes it was possible to dislocate the femoral head thereby preventing the need for cutting the pelvis. The extracted limbs were immediately placed on ice in a container, immersed in 100% ethanol.

In a pre-cleaned tissue culture hood, on a sterile petri dish, the limbs of the rat were dissected with autoclaved scissors and scalpel to remove all muscle, but leave both femur and tibia intact. The foot was removed above the ankle bones. Each limb was washed with sterile PBS or Hanks solution. 2x 10ml of full DMEM/F12 was transferred to sterile universal containers. Using an 21 gauge needle (green) and 10ml syringe, 10ml of medium was drawn into the syringe. The proximal end of the rat
Chapter 2 Methods

femur and distal end of the tibia were removed using the scalpel. The needle was inserted into the bone marrow cavity and the bones were held in a clean universal container by use of sterile forceps. By applying gentle pressure to the syringe, the marrow cells were flushed out into the container. Due to large numbers of red cells in the marrow, a red cell lysis was typically performed (see Chapter 2.3.9)

Following this, cell number was assessed by microscopical counting using a haemocytometer. One limb typically yielded $200 \times 10^6$ bone marrow cells.

2.3.2 Collection of bone marrow from mice.

A 25g male mouse (several strains used) was killed by cervical dislocation. The whole mouse was placed in a sealed container, on ice, containing 100% ethanol and transported to a sterile pre-cleaned tissue culture hood. The whole mouse was placed in a sterile petri dish and dissected in a similar way to that described for the rat. However, only the femurs were removed. The tibias were cut close to the knee joint and left in situ. Once extracted, the whole femurs were dissected using scissors and a scalpel to remove muscle. The femurs were washed with sterile PBS/Hanks solution.

10mls of full DMEM/F12 medium transferred to a universal container. 5ml of medium was drawn into a syringe and connected to a 25 gauge needle (orange). Using the scalpel blade and holding the femur close to the proximal end, the proximal 2mm of bone was removed. The bone was now held using sterile forceps over a clean universal container and the needle inserted into the shaft of the bone from its proximal end. By applying gentle pressure medium was passed into the marrow cavity to flush out the marrow cells. All 5ml was flushed through the bone. The process was repeated for the other bone.

Cell number was assessed by microscopical counting using a haemocytometer. Few red cells contaminate mouse bone marrow, therefore red cell lysis was not carried out. Typically one mouse yields 40-60 \times 10^6 cells by this method.

2.3.3 Differentiation of bone marrow (rat and mouse) to macrophages

Cells were cultured for 7 days in wells made from Teflon. This non-adherent environment was necessary for the following reasons. The majority of experiments required precise ratios of two cell types in co-culture. This was only possible by aliquoting the precise number of cells into wells immediately prior to the experiment. Cells that have been cultured and differentiated in plastic flasks/wells are notoriously difficult to remove. My personal experience is that no matter whether cells
are removed using TE, ice cold 50mM EDTA there remains a population of adherent cells that are therefore not reliably included in experiments. Furthermore, 50mM EDTA and cell scraping techniques result in many necrotic cells when re-plated. I found Teflon matured cells to be similar to adherent-matured cells in terms of histology, NO and TNFα generation in response to LPS stimulation. Anecdotally, 24h after plating into plastic wells, the Teflon matured cells are less efficient at phagocytosing apoptotic cells than those which have been matured in plastic wells.

Teflon wells were generated as described in (Chapter 2.3.8). Bone marrow cells were cultured in full DMEM/F12 with 10% L929 conditioned medium (Chapter 2.5.1) at a density of 5 x 10^6 per ml (10 x 10^6 per ml for rat cells). On day 2 and day 4, half the medium was removed and replaced with an equal volume of fresh medium. On day 6, cytospins with Diff Quik staining were used to confirm the presence of macrophages.

In some experiments it was necessary to differentiate bone marrow directly in culture plates (Falcon). To generate an approximate covering of 70% of the plastic on day 7 or 8 of culture bone marrow was plated at 4 x 10^5 cells per 1.5cm^2 (one well of 24-well plate), in 800μl of full DMEM/F12 with 10% L-929 conditioned medium. An equal volume of additional medium containing L-929 conditioned medium was added at 48h, and after washing further medium was added (1ml per 5 x 10^5 bone marrow cells) on day 4.

This method gave variable cell-density at day 7 and seemed to depend on a variety of factors including the precise batch of L-929 conditioned medium, and animal to animal variation.

2.3.4 Isolation of rat glomerular macrophages

Sprague-Dawley rats (200g) were humanely killed by CO₂ anaesthesia followed by cervical dislocation (Schedule 1). 16h prior to sacrifice they were placed in metabolic cages for collection of urine.

Immediately upon sacrifice, cardiac puncture was made for collection of serum. An abdominal midline incision was made using forceps and scissors through chlorhexidine (0.5%) cleaned skin. Kidneys were sequentially removed by cutting the vessels and the capsule removed. Small biopsies of cortex were taken for histological purposes, into neutral buffered formaldehyde (4%) or methyl Carnoy’s fixative (Appendix 1) on dry ice. The remaining renal tissue was transported in Paris buffer on ice for sieving.

Paris buffer (Appendix 1) had been prepared on the previous evening and chilled to 4°C.
Whole kidneys were chopped using a scalpel and placed in the clean sterile upper most sieve that had been moistened with buffer. The three sieves were arranged with the 250µm uppermost, the 150µm in the middle and the 63µm at the bottom. The kidney pieces were gently pressed through the top sieve using the plunger of a 10ml sterile syringe, and flushed repeatedly with buffer at 4°C.

The 250µm sieve was removed and any tissue in the middle sieve was pressed through, again with regular flushing with buffer. Finally, the glomeruli remained in the surface of the bottom sieve and were collected into cold buffer by washing them with buffer into the lip of the sieve and collecting with a sterile pipette.

Glomeruli were centrifuged at 4°C (250rpm for 2min. no brake) to remove any debris and then resuspended in 10ml of full Hanks solution (HBSS with Ca++ and Mg++). Glomeruli were centrifuged again, then resuspended in 5ml of enzyme mixture at 37°C (this contains collagenase (type I) 1.0mg/ml, trypsin (type III) 0.5mg/ml, and DNase at 1500U/ml dissolved in full Hanks solution). The glomeruli were placed on a rocker at 37°C for 20 min. The cell suspension was then centrifuged (500rpm for 5min. 4°C no brake), and the cells washed x2 with Hanks w/o by centrifugation, aspiration of supernatant and resuspension. After the final aspiration, the cells were resuspended in sterile EDTA solution (2mM in Hanks w/o) at 4°C and placed on the rocker for 20min. at 4°C.

Pelleted cells (500rpm 10min. 4°C no brake) were resuspended in a further 5ml of collagenase solution (1.0mg/ml) and rocked at 37°C for 20 min. Now, on ice, cells were aspirated into a 10ml syringe through a 20 gauge (yellow) needle and ejected from the syringe through a 24 gauge (blue) needle. This process was repeated x3. Cells were now centrifuged (1500rpm, 5min. 4°C) and resuspended in full DMEM/F12 at 4°C.

The percentage of Mφ in the single cell preparation had previously been determined by flow cytometry (ED1 immunofluorescence) for normal glomeruli and diseased glomeruli (day 2, 4, and 7 of the disease). It was possible therefore to place a known number of Mφ into wells by knowing the total cell concentration as assessed by haemocytometry.

2.3.5 Isolation of monocytes from peripheral blood mononuclear cells (PBMC) by lymphocyte depletion

Purification of PBMC by Percoll gradient was carried out as described (Chapter 2.1.2). The monocytic layer of the gradient was washed twice in PBS to remove the percoll and contaminating platelets. Cells
were counted by microscopically using a haemocytometer and populations of cells analysed, principally looking for the exclusion of granulocytes (this assessment was made by flow cytometry (FSC/SSC)) (Figure 2-3) or by cytospin with Diff-Quik staining). Pure monocytes were derived using the MACS magnetic bead monocyte cell isolation system from (Miltenyi Biotec).

![Figure 2-2: Flow cytometry-derived forward (FS) and log10 side scatter (LSS) histogram of leucocytes purified from a Percoll discontinuous gradient before (A) and after (B) lymphocyte depletion. A-lymphocytes, B-monocytes, C-granulocytes and D-platelets. Note absence selectively of the lymphocyte population after depletion.](image)

Cells were resuspended by agitation in a PBS buffer containing 0.5% BSA (filter sterilised) at a concentration of 10^7 cells per 60μl of buffer. To this, human IgG was added. The cell suspension was incubated at 6°C for 5 min. A hapten-conjugated antibody cocktail containing anti-CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies was applied to the sample and incubated at 6°C for 15 min. Unbound antibody was removed by washing x2 in buffer (20x original resuspension volume) and repelletting cells at 220g for 5 min.

Cells were then resuspended in buffer (as above). Further non-specific blocking was achieved using human IgG. To the suspension, hapten-conjugated magnetic micro beads were added and incubated at 6°C for 30 min. The whole suspension was then passed through a column of magnetic iron. The details of the composition are withheld by the manufacturers. Lymphocytes and eosinophils remained within the column. The eluted solution contained purified monocytes, which were confirmed by flow cytometry (Figure 2-2) or cytospin.
2.3.6 Isolation of monocytes from peripheral blood mononuclear cells (PBMC) by elutriation

Leucocytes were prepared as described (Chapter 2.1.1) from either 176ml or 264ml of citrated blood. During this preparation the centrifugal elutriator (Beckmann) was used (Figure 2-3).

---

**Figure 2-3**: Diagram indicating the apparatus required for elutriation of leucocytes. The rotor arm seen in cross-section is placed within a Beckman centrifuge. Enriched leucocytes are introduced into the sample chamber through the injection valve. By operating the bypass valve they are transported to the elutriation chamber within the rotor arm which is at 2500 rpm. At steady state with buffer flow through the system the leucocytes order themselves according to buoyancy. By increasing the flow rate different leucocyte populations leave the chamber separately and are collected in the effluent.

The rotor arm was assembled by initially dismantling and cleaning with ethanol and cotton buds if necessary, followed by re-assembly according to the manufacturer’s instructions. O-rings were lubricated with silicone grease and the retaining bolts loosened by half a turn before inserting rotor into
Chapter 2 Methods

to centrifuge. The rotor arm was then connected to the pump (see Figure 2-3) and pressure monitoring system. The whole circuit through which cells will pass was sterilised by passing 500mls 70% ethanol through the pump followed by 2 litres of sterile water. It was imperative that all air bubbles were removed from the system. Therefore all air locks were flushed and the rotor was accelerated (to 2500rpm) and decelerated several times to dislodge bubbles in the tubing and elutriation chamber, while sterile water was flushed through the system. Hanks (HBSS w/o) was then passed through the system and the pump was calibrated in order that the flow rate (dial) on the pump could be correlated exactly with ml/min of HBSS flowing through the chamber. This calibration was carried out with the centrifuge operating at 2500rpm. It required collection of fluid exiting the elutriator into a measuring cylinder. The pump was calibrated for flow rates of 16, 18, 20, 22, 24, 26 ml/min.

Once calibrated, with the elutriator was left in ‘steady-state’ at 2500rpm, with HBSS w/o flowing at the lowest setting (usually about 1.3 on pump or <10ml/min). Once the leucocytes were prepared, they were resuspended in 5ml of PPP (platelet poor plasma Chapter 2.1.3). PPP was added to the HBSS (final concentration 0.1%). The leucocytes were placed in the introduction chamber slowly so that speed of introduction did not exceed 15ml/min. This was carried out whilst the flow of HBSS was set to bypass the introduction chamber. Cells were then flushed through into elutriator rotor arm by switching the flow of HBSS through the introduction chamber. At the lowest flow rate (about 10ml/min.) the cells were seen to collect in the elutriation chamber within the spinning rotor arm. Once all the leucocytes were in the elutriation chamber, the flow rate was increased to 18ml/min. The effluent was collected until no more cells left the elutriation chamber. Cells in the effluent were analysed immediately (by cytospin) and would be expected to consist of red cells and lymphocytes. The flow rate was increased to 22ml/min. Further cells now left the elutriation chamber and were collected. Cytospin confirmed these cells to be pure monocytes (Figure 1.1). The remaining cells were collected by increasing the flow rate to 24 and 26 ml/min. and contained pure granulocytes. The monocyte fraction was pelleted (350g for 6min low brake) and resuspended in Iscove’s modified DMEM with 10% autologous serum (PRPDS).

2.3.7 Differentiation of human macrophages from peripheral blood monocytes

Monocytes were collected from peripheral blood of healthy donors as above. Monocytes were cultured in Iscove’s modified DME medium (containing penicillin and streptomycin 500U/ml of each) with 10% autologous serum (PRPDS). Medium was replenished every two days during the first four days by removing half the medium and replacing it with fresh medium including PRPDS. Cells were cultured
either in Teflon wells (Chapter 2.3.8) or in tissue culture plastic wells (Falcon). In the former case, the monocytes and macrophages remained non-adherent. They differentiated into MΦ and were considered mature after 7 days in culture. Culturing in Teflon resulted in complete absence of giant cells which variably comprised up to 50% of MΦ after 7d culture in plastic wells. Furthermore, cell number was much easier to control in experiments carried out after MΦ had been cultured in Teflon.

2.3.8 Preparation of Teflon wells

Teflon membrane was cut to 12 x 12cm² pieces and autoclaved. In a tissue culture hood, wearing gloves, the square was placed over the top of a sterile 50ml tube (Falcon). Using the inverted end of a clean 15ml tube (Falcon) the membrane was gently pressed into the 50ml tube to create a recess which could hold 15ml medium. This was ensured by holding the membrane close to the neck of the 50ml tube as the membrane was pressed in with the smaller tube. When completed, the neck was secured with an elastic band. 70% ethanol was placed in the well for 5min. After discarding, the well was shaken vigorously to remove as much ethanol as possible. Wells were air dried for 15min minimum before cell suspension was added. If the folds of Teflon (near the neck) remained wet, cell suspension could be subsequently lost out of the well by capillarity. Tubes containing wells were covered with the original 50ml screw-on lid and placed in a clean rack in the incubator.

2.3.9 Red cell lysis

Preparation of rat bone marrow (and occasionally other cell types) resulted in significant red cell contamination. To remove red cells, hypotonic lysis was performed. Two sterile solutions were prepared; 1.6% PBS and 0.2% PBS (Normal PBS is 0.9%). Pelleted cells were resuspended in a volume of 0.2% PBS for 15s followed by addition of an equal volume of 1.6% PBS. The resultant mixture was 0.9% PBS. After further centrifugation of cells, the supernatant should remain red, confirming lysis.
2.4 PREPARATION OF APOPTOTIC CELLS FOR INGESTION BY Mφ

2.4.1 Induction of apoptosis of rat mesangial cells

Rat MC were induced into apoptosis by ultraviolet irradiation. Sub-confluent monolayers in T75 flasks (Costar) were exposed to UV irradiation (312nm, 8W, 3min) followed by incubation for 16h. Non-adherent (apoptotic) cells were removed by agitation and collected in the supernatant. After centrifugation, apoptotic cells were further purified by washing with PBS x1 and centrifugation (190g, 5min). Apoptosis was confirmed by histology, selective uptake of Hoechst 33342 (1μg/ml), but exclusion of propidium iodide (1μg/ml). Typically, fewer than 10% of cells were PI positive and fewer that 5% did not exclude trypan blue (0.2%).

2.4.2 Percoll density gradient purification of human neutrophils

Neutrophils were collected from the lower layer of the Percoll discontinuous gradient as described in Methods 2.1.2. The cells were washed x 2 in sterile PBS to remove platelets and Percoll. Each wash comprised placing cells in 50 ml PBS in a Falcon tube followed by centrifugation (220g, 5min). Cell pellets were gently agitated to loosen before re-suspending in medium.

2.4.3 Preparation of murine peritoneal neutrophils

Murine peritoneal neutrophils were prepared by injection of 1ml of 3% autoclaved-sterile Brewers thioglycollate in PBS into C57/bl6 mice. After 16, the mice were killed by cervical dislocation and the peritoneal cavity flushed briskly by injection of 10ml of sterile saline. The fluid was then withdrawn after their creation of a sterile pocket between the anterior abdominal wall skin and the facial planes overlying the peritoneum. The peritoneum was then breached using a sharp scalpel and the peritoneal fluid withdrawn using a syringe as it collected in the pocket described. This fluid was then centrifuged and the cells resuspended in DMEM/F12 with 10% FCS. The cells were typically more than 80% neutrophils by microscopy of cytospins. Cells were cultured in wells of 6-well plates.

2.4.4 Induction of apoptosis of neutrophils

Human peripheral blood neutrophils (PMN) were isolated from the buffy coat of freshly drawn blood as described (Chapter 2.4.2). The purity (>95%) and quiescence of cells were verified by flow
Chapter 2 Methods

cytometry. Apoptosis was brought about by ageing in upright T25 or T75 flasks (Costar) for 6h if the neutrophils were resuspended in PBS with calcium and magnesium ions, at a density of 4x10⁶/ml. In some experiments PMN were aged in DMEM/F12 containing 10% FCS. In these experiments, ageing in the incubator was carried out for 16h. Alternatively apoptosis was induced by UV irradiation (312nm, 8W 10 min) in DMEM/F12 and 10% FCS (heat inactivated). Cells were utilised after 2.5h. Apoptosis, both constitutive and induced was typically seen in 40-60% of cells, as assessed by histology (Figure 2-4). Necrosis was excluded by 0.2% trypan blue staining (typically <1%).

Figure 2-4: Apoptotic neutrophils (arrows) on cytospin before (A) and after (B) Dyna bead anti CD16 purification. Note contaminating eosinophil. (C) Evidence of separation of CD16 positive and negative neutrophils by flow cytometry following Dyna bead purification.
2.4.5 CD16 enrichment of apoptotic neutrophils

Neutrophils were assessed for apoptosis, after approximately 16h incubation in medium with 10% FCS or after 6-8h incubation in PBS with Ca\(^{++}\) and Mg\(^{++}\). Assessment was made by preparation of cyto-
spins slides followed by Diff-Quik staining. Characteristic morphology was used as the criterion, and although not routinely performed, this definition has been verified against Annexin-V binding to phosphatidyl serine on the surface of apoptotic neutrophils as seen by flow cytometry (Brown & Savill 1999) (see Figure 1-3).

24h prior to culture anti-mouse IgG antibody coated magnetic beads were prepared by incubating the beads with the anti-CD16, 3G8 antibody. These beads (Dyna M450) are maintained at 4°C in a sterile suspension at 4 x 10^9/ml. 500\(\mu\)l of beads suspension were incubated with 10mls of 3G8 supernatant for 24h at 4°C. Prior to collection of apoptotic neutrophils, the beads were separated from the culture supernatant using a powerful magnet to keep them in the 10ml polystyrene tube, allowing the spent supernatant to be aspirated away. Beads were washed x 1 in 10ml PBS, then resuspended in 1ml of PBS. Half of the beads, 100 x 10^6 are sufficient to bind 100 x 10^6 live neutrophils. Therefore, in cultures where apoptosis was approaching 50%, half of the beads were incubated with the neutrophils in 10ml PBS for 30min at 4°C.

After this time, beads were separated from the supernatant using a magnet at 4°C. The supernatant contained enriched apoptotic cells. The supernatant was transferred to the remaining beads and rolled again for 40min at 4°C. After separation of the beads the supernatant contained highly enriched apoptotic cells which was confirmed by microscopy. The apoptotic neutrophil population of cells before and after CD16 purification can be seen (Figure 2-4). The cells were assessed for necrosis by trypan blue staining (see Chapter 2.7.6). Fewer than 5% of cells were trypan blue positive. Most frequently, this was less than 1%.

2.4.6 Preparation of murine thymocytes

Freshly killed 6 week old (20g) C57/B16 mice were killed by cervical dislocation. After cleaning with ethanol, the anterior rib cage was surgically lifted to reveal the prominent white lobes of the thymus. This was carefully dissected and washed in fresh medium. The thymus was then pressed through a wetted 50\(\mu\)m sieve the plunger from a 5ml syringe. The single cell preparation was washed though the sieve using 10ml of fresh medium and collected. This suspension was centrifuged (250g, 5min) and the
cells resuspended at $2 \times 10^6$ cells/ml in RPMI supplemented with glutamine and 2-mercaptoethanol in addition to 10% FCS and antibiotics. The cells were transferred to wells of a six well plate (2mls per well).

**2.4.7 Induction of apoptosis of thymocytes**

Cells were either exposed to a 5min burst of UV irradiation (312nm, 8W) followed by 2.5h culture, or dexamethasone (1μM) followed by culture for 6h. Typically >50% of induced cells were apoptotic (Annexin V binding (Boeringher Mannheim)) and permeable to Hoescht 33342 (1μg/ml)) whilst <5% of those were positive for the uptake of propidium iodide (Figure 2-5).

![Figure 2-5: Induction of apoptosis of murine thymocytes by 1μM dexamethasone or UV irradiation.](image)

- **A**: Flow cytometric histogram plot of FSC against SSC indicating a distinct homogeneous population of cells (R1)
- **B**: R1-gated cells indicating blue fluorescence (Y axis) and red fluorescence (X axis) prior to administration of Hoescht 33342 and PI.
- **C**: Thymocytes cultured overnight exposed to Hoescht and PI. Note a small (16%) population of Hoescht positive, PI negative cells.
- **D**: After overnight culture with 1μM dexamethasone 85% of cells were Hoescht positive, PI negative.
2.5 HYBRIDOMA/ CELL SUPERNATANT HARVESTING

2.5.1 Production of supernatant containing murine M-CSF (CSF-1)

Murine fibrosarcoma cell, L-929 spontaneously secrete into culture supernatant macrophage colony stimulating factor (M-CSF or CSF-1). These malignant cells grow well in DMEM/F12 medium supplemented with 10% FCS. Cells were grown in T162 flasks in 50ml medium. After 7 days they were split 1:20 and replated. The supernatant was harvested, centrifuged to remove cellular debris (250g, 5min), in 50ml Falcon tubes. Medium was then filtered (0.5μm) prior to storage at –80°C.

2.5.2 Production of supernatant containing mouse anti-human CD16 antibody

The hybridoma 3G8 releases murine anti CD16 IgG into supernatants. Cells were grown in DMEM with pyruvate, L-glutamine, and 10% FCS supplementation. Cells were grown in T162 flasks for 7 days in 50ml medium. After this duration, cells were separated from the medium by centrifugation (250g, 5min) and split 1:10. Supernatant was then passed through a 10μm filter prior to storage at –80°C. Activity was confirmed by ELISA after 6months, which was not decreased after 1yr.

2.6 CO-CULTURE

2.6.1 Rodent co-culture with bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMφ) from rats or mice were cultured for seven days in Teflon wells as described (Chapter 2.3.8) and purity assessed by cytospin. From the single cell suspension, Mφ were counted using a haemocytometer and then placed in wells of Falcon 96-well plates such that 50-70% of the well base was covered with cells. This was achieved by first placing 200ml of fresh full medium into the well, then adding a 10μl aliquot of single cell suspension containing approximately 20,000 cells. Within 2h the cells became fully adherent.

MC could now be added to the Mφ. It became apparent that the ratio of one cell to the other was very important to the outcome. The proliferating cells, the mesangial cells, had a tendency to overgrow during preliminary experiments. This overgrowth blunted the impact of Mφ on mesangial cells (see...
Chapter 2 Methods

Chapter 3). By trial, it became apparent that the best ratio to achieve a near confluent monolayer was a ratio of 1.5-2.0 Mφ per MC. This resulted on average macrophages and mesangial cells in close apposition with adequate mixing of the cell types. Furthermore, the experiments were made easier by adding macrophages prior to mesangial cells rather than the other way round due since it was easier to determine the precise number of MC at the start of the experiment. However, plating mesangial cells prior to macrophages or plating the cells simultaneously had no effect on the results.

Once ready in T75 or T25 flasks (Costar) mesangial cells were trypsinised as described for routine passaging. The non-adherent cells were resuspended in full medium and counted by haemocytometry. 13,000 cells were added to the wells of the 96-well plates containing macrophages. After 2-4h, once cells had become fully spread out and adherent, wells were washed with full medium to remove any non-adherent cells/debris prior to experimentation. Since growth factors are abundant in glomerular inflammation, platelet degranulation is an associated feature, and since cultured MC proliferate readily (another feature of glomerular inflammation) in the presence of serum growth factors, it was decided that experiments should be performed in the presence of 10% FCS. Some experiments were however performed in the presence of 1% FCS. As the concentrations of extra-vascular plasma proteins and platelet-derived growth factors will vary at different stages of inflammation it was decided to contrast the experiments in 10% FCS with those in 1% FCS.

2.6.2 Distinguishing rat mesangial cells from macrophages.

In order to distinguish macrophages from mesangial cells by microscopy, it was decided to use a fluorescent inert dye. Initial experiments showed that either cell type would take up the label without any apparent deleterious effects. The dye CellTracker green, (synonymous CMFDA) (Molecular Probes) is lipid soluble and interacts with cellular proteins. It also binds avidly to serum proteins. In preliminary experiments it appeared to have no effect on growth of MC nor was it transferred to unlabelled cells. Also in co-culture experiments there was no difference in outcome when the macrophages were labelled with the dye instead of the mesangial cells. To aid morphological distinction of apoptotic mesangial cells (red condensed nuclear material contrasts well against green cytoplasm), it was decided to label the mesangial cells with this green fluorescent dye.

To label cells, a 70-80% confluent flask of cells was aspirated to remove medium, washed x1 with 5ml of sterile medium containing no serum. This was aspirated and replaced with 5ml of serum-free medium containing 5ul of CMFDA at 1mg/ml (in DMSO). Cells were incubated for 1h, at which time the medium was aspirated away and replaced with medium containing 10% FCS (full medium) to mop
up any unbound CMFDA. If cells were to be added to co-culture immediately then cells were trypsinised and resuspended in full medium which mopped-up free dye. Contamination of unlabelled cells with dye was never a problem. The co-culture as seen by fluorescence microscopy is illustrated in Figure 3-1.

2.6.3 Rat co-culture with glomerular macrophages

Glomerular single cell supernatant containing $1 \times 10^5$ glomerular MΦ was placed in wells of 24-well plates (Costar), in full DMEM/F12. After incubation for 2h, wells were washed by piping, aspiration and replacing medium (1ml of fresh full medium per well). Non adherent cells were removed. In separate experiments (immunohistochemistry) this procedure resulted in the cells remaining containing >90% MΦ. Rat MC ($6 \times 10^4$), pre-labelled with CMFDA, were added to wells. In addition, adjacent wells without MΦ were seeded with MC as a control. Wells were returned to the incubator and were not washed again. In some experiments L-NMMA or D-NMMA (100μM) was added. No other cytokines were added at any stage however. Experiments were stopped at 8h, 16h, and 24h (Chapter 2.6.8).

2.6.4 Human co-culture

Monocyte-derived macrophages (MDM) were differentiated from peripheral blood monocytes, in Teflon wells, for seven days as described (Chapter 2.3.7). Their maturation and purity was confirmed by cytospin with Diff-Quik staining (Figure 1-1). They were plated into wells of 96-well plates to cover 50-60% of the area. This equated to $2 \times 10^4$ cells per well. The MΦ were plated in Iscove's modified DMEM containing 10% autologous human serum (this is the same medium used for differentiation of cells). Cells were usually left overnight in wells, and the following morning medium was aspirated and replaced with 200μl of full DMEM/F12 per well.

Human MC are larger than rat cells but tend to proliferate less rapidly. By trial, it was found that $0.8 \times 10^4$ to $1.0 \times 10^4$ achieved 80-90% coverage of the well surface once the mesangial cells had spread out and close apposition of the two cell types was achieved. The exact number depended on the kidney whence the mesangial cells were derived as some cells appeared a little larger than others. The ratio was 2.0-2.5 MΦ for each human MC.
Chapter 2 Methods

After 4h (not less) wells were washed by agitation of the medium using a plastic Pasteur pipette, followed by aspiration and replacement with 200μl of full DMEM/F12 per well.

2.6.5 Distinguishing human mesangial cells from macrophages

Human MC were pre-labelled with the CMFDA in an identical fashion to the rat mesangial cells (Chapter 2.6.2). If cells were cultured in T25 flasks as opposed to T75 flasks, the concentration of CMFDA in serum-free medium remained the same but the volume was reduced from 5ml to 2ml. In addition, 0.5ml of 1x trypsin-EDTA was used to detach the cells.

2.6.6 Activation of the co-culture with cytokines

Provisional experiments of rat co-culture in the presence of IFN-γ showed little effect on mesangial cells. Given that activating cytokines such as TNF-α and CD40 ligand are known to be present during inflammation and are able to activate Mϕ in the presence of IFN-γ, it was decided to prime the Mϕ for 16h prior to co-culture with IFN-γ (rat recombinant) at 100U/ml (Lake et al., 1994). Once the co-culture was established it was activated with IFN-γ (100U/ml) plus TNF-α (100U/ml). Alternatively, CD40L was used in place of TNF-α. IFN-γ priming followed by lipopolysaccharide (LPS) activation has also been widely used to activate Mϕ (Lake et al., 1994), and tends to mimic the IFN-γ/TNF-α activating regime (Lake et al., 1994; Erwig et al., 1998) (Figure 2-7). Given that during inflammation resident cells are just as likely as inflammatory leucocytes to encounter cytokines, it was decided to add cytokines to the whole co-culture instead of activating the Mϕ prior to co-culture. This ensured that both cell types would be exposed to the stimuli. For initial experiments Mϕ were primed for 16h with IFN-γ in order to differentiate the Mϕ. However, it was found that whether Mϕ were primed for 16h or not had no bearing on the ability of Mϕ to induce apoptotic death of MC or suppress mitosis. Therefore, the Mϕ-priming procedure was not used for the majority of experiments.

2.6.7 Activation of the co-culture with opsonised particles

A feature of glomerular inflammation is the deposition of immune complexes containing antigen, immunoglobulin G, sometimes immunoglobulin M, often complement factor 3bi and sometimes C1q. There has been much debate in the literature about the role of such complexes in initiating or propagating pro-inflammatory responses in glomerular disease (see Chapter 1.9). In provisional
Chapter 2 Methods

experiments, I attempted to mimic immune complexes by mixing rat IgG with rat-sheep anti-rat IgG, or human IgG with goat anti-human IgG to prepare insoluble complexes. Unfortunately these complexes did not precipitate well and failed to activate macrophages. In addition I opsonised red blood cells with anti-human erythrocyte antibodies. Such opsonised erythrocytes were poorly ingested by macrophages but again failed to induce NOS 2 or cause macrophage mediated cell death in co-culture. Finally, it was decided to opsonise zymosan (yeast wall) particles (Sigma) passively with pooled, non-heat inactivated serum. These opsonised particles are known to bind Mφ via FcγI, FcγIII receptors, complement receptor 3 (CR3) and activate macrophages through these receptor-mediated signalling pathways.

Pooled sera from greater than 10 donors was used (human serum was PRPDS from healthy donors, rodent serum was from S.A.P.U.). Every effort was made to avoid LPS contamination. Therefore, serum was pre-absorbed with Polymyxin-B-conjugated polystyrene beads in glycerol (Sigma). These beads bind LPS. Serum was mixed with an equal volume of beads suspension and rotated at 4°C overnight. Beads were readily separated by centrifugation.

Zymosan was prepared at 5mg/ml in filter-sterile low-endotoxin PBS (Sigma), and washed x3 by repeated centrifugation (500g for 2 min) and resuspension in fresh PBS. Particles were then resuspended in pooled serum at 5mg/ml and incubated with regular agitation at 37°C for 5 min. The particles were washed x2 in PBS after centrifugation and resuspended again in PBS at a final concentration of 5mg/ml.

The co-culture, once established, was activated by the addition of the 50μg of particles per well. This ensured even coverage of the bottom of the well in order that all cells were exposed to the particles.

2.6.8 Termination of co-culture

For co-culture experiments that were to be assayed morphologically by fluorescence microscopy the wells were fixed with 38% formaldehyde solution (BDH) in a linear flow cabinet, by pipetting enough solution to give a final concentration of 4% (approximately 1:10 dilution). This fixative contains methanol, which assist in cell membrane permeabilisation. In some experiments, the co-culture was assessed by fluorescence microscopy of live cells. Live co-culture was immediately assessed microscopically after addition of Hoechst 33342 (1μg/ml) and propidium iodide (1μg/ml) to differentiate apoptotic cells by their differential ability to exclude dyes from the cytoplasm. When the co-culture was assessed by flow cytometry, live co-culture was trypsinised using 1x trypsin-EDTA.
Chapter 2 Methods

The cells were then centrifuged and resuspended in ice-cold 70% ethanol in PBS overnight prior to analysis.

2.6.9 Tri-culture

In some experiments, it was necessary to interact the co-culture with cell populations containing apoptotic cells. Apoptotic rat MC and apoptotic leucocytes were prepared as described in Chapter 2.4. Cell populations that were 100% apoptotic were incubated with the co-culture based on 5 cells for every 1 Mφ in the co-culture. Apoptotic cells that were 30-60% apoptotic were incubated based on 10 cells for every 1 Mφ in the co-culture.

The co-culture was established as described (Chapter 2.6.1). Pre-prepared apoptotic cells were resuspended in full DMEM/F12 at 20 x 10^6 cells/ml if 30-60% apoptotic or 10 x 10^6/ml if 100% apoptotic. Ten microlitres of apoptotic cells was then added to wells of 96-well plate containing the co-culture. In some experiments, 10μm latex beads (5 x 10^7 /ml in ddH2O) (Polysciences) were added. This required 2μl per well of 96-well plate to achieve 5 beads for every 1Mφ.

For most experiments, apoptotic cells were incubated for 6h with the co-culture. Non-adherent cells were then washed away by agitation of the medium using a plastic Pasteur pipette, followed by aspiration of medium (see Figure 2-6). Aliquots of medium replaced that which had been aspirated and the washing procedure was repeated. After the second wash, activating cytokines, where appropriate, were added. Experimental plates were incubated for 8h and 24h before fixation (Chapter 2.6.8).

Some wells were fixed using 2% glutaraldehyde solution for 5min. The wells were washed again with PBS x2 to remove any remaining aldehyde and left dry. These wells were subsequently used to assess the percentage of cells that had phagocytosed apoptotic cells.

These wells were assessed for ingestion of apoptotic cells by staining apoptotic neutrophils for the presence of myeloperoxidase (Appendix 1). The fixed well was immersed in a substrate solution containing o-dianisidine (DMB) with hydrogen peroxide. After 10-30min, the location of PMN was seen as a brown homogenous pigment. The interaction was stopped by washing the wells with water/PBS and wells were assessed for ingestion with the aid of Nomarski optics to visualise the Mφ cell membrane. Wells were assessed for percentage Mφ ingesting apoptotic cells by counting five fields per well (see Chapter 2.7.2). Ingestion of apoptotic thymocytes and rat MC was assessed using Nomarski optics without the aid of a cell specific dye.
2.7 ASSESSMENT OF MESANGIAL CELL APOPTOSIS

2.7.1 Fluorescence microscopy

Previous studies of MC apoptosis in the group had relied on fluorescent microscopical counting of live cells in wells after staining with acridine orange (AO), a fluorescent dye that labels RNA and DNA of live cells. Unfortunately, this method was limited since it did not allow MC and MΦ to be distinguished. Also, AO was toxic to cells after 30 min, and it resulted in high background fluorescence in the medium. Propidium iodide (5µg/ml) fluoresced red with 450-490 nm excitation at the same time that CMFDA labelled cells fluoresced green. Propidium iodide labelled the DNA and RNA enabling simultaneous detection of condensed nuclear material within green cells and detection of MΦ nucleus and cytosol. Microscopically, the MC undergoing apoptosis were distinct in that they had obvious nuclear condensation, often displayed marked cytoplasmic blebbing, apoptotic bodies and cells shrinkage (Figure 2-7). To aid diagnosis (particularly when assessing human MC) Hoechst 33342 (1µg/ml) was also used to stain fixed cells. When the co-culture was excited at 365nm, cell nuclei fluoresced blue. Apoptotic cells were definable by nuclear condensation and fragmentation. This type
Figure 2-7: Mesangial cells undergoing apoptosis. (A) Four apoptotic rat MC surrounding three Mφ in fixed co-culture. Note characteristic nuclear condensation of green MC (B) Single green rat MC (thick arrow) adjacent to Mφ (thin arrow) in fixed co-culture. Note characteristic cytoplasmic blebbing. (C) In live co-culture, live green spindle shaped MCs. Four rounded up green cells can be seen excluding PI (1μg/ml) (thick arrow). Two similar cells admitting PI can be seen (thin arrow). (D) Same image of live co-culture showing Hoechst admission into rounded green cells confirming apoptosis. (E) Fixed rat MC exposed to IFN-γ/TNF-α showing many apoptotic nuclei. (F) Human fixed co-culture showing three green apoptotic MC (arrows) adjacent to a Mφ.

of assessment also enabled easy detection of cells in the different phases of mitosis; metaphase, anaphase and telophase (Figure 2-8).
Figure 2-8: Mesangial cells in different stages of mitosis (anaphase, telophase, metaphase). (A-C) Rat mesangial cells (X320, X200) in various stages of mitosis stained with PI after fixation. (D) Human MC in unactivated co-culture with CMFDA-labelled MC counterstained with PI in mitosis.

To achieve this labelling with fluorophores the fixed co-culture plates were left for at least 48h at 4°C to allow firm fixation to the monolayer of loosely adherent MC. Medium was gently tipped off the wells and a solution of PI (5μg/ml) and Hoechst 33342 (1μg/ml) in PBS was added to each well using a transfer plastic pipette. After 5min, the solution was aspirated to dryness and immediately replaced with a fluorescent-quenching mountant containing 90% glycerol, 10% PBS pH 8.6 and 1-4-diazabicyclo-2-2-2-octane (DABCO) at 5mg/ml.

2.7.2 Blinded microscopical counting

One fundamental problem with microscopical counting of apoptotic cells is observer bias. It is not feasible for one person to perform the experiments, and another person to count them. To obviate observer bias, the observer should be blinded as to the nature of the experiment in each well assessed. In addition a fixed routine for assessment of microscopical fields should be maintained; that is wells
should be counted in a stereotyped manner, and each well should be assessed in exactly the same way as the previous well. Since it is not possible to blind the observer completely as to the nature of the experimental conditions in each well (e.g. co-culture vs. MC control where in the former Mϕ are visibly present and in the latter Mϕ are not), it is mandatory that each well be assessed in an unbiased way. To achieve these maxims and avoid bias:

- All experimental plates were set up in a symmetrical fashion, with different experimental conditions occurring in a different order on separate plates.
- Once plates were processed with fluorophore labelling, plates were coded and lids were removed.
- Plates were then counted from top left (position A1) in columns and records maintained by the well code stamped on the plate. Once all wells had been counted, the lids were matched with the plates to reveal experimental conditions.
- Each well was assessed by x 32 objective for rodent studies and x 15 objective for human studies.
- Five fields were chosen for assessment from each well.
- Fields were chosen without viewing the well whilst selecting the field. The position of viewing followed from 12 o’clock to 3 o’clock to 6 o’clock to 9 o’clock, and finally a central position.
- If a selected field included the edge of the well it was reselected without being assessed
- Each field was scored regardless of whether it was devoid of cells or overcrowded with cells.
- Each condition was in triplicate wells.

### 2.7.3 Hoechst and PI staining of live cells

To further verify the nature of the cells which appear apoptotic morphologically, it was necessary to assess them functionally. Apoptotic cells but not necrotic (or secondary necrotic) cells are impermeable to propidium iodide (1μg/ml). On the other hand, live cells but not apoptotic (or necrotic) cells are impermeable to Hoechst 33342 (1μg/ml). Unlike assessment of the fixed, permeabilised cells, this assessment is a measure of membrane function. To assess the co-culture further at 24h live co-culture was exposed to the two fluorophores simultaneously. After 5min incubation at 37°C, wells were scored microscopically for Hoechst positive and PI positive cells. Both green mesangial cells and non-green (Mϕ) were assessed in this way (Figure 3-5).
2.7.4 Trypan blue staining

A further dye for assessing cells is trypan blue. This dye is excluded from all but necrotic cells. Co-culture at 24h was removed from wells by trypsinisation and an aliquot placed in a haemocytometer. An equal volume of 0.4% trypan blue was added to the haemocytometer, and the percentage of blue cells was assessed by counting.

2.7.5 Flow cytometric analysis of MC apoptosis in co-culture

Much time and effort was spent during this thesis to develop a successful flow cytometric based assay of MC apoptosis in co-culture. Unfortunately, many of the flow cytometric based assays that work well with leucocytes (Sun et al., 1992; Brown & Savill 1999) did not work well with the myofibroblast mesangial cell. FITC-conjugated Annexin V, which binds to phosphatidylserine (PS) exposed on the outer lipid bi-layer of plasma membrane of apoptotic cells, did not provide enough shift of fluorescence to be a sensitive marker of apoptotic cells. Furthermore, often the range of fluorescence of the MC was over several log orders, rendering sensitive assays impossible by this method. Other studies such as a colour shift using JC-1 (Macho et al., 1996) were also of low sensitivity.

After much work, the flow-based assay for hypodiploidy (as assessed on RNase treated ethanol fixed cells) was found to be reproducible, though reliably less sensitive than microscopical counting.

Co-culture was performed in 6-well plates. Other assays of MC alone that were induced into apoptosis were performed in 6-well plates or T25 flasks. Supernatants were removed by pipetting and placed flow cytometric polystyrene tubes (Falcon) on ice. Cells were washed with PBS, the washings kept in the chilled polystyrene tube. Adherent cells were trypsinised and all placed in the FACS tube. FACS tubes were centrifuged at 190g for 10min to pellet all cells. Supernatant was aspirated and the pellet resuspended gently in ice cold 70% ethanol, 30%PBS. Tubes were covered and stored at 4°C for 16h. Tubes were centrifuged at 190g for 5min and aspirated. After washing with 5mls PBS, and further centrifugation (190g for 10min), cells were resuspended in 200μl of PBS containing PI at 200μg/ml and RNase A at 500μg/ml, and incubated at 37°C for 1h. Cells were then assessed by flow cytometry (excitation 488nm) for DNA content.

Apoptotic MC due to their size are fragile and it was necessary to find a balance between preferentially losing apoptotic cells during centrifugation (due to smaller size) and not destroying the apoptotic cells by exerting excess force on them.
2.7.6 Terminal deoxy-uridine nick end labelling (T.U.N.E.L.) and DNA fragmentation assays

Co-culture was also assessed using TUNEL staining to highlight apoptotic cells by virtue of fragmented DNA. This assay was used with success on tissue sections. However, due to the large number of washing steps involved, in cell culture, the majority of apoptotic cells were washed away. The assay was therefore of no use.

Tritiated thymidine labelling of MC DNA was carried out, and after 24h of activated co-culture cell lysis was attempted with an alkaline buffer and Protease K digestion of the nuclear membrane. Unfortunately by virtue of the FCS present in the vast majority of experiments, the proteinase K digest was inhibited. This assay was therefore abandoned. However, should future experiments be performed in serum free conditions it could be used as it reliably indicated fragmented DNA which can be collected on separate filter papers from non-fragmented chromosomal DNA (Mooney et al., 1997).

2.8 SEMIQUANTITATIVE COLORIMETRIC ASSAYS

2.8.1 Griess assay

This assay measures nitrite concentration in a solution, and can be an indirect assay for nitric oxide generation as this is the major stable metabolic product. The acidic Griess reagent reacts to produce a deep purple colour whose absorbency is best measured at 540nm (DeShan et al., 1966; Thomsen et al., 1990). The Griess reagent is unstable and has two components, Sulfanilic acid and N-ethelenediamine. Each solution is stored separately in the dark at 4°C and mixed in equal volumes immediately prior to use.

Due to the colorimetric nature of the assay, initially medium containing no-phenol red was used. However, due to the acidic nature of the Griess reagent, medium with no nitrite appears pale yellow and does not interfere with 540nm absorbency.

The reagent is sensitive over concentration of $1\mu M$ to $100\mu M$, therefore standard curves using sodium nitrite solution were generated. A typical standard curve is shown (Figure 2-9). Samples were assayed using 96 well microplates and a plate reader.
Chapter 2 Methods

Figure 2-9: Standard curve of $A_{560}$ against concentration of sodium nitrite dissolved in DMEM/F12 (without phenol red).

2.8.2 Nitrite plus nitrate

For some experiments it was important to assay nitrite plus nitrate as nitric oxide can be converted to nitrate particularly in the presence of oxygen radicals. An enzyme purified from liver, nitrate reductase enables reduction of nitrate to nitrite so that it is detectable by the Griess reagent. The enzyme utilises NADPH as a cofactor. NADPH interferes with the Griess reagent when in high concentration therefore to keep [NADPH] low, the addition of glucose-6-phosphate and glucose-6-phosphate dehydrogenase permitted cycling of NADP back to NADPH (Granger et al., 1996; Giovannoni et al., 1997). Immediately before reduction of samples an enzyme mastermix was prepared (Appendix 1) containing these enzymes and substrates in a phosphate buffer. It was found that 80μl of sample incubated with 25μl of enzyme master-mix for one hour enabled complete reduction as assessed by reduction of standard concentrations of sodium nitrate solution.

2.8.3 Tumour necrosis factor ELISA (murine)

Attempts were made to generate our own ELISA from polyclonal antibodies, in-house biotinylation and in-house generation of peroxidase based substrates. However, the assay was unreliable with a low sensitivity. It was therefore decided to utilise the Quantikine ELISA system from R&D Systems. This kit, whilst expensive was very sensitive and reproducible. Therefore, all data utilised this system.
Chapter 2 Methods

2.8.4 Assays for estimating protein concentration

For some studies, Bradford reagent was utilised. This reagent (brown) turns blue when interacting with proteins. One part of protein solution is mixed with nine parts Bradford reagent and absorbance measured at 340nm. The effective protein concentration was obtained from a protein standard curve. This assay is limited in that SDS interferes with the assay. Therefore, in some studies, a kit based, one step solution was utilised (Biorad) and absorbencies were read in the same fashion as for the Bradford Reagent.

2.8.5 Hydrogen peroxide assay

The colorimetric reagent contained a saline/glucose buffer, with phenol red and horse radish peroxidase. This reaction mix (Appendix 1) was mixed with an equal volume (100μl) of fresh cultures supernatant that had been clarified by centrifugation (1000g, 3min, 4°C). The mix was incubated for 60min at 37°C, 5% CO₂. Five microlitres of 1N sodium hydroxide was added and the sample measured by absorbency at 610nm. Standard curves were performed revealing linearity between 5μM and 200μM hydrogen peroxide.

2.9 WESTERN BLOTTING

2.9.1 Preparation of cellular proteins

For collection of cellular proteins, a hypotonic lysis buffer was used. When collecting cellular proteins from MC for Fas and FasL analysis a Hepes based buffer was used (Appendix 1). To this lysis buffer was added Nonidet P40 (or Ipegal) (0.2%) to dissolve proteins out of membranes.

Cells in T75 flasks at sub-confluent density were transferred to ice. Medium was removed and cells were scraped in ice cold PBS. Once scraped cells were pelleted by centrifugation (250g, 5min, 4°C), the pellet was then resuspended through a fine (23G) needle in 1ml ice cold lysis buffer with detergent. The resuspended cell pellet was incubated on ice for 10min, then centrifuged (10,000g, 10min, 4°C).

Protein concentration was then calculated using the Bradford reagent (see Chapter 2.8.4).

An aliquot of cell lysate was then mixed in a 3:1 ratio with Laemmli buffer (Appendix 1), and boiled for 3min to break disulphide bridges in the proteins.
When cellular proteins were collected from MΦ the procedure was different. Due to limited numbers of MΦ, 1 x 10^6 cells were seeded into each well of 6-well plates and after 16h, experiments were performed. To collect proteins, medium was aspirated away and plate placed on ice. To each plate 500μl of hypotonic lysis buffer was added. For these experiments, a different, Hepes-based buffer was used (Appendix 1). In some experiments the detergent Triton-X 100 (0.2%) was added.

Cells were incubated for 10 min on ice and scraped with a pipette tip until all cells were detached. The aliquots were placed in chilled 1.5 ml Eppendorf tubes. In the case where Triton-X 100 was added, cells were centrifuged at 10,000g, 10min 4°C, to remove cell nuclear material and insoluble cell debris. The supernatants with the addition of 0.1% SDS were then lyophilised at 10°C. At this stage samples were resuspended and boiled for 3min in Laemmli buffer. Cell number was utilised as a surrogate for protein estimation, since wells contained equivalent cell numbers. In other experiments, membrane proteins were separated from cytosolic proteins. To achieve this, once cells were loosened from the plates, they were subjected to freeze-thaw cycles x 2 in liquid nitrogen. After sonication (4 x 5s), nuclei were then pelleted at 10,000g 10 min 4°C. The supernatant was then transferred to fresh Eppendorf tubes and centrifuged at 100,000g 10 min 4°C. The pellet now contained membranes, whereas the supernatant contained cytosolic proteins. On ice the pellets were resuspended in Laemmli buffer and boiled for 3 min.

2.9.2 Preparation of soluble supernatant proteins

For some experiments it was necessary to analyse secreted proteins by western blotting. It is possible to lyophilise supernatants but the high salt concentration remaining prevents acceptable running of proteins in acrylamide. It was therefore decided to precipitate proteins from the supernatants. Supernatants from MΦ in 6 well plates were collected into tubes on ice. Samples were centrifuged at 10,000g 10min 4°C to remove cell debris. The supernatant was then mixed with ice-cold isopropanol (60% isopropanol, 40% sample by volume). Samples were then incubated in a -20°C freezer for 15min, then centrifuged again at 10,000g for 15min 0°C to pellet precipitated proteins, remaining supernatant was aspirated and the pellets were resuspended in Laemmli buffer and boiled for 3min.

2.9.3 Preparation of SDS-PAGE

For the studies carried out in this thesis, SDS-polyacrylamide gels were prepared and analysed as follows.
Chapter 2 Methods

For large gels a BioRad assembly was used. The clean glass plates were 15 x 15 cm$^2$ and separated by 1.5mm spacers. For small gels a BioRad mini-gel system was used. Clean glass plates were 7 x 7 cm$^2$ and separated by 0.75mm spacers. The plate assembly was sealed using a sealing gel (Appendix 1). Running gels were prepared as described in Protein Electrophoresis Applications Guide (Hoefer). The proportions of constituents for different gel types can be found in Appendix 1.

Monomer acrylamide (30%) / bis-acrylamide (0.5%) solution was purchased from Sigma pre-prepared and filtered. Other constituents were prepared and stored at 4°C (Appendix 1).

Once plates were assembled, sealing gel was added to ensure sealing of the bottom of the gel plates but also the sides with the spacers. Once polymerised, running gel was added to within 2-3cm of the base of the toothed comb where samples will be loaded. The top of this gel was gently covered with 0.1% SDS solution or water saturated n-butanol to ensure even polymerisation of the top of the running gel. Once polymerised, the 0.1% SDS or butanol was poured away and allowed to evaporate. The stacking gel was poured and the toothed comb inserted. Once polymerised the gel could be used immediately or stored overnight at 4°C, in Cling-Film.

The gel(s) was transferred to the gel tank, the comb gently removed so as not to disturb the sample wells and buffer placed in top and bottom tanks (Appendix 1). Samples boiled in Laemmli buffer could now be loaded into each well.

Once loaded the gel was exposed to a current. For the large gel system, which lacked a cooling system, the gel was run for 16h on ice at 10-15mA per gel. For the mini gels with better cooling of the plates, gels were run faster (over 2-3h) at 15-20mA per gel.

2.9.4 Wet transfer of proteins

Once electrophoresed proteins were transferred to either nitro-cellulose or PVDF paper. The former required to be wetted with dH$_2$O, the latter required to be wetted with 100% methanol before usage. The membrane was cut to size taking care not to transfer any proteins (such as that on one’s hands-gloves were used), and equilibrated in Towbin transfer buffer. A transfer sandwich was made using cassette with sponges, two pieces of pre-wetted blotting paper and the membrane. The gel was removed from the glass plates, stacking gel and excess gel trimmed away and transferred by means of a stream of
Chapter 2 Methods

dH₂O into a tray containing transfer buffer with transfer sandwich. The gel was placed carefully in apposition with the membrane taking care to remove all air bubbles. As a general rule proteins are bound to SDS are negatively charged and will therefore move towards the anode (red) in an electrical field. It should be noted however, that some proteins are positively charged and will move in the opposite direction. The sandwich is then sealed and placed in the transfer tank which is filled with transfer buffer.

Proteins were transferred using BioRad system. Proteins were transferred at 1mA per cm² of transfer membrane for 6h on ice.

Upon completion of transfer, the gel was inspected for transfer of pre-stained markers to the paper. Confirmation of transfer could be obtained by transiently staining the membrane with Ponceau S and staining the gel with Coomassie brilliant blue. The membrane was not allowed to dry out but usually placed in PBS.

2.9.4 Incubation with antibodies

Membranes were placed in cylindrical tubes (sealed with Parafilm) and incubated at room temperature with blocking solution by rolling. Usually, this solution was 4% Marvel in PBS or 4% Marvel, 0.05% Tween 20 in PBS. The Marvel solution was centrifuged (200g 5min) to remove any insoluble debris.

Primary antibodies stored as recommended at 4°C or -20°C, were diluted in blocking solution and incubated with the membrane by rolling in the cylindrical tube. This was usually at 4°C overnight, though in some instances binding required 48h (see results).

After incubation membranes were washed with PBS (sometimes including 0.05% Tween 20) three times for 5min each wash and rolled to assist washing. Secondary horseradish peroxidase (HRP)-conjugated antibodies were incubated at manufacturer’s recommended dilution unless other dilutions were found superior. Sometimes further layers of secondary antibody were required. Between each layer of antibody, three washes were carried out as described above.

2.9.6 Detection of antibody binding

To achieve this, two methods were employed. Firstly, 4-chloro-1-naphthol was used as a substrate and secondly a chemi-luminescent substrate was employed in peroxidase dependent, antibody linked reactions.
Using the former, 20mg of 4-chloro-1-naphthol were dissolved in 500µl of methanol/ethanol and mixed with 50ml of 2mM Tris, 200mM NaCl pH 8.0. To this was added 600µl of 6% hydrogen peroxide. The solution was incubated at room temperature with the membrane on a rocking platform. Colour change was observed and when adequate the reaction stopped by washing with PBS.

Using chemiluminescence, the solution was purchased from Amersham and prepared according to manufacturers instructions. The wet membrane was placed in a plastic sheath and the solution poured over the surface to obtain an even thin film. The top of the plastic sheath was placed over the blot, and in a dark room photographic paper (Kodak) was placed over the blot in a cassette and exposed. To start this was usually 30s. After development, a second exposure was made as necessary.

2.9.7 Immunoprecipitation and detection of cell surface proteins

Cell surface proteins of live cells were biotinylated using 5mls PBS with 0.5mg NHS-Biotin. 10 x 10⁶ mesangial cells were incubated with the solution at 4°C for 30 min. After washing x 3 in ice cold PBS, cells were scraped, washed by centrifugation at 250g 5min in PBS, and resuspended in 1ml hypotonic lysis buffer (Hepes buffered, not Tris buffered). Effective lysis of cells was performed by freeze thaw cycles x 2 in liquid nitrogen. Cell membrane was separated from cytosol by centrifugation (14000g 10min). The supernatant was separated and the pellet resuspended in Hepes/buffer now containing Triton-X 100 0.2%. The protein solution was incubated at 4°C for 30min with 1µg of rabbit IgG and 4µl of protein A sepharose beads. After separation of the beads by centrifugation primary rabbit IgG was added (1µg/mg of protein) and incubated for 16h at 4°C. Now 4µl of Protein A sepharose beads were added and agitated for 30 min in ice. Positive and negative control antibodies were utilised and pre-cleared beads were kept for additional controls.

Beads, maintained on ice were washed x 3 in lysis buffer (no inhibitors) and then washed x1 in 0.5% SDS in 25mM Tris pH 7.4. The beads were now resuspended on 30µl of Laemmli buffer, and applied to gel electrophoresis.

2.9.8 Estimating protein concentration

To estimate protein concentration, protein solution was mixed with a colorimetric substrate. Most analysis used the Bradford reagent. This however has its weakness in that not all proteins are detected by this reagent equally and also in the presence of detergent such as SDS, the assay is unreliable. Given
Chapter 2 Methods

those findings, in some experiments a commercial colorimetric assay was used. However, in the majority of cases the Bradford reagent was sufficient (see Chapter 2.8.4).

2.9.8 Staining of membranes and gels

To view proteins in gels several methods are available including Silver stain and Coomassie brilliant blue R-250 stain. The former is more sensitive but is labour intensive. The latter is quick, simple, and particularly useful when looking for protein loading of lanes in the gel or transfer of proteins to a membrane. Gels were stained by immersing in the staining solution and covering to minimise methanol and acetic acid vapour. The gel was agitated for 4h. The stain was then replaced with destain and shaken slowly for 30min. Destain was replaced every 12h until background of the gel was clear.

The membrane itself can be stained either reversibly or permanently. The former was found to be useful as a quick test to assess whether proteins had transferred and whether loading was even. Ponceau S staining was rapidly reversible by immersing the membrane in ddH2O. To achieve staining, the membrane was incubated with Ponceau S solution for 15min and briefly destained by washing in water. Complete destaining was carried out by further washing with ddH2O.

2.10 ELECTROMOBILITY SHIFT ASSAY

2.10.1 Preparation of nuclear proteins

Sub-confluent mesangial cells growing in T162 (10x10⁶) flasks were exposed to experimental conditions. At the end of the experiment, flasks were placed on ice and the medium replaced with 10ml of chilled PBS. After scraping with a cell scraper, cells were placed in a universal container and centrifuged (250g, 5min 4°C). After decanting, the pellet was washed and resuspended with 1000μl of chilled lysis buffer A (Appendix 1). The cells were centrifuged (10,000g, 5min 4°C). After decanting, the pellet was resuspended in 100μl of buffer A containing 0.2% v/v NP 40 using a 23G needle, and incubated on ice for 10min. After centrifugation (10,000g, 10min 4°C) the supernatant was removed and stored. The pellet contained crude nuclear preparation. This was resuspended in 50-100μl of Buffer C (Appendix 1) using a 23G needle and incubated for 15min in the fridge door (>5°C). The sample was centrifuged (10,000g, 10min 4°C). The supernatant now contained soluble nuclear protein and was stored at −70°C for EMSA.
Protein concentration was calculated using the Bradford reagent in order that equal loading could be established.

15μg of nuclear protein was required for each incubation with oligonucleotide

2.10.2 Generating labelled oligonucleotides

Complementary sequences of ssDNA were prepared according to published sequences with 3’ overhangs for a known restriction enzyme. To anneal ssDNA each ssDNA oligonucleotide was diluted with ddH₂O to a final concentration of 1.75pmol/μl. The oligonucleotides were mixed and boiled in a water bath (10min). The water bath was allowed to cool to room temperature. Samples were then frozen (-20°C).

To radio-label the annealed probe (sufficient for 50 lanes) 7.0 pmole (4μl) of oligonucleotide was mixed with 1μl of 10x kinase buffer (Promega), 10U of T4 kinase and 4μl of ³²P-ATP (9.25mBq in 25μl Amershams). This mixture was incubated 37°C for 30min, then diluted with 1μl 0.5M EDTA and 89μl of Tris-EDTA (TE buffer Appendix 1). The final hot probe was frozen at -20°C until use in a lead lined container.

2.10.3 Binding reaction

This reaction needed to be optimised for each oligonucleotide used. Binding reaction buffer, therefore may be required to vary in its composition. The buffer used for NFκB and IRF-1 studies is shown (Appendix 1). 15μg of nuclear extract (usually in 2-3μl of buffer) was made up to 10μl with ddH₂O, then mixed with 1.5μl of10x reaction buffer, 1.5μl of PolydIdC (2μg/μl Promega). Finally, 2.0μl of labelled probe (10,000-100,000 cpm). The incubation was for 15min RT. In my experience nuclear protein <1μg/μl does not bind well to the oligonucleotide in vitro. Therefore choosing whether to resuspend nuclear proteins in 50μl or 100μl of Buffer C will be crucial to the outcome. 3μl of xylene cyanol/bromophenol blue loading dye (Appendix 1) was added to each incubation.

2.10.4 Gel electrophoresis

Non-denaturing polyacrylamide gels were prepared with TBE buffer, using 5% to 7% polyacrylamide (see Appendix 1).
Gels were prepared in 15 x 15 cm² plates (1.5mm spacers) with a sealing gel nut no stacking gel. Once prepared and placed in the electrophoresis apparatus, top and bottom tanks were filled with 0.25 x TBE. Behind a Perspex shield, samples were loaded with appropriate care and handling of radioactivity. Once loaded into the glass plates the gel was electrophoresed at 180V (1 plate or 2 plates) for 60-75min. Care was taken to keep the free, labelled oligonucleotide within the gel to minimise contamination.

2.10.5 Visualisation of radioactive gel

Once electrophoresed gels were placed on blotting paper and dried using a gel dryer. Free, labelled oligonucleotide in the bottom of the gel was removed and discarded appropriately. The dried gel was now exposed using photographic paper in a cassette for 16h at ~70°C. Films were then developed using standard techniques.

2.10.6 Super-shift and cold-competition assays

For cold-competition assay, the nuclear protein extract was incubated overnight with a 50 fold excess of unlabelled oligonucleotide. Following this incubation the labelled oligonucleotide was added and incubated as described in Chapter 2.10.3. The completed reaction was then electrophoresed as described. For super-shift assays, the nuclear protein extract was incubated for 16h at 4°C with the antibody of choice and as a control a non-specific antibody was incubated with another aliquot of nuclear protein. The nuclear protein was then incubated with labelled oligonucleotide as described (Chapter 2.10.3).

2.11 MOLECULAR BIOLOGICAL TECHNIQUES

2.11.1 Preparation of agar plates

Bacterial grade sterile petri dishes were assembled close to a Bunsen flame. LB agar was prepared (Appendix 1) and autoclaved. When cooled to < 55°C the appropriate antibiotic was added (Appendix 1) and the agar poured into plates. Petri dish lids were replaced to allow ventilation and when set condensation was allowed to evaporate (37°C oven or laminar flow hood). Plates were stored wrapped in Cling Film until usage.
2.11.2 Preparation of broth

LB broth and Terrific broth were prepared (Appendix 1) and autoclaved. The addition of antibiotics was made with the broth at room temperature.

2.11.3 Preparation of agarose gels

Agarose gels were prepared with Tris-Borate-EDTA (TBE) (Appendix 1) buffer. The alternative Tris-acetate-EDTA (TAE) was not regularly used. Typically percentage agarose varied from 0.7% to 1.5% depending on the size of DNA under analysis (higher % gel for smaller DNA). Gels were usually prepared in the upturned lids of microwell plates. Once the agarose had boiled, ethidium bromide was added to the gel (10μg/ml final concentration). Once the gel cooled (hand held) it was poured. Clean gel tanks were filled with 1x TBE and regularly replaced.

2.11.4 Generation of competent bacteria

DH5α competent bacteria were generated from DH5α cells. Cells were streaked onto a plate containing LB agar. Sixteen hours later, 10-15 colonies were selected and placed in 250ml of SOC medium (Appendix 1). Cells were incubated in the medium for 16h at 18-25°C with agitation at 200-250rpm, until the OD₆₀₀ was 0.6 (0.4-0.8). The culture was spun to pellet the cells (3000rpm, 4°C). The pellet was then re-suspended in 84ml of Transformation buffer (Appendix 1), on ice. This suspension was incubated for 10min on ice, then spun again (3000rpm, 4°C). The pellet was resuspended in 20ml of chilled transformation buffer and 1.5ml of DMSO was added, and again incubated on ice for 10min. The suspension was then frozen in liquid N₂ in 500μl aliquots.

2.11.5 Transformation of competent bacteria by heat shock

DH5α E. coli were routinely used in these studies. E. coli were stored at -70°C in 80μl aliquots. Bacteria were thawed on wet ice and incubated with the DNA sample (typically >10ng) for 20min on ice. They were then incubated in a block at 42°C for 1min followed by 2min on ice. 900μl of LB broth
was added and bacteria shaken and incubated at 37°C for 1h. After centrifugation (5000g 5min RT), the pellet was resuspended in 200μl of broth and applied evenly to the surface of an agar plate.

2.11.6 Selection of resistant clones

Transformed DH5α bacteria were plated onto agar plates containing antibiotics. Only those bacteria expressing the appropriate bacterial resistance gene would survive and grow as colonies. Successful transformation would result in many single colonies growing on an agar plate for 16h at 37°C. Single colonies could be picked using a flamed wire loop and transferred to bijous bottles containing 2 or 3.5ml of LB broth with appropriate antibiotics present. Bijous were shaken (200/min) for 16h. A second agar plate with a grid reference was used to streak a defined area of the agar with the picked colony so that a second population of the clone could be maintained.

2.11.7 Screening of colonies by STET preparation

Often it is necessary to screen many clones when searching for successful insertion of the gene of interest. A cheap and simple way of screening is to isolate plasmid DNA from the bacterial clonal population by lysosyme assisted hypertonic lysis.

1.5ml of bacteria in broth (or 3.0ml) was pelleted by centrifugation (5000g, 5min RT). The pellet was resuspended in 600μl of STET solution (Sucrose, Tris, EDTA, Triton X 100) (Appendix 1) and 50μl of lysosyme (10mg/ml in dH2O). Samples were then boiled in a heating block (100°C) for 1-5min until a dense white precipitate formed. Samples were centrifuged (10,000g, 10min RT). The pellet (white) was then discarded (usually by removing using a 200μl pipette tip). 600μl of isopropanol (RT) was mixed with the supernatant and inverted to mix. Samples were again centrifuged (10,000g, 15min, RT). Supernatants were discarded and samples dried by patting on blotting paper. Samples were inverted and left to dry for 5-10min. To each sample 50μl of dH2O (pH 8.0) was added (if pH of dH2O is < 7.4 consider using 10mM Tris pH 8.0) to resuspend DNA. This may require agitation and incubation at 37°C for a few min. RNase A was added (200μg/ml final concentration) and samples incubated for 15min at 37°C.

Aliquots of DNA solution (typically 5-10μl) were electrophoresed to look for gene insertion. Alternatively, diagnostic digests were made on the DNA prior to electrophoresis.
2.11.8 Purification of DNA for transfection

In the majority of cases purification of plasmid DNA was carried out using the Qiagen Tip-100 kit and lysis of pelleted bacteria was carried out according to manufacturers instructions.

However, it was not possible to obtain sufficient plasmid DNA from DH5α bacteria transformed with the 767 (see Figure 2-10) or 776 IRF-1 containing plasmids. These plasmids were very low copy number, and processing of larger volumes of broth did not improve the yield much above 2μg of DNA. It was therefore decided to use a different strategy for purifying the DNA.

![Diagram of restriction sites](image)

**Figure 2-10** The 767 vector with restriction sites demonstrated. This vector was supplied from Prof. Hauser at the Gesellschaft fur Biotechnologische Forschung. It comprises a colEl bacterial promoter, with an ampicillin resistance gene. The fusion gene IRF-1 with HER is shown. This gene construct is under the MPSV promoter which crosses the HpaI restriction site (480).

One litre of Terrific broth was inoculated and incubated with 767 plasmid and incubated until absorbency, A_{600} was 0.4-0.6. The bacteria were pelleted in 300ml flasks in a Beckman centrifuge
Chapter 2 Methods

(4000g, 10°C, 30min). The pellet (from 1 litre) was resuspended in 50ml glucose buffer. Once resuspended 100ml of lysis buffer was added and mixed by inversion, followed by incubation on ice for 5min. Subsequently, 150ml of high salt buffer was added and mixed by rapid inversion of the tube until a well-dispersed SDS/protein precipitate had formed. The mixture was incubated on ice for 10min. The mixture was aliquoted into 50ml tubes and centrifuged (4,000g, 10min 4°C). The supernatant was passed though a gauze to remove particulate matter and 100ml of isopropanol was added to the supernatant to precipitate nucleic acids. After further centrifugation (4,000g, 15min 4°C), the supernatant was drained completely leaving a pellet of small nucleic acids. After drying the tube, the pellet was resuspended in 10ml of dH2O.

To precipitate RNA an equal volume of LiCl solution (5M) was added and after mixing was incubated on ice for 30min. After centrifugation (the supernatant centrifugation (4,000g, 5min 4°C), the supernatant containing plasmid DNA was collected.

Further purification was made by mixing an equal volume of phenol:chloroform with the supernatant. After mixing and centrifugation, the upper layer containing DNA was separated and DNA precipitated using 2.5 volumes of 100% ethanol. After mixing the DNA solution/ethanol was incubated at -20°C for 1h to maximise precipitation. After centrifugation (4,000g, 15min 4°C), the pellet was washed with 70% ethanol and then resuspended dH2O containing RNase A at 15μg/ml for 1h. After a further phenol:chloroform extraction, plasmid DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH5.2 and 2.2 volumes of 100% ethanol. The DNA precipitated immediately and after further centrifugation (4,000g, 15min RT) and washing with 70% ethanol, removal of all ethanol by drying, the final pellet was resuspended in 10ml of 10mM Tris pH 8.0).

Purity and quantification of plasmid DNA was achieved by gel electrophoresis of dilutions of the stock DNA. The aliquots were run against a marker of known quantity/DNA concentration standard such as HindIII cutλ. Alternatively, an aliquot of DNA solution was checked by assessing absorbency A280 and A260. The A260 value was used to estimate DNA concentration.

2.11.9 Precipitation of DNA in solution

If DNA was soluble in a low salt solution, 0.1 volumes of 3M sodium acetate pH5.2 was added, mixed followed by the addition of 2.2 volumes of ethanol. The mixture was incubated at room temperature for 1-2h and then centrifuged at 10,000g for 15 min at room temperature. The supernatant was then decanted from the pellet and the pellet washed in 500 μl of ice-cold 70% ethanol to remove salts. After
further centrifugation at 10000g, 5min the pellet was air dried and re-dissolved on dH₂O or 0.1M Tris pH 8.0.

If DNA was already dissolved in a high salt solution, precipitation was achieved by adding 2 volumes of isopropanol and incubating at room temperature for 1-2h. Precipitated nucleic acids were pelleted, washed and resuspended as described for precipitation of DNA from low salt solutions.

2.11.10 Gel purification of DNA

Gel containing DNA was excised under UV visualisation and placed in a clean Eppendorf tube. After heating to 65°C to melt the gel it was mixed with binding buffer (Qiagen) and passed through an equilibrated Tip-100 ion exchange column according to manufacturer's instructions.

After elution in a high salt buffer, DNA was precipitated as described above (Chapter 2.11.9).

2.11.11 Ligation of insert DNA and plasmid vector

DNA was prepared and digested to generate insert DNA and vector DNA with compatible ends (either sticky-sticky or blunt-sticky). DNA was prepared by phenol:chloroform purification followed by precipitation by gel purification followed by precipitation and resuspension. Vector and insert DNA concentrations were estimated by running a known concentration on a gel against 500ng of *Hind*III cut λ (Appendix I). DNA was ligated at 1:3 vector:insert molar ratio. 100ng of vector DNA was used with the appropriate mass of insert DNA mixed. 1U of T4 DNA ligase with 1μl of 10x ligase buffer. The total volume of ligation was 10μl. The ligation was carried out in a PCR heating block. The temperature was varied every 30s from 14°C to 24°C over 100 cycles to maximise efficiency of ligation.

2.11.12 GENERATION OF A VECTOR FOR STABLE TRANSFECTION OF HERIRF-1 UNDER THE CMV PROMOTER

Previous studies of transfection of MC have indicated that the CMV promoter reliably resulted in adequate transcription of exogenous genes (Stylainou et al., 1999). Further evidence for this came from the GFP fluorescence study (Figure 2-14); the eGFP gene was linked to a CMV promoter. It was decided therefore to link the IRFHER gene to a CMV promoter. The eGFP vector contained a selection gene (neomycin) and the eGFP was under a CMV promoter.
Chapter 2 Methods

Figure 2-11: Diagram showing insertion of the IRFHER gene into the EGFP-N1 vector at the SacI restriction site in the multiple cloning site of the vector. Note the position of BglI in the MCS of the vector and insert which was used to determine correct orientation of the insert in the vector.

Furthermore, comparison of restriction-digest sites between GFP and 767 indicated that the IRFHER with its stop codon could be inserted between the CMV promoter and eGFP gene (Figure 2-11).

To achieve that, 15μg of 767 purified DNA was digested for 4h with the enzyme SacI in the presence of multicore buffer followed by 15 min heat inactivation of the enzyme at 65°C. This step generated two fragments, one 2065bp (2kb), the other 2780bp (3kb) (Figure 2-13). According to the restriction map, the 2kb fragment would contain the IRFHER gene. This 2kb fragment was gel purified and an aliquot of DNA was underwent a diagnostic digest with BamHI, which as expected revealed a 1116bp and a 953bp fragment (Figure 2-13), confirming the nature of the purified fragment. The uncut 2kb DNA was quantified by running 1μl of pure DNA along-side a 100bp ladder of known quantity. 1μl The 2kb fragment of 767 DNA was 75ng/μl. 5μg of the eGFP vector was digested with SacI in
Chapter 2 Methods

multicore buffer for 6h. The vector was seen to be linearised by the single site digest (Figure 2-12). The free ends of the digested DNA were de-phosphorylated to prevent re-ligation of vector alone during insertion of IRFHER. The vector was then purified by phenol/chloroform extraction and DNA was precipitated using sodium acetate.

![Figure 2-12: (A) Agarose gel (0.8%) showing DNA (Ethidium bromide). Lane 1, uncut 767 vector showing whole plasmid plus supercoiled form. Lane 2, 10mg of 767 vector digested with SacI for 6h producing two fragments. Lane 3 vector linearised with EcoRI indicating its size (approaching 5kb). (B) Agarose gel (1.0%). Lane 1 purified 2kb fragment from SacI digest of 767 vector. Lane 2 diagnostic digest of that fragment with BamHI to reveal two fragments (1116bp & 953bp). Lane 3 eGFP vector. Lane 4 eGFP vector linearised with SacI digest.](image)

Ligation was set up with insert : vector in a 3:1 molar ratio, using T4 ligase. Control ligation was set up with no insert. Ligation was carried out alternating between 14°C and 24°C for 30s at each temperature for 200 cycles, then was maintained for 8h at 16.5°C. This procedure was used to balance the enzyme activity against the need to DNA free ends to come into close apposition.

DH5α competent E. coli were transformed with half of the ligation product. The bacteria were plated onto agar plates containing the antibiotic kanamicin.

After incubation overnight, the plates from vector alone ligation contained few colonies, but the plates from vector plus insert ligation contained many colonies. 32 colonies were selected, isolated on a kanamicin agar plate and grown in broth. After 16h incubation, plasmid DNA was isolated from
bacteria by STET procedure. After RNAse treatment plasmid DNA was run on an agar gel to screen for inserts (Figure 2-14).

![Figure 2-14](image)

**Figure 2-13:** (A) Agarose gel (1.0%) showing plasmid DNA from colonies selected for insertion of the IRFHER cDNA. Note two of these plasmids travel more slowly than the others indicating inserted DNA. (B) Vectors selected for retardation in (A) were digested with BglII. Correct insertion of the cDNA results in a 5.2kb and 1.5kb fragment after digestion as in lanes 1,7,8.

From this initial screen, 9 colonies were selected on the basis of obvious retardation of DNA in the gel indicating insertion.

DNA (5μl of 50μl) from these clones was digested with BglII. Since both ends of the insert were cut to leave a SacI site, it would be possible for the insert to be inserted in either orientation. The new vector would only produce IRFHER mRNA if the insert was in the correct orientation. In this correct orientation, this digest would generate a 1552bp and a 5.2kb fragment. In the incorrect orientation, 527bp and 6.2kb fragments would be generated (Figure 2-13, Figure 2-11). Following this digest three colonies were identified as containing the inserted gene in the correct orientation (clones 5,25,29). Clone 5 was selected (eGFP/IRFHER(5)). 200mls of LB broth were incubated to achieve absorbencies
of 0.4. The plasmid DNA was purified by Qiagen Tip 100 columns and purity confirmed by relative absorbance (900ng/μl).

2.11.13 Generation of stably transfected rat mesangial cells

Prior to transfection, mesangial cells were treated for one week with G418 antibiotic to produce a dose response curve. Every 48h the medium containing antibiotic in medium was removed from the cells and replaced with fresh medium plus antibiotic. Cell growth and cell loss were assessed microscopically every 48h. Doses of G418 ranged from 100ng/ml to 1000ng/ml. It was found that at day 3 100ng/ml inhibited cell proliferation, 250ng induced cell death but that doses of 500ng/ml and greater resulted in almost complete cell loss. At day 6, 100ng/ml induced widespread cell death and viable cell loss. 250ng/ml of G418 resulted in virtually complete cell loss.

Prior to stable transfection, transient transfection of mesangial cells using different transfection techniques was employed to maximise transfection efficiency. A lipid transfection agent (Transfast, Promega) was compared with electroporation (Equibio) using the eGFP vector which, when expressed under the CMV promoter, the protein product fluoresces green in the cytoplasm. A series of studies were set up varying the parameters to maximise transfection. For the lipid reagent, this included cell density, amount of DNA duration of interaction, centrifuging the lipid vesicles onto the cells, and duration before assay. For electroporation, this included cell density, amount of DNA, voltage, impedance and time of electric current. At 48h cells were washed, trypsinised and assessed by flow cytometry (Figure 2-14). The histograms indicate that electroporation appeared to give more favourable results in terms of transfection efficiency.

T75 flasks of sub-confluent MC grown in medium with 10% FCS (5 x 10^6 cells), were trypsinised, and after neutralisation of trypsin-EDTA with full medium, pelletted (250g, 5min 4°C), then resuspended in 800μl of full medium containing 20μg of purified eGFP plasmid DNA (0.1M Tris pH 8.0). The cell suspension was placed in a sterile electroporation cuvette on ice and exposed to electric shock: 280V, 1500μF. Pulse time was calculated by the electroporator according to impedance. The pulse time was typically 20-30ms.

Immediately following electroporation, cells were resuspended in 10mls of chilled medium and returned to a T75 flask and returned to the incubator. Typically, more than 50% of cells survived and grew well. Typically, 20-50% of cells were transfected. (Figure 2-14). at 24h.
Figure 2-14: Fluorescence histograms of rat mesangial cells transfected with eGFP by electroporation (A) or lipid transfection reagent, transfast (B). Cells were transfected, then cultured for 24h. They were trypsinised and assessed by flow cytometry directly (C) Fluorescent micrograph (X200) of confluent rat mesangial cells P11 transfected with the eGFP vector by electroporation. Note intensely positive green fluorescent cells.

The vectors eGFP and eGFP/IRFHER(5) were linearised with the restriction enzyme AseI. Confirmation that the vectors were linearised was obtained by gel electrophoresis (not shown). Rat MC, passage, 11 were transfected with 20μg of eGFP or eGFP/IRFHER(5). After 48h, there were 20% of fluorescent cells in the control transfection (cells transfected with eGFP/IRFHER(5) were not
expected to fluoresce). Cells were now exposed to G418 at 250ng/ml and cultured for a further week, replacing the medium every third day. After 7d, colonies derived from single cells were seen. In the control wells, only 20% of these colonies fluoresced however. This suggested that in the non-fluorescing colonies either the eGFP expression was very low or that the eGFP gene had been switched off or deleted, whereas the neomycin resistance gene was being transcribed. This might suggest that a proportion of the colonies from the eGFP/IRFHER(5) transfected cells would also not significantly transcribe the IRFHER gene. Two approaches were made here. In one case, all cells from in excess of 10 colonies were ‘lumped’ together to generate a polyclonal stable line. Clearly all cells would have the neomycin gene, but many cells would not have expressed the IRFHER gene or the eGFP gene.

The other approach involved picking colonies. In the tissue culture hood, colonies were picked with sterile pipette tips and transferred to 96-well plates where they were trypsinised and re-plated into wells. Single colonies were expanded and then selected on the basis of green fluorescence or increased levels of spontaneous apoptosis as seen in the HERIRF-1 clones but not in the eGFP clones. Also some IRFHER clones that grew normally were selected. These clones were maintained in G418 at 100ng/ml, a dose sufficient to prevent growth of cells without the neomycin resistance gene.

2.11.14 Polymerase chain reaction methods for genomic DNA

When assessing gld/gld mice for a point mutation in the Fas gene, suitable primers were designed around the mutation. Primers were selected to maximise binding of primer, maximise specificity and minimise the possibility of the primer binding to itself.

To achieve this, certain maxims were observed (The Source of Discovery Protocols and Applications Guide. Third Edition. Promega). Primers should be 15-30bp. They should contain 40-60% G+C and care should be taken to avoid the possibility of a secondary structure. 3’ ends of primers should not be complementary to avoid dimerisation. Three G’s or three C’s should be avoided near the 3’ end. Ideally both primers should anneal at the same temperature which is dependent on the primer with the lowest melting temperature ($T_m$). The latter can be estimated:

$$T_m = 81.5 + 16.6 \times \log_{10}[\text{Na}^+] + 0.41 \times (\%G+C) - 675/n$$

[Na$^+$] = molar salt concentration. n = number of bases in the oligonucleotide
Chapter 2 Methods

Genomic DNA was prepared isolated from Mφ from wild type or gld/gld mice using a eukaryotic DNA extraction system from Qiagen. Following analysis of the published gene and mutation (Takahashi et al., 1994), a 311 bp segment, covering the predicted mutation site of the gene, was amplified by PCR using the primers 5'-GTGGCCTTGATCAACG-3' and 5'-CTCTGGAGTGAAGTATAAG-3'. These primers were generated at Oswel laboratories.

Due to the low G+C content of the primers, it was determined that a low annealing temperature would be required.

The PCR reaction was prepared:

5μl of DNA solution, 5 μl 10x PCR reaction buffer (Appendix 1), 2 μl dNTPs (5mM of each), 1.5 μl MgCl₂ (50mM) final concentration 1.5mM, 5μl Forward primer (100pmoles), 5μl Reverse primer (100pmoles), made up to 49μl with dH₂O.

After mixing and centrifuging, then allowing the primers to anneal 1U (1μl of Taq polymerase was added).

The following conditions were found to be optimal:

95°C, 5min, 55°C 90s followed by addition of Taq, 72°C 90s, 95°C 90s, 55°C 90s for 36 cycles.

2.11.15 Purification of mRNA from eukaryotic cells

The method used in this thesis involved extraction of total RNA which contains ribosomal as well as messenger RNA. All solutions prepared were fresh-opened, guaranteed RNase free or DEPC treated prior to usage (0.01% diethyl pyrocarbonate was added to water in RNase free glass ware and left to stand overnight, before autoclaving. Sub-confluent mesangial cells growing in a monolayer in T75 flasks were separated from overlying medium on ice, then trizol (Gibco 15596) was added to the cells using appropriate caution for phenol containing compounds. 1ml of trizol was used for each 10cm² of cells. After scraping, the cell suspension on ice was passed through a pipette several times. This cell suspension was frozen (-80°C) until required.

The suspension was incubated at room temperature for 5min, then 0.2ml of chloroform was added per ml of trizol. Tubes containing the mixture were shaken and incubated for 2/3min at room temperature. This was then centrifuged at 10000g for 15min, 4°C. The top, aqueous phase now containing RNA was separated from the lower layer containing DNA and protein and RNA was precipitated by adding 0.5ml of isopropanol per 1ml of trizol. The mix was incubated for 10min room temperature, then spun...
12,000g, 10 min 4°C. The supernatant was discarded, the pellet washed with 75% ethanol and spun at 7,500g for 5min, 4°C. The pellet was air dried for 5 min then dissolved in RNase free water or TE (20µl/1ml of trizol, and incubated at 55°C for 10min. The RNA was then frozen (-80°C) for storage.

RNA was quantified using $A_{260}$. Absorbance of 1.0 represents 40µg/ml of single stranded RNA. Purity ($A_{260}/A_{280}$) should be 2.0. Purity was also demonstrated using gel electrophoresis (Figure 2-15)

**Figure 2-15:** Agarose gel electrophoresis of RNA extracted from rat mesangial cells. Note distinct bands of rRNA (28s and 18s).

### 2.11.16 Polymerase chain reaction methods for mRNA

Moloney murine leukaemia virus reverse transcriptase (Superscript II-Gibco 18064-022) was used to produce cDNA from the mesangial cell RNA preparation. To initiate reverse transcription an oligo dT (12-14) primer was used which binds to the poly-A tail of mRNA.

In some experiments total RNA was treated with RNase free DNase (Promega) for 30min to remove any stray plasmid DNA.

1µg of total RNA was added to the oligo dT primer (0.5µg/µg RNA) The total volume was made up to 12µl with nuclease free water. This mix was heated to 70°C for 5min in a heating block, then chilled on ice to achieve annealing. To this was added:

2µl 0.1M DTT, 1µl dNTP mix (10mM of each), 4µl of 5x Superscript II buffer (250mM Tris-HCl pH8.3, 375mM KCl, 15mM MgCl$_2$)
Chapter 2 Methods

The reaction mixture was incubated at 42°C for 1h and stopped by heating to 70°C for 15min.

Murine IRF-1 primers were designed based on the cDNA sequence for murine IRF-1 and expected to give a 477bp product in a PCR reaction (Appendix 1). In preliminary experiments, using these primers with aliquots of plasmid DNA containing the IRFHER gene, PCR was maximised. The following maximised PCR system was used thereafter.

The PCR reaction was set up on ice:

2.5μl of 10X PCR reaction buffer (200mM Tris pH 8.4, 500mM KCl) was mixed with 4μl (20%) of the cDNA derived from one T75 flask of cells, 2 x 1μl of primer solutions at 10μM, made up to 25μl with water. For negative controls 1μl of RNA solution was used in place of cDNA. The mix was heated to 95°C for 2min then held at 80°C.

In a separate tube:

2.5μl of 10X PCR reaction buffer (200mM Tris pH 8.4, 500mM KCl) was mixed with 1.5μl of MgCl₂ (50mM stock) to give a final concentration of 1.5mM, 0.5μl of dNTP (20mM of each stock), Taq polymerase 0.2μl and made up to 25μl with water.

The contents of the second tube were combined with the first at 80°C and 35 cycles of PCR were initiated at 96°C (30s) followed by 60°C, (30s), followed by 74°C (45s). After these cycles the product was heated to 74°C for 5min then returned to room temperature.

2.12 IMMUNOHISTOCHEMISTRY

2.12.1 Proliferating cell nuclear antigen

Assessment of mitosis was made by specific identification of proliferating cell nuclear antigen (PCNA) in mesangial cells using PC10 antibody (DAKO) in fixed wells of 96-well plates. Briefly, following quenching of endogenous peroxidases with 3% hydrogen peroxide in methanol (5min, 20°C), and permeabilisation with 0.1% Triton-X 100, 0.1% sodium citrate in PBS (5min, 20°C), the cell monolayer was blocked for 30min at room temperature with 10% new-born calf serum (NBCS) in PBS. The primary antibody was then applied 1:50 in PBS, 10% NBCS (4h, 20°C) or the appropriate irrelevant control. After washing wells x3 with PBS containing 0.05% Tween 20, specific localisation was assured incubating with biotinylated anti-immunoglobulin (1:1000) in PBS/NBCS. After further
Chapter 2 Methods

washing steps x 3, streptavidin-peroxidase was added at 1:10000 (Sigma) in PBS, followed by the addition of the substrate AEC (Vector).

2.12.2 Anti-α-smooth muscle actin

Human, rat and mouse mesangial cells were all labelled with anti-α-smooth muscle actin (Sigma). Sub-confluent cells in wells were fixed with formaldehyde (4% in PBS) and permeabilised with ice-cold methanol (5min). After drying, wells were blocked for 30min at room temperature with 10% new-born calf serum (NBCS) in PBS. After removing, the primary antibody (1:400) in PBS/NBCS, or the appropriate irrelevant control, was applied overnight at 4°C. After washing x 3 with PBS and 0.05% Tween 20, a secondary FITC-conjugated anti-mouse IgG was applied at 1:1000, again in PBS/NBCS. After 1h, room temperature, wells were washed x 3 in PBS and then mounted with DABCO-based fluorescent mountant for viewing (Chapter 2.7.1).

2.12.3 Anti-ED-1

Rat macrophages in culture and early cultures of mesangial cells from whole glomerular outgrowths were assessed for ED-1 positive cells (macrophages). The primary antibody was anti CD120 (Serotec). Wells containing cells were fixed and permeabilised as in Chapter 2.12.2. After blocking with PBS/NBCS for 30 min room temperature the primary antibody, or the appropriate irrelevant control, was added in blocking solution (1:50) and cells were incubated overnight at 4°C. After washing steps, secondary antibody, either FITC-conjugated or TRITC-conjugated anti-mouse immunoglobulins (1:1000) was applied for 1h at room temperature. Following further washing x 3, cells were mounted as above for viewing.

2.12.4 Anti-Thy1.1

Rat mesangial cells were labelled with this antibody (Serotec) as confirmation of their phenotype. Cells were prepared as described above. However, these cells were not permeabilised due to the cell-surface location of the antigen. After blocking, cells were incubated with the antibody or appropriate control diluted 1:100 overnight at 4°C. After washing x 3 as above cells were labelled with FITC-conjugated anti mouse immunoglobulin (1:1000) in PBS/NBCS for 1h at room temperature. After further washing and mounting cells were viewed.
2.12.5 Anti-cytokeratin

Mesangial cell cultures (human and rat) were labelled with this antibody (Sigma) Cells were labelled as described above using primary antibody at 1:300. Cos cells (monkey kidney tubular epithelial cells) were used as a positive control. After washing and labelling with the secondary antibody, there was no evidence of specific binding to any cells in the mesangial cell population.

2.12.6 Anti iNOS-haem

Both macrophages and mesangial cells were labelled with this antibody. Fixed, permeabilised cells in wells were blocked with PBS/NBCS blocking solution for 30min room temperature. The rabbit anti serum was diluted 1:400 and applied in blocking solution to the cells overnight 4°C. After washing x 3 with PBS/Tween 20, cells were incubated with FITC or TRITC-conjugated anti rabbit immunoglobulins (DAKO) (1:40) diluted in blocking solution for 1h. After further washing (PBS) cells were mounted with the DABCO-based mountant.

13. FLOW CYTOMETRIC IMMUNOFLUORESCENCE

2.13.1 Anti-Fas

Human mesangial cells were grown to 80-90% confluence in 6-well plates, then trypsinised using standard TE. Immediately after loosening the cells were placed into polystyrene flow cytometry tubes (Falcon) in 50% medium containing FCS to neutralise the trypsin and EDTA effect, on ice. Suspended cells were centrifuged (200g, 5min, 4°C), and the medium overlying aspirated. The cells were then suspended in 200μl of PBS containing 10% NBCS. After 20min on ice, the primary monoclonal antibody, CH11 (TCS laboratories) was added (1μg/ml, 1:500). Appropriate IgM control was added. After incubating the live cells for 1h on ice, the polystyrene tubes were filled with 3ml of ice-cold PBS to wash, then centrifuged again as above. After gentle aspiration of medium cells were resuspended in 200μl of PBS/NBCS containing FITC-conjugated anti IgM λ at 1:1000. After 30 min on ice cells were taken to the flow cytometer for analysis after adding a further 400μl of FACS flow solution (Becton Dickinson).
Chapter 2 Methods

As a control for the effect of trypsin on Fas cleavage, Jurkat cells, known to express Fas on the cell surface treated in the same way and used as a positive control.

2.13.2 Anti TNFR1

Sub-confluent human MC proliferating in T75 flasks were trypsinised and centrifuged as described above (Chapter 2.13.1). For these experiments, cells were resuspended in ice-cold FACS flow solution. This solution is PBS, filter-sterilised containing bovine serum albumin (5%) and NaN₃ (0.1%). This solution was used in preference to the PBS/NBCS in an attempt to reduce background auto-fluorescence. Cells were re-suspended in 200µl of the FACS flow containing 10% mouse serum, on ice. The mouse serum was utilised to specifically block Ig receptors on the surface of mesangial cells which might compete with the primary antibody binding.

After 20 min blocking on ice, the primary antibody (1:200, 5µg/ml) or irrelevant control mouse IgG₁. After 1h incubation on ice, cells were washed by adding 3ml of ice-cold FACS flow to each polystyrene tube, followed by centrifugation (200g, 5min, 4°C). Cells were resuspended in 200µl of FACS flow containing FITC-labelled secondary anti mouse IgG (DAKO 1:1000) and after 30 min washed again followed by centrifugation. The cells were again resuspended and analysed by flow cytometry.

2.13.3 Anti F480

Mouse bone marrow-derived macrophages were phenotypically defined by expression of cell surface staining for the protein antigen F480. Live cells were prepared as described for cell surface labelling of mesangial cells (Chapter 2.13.2). After preparation, cells were suspended in FACS flow solution and incubated with anti-F480-FITC (Serotec) or irrelevant rat IgG at 1:50 dilution in FACS flow containing 10% rat serum. After 1h, cells in polystyrene tubes were washed with FACS flow (3mls per tube) and re-pelleted (220g, 5min, 4°C). Since the antibody was directly conjugated cells were resuspended and assessed by flow cytometry. A one log₁₀ order shift in green fluorescence was seen in the whole cell population confirming a homogenous population of macrophages compared with incubation with FITC-conjugated irrelevant IgG.
2.14 ACCELERATED NEPHROTOXIC NEPHRITIS IN THE RAT

2.14.1 Induction of accelerated nephritis

Male Sprague-Dawley rats (weight 180-200g) were immunised by injection of 1mg normal rabbit IgG, in Freund’s complete adjuvant (Difco) subcutaneously in the flanks. After seven days, the rats were injected with 1ml of nephrotoxic serum intravenously. Nephrotoxic serum was generated by repeated (x 3) injections subcutaneously of 5mg of crude extract of rat glomerular basement membrane, into rabbits. The initial dose and boosters were given at monthly intervals. The rabbits were sacrificed and serum obtained by exsanguination. The serum was pre-absorbed against rat red cells obtained from SD rats. Briefly, the serum was incubated overnight at 4°C with whole red cells, before centrifugal separation. Dilutions of serum were assayed with red cells at 37°C to determine whether anti-red cell antibodies remained. If significant red cell lysis occurred at dilutions greater than 1:3, the serum was absorbed again with rat whole red cells.

2.14.2 Assessment of proteinuria and renal function

The night prior to experimental time point, animals were placed in individual metabolic cages. Urine was collected in metabolic cages for 18h and frozen. At a later time point albuminuria was confirmed by rocket immuno-electrophoresis (performed by hospital laboratory).

2.14.3 Histology

Animals were killed, and cortical biopsies were taken for histology (paraffin sections), standard haematoxylin and eosin staining which confirmed severe proliferative glomerulonephritis. Biopsies were placed in Methyl Carnoy’s fixative and stored at -80°C, and also were placed in mountant and frozen directly in liquid nitrogen, before being transferred to -80°C for storage prior to preparation of fresh-frozen sections.

2.15 ANALYSIS OF DATA

Experimental data were expressed descriptively as mean values with standard error of mean demonstrated using error bars or preceded by ± symbol.
Experimental data were compared using student’s t test for comparison of two variables. In cases where multiple variables were tested, ANOVA was utilised.

Significance $P < 0.05$ was denoted by *, $P < 0.01$ by ** and $P < 0.001$ by ***.
Chapter 3: Results 1

Inflammatory macrophages induce apoptosis of mesangial cells
3.1 QUIESCENT OR UNACTIVATED RODENT MACROPHAGES DO NOT INFLUENCE RAT MESANGIAL CELL APOPTOSIS

Twenty thousand, seven day rat bone-marrow-derived (BMD) macrophages (MΦ) were plated into wells of 0.3cm² (96 well plate) overnight in full (i.e. containing 10% FCS) DMEM/F12 and left to spread for 16h. Rat mesangial cells (MC), pre-labelled with green CMFDA were trypsinised. Twelve thousand were added to wells containing MΦ in 200μl of medium, or to adjacent control wells containing medium with no MΦ. Once adherent (2-3h) wells were washed, and 200μl of full DMEM/F12 medium replaced. Cells were incubated at 37°C, then either fixed at 8h and 24h or assessed live at 24h. Later time-points were hindered by overgrowth of MC when contact inhibition lead to a reduction in mitotic figures seen.

Experiments carried out in this manner were assessed for apoptotic and mitotic MC by inverted fluorescence microscopy of both live cultures and formaldehyde fixed cultures by counterstaining with both propidium iodide which binds to DNA and RNA and fluoresces red, and Hoechst 33342 which binds to DNA and fluoresces blue (Figure 3-1, Figure 3-2). Mesangial cells were discernible from MΦ due to their green fluorescence from the CMFDA.

Figure 3-1: Fluorescent photomicrograph (X 200) showing fixed co-culture of CMFDA green-labelled rat MC co-cultured with rat BMD MΦ (appearing red/orange due to PI staining of cytoplasmic RNA. Note presence of MC undergoing mitosis (arrow) and an absence of apoptotic cells
Chapter 3: Results

Inflammatory macrophages induce apoptosis of mesangial cells

1. Inflammatory macrophages induce apoptosis of mesangial cells.

2. Results:
   - 3.0% apoptosis in coculture.
   - 2.0% apoptosis in MC alone.
   - 1.0% apoptosis in coculture.

3. Figure 3-2:
   - MΦ do not influence apoptosis or mitosis of proliferating MC in co-culture. Rat BMD MΦ (2x10⁴ per well) were co-cultured with CMFDA-labelled rat MC (1.2 x 10⁴ per well) in the presence of full DMEM/F12, and compared with 1.2 x 10⁴ MC per well growing alone without MΦ. (A) At 8h and 24h live co-culture was assessed by Hoechst and PI staining by fluorescence microscopy for apoptotic green MC. After fixation with formaldehyde solution and counter staining with PI and Hoechst, co-culture was scored morphologically for (B) MC apoptosis and (C) MC mitosis. Note no significant difference between co-culture and MC alone (n=4).
Performing the co-culture in 8.0cm² wells (6-well-plate) without pre-labelling mesangial cells with CMFDA enabled the whole co-culture to be assessed by flow cytometry once the cells in the well were trypsinised. It was found that the best flow cytometric assessment for mitotic and apoptotic cells was propidium iodide (PI) staining of RNAse treated, ethanol fixed cells. This labelled the nuclei of cells which the cytometer could detect by nuclear size. *n* = cells in G₀/G₁ of the cell cycle. 2n = cells in G₂/M of the cell cycle and < n = hypodiploid cells, representing those undergoing apoptosis (Figure 3-3). Other flow cytometric assays of MC apoptosis such as Annexin-V detection of phosphatidyl-serine exposure on the outer membrane was unsuccessful largely due to the high auto-fluorescence of cells and the large spread of fluorescence in the population.

![Figure 3-3: Assessment of co-culture by flow cytometry. 6 x 10⁵ mouse Mϕ were co-cultured with 3.5 x 10⁶ unlabelled rat MC in full DMEM/F12 according to Methods. After trypsinising the whole co-culture, processing and labelling nuclei with PI they were analysed by flow cytometry (A). The co-culture was compared to 3.5 x 10⁶ MC growing alone (B). Note there was no difference in the population of cells in G₂M nor hypodiploid cells. Note two diploid peaks representing rat and mouse nuclei.](image)

Data from microscopical assessment of co-culture compared with MC growing alone, and also data from flow cytometric analysis indicated that there was no evidence of significant difference in the appearance mitotic or apoptotic MC. Therefore, quiescent Mϕ did not appear to have any effect on MC (Figure 3-2, Table 3-1).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Experiments were repeated, but after washing the established co-culture, medium was replaced with DMEM/F12 containing 1% FCS. At 24h, although the background level of apoptosis in control wells had increased, there still did not appear to be any difference between co-culture and MC growing alone in the occurrence of these parameters (Table 3-2).

Table 3-1: Flow cytometric analysis of cellular DNA content in unactivated rodent co-culture

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated co-culture</th>
<th>Unstimulated MC alone</th>
<th>Unstimulated Mφ alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>% G2/M-phase cells</td>
<td>18.1±1.5%</td>
<td>19.4±1.1%</td>
<td>2.7±1.4%</td>
</tr>
<tr>
<td>% hypodiploidy</td>
<td>0.6±0.3%</td>
<td>0.7±0.2%</td>
<td>0.3±0.1%</td>
</tr>
</tbody>
</table>

Table 3-2: Microscopical assessment of rat MC apoptosis and mitosis in medium with 1% FCS.

<table>
<thead>
<tr>
<th></th>
<th>Co-culture</th>
<th>MC alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoptosis</td>
<td>10.9±0.7%</td>
<td>7.6±2.4%</td>
</tr>
<tr>
<td>mitosis</td>
<td>0.9±0.2%</td>
<td>0.5±0.3%</td>
</tr>
</tbody>
</table>

3.2 CYTOKINE ACTIVATED RODENT Mφ INDUCE APOPTOSIS AND CELL CYCLE ARREST OF RAT MC

Having established the co-culture assay, attempts were made to activate Mφ to determine whether activated Mφ had any effect on rat MC. Initially, rat recombinant IFN-γ was applied. Since in the inflamed kidney, MC adjacent to Mφ would be exposed to the same cytokines, the whole co-culture was exposed to the cytokines. This meant that controls of Mφ alone and MC alone, exposed to the same cytokines would have to be assessed.

Initial pilot studies involved priming BMD Mφ for 16h with IFN-γ rat once they were seeded into wells. One hundred U/ml and 500U/ml were tested. Once the co-culture was established and washed,
the IFN-γ was replaced. The co-culture was assessed microscopically at 8 and 24h. There appeared to be a small effect: at 24h there was a decrease in the appearance of mitotic figures. However, this effect was not uniformly reproducible. When mouse (C57Bl6) BMD Mφ were used in the same numbers in co-culture with the rat MC there was no effect from IFN-γ (rat IFN-γ is equally efficacious when binding to the murine IFN-γ receptor). Given the variability of this effect, it was possible that some of the IFN-γ effect seen with rat Mφ might be due to low grade LPS contamination (Erwig et al., 1999) acquired during collection of bone marrow.

Studies suggested that IFN-γ priming of Mφ switched the phenotype enabling other signalling molecules to activate the cell (Riches 1995; Erwig et al., 1999). To obtain full activation therefore, Mφ were primed as described above. Once the co-culture was established IFN-γ was replaced with IFN-γ plus TNF-α, IFN-γ plus lipopolysaccharide (LPS-from Escherichia coli serotype 026:B6) or IFN-γ plus CD40L. As controls, LPS, TNF-α and CD40L were assessed in co-culture alone without IFN-γ priming. In addition, MC growing alone, without Mφ were exposed to the activating cytokines. A variety of doses were assessed and it was determined that maximum effects of TNF-α were seen at 100U/ml, and for LPS this was 500ng/ml-1μg/ml of this particular preparation. 100ng/ml of LPS had approximately 20% of the effect of 1μg/ml and 1ng/ml had no discernible effect over IFN-γ alone. CD40L worked at 10ng/ml.

The activated co-culture was assessed at 8h, 16h, and 24h by fluorescence microscopy initially and later by flow cytometric analysis. At 8h there was suppression of MC mitosis, at 16h, there was additionally the appearance of apoptotic MC, which was more striking at 24h (Figure 3-4, Figure 3-5). Control wells showed no significant increase in MC apoptosis over this time-period. LPS alone activating co-culture suppressed mitosis and weakly induced apoptosis (not shown).

Assurance that the cells seen microscopically after fixation with formaldehyde and counter-staining with propidium iodide were truly apoptotic was concluded from studying live co-culture at 24h, when small rounded-up cells, loosely adherent to the monolayer were seen. In live co-culture, the rounded up cells appeared intensely green due to the presence of CMFDA. Addition of propidium iodide (PI) (1μg/ml) and Hoechst 33342 (1μg/ml) to individual wells enabled further assessment of these cells after a five minute period to allow diffusion of the reagents. Hoechst can be used to label DNA of fixed cells as it intercalates with DNA but not RNA. However, it has another property: live cells are able to exclude it from the cytosol by means of membrane pumps (Sun et al., 1992), whereas apoptotic cells (and necrotic cells) cannot. Therefore after 5min exposure, apoptotic DNA will fluoresce brilliantly (365nm excitation) but live and mitotic cells will scarcely fluoresce. PI, on the other hand, is excluded
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

from both live and apoptotic cells but can enter post apoptotic or necrotic cells due to increase membrane permeability. The results of such studies revealed three important pieces of data:

The vast majority of green rounded-up cells seen in live co-culture were able to exclude PI, but they all fluoresced with Hoechst (Table 3-3, Figure 3-6).

![Figure 3-4:](image)

**Figure 3-4:** Activated co-culture leads to MC apoptosis and suppression of mitosis. Rat BMD Mφ were co-cultured with CMFDA-labelled rat MC in a 1.5 : 1.0 ratio (Methods). Once established the co-culture was activated with IFN-γ (100U/ml) plus LPS (1µg/ml). (A) The effect of Mφ on MC apoptosis at 8h and 24h. Note induction of apoptosis at 24h. (B) The percentage of mitosis at 8h and 24h, note suppression of mitosis at both time points. (C) The effect of activated Mφ on MC number. Note a decrease in mesangial cells during the time points assayed compared with the expected increase. * P < 0.01 vs. control MC alone (n=4).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

After fixation of the same wells with formaldehyde and counter-staining with PI (now all cells admitted PI which binds to both DNA and RNA) the same cells that appeared apoptotic when assessed morphologically (fixed experiments), were scored as apoptotic when assessed functionally in the live culture (Table 3-3, Figure 3-6).

There was no difference in counting total apoptotic cells as assessed by Hoechst when compared with counting apoptotic MC only demonstrating that Mφ do not undergo apoptosis in this assay (Table 3-3).

Assessment of the whole co-culture by flow cytometry again using PI to bind DNA only, revealed an increase in hypodiploid cells that was attributable to apoptotic mesangial cells alone (since there was no evidence of Mφ apoptosis in wells of activated Mφ alone or Mφ apoptosis in co-culture when assessed microscopically). Although not all Mφ in wells were loosened when the co-culture is trypsinised for this assay, their presence is seen by the double G1 peak. These Mφ diluted the absolute level of MC apoptosis as assessed by this method (Figure 3-7, Table 3-4).

### Table 3-3: Percentage apoptotic rat MC in live co-culture by exclusion of propidium iodide but not Hoechst 33342

<table>
<thead>
<tr>
<th></th>
<th>Hoechst +ve cells</th>
<th>PI +ve cells</th>
<th>Apoptotic MC in fixed co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated co-culture</td>
<td>17.9±1.4%</td>
<td>2.7±1.4%</td>
<td>16.0±2.6%</td>
</tr>
</tbody>
</table>

Several other methods were assessed for measuring apoptosis of mesangial cells in co-culture. These included TUNEL staining (Gorczyca et al., 1993), JC-1 labelling (Macho et al., 1996), apo-3 staining and assessing cells for DNA fragmentation using a thymidine labelling, glass fibre method (Erusalimsky et al., 1996). However, these assays either did not work (apo-3 assay, and fragmentation assay due to presence of FCS) or were insensitive due to loss of delicate apoptotic cells during processing (TUNEL and JC 1). It was found that the most sensitive assay was blinded microscopical counting of live/fixed wells with verification from the hypodiploid study (which also had the advantage of assessing cells in G2/M.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-5: Fluorescent micrograph of activated rat co-culture of BMD Mφ with CMFDA-labelled MC. (A) At 24h of co-culture, after fixation and counter-staining with PI, the co-culture shows many green rounded-up cells with evidence of red nuclear condensation (X200). (B), A Mφ adjacent to a green MC showing cytoplasmic blebbing, characteristic of early apoptosis (X1000).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Table 3-4: The effect of activated rodent co-culture on percentage G2/M and hypodiploid MC

<table>
<thead>
<tr>
<th></th>
<th>unstimulated co-culture</th>
<th>activated co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>% G2/M-phase cells</td>
<td>18.1±1.5</td>
<td>10.4±1.4*</td>
</tr>
<tr>
<td>% hypodiploidy</td>
<td>0.6±0.3</td>
<td>6.2±2.2*</td>
</tr>
</tbody>
</table>

Activated MC alone showed 0.6±0.3% hypodiploidy and 18.1±1.5% G2/M cells. Mφ alone showed 0.3±0.1% hypodiploidy, with out cells in G2/M.

For further verification of the suppression of mitosis, co-cultures were stained immuno-histochemically for the proliferating-cell nuclear antigen (PCNA). The staining procedure results in loss of apoptotic cells from the wells. However, counter-labelling with anti α-smooth muscle actin to distinguish MC from Mφ enabled scoring rat MC for PCNA positivity. The results confirmed a reduction in PCNA positive MC in co-culture (Table 3-5). However, this was only seen at 24h, not 8h, which may reflect the duration of PCNA activity during the cell cycle. Assessment was made by microscopical counting of fixed, labelled wells for PCNA-positive MC. Interestingly, Mφ, which were not seen to undergo mitosis in activated co-cultures, though an occasional mitosis was observed in quiescent co-cultures, were uniformly PCNA positive. This observation may be relevant to findings of PCNA positive Mφ (by dual labelling in sections from patients with human glomerulonephritis (Lan et al., 1995). Thus PCNA Mφ might not necessarily be proliferating. The three assays of MC proliferation described, all indicate that in co-culture with activated Mφ MC are prevented from proliferating.

Table 3-5: The effect of co-culture on PCNA-positivity of rat MC at 8h and 24h

<table>
<thead>
<tr>
<th></th>
<th>unactivated co-culture</th>
<th>activated co-culture</th>
<th>activated MC alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA positive MC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8h</td>
<td>91.6±2.0%</td>
<td>87.5±0.3%</td>
<td>95.7±1.0%</td>
</tr>
<tr>
<td>24h</td>
<td>88.6±2.4%</td>
<td>12.6±4.4%**</td>
<td>90.6±1.8%</td>
</tr>
</tbody>
</table>

*P < 0.01 n=6
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

One important observation throughout was the lack of Mϕ apoptosis. Observations of the development of BMD Mϕ in wells showed a proliferative burst from days 2-4 of differentiation. Thereafter, there was a steady decline in Mϕ numbers. These cells were lost by low-grade apoptosis (Mangkornkanok et al., 1975; Daems et al., 1976). Similar loss of human monocytes during differentiation to Mϕ can be seen and similar observations have been made by others (Flad et al., 1999; Kiener et al., 1997). There are publications (Lan et al., 1997; Nitsch et al., 1997) indicating the activated Mϕ can undergo apoptosis, which may be TNF-mediated. However, when Mϕ alone were seeded to wells, washed and activated (IFN-γ plus LPS), then assessed for apoptosis at 24h, by fluorescence microscopy, there was consistently <0.5% apoptosis. In co-culture, assessment of total apoptotic cells in live co-culture (by Hoechst staining) showed no significant difference when compared to apoptotic MC as assessed by morphological assessment of fixed wells (Table 3-3). Together these results indicated that the cell type undergoing apoptosis was almost exclusively the MC.

The MC used in this assay were derived as outgrowths from whole rat glomeruli from the Sprague Dawley rat. To be sure that the effect of Mϕ on MC seen was not simply an artefact of this particular cellular outgrowth, two other outgrowths were derived; one from the Sprague Dawley rat and the other from the Wistar rat. Initially, these cells grew very slowly but after the fourth passage they grew much quicker and were duly used in the co-culture assay, as described above. On this occasion, the rat Mϕ were primed for 16h with IFN-γ, then activated using IFN-γ and LPS once the co-culture was established (Table 3-6). Both these rat MC types were induced into cell cycle arrest and apoptosis by co-culture with Mϕ, confirming this as a general effect.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-6: Confirmation of MC apoptosis in live co-culture at 24h. (A) (X 500) green rounded up MC exclude PI (i), but do not exclude Hoechst 33342 (ii), characteristic of apoptosis. After fixation and counter-staining the same cells reveal the morphological criteria of apoptosis (iii). Note some rotation of apoptotic cells during fixation. (B) At lower power (X 320) one can clearly see apoptotic cells (Hoechst positive only) but they exclude PI (i). After fixation and counter-staining the apoptotic green MC can be seen amongst live Mφ (appearing orange post apoptotic cells (ii)).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-7: Fluorescence intensity histograms showing G<sub>0</sub>/G<sub>1</sub>, G<sub>2</sub>/M and hypodiploid cell DNA content in (A) quiescent co-culture, and (B) activated co-culture. In activated co-culture, the percentage of hypodiploid cells (M1) was 9.1% compared with 0.47% in quiescent cultures. These figures correlated well with percentage apoptotic cells as assessed by fluorescent microscopy of live cultures. UV induction of MC apoptosis was used as a positive control (not shown).

Table 3-6: The effect of activated co-culture on apoptosis of primary polyclonal rat MC at 24h

<table>
<thead>
<tr>
<th></th>
<th>Unactivated co-culture</th>
<th>activated co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MC apoptosis</td>
<td>1.2±1.0%</td>
<td>7.9±1.2%*</td>
</tr>
<tr>
<td>% MC mitosis</td>
<td>2.0±0.6%</td>
<td>0.1±0.1%**</td>
</tr>
<tr>
<td>MC growing alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MC apoptosis</td>
<td>1.2±0.5%</td>
<td>1.0±0.3%</td>
</tr>
<tr>
<td>% MC mitosis</td>
<td>1.8±0.7%</td>
<td>2.5±0.4%</td>
</tr>
</tbody>
</table>

* P < 0.05 ** P < 0.01 (n=6)
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

3.3 SUPERNATANTS FROM ACTIVATED RODENT MΦ HAVE NO EFFECT ON RAT MESANGIAL CELL CYCLE OR APOPTOSIS

In order to determine the mechanism by which MΦ were inducing apoptosis and cell cycle arrest of MC initial studies focussed on whether supernatants from activated MΦ could 'carry over' the effectors from MΦ to MC. That is, whether the effectors were soluble (as opposed to membrane bound) and stable.

1.5 x 10^5 MΦ in culture in wells of 24-well plates (2.0cm^2) were cultured in 500µl of full DMEM/F12 for 24h either with IFN-γ/LPS activation or without (carriage medium only).

After 18h, the supernatants were collected, centrifuged (4°C 5min 4000g) to remove any cell debris. 200µl was transferred to rat MC that had been seeded into wells of 96-well-plates at a 70% density. Cultures were continued for 24h, and as controls fresh medium containing IFN-γ/LPS were placed on adjacent MC. At 24h, wells were fixed and assessed for MC apoptosis (Table 3-7). Supernatants transferred from MΦ to MC did not have the ability to induce MC apoptosis or cell cycle arrest over 24h, indicating the need for close apposition or contact between the two cell types.

<table>
<thead>
<tr>
<th></th>
<th>8h incubation</th>
<th>24h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activated MΦ supernatant</td>
<td>Activated MΦ supernatant</td>
</tr>
<tr>
<td>% MC apoptosis</td>
<td>4.6±3.3%</td>
<td>5.1±3.6%</td>
</tr>
<tr>
<td>% MC mitosis</td>
<td>2.3±1.7%</td>
<td>2.3±1.6%</td>
</tr>
<tr>
<td>Control supernatant</td>
<td>Control supernatant</td>
<td></td>
</tr>
<tr>
<td>% MC apoptosis</td>
<td>5.4±3.8%</td>
<td>4.0±2.8%</td>
</tr>
<tr>
<td>% MC mitosis</td>
<td>2.1±1.4%</td>
<td>2.5±2.0%</td>
</tr>
</tbody>
</table>

Note relatively high background levels of apoptosis. No significant differences.
3.4 Mφ INDUCTION OF BOTH CELL CYCLE ARREST AND APOPTOSIS IS BLOCKED BY INHIBITORS OF NITRIC OXIDE GENERATION

Given the necessity for co-culture rather than supernatant transfer, it was decided to use simple soluble inhibitors of candidate effectors, which could be added to the co-culture at the time of activation. In the literature, there are reports of nitric oxide inducing cell cycle arrest and apoptosis of mesangial cells (Muhl et al., 1996). This was obviously a candidate. Furthermore, activated Mφ are known to undergo a respiratory burst in certain circumstances (Johnson et al., 1987) and both Cook et al and Johnson et al have noted a potential role for oxygen radicals as Mφ effectors in glomerulonephritis (Cook et al., 1989; Johnson, Guggenheim et al., 1988). Initially blocking studies were set up adding L-monomethyl arginine, a competitive nitric oxide synthase inhibitor at 500μM (as suggested by Mannick et al., 1994; Waddington et al., 1996), using D-monomethyl arginine as a control (inactive) amino acid. Enzymes, degrading oxygen radical effectors, super oxide dismutase and catalase were also added at 500U/ml (effective concentrations suggested by Sugiyama et al (Sugiyama et al., 1996)) (Table 3-8). These initial studies indicated a potent effect of L-NMMA on MC apoptosis in co-culture.

Table 3-8: The effect of nitric oxide and super-oxide radical inhibitors on induction of rat MC apoptosis in co-culture with Mφ at 24h

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activated co-culture</th>
<th>Activated MC control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.4±2.4%</td>
<td>1.2±1.4%</td>
</tr>
<tr>
<td>L-NMMA (500μM)</td>
<td>2.2±2.1%*</td>
<td>1.6±1.5%</td>
</tr>
<tr>
<td>D-NMMA (500μM)</td>
<td>13.4±1.9%</td>
<td>1.9±2.5%</td>
</tr>
<tr>
<td>S.O.D. (500U/ml)</td>
<td>11.9±3.1%</td>
<td>0.9±1.1%</td>
</tr>
<tr>
<td>Catalase (500U/ml)</td>
<td>13.1±1.2%</td>
<td>1.3±0.3%</td>
</tr>
<tr>
<td>S.O.D. plus catalase (500U/ml)</td>
<td>14.1±2.1%</td>
<td>1.2±0.9%</td>
</tr>
</tbody>
</table>

*P < 0.01 (n=4)
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

To determine the effective concentration of L-NMMA activated co-cultures were set up with a range of doses of the inhibitor (Figure 3-8). This shows an IC$_{50}$ of 5.1±3.6µM and total inhibition of Mφ killing of MC with 100µM of inhibitor. At 24h the NOS inhibitor L-NMMA (100µM), but not its inactive analogue D-NMMA was able to almost completely reverse the Mφ effects on MC apoptosis and mitosis. In fact, MC grew as though Mφ were not present (Table 3-9).

![Figure 3-8](image)

Figure 3-8: Dose response curve showing the dose of L-NMMA required to inhibit rat MC apoptosis in activated co-culture at 24h (IC$_{50}$ 5.1±3.6µM) (n=4).

<table>
<thead>
<tr>
<th>L-NMMA</th>
<th>D-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co-culture</td>
</tr>
<tr>
<td>% Apoptosis</td>
<td>2.6 ± 1.2</td>
</tr>
<tr>
<td>% Mitosis</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Cell number</td>
<td>908 ± 104</td>
</tr>
</tbody>
</table>

Table 3-9: The effect of L-NMMA and D-NMMA on activated Mφ-induced induction of rat MC apoptosis and suppression of mitosis in co-culture at 24h.

After pre treating with γIFN (100U/ml 16h), Mφ were activated using γIFN (100U/ml) and TNFα (100U/ml). Either D-NMMA (100µM) or L-NMMA (100µM) was added to wells. * P < 0.05 compared with co-culture in the presence of L-NMMA (n=4).
L-NMMA is a non-specific NOS inhibitor. There are three NOS enzymes, recently renamed NOS1 (nNOS or neuronal), NOS2 (iNOS or inducible), and NOS3 (eNOS or endothelial). To be more specific a NOS2 selective inhibitor L-N6-(1-iminoethyl) lysine dihydrochloride (L-NIL) was added to co-culture at the same time as activation. This inhibitor behaved similarly to L-NMMA in that it almost completely blocked the MΦ effects on MC mitosis and apoptosis in the co-culture assay (Figure 3-9). A dose response curve for this drug gave an ED50 of 0.33±0.40µM. Ten micromolar was sufficient to maximally block the MΦ-derived effects. Note however, in these studies, there remained (not significant) a small induction of apoptosis and inhibition of mitosis.

3.5 CYTOKINE-ACTIVATED RODENT MΦ INDUCE NITRIC OXIDE SYNTHASE 2 AND ABUNDANTLY PRODUCE NO

One hundred thousand 7day BMD rat MΦ were seeded into wells of 24-well plates overnight. After 16h, MΦ were washed and 400µl of full DMEM/F12 (without phenol red) or DMEM/F12 containing no FCS was added. MΦ were activated with cytokines (Figure 3-10) and cultured for 24h. At this stage, supernatants were collected, centrifuged to removed cell debris (4000g, 5min). An equal volume of Griess reagent was added at room temperature for 5min before the sample was assessed by photospectrometry at 540nm (OD540). Purple coloration indicated nitrite, the major stable product of NO release.

Analysis of NO generation by rat MΦ indicated abundant release within 24h by activated MΦ but not quiescent MΦ (Figure 3-10A). Further, IFN-γ alone was a weak inducer of NO generation, but was necessary in switching MΦ to become sensitive to other cytokines. Indeed, in murine studies, IFN-γ alone did not bring about NO generation (Table 3-10). Thus low grade LPS contamination in the rat cultures might explain why IFN-γ alone was able to induce nitrite production. These data are in keeping with many published reports about the generation of NO by rodent MΦ (Fournier et al., 1995; Erwig et al., 1999). Mouse MΦ similarly produced abundant nitrite in supernatants using a similar experimental protocol (Table 3-10).

When MΦ were activated in the presence of the inhibitors L-NMMA and L-NIL there was a 50-60% reduction in nitrite production at inhibitor concentrations sufficient to block MΦ killing of MC (Figure 3-10B,C). Furthermore, in prolonged culture, MΦ produced most nitrite during the first 24h, less in the next 24h and thereafter produce no more nitrite. This occurred despite replenishing medium and cytokines every 24h (Figure 3-11).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-9: The effect of activated Mφ on rat MC apoptosis and mitosis is largely abrogated by the addition of the NOS 2 inhibitor L-NIL. Rat BMD Mφ, primed γIFN for 12h, then activated with γIFN (100U/ml) and LPS (1μg/ml) were cultured with mesangial cells as described in Methods. L-NIL (30μM) or carriage medium was added once the co-culture was established. (A). The effect of L-NIL on activated Mφ-induced apoptosis of mesangial cells at 24h. L-NIL almost completely abrogates the effect of co-culture on MC apoptosis. (B) The effect of L-NIL on activated Mφ-induced suppression of mitosis at 24h. L-NIL restores mesangial cell mitosis. (C) L-NIL in the co-culture enables MC to increase in number similarly to controls. * P <0.05 vs. co-culture in the presence of carriage medium (n=4).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-10: Nitrite generation in the supernatants of activated rat BMD Mφ, cultured in full DMEM/F12, expressed as nmole of nitrite per 10^6 cells per 24h. (A) A comparison of pro-inflammatory cytokines. (B) The effect of iNOS inhibitors in IFN-γ/LPS induced NO generation. (C) The effect of iNOS inhibition on IFN-γ/TNF-α induced NO generation (IFN-γ 100U/ml, TNF-α 100U/ml, LPS 1µg/ml, L-NMMA 100µM, L-NIL 10µM) (n=4).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Table 3-10: Generation of nitrite in supernatant of 1.0 x 10^5 murine Mφ in 400μl full DMEM/F12

<table>
<thead>
<tr>
<th></th>
<th>Nitrite produced over 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control IFN-γ γIFN/LPS γIFN/TNFα</td>
</tr>
<tr>
<td>Mouse BMD Mφ</td>
<td></td>
</tr>
<tr>
<td>129/sv wild type</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.7 ± 0.4 5.7±2.6 134.2 ± 38.2 74.0±19.4</td>
</tr>
</tbody>
</table>

Nitrates accumulation nmole/10^6 cells/24h

Figure 3-11: Accumulation of nitrite in supernatants of cultures of activated rat BMD Mφ with time. Fresh medium with cytokines was replaced every 24h. There was no significant loss of Mφ over the period of study. Cultures were activated with IFN-γ (100U/ml) /LPS (1μg/ml). In some cultures L-NMMA (100μM) was also added (n=4).

By indirect immunofluorescence, Mφ in 24-well plates as described above, homogeneously induce cytoplasmic iNOS, whereas unactivated or quiescent BMD Mφ do not have cytoplasmic iNOS (Figure 3-12).
3.6 CYTOKINE-ACTIVATED RODENT MC INDUCE NOS 2 AND PRODUCE LOW LEVELS OF NITRIC OXIDE

The data presented so far lead one to hypothesise that activated Mφ by their release of NO directly induce cell death and cell cycle arrest of rat MC. However, there are other possible explanations for the data. One possibility is that NO feeds back on the Mφ to maintain activation in a similar way that Riches’ group suggested TNF-α dependent feedback to maintain activation of Mφ (Lake et al., 1994). Other factors released by the Mφ would actually be the effectors. Another possibility is that MC also produce NO (perhaps to protect from Mφ mediated damage), and NO blockade selectively blocks this generation and renders the MC susceptible to other factors. Indeed, there is significant literature on the ability of rodent MC to produce NO (Nitsch et al., 1997; Shultz et al., 1994; Waddington et al., 1998; Mohaupt et al., 1994).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

To test this, 0.6 x 10^5 rat MC were cultured in wells of 24-well plates overnight, washed and 400μl of full DMEM/F12 or DMEM/F12 without FCS, was replaced. MC were then activated with a variety of cytokines to induce nitrite production in the supernatant. Supernatants were analysed 24h later after centrifugation (4000g, 5min) to remove debris.

Initial results showed no evidence of NO generation (data not shown). However, the sensitivity of the Griess assay is approximately 1μM (lower limit of assay). Following consultation with both Prof. J. Pfielschifter (Frankfurt) and Dr. V. Cattell (Imperial College, London) the three rat MC clones used in these studies were assessed more thoroughly for NO generation. Initially, by indirect immunofluorescence, it became apparent that in the presence of IFN-γ together with TNF-α iNOS was induced (Figure 3-13). Activation of Mφ with IFN-γ (100U/ml) alone, TNF-α (100U/ml) alone, LPS (1μg/ml) with and without IFN-γ and IL-1β (40ng/ml) did not induce iNOS.

**Figure 3-13:** Induction of iNOS in rat MC. 0.6 x 10^5 rat MC clone (sub-confluent) was cultured in 24-well plates with 1.0ml full DMEM/F12, and activated with IFN-γ (100U/ml) with TNF-α (100U/ml) or carriage medium for 24h. After fixation with ice cold methanol, cells were incubated overnight with anti-iNOS specific polyclonal antibody followed by appropriate secondary antibody. (A) clonal rat MC. (B) polyclonal rat MC. Note induction of cytoplasmic iNOS in cytokine activated culture. Some cells strongly express iNOS, others weakly express and others appear negative. Note cells incubated with pre-immune rabbit serum as negative control showed no specific binding (not shown).
Culture of large numbers of MC in T75 flasks for 48h confirmed the generation of small quantities of NO by Griess reagent. However, the MC only produced NO and iNOS in response to IFN-γ together with TNF-α (Figure 3-14). These experiments were performed concurrently with several clones (Figure 3-14), which showed broadly similar results, i.e. that IL-1β alone had no effect on NO generation and the IFN-γ with TNF-α were the most important cytokines in inducing NO generation. Indirect immunofluorescence of fixed wells for iNOS (Figure 3-13) showed that more cells were strongly positive for the enzyme 48h than 24h (4.7% vs. 20.2%) when using 100U/ml of each cytokine. Increasing the dose to 300U/ml of each cytokine increased the number of strongly positive cells to 16.8% at 24h and 30.4% at 48h. The remaining cells were weakly positive or and a few were negative, though this was difficult to score microscopically. The studies therefore were assayed at 48h. However, despite this result, the nitrite generated in the medium represented 2%-5% the amount of NO generated by an equal number of rodent Mϕ. By comparison, similar immunofluorescence of BMD Mϕ for iNOS (Figure 3-15), showed strong homogeneous staining of the cytoplasm at 6h which persisted at 24h.

![Graph](image-url)

**Figure 3-14:** Nitrite accumulation in supernatants of rat MC growing (clonal P8, polyclonal P6) in T75 flasks over 48h. Aliquots of rat MC (clonal or polyclonal) were seeded into T75 flasks with 5ml full DMEM/F12. After overnight culture they were activated as indicated. 48h later, supernatants collected and assayed for nitrite. Cells were trypsinised and counted by using a haemocytometer. Nitrite accumulation was normalised to cell number (TNF-α 300U/ml, IFN-γ 300U/ml, LPS 1μg/ml, IL-1β 40ng/ml).

The nitrite and iNOS data were in contrast to published work showing early rat MC primary cultures (P1-P3) produced NO in response to LPS alone (Shultz et al., 1994; Mohaupt et al., 1994).
Furthermore, Pfeilschifter’s group showed their mesangial cell clone (MZ) (derived from single cell preparation of rat glomeruli initially cultured at 1 cell per well) abundantly produced NO in response to IL-1β alone, though not to LPS (Nitsch et al., 1997). Cattell’s group had also published work on a rat MC clone producing abundant NO in response to IL-1β alone (Waddington et al., 1998), though they have struggled to produce further clones with this phenotype (Personal Communication S. Waddington). Cattell’s group demonstrated in the Thy1.1 model of mesangio-proliferative glomerulonephritis that NO released from whole glomeruli extracted from kidneys of Lewis rats with nephritis correlated closely with the presence of MΦ, not mesangial cell proliferation (Cattell et al., 1993). Furthermore, when diseased glomeruli were exposed to LPS, they produced more NO. Exogenous IL-1β also increased NO generation but this again appeared to reflect MΦ infiltration. (Cattell et al., 1993).

**Figure 3-15:** Fluorescent micrographs (X200) of confluent monolayers of early passage rat MC cultures (P2) showing induction of iNOS by LPS in some cells co-localises with ED-1 positive MΦ. 0.6 x 10^5 rat MC were cultured in 24-well plates with 1.0ml full DMEM/F12, and activated for 24h with IFN-γ (100U/ml) and LPS (1µg/ml). After fixation with ice cold methanol, cells were incubated overnight with (A) anti-iNOS specific polyclonal antibody followed by phycoerythrin-conjugated secondary antibody or (B) anti-ED-1 monoclonal antibody, followed by FITC-conjugated secondary antibody. Note in the iNOS view a MC (see different morphology) weakly staining positive next to a MΦ (arrow).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Staining of glomeruli for iNOS indicated co-localisation with ED-1 positive cells only (T Cook, Imperial College. Personal communication) These data would seem to suggest, at least in the Thy1.1 model of nephritis, Mφ, not MC were the major source of NO.

To investigate these conflicting observations further, early passage rat MC (P1-P3) revealed by immunofluorescence the presence of strongly iNOS positive cells in response to LPS alone. These positive cells appeared different from adjacent cells and dual labelling with anti-ED-1 (rat Mφ antigen) indicated significant numbers of Mφ in the culture (Figure 3-15). They represented up to 5% of cells in the culture, but were no longer present from P5 onwards. This observation, I believe explains why early passage rat MC generate low levels of NO in response to LPS (Shultz et al., 1994).

To further clarify the issue of NO generation by MC in culture, P15 MZ cells from Prof. Pfeilschifter (Nitsch et al., 1997) were obtained. These cells were cytologically a little different from any MC (human, rat or mouse) cultured in our laboratory before (Figure 3-16). Phase contrast microscopy indicated them to often lack the characteristic spindle morphology. They were, however, positive for α-smooth muscle actin by indirect immunofluorescence (data not shown). Unfortunately, there are no specific markers for rat mesangial cells as fibroblasts and interstitial myo-fibroblasts share the same markers (including Thy 1.1).

Figure 3-16:  Phase contrast photomicrographs showing (A) rat MC (X 200) cultured in our laboratory, (B) the same rat MC at higher power (X320) showing spindle morphology. (C) Rat 'MZ' cell provided by Prof. Pfeilschifter at low power and (D) the same cells at higher power showing a different morphology with less tendency to form spindle morphology.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

These MZ cells did indeed produce large amounts of NO in response to IL-1β. They produced much more in response to IFN-γ with TNF-α. LPS was ineffective (Figure 3-17). The NO generated by equivalent numbers of cells in response to cytokines, was approximately 30% that produced by Mφ. When MZ cells were co-cultured for 24h with activated Mφ, there was no specific NO-dependent killing. Indeed supernatants from the co-culture contained large amounts of NO, greater than observed from an equal number of Mφ alone. This would suggest that pro-inflammatory cytokines released from the Mφ activated the MC to produce NO. Those cells were resistant to Mφ-derived NO (Table 3-11).

Figure 3-17: Representative study of nitrite generation in the supernatant of MZ mesangial cells growing in T75 flasks over 48h. Aliquots of rat MC were seeded into T75 flasks with 5ml full DMEM/F12. After overnight culture, they were activated as indicated. 48h later, supernatants collected and assayed for nitrite. Cells were trypsinised and counted by haemocytometry. Nitrite accumulation was normalised to cell number (TNF-α 300U/ml, IFN-γ 300U/ml, LPS 1μg/ml, IL-1β 40ng/ml).

Table 3-11: Induction of apoptosis of MZ rat mesangial cells by rat BMD Mφ in activated co-culture

<table>
<thead>
<tr>
<th></th>
<th>Unactivated Co-culture</th>
<th>Unactivated Control</th>
<th>Activated Co-culture</th>
<th>Activated Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Apoptosis</td>
<td>2.4 ± 1.3</td>
<td>1.8 ± 1.2</td>
<td>3.9 ± 1.3</td>
<td>1.8 ± 1.1</td>
</tr>
<tr>
<td>% Mitosis</td>
<td>2.2 ± 1.2</td>
<td>1.8 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>2.2 ± 0.8</td>
</tr>
</tbody>
</table>
Immunofluorescence of the co-culture using MC from our laboratory indicated that despite induction of iNOS in some MC, these MC were susceptible to MØ derived NO killing. That is some of the apoptotic MC were positive for iNOS. This study was difficult due to loss of apoptotic cells during washing, however iNOS positive apoptotic cells were seen. Indeed studies detailed in the next section will highlight that MC that have been treated with IFN-γ and TNF-α to produce NO were equally susceptible to apoptosis induced by exogenous donors of NO.

3.7 RODENT MC UNDERGO CELL CYCLE ARREST AND APOPTOSIS IN RESPONSE TO HIGH LEVELS OF NO DONORS

To test the susceptibility of MC to nitric oxide, MC were plated into wells at 70% density and allowed to grow for at least 4h. This duration of growth was important, as Sterzel’s group (Yao et al., 1998) had shown that adhesion protected cells from apoptosis induced by low levels of NO. In preliminary experiments, MC that had been adherent longer than 4h (not shown) were no less susceptible compared with those plated for 4h. However, those cells that had been adherent for only 1h were much more susceptible to NO-donors. When cells were seeded into plates containing medium with NO donors (0.1mM GSNO) pre-added, they also underwent apoptosis (data not shown). Probably, these data emphasise the importance of integrin-mediated adhesion in the survival of MC and myofibroblasts in general. (Singhal et al., 1998; Jobson et al., 1998; Mooney et al., 1999).

Specific nitric oxide donors were chosen because of the availability of non-toxic, control substances: S-Nitroso glutathione (GSNO), releases glutathione (glu) and NO, S-Nitroso penicillamine (SNAP) releases penicillamine (NAP) and NO. These breakdown products were therefore used as controls on a mole for mole basis.

The NO-donors resulted in far more nitrite in the medium at 24h than MØ. When 0.1mM GSNO was incubated in solution for 24h the A₅₄₀ with the Griess reagent was 1.370. Typical A₅₄₀ of 1.0 x 10⁵ rat 118 was 0.300-0.600. The site of action of the NO donors, however, is throughout the medium in the well (there is no receptor ligand interaction). This is because the donors break down spontaneously in solution. NO release from MØ interacting with MC is at a very specific point where local concentrations will be much higher.

Cells were exposed to a range of doses of NO donors, or the appropriate control. At 24h plates were fixed and assayed subsequently using Hoechst and PI to fluorescently label DNA/RNA, and counted microscopically for apoptosis and mitosis. NO donors but not their controls induced apoptosis in the rat
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

MC (Figure 3-18). In this study, 0.5M GSNO at 24h resulted in 0.00% mitosis compared with 2.38% mitosis in the presence of 0.5M glutathione. A time course for induction of apoptosis and suppression of mitosis is also shown (Table 3-12). There was a progressive increase in apoptosis over a 24h period (in keeping with the co-culture data).

![Graph showing induction of apoptosis by donors of NO.](Figure 3-18)

**Figure 3-18:** Induction of rat MC apoptosis by donors of NO. 70% confluent rat MC clone cultured in full DMEM/F12 was exposed to 0.5, 1.0, 1.5, and 2.0mM of the NO-donors GSNO or SNAP, or appropriate control. Experiments were fixed and assayed after 24h incubation.

<table>
<thead>
<tr>
<th>NO donor</th>
<th>GSNO</th>
<th>SNAP</th>
<th>glu</th>
<th>NAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3-12:** Induction of apoptosis and cell cycle arrest of rat MC by 10mM GSNO with time

<table>
<thead>
<tr>
<th></th>
<th>8h</th>
<th>16h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO donor-GSNO 1.0mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MC apoptosis</td>
<td>5.2±1.4%</td>
<td>13.7±2.0%</td>
<td>16.3±2.3%</td>
</tr>
<tr>
<td>% MC mitosis</td>
<td>0.0±0.0%</td>
<td>0.0±0.0%</td>
<td>0.0±0.0%</td>
</tr>
<tr>
<td>control glutathione 1.0mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MC apoptosis</td>
<td>1.5±0.5%</td>
<td>1.0±0.5%</td>
<td>0.3±0.1%</td>
</tr>
<tr>
<td>% MC mitosis</td>
<td>2.3±0.5%</td>
<td>4.9±1.3%</td>
<td>2.0±0.5%</td>
</tr>
</tbody>
</table>
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Note that even without induction of apoptosis (at lower doses), there was cell cycle arrest. Flow cytometric data indicated that there was an accumulation of cells in G1/G0. (Data not shown).

Notably, the early passage primary rat MC were less susceptible to apoptosis by NO donors. Nevertheless, at higher doses the donors did induce apoptosis. This may relate the their lower rate of proliferation (as reflected by percentage mitosis), compared with later passage cells or the rat MC clone (Table 3-13).

### Table 3-13: Induction of polyclonal rat MC (P5) apoptosis by the NO-donor, GSNO at 24h

<table>
<thead>
<tr>
<th></th>
<th>Percentage apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSNO</td>
<td>6.6±1.4% 11.4±3.0% 19.0±4.9%</td>
</tr>
<tr>
<td>glutathione</td>
<td>4.1±1.5% 4.9±1.0% 3.8±1.1%</td>
</tr>
</tbody>
</table>

Prof. Pfeilschifter's group had shown that their MZ cells were resistant to NO donors when activated to produce NO (Muhl et al., 1996). To test this, our MC clone was stimulated for 24h with IFN-γ (300U/ml) plus TNF-α (300U/ml) (or mock-stimulated with carriage medium) to induce iNOS and NO generation. Activating cytokines were washed away with PBS x 3. Cells were then plated in wells at equal density, and after 4h of growth, the active NO-donor GSNO was added at a range of concentrations. Wells were assayed at 16h (Figure 3-19). Note that even after withdrawal of cytokines from the cells they continue to release NO (nitrite) into the supernatant for 24h. Nitrite in supernatant in first 24h was 12nmole/10^6 cell, and during the 24h after cytokine withdrawal was 8nmole/10^6 cells. These data indicated no protection of MC from exogenous NO following endogenous production of low levels of intracellular NO.

Rat MC were primed with IFN-γ plus TNF-α for 24h, then treated with IFN-γ, TNF-α (to maximise MC NO generation), and GSNO (1mM) to induce apoptosis. At 24h, 21.2±2.4% of primed cells compared with 11.9±2.1% of unprimed cells were apoptotic (n=4 P < 0.05). Therefore, more apoptosis was induced in the cells producing their own NO than cells which had been exposed to GSNO alone without IFN-γ plus TNF-α. This result was in contradiction to that which might be expected. Taken together with the data above (Figure 3-19), which indicated that rat MC generating their own NO were not more sensitive to NO donors, other factors must have been inducing apoptosis (Chapter 4). Corroborating evidence was obtained using the MZ cells. When primed with IL-1β (to generate MZ-
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

derived NO), then exposed to GSNO there appeared to be no difference of appearance of mitoses or apoptotic bodies compared with unprimed cells (contrary to that which was published) (Nitsch et al., 1997). However when treated with IFN-γ plus TNF-α (to generate MZ-derived NO), then exposed to GSNO there were fewer mitoses and an increase in apoptotic bodies. IFN-γ and TNF-α must be effecting apoptosis independently of NO generation.

Figure 3-19:  Representative study of induction of apoptosis of unprimed or IFN-γ/TNF-α (300U/ml) primed rat MC by the NO donor GSNO. Rat MC were primed (IFN-γ (300U/ml) TNF-α (300U/ml)) or mock-primed for 24h. Cytokines were washed away and cells were seeded into wells. After 4h, concentrations of the NO-donor GSNO were added. Wells were fixed at 16h and assayed for apoptosis

3.8 NOS 2 KNOCKOUT MURINE MΦ CAN NOT INDUCE CELL CYCLE ARREST OR APOPTOSIS OF RAT MC

The data presented so far suggested that it was most likely that MΦ induced MC apoptosis in co-culture by release of nitric oxide. The induction of iNOS and NO generation by MC in response to IFN-γ and TNF-α suggested that the simple explanation might be more complicated. However, there was little evidence that the iNOS positive MC from our laboratory were resistant, on the contrary they appeared more susceptible to apoptosis. MZ cells from Prof. Pfeilschifter’s group were resistant to MΦ killing. It remains to be determined how relevant those cells are to in vivo biology given the difficulty in generating cells from glomeruli that reproduce the phenotype of the MZ cell, and given the pattern of NO generation in the Thy 1.1 model of glomerulonephritis (Cattell et al., 1993).
To be clear as to the mechanism of MC killing, BMD Mφ from mice lacking iNOS (or NOS 2) were utilised. Previous studies had confirmed that mouse BMD Mφ (Chapter 3.2) shared the ability with rat BMD Mφ to induce apoptosis of rat MC. In collaboration with Prof. Liew at the University of Glasgow, 129/sv mice lacking a functional iNOS gene were obtained for use (Wei et al., 1995). Bone marrow was harvested from five gene-targeted animals and five wild-type controls. Following maturation in Teflon wells, Mφ differentiation was confirmed by cytology (Figure 3-20). Cells were tested for NO generation by collection of supernatants from wells of 24-well plates (Table 3-14). These data confirmed a dramatic reduction in NO generation over 24h, compared with same strain wild type Mφ. Interestingly, however, the cells could still be induced to produce some nitrite. There is evidence that eNOS can also be induced, but that it is less efficient at NO generation (Weiner et al., 1994 Wei et al., 1995). It may be an adaptive response by the animal that eNOS partially compensates for the lack of iNOS.

Figure 3-20: Photomicrograph (X 400) of Diff-Quik stained cytopsins of murine bone marrow derived Mφ from iNOS knockout 129/sv mice. Note characteristic morphology of macrophages. There were no morphological differences compared with the wild type (not shown).

Mφ were co-cultured with rat MC as previously described and assayed at 8h, 24h and 48h. At 24h wild type Mφ induced apoptosis of rat MC (Figure 3-21), yet the knockout Mφ were incapable of doing so. This difference was marked and clear. In addition, whilst mitosis was suppressed by wild type Mφ, the knockout Mφ did not suppress MC mitosis (Figure 3-21).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Table 3-14: Nitrite generation by wild type and iNOS knockout murine BMD Mφ

<table>
<thead>
<tr>
<th></th>
<th>129/sv wild type</th>
<th>NOS 2 -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite produced over 24h</td>
<td>3.7 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>134.2 ± 38.2</td>
<td>32.1 ± 5.6</td>
</tr>
</tbody>
</table>

Figure 3-21: The effect of Mφ from iNOS knockout mice on rat MC apoptosis and mitosis. Co-culture with CMFDA-labelled MC was established in a 1.0 MC:1.5 Mφ ratio. After washing some experiments were activated with IFN-γ (100U/ml) and LPS 1μg/ml. After 24h incubation wells were fixed and scored for (A) MC apoptosis and (B) MC mitosis. iNOS knockout Mφ were compared with wild type Mφ and MC growing alone (n=4).
3.9 Mφ FROM INFLAMED RAT GLOMERULI SPONTANEOUSLY PRODUCE COMMENSURATE AMOUNTS OF NO

Whilst it was clear that Mφ derived from rat or mouse bone marrow induced apoptotic cell death of MC by release of nitric oxide, it was important to determine how relevant this was to glomerular inflammation. During the course of the thesis, it has become apparent that the models of glomerulonephritis available, vary widely in phenotype and presence of cell type, depending on many factors including strain of animal, specific batch of inducing agent and possible low grade pathogens harboured by the animals used.

In collaboration with Prof. Rees (University of Aberdeen), the question was asked whether Mφ from glomeruli of Sprague Dawley rats with a macrophage-rich form of telescoped nephrotoxic nephritis shared similar properties with activated BMD Mφ.

To achieve this telescoped NTN was induced. This particular disease model is believed to be a largely cell-mediated disease, brought about by cognate and non-cognate CD4 T cells with Mφ as the final effectors (Rosenkranz et al., 2000; Erwig et al., 1999). This disease model results in proteinuria and diminution of renal function within 48h, and progressive deterioration over succeeding weeks. On day 2, there were 21.4±3.1 Mφ per glomerular cross section and by day 4, there were 16.1±1.5 Mφ seen (approximately 80 cells per glomerular cross section).

To isolate Mφ, disease was induced, upon sacrifice of the animal, kidneys were removed onto ice. Glomeruli were isolated from kidneys by sieving and then digested using enzymes to a single cell suspension. The single cell suspension was then plated into wells of 24 well plates, in full DMEM/F12 and washed to remove non-adherent cells after 2h. Mφ, by their adherent properties were selected by this process with > 90% purity.

To compare the effects of glomerular Mφ with BMD Mφ, aliquots of single cell suspension were placed in wells from control animals, and animals from 2d, 4d and 7d of disease such that equal numbers of Mφ remained in wells (1.5 x 10^5), as calculated from previous experiments. 400μl of medium was placed on the adherent cells and cultured without the addition of cytokines for 24h.

Supernatants were assayed for nitrite generation at 24h (Figure 3-22). It can be seen that Mφ from this model of NTN spontaneously produced nitric oxide without the addition of activating cytokines. By comparison, Mφ from control or normal glomeruli did not produce NO spontaneously. When BMD Mφ
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

were activated for the 24h period with IFN-γ (100U/ml) and LPS (1μg/ml), or IFN-γ plus TNF-α they produced similar amounts of nitrite compared with the glomerular Mφ from diseased animals (Figure 3-22). Further, resident Mφ from normal glomeruli responded similarly to pro-inflammatory stimuli. These results suggested that Mφ from this model of NTN might induce MC apoptotic death by NO release in vivo.

Figure 3-22: Spontaneous nitrite generation by glomerular Mφ from telescoped nephrotoxic nephritis compared with cytokine-activated resident glomerular and BMD Mφ. 1.5 x 10⁵ Mφ were seeded to wells with 400μl of medium and cultured for 24h. Where indicated exogenous cytokines were added. i. BMD Mφ, ii. activated BMD Mφ (IFN-γ 100U/ml LPS 1μg/ml), iii. activated BMD Mφ (IFN-γ 100U/ml TNF-α 100U/ml), iv. resident glomerular Mφ, v. activated resident glomerular Mφ (IFN-γ 100U/ml TNF-α 100U/ml), vi. glomerular Mφ day 2 NTN, vii. glomerular Mφ day 4 NTN (n=5).

3.10 Mφ FROM INFLAMED BUT NOT NORMAL RAT GLOMERULI INDUCE CELL CYCLE ARREST AND APOPTOSIS OF RAT MC

To determine the role of NO in inducing MC apoptosis in vivo, NO generation by Mφ in the glomeruli should be specifically blocked in vivo. However, this process is technically very difficult to achieve for a variety of reasons. Firstly, there are no truly specific inhibitors of iNOS, i.e. all the inhibitors affect eNOS which plays a major role in vascular tone and local circulation. In inflammation endothelial cells are induced to express iNOS which plays a major role in modulating vascular tone and local haemodynamics (Bouloumie et al., 1997; Cooke 1998). Also, iNOS blockade in vitro is followed by
rebound effect when the drug is removed (data not shown). If local concentrations of iNOS inhibitors were to decline during an in vivo experiment due to metabolism, then opposite effects might be seen. Furthermore, as discussed later (see Chapter 8), each published model of nephrotoxic nephritis differs in the contribution of immune complexes and cell mediated immunity to Mφ activation. At certain time points in the disease model, neutrophil and Mφ oxygen radical mediated damage might predominate (see Chapter 5). Since NO can counteract the effects of oxygen radicals, paradoxical effects might be seen.

To obviate these problems, it was decided to perform an ex vivo co-culture, 1.0 x 10⁵ Mφ were isolated from tNTN glomeruli in wells of 24-well plates. CMFDA pre-labelled MC were added in a 1MC:1.5 Mφ ratio (0.6 x 10⁵ MC), and cultured in 1.0ml of full DMEM/F12 for 8, 16, and 24h. Wells were assayed at this time point for the appearance of apoptotic MC (Figure 3-23).

The data presented indicate that these Mφ spontaneously inhibited MC mitosis and induced apoptosis, reminiscent of the effect of activated BMD Mφ. The combined effects on mitosis and apoptosis might suggest an NO-dependent effect. However, the data were notably different from the co-cultures with BMD Mφ. Whilst in the BMD co-cultures there was a progressive increase in induction of apoptosis from 8h to 24h, these experiments showed induction of apoptosis at 8h which increased at 16h, but was diminished by 24h. Mitosis was suppressed at 8h, and at 16h, but by 24h the effect was diminished. Importantly, however, Mφ from each of the three experimental time points (2d, 4d, and 7d) displayed a similar pattern of effect on the MC (Table 3-15). By contrast, equal numbers of resident glomerular Mφ did not significantly induce MC apoptosis or suppress the increase in cell number (Table 3-15). Mitosis appeared to be suppressed but this did not occur at 8h.

Why should these differences exist? Firstly, the experimental protocol was necessarily different since the Mφ were already activated in the wells. Therefore when seeded, MC were immediately exposed to NO (and other cytokines). In the BMD co-cultures, Mφ were not activated, therefore seeded MC would not be exposed to NO and cytokines prior to adhesion and laying down matrix. The increased susceptibility of MC to NO when not adherent is described (Chapter 3.7). Furthermore, the co-culture was not washed after cells were plated. Since there is always some attrition during seeding, these factors probably explain why there is early appearance of apoptosis and why control wells show increased apoptosis at 8h than later time points.

Secondly, control wells of glomerular Mφ alone showed that at 24h, the majority of cells appear small and rounded up rather than adherent and spread out. Unfortunately, Hoechst staining was not available at the time of completing these experiments, but these cells appeared to spontaneously undergo
apoptotic cell death at 24h. It remains possible that they were a loosely adherent type of Mφ. However, my experience has been that activated Mφ adhere better than their quiescent counterparts. (Figure 3-24 Mφ alone and co-culture). Rubin-Kelley’s group describe the necessity for CSF-1 to allow glomerular Mφ survival in early glomerulonephritis seen in the MRL lpr/lpr mouse (Rubin-Kelley et al., 1994). Perhaps the same is true for Mφ in this model of nephritis.

**Figure 3-23:** The effect of Mφ from glomeruli of rats with day 2 telescoped NTN on MC apoptosis and mitosis. Glomerular single cell suspension and Mφ isolation yielded 50-60% confluent monolayer of Mφ. Immediately following isolation, co-culture was established with CMFDA labelled MC without the addition of cytokines. (A) The effect of Mφ on mesangial cell apoptosis at 8h, 16h and 24h. Note induction of apoptosis at 8h, peaking at 16h and much reduced at 24h. (B) The effect of Mφ on mesangial cell mitosis at the same time points: mesangial cell mitosis was suppressed at 8h and 16h. (C) Mφ suppressed the expected increase in MC number at 16h. This effect was more marked at 24h.* P < 0.05 compared with control MC (n=3).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

It seemed likely therefore, that the reason that Mφ effects on MC declined at 24h was Mφ death. Since this occurred in the cultures of Mφ alone, two questions arose; firstly did glomerular Mφ die due to the enzymatic digest or did they die due to lack of cytokines such as CSF-1? Secondly, was the reason for poor induction of apoptosis of MC in co-culture, due to death of Mφ?

Table 3-15: The effect of resident glomerular Mφ and diseased glomerular Mφ from rats with NTN at 2d, 4d and 7d, upon MC apoptosis, mitosis and cell number.

<table>
<thead>
<tr>
<th></th>
<th>2d Mφ</th>
<th>4d Mφ</th>
<th>7d Mφ</th>
<th>resident Mφ</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoptosis at 16h</td>
<td>493 ± 23</td>
<td>261 ± 11</td>
<td>487 ± 16</td>
<td>58±35</td>
</tr>
<tr>
<td>% of control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitosis at 16h</td>
<td>19 ± 12</td>
<td>38 ± 60</td>
<td>23 ± 29</td>
<td>52±25</td>
</tr>
<tr>
<td>% of control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC number at 24h</td>
<td>73 ± 9</td>
<td>85 ± 6</td>
<td>81 ± 9</td>
<td>99±8</td>
</tr>
<tr>
<td>% of control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Control MC apoptosis 0.68% ± 0.04, mitosis 3.8% ± 0.7 and MC number 1729 ± 68.

To answer these questions, 7d BMD rat Mφ in Teflon were activated for 6h with IFN-γ (100U/ml) and LPS (1μg/ml). They were washed x 2 and exposed to the same enzymatic digest procedure as used to extract Mφ from glomeruli. These Mφ were then plated into wells of 24-well plates at 1.0 x 10^5, followed by 0.6 x 10^5 CMFDA-labelled MC. The experiment was assayed at 8h, 16h and 24h (Figure 3-25.). Mφ treated with digestive enzymes after activation were less able to induce apoptosis than BMD Mφ in the co-culture described above. Inhibition of mitosis was present at 8h and 16h, but not at 24h, and MC number was slightly lower than controls at 24h. They therefore behaved similarly to glomerular Mφ. Furthermore, the cultures at 24h showed many apoptotic Mφ, indicating that the extraction procedure of glomerular Mφ can inflict substantial damage to the Mφ (Figure 3-24).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-24: Fluorescent micrographs (X200) from co-culture using glomerular Mφ from NTN. (A) Co-culture of green-labelled rat MC with rat glomerular Mφ from nephrotoxic nephritis (day 4) fixed and counterstained with PI at 24h. Note apoptotic rat MC (arrows). (B) Similar photomicrograph of glomerular Mφ growing alone fixed and stained with PI, showing many small rounded cells (suggestive of apoptosis). Rat BMD Mφ activated with IFN-γ and LPS for 4h show very few small rounded cells (C) but if treated with collagenase digest after activation, 24h later many become small and round (D).

Figure 3-25: Induction of rat MC apoptosis by pre-activated rat BMD Mφ that had been pre-treated with a collagenase digest. Mφ and MC were plated in 24-well plates in a 1.0 MC:1.5Mφ ratio. No activating cytokines were added. Wells were fixed, labelled and assayed by microscopical counting (n=3).
Chapter 3: Results

1. Inflammatory macrophages induce apoptosis of mesangial cells

3.11 GLOMERULAR MΦ INDUCED DEATH OF RAT MC IS BLOCKED BY INHIBITORS OF NO GENERATION

To determine the aetiology of the effects of glomerular MΦ on MC in co-culture, L-NMMA or the inactive isomer D-NMMA was added to wells at 100μM, at the time of seeding wells. When these experiments were assayed (Figure 3-26) there appeared to be inhibition of the MΦ effects on MC. When L-NMMA was applied to 4d-co-culture, results showed a reversal of suppression of mitosis and mesangial cell number increased in line with control MC growing alone. MC apoptosis was not different from control wells at any time point, though background levels were elevated. The results for co-culture with 2d MΦ and 7d MΦ in the presence of L-NMMA were less convincing, though in each case there appeared to be some suppression of the MΦ-derived effects. It remains unclear whether this reflects other, non NO-dependent mechanisms, or whether the sensitivity of the assay was insufficient to indicate differences clearly. Unfortunately, whilst in retrospect it would have been interesting to look at NO\textsuperscript{2}/NO\textsuperscript{3} ratios and hydrogen peroxide levels in supernatants from the glomerular MΦ, these assays were not performed and no samples remain from those studies. Cook et al (Cook et al., 1989) and Holdsworth et al (Holdsworth et al., 1988) reported oxygen radical generation in a rabbit models of tNTN using luminol. NO however, also causes a positive response with luminol (Wang et al., 1991).

3.12 L-ARGININE, THE PRINCIPAL NOS 2 SUBSTRATE IS NECESSARY FOR MΦ-DEPENDENT EFFECTS ON RAT MC

Albina et al have reported in a model of healing skin wounds in the rat, that the concentration of L-arginine, the substrate for iNOS, falls to a level well below that found in plasma. This fall occurs swiftly following the early stages of the wound generation (Albina et al., 1990; Reichner et al., 1999). The fall in L-arginine is attributed to a rise in arginase levels, another enzyme which utilises L-arginine. This arginase does not appear to be derived from MΦ, though activated MΦ are known to synthesise a form of arginase, whose activity lags behind iNOS activity. In addition, in low oxygen tensions as might be found when capillary flow is compromised, iNOS might not function efficiently since it is also requires O\textsubscript{2} as a substrate (Albina et al., 1990) (see Figure 1-2). Indeed activated MΦ, found in the Albina wound model, show iNOS enzyme induction but no evidence of NO generation (as evidenced by accumulation of L-citrulline (see Figure 1-2)). Unfortunately, this wound-healing model is artificial in that it involves dramatic disruption of tissue architecture with consequent diminished local blood flow. This model might more accurately therefore reflect an ischaemic, inflamed tissue.
When L-arginine levels are low, the enzyme eNOS has been described to generate superoxide radicals and possibly peroxynitrite (ONOO-) instead of NO (Xia et al., 1996; Xia et al., 1998).

**Figure 3-26:** The effect of Mφ from glomeruli of rats with day 4 telescoped NTN on MC apoptosis and mitosis in the presence of L-NMMA. Macrophages from inflamed glomeruli were co-cultured with CMFDA-labelled rat MC as shown in Figure 3-24. However, during co-culture the medium contained L-NMMA 100μM. Apoptosis and mitosis were scored microscopically at 24h (n=3).
Such dysfunctional eNOS has become a topic of avid research recently in the context of abnormal vascular relaxation. The role of the highly unstable peroxynitrite, which is said to be toxic to cells, I believe remains putative (Xia et al., 1997; Bank et al., 1998; Heeringa et al., 2001). Using electron paramagnetic resonance spin trapping for detecting oxygen radicals and nitric oxide Xia et al (Xia et al 1996; Xia et al., 1997) have suggested that both nNOS and iNOS also can generate super-oxide in low oxygen tensions. Superoxide generation is inhibitable by both L-NMMA and SOD. They show by immunostaining activated murine macrophages for nitritosine that nitroosylated tyrosine only occurs in activated MΦ in L-arginine depletion. These data suggest the presence of peroxynitrite (Figure 3-27).

Whilst the glomerulus has an extremely good blood flow, during inflammation, there is often capillary thrombosis and destruction of the normal glomerular capillary bed. It is possible that relative hypoxia and poor transfer of plasma factors may occur in these situations. To take these observations further,
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Mφ:MC co-culture was performed in the presence of low levels of L-arginine, achieved by combining DMEM/F12 containing no L-arginine with 10%FCS that contains low levels of L-arginine (estimated at 6μM). The L-arginine levels for these experiments was not measured but one can assume that it is close to normal calf serum levels as reported previously (Albina et al., 1989). DMEM/F12 medium normally contains 0.6mM, RPMI contains 1.2mM and normal human plasma contains 0.1mM.

For these experiments, co-culture was established as described in Methods (Chapter 2.6). After 2-4h, the co-culture was washed and replaced with full DMEM/F12 (L-arginine deplete). Co-culture was activated with IFN-γ (100U/ml) and LPS (1μg/ml). At 24h experiments were assayed for MC apoptosis (Figure 3-28). One might have expected MC necrosis or possibly increased MC apoptosis due to oxygen radical release or peroxynitrite release (Xia et al., 1997; Bank et al., 1998; Albina et al., 1989). However, in stark contrast, MC apoptosis was markedly decreased to levels just exceeding control levels. As a positive control for the experiments, adjacent wells prepared identically were executed in medium containing normal levels of L-arginine. At the same time mitosis was equally suppressed in the L-arginine deficient experiments compared with the L-arginine replete experiments previously demonstrated (not shown). To investigate the mechanism further, the absolute level of apoptosis was enhanced by increasing the ratio of Mφ to MC from 1.5:1 to 3:1 (see next section for details). Inhibitors of NOS and oxygen radicals were tested to see whether the lower level was inhabitable. The excess apoptosis was inhibited by the addition of L-NMMA) (Figure 3-29) (albeit higher doses were required as 100μM of L-NMMA had no effect not shown), but not inhibitors of hydrogen peroxide or superoxide radicals; catalase and SOD. Furthermore, no increased levels of Mφ apoptosis were observed.

These data would imply that in conditions where the concentration L-arginine was limited, iNOS expression in Mφ does not necessarily lead to cell death of neighbouring MC. The role of peroxynitrite is brought into question by these data as one would have expected MC apoptosis or necrosis mediated by peroxynitrite. This did not happen. Interestingly, when the co-culture was repeated using 1% FCS in the absence of L-arginine MC apoptosis was induced (17.4±2.4% (normal L-arginine) vs 16.9±2.2% (L-arginine deplete)) (see Chapter 4).
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-28: The effect of low levels of L-arginine on Mφ killing of MC in co-culture. Rat BMD Mφ were co-cultured with CMFDA green MC. After washing medium was replaced with L-arginine depleted full DME/F12 medium and activated with IFN-γ (1000 U/ml) and LPS (1 μg/ml). MC apoptosis was assessed at 24h. As a positive control, induction of MC apoptosis in adjacent wells using medium with normal (plasma) levels of L-arginine is shown (n=5).

Figure 3-29: L-NMMA, but not catalase or superoxide dismutase inhibits Mφ killing of MC in L-arginine depleted conditions. Activated co-culture was established in low L-arginine tensions. At the time of activation with IFN-γ and LPS soluble inhibitors were added. MC apoptosis was assessed at 24h (n=5).
3.13 THE RATIO OF RAT Mφ TO RAT MC INFLUENCES Mφ DEPENDENT KILLING

To define further the killing effect of Mφ on MC experiments were designed to find the maximum effect of Mφ on MC. In vivo Mφ may contribute as many as 50% of cells in the glomerulus during inflammation (Nolasco et al., 1987; Cockwell et al., 1998). However, it is difficult to translate the interaction of the two cells in vivo (three dimensions, local blood flow, oxygen tension) directly to the situation in vitro (two dimensions, no blood flow, optimal pH, oxygen tensions). It is clear that close proximity of cells is required for the killing effect though the co-culture does not directly indicate whether cell: cell contact is required. The studies reported thus far indicated that over 24h, up to fifteen percent of MC underwent apoptosis. Were the remaining cells recalcitrant or would an increase in Mφ:MC ratio result in further apoptosis of cells? Series of experiments were initiated to vary the ratio of cells, but maintain the overall coverage of the well at 90-100% at the start of the co-culture (Figure 3-30). At higher Mφ:MC ratios there was a greater induction of MC apoptosis which reached a plateau at < 40%. When Mφ:MC ratios were 0.5:1, there was a reduction in killing compared with higher ratios but this was still greater than control wells. The data suggest that local interactions of Mφ with MC might be important, indeed individual glomeruli show areas of intense Mφ infiltration with apoptosis present (Figure 1-10).

![Graph](image-url)

*Figure 3-30:* The effect of increasing Mφ to MC ratio on induction of apoptosis of MC in activated co-culture at 24h. Co-culture was established by altering the ratio of cell types but keeping the total number of cells constant (3.5 x 10^4 cells per 0.3cm² well). Co-culture was assessed 24h after activation. Note that MC apoptosis rises sharply but plateaux at ratios above 4Mφ:1MC with induction of approximately 40% MC apoptosis. Average MC apoptosis in control wells of MC alone plus cytokines was 1.9±1.1% (n=4).
3.14 QUIESCENT HUMAN MONOCYTE DERIVED Mφ DO NOT INFLUENCE HUMAN MC APOPTOSIS

The studies so far have described experiments using rodent bone marrow Mφ and rat MC. They have been very informative in understanding the interaction between activated Mφ and MC. Parallel studies were carried out using human cells. These were more difficult to achieve satisfactorily, largely because of the difficulty in reproducibly growing human MC. Many observations made early in the thesis were not investigated until the latter stages of the thesis due to lack of suitable human MC.

However, in the end a surprisingly coherent pattern developed. Of note, the Mφ used in these experiments were derived from peripheral blood monocytes from healthy individuals. They were differentiated in autologous serum (believed to be under the influence of GM-CSF (Daems et al., 1976)). One must be cautious comparing and contrasting the data obtained from the rodent with the human. Also, the human MC were obtained from 50y (average) old kidneys. Whilst, the cell doubling time approached that of rat clonal MC during some stages, that was not sustained and cells did not grow for more than 13 passages (usually less than 10). The rat cells can grow for more than 100 passages (Personal communication Dr. M Kitamura, Imperial College).

Bearing these differences in mind, 7d monocyte derived Mφ (MDM), matured in Teflon wells (purified by either elutriation or MACS bead column), were plated into wells to cover 50% of the surface (2 x 10^4) per well of 96-well plate. The Mφ culture medium was Iscove's modified DMEM with 10% autologous PRPDS. After 4h, medium was removed and replaced with full DMEM/F12. Cells were left for 12h in wells. Sub-confluent flasks of human MC were labelled with green CMFDA, washed, trypsinised and added to wells in a 2:1 ratio (small cells) or 2.5:1 ratio (larger cells). After 4h cells were washed and medium was replaced with full DMEM/F12. Cells were cultured for 8h, 24h and 48h, and assayed for human MC apoptosis and mitosis

This was assessed by fluorescence microscopy of live and formaldehyde fixed wells using PI (1µg/ml) and Hoescht 33342 (1µg/ml). Due to the limited supply of human MC they were used sparingly. Flow cytometric assays require large numbers of cells that would have precluded many of the experiments presented being performed. The microscopical assay demonstrated that Mφ had no significant effect on either mitosis or apoptosis over a 48h period (8h, 24h, 48h) (Figure 3-31 Figure 3-32).
Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-31: The effect of co-culture on human MC mitosis and apoptosis at 24h. Co-culture was established as described. Here, the assay was fixed and MC apoptosis and mitosis assessed microscopically. Note there was no induction of MC apoptosis (n=5).

This result is in contrast to data reported by Mene & Cinotti (Mene & Cinotti 1996) where they showed an early lysis of human MC induced by adhesion of U937 myelomonocytic cells. It is possible that these U937 cells are activated. Indeed U937 cells have been shown to have cell surface TNFα suggesting they display an activated phenotype. However, even at 3h after initiation of co-culture in these studies there was no evidence of induction of cell death by apoptosis nor any evidence of necrotic cells (as assessed by uptake of PI by live co-culture).

In limited studies, once the co-culture was established, medium was replaced using DMEM/F12 containing only 1% FCS. In these studies, there was no induction of apoptosis of MC. Importantly, MC mitosis did not appear to differ greatly in co-culture from growing alone (MC apoptosis; co-culture 1.2%, MC alone 1.7%. MC mitosis; co-culture 0.21%, MC alone 0.13%). This result is, perhaps in contrast to that shown by Singhal (Sharma et al., 1996; Singhal et al., 1996) where supernatants from Mφ were able to promote thymidine uptake in labelled human MC, suggesting that Mφ might release growth factors such as PDGF to promote MC growth. There was no obvious indication of this from these studies, though 1% FCS might surpass any effect from growth factors released from Mφ. Further, there is good evidence (Abbott et al., 1991; Abbott et al., 1992) that cultured human MC generate their own growth factors.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Further, in limited studies, seeding 0.5 $\times$ 10$^5$ human MC (to achieve 50% covering of the well) onto confluent monolayers of Mφ (5.0 $\times$ 10$^5$ Mφ per 1.5 cm$^2$), lead to suppression mitosis of MC at 24h assessed microscopically (Table 3-16). Similar experiments in the rodent system had no effect on MC mitosis (Chapter 3.1). Note, in the human co-culture, there was also a small induction of apoptosis. This data might be compared with studies by Bennett et al (Boyle et al., 1998; Bennett 1999) who co-cultured MDM with human vascular smooth muscle cells (VSMC) and showed over 7d a dramatic loss of VSMC induced by Mφ. The Mφ were not activated with cytokines. It should be emphasised that these studies were also carried out on early passage cells (P4-P6) which explains why mitotic levels were high.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Table 3-16: The effect of a confluent monolayer of Mφ on human MC proliferation

<table>
<thead>
<tr>
<th></th>
<th>Confluent Mφ</th>
<th>plastic well</th>
</tr>
</thead>
<tbody>
<tr>
<td>mitosis</td>
<td>0.62%</td>
<td>3.59%</td>
</tr>
<tr>
<td>apoptosis</td>
<td>3.83%</td>
<td>0.83%</td>
</tr>
</tbody>
</table>

3.15 CYTOKINE ACTIVATED HUMAN Mφ INDUCE HUMAN MC APOPTOSIS

Similar co-cultures were prepared to those described in the preceding section. Once established, and after washing, the co-culture was activated with human IFN-γ (500U/ml) and LPS (1μg/ml), after 24h of co-culture there was an increase in MC apoptosis (Figure 3-33, Figure 3-34) at 24h. In live activated co-cultures there were 7.2±1.6 cells positive for Hoechst uptake. Only 6.9% of these took-up PI as well, indicating that the majority of cells were apoptotic as opposed to necrotic. Similar increases in MC apoptosis were also present at 48h (data not shown). Like the rodent studies, there was a decrease in human MC mitosis. However, in the control wells (MC alone with activating cytokines IFN-γ and LPS), mitosis was also suppressed. It appeared that IFN-γ was a potent suppressor of mitosis in the human MC, though was not sufficient to induce apoptosis. In limited studies, when co-culture was activated in DMEM/F12 containing 1% FCS only, there was a small augmentation of apoptosis (9.6% MC apoptosis at 24h in activated co-culture vs. 2.5% in MC controls) compared with cultures in 10% FCS. Importantly, in similar co-culture using human MC of earlier passage (P4-P6 compared with P7-P10) the induction of apoptosis was more impressive. In those studies (n=4) 16.4% of MC were apoptotic at 24h compared with 2.8% in MC control wells and 4.0% in unactivated co-culture. Since similar ratios of MC: Mφ were used in all studies, one explanation for this observation is the higher rate of mitosis of the earlier passage cells (2.3% compared with 1.3%). The relationship between cell cycle and apoptosis is explored (Chapter 6, Chapter 8) further.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Figure 3-33: The effect of activation of human co-culture with IFN-γ (500U/ml) and LPS (1μg/ml) on MC mitosis and apoptosis at 24h. Co-culture was established in a 2.5 (MΦ): 1.0 (MC) ratio using human MC of passage 7-10. After 24h incubation cultures were fixed and assayed by fluorescence microscopy for MC mitosis and apoptosis (n=5).

3.16 HUMAN MΦ KILLING OF HUMAN MC IS NOT BLOCKED BY INHIBITORS OF NO GENERATION

When L-NMMA (100μM) was added to the co-culture at the time of activation there was no effect on either MC apoptosis or mitosis (Table 3-17), that is L-NMMA was not effective in counteracting the effects of activated human MDM on human MC

Table 3-17: The effect of L-NMMA (100μM) in cytokine-activated human co-culture

<table>
<thead>
<tr>
<th></th>
<th>No inhibitor</th>
<th>L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoptosis</td>
<td>7.6±2.6%</td>
<td>6.9±1.9%</td>
</tr>
<tr>
<td>mitosis</td>
<td>0.3±0.1%</td>
<td>0.3±0.1%</td>
</tr>
</tbody>
</table>
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

3.17 HUMAN MΦ DO NOT PRODUCE NITRIC OXIDE

To investigate why L-NMMA was ineffective, a search of the literature uncovered many publications on the relative inability of human MΦ to generate NO (Albina 1995; Weinberg et al., 1995). Authors had published data conclusively showing that human MDM produced iNOS in response to pro-

Figure 3-34: Fluorescent micrograph of activated human co-culture. Fixed wells (X 200) showing green MC and red MΦ. Note rounded up green cells with condensed nuclear material, indicative of apoptosis (arrows). (i) Live co-culture with Hoechst and PI present in the medium (X640) showing a green rounded up cell with cytoplasmic blebbing (arrow). The same cell fluorescing by different excitation wavelength (ii) shows the green, rounded cell admitting Hoechst only, not PI and binding to the condensed chromatin. (iii) Fixed co-culture (X200) with PI and Hoescht. A composite view showing red MΦ, green MC and two apoptotic MC bodies. Nuclear condensation is seen in the left-hand body.
inflammatory cytokines such as IFN-γ with LPS (Weinberg et al., 1995; Kashem et al., 1996). iNOS positive Mφ in inflamed human glomeruli have been demonstrated (Kashem et al., 1996; Furusu et al., 1998; Wang et al., 1997; Heeringa et al., 2001). However, studies of NO release in the form of nitrite have indicated that human MDM do not produce NO despite induction of iNOS. There are many possibilities for this (Figure 3-35). For example arginase II which is induced in Mφ might predominate (Waddington et al., 1998), other co-factors or substrates might be lacking (Albina 1995). However, a very comprehensive study by Weinberg (Weinberg et al., 1995) indicated that even in vitro enzyme studies of human MDM iNOS enzyme failed to result in NO production. Furthermore, Mφ derived from human bone marrow also fail to produce NO (data not shown). It should be noted however, that U937 cells (human monocytic cell line) which also do not produce NO in response to cytokines, can be induced to become abundant producers by transfection with the human iNOS gene (Bertholet et al., 2000).

![Diagram](image-url)

**Figure 3-35:** Diagram indicating the factors influencing the ability of human iNOS to generate NO in mesangial cells. The presence of arginase II might deplete intracellular arginine, the availability of oxygen, and co-factors such as BH4 will influence NO generation, as will the prevailing intracellular and extracellular levels of ROI.
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

Using the particular system described for maturing MDM, differentiated MΦ were plated (1.5 x 10^5) per well of 24 well plate and once adherent medium was replaced with full DMEM/F12 and cells were cultured overnight. Pro-inflammatory cytokines were added and cells were incubated for 24h (Table 3-18). Supernatants were assayed for nitrite using the Griess reagent. In none of these cases was there release of nitrite into the medium. Interestingly, when human cells ingested opsonised zymosan particles there was a small release of nitrite (Table 3-18). These levels compared favourably with nitrite released from rat BMD MΦ when ingesting rat serum-opsonised particles. Clearly human MDM have the ability to produce some NO in response to the appropriate stimulus.

To investigate this further, MΦ were seeded into wells and cultured for protracted periods (72h and 7d) with a variety of cytokines (LPS, IFN-γ/LPS, IL-4, IFN-γ/IL-4), which had been reported to induce substantial nitrite release from MDM (Paul-Eugene et al., 1995; Panaro et al., 1999; Vouldoukis et al., 1995; Kashem et al., 1996). None of these were able to induce NO release. Furthermore, supernatants from MΦ cultured in Teflon wells for 7d also did not produce nitrite. This final result is in contrast to data from Bennett’s group who indicated nitrite generation by MDM over 7d in culture (Boyle et al., 1998).

**Table 3-18: Nitrite generation by human monocyte derived MΦ over 24h**

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>LPS</th>
<th>IFN-γ</th>
<th>IFN-γ/LPS</th>
<th>OZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>1.21</td>
<td>2.84</td>
<td>2.14</td>
<td>1.37</td>
<td>10.36</td>
</tr>
<tr>
<td>±1.49</td>
<td>±1.14</td>
<td>±1.53</td>
<td>±1.41</td>
<td>±1.31</td>
<td>±2.69</td>
</tr>
</tbody>
</table>

Nitrite is expressed as nmole/10^6 cells/24h
N/A no additive, IFN-γ 500U/ml, LPS 1μg/ml, TNF-α 300U/ml, IL-1β 10ng/ml, opsonised zymosan (OZ) 25μg per well.
In concurrent experiments, for comparison rat BMD MΦ stimulated with IFN-γ/LPS generated 104.6 units of nitrite, and stimulated with OZ generated 13.8 units of nitrite.

**3.18 HUMAN MC DO NOT PRODUCE NITRIC OXIDE**

Proliferating human MC (three different growths) were grown to sub-confluence in T75 flasks in full DMEM/F12, then stimulated with cytokines for 48h. There was no release of nitrite detected by the Griess assay (Table 3-19). However, workers in Aberdeen had reported three human MC growths to
Chapter 3: Results 1. Inflammatory macrophages induce apoptosis of mesangial cells

release nitrite into supernatants in response to pro-inflammatory cytokines (Nicolson et al., 1993). The levels of NO reported were in keeping with the data for rat MC (but not MZ cells) (Chapter 3.6). Their data indicated combinations of IFN-γ/TNF-α and LPS did not induce nitrite (as confirmed by Table 3-19), but in addition, IL-1β was necessary. The human MC reported here were not exposed to a similar cytokine combination. The rat MC studies (Chapter 3.6) would suggest, however that the levels of NO reported by Nicolson et al would be insufficient to protect human MC from high levels of NO that could potentially be released from neighbouring cells in vivo. Immunohistochemistry of human IgA nephritis indicated that both Mφ and MC induce iNOS enzyme. In retrospect it would have been useful to assay supernatants from activated human co-culture for release of MC derived nitrite. Unfortunately, none were stored in suitable conditions for that purpose.

<p>| Table 3-19: Nitrite generation by human MC over 48h |
|-----------------|------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>NA</th>
<th>IFN-γ</th>
<th>IFN-γ/TNF-α</th>
<th>IFN-γ</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.56</td>
<td>0.15</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>±0.21</td>
<td>±0.41</td>
<td>±0.07</td>
<td>±0.39</td>
<td></td>
</tr>
</tbody>
</table>

nitrite is expressed as nmole/10^6 cells/48h

3.19 HUMAN MC UNDERGO CELL CYCLE ARREST AND APOPTOSIS IN RESPONSE TO HIGH LEVELS OF NO DONORS.

Whilst there is clear evidence above that NO plays no obvious role in human Mφ control of human MC in vitro, there may be a role for Mφ NO in vivo, that is not manifest in vitro. Unfortunately extraction of human Mφ from tissue is not possible due to the small amounts of tissue at biopsy and the uncertainty of Mφ presence at the time of biopsy sampling. Nevertheless, it was relevant to inquire whether proliferating human MC were susceptible to apoptosis and cell cycle arrest in response to the NO donor GSNO. Wells of 96-well plates were seeded with human MC (8,000 per well, left overnight, washed and replaced with full DMEM/F12. GSNO was added over a range of concentrations and the experiments were fixed and assayed microscopically at 24h (Table 3-20). These data indicate that NO
donors have similar effects on human MC as those seen on rat MC confirming NO-dependent killing is a possibility.

### Table 3-20: Induction of apoptosis of human MC in response to the NO donor, GSNO

<table>
<thead>
<tr>
<th></th>
<th>Gutathione</th>
<th>GSNO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0mM</td>
<td>0.5mM</td>
</tr>
<tr>
<td>apoptosis</td>
<td>2.1±1.6%</td>
<td>2.6±1.8%</td>
</tr>
<tr>
<td>mitosis</td>
<td>1.6±0.4%</td>
<td>0.1±0.3%</td>
</tr>
</tbody>
</table>

### 3.20 SUMMARY

- Quiescent rodent and human Mφ do not induce apoptosis of MC
- Cytokine activated rodent and human Mφ induce apoptosis and cell cycle arrest of MC
- In the rodent co-culture, iNOS inhibitors block, and iNOS knockout Mφ are incapable of, induction of apoptosis and cell-cycle arrest
- Mφ from inflamed glomeruli of rats also induce iNOS-dependent cell-cycle arrest and apoptosis of MC ex vivo
- Rat MC can also induce iNOS and produce low levels of NO
- In those MC that generate large amounts of NO, there is resistance to Mφ-mediated induction of apoptosis
- Human Mφ produce little NO
Chapter 4: Results 2

Regulation of mesangial cell apoptosis in co-culture by interferon-γ
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

4.1 IN CYTOKINE-ACTIVATED CO-CULTURE WITH RODENT MΦ, IFN-γ PRIMED RAT MC ARE MORE SUSCEPTIBLE TO APOPTOSIS THAN UNPRIMED CELLS

Both in vitro (Chapter 3) and in vivo, there is evidence that MC produce iNOS (Furusu et al., 1998). IgA nephropathy, a human glomerular disease can develop into a crescentic glomerulonephritis with inflammatory infiltrate. Immunohistochemistry of glomeruli from patients with such disease shows the presence of iNOS in both infiltrating MΦ and in MC, indicating that MC can potentially produce NO.

Experiments initially designed to investigate whether rat MC producing NO after IFN-γ plus TNF-α treatment would be protected from MΦ-derived NO killing, revealed an unexpected observation. Rather than MC being protected from apoptosis induced by MΦ, on the contrary, MC that were pre-treated with IFN-γ (300U/ml) and TNF-α (300U/ml) for 24h before co-culture underwent more apoptosis than unprimed cells in concurrent experiments. The increase was approximately a doubling (Figure 4-1). The presence of mitotic figures in the co-culture was completely suppressed, suggesting that MΦ-derived NO was still able to act on the NO producing MC (not shown). The observation that MC that were producing NO themselves were still susceptible to NO released from MΦ was seen in Chapter 3.7 where GSNO induced apoptosis and cell cycle arrest of IFN-γ/TNF-α primed MC.

![Figure 4-1: The effect of priming of rat MC on MΦ induced apoptosis in activated co-culture at 24h. Rat MC were primed with IFN-γ (300U/ml) and TNF-α (300U/ml) for 24h prior to co-culture. After washing they were labelled with CMFDA and were then cultured in a 1.5 MΦ : 1.0 MC ratio. After washing, the co-culture was activated (IFN-γ 100U/ml plus LPS 1μg/ml) and assayed for MC apoptosis at 24h (n=5 * P < 0.05).](image_url)
4.2 L-NMMA PARTIALLY INHIBITS Mφ MEDIATED APOPTOSIS OF PRIMED RAT MC

Mesangial cells primed with IFN-γ (300U/ml) plus TNF-α (300U/ml) were co-cultured with rat BMD Mφ and the co-culture was activated with IFN-γ (100U/ml) and LPS (1μg/ml). The NOS competitive inhibitor L-NMMA or its inactive analogue D-NMMA was added at 100μM simultaneously with activation. There was induction of apoptosis by Mφ at 24h. In co-cultures with L-NMMA present, the percentage MC apoptosis induced was reduced compared with experiments including D-NMMA in the medium (Figure 4-2). However, unlike co-culture with unprimed MC where L-NMMA completely blocked killing (Table 3-9), in these experiments, the effect was limited, not exceeding a fifty percent reduction. Experiments were also performed with L-NMMA at 500μM. Despite the high dose of NO competitive inhibitor, the primed MC still underwent apoptosis compared with control wells of primed MC with cytokines i.e. 500μM was no more effective than 100μM L-NMMA.

Figure 4-2: The effect of priming rat MC with γIFN plus TNFα on susceptibility to apoptosis by activated rat BMD Mφ in the presence of iNOS inhibitors. MC were pre-treated with γIFN (300U/ml) plus TNFα (300U/ml) (primed), or PBS carriage medium (unprimed), for 36h. BMD Mφ were added to wells and primed with γIFN (100U/ml) for 12h. Co-culture was established and Mφ activated with γIFN (100U/ml) plus LPS (1μg/ml) according to Methods. L-NMMA (100μM) or D-NMMA (100μM) was added to the co-culture with the activating cytokines. At 24h, unprimed MC (left hand columns) underwent apoptosis in the presence of D-NMMA, but in the presence of L-NMMA this was abrogated. Primed MC (right hand columns) also underwent apoptosis, but the frequency was increased in the presence of D-NMMA, and L-NMMA was only partially able to abrogate this effect. * P < 0.01 vs. unprimed co-culture with D-NMMA. ** P < 0.01 vs. control primed MC with L-NMMA (n=5).
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

4.3 BMD Mϕ from NOS 2 Knockout Mice Can Induce Apoptosis of Primed MC

The rat co-culture experiments described suggested non-NO dependent killing of MC by Mϕ was occurring when the MC had been exposed to a synergistic combination of IFN-γ with TNF-α over the preceding 24h. However, the generation of NO by both Mϕ and MC renders this interpretation of the results complicated. To clarify the mechanism, BMD Mϕ from 129s/v iNOS knockout mice were employed in the co-culture and compared with littermate wild type controls (Figure 4-3). The results re-iterate the data derived from similar experiments shown above with the iNOS inhibitor L-NMMA (Chapter 4.2); that is that Mϕ can induce apoptosis of MC by a mechanism independent of NO generation by the Mϕ.

![Figure 4-3](image_url)

**Figure 4-3:** The effect of activated Mϕ from NOS 2 knockout mice on unprimed and primed MC apoptosis. BMD Mϕ from NOS 2 -/- 129/sv and wild type mice were matured as described in Methods. MC were primed or not primed with IFN-γ (300U/ml)/TNF-α (300U/ml), and the co-culture was established and activated as before. At 24h of co-culture there was induction of MC apoptosis when wild type mouse Mϕ were employed. NOS 2 -/- Mϕ had a greatly reduced capacity to induce apoptosis of unprimed cells. Wild type Mϕ induced increased apoptosis of primed MC at 24h. However, NOS 2 -/- Mϕ were also able to induce a more modest degree of apoptosis of primed MC. * P < 0.01 vs. NOS 2 -/- Mϕ in co-culture with unprimed MC. ** P < 0.001 vs. control primed MC. n=4.

These experiments were repeated using MC that had been primed with IFN-γ alone for 24h prior to co-culture (Table 4-1). The MC therefore were not generating NO prior to the co-culture (Chapter 3.6.). MC that had been primed with IFN-γ alone were also more susceptible to apoptosis than unprimed cells and the additional susceptibility was independent of NO generation by Mϕ (not shown).
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

Table 4-1: Augmented induction of rat MC apoptosis by activated Mφ following MC priming with IFN-γ (300U/ml) alone

<table>
<thead>
<tr>
<th></th>
<th>Primed MC</th>
<th>unprimed MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>% MC apoptosis at 24h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activated co-culture</td>
<td>28.9±1.6%</td>
<td>18.1±3.1%**</td>
</tr>
<tr>
<td>Activated MC alone</td>
<td>4.5±1.5%</td>
<td>2.1±1.4%</td>
</tr>
</tbody>
</table>

**P < 0.01 n=5

4.4 SUPERNATANT TRANSFER FROM ACTIVATED Mφ TO PRIMED MC INDICATED CLOSE APPPOSITION OF CELLS IS REQUIRED FOR KILLING

To test whether the additional factor that enabled killing of primed MC (by apoptosis) by cytokine activated Mφ supernatant transfer experiments were established comparable to those described (Chapter 3.3).

One hundred and fifty thousand BMD Mφ were cultured in wells of 24-well plates (2.0cm²) in 500μl of full DMEM/F12 for 24h. They were then activated with either IFN-γ (100U/ml) plus LPS (1μg/ml), or with carriage medium only (not activated). After 18h, the supernatants were collected, centrifuged (4°C 5min 4000g) to remove any cell debris. Mesangial cells which had been primed with IFN-γ/TNF-α (300U/ml) for 24h were washed, trypsinised and seeded into wells of 96-well-plates at a 70% density 4h before supernatant transfer. Two hundred microlitres of freshly isolated Mφ-conditioned medium were transferred to rat MC. Cultures were continued for 8h and 24h. As controls, fresh medium containing IFN-γ/LPS was placed on adjacent MC. At 8h, and 24h, wells were fixed and assessed for MC apoptosis.

Conditioned medium containing 10% FCS did not induce apoptosis of MC (0.85% vs control 1.05% (fresh full medium with IFN-γ plus LPS), nor was there any effect on mitosis. When the experiments were repeated using DMEM/F12 without FCS (i.e. serum free medium) the conditioned media from activated Mφ now induced apoptosis over and above controls (Table 4-2). This data suggests the need for close apposition in the NO-independent induction of apoptosis. However, it is possible that since the effective dose of this apoptosis-inducing reagent will be much lower in a supernatant than at the co-
culture monolayer, it will be ineffective and therefore an artefact of cell culture. In low FCS conditions soluble factors might be more effective (see Chapter 8).

Table 4-2: The effect of conditioned medium from activated Mφ on MC apoptosis and mitosis in the absence of FCS at 24h

<table>
<thead>
<tr>
<th></th>
<th>Unactivated Mφ</th>
<th>Activated Mφ</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoptosis</td>
<td>3.7±1.0%</td>
<td>10.4±0.9%*</td>
</tr>
<tr>
<td>mitosis</td>
<td>1.8±0.3%</td>
<td>1.6±0.2%</td>
</tr>
</tbody>
</table>

Control medium with activating cytokines (IFN-γ 100U/ml plus LPS 1μg/ml) induced 5.7% apoptosis. Mitosis was 1.4% (* P < 0.05 n=4).

4.5 RAT MC PRIMED WITH IFN-γ PLUS TNF-α ARE SUSCEPTIBLE TO APOPTOSIS BY SOLUBLE RECOMBINANT FAS LIGAND

Rat MC were plated into wells at 70% density and exposed to soluble human recombinant Fas ligand (sFasL). There was no effect on MC apoptosis at 24h as assessed by Hoechst and PI staining of fixed wells. However, when rat MC that had been primed for 24h with IFN-γ and TNF-α in combination, prior to plating into wells for experimentation, sFasL (100ng/ml) induced apoptosis of MC (Figure 4-4). To be sure this observation was not an artefact of the clonal cells, other polyclonal cells were compared (Figure 4-4) and showed similar induction of apoptosis after similar priming. Further, other rat MC clones were susceptible to sFasL only after IFN-γ/TNF-α priming, as was the MZ clone from Prof. Pfeilschifter’s group (18.0% vs 0.7% at 16h). Priming with IFN-γ alone resulted in 0.5% apoptosis vs. 0.0% (control), and after IL-1β priming 0.4% vs. 0.0% (control).

The pattern of induction of apoptosis by FasL was different from that induced by NO; firstly, mitotic cells were observed in wells where apoptosis was induced (though this was reduced compared with control wells). Secondly, induction of apoptosis occurred earlier and peaked (Table 4-3) earlier than induction of apoptosis in co-culture (Chapter 3.2) or upon administration of exogenous GSNO (Chapter 3.7). Peak apoptosis with FasL occurred at 16h.

Using human MC, Gomez (Gonzalez-Cuadrado et al., 1996) reported that IFN-γ priming alone of human MC was sufficient to augment susceptibility of these cells to apoptosis by the agonistic anti-Fas
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-\(\gamma\)

antibody CH11. The experiments reported in this thesis indicated that rat M\(\phi\) primed with IFN-\(\gamma\) alone were not rendered susceptible to apoptosis by sFasL. However, 24h of priming with TNF-\(\alpha\) (300U/ml) alone did render cells slightly susceptible (Figure 4-5). This difference between human and rat cells might relate to activation of the IRF-1 (interferon regulatory factor-1) promoter which is investigated in Chapter 6. As a positive control, Jurkat cells (a T cell lymphocytic leukaemia cell line), with known susceptibility to Fas-mediated apoptosis were cultured with sFasL at 100ng/ml. At 15h, there was 31\(\pm\)1.7\% apoptosis vs 3.9\(\pm\)0.2\% in controls without sFasL. Apoptotic morphology was assessed by cytopsin following Diff Quik staining. This result is in keeping with reports from Jurg Tschopp’s group (Schneider et al., 1998). Interestingly when CH11 anti-Fas IgM antibody at 50ng/ml was applied to Jurkat cells over a similar time period apoptosis was > 95\%.

![Graph showing percentage apoptosis](image)

**Figure 4-4:** The effect of priming rat MC with \(\gamma\)IFN plus TNF\(\alpha\) for 24h on susceptibility to Fas ligand (Apo-1 ligand) and expression of Fas protein. These studies employed both cloned primary MC (filled bars) and conventional multiclonal primary cultures (unfilled bars). Apoptosis assessed at 16h. Note that unprimed MC (two sets of bars on the left) were not susceptible to soluble FasL at 100ng/ml (NA = no additives). However note a significant increase in apoptosis after exposure of cytokine primed MC to FasL. Note also similar results using cloned MC or primary cultures * \(P < 0.01\) vs unprimed with FasL (\(n=3\)).

During the course of this work, Schlondorff’s group in Germany demonstrated identical findings using an SV40 transformed mouse mesangial cell line: the agonising anti-Fas antibody, Jo-1, had no effect on the mouse MC. However, when primed with IFN-\(\gamma\) plus TNF-\(\alpha\) the cells were now sensitised to Fas-mediated apoptosis (Berger et al., 1998).
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

Table 4-3: Time course for induction of apoptosis of primed rat MC by soluble Fas ligand at 100ng/ml

<table>
<thead>
<tr>
<th>Time</th>
<th>8h</th>
<th>16h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>21.1±3.7%</td>
<td>40.5±4.4%</td>
<td>15.0±5.3%</td>
</tr>
<tr>
<td>apoptosis</td>
<td>0.5±0.3%</td>
<td>1.2±0.8%</td>
<td>1.9±0.7%</td>
</tr>
<tr>
<td>mitosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.5±0.5%</td>
<td>1.0±0.5%</td>
<td>0.3±0.1%</td>
</tr>
<tr>
<td>apoptosis</td>
<td>2.3±.5%</td>
<td>4.9±1.3%</td>
<td>2.0±0.5%</td>
</tr>
<tr>
<td>mitosis</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4-5: Dose response curve for induction of apoptosis by sFasL on IFN-γ/TNF-α primed rat MC. Rat MC were primed for 24h with either IFN-γ (300U/ml) with TNF-α (300U/ml) or TNF-α alone. Cells were washed, trypsinised and re-plated at 70% confluency. After 4h FasL was added. Cells were fixed and scored for apoptosis at 16h. Note priming with IFN-γ alone had no effect. Note, FasL at 200ng/ml was no more effective than 100ng/ml.

To determine the efficacy of sFasL a dose response profile was generated using primed rat MC cells (Figure 4-5). This study indicates the relatively high concentrations of FasL required for induction of apoptosis compared with the actions of other cytokines (such as TNF-α). The dependency on high
concentrations of sFasL may reflect the need for trimerization of the molecule for it to be effective at activating Fas (Schneider et al., 1998). There is a wealth of literature on the role of soluble versus membrane-bound Fas ligand in bringing about apoptosis. Much of this data has been published during the generation of this thesis. The debate has hinged on whether FasL released from the surface of leucocytes can effectively trimerize the receptor Fas (Schneider et al., 1998; Cheng et al., 1994; Serrao et al., 2001; Brown & Savill 1999). However, recent data from cells multiply transfected with cDNA for Fas-tagged with different fluorophores, indicates that Fas (and other death receptors) exist as preformed trimers in the plasma membrane as do their ligands. The possible role of multiple trimeric receptor clustering might therefore be more important in death signalling. Data from our laboratory suggest that sFasL released from human MΦ can kill human neutrophils (Brown & Savill 1999), probably in conjunction with other cytokines, The data are supported by others (Kiener et al., 1997; Mangan et al., 1991). However, there are significant data suggesting other roles for sFasL such as blockade of Fas-induced apoptosis (Schneider et al., 1998; Cheng et al., 1994 Tanaka et al., 1998), or as a neutrophil chemokine (Allison et al., 1997; Seino et al., 1997; Kang et al., 1997; Chen et al., 1998). It is possible that soluble recombinant FasL is better able to self trimerize/induced receptor clustering at high concentrations that endogenous sFasL which is cleaved from eukaryotic cells.

When experiments on rat MC were repeated in serum free conditions, cells were marginally susceptible to apoptosis by sFasL, above control levels (control 6.1% vs sFasL 13.7% at 16h). When TNFα was also added there was a dramatic induction of apoptosis that was not mediated by TNF-α alone (TNF-α 9.8% vs TNF-α plus sFasL 20.8% at 16h), indicating synergy. The fact that TNF-α signalling through TNFR1 recruits DISC proteins might allow cross-talk with Fas, since Fas utilises the same DISC proteins (Varfolomeev et al., 1996).

These data indicate that in some way priming renders cells more susceptible to Fas ligand.

4.6 PRIMED RAT MC INCREASE EXPRESSION OF THE DEATH RECEPTOR FAS

Numerous regulatory steps in caspase 8-dependent signalling and DAXX-dependent signalling following Fas trimerization have now been described, principally in lymphocytes (Holler et al., 2000; Yanagisawa et al., 1997; Ungefroren et al., 1998; Boise et al., 1997; Duckett et al., 1998) (Figure 1.8). These factors seem to regulate a cell’s susceptibility to death. It appears that Fas (and FasL) are fairly ubiquitous, so clearly Fas mediated apoptosis must depend on other factors rendering cells susceptible.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

One factor might simply be the density of cell surface receptors since trimerization of Fas is required for signalling, another might be cell to cell receptor-ligand co-factors.

To investigate further, antibodies were selected to assess cell-surface Fas expression in rat mesangial cells. Unfortunately, no commercially available antibodies have been raised against rat Fas, they are all against human and mouse Fas, with some reported to display cross-reactivity. The most widely used human (CH11, ZB4) and mouse (Jo1) antibodies do not cross-react with rat Fas. Two antibodies were selected for flow cytometry (Transduction labs F22120, Pharmingen). In repeated studies, however neither showed selective binding to unprimed or primed rat cells (data not shown). Instead, membrane fraction lysates from primed and unprimed rat mesangial cells were prepared. Using the AB-1 anti-Fas antibody (Calbiochem) which recognises the trans-membrane domain of rat Fas (Kimura et al., 1997) a band at 45kD was faintly present in unprimed cells and increased in primed cells (Figure 4-6). Equal loading was ensured by Coomassie staining of the polyacrylamide gel. Since the ubiquitous actin also lies at about 45kD, adjacent lanes containing the same amount of protein were blotted for β-actin. Clearly this intense band migrates slightly further than the Fas band. The results suggest therefore that it is likely that cell surface Fas is increased. This issue was clarified further with human MC (Chapter 4.9).

**Figure 4-6:** Immunoblot showing increased expression of the Fas protein with IFN-γ/TNF-α priming of rat MC for 24h. Whole cell Triton-X 100 lysates were prepared from unprimed cells (Lanes 1 & 3) or cells primed with IFN-γ/TNF-α (Lanes 2 & 4). After normalisation for protein loading, the gel was electrophoresed, and blotted. The membrane was incubated with AB-1 (1:40) anti-Fas antibody overnight. Visualisation used a peroxidase conjugated secondary antibody and enhance chemiluminescence. Adjacent lanes with the same loading were separated and incubated for β-actin to demonstrate a different migratory pattern. The blot indicates increased Fas expression after priming.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

4.7 RED-OX POTENTIAL AND FAS-MEDIATED KILLING

There has been significant interest in the red-ox potential of cells in regulating susceptibility to Fas mediated killing. Human lymphocytes that are induced to express iNOS and release of low levels of NO are rendered susceptible to Fas-mediated killing (Mannick et al., 1997; Sciorati et al., 1997). This susceptibility is blocked by the NOS inhibitor L-NMMA. Indeed, unpublished data from our laboratory indicates that in the presence of the iNOS inhibitor L-NIL, human PMN are protected from apoptosis induced by the agonistic anti-Fas antibody, CH11 (Dimmeler et al., 1998; S. Brown Personal Communication). It has been reported conversely that superoxide radicals also can protect cells from Fas mediated death (Clement et al., 1996). Human monocytes, activated with IL-1β or TNF-α however, undergo apoptosis upon Fas ligation using anti-Fas CH11 antibody. This effect is abrogated using N-acetylcysteine or glutathione (Um et al., 1996). The MC used here, when primed with IFN-γ and TNF-α became susceptible to apoptosis by sFasL. However, they also produced NO through iNOS. Experiments were designed to block NO generation to determine whether Fas-mediated killing could be suppressed. Rat MC were primed with IFN-γ and TNF-α for 24h in the presence of L-NIL (30μM). Cells were transferred to wells and exposed to sFasL in the continued presence of L-NIL 30μM. There was no difference in the ability of sFasL to induce apoptosis at 16h (28.4±4.6% vs 30.2±3.9%) after 16h. There was also no difference at 8h and 24h (not shown).

4.8 IFN-γ PLUS TNF-α TREATED HUMAN MC ARE ALSO SUSCEPTIBLE TO FAS-MEDIATED APOPTOSIS

Gonzalez-Cuadrado (Gonzalez-Cuadrado et al., 1996) had reported that human MC can undergo apoptosis after exposure to high doses of the agonistic IgM anti-Fas antibody CH11. The percentage apoptosis increased after treatment with IFN-γ. The same group went on to study (Gonzalez-Cuadrado et al., 1997) the fate of mesangial cells in vivo mice after bolus of the agonistic anti-Fas antibody Jo1. The treated mice underwent a very rapid mesangiolysis (presumably by apoptosis), suggesting that in vivo MC apoptosis by Fas might be biologically important. Of note, however, similar studies by Nagata’s group (Ogasawara et al., 1993; Lacronique et al., 1996) confirmed hepato-cellular death after bolus administration of the Jo 1 antibody but did not report pathology in the kidney.

Since the rat MC in vitro required priming with a combination of cytokines to render them susceptible, human MC were primed with IFN-γ plus TNF-α for 24h. After trypsinising, re-plating and washing,
either sFasL (100ng/ml) or the agonistic antibody CH11 (500ng/ml) was added to wells which were assayed at 16h for apoptosis (Figure 4-7). It was evident that the priming step rendered these cells very sensitive to apoptosis by CH11, but in adjacent wells sFasL at 100ng/ml was ineffective. Isotype control mouse IgM had no effect. Even at 500ng/ml human sFasL induced only 0.9±1.1% apoptosis of IFN-γ/TNF-α primed cells by 24h. Whilst sFasL was ineffective on human MC, concurrent experiments with the MZ rat MC clone confirmed the bioactivity of this agent.

The data indicated that unlike the rat cells, human MC that had been primed with IFN-γ alone or IFN-γ plus LPS were also susceptible to CH11 mediated apoptosis. TNF-α priming and IL-1β priming were ineffective at inducing susceptibility in human MC (Figure 4-7).

Further evidence that red-ox potential is not involved in regulating MC Fas-mediated apoptosis can be implied from these data. Human MC treated with the cytokines in Figure 4-7. did not produce measurable NO (unlike the rat MC) (Chapter 3.19). Therefore low level NO generation does not regulate Fas susceptibility in MC unlike that shown for human lymphocytes (Mannick et al., 1994).

![Figure 4-7: Induction of apoptosis by CH11 anti-Fas antibody after priming of human MC. Proliferating human MC were primed with cytokines as shown (IFN-γ 500U/ml, TNF-α 300U/ml, LPS 1μg/ml) for 24h. They were trypsinised seeded in full medium into wells at 70% confluence. After 4h they were exposed to CH11 500ng/ml or IgM control and assayed for MC apoptosis at 24h. Note IgM control did not exceed 0.9±1.1% apoptosis (n=3).]
4.9 PRIMED HUMAN MC HAVE INCREASED EXPRESSION OF CELL SURFACE FAS

Proliferating human MC (P5-7) growing in six-well plates were primed with IFN-γ and TNF-α for 24h, or primed with carriage medium only (unprimed). Cells were trypsinised and placed on ice with the anti-Fas antibody CH11 at 500ng/ml. An isotype control of mouse IgM was used on some cells. As a positive control, Jurkat cells were exposed to trypsin and after neutralisation were also incubated with IgM control or CH11 for 1h on ice.

**Figure 4-8:** Immunofluorescence flow cytometry histograms showing induction of Fas on the cell surface of human MC following priming with IFN-γ plus TNF-α. Proliferating human MC were trypsinised, pelleted and resuspended in blocking buffer alone (red), with the addition of CH11 at 2μg/ml (green) or IgM control (black). After 1h on ice, cells were washed, and a secondary FITC-conjugated antibody added. After 30min on ice cells were assessed by flow cytometry. (A) unprimed MC, (B) MC that had been primed with IFN-γ (500U/ml) plus TNF-α (300U/ml) for 24h and (C) trypsinised Jurkat cells as a positive control.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

After washing and addition of FITC-conjugated anti-mouse IgM antibody (1:400), samples were analysed by flow cytometry (Figure 4-8). The data indicate the presence of Fas on human MC. The expression is markedly increased by priming.

4.10 HUMAN AND RAT MΦ HAVE FAS LIGAND, BUT ONLY PARTICULATE PHAGOCYTOSIS RESULTS IN RELEASE OF SOLUBLE FAS LIGAND

It has been reported that MΦ can utilise FasL/Fas interaction to induce cell death of neighbouring cells (Badley et al., 1997; Brown & Savill 1999; Hughes et al., 1999). This interaction is well-established in lymphocyte interactions (Takahashi et al., 1994; Ashany et al., 1995; Waldner et al., 1997), though the role of FasL in MΦ cell mediated toxicity is more controversial. Clearly for MΦ in the co-culture (and also in vivo) to induce MC apoptosis by Fas/FasL interaction, they must express FasL at the cell surface. Reports of FasL expression by flow cytometry (Badley et al., 1997; Kiener et al., 1997) indicate both very low levels which require biotinylation of the primary antibody to achieve a detectable signal and large intracellular stores which are readily mobilised. In our own laboratory, mature human MDM have been shown to have cell surface FasL only using the anti FasL antibody, clone 33 (Pharmingen) (S. Brown unpublished observations). These discrepancies may relate to the use of antibodies which had been reported to detect Fas ligand (Santa Cruz) (Smith et al., 1998; Restifo 2000) have been shown to cross react with other cell surface proteins.

Given these difficulties in detection, a western blot approach was chosen using the clone 33 antibody. To clarify the presence of Fas ligand in/on macrophages a western blot approach was taken since the clone 33 antibody has been used successfully in the department with Jurkat cell controls (Brown & Savill 1999).

Whole cell Triton-X 100 lysates were prepared from human monocyte derived and rat BMD MΦ cultured in 6 well plates (approximately 1 x 10⁶ cells per well). MΦ had been cultured in Teflon for 7d and seeded into wells 16h earlier. Cells were activated with IFN-γ/TNF-α, IFN-γ/LPS or opsonised zymosan particles for 16h. Supernatants were collected on ice and simultaneously MΦ in wells were lysed with a Triton-X 100 lysis buffer (Chapter 2). Soluble cellular proteins were isolated by centrifugation of lysate at 10,000g for 10min (4°C). Supernatants were centrifuged at 1000g for 10min (4°C) to remove cell debris and then mixed with an equal volume of ice cold ethanol. The mixture was incubated at minus 20°C for 15min and then centrifuged at 0°C for 20min 15,000g. The protein precipitate was removed from the supernatant by decanting and resuspended in Laemmli buffer.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

The whole cell lysates were boiled with 0.1% SDS, then lyophilised at 4°C and resuspended in Laemmli buffer. Samples were run on a 12.5% SDS PAGE, transferred onto PVDF membrane and, after blocking with 5% marvel in PBS with 0.05% Tween 20, the membrane was incubated at 4°C for 48h with Clone 33 anti-human FasL antibody (1:200). After washing and the addition of secondary peroxidase-conjugated antibodies the membrane was developed with 4-chloro-1-naphthol. The membrane (Figure 4-9) indicates that both human and rat M(φ) have membrane bound FasL. The amount of protein does not appear to increase with cytokine activation. Rather, M(φ) that have ingested opsonised zymosan particles may have less membrane bound protein. The blot was re-incubated with anti β-actin to show protein loading. The blot would suggest that rat M(φ) produce rather less FasL than human M(φ), though that might reflect a greater avidity of the antibody for human FasL over rat FasL.

![Immunoblot for Fas ligand from Triton-X soluble extracts from human (lanes 1-3) and rat M(φ) (lanes 4-8).](image)

The blot of soluble protein isolated from supernatants (Figure 4-10) indicates that M(φ) release soluble FasL which is augmented in response to ingestion of opsonised zymosan particles. Cytokine stimulation did not promote sFasL release. Despite centrifugation the blots indicate significant membrane bound FasL was found in the supernatant. The amount of mFasL in supernatant might be increased following M(φ) ingestion of opsonised zymosan. This might reflect release of micro vesicles by the M(φ). These vesicles have been shown to have biological effects on neighbouring cells including induction of apoptosis (Martinez-Lorenzo et al., 1999). The rat M(φ) did not appear to release any
soluble FasL. This may reflect a true biological difference or might simply indicate a lower affinity of the clone 33 antibody for rat sFasL.

Figure 4-10: Immunoblots of soluble FasL in protein extracts from supernatants of $1 \times 10^6$ macrophages. At 16h supernatants were harvested and soluble proteins were precipitated (see Methods). After electrophoresis and transfer of proteins FasL was visualised with anti-FasL antibody (clone 33 1:200) (A) Supernatants from human MDM 16h after treatment with no agent (lane 1), IFN-γ (500U/ml) plus TNF-α (300U/ml) (lane 2), or opsonised zymosan (lane 3). (B) Supernatants from rat BMD macrophages 16h after treatment with no agent (lane 1), IFN-γ (300U/ml) plus TNF-α (300U/ml) (lane 2), IFN-γ (300U/ml) plus LPS (1µg/ml) (lane 3) zymosan (lane 4) or opsonised zymosan (lane 5). Note human MDM release the 27kDa sFasL spontaneously and this is augmented by opsonised zymosan. Rat Mφ do not release sFasL in this assay. Note release of mFasL.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

4.11 RAT MC EXPRESS FAS LIGAND

During the course of this study, several groups (Hughes et al., 1999; Koide et al., 1999) indicated that proximal tubular cells of the kidney produced both in vitro and in vivo Fas ligand. This observation appeared surprising given the known pro-apoptotic effects of this cytokine. It was suggested that tubular cells might regulate their population by ‘fratricide’ i.e. neighbouring cells might induce death on others by Fas/FasL interactions. Others had shown increases in Fas protein during stresses such as hypoxia (Tanaka et al., 1994; Khan et al., 1999) in tubular cells but also in cardiac myocytes, and indicated that Fas/FasL interactions might regulate hypoxia-induced apoptosis. These ideas were expanded on by Bennett using vascular smooth muscle cells suggesting Fas/FasL cell surface interactions might be important in P53-apoptosis (Bennett et al., 1998). Indeed, work from our own group using gld/gld mice indicated that Fas/FasL interactions did play a role in induction of apoptosis of distal tubular cells during the inflammatory response to acute ureteric obstruction (Hughes et al., 1999).

It was therefore of interest to ascertain whether MC might also express FasL. Rat MC were cultured in T75 flasks and primed with IFN-γ/TNF-α for 24h, or left unprimed. At 80% confluence cells were lysed with a NP-40 lysis buffer on ice, and scraped from the flasks. Soluble proteins were isolated in the supernatant after centrifugation and after re-suspending and boiling in Laemmli buffer were analysed by SDS-PAGE. After incubating with the clone 33 antibody, discrete bands were visualised after peroxide-conjugated secondary antibody binding (Figure 4-11). The blot clearly indicates specific binding at 33kD indicating the presence of FasL.

![Figure 4-11: Immunoblot for Fas ligand in rat MC.](image)

Rat MC were unstimulated or stimulated with IFN-γ/TNF-α for 24h. Soluble proteins were extracted in a buffer containing a detergent NP40. After electrophoresis (12% PA gel) and transfer of proteins, the PVDF membrane was incubated with clone 33 mAb (1:200), followed by 2nd peroxidase antibody. This membrane was visualised with enhanced chemiluminescence. Discrete 37kDa bands can be seen indicating the presence of FasL in rat MC. No other bands were visualised on this membrane.
4.12 FAS LIGATION IS NOT RESPONSIBLE FOR Mφ MEDIATED KILLING OF PRIMED RAT MC

One attractive hypothesis for the increased killing by Mφ of MC that have been primed, is that Mφ induce MC apoptosis by ligation Fas either by release of sFasL or by membrane interactions. In support of this, evidence of FasL/Fas interactions inducing target cell apoptosis by Mφ is provided from (Brown & Savill 1999; Badley et al., 1997). Further, it is clear that mouse MC can undergo apoptosis through Fas ligation in vivo (Hughes et al., 1999). However, against this hypothesis is that Triton-X 100 lysates showed that unactivated Mφ also have FasL, whereas unactivated Mφ do not have TNF-α for example. Also the target cells (MC) have FasL as well as Fas. To investigate further BMD Mφ were developed from gld/gld mice on the C3H/HEJ background. This spontaneous point mutation renders FasL unable to bind to Fas (Takahashi et al., 1994) and the mice susceptible to the lupus syndrome. However, on the C3H/HEJ background the organs are normal (i.e., there is no inflammation). The Mφ were assessed for NO production in response to IFN-γ/LPS; gld/gld Mφ produced 85.6±4.4 nmole/24h/10⁶ cells, wild type Mφ produced 103.7±9.9nmole/24h/10⁶ cells. Co-cultures were performed using the mutated mouse Mφ and the wild type littermate Mφ. The co-cultures were performed with primed and unprimed cells (Figure 4-12) and assayed at 24h. The data indicated that both primed and unprimed rat MC were induced into apoptosis to the same degree whether the Mφ can produce functional FasL or not. It is obviously possible that due to the species heterogeneity (mouse and rat) that mouse FasL might not bind well to rat Fas. However, the observations of increased killing of rat MC that had been primed occurred whether the Mφ were derived from rat or mouse (Chapter 4.2, 4.3). The animals used were purchased from Jackson Laboratories USA. They were not routinely genetically screened. Given the lack of histology to identify clearly the mice as mutated, it was important to be certain of the mice were indeed gld/gld. To this end, DNA was isolated from Mφ from the knockout and wild type animals. A 311bp segment, covering the predicted mutation site of the gene (Takahashi et al., 1994), was determined to be amplified by PCR using the primers 5'-GTGCGCTTGTGATCAACG-3' and 5'-CTCTGGAGTGAAGTATAAG-3' which were designed around the point mutation. Purified genomic DNA was then subjected to PCR (Chapter 2). The products were sequenced by a fluorescent dideoxy method. The sequence analysis (Figure 4-13) indicates the published point mutation in this animal was indeed present in the animals used for these experiments, but not present in the wild type controls.

These data indicate that at least in the co-culture system Mφ do not employ Fas ligand in inducing target cell death. Interestingly, Rubin-Kelley’s group published experiments (Wada et al., 1999) where
kidneys from wild type MRL mice had been transplanted into lpr/lpr MRL mice (i.e. a Fas mutated mouse that develops glomerulonephritis). The transplanted kidneys were not rejected (consanguineous) but the non-mutated kidneys developed glomerulonephritis and renal damage at the same rate as mutated kidneys. This result supports the data shown that suggest that Fas/FasL interactions are not important in deletion of MC in inflammation.

**Figure 4-12:** The effect of murine BMD Mφ with mutated, non-functional Fas ligand (gld/gld) in co-culture with unprimed and primed MC. BMD Mφ from the CH3/HEJ mouse were matured according to Methods. MC were primed or unprimed, and the co-culture was established and activated as described. At 24h, activated wild type and gld/gld Mφ induced similar amounts of apoptosis in unprimed MC. Primed MC were more susceptible to the apoptosis-inducing effects of these Mφ. However the gld/gld Mφ were not significantly different (n=5).

**Figure 4-13:** Fluorescent dideoxy sequencing of PCR products from genomic DNA from gld/gld and wild type macrophages using primers for the murine FasL gene. Genomic DNA was subjected to PCR using primers designed to incorporate the published point mutation. Excerpts from the gld/gld (1) and wild type (2) sequences confirm the published mutation resulting in phenylalanine to leucine conversion.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

4.13 IN CYTOKINE ACTIVATED RODENT CO-CULTURE WITH IFN-γ PRIMED MC, BLOCKADE OF TUMOUR NECROSIS FACTOR RECEPTOR 1 INHIBITS MC APOPTOSIS

The investigation of the NO-independent, Mφ-mediated induction of apoptosis, had not found an explanation in Fas ligand. However, one piece of evidence perhaps held an important clue. When Mφ were primed with IFN-γ alone (without co-incubation with TNF-α) the extra apoptosis seen in co-culture was still present. A number of other death receptors and their ligands are found on Mφ and peripheral cells. One common feature for some death receptors been that IFN-γ treatment of a variety of tumour cells renders them susceptible to apoptosis by ligation of these death receptors whereas otherwise the tumour cells would be resistant (see Chapter 1). Since the co-culture indicated a role for IFN-γ alone in modulating susceptibility of the MC it seemed logical to investigate other receptors and ligand in more detail. These receptors and their ligands are stylised in cartoon form (Figure 1.12). Although not included in this study, TRAIL, another TNF family of death ligands can ligate TR2 on target cells. The latter has been found on proximal tubular cells of the kidney (Choi et al., 2000). All these receptors can activate NFkB pathway and/or activate the caspase pathway.

To determine their role in Mφ:MC interactions, in collaboration with Carl Ware (La Jolla, USA) we were able to use chimeric soluble receptors as blocking agents in the co-culture. These proteins were generated by isolation of cDNA (Crowe et al., 1994), then fusion to the Fc portion of human IgG. The proteins were expressed in a baculovirus expression system and secreted by insect cells. They were purified from cell culture supernatants by Protein A sepharose columns. Proteins were provided in serum-free Hanks solution.

In the presence of competitive inhibitors of NO generation in the co-culture of activated Mφ with IFN-γ primed MC, the remaining kill would be due to factors other than NO. This model was used for addition of soluble receptors.

Co-culture was established and at the time of activation with IFN-γ and LPS L-NIL (30μM) and the soluble receptor (10μg/ml) was added. As controls human IgG, or no receptor were used. Experiments were assayed at 24h, though some experiments were extended to 48h, when the results were very similar (Figure 4-14). The results showed that TNFR1 blockade was effective at reducing the ability of Mφ to kill MC. The inhibition was not complete however, so a dose response curve was generated to determine whether higher concentrations of TNFR1-Fc would increase inhibition further (Figure 4-14). Whilst 50μg/ml of TNFR1-Fc was slightly more potent than 10μg/ml, the effect was still not complete inhibition. It remained possible that other receptor ligand interactions were relevant. However,
blockade of LTBR and HVEM (or both together (not shown) did not have any effect at 24h or 48h, suggesting these receptor-ligand interactions were not important for MC apoptosis.

Figure 4-14: Soluble tumour necrosis factor receptor 1 inhibits Mφ killing of primed rat MC. (A) Murine BMD Mφ were co-cultured with MC as described in Methods. Experiments were activated with IFN-γ and LPS as described, but in addition L-NIL (30μM) was added to block NOS 2-mediated killing, and were assayed at 24h. Additionally, soluble Fc-fusion proteins TNFR1-Fc, LTBR-Fc, HVEM-Fc, or isotype control IgG1 were added in culture medium at 10μg/ml. Control wells containing MC but no Mφ received all reagents. Note a reduction in the capacity of Mφ to induce apoptosis in the presence of TNFR1-Fc. * P < 0.05 by single factor ANOVA (n=3). (B) Using the same co-culture assay of MC apoptosis, a range of doses of TNFR1-Fc was added to wells and apoptosis quantified, compared with 50μg/ml of IgG1 control shown as zeroμg/ml of TNFR1-Fc on the curve (n=3).
4.14 TNF-α/TNF-β DOUBLE KNOCKOUT MOUSE Mφ SHOW IMPAIRED ABILITY TO INDUCE APOPTOSIS OF IFN-γ PRIMED MC

The fusion protein studies did not definitively determine the precise role of TNF in regulating MC apoptosis. They did however point to TNFR1 ligation as the likely candidate in bringing about MC apoptosis. In collaboration with Prof. Ryffel at University of Cape Town, murine BMD Mφ, deficient in TNFα (Yoshioka et al., 1993; Takemura et al., 1994) and because TNFβ has a similar affinity for TNFR1 as TNFα, mice deficient in both gene products were used (Rudin et al., 1997). Preliminary studies showed both knockout and wild type Mφ produced similar amounts of NO (nitrite) in response to IFN-γ with LPS (50.0±4.6 [WT] compared with 47.0±3.3 [TNF KO] measured in nmoles per 10⁶ cells per 24h). In co-culture, both wild type and knockout Mφ had similar capacity to induce apoptosis of unprimed or quiescent MC at 24h. MC apoptosis was 20.6%±1.0 at 24h induced by wild type Mφ and 18.1%±3.1 induced by knockout Mφ.

However, in co-culture with primed MC the knockout Mφ were significantly less able to induce apoptosis at 24h than the wild type littermates (Figure 4-15).

Figure 4-15: The comparative effect of activated wild type and TNFα/TNFβ knockout murine Mφ on apoptosis of MC primed with IFN-γ. MC were primed as described for 24h in the presence of IFN-γ (300U/ml). Co-culture was established using BMD Mφ from either TNFα/β knockout mice or WT littermate controls and activated according to Materials and Methods. Experiments were assayed after 24h. Note that the knockout Mφ were less able to induce MC apoptosis than the WT controls. * P < 0.05 vs WT co-culture (n=5).
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

To determine further whether the additional ‘kill’ of MC was solely due to TNF release similar experiments were performed in the presence of L-NIL 30μM. In these experiments induction of MC apoptosis by knockout Mφ was no different from control MC alone with activating cytokines, implying the NO and TNF alone account for all the killing effect of Mφ on MC (Figure 4-16).

Supernatants from activated Mφ (IFN-γ plus LPS) cultured in 24-well plates, confirmed that the knockout Mφ were completely unable to produce TNF-α as assessed by ELISA (WT Mφ 3982 pg/ml (n=3), TNF KO Mφ < 0.00 pg/ml at 24h (n=3)).

![Bar chart showing percentage apoptosis in wild type and TNF KO Mφ](image)

**Figure 4-16:** Mφ directed killing in the absence of NO is TNF restricted. In a separate series of experiments to those in 2.14.1, co-culture was established with primed MC in co-culture with wild type and TNF-α/TNF-β knockout Mφ. In addition to activation, L-NIL (30μM) was added to wells to block NOS 2 mediated killing. Again, MC apoptosis was assayed at 24h. In these conditions, whilst the WT Mφ were able to induce MC apoptosis, the knock out animals incapable of inducing apoptosis greater than that seen in control wells. **P < 0.01 vs WT co-culture (n=5).**

4.15 IN LOWER SERUM CONDITIONS, TNF-DIRECTED KILLING OF RAT MC PREDOMINATES OVER NITRIC OXIDE.

All the co-culture experiments had been performed with 10% FCS in the medium (i.e. full DMEM/F12). This was in keeping with a model of glomerular inflammation where it is known that there can be an abundance of growth factors and the mesangium is exposed to plasma proteins.
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

However, when co-culture of BMD MΦ and unprimed MC was carried out in medium containing 1% FCS, there was a substantial increase in the ability of wild type activated MΦ to induce apoptosis compared with the TNF knockout MΦ (Figure 4-17). This was not due to different NO release since in DMEM/F12 without FCS wild type and TNF KO MΦ produced similar amounts of nitrite (40.6±8.2 [WT] compared with 35.4±1.6 [TNF KO] measured in nmoles per 10^6 cells per 24h).

These data contrast sharply with similar experiments carried out in the presence of 10% FCS where there was no such difference (see above). It seems likely therefore that in conditions of low FCS, the predominating mechanism of MΦ mediated death is by TNF release rather than NO.

![Graph](image)

**Figure 4-17:** The effect of wild type and TNF-α/TNF-β knockout MΦ on unprimed MC in low-serum conditions. Co-culture was established as described in Methods using BMD MΦ derived from WT or TNF-α/TNF-β knockout animals. Once established medium was removed and replaced with similar medium containing only 1% FCS. Cultures were activated as described with IFN-γ (100U/ml) and LPS (1μg/ml) and were assayed at 24h. WT MΦ were more potent in their ability to induce MC apoptosis. **P < 0.01 vs WT co-culture (n=5).**

4.16 IN CYTOKINE-ACTIVATED CO-CULTURE WITH HUMAN MΦ, IFN-γ PRIMED HUMAN MC ARE MORE SUSCEPTIBLE TO APOPTOSIS THAN UNPRIMED CELLS

In Chapter 4.8, it was shown that human MC that were primed were susceptible to Fas mediated induction of apoptosis. The data indicated that IFN-γ priming alone seemed to be sufficient to enable Fas mediated killing though this was augmented by priming with both IFN-γ with TNF-α. The results of co-culture of cytokine activated human MΦ with unprimed human MC also demonstrated MΦ-dependent killing that was independent of NO generation. Despite the results in rat co-culture
indicating no role for FasL/Fas interactions, Fas mediated killing of human MC seemed to be a possible candidate. Furthermore, given that data on co-culture of rodent cells, TNFR1 ligation also seemed a strong candidate.

Initially human MC were primed for 24h with human IFN-γ (500U/ml) or left unprimed and then established in co-culture. The co-culture was activated with IFN-γ and LPS and assayed at 24 and 48h. MC apoptosis at 24h was increased in those experiments using primed MC, again indicating that priming of MC increases their susceptibility to Mφ mediated killing (Figure 4-18).

**Figure 4-18:** Interferon-γ augments killing of MC by apoptosis in activated human co-culture. Human MC were either primed with IFN-γ (500U/ml) for 24h or left unprimed. Co-culture was established with human MDM after labelling MC with green CMFDA. After 4h to allow firm adhesion of cells cultures were washed, activated with IFN-γ (500U/ml) plus LPS (1μg/ml), then incubated for 24h. Note that activated Mφ induce apoptosis of MC but the absolute percentage of apoptosis is increased significantly (*P < 0.05, n=5) in cultures with primed human MC.

### 4.17 TNFR1 BLOCKADE RATHER THAN FAS BLOCKADE INHIBITS HUMAN Mφ-DERIVED KILLING OF BOTH PRIMED AND UNPRIMED HUMAN MC

Since no patients with Fas deficiency were available (Wu et al., 1996) it was decided to test whether human Mφ utilise FasL to induce MC apoptosis by blocking studies using the anti-Fas blocking antibody, ZB4. Unfortunately, in preliminary cultures of human MC alone, this antibody induced apoptosis of IFN-γ/TNF-α primed human MC. It was equally efficacious as CH11, the agonist anti-Fas antibody (see Chapter 4.8). This paradox might be explained by the possibility of immunoglobulin
aggregates forming in the antibody stock, since ZB4 and CH11 bind the same epitope on human Fas (Fadeel et al., 1997; Komada et al., 1999). However, their difference in function has been attributed to CH11 polymerising Fas whereas the IgG, ZB4 prevents polymerisation. After consultation with Prof. Nagata (Osaka Bioscience Institute, Japan), it was decided to use the blocking anti-Fas ligand antibody which has been shown to be at least ten fold more potent than soluble Fas-Fc fusion proteins. Unfortunately, the isotype of type of hamster anti-FasL IgG produced by the clone is not known. Therefore, no isotype control antibody was used in these studies.

Concurrent experiments using TNFR1-Fc, LTβR-Fc and HVEM-Fc along with human IgG, isotype control were carried out to try to elucidate the mechanism of Mφ killing of primed human MC.

At 24h (Figure 4-19), anti FasL IgG (1μg/ml) had no effect on human MC apoptosis. HVEM-Fc and LTβR-Fc at 10μg/ml were also without effect. However, TNFR1-Fc (10μg/ml) inhibited killing by more than 50%. At 48h (Table 4-4) the pattern was the same, i.e. only TNFR1-Fc blocked MC apoptosis.

![Figure 4-19: Induction of apoptosis of human mesangial cells by human monocyte-derived Mφ is inhibitable by TNF receptor 1 blockade. Co-culture was established using primed human MC and Mφ. Upon activation with IFN-γ plus LPS, soluble Fc-fusion proteins TNFR1-Fc, LTβR-Fc, HVEM-Fc, or isotype control IgG1 (10μg/ml) were added in culture medium at 10μg/ml. Also, the blocking anti-Fas ligand antibody (1μg/ml) was applied to adjacent wells. Control wells containing human MC but no Mφ received all reagents. Note a reduction in the capacity of Mφ to induce apoptosis in the presence of TNFR1-Fc, but not in the presence of any of the other reagents (** P < 0.01 vs activated co-culture plus IgG1 (n=4)).](image-url)
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

Studies of cytokine activated co-culture with unprimed cells showed that TNFR1 was effective at blocking MC apoptosis without the need for priming (Table 4-5).

Table 4-4: Inhibition of primed human MC apoptosis induced by activated human MØ at 48h

<table>
<thead>
<tr>
<th>MC apoptosis at 24h</th>
<th>Activated plus IgG control</th>
<th>Activated plus TNFR1-Fc control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FCS</td>
<td>12.4±%</td>
<td>2.7±%</td>
</tr>
<tr>
<td></td>
<td>4.1±%∗</td>
<td>1.8±%</td>
</tr>
</tbody>
</table>

(∗∗ P < 0.01 n=5)

Table 4-5: Inhibition of the induction of unprimed human MC apoptosis by human MØ at 24h by TNFR1-Fc

<table>
<thead>
<tr>
<th>MC apoptosis at 24h</th>
<th>Activated plus IgG control</th>
<th>Activated plus TNFR1-Fc control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FCS</td>
<td>7.2±0.4%</td>
<td>2.5±0.8%</td>
</tr>
<tr>
<td></td>
<td>4.1±0.9%∗</td>
<td>1.8±1.0%</td>
</tr>
</tbody>
</table>

(∗∗ P < 0.01 n=5)

Whilst these studies did not show complete blockade of MC apoptosis, limited mesangial cell tissue and blocking agents rendered further experiments not possible. However, the rodent studies (Chapter 4.13, 4.14) indicated that whilst TNFR1-Fc brought about 50% blockade, when knockout MØ were utilised complete blockade was seen. The data suggest therefore that at least over 48h, TNF signalling to cells that have also exposed to IFN-γ is responsible for human MC apoptosis.

4.18 LOW SERUM CONDITIONS DO NOT AUGMENT HUMAN MØ-DERIVED KILLING OF HUMAN MC

Studies of rat co-culture indicated that low serum conditions rendered MC more susceptible to TNF mediated apoptosis. It is noteworthy that background (controls) levels of apoptosis was also elevated
in those experiments, indicating the dependence on serum factors for survival (Mooney et al., 1997). However, when human co-cultures both primed and unprimed, were performed in low serum (1%) conditions there did not appear to be an augmented effect, nor did baseline levels of apoptosis increase (Table 4-6). This was regardless of whether the cells had been primed.

**Table 4-6:** Induction of human MC apoptosis in the presence of 10% FCS compared with 1% FCS

<table>
<thead>
<tr>
<th></th>
<th>Unprimed MC apoptosis at 24h</th>
<th>IFN-γ primed MC apoptosis at 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>co-culture</td>
<td>control</td>
</tr>
<tr>
<td>1% FCS</td>
<td>7.1±0.4%</td>
<td>2.5±0.8%</td>
</tr>
<tr>
<td>10% FCS</td>
<td>6.7±1.6%</td>
<td>1.8±0.4</td>
</tr>
</tbody>
</table>

n=4

### 4.19 TNFR1 CELL SURFACE EXPRESSION IS NOT INCREASED BY IFN-γ PRIMING OF HUMAN MC

Since IFN-γ/TNF-α priming of mesangial cells renders cells susceptible to death by Fas ligation and increases cell surface expression of the receptor, it seemed important and logical to ask whether IFN-γ priming alone of MC increased TNFR1 expression, since IFN-γ renders MC more susceptible to TNF-α mediated apoptosis. Unfortunately, due to a lack of specific antibodies against rat TNFR1, the studies were performed on human MC (127). T75 flasks of proliferating MC were either unprimed or primed for 24h with human IFN-γ for 24h. After trypsinisation, 1x10^6 cells were incubated at on ice for 1h with no antibody, mouse IgG1 control or mouse anti human TNFR1 antibody. Cells were washed x1 and incubated for 30min. with a FITC conjugated Fab of goat anti human IgG. After a further wash, cells were analysed by flow cytometry (Figure 4-20). The histograms highlight the difficulties in studying these cells by flow cytometry due to the four log_{10} order spread of auto-fluorescence. However, the results demonstrate specific binding of TNFR1 antibody which is not augmented by IFN-γ treatment.
Chapter 4: Results

2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

Figure 4-20: Fluorescence histogram of human MC showing TNF receptor 1 expression on unprimed and primed cells. (A) Unprimed cells, showing autofluorescence (black curve), murine IgG, plus FITC goat anti mouse IgG (red curve), and specific binding of mouse anti human TNFR1 plus secondary anti-IgG (green curve). (B) Cells were primed with IFN-γ (500U/ml) for 24h prior to analysis. Coloured curves represent the same conditions as in (A). Note there is low level expression of TNFR1. P < 0.001 (K-S statistics anti-TNFR1 vs. irrelevant IgG), but this is not increased after priming.

4.20 SUMMARY

- IFN-γ is able to augment the susceptibility of MC to killing by Mφ whether the cells are rodent or human
- MC treated with IFN-γ (plus TNF-α in rodents) are susceptible to Fas mediated killing, Mφ did not induce Fas-mediated death of MC despite the presence of ligands and receptors
Chapter 4: Results 2. The regulation of mesangial cell apoptosis in co-culture by interferon-γ

- Blocking studies in co-culture showed that TNFR1 ligation was important in rodent co-culture and fundamental in human co-culture.

- In the absence of NO generation (with iNOS inhibition or iNOS knockout mice for rodents) TNF mediated signalling from Mφ results in MC apoptosis in both humans and rodents

- The data point to a pivotal role of IFN-γ in controlling Mφ:MC interactions.
Chapter 5: Results 3

Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles
5.1 OPSONISED ZYMOSAN-ACTIVATED RODENT M\(\Phi\) INDUCE APOPTOSIS BUT NOT CELL CYCLE ARREST OF RAT MC

Co-cultures of rat BMD M\(\Phi\) and rat CMFDA-labelled MC in a 1.5:1.0 ratio were established in wells of 96-well plates in full medium for 4h. As a model of particulate immune complex activation of the co-culture, zymosan pre-opsonised with pooled rat sera was added (0.5mg/ml; 0.1mg per well) (see Chapter 1.7). Careful attention to elimination of contaminating LPS was made during the preparation of the opsonised zymosan (Chapter 2.6.7) (Johnston et al., 1985). Twenty-four hours later, the co-cultures were assayed for MC apoptosis both in live co-culture using Hoechst with propidium iodide and after fixation and counter-staining (Figure 5-1). As controls, wells containing MC alone with opsonised particles were assayed. In these studies, M\(\Phi\) readily ingested the opsonised zymosan (OZ). It was unclear whether MC also ingested particles. They developed large numbers of intracellular vesicles in the proximity of the Golgi apparatus, but it was not obvious that these contained OZ, unlike obvious particles seen within the M\(\Phi\).

The results demonstrated that co-culture, activated by OZ, induced M\(\Phi\) to bring about apoptotic cell death of MC. (Figure 5-2). Furthermore, unlike the cytokine activated rodent co-culture, mitoses were still observed (Figure 5-1, Figure 5-2), albeit the frequency was reduced, suggesting that nitric oxide might not be abundant.

When rat M\(\Phi\) were replaced in co-culture with mouse bone marrow-derived M\(\Phi\) and the zymosan was opsonised with mouse serum, similar effects were observed at 24h of co-culture (not shown).

5.2. OZ ACTIVATED RODENT M\(\Phi\) RELEASE LITTLE NO BUT ABUNDANTLY RELEASE H\(\text{H}_2\text{O}_2\)

To investigate this killing mechanism further, OZ-activated rat M\(\Phi\) were assayed for NO production (Table 5-1). The Griess reaction indicated much lower release of NO in the form of nitrite, than that released from cytokine activated M\(\Phi\). The assay was repeated, following nitrate reduction of samples, which indicated that a proportion of the NO had been converted to nitrate (Table 5-1), indicative of an environment rich in oxygen radicals (Waddington et al., 1996; Sandau et al., 1997).
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

Figure 5-1: Co-culture of CMFDA labelled rat mesangial cells with rat bone marrow-derived Mφ 24 h following exposure to opsonised zymosan. (A) In co-culture of unprimed MC with Mφ there is induction of apoptosis of MC apoptosis (X200). (B) Co-culture of IFN-γ/TNF-α primed MC with Mφ, there is still MC apoptosis but this is reduced. (C) In unprimed co-culture exposed to opsonised zymosan in the presence of catalase (100U/ml) killing of MC is abrogated (X320). (D) High power view of MC apoptosis (E) Mitotic MC (telophase) in activated co-culture.
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

Figure 5-2: The effect of opsonised zymosan activated rat Mφ on mesangial cell (A) apoptosis and (B) mitosis. Co-culture of CMFDA-labelled rat MC and rat Mφ in a 1.5:1.0 ratio was established. The co-culture was activated with rat serum-opsonised zymosan (0.5mg/ml). The culture was incubated for 24h, fixed, then assayed for MC apoptosis and mitosis (** P <0.01 vs MC alone n=5)

Table 5-1: Nitrite generation by rat BMD Mφ following binding and phagocytosis of opsonised zymosan.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nitrite</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unactivated</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>IFN-γ/LPS</td>
<td>25.5</td>
<td>21.3</td>
</tr>
<tr>
<td>Ops. zym.</td>
<td>2.5</td>
<td>10.0</td>
</tr>
</tbody>
</table>

nmol/24h/10^6 cells
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

After accounting for the additional NO that had been converted to nitrate, the OZ-activated Mφ generated much less NO than cytokine activated cells, however.

To investigate further the mechanism of Mφ killing, supernatants were assayed for hydrogen peroxide (H₂O₂) generation (Table 5-2). In contrast to cytokine-activated Mφ, OZ-activated Mφ produced large quantities of H₂O₂. Evidence in the literature of H₂O₂-generating Mφ in glomerular disease can be seen in work by Cook (Cook et al., 1989), Johnston (Johnson et al., 1987, Johnson et al., 1995) and Holdsworth (Holdsworth et al., 1985), where in both rabbit NTN and Thy 1.1 nephritis there is evidence of Mφ derived H₂O₂. However, these data should be interpreted cautiously since NO can produced false positive results in luminol assays (Kikuchi et al., 1993). However, Waddington and colleagues (Waddington et al., 1996) augmented injury in early heterologous phase NTN with arginase therapy, which blocks NO production. One interpretation of this result is that inhibiting NO production results in less quenching of ROI. Together with the earlier reports, this information would be supportive of a role for oxygen radical production by Mφ in vivo at least in the early stages of glomerular inflammation.

<table>
<thead>
<tr>
<th>Table 5-2: Peroxide generation by activated macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>No priming of Mφ</td>
</tr>
<tr>
<td>IFN-γ prime</td>
</tr>
<tr>
<td>LPS prime</td>
</tr>
</tbody>
</table>

Hydrogen peroxide (nmolar) assayed at 1h (PMA 10ng/ml, OZ 0.5mg/ml.)

In some experiments the Mφ were primed with cytokines prior to activation with OZ

5.3. INHIBITORS OF H₂O₂ RELEASE, BLOCK OZ-ACTIVATED Mφ-DEPENDENT KILLING OF RAT MC

Apoptosis seen in the co-culture assay at 24h was subjected to inhibition by L-NMMA (not shown), catalase and super oxide dismutase (SOD) (Figure 5-3). L-NMMA was ineffective at 500μM, as was SOD (500U/ml), but catalase abrogated Mφ killing of MC. Sugiyama (Sugiyama et al., 1996) was able to induce apoptosis of human MC by addition of exogenous H₂O₂ to cultures growing in full medium.
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

In his experiments, exogenous H$_2$O$_2$-mediated killing was blocked with catalase at 500U/ml; SOD was without effect. Here, 100U/ml of catalase was sufficient to block Mφ-mediated killing of rat MC, and 500U/ml showed no additional effect. The data clearly indicate a major role for H$_2$O$_2$ in bringing about apoptosis of rat MC. Of note, SOD did not appear to play any significant role in inhibiting apoptosis either alone or in conjunction with catalase (not shown). Rehan (Rehan et al., 1985) showed a role for hydrogen peroxide in the genesis of NTN in the rat, rather than other oxygen radicals. Catalase blocked generation of the disease whilst SOD had no effect. This inhibition was believed to be due to blockade of neutrophil mediated damage to endothelial cells at the onset of disease (Rehan et al., 1985 Johnson et al., 1987). However, as described in the preceding section, there is no reason to suppose that Mφ, also present in the acute inflammatory response in these nephritis models could not be activated to produce H$_2$O$_2$.

Figure 5-3: Induction of MC apoptosis by Mφ in opsonised zymosan activated co-culture is inhibitable by catalase. Co-culture was established as before and activated with opsonised zymosan. At 24h co-culture was fixed and after counterstaining was scored for MC apoptosis. In some wells the inhibitors S.O.D or catalase were added. These enzymes were suspended in PBS (** P<0.01 n=4).
5.4. RAT MC, PRIMED WITH IFN-γ PLUS TNF-α ARE RESISTANT TO H₂O₂ MEDIATED INDUCTION OF APOPTOSIS

This experiment was initially designed to test whether Fas ligand release from Mφ might induce mesangial cell death. Mφ that ingest OZ release FasL (see Chapter 4.10 and Brown & Savill 1999), which in some circumstances induces apoptosis of target cells. Rat MC that have been primed with IFN-γ and TNF-α are susceptible to apoptosis induced by FasL (see Chapter 4.5). Thus, it seemed plausible that Fas/FasL interaction might be responsible for MC death under these experimental conditions. Therefore one might have expected to observe increased MC apoptosis compared with unprimed cells due to an additional Fas mediated death signal.

Rat BMD Mφ were co-cultured with rat MC that had been primed with IFN-γ (300U/ml) plus TNF-α (300U/ml). The priming cytokines, TNF-α/IFN-γ were removed by washing. The co-culture was exposed to OZ (0.5mg/ml). Apoptosis was assessed at 24h.

Rather than demonstrate any additional role for Mφ-derived FasL in killing of MC by apoptosis, the rat MC primed with IFN-γ plus TNF-α for 24h prior to co-culture were more resistant to apoptosis (Figure 5-4). This result was opposite to that seen in the cytokine activated co-culture (Chapter 4.1) where priming of mesangial cells was able to augment Mφ directed killing. Blockade of the killing effect seen in Figure 5-4 with catalase, SOD or L-NMMA had no effect (Table 5-3). Thus the MC were rendered resistant to peroxide mediated apoptosis by priming.

Importantly, when MC were primed with IFN-γ alone (i.e. priming without TNF-α) they were no longer protected from peroxide mediated death (not shown). Thus, cells were only rendered resistant to H₂O₂-mediated apoptosis by priming with both IFN-γ plus TNF-α. These unexpected results might be explained by the generation of NO by mesangial cells primed with IFN-γ with TNF-α (see Chapter 3.6). As well as being cytotoxic, NO can be protective due to its ability to quench oxygen radicals, resulting in nitrate production. Evidence for this cytoprotective role can be seen in atherosclerosis, and UV damage of keratinocytes in the skin (Cooke et al., 1998; Bauersachs et al., 1999; Yamaoka et al., 2000). It seems therefore that in rodents, whilst IFN-γ/TNF-α primed cells are protected from peroxide mediated cell death, they remain susceptible to NO mediated death (Chapter 4), and are rendered susceptible to TNF-mediated death.
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

Figure 5-4: The effect of priming rat MC with IFN-γ and TNF-α for 24h prior to co-culture on the susceptibility of MC to killing by opsonised zymosan activated Mφ. Co-culture was established as before. MC were primed with IFN-γ (300U/ml) plus TNF-α (300U/ml) for 24h prior to co-culture (primed) or with carriage medium alone (unprimed). After 24h of co-culture MC apoptosis in co-culture and control wells was assessed (* P <0.05 n=5).

Table 5-3: The effect, in rodent co-culture of oxygen radical scavengers on OZ-activated macrophage-mediated killing of IFN-γ/TNF-α primed rat MC at 24h

<table>
<thead>
<tr>
<th></th>
<th>N/A</th>
<th>ops zym</th>
<th>+ catalase</th>
<th>+SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-culture</td>
<td>1.4±1.0%</td>
<td>12.9±2.2%</td>
<td>14.2±0.9%</td>
<td>12.2±2.4%</td>
</tr>
<tr>
<td>Control</td>
<td>0.9±0.7%</td>
<td>1.4±1.0%</td>
<td>1.0±1.2%</td>
<td>1.1±0.8%</td>
</tr>
</tbody>
</table>

Activated neutrophils undergo a respiratory burst and rapidly induce MC apoptosis by a catalase inhibitable mechanism (Table 5-4). MC that generate NO after priming are protected from this neutrophil mediated death. Clearly, it might be advantageous for MC to be protected from excessive cell death brought about by Mφ or neutrophil interactions.
Table 5-4: Catalase inhibitable induction of MC apoptosis by activated neutrophils

<table>
<thead>
<tr>
<th></th>
<th>LPS activated % apoptosis</th>
<th>LPS plus catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprimed MC</td>
<td>24.7±3.2%</td>
<td>3.4±1.8%</td>
</tr>
<tr>
<td>Primed MC</td>
<td>4.1±1.4%</td>
<td>ND</td>
</tr>
</tbody>
</table>

Co-culture was established in 96-well plates by seeding 1.2x10^4 CMFDA-labelled rat MC into wells. MC had been primed with IFN-γ (300U/ml) plus TNF-α (300U/ml) for 24h or left unprimed. An equal number of human blood-derived neutrophils was added to wells, then activated with LPS (1μg/ml) with or without catalase (500U/ml). The experiment was fixed with 4% formaldehyde then counter-stained with PI and apoptosis scored morphologically.

5.5 OZ ACTIVATED Mφ FROM GLD/GLD AND WILD TYPE MICE INDUCE CELL DEATH OF PRIMED RAT MC EQUALLY

When Mφ are activated by binding and ingestion of OZ, they release soluble FasL (see Chapter 5.4). The data above indicate that whilst IFN-γ/TNF-α primed MC were resistant to H₂O₂-mediated apoptosis they were still induced to die by Mφ through another mechanism. To test whether Mφ release of FasL had any role in this killing of MC, MC were rendered susceptible by priming with IFN-γ plus TNF-α for 24h. Wild type mouse Mφ and FasL deficient, gld/gld Mφ (described in Chapter 4.12) were prepared. Co-culture was established with IFN-γ/TNF-α primed rat MC and either WT or gld/gld Mφ in the presence of OZ (0.5mg/ml). These mesangial cells previously confirmed as susceptible to apoptosis by recombinant sFasL (Chapter 4.5). At 24h, MC apoptosis was assessed. The level of apoptosis was no different in wild type compared with knockout Mφ (Table 5-5). Adjacent wells of primed MC alone with exogenous sFasL (100ng/ml) displayed 13.4% apoptosis at 24h whilst primed cells without sFasL displayed 1% apoptosis.

These results indicate that whilst recombinant human sFasL can induce apoptosis in susceptible cells, when it is released from Mφ it is not effective as a pro-apoptotic agent.
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

Table 5-5: The effect of OZ-activated wild-type and gld murine Mφ on killing of IFN-γ/TNF-α primed rat MC at 24h

<table>
<thead>
<tr>
<th>Co-culture</th>
<th>MC control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>gld/gld</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>14.5±1.1%</td>
</tr>
<tr>
<td>Mitosis</td>
<td>1.5±0.3%</td>
</tr>
</tbody>
</table>

n=5

5.6 KILLING OF PRIMED RAT MC BY OZ-ACTIVATED Mφ DERIVED FROM TNF-α/β DEFICIENT MICE IS ATTENUATED.

Co-culture of mouse Mφ (derived from c57/bl6 mouse) and IFN-γ/TNF-α primed rat MC was established as before. Simultaneously with activation, either human IgG1 or TNFR1-Fc at 10μg/ml was added to wells. Apoptosis of MC was assessed at 24h and compared with controls of Mφ growing alone, but exposed to the same reagents. Table 5-6 indicates that killing of IFN-γ/TNF-α primed MC by Mφ, activated with OZ, was partially inhibitable by TNFR1-Fc.

Table 5-6: Inhibition of opsonised zymosan killing of primed rat MC by soluble TNF receptor 1

<table>
<thead>
<tr>
<th>Co-culture</th>
<th>MC control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 control</td>
<td>TNFR1-Fc</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>12.0±0.9%</td>
</tr>
<tr>
<td>Mitosis</td>
<td>0.6±0.2%</td>
</tr>
</tbody>
</table>

TNFR1-Fc or IgG1 used at 10μg/ml
* P < 0.01 n=4
To better define the mechanism of killing, co-culture, activated by OZ, was performed with BMD Mϕ from either TNFα/β KO mice or WT littermates and assayed for MC apoptosis in fixed wells after 24h incubation. As in the equivalent co-culture studies using cytokine activated Mϕ (Results 4.2), the TNF KO Mϕ were much less able to induce apoptosis of the mesangial cells (Figure 5-5), confirming a predominant role for TNF signalling apoptosis. Interestingly, there was residual killing at 24h compared with MC growing alone. The aetiology of this residual kill was not investigated.

![Graph showing percentage apoptosis in wild type and TNF KO conditions.](image)

**Figure 5-5**: The effect of TNF-α/β knockout or wild type mouse BMD-Mϕ activated by opsonised zymosan on the killing of IFN-γ/TNF-α primed MC in co-culture at 24h. Co-culture with primed MC was established as before. Murine BMD-Mϕ were utilised from either TNF-α/β KO or wild type (C57bl6/129/sv) mice. After 24h of activated co-culture, MC apoptosis was scored microscopically (P<0.01 vs wild type co-culture. n=5)

### 5.7 OZ-ACTIVATED HUMAN Mϕ INDUCE APOPTOSIS OF HUMAN MC THROUGH RELEASE OF H$_2$O$_2$ BUT NOT FAS LIGAND

Given the observed differences between human and rodent cytokine-activated co-culture (Chapter 4), it was necessary to investigate OZ-activated human co-culture further. Human MDM were cultured and matured for 7d in Teflon. Initially in culture, they were assessed for NO generation (Chapter 3) and hydrogen peroxide generation. The human MDM behaved similarly to rodent BMD Mϕ in peroxide generation and NO generation in response to OZ. Human co-culture was then assessed. Mature MDM were seeded into wells for 16h (2 x 10$^4$ per well of 96-well plate). 1 x 10$^4$ CMFDA green labelled human MC were added into full DMEM/F12 medium. After 4h, wells were washed and human-opsonised zymosan was added (0.5mg per well). Wells were assayed at 24h and 48h for human MC
apoptosis (Figure 5-6). Human Mφ induced human MC apoptosis in OZ-activated co-culture. Data from 48h studies provided similar results to those seen at 24h (not shown). To investigate the mechanism, inhibitors of H₂O₂ generation, and blocking antibodies to Fas ligation were added to the co-culture. The induction of apoptosis was inhibited by catalase (Figure 5-6), but not by blocking antibodies to human Fas ligand.

![Graph showing percentage apoptosis](image)

**Figure 5-6:** The effect of inhibitors of Fas ligation and oxygen radical release on opsonised zymosan-activated human Mφ killing of human mesangial cells. Catalase and SOD were used at 100U/ml, and anti FasL at 1μg/ml. Opsonised zymosan was added at 0.5mg/ml to co-cultures. (* P<0.05 compared with no inhibitor. n=3)

### 5.8 IFN-γ PRIMED HUMAN MC UNDERGO APOPTOSIS IN CO-CULTURE WITH OZ-ACTIVATED HUMAN Mφ BY A TNF-INHIBITABLE MECHANISM

When human MC were primed with IFN-γ (500U/ml) alone for 24h, then co-cultured there was induction of MC apoptosis. This induction was not inhibited by catalase, suggesting that these cells were now resistant to peroxide mediated apoptosis (Table 5-7). However, the killing observed was partially inhibited by TNFR1-Fc at 24h (Table 5-7), but not by anti-human FasL antibody. These data indicate that primed human mesangial cells are relatively resistant to peroxide mediated apoptosis, but are susceptible to TNF-mediated apoptosis. The data mirror those described above when using rodent cells in co-culture.
Chapter 5: Results 3. Triggering of mesangial cell apoptosis by macrophages ingesting opsonised particles

Table 5-7: The effect of candidate inhibitors on OZ-activated macrophage-mediated killing of IFN-γ primed human MC at 24h of co-culture

<table>
<thead>
<tr>
<th></th>
<th>N/A</th>
<th>OZ</th>
<th>+ catalase</th>
<th>+ anti FasL</th>
<th>+TNFR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-culture</td>
<td>3.1±1.1%</td>
<td>9.5±2.1%</td>
<td>7.8±1.9%</td>
<td>10.2±2.4%</td>
<td>*5.6±1.1%</td>
</tr>
<tr>
<td>Control</td>
<td>1.6±0.4%</td>
<td>1.9±1.4%</td>
<td>1.8±0.9%</td>
<td>2.1±0.6%</td>
<td>2.1±0.8%</td>
</tr>
</tbody>
</table>

Catalase 500U/ml, anti FasL antibody 1µg/ml, TNFR1-Fc 10µg/ml
* P < 0.05 ANOVA n=3

5.9 SUMMARY

To model activation by Fc receptor/complement receptor phagocytosis by Mφ, co-culture was devised with mesangial cells and activated with OZ particles.

- Killing of MC by Mφ was observed in both rodent and human systems
- Catalase-inhibitable, H₂O₂-dependent killing was observed. No role for NO was found
- When rat or human MC were primed with IFN-γ/TNF-α (IFN-γ alone for human MC), killing was diminished and catalase independent
- The persistent killing was not caused by Fas ligation
- The persistent killing was inhibitable by TNF-R1/TNF-α/β KO Mφ in rodents and humans
Chapter 6: Results 4

The regulation of TNF-mediated apoptosis in mesangial cells.
6.1 INTRODUCTION

The co-culture data described in Chapter 4 indicated that Mφ directed killing of MC involved TNFR1 ligation, but that IFN-γ was a co-factor. Indeed, in circumstances where TNF release by Mφ occurred without IFN-γ rendering the target rat MC susceptible, the cells were resistant to apoptosis in response to this cytokine. In the human studies, MC were susceptible to TNF-α mediated killing in co-culture without priming of MC with IFN-γ, but this killing was augmented following priming with IFN-γ. The data suggest that IFN-γ might somehow alter TNFR1 signalling to enable initiation of the death programme. Importantly, supernatant transfer experiments indicated that soluble TNF-α from Mφ was not effective at inducing death, implying that close cell-cell contact was required, possibly indicating a role for mTNF-α.

6.2 IFN-γ ENABLES TNF INDUCTION OF APOPTOSIS IN RAT MC

To confirm this interpretation of the data, experiments were designed to investigate the role of synergy of IFN-γ and TNF-α. Rat MC, 70% confluent in 96-well plates, were exposed to recombinant IFN-γ, TNF-α or both for up to 36h, in the presence of 0% FCS, 1% FCS or 10% FCS. They were assayed at 8, 16, 24 and 36h for MC apoptosis (Table 6-1, Figure 6-1, Figure 6-2). IFN-γ alone had no effect on the rat MC apoptosis or mitosis with time (not shown). This observation was reproduced using the other rat MC growths available (not shown). TNF-α alone had no effect on rat MC apoptosis in 10% FCS, but in 0% FCS there was a small increase in apoptosis at 16h (8.6±3.5%). This result is similar to that shown by Sugiyama and colleagues (Sugiyama et al., 1999) utilising the same cells. However when cells were treated with IFN-γ and TNF-α there was clear synergy. Indeed, even in medium with 10% FCS there was still induction of apoptosis, albeit delayed. This delay was not simply due to the period required to metabolise FCS-derived growth factors since, replacing the medium at 24h, did not further retard the induction of death. One interpretation is that in the presence of FCS growth factors, the modulating effect of IFN-γ is hindered/retarded.

These data contrast with the supernatant transfer experiments (Chapter 4.4) where transfer of supernatants from activated (IFN-γ/LPS) Mφ did not induce apoptosis of primed MC, yet the supernatants contained several ng/ml of TNF-α when assessed by ELISA (Chapter 4.14). Notably, whilst the recombinant TNF-α shown here could only induce 7.8% apoptosis at 36h, in co-culture with
activated Mφ, the TNF-dependent kill was often in excess of 20% at 24h (Chapter 4). It appears therefore that soluble native TNF-α is without potency, recombinant TNF-α has some potency, when close cell contact is achieved the TNF-dependent induction of apoptosis is much greater.

Table 6-1: The synergistic effect of IFN-γ on recombinant TNF-α-induced apoptosis of rat MC

<table>
<thead>
<tr>
<th></th>
<th>Percentage apoptosis of MC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8h</td>
</tr>
<tr>
<td>experimental</td>
<td></td>
</tr>
<tr>
<td>0% FCS</td>
<td>5.7±2.7</td>
</tr>
<tr>
<td>1% FCS</td>
<td>1.0±0.8</td>
</tr>
<tr>
<td>10% FCS</td>
<td>0.9±1.1</td>
</tr>
</tbody>
</table>

control = carriage medium only
TNF-α alone did not induce apoptosis after 36h in 10% FCS cultures, but induced 8.6±3.5% apoptosis at 16h in 0% FCS. IFN-γ alone had no effect on apoptosis.

Figure 6-1: Fluorescent photomicrograph (X200) showing apoptotic rat MC due to the synergistic effect of IFN-γ plus TNF-α. MC were cultured with IFN-γ (300U/ml) plus TNF-α (300U/ml) for 16h in medium with 1% FCS. After fixing and staining with propidium iodide, cells were visualised. Note condensed nuclei of apoptotic cells (arrows) and a cell in early apoptosis (with cytoplasmic blebbing) following aborted mitosis (block arrow).
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

The appearance of mitotic figures was also reduced when IFN-γ and TNF-α synergised; at 36h in the presence of IFN-γ plus TNF-α, mitotic figures were 4.1% (control) compared with 1.9% (experimental). Data drawn from different experiments suggested that this may be because cells in G₂M phase of the cell cycle were more susceptible to TNF-α mediated apoptosis than cells in other stages of the cycle. When studying the cell cycle by flow cytometry, rat MC that were treated with IFN-γ and TNF-α exhibited a clear reduction of the proportion of cells in G₂M (Figure 6-2). This was in keeping with the qualitative microscopical data where some apoptotic cells appeared to be cells which had aborted mitosis (Figure 6-1). Furthermore, similar studies on human MC (Table 6-2), which showed similar synergy of IFN-γ with TNF-α, indicated that when the cells were proliferating well (in the early passages), there was more TNF dependent induction of apoptosis than when they were proliferating slowly (see Chapter 3.15). When the mitotic rate of HMC growing alone was 2.3±0.8%, cytokine-activated human Mø co-cultured with human MC induced 16.4±2.9% apoptosis. When the mitotic rate was 1.8±0.1%, the induction of apoptosis in co-culture was 6.9±0.3%. Further evidence of a link between cell cycle and TNF-mediated apoptosis came from mouse MC cultures. Mouse MC divide very slowly in culture and do not undergo apoptosis in response to IFN-γ with TNF-α despite evidence of intracellular signalling (induction of iNOS). This negative result was seen whether experiments were performed in 0% FCS or 10% FCS, over a 36h period (Chapter 6.7).

Table 6-2: The effect of recombinant IFN-γ plus TNF-α on IFN-γ primed and unprimed rat and human MC at 24h

<table>
<thead>
<tr>
<th></th>
<th>Percentage apoptosis of MC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unprimed</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Rat mesangial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9±0.4%</td>
</tr>
<tr>
<td>Human mesangial cells</td>
<td>1.3±1.0%</td>
</tr>
</tbody>
</table>

IFN/TNF = IFN-γ 300U/ml plus TNF-α 300U/ml. cont = control
IFN-γ plus TNFβ (300U/ml) induced 8.1±2.0% apoptosis of primed rat MC at 24h.
6.3 INHIBITORS OF NITRIC OXIDE GENERATION DO NOT BLOCK IFN-γ/TNF-α INDUCTION OF MC APOPTOSIS

The role of MC-derived NO was investigated in either induction of apoptosis of MC or protection from Mφ-derived NO (Chapter 3.6). NO generated by rat MC, did not protect the MC from Mφ-derived NO. However, in this study, MC were cultured with IFN-γ and TNF-α for 48h to allow induction of MC apoptosis. Adjacent wells also contained high concentration NO inhibitors, L-NMMA (500μM) or L-NIL (30μM). MC apoptosis was assessed by fluorescent microscopy (Figure 6-3). These results suggest that NO had no role in regulating TNF-α mediated apoptosis of rat MC.

Figure 6-2: Fluorescent histograms of PI stained RNase treated rat MC indicating DNA content. (A) In untreated cells there were 19.2% in G2/M of the cell cycle and 2.5% of hypodiploid cells whereas (B) in cells stimulated with IFN-γ plus TNF-α for 24h, there were 12.7% of cells in G2/M and 22.5% of hypodiploid cells. Note that DNA content apparently greater than 2n represents cellular aggregates.
Chapter 6: Results

4. The regulation of TNF-mediated apoptosis in mesangial cells

5.0% - 4.0%

Figure 6-3: Induction of apoptosis by IFN-γ/TNF-α in rat MC is not suppressed by iNOS inhibitors. In full medium, 70% confluent MC were treated with IFN-γ plus TNF-α and assayed for apoptosis after 24h. In some wells, iNOS inhibitors were added in addition to cytokines, in high concentration in an attempt to block mediated TNF-killing. (IFN-γ [300U/ml], TNF-α [300U/ml], L-NMMA [500μM], L-NIL [50μM]).

6.4 INHIBITORS OF INTRACELLULAR OXYGEN RADICAL GENERATION DO NOT INHIBIT TNF-MEDIATED KILLING OF IFN-γ PRIMED RAT MC

As with Fas signalling, there is evidence that intracellular red-ox potential is important in modulating TNFR1 signalling (Kim et al., 1997; Sidoti-de Fraisse et al., 1998; Spanaus et al., 1998; Bradham et al., 1998). Indeed, there appear to be two, sequential mitochondrial-derived oxygen radical bursts in some cells after TNFR1 ligation. In some studies, intracellular blockade of the oxygen burst inhibits TNFR1 induction of apoptosis (Um et al., 1996; Sidoti-de Fraisse et al., 1998). The oxygen radical inhibitors, catalase and SOD, only act extracellularly. Therefore, N-acetyl cysteine, reduced glutathione and a synthetic inhibitor BHA were all utilised to block the intracellular oxygen spike. None of these inhibitors had any effect on blocking TNFR1 mediated apoptosis of MC (Figure 6-4) either in 10% FCS or 1% FCS. There are reports that cell surface glutathione pumps may determine whether this oxygen spike can be blocked with such quenching agents. In the case of high pump activity, red-ox status in such cells will not be determined by application of exogenous agents (de Bittencourt Junior et al., 1998; Hampton et al., 1998; van den Dobbelsteen et al., 1998).
6.5 IFN-γ PRIMED MC TRANSLOCATE MORE NFκB TO THE NUCLEUS THAN UNPRIMED CELLS UPON EXPOSURE TO TNF-α

Flow cytometric analysis of human MC suggested that unlike Fas, TNFR1 was not upregulated on the cell surface in cells susceptible to undergo apoptosis (Chapter 4.19). Early work on TNFR1 signalling into the caspase pathway had suggested that there was a dichotomy in signalling either into the NFκB pro-inflammatory pathway or into the caspase pathway (Hsu, Huang et al., 1996; Hsu, Shu et al., 1996; Liu et al., 1996; Wang et al., 1998). This is of course an oversimplification since TNFR1 ligation appears to signal into many pathways (Liu et al., 1996). However, studies in which TNFR1 induction of NFκB activity was blocked (Beg et al., 1995; Beg et al., 1996; Brown et al., 1995; Liu et al., 1996; Hirahashi et al., 2000) indicated that TNFR1 then signalled predominantly to induce apoptosis. Indeed studies from our own group (Sugiyama et al., 1999) on rat MC using an IκB over-expressing stable cell line confirmed just that; TNFR1 ligation alone, readily induced MC apoptosis, even in the presence of 10% FCS. Whether this observation reflects inhibition of the survival factors such as the IAPs or whether it alters the red-ox potential of the cell is unclear (Schreck et al., 1991).

Given the observations above, one might expect that IFN-γ treatment would inhibit NFκB signalling by TNFR1 ligation in MC, thereby ‘encouraging’ signalling from TNFR1 into the death pathway. Indeed, Manna and colleagues (Manna et al., 2000) showed that interferon-α down regulates NFκB in Jurkat T cells, enabling TNF-mediated apoptosis.

To investigate further, nuclear proteins were collected from rat MC, which had been primed with IFN-γ (300U/ml) for 24h, and then treated with TNF-α for 30min, 6h or 24h. This was compared with cells treated with TNF-α but not primed with IFN-γ. The nuclear proteins were then incubated with a short P32-conjugated sequence of dsDNA, the sequence of which corresponded to the NFκB binding site for the human IL-6 gene (Stylainou et al., 1999). The nuclear proteins were also incubated with a similar consensus oligomer (Sugiyama et al., 1999; Stylianou et al., 1999). Once incubated the DNA protein mix was electrophoresed through a non-denaturing polyacrylamide. Retardation of the oligomer running in the gel indicated binding of nuclear protein and therefore nuclear protein activity. This is the basis for the Electromobility Shift Assay (EMSA).
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

Figure 6-4: Induction of apoptosis and suppression of mitosis by IFN-γ/TNF-α in rat MC is not suppressed by intracellular oxygen radical scavengers. (A) rat MC were cultured for 36h in full DMEM/F12 with IFN-γ/TNF-α (300U/ml) in the presence of free radical scavengers, reduced glutathione (10μM), N-acetyl cysteine (10μM) and BHA (100μM). (B) A similar experiment was carried out in DMEM/F12 and 1% FCS. Apoptosis was scored at 24h.

A representative mobility shift assay (Figure 6-5) demonstrated that IFN-γ treatment alone did not lead to induction of NFκB activity. TNF-α treatment however led to a rapid recruitment of a proabaly two NFκB species. Supershift studies (using the NFκB consensus) suggested P65 induction without obviously P50, though the two induced bands are likely to be (P65)2, and P65/P50. (Figure 6-6). This induction was sustained for at least 36h. However, when MC had been primed with IFN-γ, TNF-α treatment led to a much greater translocation of NFκB to the nucleus. Supershift studies, and studies using mutated sequences (Sugiyama et al., 1999) indicated that the proteins were specifically binding the NFκB sequences.
This observation is contrary to that which one might expect from previous studies since NFkB has been shown to protect many cells from apoptosis. Although one explanation for augmented (P65)\textsuperscript{2} translocation to the nucleus might be increased TNFR1 expression on the cell surface, no evidence for this was found. Unfortunately, the commercially available anti rat TNFR1 antibodies employed in flow cytometry and western blotting did not specifically locate TNFR1 (not shown). However, flow studies of human MC which behaved similarly to rat MC, showed no evidence of increased TNFR1 expression following IFN-\(\gamma\) priming (Chapter 4.19).

6.6 INTERFERON REGULATORY FACTOR 1 IS TRANSLOCATED TO THE NUCLEUS UNDER THE INFLUENCE OF IFN-\(\gamma\) WITH TNF-\(\alpha\)

This surprising result required some explanation. Contrary to much published work, rat MC undergo TNFR1-apoptosis in the face of increased NFkB activity as well as when NFkB activity is blocked. Studies (albeit on human MC) showed that IFN-\(\gamma\) did not render cells susceptible to TNF-mediated
apoptosis by increasing TNFR1 expression. Was it possible therefore that TNFR1 was signalling apoptosis by another mechanism?

![Figure 6-6: EMSA for NFkB using nuclear extract from TNF-α (300U/ml) (4h) stimulated rat MC. The nuclear extracts were pre-incubated with antibodies against candidate NFkB species (Santa Cruz Biotechnology) and the nuclear factor AP-1. Note absence of the specific induced band when nuclear extracts were incubated with anti P65 (Produced in conjunction with Dr. Hitoshi Sugiyama).](image)

Studies in other cell types for many years have observed the anti-proliferative effects of IFN-γ, and have commented that it has been augmented by TNF-α (Kirchkoff et al., 1993; Pine 1997; Ossina et al., 1997; Spanaus et al., 1998; Burke et al., 1999; Ohta et al., 2000). Many of these studies just looked at cell counts or H3-thymidine incorporation and did not look for apoptosis to explain a lack of growth. However, it was highlighted more than a decade ago that interferon regulatory factor-1 (IRF-1) might be an important nuclear factor in determining this synergistic effect of IFN-γ with TNF-α. This nuclear factor is under the regulation of several nuclear proteins, including NFkB family and STAT 1α, a nuclear protein translocated by IFN-γ receptor ligation (Pine 1997; Gupta et al 1998).

Early studies indicated that when myeloid tumour cells over-expressed IRF-1 in the nucleus, the cells did not grow (Kirchkoff et al., 1993). Indeed, in a transgenic mouse where IRF-1 was linked to the immunoglobulin promoter, the animals were grossly deficient in the B cell lineage (Yamada et al., 1991).
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

Renewed interest has developed recently since it has been found that the NFκB binding site in the IRF-1 promoter is a novel binding site that synergistically binds NFκB proteins only when STAT 1α is also present (Pine 1997; Gupta et al., 1998). Furthermore, many pro-inflammatory genes require IRF-1 to bind to their promoters (such as iNOS (Martin et al., 1994; Kamijo et al., 1994) in addition to NFκB species for efficient transcription.

To determine whether IRF-1 was relevant in the rat MC, nuclear proteins were isolated from cells exposed to IFN-γ, TNF-α or both. EMSAs were performed using a IRF-1 consensus dsDNA binding sequence (Kamijo et al., 1994) and another IRF-1 binding sequence from the protein ISG-15 (Pine 1997), a protein of unknown function that is induced by IFNγ.

The shift assay using ISG15 IRF-1 binding sequence (Figure 6-7) indicates that in this cell-type, IRF-1 is synergistically active in the nucleus under the influence of IFN-γ and TNF-α. Note that IFN-γ alone was able to weakly induce IRF-1 activity, but TNF-α was completely unable to. Together, induction of the nuclear protein was augmented.

Using 50X excess cold oligonucleotide almost totally prevented specific binding (Figure 6-8). Furthermore, rabbit polyclonal anti-IRF-1 IgG but not anti-IRF-2 IgG was able to inhibit specifically binding of IRF-1 to the oligonucleotide. A scrambled sequence of the ISG15 did not bind IRF-1 in induced cell extracts. Similar studies to those shown (Figure 6-7 and 6-8.) were performed using the IRF-1 consensus sequence and confirmed the results (not shown).

6.7 WILD TYPE AND IRF-1 KNOCKOUT CULTURED MOUSE MESANGIAL CELLS DO NOT UNDERGO APOPTOSIS IN RESPONSE TO IFN-γ AND TNF-α

To investigate the pro-apoptotic role of IRF-1 further, glomeruli were isolated from IRF-1 KO mice and wild type littermates, and mesangial cells were cultured from these glomeruli (see Chapter 2). The hypothesis was that the knockout cells might be resistant to TNF-α mediated apoptosis following treatment with IFN-γ. The wild type cells would be susceptible to TNF-mediated apoptosis much like the rat and human MC.

Once cells were established, it was noted that they proliferated very slowly, more slowly than both human and rat MC. The doubling time was in the order of 96h compared with less than 24h for rat and human MC. When wild type cells were treated with rat IFN-γ and human TNF-α there was no induction of apoptosis, nor was there suppression of mitosis nor alteration of the G2/M population by
flow cytometry (not shown). Induction of iNOS however was readily detected in the supernatant as nitrite, thus confirming that the cytokines were active. There were several possibilities for this observation. Firstly, human recombinant TNF-α is said not to bind to murine TNFR-2 and TNFR-2 may be important in apoptosis signalling (Chan et al., 2000). Secondly, the cell cycle might be too slow if the only cells which undergo TNF-dependent apoptosis are the cells which are cycling into G₂/M (see Chapter 3.15). Finally, if membrane bound TNF-α is the most potent form for inducing apoptosis, the mouse MC might be resistant to recombinant TNF-α, which tends to be mainly in soluble trimeric form. It was not possible therefore to show that IRF-1 was necessary for TNF signalling of apoptosis using the knockout cells.

![Figure 6-7: Representative band shift using the ISG15 promoter IRF-1 binding sequence. Lanes 2-4 from cells treated for 30 min. Lane 1 nuclear extract from unstimulated cells. Lane 2 from cells treated with IFN-γ (300U/ml). Lane 3 cells treated with TNF-α (300U/ml). Lane 4 cells treated with both IFN-γ and TNF-α. Lanes 5-7 cells were treated for 6h. Lane 5 from cells treated with IFN-γ (300U/ml). Lane 6 cells treated with TNF-α (300U/ml). Lane 7 cells treated with both IFN-γ and TNF-α. Note synergistic induction of IRF-1 activity with the combination of cytokines.](image-url)
6.8 TRANSIENT TRANSFECTION OF RAT MC WITH IRF-1 UNDER THE CMV PROMOTER RESULTS IN EXPRESSION OF IRF-1 mRNA

Since the mouse cells did not display IFN-γ/TNF-α synergism in apoptosis induction, it was decided to employ an over-expression system to investigate the role of IRF-1 in rat MC apoptosis. In collaboration with Prof. H. Hauser (Germany) vectors containing the mouse cDNA for IRF-1 were obtained. This gene was fused with the 3’ end of the human oestrogen receptor (HER), under control of the myeloproliferative sarcoma virus (MPSV) promoter, which had been shown in myeloid cells to achieve high levels of transcription. The fusion protein IRFHER had been shown to reside in the cytosol (in myeloid cell lines), but on application of an oestrogen, be translocated to the nucleus where the IRF-1 bound to DNA and was functionally active (Kirchkoff et al., 1993) (vector 767 Figure 2-10). A similar vector containing the oestrogen gene but without the IRF-1 cDNA was provided as a negative control (vector 776).

Unfortunately, no simple assay for IRF-1 activity in transfected cells, such as a luciferase linked IRF-1 dependent promoter that could be co-transfected, was available.

![Figure 6-8: Representative band shift using the ISG15 promoter sequence for IRF-1 binding. Lane NS contains no nuclear protein extract. ‘u’ = nuclear extract from unstimulated cells and, ‘a’ = nuclear extract from cells activated with IFN-γ (300U/ml) plus TNF-α (300U/ml) for 4h. Lanes 1, extract was incubated with cold probe. Lanes 2, extract was incubated with hot probe. Lanes 3, hot probe was competed out with 50X excess of cold probe. Lanes 4, nuclear extract was incubated overnight with polyclonal anti-IRF-1 antibodies, lanes 5 nuclear extract was incubated with anti-IRF-2 antibody. Lanes 6, a mutated probe sequence was employed.](image-url)
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

The rat MC were normally cultured in 10% FCS. However, FCS contains oestrogens. To perform experiments on rat MC in FCS using this construct it was necessary to deplete all FCS of oestrogens (Chapter 2.2.9). All experiments utilising transfected cells were cultured in medium supplemented with depleted FCS.

Rat MC were now transfected with vectors 767 and 776. EGFP-N1 (vector containing the green fluorescent protein gene under the CMV promoter) was utilised as a control for transfection efficiency. At 48h, by flow cytometry, 32% of cells were fluorescent. The 767-transfected MC appeared to be growing as well as the 776 transfected cells. These cells did not undergo apoptosis in response to estradiol at 5μM (an oestrogen), TNF-α (300U/ml) nor a combination of both (1.33% compared with 1.28% for untreated cells).

To determine why the transiently transfected cells did not undergo apoptosis, mRNA was extracted from the cells, RT-PCR was performed for IRF-1 cDNA and analysed on a 1.5% agarose gel (Figure 6-9 lane 3). These data clearly indicated that the transfected cells did not contain any IRF-1 mRNA. The most likely conclusion from this study was that the gene in mesangial cells is not transcribed under the MPSV promoter.

Figure 6-9: Agarose gel (1.0%) showing PCR products from cDNA from transiently transfected rat MC when using IRF-1 primers (top panel) and actin primers (bottom panel). Lanes 1 cells transfected with eGFP/IRFHER, lanes 2 with EGFP-N1, lanes 3 with 767 vector, lanes 4 with 776 vector. Lane 5 shows PCR products from eGFP/IRFHER vector alone. NS -no DNA sample. ‘a’ denotes omission of reverse transcriptase. Note only when cells are transfected with eGFP/IRFHER is there evidence of gene transcription (lane 1).

The HERIRF-1 gene construct was therefore excised from the 767 vector and inserted into the EGFP-N1 vector under the control of a CMV promoter. Previous studies of transfection of mesangial cells
have indicated that the CMV promoter reliably resulted in adequate transcription of exogenous genes (Stylianou et al., 1999). Also, the EGFP-N1 vector resulted in strong green fluorescence in the rat MC, confirming CMV promoter activity.

After generation and purification of the new vector (Chapter 2.11), 5x 10^6 rat MC were transiently transfected with 20μg of eGFP/IRFHER(5), or 20μg of control EGFP-N1 vector by electroporation. At 48h, RNA was purified and after reverse transcription, PCR was performed. This study now confirmed production of mRNA for IRF-1 (Figure 6-9 lane 1). Furthermore, in preliminary studies of the transiently transfected cells, those with IRFHER appeared to undergo apoptosis in the presence of estradiol (an oestrogen) and TNF-α whereas the eGFP-transfected cells did not (not shown).

6.9 OVER-EXPRESSION OF IRF-1 IN RAT MC RENDERS THEM SUSCEPTIBLE TO TNF-MEDIATED APOPTOSIS WITHOUT THE REQUIREMENT FOR IFN-γ

Following stable transfection of rat MC with the new vector and the control EGFP-N1 vector, polyclonal (>10 colonies) and monoclonal cell cultures were generated. Initial observations suggested that the polyclonal cell cultures appeared to grow normally, but some of the single colony cultures grew abnormally: IRFHER clones 8,23,25,27,33 all showed increased levels of spontaneous apoptosis. Whereas, IRFHER clones, 6,11,16,17,18 showed normal growth. The high-expressing eGFP colonies were maintained (6,5,3,7) and all showed normal growth with normal low levels of spontaneous apoptosis. From the cells transfected with EGFP-N1, seven other clones were derived that produced no detectable GFP.

Study of the polyclonal cell lines by morphology and flow cytometry of fixed, PI stained cells, revealed firstly that the cells exhibited increased levels of spontaneous apoptosis (Table 6-3, Figure 6-10). Secondly, estradiol alone (5μM) had no marked effect. However, TNF-α alone was able to induce apoptosis of the polyclonal cells without the necessity for IFN-γ. (Table 6-3 shows this most clearly). When estradiol and TNF-α were applied together, there appeared to be a small increase in the induction of apoptosis. Note, the control eGFP-expressing polyclonal cells exhibited no more than 0.9% hypodiploidy even in the presence of TNF-α plus estradiol (5μM).
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

Table 6-3: Polyclonal IRFHER expressing MC are susceptible to TNF-α mediated apoptosis when assessed microscopically at 24h.

<table>
<thead>
<tr>
<th></th>
<th>N/A</th>
<th>estradiol</th>
<th>TNF-α</th>
<th>estradiol +TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyIRFHER(1)</td>
<td>2.1±1.1%</td>
<td>2.3±0.9%</td>
<td>8.4±1.5%</td>
<td>10.7±2.1%</td>
</tr>
<tr>
<td>PolyIRFHER(2)</td>
<td>1.6±0.9%</td>
<td>2.4±1.5%</td>
<td>3.5±1.7%</td>
<td>5.6±2.2%</td>
</tr>
<tr>
<td>PolyGFP(1)</td>
<td>0.6±1.0%</td>
<td>0.7±0.3%</td>
<td>0.9±0.9%</td>
<td>0.7±1.2%</td>
</tr>
</tbody>
</table>

Estradiol 5μM, TNF-α 300U/ml, N/A = estradiol carriage medium

Figure 6-10: Polyclonal HERIRF-1 stably transfected rat mesangial cells undergo increased apoptosis in response to tumour necrosis factor alone (A) Unstimulated cells after 24h in culture as assessed by flow cytometry of PI-stained fixed cells and showed 2.1% hypodiploidy. (B) Cells were exposed to estradiol (5μM) for 24h resulting in 2.6% hypodiploidy. (C) Cells were treated with TNF-α (300U/ml) which alone induced 6.8% hypodiploidy and (D) when cells were treated with estradiol and TNF-α in combination hypodiploidy increased to 8.1%.
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

The individual clones were now assessed for apoptosis. All the clones which displayed increased spontaneous apoptosis, also exhibited TNF-α but not estradiol induced apoptosis (Table 6-4). The clones that did not exhibit increased spontaneous apoptosis were resistant to the effects of TNF-α (6,11,16,17,18), and behaved similarly to the stable eGFP-expressing clones (6,5,3,7). Importantly, when the IRFHER clones that were sensitive to TNF-α were studied by flow cytometry, some of the clones had abnormalities of cell cycle progression (Figure 6-11). Unfortunately, these clones were not assessed for IRF-1 expression either quantitatively or by intracellular compartment. Further, no studies were performed to determine the effect of exogenous estradiol on IRF-1 location within the mesangial cells.

Table 6-4: IRFHER transfected MC clones are susceptible to TNF-α mediated apoptosis when assessed microscopically

<table>
<thead>
<tr>
<th>Clone</th>
<th>N/A</th>
<th>estradiol</th>
<th>TNF-α</th>
<th>estradiol +TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>0.6±0.6%</td>
<td>0.6±1.0%</td>
<td>4.9±0.5%</td>
<td>5.2±1.3%</td>
</tr>
<tr>
<td>25</td>
<td>2.1±1.4%</td>
<td>2.6±1.4%</td>
<td>12.4±2.4%</td>
<td>12.9±1.8%</td>
</tr>
<tr>
<td>33</td>
<td>5.5±1.4%</td>
<td>4.9±2.2%</td>
<td>8.9±1.0%</td>
<td>9.4±1.2%</td>
</tr>
<tr>
<td>23</td>
<td>2.4±1.1%</td>
<td>1.9±0.6%</td>
<td>6.6±1.2%</td>
<td>7.4±1.2%</td>
</tr>
<tr>
<td>8</td>
<td>1.6±0.3%</td>
<td>1.4±1.0%</td>
<td>4.8±1.6%</td>
<td>5.7±1.6%</td>
</tr>
</tbody>
</table>

Estradiol 5μM, TNF-α 300U/ml, N/A = estradiol carriage medium

6.10 CASPASE INHIBITORS DO NOT BLOCK TNF-MEDIATED KILLING OF IFN-γ PRIMED RAT MC

Rat MC, 70% confluent, primed with IFN-γ (300U/ml) for 24h, were then cultured with 100μM of DEVD or LEHD (Nicholson et al., 1995; Garcia-Calvo et al., 1998) caspase inhibitors at the same time as addition of recombinant TNF-α. After 24h there was induction of similar apoptosis in cells that had and had not been treated with the caspase inhibitors (8.4% LEHD, 7.9% DEVD, 8.9% no inhibitor). In control wells without TNF-α, there was no difference in background mitosis/ apoptosis levels when the inhibitors were present. Unfortunately, no suitable positive control was used.
To determine whether caspase 3 was cleaved, western blots were performed with two different antibodies, one polyclonal, the other monoclonal (Figure 6-12). The former did not give significant bands for the protein. The latter (anti-mouse) gave a sharp band for the positive control, but the rat MC did not appear to have detectable caspase 3. Whilst neither of these two pieces of data were definitive, they do suggest that rat MC do not have caspase 3.

Figure 6-11: Rat MC clones over-expressing IRF-1 have abnormalities of cell cycle progression as seen by flow cytometry for DNA content. The four clones shown (25,33,23,8) all exhibit increased levels of spontaneous hypodiploid cells compared with the control eGFP expressing cells (e7). Furthermore, the IRF-1 expressing cells all have an accumulation of cells in G2/M phase of the cell cycle and a relative loss of cell in the G0/G1 phase of the cycle. This is most prominent in the clone 23.
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

Figure 6-12: Rat MC do not obviously have Caspase 3 in the cytosol. Rat MC were treated for 24h with IFN-γ and TNF-α, then cytosolic proteins were harvested in lysis buffer with NP-40 (lane 1). 100μg of soluble protein was electrophoresed in a 10% gel along with (lane 2) 10μg protein extract from murine RZW-3T3 cells, stably over-expressing caspase 3. The subsequent blot was incubated with anti-mouse caspase 3 antibody C76920 (Transduction labs), which cross reacts with rat caspase 3. After secondary antibody, the blot was visualised using ECL. Note the non-specific bands at around 40 and 65kDa.

6.11 SUMMARY

- TNF-α mediated apoptosis of mesangial cells is regulated by IFN-γ.
- Soft evidence indicates that cells moving into G₂M are more susceptible to TNF-α killing than cells in G₀/G₁.
- The mechanism of TNF-α intracellular signalling appears to be nitric oxide and ROI independent
- IFN-γ priming of mesangial cells augments NFkB induction upon TNFR1 ligation.
- IRF-1 is synergistically induced by IFN-γ/TNFα
- Over-expression of this IRF-1 alone, appears to be sufficient to induce low level apoptosis of mesangial cells and block their cell-cycle progression
- IRF-1 appears to permit TNF-α signalling apoptosis without the requirement for IFN-γ
Chapter 6: Results 4. The regulation of TNF-mediated apoptosis in mesangial cells

- The role of caspases in TNF-α induced apoptosis of rat MC remains unclear and requires further work.
Chapter 7: Results 5

Macrophage ingestion of apoptotic cells blocks TNF-dependent killing
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

7.1 IN CO-CULTURE, ACTIVATED Mϕ PHAGOCYTOSE MC THAT HAVE BEEN INDUCED INTO APOPTOSIS

From the co-culture studies reported in Chapters 3, 4, and 5, it was notable that a proportion of the MC induced into apoptosis by Mϕ were subsequently phagocytosed by the Mϕ. The proportion of MC that had been phagocytosed at 24h was variable, but in studies where this was assessed semiquantitatively, it amounted to around 10%. This proportion is low given the known avidity of macrophages for ingestion of apoptotic cells (Savill et al., 1989). However, in vitro studies where the proportion of Mϕ that have phagocytosed is much higher (30-40% of Mϕ containing at least one apoptotic cell) have experimental designs that maximise the chance of phagocytosis. In vivo also, where Mϕ are believed to avidly phagocytose, there is much greater cell to cell contact, thereby maximising phagocytic recognition (Baker et al., 1994). In the 2D co-culture monolayer the majority of apoptotic MC were not ingested by Mϕ, but remained loosely adherent to the monolayer, often microscopically in a focal plane just above the live, adherent cells. For stoichiometric reasons, therefore, the assay was not best designed to promote binding and phagocytosis of such apoptotic cells.

7.2 INGESTION OF APOPTOTIC MC BY Mϕ IN CO-CULTURE INHIBITS FURTHER INDUCTION OF MC APOPTOSIS

To address the hypothesis that, in the co-culture model of glomerular inflammation, macrophages that have ingested apoptotic cells are no longer able to kill live cells, the co-culture was redesigned to augment or maximise Mϕ-phagocytosis of apoptotic MC. This was achieved by overlaying established co-culture of CMFDA-green labelled rat MC and BMD-Mϕ with a confluent layer of pre-formed (unlabelled) apoptotic MC (Chapter 2.6). For this, large numbers of apoptotic MC were required, and were generated by UV induction of confluent monolayers. Care was taken to promote apoptosis rather than necrosis. In all experiments, fewer than 10% of such apoptotic MC admitted propidium iodide and none admitted trypan blue (Chapter 2.4).

The rodent co-culture was established as previously described in 96-well plates, using primed rat MC (since these were susceptible to both NO and TNF killing). At the start of the experiment (at the same time as activation with IFN-γ and LPS), apoptotic rat MC were added such that there were 5x the number of apoptotic MC as Mϕ in the well (total 1 x 10⁵). Live MC were labelled green with CMFDA and were therefore easily distinguishable from the UV induced apoptotic MC. As a control for the
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

Apoptotic cells, an aliquot of inert latex beads (10μm) diameter was added to separate wells of co-culture. In other wells of co-culture and Mφ alone, to which aliquots of apoptotic cells and latex beads had been added, the experiment was stopped by fixation at 4h. These wells were assessed for ingestion of apoptotic MC using Nomarski optics. Assessing ingestion in this way revealed that many pre-formed apoptotic MC had been ingested, and that 30±6.7% of Mφ had ingested at least one apoptotic MC. Many Mφ had ingested two or more apoptotic MC (In the co-culture, the majority of phagocytosing cells were Mφ though some CMFDA-labelled MC also ingested apoptotic MC). Further, 67±8.4% of Mφ had ingested latex beads. Again, in co-culture, Mφ were the predominant cell-type to phagocytose the beads. When pre-formed apoptotic MC were added to established quiescent co-culture and incubated for 4h, 27±9.4% of Mφ ingested apoptotic cells.

The co-culture was incubated for 24h, then fixed and assessed microscopically for green CMFDA MC apoptosis (Figure 7-1). There was little difference between the proportion of apoptotic rat MC in co-cultures when Mφ had not ingested, compared with when they ingested latex beads and when they ingested apoptotic MC.

![Figure 7-1: Ingestion of apoptotic MC by activated Mφ does not prevent Mφ induced killing of live primed MC in co-culture. Activated co-culture with IFN-γ primed MC was established and activated with IFN-γ and LPS. A five-fold excess of pre-formed apoptotic MC was added to the co-culture at the time of activation, Control latex beads were added to other wells. Co-culture where Mφ had ingested the apoptotic cells showed a small reduction in Mφ-derived killing but this was not significant (n=5).](image)
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

However, when the experiments were repeated in the presence of L-NMMA at 200μM (added simultaneously with activating cytokines to block the NO-induced killing), wells with Mφ that had selectively phagocytosed apoptotic MC were no longer able to kill CMFDA-green MC (Figure 7-2).

![Graph showing percentage apoptosis](image)

**Figure 7-2:** Ingestion of apoptotic MC by activated Mφ blocks TNF-dependent killing of live MC in co-culture. Activated co-culture was established and activated in the presence of L-NMMA (200μM). A five-fold excess of pre-formed apoptotic MC was added to the co-culture at the time of activation. Control latex beads were added to other wells. Co-culture where Mφ had ingested the apoptotic cells showed no evidence of Mφ-derived killing (n=5 *** P<0.001).

The experimental design was modified in order that Mφ could ingest apoptotic cells prior to activation with IFN-γ plus LPS. The co-culture was established, then covered with aliquots of pre-formed apoptotic MC or beads as above. After 4h, the co-culture was then gently washed to remove any apoptotic MC or beads that had not been ingested. After washing and replacing full medium, the co-culture was activated with IFN-γ and LPS and, in half the experiments, L-NMMA (200μM) was added. In these experiments, TNF-dependent killing of live CMFDA-green MC by activated Mφ was still inhibited by prior ingestion of apoptotic cells (Table 7-1).
Table 7-1: The effect of ingestion of apoptotic MC by Mφ in co-culture with IFN-γ primed MC on Mφ-induction of MC apoptosis at 24h

<table>
<thead>
<tr>
<th>Particle ingested</th>
<th>% mesangial cell apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No L-NMMA</td>
</tr>
<tr>
<td></td>
<td>no φ</td>
</tr>
<tr>
<td>apoptotic mesangial cell (UV)</td>
<td>17.9±2.2</td>
</tr>
<tr>
<td>latex beads (10μm)</td>
<td>20.9±1.8</td>
</tr>
</tbody>
</table>

UV = ultraviolet, φ = phagocytosis

Rodent co-culture with IFN-γ primed CMFDA-labelled MC was established as described in Chapter 2. Apoptotic cells were incubated with the co-culture for 4h. Mφ were activated with IFN-γ plus LPS in the presence of L-NMMA 200μM after washing away non-ingested cells. Note that control cultures of MC alone exposed to apoptotic cells followed by activating cytokines underwent no greater than 3.42±0.2% apoptosis.

** P < 0.01, compared with no phagocytosis

7.3 INGESTION OF APOPTOTIC LEUCOCYTES BY Mφ IN ACTIVATED CO-CULTURE INHIBITS THE INDUCTION OF MC APOPTOSIS BY Mφ.

To investigate whether selective inhibition of Mφ-derived, TNF-dependent killing following ingestion of apoptotic MC, was an effect unique to apoptotic MC, apoptotic neutrophils (PMN) and lymphocytes (thymocytes) were prepared. Rodent neutrophils were difficult to obtain but human PMN were plentiful from peripheral blood donation. Neutrophils undergo spontaneous apoptosis. Alternatively, they can be induced by UV irradiation into apoptosis. The thymus of mice represented an easy source of large numbers of murine single cells that were easily induced into apoptosis by steroids or UV irradiation. As a control particle in these experiments, live thymocytes were utilised. Both lymphocytes and neutrophils are cells found in the glomerulus during nephritis.

Neutrophils were incubated either for 16h in full DMEM/F12 or for 6h in Dulbecco’s PBS (containing Ca++ and Mg++). Both treatments typically yielded 30-50% apoptosis when assessed by light microscopy (see Figure 2-4). Care was taken to make sure that the population did not contain necrotic cells. To that end, cells were always assessed for trypan blue positivity and in some experiments assessed by two colour flow cytometry for FITC-conjugated Annexin V binding and PI positivity. PI positive cells did not exceed 5% (not shown).
Thymocytes were cultured in full medium and treated with UV irradiation, or dexamethasone. Those cells that were not treated, progressively became apoptotic such that at 16h there were 14% apoptotic. However, after 4h incubation, apoptosis was less than 5% when assessed by flow cytometry (Figure 2-5). Six hours of culture with dexamethasone and 4h culture after UV irradiation yielded apoptotic cells without significant numbers of post-apoptotic cells.

Live neutrophils exposed to LPS undergo a brisk respiratory burst and can induce bystander MC apoptosis by a catalase inhibitable mechanism (see Chapter 5.4). It was therefore important that no live neutrophils remained in the co-culture as this would affect the killing of MC by Mφ. In the case of thymocytes, theoretically live cells might interact with activated Mφ and modulate their response, though these studies showed no evidence of this being the case. Given these potential problems, the co-culture was established and apoptotic cells were added (5 x the number of Mφ) for 4h. At this point non-adherent, non-ingested cells were washed away by very gentle pipetting to leave only the adherent co-culture, with ingested or bound apoptotic cells. The co-culture was assessed for uptake of apoptotic leucocytes. This indicated 35±8.1% of Mφ had ingested neutrophils (Figure 7-3), and 31.8±6.4% of Mφ had ingested apoptotic thymocytes. Some Mφ had ingested up to 10 thymocytes (smaller than neutrophils). Live thymocytes were not obviously ingested by Mφ. Non-ingested apoptotic and live thymocytes were readily washed away. However, it was not possible to wash away all neutrophils. It appeared that some of the live neutrophils remained adherent to the co-culture. Thus it was necessary to purify the apoptotic PMN from the live PMN, since the apoptotic PMN were not able to generate a respiratory burst (personal communication Dr. A Rossi, University of Edinburgh). To achieve this, aged or UV-induced apoptotic PMN were incubated with magnetic beads bearing anti CD16. Live but not apoptotic PMN have cell-surface CD16. By this method live and apoptotic cells could be separated (Chapter 2.4). Effective separation was ensured by flow-cytometry and morphology (Figure 2-4).

Now CD16-purified apoptotic PMN, live and apoptotic thymocytes were added to the established co-culture to enable phagocytosis by Mφ. After washing, the co-cultures were activated by IFN-γ/LPS and, after 24h, were assessed for green CMFDA labelled apoptotic MC (Figure 7-4). All the studies with apoptotic PMN and thymocytes suggested a reduction in MC apoptosis, but none of the results was significant. However, in the presence of L-NMMA (200μM) (Figure 7-5), interaction with apoptotic cells but not live thymocytes blocked induction of MC apoptosis. The method of induction of apoptosis did not have bearing on the suppression of Mφ in co-culture. Thus, there was no difference between UV-induced and dexamethasone induced thymocytes, and there was no difference between
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

aged and UV induced apoptotic PMN. Furthermore, PMN that were aged in PBS only, induced similar reductions to those aged in medium with FCS, or human serum (not shown).

![Image](image.jpg)

**Figure 7-3:** Ingestion of apoptotic neutrophils by quiescent BMD Mφ viewed by phase contrast microscopy (X400). A sub-confluent layer of Mφ was interacted with a confluent monolayer of aged human PMN for 2h. After incubation, cells were washed and the remaining, adherent cells were fixed with glutaraldehyde (0.5%). After washing with PBS the cells were incubated with DMB and H₂O₂. Myeloperoxidase activity (in PMN) results in brown staining.

### 7.4 NITRIC OXIDE RELEASE BY ACTIVATED Mφ IS AUGMENTED BY INGESTION OF APOPTOTIC CELLS BUT NOT LATEX BEADS

The data from co-culture indicated that the inhibition of Mφ killing of MC was best achieved in the presence of L-NMMA. To determine further the mechanism, supernatants were collected from wells of Mφ (24-well plates) that had first ingested apoptotic cells, then been activated with IFN-γ (100U/ml) and LPS (1μg/ml). Care was taken to ensure that equal numbers of Mφ were in each well, an equal volume of medium was applied, and washing of control wells (with latex beads or no apoptotic cells) was the same as wells with apoptotic cells. All conditions were prepared in triplicate and each experimental condition was compared with wells of Mφ that had not phagocytosed, on the same plate.
Reiter and colleagues (Reiter et al., 1999) showed that when LPS-activated murine Mφ ingested apoptotic tumour cells, there was a small reduction in NO release as assessed by nitrite generation. Similar reductions in NO release were reported from trypanosome (Protozoan) infected peritoneal mouse Mφ following ingestion of lymphocytes (Freire-de-Lima et al., 2000) induced into 'apoptosis' by heating (note that heating would normally induce a non-apoptotic phenotype and is used elsewhere as a model of necrosis).

Figure 7-4: In activated co-culture, prior ingestion of apoptotic leucocytes by Mφ does not inhibit Mφ induction of MC apoptosis. Co-culture of murine Mφ and CMFDA-labelled, IFN-γ primed rat MC was established. A monolayer of apoptotic leucocytes was added (A) thymocytes, or (B) neutrophils. After 4h incubation, the co-culture was washed to removed non-ingested cells and then activated with IFN-φ 100U/ml and LPS (1μg/ml). CMFDA-labelled MC apoptosis was assessed at 24h. Co-culture was compared with CMFDA-labelled MC cultured without Mφ but exposed to the same apoptotic cell monolayer, washing and activating cytokines. There was no significant difference in MC apoptosis when Mφ were interacted with apoptotic cells.
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

Figure 7-5: In activated co-culture, prior ingestion of apoptotic leukocytes by Mφ inhibits TNF-restricted Mφ induction of MC apoptosis. Co-culture of murine Mφ and CMFDA-labelled, IFN-γ primed rat MC was established. A monolayer of apoptotic leukocytes was added (A) thymocytes, or (B) neutrophils. After 4h incubation, the co-culture was washed to removed non-ingested cells and then activated with IFN-φ 100U/ml and LPS (1μg/ml) plus the NOS inhibitor L-NMMA at 200μM. CMFDA-labelled MC apoptosis was assessed at 24h. Co-culture was compared with CMFDA-labelled MC cultured without Mφ but exposed to the same apoptotic cell monolayer, washing and activating cytokines. When Mφ had ingested apoptotic cells there was a reduction in induction MC apoptosis by the Mφ (P < 0.001 vs no cells. P < 0.05 vs no ingestion).

Since any live PMN that might contaminate the culture (after interaction of apoptotic PMN with Mφ) would produce oxygen radicals after LPS treatment, it was decided to analyse NO release from all cultures by assessing nitrite plus nitrate (since oxygen radicals promote oxidation of NO^2⁻ to NO^3⁻). To achieve this, once harvested supernatants had been clarified by centrifugation, they were reduced by nitrate reductase (see Chapter 2.8). To ensure all nitrate was reduced to nitrite, assay plates had sodium nitrate controls. Before reduction nitrate does not effect colour change in the Griess reaction. By
comparing equimolar concentrations of sodium nitrite, one could be sure of complete reduction. Total nitrite was then assayed by Griess reaction and absorbance at 540nm.

When NO was assayed from quiescent M\(\phi\) that had ingested apoptotic rat MC there was no significant generation of NO. Also, apoptotic rat MC, in the presence of IFN-\(\gamma\)/LPS did not generate NO. However, surprisingly, when M\(\phi\) had ingested apoptotic rat MC and were then activated there was an augmented release of NO compared with M\(\phi\) that had not phagocytosed (assessed at 24h) (Figure 7-6).

When apoptotic thymocytes were ingested by M\(\phi\), there was a similar pattern. That is, ingestion of apoptotic cells did not activate M\(\phi\) per se, and apoptotic thymocytes in the presence of IFN-\(\gamma\) and LPS did not lead to NO generation. However, when M\(\phi\) ingested apoptotic thymocytes (induced by dexamethasone, or UV irradiation), and were then activated, there was augmentation of NO release (Figure 7-6). As a control, fresh thymocytes (at 2h incubation) which contained few apoptotic cells, did not have this effect. However, aged thymocytes that had been cultured for 16h displayed up to 20% apoptosis (see Chapter 2.4). When these cells were incubated with M\(\phi\) prior to activation, there was a 22.4±5.6% increase in NO generation.

![Figure 7-6](image)

**Figure 7-6:** M\(\phi\) that have ingested apoptotic cell produce more NO when activated by IFN-\(\gamma\)/LPS than M\(\phi\) which have ingested inert particles or have not ingested any particle. M\(\phi\) were exposed to a five fold excess of pre-formed apoptotic leucocytes or apoptotic MC for 4h in culture. Wells were washed to remove non-ingested apoptotic cells, medium replaced, then activated with IFN-\(\gamma\) (100U/ml) plus LPS (1\(\mu\)g/ml). At 24h, medium was aspirated, clarified and assayed for [nitrate] plus [nitrite] (n=5 ** P < 0.01).
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

Human neutrophils (PMN) when aged in Dulbecco’s-PBS for 6h or overnight in full DMEM/F12 were also phagocytosed. Apoptotic PMN alone, treated with IFN-γ plus LPS did not induced NO generation. Phagocytosis of apoptotic PMN by Mφ did not activate the Mφ to produce NO (Figure 7-6). However, Mφ that had phagocytosed apoptotic PMN, upon activation with IFN-γ plus LPS, exhibited augmented release of NO. The method of induction of PMN apoptosis and the type of medium the PMN were cultured in during induction of apoptosis did not significantly influence these results.

Ten micrometre latex beads did not induce NO release by quiescent Mφ but significantly, after ingestion, did not influence the generation of NO release by Mφ following activation with IFNγ plus LPS.

7.5 TNF-α RELEASE BY ACTIVATED Mφ IS INHIBITED BY INGESTION OF APOPTOTIC CELLS, BUT NOT LATEX BEADS

Diminution of soluble TNF-α release has been reported from LPS-activated human MDM (and murine J774 Mφ cell line) in response to ingestion of apoptotic peripheral blood lymphocytes or human UV induced PMN (Fadok et al., 1998; McDonald et al., 1999). It seemed likely that since NO release was not inhibited by ingestion of apoptotic cells, it was likely that the other ligand identified as important in Mφ-directed MC apoptosis, TNF-α, would be inhibited. The same supernatants assayed for NO release were also assayed for murine TNF-α by sandwich ELISA.

The same supernatants, analysed in Figure 7-6, were also analysed for TNF-α (Figure 7-7). Phagocytosis of apoptotic cells, latex beads and necrotic cells did not increase TNF-α release from quiescent Mφ (open bars in Figure 7-7). None of the apoptotic cell types (alone) treated with IFN-γ and LPS caused TNF-α release into the supernatant (not shown). However, ingestion of apoptotic cells by Mφ, followed by activation of Mφ with IFN-γ plus LPS led to decreases in TNF-α release assessed at 24h. Latex beads did not influence activated Mφ TNF-α release. Furthermore, all apoptotic cells studied were able to decrease TNF-α release.

Interestingly, though release was diminished by ingestion of apoptotic rat MC, it was not completely blocked, yet in co-culture Mφ that had ingested apoptotic rat MC were unable to kill live MC by TNF-dependent mechanisms. One explanation for this is the predominant role of membrane bound TNF-α in killing by apoptosis. Indeed, there is evidence from Mφ cytolysis of tumour cells that membrane-bound TNF-α is more potent than the predominant soluble form (Decker et al., 1987; Peck et al., 1989; Perez
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

et al., 1990; Duerksen-Hughes et al., 1992; Day et al., 1994; Lazidins et al., 1997; Kusters et al., 1997; Weiss et al., 1998). Preliminary flow-cytometric based studies of murine BMD-Mϕ have located low levels of mTNF-α on the cell surface of activated cells (Lynda Stuart personal communication) and mTNF-α has been clearly demonstrated by western blotting techniques (Jue et al., 1990). Further work is required to elucidate whether ingestion of apoptotic cells down-regulates mTNF-α expression on activated Mϕ, or whether co-factors such as integrins are down-regulated (Sieg et al., 1999; Meszaros et al., 2000).

![Figure 7-7](image)

**Figure 7-7:** Mϕ that have ingested apoptotic cell release less TNF-α when activated by IFN-γ/LPS than Mϕ which have ingested inert particles or have not ingested any particle. Mϕ were exposed to a five fold excess of pre-formed apoptotic leucocytes or apoptotic MC for 4h in culture. Wells were washed to remove non-ingested apoptotic cells, medium replaced, then activated with IFN-γ (100U/ml) plus LPS (1μg/ml). At 24h, medium was aspirated, clarified and by ELISA for TNF-α (n=5 * P < 0.05, ** P < 0.01).

### 7.6 TRANSFORMING GROWTH FACTOR-β RELEASE IS AUGMENTED BY Mϕ AFTER INGESTION OF APOPTOTIC CELLS

Although, only one third of Mϕ in these studies ingested apoptotic cells, the effect of binding/ingestion of apoptotic cells by Mϕ appeared to be able to influence all Mϕ, given the complete suppression of TNF-dependent killing. This observation might suggest the role of soluble or paracrine factors. In limited studies, the cytokine transforming growth factor beta (TGFβ) was assessed in the supernatants from Mϕ that had ingested apoptotic cells, since active TGFβ is a candidate cytokine responsible for
inhibiting pro-inflammatory responses following IFN-\(\gamma\)/LPS activation. Further, Fadok and colleagues showed that this cytokine could be released from M\(\phi\) upon ingestion of apoptotic neutrophils (Fadok et al., 1998; McDonald et al., 1999). Because FCS contains large amounts of inactive bovine TGF\(\beta\), which would interfere with an ELISA assay, experiments were performed as described in Chapter 7-4, except that the medium used contained no FCS. In parallel studies to those described in Chapter 7-4, M\(\phi\) that had ingested apoptotic cells were studied for TGF-\(\beta\) release. McDonald and colleagues (McDonald et al., 1999) suggested that when J774 cells (M\(\phi\) cell line) ingested apoptotic neutrophils all TGF\(\beta\) was released within 2h of interaction, therefore in these studies M\(\phi\) were not washed to remove non-phagocytosed cells after the interaction period.

Table 7-2 documents TGF-\(\beta\) release into culture supernatants from murine BMD M\(\phi\) following ingestion of apoptotic cells or beads. It indicates that there were small increases in TGF-\(\beta\) release by the BMD M\(\phi\) used in this study. However, non-activated and IFN-\(\gamma\) LPS activated M\(\phi\) produced TGF-\(\beta\) without ingestion. It is clear however, that the percentage increase in TGF\(\beta\) release corresponded closely with the degree of TNF-\(\alpha\) decrease and NO increase documented in the parallel studies of Chapter 7.4 and 7.5. Whether these small increases in cytokine release have functional paracrine or autocrine effects remains to be explored by blocking studies.

<table>
<thead>
<tr>
<th>Ingestion</th>
<th>None</th>
<th>apop. PMN</th>
<th>apop. Thym.</th>
<th>beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unactivated</td>
<td>18.2±0.8</td>
<td>32.5±11.1</td>
<td>33.8±7.4</td>
<td>12.5±5.4</td>
</tr>
<tr>
<td>Activated</td>
<td>23.2±4.3</td>
<td>45.4±10.4</td>
<td>37.2±6.4</td>
<td>17.6±5.8</td>
</tr>
</tbody>
</table>

PMN were induced into apoptosis by ageing, thymocytes by UV treatment. Apoptotic cells alone did not produce significant levels of TGF\(\beta\).
Chapter 7: Results 5. Macrophage ingestion of apoptotic cells blocks TNF-dependent killing

7.7 SUMMARY

- As well as induction of apoptosis of neighbouring MC, the same Mφ are able to phagocytose the MC induced into death
- In the presence of the NOS inhibitor L-NMMA, activated rodent Mφ that have ingested apoptotic MC (or leucocytes) are unable to induce TNF-dependent killing of further MC
- Ingestion of apoptotic cells augments NO release by activated rodent Mφ
- Ingestion of apoptotic cells inhibits sTNF-α release by activated rodent Mφ
- Ingestion of apoptotic cells induces TGFβ release from Mφ
Chapter 8

Discussion
8.1 SUMMARY

The data demonstrate in vitro that macrophages activated by pro-inflammatory cytokines and opsonised particles can induce proliferating MC from the glomerulus of the kidney into apoptosis. This provides a crucial link between the occurrence of apoptosis in inflammation with presence of infiltrating Mφ. Secondly the data indicate for the first time that ingestion of cells that have undergone apoptosis can regulate the capacity of macrophage populations to induce apoptosis; a form of negative feedback built into the inflammatory response. Thirdly, the studies have attempted to delineate the mechanisms involved in bringing about the Mφ effects on MC. Amongst the myriad of cell surface and soluble ligands generated by Mφ, the studies demonstrate roles for nitric oxide, tumour necrosis factors alpha and possibly beta, and also a potential role for hydrogen peroxide. Investigation of these factors has revealed complex interplay between NO released from both Mφ and MC, and the potential neutralising effects of NO by oxygen radicals. Further, a striking and potentially important absence of NO release from human Mφ was shown, compared with equivalent rodent Mφ. Studies of TNF have indicated subtle synergy between this ligand and the lymphokine IFN-γ, a cytokine that may be a crucial regulator of Mφ directed manifestations of the inflammatory process. Furthermore, the data point to a predominant role of membrane bound TNF over soluble TNF in bringing about mesangial cell killing. Cell signalling of apoptosis by TNF-α is at least in part dependent on the nuclear factor IRF-1. Finally, Fas ligand has no role in macrophage effects upon mesangial cell death or cell cycle. The data have many implications for understanding inflammation in the kidney, but striking similarities in inflammatory settings suggest many of the findings here are relevant to the inflammatory process per se and reveal new findings about Mφ biology.

8.2 MACROPHAGES KILL RESIDENT CELLS; A SPECIFIC OR GENERAL PHENOMENON?

The thesis demonstrates that inflammatory Mφ can kill proliferating resident cells of the glomerulus in an ex vivo environment (Figure 8-1). The role of Mφ in the sterile inflammatory setting has attracted relatively little scrutiny. Inflammation leading to pathology is often predominated by lymphocytes, the paradigmatic example being acute allograft rejection. However, as described in the introduction, the
normal resolving inflammatory infiltrate is characterised by neutrophils, followed by Mφ, with only few lymphocytes influxing. If one considers the normal, resolving inflammatory process as the first building block of the pathological inflammatory response, Mφ become key players. Pathological inflammation in the kidney and in particular the glomerulus is characterised by a relative paucity of lymphocytes and an abundance of Mφ, and scrutiny of inflammation elsewhere reveals Mφ as an abundant cell type. This is evident in autoimmune diabetes where Mφ comprise a major cell type in the pancreatic islets (Jun, Santamaria et al., 1999; Jun, Yoon et al., 1999; Suk et al., 2001; Suarez-Pinzon et al., 2000; Thomas et al., 2000; Liu et al., 2000), and during relapsing and remitting multiple sclerosis where both BMD Mφ and oligodendrocytes, which are of the Mφ lineage, are major contributors (Okuda et al., 1997; Tada et al., 1997). Progressive studies of atherosclerotic plaques have confirmed the inflammatory nature of this disease and confirmed monocyte derived Mφ as crucial players in disease progression (Szabo et al., 1996; Boyle et al., 1998; Bennett 1999). Ongoing studies of resolving liver fibrosis have shown a striking correlation between the presence of activated Mφ in the resolving scar tissue and the presence of hepatic stellate cell apoptosis (liver myofibroblasts) (J. Duffield & J. Iredale unpublished observations).

Figure 8-1: Schematic of the mechanisms by which Mφ induce apoptosis of MC
However, the presence of such Mφ does not necessarily equate with a killing capacity. Indeed, it may be that Mφ are crucial to the survival of certain cell types (Chang et al., 2001; Reveneau et al., 1999). As well as death inducing factors, Mφ can release a host of pro-survival cytokines. This is well-illustrated in Mφ-lymphocyte interactions. In the diabetes mellitus mouse model, there is evidence that local Mφ producing IL-12 are crucial for survival and proliferation of cytotoxic T lymphocytes. In addition the Mφ release NO and TNF-α, both of which have been shown to be crucial factors in inducing cell death of pancreatic β cells (Jun, Yoon et al., 1999). Whether the same individual Mφ cells are responsible for both effects is unclear. In atherosclerosis, Mφ plus platelet knockout studies in mice prone to atherosclerosis (Apo E mice) suggest controversially that Mφ in the plaque are at least partly responsible for pro-proliferative signals encouraging smooth muscle cells (myofibroblasts known as VSMCs) to grow (Ross et al., 1990; Wilcox et al., 1994). However, in vitro, monocyte derived Mφ cultured with VSMCs induce apoptosis of the latter (Boyle et al., 1998; Wilcox et al., 1994; Szabo et al., 1996). Whether the conflicting nature of Mφ in plaques reflects different populations or different types of disease activity in the plaques is unclear.

Very recently, in elegant ex vivo co-cultures Meszaros and colleagues (Meszaros et al., 2000) have demonstrated that wound Mφ not only phagocytose wound neutrophils, but they also induce neutrophil apoptosis by a contact dependent mechanism. In the kidney, in a mouse model of glomerulonephritis with associated tubulo-interstitial nephritis, Mφ can be clearly localised to tubular cells undergoing apoptosis. The cells undergoing apoptosis are MCP-1 expressing. In the MCP-1 knockout mouse, many fewer cells undergo apoptosis and there are many fewer Mφ localised to the tubular cells. Clearly, in this model Mφ are inducing apoptosis of tubular cells (Tesch et al., 1999). However, in the glomerulus, there is evidence in other disease models that Mφ can induce proliferation of MC (Oldroyd et al., 1999). The studies presented here indicate that even the presence of low levels of serum growth factors Mφ have no additive pro-proliferative effect on MC. This might be because MC produce their own growth factors which act in an autocrine fashion (Mene & Cinotti 1989; Abbott et al., 1992; Floege et al., 1994).

Further afield, tumour biology showed initially in vitro but later in vivo that Mφ can induce (Hajri et al., 1998; Nishikawa et al., 1998; Gal et al., 1997) tumour cell apoptosis by a variety of mechanisms including nitric oxide, TNF, TRAIL and Fas ligation. The immune response against tumour cells shares many features with that described in inflammation in the kidney.

These studies taken together with the work presented in this thesis and the work of Lang and colleagues (Lang & Bishop 1993; Lang 1997) showing the necessity in vivo for Mφ in deleting excess
cells in the developing eye would indicate that a major role of Mφ is in the regulation of cell populations.

### 8.3 THE INTERPLAY OF CELL CYCLE AND APOPTOSIS

Throughout this study, apoptosis of MC has been linked to cell cycle. Firstly MC interacting with activated rodent Mφ are prevented from entering mitosis, described here as cell cycle arrest. DNA studies indicate this to be a G1/S arrest. Secondly, many rat MC undergoing apoptosis by TNF-α appear microscopically to be cells that had attempted mitosis, but had aborted part-way through, suggesting that the cells were more susceptible to apoptosis in G2/M of the cell cycle. The DNA content data is difficult to interpret for IFN-γ/TNF-α mediated MC apoptosis but would be compatible with cell death occurring at G2/M. However, cells over-expressing IRF-1, which appears to be important in TNF-signalling of apoptosis, show an unequivocal G2/M block, evidenced by accumulation of cells in with 2n DNA content. During experiments with human MC, IFN-γ alone appeared to block MC mitosis. This was not seen with the rat or mouse MC. The effect was seen with different growths of MC suggesting a true species difference rather than a heterotypic difference. The anti-proliferative effects of IFN-γ are well-described (Kano et al., 1999; Burke et al., 1999). Indeed there is evidence of this cytokine playing such a role in models of glomerular disease (Johnson et al., 1995; Oldroyd et al., 1999). There is clear evidence elsewhere that IRF-1 is important in both ‘stress’ induced and cytokine induced cell cycle arrest and apoptosis (Tamura et al., 1995; Prost et al., 1998; Kano et al., 1999). The data shown here for MC confirm that IRF-1 does play a role in both these functions and for the first time is shown to play a role at the G2/M checkpoint (Figure 8.2).

One other striking correlation in all the studies presented here was that the more cells proliferated, the greater their tendency to undergo apoptosis in the presence of activated Mφ. This observation might also indicate the role of cell cycle in susceptibility to apoptosis. It is backed up in vivo, in the model of Thy1.1 nephritis, and elsewhere, where there was a correlation between number of apoptotic and mitotic cells in individual glomeruli (Baker et al., 1994; Ophascharoensuk et al., 1998; Hiromura et al., 1999). It may be that there are checkpoints in the cell cycle at both the G1/S border and the G2/M border (Shankland 2000; Doostzadeh-Cizeron et al., 2001) in mesangial cells that regulate apoptosis.
P53, a nuclear factor important in regulating the cell cycle has also been shown to be important in regulating cell death by apoptosis in the presence of DNA damage. Prior to DNA synthesis (S phase) damaged DNA must be repaired. Agents such as NO and oxygen radicals can induce direct DNA damage. P53 regulates this process by inducing blockade of cell cycle progression prior to new synthesis. If, after a given period, repair is unsuccessful, apoptosis is triggered (Mercer 1998; Schwarz & Rotter 1998). One can imagine how important this is in the prevention of propagation of mutations and the development of carcinomata. However, this pattern of mitotic blockade followed by apoptosis is that which one sees in Mφ-derived iNOS mediated death in co-culture and the NO-induced G1 block is well described in the literature (Messmer & Brune 1996; Sandau et al., 1997; Schwarz & Rotter 1998). Interestingly, hydrogen peroxide mediated death as described in Chapter 5 is described to induce P53 but does not lead to cell cycle arrest suggesting a different intracellular process at work. Again, frequently these apoptotic cells appear to be mitotic cells that have defaulted into apoptosis, possibly at the G2/M border. Whilst not investigated in this thesis, it is noteworthy that P53 also exerts a G2/M checkpoint (Taylor et al., 2001; Bache et al., 1999; Chien et al., 2000). The role of the G2/M checkpoint in peroxide mediated cell death by apoptosis should be sought.
Bcl-2, is an intracellular protein that is the archetypal family member of a series of proteins that regulate susceptibility to apoptosis (Korsmeyer 1992). Recent data indicate that Bcl-2 family proteins are important in regulating cytochrome C release from the mitochondria (Yang et al., 1997). Bcl-2 is a pro-survival factor that hinders successful signalling through the caspase pathway in certain cell types. It is de-activated when phosphorylated, thereby tendering the cell more susceptible to apoptosis. In Jurkat T cells Bcl-2 is phosphorylated during G2/M, and these cells are more susceptible to a variety of pro-apoptotic physiological stimuli at this time. Inhibition of phosphorylation renders the cells more resistant to apoptosis (Yamamoto et al., 1999). This makes biological sense since damage to DNA during mitosis would render progeny mutated without opportunity for repair since the condensed chromatin is inaccessible to nuclear enzymes (see Figure 1.4).

Studies of γ irradiation induced apoptosis of mouse thymocytes indicated apoptosis occurred following the G2/M checkpoint. In these cells however, when Cyclin B, a regulator of mitosis along with cdc2 kinase, is prevented from accumulating, γ irradiation induced apoptosis is prevented. Conversely over-expression of Cyclin B induces apoptosis (Porter et al., 2000) (Figure 8-2) It appears that the proteins regulating the cell cycle can now be directly linked to those involved in apoptosis.

Thus, one can see that proliferating cells have a variety of checkpoints where they are susceptible to apoptosis, as a way of preventing mutation of the genome. Toxic factors such as NO and H2O2 can impinge on these checkpoints and death receptor signalling may be most influential at these phases of the cycle. It seems likely that cells can die by apoptosis at any stage of the cycle, but that susceptibility varies with cell cycle progression. Experiments to test whether MC have differential susceptibility to apoptotic stimuli at stages of cell cycle progression were sought by synchronising MC with a double thymidine block. However, time constraints have prevented their completion.

8.4 NITRIC OXIDE IN INFLAMMATION - JANUS

This study has identified NO in Mφ-derived induction of cell cycle arrest and apoptosis of MC in the rodent system. It seems plausible that Mφ-derived NO will be important in vivo in the inflamed glomerulus; NO only acts over short distances, both proliferating MC and Mφ are found in close apposition in glomerular inflammation (see Figure 1.10). There are now clear examples in vivo of NO-
directed killing of cells. However, these have been a long time coming (Okuda et al., 1997; Hajri et al., 1998; Nishikawa et al., 1998; Koglin et al., 1999; Cobb et al., 1999).

However, studies using iNOS knockout mice or competitive inhibitors of iNOS, in in vivo models of glomerular inflammation have not provided a clear picture:

In a mouse model of NTN using the iNOS mouse over the first 48h of disease, there was no difference in disease parameters compared with control (Cattell et al., 1998). However, this disease appeared mild, heterologous phase disease, with some neutrophil influx, but only occasional Mφ (Figure 8-3). In addition, apoptosis was not assessed. Unfortunately, the limited tissue remaining from that study was insufficient to assess adequately apoptosis (Personal observations in conjunction with Dr. V. Cattell).

Other studies have looked at NTN in the rat, where significant Mφ influx is readily achievable. The use of L-NIL as an inhibitor of NO generation had no real effect on histological parameters, but augmented proteinuria (Lianos et al., 1998). In this study, Mφ influx was not quantified. However, it is likely that there were significant Mφ present. Three issues are raised by this study however; firstly, the disease model is initiated by immune complex and complement deposition in the glomerular basement membrane. In vitro studies from Chapter 5 indicate these to be potential ligands for ROI release from
MΦ. Investigation of heterologous phase NTN has shown the importance of hydrogen peroxide in initiation of the disease by neutrophil damage to the endothelium (Johnson et al., 1987; Rehan et al., 1984; Cook et al., 1989). Secondly, from the data in Chapter 3, it is likely that during the switch from heterologous to autologous phase disease glomerular MΦ populations switch from producers of oxygen radicals to generators of NO. Further supportive evidence of this comes from studies by Waddington who specifically examined the role of iNOS-derived NO in heterologous phase NTN (Waddington et al., 1996). iNOS inhibitors tend to lack specificity, but are selectively dependent on extracellular L-arginine concentrations. By inhibiting arginase (the other metabolic pathway for L-arginine (see Figure 1.2)) or by infusing L-arginine, he was able to regulate arginine availability at the site of inflammation. The studies suggested that NO had little effect on histological parameters. There appeared to be increases in proteinuria when L-arginine availability was limited, suggesting that NO might be protective, or anti-inflammatory in some way. Proliferation and apoptosis were not assessed. One might interpret these observations in two ways; firstly, (assuming that NO-mediated apoptosis of resident cells is occurring) that NO-induced apoptosis is protective. Secondly, that NO might be nullifying the effect of neutrophil and MΦ release of oxygen radicals.

In favour of the latter is a wealth of evidence that eNOS-derived NO is important in protecting the endothelium from oxidative stress damage. These observations indicate that NO reacts with radicals to produce stable, inert nitrate. The eNOS studies however indicate that in conditions of L-arginine depletion eNOS can paradoxically generate O₂⁻ itself, and in addition, a putative compound peroxynitrite (Xia et al., 1996; Xia & Zweier 1997; Xia et al 1998). Sandau and colleagues (Sandau et al., 1997) showed that oxygen radicals and NO donors ‘cancel each other out’ when applied concurrently to rat MC cultures and MC apoptosis was assessed. Waddington’s studies might be best explained by hypothesising that early oxidative damage from principally neutrophils was being counteracted by NO generation from both neutrophils and MΦ.

The role of iNOS-derived NO at the site of inflammation is complicated by two further observations:

Firstly, Albina’s group indicated that tissue L-arginine concentrations are very low in a model of a skin wound and also in samples of muscle injected with sterile irritants (Albina et al., 1990; Reichner et al., 1999). It is conceivable that L-arginine levels are low in glomerular inflammation, though high capillary blood flow might counteract arginine depletion. Using the in vitro model, MΦ killing of MC by NO was inhibited by reducing L-arginine in the culture medium (Chapter 3.12). Rather than generating ‘toxic’ oxygen radicals and peroxynitrite as suggested by Xia and colleagues (Xia et al., 1998), there was no evidence of MC necrosis and both apoptosis and cell cycle arrest were diminished.
Chapter 8. Discussion

The remaining induction of apoptosis was inhibited by L-NMMA, not SOD or catalase, suggesting that the remaining kill was still mediated by lower levels of NO release. These data suggest that unlike eNOS, iNOS does not appear to induce oxygen radical and peroxynitrite generation or that peroxynitrite does not kill mesangial cells (Bank et al., 1998; Heeringa et al., 2001).

Secondly, it appears that during inflammation, endothelial cells induce iNOS and this enzyme can 'take over from eNOS' in regulating vascular tone (Cadogan et al., 1999; Heeringa et al., 2001). Thus, any attempt to modulate iNOS release of NO will necessarily influence local blood flow and local haemodynamics. If iNOS is inhibited, arterioles will vasoconstrict, and may contribute to ischaemia and hypoxia, which are undoubtedly factors in glomerular scarring in their own right (Shanley 1996). Therefore, in vivo studies of the role of NO in regulating MC proliferation and apoptosis will probably prove impossible to interpret unless iNOS can be selectively regulated in the Mφ.

Yet we know that Mφ in the glomerulus produce iNOS and (in rodents ex vivo) nitric oxide (Cattell et al., 1993; Kashem et al., 1996; Furusu et al., 1998; Heeringa et al., 2001), and detailed studies in Class IV human lupus glomerulonephritis have indicated close correlation between iNOS and neighbouring apoptosis (Wang et al., 1997). We now also know that Mφ ex vivo from the rat tNTN model can induce MC apoptosis by iNOS dependent mechanisms. Further Weinberg (Weinberg et al., 1994) has indicated that NO must have a role in development of nephritis since the MRL lpr/lpr, lupus nephritis prone mouse, did not develop nephritis when fed L-NMMA in the diet. Noble's group have indicated that iNOS is crucial in the initial mesangial apoptosis seen in their model of Thy1.1 nephritis (Narita et al., 1995), but this work has not been reproduced successfully (K Mosely personal communication and Mosely et al., 1999).

One possible future avenue is the iNOS knockout mouse. In studies of transplanted cardiac rejection, the iNOS knockout mouse shows less apoptosis of cardiomyocytes than wild type controls, indicating an unequivocal for Mφ-derived NO in inducing resident cell apoptosis (Koglin et al., 1997). However, Weinberg (Gilkeson et al., 1997) crossed the MRL lpr/lpr with the iNOS KO mouse, to find that progression of spontaneous glomerular disease (which does have glomerular inflammatory Mφ) was not different from controls. Vasculitic lesions in the animals that lacked iNOS did not develop, however. This disappointing result is hard to reconcile with the earlier work using L-NMMA (Weinberg et al., 1994) and might suggest that if iNOS is important in determining glomerular disease progression that in the knockout animal other, non-NO pathways can take its place (redundancy). Furthermore, there is evidence that during ontogeny NO (like Fas, TNF-α, LT-α) is important in regulating lymphocyte subset proliferation rendering the knockouts prone to more vigorous TH1
Chapter 8. Discussion

responses. Therefore studying this role of iNOS in MΦ in vivo might require conditional cell-specific knockout animals.

To conclude, iNOS derived NO at the site of glomerular inflammation can theoretically have the following, often opposing influences:

1) induction of apoptosis and cell cycle arrest of resident cells
2) Negating the effect of oxygen radicals released from neutrophils and MΦ
3) Regulating microvascular tone

One can see from in vivo glomerulonephritis studies that often the disease comprises different phases of immune activation whereby high levels of oxygen radicals might be released and only later high levels of NO would be generated. It also appears that each model of nephrotoxic nephritis has different characteristics perhaps dependent on the different severity and duration of the heterologous phase of disease compared with autologous phase.

Finally, evidence from Chapter 3 indicates that MC can also generate NO. It is possible that high levels can be generated, by some MC in some circumstances, enough to protect that MC from NO-mediated damage. However, more likely is the somewhat lower level of MC NO generation as demonstrated from MC from our laboratory. In studies of co-culture where MC were primed to generate their own NO and MΦ were activated by opsonised particles to generate oxygen radicals, the MC were relatively protected from peroxide mediated apoptosis. This observation has been mirrored in the vascular endothelium and in UV-induced inflammation in the skin (Yamaoko et al., 2000; Cooke 1998).

Thus, NO is a molecule with diverse biological effects, which may be both protective and inductive in immune mediated apoptosis.

8.5 FAS AND ITS ROLE IN APOPTOSIS

Fas/FasL interactions have been recognised in regulating lymphocyte populations for some years. This is most clearly seen in the MRL lpr/lpr or gld/gld mice which have non-functional mutations in Fas
Chapter 8. Discussion

and FasL respectively. These animals are characterised by a generalised lymphoproliferative disease with excess CD4-, CD8- lymphocytes, cells which are normally deleted (Takahashi et al., 1994). One characteristic of the Fas/FasL deficient animals is the development of glomerulonephritis. The animals have a lupus-like syndrome with auto-antibodies including anti dsDNA antibodies.

The glomerulonephritis is similar to that seen in human lupus, with linear immune complex deposition on the glomerular basement membrane, mesangial proliferation and influx of Mφ mainly and some lymphocytes. Often there is intense focal lymphocyte infiltration to areas of the interstitium also.

This disease brings into question the role of Fas/FasL signalling apoptosis in development and progression of glomerular disease. Since the early-1990’s it was recognised that in addition to lymphocyte regulating properties of Fas/FasL signalling ligation of Fas on resident hepatocytes could induce widespread apoptosis (Ogasawara et al., 1993). Gonzalez-Cuadrado first showed that Fas ligation with agonistic antibodies could induce apoptosis of human mesangial cells in the presence of IFN-γ. They went on to describe mesangial lysis (due to mesangial cell apoptosis) in mice after administration of systemic agonistic anti-Fas antibodies (Gonzalez-Cuadrado et al., 1996; Gonzalez-Cuadrado et al., 1997). In addition there was widespread hepatocyte apoptosis. This glomerular observation was not reported in earlier, similar studies showing similar hepatic cell apoptosis to that noted in the Gonzalez-Cuadrado study. However, given this result and the observations reported in Chapter 4.5-4.10 it seemed likely that not only was Fas important in lymphocyte regulation but also in resident tissue regulation in inflammation.

In the MRL lpr/lpr mouse model of spontaneous progressive glomerulonephritis, Rubin-Kelley’s group highlighted the importance of CSF-1 (Mφ survival and differentiation cytokine) and the chemokine MCP-1 (monocyte chemo-attractant protein) in glomerular disease progression (Rubin-Kelley et al., 1994; Wada et al., 1997; Tesch et al., 1999). These data heavily implicate the Mφ as the key mediator in disease progression in this model.

The data from Chapter 4 confirm that in vitro, MC primed with IFN-γ plus TNF-α (in rodents) and primed with IFN-γ alone (humans) was sufficient to enable Fas-mediated apoptosis of proliferating cells. This feature of myofibroblast-like MC is echoed in similar studies of other myofibroblasts such as human vascular smooth muscle cells (VSMC) (Bennett et al., 1998) where Fas ligation also mediates apoptosis in primed cells. Furthermore, Fas induced apoptosis of tubular epithelial cells of the kidney has been widely reported (Schelling et al., 1998; Lorz et al., 2000; Hughes et al., 2000). Thus interstitial cells, tubular cells and mesangial cells from the kidney might all be susceptible to Fas-mediated killing.
In 1997, Badley and colleagues (Badley et al., 1997) using ex vivo modelling, indicated that human Mϕ could utilise FasL to bring about apoptosis of bystander CD4+ lymphocytes, providing a model of CD4+ cell loss in HIV disease by HIV infected Mϕ. TNF-α was also implicated as a factor in bringing about this apoptosis. Studies from our own group also indicated that supernatants from Mϕ activated by opsonised particles were able to bring about neutrophil apoptosis by a mechanism inhibitable by blockade of Fas binding (Brown & Savill 1999). Thus, it seemed plausible that activated Mϕ might regulate MC populations utilising FasL.

The co-cultures shown in this thesis (Chapter 4, Chapter 5) indicate that whilst Mϕ are known to have FasL both on the plasma membrane (Dr. S. Brown-personal communication) (Bennett 1999) and released into the supernatant, following cleavage, they do not appear to utilise it in inducing apoptosis of susceptible MC. More curiously is that MC themselves (Chapter 4.11), VSMC and tubular epithelial cells of the kidney also have FasL, yet there is no in vitro evidence of an apoptosis inducing role for this ligand.

This lack of Mϕ killing by apoptosis is in contrast to in vivo studies by Hughes & Johnson (Hughes & Johnson 1999) where inflammatory Mϕ influx induced by ureteric ligation appears to be a factor responsible for induction of apoptosis of distal tubular cells. When experiments were performed in lpr/lpr mice (C3H/HEJ background which lacks glomerulonephritis), there were fewer apoptotic distal tubular cells, though this did not affect other disease parameters.

However, in keeping with the negative data presented in this thesis, Rubin-Kelley's group were able to transplant kidneys from MRL lpr +/- mice into MRL lpr -/- mice and show that the onset and progression of glomerulonephritis was no different from the native kidneys over a 12 wk period (there was no rejection). This would indicate that in a Mϕ-rich progressive disease, FasL had no bearing on disease outcome.

The role of FasL in vivo appears complicated. There is very good evidence that FasL is a neutrophil chemokine (Kang et al., 1997, Otonello et al., 1999, Suarez-Pinzon et al., 2000, O’Connell 2000). It may be that expression of FasL by resident cells in culture (activated de-differentiated phenotype) has more to do with chemokine function than apoptosis. Indeed MC in culture are known to release an array of chemokines (Banas et al., 1999). Unfortunately, in the studies in this thesis, soluble FasL released by MC was not assessed.
Chapter 8. Discussion

Notably, Rubin-Kelly's group purified lymph node lymphocytes from diseased (lpr/lpr) animals and found that renal proximal tubular cells underwent apoptosis in co-culture, indicating that FasL was not crucial for lymphocyte mediated induction of apoptosis (Schwarting et al., 1998).

There has been some debate about the respective roles of soluble over membrane bound FasL in bringing about apoptosis. This will be explored elsewhere (Chapter 8.6).

Pancreatic β cells in the islets of Langerhans in the pancreas are a target for immune mediated destruction by apoptosis in type 1 diabetes mellitus and in the spontaneously diabetic (non-obese diabetic) mouse. A role for Fas/FasL has been postulated with significant in vivo evidence from the lpr/lpr mouse crossed with the NOD mouse since these mice do not develop disease (Signore et al., 1998; Thomas et al., 2000). Pancreatic β cells are rendered susceptible to FasL by similar cytokine administration to that shown in this thesis for mesangial cells. However, despite intense influx of Mφ and cytotoxic lymphocytes in this disease model, it appears that in Fas deficient NOD mice, the attenuated disease phenotype is due impaired proliferation of cytotoxic and effector T cells, not due to impaired apoptosis of β cells (Kim et al., 1999; Kim et al., 2000). Like the situation for kidney disease whilst Fas/FasL appeared an attractive target for manipulating disease progression through regulating target cell death, it appears that in the pancreas Fas/FasL plays no role clear role in apoptosis of target cells, but may regulate lymphocyte populations.

In contrast, Hughes and Johnson (Hughes 1999) have confirmed a small but definitive role for Mφ derived FasL in inducing distal tubular cell apoptosis in kidney inflammation induced by ureteric obstruction. Also, the Con A model of hepatitis is clearly inhibitable by anti-FasL antibodies (Kim et al., 1999). Whether this is due to down-regulation of the immune response or blockage of target cell killing is unclear (Ogasawara et al., 1993). However, more detailed studies of hepatocyte injury indicate IFN-γ and TNF-α to be indispensable whereas absence of Fas had no bearing on the disease process (Ohta et al., 2000).

Thus whilst there are clear in vitro examples of Mφ killing target cells by FasL/Fas ligation, there was no in vitro evidence from this thesis of Mφ killing MC by this mechanism. The in vivo data would suggest that Fas/FasL is an important regulator of lymphocyte populations but it has little role in regulation of resident cell apoptosis in inflammation.
8.6 TUMOUR NECROSIS FACTOR AS A GENERAL REGULATOR OF CELL POPULATIONS

Despite clear evidence in vivo and in vitro of FasL regulating lymphocyte populations, the evidence of regulation of resident cell populations is limited. The data presented here indicate that MC apoptosis can be mediated by TNFR1 ligation. In the case of human MC, it appears to be the major mechanism of MΦ-directed MC apoptosis in the absence of NO release by MΦ. The rodent and human data indicate a role for IFN-γ in modulating MC susceptibility to apoptosis by TNF signalling. Thus TNF alone appears insufficient to induce cell death; rather cells require prior exposure to IFN-γ in order that TNF can successfully trigger apoptosis.

As stated in Chapter 8.5, there is clear evidence for an in vivo role for TNF-α in directing target cell apoptosis in models of viral induced hepatitis (Ohta et al., 2000). TNF-α is also strongly implicated in immune destruction of the pancreas in the NOD mouse (Suk et al., 2001). There is an abundance of evidence that TNF-α has an important role in the immune response to tumour cells (see Chapter 8.7). In vitro studies have also indicated TNF-α to have a role in endothelial cell apoptosis and CD4 lymphocyte apoptosis in models of HIV disease (Lindner et al., 1997; Badley et al., 1997).

Unfortunately, like studies of NO in vivo, the disease outcome following modulation of the TNF/TNFR1 axis is complicated by the pleiotropic effects of TNF in the inflammatory response. For example, there are unequivocal studies showing NTN in rats and mice is markedly abrogated in TNF-α KO mice or in rats concurrently treated with anti-TNF-α antibodies (Le Hir et al., 1998; Karkar et al., 2001). However, the authors suggest that the major reason for this is that TNF-α is crucial in disease initiation through either platelet activation and degranulation or its role in upregulation of integrins on the endothelium, which enable leucocyte diapedesis. This being the case it is not possible to comment on whether TNF in vivo is playing a role in resident cell apoptosis. Conditional knockout animals might be required to identify the role of TNF at different stages of the inflammatory process in vivo, given that its requirement early in inflammation prevents testing its function in later inflammation in knockout models.

The TNF-α knockout mouse exhibits significant abnormalities of lymph node development with certain cell types being under-represented or absent (Eugster et al., 1996). It appears that TNF-α/β have roles in regulating lymphocyte populations.
Chapter 8. Discussion

8.7 MEMBRANE OR SOLUBLE TNF?

The co-culture studies in this thesis show that whilst MC death in humans and rodents is TNF-dependent, transfer of clarified supernatants from activated Mφ has no killing effect on susceptible mesangial cells, despite the presence of micromolar quantities when assessed by ELISA. The TNF-dependent killing in co-culture amounted to up to 20% of MC at 24h. Notably, recombinant TNF-α (rTNF-α) in susceptible cells had some killing effect but it did not match the direct Mφ-co-culture effect. Thus in a hierarchy of killing mTNF > rTNF > sTNF. There is evidence that rTNF-α can exist in polymeric form. Interestingly, the studies of the killing effect of FasL in this thesis would indicate that CH11 IgM antibody > rFasL > endogenous sFasL in killing of MC, and studies of other cell types would confirm that mFasL >> sFasL in inducing apoptosis (Schneider et al., 1998). Again there is evidence that the recombinant sFasL can exist in a polymeric form in solution. This potency for mTNF-α in inducing apoptosis has recently been highlighted in studies from Albina’s group of wound Mψ inducing apoptosis of wound inflammatory neutrophils by a TNF-dependent mechanism. This killing effect was contact dependent, soluble TNF-α was ineffective (Meszaros et al., 2000). Other studies of peripheral blood neutrophils has indicated their selective susceptibility to apoptosis by mTNF-α (Murray et al., 1997).

There is however, a president for mTNF-α having a more potent role in killing of target cells in other in vitro and in vivo studies (Decker et al., 1987; Peck et al., 1989; Perez et al., 1990; Duerksen-Hughes et al., 1992; Day et al., 1994; Lazidins et al., 1997; Kusters et al., 1997; Weiss et al., 1998). The data of FasL seem to reiterate this paradigm. Importantly, in studies of the killing effect of both mTNF-α and mFasL there is evidence of a synergistic role for adhesion molecules ICAM-1, α,β, and CD36. Whether they act purely as functional anchors or whether they provide co-stimulation has not been investigated (Meszaros et al., 2000; Seig et al., 1998).

In the studies of Chapter 7, it was notable that it was possible to abrogate completely, TNF-dependent killing of MC following Mψ-ingestion of apoptotic MC. At the same time, sTNF-α release by the same Mψ was only partially inhibited. This paradox would fit well with the hypothesis that Mψ-membrane TNF was dependent for killing. Further studies should be directed at understanding co-factors in mTNF killing such as integrins as suggested by Meszaros and Seig.
8.8 INTERFERON-γ; A CONTROLLING CYTOKINE

Throughout this thesis, IFN-γ can be viewed as a fundamental co-factor in Mφ activation, as a modulator of Mφ activation states, and importantly as a crucial factor controlling both MC susceptibility to apoptosis and cell cycle progression. It can be seen that for effective Mφ activation to stimuli such as TNF-α, CD40L and LPS (and IL-1β), IFN-γ was a crucial co-factor. When Mφ were exposed to immune complexes (opsonised zymosan), they responded with a brisk respiratory burst. However, IFN-γ pre-treatment in rodent Mφ modulated this response (Chapter 5.2 and Johnston et al., 1985). When, proliferating MC had been exposed to IFN-γ there was augmentation of Mφ-dependent killing, which in both humans and rodents was TNF-dependent. IFN-γ was therefore important in TNF-dependent apoptosis. Interestingly, IFN-γ was crucial in protection of MC from peroxide-dependent killing of MC, possibly by the induction of MC to produce low levels of NO.

IFN-γ is the archetypal lymphokine, and certainly there is much evidence in vivo of the presence of activated lymphocytes in glomerular inflammation. There is evidence in telescoped nephrotoxic nephritis (Huang et al., 1997; Kitching et al., 1999; Cunningham et al., 1999; Rosenkranz et al., 2000) and the diffuse proliferative glomerulonephritis of lpr/lpr MRL mice (Schwarting et al., 1998) of IFN-γ secreting, proliferating lymphocytes. However, there is also now substantial evidence of IFN-γ release by activated Mφ (Schindler et al., 2001). Whether the Mφ used in the experiments shown in this thesis were producing IFN-γ was not tested, but the efficacy of adding IFN-γ to these cultures suggests Mφ-derived IFN-γ levels was low, if present at all. The reports of Mφ-derived IFN-γ at least suggest that IFN-γ secreting T cells/ NK cells might not be necessary at the site of inflammation for the effects observed in these studies.

Interestingly, two groups reported opposite effects from IFN-γ blockage in nephrotoxic nephritis in mice. In one, from Tipping’s group (Kitching et al., 1999) telescoped NTN was compared in IFN-γ CS7B16 KO mice and wild type mice. This intense crescentic glomerulonephritis, which the group have shown to be Th1 cell-mediated disease, was dramatically abrogated in the IFN-γ KO mice. The data indicated a requirement for locally produced IFN-γ in the glomerulus for disease progression. This result can be readily explained from the models in this thesis whereby glomerular Mφ are activated largely by lymphokines from antigen specific T cells. Excessive Mφ number and activation would lead to excessive mesangial cell loss, through the mechanisms delineated in this thesis, scarring and glomerular injury, whereas the KO mice would be much less able to activate Mφ thereby limiting
damage. Similar studies of the lupus like GN seen in the lpr/lpr mice have shown dependence on IFN-γ locally produced in the glomeruli as crucial for disease progression.

Another group (Ring et al., 1999), also induced telescoped NTN in C57bl6 mice, compared disease in WT animals, WT treated with anti-IFN-γ antibodies, or disease in IFN-γ KO mice. When IFN-γ was deficient or blocked, there was augmentation of disease, the opposite of that reported above. On further examination of the disease model, it could be seen that the disease was mild, with marked immune complex formation, and a mild Mφ and neutrophil influx suggestive of a humoral-mediated disease.

From the models developed in this thesis, one might anticipate that many of the Mφ in the inflamed tissue might be activated by immune complexes, not lymphokines. It would therefore follow that IFN-γ acting on proliferating MC might protect them from peroxide mediated injury derived from neutrophils and Mφ. Thus blockade of IFN-γ would lead to failed MC protection and therefore, excessive apoptosis, scarring and damage to glomeruli.

8.9 HYDROGEN PEROXIDE, A FACTOR IN MACROPHAGE-DIRECTED KILLING?

The data presented here point to a role for hydrogen peroxide in Mφ-mediated deletion of MC by apoptosis. The importance of hydrogen peroxide in disease initiation in a variety of inflammatory lesions of the kidney has been demonstrated by others (Holdsworth et al., 1988; Johnson Couser et al., 1987; 1988, Johnson, 1994; Rehan et al., 1994; Heeringa et al., 2001). It is clear that neutrophil-mediated endothelial and basement damage is catalase inhibitable in these models. Cattell’s group emphasised the role of hydrogen peroxide (Mosely et al., 1999) in the initial mesangiolysis (mesangial cell apoptosis) seen in the Thy1.1 model of mesangiproliferative GN. This is likely to be mediated by both neutrophils and Mφ that are rapidly recruited to the mesangium.

That immune complexes are crucial in the early pro-inflammatory stages of the immune response has been unequivocally demonstrated using Fc-γ receptor knockout mice (Suzuki et al., 1998; Wakayama et al., 2000; Launay et al., 2000; Gomez-Guerrero et al., 2000). However, whether they are important in the perpetuation of injury or remodelling is much more controversial. Waddington’s studies of early, heterologous rat NTN suggested strongly a predominant role for oxygen radicals in disease perpetuation at least over the first 24h of disease (Waddington et al., 1996). Furthermore, Johnson has indicated that a proportion of Mφ in the resolving stages of Thy 1.1 nephritis are myeloperoxidase positive (Johnson et al., 1995), suggesting that in later, resolving stages of this disease that Mφ may be generating ROI. However, there is much data that point to the role of cell mediated immunity in
disease progression in inflammatory models such as nephROTOXIC nephritis, possibly without the requirement for immune-complex activation of the immune system (reviewed by Rosenkranz et al., 2000). Indeed, the Mφ from day 2 and day 4 of rat telescoped NTN presented here show their phenotype to be similar to lymphokine activated Mφ rather than immune complex activated cells (Chapter 3.9). In the telescoped model of NTN used here it appears therefore that the predominating Mφ phenotype is one activated by lymphokines rather than immune complexes (see Figure 8-3). The in vitro studies here indicated that Mφ activated by opsonised particles can abundantly release H2O2. However, Albina's group (Nessel et al., 1999) have questioned the ability of wound Mφ to generate a respiratory burst. Unlike resident peritoneal Mφ, Mφ from wounds were less able to generate peroxide and radicals in response to PMA or zymosan. It is possible that one explanation for this observation is that the wound Mφ have already undergone a respiratory burst and are therefore no longer able to respond.

8.10 IRF-1 APPEARS TO HAVE ROLES IN APOPTOSIS SIGNALLING PER SE, AND ALSO SUSCEPTIBILITY TO TNF-DIRECTED APOPTOSIS

The data in this thesis point to an important potential role for IRF-1 in kidney disease as a regulator not only of Mφ activation (such as a co-factor in iNOS gene transcription (Kamijo et al., 1994; Martin et al., 1994; Tada et al., 1997)), but also a crucial factor in regulating MC progression through the cell-cycle and susceptibility to apoptosis. There is no published work on the role of IRF-1 in glomerular injury, but it appears plausible that at least in some MC, TNF-α might regulate the MC population by activating an IRF-1 dependent check point in the cell cycle and overriding the cell into apoptosis. Most work on apoptosis has used cell lines and in particular haematopoetic cell lines. Relatively little attention has been paid to primary tissue-derived cells. However, important studies in the inflamed endocrine pancreas and in hepatitis point to a role for IRF-1 dependent apoptosis in pancreatic β cells and hepatocytes (Suk et al., 2001; Pavlovic et al., 1999; Kano et al., 1999). The studies of β cell apoptosis in endocrine pancreatic inflammation co-localised IRF-1 positive β cells with TUNEL positivity indicating those cells undergoing apoptosis were IRF-1 expressing. The data in Chapter 6 suggest that the MC did not utilise caspase 3 and possibly other caspases in TNF-dependent death. This would appear incompatible with the orthodox model of caspase dependent apoptosis shown in Figure 1.4, but increasingly there are many reports of caspase independent death in a variety of cell types (Green & Kroemer 1998; Hildeman et al., 1999; Huang et al., 2000; Doerfler et al., 2000; Fitch et al., 2000; Honarpour et al., 2000). It increasingly appears that there is no universal death pathway in
mammalian cells. The studies in Chapter 6 provide no evidence for an intracellular ROI flux being important in signalling apoptosis following TNFR1 trimerization, whilst it has been demonstrated to be important in certain tumour cells, and many other cells investigated. Whether this relates to glutathione pumps preventing accumulation of ROI quenchers from accumulating intracellularly remains unanswered (de Bittencourt Junior et al. 1998; Hampton et al. 1998; van den Dobbelsteen et al. 1998). It is striking that whilst certain cells such as the L929 mouse fibrosarcoma cell line are exquisitely sensitive to soluble TNF-α, only a proportion of MC undergo apoptosis. I have suggested elsewhere that in MC it is likely to relate to the number of cells entering the G2/M phase of the cell-cycle. TNF-α mediated death in L-929 cells, due to its widespread killing effect is unlikely to be cell cycle dependent, is both caspase and ROI dependent. Could it be that TNF-mediated apoptosis in MC is both caspase and ROI independent?

The data from the IRF-1 over-expression studies (Chapter 6) show firstly that such over-expressing cells have an increased rate of spontaneous apoptosis. This would implicate IRF-1 directly in the apoptotic signalling process. When the IRF-HER gene construct was expressed in myeloid cells, increased spontaneous loss of these myeloid cells was only reported when estradiol was present (Kirchoff et al., 1993). This was not the case in the transfected MC. One possibility for this difference is that the system is 'leaky' in the MC, i.e. active IRF-1 leaks into the nucleus without the need for estradiol. This could be tested by EMSA, and is required to establish why spontaneous apoptosis is increased. Providing that is confirmed, one can assume that IRF-1 has a role in apoptosis. Secondly, many of the clones exhibited retarded progression through G2/M, indicating a G2/M block. Again, assuming the gene construct in MC is 'leaky', this would indicate that IRF-1 does play a role in cell surveillance at the G2/M checkpoint (Figure 8-2). This has not previously been demonstrated. Thirdly, when TNF-α was applied to these cells, apoptosis was augmented. It is unlikely that TNF-α application would significantly augment active IRF-1 levels in the MC. More likely is that IRF-1 has rendered the MC susceptible to TNF-mediated apoptosis. Thus IRF-1 may well be the pathway required by IFN-γ for achieving susceptibility, as has been reported for hepatocytes (Kano et al., 1999). These data fit in with evidence that IRF-1 is essential for some types of apoptosis in lymphocytes (Yamada et al., 1991; Matsuyama et al., 1993; Tamura, Ishihara et al., 1995) and hepatocytes (Kano et al., 1999).

Whilst it is purely speculation, and several areas of investigation require clarification, it appears logical to pursue IRF-1 dependent signalling in the regulation of tissue homeostasis and remodelling in kidney
Chapter 8. Discussion

disease. Investigation of models of glomerular injury in the IRF-1 KO mouse chimeric for wild type bone marrow would be a potential mechanism for delineating the role of IRF-1 more clearly.

8.11 MACROPHAGE ACTIVATION STATES

The data from this thesis emphasise that Mφ are not simply ‘activated’ but that activation can result in different patterns of cytokine production and toxic metabolite release. These patterns of activation are clearly seen in Mφ activated by IFN-γ plus another pro-inflammatory cytokine such as LPS, compared with Fc-γR-mediated activation. In the former, NO is abundantly produced, in the latter oxygen radicals released. Investigations by others of cytokine release from Mφ or array data of mRNA transcripts demonstrates that different stimulatory factors result in different patterns of cytokine release such as high IL-12/IL-18 following toxoplasma surface antigen exposure compared with much lower IL-12 release following IFN-γ plus LPS (Yap & Sher 1999). In this thesis Mφ taken directly from inflamed glomeruli were high NO and high TNF-α producing cells. Whether all Mφ were producing NO and TNF-α was not tested. Single cell data were not collected for Mφ cytokine profiles to see whether there were mixed populations. For example, it would be valuable to know whether they were individual high H2O2 producing cells amongst the glomerular Mφ from the rat t NTN model (Chapter 3). Interestingly, immunohistochemical studies of inflamed glomerular Mφ suggest that the glomerular population of Mφ might be phenotypically diverse (Lan et al., 1995; Furusu et al., 1998; Heeringa et al., 2001).

Over recent years it has also been appreciated that naïve Mφ can be classically activated and alternately activated (Goerdt & Orphanos 1999). These latter cells are alternatively activated in that they are producing an array of cytokines such as IL-10 and TGF-β, but not pro-inflammatory cytokines such as IL-1, TNF, IL-12, IL-6. The spectrum of cytokine production is not subtly different, but quite different from the IFN-γ/LPS activated Mφ. It is clear that initial cytokine exposure of naïve Mφ or monocytes to cytokines such as IL-4, IL10, IL-13, TGF-β or glucocorticoids can induce the alternatively activated phenotype (Erwig et al., 1999; Goerdt & Orphanos 1999).

There is increasing evidence from the microbiological field that Mφ, potently activated by the adaptive immune system can be down-regulated by concomitant signalling from receptors ligated by components of the innate system. This has been elegantly displayed by Mosser’s group, whereby Mφ activated by LPS are alternatively activated by Fc-γRI ligation and signalling (Gerber et al., 2001).
Chapter 8. Discussion

Rather than release IL-12, they release IL-10, and for that reason might be thought of as alternatively activated. Others have linked infectious agents which notoriously evade the immune system, such as the measles virus, with similar activation of innate immune receptors that render the Mφ de-activated in terms of pro-inflammatory cytokine release. This deactivation correlates with disease perpetuation (Atabani et al., 2001; Karp & Wills-Karp 2001; Braun et al., 2001).

These alternatively activated Mφ appear to have different functions. They express preferentially, receptors of the innate immune system such as mannose receptor, scavenger receptor type 1. They appear to suppress production of oxygen radicals and are said to predominantly metabolise arginine through the arginase pathway. They express highly, Class II molecules, but express limited chemokines. Thus they appear well equipped for anti-inflammatory responses, antigen presentation and efficient phagocytosis (see Figure 8-4) (Goerdt & Orphanos 1999). The inflammatory process involves the pro-inflammatory response with positive feedback and local destruction of tissue. However, for healing and resolution the pro-inflammatory response has to abate. It is possible that alternatively activated Mφ are an essential component of the normal healing response in any organ, in particular the kidney. Thus imbalance in the types of Mφ involved in the inflammatory response might explain excessive scarring or a failure to resolve the inflammatory process (Kitamura et al., 1997; Sime et al., 1997).

![Figure 8-4: Differentiation of monocytes to classically activated or alternatively activated Mφ](image)
8.12 APOPTOTIC CELLS AS LIGANDS AND MODULATORS OF MΦ FUNCTION

For some years, it has been clear that unlike many other particles, when apoptotic cells are phagocytosed they do not exert a pro-inflammatory response from the phagocytosing cell such as MΦ (Meagher et al., 1992). The perception has been that apoptotic cells are effete bodies that need to be removed from the inflammatory site lest they should spill their contents and provoke further inflammation. This is certainly true for apoptotic granulocytes (Stern et al., 1996). However, that view does not hold so tight when considering apoptotic resident cells. Firstly, they tend not to contain granules with potent tissue-destroying enzymes, and secondly in limited experiments (not shown) I found no evidence that post-apoptotic MC nor necrotic debris incited pro-inflammatory responses in MΦ (not shown). More recently data has indicated that binding/phagocytosis of apoptotic cells appears to alter their function. MΦ have variously been reported to show a decreased capacity to induce cytolysis of tumour cells, release TGF-β, IL-10, decreased TNF-α, IL-1β, decreased NO release and decreased chemokine production. In addition, it appears that other anti-inflammatory factors such as prostaglandins are upregulated (Voll et al., 1997; Fadok et al., 1998; Reiter et al., 1999; McDonald et al., 1999; Freire-de-lima et al., 2000). Thus it appears that apoptotic cells are not inert but actually send signals to the MΦ or non-professional phagocytosing cell, to alter the function. In the case of the MΦ this signal bears some of the hallmarks of the alternatively activated MΦ. The data presented in Chapter 7 indicate for the first time that MΦ killing MC can be switched off from further killing by ingesting apoptotic MC. This is a clear case of negative feedback in the inflammatory response; the sort of process one would expect to see in the normal inflammatory response (Figure 8.5). This new hypothesis needs to be tested in vivo. For it now provides a novel site for therapeutic intervention. Clearly, removal of angry MΦ or exogenous agents that alter their function will alter the inflammatory process (such as corticosteroids). Modulation of TNF signalling of apoptosis (or NO or H₂O₂) would alter the disease, and modulation of the binding of phagocytic cells might alter the disease. Further, it is clear now that of the increasing cell-surface ligands important in phagocytosis of apoptotic cells (see Savill and Fadok 2000) some will be important in cell signalling. Exploiting these signal pathways should reveal new disease modifying and therapeutic targets.

The data from Chapter 7 provide an unexpected observation. Both Reiter and colleagues (Reiter et al., 1999) and Freire-de-lima and colleagues (Freire-de-lima et al., 2000) show data of modest reductions in NO release (nitrite) when phagocytes ingest apoptotic cells (in the latter case they might have been necrotic). The data from Chapter 7.4 show augmented NO release by MΦ following ingestion of apoptotic cells. This contrary data requires further investigation, but it brings us back to the question of
whether NO is pro-inflammatory or anti-inflammatory. I have provided much evidence that NO in inflammation often acts to counteract pro-inflammatory events (Chapter 8.4). It might be entirely appropriate in vivo in certain situations that the alternatively activated Mϕ should have augmented NO generation.

![Diagram](image)

**Figure 8-5:** Negative feedback in killing of MC by Mϕ by ingestion of apoptotic MC

The question of failed clearance of apoptotic cells enters a new era if it is true that post apoptotic cells or necrotic cells (apart from granulocytes) are not pro-inflammatory to the Mϕ. It is possible that post apoptotic or necrotic cells fail to deactivate Mϕ, and it is possible that ingestion post apoptotic cells could lead to inappropriate presentation of self to the immune system (Gallucci et al., 1999). The C1q knockout mouse exhibits delayed or failed apoptosis and develops autoimmunity (Taylor et al., 2000). It is likely that this autoimmunity relates directly to inappropriate presentation of self to the immune system.
Chapter 8. Discussion

9.13 FUTURE WORK

The two main conclusions from this Thesis are firstly that Mφ, by a variety of mechanisms are able to bring about apoptosis of proliferating mesangial cells, and secondly that binding/ingestion of those cells induced into apoptosis serves as an off signal to the inflammatory Mφ, turning it into a reparative healing Mφ.

These observations need to be tested in vivo. The balance between classically activated and reparative Mφ should be modified and the absolute number of inflammatory Mφ should be selectively modified in glomerulonephritis to determine the effect on mesangial cells.

I believe that further investigations are warranted into the role of IRF-1 and caspases/ROI signalling of death. I feel this section of the thesis received unsatisfactory attention, since there appear to be important signalling pathways controlling MC susceptibility to death.

The roles of mTNF can be clarified further with simple in vitro experiments and there remains the question of the role of NO which can be tested with the right in vivo experiments.

Finally, a whole new avenue has been opened up by considering modifying phagocytosis of apoptotic cells. There are now knockout mice with clear defects in clearance of inflammatory apoptotic cells. These animals might well provide proof of concept and lead to manipulate strategies for control of inflammation.


Bibliography


Fadeel, B., Thorpe, C. J., Yonehara, S. and F. Chiodi. Anti-Fas IgG1 antibodies recognizing the same epitope of Fas/APO-1 mediate different biological effects in vitro. *Int Immunol* *9*: 201-9, 1997


Bibliography


Gresham, H. D., J. A. McGarr, P. G. Shackelford, and E. J. Brown. Studies on the molecular mechanisms of human Fc receptor-mediated phagocytosis. Amplification of ingestion is dependent on


STANDARD SOLUTIONS

Phosphate buffered saline

0.2g KCl
8.0g NaCl
0.2g NaH₂PO₄
1.15g Na₂HPO₄
Add components one at a time to 900ml dH₂O. Adjust pH to 7.4. Make up to 1.0 litre

Dulbecco’s phosphate buffered saline

0.2g KCl
0.2g KH₂PO₄
0.132g CaCl₂.2H₂O
0.1g MgCl₂.6H₂O
1.15g Na₂HPO₄
8.0g NaCl
Add components one at a time to 900ml dH₂O. Adjust pH to 7.4. Make up to 1.0 litre

Tris-buffered saline

Dissolve 8g of NaCl, 0.2g of KCl, and 3g of Tris base in 800ml of distilled H₂O. Add 0.015g of phenol red (optional). Adjust the pH to 7.4. Make up to 1 litre

1x Trypsin EDTA (supplied by Life Technologies)

0.05% (w/v) trypsin
0.53mM EDTA
made up in Ca²⁺, Mg²⁺ free solution (Hanks w/o)

DMEM/F12 (1 litre)

116.6mg CaCl₂
3151mg D-glucose
Appendix 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0013mg</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$.9H$_2$O</td>
<td>0.05mg</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.417mg</td>
</tr>
<tr>
<td>KCl</td>
<td>311.8mg</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>61mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>6996mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1200mg</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>134mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.432mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>62.5mg</td>
</tr>
</tbody>
</table>

3075.4mg HEPES
2.39mg Na hypoxanthine
0.042mg Linoleic acid
0.105mg DL-68 Thiocitic acid
8.1mg Phenol red
0.081mg Sodium putrescine.2HCl
55mg sodium pyruvate

0.0035mg Biotin
2.24mg D-Ca pantothenate
8.98mg Choline chloride
2.65mg Folic acid
12.6mg I-nositol
2.02mg Nicotinamide
2.031mg Pyridoxine HCl
0.219mg Riboflavin
2.17mg Thiamine HCl
0.365mg Thymidine
0.68mg Vitamin B12
### Appendix 1

**RPMI (1 litre)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mg Ca(NO₃)₂.4H₂O</td>
<td></td>
</tr>
<tr>
<td>400mg KCl</td>
<td></td>
</tr>
<tr>
<td>100mg MgSO₄.7H₂O</td>
<td></td>
</tr>
<tr>
<td>6.0g NaCl</td>
<td></td>
</tr>
<tr>
<td>2.0g NaHCO₃</td>
<td></td>
</tr>
<tr>
<td>800mg Na₂HPO₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>240mg L-arginine.HCl</td>
<td></td>
</tr>
<tr>
<td>50mg L-asparagine</td>
<td></td>
</tr>
<tr>
<td>20mg L-aspartic acid</td>
<td></td>
</tr>
<tr>
<td>50mg L-cystine</td>
<td></td>
</tr>
<tr>
<td>20mg L-glutamic acid</td>
<td></td>
</tr>
<tr>
<td>300mg L-glutamine</td>
<td></td>
</tr>
<tr>
<td>10mg Glycine</td>
<td></td>
</tr>
<tr>
<td>15mg L-Histidine</td>
<td></td>
</tr>
<tr>
<td>50mg L-Isoleucine</td>
<td></td>
</tr>
<tr>
<td>50mg L-leucine</td>
<td></td>
</tr>
<tr>
<td>40mg L-Lysine HCl</td>
<td></td>
</tr>
<tr>
<td>15mg L-methionine</td>
<td></td>
</tr>
<tr>
<td>15mg L-phenylalanine</td>
<td></td>
</tr>
<tr>
<td>20mg L-proline</td>
<td></td>
</tr>
<tr>
<td>30mg L-serine</td>
<td></td>
</tr>
<tr>
<td>20mg L-threonine</td>
<td></td>
</tr>
<tr>
<td>5mg L-tryptophan</td>
<td></td>
</tr>
<tr>
<td>20mg L-tyrosine</td>
<td></td>
</tr>
<tr>
<td>20mg L-Valine</td>
<td></td>
</tr>
<tr>
<td>20mg L-hydroxyproline</td>
<td></td>
</tr>
<tr>
<td>2.0g D-glucose</td>
<td></td>
</tr>
<tr>
<td>1.0mg glutathione (reduced)</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td></td>
</tr>
<tr>
<td>5mg Phenol red</td>
<td></td>
</tr>
<tr>
<td>0.3mg D-Ca pantothenate</td>
<td></td>
</tr>
<tr>
<td>3.0mg Choline chloride</td>
<td></td>
</tr>
<tr>
<td>1.0mg Folic acid</td>
<td></td>
</tr>
<tr>
<td>35mg L-inositol</td>
<td></td>
</tr>
<tr>
<td>1.0mg Pyridoxal HCl</td>
<td></td>
</tr>
<tr>
<td>0.2mg Riboflavin</td>
<td></td>
</tr>
<tr>
<td>1.0mg Thiamine HCl</td>
<td></td>
</tr>
<tr>
<td>0.2mg Biotin</td>
<td></td>
</tr>
<tr>
<td>0.005mg Vitamin B12</td>
<td></td>
</tr>
<tr>
<td>1.0mg para-aminobenzoic acid</td>
<td></td>
</tr>
<tr>
<td>1.0mg nicotinamide</td>
<td></td>
</tr>
</tbody>
</table>
Hanks balanced salt solution

0.185g CaCl₂H₂O
0.4g KCl
0.06g KH₂PO₄
0.1g MgCl₂.6H₂O
0.1g MgSO₄.7H₂O
8.0g NaCl
0.048g Na₂HPO₄
0.35g NaHCO₃
1.0g D-glucose
0.01g phenol red
Make up to 1.0litre

Hanks balanced salt solution without Ca²⁺ and Mg²⁺

0.4g KCl
0.06g KH₂PO₄
8.0g NaCl
0.048g Na₂HPO₄
0.35g NaHCO₃
1.0g D-glucose
0.01g phenol red
Make up to 1.0litre

Paris buffer

20mM Tris HCl 2.42g
125mM NaCl 7.31g
10mM KCl 0.74g
10mM Na acetate 0.82g
5mM glucose 0.9g
Make up to 900ml with dH₂O
PH to 7.4 with 0.1M HCl, then make up to 1000ml
Appendix 1

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Lysis buffers

1) For mesangial cells

10mM HEPES pH 7.9
1.5mM MgCl₂
10mM KCl
50μM DTT
100μM phenanthroline
1μg/ml pepstatin
100μM 1-trans-epoxysuccinyl-leucylamide (4-guanido)butane
100μM 3,4-dichloroisocoumarin
10mM sodium fluoride
100μM sodium orthovanadate
25mM β-glycerophosphate

2) For macrophages

10mM HEPES pH 7.4
1mM EDTA
1.57μg/ml Benzamidine HCl
0.5μg/ml leupeptin
6.9μg/ml pepstatin A
1mM ortho-phenantholine
4.3μg/ml leupeptin
1.8mM PMSF

Sealing gel

500μl 30%/0.5% acrylamide solution
2.0ml dH₂O
Appendix 1

10μl TEMED
20μl 10% APS

4 x running buffer

36.3g Tris base
Add 150ml ddH₂O
Adjust to pH 8.0 with HCl
ddH₂O make up to 200ml
Store 4°C in the dark (3months)

4 x stacking buffer

3.0g Tris base
Add 40ml ddH₂O
Adjust to pH 6.8 with HCl
Store 4°C in the dark (3months)

Laemmli buffer

1.0ml 8M urea
250μl 0.5M Tris pH 6.8
200μl glycerol
400μl 10% SDS
100μl 2-mercaptoethanol (can be substituted)
50μl ddH₂O
Trace of bromophenol blue

Top tank buffer

57.6g glycine
12.0g Tris-HCl
2.0g SDS
Appendix 1

made up to 2 litres with dH₂O

**Bottom tank buffer**

28.8g glycine  
6g Tris-HCl  
2.0g SDS  
made up to 2 litres with dH₂O

**Transfer buffer**

3g Tris-HCl  
14.4g glycine  
1.0g SDS  
100ml methanol  
made up to 1 litre with dH₂O (pH should be 8.2-8.4)

**Coomassie brilliant blue stain**

2.5g Brilliant blue G  
1250ml Methanol  
750ml H₂O  
500ml acetic acid

**Destain**

50ml Formic acid  
1500ml methanol  
make up to 5 litres with water
Appendix 1

Ponceau S

0.1% (w/v) Ponceau S in 5% acetic acid
1.0g Ponceau S
50ml acetic acid
Make up to 1litre with ddH₂O
Table M1

<table>
<thead>
<tr>
<th></th>
<th>5%</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer sol⁹</td>
<td>10ml</td>
<td>15ml</td>
<td>20ml</td>
<td>25ml</td>
<td>30ml</td>
</tr>
<tr>
<td>4 x running buffer</td>
<td>15ml</td>
<td>15ml</td>
<td>15ml</td>
<td>15ml</td>
<td>15ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>600μl</td>
<td>600μl</td>
<td>600μl</td>
<td>600μl</td>
<td>600μl</td>
</tr>
<tr>
<td>dd H₂O</td>
<td>34.1ml</td>
<td>29.1ml</td>
<td>24.1ml</td>
<td>19.1ml</td>
<td>14.1ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>300μl</td>
<td>300μl</td>
<td>300μl</td>
<td>300μl</td>
<td>300μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
</tbody>
</table>

Stacking gel 4% acrylamide

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer sol⁹</td>
<td>2.66ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x stacking buffer</td>
<td>5ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>200μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dd H₂O</td>
<td>12.0ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% APS</td>
<td>100μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>10μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ELECTROMOBILITY SHIFT ASSAY

Buffer A

<table>
<thead>
<tr>
<th>Final conc.</th>
<th>Stock conc.</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM HEPES pH 7.9</td>
<td>1M</td>
<td>100μl</td>
</tr>
<tr>
<td>1.5mM MgCl₂</td>
<td>1M</td>
<td>15μl</td>
</tr>
<tr>
<td>10mM KCl</td>
<td>1M</td>
<td>100μl</td>
</tr>
<tr>
<td>50μM DTT</td>
<td>1M</td>
<td>5μl</td>
</tr>
<tr>
<td>100μM phenanthroline</td>
<td>10mM</td>
<td>100μl</td>
</tr>
<tr>
<td>1μg/ml pepstatin</td>
<td>1mg/ml</td>
<td>10μl</td>
</tr>
<tr>
<td>100μM 1-trans-epoxysuccinyl-leucylamide</td>
<td>5mM</td>
<td>200μl</td>
</tr>
<tr>
<td>(4-guanido)butane (antipain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100μM 3,4-dichloroisocoumarin (DCI)</td>
<td>30mM</td>
<td>33μl</td>
</tr>
<tr>
<td>10mM sodium fluoride</td>
<td>0.5M</td>
<td>200μl</td>
</tr>
<tr>
<td>100μM sodium orthovanadate</td>
<td>5mM</td>
<td>200μl</td>
</tr>
<tr>
<td>25mM β-glycerophosphate</td>
<td>250mM</td>
<td>1000μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>8037μl</td>
</tr>
</tbody>
</table>

296
Appendix 1

Buffer C

<table>
<thead>
<tr>
<th></th>
<th>Final conc.</th>
<th>Stock conc.</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM Hepes pH 7.9</td>
<td>1M</td>
<td></td>
<td>200μl</td>
</tr>
<tr>
<td>420mM NaCl</td>
<td>5M</td>
<td></td>
<td>840μl</td>
</tr>
<tr>
<td>1.5mM MgCl₂</td>
<td>1M</td>
<td></td>
<td>15μl</td>
</tr>
<tr>
<td>0.2mM EDTA</td>
<td>0.5M</td>
<td></td>
<td>2μl</td>
</tr>
<tr>
<td>25% v/v glycerol</td>
<td>100%</td>
<td></td>
<td>2500μl</td>
</tr>
<tr>
<td>100μM DCI</td>
<td>30mM</td>
<td></td>
<td>33μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td></td>
<td>6408μl</td>
</tr>
</tbody>
</table>

Tris-EDTA (TE)

10mM Tris-HCl (500μl 1M Tris pH 7.4)
1mM EDTA (100μl 0.5M EDTA)

pH 7.4
make up to 50ml with ddH₂O

Table M2

<table>
<thead>
<tr>
<th></th>
<th>5%</th>
<th>6%</th>
<th>7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%/0.5% acrylamide solution</td>
<td>8.33ml</td>
<td>10.0ml</td>
<td>11.67ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>38.8ml</td>
<td>37.1ml</td>
<td>35.5ml</td>
</tr>
<tr>
<td>5x TBE</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>320μl</td>
<td>320μl</td>
<td>320μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>40μl</td>
<td>40μl</td>
<td>40μl</td>
</tr>
</tbody>
</table>

NITRIC OXIDE ASSAYS

Griess Reagent

2% Sulfanilic acid solution:

5% orthophosphoric acid was prepared from 85% orthophosphoric acid (< 0.0005% nitrite) and distilled water. 470mls of ddH₂O was added to 30mls of orthophosphoric acid stock. To this were dissolved 10g of sulfanilamide. The solution was stored at 4°C.
Appendix 1

0.2% N-(1-naphthyl)ethelendiamide (NED) solution:
1g of NED was dissolved in 500ml of distilled water and stored at 4°C in the dark.

Nitrate reductase

Phosphate buffer
1 litre of ddH₂O
8.00g sodium chloride
0.20g Potassium chloride
0.20g Potassium dihydrogen orthophosphate (KH₂PO₄)
1.15g Disodium hydrogen orthophosphate (Na₂HPO₄)
pH 7.4, filter sterilised

Nitrate reductase and glucose-6-phosphate dehydrogenase were dissolved in 50% glycerol 50% phosphate buffer and stored at –20°C. NADPH was dissolved in phosphate buffer and stored at –20°C.

Enzyme mastermix

Phosphate buffer 1ml
Nitrate reductase (59U/ml) 10µl
Glucose-6-phosphate dehydrogenase (125U/ml) 10µl
Glucose-6-phosphate 1.14mg
NADPH 10mM (10µl)

MOLECULAR BIOLOGY

LB broth

10g NaCl
10g bactotryptone
5g yeast extract
Make up to 1 litre with dH₂O
pH 7.5
Autoclave
Appendix 1

**LB agar**

10g NaCl
10g bactotryptone
5g yeast extract
15g LB agar
Make up to 1 litre with dH₂O
pH 7.5
Autoclave

**Terrific broth**

12g bactotryptone
24g bacto-yeast extract
4ml glycerol
900ml dH₂O
Autoclave
Cool to 55°C
100mls of buffer

**SOB medium**

20g bactotryptone
5g yeast SOB
0.5g NaCl
10ml 250mM KCl
5ml 2M MgCl₂
10ml 2M glucose
Make upto 1000mls. Autoclave.

**Antibiotic concentrations for agar plates and broths**

Final concentration for agar plates:
Ampicillin 50-100µg/ml (used 60µg/ml)
Appendix 1

Kanamycin 30µg/ml

Stock solutions for broths:
Ampicillin 100mg/ml in dH₂O (sterile filtered)
Kanamycin sulphate 30mg/ml in dH₂O (sterile filtered)

STET solution

4g Sucrose
1.25ml Tris-HCl 2M pH 8.0
5ml EDTA 0.5M
2.5ml Triton-X 100
made up to 50ml with dH₂O
Stored at RT

Transformation buffer

3.0g PIPES
2.0g CaCl₂. 2H₂O
18.6g KCl

Dissolve into 950ml of H₂O. Adjust pH to 6.7
Add 10.9g of MnCl₂.4H₂O, then make up to 1000ml with H₂O. Filter with 0.22μm membrane. Store at 4°C.

10x Tris borate EDTA (TBE)

107.8g Tris base
55g boric acid
7.44g disodium EDTA . 2H₂O

Add components in order to 800ml of dH₂O. Use slightly less than full amount of boric acid. Check pH and adjust to 8.3 with boric acid. Bring up final volume to 1litre
DEPC treated water

Diethyl pyrocarbonate (DEPC) 0.01% in dH2O (note carcinogenic)
Leave overnight at RT
Autoclave for 20min

Gel loading buffer

250mM Tris-HCl pH 7.5
0.2% bromophenol blue
0.2% xylene cyanol
40% glycerol

Plasmid mega (midi) prep

Glucose buffer

1.25ml 2M glucose
1.25ml 1M Tris pH 8.0
1ml 0.5M EDTA
make up to 50ml with dH2O

Lysis buffer

5ml 2N NaOH
5ml 10% SDS
make up to 50ml with dH2O

High salt buffer

150ml 5M potassium acetate
28.75ml acetic acid
make up to 250ml with dH2O
Lithium Chloride solution

53g LiCl
2.6g MOPS
200ml dH₂O adjust pH to 8.0
make up to 250ml

RNAse stock solution

100mg pancreatic RNAse A (bovine pancreas)
10ml T₁₀S₁₅ (pH 7.5)
Heat 100°C for 15min. Cool slowly to RT.
Store at −20°C

HISTOLOGY/CYTOLOGY

Methyl Carnoy’s fixative

60ml methanol
30ml chloroform
10ml glacial acetic acid
use within 72h

Apoptotic neutrophil stain

15ml PBS pH 6.2
15ml Hanks w/o
300μl DMB (o-dianisidine (Sigma)1.25mg/ml in dH₂O. Aliquots stored at −20°C)
2μl H₂O₂ (30% solution)
All tissue culture plastic flasks were from Costar (Cambridge MA)

All tissue culture plates for experiments were from Becton Dickinson (Falcon; Franklin Lakes NJ)

Tissue culture reagents were from Life Technologies (Paisley, Scotland)

Cytokines were from R & D systems unless stated otherwise

All enzymes used in molecular biology were from Promega (Madison WI) unless stated otherwise

Oligonucleotides were from MWG-Biotech AG

All other reagents were from Sigma-Aldrich unless stated otherwise

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
</tr>
<tr>
<td>CH11 anti-Fas (human)</td>
<td>Upstate biotechnology</td>
</tr>
<tr>
<td>ZB4 anti-Fas (human)</td>
<td>Upstate biotechnology</td>
</tr>
<tr>
<td>F22120 Anti Fas (for rat)</td>
<td>Transduction Labs</td>
</tr>
<tr>
<td>Anti Fas (for rat)</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Ab-1 anti Fas (for rat)</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>4H9 Anti FasL</td>
<td>MBL</td>
</tr>
<tr>
<td>Clone 33 anti FasL</td>
<td>Transduction Labs</td>
</tr>
<tr>
<td>C76920 anti caspase 3</td>
<td>Transduction Labs</td>
</tr>
<tr>
<td>Anti iNOS-haem</td>
<td>Gift from Prof J Pfeilschifter</td>
</tr>
<tr>
<td>C-20 anti IRF-1</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>C-19 anti IRF-2</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti P65</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti P50</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti c-rel</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti rel B</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti rat CD90 (Thy-1.1)</td>
<td>Serotec</td>
</tr>
<tr>
<td>Anti ED-1</td>
<td>Serotec</td>
</tr>
<tr>
<td>Anti F4-80</td>
<td>Serotec</td>
</tr>
</tbody>
</table>
## Appendix 2

### Soluble receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFR1-Fc</td>
<td>Gift from Prof C. Ware</td>
</tr>
<tr>
<td>LTβR-Fc</td>
<td>Gift from Prof C. Ware</td>
</tr>
<tr>
<td>HVEM-Fc</td>
<td>Gift from Prof C. Ware</td>
</tr>
</tbody>
</table>

### Cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IFN-γ</td>
<td>Peprotech</td>
</tr>
<tr>
<td>Human TNF-β</td>
<td>Peprotech</td>
</tr>
<tr>
<td>Soluble recombinant human Fas ligand</td>
<td>Calbiochem (oncogene research products)</td>
</tr>
</tbody>
</table>

### Miscellaneous

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynabeads M450 sheep anti-mouse IgG</td>
<td>Dynal</td>
</tr>
<tr>
<td>Latex beads</td>
<td>Polysciences UK</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Superscript II</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Ase 1</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>Sea 1</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Nhe 1</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>L-NIL</td>
<td>Gift from Dr. V. Cattell</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>D-NMMA</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>
Activated Macrophages Direct Apoptosis and Suppress Mitosis of Mesangial Cells

Jeremy S. Duffield,† Lars-Peter Erwig,† Xiao-quing Wei,‡ Foo Y. Liew,‡ Andrew J. Rees,‡ and John S. Savill*†

During inflammation in the glomerulus, the complement of resident myofibroblast-like mesangial cells is regulated by mitosis and apoptosis, but the cellular mechanisms controlling the size of mesangial cell populations have remained obscure. Prompted by studies of development, we sought evidence that macrophages regulate mesangial cell number. Rat bone marrow-derived macrophages primed with IFN-γ then further activated in coculture with LPS or TNF-α elicited a 10-fold induction of rat mesangial cell apoptosis and complete suppression of mitosis, effects inhibitable by the NO synthase inhibitors L-monomethyl arginine and L-N^4-(1-iminoethyl) lysine dihydrochloride. Complete dependence upon macrophage-derived NO was observed in comparable experiments employing activated bone marrow macrophages from wild-type and NO synthase 2^−/− mice. Nevertheless, when mesangial cells were primed with IFN-γ plus TNF-α, increased induction by activated macrophages of mesangial apoptosis exhibited a NO-independent element. The use of gld/gld macrophages excluded a role for Fas ligand in this residual kill, despite increased expression of Fas and increased susceptibility to soluble Fas ligand exhibited by cytokine-primed mesangial cells. Finally, activated macrophages isolated from the glomeruli of rats with nephrotoxic nephritis also induced apoptosis and suppressed mitosis in mesangial cells by an L-monomethyl arginine-inhibitable mechanism. These data demonstrate that activated macrophages, via the release of NO and other mediators, regulate mesangial cell populations in vitro and may therefore control the mesangial cell complement at inflamed sites. The Journal of Immunology, 2000, 164: 2110-2119.

Remodeling of tissues occurs during successful resolution of the inflammatory response induced by tissue injury. Until recently, the mechanisms involved have been obscure. Although accumulation of cells with myofibroblast features is a familiar feature of the response to injury by many tissues, it is only recently that apoptosis (programmed cell death) has been identified as the major mechanism by which excess myofibroblast-like resident cells are eliminated during successful repair of inflammatory injury. We established this concept for myofibroblast-like mesangial cells (MC) in the glomerulus of self-limited proliferative glomerulonephritis induced by anti-MC Ab (Thy-1.1) (1). Subsequent reports have confirmed a comparable remodeling role for myofibroblast apoptosis in the healing of skin wounds (2) and the resolution of hepatic inflammatory injury (3).

However, little is understood of the cellular control mechanisms that regulate apoptosis of myofibroblast-like resident cells during resolution of inflammation. Given that repair of tissue injury may reiterate processes involved in tissue development, we have become interested in the possibility that similar cellular mechanisms might regulate remodeling by apoptosis in both developmental and inflammatory contexts. Seminal work by Lang and colleagues (4) has demonstrated a key role for macrophage (Mφ)-directed apoptosis in the elimination of unwanted capillaries in neonatal development of the mouse and rat eye (5). Mφ dock on to the microvascular endothelial cells and trigger their apoptosis in this model of tissue remodeling, but the cell-killing mechanisms deployed are as yet unclear. The probable relevance of this observation to regulation of cell populations at inflamed sites has been underscored recently by Tesch and colleagues (6), who, using a nephrotoxic serum model of glomerulonephritis, demonstrated decreased resident tubular cell apoptosis in the monocyte chemotactant protein-1 (MCP-1) knockout animal compared with wild type. During tubular inflammation, there was an MCP-1-directed influx of Mφ to the interstitium. In the knockout animal, the influx of Mφ was reduced. It appears, therefore, that Mφ in this model are inducing tubular cell apoptosis. The killing mechanisms in both models, however, remain to be defined, although in the tubular injury model, in vitro experiments suggested that Mφ can release soluble factors capable of triggering tubular cell death.

Furthermore, there have been indications that Mφ might have the capacity to direct apoptosis of glomerular MC, which assume a myofibroblast-like phenotype in situations in which they threaten to promote the persistence of glomerular inflammation by secretion of cytokines, or progression to scarring by excess deposition of abnormal extracellular matrix. First, Mone and colleagues (7) showed that U937 promonocytic cells were able to induce cytolysis of cultured MC, although it was not determined whether this represented apoptosis or necrosis. Second, Hruby et al. (8) reported

---

*Centre for Inflammation Research, Department of Clinical and Surgical Sciences (Internal Medicine), Royal Infirmary, University of Edinburgh, Edinburgh, United Kingdom; †Department of Immunology, University of Glasgow, Glasgow, United Kingdom; ‡Department of Medicine and Therapeutics, University of Aberdeen, Foresterhill, Aberdeen, United Kingdom

Received for publication May 24, 1999. Accepted for publication December 3, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work is supported by the award to J.S.D. of a clinical training fellowship from the U.K. Medical Research Council, and also grants from the Wellcome Trust (031558, 039108, 047273).

Address correspondence and reprint requests to Dr. J. S. Duffield, Centre for Inflammation Research, Department of Clinical and Surgical Sciences (Internal Medicine), Royal Infirmary, University of Edinburgh, Edinburgh, EH3 9YW, U.K. E-mail address: jduffield@ed.ac.uk

Abbreviations used in this paper: MC, mesangial cell; BMD, bone marrow-derived; pMMMA, p-monomethyl arginine; FasL, Fas ligand; gld, generalized lymphoproliferative disease; LNIL, L-N^4-(1-iminoethyl) lysine dihydrochloride; LNMMMA, L-monomethyl arginine; Mφ, macrophage; MCP, monocyte chemotactant protein; NOS, NO synthase; PCNA, proliferating cell nuclear antigen; PI, propidium iodide.

Copyright © 2000 by The American Association of Immunologists
similar findings in cocultures of rat peritoneal Mφ and MC, implicating NO in the phenomenon by specific inhibition of MC disintegration with L-NMMA. Further evidence that Mφ might have the capacity to induce apoptosis in neighboring cells by the release of NO comes from coculture studies indicating that cytokine-activated peritoneal Mφ can trigger apoptosis in proliferating breast tumor cells by a mechanism inhibitable by L-NMMA (9, 10). However, in no preceding study has there been a direct demonstration that Mφ can trigger apoptosis in glomerular MC.

In this study, we have tested the hypothesis that Mφ activated to express inducible NO synthase (also known as NOS-2) can trigger apoptosis in cultured MC, which are well known to reiterate the myofibroblast features displayed in the injured glomerulus in vivo. We report that in contrast to quiescent cells, Mφ activated in vitro or isolated from inflamed glomeruli not only inhibited MC mitosis, but did induce apoptosis in MC. Cytokine stimulation of MC was able to increase the kill. Furthermore, use of specific NOS-2 inhibitors and NOS-2 knockout Mφ clearly implicated Mφ-derived NO as a key mediator inducing apoptosis and inhibiting mitosis in glomerular cells. The data establish a new facet to the multifunctional role of the Mφ in inflammation: the capacity to directly remodel by coordinate induction of apoptosis and inhibition of mitosis in resident tissue cells.

Materials and Methods

Materials

Tissue culture reagents were from Life Technologies (Paisley, Scotland), and tissue culture plastics from Costar (Cambridge, MA). Experimental culture wells were from Becton Dickinson (Falcon; Franklin Lakes, NJ). Cytokines were from Genzyme (Cambridge, MA), and all other reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Preparation of BMD Mφ

Femurs and tibias were removed from 200- to 250-g rats (Wistar). Bone marrow was isolated from these by standard sterile techniques (11) and matured for 7 days in Teflon wells in using DMEM/F12 medium with 10% FCS, penicillin (100 U/ml), and streptomycin (100 mg/ml), conditioned with M-CSF from L929 cells. In some experiments, BMD Mφ were derived from knockout NOS-2 mice (129sv-NOS-2−/−) (12) and wild-type controls (8 wk). In others, BMD Mφ were obtained from mice bearing an inactivating point mutation of Fasl (C3H/HeJ-hldgl/gld) and wild-type controls (8 wk). BMD Mφ were purchased from The Jackson Laboratory (Bar Harbor, ME).

Establishment of MC cultures

MC were derived by clonal selection from glomerular single cell preparation (gift of Dr. M. Kitamura, Department of Medicine, University College, London, U.K.) and from primary outgrowths from isolated whole glomeruli (13). MC were verified, and purity established, as described (1, 13, 14). Cells were used from passages 4 to 12. Primary cultures were maintained in DMEM/F12 medium with 16% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Clonal cultures were maintained in the same medium with 10% FCS. MC were maintained in the logarithmic growth phase.

Mφ coculture with MC

Matured (7–10 day) Mφ were plated in 96-well plates initially at a density to cover 60–70% of the well surface; typically, this required 2 × 104 cells. MC were prelabelled with CellTracker Green CMFDA (Molecular Probes, Eugene, OR): cell cultures, 70–80% confluent, were washed with medium lacking serum and then incubated for 1 h in serum-free medium containing CMFDA at 5 ng/ml. Cells were washed in medium containing 10% FCS to remove any unbound CMFDA, then trypsinized and added to cultured Mφ in 1:1 Mφ:MC ratio. Experiments were conducted in DMEM/F12 medium containing 10% FCS. Once cells had become adherent, typically 2–4 h, wells were washed to remove nonadherent cells. Preliminary experiments mixing unlabeled and labeled cells showed no evidence of transfer of CMFDA from labeled to unlabeled cells (data not shown).

Activation protocols

When desired, Mφ were primed for 12 h with IFN-γ (100 U/ml) before coculture. After coculture was established, Mφ were activated using either IFN-γ (100 U/ml) plus LPS (1 μg/ml), or IFN-γ (100 U/ml) plus TNF-α (100 U/ml). Unactivated or quiescent cells were exposed to carriage medium only (PBS, 0.1% BSA).

In some experiments, MC were primed in flasks for 36 h with a combination of IFN-γ (300 U/ml) plus TNF-α (300 U/ml). Cells were washed thoroughly and trypanized before establishing coculture. Macrophages were grown in flasks containing carriage medium only (PBS, 0.1% BSA).

Assessment of apoptosis and mitosis

Morphological criteria. At the end of the experiment, wells were fixed by adding formaldehyde (4% final concentration), stored at 4°C for 48 h to ensure firm adherence of the fixed apoptotic cells to the monolayer. Subsequently, medium was removed and propidium iodide (PI) in PBS (5 μg/ml) was added for 5 min to stain both Mφ and MC. This reagent was then discarded and wells were covered with a fluorescent mountant. Using inverted fluorescent microscopy, five fields were randomly and blindly selected from each well so that at least 300 MC were counted in each well. Wells were counted nonconsecutively. MC labeled with CellTracker Green CMFDA were discernible from Mφ by their green fluorescence (Mφ appeared red due to PI binding to RNA as well as DNA). Apoptotic cells were clearly distinguishable by characteristic morphology (cytoplasmic blebbing, cell shrinkage, nuclear condensation, and fragmentation); in our previous studies of MC apoptosis (1, 14), these morphological criteria have been extensively validated against quantitative and qualitative measures of apoptosis. Mitotic cells were easily discernible by characteristic morphology and shape change.

Functional criteria. MC apoptosis was also assayed in live cells by addition of Hoechst 33342 (bisbenzimide dye) to wells at a final concentration of 5 μg/ml. This dye is actively excreted by live, but not apoptotic cells (15). After 15 min at 37°C, wells were counted as described above for apoptotic cells. PI was also added to live wells (1 μg/ml) to exclude necrotic cells (see Fig. 1). Furthermore, 99.8% of cells in the live coculture were able to exclude trypan blue assessed, as previously described (10).

Flow-cytometric assay. Coculture was performed as described above in a 1 MC:1.5 Mφ ratio in six-well plates (Becton Dickinson). After 24 h, the coculture or controls were washed, followed by the application of 1× trypan-blue-EDTA. All washings and eluted cells were collected, and after centrifugation (300 × g), they were permeabilized with 70% ethanol at 4°C overnight. After washing with PBS, cells were treated with RNase (100 μg/ml) and PI (40 μg/ml) for a minimum of 30 min. Cells were then detected by forward and side scatter, then assessed by excitation at 488 nm and measuring fluorescence at 639 nm using FACSCalibur (Becton Dickinson). The characteristic distribution of DNA content enabled accurate assessment of diploid, G1/M phase, and hypodiploid populations (14).

Immunohistochemistry. Further assessment of mitosis was made by specific identification of proliferating cell nuclear Ag (PCNA) in MC using PC10 Ab (Dako, Carpinteria, CA) in fixed wells. Briefly, following quenching of endogenous peroxidases with 3% hydrogen peroxide in methanol (5 min, 20°C) and permeabilization with 0.1% Triton X-100, 0.1% sodium citrate in PBS (5 min, 20°C), the primary Ab was applied 1:50 in PBS, 10% NBCS (4 h, 20°C). Specific localization was assayed by biotinylated anti-lg and streptavidin-peroxidase using the substrate AEC (Vector). MC were distinguished by the subsequent labeling with FITC-conjugated α smooth muscle actin Ab.

All experimental conditions were prepared in triplicate. All experiments and conditions were observer blinded. Experiments were performed on at least three separate occasions. Mφ and primary MC cultures were derived from at least three different animals.

Mφ-conditioned supernatant transfer

A total of 106 mature BMD Mφ was seeded to six-well plates with medium containing 10% FCS. Cells were activated with IFN-γ and LPS, as described in activation protocols. After 16 h, supernatant was removed and clarified at room temperature by centrifugation at 350 × g for 5 min, followed by centrifugation at 10,000 × g for 3 min. Two hundred microliters of supernatant were transferred directly to MC established 24 h previously (covering 70–80% of the well surface) in 96-well plates. After 8, 16, and 24 h, apoptosis, mitosis, and cell number were assayed morphologically by PI staining of fixed monolayers.
Isolation of single cells from glomeruli

The number of cells isolated was confirmed by ED-1 immunostaining. Isolation of resident Mø from control kidneys (normal histology) by the same method required pooling of the single cell suspension from all control animals due to the scarcity of this cell type (<5% of glomerular cells).

Western blot analysis

Cytosolic extracts from cells were prepared using a hypotonic lysis buffer (10 μM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 50 μM DTT, 100 μM phenanthroline, 1 μM pepstatin, 100 μM 1-trans-epoxyisuccinyl-

Induction of accelerated autologous phase of nephrotropic nephritis

Nephritis was induced using a standardized protocol (17). Briefly, male Sprague Dawley rats (weight 180–200 g) were immunized by injection of 1 mg normal rabbit IgG, in Freund’s complete adjuvant (Difco, Detroit, MI). After 7 days, the rats were injected with 1 ml of nephrotropic serum i.v. They were sacrificed, and kidneys were prepared as below. Biopsies were taken for histology, which confirmed severe proliferative glomerulonephritis (18). Urine was collected in metabolic cages for 18 h, and albuminuria was confirmed by rocket immunoelectrophoresis.

Isolation of single cells from glomeruli

Glomeruli were isolated using a standard sieving technique. Isolated glomeruli were enzymatically digested to single cell suspensions using trypsin, collagenase, DNase, and EDTA, as described (19, 20). Mø were isolated by adherence in 24-well plates. Aliquots of the single suspension containing 1 × 10^5 Mø were added to wells in full medium with serum. After 2 h, wells were thoroughly washed to leave adherent Mø only. Purity

Effects of Mø on the proportion of cells exhibiting DNA content of G2/M-phase of the cell cycle or hypodiploid DNA content

<table>
<thead>
<tr>
<th>Condition</th>
<th>Unstimulated</th>
<th>Activated</th>
<th>Activated + 1-nIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% G2/M-phase cells</td>
<td>18.1 ± 1.5</td>
<td>10.4 ± 1.4*</td>
<td>20.9 ± 1.5</td>
</tr>
<tr>
<td>% Hypodiploidy</td>
<td>0.6 ± 0.3</td>
<td>6.2 ± 2.2*</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

* Cells were prepared and analysed according to Materials and Methods with 1.0 MC1: 1.5 Mø. Mø were primed with IFN-γ (100 U/ml), and once coculture was established, activated with IFN-γ and LPS (1 μg/ml). Note data shown refer to whole coculture. MC controls were activated with the same agents and showed 19.2 ± 0.7% in G/M, 0.3 ± 0.1% hypodiploidy. Activated Mø alone demonstrated 0.3 ± 0.1% hypodiploidy. No Mø had increased DNA content.

p < 0.05 compared with unstimulated coculture (n = 3).

Results

Activated but not quiescent Mø coordinately inhibit mitosis and stimulate apoptosis in MC

Mature rat BMD Mø were cocultured with rat MC in the presence of medium containing 10% FCS. Prelabelling of MC with Cell-Tracker Green CMFDA, and staining of fixed mononuclears with PI enabled selective assessment of MC. Extensive work in our laboratory has shown that morphological assays of apoptosis on the basis of typical cell shrinkage and nuclear condensation have advantages over other techniques, with which this approach has been compared and validated (1, 14). First, because apoptotic MC remain intact and closely associated with the mononuclear, it is possible to assay definitively cell death by apoptosis without disturbing the culture and risking underestimation of apoptosis due to cell damage or loss, which we have found to be an inherent problem with certain assays of apoptosis. Second, microscopic assessment of the cell culture allows simultaneous detection of cells in mitosis. Nevertheless, to verify the data, we included live cells in coculture before fixation with Hoechst 33342 and counted cells, admitting the dye so that nuclei were stained; this fluorescent dye is excluded by the plasma membrane of healthy but not apoptotic cells (14). Interestingly, all stained cells demonstrated typical condensed apoptotic nuclei, indicating synchronicity of plasma membrane and nuclear changes of apoptosis in this cell type. No significant difference between the proportion of cells admitting Hoechst 33342 and those demonstrating nuclear condensation on PI staining after fixation of coculture was seen in activated (in one series at 24 h, there were 35.3 ± 6.2 Hoechst-positive cells per field compared with 31.5 ± 9 apoptotic MC after fixation and counterstaining (representing 16.1 ± 2.6% MC per field), n = 9, p = NS) or unstimulated coculture. To verify further our observations, PI was used to label DNA in fixed cells from the coculture and assess the proportion of cells in G2/M phase or with hypodiploid content of DNA characteristic of apoptosis by flow cytometry (Table I). Furthermore, confirmatory evidence of suppression of mitosis was obtained by immunohistochemistry of fixed coculture for PCNA; the percentage of PCNA-positive MC after 24 h in quiescent coculture was 88.6 ± 3.7%, but this was markedly reduced to 12.6 ± 4.4% in MC cocultured with Mø under activating conditions.
Using these approaches, we found that coculture with unstimulated rat BMD Mø had no effect on MC mitosis or apoptosis over a 24-h period compared with control (Fig. 1A, Table I). However, when Mø were primed for 12 h with IFN-γ, and then the coculture was activated with IFN-γ plus LPS, suppression of MC mitosis was observed as early as 8 h (Fig. 2). At 24 h, there was near total cessation of mitosis compared with control culture of MC growing alone, stimulated with IFN-γ and LPS. In addition, there was almost a 10-fold induction of MC apoptosis (Figs. 1B and 2). These results were supported by flow-cytometric DNA content data (Table I), which showed a 10-fold induction of hypodiploid cells in the whole coculture when activated, and a 50% reduction of all cells with double DNA content (G2/M). The combination of these effects was, as expected, associated with a decrease in MC number over this time period, whereas when cultured under control conditions with FCS, MC exhibited the expected increase in number (Fig. 2). Replacing LPS with TNF-α in the activating regimen in coculture elicited similar results to those in Fig. 2. Control cultures of MC alone, stimulated with IFN-γ and TNF-α, did not undergo apoptosis (data not shown). Primary cultures of MC also exhibited

FIGURE 1. Fluorescence microcopy of rat BMD Mø and rat MC in coculture (×200). A. Coculture was established as described in Materials and Methods, but was not activated. At 24 h, after fixation and counterstaining, green MC with pale red nuclei are easily distinguished from Mø, which are seen as red cells with red nuclei. Note mitotic MC (arrow). B. The coculture was activated by priming Mø with IFN-γ (100 U/ml) for 12 h; then once the coculture was established, IFN-γ (100 U/ml) plus LPS (1 μg/ml) were added. At 24 h, note apoptotic MC with nuclear condensation and cell shrinkage (large arrows). Also note absence of mitotic cells. i. (×500) Green rounded-up MC can be seen in live coculture at 24 h. These cells are excluding PI in the medium (1 μg/ml). ii. The same cells are selectively failing to exclude Hoechst 33342 (5 μg/ml), which simultaneously reveals characteristic apoptotic nuclei. iii. The same cells after fixation, permeabilization, and counterstaining with PI show typical nuclear condensation (note that there has been some rotation of MC during fixation).
similar responses to coculture with activated MΦ. After 24 h of coculture, MC apoptosis was 7.9% (coculture) compared with 1.2% in controls \((p < 0.05)\), and MC mitosis was 0.1% and 2%, respectively \((p < 0.01)\). Activated MΦ underwent very low levels of apoptosis after 24 h \((<0.5\%)\). This low level was seen whether activated MΦ were cultured alone or with MC.

By increasing the ratio of MΦ to MC in coculture, a progressively greater proportion of MC was apoptotic \((39.3 \pm 3.5\% \text{ at } 24 \text{ h, ratio } 10:1)\). However, at ratios higher than 4:1, a small proportion of apoptotic cells in live cocultures did not exclude PI. A 3-fold reduction in the number of MΦ in coculture still demonstrated significant induction of apoptosis \((6.1 \pm 2.4\% \text{ at } 24 \text{ h MΦ control } 1.2 \pm 0.4\%, p < 0.05)\) and inhibition of mitosis \((0.83\% \pm 0.15 \text{ at } 24 \text{ h MΦ control } 2.3 \pm 0.4\%, p < 0.05)\), although this was reduced compared with 1.5:1 ratio.

**NOS-2 mediates inhibition of mitosis and induction of apoptosis in MC cocultured with activated MΦ**

The addition of the nonspecific NOS competitive inhibitor L-NMMA at 100 μM was able largely to abrogate the MΦ-induced effects on MC proliferation and apoptosis \((IC_{50} \text{ for apoptosis } 5.1 \pm 3.6 \mu M)\). Higher concentrations \((200 \text{ and } 500 \mu M)\) had no additional effect. Its inactive analogue \((a\text{-NMMA})\) had no discernible effect in cocultures with activated BMD MΦ at 24 h (Table II). L-N'-(1-iminoethyl) lysine dihydrochloride \((L-NIL)\), a specific competitive inhibitor of NOS-2, was also able largely to abrogate these MΦ effects at 24 h \((IC_{50} \text{ for apoptosis } 0.33 \pm 0.40\%)\) (Fig. 3, Table I). Transfer of conditioned supernatants directly from Mϕ \((10^5/ml)\) activated for 16 h with IFN-γ \((300 \mu M)\) plus LPS \((1 \mu g/ml)\) had no effect on apoptosis or mitosis of MC compared with control (medium containing IFN-γ and LPS) (data not shown). These results are in keeping with NO \((\text{a short-lived unstable molecule})\), signaling apoptosis from the MΦ to the MC.

**Proinflammatory cytokines increase MC susceptibility to apoptosis induced by activated MΦ or FasL**

Using the morphological assay for assessing MC apoptosis, MC pretreated with IFN-γ and TNF-α for 36 h were rendered susceptible to apoptosis induced by soluble recombinant FasL (Fig. 4A). This effect was not observed following pretreatment with IFN-γ alone (data not shown). In keeping with previous work on human MC demonstrating that similar pretreatment increased the expression of Fas (Ref. 21 and our unpublished observations), rat MC Triton X lysates were prepared for Western blot analysis, which confirmed increased expression of Fas after pretreatment with IFN-γ and TNF-α (Fig. 4B).

To assess whether proinflammatory cytokines also increased MC susceptibility to MΦ-induced kill, following pretreatment with IFN-γ and TNF-α for 36 h, MC were washed, trypsinized, and cocultured with IFN-γ-primed rat BMD MΦ in the presence of IFN-γ plus LPS. After 24 h (Fig. 5), there was a significant increase in MC apoptosis compared with concurrent experiments using mock-pretreated MC. This effect was only partially reversible by adding the NOS-2 competitive inhibitor L-NMMA, suggesting that mediators other than NO might contribute to the induction of apoptosis in primed MC. Quiescent MΦ had no effect on primed MC, and the latter were no more susceptible to the suppression of mitosis by activated MΦ (see Fig. 6B).

---

**Table II. Effect of \(L\)-NMMA and \(d\)-NMMA on activated MΦ-induced induction of MC apoptosis and suppression of mitosis in rat MC**

<table>
<thead>
<tr>
<th></th>
<th>L-NMMA</th>
<th></th>
<th>d-NMMA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coculture</td>
<td>Control</td>
<td>Coculture</td>
<td>Control</td>
</tr>
<tr>
<td>% Apoptosis</td>
<td>2.6 ± 1.2</td>
<td>2.9 ± 1.9</td>
<td>16.3 ± 4.3*</td>
<td>3.5 ± 2.1</td>
</tr>
<tr>
<td>% Mitosis</td>
<td>2.9 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>0.2 ± 0.2*</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Cell number</td>
<td>908 ± 104</td>
<td>911 ± 86</td>
<td>549 ± 78*</td>
<td>820 ± 19</td>
</tr>
</tbody>
</table>

* Coculture was established as described in Materials and Methods. After priming with IFN-γ, MΦ were activated using IFN-γ \((100 \mu M)\) and TNF-α \((100 \mu M)\). Either \(d\)-NMMA \((100 \mu M)\) or \(L\)-NMMA \((100 \mu M)\) was added to well. Experiments were assessed at 24 h. Data are given as mean ± SEM.

* \(p < 0.05\) compared with coculture in the presence of \(L\)-NMMA \((n = 4)\).
$M_b$ expression of NOS-2, but not FasL, determines the capacity to inhibit mitosis and induce apoptosis in MC

Previous work has indicated that both $M_b$ and MC produce NO upon cytokine induction of NOS-2 (22, 23). We confirmed that rodent BMD $M_b$ produced NO (as assessed by the use of Griess reagent to measure accumulated nitrate in culture medium) when challenged with proinflammatory cytokines (Table III). MC produced much smaller amounts of NO in response to IFN-γ plus TNF-α (Table III). Such cells stained weakly for NOS-2 by indirect immunofluorescence compared with the bright staining of all $M_b$ treated with cytokines (not shown).

These data indicated that competitive NOS-2 inhibitors in the coculture might therefore have exerted their effects on MC NOS-2, $M_b$ NOS-2, or both. To differentiate between these possibilities, BMD $M_b$ from NOS-2 knockout mice and wild-type control mice were prepared. The NOS-2$^{−/−}$ $M_b$ produced a little NO in response to proinflammatory stimuli (Table III) such as IFN-γ plus LPS when compared with wild type, in keeping with previous work suggesting that other NOS may be up-regulated by proinflammatory cytokines (12, 24). Activated 129/sv wild-type mouse $M_b$ behaved identically to rat $M_b$ in the coculture assay, suppressing mitosis (8 and 24 h) and inducing apoptosis (24 h) in both cytokine-primed or unprimed MC (Fig. 6a). However, activated NOS-2$^{−/−}$ 129/sv BMD $M_b$ exhibited a significantly diminished capacity to induce MC apoptosis. However, the magnitude of this reduction was much smaller when MC were primed. This residual kill did not appear to be due to priming rendering MC susceptible to the low levels of NO produced by activated NOS-2$^{−/−}$ $M_b$; in that no further reduction in apoptosis was brought about by adding $l$-NMMA to the coculture (up to $500 \mu M$). The ability to suppress mitosis was completely absent when activated NOS-2$^{−/−}$ $M_b$ were cultured with unprimed MC, but by contrast with apoptosis, there was no residual effect when NOS-2$^{−/−}$ $M_b$ were incubated with primed MC; there was still no suppression of mitosis (Fig. 6b). These data support the possibility suggested above (Fig. 5) that $M_b$-derived mediators in addition to NOS-2-derived NO may contribute to the killing of primed rather than unprimed MC. Furthermore, in separate experiments (see Materials and Methods), clarified supernatants transferred from activated rat $M_b$ directly to primed MC demonstrate that this additional factor was soluble and partially transferable: 24 h after transfer of conditioned supernatant to primed MC, apoptosis was $10.4 \pm 0.9\%$, control (medium alone with activating cytokines) $3.7 \pm 1\%$ ($n = 3$).

Given the increased susceptibility of cytokine-primed MC to FasL-induced apoptosis demonstrated above (Fig. 4a), and the recently reported capacity of activated human $M_b$ to release FasL capable of triggering apoptosis in bystander cells (25, 26), we were
isolated from inflamed rat BMD and uli were ED-1 by this immunohistochemistry. Animals from days 2, 16.15 mesangium During have been mediated M$ with MC (Fig. 7). Nevertheless, the primed identically, whether cocultured with unprimed or primed MC (Fig. 7). Nevertheless, the primed MC used in these experiments were susceptible to apoptosis by soluble FasL (100 ng/ml) (13.4% at 24 h), whereas unprimed were not (1% at 24 h). The residual MC apoptosis observed incubating activated NOS-2−/− M$ with cytokine-primed MC therefore appears most unlikely to have been mediated by FasL.

M$ isolated from inflamed glomeruli also inhibit mitosis and induce apoptosis in cultured MC by an NO-mediated mechanism

To assess the likely in vivo relevance of these observations, telecoped nephrotoxic nephritis was induced in Sprague Dawley rats. During the autologous phase, there was predominance of M$ in the mesangium (21.4 ± 3.1 M$ per glomerular cross section on day 2, 16.1 ± 1.5 on day 4, 1.4 ± 0.6 in controls by ED-1 immunohistochemistry). Animals from days 2, 4, and 7 after disease induction were sacrificed, kidneys were removed, and glomeruli isolated by sieving. Glomeruli were then exposed to an enzymatic digest, resulting in a single cell suspension. M$ were isolated from this by rapid adhesion of these cells to plastic wells (>99% purity by ED-1 immunohistochemistry). M$ from these diseased glomeruli were immediately cocultured with CellTracker Green-labeled MC, and the coculture was assessed at 8, 16, and 24 h. Inflammatory glomerular M$ released similar amounts of NO to activated rat BMD M$ (Table III). Resident M$ from normal glomeruli produced little NO, but when challenged with proinflammatory cytokines, released NO in similar quantities to BMD M$ treated with the same cytokines.

M$ from inflamed glomeruli (activated in vivo) from day 2 of the disease were also able to induce effects comparable with activated BMD M$ upon coculture with MC (Fig. 8). Induction of apoptosis in coculture compared with controls, and suppression of mitosis was present at 8 and 16 h, but diminished at 24 h. However, the combination of these effects led to a decrease in cell number. Results from coculture using M$ isolated from days 4 and 7 of the glomerulonephritis (Table IV) showed similar findings to M$ from day 2. The addition of t-NMMA was also able to abrogate partially the M$-mediated effects (Table IV). d-NMMA had no effect. At 16 h, there was no significant difference in apoptosis and mitosis compared with controls.

The difference in tempo and magnitude of effects seen using ex vivo M$ appeared likely to be due to two factors. First, the ex vivo M$ were already activated, whereas the BMD M$ employed above (Fig. 2) were used under conditions in which full activation would only have been expected after the coculture had been established. Second, ex vivo M$ underwent programmed cell death (>70% at 24 h), which appeared to be related to the enzymatic digest. To investigate these issues further, BMD M$ were activated with IFN-γ (100 U/ml) and LPS (1 μg/ml) for 6 h, then washed. They were exposed to the same enzyme protocol as glomerular cells, then immediately used in coculture. This approach
gave comparable results to the ex vivo glomerular Mφ on MC (data not shown).

Discussion

In this study, we have demonstrated for the first time that activated but not quiescent Mφ coordinately induce apoptosis and suppress mitosis in cultured glomerular MC, which are well known to exhibit myofibroblast-like features. The use of competitive inhibitors (l-NMMA and l-NIL) and Mφ from gene-targeted animals lacking NOS-2 provided definitive evidence that Mφ-derived NO was critical in both induction of apoptosis and suppression of mitosis. When MC susceptibility to these Mφ-induced effects was deliberately increased by pretreating MC with proinflammatory cytokines, a proportion of the Mφ-induced kill was independent of NOS-2. However, despite evidence of sensitization to FasL by treatment of MC with proinflammatory cytokines, the use of activated gld/gld Mφ demonstrated that this residual kill was not me-

FIGURE 7. The effect of Mφ with mutated, nonfunctional FasL (gld/gld) in coculture with unprimed and primed MC. BMD Mφ from the C57/HEJ mouse were matured according to Materials and Methods. MC were primed or unprimed, and the coculture was established and activated as described before. At 24 h, activated wild-type and gld/gld Mφ induced similar amounts of apoptosis in unprimed MC. Primed MC were more susceptible to the apoptosis-inducing effects of these Mφ. However, the gld/gld Mφ were not significantly different from the wild-type control Mφ (n = 4).

FIGURE 8. The effect of Mφ from the inflamed glomeruli of rats with nephrotoxic nephritis (NTN) on MC apoptosis and mitosis. Glomerular single cell suspension and Mφ isolation were carried according to Materials and Methods. Immediately following isolation, glomerular Mφ from day 2 of nephrotoxic nephritis were cocultured with MC without the addition of cytokines. A, The effect of Mφ on MC apoptosis at 8, 16, and 24 h. Note induction of apoptosis at 8 h, peaking at 16 h, and much reduced at 24 h. B, The effect of Mφ MC mitosis at the same time points: MC mitosis was suppressed at 8 and 16 h. C, Mφ suppressed the expected increase in MC number at 16 h. This effect was more marked at 24 h. *, p < 0.05 compared with control MC (n = 3).
Table IV. Effect of glomerular MΦ isolated from rats with NTN at days 2, 4, and 7 upon MC apoptosis, mitosis, and cell number.a

<table>
<thead>
<tr>
<th></th>
<th>Day 2 MΦ</th>
<th>Day 2 MΦ + l-NMMA</th>
<th>Day 4 MΦ</th>
<th>Day 7 MΦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis at 16 h ( % of control)</td>
<td>493 ± 23</td>
<td>136 ± 5*</td>
<td>261 ± 11</td>
<td>487 ± 16</td>
</tr>
<tr>
<td>Mitosis at 16 h ( % of control)</td>
<td>19 ± 12</td>
<td>83 ± 18*</td>
<td>38 ± 60</td>
<td>23 ± 29</td>
</tr>
<tr>
<td>MC number at 24 h ( % of control)</td>
<td>73 ± 9</td>
<td>99 ± 10*</td>
<td>85 ± 6</td>
<td>81 ± 9</td>
</tr>
</tbody>
</table>

a Control MC apoptosis 0.68 ± 0.04%, mitosis 3.8 ± 0.7%, and MC number 1729 ± 68%. The addition of l-NMMA (100 µM) to cocultures with day 2 MΦ shows partial abrogation of the MΦ-induced effects on MC. l-NMMA in coculture with day 2 MΦ were comparable to no additive and the data have been omitted for clarity. *: p < 0.05 compared to day 2 MΦ (n = 3).

diated by FasL from MΦ. Finally, glomerular MΦ activated in vivo during experimentally induced glomerulonephritis also induced apoptosis and suppressed mitosis when cultured ex vivo with MC, by an l-NMMA-inhibitable mechanism. We conclude that by virtue of release of NO, activated MΦ can regulate MC number by both induction of apoptosis and suppression of mitosis.

We believe that these data provide evidence of a new concept in inflammation: that of MΦ-directed regulation of resident cell populations by the coordinated induction of apoptosis and inhibition of mitosis. This control may have a pivotal role in the outcome of acute inflammatory responses, either progression to a hypocellular scar, or healing by restoration of the normal cell complement and phenotype. The ex vivo data argue strongly that MΦ-directed regulation of MC complement is likely to occur in glomerulonephritis in vivo, but it is clear that future experiments will need to test the population-regulating role of MΦ in greater detail. Evidence of MΦ-directed tubular cell apoptosis already exists in nephrotoxic nephritis induced in MCP^-/-^ and wild-type mice (6), in which MΦ accumulation in the tubulointerstitium was much reduced in the chemokine-deficient mice, and was associated with a marked decrease in tubular cell apoptosis. Furthermore, consistent with data suggesting that many cell types exhibit myofibroblast-like features at sites of tissue injury, preliminary data from Bennett’s group (27) have linked MΦ with apoptosis of vascular smooth muscle cells in vitro and in atherosclerotic plaques in vivo. Therefore, we suggest that MΦ-mediated regulation of resident cell populations should be sought in a wide range of disease settings.

The competition and knockout MΦ experiments indicated that MΦ-derived NO plays a major role in inducing apoptosis and suppressing mitosis in MC. The paucity of NO production by rat MC compared with MΦ is in keeping with work on whole glomeruli from rats with Thy-1.1 nephritis, in which NO release correlated with the presence of MΦ rather than proliferating MC (28). However, the only available study of glomerulonephritis in NOS-2^-/-^ mice was in a model of mild nephrotoxic nephritis with little MΦ infiltration of glomeruli (29), and apoptosis was not assessed. Furthermore, the equivocal data on NO in glomerulonephritis published to date (in which two studies show amelioration of disease (30, 31); two show no difference (29, 32), and one shows exacerbation (33)) have concentrated on conventional parameters of glomerular injury rather than apoptosis or remodeling. Therefore, additional experiments will be required to confirm a role for MΦ-derived NO in regulating MC number in glomerulonephritis models exhibiting degrees of MΦ infiltration comparable with that observed in human disease (34, 35), in which MΦ number may approach MC number (comparable with the in vitro ratio employed in our experiments). It is, however, difficult to extrapolate directly from in vitro ratios, in which cell interaction is in one plane, to the in vivo situation. Nevertheless, expression of NOS-2 by MΦ in human glomerulonephritis (36, 37), despite notorious difficulties in achieving NO production in vitro (38, 39), suggests again that NO-mediated apoptosis may be relevant in vivo.

However, the competitor and NOS-2^-/-^ MΦ studies employing MC treated with proinflammatory cytokines indicated that, in addition to NO, there is at least one other mechanism by which MΦ can direct MC killing. Despite evidence in human monocye-derived MΦ of FasL-dependent killing of neighboring cells (25, 26), the data from gld/gld mice argue strongly against this subsidiary MΦ-directed killing mechanism being mediated by rodent MΦ-derived FasL. To what extent the cytokine-treated or primed in vitro MC represents a MC in vivo during inflammatory responses is unclear. In this study, we found that primed cells in vitro were weakly NOS-2 positive, and produced relatively small amounts of NO. Immunohistochemistry of glomerular sections from nephrotoxic nephritis suggested that MΦ are the principal cell type expressing NOS-2 (40), although similar studies of human disease demonstrated that some MC in inflamed glomeruli were NOS-2 positive (37). Furthermore, we observed increased rat MC Fas protein following cytokine priming, in keeping with studies of human MC (21). This correlates with work showing that some human MC during acute glomerular disease have increased Fas protein (41). Together, these data indicate that at least some MC in vivo share phenotypic features with cultured MC primed with cytokines in vitro. Therefore, it would appear desirable for future studies to investigate further whether such cells are rendered susceptible to ligation of receptors other than Fas that signal into the caspase (intracellular death signaling) pathway. Preliminary experiments (J. Duffield, C. Ware, and J. Savill, unpublished data) employing TNFRI-Fc chimeras to block TNFRI on MC point to a role for MΦ-derived TNF-α/TNF-β in mediating NO-independent residual kill of cytokine-primed MC, but a range of ligands for the expanding TNFR family will need to be assessed before definitive conclusions can be drawn.

The major focus of this study was to determine the potential of activated MΦ to direct apoptosis of myofibroblast-like MC. However, the capacity to limit the growth of MC populations was clearly enhanced by concomitant suppression of MC mitosis. Indeed, exogenous NO donors have been reported to induce DNA fragmentation and inhibit mitosis in MC (42, 43). It is known that when MC are treated with the NO donor, S-nitroso-glutathione, the nuclear protein p53, whose effects on the cell cycle are well recognized, is induced (42). Furthermore, exogenous NO donors activate caspase 8, the most upstream member of the caspase pathway, by cleavage, in lymphoid cell lines (44). Future studies should address the molecular mechanisms by which NO coordinates suppresses mitosis and promotes apoptosis in MC.
To conclude, this study implicates NO derived from activated Mdp in regulation of MC populations by inducing apoptosis and suppression of mitosis in this resident glomerular cell type. We speculate that this may be a generally relevant mechanism for regulation of myofibroblast-like cell populations at sites of tissue injury.

Acknowledgments
We thank Dr. J. Pfeilschifter for helpful discussions and the mouse anti-
inOS- haem Ab; Drs. Z. Hruby, R. A. Lang, and V. Cattell for helpful comments; and Dr. S. Brown for endless ideas and criticism.

References
Suppression by Apoptotic Cells Defines Tumor Necrosis Factor-Mediated Induction of Glomerular Mesangial Cell Apoptosis by Activated Macrophages

Jeremy S. Duffield,* Carl F. Ware,† Bernhardt Ryffel,‡ and John Savill*

From the Medical Research Council Centre for Inflammation Research,* University of Edinburgh, Edinburgh, United Kingdom; the La Jolla Institute for Allergy and Immunology,† University of California, San Diego, California, and the Department of Medicine,‡ University of Cape Town, Cape Town, South Africa

Activated macrophages (Mφ) isolated from inflamed glomeruli or generated by interferon-γ and lipopolysaccharide treatment in vitro induce glomerular mesangial cell apoptosis by hitherto incompletely understood mechanisms. In this report we demonstrate that nitric oxide-independent killing of co-cultured mesangial cells by interferon-γ/lipopolysaccharide-activated Mφ is suppressed by binding/ingestion of apoptotic cells and is mediated by tumor necrosis factor (TNF). Thus, soluble TNF receptor-1 significantly inhibited induction of mesangial cell apoptosis by 1) rodent Mφ in the presence of nitric oxide synthase inhibitors or 2) human Mφ, both situations in which nitric oxide release was minimal. Furthermore, murine TNF knockout Mφ were completely unable to induce mesangial cell apoptosis in the presence of nitric oxide synthase inhibitors. We conclude that TNF-restricted Mφ-directed apoptosis of glomerular mesangial cells can be down-regulated by Mφ binding/ingestion of apoptotic cells, suggesting a new mechanism for negative feedback regulation of Mφ controls on resident cell number at inflamed sites. (Am J Pathol 2001, 159:000–000)

Proliferation of resident cells is a prominent feature of inflammatory responses. In glomerular inflammation there is typically an increase in number of resident glomerular mesangial cells that adopt a myofibroblast-like phenotype, lay down excess abnormal matrix, and thereby threaten progression to scarring.1 However, in self-limited nephritis excess mesangial cells are deleted by apoptosis and the glomerular cell complement returns to normal.2,3 Until recently the mechanisms mediating deletion of myofibroblast-like mesangial cells have been obscure. However, we showed that activated macrophages (Mφ) can direct apoptosis of such cells,4 mesangial cell killing being mediated by nitric oxide (in a rodent cell system) and another unknown factor. Because inflammatory Mφ can delete neutrophils5 and a range of tumor cells6–8 by tumor necrosis factor (TNF)-mediated mechanisms, there was a strong possibility that activated Mφ might use TNF to induce mesangial cell apoptosis, particularly in human cell systems in which Mφ production of nitric oxide is notoriously difficult to detect.9

Furthermore, the demonstration that activated/inflammatory Mφ can kill resident glomerular cells immediately begs the question as to how the killing capacity of Mφ might be regulated. Importantly, work from Reiter and colleagues10 demonstrated that the capacity of rodent bone marrow-derived Mφ stimulated with interferon (IFN)-γ and lipopolysaccharide (LPS) to induce tumor cell apoptosis was diminished to ~30% of control by ingestion of apoptotic cells. However, although this was associated with a modest reduction in nitric oxide production to ~75% of control, the Mφ killing mechanism(s) suppressed by ingestion of apoptotic cells was/were not characterized further.10

In this study we set out to determine whether activated Mφ induction of glomerular mesangial cell apoptosis was suppressed by Mφ ingestion of apoptotic cells and to determine which Mφ mechanisms for triggering apoptosis in neighboring mesangial cells were subject to such control.

Materials and Methods

Materials

Media and fetal calf serum (FCS) were purchased from Life Technologies (Paisley, UK). Tissue culture plastic was from Falcon (Becton Dickinson, Mountainview, CA) and Costar (Cambridge, MA) as stated in the text. Cyto-

Supported by a Medical Research Council Clinical Training Fellowship (no. G84/4757, to J. S. D.) and a Wellcome Trust Program Grant (no. 047273, to J. S.).

Accepted for publication July 5, 2001.

Address reprint requests to Dr. J. S. Duffield, M. R. C. Center for Inflammation Research, Medical School, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, UK. E-mail: j.duffield@ed.ac.uk.
kines were purchased from R&D Systems (Minneapolis, MN) and all other reagents from Sigma (St. Louis, MO) unless otherwise stated.

Cell Isolation and Preparation

Human mesangial cells were obtained from the cortex of fresh nephrectomy specimens. Mesangial cells were purified from outgrowths of whole, purified glomeruli and passages according to standard techniques in full Dulbecco's modified Eagle's medium (DMEM)/F12 (with 10% FCS, and supplemented with penicillin and streptomycin (Life Technologies). Rat mesangial cells were derived from outgrowths of whole glomeruli as described. They were passaged according to standard techniques in full DMEM/F12, and were used between passage 6 and 14. Rodent macrophages were derived from bone marrow taken from the femur of Wistar rats or from murine strains (TNFαβ−/−) and wild-type littermates (C57BL6) and cultured in Iscove's-modified DMEM with 10% fetal bovine serum (FBS). Cells were washed twice in PBS, detached with Trypsin-EDTA (2X), and resuspended in DMEM/F12 containing 10% FBS, 50 µg/ml methasone, and 1 µg/ml of LPS (1 µg/ml). Cells were counted using a Beckman Coulter SXT Automated Cell Counter and adjusted to 10^5 cells/ml before use.

Generation of Apoptotic Cells

Rat mesangial cells were induced into apoptosis by ultraviolet irradiation. Subconfluent monolayers in T75 flasks (Costar) were exposed to UV irradiation (312 nm, 8 W, 3 minutes) followed by incubation for 16 hours. Non-adherent (apoptotic) cells were removed by agitation and collected in the supernatant. After centrifugation, apoptotic cells were further purified by washing with phosphate-buffered saline (PBS) 1x and centrifugation (190 x g, 5 minutes). Apoptosis was confirmed by histology, selective uptake of Hoechst 33342 (1 µg/ml), but exclusion of propidium iodide (PI) (1 µg/ml). Typically, <10% of cells were PI-positive and <5% did not exclude trypan blue (0.2%).

Mouse thymocytes were prepared from 20 g C57/BL6 mice by pressing the thymus through a 50-µm sieve. The single cell supernatant was resuspended (2 x 10^8 cells/ml) in RPMI supplemented with glutamine and 2-mercaptoethanol in addition to 10% FCS and antibiotics. Cells were either exposed to a 5-minute burst of UV irradiation (312 nm, 8 W) followed by 2.5 hours of culture, or dexamethasone (1 µmol/L) followed by culture for 6 hours. Typically >50% of induced cells were apoptotic (Annexin V binding; Boehringer Mannheim, Mannheim, Germany) and permeable to Hoechst 33342 (1 µg/ml) whereas <5% of those were positive for the uptake of PI (flow cytometric analysis, data not shown).

Co-Culture of Mφ and Mesangial Cells

For a detailed description, see our earlier work. Matured rodent bone marrow-derived macrophages were plated in 96-well plates initially at a density to cover 60 to 70% of the well surface; typically this required 2 x 10^5 cells per well. Rat mesangial cells were prelabeled with CellTracker Green CMFDA (Molecular Probes, Eugene, OR); cell cultures, 70 to 80% confluent, were washed with medium lacking serum and then incubated for 1 hour in serum-free medium containing CMFDA at 5 ng/ml. Cells were washed in medium containing 10% FCS to remove any unbound CMFDA; then trypsinized and added to cultured rodent Mφ in a 1.5 Mφ:1.0 mesangial cell ratio, previously shown to be optimal for demonstration of macrophage-directed mesangial cell apoptosis. Experiments were performed in DMEM/F12 medium containing 10% FCS. Once cells had become adherent, typically 2 to 4 hours, wells were washed to remove nonadherent cells. In our earlier work, mixing unlabeled and labeled cells showed no evidence of transfer of CMFDA from labeled to unlabeled cells. Rodent co-cultures were activated with IFN-γ (100 U/ml) plus LPS (1 µg/ml).

In some experiments, unlabeled apoptotic cells (1 x 10^5 per well of a 96-well plate) were added to the established co-culture and to the control wells of mesangial cells alone, at the same time as activating cytokines. As a control for apoptotic cells, aliquots of 10-µm diameter sterile latex beads (Polysciences, UK) were added to adjacent co-culture. When thymocytes were used as apoptotic cells, aliquots were added to co-culture for 6 hours, then noningested cells gently washed away, before activation. In separate wells containing Mφ alone, apoptotic cells or latex beads were also incubated for 6 hours, then washed off, and wells were fixed for quantification of percentage Mα that had phagocytosed apoptotic cells. This was assessed by phase contrast microscopy. Mφ (34 ± 5.6%) phagocytosed at least one apoptotic thymocyte and 30 ± 6.7% phagocytosed apoptotic mesangial cells. Mφ (67 ± 8.4%) ingested latex beads. By contrast only 1 ± 1% of Mφ phagocytosed the population of live thymocytes in this assay.

For human co-culture, human monococyte-derived Mφ were plated at 2 x 10^4 per well as above. Cycling human mesangial cells were primed with IFN-γ (500 U/ml) for 24 hours, then prelabeled with CMFDA as described for rat mesangial cells. Once washed and trypsinized, they were added to wells in a 2:1 ratio (because of the larger size of human mesangial cells). After 4 hours, wells were washed and replaced with full DMEM/F12. Human co-cultures were activated with human IFN-γ (Peprotech) (500 U/ml) and LPS (1 µg/ml).
Assessment of Mesangial Cell Apoptosis in Co-Culture

For a detailed description, see our earlier work. At the end of co-culture experiments, wells were either fixed with formaldehyde (4% final concentration) and stored for 48 hours at 4°C to allow firm adhesion of apoptotic cells to the plate, or they were assessed live by fluorescence microscopy after the addition of Hoechst 33342 (1 μg/ml) and PI (1 μg/ml). For assessment of live cells, green rounded-up cells were scored as apoptotic if they also were positive for Hoechst uptake, but excluded PI. For assessment of fixed (and permeabilized) cells by morphology, plates were first counterstained with PI (1 μg/ml) and Hoechst (1 μg/ml) in PBS for 5 minutes (which stains both Mφ and mesangial cells). After discarding the stain, wells were covered with a fluorescent mountant. Apoptotic mesangial cells were easily discernible by green fluorescence and characteristic morphology. For both live and fixed wells, apoptosis of mesangial cells was assessed blindly and five fields per well were randomly chosen without observer bias. Each experiment was performed in triplicate. Previous studies have shown this method to give similar results to flow cytometric assays. However, the microscopical assay is reproducibly more sensitive because apoptotic mesangial cells tend to disintegrate during centrifugation once they have been fixed. Note that when exogenous apoptotic cells were added these were not labeled with fluorescent dyes, enabling confident identification of apoptosis in the previously healthy target mesangial cells.

Assays of TNF-α and Nitric Oxide

Culture supernatants (free from phenol red) were harvested, clarified by centrifugation (4000 x g, 5 minutes) then stored at -20°C. After complete thawing, 50 μl of each sample was assayed by Quantikine mouse TNF-α enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. A standard curve with absorbencies from 0.1 to 1.2 was achieved on each occasion. Samples (50 μl) were assayed for nitrite by mixing with an equal volume of the Griess reagent as previously described and measuring absorbency at 540 nm and comparing with a sodium nitrite standard curve.

Chimeric Soluble Death Receptors

Fusion proteins were constructed using cDNAs for the extracellular domains of human receptors fused with the Fc portion of human IgG3. Proteins were expressed in insect cells infected with recombinant baculoviruses. Protein secreted into the culture supernatant was then purified by protein A-Sepharose column affinity. The protein was stored at -20°C in Hanks' salt solution.

Knockout Mice

TNF-α/TNF-β double-knockout mice were generated by inserting a targeting vector between exon 1 and 2 of the murine TNF-β gene and the middle of exon 4 of the adjacent murine TNF-α gene of chromosome 17 of GS1 mouse embryonic stem cells. Mutant embryonic stem cells were selected and injected into C57/BL6 blastocysts.

Statistics

All experiments were performed on at least four separate occasions using at least four animals. The data were expressed as mean values with the SE of mean. Paired data were compared using the t-test and multiple comparisons using analysis of variance.

Results

Ingestion of Apoptotic Cells by Activated Rodent Mφ Selectively Suppresses Nitric Oxide-Independent Induction of Mesangial Cell Apoptosis

In our previous studies of IFN-γ/LPS-activated rodent bone marrow-derived Mφ killing of rodent mesangial cells primed with IFN-γ (Figure 1) we demonstrated that at least half of the mesangial cell killing was independent of Mφ-derived nitric oxide, being unaffected by inhibitors of inducible nitric oxide synthase (iNOS) such as L-NMMA at 500 μmol/L. We also observed that the vast majority of mesangial cells induced into apoptosis by activated Mφ were not ingested, presumably because of kinetic and spatial restraints in a two-dimensional culture system in which IFN-γ-primed mesangial cells were added above a sparse monolayer of Mφ before the co-culture was activated by addition of IFN-γ and LPS.

Therefore, to test whether ingestion of apoptotic cells diminished Mφ capacity to trigger apoptosis in mesangial cells, an established co-culture of IFN-γ-primed rat mesangial cells with rat Mφ was exposed to a fivefold excess of mesangial cells induced into apoptosis by UV irradiation; the apoptotic cells were administered at the same time as activation of the co-culture with IFN-γ and LPS. Under standard conditions, there was no significant effect of apoptotic cells on Mφ induction of mesangial cell apoptosis. However, in the presence of 200 μmol/L L-NMMA, apoptotic mesangial cells exerted a dramatic inhibitory effect on Mφ induction of mesangial cell apoptosis (Figure 2). Addition of the same number (1 x 10⁵ per well) of 10-μm latex beads, as a control phagocytic particulate, had no inhibitory effect whether L-NMMA was present or not (Figure 3). Furthermore, suppression of nitric oxide-independent triggering of apoptosis was observed with rodent thymocytes induced into apoptosis by UV or dexamethasone, but not when freshly isolated non-apoptotic thymocytes were used as controls (Table 1).

Finally, similar results were observed whether apoptotic cells were added simultaneously with activation of coculture, as above, or if apoptotic cells were administered to co-culture before activation (Table 1). This indicates that the suppressive effect was mediated by effects of

Phagocytosis of Apoptotic Cells Inhibits Macrophage Killing

AJP October 2001, Vol. 159, No. 4
Figure 1. Fluorescent micrograph (original magnification, ×320) showing activated co-culture of CMFDA green-labeled mesangial cells with M6. The co-culture was established according to Materials and Methods and activated with IFN-γ (100 U/ml) plus LPS (1 µg/ml) after 4 hours of exposure to preformed unlabeled apoptotic mesangial cells. At 24 hours, the co-culture was fixed and later counterstained with PI (1 µg/ml). Apoptotic green mesangial cells are clearly seen with red/orange pyknotic nuclear material and are readily distinguished from Mψ (arrows). Before fixation, co-culture was exposed to Hoechst 33342 (1 µg/ml) for confirmation of apoptosis of the green mesangial cells (see inset). Note inset field and arrow-marked apoptotic cells correspond to central field of main figure. Note also two apoptotic mesangial cells showing cytoplasmic blebbing (large arrows).

Figure 2. Induction of rodent mesangial cell apoptosis by activated Mψ is blocked by simultaneous phagocytosis of apoptotic mesangial cells when iNOS inhibitors are present. Established co-cultures of rodent Mψ and IFN-γ primed, CMFDA-labeled, mesangial cells, or mesangial cells growing alone (as control), were activated with IFN-γ (100 U/ml) and LPS (1 µg/ml). Simultaneously to activation, a fivefold excess of unlabeled apoptotic rodent mesangial cells was added (1 × 10⁶ cells per well). In addition, to some wells (hatched bars), L-NMMA (200 µmol/L) was added at the same time as activation. After 24 hours of incubation, induction of CMFDA-labeled mesangial cells was scored in all wells. ***, P < 0.001 versus no ingestion (n = 5).

Figure 3. Induction of rodent mesangial cell apoptosis by activated Mψ is not blocked by simultaneous phagocytosis of latex beads when iNOS inhibitors are present. Both established co-cultures of rodent Mψ- and IFN-γ-primed, CMFDA-labeled, mesangial cells, and wells of mesangial cells alone were activated with IFN-γ (100 U/ml) and LPS (1 µg/ml). Simultaneously a fivefold excess of unlabeled sterile 5-µm latex beads was added (1 × 10⁶ cells per well). In addition, to some wells (hatched bars), L-NMMA (200 µmol/L) was added. After 24 hours of incubation, induction of CMFDA-labeled mesangial cells was scored (n = 5).
Table 1. The Effect of Co-Culture with Apoptotic Cells upon Murine Mϕ Induction of Apoptosis in IFN-γ Primed Mesangial Cells at 24 Hours in the Presence of Nitric Oxide Synthesis Inhibitor

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No added cells</th>
<th>Cells added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic mesangial cells (UV)</td>
<td>10.7 ± 2.1</td>
<td>3.3 ± 1.0*</td>
</tr>
<tr>
<td>Apoptotic thymocytes (UV)</td>
<td>16.1 ± 1.4</td>
<td>8.2 ± 0.1†</td>
</tr>
<tr>
<td>Apoptotic thymocytes (Dex)</td>
<td>16.1 ± 1.4</td>
<td>8.3 ± 0.6†</td>
</tr>
<tr>
<td>Nonapoptotic thymocytes</td>
<td>16.1 ± 1.8</td>
<td>14.2 ± 1.3</td>
</tr>
</tbody>
</table>

UV, Ultraviolet; Dex, dexamethasone.

Rodent co-culture was established as described in Materials and Methods. Apoptotic cells were incubated with the co-culture for 6 hours. Mϕ were activated with IFN-γ plus LPS in the presence of L-NMMA 200 μmol/L, after washing away noningested cells. Note that control cultures of mesangial cells alone exposed to apoptotic cells followed by activating cytokines underwent no greater than 3.42 ± 0.2% apoptosis. †P < 0.01 compared with no added cells. *P < 0.001 compared with no added cells.

apoptotic cells on the Mϕ most like likely consequent on ingestion of apoptotic cells, but not excluding an effect of binding alone.

Blockade of TNFR1 Also Inhibits Nitric Oxide-Independent Macrophage Induction of Mesangial Cell Apoptosis

Exposure of activated monocytes/macrophages to apoptotic cells exerts a well-established inhibitory effect on secretion of TNF. It therefore seemed especially likely that nitric oxide-independent induction of IFN-γ-primed mesangial cell apoptosis by activated Mϕ, which we had found to be inhibited by apoptotic cells (Figure 2), was also mediated by Mϕ-derived TNF.

To seek a role for TNF in Mϕ directed killing of mesangial cells, we examined the effect of soluble chimeric death receptors (fused with the Fc portion of human IgG1) on activated rat macrophage killing of primed rat mesangial cells in the presence of inhibitors of nitric oxide synthesis (in these experiments the selective iNOS inhibitor L-NIL at 30 μmol/L, as validated in our earlier work). A role for Mϕ-derived FasL had already been ruled out in rodent co-culture by using bone marrow Mϕ from gld/gld mice that lack active FasL. Therefore soluble inhibitors of FasL/FasL interaction were not used. Induction of rat mesangial cell apoptosis in activated co-culture was assessed at 24 hours. TNFR1-Fc (10 μg/ml) was able to reduce significantly the killing capacity of Mϕ in co-culture (Figure 4) whereas lymphotoxin β-receptor fusion protein (LTβR-Fc) and herpesvirus entry mediator fusion protein (HVEM-Fc) had no effect. At higher concentrations, TNFR1-Fc (50 μg/ml) was able to increase further the reduction in killing capacity (14.3 ± 3.3% rat mesangial cell apoptosis in activated co-culture with IgG1 versus 3.9 ± 0.7% mesangial cell apoptosis with 50 μg/ml TNFR1-Fc), but the reduction was not complete. Control rat mesangial cells growing alone with 50 μg/ml TNFR1-Fc showed 1.3 ± 0.7% apoptosis at 24 hours. The IC₅₀ for this fusion protein was 5.0 ± 1.2 μg/ml (Figure 5).

We were also interested in determining whether TNFR1 blockade might similarly inhibit activated human Mϕ induction of mesangial cell apoptosis, because human Mϕ production of nitric oxide is notoriously difficult to elicit. Indeed, we confirmed that the IFN-γ/LPS activation regimen had no significant stimulatory effect on human monocyte-derived Mϕ production of nitric oxide (data not shown) and that L-NMMA had no effect on activated human Mϕ killing of IFN-γ-primed human mesangial cells (14.7 ± 2.1% apoptosis at 24 hours with 200 μmol/L L-NMMA versus 15.7 ± 1.9% under control conditions). In this physiologically nitric oxide-independent human cell system, soluble TNFR1 again demonstrated selective inhibition of activated Mϕ direction of mesangial cell apoptosis (Figure 6).
Nitric Oxide-Independent Induction of Mesangial Cell Apoptosis Is Restricted by Macrophage TNF

Although the foregoing studies provided strong evidence that ligation of TNFR1 played a major role in nitric oxide-independent MΦ killing of mesangial cells, the data did not provide direct evidence that MΦ-derived ligands for TNFR1 (TNF-α and TNF-β) were involved. Furthermore, the failure of sTNFR1 to exert complete inhibition of killing might have reflected its physical exclusion from regions of close contact between macrophages and mesangial cells. To examine this question definitively, we prepared BMD MΦ from double-knockout Tnfα−/−, Tnfb−/− mice, and wild-type littermate controls; all animals used were C57BL/6 x 129sv to ensure that strain differences did not complicate interpretation.13 Preliminary studies showed both knockout and wild-type MΦ produced similar amounts of nitric oxide in response to IFN-γ with LPS (50 ± 4.6 [wild type] versus 47.0 ± 3.3 [Tnfo/β−/−] nmol nitrite per 10⁶ cells per 24 hours) and that this was almost completely inhibited by the iNOS inhibitor L-NIL at 30 μmol/L (to 3.7 ± 2.1 and 2.9 ± 1.9 nmol nitrite per 10⁶ cells per 24 hours, respectively). Supernatants from MΦ activated with IFN-γ and LPS confirmed that the Tnfo/β−/− MΦ were completely unable to produce TNF-α, as assessed by enzyme-linked immunosorbent assay of culture supernatants at 24 hours (wild-type MΦ 3382 pg/ml Tnfo/β−/− MΦ < 0.05 pg/ml). Rat mesangial cells were primed for 24 hours with IFN-γ then co-cultured with either wild-type MΦ or Tnfo/β−/− MΦ. The co-cultures were activated with IFN-γ (100 U/ml) and LPS (1 μg/ml) in the presence of L-NIL (30 μmol/L). In these experiments, knock-out MΦ were completely unable to induce mesangial cell apoptosis compared with controls of mesangial cells growing alone in the presence of cytokines (Figure 7). Wild-type MΦ induced mesangial cell apoptosis as expected. These data demonstrate that when nitric oxide synthesis was inhibited, and therefore used as a model of human MΦ-directed apoptosis, MΦ production of TNF-αβ accounted for almost all of the killing effect of rodent MΦ on mesangial cells.

Discussion

Although it has been well documented that MΦ can kill tumor cells, it has only very recently become apparent that macrophages can also direct tissue remodeling by inducing physiological cell death, in nontransformed resident cells.4-19,20 Thus we have shown previously that activated MΦ, whether isolated directly from experimentally inflamed glomeruli or generated by IFN-γ/LPS activation in vitro can induce apoptosis in cultured primary mesangial cells.4 In this report, we demonstrate for the first time that activated MΦ induction of apoptosis in nontransformed cells can be profoundly inhibited by interaction of MΦ with apoptotic cells. However, in a rodent system this effect could only be demonstrated when nitric oxide production was inhibited. Nevertheless, such nitric oxide-independent direction of mesangial cell apoptosis was inhibitable by soluble TNF receptor in both a rodent cell culture system and a physiologically nitric oxide-independent human cell culture system. Indeed, a major role for TNF in the rodent system was confirmed by the failure of knockout MΦ to induce nitric oxide-independent mesangial cell apoptosis. Therefore, our key conclusion is that macrophage-derived TNF may play a major role in directing apoptosis of primary, nontransformed glomerular mesangial cells.

We also regard it as interesting and important that TNF-restricted MΦ direction of mesangial cell apoptosis was selectively suppressed by interaction of MΦ with apoptotic cells but not when healthy cells or latex beads were used as control particles. These data are in keeping with the marked suppressive effects of apoptotic cells on activated rodent bone marrow-derived MΦ killing of tumor.
cells, in which the reported data favor a major role for a nitric oxide-independent cell killing mechanism because nitric oxide release was only modestly suppressed.19 Both the latter study and the current work may have methodological differences from experiments in which nitric oxide-directed parasite killing was suppressed by M6 binding of apoptotic cells.21 Nevertheless, a second key conclusion of our work is that interaction with apoptotic cells can suppress TNF-restricted M6 direction of mesangial cell apoptosis.

We propose that further work should examine the likelihood that activated M6 deletion of resident cells at inflamed sites may be subject to negative feedback control, in which ingestion of apoptotic cells down-regulates M6 capacity to induce apoptosis in resident cells. This new concept suggests additional deleterious consequences should M6 clearance of apoptotic cells be defective. Previously it has been proposed22,23 that reduced M6 capacity for clearance of leukocytes and other cells undergoing apoptosis, as now demonstrated in C1q−/− knockout mice,24 threatens tissue injury because of the likelihood that cellular contents escaping from noningested apoptotic cells undergoing secondary necrosis will injure tissues directly and indirectly by stimulating M6 release of injurious mediators. Our new data indicate that failure to ingest apoptotic cells might deprive activated M6 of a crucial off-signal resulting in unduly prolonged capacity to direct death of neighboring cells by TNF-restricted mechanisms. Nevertheless, it is likely to be some time before this hypothesis can be tested in vivo, because a growing body of evidence argues that redundancy in clearance mechanisms may require that a number are disabled before sustained defects in clearance of apoptotic cells can be demonstrated.25,26

Furthermore, future work will also need to address whether M6 ingestion of apoptotic cells down-regulates M6 membrane TNF-α cell-surface expression, or whether integrin co-factors, necessary for successful transduction of the apoptotic signal, are down-regulated.9 Preliminary data suggest that membrane-bound rather than soluble TNF-α is responsible for M6-directed apoptosis of mesangial cells (J. Duffield and J. Savill, unpublished data), as reported for M6-directed leukocyte apoptosis.9

To conclude, when macrophage release of nitric oxide is minimal, as may be expected in human inflammation, these data demonstrate a major role for TNF in activated M6 killing of cytokine-primed mesangial cells by apoptosis. Furthermore, inhibition of the capacity to direct apoptosis consequent on M6 binding/ingestion of apoptotic cells reveals a new and potentially important negative feedback control mechanism in remodeling of the inflamed site.

Acknowledgments

We thank Mrs. Carolyn Gilchrist for expert secretarial assistance, Ms. Shonna MacCall for technical assistance, and Drs. Adam Lacy-Hulbert and Ian Dransfield for providing invaluable advice.

References


AQ: H