DECLARATION

This declaration is to state that the contents of this thesis have been composed solely by myself, and that the work described is entirely my own.

Chapter 3 is a manuscript co-authored with M.D. Taylor, M.S. Wilson and R.M. Maizels, and submitted for publication in the European Journal of Immunology.

Constance Finney

1st October
ACKNOWLEDGEMENTS

Wow.
It's over.
I did it.

Well, actually, what I should say is we did it. As cheesy and American as that sounds, this thesis could never have been written without all the support I received from so many people.

This is supposed to be the bit where I thank my primary/secondary school biology teacher for leading me into the field and helping me discover the marvels of biology. However, the people I truly have to thank are my parents. I've always wanted to study biology, and instead of pushing me towards engineering, as is the custom in most French families for anyone with decent grades, they supported me in my choice every step of the way and have even become nematode fans! Merci maman, merci papa.

I also need to thank my sisters, Lives and Aggs, as well as Mylene. You girls have really helped me through my PhD and made sure I became a good scientist rather than a true geek! I know it's been tough, especially with my tendencies to hang around libraries, but you've done a great job. Thank you for always being there to make me laugh and realise things aren't all that bad.

Ah, the office...what would I have done without you? We've had some good times in our small windowless space. Over the 3 years, you've kept me sane, you've been there for me, and most importantly, you've made me laugh and laugh and laugh. Anjie, Iain,
Karen, Katie and Henry, you made the bad days seem OK, which is no easy task. Of course, the constant stream of home-baked cakes, chocolate goodies and tea did help in that respect...

I also need to thank Rick, my supervisor, who, apart from his lack of time keeping (!), has taught me how to be a good scientist. The training I received in his lab has provided me with the tools I will need in my future career. Thank you for believing in my work, despite my best efforts to undermine it! A big thank you as well to everyone in the labs who has helped me scientifically and just made my life in Edinburgh a much more enjoyable experience: Simmi and Arvind, Matt, Simon, Marieke, Irma and Michael, John G., James, Lidia, Janice, Adam, Paul, Grainne, Andrea, Judi, the animal staff, the Gray lab, the Anderton lab, Mark W., Mark B... The list goes on and on, and I am especially indebted to Karen, Yvonne H., Yvonne G. and Meera, without whose help many of my experiments could not have been conducted, as well as Marieke, Karen, Henry and James who were invaluable proof-readers.

Friends in Edinburgh (Tina, Alix, Amy, Matt, Ross, Chippo, Philippa, Lina), London (Vuk, Becky, Richard, Ig, Etienne and Natalie), Paris (Caro, Charlotte, Stephanie), and all over the world (Tania, Meera) have had to put up with my moaning and whining over the past 3 years. Thank you for all your encouragement, and still being there for me now.

Finally, James. Thank you for everything. You made it all so much easier. Your encouragement and belief in me kept me going when things were really getting me down, as did the thought of our new life in Toronto...
ABSTRACT

We have found that a primary infection with the gastrointestinal nematode *Heligmosomoides polygyrus* elicits a potent CD4^+^CD25^+^ regulatory T cell population within a generalised Th2 environment. This indicates that long-lived helminth parasites may exploit the host regulatory network to suppress protective immunity.

We followed the expansion of CD4^+^CD25^+^ Treg cells within the mesenteric lymph nodes (MLN) and spleen over 70 days of infection. Over the time course, increased levels of IL-4, IL-5, IL-9, IL-13 and IL-10 were detected in both these sites by *in vitro* recall response to parasite antigen. In infected animals, regulatory T cell markers, such as surface-bound TGF-β1 and CD103 were upregulated on CD4^+^CD25^+^ MLN cells. However, surprisingly, FoxP3, a transcription factor necessary for Treg development and function, was not upregulated on CD4^+^CD25^+^ in infected animals.

Additionally, we have demonstrated that this regulatory population has potent *in vitro* and *in vivo* suppressive activity. CD4^+^CD25^+^ MLN cells from infected animals (day 28) can suppress mitogen-induced proliferation by both naïve and infected CD4^+^CD25^-^ cells, whilst naïve CD4^+^CD25^+^ MLN cells can only suppress naïve CD4^+^CD25^-^ cells. Moreover, in a model of allergic airway inflammation, we observed that allergic inflammation was decreased in both infected animals and those cured of infection before airway challenge. Thus infection is required to induce but not maintain this regulatory function. However, the mechanisms by which suppression is maintained may not solely rely on CD4^+^CD25^+^ cells.

Finally, we found that concomitant *H. polygyrus* infection led to reduced expulsion of the helminth *Nippostrongylus brasiliensis* in co-infected hosts. Regulatory markers, CD103 and TGF-β1, as well as Th2 cytokines were elevated in co-infected animals as compared to those with a single *N. brasiliensis* infection. However re-infection with *H. polygyrus* of both cured and infected animals led to important decreases in worm burden, so although the regulatory network generated by this parasite can increase survival of other nematodes, it does not suppress immune responses to itself upon re-infection.
ABBREVIATIONS

AHR – airway hyperresponsiveness
AIDS – acquired immunodeficiency syndrome
AIG – autoimmune gastritis
ANOVA – analysis of variance
APC – allopochocyanin
APC – antigen presenting cell
BALF – bronchoalveolar lavage fluid
BCG – Bacillus of Calmette-Guerin
BSA – bovine serum albumin
CFSE – carboxy fluorescein succinimidyl ester
CTLA-4 – cytotoxic T lymphocyte antigen 4
CC10 – Clara cell 10 kDa protein
CXCL10 – chemokine from the CXC chemokine family
d – day
DC – dendritic cell
Der P1 – house dust mite (Dermatophagoides pteronyssinus) allergen
EAE – experimental allergic encephalitis
ELISA – enzyme-linked immunosorbent assay
FCS – foetal calf serum
FHA – filamentous hemagglutinin
FITC – fluorescein
FIZZ – found in inflammatory zones
GATA-3 – transcription factor
gfp – green fluorescent protein
GITR – glucocorticoid-induced tumour necrosis factor receptor
GITRL – glucocorticoid-induced tumour necrosis factor receptor ligand
HCV – hepatitis C virus
HES – H. polygyrus excretory/secretory products
HIV – human immunodeficiency virus
Hp antigen – H. polygyrus antigen
HRP – horseradish peroxidase
IBD – inflammatory bowel disease
IDO – indoleamine 2, 3-dioxygenase
Ig – immunoglobulin
IL – interleukin
IL-4Rα – interleukin 4 receptor alpha
IL-10R – interleukin 10 receptor
iNOS – inducible nitric oxide synthase
i.p. – intraperitoneal
i.t. – intratracheal
IVC – individually ventilated cages
L3 – third larval stage
LC – Langerhan cell
MBP – myelin basin protein
MFI – mean fluorescence index
MHC – major histocompatibility complex
MLN – mesenteric lymph node
MLNC – mesenteric lymph node cell
MMLV – murine Maloney leukaemia virus
MPC – magnetic particle concentrator
Nb antigen – *N. brasiliensis* antigen
OVA – ovalbumin
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PE – phycoerythrin
PMA – a PKC activator
pNPP – p-nitrophenylphosphate
RAG – recombination activating gene
RELM – resistin-like molecule
RT-PCR – reverse-transcription PCR
SCID – severe combined immunodeficiency
SEM – standard error of the mean
Smad – MAD (mothers against decapentaplegic)-related proteins
SPF – specific pathogen free
SMM – spent mite medium
Stat – signal transducer and activator
TB – tuberculosis
T-BET – T-box expressed in T cells
TBS – Tris buffered saline
TCR – T cell receptor
TGF – transforming growth factor
TGF-βR – transforming growth factor beta receptor
Th – T helper
TNF – tumour necrosis factor
TrI – T regulatory I
Treg — regulatory T cell
v. — versus
WHO — World Health Organisation
ORGANISMS CITED

Bacteria

- *Bordetella pertussis* (β-proteobacter)
- *Campylobacter jejuni* (ε-proteobacter)
- *Citrobacter rodentium* (γ-proteobacter)
- *Chlamyphila abortus* (Chlamydiae)
- *Helicobacter pylori* (ε-proteobacter)
- *Mycobacterium vaccae* (actinobacter)
- *Mycobacterium avium* (actinobacter)

Nematoda (roundworm)

- *Ancylostoma duodenale*
- *Ascaris lumbricoides* (strongylid)
- *Brugia pahangi*
- *Heligmosomoides polygyrus* (strongylid)
- *Litomosoides sigmodonitis*
- *Necator americanus* (strongylid)
- *Nippostrongylus brasiliensis* (strongylid)
- *Strongyloides ratti* (strongylid)
- *Trichinella pseudospiralis* (trichinellid)
- *Trichinella spiralis* (trichinellid)
- *Trichuris muris* (trichinellid)
- *Trichuris suis* (trichinellid)
Cestoda

- *Schistosoma mansoni*

Trematoda (flatworm)

- *Fasciola hepatica*

Kinetoplastid (protozoa)

- *Trypanosoma cruzi*

Apicomplexa (protozoa)

- *Leishmania infantum*
- *Leishmania major*
- *Plasmodium chabaudi chabaudi*
- *Plasmodium berghei*
- *Toxoplasma gondii*
# TABLE OF CONTENTS

1. INTRODUCTION.................................................................1

1.1. Helminth disease............................................................1

1.2. The characteristics of helminth infection.................................4

   1.2.1. *H. polygyrus*..........................................................5
   1.2.2. *N. brasiliensis*........................................................6

1.3. Immune responses to helminth infections......................................7

1.4. Mechanisms of worm expulsion................................................9

   1.4.1 IL-4 and IL-13..........................................................11
   1.4.2. Goblet cells, intelectins and the FIZZ family......................13
   1.4.3. Epithelial cells..........................................................15
   1.4.4. Mast cells and IL-9....................................................17
   1.4.5. Neutrophils, eosinophils and IL-5...................................18
   1.4.6. B cells and antibodies.................................................19
   1.4.7. Summary.................................................................22

1.5. Regulation during helminth infections.......................................23

   1.5.1. Regulatory T cells and their markers..............................24
   1.5.1.1. CD25.................................................................28
   1.5.1.2. Foxp3.................................................................29
   1.5.1.3. CTLA-4............................................................31
   1.5.1.4. GITR.................................................................32
   1.5.1.5. TGF-β..............................................................35
   1.5.1.6. IL-10.................................................................37
1.5.1.7. CD103 ................................................................. 39
1.5.2. Regulatory T cell function ........................................ 40
1.5.3. Summary ................................................................. 42

1.6. The effect of helminth-induced regulation on bystander responses ........ 43
1.6.1. The hygiene hypothesis ............................................. 43
1.6.2. The mechanisms involved .......................................... 45
1.6.3. The impact of nematode infections on bystander responses ...... 46
1.6.4. Summary ................................................................. 47

1.7. The effect of helminth-induced regulation on concurrent infections .... 47
1.7.1. Negative effects of helminths on co-infection .................... 48
1.7.2. Beneficial impact of helminths on co-infection .................. 49
1.7.3. Summary ................................................................. 50

1.8. Thesis plan ................................................................. 52

2. MATERIALS AND METHODS ............................................ 55

2.1. Animals ................................................................. 55

2.2. Parasites, antigens and allergens .................................... 55
2.2.1. Parasite maintenance and life cycles ............................ 55
2.2.2. Parasite antigen and allergen preparations .................... 56
2.2.2.1. H. polygyrus and N. brasiliensis antigen ................. 56
2.2.2.2. Der p1 .......................................................... 56
2.2.2.3. Ovalbumin .................................................... 58

2.3. Models ................................................................. 58
2.3.1. H. polygyrus infection ............................................ 57
2.3.1.1. H. polygyrus time course.................................................................58
2.3.1.2. H. polygyrus infection and in vivo depletion.................................59

2.3.2. H. polygyrus infection and allergen-induced airway inflammation........62
2.3.2.1. Cure after sensitisation.................................................................62
2.3.2.2. Cure prior to sensitisation.............................................................64
2.3.2.3. αCD25 treatment prior to sensitisation............................................65

2.3.3. Re-infection and co-infection.............................................................65
2.3.3.1. Re-infection.................................................................................65
2.3.3.2. Co-infection.................................................................................66

2.4. Differential cell counts..........................................................................68

2.5. Proliferation assays and cell culture.......................................................69
2.5.1. Proliferation assays............................................................................69
2.5.2. Cell expansion...................................................................................70

2.6. Detection of chemokines, cytokines and antibodies by ELISA...............70
2.6.1. Cytokine and chemokine detection by ELISA.......................................70
2.6.2. Antigen-specific isotype detection by ELISA.......................................73

2.7. Cell isolation using magnetic beads.......................................................74
2.7.1. CD4⁺ T cell isolation..........................................................................74
2.7.2. Real-time PCR..................................................................................75
2.7.3. CD4⁺CD25⁺ and CD4⁺CD25⁻ isolation................................................76

2.8. Flow cytometric analysis.......................................................................77

2.9. Statistical analysis.................................................................................79
# 3. CHARACTERISATION OF TREGS DURING *H. POLYGYRUS* INFECTION

## 3.1. Introduction

## 3.2. Results

- **3.2.1. *H. polygyrus* generates a typical Th2 response early in infection**
- **3.2.2. Regulatory cytokine dynamics**
- **3.2.3. Expansion of CD25⁺ T cells during infection**
- **3.2.4. *In vitro* suppressive activity by CD25⁺ Tregs**
- **3.2.5. FoxP3 expression levels remain relatively constant during *H. polygyrus* infection**
- **3.2.6. Increased frequency of CTLA-4 and GITR expression levels on both CD4⁺CD25⁻ T cells during *H. polygyrus* infection**
- **3.2.7. CD103 and TGF-β expression is raised in frequency and intensity by *H. polygyrus* infection**
- **3.2.8. Increased CD103 expression is more marked in CD25⁺FoxP3⁺ cells, while increased TGF-β⁺ expression is restricted to the CD25⁺FoxP3⁻ population**

## 3.3. Discussion

---

# 4. *IN VIVO* DEPLETION OF REGULATORY MARKERS

## 4.1. Introduction

- **4.1.1. TGF-β**
- **4.1.1.1. General characteristics**
- **4.1.1.2. Effect of TGF-β on leukocytes**
- **4.1.1.3. TGF-β and immunoregulation**
- **4.1.2. IL-10**
4.1.2.1. General characteristics .............................................................. 130
4.1.2.2. Effect of IL-10 on leukocytes ........................................................... 132
4.1.2.3. IL-10 and immunoregulation ...................................................... 133
4.1.3. CD25 ............................................................................................. 135
4.1.4. Hypotheses and aims ........................................................................ 137

4.2. Results .................................................................................................... 138

4.2.1. TGF-β is involved in the regulatory networks apparent during H. polygyrus infection.......................................................... 138
4.2.2. IL-10 does not play a unique role during H. polygyrus infection .............. 141
4.2.3. αCD25 treatment alone during H. polygyrus infection does not alter worm burdens but does affect regulatory markers .................................................................. 146
4.2.4. αCD25 and αTGF-β treatments during H. polygyrus infection do not alter worm burdens but do affect regulatory markers ................. 155

4.3. Discussion ............................................................................................... 163

4.3.1. TGF-β ........................................................................................ 163
4.3.2. IL-10 ............................................................................................ 168
4.3.3. TGF-β and IL-10 ........................................................................ 171
4.3.4. CD25 ............................................................................................ 173
4.3.5. CD25 in combination with other Treg markers ..................................... 176
4.3.6. Concluding remarks and ideas for future research ............................... 177

5. HELMINTH INFECTION AND ALLERGY ...........................................................179

5.1. Introduction ............................................................................................. 179

5.1.1. Allergy — a brief introduction ................................................................ 179
5.1.2. Infection and allergy ............................................................................ 182
5.1.3. Regulation during infection and allergy.................................185
5.1.4. Tregs during infection and allergy..........................................187
5.1.5. Hypotheses and aims.............................................................188

5.2. Results......................................................................................189

5.2.1. Allergic airway inflammation in *H. polygyrus*-infected animals treated with anthelmintics after sensitisation.................189
5.2.2. Cytokine responsiveness in mice treated with anthelmintics after sensitisation does not differ significantly from non-treated animals.........................................................194
5.2.3. Effect of allergic airway inflammation, in treated and untreated *H. polygyrus*-infected mice, on CD25, FoxP3 and CD8 expression in TLN and MLN.................................................199
5.2.4. CD103 expression is upregulated in mice with a prolonged *H. polygyrus* infection, and mice cured of infection prior to airway challenge.........................................................203
5.2.5. The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is not affected by helminth eradication prior to sensitisation with OVA.................206
5.2.6. Cytokine responsiveness in mice treated with anthelmintics prior to sensitisation does not differ significantly from non-treated animals.........................................................209
5.2.7. *H. polygyrus* infection, whether it has or has not been eradicated prior to sensitisation, does not affect CD25 and FoxP3 expression in TLN cells, after allergic airway sensitisation and challenge.........................................................213
5.2.8. CD103 expression increases in TLN and MLN from sensitised mice infected with *H. polygyrus*, but not in animals cleared of infection prior to sensitisation.................................................216
5.2.9. Effect of αCD25 treatment on *H. polygyrus*-induced reduction of airway sensitivity.................................................................................................................................219
5.2.10. Cytokine responsiveness increases in mice treated with αCD25 after eradication of *H. polygyrus* infection prior to sensitisation with OVA.........................................................223
5.2.11. CD103 expression increases in TLN cells from animals having been treated with αCD25 after administration of anthelmintics.................................................................227

5.3. Discussion................................................................................230
5.3.1. Timing of infection ............................................................... 230
5.3.2. The effect of anthelmintic treatment ........................................ 232
5.3.3. Treg involvement ............................................................... 233
5.3.4. T cell trafficking ............................................................... 235
5.3.5. The timing of anthelmintic treatment ...................................... 236
5.3.6. Concluding remarks and ideas for future research .................... 238

6. SECONDARY INFECTION WITH \textit{H. POLYGYRUS} .................. 239

6.1. Introduction ........................................................................... 239
   6.1.1. IL-4 and IL-13 ............................................................... 241
   6.1.2. The gut mucosa ............................................................. 244
   6.1.3. IL-4 and IgE ................................................................. 245
   6.1.4. IL-3, IL-4, IL-9 and mastocytosis .................................... 247
   6.1.5. IL-5 and eosinophilia .................................................... 248
   6.1.6. Hypotheses and aims ..................................................... 250

6.2. Results ..................................................................................... 251
   6.2.1. Secondary \textit{H. polygyrus} infections leads to reduced worm burdens as compared to primary infections ................................................................. 251
   6.2.2. Cytokine responsiveness in \textit{H. polygyrus} infection, after re-infection ............ 255
   6.2.3. CD25 and CD103 expression increases in both re-infected groups .............. 259

6.3. Discussion ............................................................................... 262
   6.3.1. Worm burdens during secondary \textit{H. polygyrus} infections ................. 262
   6.3.2. Cytokine responses during secondary \textit{H. polygyrus} infections .............. 265
6.3.3. Treg markers during secondary H. polygyrus infections

6.3.4. Concluding remarks and ideas for future research

7. CO-INFECTION WITH H. POLYGYRUS

7.1. Introduction

7.1.1. Helminth – virus co-infection

7.1.2. Helminth – bacteria co-infection

7.1.3. Helminth – apicomplexan co-infection

7.1.4. Helminth – helminth co-infection

7.1.5. Hypotheses and aims

7.2. Results

7.2.1. Pre-existing H. polygyrus infection delays expulsion of N. brasiliensis

7.2.2. Cytokine responsiveness in H. polygyrus infection, N. brasiliensis infection and co-infection

7.2.3. After 7 days, CD25, CD103 and TGF-β are increased in co-infected animals compared to animals with N. brasiliensis infection alone

7.2.4. By d42 post-co-infection, there is no parasite-specific IL-4 production by CD4⁺ T cells from any of the groups, however in H. polygyrus-infected and co-infected animals, H. polygyrus-specific IL-10 was produced

7.3. Discussion

7.3.1. Effect of co-infection on worm burdens

7.3.2. Effect of co-infection on cytokine production

7.3.3. Effect of co-infection on Treg markers

7.3.4. Effect of co-infection on CD4⁺ T cell cytokine production

7.3.5. Concluding remarks and ideas for future research
8. CONCLUDING DISCUSSION ................................................................. 307
8.1. Regulation induced by helminths .......................................................... 307
8.2. The impact of helminths in the field .................................................... 312
9. REFERENCES ............................................................................. 315
10. PUBLISHED RESEARCH ................................................................. 341
LIST OF FIGURES

Chapter 1

Figure 1.1 – *H. polygyrus* lifecycle.................................................................5
Figure 1.2 – *N. brasiliensis* lifecycle.................................................................6
Figure 1.3 – The three main types of adaptive immune responses.......................8
Figure 1.4 – Summary diagram of the role of Tregs during *H. polygyrus* infection ....51

Chapter 2

Figure 2.1 – *H. polygyrus* infection and *in vivo* depletion...............................60
Figure 2.2 – *H. polygyrus* infection and allergen-induced airway inflammation ....63
Figure 2.3 – *H. polygyrus* infection and other infections..................................67

Chapter 3

Figure 3.1 – Elevated Th2 cytokine responsiveness in *H. polygyrus* infection........86
Figure 3.2 – Nascent Th1 responsiveness in *H. polygyrus* infection....................89
Figure 3.3 – *H. polygyrus* generates a strong regulatory cytokine response ........91
Figure 3.4 – Expansion of CD25-expressing cells within the total CD4⁺ T cell population during *H. polygyrus* infection.................................................................94
Figure 3.5 – CD4⁺CD25⁺ T cells from *H. polygyrus*-infected mice show increased potency of suppression of CD25⁻ effector cell proliferation ..............................................97
Figure 3.6 – CD4⁺ T cell Foxp3 expression levels remain relatively constant during *H. polygyrus* infection ......................................................................................100
Figure 3.7 – CTLA-4 and GITR expression levels increase on CD4⁺CD25⁻ cells, but not on CD4⁺CD25⁺ cells, during *H. polygyrus* infection .................................104
Figure 3.8 - CD103 and TGF-β expression is raised in frequency and intensity by infection...

Figure 3.9 - By day 28 of infection, CD103*FoxP3* cells increase whilst TGF-β*FoxP3* do not.

Chapter 4

Figure 4.1 - αTGF-β treatment during H. polygyrus infection significantly decreases Treg marker expression.

Figure 4.2 - αIL-10R treatment during H. polygyrus infection has no significant effect.

Figure 4.3 - αCD25 treatment before or during H. polygyrus infection does not affect worm burdens.

Figure 4.4 - Cytokine responsiveness in H. polygyrus infection, after αCD25 treatment.

Figure 4.5 - CD103 expression increases in CD4*CD25* but not CD4*CD25* cells after αCD25 treatment prior or during H. polygyrus infection.

Figure 4.6 - αTGF-β and αCD25 treatment early in H. polygyrus infection do not affect worm burden.

Figure 4.7 - Cytokine responsiveness in H. polygyrus infection, after αCD25 and αTGF-β treatment.

Figure 4.8 - CD103 expression increases in CD4*CD25* cells from αCD25-treated animals, but decreases in CD4*CD25* cells from animals having received αCD25 and αTGF-β treatment.
Chapter 5

Figure 5.1 – The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is also observed in animals treated with anthelminthics after sensitisation with OVA or Derp1..........................................................193

Figure 5.2 – Cytokine responsiveness in mice treated with anthelminthics after sensitisation does not differ significantly from non-treated animals..............................198

Figure 5.3 – Effect of allergic airway inflammation, in treated and untreated *H. polygyrus*-infected mice, on CD25, Foxp3 and CD8 expression in TLN and MLN..........................................................202

Figure 5.4 – CD103 expression, independently of Foxp3 expression, is increased in the TLN and MLN from mice with a prolonged *H. polygyrus* infection as well as mice having been cured from the infection, prior to airway challenge...........................................205

Figure 5.5 – The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is also observed in animals treated with anthelminthics prior to sensitisation with OVA..........................................................208

Figure 5.6 – Cytokine responsiveness in mice treated with anthelminthics prior to sensitisation does not differ significantly from non-treated animals...........................................212

Figure 5.7 – *H. polygyrus* infection, whether it has or has not been eradicated prior to sensitisation, does not affect CD25 or Foxp3 expression in TLN cells after allergic airway sensitisation and challenge........................................................................215

Figure 5.8 – CD103 expression increases in TLN and MLN from sensitised mice infected with *H. polygyrus*, but not in animals cleared of infection prior to sensitisation..........................................................218

Figure 5.9 – Effect of αCD25 treatment on *H. polygyrus*-induced reduced airway sensitivity...........................................................................................................................222

Figure 5.10 – Cytokine responsiveness increases in mice treated with αCD25 after eradication of *H. polygyrus* infection prior to sensitisation with OVA .........................226
Figure 5.11 - CD103 expression increases in TLN cells from animals having been treated with αCD25 after administration of anthelminthics..................229

Chapter 6

Figure 6.1 - Secondary *H. polygyrus* infections led to reduced worm burdens as compared to primary infections.................................254
Figure 6.2 - Cytokine responsiveness in *H. polygyrus* infection, after re-infection........258
Figure 6.3 - CD25 and CD103 expression increases in both re-infected groups..........261

Chapter 7

Figure 7.1 - Pre-existing *H. polygyrus* infection delays expulsion of *N. brasiliensis*........284
Figure 7.2 - Cytokine responsiveness in *H. polygyrus* infection, *N. brasiliensis* infection and co-infection.................................................................288
Figure 7.3 - After 7 days, CD25, CD103 and TGF-β are increased in co-infected animals compared to animals with a single *N. brasiliensis* infection..............................................291
Figure 7.4 - By d42 post-co-infection, there is no parasite-specific IL-4 production by CD4+ T cells from any of the groups, however in *H. polygyrus*-infected and co-infected animals, *H. polygyrus*-specific IL-10 was produced..............................295
LIST OF TABLES

Chapter 1

Table 1.1 – Cells and molecules associated with helminth resistance.............................10
Table 1.2 – Treg-associated markers.......................................................................27

Chapter 2

Table 2.1 – Characteristics of antibodies used for detection of cytokines and chemokines by ELISA.................................................................72
Table 2.2 – Characteristics of antibodies used for flow cytometry.................................78

Chapter 4

Table 4.1 – Pleitropic effects of TGF-β on leukocytes (adapted from Li et al., 2006)........126
1. INTRODUCTION

1.1. Helminth disease

When listing major global afflictions, helminths (parasitic worms belonging to the Nematoda and Platyhelminth phyla) are not usually highly ranked, and in comparison to malaria, human immunodeficiency virus (HIV) and tuberculosis (TB), they do indeed have far lower mortality rates. However, their impact on health should not be underestimated: helminths severely affect human health as well as livestock. Over 2 billion individuals are infected with helminths; 300 million of those are severely ill with children and young women most affected (Anonymous, 2004). Infection is often chronic and re-infection frequent; long-lasting immunity is rare. Helminthiasis are a group of diseases with highly variable symptoms. They are mainly found in impoverished communities where levels of sanitation are low. Indeed, 80 % of schistosomiasis cases occur in Africa (Southgate et al., 2005), and studies in South Africa have shown that the main source of helminth infection is most probably involuntarily exposure through contamination of food and drinking water (Adams et al., 2005).

Numerous studies have demonstrated the risk of serious disease resulting from helminth infection in children (Bundy & Cooper, 1989; Crompton et al., 2003). Symptoms range from growth retardation, to reduced physical activity and impaired educational performance. However, the World Bank has stated that cost effective
interventions could efficiently control the morbidity in these individuals (Crompton et al., 2003).

In order to combat helminth disease, the World Health Assembly resolution 54.19 was passed in May 2001 declaring the intent of the World Health Organization (WHO) member states to implement a combined strategy for the control of morbidity caused by schistosomiasis and soil-transmitted nematodes (Colley & Evans, 2004; Crompton et al., 2003). The WHO proposed regular anthelmintic treatment for individuals at greater risk of high morbidity, including children, women and those exposed occupationally. Interestingly, it has been postulated that anthelmintic treatment not only clears parasites, but also affects immunologic changes to the normal host-parasite relationship. Resulting immune responses are thought to lead to both increased resistance (protection against re-infection), and increased immunoregulatory mechanisms that control morbidity upon subsequent re-infections (Colley & Evans, 2004).

Helminth infection may also significantly impact on host susceptibility to other pathogens. Evidence from human field studies suggests that treating with anthelmintics increases general health, and may lead to stronger immune responses to other pathogens such as malaria, HIV and TB (Anonymous, 2004). Recent research conducted on the interactions between helminth infections and HIV indicates their relationship may have created an opportunity for more rapid infection by HIV, as well as quicker progression to AIDS in areas where helminths are endemic (Fincham et al., 2003). Also, responses to BCG vaccinations, which provide
protection against TB, are reduced in helminth-infected people (Elias et al., 2001), and mouse models have shown that anthelmintic treatment prior to vaccination was beneficial to the long lasting effects of a malaria vaccine (Su et al., 2006). Immune down-modulation by helminths therefore has important implications for the treatment of other diseases in helminth-endemic areas.

For successful treatment, an organised strategy is required involving well-planned and careful use of high quality anthelmintic drugs by trained personnel (Crompton et al., 2003). Although treating pregnant women and children is made easier by their attendance of hospitals and schools, not all aspects are as simple. Countries affected by helminths tend to have reduced healthcare budgets and minimal infrastructure, which can prove a major obstacle in trying to implement eradication/treatment programs (Gyapong & Twum-Danso, 2006). Also, generic drugs produced in these countries, whilst cheap as well as easy to manufacture and store, are not always up to standard: some have been found to contain little or no active compound (Savioli et al., 2004). Problems with drug resistance in the near future may add further difficulties, although some groups (Southgate et al., 2005) are already looking into alternatives to praziquantel, a compound commonly used to treat schistosomiasis (reviewed in Utzinger & Keiser, 2004). All these factors emphasise the urgent need for research into the effects of helminths on the immune system.
1.2. The characteristics of helminth infection

Helminths are a polyphyletic group comprised of nematodes (round worms) and platyhelminths (flat worms). The phylum Nematoda is composed of 20,000 species, with varied habitats and life strategies. Of the 20,000 known species, 15,000 are parasites. Parasitic nematode species infect both plants and animals, and have highly diverse life-cycles. Many nematode species are parasites of mammals, including the intensely studied Strongyloidea, such as *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* and Trichinellida including *Trichuris muris* and *Trichinella spiralis*.

Murine hosts are appropriate systems to model immune regulation of parasitic nematode infection (Artis, 2006). Two species commonly used in murine infection models of helminthiasis are *Heligmosomoides polygyrus* (gut-dwelling species) and *Nippostrongylus brasiliensis* (tissue-migrating species) (Gause *et al.*, 2003).
1.2.1. *Heligmosomoides polygyrus*

*H. polygyrus* is a strongylid nematode which causes chronic infection in its murine host (Figure 1.1). Free-living third stage larvae are ingested orally and encyst into the intestinal mucosa, forming granulomata – particularly in resistant mice – where they develop into fourth stage larvae, feeding on host tissue during their development (Sukhdeo *et al.*, 1984). This phase is characterised by a strong inflammatory response. Adults emerge into the gut lumen in the proximal third of the small intestine (Monroy & Enriquez, 1992), where they remain attached to the mucosa and feed on living tissue (Bansemir & Sukhdeo, 1994). Adult parasites can survive in the gut for several months – depending on the mouse strain infected – producing eggs, which are expelled in the faecal content. The eggs require a moist environment to develop into free-living, third stage larvae which await ingestion by a murine host. Secondary infections into the same host leads to diminished worm burdens (Urban *et al.*, 1991a).

Figure 1.1 – *H. polygyrus* lifecycle
1.2.2. *Nippostrongylus brasiliensis*

*N. brasiliensis* is a natural parasite of rats but can, albeit less successfully, also infect mice (up to 47% larvae only reach maturity, as compared to 75% in rats) (reviewed in Ogilvie & Jones, 1971). Unlike *H. polygyrus*, it causes acute infection (Figure 1.2) with similar infection and migration patterns to the hookworm species *Ancylostoma duodenale* and *Necator americanus* (Gause et al., 2003). After percutaneous (or experimental subcutaneous) infection, third stage larvae migrate to the lung. Here, the larvae moult into fourth stage larvae and by the third day, travel to the intestine via the trachea and oesophagus. Adults develop by day 7 and females start to produce eggs. Worms feed by absorption from the gut. Egg production continues until day 10, whereby parasites are expelled and hosts are immune to re-infection. The eggs require a moist environment to develop into free-living, third stage larvae which await percutaneous infection into a host (Ogilvie & Jones, 1971).

![Figure 1.2 - N. brasiliensis lifecycle](image-url)

Figure 1.2 - *N. brasiliensis* lifecycle
Both these nematode species have been intensely researched to understand the effects of helminths on their host. This has been achieved mostly through the study of resistance mechanisms.

1.3. Immune responses to helminth infections

Immune responses to pathogens have typically been characterised as either Th1 or Th2, although regulatory mechanisms are now also known to play an important part in these processes (Figure 1.3).

Briefly, Th1 responses are generally initiated by the host in response to intracellular pathogens such as bacteria (*e.g.* *Mycobacteria*), viruses and protozoa. However, Th1 responses are also responsible for perpetuating autoimmune diseases. These proinflammatory reactions are characterised mainly by the production of inflammatory cytokines such as IFN-$\gamma$, and the activation of Th1 cells which ultimately lead to the recruitment of cytotoxic lymphocytes, macrophages and natural killer cells, and induce the death of the infected cells.

Th2 responses (*c.f.* Section 1.4) are mainly generated in the presence of parasitic worms, and as a result of hypersensitivity reactions. The Th2 immune responses lead to the activation of Th2 T cells, which produce such cytokines as IL-4, IL-5, IL-9, and IL-13, and the production of IgE as well as the activation of eosinophil, mast cells and goblet cells. These mechanisms promote parasite clearance and, if directed against an environmental antigen, lead to the development of allergy.
Intracellular pathogens

Th1 RESPONSE
- Proinflammatory cytokines
- Th1 T cell
- Cytotoxic lymphocytes
- Macrophages
- Natural killer cells
- Infected-cell death
- Parasite clearance

Pathogens

Treg RESPONSE
- Regulatory cytokines
- Tregs
- Reduced inflammation
- Parasite persistence

Extracellular pathogens

Th2 RESPONSE
- IL-4, IL-5, IL-9, IL-13
- IgE
- Eosinophils
- Mast cells
- Goblet cells
- Parasite clearance

Figure 1.3 – The three main types of adaptive immune responses
Finally, regulatory mechanisms involve the regulatory cytokines TGF-β and IL-10, as well as the activation of regulatory T cells (Tregs) (c.f. Section 1.5). These cells can dampen both excess inflammatory and parasitic-specific immune responses thus limiting pathology to the host but promoting parasite survival.

1.4. Mechanisms of worm expulsion

Many investigators have shown that increased worm expulsion from the gut is reliant upon a coordinated response from numerous cell types and their associated humoral products (reviewed in Else & Finkelman, 1998; c.f. Table 1.1). However, different mechanisms have been linked to different helminth species (reviewed in Maizels & Holland, 1998). Cell types involved include both non-immune cells such as goblet cells and gut epithelial cells as well as immune system cells including lymphocytes, mast cells, eosinophils and neutrophils. Recently macrophages have also been implicated in resistance to re-infection (Anthony et al., 2006). Production of molecules such as intelectins, FIZZ proteins, antibodies and cytokines has proved vital in generating the appropriate immune response. Indeed, Th2 responses, characterised by the expression of cytokines such as interleukin-4 (IL-4), IL-5, IL-9, and IL-13, are important in host protective immunity to many intestinal nematode infections (Kringel et al., 2006; reviewed in Finkelman et al., 1997; Grecis, 1997 and Else & Finkelman, 1998). However, variations between the parasite infection studied and models used (species/strain/gender) have made teasing out their individual contribution a difficult task.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Associated Molecules</th>
<th>Nematode Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cell</td>
<td>IgE, IgG2a</td>
<td>S. mansoni, T. muris</td>
<td>Hernandez et al., 1997 Blackwell &amp; Else, 2001</td>
</tr>
<tr>
<td>Gut epithelial cell</td>
<td>IL-4, IL-13</td>
<td>H. polygyrus, T. muris</td>
<td>Reviewed by Artis, 2006</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>IL-5</td>
<td>Angiostrongylus cantonensis, S. mansoni, Strongyloides venezuelensis, T. spiralis</td>
<td>Reviewed in Maizels &amp; Balic, 2005</td>
</tr>
<tr>
<td>Goblet cell</td>
<td>FIZZ proteins and intelectins</td>
<td>N. brasiliensis, Strongyloides ratti, T. spiralis, T. muris</td>
<td>Reviewed in Khan &amp; Collins, 2004; Artis, 2006</td>
</tr>
<tr>
<td>Macrophage</td>
<td>IL-4R</td>
<td>H. polygyrus</td>
<td>Anthony et al., 2006</td>
</tr>
<tr>
<td>Mast cell</td>
<td>IL-3, IL-4, IL-9</td>
<td>N. brasiliensis, T. muris</td>
<td>Madden et al., 1991 Else et al., 1992; Khan et al., 2003</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>In combination with serum</td>
<td>H. polygyrus</td>
<td>Prowse et al, 1978</td>
</tr>
<tr>
<td>T cell</td>
<td>IL-4, IL-5, IL-9, IL-10, IL-13</td>
<td>H. polygyrus, N. brasiliensis, T. spiralis, T. muris</td>
<td>Reviewed in Else &amp; Finkelman, 1998</td>
</tr>
</tbody>
</table>

Table 1.1 – Cells and molecules associated with helminth resistance
Chapter 1 – Introduction

1.4.1 IL-4 and IL-13

Numerous models whereby IL-4 and IL-13 cytokines were depleted have confirmed their importance during infection. In *H. polygyrus* infection, treatments with a monoclonal antibody to IL-4 (αIL-4) and to the receptor subunit for both IL-4 and IL-13 (αIL-4Rα) during primary or secondary infections have demonstrated a strong role for both these cytokines during worm expulsion (Urban *et al.*, 1991a; Urban *et al.*, 1998). However, the role of cytokines during worm infections varies greatly according to the species studied. Indeed, IL-4 is necessary but insufficient in limiting *H. polygyrus* infection. Treatment with αIL-4 or αIL-4Rα limits worm expulsion (Urban *et al.*, 1991a) and administration of IL-4 induces parasite clearance (Urban *et al.*, 1995). However, the high levels of IL-4 generated during infection are insufficient to clear the nematode (Svetic *et al.*, 1993; Finney *et al.*, submitted; Chapter 3). During *N. brasiliensis*, the opposite is true: IL-4 is sufficient but not necessary to terminate infection. Treatment with IL-4 induces worm expulsion (Urban *et al.*, 1995), whereas administration of αIL-4 does not increase parasite survival within the host (Madden *et al.*, 1991).

It has been postulated that IL-4 and IL-13 act in concert to initiate rapid Th2-cell driven responses, and that although their functions overlap, they perform additive roles (McKenzie *et al.*, 1999). In a model of *T. muris* (mouse whipworm) infection, both IL-4⁻/⁻ (deficient in IL-4) and IL-13⁻/⁻ animals are susceptible to infection, unlike their wild-type littermates who expel the parasites. However, the phenotypes are different with IL-4⁻/⁻ animals showing diminished Th2 responses during the
course of infection, whilst IL-13\(^{-/-}\) animals are capable of generating a Th2 response at later time points. Both these cytokines therefore play important yet different roles in mediating immunity to intestinal helminths (Bancroft et al., 1998). Indeed, research using *N. brasiliensis* as a parasite model has uncovered a unique role for IL-13. This cytokine appears responsible for mucus production by goblet cells (Mckenzie et al., 1998), which are strongly associated with nematode expulsion (Miller, 1987; Pemberton et al., 2004a; Artis et al., 2004).

However, it is important to remember that the *IL-4* and *IL-13* genes are very closely linked (McKenzie et al., 1999). Therefore, in single knock-out animals, the gene which was not knocked out may have been affected, thus confounding the role of both these cytokines. Guo and colleagues have shown that, in their IL-13\(^{-/-}\) animals, IL-4 production is decreased due to cis effects (Guo et al., 2001).

Mouse strain and gender also strongly influence cytokine-mediated immunity during nematode infections. In a model of *T. muris* infection, IL-4 and IL-13 were found to be important for expulsion. However, strong differences exist as to their roles depending on the sex and strain of mouse used: female IL-4\(^{-/-}\) C57BL/6 and male IL-4\(^{-/-}\) BALB/c are naturally susceptible to infection whilst female IL-4\(^{+/+}\) BALB/c animals are naturally resistant. Effects of sex hormones on the Th1/Th2 balance were postulated to account for the gender differences observed. Thus, strain and sex need to be taken into consideration when designing experiments (Bancroft et al., 2000).
1.4.2. Goblet cells, intelectins and the FIZZ family

Goblet cells are mucus-producing cells found in both the gut and the lung (reviewed in Specian & Oliver, 1991 and Rogers, 1994). Indeed, goblet cells and mucus production have long been associated with the expulsion of nematode worms (Miller & Nawa, 1979; Miller, 1987; Koninkx et al., 1988; reviewed in Levy & Frondoza, 1983 and Khan & Collins, 2004). More recently, goblet cells have also been found to produce two molecules important during nematode infection: intelectins (Pemberton et al., 2004a) and FIZZ proteins (also known as RELM (resistin-like molecule) proteins, Steppan et al., 2001; Artis et al., 2004). Recent microarray analyses have shown that both intelectins and FIZZ proteins are highly expressed in mice resistant to *T. muris* and *T. spiralis*, but not in susceptible strains (Artis et al., 2004; Knight et al., 2004; Pemberton et al., 2004a; Datta et al., 2005).

Lectins perform a number of biological functions, which include the regulation of cell proliferation, the recognition of tumour antigens and the innate recognition of carbohydrates present in pathogen cell walls (Wang et al., 2005; Elola et al., 2005; reviewed in Apostolopoulou & McKenzie, 2001; East & Isacke, 2002 and Iwanaga & Lee, 2005). Intelectins are a family of calcium-dependent galactose-binding lectins implicated in mucus secretion (Komiya et al., 1998). Two types of intelectins have been identified, intelectin 1 and 2, found in both mouse and human (reviewed in Artis, 2006). However, only intelectin 2 is produced by goblet cells and increased in resistant mice during *T. spiralis* infection; intelectin 1 is constitutively expressed (Pemberton et al., 2004b). Intelectins are localised in the excretory vesicles within
goblet cells suggesting that they are targeted for secretion into the intestinal lumen (reviewed in Artis, 2006). There is strong evidence for intelectin contribution to parasite expulsion and host resistance in nematode infections, although the effect is considered to be indirect rather than direct (reviewed in Artis, 2006).

FIZZ proteins are small, cysteine-rich proteins (Steppan et al., 2001), which act as effector/regulatory molecules during Th2 immune responses (reviewed in Artis, 2006). Both FIZZ 1 (RELMα) and FIZZ 2 (RELMβ) expression are increased during nematode infections, with FIZZ 1 expressed in macrophages (Loke et al., 2002; Nair et al., 2005; Knight, et al., 2004; Wang et al., 2005), and FIZZ 2 in goblet cells (Zimmermann et al., 2004, Steppan et al., 2001; Artis et al., 2004). It has been postulated that FIZZ proteins may directly bind to the chemosensory organs of the parasites and thus disorientate them (Artis et al., 2004). Also, during nematode infection, induction of FIZZ 2 expression is a highly Th2-cytokine-dependent intestinal response. Although FIZZ 2 expression is not altered in IL-4−/− animals, IL-4R−/− animals show decreased FIZZ 2 expression leading to persistent infections; this implicates IL-13 (Artis et al., 2004).
1.4.3. Epithelial cells

Gut epithelial cells play an important role in the expulsion of nematode worms (reviewed in Artis, 2006). Epithelial cell proliferation can be severely disrupted following helminth infection. In a *T. muris* model, susceptible mouse strains have significantly more proliferating epithelial stem and transit cells during infection, leading to dysregulated epithelial cell proliferation (Artis *et al.*, 1999). However, later studies have shown that this observed increase in cells is not due to increased proliferation, but rather impaired migration (Cliffe *et al.*, 2005). Mouse strains resistant to *T. muris* have a more rapid rate of epithelial cell migration up the crypt column during worm expulsion (Cliffe *et al.*, 2005). In severely combined immunodeficiency (SCID) animals, worm expulsion is observed when cell migration is increased through the blocking of CXCL10 (a chemokine), demonstrating that immunity to nematodes is possible in the absence of adaptive immunity and that regulation of cell turnover alone is enough for worm expulsion (Cliffe *et al.*, 2005).

The idea of an ‘epithelial escalator’ has been proposed whereby increased epithelial cell migration facilitates worm expulsion. Using both IL-4/− and IL-13/− animals, it was demonstrated that IL-13, but not IL-4, plays an important role in these mechanisms (Cliffe *et al.*, 2005). However, this process was described for nematodes present in the large intestine. *H. polygyrus* and *N. brasiliensis* reside in the small intestine, where other mechanisms may play a greater role.
Changes in IL-4 and IL-13 have also been linked to epithelial cell function, which may contribute to worm expulsion (Urban et al., 1998). Worm burdens in animals re-infected with *H. polygyrus* are decreased compared to those with a primary worm infection (Urban et al., 1991a), and is characterised by increased fluid secretion and decreased fluid adsorption in the gut (Shea-Donohue et al., 2001). These changes increase the net movement of fluid and ions in the gut lumen, interfering with the ability of worms to feed on gut mucosa (Bansemir & Sukhdeo, 1994) as well as dislodging the parasites. However, treatment with αIL4-R blocks these changes (Urban et al., 1998), implicating IL-4 and IL-13 in these mechanisms. Recently, work by Anthony and colleagues has suggested that resistance mechanisms to secondary *H. polygyrus* infection also involve macrophages which express the IL-4R (alternatively activated macrophages) (Anthony et al., 2006).

IL-4 and IL-13, albeit by different mechanisms (Zhao et al., 2003), have also been associated with increases in smooth muscle contractility in models of *H. polygyrus* and *T. muris* infection (Goldhill et al., 1997; Shea-Donohue et al., 2001; Khan et al., 2001). This suggests that Th2 cytokines, namely IL-4 and IL-13, may promote worm expulsion by contributing to the ‘weep and sweep’ response to nematode infections (Shea-Donohue et al., 2001).
1.4.4. Mast cells and IL-9

Mast cells play an important role in allergy and anaphylaxis, as well as being involved in wound healing and defense mechanisms against pathogens. Upon activation, they release granules rich in histamine and heparin, although the exact mechanisms by which this occurs remain unknown (reviewed in Sim et al., 2006).

The role of mucosal mast cells in nematode infection has been controversial. Mast cell numbers increase in response to parasite infection in some species (*N. brasiliensis*, Madden et al., 1991), but not in others (*H. polygyrus*, Dehlawi et al., 1987). Their involvement in resistance mechanisms has also differed according to the parasite model used (Dehlawi et al., 1987; Crowle & Reed, 1981; Donaldson et al., 1996; Betts & Else, 1999).

Th2 cytokines are strongly linked to mastocytosis. IL-3 and IL-4 are essential in this process. However, studies depleting these molecules during *N. brasiliensis* infection have found no effect on worm expulsion despite the abrogation of mastocytosis (Madden et al., 1991).

IL-9 plays a critical role in a variety of immunological responses, including intestinal mastocytosis and enhanced resistance to nematode infection (Else et al., 1992; Faulkner et al., 1997; Faulkner et al., 1998). IL-9, with IL-4 and IL-13, influences muscle contractibility. However, contributions of IL-9 to host protection have differed according to nematode infection (Khan et al., 2003).
Neutrophilia and eosinophilia have been directly associated with intestinal inflammatory responses to tissue larvae. Resistance to *H. polygyrus* (reviewed in Monroy & Enriquez, 1992) infection has been transferred to naïve recipients by the administration of immune neutrophils in combination with immune serum (Prowse *et al.*, 1978); IL-5 depletion, leading to decreased eosinophilia, however has no effect on *H. polygyrus* infection (Urban *et al.*, 1991a). The reverse is true of schistosome infection. Depletion of neutrophils during schistosomiasis does not affect resistance of immune animals, whereas depleting eosinophils renders immune animals susceptible (Mahmoud *et al.*, 1975).

Interestingly, neither the neutrophils or eosinophils alone nor serum alone are sufficient to kill *H. polygyrus* larvae. Thus, antibody-dependent eosinophil- and neutrophil-mediated resistant mechanisms may not be efficient in protecting hosts from primary nematode infection due to the lack of protective antibody early in infection (reviewed in Monroy & Enriquez, 1992). Protection against nematodes may therefore require both antibody-mediated and cell-mediated mechanisms aimed at reducing re-infection as well as Th2 cytokine-mediated mechanisms aimed at expelling adult worms.

Although IL-4 and IL-13 are the major cytokine players during helminth infection, other cytokines may also have important roles. IL-5 has pleiotropic functions which include stimulating eosinophil differentiation and activation (Sanderson, 1988). IL-5
and eosinophilia have long been associated with helminth infections, although their role during worm expulsion has been the subject of much debate (reviewed in Maizels & Balic, 2004). However, IL-5 and eosinophils appear to operate against incoming larvae rather than existing worm burdens (reviewed in Maizels & Balic, 2004). Indeed, in vivo studies with numerous helminth models have not confirmed IL-5 or eosinophils as major players in resistance to helminth infection despite their increased levels during infection (Sher et al., 1990a; Urban et al., 1991a; Herndon & Kayes, 1992; Betts & Else, 1999), but they have been implicated in immunity to the larvae of tissue migrating species such as *N. brasiliensis* (Shin et al., 1997; Daly et al., 1999), *Litomosoides sigmodontis* (Martin et al., 2000) and *Strongyloides ratti* (Watanabe et al., 2003).

1.4.6. B cells and antibodies

Although antibody-mediated eosinophils and neutrophils may partly mediate resistance to re-infection (Monroy & Enriquez, 1992), B cells do not appear in general to have a prominent role in resistance to nematode infection (reviewed in Gause et al., 2003 and Else & Finkelman, 1998). However, results have varied in response to the models used.

In *T. muris* (Blackwell & Else, 2001), and *Schistosoma mansoni* infection (Hernandez et al., 1997), B cells are involved in mechanisms of parasite resistance. Indeed, B cell-deficient mice become susceptible to *T. muris*, whilst administering B cells to these animals restores resistance (Blackwell & Else, 2001). During *S.*
mansoni infections, B cells are required for the generation of Th2 responses (Hernandez et al., 1997). Infection with N. brasiliensis however, does not require B cells for parasite clearance; B cell-deficient animals, just as wild-type mice, clear the parasite within 2 weeks (Liu et al., 2002). The role of B cells during H. polygyrus infection has proved less clear. B cell numbers increase early in infection but not once adults are present. B cells also produce Th2 cytokines during infection (Harris et al., 2000). However, the precise role of these cells in host resistance has not yet been defined.

B cells may play a role through the production of antibody. It has long been shown that protective immunity to helminths can be achieved through the administration of immune serum early during infection (reviewed in Wakelin, 1978). Indeed, the production of parasite-specific IgE (Dessein et al., 1981) and IgG1 (Pritchard et al., 1983) during helminth infections has been implicated in nematode resistance. However, in these early studies, whole serum may have contained cytokines such as IL-4 and IL-13 which could have contributed to the protective immunity observed. Also, in studies where antibody has been absent (Else & Grencis, 1996), or animals have been depleted of their antibody response (Jacobson et al., 1977), nematode expulsion is still observed. Finally, antibody responses generated in the absence of IL-4 and IL-13 are not sufficient to expel parasites (Urban et al., 1998). Indeed, IgE production has been strongly linked to IL-4 production in numerous models (Finkelman et al., 1988; Coffman et al., 1989; Sher et al., 1990b; Urban et al., 1991a; McKenzie et al., 1999).
As with murine models, human studies have not provided clear answers as to the role of antibodies in nematode resistance. Some have found antibody titers to correlate with protection (Hagan et al., 1991; Faulkner et al., 2002; Bethony et al., 2005; reviewed in Hagan, 1993), whilst others have not (Seet et al., 2005; reviewed in Watanabe et al., 2005). However, much research is being conducted into vaccination strategies against helminths, especially for veterinary parasites (reviewed in Hein & Harrison, 2005), since although antibodies may not represent the unique source of protection from re-infection, they do appear to participate in this process.

CD80 (B7.1) and CD86 (B7.2) are two surface markers expressed by B cells required for co-stimulation and the expansion of T cell responses (reviewed in Chambers & Allison, 1997 and McAdam et al., 1998). Although antibody production may not play a major role in nematode resistance, studies with CD80- and CD86-deficient animals have demonstrated that B cells may have a significant influence as antigen presenting cells (APC) or Th2 cytokine producers, rather than antibody producers. Indeed, a role has also been demonstrated for both markers in Th2 cell priming (Corry et al., 1994), and during nematode infection (Lu et al., 1994; Greenwald et al., 1997; Subramanian et al., 1997; Urban et al., 2000).
1.4.7. Summary

Numerous studies have demonstrated the role of Th2 cytokines in resistance mechanisms to nematode infection, and as we study more cytokines and their potential roles in nematode infection, the proposed models for nematode resistance mechanisms become more detailed (Fallon et al., 2006). However, in chronic infections such as with *H. polygyrus*, Th2 cytokines and their associated effects are not sufficient to clear the parasite. Regulatory mechanisms must exist which dampen the Th2 responses and allow the parasite to maintain itself within the host.
1.5. Regulation during helminth infections

Chronic helminth parasites develop an intriguing balance with their host’s immune system (reviewed in Maizels & Yazdanbakhsh, 2003 and Mountford & Trottein, 2004). They interfere with immune activation and attack, so promoting their continued survival in an immunologically sufficient environment (Harnett et al., 2005; Smith et al., 2005). There is increasing evidence that the infected host develops a form of immunological ‘tolerance’ to parasite antigens, mediated in part by regulatory T cells, which may selectively mute certain effector mechanisms (King et al., 1993; Hoerauf & Brattig, 2002; Steel & Nutman, 2003; Babu et al., 2006; reviewed in Maizels & Yazdanbakhsh, 2003). Tregs have been associated with the persistence and reactivation of parasitic infections (Hisaeda et al., 2004; Mendez et al., 2004). Indeed, in numerous models of helminth infection (Gillan & Devaney, 2005; Wilson et al., 2005; Baumgart et al., 2006; Taylor et al., 2006; Finney et al., submitted; Chapter 3) the development of Treg phenotype populations has been reported following infection.
1.5.1. Regulatory T cells and their markers

Three main different Treg phenotypes have currently been characterised (reviewed in Akbari et al., 2003): Th3 cells, Tr1 cells and CD4\(^+\)CD25\(^+\) cells. Th3 cells were discovered in the mesenteric lymph nodes (MLN) when CD4\(^+\) cells were induced by oral antigen (Chen et al., 1994). They were found to produce TGF-β, and various amounts of IL-4 and IL-10. Tr1 cells were generated when both human or murine CD4\(^+\) cells were chronically activated in the presence of IL-10 (Groux et al., 1997). Finally, CD4\(^+\)CD25\(^+\) cells were first described as a small fraction of the CD4\(^+\) cell population, and play a critical role in the maintenance of self-tolerance (Sakaguchi et al., 1995).

Natural Tregs express CD25 and FoxP3, employ cell contact-dependent suppressive mechanisms (reviewed in Bluestone & Abbas, 2003) and are a functionally and phenotypically distinct immunoregulatory T cell subpopulation (Kuniyasu et al., 2000). These cells arise during the normal process of maturation in the thymus. They are selected on the basis of specificity for self-antigens, through processes of selective survival and instructive induction (reviewed in Kim & Rudensky, 2006). It has been proposed that in contrast to high affinity interactions with self-peptides, which are likely to favour deletion of autoreactive CD4\(^+\) T cells, lower affinity interactions might favour regulatory T cell formation (reviewed in Shevach, 2000). However, evidence now suggests that the thymic selection of CD4\(^+\)CD25\(^+\) regulatory T cells is extremely sensitive to and dependent upon high affinity interactions between thymocytes and self-peptide MHC (major histocompatibility complex).
complexes (Jordan et al., 2001; reviewed in Picca et al., 2006). These interactions are thought to initiate signalling cascades that induce FoxP3 expression and commit the thymocytes to the regulatory T cell lineages as well as enhancing their survival (reviewed in Kim & Rudensky, 2006).

In contrast, adaptive Tregs are thought to develop from naive (CD25\(^-\), Th0) mature peripheral populations in response to specific stimulatory conditions such as sub-optimal signalling from accessory cells (reviewed in Bluestone & Abbas, 2003). Adaptive Tregs include both Tr1 (Groux et al., 1997) and Th3 (Chen et al., 1994) cells. They produce IL-10 or TGF-\(\beta\), and have induced CD25 expression. Whether these cells also initiate FoxP3 expression remains controversial (Vieira et al., 2004), although \textit{in vitro} CD4\(^+\)CD25\(^-\) T cells can be induced to express CD25 and FoxP3 following stimulation with TGF-\(\beta\) (Chen et al., 2003; Fantini et al., 2004; Wan et al., 2005).

The specificity of Tregs is currently the source of much debate. Natural Tregs express a polyclonal suite of TCRs and are thought to recognise a wide array of self-antigens (Cozzo et al., 2003; Hsieh et al., 2006). However, whether Tregs can recognise foreign antigen, and the extent of their repertoire for such antigens remains unknown. Strong evidence from both murine infection models (schistosomiasis: McKee & Pearce, 2004; Hesse \textit{et al.}, 2004, leishmaniasis: Belkaid \textit{et al.}, 2002 and \textit{Helicobacter pylori} infection: Raghavan \textit{et al.}, 2004) and human studies (HIV: Kinter \textit{et al.}, 2004; Weiss \textit{et al.}, 2004 and hepatitis C virus (HCV): MacDonald \textit{et al.}, 2002; Cabrera \textit{et al.}, 2004) demonstrates that natural Tregs can recognise specific
pathogen-derived antigens and that such recognition is essential to their function. It has also been postulated that, due to tissue damage during infection, self-antigens could potentially be presented in a non-tolerogenic way; TCRs selected for self-reactivity could thus cross-react with certain foreign specificities (reviewed in Belkaid & Rouse, 2005 and Rudensky & Campbell, 2006). However, although activation of Tregs is likely to be antigen-specific, the suppression they exert need not require cognate antigen: antigen-specific Tregs can act in a non-specific manner (McGuirk et al., 2002).

Tregs are members of what may be a unique lineage of professional immunoregulatory T cells (Suri-Payer et al., 1998) although no cellular markers are known to be truly specific to Treg cells at present (Fietta & Meloni, 2004). However, a number of markers, such as CD25, FoxP3, CTLA-4, GITR, TGF-β, IL-10 and CD103 have been linked to Treg phenotype and function (Table 1.2).
<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25</td>
<td>Alpha subunit of the IL-2R</td>
<td>Natural Tregs</td>
<td>Asano et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transiently on all activated T cells</td>
<td>Reviewed in Greene &amp; Leonard, 1986</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Transcription factor</td>
<td>Tregs</td>
<td>Review ed in Ziegler, 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some debate as to other cell types</td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Negative co-stimulatory molecule Binds CD80/CD86</td>
<td>Tregs</td>
<td>Review ed in Sansom &amp; Walker, 2006</td>
</tr>
<tr>
<td></td>
<td>Positive co-stimulatory molecule Binds GITRL</td>
<td>Tregs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Precise role unknown</td>
<td>Activated T cells</td>
<td></td>
</tr>
<tr>
<td>GITR</td>
<td></td>
<td>Tregs</td>
<td>Review ed in Shevach &amp; Stephens, 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activated T cells</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Suppressive Th1 cytokine Th2 promoting cytokine</td>
<td>Tregs</td>
<td>Review ed in Moore et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Binds IL-10R</td>
<td>Th2 cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>APCs</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cells</td>
<td></td>
</tr>
<tr>
<td>CD103 (αβ7)</td>
<td>Integrin Binds E-cadherin</td>
<td>Mucosal and skin-homing T cells</td>
<td>Suffia et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Correlates with most active Tregs</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 – Treg-associated markers
Sakaguchi and colleagues were the first to show that CD25 (the α chain of the IL-2 receptor) could be used as a marker for regulatory T cells. They demonstrated that, in vivo, a minor population of T cells (approximately 10% of peripheral CD4+ T cells) which co-expressed the CD25 receptor was crucial in the control of autoreactive T cells (Sakaguchi et al., 1995).

CD25 remains the most widely-used marker for identification of Tregs. However, this marker is expressed by all recently activated T cells and thus is not always associated with the suppressor activity which characterises Tregs (Thornton & Shevach, 1998); induction of CD25 expression on CD25- cells in vitro or in vivo has been shown not to result in suppressive activity (Lehmann et al., 2002) and the stability of its expression remains unknown (Thornton & Shevach, 1998). For these reasons, it is important to analyse additional functional and phenotypic markers, such as the intracellular transcription factor FoxP3, which is essential for Treg development (Fontenot et al., 2003), and the inhibitory/stimulatory co-receptors CTLA-4 (reviewed in Egen et al., 2002) and GITR (Shimizu et al., 2002; McHugh et al., 2002). Expression of FoxP3, as well as the production of the suppressive cytokines IL-10 and TGF-β, are also considered to distinguish subsets of Tregs which have been described as natural or adaptive (reviewed in Bluestone & Abbas, 2003). Finally CD103 is important in Treg migration (Suffia et al., 2005).
1.5.1.2. FoxP3

The transcription factors controlling Treg development have not been currently identified. However, FoxP3 is a transcription factor specifically expressed by Tregs, (reviewed in Ziegler, 2006) and necessary for Treg function and development (Fontenot et al., 2003; Khattri et al., 2001). FoxP3 blocks IL-2, IL-4, or IFN-γ cytokine production in CD4+ T cells in response to T cell receptor (TCR) stimulation, most likely by inhibiting the activity of two other transcription factors, NF-AT and NF-κB (Wu et al., 2006; reviewed in Carson et al., 2006 and Rudensky et al., 2006).

At present, FoxP3 is the only marker used to definitively identify Tregs (Hori et al., 2003); it is a more specific marker than the currently used surface molecules, such as CD25, CTLA-4 and GITR, which are unable to completely discriminate between regulatory T cells and activated, effector or memory T cells (Hori et al., 2003). Indeed, FoxP3 is stably expressed in CD4+CD25+ cells (Fontenot et al., 2003; Khattri et al., 2001) and its expression in naïve T cells can convert these cells to a regulatory phenotype (Chen et al., 2003). It has been proposed that FoxP3 expression in CD4+CD25+ Tregs may be induced by specialised populations of APCs (Powrie & Maloy, 2003). Thus FoxP3 may be a master regulatory gene for cell-lineage commitment or developmental differentiation of Tregs (Hori et al., 2003).

Recently, a FoxP3 reporter system (Fontenot et al., 2005a) has been used to further study the role of this transcription factor in immunoregulatory mechanisms. The green fluorescent protein (gfp) coding sequence has been inserted within exon 2 of
the FoxP3 gene, thus facilitating identification and isolation of FoxP3+ cells. Experiments using the reporter system have found that in naïve mice, FoxP3 expression is mostly limited to CD4+ cells, of which 60-88% expressed high levels of CD25 (Fontenot et al., 2005a). The Rudensky group also demonstrated that FoxP3 expression in T cells correlates with suppressor activity, irrespective of CD25 expression, confirming that FoxP3 is a better marker for Tregs than CD25. However, although IL-2 signalling, and thus CD25, does not appear to be directly required for Treg-mediated suppression, it has been found necessary for sustaining Treg population sizes required for maintaining immune homeostasis and tolerance to self, which helps explain the high percentage of CD25+FoxP3+ cells (Fontenot et al., 2005b).

Small subsets of CD8+FoxP3+ cells, as well as CD4−CD8+FoxP3+ cells were also identified using the FoxP3-gfp reporter system (Fontenot et al., 2005a). However, the suppressive activity of these cell populations has not yet been determined and due to their small size, their contribution to regulatory mechanisms is deemed negligible. Indeed, T cell-specific deletion of FoxP3 is sufficient to induce the identical lymphoproliferative syndrome observed in mice with germline FoxP3 deficiency, confirming the vital role played by FoxP3+ Tregs in maintaining dominant tolerance to self (Fontenot et al., 2005a).
1.5.1.3. **CTLA-4**

CTLA-4 is an inhibitory component of the co-stimulation machinery associated with TCR signalling. It is thought to act by competing for ligand (CD80 and CD86) with the CD28 stimulatory receptor, as well as by raising the activation threshold of T cells through the immunological synapse (reviewed in Egen et al., 2002). The importance of CTLA-4 as a negative regulator of lymphocytes has been demonstrated by the fact that CTLA-4-deficient mice die within 3 to 4 weeks of age from a profound lymphoproliferative disease, which results in immune-mediated damage to multiple organs (Waterhouse et al., 1995; Tivol et al., 1995). However, the mechanisms responsible have not yet been fully defined.

CTLA-4 has been linked to CD4⁺CD25⁺ Treg suppressive activity (Walunas et al., 1994; Takahashi et al., 2000). Indeed, CD4⁺CD25⁺ T cells are the only lymphocytes to constitutively express this marker (Shevach, 2002), and *in vitro* experiments have shown that CTLA-4 signalling is required for the suppressive properties of these cells (Read et al., 2000; Takahashi et al., 2000). However, CTLA-4⁻/⁻ mice possess cells which express FoxP3 and are capable of suppression (Tang et al., 2004). Although this does not rule out a role for CTLA-4 during suppression, it demonstrates that the role is not essential.

Rather than directly mediating suppression, CTLA-4 may also indirectly affect regulation by triggering a suppressive pathway in APCs through the enzyme indoleamine 2,3-dioxygenase (IDO) (Fallarino et al., 2003; reviewed in Mellor et al.,...
2004), resulting in the breakdown of tryptophan which is an essential amino acid for T cell proliferation (Munn et al., 2002).

CTLA-4 has also been implicated in suppressive mechanisms during helminth infection. When CTLA-4 is blocked by antibody treatment in vivo, parasite expulsion is accelerated (McCoy et al., 1997). Interestingly, during helminth infection in humans (Steel & Nutman, 2003) and mouse models (Taylor et al., 2005; Finney et al., submitted; Chapter 3), the CD25−CTLA-4+ phenotype is increased. These cells represent anergic effector cells and their increase during infection may therefore provide another mechanism by which effector responses to helminth parasites are down-modulated by parasites.

1.5.1.4. GITR

GITR is a member of the tumour necrosis factor (TNF) receptor superfamily, and functions as a co-stimulatory molecule for T cell activation (Kanamaru et al., 2004). It is constitutively expressed at high levels on Tregs (McHugh et al., 2002; Shimizu et al., 2002); it is also expressed at low levels on CD4+CD25− cells (Shimizu et al., 2002; Kanamaru et al., 2004; Kohm et al., 2004) as well as B cells, macrophages and dendritic cells (DCs, reviewed in Shevach & Stephens, 2006). Indeed, GITR expression, as with CD25 expression, is not specific to the Treg lineage, although the functional relevance of GITR expression by non-T-cells has yet to be fully understood (reviewed in Shevach & Stephens, 2006).
GITR ligand (GITRL), unlike other TNF receptor ligands which are only expressed at very low levels under steady state conditions (reviewed in Croft, 2003), is constitutively expressed by APCs in the secondary lymphoid organs (Kim et al., 2003; Tone et al., 2003; Stephens et al., 2004). Although the ligation of GITR to GITRL has been implicated in abrogating Treg-mediated suppression (McHugh et al., 2002; Shimizu et al., 2002; Stephens et al., 2004; Ronchetti et al., 2004), the cells and mechanism involved are still the topic of much debate, especially since GITR\(^{-/-}\) mice possess functional Tregs (Ronchetti et al., 2004).

Both \textit{in vitro} and \textit{in vivo} murine studies have demonstrated that the GITR/GITRL signalling pathway is transiently active on T cells following antigen priming (reviewed in Shevach & Stephens, 2006). Addition of agonistic GITR-specific antibodies (McHugh et al., 2002; Shimizu et al. 2002; Stephens et al., 2004) or GITRL (Kim et al., 2003) to co-cultures of suppressor and responder T cells has led to the apparent abrogation of Treg suppressive activity, although it has been argued that GITR ligation in fact promotes the proliferation of Tregs in culture (Stephens et al., 2004). Also, blocking GITRL \textit{in vitro} leads to decreased responder T cell proliferation (Stephens et al., 2004; Kanamaru et al., 2004), whilst promoting GITR signalling with agonistic GITR antibody increases T cell proliferation (McHugh et al., 2002; Kanamaru et al., 2004; Kohm et al., 2004; Ronchetti et al., 2004). Thus, rather than directly turning off Tregs, GITR signalling appears to promote the resistance of responder cells to Treg-mediated suppression (reviewed in Shevach & Stephens, 2006).
Chapter 1 – Introduction

It has been postulated (reviewed in Shevach & Stephens, 2006) that since following TCR engagement, expression of both GITR and GITRL is upregulated on T cells and APCs respectively, initial interactions between GITR and GITRL may promote T cell survival, IL-2 production and effector cell differentiation. As immune responses progress, non-antigen-specific expansion of Tregs could arise due to GITR/GITRL interactions between Tregs (which constitutively express high level of GITR) and APCs in the presence of IL-2, whilst expression of GITR and GITRL returned to basal levels on activated T cells and APCs. The expanded Tregs could therefore suppress the activated effector cells, which display low levels of GITR.

Much research is currently focused on understanding and manipulating the GITR/GITRL interactions, especially in overcoming the host unresponsiveness that often accompanies chronic infection (reviewed in Shevach & Stephens, 2006). Indeed, studies with *L. sigmodontis* have shown that treating infected animals with agonistic GITR antibody in conjunction with αCD25 (CD25-depleting antibody) leads to decreased worm burdens, attributed to increased effector cell function in the Treg-depleted environment (Taylor *et al.*, 2005).
TGF-β has been strongly implicated in immunoregulatory mechanisms and as such has been intensely studied in this context. It has been suggested that the main mechanism by which CD4⁺CD25⁺ Tregs mediate suppression is through their surface bound TGF-β (Nakamura et al., 2001; Nakamura et al., 2004). TGF-β has been linked to the induction and survival of Tregs (Green et al., 2003), and the down-modulation of effector T cell populations (reviewed in Li et al., 2006). It has been found essential for Treg-mediated suppression in numerous models of colitis (Powrie et al., 1994; Read et al., 2000 Liu et al., 2003; Nakamura et al., 2004; Huber et al., 2004) and diabetes (Du et al., 2006). Increases in TGF-β levels during nematode infection have also been reported (Su et al., 2005; Finney et al., submitted; Chapter 3). Most recently, reduction in egg production and worm survival was observed in animals depleted of TGF-β (Doligalska et al., 2006), indicating an important role for this molecule in parasite immune evasion. Also, TGF-β levels have been shown to affect FoxP3 expression (Peng et al., 2004; Schramm et al., 2004; Marie et al., 2005), confirming the model linking TGF-β, FoxP3 and Smad7 – an intracellular protein required for TGF-β signalling (Nakao et al., 2002) – in a negative feedback mechanism (Chen et al., 2003; Coffer & Burgering, 2004). Experiments with models using FoxP3 reporter systems (Wan & Flavell, 2005; reviewed in Kim & Rudensky, 2006) have demonstrated TGF-β-dependent FoxP3 induction in vitro.
However, results demonstrating a role for TGF-β during regulation have been strongly contested. Tregs from TGF-β1−/− animals have all the characteristics of natural Tregs from wild type mice, with markers such as GITR, CTLA-4 and FoxP3 (Mamura et al., 2004) as well as suppressive function in models of colitis (Piccirillo et al., 2002; Mamura et al., 2004; Kullberg et al., 2005). Also, treatment with neutralising αTGF-β does not abrogate Treg-mediated protection (Piccirillo et al., 2002). Thus, despite TGF-β-deficiency, suppressive activity is still apparent. The discrepancies between studies as to the role of TGF-β during regulation appear to be a factor of experimental protocols (Nakamura et al., 2004). Indeed, neutralising αTGF-β may not affect surface-bound protein and thus treatment could impact models differently depending on the importance of the surface-bound protein in mediating suppression. However, although results have been contradictory in studies of autoimmunity, work with helminths has shown that TGF-β is a mediator of suppression during infection (reviewed in Maizels et al., 2004 and Belkaid & Rouse, 2005).
Treg-derived IL-10 is a major determinant in systems where Th1 immune responses are protective: IL-10 was first characterised as a factor generated by Th2 cells that prevents cytokine production by Th1 cells (Fiorentino et al., 1989). IL-10 plays a critical role in downregulating cell-mediated immune hyperactivity. The effects of IL-10 are mainly anti-inflammatory, such as the inhibition of cytokines including IL-1, IL-6, IL-8 and TNF-α (reviewed in Moore et al., 2001). The cytokine has a prominent role in limiting pathology in autoimmune diseases such as colitis (Hagenbaugh et al., 1997; Asseman et al., 1999; Liu et al., 2003), bacterial infections of the gut such as Helicobacter hepaticus infection (Maloy et al., 2003), and also parasitic infections where Th1 responses are important. Due to deregulated production of Th1 cytokines, IL-10−/− mice are unable to clear numerous avirulent strains of both nematodes such as T. muris (Schopf et al., 2002), and protozoa including Toxoplasma gondii (Gazzinelli et al., 1996; Neyer et al., 1997), Plasmodium chabaudi chabaudi (Li et al., 1999), Trypanosoma cruzi (Hunter et al., 1997) and Leishmania major (Belkaid et al., 2001).

However, despite fulfilling a purely downregulatory role in the Th1 setting, IL-10 proves to be a critical promoter of strong Th2 responses in many helminth systems. During T. spiralis infection, IL-10 specifically promotes mast cell maturation, upon which expulsion is dependent (Helmby & Grencis, 2003). In both IL-10−/− animals and fast responders to T. spiralis (animals expel worms within 10 to 12 days) treated with αIL-10, expulsion is delayed. IL-10 also regulates Th1 and Th2 cytokines in
this model: IFN-γ as well as IL-4 and IL-13 levels are elevated in *T. spiralis*-infected IL-10−/− animals. Both Th1 and Th2 responses develop simultaneously without inhibiting or preventing the development of the other in the absence of IL-10.

However, in schistosome infections, which drive dominant type-2 responsiveness, IL-10 does not play a prominent role in Treg-mediated suppression of egg-induced Th cell responses (Baumgart *et al.*, 2006; Taylor *et al.*, 2006). Studies with nematode infections have had similar findings in that they have found no essential role for IL-10 in mediating suppression exerted by parasite-induced Tregs in a model of allergic asthma (Wilson *et al.*, 2005) or inflammatory bowel disease (IBD, Elliott *et al.*, 2004). During *L. sigmodontis* infection, αIL-10 receptor (IL-10R) treatment did not affect filarial survival (Taylor *et al.*, 2005). These results indicate that in the mouse at least, IL-10 is not a primary mechanism for helminth-associated Treg function.
1.5.1.7. CD103

The αE integrin CD103 (Nakamura et al., 2004) is a homing marker expressed by 20-30% of CD4+CD25+ cells in lymphoid organs (McHugh et al., 2002). CD103 interacts with E-cadherin (Cepek et al., 1994) and, unlike CD25, is not induced upon activation (Lehmann et al., 2002). Both CD103+CD25+ and CD103+CD25− subsets have been described as suppressive cells (McHugh et al., 2002; Banz et al., 2003; Huehn et al., 2004). CD103, therefore, may not be a mechanistic mediator of suppression, but rather a pre-requisite for Tregs to traffic into, and remain at, sites of inflammation (Suffia et al., 2005). Hence, CD103 does not define a lineage of CD25+ Tregs with distinct properties, but rather a subset capable of homing into the site of infection. The expression of CD103 is positively regulated by TGF-β (Kilshaw & Murant, 1991; Smith et al., 1994; Hadley et al., 1997; Robinson et al., 2001) and has been strongly correlated with CTLA-4 expression (Lehmann et al., 2002). Therefore, at present, the precise role of CD103 remains undefined, although it has been suggested that CD103 Tregs fulfill the unique function of transferring tolerogenic potential by shuttling between epithelial and lymphoid sites (Lehmann et al., 2002; Suffia et al., 2005).
1.5.2. Regulatory T cell function

CD4⁺CD25⁺ regulatory cells have both anergic (non-proliferative) and suppressive characteristics (Thornton & Shevach, 1998; Takahashi et al., 2003). *In vitro* studies have demonstrated that these cells are hypo-responsive (fail to proliferate) to allogeneic or polyclonal activation (Thornton & Shevach, 1998) and that the suppression (as measured by the decreased proliferation of CD4⁺CD25⁻ cells in the presence of CD4⁺CD25⁺ regulatory cells) they exert is independent of the antigen specificity of the responding T cell population (Thornton & Shevach, 2000).

The anergic state (measured as the non-proliferation of cells in response to TCR stimulation) of CD4⁺CD25⁺ cells observed *in vitro* can be broken by high doses of IL-2 or αCD28 (Itoh et al., 1999). This also abolishes their suppressive properties. However, removal of the IL-2 or αCD28 allows the cells to revert back to their original anergic/suppressive state (Thornton & Shevach, 1998; Takahashi et al., 2003). *In vitro*, the suppressive effect of Tregs on CD4⁺CD25⁻ cell proliferation can also be overcome through the ligation of Toll-like receptors on DCs and production of IL-6 by these same cells (Pasare & Medzhitov, 2003).

The expression of other molecules by DCs also affects regulation: IDO for example has been shown to induce tolerance by altering the proliferation and differentiation programmes of responding T cells. IDO metabolises free tryptophan, essential to T cell proliferation, and also alters translational mechanisms to a more regulatory profile (reviewed in Mellor & Munn, 2004). Thus DCs may partly regulate the
suppression induced by Tregs (reviewed in Powrie & Maloy, 2003).

Suppression by CD4\(^+\)CD25\(^+\) cells *in vitro* requires cell to cell contact, and is independent of soluble factors (Thornton & Shevach, 1998; O'Garra & Vieira, 2003; Ostroukhova *et al.*, 2004). However, the molecular basis of suppression is still currently unknown (O'Garra & Vieira, 2003), and it is not yet clear whether the suppressors target responder cells or APCs (Shevach, 2002). CD4\(^+\)CD25\(^+\) cells appear to suppress proliferation by specifically inhibiting the production of IL-2 in the responder cells (Thornton and Shevach 1998; Shevach, 2002). However, it has been suggested that *in vitro*, the suppressor cells also require activation through their TCR in order to exert suppression (Thornton and Shevach 1998).

At present, the main issue to be resolved is the identification of the molecular pathways responsible for mediating suppression (Shevach, 2002). Indeed, discrepancies have been found between the *in vitro* and *in vivo* work. *In vivo*, suppression is not cell contact dependent but rather due to the secretion of cytokines (reviewed in Thompson & Powrie, 2004). The differences observed may be due to the existence of different CD4\(^+\)CD25\(^+\) subsets, which would suppress by cell contact or secretion of cytokines depending on the milieu within which they find themselves (Shevach, 2002).
Further characterisation of Tregs and their modes of action is imperative, especially in the context of helminth infection. Much work has been carried out in autoimmunity studies (Sakaguchi et al., 2001a; Sakaguchi et al., 2001b), and more research is required in the field of parasitology. Immunological downmodulation during infection can prove important in protecting the host from the more pathological outcomes of infection. A further understanding of these processes will prove vital in developing appropriate treatment strategies for helminth-infected and co-infected individuals living in the developing world.
1.6. The effect of helminth-induced regulation on bystander responses

1.6.1. The hygiene hypothesis

It has previously been observed that, in developed countries, allergies and autoimmune diseases are on the increase, whilst childhood infections are decreasing (reviewed in Rook & Stanford, 1998). The hygiene hypothesis, based on these observations, supposes that the decrease in childhood exposure to common bacterial infections results in a deregulation of the immune system and immune responses (Strachan, 1989; Gerrard et al., 1976). A lack in childhood infections was suggested to impair the development of a Th1 response, allowing exaggerated Th2 responses, such as allergies, to be generated (Strachan, 1989; reviewed in Rook & Stanford, 1998). However, later epidemiological studies have indicated that parasitic helminth infections (Th2-inducing) as well as bacterial and viral infections (Th1-inducing) may also play a role. Indeed, in developing countries, where chronic helminth parasitic infections are prevalent, it has been demonstrated that infected children show reduced allergic sensitivities (reviewed in Yazdanbakhsh et al., 2002). The revised hypothesis therefore includes both Th1- and Th2-inducing childhood infections as having a determining role in regulatory mechanisms which affect the later generation of allergies.
The hygiene hypothesis has been supported by numerous human studies. Helminth infections have been associated with decreased allergic responses in the Gabon (van den Biggelaar et al., 2000), the Gambia (Nyan et al., 2001), Venezuela (Lynch et al., 1993) and Brazil (Araujo et al., 2004). The findings are not limited to the effect of helminths as decreases in allergic phenotype have also been obtained using treatment with suspensions of the bacterium *Mycobacterium vaccae* (Arkwright et al., 2001). Instances also exist whereby helminth infections exacerbate allergic phenotypes (reviewed in Mao et al., 2000). However, discrepancies in the findings could be partly attributed to differences in the level and duration of natural exposure to both allergens during infection and the infection itself. Indeed, in human studies, these variables are extremely difficult to determine, but may play an important part in determining the effect of pathogens on bystander immune responses.

Animal experiments have also found that chronic nematode infections can dampen dysregulated immune responses such as allergic asthma (Wang et al., 2001; Wilson et al., 2005; Kitagaki et al., 2006), diabetes (reviewed in Zaccone et al., 2006), a model of multiple sclerosis in rats (Boles et al., 2000) and IBD (Neurath et al., 1995; Khan et al., 2002; Elliott et al., 2003; Moreels et al., 2004).
1.6.2. The mechanisms involved

Numerous hypotheses have been postulated as to why responses to allergens are inhibited in individuals living in helminth endemic areas (reviewed in Yazdanbakhsh et al., 2002 and Maizels et al., 2004). For some, production of parasite-induced IgE is a key factor and they believe competition exists between helminth-induced polyclonal IgE and aeroallergen-specific IgE for the high-affinity receptors present on mast cells (Hagel et al., 1993). However, recently both human (Selassie et al., 2000) and murine (Wilson et al., 2005) studies have shown that IgE may not explain helminth-induced regulation (c.f. Section 5.1).

It is now widely believed that high levels of regulatory cytokines, such as IL-10 and TGF-β, produced during helminth infections, suppress the immune response to unrelated antigens (reviewed in Yazdanbakhsh et al., 2001 and Yazdanbakhsh et al., 2002). Human studies have found strong negative associations between parasite-specific IL-10 production and allergic reactivity (van den Biggelaar et al., 2000). Murine studies in IL-10−/− mice (Wohleben et al., 2004), or where IL-10 and TGF-β were depleted (Zuany-Amorim et al., 2002) have confirmed a regulatory function for both these molecules. However, these findings have been contested in studies where IL-10 was not found to be a major player in the regulatory network (Wilson et al., 2005; Furze et al., 2006).
Despite the uncertainty as to the involvement of IL-10 in suppressing bystander responses during helminth infections, Tregs have been implicated (Wilson et al., 2005; Kitagaki et al., 2006). It has been postulated that Tregs from chronically infected mice migrate to sites of inflammation, either to directly suppress host immune responses, or to conscript resident cells to a regulatory phenotype (Wilson et al., 2005). However, their regulatory activity on innate mechanisms may be limited (Hadeiba et al., 2003).

1.6.3. The impact of nematode infections on bystander responses

Nematode infections have a profound impact on the immune responses of the host and its ability to respond to bystander antigens/allergens. They also have an effect on the children of helminth-infected mothers (Malhotra et al., 1999). This implies that helminth parasites impact on vaccination strategies in helminth-endemic areas. The regulation induced by these parasites has detrimental effects on the efficacy of vaccines in humans (Sabin et al., 1996; Cooper et al., 1999; Elias et al., 2001; reviewed in Nacher, 2001) and mice (Su et al., 2006). Although responses have been improved by anthelmintic treatment prior to vaccination in some models (Su et al., 2006), others have shown that in human studies, treating with anthelmintics can improve or decrease an individual’s ability to respond to bystander allergens (Lynch et al., 1993; Lynch et al., 1997). These types of study have major implications on the anthelmintic strategies in the developing world.
1.6.4. Summary

In helminth endemic areas, bystander responses are severely affected by parasite-induced regulation in infected hosts. Since drug cures do not protect from subsequent infections, and re-infection or co-infection with other parasites are common in these areas, it is vital we understand what role nematodes play so as to design appropriate vaccination/treatment programs.

1.7. The effect of helminth-induced regulation on concurrent infections

The regulatory environment generated by helminths not only impacts bystander responses in the host, but also affects responses to concurrent infection. Most helminth-infected individuals live in poverty-stricken areas where disease is rife, and are often harbouring more than one parasite. Helminths modulate immune responses such that responses to other pre-existing or incoming parasites are strongly affected, which can be harmful or prove beneficial to the host.
1.7.1. Negative effects of helminths on co-infection

Underlying helminth infections strongly affect the outcome of other infections, including viruses, bacteria, protozoans and helminths. Indeed, studies have shown that the presence of helminths leads to increased severity and viral persistence in both HIV infection (Actor et al., 1993; Borkow et al., 2001; reviewed in Bentwich et al., 1999) and HCV disease progression (Kamal et al., 2001).

Nematodes can also exacerbate concurrent bacterial infections (Brady et al., 1999; Chen et al., 2005). This has been attributed to helminth-induced Th2 responses – IL-4 and IL-13 have been implicated – which impair host-protective immunity by downregulating pro-inflammatory Th1 responses in co-infected animals. Impaired Th1 responses, as well as impaired macrophage-mediated parasite-killing are also responsible for the exacerbation of leishmaniasis in nematode-infected animals (La Flamme et al., 2002).

Nematode-malaria co-infection results in exacerbation of malaria in both human (Spiegel et al., 2003) and murine (Graham et al., 2005; Su et al., 2005) studies. Surprisingly, this was not attributed to increased helminth-specific Th2 cytokines, but rather to increased TGF-β and IL-10 levels. The regulatory network induced by the parasite leads to impaired immune protection (Su et al., 2005).

Due to highly dysregulated cytokine responses, co-infection can also lead to unforeseen results such as severe pathology in pigs infected with *Trichuris suis* and
the bacterium *Campylobacter jejuni* (Mansfield *et al.*, 2003) as well as increased mortality rates in mice infected with *S. mansoni* and *T. gondii* (Kloetzel *et al.*, 1977; Marshall *et al.*, 1999). The observations were not associated with increased parasitaemia.

### 1.7.2. Beneficial impact of helminths on co-infection

Although some studies have found helminth infections to be detrimental to concomitant infections, this is not always the case. Treating HIV patients of their nematode parasites has no beneficial effect on HIV disease progression (Lawn *et al.*, 2000, Elliott *et al.*, 2003 Brown *et al.*, 2004). Thus helminths do not directly impact disease progression in HIV-infected patients. Helminths can even improve the pathology of concurrent infections such as bacterial infections (Fox *et al.*, 2000; Buendia *et al.*, 2002) even when these are pre-existing to the parasite infection (Sacco *et al.*, 2002).

These beneficial effects have been attributed to the skewing of immune responses towards a Th2 bias by helminths, as well as the generation of parasite-specific IgE. Indeed, leishmaniasis is improved with concurrent helminth infection through the production of parasite-specific IgE (Rousseau *et al.*, 1997), which initiates parasite-killing mechanisms (Vouldoukis *et al.*, 1995). Human and murine studies have also demonstrated a beneficial role for helminths in the context of cerebral malaria infections which was linked to parasite specific IgE (Yan *et al.*, 1997; Nacher *et al.*, 2000). In the human study (Nacher *et al.*, 2000), a dose-dependent association
between *Ascaris lumbricoides* and protection from cerebral malaria was observed and attributed to decreased cytoadherence as well as increased IgE levels. In the murine study (Yan *et al.*, 1997), animals co-infected with *Brugia pahangi* and a lethal strain of *Plasmodium berghei* showed prolonged survival as a result of increased parasite-induced Th2 responses, including increased levels of IgE.

1.7.3. Summary

Pre-existing helminth infections have a profound influence on immune responses to other incoming or pre-existing pathogens, whether it be beneficial or detrimental to the host. This critical balance between benefit and harm depends on pathogens which are likely to have evolved strategies to favour Treg priming, recruitment and survival (reviewed in Bradley & Jackson, 2004 and Belkaid & Rouse, 2005). The regulatory network, induced by helminths, biases immune responses to incoming parasites (Figure 1.4). Understanding the characteristics of this network is necessary in order to determine how best to deal with disease symptoms in co-infected individuals.
Figure 1.4 - Summary diagram of the role of Tregs during H. polygyrus infection.
When considering susceptibility, pathology or immunity to pathogens, the contribution of Treg populations can prove decisive. Indeed, these cells, which are known to be induced by helminth parasites, can strongly bias and suppress immune responses. A delicate balance between immune regulatory and effector mechanisms is required to maintain host-parasite homeostasis. Much work has been conducted in trying to use Tregs to treat dysregulations of the immune system such as autoimmune diseases (reviewed in Horwitz et al., 2003 and Ziegler, 2006). However, could these cells also be used for controlling the immunopathology resulting from parasite infection by altering the balance in favour of the host? Answering these questions requires more research into regulatory immune mechanisms in the context of parasite infection, which forms the basis of this thesis.

The strongylid nematode *H. polygyrus* elicits a potent Th2 response in mice, currently thought to be dampened/suppressed by a regulatory population of CD4\(^+\)CD25\(^+\) cells (Finney et al., submitted; Chapter 3). I investigated the role played by these regulatory T cells in downregulating immune responses generated during chronic nematode infections, specifically how these cells are involved in aiding the survival of helminth parasites within their host.

My project has demonstrated that these cells show *in vitro* suppressive activity accompanied by increased levels of surface bound TGF-β1 and CD103. Other regulatory T cell markers, such as CTLA-4 and GITR are not upregulated on
CD4⁺CD25⁺ T cells from infected animals; expression of the Treg-associated transcription factor FoxP3 in this population was not altered by infection (Chapter 3).

Having demonstrated the suppressive nature of these cells \textit{in vitro}, I also studied the mechanisms by which they achieve immunosuppression \textit{in vivo}. I conducted experiments that block CD25, TGF-β and IL-10 signalling in order to determine the key markers for suppression activity \textit{in vivo} (Chapter 4).

Previous work has shown that during infection, CD4⁺CD25⁺ cells can down-modulate bystander responses, such as within a model of allergic airway inflammation (Wilson \textit{et al.}, 2005). I have shown that such regulation is still observed once infection has been cured, indicating that infection imprints immunity and that infection is required to induce but not maintain this regulatory function. In addition, I also investigated the imprinting mechanism by using animals being both sensitised and challenged after they had been cured from infection. This helped determine whether the presence of infection was required during the sensitisation stage or if the infection generated a regulatory response which could then act in the absence of infection to dampen allergic responses (Chapter 5).

Finally, I investigated the effects of both re-infection (Chapter 6) and co-infection (Chapter 7) on the regulatory environment generated through primary \textit{H. polygyrus} infection. This aspect of the project was aimed at understanding the impact of helminths on the immune system of hosts undergoing numerous parasite challenges, as is the case in human patients.
The overall aim of my thesis was to further knowledge into the regulatory mechanisms induced by helminths. These parasites are endemic in many parts of the world where sanitation levels are low, disease prevalence is high and vaccination appears to have little effect. Understanding the impact of the worms on the immune system is key to providing appropriate healthcare for people living in these regions. Also, manipulating regulatory networks may prove vital in treating patients for autoimmune diseases or inflammatory conditions such as IBD or asthma. Thus understanding how helminths achieve this could provide insights into possible treatment strategies.
2. MATERIALS AND METHODS

2.1. Animals

Female BALB/c and C57BL/6 mice, 6-8 weeks of age, were purchased from Harlan (Oxon, UK) or bred in-house (University of Edinburgh, UK). All mice were housed in individually ventilated cages (IVC); all experiments complied with the Home Office 1986 Animals Scientific Act. A minimum of 5 mice per group were used in each experiment.

2.2. Parasites, antigens and allergens

2.2.1. Parasite maintenance and life cycles

_Heligmosomoides polygyrus bakeri_ infective third stage larvae (L3) were kindly donated by Dr. J. Behnke, (University of Nottingham, UK). BALB/c mice were orally gavaged with 200 L3s. Fresh faecal pellets were collected from infected mice from 14 days post-infection and mixed with tap water and charcoal into a thin slurry. The faecal slurry was pasted onto moist filter paper and placed into a large glass Petri dish. The cultures were covered and left at room temperature to stimulate hatching. After 7 days, L3s were collected from the filter paper by rinsing it with...
water. Infective, ensheathed L3s were washed and stored at 4°C until use. *Nippostrongylus brasiliensis* larvae were donated by Yvonne Harcus.

### 2.2.2. Parasite antigen and allergen preparations

#### 2.2.2.1. *H. polygyrus* and *N. brasiensis* antigen

For both *H. polygyrus* and *N. brasiensis* antigen, somatic extracts of mixed sex adult worms were taken up in 1 ml of phosphate-buffered saline (PBS) in the absence of protease inhibitors and homogenised using a ground-glass homogeniser (Jencons, H103/32/324), followed by centrifugation at 10,000 g for 20 minutes. The soluble supernatant fractions were passed through a 0.2 μm filter (Millipore) prior to determining protein concentration using the Coomassie Plus protein assay (Pierce). Antigen was stored at -80°C until use for *in vitro* re-stimulation assays and cell culture. *N. brasiensis* antigen was kindly donated by Yvonne Harcus.
2.2.2.2. Der p1

House dust mite allergen, Der p1, was affinity-purified from spent mite medium (SMM) using the monoclonal antibody 4C1 (INDOOR biotechnologies Ltd, UK). Der p1 was isolated by affinity chromatography from SMM of Dermatophagoides pteronyssinus (provided by the Commonwealth Serum Laboratories, Victoria, Australia) by Andrew Jeske and Dr Melanie Leech (University of Edinburgh). In summary, a 20 % SMM solution with PBS was prepared and stirred overnight at 4°C. Mite extract was collected after centrifugation at 3,000 rpm (1,400 g) for 30 minutes at 4°C, filtered through 0.2 μm filter paper and supplemented with 0.05 % sodium azide. The solution was passed through a sepharose 4B column coupled with Der p1-specific monoclonal antibody 4C1. 4C1 was kindly provided by Dr. M Chapman (Charlottesville, Virginia, USA). Der p1 was allowed to bind for one day, and then rinsed with 5 bed volumes of PBS containing 0.5 M NaCl and 0.05 % sodium azide. Der p1 was eluted using ammoniated water (NH₄OH, elution buffer) at pH 11 and collected directly into fractions of 30 % sterile 0.2 M sodium dihydrogen orthophosphate, to counteract the alkalinity of the elution buffer.

The concentration of Der p1 was calculated using a Bradford protein assay and, if necessary, dialysed in PBS overnight and placed on polyethylene glycol crystals. Der p1 was stored at -80°C until use. For my project, Der p1 was purified by Drs Mark Wilson and Melanie Leech.
2.2.2.3. Ovalbumin

Grade V chicken egg ovalbumin (OVA, Sigma-Aldrich, 9006-59-1) was used for experiments.

2.3. Models

2.3.1. H. polygyrus infection

2.3.1.1. H. polygyrus time course

Mice were infected orally with 200 infective ensheathed L3s using a gavage needle. Following oral infection H. polygyrus resides in the intestinal tract (Monroy & Enriquez, 1992) and remains an enteric dwelling nematode, without migrating from the intestinal tract and immediate tissues. Infection was studied at day 7 (d7), 14, 21, 28 and 70; worm burdens were assessed at necropsy by individually counting all live adult worms in the intestinal tract.
2.3.1.2. *H. polygyrus* infection and in vivo depletion

Mice were infected orally with 200 *H. polygyrus* larvae for 28 days. Antibody treatments were prepared in PBS and administered intraperitoneally (i.p.) as follows (Figure 2.1 A, B, C, D):

- αTGF-β: 5 doses of 50 µg, one every two days from d14 (Figure 2.1 A).
- αIL-10: 5 doses of 250 µg, one every two days from d14 (Figure 2.1 B).
- αCD25: one dose of 1mg, 7 days prior to infection, or 14 days after infection (Figure 2.1 C).
- αTGF-β + αCD25: one dose of 1mg of αCD25, 3 days prior to infection, followed by 5 doses of 50 µg of αTGF-β, one every two days from d0 (Figure 2.1 D).

For all antibody-depleting experiments, rat IgG (Sigma-Aldrich, I4131-100MG) in PBS was administered i.p. to the control animals. The control antibody dose matched the depleting antibody dose used.
Figure 2.1 – *H. polygyrus* infection and *in vivo* depletion
Rat α-mouse CD25 monoclonal IgG1 antibody (PC61) was produced in-house from cells kindly provided by Fiona Powrie (University of Oxford). Rat α-mouse IL-10R monoclonal IgG1 antibody was obtained from BD Pharmingen. (1B1.3a, BD Pharmingen). Mouse α-mouse TGF-β (1011.16) was produced in-house from cells kindly provided by Jenny Philips (University of Cambridge). All antibodies were grown in serum-free media in a Vectra cell growth bag (Bio-Vectra), purified on a protein G-sepharose column and dialysed against PBS.

Worm burdens were assessed at necropsy. Faecal egg counts were carried out during infection using a McMaster Chamber (Weber Scientific International Ltd). Briefly, fresh faecal samples were obtained from each animal. Samples were weighed and incubated for one hour at room temperature with 22.5 ml water per gram of faeces. An equal volume of saturated salt solution was added to the mixtures after an hour, and 800 μl of the resulting solution was placed in the chamber and left for 1 minute to allow eggs to float to the surface. Eggs were counted using a dissection microscope at maximum magnification (X 50).
2.3.2. *H. polygyrus* infection and allergen-induced airway inflammation

2.3.2.1. *Cure after sensitisation*

Mice were infected orally with 200 *H. polygyrus* larvae. Mice were immunised by i.p. inoculation with 10 μg OVA (BALB/c) or Der p1 (C57BL/6) adsorbed to 9 % potassium alum (Sigma-Aldrich, A7167) on day 28 of infection, and boosted again with 10 μg OVA or Der p1 in alum i.p. 14 days later (d42). From day 47, mice were treated three times (at two day intervals) with an anthelmintic (pyrantel embonate, Pfizer). Faecal pellets were collected and examined for *H. polygyrus* eggs on the final day of treatment to ensure parasite clearance. A third sensitisation was administered 2 weeks after the first anthelmintic treatment (d65). On days 79 and 82, mice were anaesthetised i.p. with 13 μl/g body weight of avertin (a tribromoethanol anaesthetic). Mice were subsequently given two airway challenges with 10 μg of OVA or Der p1 in PBS by the intratracheal (i.t.) route. Mice were killed 24 hours after the final airway challenge to assess airway inflammation (Figure 2.2 A). The reagents, doses and kinetics were used following characterisation within the Lamb group (Jarman & Lamb, 2004). The duration of infection (28 days) prior to the first allergen sensitisation was established by our group to address questions relating to chronic helminth infections and allergic reactivity (Wilson *et al.*, 2005).
Figure 2.2 – *H. polygyrus* infection and allergen-induced airway inflammation
2.3.2.2. Cure prior to sensitisation

Mice were infected orally with 200 *H. polygyrus* larvae. From day 28 of infection, mice were treated three times (at two day intervals) with an anthelmintic pyrantel embonate. Faecal pellets were collected and examined for *H. polygyrus* eggs on the final day of treatment to ensure parasite clearance. Mice were immunised by i.p. inoculation with 10 µg OVA adsorbed to 9 % potassium alum a week later, and boosted again with 10 µg OVA in alum i.p. 14 days later (d50). On days 63 and 66, mice were anaesthetised i.p. with 13 µl/g body weight of avertin. Mice were subsequently given two airway challenges i.t. with 10 µg of OVA in PBS. Mice were killed 24 hours after the final airway challenge to assess airway inflammation (Figure 2.2B).

Experiments were conducted in BALB/c animals sensitised and challenged with OVA even though this model proved more variable than the C57BL/6 model using Der p1 as an allergen. However, Der p1 is an expensive compound and could not be used for further experiments. Ragweed was experimented with so as to replace Der p1: it is cheaper, more widely available and also a natural allergen which can be used to sensitise and challenge C57BL/6 mice. Unfortunately, ragweed, mainly due to its formulation, proved a weak stimulus and high levels of allergic airway inflammation were never observed using this allergen (data not shown).
2.3.2.3. \( \alpha \text{CD25} \) treatment prior to sensitisation

Mice were infected orally with 200 \( H. \text{polygyrus} \) larvae. From day 28 of infection, mice were treated on three consecutive days with pyrantel embonate. Previously, treatment was administered at two-day intervals for reasons of dosage. However, treating on consecutive days was later found to be safe and effective. Faecal pellets were collected and examined for \( H. \text{polygyrus} \) eggs on the final day of treatment to ensure parasite clearance. Treatment with 1mg of \( \alpha \text{CD25} \) (or rat IgG control, Sigma-Aldrich) was administered on day 53. Three days later, mice were immunised by i.p. inoculation with 10 \( \mu \text{g} \) OVA adsorbed to 9 % potassium alum, and boosted again with 10 \( \mu \text{g} \) OVA in alum i.p. 14 days later (d70). On days 84 and 87, mice were anaesthetised i.p. with 13 \( \mu \text{l/g} \) body weight of avertin. Mice were subsequently given two airway challenges i.t. with 10 \( \mu \text{g} \) of OVA in PBS. Mice were killed 24 hours after the final airway challenge to assess airway inflammation (Figure 2.2 C).

2.3.3. Re-infection and co-infection

2.3.3.1. Re-infection

Mice were infected orally with 200 infective \( H. \text{polygyrus} \) larvae. Twenty eight days later animals were treated for infection on three consecutive days with pyrantel embonate. Faecal pellets were collected and examined for \( H. \text{polygyrus} \) eggs on the final day of treatment to ensure parasite clearance.
Ten days after the first treatment, animals were re-infected with 200 *H. polygyrus* larvae. Experiments were terminated 14 days after the secondary infection; worm burdens were assessed at necropsy (Figure 2.3 A).

2.3.3.2. Co-infection

Mice were infected orally with 200 infective *H. polygyrus* larvae. Twenty eight days later, animals were infected subcutaneously with 300 infective *N. brasiliensis* larvae. Co-infection was studied after 7, 14 and 42 days of *N. brasiliensis* infection; worm burdens were assessed at necropsy (Figure 2.3 B).
Cure Infection with *H. polygyrus* 1.3 larvae

**Figure 2.3** – *H. polygyrus* infection and other infections
2.4. Differential cell counts

In the models of allergic airway inflammation, 24 hours after the final airway challenge, mice were terminally anaesthetised with ketamine (Vetalar V, Pharmacia & Upjohn) and Xylazine (Rompun 2%, Bayer). The trachea was cannulated using an Abbocath-T sheath (Abbott, Ireland) and internal airspaces were initially lavaged with 500 μl of sterile PBS, followed by two 350 μl washes.

Bronchioalveolar lavage fluids (BALF) were centrifuged at 300 g, and pellets recovered for cellular analysis. The supernatants of the initial 500 μl of BALF were stored at -80°C for biochemical analyses. Cytospins were prepared by spinning 5 x 10^5 cells onto poly-(L-lysine) coated slides (BDH, UK) followed by Diff Quick® (Boehringer, UK) staining. Differential cell counts were performed by microscopy at 100 X magnification. The identification of eosinophils, neutrophils, macrophages and lymphocytes was based on their morphology and staining characteristics; a minimum of 200 cells were counted for each slide.
Chapter 2 – Materials and methods

2.5. Proliferation assays and cell culture

2.5.1. Proliferation assays

All *in vitro* cultures were performed in RPMI 1640 medium supplemented with penicillin (100 U/ml, Gibco UK, 15140-122), streptomycin (100 μg/ml, Gibco UK, 15140-122) and glutamine (4 mM, Gibco UK, 25030-024), as well as 10 % foetal calf serum (FCS) (Gibco UK, 21985-023), unless otherwise stated. Single cell suspensions from lymph nodes or spleen were incubated for 48 hours at 37°C; this time point was chosen for consistency with previous work from our group (Wilson *et al.*, 2005). Quadruplicate samples (or triplicate if cell numbers were low) were incubated in 200 μl, at 1x10⁶ cells/well of a round-bottomed 96-well plate. Proliferation in response to media (unstimulated control), *H. polygyrus* antigen (10 μg/ml), *N. brasiliensis* antigen (10 μg/ml), OVA (Sigma-Aldrich, 10 μg/ml), Der p1 allergen (10 μg/ml), or Concanavalin A (mitogen used as positive control, Sigma-Aldrich, C-0412, 1 μg/ml) was measured by the addition of 1 μCi of [³H] thymidine for 18 hours after a 48-hour incubation. The timing of thymidine addition and the cell densities used were previously established and optimised by our group (Wilson *et al.*, 2005). Supernatants were collected after 48 hours of culture, and stored at -80°C for cytokine analysis. [³H]-thymidine incorporation was used as an indication of cellular proliferation: [³H]-thymidine incorporation into cellular DNA was captured on glass fibre filter mats (PerkinElmer/Wallac-1450-421) coated with
scintillator sheets (PerkinElmer/Wallac-1450-411) and measured in a scintillation counter.

2.5.2. Cell expansion

All in vitro cell expansion cultures were performed in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and glutamine (4 mM), as well as 10 % FCS, unless otherwise stated. Single cell suspensions from mesenteric lymph nodes (MLN) were incubated for 72 hours at 37°C in order to obtain sufficient antigen-specific cytokine-producing cells. Cells were spun down and collected for staining (c.f. Section 2.8).

2.6. Detection of chemokines, cytokines and antibodies by ELISA

2.6.1. Cytokine and chemokine detection by ELISA

Cytokine levels were determined in culture supernatants collected after 48 hours of incubation, and in ex-vivo BALF samples. Cytokines were measured by enzyme-linked immunosorbent assay (ELISA) according to suppliers' guidelines. Between each step the plates were washed five times with PBS/Tween.

ELISA capture monoclonal antibodies were prepared in 0.06 % carbonate buffer (0.1 mM NaHCO₃, pH 9.6) and 50 µl were added per well of an ELISA plate (NUNC,
Immunoplate MaxiSorp, NUNC, Denmark). Plates were incubated overnight at 4°C (for concentrations, cf. Table 2.1). Following incubation, plates were blocked with 200 µl/well of 5% bovine serum albumin (BSA) in carbonate buffer and incubated for 2 hours at 37°C.

Standards were prepared for each cytokine (Table 2.1) using doubling dilutions of recombinant cytokine for IL-4, IL-10, IFN-γ (R&D Systems, Minneapolis, MN), IL-5, IL-9, IL-13, and eotaxin (BD Pharmingen). For each plate, a standard consisting of 16 doubling dilutions was added. As a negative control, 50 µl of media or PBS was added to represent culture supernatants and BALF samples respectively. Finally, 50 µl of sample (cell culture supernatant or BALF) was added to remaining wells.

Following incubation and washing, 50 µl/well of matched biotinylated detection antibody (Table 2.1) was added and incubated at 37°C for 2 hours. Streptavidin-alkaline phosphatase (50 µl/well at 1 µg/ml for IL-4, IL-5, IL-10, IFN-γ and eotaxin) or streptavidin-HRP (50 µl/well at 1 µg/ml for IL-9 and IL-13) were added and incubated at 37°C for a further hour.

Finally, plates were washed and 100 µl of p-nitrophenyl phosphate substrate (Sigma-Aldrich) was added to plates previously incubated with streptavidin-alkaline phosphatase (IL-4, IL-5, IL-10, IFN-γ and eotaxin) and 50 µl of ABTS peroxidase substrate system (KPL, 50-62-00) was added to plates previously incubated with streptavidin-HRP (IL-9 and IL-13).
<table>
<thead>
<tr>
<th>Monoclonal Capture Antibodies (concentration for coating plate)</th>
<th>Clone and Supplier</th>
<th>Top Standard Concentration</th>
<th>Biotinylated Monoclonal Detection Antibodies (concentration for coating plate)</th>
<th>Clone and Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (2 μg/ml),</td>
<td>11B11, in house</td>
<td>8 ng/ml</td>
<td>5 μg/ml</td>
<td>BVD6-24G2, Pharmingen</td>
</tr>
<tr>
<td>IL-5 (1 μg/ml),</td>
<td>TRFK5, Pharmingen</td>
<td>25 ng/ml</td>
<td>2 μg/ml</td>
<td>TRFK4, Pharmingen</td>
</tr>
<tr>
<td>IL-9 (5 μg/ml)</td>
<td>229.4, donated</td>
<td>40 ng/ml</td>
<td>1 μg/ml</td>
<td>D9302C12, Pharmingen</td>
</tr>
<tr>
<td>IL-10 (4 μg/ml)</td>
<td>JES5-2A5, Pharmingen</td>
<td>10 ng/ml</td>
<td>2 μg/ml</td>
<td>SXC-1, Pharmingen</td>
</tr>
<tr>
<td>IL-13 (2 μg/ml)</td>
<td>38213, R&amp;D</td>
<td>16 ng/ml</td>
<td>0.1 μg/ml</td>
<td>Rabbit polyclonal, PeproTech</td>
</tr>
<tr>
<td>IFN-γ (2 μg/ml)</td>
<td>R46A2, in house</td>
<td>100 ng/ml</td>
<td>0.5 μg/ml</td>
<td>XMG1.2, Pharmingen</td>
</tr>
<tr>
<td>Eotaxin (0.4 μg/ml)</td>
<td>CCL11, R&amp;D</td>
<td>2 ng/ml</td>
<td>0.4 μg/ml</td>
<td>CCI11, R&amp;D</td>
</tr>
</tbody>
</table>

Table 2.1 – Characteristics of antibodies used for detection of cytokines and chemokines by ELISA
Once colour had developed, optical densities were read at 405 nm. Cytokine concentration was determined by intrapolation from the standard curve. Levels measured are expressed in nanograms per millilitre $\pm$ standard error of the mean (SEM), unless otherwise stated. The buffers, substrates and concentrations of reagents used were adopted from previously published protocols from this laboratory (Lawrence et al., 1995) or from in house testing and optimisation.

2.6.2. Antigen-specific isotype detection by ELISA

Antigen-specific IgG1 and IgG2a antibodies were determined by ELISA. Unless otherwise stated, serum samples were collected on days 7 and 28 after infection and diluted in Tris-buffered saline (TBS)/Tween. Wells were washed five times between each incubation step with TBS/Tween. Flat-bottomed 96-well ELISA plates were coated with 4 $\mu$g/ml of *H. polygyrus* antigen diluted in carbonate buffer (0.1 mM NaHCO$_3$, pH 9.6). After washing, non-specific binding was blocked by incubating wells with 5 % BSA in carbonate buffer, for 2 hours at 37°C. Doubling dilutions of serum from 1:10 to 1:1,280 were then added and plates were incubated overnight at 4°C. For IgG1, HRP-conjugated rat $\alpha$-mouse IgG1 (BD Pharmingen, UK) was added at 1:6,000 dilution, HRP-conjugated rat $\alpha$-mouse IgG2a (BD Pharmingen, UK) was used at 1:4,000 dilution. Following a 2-hour incubation at 37°C, 50 $\mu$l/well of ABTS peroxidase substrate system was added, plates were left for colorimetric change to develop and later monitored at 405 nm.
2.7. Cell isolation using magnetic beads

2.7.1. CD4⁺ T cell isolation

For CD4⁺ T cell purification, 3 x 10⁷ cells in a single cell suspension were incubated with 30 µl of αCD4 (L3T4) microbeads (Miltenyi Biotech, 130-049-201) in HBSS medium (Sigma-Aldrich, H9394) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and glutamine (4 mM), as well as 2 % FCS. Cells were incubated for 15 minutes at 4°C and washed. Bead-bound cells were separated using the mini-MACS magnets with MACS MS separation columns (Miltenyi Biotech, 130-042-201) with pre-separation filters (Miltenyi Biotech, 130-041-407). All Miltenyi Biotech reagents were used in accordance with the manufacturer’s recommendations. Positively selected cells (CD4⁺ cells) were retained on the magnetic column, and subsequently eluted in sterile HBSS medium. Samples of purified CD4⁺ cells were stained with fluorescently labelled antibodies for flow cytometry (c.f. Section 2.8) to determine purity; cells were typically greater than 95 % pure.
2.7.2. Real-time PCR

Cells were washed and spun at 13,000 rpm for 20 minutes. RNA was recovered from purified CD4⁺ cells by the direct addition of Trizol (Invitrogen, 1 ml/10⁶ cells), and later extracted following the manufacturer’s protocol. Using standard techniques, reverse-transcription PCR (RT-PCR) was performed with 1 µg RNA using MMLV reverse transcriptase (Stratagene) and oligo dT primers (Promega). Relative quantification of the genes of interest was measured by real-time PCR, using the LightCycler (Roche Molecular Biochemicals). Real-time PCR of the housekeeping gene β-actin allowed normalisation of the expression of the genes of interest.

PCR amplifications were performed in 10 µl, containing 1 µl cDNA, 1.2 µl MgCl₂ (25 mM), 0.3 µl (10 µM) primers and 1 µl LightCycler-DNA SYBR Green I mix (10 X). The amplification of β-Actin (5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'), T-bet (5'-GCC AGG GAA CCG CTT ATA TG-3', 5'-GAC GAT CAT CTG GGT CAC ATT GT-3') and Gata-3 (5'-CTA CGG TGC AGA GGT ATC C-3', 5'-GAT GGA CGT CTT GGA GAA GG-3') was performed in the following conditions: 30 s denaturation at 95°C, 5 s annealing of primers at 55°C and 12 s elongation at 72°C, for 40-60 cycles. For T-bet the acquisition temperature was reduced to 84°C. Conventional curve analyses were performed according to the LightCycler instruction kit. Products were run on agarose to ensure no genomic DNA amplification occurred.
2.7.3. CD4^+CD25^+ and CD4^+CD25^- isolation

For CD4^+CD25^+ cell enrichment, CD4^+ cells were first negatively isolated using sheep α-rat IgG beads (M540, Dynal) and biotinylated αMAC1 (0.5 μl/10^7 cells, M1/70.15), αCD8a (0.5 μl/10^7 cells, 53-6.72), αMHC-class II (1 μl/10^7 cells, M5114), αB220 (0.5 μl/10^7 cells, RAB632) and αGR1 (3.33 μl/10^7 cells, RB6-8C5, BD Pharmingen). Cells in HBSS medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (4mM), as well as 2 % FCS were incubated with antibodies for 30 minutes at 4°C. Antibody-bound beads and cell solutions were separated on a magnetic particle concentrator (MPC, Dynal). Positive selection of CD25^+ cells was achieved with PE-conjugated αCD25 (Miltenyi Biotech, 130-091-013) and PE microbeads (Miltenyi Biotech, 130-048-801), on MACS LS separation columns (Miltenyi Biotech, 130-042-401) with pre-separation filters. CD25^- cells obtained were stained with CFSE (5 μM, Sigma-Aldrich) following the method described in Current Protocols in Immunology (Coligan et al., 2003); they were cultured with or without 5 x 10^4 CD4^+CD25^+ cells for 4 days in a 1:1 ratio, with 1 x 10^5 CD4^- irradiated antigen presenting cells (APCs) and in the presence of 1 μg/ml Concanavalin A.
2.8. Flow cytometric analysis

Lymph node and spleen cell suspensions were prepared for flow cytometry at $1 \times 10^7$ cells/ml in supplemented RPMI 1640 medium. Antibodies (Table 2.2) were diluted in PBS, 0.5% BSA, 0.05% sodium azide, and added to cell suspensions (1-2 x $10^6$ total cells) for 20 minutes at 4°C. For detection of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells, rat α-mouse CD4 (L3T4, clone RM4-5, IgG2a, 1/100) and rat α-mouse CD25 (clone PC61, IgG1, CALTAG, 1/100) monoclonal antibodies were used at the dilutions indicated.

For staining intracellular IL-4, IL-10 and CTLA-4, cells were permeabilised in cytofix/cytoperm, washed in perm/wash buffer (BD Pharmingen) and stained with rat α-mouse IL-4 (11B11, rat IgG1, 1/50), rat α-mouse IL-10 (JES5-16E3, rat IgG1, 1/50) or rat α-mouse CTLA-4 (UC10-4F10-11, 1/10) for 30 min. For IL-4 and IL-10 staining, single cell suspensions were stimulated with 50 ng/ml phorbol myristic acid (PMA, a protein kinase C (PKC) activator, Sigma-Aldrich), 1 μg/ml ionomycin (a calcium ionophore, Sigma-Aldrich) and 20 μg/ml brefeldin A (Sigma-Aldrich) for 5 hours at 37°C. Cells were stained for surface markers, IL-4 and IL-10 following incubation.

For staining intracellular FoxP3, cells were permeabilised in cytofix/cytoperm for 1 hr, washed in perm/wash buffer (eBiosciences) and stained with rat α-mouse FoxP3 (FJK-16s, rat IgG2a, eBiosciences, 1/50) for 30 minutes.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone and Supplier</th>
<th>Dilution</th>
<th>Isotypes</th>
<th>Fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>L3T4, RMA-4, BD Pharmingen</td>
<td>1/100</td>
<td>IgG2a</td>
<td>APC-Cy7</td>
</tr>
<tr>
<td>CD25</td>
<td>PC61, Caltag;7D4, BD Pharmingen</td>
<td>1/100; 1/25</td>
<td>IgG1; IgM</td>
<td>PE; Biotin</td>
</tr>
<tr>
<td>GITR</td>
<td>DTA-1, produced in house</td>
<td>1/250</td>
<td>IgG2a</td>
<td>FITC</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>UC10-4F10-11</td>
<td>1/10</td>
<td>NA</td>
<td>PE</td>
</tr>
<tr>
<td>CD103</td>
<td>M290, BD Pharmingen</td>
<td>1/100</td>
<td>IgG2a</td>
<td>FITC</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>A75-3, BD Pharmingen</td>
<td>1/25</td>
<td>IgG2a</td>
<td>Biotin</td>
</tr>
<tr>
<td>FoxP3</td>
<td>FJK-16s, eBiosciences</td>
<td>1/50</td>
<td>IgG2a</td>
<td>APC</td>
</tr>
<tr>
<td>IL-4</td>
<td>11B11, BD Pharmingen</td>
<td>1/50</td>
<td>IgG1</td>
<td>APC</td>
</tr>
<tr>
<td>IL-10</td>
<td>JES5-16E3, BD Pharmingen</td>
<td>1/50</td>
<td>IgG1</td>
<td>PE</td>
</tr>
</tbody>
</table>

Table 2.2 – Characteristics of antibodies used for flow cytometry
Surface bound TGF-β, CD103 and GITR, were detected using rat α-mouse TGF-β1 (A75-3, IgG2a, 1/25), rat α-mouse CD103 (M290, IgG2a, 1/100), rat α-mouse CD8 (53-76, IgG2a, 1/100) and rat α-mouse GITR (DTA-1, IgG2a, produced in-house, 1/250). The expression of surface and intracellular markers was analysed on an LSR II flow cytometer using FlowJo software (Tree Star).

2.9. Statistical analysis

For comparisons involving more than two data sets, data were transformed (log_{10}) and ANOVAs performed if transformation allowed equal variances. When p-values were less than 0.05, results were considered significant and Bonferroni post-tests were performed on the pairs of data sets of interest. Mann Whitney U and Kruskal-Wallis tests were used as indicated for statistical comparisons between groups with unequal distributions, even when transformed. All calculations were performed using GraphPad Prism.
3. CHARACTERISATION OF TREGS DURING H. POLYGYRUS INFECTION

3.1. Introduction

Long-lived helminth parasite infections develop an intriguing balance with the immune system of their host (reviewed in Maizels & Yazdanbakhsh, 2003 and Mountford & Trottein, 2004). Their continued survival in an immunologically sufficient environment may be ascribed in part to interference with immune activation and attack (Harnett et al., 2005; Smith et al., 2005). However, there is increasing evidence that the infected host develops a form of immunological ‘tolerance’ to parasite antigens, which may selectively mute certain effector mechanisms. The possibility that susceptibility to helminth infections may be mediated, in part, by regulatory T cells is supported by recent work showing that antibody treatment to Treg markers results in heightened anti-parasite responsiveness and clearance of adult worms in Litosomosoides sigmodontis infection (Taylor et al., 2005).

Immunological down-modulation during infection is also important in protecting the host from the more pathological outcomes of infection. In the case of schistosomiasis, the release of eggs from vascular worms can cause hepatic granulomatous disease in chronically-infected hosts (Wynn et al., 2004). Immunopathology is controlled initially by the regulatory cytokine IL-10, as IL-10-
deficient (IL-10\(^{-}\)) mice succumb to acute liver inflammation (Wynn et al., 1998), while chronic granulomatous fibrosis can be suppressed by T cells transfected with the transcription factor FoxP3 (Singh et al., 2005) which is functionally associated with regulatory T cell activity (Fontenot et al., 2003; Hori et al., 2003).

The ability of helminth infection to alter the quality of responses to unrelated bystander antigens is well established (Jarrett & Stewart, 1972; Kullberg et al., 1992). More recently, it has been recognised that infection can impact upon the pathologic outcome of autoimmune (Cooke et al., 1999; La Flamme et al., 2003) and allergic (Wang et al., 2001) challenge. In this regard, we recently showed that infection with the murine gastrointestinal nematode *Heligmosomoides polygyrus* dampens immune responsiveness to unrelated allergens (OVA and Der p1) in a manner dependent upon CD25\(^{+}\) T cell activity, and transferrable with CD25\(^{+}\) T cells into a sensitised but uninfected recipient (Wilson et al., 2005). Significantly, the presence of a Th2-biasing helminth infection did not switch the anti-allergen response away from the Th2 phenotype, but suppressed IL-5 and other effector molecules associated with the airway inflammatory response. Moreover, the amelioration of allergy was achieved in the absence of IL-10 (Wilson et al., 2005).

*H. polygyrus* infection has previously been characterised as driving a strong Th2 response (Urban et al., 1991a; Monroy & Enriquez, 1992; Svetić et al., 1993; Wahid et al., 1994). However, chronic infection is also associated with potent down-regulation of immune responsiveness *in vivo* (Fox et al., 2000; Bashir et al., 2002;
Elliott et al., 2004). Our recent finding that CD4⁺CD25⁺ T cells, predominantly positive for FoxP3, from *H. polygyrus*-infected mice can suppress airway allergy (Wilson et al., 2005) confirmed that an active regulatory T cell response was being generated. In the present study, we follow in detail the evolution of both Th2 and Treg parameters over the course of infection. We show that the expanded CD4⁺CD25⁺ population exerts a heightened activity of functional suppression, and that levels of CTLA-4 (CD152) are increased in both CD4⁺CD25⁺ and CD4⁺CD25⁻ cell types. Within the CD25⁺ population, both FoxP3⁺ and FoxP3⁻ subsets appear activated by infection, through heightened expression of CD103 and TGF-β respectively. Thus, this helminth infection expands cell populations with the phenotypic and functional characteristics of regulatory T cells, as well as inducing potentially important changes in other T cell populations.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

### 3.2. Results

#### 3.2.1. *H. polygyrus* generates a typical Th2 response early in infection.

We first examined the cytokine profile of mesenteric lymph node (MLN) and spleen cells in response to parasite antigen challenge *in vitro*, over the course of a 10-week infection of BALB/c mice with *H. polygyrus*. Within two weeks there is a substantial Th2-type response marked by antigen-specific IL-4 (Figure 3.1 A), IL-5 (Figure 3.1 B), IL-9 (Figure 3.1 C) and IL-13 (Figure 3.1 D) evident in the MLN. Th2 responsiveness remains for the life of the infection although there is a general down-modulation after day 21. Responses from the spleen are slower to evolve but follow a similar profile and the overall pattern of Th2 responsiveness to this infection is in accord with previous reports (Urban *et al.*, 1991a; Monroy & Enriquez, 1992; Svetić *et al.*, 1993; Shi *et al.*, 1998).
Figure 3.1 – Elevated Th2 cytokine responsiveness in *H. polygyrus* infection.

Parasite antigen-specific cytokine responses are presented from MLN (Left panels) and spleen (Right) cells taken at days 7-70 post-infection. Cells were cultured for 48 hours in medium alone (open symbols) or *H. polygyrus* antigen (solid symbols). Panels A-D present respectively IL-4, IL-5, IL-9 and IL-13. Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point). The experiment was performed four times.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

A. MLN

B. Spleen

C. Time Post-infection (in days)

D. Time Post-infection (in days)

Figure 3.1 – Elevated Th2 cytokine responsiveness in *H. polygyrus* infection
In contrast, Th1-type responses are relatively feeble. Parasite-specific IFN-\(\gamma\) responses \textit{in vitro} are negligible until the final time point, d70, when infection is waning (Figure 3.2 A). A nascent Th1 reaction at day 7 can be detected at the mRNA level, evident by raised T-BET expression in Real-time PCR (Figure 3.2 B), most probably caused by the exit of worms from the gut mucosa to the lumen resulting in local inflammation. However, by day 28 this Th1-promoting transcription factor has been entirely replaced by the Th2-promoting factor GATA-3 (Figure 3.2 B). A further reflection of the polarisation away from Th1 is in the isotype balance of anti-\(H.\) \textit{polygyrus} serum antibodies. As previously reported (Pritchard \textit{et al.}, 1983), infection stimulates high levels of IgG1, but no detectable IgG2a (Figure 3.2 C).

3.2.2. Regulatory cytokine dynamics.

As we have previously reported (Wilson \textit{et al.}, 2005), MLN cells (MLNC) from \(H.\) \textit{polygyrus} mice respond briskly to parasite antigen challenge \textit{in vitro} with IL-10 release (Figure 3.3 A), which remains elevated over the course of infection. Similar results can be obtained by \textit{ex vivo} staining of MLNC from infected mice for intracellular IL-10 or for surface-bound TGF-\(\beta\) (Figure 3.3 B), each of which are significantly increased at d28 post-infection. These findings are consistent with other reports of elevated antigen-specific and serum TGF-\(\beta\) in mice infected with \(H.\) \textit{polygyrus} (Su \textit{et al.}, 2005; Su \textit{et al.}, 2006).
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

Figure 3.2 – Nascent Th1 responsiveness in *H. polygyrus* infection.

A: IFN-γ release from MLN and spleen cells directly stimulated *ex vivo*, taken at days 7-70 post-infection and cultured for 48 hours in medium alone (open symbols) or *H. polygyrus* antigen (solid symbols). Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point). The experiment was performed four times.

B: T-BET and GATA-3 Real-time PCR in MLN from naïve and infected mice. CD4⁺ T cells were purified from MLN, RNA was extracted and Real-time PCR performed on the resulting cDNA. Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point). The experiment was performed twice.

C: *H. polygyrus*-specific serum antibodies of IgG1 and IgG2a isotypes measured by ELISA. Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point). The experiment was performed once.
Figure 3.2 – Nascent Th1 responsiveness in *H. polygyrus* infection
Figure 3.3 – *H. polygyrus* generates a strong regulatory cytokine response.

**A:** IL-10 release from MLN (Left) and spleen (Right) cells directly stimulated *ex vivo*, cultured as in Figure 3.2 A. The experiment was performed four times.

**B:** Intracellular IL-10 (Left) and surface TGF-β (Right) staining in naïve and d28-infected MLN CD4⁺ T cells determined by flow cytometry. Data represent means and standard errors from groups of 5 mice assayed individually. The experiment was performed once.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

Figure 3.3 – *H. polygyrus* generates a strong regulatory cytokine response
3.2.3. Expansion of CD25$^+$ T cells during infection.

Expression of the IL-2Rα chain, CD25, is a widely-used but not exclusive marker for regulatory T cells (Shevach, 2002). Using flow cytometry, we found that total CD4$^+$CD25$^+$ cell numbers expand dramatically in the draining MLN (Figure 3.4 A), although total lymph node cell counts are expanding during the same period. A smaller rise in CD25$^+$ cells is observed in the spleen, which attains statistical significance from d21 onwards (Figure 3.4 A). The intensity of CD25 expression on positive cells does not alter appreciably (Figure 3.4 B). When considered as a proportion, it can be seen that CD25$^+$ cells also outgrow CD4$^+$CD25$^-$ cells from day 7 onwards (from d14 in the spleen) and remain in significant excess over uninfected controls throughout infection (Figure 3.4 C).
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

Figure 3.4 – Expansion of CD25-expressing cells within the total CD4^+^ T cell population during *H. polygyrus* infection.

A: Total numbers (10^6^) of CD4^+^CD25^+^ T cells from d7-70 post infection in MLN (Left) and spleen (Right). Data represent means and standard errors from groups of 5 mice assayed individually. D0 represents all naïve mice (5 for each time point). Mann-Whitney tests were performed (n.s. = no significant difference, ^*^ = p<0.05, ^**^ = p<0.01). The experiment was performed four times.

B: Representative histograms of CD25 expression within CD4^+^ cells from naïve (grey line) and d28-infected (thick black line) MLNC (Left) and spleen cells (Right); isotype controls are shown as thin black lines.

C: Changes in proportion of CD25-expressing CD4^+^ T cells relative to total CD4^+^ cells over 70 days of infection. For each point in time, the percentages of CD4^+^ T cells which also expressed CD25 among MLN (Left) and spleen (Right) cells from infected and naïve animals were determined; the arithmetic difference between infected and naïve in percentage frequency at each time point was then calculated. Mann-Whitney tests were performed (^*^ = p<0.05, ^**^ = p<0.01). Data represent means and standard errors from groups of 5 mice assayed individually. D0 represents all naïve mice (5 for each time point). The experiment was performed four times.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

**Figure 3.4** – Expansion of CD25-expressing cells within the total CD4+ T cell population during *H. polygyrus* infection
3.2.4. *In vitro* suppressive activity by CD25\(^+\) Tregs.

A common test of functional Treg activity is their ability to block the proliferative response of effector T cell populations to antigen or mitogen stimulation (Thornton & Shevach, 2000). We accordingly assessed whether bead-enriched CD4\(^+\)CD25\(^+\) MLNC from infected animals were able to suppress the proliferation of CFSE-loaded naïve, CD4\(^+\)CD25\(^-\) MLNC, responding to Concanavalin A. We observed that, on a per-cell basis, CD4\(^+\)CD25\(^+\) MLNC, taken 28 days post-infection, were substantially more suppressive than similar phenotype cells from naïve animals (Figure 3.5 A, B).

In parallel experiments we also noted that CD4\(^+\)CD25\(^-\) T cell populations from infected mice showed greater resistance to proliferative inhibition, and indeed were largely refractory to suppression by CD4\(^+\)CD25\(^+\) MLNC from naïve mice (Figure 3.5 C). However, CD4\(^+\)CD25\(^-\) effectors from *H. polygyrus*-infected animals remained susceptible to inhibition by the more 'activated' regulatory cells from infected mice (Figure 3.5 D).
Figure 3.5 – CD4⁺CD25⁺ T cells from H. polygyrus-infected mice show increased potency of suppression of CD25⁻ effector cell proliferation.

Proliferation of 5x10⁴ CD4⁺CD25⁻ T cells in response to in vitro Con A stimulation was measured by CFSE (blue lines), in populations from naïve (top panels = A, B) and d28-infected (bottom panels = C, D) MLNC. In parallel cultures, an equal number of CD4⁺CD25⁺ MLNC were added to the CD4⁺CD25⁻ CFSE-loaded T cells. MLNC from infected mice (red lines, right hand panels = B, D) show more profound suppression than equal numbers of cells from uninfected controls (red lines, left hand panels, A, C). Data represent pooled cells from groups of 5 mice. The experiment was performed three times.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

**A** - Naïve CD4⁺CD25⁻

Naïve CD4⁺CD25⁺

**B** - Naïve CD4⁺CD25⁻

*H. polygyrus* CD4⁺CD25⁺

**C** - *H. polygyrus* CD4⁺CD25⁻

Naïve CD4⁺CD25⁺

**D** - *H. polygyrus* CD4⁺CD25⁻

*H. polygyrus* CD4⁺CD25⁺

---

**Figure 3.5** - CD4⁺CD25⁺ T cells from *H. polygyrus*-infected mice show increased potency of suppression of CD25⁺ effector cell proliferation
3.2.5. FoxP3 expression levels remain relatively constant during *H. polygyrus* infection.

A key marker for natural Tregs is the transcription factor FoxP3, which is expressed by the majority of CD25⁺ T cells (Fontenot *et al.*, 2003; Hori *et al.*, 2003; Fontenot *et al.*, 2005a; reviewed in Sakaguchi, 2005). As previously reported (Wilson *et al.*, 2005), we found that *H. polygyrus* infection results in a modest increase in CD4⁺CD25⁺FoxP3⁺ cells (from 7.4% of all CD4⁺ at d0 to 9.7% at d28; Figure 3.6 A). This expansion occurs rapidly following infection (Figure 3.6 B) although, due to parallel expansion of CD25⁺FoxP3⁻ cells in infection, there is in fact a small diminution in the proportion of CD4⁺CD25⁺ cells which express FoxP3 (Figure 3.6 C). It was also apparent that the intensity of FoxP3 expression within the CD4⁺CD25⁺FoxP3⁺ T cell population does not increase (Figure 3.6 D). Thus, the increment in CD25⁺ cells during infection is not accompanied by preferential FoxP3 induction and represents an expansion of both CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁺FoxP3⁻ T cells. This latter phenotype may represent either an activated effector cell (which could be stimulated in the environment of chronic infection), or a FoxP3⁻ ‘adaptive’ or inducible regulatory T cell.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

Figure 3.6 – CD4⁺ T cell FoxP3 expression levels remain relatively constant during *H. polygyrus* infection.

A: Representative plots of bivariate flow cytometry analysis for CD25 and FoxP3 expression in CD4⁺ T cells in naïve (Far Left), d7-infected (Left) and naïve (Right) and d28-infected (Far Right) MLNC. The experiment was performed twice.

B: Time course of expression of CD25⁺FoxP3⁺ as percentage of total CD4⁺ cells in MLN and spleen from 7-70 days of infection. Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point). Mann-Whitney tests were performed (n.s. = no significant difference, * = p<0.05, ** = p<0.01).

C: FoxP3⁺ T cells as proportion of total CD4⁺CD25⁺ in naïve and d28-infected MLN and spleen. Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point). Mann-Whitney tests were performed (n.s. = no significant difference, ** = p<0.01).

D: FoxP3 expression intensity over the course of infection. The percentage difference in mean fluorescence intensity (MFI) relative to uninfected values for FoxP3 was calculated. Mann-Whitney tests were performed (n.s. = no significant difference, * = p<0.05, ** = p<0.01). Data represent means and standard errors from groups of 5 mice assayed individually; d0 represents all naïve mice (5 for each time point).
Figure 3.6 (A, B) - CD4+ T cell FoxP3 expression levels remain relatively constant during *H. polygyrus* infection.
Figure 3.6 (C, D) - CD4+ T cell FoxP3 expression levels remain relatively constant during *H. polygyrus* infection.
3.2.6. Increased frequency of CTLA-4 and GITR expression levels on both CD4\(^+\)CD25\(^-\) T cells during *H. polygyrus* infection.

Two surface markers closely associated with the Treg phenotype are CTLA-4 (Tang *et al.*, 2004) and GITR (Shimizu *et al.*, 2002). Both were measured in MLN and spleen cells over the 70-day timecourse. At day 28, for example, CTLA-4 is expressed by 23% of CD4\(^+\) T cells, compared to 12% in naïve controls (Figure 3.7 A). This increase is represented by a relatively constant per-cell level within the CD4\(^+\)CD25\(^+\) population, together with a significant rise in expression among CD4\(^+\)CD25\(^-\) cells (Figure 3.7 A). Moreover, within the CD4\(^+\)CD25\(^+\) compartment (Figure 3.7 B) CTLA-4 staining increases more than twofold among the FoxP3\(^-\) subset, while actually declining in FoxP3\(^+\) cells. A similar, though less marked, trend is seen with GITR staining; overall GITR expression within the CD4\(^+\) T cell population rises from 8.5% in naïves to 13.8% in d28-infected mice (Figure 3.7 C). Again there is a significant rise in the proportion of CD4\(^+\)CD25\(^-\) cells expressing GITR, and within the CD4\(^+\)CD25\(^+\) subset the expansion in GITR\(^{\text{high}}\) cells occurs with the FoxP3\(^-\) population (Figure 3.7 D). Thus, the uplift in CTLA-4 and GITR is observed primarily, if not totally, within cells of a non-regulatory phenotype.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

Figure 3.7 – CTLA-4 and GITR expression levels increase on CD4⁺CD25⁻ cells, but not on CD4⁺CD25⁺ cells, during *H. polygyrus* infection.

**A:** CTLA-4 expression measured by flow cytometry on MLNC. Top panels, CTLA-4 and CD25 bivariate plot from naïve and d28-infected mice; Centre panels, percentage of CTLA-4^{high} T cells in the CD4⁺CD25⁻ and CD4⁺CD25⁺ subsets. Bottom, frequency of CTLA-4 expression in CD4⁺CD25⁻ cells over the course of infection. Data represent means and standard errors from groups of 5 mice assayed individually. Mann-Whitney tests were performed (n.s. = no significant difference, ** = p<0.01, *** = p<0.001). The experiment was performed twice.

**B:** CTLA-4 expression measured by flow cytometry on MLNC. Top panels, CTLA-4 and FoxP3 bivariate plot from naïve and d28-infected mice; Centre panels, percentage of CTLA-4^{high} T cells in the CD25⁺FoxP3⁺ and CD25⁺FoxP3⁻ subsets. Data represent means and standard errors from groups of 5 mice assayed individually. Mann-Whitney tests were performed (** = p<0.01). Bottom panels, representative histograms of CTLA-4 and FoxP3 expression on CD4⁺CD25⁻ cells (thick red line represents infected animal, thick blue line represents naïve animal, thin black line represents isotype controls).

**C:** GITR expression, measured as for CTLA-4 in Figure 3.7 A.

**D:** GITR expression, measured as for CTLA-4 in Figure 3.7 B.
Figure 3.7 (A, B) - CTLA-4 and GITR expression levels increase on CD4⁺CD25⁺ cells, but not on CD4⁺CD25⁻ cells, during *H. polygyrus* infection.
Figure 3.7 (C, D) – CTLA-4 and GITR expression levels increase on CD4⁺CD25⁻ cells, but not on CD4⁺CD25⁺ cells, during *H. polygyrus* infection.
3.2.7. CD103 and TGF-β expression is raised in frequency and intensity by *H. polygyrus* infection.

We also examined expression of CD103 (the integrin αEβ7) and of the regulatory cytokine TGF-β in the CD4⁺ populations. In the MLN, there were significant increases in the proportion of CD4⁺CD25⁺ T cells which express CD103, returning to control levels by d70 of infection (Figure 3.8 A); a similar pattern was observed in splenic populations (data not shown). While most CD103⁺ cells were in the CD4⁺CD25⁺ subset, there were also small but significant increases in the frequency of CD103⁺ among CD25⁻ cells. Moreover, infection induced a substantial upshift in intensity of CD103 expression, reaching levels approximately 50% higher than in naïve populations (Figure 3.8 B).

A similar profile was observed in staining for surface-bound TGF-β. A modest, but significant, rise in TGF-β staining occurred in both MLN and splenocytes, peaking at d21-28; thus by d28 nearly 10% of all CD4⁺CD25⁺ T cells were TGF-β⁺, although no change was seen in the very low levels of TGF-β among CD4⁺CD25⁻ cells (Figure 3.8 C). There was also a measurable rise in fluorescence intensity, evident only in the CD4⁺CD25⁺ population, over the first 28 days of infection (Figure 3.8 D).
MLN cells were stained for CD4, CD25, CD103 and TGF-β and analysed by flow cytometry. For CD103 and TGF-β, the percentages within total CD4^+CD25^+ T cell populations were calculated, as well as the percentage change in MFI for each infected group compared to the corresponding naïve group; Mann-Whitney tests were performed (n.s. = no significant difference, * = p<0.05, ** = p<0.01). Data represent means and standard errors from groups of 5 mice assayed individually. DO represents all naïve mice (5 for each time point). The experiment was performed four times.

A: Time course of expression of CD103^+ as percentage of total CD4^+CD25^+ cells in MLN (Left), and as proportion of total CD4^+CD25^+ or CD4^+CD25^- T cells in naïve and d28-infected MLN cells (Right).

B: CD103 expression levels over the time course (Left) and in representative MLN cells from naïve (blue line) and d28-infected (red line) mice (Right); isotype controls are shown as thin black lines.

C: TGF-β expression, measured as for CD103 in Figure 3.8 A.

D: TGF-β expression, measured as for CD103 in Figure 3.8 B.
Figure 3.8 (A, B) - CD103 expression is raised in frequency and intensity by infection
Figure 3.8 (C, D) - TGF-β expression is raised in frequency and intensity by infection
3.2.8. Increased CD103 expression is more marked in CD25⁺FoxP3⁺ cells, while increased TGF-β⁺ expression is restricted to the CD25⁺FoxP3⁻ population.

Since the CD4⁺CD25⁺ population from infected mice displayed increases in both CD103 and TGF-β, co-staining was performed for these markers and for the FoxP3 transcription factor. These analyses showed, firstly, that in naïve MLNC approximately two-thirds of the CD103⁺ cells are FoxP3⁺ (Figure 3.9 A), as are a similar proportion of the TGF-β⁺ cells (Figure 3.9 B). However, infection generated a substantial and significant increase in CD25⁺CD103⁺FoxP3⁺ cells (Figure 3.9 A), while the frequency of TGF-β⁺FoxP3⁺ cells did not differ between naïve and infected mice (Figure 3.9 B). Moreover, we found that both CD103 and TGF-β staining occurs on a significant subset of CD25⁺FoxP3⁻ cells, and indeed comparison of naïve and infected MLN shows significant increases in CD103⁺FoxP3⁻ (Figure 3.9 A) and in TGF-β⁺FoxP3⁻ cells (Figure 3.9 B).
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

Figure 3.9 – By day 28 of infection, CD103\(^*\)FoxP3\(^*\) cells increase whilst TGF-\(\beta\)^*FoxP3* do not.

Cells were stained for CD4, CD25, CD103, FoxP3 and TGF-\(\beta\). Levels of CD103 and TGF-\(\beta\) and FoxP3 expression were determined within the CD4\(^*\)CD25\(^*\) population. Mann-Whitney tests were performed (n.s. = no significant difference, ** = \(p<0.01\)). The experiment was performed twice.

A: CD103 expression plotted by bivariate analysis against FoxP3 staining, in naïve and d28-infected MLNC (Left panels), and percentage expression of CD103\(^*\)FoxP3\(^*\) and CD103\(^*\)FoxP3\(^-\) cells within CD4\(^*\)CD25\(^*\) T cell populations (Right).

B: Surface TGF-\(\beta\) expression plotted by bivariate analysis against FoxP3 staining, in naïve and d28-infected MLNC (Left panels), and percentage expression of TGF-\(\beta\)^*FoxP3* and TGF-\(\beta\)^*FoxP3* cells within CD4\(^*\)CD25\(^*\) T cell populations (Right).
Figure 3.9 - By day 28 of infection, CD103⁺FoxP3⁺ cells increase whilst TGF-β⁺FoxP3⁺ do not
3.3. Discussion

It is now evident that Tregs are active at many points in the control of immune responses against pathogens (Belkaid et al., 2002; Suvas et al., 2003; reviewed in Maloy & Powrie, 2001; McGuirk & Mills, 2002; Mills, 2004; Rouse & Suvas, 2004 and Belkaid & Rouse, 2005). CD4+CD25+ Tregs block protective immunity in animal models of malaria (Hisaeda et al., 2004) and filariasis (Taylor et al., 2005), but are also required to minimise pathology caused by the response to pathogen invasion (Kullberg et al., 2002; Raghavan et al., 2003; Hesse et al., 2004). This critical balance between benefit and harm is played out against a backdrop of pathogens which are likely to have evolved strategies to favour Treg priming, recruitment and survival (reviewed in Belkaid & Rouse, 2005). For the host, the optimal regulatory response may in some cases permit a residual parasite population to survive, so providing ongoing antigenic stimulation without disease (Belkaid et al., 2002). Thus, whether considering susceptibility, pathology or immunity to pathogens, the contribution of Treg populations can prove decisive.

Helminth parasitic diseases are typically slowly-evolving and chronic in nature, often associated with immune downregulation (reviewed in Maizels & Yazdanbakhsh, 2003 and Maizels et al., 2004). Epidemiological and cellular evidence from human lymphatic filariasis, onchocerciasis and schistosomiasis defines a specific downmodulation of immunity which is consistent with the activity of Treg-like cells, with involvement of IL-10, TGF-β and CTLA-4 (King et al., 1993; Babu et al.,...
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

2006; reviewed in Hoerauf & Brattig, 2002; Maizels & Yazdanbakhsh, 2003 and Steel & Nutman, 2003). In animal models of filariasis (Gillan & Devaney, 2005) and schistosomiasis (Baumgart *et al.*, 2006; Taylor *et al.*, 2006) the development of Treg phenotype populations has been reported following infection, whilst in the murine gastrointestinal nematode *H. polygyrus* (Wilson *et al.*, 2005), functional regulation by CD4⁺CD25⁺ T cells exerted a suppressive effect on the bystander response to an allergic provocation. In the current study, we also show that this phenotype is associated with an enhanced capacity, following infection, to suppress the polyclonal proliferative response of CD25⁻ effector cells. However, the origin and specificity of the CD4⁺CD25⁺ population generated by this or other helminth infections have yet to be delineated.

CD25, the α chain component of the surface IL-2 receptor, is not a unique marker for Tregs, being also present on activated effector T cells (Thornton & Shevach, 1998). For this reason, it is important to analyse additional functional and phenotypic markers, such as the intracellular transcription factor FoxP3, which is essential for Treg development (Fontenot *et al.*, 2003), the inhibitory/stimulatory co-receptors CTLA-4 (reviewed in Egen *et al.*, 2002) and GITR (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). Expression of FoxP3, as well as the production of the suppressive cytokines IL-10 and TGF-β, are also considered to distinguish subsets of Tregs which have been described as natural or adaptive (reviewed in Bluestone & Abbas, 2003).
Broadly, natural Tregs arise during the normal process of maturation in the thymus, are selected on the basis of specificity for self-antigens, express surface CD25 and FoxP3 (reviewed in Fontenot & Rudensky, 2005), and employ cell contact-dependent suppressive mechanisms. In contrast, adaptive Tregs are thought to develop from naïve (CD25⁻, Th0) mature peripheral populations in response to specific stimulatory conditions such as sub-optimal signalling from accessory cells. Adaptive Tregs include those designated Tr1 (Groux et al., 1997) and Th3 (Chen et al., 1994), produce IL-10 or TGF-β, and have induced CD25 expression. Whether these cells also initiate FoxP3 expression is controversial: no induction could be found either in Tr1 cells induced in vitro with IL-10, or in vivo by intranasal tolerisation (Vieira et al., 2004). However in vitro CD4⁺CD25⁻ T cells can be induced to express CD25 and FoxP3 following stimulation with TGF-β (Chen et al., 2003; Fantini et al., 2004; Wan & Flavell, 2005), and in a TCR transgenic, thymectomised mouse, de novo FoxP3 expression is induced in a regulatory CD25⁺ population exposed to low-dose antigen delivered by osmotic pump (Knoechel et al., 2005). Hence, FoxP3 expression delimits a subset containing all naturally-arising Tregs and, possibly, a proportion of adaptive Treg cells.

In *H. polygyrus* infection, there is a preferential expansion of CD25⁺ cells without a proportional increase in FoxP3⁺ cells, and yet the functional regulatory activity of the CD25⁺ T cell population is greatly amplified in infected mice. Hence, the increment in CD25⁺FoxP3⁻ T cell numbers is unlikely to represent effector cell expansion alone. A plausible hypothesis is that many of the CD25⁺FoxP3⁻ cells are adaptive
Tregs, with specificity for parasite antigens, which have arisen from naïve precursors with induction of expression of CD25 rather than that of FoxP3. In addition, there is good evidence from our work and that of others, that production of IL-10 and TGF-β is substantially heightened in *H. polygyrus* infection (Su et al., 2005; Wilson et al., 2005).

Treg-derived IL-10 is known to be a major determinant in systems where Th1 immune responses are protective, such as murine infection with *Leishmania major* (Belkaid et al., 2002). In schistosome infections which drive dominant type-2 responsiveness, however, only a small proportion of the IL-10 emanates from CD25⁺FoxP3⁺ T cells (Baumgart et al., 2006; Taylor et al., 2006), and even though IL-10 is important in the overall control of immune pathology in schistosomiasis, granuloma modulation is IL-10-independent (Wynn et al., 1998). Moreover, IL-10 proves to be a critical promoter of strong Th2 responses in many helminth systems (Helmby & Grencis, 2003), and unlike the Th1 setting does not fulfill a purely downregulatory role. In the specific case of *H. polygyrus*, we have previously shown that the ability of CD25⁺ Tregs to suppress airway allergy in infected mice is undiminished by the administration of αIL-10R antibody, and that MLN cells from IL-10⁻/⁻ infected mice can transfer suppression of allergy into uninfected animals (Wilson et al., 2005). Hence, in the mouse at least, IL-10 does not appear to be a primary mechanism for helminth-associated Treg function.
TGF-β, however, remains a credible candidate for the functional Treg product in this system, both with respect to the induction and survival of Tregs (Green et al., 2003), and the downmodulation of effector T cell populations (reviewed in Li et al., 2006). We found significantly raised surface TGF-β staining over the course of infection, and others have reported parasite antigen-specific TGF-β release in similar experiments (Su et al., 2006). Most recently, Doligalska and colleagues have reported that αTGF-β antibody treatment greatly reduces egg production and worm survival in mice (Doligalska et al., 2006), indicating an important role for TGF-β in parasite immune evasion. Interestingly, in our experiments, increased TGF-β was essentially observed within the CD25⁺FoxP3⁻ subset, and not among CD25⁺FoxP3⁺ cells. Hence, the cells induced to express TGF-β may be adaptive Tregs most similar to the Tr1/Th3 type described in other systems, in particular the TGF-β-secreting cells derived from the MLN of orally tolerised mice (Chen et al., 1994).

We also observed a marked increase in CTLA-4 expression among T cells from infected mice. CTLA-4 is an inhibitory component of the co-stimulation machinery associated with T cell receptor signalling, and may act by competing for ligand with the CD28 stimulatory receptor, and by raising the activation threshold of T cells through the immunological synapse (reviewed in Egen et al., 2002). Hence, when CTLA-4 is blocked by antibody treatment in vivo, parasite expulsion is accelerated (McCoy et al., 1997). Interestingly, the more substantial upshift in CTLA-4 staining was seen on CD25⁻ cells, and this change occurred later in the course of infection than the increases in CD25⁺ cell activity. It is plausible, therefore, that the CD25⁻
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

The **CTLA-4** phenotype represents an anergic effector cell, as postulated in both human (reviewed in Steel & Nutman, 2003; Babu *et al.*, 2006) and mouse (Taylor *et al.*, 2005) helminth infections, which develops subsequent to and under the influence of, the more rapidly arising Treg response to infection.

**GITR**, originally identified as a marker up-regulated on Treg cells, is a receptor thought to activate both regulatory and effector cells on ligation. In *H. polygyrus* infection, levels of **GITR** expression increase albeit less than observed for **CTLA-4**. In particular, **CD25** T cells as well as **FoxP3**CD25 cells show small but significant uplifts in the frequency of **GITR** expression. The induction of **GITR** on the **CD25** population is similar to that observed in the tissue helminth infection, *L. sigmodontis* (Taylor *et al.*, 2005).

Tregs may act either, or both, at the induction stages of the immune response and at the inflammatory phase in the tissues. In the *H. polygyrus* model, it has been shown that priming to allergens is unaffected by infection, and that infection-generated Tregs can act in the fully-primed environment to suppress inflammation in the lung (Wilson *et al.*, 2005). The proposition that priming is unaffected in infection is also supported by the prompt and powerful responses to *H. polygyrus* antigens we observed in the current study.
Chapter 3 – Characterisation of Tregs during *H. polygyrus* infection

If infection-induced Tregs act in tissue sites of inflammation rather than in the lymph node foci of induction, this may be reflected in the pattern of homing marker expression (Siegmund *et al.*, 2005; reviewed in Huehn & Hamann, 2005). In this context, the marked upregulation of CD103 on the infected CD4^+^CD25^+^ T cell subset may have particular functional significance. CD103 is a homing marker and is expressed on 20-30% of Tregs in lymphoid organs (McHugh *et al.*, 2002). CD103, therefore, may not be a mechanistic mediator of suppression, but rather a prerequisite for Treg to traffic into, and remain at, sites of inflammation. In a model of leishmaniasis, CD103 is induced and maintained on Tregs following or just prior to their arrival in inflammed tissues (Suffia *et al.*, 2005). Hence, CD103 does not define a lineage of CD25^+^ Tregs with distinct properties, but rather a subset capable of homing into the site of infection. To a lesser extent, we also see increases in CD103^+^CD25^-^ cells (data not shown). Indeed, both CD103^+^CD25^+^ and CD103^+^CD25^-^ subsets have been described as suppressive cells (Banz *et al.*, 2003; Huehn *et al.*, 2004). The expression of CD103 is positively regulated by TGF-β (Robinson *et al.*, 2001). Surface TGF-β levels increase during infection in our system, and this may provide a mechanism by which CD103 is continuously upregulated.

The specificity of Tregs is currently the source of much debate. Natural Tregs express a polyclonal suite of TCRs and are thought to recognise a wide array of self antigens (Cozzo *et al.*, 2003; Hsieh *et al.*, 2006). However, it is still possible that TCRs selected for self-reactivity will cross-react with certain foreign specificities. Evidence from leishmaniasis and schistosomiasis models, as well as human studies with HIV, HCV, and *H. pylori* point to this (reviewed in Belkaid & Rouse, 2005). In
the case of *Leishmania major* infection, natural Tregs (defined as cells expressing CD25 prior to infection) respond to *L. major* antigen by producing IL-10. Furthermore, Treg lines derived from these cells can reactivate disease when transferred into animals carrying a latent *L. major* infection, but not when transferred into *Toxoplasma gondii* infected mice (Suffia et al., 2006). At present the antigen specificity of the Tregs elicited by *H. polygyrus* has not been established. Studies currently under way have indicated *in vitro* expansion of Tregs in response to parasite antigens, but more thorough investigations are required.

Whilst activation of Tregs is likely to be antigen-specific, the suppression they exert need not require cognate antigen. FHA-specific Tr1 cells can suppress Th1 responses to *Bordetella pertussis* and unrelated pathogens (McGuirk et al., 2002), and many other examples exist of antigen-specific Tregs acting in a non-specific manner. Tregs generated during *H. polygyrus* infection appear to have similar characteristics, as CD4<sup>+</sup>CD25<sup>+</sup> T cells from *H. polygyrus*-infected allergen-naive mice can reduce the severity of allergic reactions in uninfected, allergen-sensitised recipients (Wilson et al., 2005).

In conclusion, we show here that *H. polygyrus* induces significant phenotypic changes in distinct subsets of cells, including one with functional suppressive properties and some characteristic markers associated with Tregs. It may be significant that FoxP3<sup>+</sup> population is only one of the players in the system, and we cannot yet distinguish whether ‘natural’ and ‘adaptive’ Tregs expand independently
in the context of this infection, or if the evolution of the adaptive phenotype is dependent upon the pre-existing natural Treg population. Further characterisation of these regulatory cells and their mechanisms is therefore imperative, as is the analysis of the potentially anergic effector T cell population which we have postulated. Nematode infections are of particular importance since they affect over 2 billion people worldwide, mostly in poverty-stricken regions where numerous other infections are rife. Unravelling the effects of dampened immune responses, due to increased regulatory mechanisms triggered by worms, on disease progression and outcome in co-infected people would open new avenues for treatment, control and eradication of these prevalent diseases.
4. IN VIVO DEPLETION OF REGULATORY MARKERS

4.1. Introduction

It has previously been shown that resolution of gastrointestinal helminthiasis is a T cell-dependent process (Jacobson & Reed, 1977; Katona et al., 1988; reviewed in Else & Finkelman, 1998 and Gause et al., 2003). In this Chapter, we attempted to disrupt T cell-dependent regulatory processes to demonstrate their involvement in maintaining parasite numbers during chronic infection. In order to achieve this, we focused on three main targets: TGF-β, IL-10 and CD25.

4.1.1. TGF-β

4.1.1.1. General characteristics

The TGF-β protein family is composed of a set of pleiotropic, multifunctional, secreted signalling molecules which are produced as precursors, later proteolytically cleaved to generate active proteins with unique and potent immunoregulatory properties. The proteins are produced by and act on a wide variety of cell types; they regulate such processes as the control of cell growth and differentiation as well as the regulation of both cellular function and target gene activity (reviewed in Letterio & Roberts, 1998 and Li et al., 2006).
TGF-β proteins belong to the TGF-β superfamily. Three isoforms of TGF-β (TGF-β1, 2 and 3) exist in mammals and share the same receptor complex consisting of a member of each of the TGF-βR1 and TGF-βR2 families; the three isoforms have near identical cellular targets. However, each isoform is under the control of a unique promoter and expressed in a distinct pattern (reviewed in Attisano & Wrana, 1996; Letterio & Roberts, 1998 and Li et al., 2006).

The TGF-β proteins are best known for their roles in development, epithelial growth and differentiation, and in the process of carcinogenesis. However, TGF-β1 is also produced by every leukocyte lineage and is the predominant isoform produced by these lineages. Both its autocrine and paracrine expressions control differentiation, proliferation, and the state of activation of immune cells (reviewed in Letterio & Roberts, 1998 and Li et al., 2006).

TGF-β signalling controls diverse sets of cellular processes such as cell proliferation, recognition, differentiation, apoptosis and specification of developmental fate, in the metazoan phyla (reviewed in Patterson and Padgett, 2000; ten Dijke et al., 2002 and Massague et al., 2000). TGF-β receptor complexes are formed from a heterodimer of two serine/threonine kinase receptors, TGF-βRI and TGF-βRII. TGF-β brings together members from the two families, resulting in the activation of the type I receptor by the type II receptor. The activated type I receptor propagates the signal by phosphorylating Smad proteins (reviewed in Attisano & Wrana, 1996; Massague et al., 1998 and Massague et al., 2000), leading to changes in gene transcription, such as that of FoxP3 (reviewed in Coffer & Burgering, 2004). However, latent
forms of TGF-β (bound to other proteins such as the latency-associated protein) are blocked from receptor binding, and activation of these molecules is an important post-transcriptional control point in both physiological and pathological actions of TGF-β (reviewed in Letterio & Roberts, 1998).

4.1.1.2. Effect of TGF-β on leukocytes

TGF-β has many effects on the different leukocyte cell types (Table 4.1), both stimulatory and inhibitory. TGF-β signalling strongly affects cell proliferation, differentiation and survival, and as such is considered a strong immunomodulator of T and B cells, dendritic cells (DC) and monocytes/macrophages – TGF-β also affects other leukocytes such as natural killer cells, eosinophils, polymorphonuclear cells and mast cells, which are not discussed here.
Chapter 4 – *In vivo* depletion of regulatory markers

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>TGF-β Stimulates</th>
<th>TGF-β Inhibits</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>Survival</td>
<td>Proliferation</td>
</tr>
<tr>
<td></td>
<td>Differentiation (Th17 cells, Tregs)</td>
<td>Effector function</td>
</tr>
<tr>
<td>B cell</td>
<td>IgA switching</td>
<td>Proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Survival</td>
</tr>
<tr>
<td>DC</td>
<td>Langerhans cell development</td>
<td>Maturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>Monocyte</td>
<td>Chemotaxis</td>
<td>Scavenger function</td>
</tr>
<tr>
<td>Macrophage</td>
<td></td>
<td>Effector functions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigen presentation</td>
</tr>
</tbody>
</table>

Table 4.1 – Pleiotropic effects of TGF-β on leukocytes (adapted from Li *et al.*, 2006)

TGF-β influences the differentiation, activation and proliferation of T lymphocytes at all stages of their development. TGF-β typically inhibits IL-2-dependent T cell proliferation (*Kehrl et al.*, 1986). TGF-β can inhibit the production and response to cytokines associated with both the Th1 and Th2 cell subsets, and the production of TGF-β by antigen-specific cells may mark a unique subset, referred to as Th3 (*Chen et al.*, 1994; *Fukaura et al.*, 1996; *Bridoux et al.*, 1997). However, depending on the differentiation or activation status of the cell, TGF-β can also enhance the growth of T cells.

TGF-β inhibits B cell proliferation and may induce apoptosis in both B cells (*Holder et al.*, 1992; *Lomo et al.*, 1995) and in the fully differentiated plasma cells. In addition to its effects on B cell proliferation, TGF-β controls several aspects of the
normal maturation and differentiated functions of B cells, which include the regulation of expression of surface molecules such as the inhibition of immunoglobulin switching to IgG isotypes and the induction of the MHC II receptor on both pre-B and mature B cells. In a broad spectrum of diseases, active TGF-β1/IgG complexes secreted by B cells and plasma cells may also modulate B cell responses and thus mediate immunosuppressive effects on both T cells and neutrophils (Caver et al., 1996; reviewed in Letterio & Roberts, 1998 and Li et al., 2006). Despite its ability to inhibit immunoglobulin synthesis and secretion, under certain conditions, low levels of autocrine TGF-β may also enhance production and secretion of these molecules (reviewed in Letterio & Roberts, 1998 and Li et al., 2006).

TGF-β also influences DC development and function. In TGF-β1+/− animals, epidermal Langerhans cells (LC, a type of dendritic cell) are completely absent (Borkowski et al., 1996). However, normal LC progenitors are found in TGF-β1+/− animals, and paracrine sources of TGF-β1 are sufficient for LC development and perhaps even their migration to the epidermis (Borkowski et al., 1997). Thus, the absence of epidermal LCs cannot be attributed to defects in progenitor populations or homing mechanisms, but rather could be due to TGF-β1 facilitating LC survival or localisation in the epidermis (Borkowski et al., 1996).

TGF-β is secreted by both monocytes and macrophages, and it can regulate a broad spectrum of their activities. These include chemotaxis, host defence, as well as activation and deactivation mediated by the expression of cytokines, their receptors
and small effector molecules such as reactive oxygen species and nitric oxide (reviewed in Bogdan & Nathan, 1993). As such, these cells mediate many of the direct and indirect effects of TGF-β on inflammation (reviewed in Wahl, 1992; McCartney-Francis & Wahl, 1994 and Border & Noble, 1994). TGF-β contributes both to the formation of inflammatory foci by direct effects on chemotaxis and adhesion, as well as to the resolution of acute inflammatory reactions and restoration of homeostasis by increasing the phagocytic activity of macrophages towards inflammatory cells and damaged parenchymal cells.

In contrast to the activating effects of TGF-β on peripheral blood monocytes, its actions on tissue macrophages are generally suppressive, through modulation of the cytokine profile, and contribute to the resolution of an inflammatory response. This has been attributed in part to differences in the pattern of receptor expression: resting monocytes express high levels of TGF-β receptors, whereas, as cells mature, receptor levels decline (reviewed in McCartney-Francis & Wahl, 1994). Activation of latent TGF-β by macrophages is also key to both its physiological and pathological actions on immune cells.

The ability of TGF-β to limit the production of cytotoxic reactive oxygen and nitrogen intermediates represents an important method of suppressing macrophage function. The enzyme responsible for the production of nitric oxide is an inducible form of inducible nitric oxide synthase (iNOS), and regulation of this enzyme by cytokines including TGF-β is now known to underlie the control of antimicrobial and
tumoricidal pathways of macrophages and of immune responses in general (reviewed in Bogdan & Nathan, 1993).

### 4.1.1.3. TGF-β and immunoregulation

TGF-β is a molecule which has been strongly linked to immunoregulatory mechanisms (c.f. Section 1.5.1.5; Chapter 3), and as such has been intensely studied in this context. Indeed, many lines of evidence implicate TGF-β in the pathogenesis of autoimmune disease, as well as an important immunoregulator and parasite escape mechanism (reviewed in Letterio & Roberts, 1998 and Li et al., 2006). Several methods have been developed to best study this molecule’s role in different systems.

TGF-β has pleiotropic effects such that by three to five weeks of age TGF-β−/− mice develop a fatal multiorgan inflammatory phenotype (Kulkarni et al., 1993). This renders it difficult to work with these transgenic animals, and due to the major impact of the phenotype on immune responses, it may not represent the best model for teasing out the complex relationships between different regulatory molecules during nematode infection.

Most models which implicate TGF-β as having a strong role during immunomodulation have used αTGF-β treatments or dominant negative TGF-βR transgenic animals rather than TGF-β± animals. Since the dominant negative TGF-βR transgenic animals were unavailable to us at the time of experiments, we used
αTGF-β treatments, ensuring that the immune environment of these animals up until treatment was as close to that the naïve controls as possible.

However, in future, experiments will be performed using our recently acquired dominant negative TGF-βR transgenic animals. Indeed, the Massague group characterised mutations within the TGF-βR which allow binding of TGF-β without subsequent signalling (Wieser et al., 1993). These mutated receptors can in essence ‘mop up’ active TGF-β, thus decreasing its immunomodulatory effect. Gorelik & Flavell used these results to create a CD4 dnTGF-βR2 mouse which carries such mutations in an extra copy of the TGF-βR on CD4+ cells. The effects of TGF-β can therefore be studied specifically in the CD4+ cell subset (Gorelik & Flavell, 2000).

4.1.2. IL-10

4.1.2.1. General characteristics

The main function of IL-10 is limiting, and ultimately terminating inflammatory responses. However, IL-10, like TGF-β has pleiotropic effects. Numerous studies, both in vitro and in vivo, with recombinant cytokine and neutralising antibodies have revealed the activities of IL-10. IL-10 is a growth and differentiation factor for many cell types including B cells (Go et al., 1990; Rousset et al., 1992), T cells (MacNeil et al., 1990; Chen & Zlotnik, 1991; de Waal malefyt et al., 1993) and mast cells (Thompson-Snipes et al., 1991). IL-10 can also indirectly inhibit cytokine production
by both T and NK cells, via inhibition of accessory cell function (de Waal malefyt et al., 1991; Fiorentino et al., 1991; Hsu et al., 1992; Ding & Shevach, 1992).

IL-10 can be expressed by a variety of cells usually in response to an activation stimulus. However, its expression is regulated by different mechanisms in different cell types. Transcription of *IL-10* is constitutive to some degree and subject to control by alteration of post-transcriptional RNA degradation mechanisms (reviewed in Moore et al., 2001).

The IL-10 receptor (IL-10R) is composed of at least 2 subunits, IL-10R1 and IL-10R2, members of the interferon receptor family. IL-10R1, the ligand binding subunit expressed constitutively by most hematopoietic cells, binds to IL-10 with high affinity (Tan et al., 1993; Ho et al., 1993; Liu et al., 1994; Carson et al., 1995). IL-10R1 expression has also been observed on non-hematopoietic cells although, rather than being constitutive, expression is more often induced. IL-10R2 is the accessory subunit used for signalling and contributes little to IL-10 binding affinity (reviewed in Moore et al., 2001). The principal function of IL-10R2 is the recruitment of a Jak kinase into the signalling complex (Kotenko et al., 1997). In most cells and tissues examined, IL-10R2 is constitutively expressed (Gibbs & Pennica, 1997), and since there is no evidence for activation-associated regulation of IL-10R2 expression in immune cells, stimuli activating IL-10R1 should suffice to render most cells responsive to IL-10 (reviewed in Moore et al., 2001).
Chapter 4 – *In vivo* depletion of regulatory markers

Although not the unique signalling pathway, the best characterised IL-10R signal transduction pathway is through the Jak/stat system (reviewed in Moore *et al.*, 2001). Briefly, the IL-10/IL-10R interaction engages the Jak family tyrosine kinases, Jak1 and Tyk2 (Finbloom & Winestock, 1995; Ho *et al.*, 1995) constitutively associate with IL-10R1 and IL-10R2 respectively. IL-10 induces tyrosine phosphorylation and activation of the latent transcription factors stat-3, stat-1 and in non-macrophage cells, stat-5 (Finbloom & Winestock, 1995; Lai *et al.*, 1996; Wehinger *et al.*, 1996; Weber-Nordt *et al.*, 1996), which lead to changes in gene expression.

4.1.2.2. Effect of IL-10 on leukocytes

The inhibitory effects of IL-10 on pro-inflammatory cytokine production and the physiology of individual cell types suggests it has potent anti-inflammatory activities *in vivo* (reviewed in Moore *et al.*, 2001). Even though the inhibitory effects of IL-10 on individual processes may be modest, IL-10 inhibits many of the individual steps in the inflammatory pathway of antimicrobial immunity, thus resulting in profound inhibition of the ultimate effector functions.

IL-10 modulates the expression of cytokines, soluble mediators and cell surface molecules by cells of myeloid origin (including monocytes, macrophages and DCs). This has important consequences for their ability to activate and sustain immune and inflammatory responses. Indeed, IL-10 not only inhibits the production of effector molecules by transcriptional and post-transcriptional mechanisms (Bogdan *et al.*, 2001).
Chapter 4 - In vivo depletion of regulatory markers

1991; Brown et al., 1996; Aste-Amezaga et al., 1998), but also enhances the expression of their natural antagonists (reviewed in Moore et al., 2001).

Inflammatory responses can be limited through the effect of IL-10 on DCs either by inhibitory effects on ‘inflammation-inducing’ DCs or by induction of anti-inflammatory T cell populations by IL-10-producing DCs. IL-10 also limits the duration and harmful pathology of inflammatory responses by inhibiting the production of chemokines, proinflammatory cytokines and mediators of granulocyte survival by neutrophils.

Finally, IL-10 can also limit inflammatory responses by inducing regulatory T cells. Activation of T cells in the presence of IL-10 can induce non-responsiveness or anergy. IL-10-mediated anergy can be associated with the induction of a population of regulatory T cells that produce high levels of IL-10 and can suppress antigen-specific responses in vivo and in vitro (Groux et al., 1997; Asseman et al., 1999; Doetze et al., 2000; reviewed in Cobbold & Waldmann, 1998).

4.1.2.3. IL-10 and immunoregulation

IL-10 plays a critical role in infectious disease by modulating the pathological consequences of inflammatory responses to microbial pathogens. A successful response to invading pathogens must balance protection and pathology. Although, IL-10 production usually imposes some limits on the effectiveness of anti-pathogen immune responses, this cost is often outweighed by the ability of IL-10 to protect the
host from immunopathology by antimicrobial cytokines and effector molecules (reviewed in Moore et al., 2001). Indeed, IL-10 plays a central role in peripheral tolerance, protection against autoimmunity, allergic responses, cancer and graft rejection although outcome is very dependent on the source, level, timing, and duration of IL-10 expression (reviewed in Moore et al., 2001).

Since IL-10 production tends to limit the effectiveness of the immune response to most pathogens, resistance to infection can nearly always be improved by reducing IL-10 levels. Our experiments were conducted to as to determine the effect of reducing IL-10 signalling during helminth infection.

IL-10 is considered an essential immunoregulator in the intestinal tract. IL-10−/− mice, generated by gene targeting, develop spontaneous chronic enterocolitis even though they show normal lymphocyte development and antibody responses (Kuhn et al., 1993). Berg and colleagues further characterised these animals and demonstrated that the mucosal gut inflammation is due to increased and dysregulated Th1 responses. They also showed that treating weanlings with IL-10 cured animals of colitis, whilst treatment in adults reduced pathology. This confirms that the colitis observed in the IL-10−/− animals is directly linked to the lack of IL-10 (Berg et al., 1996).

However, when the IL-10−/− mice are kept in specific pathogen free (SPF) conditions, only local inflammation of the colon develops. These results indicate that the colitis seen in the animals results from uncontrolled immune responses stimulated by
Chapter 4 – In vivo depletion of regulatory markers

enteric antigens. Results from the Sher group have confirmed this by demonstrating that IL-10−/− mice kept in SPF conditions infected with Helicobacter hepaticus developed colitis, unlike their wild type counterparts. The infected animals showed increased Th1 responses whilst the wild type animals had strong IL-10 responses (Kullberg et al., 1998).

Due to the severely altered intestinal environment of IL-10−/− mice, our experiments were conducted using depleting αIL-10R antibody (reviewed in Moore et al., 2001). Rather than administering αIL-10, αIL-10R was used to ensure that IL-10 signalling was affected.

4.1.3. CD25

CD25, the IL-2Rα subunit, remains the most widely-used marker for identification of Tregs. Sakaguchi and colleagues have demonstrated that cell suspensions depleted of CD25+ cells, when injected into athymic nude mice, cause severe autoimmune diseases in these animals. Disease development was prevented when mice were also injected with CD25+ cells. The group was the first to demonstrate the role of CD25 in maintaining self tolerance by downregulating immune responses to self and non-self antigens in an antigen non-specific way (Sakaguchi et al., 1995). Confirming this, the Sakaguchi group also found that mice, thymectomised 3 days after birth, developed autoimmune diseases. Disease was prevented by inoculating animals with CD25+ but not CD25− cells (Asano et al., 1996). However, McHugh & Shevach have found that although CD4+CD25+ Tregs induce protection against the development of
autoimmune diseases, they are not the sole contributors. In their experiments, transferred CD4^+CD25^+ Tregs protected animals from autoimmunity. However, animals only rarely developed autoimmune diseases after Treg depletion. Another signal was required to activate autoreactive effector CD4^+CD25^- cells. These results were obtained even when using high doses of depleting antibody, therefore lack of autoimmunity was not attributed to low antibody dose (McHugh & Shevach, 2002). However, CD25 is not always associated with the suppressor activity which characterises Tregs (Lehmann et al., 2002; Fontenot et al., 2005a); it is expressed by all recently activated T cells and the stability of its expression remains unknown (Thornton & Shevach, 1998).

New findings have made studying the effect of CD4^+CD25^+ cells on regulation more complex. Recently, the Miller group have found that exacerbated autoimmune disease in αCD25-treated animals was the result of functional inactivation rather than the depletion of Tregs (Kohm et al., 2006). Supporting this, treated mice had lower numbers of CD4^+CD25^+ T cells but no change in the number of CD4^+FoxP3^+ cells. Treatment with αCD25 also failed to both reduce the number of Thy1.1^+ congenic CD4^+CD25^+ Treg cells or alter levels of CD25 mRNA expression in treated recipients. Thus depleting antibodies may not be the most reliable method by which to study Tregs (Kohm et al., 2006). However, in our experiments, we used the PC61 clone of CD25 for CD25^+ cell depletion, rather than the 7D4 clone with which the Miller group obtained most of their results (Kohm et al., 2005; Kohm et al., 2006). PC61, unlike 7D4 for which the effects are thought only to last two weeks (Kohm, et al., 2005), has effects lasting up to one month (Shimizu et al., 1999) after
administration. Since our experiments ran for 28 days, depleting with the PC61 antibody was determined to be the best method available for studying of Tregs in our system.

4.1.4. Hypotheses and aims

In Chapter 3, we demonstrated elevated levels of TGF-β, IL-10 and CD25 during *H. polygyrus* infection. From these results, we postulated the molecules play a significant role in maintaining a regulatory environment and allowing parasite persistence. In this chapter, we determine the effects on regulation of removing these molecules *in vivo* using depleting antibodies.
4.2. Results

4.2.1. TGF-β is involved in the regulatory networks apparent during *H. polygyrus* infection.

Animals were treated with 5 doses of 50 μg of αTGF-β from d14 of infection (Figure 4.1 A). The dose used was adapted from a protocol used by the Riley group (Omer et al., 2003). Since active TGF-β is very short lived (Wakefield *et al.*, 1990), in order to increase potency, 5 small doses were administered rather than a single large dose. The timing of the antibody treatment was specifically chosen since by d21 surface TGF-β is increased on the CD4⁺CD25⁺ cells from the MLN (Finney *et al.*, submitted; Chapter 3). At d14 this increase has yet to become apparent, and treatment at d14 was hypothesised to block this increase, thereby affecting infection.

After treating animals with αTGF-β during infection, worm burdens were decreased by d28 (Figure 4.1 A). The result was not significant by statistical analysis although this can be attributed to the large spread in worm burdens in the IgG control group. A repeat of the experiment, which was only conducted once, is necessary to ensure the decrease observed is reproducible. Also, prolonging the experiment to d42 instead of d28 might yield more significant results as it would allow more time for worm expulsion to take place; here, the last dose of antibody was administered on d22 and worm burdens were calculated on d28.
Chapter 4 – In vivo depletion of regulatory markers

Figure 4.1 – αTGF-β treatment during H. polygyrus infection significantly decreases Treg marker expression.

A: Experimental protocol for αTGF-β treatment in vivo (Left), performed once. Mice were infected on d0, and treated five times with 50 μg of αTGF-β i.p. at two-day intervals from d14. Right, worm burdens for naïve, infected and treated animals after 28 days of infection. Data represent means from groups of a minimum of 4 mice; data were transformed and Student’s T-test performed on IgG control v. αTGF-β treated animals (n.s. = no significant difference).

B: Representative plots of bivariate flow cytometry analysis for CD25 and CD103 expression in CD4+ T cells from MLN in naïve (Left), d28-infected (Centre) and αTGF-β-treated (Right) animals.

C: CD25+ (Left) and CD103+ (Centre) T cells as proportion of total MLN CD4+ cells and CD103+ T cells as proportion of CD4+CD25+ (Right) in naïve, d28-infected and αTGF-β-treated animals. Data represent means and standard errors from groups of a minimum of 4 mice assayed individually; data were transformed and one-way ANOVAs performed using Bonferroni post-tests on IgG control v. αTGF-β treated animals (n.s. = no significant difference, ** = p<0.01).

D: IL-10+ (Left) and TGF-β+ (Right) T cells as proportion of total CD4+ in naïve, d28-infected and αTGF-β-treated MLN. Data represent means and standard errors from groups of a minimum of 4 mice assayed individually; data were transformed and one-way ANOVAs performed using Bonferroni post-tests on IgG control v. αTGF-β-treated animals (n.s. = no significant difference, ** = p<0.01).
Chapter 4 – *In vivo* depletion of regulatory markers

---

Figure 4.1 – αTGF-β treatment during *H. polygyrus* infection significantly decreases Treg marker expression
However, the decrease in worm burden was also accompanied by a significant decrease in regulatory markers. CD103 reverted back to naïve levels in both the total CD4\(^+\) population and in the CD4\(^+\)CD25\(^+\) cell subset (Figure 4.1 B, C). CD25 appears to have also followed this trend, although results were not significant. The antibody treatment did therefore have a significant effect on regulatory markers, confirming that the regulatory environment during infection was altered and affected parasite survival.

IL-10 produced by MLN CD4\(^+\) cells \textit{ex vivo} also returned to background levels (Figure 4.1 D) and surface TGF-\(\beta\) levels appeared decreased in treated animals (Figure 4.1 D) demonstrating that the antibody had some effect on its target molecule. However, the signal for both surface (Figure 4.1 D) and soluble (data not shown) TGF-\(\beta\) was close to background which may explain why the observed decrease was not significant.

4.2.2. IL-10 does not play a unique role during \textit{H. polygyrus} infection.

Animals were treated with 5 doses of 250 \(\mu\)g of \(\alpha\)IL-10R from d14 of infection (Figure 4.2 A). The dose used was adapted from a protocol used by the Riley group (Omer \textit{et al.}, 2003). The timing of the antibody treatment was specifically chosen since it was to be administered in conjunction with \(\alpha\)TGF-\(\beta\). However, the \(\alpha\)TGF-\(\beta\) batch used in this particular experiment was contaminated with FCS protein, and found not to be effective, explaining why only results for \(\alpha\)IL-10R treatment alone
were analysed – no control was available for the control of the efficacy of the IL-10R antibody.

IL-10 is increased during *H. polygyrus* infection: both antigen-specific IL-10 produced by MLN cells grown in culture and IL-10 produced by MLN CD4\(^+\) cells *ex vivo* were increased on d28 of infection (Finney *et al.*, submitted; Chapter 3). It was hypothesised that IL-10 may play a significant role during infection. However, treating animals with αIL-10R antibody did not lead to a decrease in worm burden (Figure 4.2 A). The worm burdens were slightly, but not significantly, increased. The egg counts did not differ between the IgG control- and IL-10R-treated groups over the two weeks after the initial treatment, indicating that worm burdens remained stable over that period. By day 26, the αIL-10R-treated group did have significantly more eggs in their faeces (transformed data, Student’s T-test, p<0.01) but it is believed this result only reflects the slightly increased worm burden in this group.
Figure 4.2 – αIL-10R treatment during *H. polygyrus* infection has no significant effect.

A: Experimental protocol for αIL-10R treatment *in vivo* (Left), performed once. Mice were infected on d0, and treated five times with 250 μg of αIL-10R i.p. at two-day intervals from d14. The centre panel shows worm burdens for naïve, infected and treated animals after 28 days of infection. Data represent means from groups of 5 mice; data were transformed and Student’s T-test performed on IgG control v. αIL-10R-treated animals (n.s. = no significant difference). Right, egg counts taken from faeces collected at two-day intervals from d14 to d26 of infection. No eggs were detected in naïve samples. Data represent means of 3 readings per time point from groups of 5 mice sampled individually.

B: Representative plots of bivariate flow cytometry analysis for CD25 and CTLA-4 expression in CD4+ T cells from MLN in naïve (Left), d28-infected (Centre) and αIL-10R-treated (Right) animals.

C: CD25+ (Left) and CTLA-4high (Right) T cells as proportion of total CD4+ cells in naïve, d28-infected and αIL-10R-treated MLN. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed. No significant differences were detected.
Figure 4.2 - αIL-10R treatment during *H. polygyrus* infection has no significant effect.
CD25 and CTLA-4 levels on CD4\(^+\) cells, although slightly decreased compared to infected groups, were not significantly affected by \(\alpha\)IL-10R treatment (Figure 4.2 B, C). On the CD4\(^+\)CD25\(^+\) cell subset, we have previously shown that CTLA-4 is decreased by d28 of infection (Finney et al., submitted; Chapter 3). The result was confirmed in this experiment and the decrease observed was unaltered by \(\alpha\)IL-10R treatment (Figure 4.2 B).

\(\alpha\)IL-10R treatment did not significantly alter regulatory markers and this was reflected in the worm burdens, which were not decreased. However, although IL-10 does not appear to be a key player during infection, it may be acting in conjunction with a number of other molecules. Treating animals with both \(\alpha\)IL-10R and \(\alpha\)TGF-\(\beta\) would provide new insights into possible interactions between both these regulatory molecules.
4.2.3. αCD25 treatment alone during *H. polygyrus* infection does not alter worm burdens but does affect regulatory markers.

Animals were treated with 1 dose of 1 mg of αCD25 (Figure 4.3 A) as in previous experiments by our group (Taylor *et al.*, 2005; Wilson *et al.*, 2005). αCD25 was administered at two different time points to study the effect on infection of natural Tregs (early time point) and possible induced Tregs (late time point) (Figure 4.3 A). However, neither worm burdens nor egg counts were significantly altered by either treatment (Figure 4.3 A). To ensure the antibody was depleting CD25⁺ cells, blood samples were taken 6 days after the early αCD25 treatment and assayed for the CD25 surface marker. A significant decrease was observed in the treated animals (Figure 4.3 B) confirming the reduction in CD25 expression.
Figure 4.3 – αCD25 treatment before or during *H. polygyrus* infection does not affect worm burdens.

A: Experimental protocol for αCD25 treatment *in vivo* (Left), performed once. Mice were infected on d0, and treated once with 1 mg of αCD25 i.p. either 7 days before (early) or 14 days after infection (late). Centre, worm burdens for naïve, infected and treated animals after 28 days of infection. Data were transformed and a one-way ANOVA performed on all infected groups. No significant differences were detected. Right, egg counts taken from faeces collected at two-day intervals from d14 to d26 of infection. No eggs were detected in naïve samples. Data represent means of 3 readings per time point from groups of 5 mice sampled individually.

B: Representative plots of bivariate flow cytometry analysis for CD25 and CD4 expression (pre-gated on CD4) in blood samples obtained 6 days after early treatment of αCD25, in naïve (Far Left), d28-infected (Left) and αCD25-treated (Right) animals. Far Right, CD25⁺ T cells as proportion of total CD4⁺ in naïve, IgG control- and αCD25-treated animals. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed using Bonferroni post-tests on all groups (n.s. = no significant difference, * = p<0.05, ** = p<0.01).
Figure 4.3—αCD25 treatment before or during H. polygyrus infection does not affect worm burdens.
Constitutive cytokine release by MLN cells cultured for 48 hours in media was increased in animals having received αCD25 (Figure 4.4 A, B, C). This was another indication that treatments had been successful. However, due to this constitutive increase, no differences in Hp-specific release were observed for any of the cytokines; αCD25 treatment dysregulated cytokine production so that constitutive cytokine production was greatly enhanced masking any changes in Hp-specific responses. For IL-5 and IL-13, increased Hp-specific levels in the IgG control-treated animals were decreased compared to constitutive levels in both αCD25-treated groups. IL-5 has been strongly linked with anthelmintic protective immunity (Butterworth et al., 1975; Mahmoud et al., 1975) and it is currently thought that IL-5-dependent mechanisms can operate to kill helminth parasites (reviewed in Maizels & Balic, 2004). IL-13 has been strongly linked to worm expulsion mechanisms (Shea Donohue et al., 2001). The fact that the increase in Hp-specific IL-13 and IL-5 production is lost with αCD25 treatment is difficult to explain as it was hypothesised that depleting animals of Tregs would allow mechanisms of worm expulsion to clear parasites.
Parasite antigen-specific cytokine responses are presented from MLN cells. Cells were cultured for 48 hours in medium alone (open bars) or *H. polygyrus* (Hp) antigen (solid bars). Naïve levels were undetectable. Panel A presents IL-4 (Left), IL-5 (Centre) and IL-13 (Right). Panels B and C present respectively IFN-γ and IL-10. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed for IL-4 and IL-10 on all groups of cells cultured in Hp antigen. No significant differences were detected. One-way ANOVAs were not performed for IL-5, IL-13 and IFN-γ as no antigen-specific cytokine production was detected. The experiment was performed once.
Figure 4.4 - Cytokine responsiveness in *H. polygyrus* infection, after αCD25 treatment
Regulatory markers on CD4$^+$CD25$^-$ cells were not altered by the αCD25 treatments (Figure 4.5 A). However in CD4$^+$CD25$^+$ cells, CD103 levels were increased in both groups, independently of FoxP3 expression (Figure 4.5 B, C). Therefore, although the CD4$^+$CD25$^-$ cells did not alter through treatment, the repopulating CD4$^+$CD25$^+$ cells had a greater proportion of CD103$^+$ cells. CD103 is a homing marker and therefore the increased expression of this marker in the repopulating CD4$^+$CD25$^+$ subset could be so that these cells are directed to the site of infection. The timing of the antibody treatment did not seem to have an effect: both early- and late-treated animals showed an increase in CD103.

Although treatment with αCD25, whether before or during infection, does not appear to have greatly affected the establishment and/or maintenance of infection, the regulatory milieu was altered by treatment. Cytokine production was dysregulated to such an extent that Hp-specific responses were lost and repopulating CD4$^+$CD25$^+$ cells showed increases in CD103 expression. Whether these effects would have lead to decreased worm burdens had the experiment continued remains to be investigated.
Figure 4.5 – CD103 expression increases in CD4^+CD25^+ but not CD4^+CD25^- cells after αCD25 treatment prior or during *H. polygyrus* infection.

A: CD103^- (Left) CD103^-FoxP3^- (Centre) and CD103^-FoxP3^- (Right) T cells as proportion of total CD4^-CD25^- MLN cells in naïve, d28-infected, early- and late-treated animals. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed on all infected groups. No significant differences were detected. The experiment was performed once.

B: CD103^- (Left), CD103^-FoxP3^- (Centre) and CD103^-FoxP3^- (Right) T cells as proportion of total CD4^-CD25^- MLN cells in naïve, d28-infected, early- and late-treated animals. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed on all infected groups using Bonferroni post-tests between IgG control- and αCD25-treated animals (n.s. = no significant difference, * = p<0.05, ** = p<0.01).

C: Representative plots of bivariate flow cytometry analysis for CD25 and CD103 expression in CD4^-CD25^- T cells from MLN in naïve (Far Left), d28-infected (Left), early αCD25-treated (Right) and late αCD25-treated (Far Right) animals.
Figure 4.5 – CD103 expression increases in CD4⁺CD25⁺ but not CD4⁺CD25⁻ cells after αCD25 treatment prior or during *H. polygyrus* infection.
4.2.4. αCD25 and αTGF-β treatments during *H. polygyrus* infection do not alter worm burdens but do affect regulatory markers

To determine whether CD25 and TGF-β are required very early in infection and affect chronicity and/or outcome of infection, treatment with either or both antibodies was administered just prior to infection (Figure 4.6). However, worm burdens were not altered by either the single treatments or the combination treatment, indicating that this is not the case.

Again, as seen with prior experiments (Figure 4.4), depleting CD25 and/or TGF-β had no real effect on Hp-specific cytokine production in both the MLN and spleen (Figure 4.7 A, B, C, D, E). Interestingly, unlike when treating with TGF-β during infection (Figure 4.1), the IL-10 response was not affected by antibody treatment early in infection. The only cytokine affected by treatment was IL-5, where levels were reduced in all treated groups in the MLN but not in the spleen (Figure 4.7 B).
Figure 4.6 – αTGF-β and αCD25 treatment early in *H. polygyrus* infection do not affect worm burden.

Experimental protocol for αTGF-β and αCD25 treatment *in vivo* (Left), performed once. Mice were infected on d0, and treated with either or both of one dose of 1 mg of αCD25 i.p. 3 days pre-infection and five doses, at two-day intervals, of 50 µg of αTGF-β i.p. from d0. Left, worm burdens for naïve, infected and treated animals after 28 days of infection. Data represent means from groups of 5 mice; data were transformed and a one-way ANOVA performed on infected groups. No significant difference was detected.
Chapter 4 – *In vivo* depletion of regulatory markers

Figure 4.6 – αTGF-β and αCD25 treatment early in *H. polygyrus* infection do not affect worm burden.
Figure 4.7 – Cytokine responsiveness in *H. polygyrus* infection, after αCD25 and αTGF-β treatment.

Parasite antigen-specific cytokine responses are presented from MLN (Left panels) cells and Spleen (Right panels) cells. Cells were cultured for 48 hours in medium alone (open bars) or *H. polygyrus* antigen (solid bars). Naïve levels were undetectable. Panels A-C present Th2 cytokines (respectively IL-4, IL-5, IL-13), panel D presents the Th1 cytokine IFN-γ and panel E presents the regulatory cytokine IL-10. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed for all five cytokines on all groups of cells cultured in Hp antigen relative to background media levels. No significant differences were detected. The experiment was performed once.
Figure 4.7 - Cytokine responsiveness in *H. polygyrus* infection, after αCD25 and αTGF-β treatment
CD103 levels were also examined as these were elevated in the αCD25-treated animals in the previous experiment (Figure 4.5). Here, in the CD4⁺CD25⁻ subset, CD103 levels were not altered compared to the IgG control-treated group for either of the single depleting antibody treatments. However, administering both αCD25 and αTGF-β significantly reduced CD103⁺ cells, independently of FoxP3 expression (Figure 4.8 A). These results support studies stating that TGF-β expression regulates CD103 expression (Robinson et al., 2001).

CD103 levels in the CD4⁺CD25⁺ cells were only increased in the group having received a single αCD25 treatment; the increase was independent of FoxP3 expression (Figure 4.8 B). This confirms the results obtained for the previous experiment (Figure 4.5) and demonstrates that, surprisingly, treating with αCD25 and αTGF-β in conjunction has no effect on the repopulating CD4⁺CD25⁺ population.

Although treating with αCD25 and αTGF-β early in infection showed some effects, worm burden was not decreased. However, since active TGF-β has short half-life (Wakefield et al., 1990), a more effective protocol may be to deplete CD25 and TGF-β at d14. Indeed, an effect on worm burden was obtained with administration of αTGF-β at this time point. Treating in conjunction with αCD25 may amplify this result.
Figure 4.8 – CD103 expression increases in CD4^+CD25^+ cells from αCD25-treated animals, but decreases in CD4^+CD25^- cells from animals having received αCD25 and αTGF-β treatment.

A: Representative plots of bivariate flow cytometry analysis for CD25 and CD103 expression in CD4^+CD25^- T cells from MLN in naïve (Far Left), d28-infected (Left), αCD25-treated (Centre), αTGF-β-treated (Right) and αCD25 + αTGF-β-treated (Far Right) animals.

B: Representative plots of bivariate flow cytometry analysis for CD25 and CD103 expression in CD4^+CD25^+ T cells from MLN in naïve (Far Left), d28-infected (Left), αCD25-treated (Centre), αTGF-β-treated (Right) and αCD25 + αTGF-β-treated (Far Right) animals.

C: CD103^+ T cells as proportion of total CD4^+CD25^- (Left) and CD4^+CD25^+ (Right) MLN cells in naïve, d28-infected, αCD25-, αTGF-β- and αCD25 + αTGF-β-treated animals. Data represent means and standard errors from groups of 5 mice assayed individually; data were transformed and one-way ANOVAs performed on all infected groups using Bonferroni post-tests between IgG control- and depleting antibody-treated animals (n.s. = no significant difference, ** = p<0.01). The experiment was performed once.
Figure 4.8 - CD103 expression increases in CD4⁺CD25⁺ cells from αCD25-treated animals, but decreases in CD4⁺CD25⁻ cells from animals having received αCD25 and αTGF-β treatment.
4.3. Discussion

4.3.1. TGF-β

In Chapter 3, we postulated that TGF-β, which is increased during *H. polygyrus* infection on CD4⁺CD25⁺ cells (*c.f* Chapter 3), was a highly plausible candidate for the functional Treg product in our system. Depleting this molecule mid-infection provided some support for this hypothesis, as regulatory markers (CD25, CD103 and secreted IL-10) were all found to revert to naïve levels after antibody treatment. Worm burdens were also decreased in treated animals. However, when animals were treated early in infection (from d0 to d8 post-infection), these effects disappeared. Thus, the establishment of infection may not require host TGF-β, although the later regulatory changes do appear to need this cytokine.

The involvement of TGF-β in regulatory mechanisms both *in vitro* as well as *in vivo* has been the source of much controversy (*c.f* Section 1.5.1.5). *In vitro* results have already been discussed in detail (*c.f* Section 1.5.1.5; Chapter 3), and this chapter will concentrate on the effects of this molecule in *in vivo* models.

The effect of altering levels of TGF-β has been studied in numerous models, with differing results as set out below. In some systems, TGF-β has been found not to be essential for regulation whilst in others it has been found that the molecule is an essential component of the regulatory mechanisms.
Arguing that TGF-β is dispensible, Mamura and colleagues have demonstrated that Tregs from TGF-β⁻/⁻ animals show all the characteristics of natural Tregs from wild type mice, including characteristic Treg markers (GITR, CTLA-4 and FoxP3) as well as being functionally suppressive in a model of colitis (Mamura et al., 2004). Both the Piccirillo and Shevach groups have confirmed this by demonstrating that in models of colitis and autoimmune gastritis (AIG) respectively, CD4⁺CD25⁺ cells from both TGF-β⁺/+ and TGF-β⁻/⁻ animals were able to suppress disease in recipient animals (Kullberg et al., 2005; Piccirillo et al., 2002). While Kullberg and colleagues conceded that TGF-β clearly plays a role in protection against inflammation, its production by CD4⁺CD25⁺ Tregs is not essential for suppressive function in their model (Kullberg et al., 2005). The Shevach group also showed that when recipient mice were treated with neutralising αTGF-β, Treg-mediated protection was not abrogated. Thus, in the absence of TGF-β, suppressive activity was still apparent (Piccirillo et al., 2002).

However, these results demonstrating that TGF-β does not play a significant role during regulation have been strongly contested. Work by Nakamura and colleagues directly contradicts Kullberg’s research, whereby CD4⁺CD25⁺ cells from TGF-β⁻/⁻ animals, although suppressive, were not able to protect animals from colitis (Nakamura et al., 2004). Explanations for these discrepancies have yet to be elucidated.

Further strong evidence for TGF-β-mediated regulation has emerged from the Powrie group who have shown that while transfer of CD4⁺CD45RB⁺ T cells into
severe combined immunodeficiency (SCID) or recombination activating gene knockout (RAG<sup>-/-</sup>) recipient mice leads to colitis, transfer of these cells with CD4<sup>+</sup>CD45RB<sub>B</sub><sup>low</sup> T cells prevents colitis (Powrie <em>et al.</em>, 1996). However, this effect was completely ablated when recipient animals were treated with αTGF-β (Powrie <em>et al.</em>, 1996; Read <em>et al.</em>, 2000). By treating animals with αTGF-β, the Liew group also demonstrated that TGF-β is required for Treg suppressive activity in a model of colitis (Liu <em>et al.</em>, 2003). Huber and colleagues obtained similar results using the CD4 dnTGF-βR2 mice, showing that cells from such animals failed to expand <em>in vivo</em> and to suppress colitis (Huber <em>et al.</em>, 2004). In their model, TGF-β signalling in CD4<sup>+</sup>CD25<sup>+</sup> Treg cells was required for their <em>in vivo</em> expansion and suppressive capacity. It would have been interesting to assess the suppressive ability of the CD4<sup>+</sup>CD25<sup>+</sup> Treg in our experiments after αTGF-β treatment by performing suppression assays. Determining whether the suppressive ability of the Tregs was altered after treatment would help elucidate whether TGF-β has a significant impact on Treg function during <em>H. polygyrus</em> infection. Infecting CD4-dnTGF-βR2 animals (Gorelik & Flavell, 2000, <em>c.f</em>. Section 4.1.1.3), which carry an extra but non-functional copy of the TGF-βR on CD4<sup>+</sup> cells thus limiting TGF-β signalling in this subset, would also help determine the role played by TGF-β during infection.

The use of αTGF-β by some groups has not led to an alleviation of suppression (<em>c.f</em>. Section 1.5.1.5). Although this could be due to the cytokine not playing a major role in all models, the Strober group have attributed the results to two main factors (Nakamura <em>et al.</em>, 2004). First, the antibody dose used may be too low. Indeed, the dose administered in our experiments, based on a protocol by the Riley group (Omer
et al., 2003) appears low in relation to other models. We used 5 doses of 50 μg, but it appears weekly doses of 1 mg are the norm. This may help explain why no effect was seen when animals were treated early in infection, and why effects on worm burdens after treatment mid-infection were not significant. These experiments should be repeated with increased antibody doses.

The second factor which may be responsible for not obtaining ablation of suppression when treating with αTGF-β is the blocking activity of the antibody. Either the antibody used does not block the ability of latent TGF-β to bind to sites where conversion to an active form must occur, or it fails to block the ability of TGF-β to bind to TGF-βR after its conversion to an active form (Nakamura et al., 2004). We believe this not to be the case with our experiments since effects from treatment with αTGF-β were apparent in this work, as well as work from other members of the group (Dr M.D. Taylor, unpublished results).

Defects in TGF-β signalling have been shown to lead to decreases in both FoxP3 expression and suppressive functional activity in peripheral Tregs (Marie et al., 2005). Since TGF-β, Smad7 and FoxP3 have all been linked in a negative feedback mechanism (Chen et al., 2003; reviewed in Coffer & Burgering, 2004), this is of no surprise. Indeed, a transient pulse of TGF-β into pancreatic cell islets has been shown to inhibit the onset of diabetes by increasing the number of intra-islet CD4⁺CD25⁺FoxP3⁺ suppressor T cells (Peng et al., 2004). Also, overexpression of active TGF-β under the human CD2 promoter in mice results in increased numbers of CD4⁺CD25⁺ cells in the periphery (Schramm et al., 2004). When FoxP3 levels...
were studied in these animals as well as in hCD2-ΔkTβR2 transgenic animals (animals expressing an extra but non-functional copy of the TGF-βR on T cells driven by a human CD2 promoter, Schramm et al., 2003), levels were increased in the former and decreased in the latter, demonstrating that in vivo, FoxP3 appears to be regulated by TGF-β (Schramm et al., 2004). It would have been interesting to stain cells for FoxP3 in our experiments (the antibody was unavailable at the time of experimentation) to determine whether its expression was altered by αTGF-β treatment. However, on d28 of infection we observed increased TGF-β levels on CD4⁺CD25⁺ cells (Finney et al., submitted; Chapter 3), decreased Smad7 levels in CD4⁺ cells (data not shown) but no change in FoxP3 expression on CD4⁺CD25⁺ cells (Finney et al., submitted; Chapter 3). Schramm and colleagues measured FoxP3 by real-time PCR (Schramm et al., 2004). In our model we have found discrepancies between real-time PCR results, where levels of FoxP3 were dramatically increased by d28 (data not shown), and intracellular staining where levels remain at naïve levels (Finney et al., submitted; Chapter 3). These have yet to be elucidated, but imply that TGF-β may regulate FoxP3 mRNA production rather than its protein expression.
4.3.2. IL-10

As with work on TGF-β, the involvement of IL-10 in regulation has been the subject of much debate. Our results show that during *H. polygyrus* infection, IL-10 appears redundant; no changes in either worm burdens or regulatory markers (CD25 and CTLA-4) were obtained with αIL-10R treatment. However, as with αTGF-β treatment, timing and dose are of the essence when attempting to disrupt such a closely regulated system. Doses could be increased in future experiments to verify the apparent lack of effect of αIL-10R treatment during *H. polygyrus* infection. Timing of IL-10R treatment may also have been a factor, although an effect was observed with αTGF-β treatment administered over the same time period.

Previous work by our group (Wilson *et al.*, 2005) and by the Weinstock group (Elliott *et al.*, 2004) support our results that *H. polygyrus*-induced regulation appears to be IL-10 independent. They demonstrated that, in a model of chronic IBD, *H. polygyrus* could reverse previously established, ongoing chronic intestinal inflammation in IL-10−/− mice by inhibiting mucosal Th1 cytokine production. Adoptive transfer of MLN T cells from infected IL-10−/− colitis-free donors into IL-10−/− colitic recipients also reversed the inflammation, suggesting that the protection was T cell dependent. Since *H. polygyrus* resides in the duodenum, a site distinct from the colon or ileum where colitic inflammation occurs, this suggests that *H. polygyrus* induces regulatory-type cells that migrate to the colon and inhibit the intestinal inflammation in an IL-10 independent fashion (Elliott *et al.*, 2004). Confirming this, we have previously shown that the ability of CD4+CD25+ Tregs to
suppress airway allergy in infected mice is undiminished by the administration of αIL-10R antibody, and that MLN cells from IL-10\(^{−/−}\) infected mice can transfer suppression of allergy into uninfected animals (Wilson et al., 2005). Hence, in the mouse at least, IL-10 does not appear to be a primary mechanism for helminth-associated Treg function. Evidence by Bashir and colleagues appears to contradict these findings, by demonstrating that reduction of allergic symptoms to peanut allergen in \(H. \text{polygyrus}\)-infected mice is IL-10 dependent (Bashir et al., 2002). Although the group used αIL-10 antibody rather than αIL-10R for depletion, this is unlikely to explain discrepancies in the findings.

Results similar to ours were also obtained in a murine model of filariasis, whereby treatment with αIL-10R did not restore the ability of the immune system to kill parasites. This suggests that IL-10 does not play a key role in promoting parasite survival in this model and that T cells also act in an IL-10 independent manner to suppress host immunity to filariasis (Taylor et al., 2005).

Other experimental models involving IL-10 manipulation, however, have defined circumstances in which it plays an essential role during suppression by regulatory T cells. The Powrie group showed that αIL-10R treatment of recipients having received protective wild type CD\(^{4+}\)CD45RB\(^{\text{high}}\) cells abrogated protection from colitis. Also, unlike wild type cells, CD\(^{4+}\)CD45RB\(^{\text{low}}\) cells from IL-10\(^{−/−}\) mice did not protect recipient animals from colitis. Thus, IL-10 appears necessary for Treg effector function in this model (Asseman et al., 1999). Confirming this in a similar model of colitis whereby SCID animals were injected with CD\(^{4+}\)CD25\(^{−}\) cells in order
to develop spontaneous disease, the Liew group found that the therapeutic effect of Tregs was completely blocked by αIL-10R treatment of recipient animals (Liu et al., 2003). Finally, in a model of *H. hepaticus* infection, treatment with αIL-10R again ablated the ability of Tregs to inhibit *H. hepaticus*-induced intestinal inflammation, and T cells from IL-10−/− mice failed to suppress (Maloy et al., 2003).

The prominent role of IL-10 during regulation has been further demonstrated in experiments in which IL-10 was overexpressed in T cells. Mice overexpressing IL-10 driven by a T-cell-specific IL-2 promoter show no defects in growth or development (Hagenbaugh et al., 1997). However, CD4+CD45RBhigh cells from these animals, which when taken from wild type mice and transferred into SCID or RAG2−/− recipients induce colitis, do not induce colitis under the same circumstances. When cells from overexpressing animals were transferred with wild type CD4+CD45RBhigh cells, colitis was again ablated in recipient animals; it appears overexpressing IL-10 in the donor animals modulates their effector cells such that they can show regulatory activity (Hagenbaugh et al., 1997).

IL-10, as well as having a prominent role in autoimmune diseases such as colitis, also strongly affects the outcome of some parasitic infections. IL-10−/− mice appear unable to clear normally avirulent infection of *Trichuris muris* (Schopf et al., 2002), *Toxoplasma gondii* (Gazinelli et al., 1996; Neyer et al., 1997), *Plasmodium chabaudi chabaudi* (Li et al., 1999) or *Trypanosoma cruzi* (Hunter et al., 1997). The infections led to death in most of the animals, attributed to increased deregulated production of Th1 cytokines, rather than increased parasitaemia. The results emphasise the role
played by IL-10 in resistance to these infections. Neyer and colleagues demonstrated that, in a model of *T. gondii* infection, death could be prevented with early IL-10 treatment (Neyer *et al.*, 1997). Thus it appears that IL-10 plays a critical role in down regulating cell-mediated immune hyperactivity.

Work by the Sacks group in a model of leishmaniasis illustrates a very different role for IL-10 in maintaining a low but stable level of parasite persistence. Infection of IL-10⁻/⁻ mice, or treatment of chronically infected mice with αIL-10R resulted in complete elimination or sterile cure (Belkaid *et al.*, 2001).

In conclusion, the role of IL-10 appears very dependent on the model system studied. In *H. polygyrus* infection we find that IL-10 is not required for Treg suppression induced by the parasite although IL-10 is certainly increased by infection. This has been confirmed by other findings from our group as well as those from other investigators (Elliott *et al.*, 2004). Future experiments downregulating both TGF-β and IL-10 are now necessary to determine if there is redundancy or cross-talk between these two potential regulatory molecules.

### 4.3.3. TGF-β and IL-10

The finding that spontaneous pathology in IL-10⁻/⁻ mice is only found in their intestine, whilst TGF-β⁻/⁻ mice develop multi-organ disease, implies that IL-10 is not required for TGF-β production (Asseman *et al.*, 1999).
Although much controversy exists over the roles of TGF-β and IL-10 during regulation, few groups have studied the effect of disrupting both molecules in the same system. This is probably because the purpose of most investigators is to tease out the intricate role of the molecule playing a greater role in their system, be it TGF-β or IL-10. Unfortunately, although treating with both αTGF-β and αIL-10R was attempted in our experiments, the αTGF-β batch used contained a significant level of FCS-derived IgG and therefore the dose administered was probably not sufficient to repeat the effects seen when treating with αTGF-β alone during infection. Treating with both would indicate whether a redundant role played by IL-10 would be evident only in the absence of TGF-β, thus helping to discern intricate patterns of regulation.

The Riley group were unable to reverse the mortality of malaria-infected mice by neutralising with αTGF-β alone. However, when the antibody was administered early and in combination with αIL-10R, both parasitaemia and mortality were reduced (Omer et al., 2003).

IL-10 and TGF-β are both regulatory cytokines, and it appears that TGF-β, unlike IL-10, plays a non-redundant role during *H. polygyrus* infection. However, αTGF-β treatment did not effect any numerical reduction in Tregs in the system. Both αCD25 and αTGF-β were therefore used to tease out the specific effects of Tregs during *H. polygyrus* infection.
4.3.4. CD25

Depleting the majority of CD25⁺ T cells in *H. polygyrus* infected mice, whether before or during infection, did not affect worm burdens or the CD4⁺CD25⁻ population, but did affect the phenotype of the remaining/repopulating CD4⁺CD25⁺ cells. CD103 expression was increased within this subset in animals having been treated with αCD25 compared to IgG-treated controls. CD103 is increased by d28 on CD4⁺CD25⁺ cells during *H. polygyrus* infection (Finney *et al.*, submitted; Chapter 3, Figure 4.8), and this increase was exaggerated in αCD25-treated animals. CD103, as described in Chapter 3, is a homing marker thought to define a population of cells capable of infiltrating the site of infection (Suffia *et al.*, 2005). Thus the remaining/repopulating CD4⁺CD25⁺ cell population in our experiments appears to be enriched in Tregs capable of homing to the MLN and gut mucosa, and therefore capable of regulating infection. Indeed, CD103 expression is upregulated by TGF-β (Robinson *et al.*, 2001), and increases in surface TGF-β expression were found in CD4⁺CD25⁺ cells from αCD25 groups, although not to a significant level (data not shown), a point that should be explored. In future experiments however, secreted as well as surface TGF-β levels should be studied, so as to determine the impact of TGF-β signalling during infection.

It was postulated that differences between early and late treated groups would be apparent in our experiments since early treatment only targets natural Tregs, whilst, treating with αCD25 during infection affects both effector and regulatory cells. Gillan & Devaney found that, when depleting CD25⁺ cells prior to *Brugia pahangi*
infection, no decrease in CD4^+CD25^+CTLA-4^- effector cells was later detected, but a strong decrease in CD4^+CD25^+CTLA-4^- regulatory cells occurred (Gillan & Devaney, 2005). Surprisingly, no significant differences in Treg marker expression or Th2 cytokine expression were obtained between the early- and late-treated groups in our experiments, although trends indicated CD103 expression was elevated in the late-treated group. With αCD25 treatment lasting up to a month (Shimuzu et al., 1999), it is hypothesised that differences between these two groups (early- and late-treated) were due to antibody effects waning in the early-treated group. Thus, in our model, it appears that treating with αCD25 alone does not have a major impact on infection. However, in previous work from our group and in other models treatment with αCD25 can exert a range of effects on ensuing immune responses.

Previous studies from our laboratory have demonstrated that, in a murine model of allergic airway inflammation, infection with *H. polygyrus* limited the severity of allergic symptoms (Wilson et al., 2005). However, treatment with αCD25 prior to antigen challenge abrogated this protection. This strongly indicates that the CD4^+CD25^+ cells play a major role in the bystander regulation observed in this system. These findings do not contradict our results since no conclusions were drawn as to the impact of αCD25 treatment on infection itself.

Other groups have demonstrated an important role for CD4^+CD25^+ cells in other contexts. In cancer models, Onizuka and colleagues found that, in six out of eight tumours studied, αCD25 treatment led to tumour rejection, despite using low doses (0.125 mg) of antibody (Onizuka et al., 1999). Research by the Sakaguchi group
provided parallel evidence for these findings as they also found rejection of tumour was induced with αCD25 treatment (Shimizu et al., 1999).

In other parasite infection models, αCD25 treatment has also been found to exert a strong effect. In a mouse model of malaria, Long and colleagues showed that treatment with αCD25 delayed the rise in parasitaemia (Long et al., 2003). Similar results were obtained in another model of malaria, where depletion of CD25 protected animals from a lethal strain (Hisaeda et al., 2004). This confirms the hypothesis that regulatory mechanism delay protective immunity to malaria, allowing parasite growth and disease progression, and thus parasite escape from host immune responses.

Gillan & Devaney showed that αCD25 treatment during Brugia pahangi infection led to elevated antigen-specific proliferation and Th2 cytokine (IL-4) production by T cells in vitro, indicating that αCD25 treatment abrogated the T cell hyporesponsiveness associated with filariasis (Gillan & Devaney, 2005). Th2 cytokine production was also increased in T cells after αCD25 treatment in a murine model of schistosomiasis. Depletion of natural Tregs 6 days prior to egg-immunisation strongly increased the frequency of IL-4 producing CD4⁺ cells (Baumgart et al., 2006). Finally, the Sacks group have shown that Tregs are important and necessary in the persistence of Leishmania infection (Belkaid et al., 2002), indicating that CD4⁺CD25⁺ cells play an important role in the maintenance of low levels of parasites during chronic infections.
4.3.5. CD25 in combination with other Treg markers

In a number of models, however, depleting CD25 alone has not yielded significant changes in immune function. Indeed, research by the Melief group on cancer, Beilharz and colleagues on murine AIDS, and the Maizels group on filariasis, all concluded that combination treatments with other Treg markers such as GITR or CTLA-4 have greater effects than depleting CD25 alone (Sutmuller et al., 2001; Beilarz et al., 2004; Taylor et al., 2005).

Combination treatments were therefore attempted in our experiments. Although combined depletion of CD25 and TGF-β did not affect worm burdens, significant decreases in CD103 expression in CD4⁺CD25⁻ cells were obtained (Figure 4.8) attributed to the regulation of CD103 expression by TGF-β (Robinson et al., 2001). Strangely, the decrease was not observed in the αTGF-β single treatment group, and it was not accompanied by a significant decrease in surface TGF-β levels (data not shown). This could be due to the fact that the antibody used neutralises secreted rather than surface antibody or that most of the TGF-β is secreted by the CD4⁺CD25⁺ subset. Therefore, it appears that by targeting the cells thought to be directly involved in regulation (CD25⁺TGF-β⁺), migration of other cell subtypes (CD103⁺CD25⁻) to the draining lymph nodes was also affected. Interestingly, the group treated with αCD25 alone showed increases in CD103 in the repopulating CD4⁺CD25⁺ cells, which was not observed in the group treated with both depleting antibodies. This can again be explained by CD103 upregulation being dependent on
TGF-$\beta$.

4.3.6. Concluding remarks and ideas for future research

Experiments conducted in this chapter have provided an insight into the possible regulatory mechanisms involved during *H. polygyrus* infection. To date, only limited explanation of different timings and antibody combinations have been possible. Clearly, timing and antibody dose require further consideration due to the pleiotropic nature of the molecules studied.

One area of further study would be determining markers of worm expulsion mechanisms. Understanding these processes may yield more information as to whether relieving regulation allows parasite clearance, or whether the responsible effector mechanisms require triggering by other factors.

Finally, different depleting antibody combinations could be tested, such as depleting both TGF-$\beta$ and CTLA-4, CD25 and CTLA-4, or TGF-$\beta$ and CD103, in order to determine the role other Treg markers play in our system. A range of knock-out animals could also be included (such as CD103<sup>−/−</sup> mice (Schon *et al.*, 1999)) and tested by antibody depletion of additional components to tease out the role of each mediator during regulation.
5. HELMINTH INFECTION AND ALLERGY

5.1. Introduction

5.1.1. Allergy – a brief introduction

Allergy is defined as a set of hypersensitivity mechanisms (Kay, 2006), such as IgE-mediated excessive activation of mast cells and eosinophils in allergic diseases which result in immunopathology. The two major cell types involved in inflammatory allergic responses are eosinophils and mast cells (reviewed in Holt et al., 1999). Due to the rapid expansion in eosinophil numbers and their easy identification through eosin staining or immunohistochemistry, this cell type in particular has long been used to assess allergic phenotypes and has been well established as a hallmark of allergic inflammation, as has the eosinophil chemoattractant cytokine IL-5 (reviewed in Kay, 2005). Work by the Kay group using both immunohistochemistry and in situ hybridization, has indicated that cytokines, such as IL-5 are instrumental to local eosinophil accumulation during allergen-induced asthma (Bentley et al., 1993).

The Kay group has also found that IL-5 expression is increased in the bronchi of atopic asthma patients (Robinson et al. 1992). Murine studies have confirmed the importance of this cytokine: αIL-5 treatment prior to or five days after airway...
challenge results in decreased eosinophilia (Kung et al., 1995). These results implicate IL-5 in the development of eosinophilic inflammation of the airways and in the migration of eosinophils from the bone marrow into blood in response to antigen challenge (Kung et al., 1995).

CD3+ cells were found to be the principal cellular source of both IL-5 and IL-4 cytokines in the nasal and bronchial tissue of patients with atopic asthma or allergic rhinitis (Kay et al., 1995). Data from human studies strongly implicate IL-5 as a major molecular target in atopic asthma; the role of IL-4 was less clear in this work (Humbert et al., 1997).

However, in a murine model of OVA-induced airway inflammation, Grunig and colleagues confirmed the importance of IL-4, and a related cytokine IL-13 (Grunig et al., 1998). IL-13, like IL-4 is a Th2 cytokine; both IL-4 and IL-13 bind a receptor sharing the same α subunit, IL-4Rα (Zurawski et al., 1995; Lin et al., 1995). Grunig and colleagues showed that when sensitised with ovalbumin (OVA), both IL-4−/− and IL-4Rα+/+ mice displayed attenuated asthmatic phenotypes compared to wild-type controls. When soluble IL-13 was administered to animals using the same model, allergy was exacerbated (Grunig et al., 1998). Studies by Zhu and colleagues also support the importance of IL-13 in the pathogenesis of the acute and chronic manifestations of asthma. This group showed that the targeted pulmonary expression of IL-13, using transgenes containing both the CC10 promoter (targets genes to pulmonary tissues) and murine IL-13, ultimately led to allergic inflammation characterised by an eosinophilic inflammatory response, as well as eotaxin
production (Zhu et al., 1999). However, although IL-13 appears capable of inducing the entire allergic asthmatic phenotype, neutralising the cytokine through the administration of a soluble IL-13 fusion protein – which binds to and neutralises IL-13 – prior to airway challenge with OVA has clearly shown that IL-13-dependent airway hyperresponsiveness (AHR) can occur by mechanisms that are independent of eosinophils (Wills-Karp et al., 1998), thus indicating that IL-13 may act through mechanisms other than those classically implicated in allergic responses.

Eotaxin is an eosinophil chemoattactant, identified by Ganzolo and colleagues in the mouse (Ganzalo et al., 1996). In OVA-induced allergic inflammation, decreased numbers of eosinophils are present in the BALF of animals treated with antibodies to eotaxin (Gonzalo et al., 1996). Eosinophilic recruitment therefore appears to rely on eosinophilic chemoattractants such as eotaxin. Indeed, IL-5, IL-13 and eotaxin have all been implicated in eosinophil recruitment in various allergy models. We therefore measured each of these components in our experiments to assess the effect of helminth infection on eosinophil recruitment in an established model of allergic airway inflammation.
5.1.2. Infection and allergy

The hygiene hypothesis (as reviewed by Wills-Karp et al., 2001 and Yazdanbakhsh et al., 2002) postulates that in the developed world, decreases in childhood diseases have led to an increased prevalence in allergy (c.f. Section 1.5.1). Epidemiological studies have indeed indicated that lack of exposure to parasitic helminth infections (Th2-inducing) as well as bacterial and viral infections (Th1-inducing) may also play a role. In developing countries, where chronic helminth parasitic infections are prevalent, it has been demonstrated that infected children show reduced allergic sensitivities (reviewed in Yazdanbakhsh et al., 2002), and that sensitivities increase in response to anthelminthic treatment (Lynch et al., 1993). Many murine studies have also found associations between worm infections and decreased allergic phenotypes. For example, Wang and colleagues showed that infection with Strongyloides stercoralis prior to OVA exposure resulted in decreased eotaxin levels in the BALF, and a general decrease in pulmonary allergic responses to the allergen (Wang et al., 2001). Previous results from our group (Wilson et al., 2005), and more recently by others (Kitagaki et al., 2006) have shown that infection with H. polygyrus ameliorates airway inflammation, as compared to uninfected controls.

A significant extension of the hygiene hypothesis is that autoimmune diseases may also be increasing in inverse proportion to infection (Bach, 2002). Helminths have been shown to affect autoimmune diseases, such as in a model of multiple sclerosis, the symptoms of which were limited by Trichinella pseudospiralis infection (Boles et al., 2000) and in models of inflammatory bowel disease (IBD) whereby helminths
were shown to decrease the onset and progression of disease (Neurath et al., 1995; Moreels et al., 2004; Khan et al., 2002; Elliott et al., 2004).

Numerous human studies corroborate these results. Skin test reactivity to common allergens was decreased in helminth-infected compared to non-infected individuals in studies in the Gabon (van den Biggelaar et al., 2000), the Gambia (Nyan et al., 2001), Venezuela (Lynch et al., 1993) and Brazil (Araujo et al., 2004). In clinical trials, treating patients for IBD using the pig nematode *Trichuris suis* had positive results (Summers et al., 2005). These findings are not limited to the effect of helminths. Similar results were obtained using treatment with suspensions of the bacterium *Mycobacterium vaccae* (Arkwright & David, 2001) which improved dermatitis in treated children as well as mice; treatment with the suspension conferred protection against allergic inflammation in an OVA-induced murine model of allergic asthma (Zuany-Amorim et al., 2002).

However, instances also exist whereby helminth infections exacerbate allergic phenotypes (reviewed by Mao, 2000). Confounding factors such as helminth type and burden as well as socio-economic status may account for these findings. Therefore more studies are required in order to obtain definitive conclusions on the relationship between helminth infections and atopic disorders.

Numerous hypotheses have been postulated as to why responses to allergens are inhibited in individuals living in areas where helminths are endemic (reviewed in Yazdanbakhsh et al., 2002 and Maizels et al., 2004). One hypothesis is that
production of parasite-induced IgE is a key factor, suggesting that competition exists between helminth-induced polyclonal IgE and aeroallergen-specific IgE for the high-affinity receptors present on mast cells. In support of this, *Nippostrongylus brasiliensis* infection was found to depress hypersensitivity responses in the low-IgE-responder rats, but infection was without effect in high-IgE-responder animals (Turner et al., 1985). Work by Hagel and colleagues with children from the slums in Caracas suggests that polyclonal production of IgE stimulated by helminthic infection can suppress the allergic response to environmental and parasite allergens via both mast cell saturation and inhibition of specific IgE production (Hagel et al., 1993). Indeed, aeroallergen-specific IgE synthesis could be inhibited by high levels of polyclonal IgE. Turner and colleagues proposed that parasitic infections (*Ascaris lumbricoides* and/or *Necator americanus*) in both man and laboratory animals potentiate the production of high levels of IgE with specificity unrelated to the parasite antigens. While the specificity of the potentiated IgE was not established in their studies, it was not directed towards inhalant allergens (Turner et al., 1979).

However, in work by the Turner group, an *Ascaris*-induced immune response was associated with enhanced IgE-mediated reactivity to common inhaled allergens in both allergic and clinically non-allergic subjects (Joubert et al., 1980). Recent studies by our group also indicate that IgE levels may not explain helminth-induced regulation of allergy. Levels of both allergen-specific and polyclonal IgE in mice displaying suppressed allergy following receipt of MLN cells from *H. polygyrus*-infected donors were measured. These IgE levels were comparable to those of control animals. Thus, in this transfer model at least, it is unlikely that the reduction
of allergy was due to saturation of mast cell receptors with allergen-unreactive IgE antibodies (Wilson et al., 2005).

Indeed, recent human data from the Nutman group have shown that saturation of IgE binding sites by polyclonal IgE does not explain the protective effect of helminth infections against atopy. In filaria-infected patients, the ratios of polyclonal IgE to allergen-specific IgE rarely reach the levels required to inhibit allergen-specific IgE receptor binding and to suppress allergen-induced degranulation of mast cells and basophils. Thus, while saturation of IgE binding sites by high ratios of polyclonal to specific IgE is physically possible \textit{in vitro}, this phenomenon likely occurs only rarely in helminth-infected patients, and is not the primary mechanism by which most helminth-infected patients are protected from allergy and asthma (Mitre et al., 2005).

5.1.3. Regulation during infection and allergy

It is now widely accepted that, in both mouse and Man, high levels of regulatory cytokines, such as IL-10, produced during helminthic infections suppress the immune response to unrelated antigens (reviewed in Yazdanbakhsh et al., 2001 and Yazdanbakhsh et al., 2002). In addition, IL-10 has been strongly implicated in allergic asthma regulation (reviewed in Umetsu & DeKruyff, 1999 and Wills-Karp et al., 2001).
Studies by the Erb group have shown that infection of mice with *N. brasiliensis* four weeks prior to allergen sensitisation leads to a decrease in allergic symptoms on challenge. However, this decrease was not apparent in IL-10<sup>−/−</sup> mice, implicating IL-10 in the regulatory mechanisms involved in suppressing allergic symptoms (Wohlleben *et al.*, 2004). Zuany-Amorim and colleagues support a role for both IL-10 and TGF-β in their model system. Treatment with antibodies against both these molecules abrogated protection against airway inflammation observed in mice treated with a suspension of heat-killed *Mycobacterium vaccae* (Zuany-Amorim *et al.*, 2002). In human studies, the Yazdanbakhsh group gave further support to the importance of IL-10. They found a strong negative association between schistosome-specific IL-10 production and positive skin-test reactivity to dust mite in Gabonese children (van den Biggelaar *et al.*, 2000).

However, previous work from our group does not agree with these findings. The decreased allergic phenotype in mice infected with *H. polygyrus* was not affected by IL-10. Indeed, transfer of MLN cells from IL-10<sup>−/−</sup> mice was found to mediate suppression of allergy, while treatment with IL-10R prior to allergen challenge had no effect on allergic inflammation (Wilson *et al.*, 2005). Interestingly, the Selkirk group also demonstrated that IL-10 producing cells were not expanded in their model of *T. spiralis* infection in mice infected with influenza virus (Furze *et al.*, 2006).
5.1.4. Tregs during infection and allergy

The regulatory cytokine IL-10 was shown to be redundant in our allergic airway inflammation model. However, transfer of CD4⁺CD25⁺ regulatory T cells from *H. polygyrus* infected mice significantly decreased the allergic phenotypes of recipients (Wilson *et al.*, 2005). Thus, Tregs generated/expanded by parasitic infection appear to play a vital role in the regulation of this asthma model. Work by the Wahl group has corroborated these findings as transfer of TGF-β-induced CD4⁺ suppressor cells into an antigen-induced allergic model prevented house dust mite-induced allergic pathogenesis (Chen *et al.*, 2003). Zuany-Amorim and colleagues showed that administration of a *Mycobacterium vaccae* suspension generated allergen-specific CD4⁺CD45RBlo Tregs. Transfer of these cells, from OVA-immunised mice into donor animals 24 hours prior to airway challenge conferred protection to OVA-induced eosinophilic inflammation and bronchial hyperresponsiveness in an allergen-specific manner in recipients (Zuany-Amorim *et al.*, 2002). Conversely, Jaffar and colleagues transferred D011.10 Th2-derived-CD4⁺CD25⁻ cells to recipients which were subsequently exposed to OVA. They found increased eosinophilic airway inflammation in response to OVA inhalation, as compared to recipients of total CD4⁺ cells, indicating that this subset is not responsible for the regulatory activity observed (Jaffar *et al.*, 2004).

Akdis and colleagues postulate that the delicate balance between Tregs and effector cells determines either healthy or allergic immune responses to common environmental proteins. They believe that Tregs display antigen-specific suppressor
activity when in small numbers. However, if the number of cells exceeds a certain threshold, non-specific suppression is achieved, explaining the spontaneous development and resolution of allergic diseases (Akdis et al., 2004).

5.1.5. Hypotheses and aims

In our experiments, we wanted to further our understanding of the role of Tregs during helminth infection in a model of allergic asthma inflammation. We were interested in Treg markers at the local sites (MLN and TLN) as well as systemically (spleen) during infection and allergy. We also wanted to determine the dynamic relationship between infection and decreased airway inflammation, and wished to answer whether helminth presence, or merely helminth-induced immunity, was required to maintain the regulation observed. Finally, we were keen to understand the role of CD4⁺CD25⁺ cells within our system, and performed experiments in which these cells were removed by depleting antibodies prior to sensitisation.
5.2. Results

5.2.1. Allergic airway inflammation in *H. polygyrus*-infected animals treated with anthelmintics after sensitisation.

Prior experiments by our group (Wilson *et al.*, 2005) have demonstrated that *H. polygyrus*-infected animals show reduced allergic airway inflammation compared to uninfected controls in a murine model of allergic asthma. We have investigated whether this is also true for animals cured of the infection subsequent to two allergen sensitisations.

The protocol used was similar to that previously described (Wilson *et al.*, 2005), except for an additional third sensitisation, administered 14 days after the final cure in order that the interval between the last sensitisation and the first airway challenge remain constant. The anthelminthic protocol itself was kindly provided by Professor J. Behnke, and to ensure treatment had been successful, faecal egg counts were taken after administration of the final treatment dose. The anthelminthic compound is an acetylcholine agonist and depolarising neuromuscular blocking agent. It produces rigid contraction of the muscles and appears to act on the nerve ganglia and nematode muscle cell membranes. As such, the drug is not thought to affect host immune mechanisms (Fallon *et al.*, 1996). However, in order to ascertain this, initial experiments were conducted with controls for the anthelminthic treatment; no effect of the treatment on host immune responses was discernible (data not shown).
Experiments were conducted in both C57BL/6 and BALB/c mice (Figure 5.1), and in each case, reduced (although not significantly) allergic inflammation was observed irrespective of the use of curative anthelminthic. Total cell infiltrates in the BALF were reduced for both infected and cured C57BL/6 mice and this same trend was observed for BALB/c animals (Figure 5.1 B). Eosinophil numbers were also reduced in both infected and cured groups (Figure 5.1 C). Both eotaxin (Figure 5.1 D) and IL-5 (Figure 5.1 E) levels were similar in infected and cured animals, but both decreased compared to allergic animals.

The decreases in allergic airway inflammation in infected and cured animals were more robust in the C57BL/6 model using Der p1 as an allergen. In BALB/c animals, in which OVA was used as the allergen, the decrease was also apparent but the data were more variable (Figure 5.1 B, C, D, E). It should be noted that more red blood cells contaminated the BALF in the BALB/c experiments, explaining the high levels of BALF cells in these animals compared to the C57BL/6 mice (Figure 5.1 B).
Figure 5.1 – The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is also observed in animals treated with anthelmintics after sensitisation with OVA or Der p1.

A: Experimental protocol for allergic airway inflammation. Mice were infected on d0, and sensitised with 10μg OVA (BALB/c) or Der p1 (C57BL/6) i.p. 28 days later. A second sensitisation was administered on day 42 (14 days after the first), and five days later mice were treated three times (at two day intervals) with an anthelmintic. A third sensitisation was administered two weeks after the first anthelmintic treatment (d65). Mice were challenged i.t. with 10μg OVA or Der p1 on days 79 and 82. Animals were sacrificed 24 hours after the final challenge, and the following groups were studied: naïve animals, allergic animals having been sensitised and challenged to the allergen, infected animals having been infected with *H. polygyrus* prior to sensitisation and challenge and finally the cured group having been infected with *H. polygyrus*, sensitised twice and artificially cleared of parasites with an anthelmintic prior to the third sensitisation and two challenges. The experiment was successfully performed twice, once in C57BL/6 mice and once in BALB/c mice.

B: Total BALF cells from C57BL/6 (Left) and BALB/c (Right) animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and ANOVAs performed; due to variation within the groups, no significance was obtained.

C: Total eosinophils in the BALF from C57BL/6 (Left) and BALB/c (Right) animals, counted from cytopspins stained with Diff Quick (Boehringer, UK). Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and ANOVAs performed; no significance was obtained.
D: Eotaxin levels measured by ELISA in the BALF of C57BL/6 (Left) and BALB/c (Right) mice. Data represent means from groups of 5 mice assayed individually. Dotted line represents the limit of detection. OD values are given when standard curves were not available. Data were transformed and ANOVAs performed; significance was obtained for the experiments performed in C57BL/6 animals but not in BALB/c mice.

E: IL-5 levels measured by ELISA in the BALF of C57BL/6 (Left) and BALB/c (Right) animals. Data represent means from groups of 5 mice assayed individually. Dotted line represents the limit of detection. Data were transformed and ANOVAs performed; no significance was obtained.
Figure 5.1 - The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is also observed in animals treated with anthelmintics after sensitisation with OVA or Der p1.
5.2.2. Cytokine responsiveness in mice treated with anthelmintics after sensitisation does not differ significantly from non-treated animals.

Cytokines were measured in cell culture supernatants from TLN, MLN and spleen cells cultured for 48 hours with or without Hp antigen or OVA allergen. Interestingly responses to OVA in the MLN and responses to Hp antigen in the TLN were at background levels (data not shown). This indicates that responses to antigen/allergen remained within their local lymph nodes (MLN for Hp antigen since *H. polygyrus* is an intestinal parasite, and TLN for OVA allergen, administered i.t.). Although we observed responses to both OVA and Hp antigen in the spleen demonstrating that systemic responses did occur, in the draining lymph node, responses were limited to the antigen/allergen present at the local site of inflammation.

Due to limited supernatant from the TLN cultures, only IL-5, IL-10 and IL-13 were measured. It was hypothesised that the hallmark cytokines of allergy, IL-5 and IL-13, would be reduced, whilst the regulatory cytokine IL-10 would be increased in the infected and the cured animals. For the MLN and spleen, more supernatant was available and IL-4 was also measured as it is a key Th2 cytokine involved in nematode infection (*Urban et al.*, 1991a).

Surprisingly, in the TLN, cytokine production from cultures of cells from allergic, infected and cured mice was not significantly different (Left Panels, Figure 5.2 B, C, D). This contradicts previous results showing that in both the BALF and cells from the TLN, IL-5 and IL-13 responses are significantly reduced in infected compared to
uninfected allergic animals (Wilson, 2005). However, TLN cytokine levels do not always mirror BALF cytokine levels and, the eosinophil and BALF cytokine data from our experiments show clear differences between the allergic and both the cured and the infected groups (Figure 5.1 D, E).

As postulated, in the MLN, responses to *H. polygyrus* were only observed in mice having harboured the parasite, with no significant differences between infected and cured animals (Centre Panels, Figure 5.2 A, B, C, D). Both these groups had similar MLNC proliferative responses to *H. polygyrus* (data not shown), partly explaining these results.

In the spleen, cell culture responses to both OVA and Hp antigen were measured. As hypothesised, responses to Hp antigen were only increased in the infected and the cured groups (Right Panels, Figure 5.2 A, B, C, D). The only significant difference observed between these two groups were the elevated levels of IL-13 in the spleens of the infected group (Right Panel, Figure 5.2 D). IL-13 has been strongly linked to mechanisms of worm expulsion (McKenzie *et al.*, 1999). By the end of the experiment (d83), infected mice still harboured parasites, although few in number, whilst the cured mice had been cleared of worms by d51 (determined by faecal egg counts on the last day of anthelminthic treatment). Elevated levels of the IL-13 cytokine in the local lymph nodes may therefore have occurred earlier and only transiently explaining why this increase in IL-13 was only observed in the spleen and not the lymph nodes, where responses tend to occur later and last longer (*c.f.* Chapter 3; Finney *et al.*, submitted).
Responses to OVA in the spleen cultures were not as strong as the Hp antigen responses (Right Panels, Figure 5.2 A, B, C, D). No significant differences were observed for any cytokine between any of the groups, except for IL-5, where levels in allergic mice were greatly increased compared to the infected (significantly) and the cured animals (non-significantly) (Right Panel, Figure 5.2 B). The strong involvement of IL-5 in allergic responses may help explain the higher levels found within this group. Indeed, increased levels of this cytokine were also found in the BALF. Why this was not reflected at the TLN level is as yet not clear.
Figure 5.2 – Cytokine responsiveness in mice treated with anthelmintics after sensitisation does not differ significantly from non-treated animals.

Parasite antigen-specific and OVA allergen-specific cytokine production in cultures are presented from TLN (Left Panels), MLN (Centre Panels) and spleen (Right Panels) cells. Cells were cultured for 48 hours in medium alone (open bars), or with *H. polygyrus* antigen (solid bars) or OVA allergen (hatched bars). No parasite-specific responses in the TLN or OVA allergen-specific responses in the MLN were greater than constitutive levels, as measured by cells cultured in media. Panel A-D present respectively IL-4, IL-5, IL-10 and IL-13. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all cytokines. No differences were detected between antigen/allergen specific responses in infected and cured animals, except for when stated on graph (* = p<0.05, *** = p<0.001). This experiment was successfully performed once in BALB/c mice.
Figure 5.2 - Cytokine responsiveness in mice treated with anthelmintics after sensitisation does not differ significantly from non-treated animals.
5.2.3. Effect of allergic airway inflammation, in treated and untreated *H. polygyrus*-infected mice, on CD25, FoxP3 and CD8 expression in TLN and MLN.

To assess the role played by Tregs in the described allergy model (Figure 5.1 A), cells from the TLN and MLN of BALB/c mice were stained for CD4, CD8, CD25, and FoxP3. Cells from the spleen were also stained for these markers, however, at this site, no differences were found between any of the groups in the populations studied (data not shown).

In the TLN, the proportion of CD4^{+}CD25^{+} cells, as well as of the CD4^{+}CD25^{+}FoxP3^{+} subset, were significantly decreased in the cured group compared to the allergic group (Left Panels, Figure 5.3 A, B). This was not true for the infected animals. No differences were found between the three groups for FoxP3 expression within the CD4^{+}CD25^{+} population (Left Panel, Figure 5.3 C). Why such a decrease in CD25^{+} cells was observed for cured animals is not certain. Although this experiment was undertaken three times, flow cytometry staining was only possible once; the results need repeating to ensure the decrease is real, particularly as there is no clear explanation as to why the TLN of the cured group should be depleted in these cells.

In the MLN, few differences were observed between the groups for any of the markers (Right Panels, Figure 5.3 A, B, C). CD25 expression was increased in infected animals compared to allergic animals (Right Panel, Figure 5.3 A). We have previously
shown increases in CD25 expression after 70 days post-infection in *H. polygyrus*-infected animals (*c.f.* Chapter 3; Finney *et al.*, submitted). However, for cured mice, this increase was no longer apparent (Right Panel, Figure 5.3 A). Infected animals appear to have decreased levels of FoxP3 (Right Panel, Figure 5.3 B). However, we hypothesise that the significance of this result appears only due to the slightly elevated levels of FoxP3 expression in the allergic compared to naïve animals. No other differences were observed in the MLN.

The CD8^+^FoxP3^+^ cell population was also studied in the lymph nodes as CD8^+^ regulatory cells have been implicated during *H. polygyrus* infection (Metwali *et al.*, 2006). However, although staining was successful, no differences were detected between the allergic, infected and cured groups in either the TLN or MLN (Figure 5.3 D, E).
Figure 5.3 – Effect of allergic airway inflammation, in treated and untreated *H. polygyrus*-infected mice, on CD25, FoxP3 and CD8 expression in TLN and MLN.

**A:** CD4^+CD25^+ T cells from TLN (Left) and MLN (Right) as a proportion of total CD4^+ cells in naïve, allergic, infected and cured animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. Bonferroni post-tests were conducted comparing allergic animals to infected and cured animals (n.s. = no significant difference, * = p<0.05, ** = p<0.01). The experiment was performed once.

**B:** CD4^+CD25^+FoxP3^+ T cells from TLN (Left) and MLN (Right) as a proportion of total CD4^+CD25^+ cells in naïve, allergic, infected and cured animals, as for Figure 5.3 A.

**C:** CD4^+CD25^+FoxP3^+ T cells from TLN (Left) and MLN (Right) as a proportion of total CD4^+ cells in naïve, allergic, infected and cured animals, as for Figure 5.3 A.

**D:** Representative plots of bivariate flow cytometry analysis of CD8 and FoxP3 expression in lymphocytes from TLN in naïve (Far Left), allergic (Left) and infected (Right) and cured (Far Right) animals.

**E:** CD8^+FoxP3^+ T cells from TLN (Left) and MLN (Right) as a proportion of total CD4^+ cells in naïve, allergic, infected and cured animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. No overall differences were detected.
Figure 5.3 – Effect of allergic airway inflammation, in treated and untreated *H. polygyrus*-infected mice, on CD25, FoxP3 and CD8 expression in TLN and MLN
5.2.4. CD103 expression is upregulated in mice with a prolonged *H. polygyrus* infection, and mice cured of infection prior to airway challenge.

CD103 and FoxP3 levels were measured in lymph node populations by flow cytometry in order to determine whether homing of Tregs after allergic sensitisation and challenge was affected by *H. polygyrus* infection. Significantly increased levels of CD103 expression were observed in both infected and cured groups compared to naïve and allergic groups in the TLN (Figure 5.4 A, B) and MLN (Figure 5.4 C, D). Increases in CD103 expression occurred in both FoxP3\(^+\) (Centre Panel, Figure 5.4 B, D) and FoxP3\(^-\) (Right Panel, Figure 5.4 B, D) subpopulations. However, although in the TLN the cured and infected groups showed very similar results, in the MLN increases were greater in the infected group, than in the cured group. This may indicate that in the MLN of cured mice where treatment had eradicated worms 51 days post-infection, levels had started to return to basal levels. It would be interesting to repeat the experiment leaving more than 14 days between cure and sensitisation so as to assess whether the effect of infection is long-lasting in animals treated with anthelmintics.

Increases in the levels of CD103 in the lymph nodes of infected and cured animals indicate increased levels of homing capacity within the Treg populations of these two groups. This further implies that cells involved in immune regulation in the gut can recirculate to a new site where regulation is required, and exert suppressive activity at this site.
Figure 5.4 – CD103 expression, independently of FoxP3 expression, is increased in the TLN and MLN from mice with a prolonged *H. polygyrus* infection as well as mice having been cured from the infection, prior to airway challenge.

A: Representative plots of bivariate flow cytometry analysis of FoxP3 and CD103 expression in CD4^+^CD25^+^ T cells from TLN in naïve (Far Left), allergic (Left), infected (Right) and cured (Far Right) animals. The experiment was performed once.

B: CD103^+^ (Left), CD103^+^FoxP3^+^ (Centre) and CD103^+^FoxP3^- (Right) cells from TLN as a proportion of total CD4^+^CD25^+^ cells in naïve, allergic, infected and cured animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. Bonferroni post-tests were conducted comparing allergic animals to infected and cured animals (n.s. = no significant difference, * = p<0.05, ** = p<0.01, *** = p<0.001).

C: As Figure 5.4 A, using MLN cells.

D: As Figure 5.4 B, using MLN cells.
Figure 5.4 – CD103 expression, independently of FoxP3 expression, is increased in the TLN and MLN from mice with a prolonged *priceurus* infection as well as mice having been cured from the infection, prior to airway challenge.
5.2.5. The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is not affected by helminth eradication prior to sensitisation with OVA.

As shown in Figures 5.1 to 5.4, treating animals with anthelmintics after sensitisation did not affect the decreased allergic airway inflammation observed in infected compared to allergic animals. However with this protocol (Figure 5.1 A), antigen and allergen were both present between the times of the first sensitisation (d28) and the first cure (d47). In the following alternative protocol (Figure 5.5 A), animals were cured prior to sensitisation, in order that infection and allergen never be contemporaneously present.

Using this latter protocol, all three markers for allergy in the BALF (eosinophilia, eotaxin and IL-5 levels) were reduced in the infected and the cured groups compared to the allergic animals. Thus, readings of eosinophilia and BALF cytokines indicate that treating infected mice with anthelmintic prior to sensitisation did not affect their ability to limit allergic airway inflammation.
Figure 5.5 – The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is not affected after helminth eradication prior to sensitisation with OVA.

A: Experimental protocol studying allergic airway inflammation. Mice were infected on d0, and treated three times (on consecutive days) with an anthelminthic 28 days later. A week after the first treatment, mice were sensitised with 10μg OVA i.p, followed by second sensitisation on day 50 (14 days after the first). Mice were challenged i.t. with 10μg OVA on days 63 and 66. Animals were sacrificed 24 hours after the final challenge. The experiment was successfully performed once.

B: Total eosinophils in the BALF from BALB/c animals, counted from cytopsins stained with Diff Quick (Boehringer UK). Data represent means from groups of 5 mice assayed individually.

C: Eotaxin (Left) and IL-5 (Right) levels measured by ELISA in the BALF of naïve, allergic, infected and cured animals. Data represent means from groups of 5 mice assayed individually. Dotted line represents the detection level. Data were transformed and ANOVAs performed; for eotaxin, p<0.001, while no significance was obtained for IL-5.
Figure 5.5 – The reduced allergic airway inflammation observed in *H. polygyrus*-infected animals is also observed in animals treated with anthelmintics prior to sensitisation with OVA.
5.2.6. Cytokine responsiveness in mice treated with anthelmintics prior to sensitisation does not differ significantly from non-treated animals.

Cytokine levels were measured in the TLN, MLN and spleen as previously described in Section 5.2.2; responses to OVA in the MLN and to Hp antigen in the TLN were at background levels.

In the TLN cultures, cytokine production by the cells from the allergic group was significantly increased compared to those from infected and cured mice (Left Panels, Figure 5.6 B, C, D). This goes against our previous findings described in Section 5.2.2 (Figure 5.2) but corroborates our previous study where IL-5, IL-10 and IL-13 were significantly increased in the TLN of allergic mice (Wilson, 2005). Levels of these three cytokines remained at background levels in infected and cured animals. Interestingly, as seen in Section 5.2.2, no differences were observed between infected and cured animals, indicating that treating mice with anthelmintics prior to (Figure 5.5 A) rather than after sensitisation (Figure 5.1 A) did not affect the infection-induced decrease in allergic airway inflammation.

In the MLN, responses to *H. polygyrus* were only observed in mice having harboured the parasite. For all cytokines, it appeared that levels in cured animals were decreased compared to those of infected animals (Centre Panels, Figure 5.6 A, B, C, D). Indeed, cured animals were treated with anthelmintics on d28 of infection and by day 67, their MLNC proliferative responses to *H. polygyrus* antigen were not as elevated
as those from infected animals (data not shown), which could account for lower cytokine production.

In the spleen, cytokine production in response to both OVA and Hp antigen was measured. Responses to Hp antigen were only measurable in the infected and cured groups (Right Panels, Figure 5.6 A, B, C, D). As with the MLN, it appeared that cytokine levels were elevated in the infected compared to the cured group. This decreased production by cells from the cured animals was observed for all cytokines except IL-10 and was most probably a result of lower proliferative responses to Hp antigen in the spleen cells from cured animals (data not shown).

In the spleen, responses to OVA were not as strong as the Hp antigen responses (Right Panels, Figure 5.6 A, B, C, D). No differences in OVA-specific responses were observed for any cytokine between any of the groups, except for IL-13, where levels in allergic mice were increased (non-significantly) compared to the infected and the cured animals (Right Panel, Figure 5.6 D).
Figure 5.6 – Cytokine responsiveness in mice treated with anthelmintics prior to sensitisation does not differ significantly from non-treated animals.

Parasite antigen-specific and OVA allergen-specific cytokine responses are presented from TLN (Left Panels), MLN (Centre Panels) and spleen (Right Panels) cells. Cells were cultured for 48 hours in medium alone (open bars), or with *H. polygyrus* antigen (solid bars) or OVA allergen (hatched bars). No parasite-specific responses in the TLN and OVA allergen-specific responses in the MLN were greater than constitutive levels, as measured by cells cultured in media. Panel A-D present respectively IL-4, IL-5, IL-10 and IL-13. Data represent means and standard errors from groups of 5 mice assayed individually. The experiment was performed twice.
Figure 5.6 - Cytokine responsiveness in mice treated with anthelmintics prior to sensitisation does not differ significantly from non-treated animals.
5.2.7. *H. polygyrus* infection, whether it has or has not been eradicated prior to sensitisation, does not affect CD25 and FoxP3 expression in TLN cells, after allergic airway sensitisation and challenge.

In order to characterise regulatory T cells in an allergy model where infection with *H. polygyrus* was not contemporaneous with allergen sensitisation (as opposed to the model described in Figure 5.1A where this was the case), cells from the TLN, MLN and spleen were stained for CD4, CD25 and FoxP3. Cells were not stained for CD8 since no significant differences had been observed in the previous experiment (Figure 5.3D, E). Results are shown only for the TLN cells as no differences were found between any of the groups in the MLN and spleen populations studied (data not shown).

Allergic sensitisation with or without infection did not alter the frequencies of CD25 and FoxP3 expression. Moreover, unlike when animals were treated with anthelminthic after sensitisation (Figure 5.1A), no reduction was observed as a result of treatment here (Figure 5.7A, B, C). Thus no difference in the proportion of Tregs (defined as CD4+CD25+FoxP3+ cells) was detected between any of the three groups. However, total TLN cell and thus Treg numbers were greater in the allergic group than in the infected and cured groups (data not shown). This indicates that either Tregs are not involved in the decrease in allergic airway inflammation observed in the infected and cured groups, or that shifts in cell numbers, rather than in cell proportions, are sufficient for this process.
Figure 5.7 – *H. polygyrus* infection, whether it has or has not been eradicated prior to sensitisation, does not affect CD25 or FoxP3 expression in TLN cells after allergic airway sensitisation and challenge.

A: CD4<sup>+</sup>CD25<sup>+</sup> T cells from TLN as a proportion of total CD4<sup>+</sup> cells in naïve, allergic, infected and cured animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. No significant differences were detected. The experiment was performed once.

B: CD4<sup>+</sup>FoxP3<sup>+</sup> T cells from TLN as a proportion of total CD4<sup>+</sup>CD25<sup>+</sup> cells in naïve, allergic, infected and cured animals, as for Figure 5.7 A.

C: CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells from TLN as a proportion of total CD4<sup>+</sup> cells in naïve, allergic, infected and cured animals, as for Figure 5.7 A.
Figure 5.7 — *H. polygyrus* infection, whether it has or has not been eradicated prior to sensitisation, does not affect CD25 or FoxP3 expression in TLN cells after allergic airway sensitisation and challenge.
5.2.8. CD103 expression increases in TLN and MLN from sensitised mice infected with *H. polygyrus*, but not in animals cleared of infection prior to sensitisation.

In order to study Tregs in a model of allergic inflammation where infection and allergen were never contemporaneous (as described in Figure 5.5 A), CD103 and FoxP3 levels were measured by flow cytometry. Cured animals did not show the increased levels of CD103 expression (appearing primarily in FoxP3⁻ cells) characteristic of infected mice (Figure 5.8). In animals having been treated prior to sensitisation, levels of CD103 expression were similar to those in uninfected mice, whether naïve or allergic. This was in contrast to findings from the previous model in which anthelminthic treatment was given after sensitisation (Figure 5.1 A).

Interestingly, although CD103 levels were not increased in the cured group, the main markers of allergic airway inflammation were decreased similarly to infected animals, compared to allergic animals (Figure 5.5). CD103⁺ cells may therefore not be involved in the mechanisms which downregulate allergic inflammation in the airways of these mice. However, timing may have played a role in these results. The decrease in CD103⁺ cells in the lymph nodes may have been recent, such that sufficient numbers of regulatory cells were present in the airways to maintain regulation – in future experiments, CD103 staining in the lung would determine whether this is the case. Had the time lapse between the cure and sensitisation been greater, markers of allergy may have been increased in the cured group.
Figure 5.8 – CD103 expression increases in TLN and MLN from sensitised mice infected with *H. polygyrus*, but not in animals cleared of infection prior to sensitisation.

A: Representative plots of bivariate flow cytometry analysis of FoxP3 and CD103 expression in CD4⁺CD25⁺ T cells from TLN in naïve (Far Left), allergic (Left) and infected (Right) and cured (Far Right) animals. The experiment was performed twice.

B: CD103⁺ (Left), CD103⁺FoxP3⁺ (Centre) and CD103⁺FoxP3⁻ (Right) cells from TLN as a proportion of total CD4⁺CD25⁺ cells in naïve, allergic, infected and cured animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. Bonferroni post-tests were conducted comparing allergic animals to infected and cured animals (n.s. = no significant difference, * = p<0.05, ** = p<0.01, *** = p<0.001). The experiment was performed twice.

C: As Figure 5.8 A, using MLN cells.

D: As Figure 5.8 B, using MLN cells.
Figure 5.8 – CD103 expression increases in TLN and MLN from sensitised mice infected with *H. polygyrus*, but not in animals cleared of infection prior to sensitisation.
5.2.9. Effect of αCD25 treatment on *H. polygyrus*-induced reduction of airway sensitivity.

As shown in Figure 5.5, treating animals with anthelmintics prior to sensitisation did not abolish the decrease in airway inflammation observed in infected compared to allergic animals. This indicates that helminth presence is not necessary, but that helminth-induced immunity is sufficient in downmodulating airway inflammation. In order to determine whether the observed downregulation was due to CD4⁺CD25⁺ cells, CD25 depleting antibody (PC61) was administered 4 days prior to the first sensitisation (Figure 5.9 A).

Following allergen challenge, total BALF cellular infiltrates were measured and found to contain increased cell numbers in the cured/αCD25-treated animals compared to cured/IgG control-treated mice (Figure 5.9 B). However, cell numbers remained below those seen in allergic animals. More importantly eosinophil infiltration was as low as in the αCD25-treated as in the IgG control-treated animals (Figure 5.9 B), so it would appear that CD4⁺CD25⁺ cells do not account for the reduction in eosinophils observed in animals cured of infection (Figure 5.5 B).

Eotaxin levels in the BALF were elevated in cured/αCD25-treated animals as compared to all other groups. However, contrary to previous findings (Figure 5.1 C, Figure 5.5 C, Wilson et al., 2005), levels in allergic animals remained low in this experiment. Levels of IL-5 were increased in allergic animals but in cured/αCD25-treated animals, levels were undetectable (Figure 5.9 C). Therefore, although much
variability was observed in this experiment, changes were apparent in the cured/αCD25-treated compared to the cured/IgG control-treated animals. This implies that CD4^+CD25^+ cells, although not the sole contributors, may play a role in the immunoregulation observed in animals having been cured of infection prior to allergen sensitisation and challenge.
Figure 5.9 - Effect of αCD25 treatment on *H. polygyrus*-induced reduced airway sensitivity.

A: Experimental protocol for allergic airway inflammation. Mice were infected on d0, and treated three times (on consecutive days) with an anthelminthic 28 days later. Treatment with 1mg of αCD25 (or IgG control) was administered on day 53, and three days later, mice were sensitised with 10µg OVA i.p, followed by a second sensitisation on day 70 (14 days after the first). Mice were challenged i.t. with 10µg OVA on days 84 and 87. Animals were sacrificed 24 hours after the final challenge. The experiment was performed once.

B: Total BALF cells (Right) and eosinophils (Left) within the BALF were counted from cytospins stained with Diff Quick (Boehringer, UK). Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. No significant differences were detected.

C: Eotaxin (Left) and IL-5 (Right) levels were measured by ELISA in the BALF in naïve, allergic, cured/IgG control-treated and cured/αCD25-treated animals. Data represent means from groups of 5 mice assayed individually. Dotted lines represent the detection levels. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. No significant differences were detected.
Figure 5.9 – Effect of αCD25 treatment on *H. polygyrus*-induced reduced airway sensitivity
5.2.10. Cytokine responsiveness increases in mice treated with αCD25 after eradication of *H. polygyrus* infection prior to sensitisation with OVA.

So as to determine the effect of αCD25 treatment (Figure 5.9 A) on TLN, MLN and spleen cell responses, cytokine levels were measured as for Section 5.2.2 (Figure 5.2). Responses to OVA in the MLN and to Hp antigen in the TLN were at background levels.

In the TLN cultures, cytokine production in the infected/cured (IgG control-treated) group differed significantly from uninfected/allergic mice (Left Panels, Figure 5.10 B, C, D). However, OVA-specific levels of IL-5, IL-13 and IL-10 all approached allergic levels in the αCD25-treated animals, although the constitutive expression of these cytokines also increased in the αCD25-treated animals. Depleting natural Tregs prior to sensitisation did therefore affect the cytokine responses in the TLN.

In the MLN, responses to *H. polygyrus* were only observed in mice having harboured the parasite. Administration of αCD25 increased cytokine production as compared to the IgG control-treated mice. However, again, all constitutive levels were greatly raised in the αCD25-treated animals. Therefore, treating with αCD25 increases cytokine production (*c.f.* Chapter 4), but not in a regulated fashion, which may explain why all cytokine levels were increased here.

In the spleen, responses to both OVA and Hp antigen were measured. Again, as hypothesised, responses to Hp antigen were high in the infected and cured groups
only (Right Panels, Figure 5.10 A, B, C, D). As with the MLN, it appeared that cytokine levels were elevated in the cured/αCD25-treated animals compared to the cured/IgG-control-treated group. However, this was solely due to constitutive production; no parasite or allergen-specific responses were observed in the spleen as a result of CD25 depletion.

In the spleen cultures, as postulated, Hp-specific cytokine responses were low in allergic animals but increased in the cured/IgG control-treated animals (Right Panel, Figure 5.10 A, B, C, D). Cytokine levels in the cured/αCD25-treated animals were also elevated but to the same degree as constitutive responses (Right Panel, Figure 5.10 A, B, C, D). No differences in OVA-specific cytokine production were observed between either of the allergic or cured/IgG control-treated groups, except for IL-5, where levels in allergic mice were significantly increased compared to other groups (Right Panel, Figure 5.10 B). However, cytokines levels in the cultures from spleen cells from cured/αCD25-treated animals were greatly increased (Right Panel, Figure 5.10 A, B, C, D). This does not reflect a marked upregulation of OVA-specific responses in these mice since constitutive cytokine levels (measured in the media cultures) were increased to the same degree, indicating that administration of αCD25 leads to a dyregulation of cytokine responses in the spleen.
Figure 5.10 – Cytokine responsiveness increases in mice treated with αCD25 after eradication of *H. polygyrus* infection prior to sensitisation with OVA.

Parasite antigen-specific and OVA allergen-specific cytokine responses are presented from TLN (Left Panels), MLN (Centre Panels) and spleen (Right Panels) cell cultures. Cells were cultured for 48 hours in medium alone (open bars), or with *H. polygyrus* antigen (solid bars) or OVA allergen (hatched bars). No parasite-specific responses in the TLN and OVA allergen specific responses in the MLN were greater than constitutive levels, as measured by cells cultured in media. Panels A-D present respectively IL-4, IL-5, IL-10 and IL-13. All responses measured were intrapolated from standard curve values. Data represent means and standard errors from groups of 5 mice assayed individually. The experiment was performed once.
Figure 5.10 – Cytokine responsiveness increases in mice treated with αCD25 after eradication of *H. polygyrus* infection prior to sensitisation with OVA.

A  
**MLN**

**Spleen**

B  
**TLN**

C

D
5.2.11. CD103 expression increases in TLN cells from animals having been treated with αCD25 after administration of anthelmintics.

CD4, CD25 and CD103 expression on TLN cells was measured by flow cytometry staining. CD103 expression on both CD4⁺CD25⁻ and CD4⁺CD25⁺ populations were increased in the αCD25-treated group compared to those observed in the IgG-control-treated group, but not changed from the allergic group (Figure 5.11). Thus CD103⁺ cells may contribute to the mechanisms involved in maintaining reduced airway inflammation in the cured animals. However, whether CD103 is important for suppression or migration requires further investigation.
Figure 5.11 – CD103 expression increases in TLN cells from animals having been treated with αCD25 after administration of anthelmintics.

A: Representative plots of bivariate flow cytometry analysis of CD25 and CD103 expression in CD4⁺ T cells from TLN in cured/IgG control-treated (Left) and cured/αCD25-treated (Right) animals. The experiment was performed once.

B: CD103⁺ cells as a proportion of total CD4⁺CD25⁻ (Left) and CD103⁺ cells as a proportion of total CD4⁺CD25⁺ cells (Right) from TLN in naïve, allergic, IgG Control and αCD25-treated animals. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed and one-way ANOVAs performed on all groups except for naïve animals. Bonferroni post-tests were conducted comparing allergic animals to infected and cured animals (n.s. = no significant difference, ** = p<0.01).
Figure 5.11 – CD103 expression increases in TLN cells from animals having been treated with αCD25 after administration of anthelmintics
5.3. Discussion

The results obtained from the experiments described in this chapter confirm those previously published by our group (Wilson et al., 2005) and more recently by Kitagaki and colleagues (Kitagaki et al., 2006), stating that *H. polygyrus*-induced infection (prior to allergy onset) can reduce symptoms of allergic inflammation. However, it also appears that eradicating infection does not affect the ability to downmodulate allergic inflammation, and that this process does not solely rely on CD4^+^CD25^+^ Tregs.

5.3.1. Timing of infection

In many models of infection, the timing of infection has proved an important factor in regulating the suppression of allergic inflammation. Work by Boles and colleagues demonstrated that in rat experimental allergic encephalitis (EAE) triggered by injection of myelin basic protein (MBP), symptoms were ameliorated when MBP was injected while *Trichinella pseudospiralis* was undergoing its migratory phase of infection. Such results were not obtained when the intestinal phase of infection was used (Boles et al., 2000). The Erb group similarly confirmed the importance of infection stage by showing that a decrease in eosinophilia and eotaxin was apparent in the airways of mice infected with *Nippostrongylus brasiliensis* 4 and 8 weeks prior to allergy, but not one or two weeks prior to OVA challenge (Wohlleben et al., 2004). Also, infection with *Brugia pahangi* only affected responses to PPD (soluble...
Mycobacterium extract) if it preceded the PPD treatment; when administered simultaneously, the helminth had no effect on the PPD response (Pearlman et al., 1993). Finally, the Selkirk group recently reported that influenza symptoms were alleviated during initial infection with *Trichinella spiralis* while this was not the case when parasites were encysted in the skeletal muscle (Furze et al., 2006). They postulated that immunmodulatory effects generated by the parasites may have evolved to minimise tissue damage during parasite migration, to protect the relatively vulnerable larval stages when they are most exposed to immune attack, so that dispersal of eggs and larval stages is ensured (Furze et al., 2006).

Since infection time lines have such profound effects on suppressive potential, it would be interesting to assess how early in infection suppression to airway allergic inflammation can be observed in the *H. polygyrus* model. Animals used in our experiments were infected 28 days prior to cure and/or sensitisation. However, could suppression be observed using animals infected for only 21 days prior to this, a time where both TGF-β and CD103 Treg markers are increased, or even 14 days, when increases in CD103 only are observed (c.f. Chapter 3; Finney et al., submitted)? Similar questions can be asked for the anthelminthic protocol. In the group of animals cured after sensitisation, anthelminthic treatment was administered on d47, when the majority, but not all, parasites would have been cleared naturally (c.f. Chapter 7). It would be interesting to eradicate parasites at an earlier time point, as this may reveal the critical time point after which regulatory mechanisms induced by infection have been generated.
However, the initial time point of infection is impossible to determine in the field which may explain discrepant reports on the suppressive effects of helminths on allergy in human studies (c.f. Section 5.1). Notably, few of these studies were long term. For example, work by Arkwright & David showed an improvement in atopic dermitis in children who were administered a suspension of *Mycobacterium vaccae*. The effects were observed for up to 3 months after administration, but longer term effects were not assessed (Arkwright & David, 2001). Therefore, although timing of infection is of interest, determining the effect of anthelminthic treatment on the longevity of helminth-induced regulation is more informative for assessing the effect of helminths in the context of human allergic disease.

5.3.2. The effect of anthelminthic treatment

Our results indicate that treating infected mice with anthelmintics had no effect on their ability to downregulate symptoms of allergic airway inflammation. In the models presented therefore, infection is required for initiating regulation, but parasite presence is not required for maintaining it.

Data from human studies relating to this aspect have been contradictory. Lynch and colleagues found that curing patients of helminth infections ablated their protection from allergic symptoms 22 months after treatment (Lynch *et al.*, 1993). However, in a later study, they found that anthelminthic treatment alleviated asthmatic symptoms (Lynch *et al.*, 1997).
Timing of treatment may explain some of these discrepancies. In our murine model, animals were challenged with OVA no later than one month after treatment. Had the experiments ran for longer, we may have seen that, in the cured animals, the suppressive effect of infection on allergy was abolished. Indeed, future experiments where animals are left for 6 months or a year after treatment before the induction of allergy would help elucidate whether active infection is required to maintain long-term protection from allergic conditions.

5.3.3. Treg involvement

Tregs have been strongly implicated in regulating allergic responses (c.f. Section 5.1). However, in our experiments we found no increase in the number of CD25⁺FoxP3⁺ cells or FoxP3 expression on CD4⁺CD25⁺ cells in the TLN of either infected or cured mice. No increases were apparent in the MLN or spleen. CD8⁺FoxP3⁺ have also been implicated in regulatory mechanisms induced by *H. polygyrus* (Metwali *et al.*, 2006). Again, no increases in the frequency of this phenotype in the TLN, MLN or spleen were found for this subset. Thus the ability of infected/cured mice to suppress subsequent allergic response is not mediated through Tregs in our model.

Our results contradict recent findings by Kitagaki and colleagues, who reported an increase in the percentage of CD4⁺CD25⁺ cells expressing FoxP3 in the TLN of infected mice. However, it has still not been determined whether changes in CD4⁺CD25⁺FoxP3⁺ cell proportions in the draining lymph nodes (thus preferential
influx) are necessary for regulatory mechanisms to take place, or if shifts in total cell numbers, as in our experiments, could be sufficient. Also, data presented by Kitagaki and colleagues did not include flow cytometry plots and so it was difficult to directly compare the two sets of data. For example, in our hands, intracellular FoxP3 staining required much optimisation before clear and repeatable results were obtained.

The data from experiments in which animals were treated with αCD25 just prior to sensitisation (Figure 5.9, 5.10, 5.11), indicate that natural CD4⁺CD25⁺ cells are involved, but probably not the main players, in the regulatory mechanisms which downregulate allergic airway inflammation. Indeed, total cell numbers and eotaxin levels were increased in the BALF of cured/αCD25-treated animals, as compared to cured/IgG-treated controls. Also, cytokine (IL-5, IL-10 and IL-13) levels in the TLN, which were decreased in cured/IgG control-treated animals compared to allergic animals were increased in cured/αCD25-treated animals. However, we found no increases in FoxP3 in the TLN, MLN or spleen and IL-5 levels and eosinophil numbers in the BALF were not altered by αCD25 treatment, indicating that other cell types, rather than CD4⁺CD25⁺ Tregs, may be involved in maintaining the regulatory environment. This was confirmed by work from the Locksley group whereby Tregs, challenged with exogenous antigens, whilst remaining efficient in blocking Th2 effector functions by CD4⁺ cells, were unable to curtail the induction of airway hyperreactivity. This group postulated that CD4⁺CD25⁺ cells may have limited regulatory activity on innate mechanisms such as mucus secretion during complex, chronic conditions, and that other cells may play a substantial role in these conditions (Hadeiba & Locksley, 2003).
However, work by our group suggests that *H. polygyrus*-stimulated CD4⁺CD25⁺ cells play a very important role in regulating airway inflammation (Wilson *et al.*, 2005). When these cells were transferred from infected animals into sensitised recipients, allergy symptoms were reduced in the latter. Depleting CD25 prior to allergen challenge also ablated protection provided by helminth infection in this model (Wilson *et al.*, 2005). It would have been interesting to study the effect of the CD25 depletion on allergy itself in our experiments. Uninfected animals receiving αCD25 treatment should be studied in future to determine whether the effects observed here were truly due to regulatory cells generated by infection.

5.3.4. T cell trafficking

A recent study by our group demonstrated that cells transferred from *H. polygyrus* infected mice into OVA sensitised mice by i.p. injection, 7 days prior to allergen challenge, were found to have migrated to the draining lymph nodes. It was postulated that donor cells from chronically infected mice migrate to sites of inflammation, whether to directly suppress host immune responses, or to conscript resident cells to a regulatory phenotype (Wilson *et al.*, 2005).

In the experiments described in this chapter, we showed that CD103 expression was increased in both TLN and MLN CD4⁺CD25⁺ cell populations in infected and cured – after sensitisation (Figure 5.1 A) – animals as compared to allergic mice. This was independent of FoxP3 expression. Since CD103 is known to be a homing marker, the
increased expression could reflect enhanced cell migration capacity to the draining lymph nodes and/or tissue.

CD103 expression was also increased in both CD4⁺CD25⁺ and CD4⁺CD25⁻ cells after αCD25 treatment compared to IgG control treatment in our experiments. After the depletion of regulatory cells, regulatory mechanisms are greatly disrupted and this is thought to result in the general cell migration observed towards the TLN, local to the site of inflammation.

To gain more insight into the homing of regulatory cells in our model, it would be interesting to use CD103⁻/⁻ animals. Experiments conducted in these animals could help determine whether CD103 is required for the migration of Tregs to inflammation sites. Transferring CD103⁺ or CD103⁻ cells expressing congenic markers such as Ly5.1, would also provide clues as to the role of CD103 in cell migration and the regulation of inflammation and allergy.

5.3.5. The timing of anthelminthic treatment

In the models of allergic inflammation used here, no differences in allergic phenotype were obtained for mice cured prior to (Figure 5.5 A) or after sensitisation (Figure 5.1 A): allergy was reduced compared to non-infected animals in both protocols. By studying the differences between these protocols, we wanted to gain an understanding of the importance of Treg specificity. Could regulation of allergy still occur, even though infection and the allergen had never been present at the same
time? In all our experiments, reduced allergic airway inflammation was observed in animals cured of infection (determined by faecal egg counts post-treatment), be it before (Figure 5.5 A) – a week or a month – or after sensitisation (Figure 5.1 A). Therefore, despite Treg activation being an antigen-dependent driven process (c.f. Section 1.4.1), the pleiotropic suppressive effects of Tregs generated by infection are not and could be targeted to allergens to which they had not been previously exposed. However, in follow-up studies, more time should be left between the cure and sensitisation to ensure that the effects we observed can be long lasting.

Our findings on the effects of nematodes on bystander responses have implications for vaccination strategies in helminth-endemic areas. Indeed, as early as over 30 years ago, *H. polygyrus* infection was found to alleviate influenza disease symptoms in mice (Chowaniec *et al.*, 1972). However, it was postulated the parasite infection may have an impact on vaccination efficiency. Indeed, data from human studies have revealed that helminths have detrimental effects on the efficacy of vaccines (tetanus vaccine: Sabin *et al.*, 1996; Cooper *et al.*, 1999, BCG: Elias *et al.*, 2001, malaria vaccine: reviewed in Nacher, 2001). Experiments in murine models agree with these findings, and in the case of *H. polygyrus* infection, it was found that protection conferred by immune activation with a malaria vaccine which normally improved parasitaemia and survival, was of diminished magnitude (Su *et al.*, 2006). However, anthelminthic treatment prior to vaccination induced strong protective immunity in this murine model of malaria. These findings have implications on the anthelminthic strategies in the developing world as they indicate that helminth infection is detrimental to vaccine efficiency. Our results, albeit in a mouse model, demonstrate
that careful thought and consideration as to the timing of treatment should be taken since it appears that the regulatory effects induced by helminths are maintained in cured animals, even if sensitised after the treatment protocol.

5.3.6. Concluding remarks and ideas for future research

Our work in this chapter, in corroboration with previous studies from our group (Wilson et al., 2005), has shown that Tregs generated by parasite infection have a profound impact on bystander immune responses. Future experiments using much longer time lines should be carried out in order to determine the long lasting suppressive effects of chronic infections on immune responses.

Moreover, to gain information on the effect of parasite-induced regulation on the developing immune system, infections in neonatal animals should be performed. Work in the field has demonstrated that children born to schistosome-infected mothers show decreased responses to BCG in early life (Malhotra et al., 1999). Therefore, in animal models, infection of pregnant females should be studied to determine how parasite infection in the parent can affect the uninfected offspring. Imprinting may have significant effects on long term immune responses, and as such experiments like these must be conducted for better understanding of the long lasting effects of helminth infections on the immune system. Since parasites are ubiquitous in poverty-stricken regions and drug cures do not protect from re-infection, it is vital we understand how they could affect immune responses to other pathogens/allergens/antigens in order to design vaccination/treatment programs accordingly.
6. SECONDARY INFECTION WITH *H. POLYGYRUS*

6.1. Introduction

Re-infection with helminths is common in endemic areas; drug clearance does not result in protection from future infections. However, in some individuals, acquired immunity is generated and strengthens with age resulting in less severe pathology with secondary infections. Understanding how acquired immunity is generated through the study of secondary infections is of great relevance in designing disease control and vaccination programs.

Secondary infections with *H. polygyrus* are characterised by strongly decreased worm burdens, compared to primary infections (Urban et al., 1991a). However, the exact mechanisms involved in this process, and the parasitic stage targeted (larvae or adult) is still a question of debate. Possible candidates include Th2 cytokines, IgE, the gut mucosa, mast cells and eosinophils (c.f. Section 1.4). Although recently, using immuno-histochemistry and immunofluorescent laser capture microdissection, IL-4R-expressing macrophages have also been strongly implicated in protection from *H. polygyrus* (Anthony et al., 2006).

The effector mechanisms responsible for increased expulsion are highly dependent on Th2 cytokines. Indeed, Th2 responses, characterised by the expression of cytokines such as IL-4, IL-5, IL-9, and IL-13, are essential for host protective immunity.
to many intestinal nematode infections (reviewed in Finkelman et al., 1997; Grencis, 1997 and Else & Finkelman, 1998). For example, in *Trichuris muris* infection, IL-4, IL-5 and IL-9-producing Th2-type cells have been associated with host resistance, whilst IFN-γ-producing Th1 cells have been associated with susceptibility (Else et al., 1992). These Th2 cytokines are also increased during primary *H. polygyrus* infection (Svetic et al., 1993; Finney et al., submitted; Chapter 3). However, why these cytokines are not protective in primary infections has yet to be fully determined. In secondary infections, quantitative and qualitative changes such as a more rapid rise in cytokine levels and the presence of mast cells (Dehlawi et al., 1987) respectively, may account for the differences observed in worm burdens, although more research is required to fully understand the mechanisms of parasite expulsion.
6.1.1. IL-4 and IL-13

IL-4 is a major player in controlling *H. polygyrus* infection, as shown by decreased worm fecundity and more efficient expulsion in hosts treated with exogenous IL-4 during primary infection (Urban *et al.*, 1995). Even though IL-4 is produced by animals with a primary infection, the ability of exogenous IL-4 to terminate infection suggests that high levels are required for complete clearance (Urban *et al.*, 1995). Treatment with exogenous IL-4 during the larval stages has no real effect on parasite survival or fecundity (Urban *et al.*, 1995), indicating that IL-4 has little effect on the early phases of primary infection. Also, αIL-4 treatment appears to block polyclonal IgE responses and abrogate protection observed during secondary *H. polygyrus* infection (Urban *et al.*, 1991a).

Treatment with αIL-4R, a receptor subunit for both IL-4 and IL-13, also abrogates protection to secondary *H. polygyrus* infection. Worm burdens and fecundity were increased in mice with a secondary *H. polygyrus* infection when treatment with αIL-4R was administered during primary infection. Results did not depend on whether animals were cured prior to secondary infection (Urban *et al.*, 1991a). In a model of *T. muris* infection, a role for IL-13 in particular was demonstrated using IL-4*−/−* animals, whereby administering IL-13 significantly reduced worm burdens. However, results were strongly dependent on the strain and sex of animals used (Bancroft *et al.*, 2000).
In infection models whereby IL-4 and IL-13 cytokines are deleted or depleted, immunity is often ablated. In *H. polygyrus* infection, treatments with αIL-4 and αIL-13R antibodies during primary or secondary infections have resulted in failure to expel parasites (Urban *et al.*, 1991a; Urban *et al.*, 1995). However, the role of individual cytokines during worm infections varies according to the species studied. During *H. polygyrus* infection, the high levels of IL-4 generated during infection are insufficient to clear the nematode (Finney *et al.*, submitted; Chapter 3). During *Nippostrongylus brasiliensis*, the opposite is true: IL-4 is sufficient but not necessary to terminate infection. Treatment with IL-4 induces worm expulsion (Urban *et al.*, 1995), whereas administration of αIL-4 does not increase parasite survival within the host (Madden *et al.*, 1991).

A role for both IL-4 and IL-13 during *T. muris* infection was confirmed in studies by the Grencis group (Bancroft *et al.*, 1998). Both IL-4+/− and IL-13+/− animals were susceptible to infection, unlike their wild-type littermates who expelled the parasites. IL-4+/− animals showed diminished Th2 responses during the course of infection, whilst IL-13+/− animals were capable of generating a Th2 response at later time points. Since IL-4+/− and IL-13+/− animals had different phenotypes, the group hypothesised that both these cytokines play important yet different roles in mediating immunity to intestinal helminths (Bancroft *et al.*, 1998).

In a study of *N. brasiliensis* infection, a specific role for IL-13 was uncovered (McKenzie *et al.*, 1998). IL-13+/− animals, unlike IL-4+/−, failed to clear infection and showed no goblet cell hyperplasia associated with worm expulsion. When exogenous
IL-13 was administered to IL-13+/− animals, worm burdens were decreased. Thus it appears that during *N. brasiliensis* infection, IL-13 is required for worm expulsion and may be responsible for increased mucus production by goblet cells (McKenzie *et al.*, 1998), which is associated with this process (Miller *et al.*, 1987). However, later studies have revealed that in the IL-13−/− mutants, disrupted IL-13 diminishes IL-4 production from the linked *IL-4* gene (Guo *et al.*, 2001), indicating that the first study may have not revealed a unique role for IL-13.

The McKenzie group have also shown that, in a model of pulmonary granuloma induced by *Schistosoma mansoni* eggs, both IL-4−/− and IL-13−/− single mutants have significantly lower levels of IgE, IL-5 and eosinophil infiltration. However, these responses were only altogether abolished in animals deficient in both cytokines. The IL-4+/−IL-13−/− double mutant animals also displayed an impaired ability to expel *N. brasiliensis* worms in this same study. The group therefore postulate that IL-4 and IL-13 act in concert to initiate rapid Th2-cell driven responses, and although their functions overlap, they perform additive roles (McKenzie *et al.*, 1999).

In a model of *T. muris* infection, IL-4 and IL-13 were both found to be important for expulsion. Indeed, neutralising IL-13 in resistant female BALB/c IL-4+/− mice prevented parasite expulsion, and treating susceptible male BALB/c IL-4+/− animals with recombinant IL-13 protein led to a significant reduction in worm burden. However, the importance of strain and gender in influencing the cytokine-mediated immunity was demonstrated since female IL-4+/− BALB/c mice are resistant to infection whereas male IL-4+/− BALB/c mice, similarly to female IL-4+/− C57BL/6
mice, are susceptible. Effects of sex hormones on the Th1/Th2 balance were postulated to account for the gender differences observed. Thus, strain and sex need to be taken into consideration when designing experiments (Bancroft et al., 2000).

6.1.2. The gut mucosa

The Urban group have postulated that IL-4 and IL-13 can act directly on gut epithelial cell function, thereby contributing to worm expulsion. Treatment with αIL4-R blocked several changes that are normally observed during secondary H. polygyrus infection, such as increased fluid secretion and decreased fluid adsorption (Shea-Donohue et al., 2001). These changes increase the net movement of fluid and ions in the gut lumen, interfering with the ability of worms to feed on gut mucosa (Bansemir & Sukhdeo, 1994) as well as dislodging the parasites.

Although the ability of IL-4 and IL-13 to decrease mucosal barrier function may be due to a direct effect on epithelial cells, the actions of IL-4 appear also to be neurally-mediated, involving an interaction between nerves and mast cells. Goldhill and colleagues observed a neurally-mediated, IL-4-dependent increase in in vitro intestinal smooth muscle contractility during secondary infections with H. polygyrus (Goldhill et al., 1997). The changes were thought to be mast cell dependent since they were not observed in mast cell deficient animals. Thus, the ability of IL-4 and IL-13 to increase epithelial cell secretion and/or smooth muscle contractility may
promote worm expulsion by contributing to the “weep and sweep” response to nematode infections (Shea-Donohue et al., 2001).

In *Trichinella spiralis* infection, Th2 cells producing both IL-4 and IL-13 also promote intestinal muscle hypercontractility, so contributing to worm expulsion from the gut. This effect is partially dependent on a signal transducer and activator of transcription factor 6 (Stat-6), involved in the IL-4/IL-13 signal transduction cascade (Khan et al., 2001). Stat-6-dependent mechanisms have also been associated with muscle contractibility during *H. polygyrus* and *N. brasiliensis* infection (Zhao et al., 2003).

IL-9 has also been shown to influence muscle contractibility (Khan et al. 2003). Mice infected with *T. spiralis* and treated with IL-9 show increased muscle contractibility and worm expulsion, as well as increased IL-4, IL-13, goblet cells and serum mast cell proteases; treatment with αIL-9 has no effect (Khan et al., 2003). However, it appears the contributions of IL-9 to host protection may differ according to nematode infection, since treatment with αIL-9 during a *T. muris* infection led to attenuated worm expulsion, with no changes in goblet cell numbers or mast cell protease levels (Khan et al., 2003). During *T. spiralis*, infection, IL-4 and IL-13 are therefore required and sufficient for hypercontractility, whilst IL-9 is required for an optimal response to *T. muris* infection.

6.1.3. IL-4 and IgE

Parasite-specific IgE is generated during helminth infections and has been postulated to be a major component of acquired immunity to human helminths (Hagan et al., 1991; reviewed in Hagan, 1993; Faulkner et al., 2002; Bethony et al., 2005). In
animal models, Dessein and colleagues demonstrated that IgE could influence infection outcome. They showed that by depleting IgE in vivo, resistance to *T. spiralis* in rats was greatly diminished. However, a decrease in tissue and blood eosinophilia was also observed; the effects of IL-4 were not studied (Dessein et al., 1981). More recently, in a *T. muris* model, increased IL-9 levels were also linked to production of IgE: infected mice injected with an IL-9-secreting cell line produced more IgE than control animals (naïve, infection alone, injection alone). Again, the effects of IL-4 were not studied (Faulkner et al., 1998).

IgE production was first linked to IL-4 by Finkelman and colleagues in studies with *N. brasiliensis*. They found that αIL-4 treatment inhibited primary polyclonal IgE responses by 99 % in infected mice, but had no effect on IgG1 and IgG2a production. IL-4 was also important in generating secondary antigen-specific IgE responses, as well as maintaining established IgE responses (Finkelman et al., 1988). The results were confirmed in later studies by Coffman and colleagues, Sher and colleagues as well as the Finkelman group, whereby treatment with αIL-4 led to diminished production of parasite-induced IgE during *N. brasiliensis*, *S. mansoni* and *H. polygyrus* infection respectively (Coffman et al., 1989; Sher et al., 1990b; Urban et al., 1991a). The McKenzie group also showed diminished IgE levels in IL-4−/− animals in a model of schistosomiasis (McKenzie et al., 1999). However, levels were only completely abolished in animals deficient for both IL-4 and IL-13, suggesting these two cytokines cooperate during Th2 responses.
6.1.4. IL-3, IL-4, IL-9 and mastocytosis

Several cytokines are known to promote mast cell expansion, although the generation of transgenic mice over-expressing IL-9 has highlighted this cytokine as the most critical for intestinal mastocytosis and enhanced resistance to nematode infection (Faulkner et al., 1998). Other cytokines, such as IL-3 and IL-4 have also been linked to mastocytosis. Treatment with αIL-3 or αIL-4 during *N. brasiliensis* infection significantly suppressed the mucosal mast cell response by 40-50%. Combination treatment suppressed the response by 85-90%, but did not prevent worm expulsion (Madden et al., 1991). The role of mast cells in worm expulsion mechanisms has been controversial, and as yet, no specific function has been associated with this cell type during nematode infection (reviewed by Else & Finkelman, 1998).

High levels of IL-9 have been shown to arise during *H. polygyrus* infection (Svetic et al., 1993; Finney et al., submitted, Chapter 3). However, mastocytosis has not been associated with *H. polygyrus* infection. Studies by Dehlawi and colleagues demonstrated that *H. polygyrus* infection, unlike infections with *N. brasiliensis* and *T. spiralis*, failed to induce a mucosal mast cell response, independently of host strain or sex. Only the SJL strain of mouse, which expel primary as well as secondary infections of *H. polygyrus*, develop a strong mastocytosis during secondary infections (Dehlawi et al., 1987). Betts & Else also found that mast cells do not play a role in resistance to *T. muris* infection (Betts & Else, 1999).
6.1.5. IL-5 and eosinophilia

The McKenzie group have found a feature that appears to be unique to intestinal responses to nematode infections. In the absence of both IL-4 and IL-13, expansion of IL-5-producing cells is possible if they receive sufficient costimulation (McKenzie et al., 1999). Thus although IL-4 and IL-13 are the major players during helminth infection, other cytokines may also have important roles.

IL-5 has pleiotropic functions which include stimulating eosinophil differentiation and activation (Sanderson et al., 1988). IL-5 and eosinophilia have long been associated with helminth infections, although their role during worm expulsion has been the subject of much debate (reviewed by Maizels & Balic, 2004). In vitro studies suggested that eosinophils could kill antibody-covered schistosomes (David et al., 1980) and work by Else & Grencis has suggested that resistance to T. muris involves the induction of IL-5-secreting cells (Else & Grencis, 1991). However, in vivo studies with numerous helminth models have not confirmed IL-5 or eosinophils as major players in resistance to established helminth infection (Sher et al., 1990a; Urban et al., 1991a; Herndon & Kayes, 1992; Betts & Else, 1999), although both have been implicated in immunity to the larvae of tissue migrating species such as N. brasiliensis (Shin et al., 1997; Daly et al., 1999), Litomosoides sigmodontis (Martin et al., 2000) and Strongyloides ratti (Watanabe et al., 2003). Indeed, when infected with N. brasiliensis, animals engineered to over-express IL-5 have fewer, smaller, less fecund worms than wild-type controls both three and five days post-infection (Dent et al., 1999).
Although increased levels of IL-5 have been observed during _H. polygyrus_ infection (Finney _et al._, submitted; Chapter 3), treatment with αIL-5 was reported to have had no effect on worm burdens (Urban _et al._, 1991a). Treatment with αIL-5 results in sharply decreased eosinophilia (Coffman _et al._, 1989; Sher _et al._, 1990b; Urban _et al._, 1991a; Herndon & Kayes, 1992). However, αIL-5 treatment does not alter the vaccinated status of mice in a model of schistosomiasis, indicating that neither IL-5 nor eosinophilia are necessary for resistance to schistosomiasis (Sher _et al._, 1990a). Studies by Herndon & Kayes have confirmed this, whereby αIL-5 treatment did not affect the control of _T. spiralis_ infection. In their studies, worm burdens were unaffected by treatment, as was resistance to challenge infection (Herndon & Kayes, 1992). Finally, in a model of _T. muris_, Betts & Else also found that αIL-5 treatment had no effect on infection. Resistant mouse strains treated with the antibody remained resistant to infection (Betts & Else, 1999).

In order to elucidate the individual contributions of Th2 cytokines during _N. brasiliensis_ infection, Fallon and colleagues created animals deficient in up to four Th2 cytokines (Fallon _et al._, 2002). While their studies found no role for IL-5 in worm expulsion in mice deleted of IL-5 alone, the absence of IL-5 in conjunction with IL-4 and IL-13 significantly delayed worm expulsion, with more worms surviving in the IL-4/5/13−/− animals when compared to IL-4/13−/− mice. Thus, although IL-4 and IL-13 appear to be the primary cytokines involved in worm expulsion, other cytokines may also play a role (Fallon _et al._, 2002). In IL-4/5/9/13−/− animals, the additional deletion of IL-9 results in a further delay in the expulsion of parasites from the intestine (Fallon _et al._, 2002). IL-9 has also been found to contribute to worm expulsion during _T. spiralis_ infection (Faulkner _et al._, 1998); this
was in conjunction with increases in IL-4 and IL-13 (Khan et al., 2003).

6.1.6. Hypotheses and aims

Numerous studies have been conducted to determine the intricate mechanisms involved in resistance to helminth pathogens. Our experiments aimed to elucidate Treg involvement during re-infection with *H. polygyrus*. Re-infection with this parasite leads to significant decreases in parasite burden (Urban et al., 1991a). We wanted to determine whether this involved changes in Treg populations since we have shown these cells are elicited during primary infection. Also, we were interested in whether the characteristics of the secondary infection differed in animals treated with anthelmintics prior to re-infection as compared to animals being re-infected whilst they had an ongoing infection, as each protocol reflects an important situation in disease control in humans.
6.2. Results

6.2.1. Secondary *H. polygyrus* infections leads to reduced worm burdens as compared to primary infections.

Animals were infected with *H. polygyrus* for 28 days, at which time they received, or not, an anthelmintic treatment to clear parasites. Ten days after the final cure, animals were, or were not, re-infected with *H. polygyrus* (Figure 6.1 A). All mice were sacrificed on d56, and the following groups were studied:

- **Negative Control**: naïve animals.
- **Short primary infection**: I (d14), animals infected for 14 days (from day 42-56).
- **Chronic primary infection**: I (d56), animals infected for the entire 56 days.
- **Re-infection without cure**: I (d42) + I (d14), animals infected for 42 days, and re-infected for 14 days (from day 42-56).
- **Control for cure**: I (d28) + Cure, animals infected for 28 days and cured, thus free of worms from day 28-56.
- **Re-infection with cure**: I (d28) + Cure + I (d14), animals infected for 28 days, cured and re-infected for 14 days (from day 42-56).
Worm burdens were determined for each group on d56 and significant decreases were found in both re-infected groups compared to the 14-day-infected group (Figure 6.1 B). However, the decreases in the re-infected group without cure were no different to the d56-infected animals. This could be due to worms from the primary infection maintaining themselves whilst worms from the secondary infection were expelled, thus mice becoming resistant to incoming parasites. Another explanation for the variation in the data obtained is that if mice had few worms by d56, they were more likely to retain worms from the second infection, whilst mice still harbouring high levels of *H. polygyrus* by d42 would reject all worms from the secondary infection.

Although worm burdens in the re-infected groups were significantly different from the d14-infected group, this was not reflected in the number of MLN or spleen cells. Both MLN and spleen cell numbers were increased in d14-infected and re-infected groups compared to naïve, d56-infected and the cured animals (Figure 6.1 C). The decrease in worm burdens observed can therefore not be attributed to decreased immune responses since both at the local (MLN) and systemic (spleen) level, re-infected animals showed similar cell infiltration to d14 infection animals.
Figure 6.1 – Secondary *H. polygyrus* infections led to reduced worm burdens as compared to primary infections.

A: Experimental protocol for re-infection. Mice were infected on d0, and treated three times with anthelmintic at two-day intervals from d28. The efficacy of drug therapy was verified by faecal egg counts after the third and final dose; no eggs were detected in the faeces of treated animals. Ten days after the final treatment animals were re-infected with *H. polygyrus* and left for 14 days. The experiment was performed twice.

B: Worm burdens for naïve, infected (d14 and d56), cured (28 days of infection followed by cure) and re-infected (with and without cure prior to re-infection) animals. Data represent means from groups of 5 mice. Data were transformed for statistical analysis; a one-way ANOVA was performed using all data sets - except for the naïve group - and a Bonferroni post-test compared differences between d14 infected animals and both re-infected groups (* = p<0.05).

C: Total cell numbers from MLN (Left) and spleen (Right). Data represent means from groups of 5 mice. Data were transformed for statistical analysis; a one-way ANOVA was performed using all data sets - except for the naïve group - and a Bonferroni post-test compared differences between d14 infected animals and both re-infected groups (n.s. = no significant difference).
Chapter 6 – Secondary infection with *H. polygyrus*

Figure 6.1 – Secondary *H. polygyrus* infections led to reduced worm burdens as compared to primary infections.
6.2.2. Cytokine responsiveness in *H. polygyrus* infection, after re-infection.

Cytokine production by MLN and spleen cells cultured with Hp antigen for 48 hours was measured. For IL-4, the only difference observed was between the cured (I (d28) + Cure) and re-infected (I (d28) + Cure + I (d14)) groups (Figure 6.2). However, trends in both MLN and spleen show that in general, IL-4 levels are high by d14, but have decreased by d56 and that re-infection causes an increase in levels. This is true of all the other cytokines in the MLN, although significance was only observed for IL-5 and IL-13. Also, IL-5 and IL-13 are the only cytokines for which a difference was observed between the re-infected groups and the d14-infected groups (although this is only significant for IL-5), indicating that these cytokines are being downmodulated. The differences were not reflected in the spleen, which implies that cytokines at the local level may be more tightly regulated than at the systemic level, or that timings of local versus systemic responses should be considered. Responses in the spleen are usually delayed compared to those of local lymph nodes during *H. polygyrus* infection (Finney et al., submitted; Chapter 3) and the differences observed in the MLN may not yet have been evident in the spleen.

Interestingly IL-5 and IL-13 were decreased in the re-infected groups compared to d14-infected animals. IL-13 has been linked to worm expulsion and it was expected to have increased in the re-infected groups as with previous studies of *H. polygyrus* infection (Finkelman *et al.*, 2000). However, in our experiments, we found no evidence of a ‘memory effect’, where cytokine levels increase more rapidly and to a
higher level due to previous antigen exposure. Moreover, for both IL-5 and IL-13, we repeatedly found a ‘negative memory effect’, whereby the cytokine levels were decreased upon re-infection as compared to a primary 14-day infection.

Another surprising result was the lack of differences between cured and uncured re-infected groups. Apart from IL-4 in the MLN, which was increased in the group having been cured, all other results were similar. Therefore a single non-protective cure did not significantly alter immune responses after re-infection.
Figure 6.2 – Cytokine responsiveness in H. polygyrus infection, after re-infection.

Parasite antigen-specific cytokine responses are presented from MLN cells. Cells were cultured for 48 hours in medium alone (open bars) or H. polygyrus antigen (solid bars). Panels A-E present IL-4, IL-5, IL-9, IL-10 and IL-13. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed for statistical analysis; one-way ANOVAs were performed using all data sets - except for the naïve group - and Bonferroni post-tests compared differences between d14-infected animals and both re-infected groups, as well as each re-infected group with its own control (I (d56) v. I (d42) + I (d14), and I (d42) + Cure v. I (d28) + Cure + I (d14)), (n.s. = no significant difference, * = p<0.05, ** = p<0.01, *** = p<0.001). For IL-13 levels in the spleen, since equal variances were not obtained by transforming the data, a non-parametric Kruskal-Wallis test was performed, using a Dunn's post-test. The experiment was performed twice.
Chapter 6 — Secondary infection with *H. polygyrus*

Figure 6.2 — Cytokine responsiveness in *H. polygyrus* infection, after re-infection
6.2.3. CD25 and CD103 expression increases in both re-infected groups.

Cells from the MLN were stained for CD25 and CD103 and it was observed that both these markers were increased in re-infected animals compared to their control single infections (Figure 3 A, B). This was also true for CD103 intensity on CD4^+CD25^+ cells (data not shown). However, the markers were not decreased compared to the d14-infected animals. Therefore, no differences were apparent in CD103 expression between secondary d14-infection compared to primary d14-infection; re-infection did not affect CD103 expression.

The result was unexpected since CD103, being a regulatory marker, was thought to be downregulated during secondary infections in which worm burdens are significantly decreased compared to animals harbouring a primary infection. However, this was not the case, although CD103 levels may change at time points other than those assayed.
Figure 6.3 – CD25 and CD103 expression increases in both re-infected groups.

A: Bivariate flow cytometry analysis of CD25 and CD103 in MLN from animals infected for 56 days (Far left), re-infected without cure (Left), infected and cured (Right) and re-infected after cure (Far right). The experiment was performed once.

B: CD25+ T cells as proportion of total CD4+ MLN cells (Left) and CD103+ T cells as a proportion of total CD4+CD25+ MLN cells (Right) in naïve, d14-infected, d56-infected, re-infected, d28-infected + cured and animals re-infected after cure. Data represent means and standard errors from groups of 5 mice assayed individually. Data were transformed for statistical analysis; one-way ANOVAs were performed using all data sets - except for the naïve group - and Bonferroni post-tests compared differences between d14-infected animals and both re-infected groups, as well as each re-infected group with its own control (I (d56) v. I (d42) + I (d14), and I (d28) + Cure v. I (d28) + Cure + I (d14)), (n.s. = no significant difference, * = p<0.05, *** = p<0.001).
Figure 6.3 - CD25 and CD103 expression increases in both re-infected groups
6.3. Discussion

6.3.1. Worm burdens during secondary *H. polygyrus* infections

Our results, in concordance with previous work (Urban *et al.*, 1991a), clearly show that re-infection with *H. polygyrus* leads to decreased worm burdens as compared to primary infections. The research by Urban and colleagues demonstrated that by drug clearance of a primary *H. polygyrus* infection, new adult worm development was reduced by 70-80\%, and their fecundity by 90\%. Our studies have also shown that this reduction in worm burden is apparent when no drug treatment was administered prior to re-infection.

Interestingly, few worms were present in d56-infected animals. However, BALB/c mice are considered intermediate responders to *H. polygyrus* (Su & Dobson, 1997). Worm burdens vary greatly according to the mouse strain used (Robinson *et al.*, 1989; Wahid *et al.*, 1994) and intermediate responders, by week 8 of infection (d56), have been shown to carry very few worms (Ben-Smith *et al.*, 2003).

In our experiments, the I (d42) + I (d14) animals had very similar worm burdens to the I (d56) animals indicating that adults from the primary infection remained in the gut whilst incoming parasites were expelled. However, in view of the spread in worm burdens, another explanation exists. The intensity of infection prior to secondary infection may play an important role in protection to re-infection. Animals
habouring many worms prior to secondary infection may, upon re-infection, expel most of their worms due to a strong protective immune response. However, those animals with few worms prior to secondary infection, may, upon re-infection, only expel part of their parasite load. Future experiments in which different doses of parasites are administered during primary infections could help determine whether this is the case.

Upon re-infection, the animals did retain adult parasites, but to a lesser degree than a primary 14-day-infection. In the cured and re-infected animals, resistance to re-infection was less strong than with re-infected animals with an ongoing infection. However, in both cases, the spread in worm burden within the groups rendered it difficult to draw solid conclusions.

Total cell numbers in the MLN and spleen indicate that the decreased worm burdens observed were not due to decreased trafficking to these sites. Both re-infected groups had similar numbers of total cells within their MLN and spleens as the d14-infected animals; no significant differences were detected. It therefore appears some other mechanism is involved in worm expulsion in the re-infected groups.

Previous work has disagreed on the nature of the mechanisms involved in resistance to *H. polygyrus*. Work by Ey suggests that the development of surviving larvae is significantly retarded in mice that have experienced one or more previous infections. The author also found that adult worms arising from a challenge infection were stunted and appeared anaemic (Ey, 1988). Chaicumpa and colleagues also concluded
that immunity is directed not against the adult worm but against third-stage larvae, following their penetration of the wall of the intestine. They showed that mice could be immunised against re-infection with *H. polygyrus* by oral, intravenous, intraperitoneal and subcutaneous administration of live third-stage larvae (Chaicumpa *et al.*, 1977). Strong resistance to secondary infection has been observed in mice infected for only 12-36 hours (thus having only harboured L3 parasites), although when infection was terminated later, 4-6 days after initial inoculation (fourth stage larvae present), protection was 95-100 % effective (Wahid & Behnke, 1992). Finally, the Enriquez group found that during re-infection, L3s were less able to penetrate the intestinal wall, and those that did showed retarded development (Cypess *et al.*, 1988). Indeed recently, alternatively activated macrophages have been implicated in resistance to re-infection by *H. polygyrus* larvae (Anthony *et al.*, 2006). These studies indicate that larval rather than adult stages may be the main target of host immunity during re-infection.

In our experiments, we did not find any damaged parasites. All adult worms identified at necropsy appeared healthy. However, in future experiments, assessing the worms’ fecundity would help determine whether adults were affected during re-infection, as has previously been observed (Urban *et al.*, 1991a). From our experiments, we cannot conclude whether protection was targeted against larval or adult stages. However, from the cytokine and Treg marker data, it appears that regulation was induced by secondary infection similarly to primary infection, implying that while larval stages were more likely to be the target of resistance
mechanisms, surviving adults provoke a comparable Treg response on naïve or immune mice alike.

6.3.2. Cytokine responses during secondary *H. polygyrus* infections

It has been well documented that IL-4, IL-5, IL-9, IL-10 and IL-13 are involved in helminth infections (reviewed by Else & Finkelman, 1998). Indeed, studies by Svetic and colleagues demonstrated that *H. polygyrus* infection induces Th2 cytokine gene expression, which was decreased when animals were treated with αCD4, on days 6 and 8 post-infection (Svetic *et al.*, 1993). Work by the Finkelman group has also shown that αCD4 treatment abrogates the protective response observed during a secondary infection of *H. polygyrus* (Urban *et al.*, 1991b), indicating that cytokines produced by CD4⁺ cells may play an important role during re-infection.

Our results demonstrate an increase in Hp antigen-specific Th2 cytokines produced by MLN and spleen cells over the course of infection (Finney *et al.*, submitted; Chapter 3). Numerous studies have shown that in transgenic animals where these cytokines are absent, worm expulsion is affected (reviewed by Else & Finkelman, 1998). Also, in human studies, when age and sex are accounted for, Th2 cytokine responses (especially IL-5) immediately before deworming have a significant negative effect on the probability of re-infection 8-9 months later (Jackson *et al.*, 2004).
Our results, in agreement with the work of Urban and colleagues, also show an increase in IL-4 levels in re-infected animals (subsequent to cure) compared to that of cured animals (Finkelman et al., 2000). IL-4 levels in both re-infected groups were no different to those found in d14-infected animals. In the MLN, elevated levels of IL-4 were observed in all infected groups, but not in naïve or cured animals, indicating that this cytokine plays an important role during infection. However, such increases were not observed in the spleen during re-infection, and this was true for all studied cytokines. Therefore upon re-infection with *H. polygyrus*, immune responses to the parasite are local and controlled.

More rapid production of IL-4 and IL-13 has been linked to host protective immunity during re-infection (Urban et al., 1991a; Finkelman et al., 2000). However, in our experiments, levels of IL-4 and IL-13 were not significantly different in re-infected groups as compared to a 14-day primary infection, although for IL-13 a non-significant decrease was repeatedly observed. In future experiments, cytokine levels should be measured earlier (at d7 and d9) to determine if cytokines are produced more rapidly upon re-infection, and whether this production is dependent on adult worms (at d7, adults are not yet developed). Also, Urban and colleagues did not measure cytokine levels, and Finkelman and colleagues used a different strain of mouse which may help explain the discrepancy observed.

Work by the Finkelman group on the role of IL-5 during infection concluded that treatment with αIL-5 alone or in conjunction with IL-4 abolished eosinophilia but had no specific effect on protection (Urban et al., 1991a). However, in our model, we
see a significant decrease in IL-5 levels in the MLN of re-infected animals compared to d14 infected animals. This was not observed in the spleen.

As for IL-9, no significant differences in IL-9 levels between re-infected animals and their controls. However, although non-significant, trends for increased levels in both re-infected and d56-infected groups were observed. In these three groups, worm burdens were significantly decreased compared to d14-infected animals, and thus worm expulsion mechanisms were in place.

IL-10 has not been as intensely studied as IL-4 and IL-13 in the context of re-infection. However, we find IL-10 levels increased in all groups exposed to infection 14 days earlier irrespective of previous infection history, so the IL-10 response is intact, even in mice able to expel most parasites. IL-10 is strongly linked with regulatory mechanism, and this may help explain the lack of increase in the levels of other cytokines involved in worm expulsion. Indeed, it has been shown that IL-10 counter-regulates IL-4-dependent effector mechanisms in murine filariasis (Specht et al., 2004). Also, if the protection to re-infection is mostly aimed at the larvae rather than the adults, cytokine levels at d14 in the MLN may not reflect the levels required for worm expulsion.
6.3.3. Treg markers during secondary *H. polygyrus* infections

Our results indicate that both CD25 and CD103 were not affected by re-infection. CD25 and CD103 expression in the MLN were no different in the re-infected groups as compared to the d14-infected animals. This was also true of TGF-β expression in the MLN, and of CD25 and CD103 expression in the spleen as well as CD103 intensity on CD4^+^CD25^+^ cells (data not shown) – no FoxP3 staining was performed for these experiments since, at the time of experimentation, the FoxP3 antibody was not available. Re-infection had no effect on the Treg markers studied. If protection from re-infection is indeed directed mainly against the larval form of the parasite, the fact that Treg markers generated by infection did not differ between primary and secondary infection was to be expected. The Treg markers are associated with adult parasites since they do not increase until at least d14 of infection (Finney *et al.*, submitted; Chapter 3). Also, Treg markers were increased in the re-infected groups in comparison to the single chronic infection and the cured group, indicating that the increase in the re-infected groups was not due to the primary infection.
6.3.4. Concluding remarks and ideas for future research

Re-infection with *H. polygyrus* led to decreased worm burdens as compared to primary infections. However, since the process of protection from re-infection may be directed at larval stages rather than adult parasites, it is not surprising that we did not find an effect of re-infection on Treg markers.

Future work should concentrate on studying reactions at the site of infection. Attempts were made to study Treg markers in intra-epithelial lymphocytes and lamina propria cells from the gut mucosa. However, isolation of these cells proved difficult and staining was unsuccessful. Changes in gut mucosa such as mucin production would also be of interest since mucins secreted by goblet cells have been shown to play an important role in trapping and removing intestinal nematodes from the gut (Miller, 1987).

Since IL-4 is required to generate and maintain IgE responses during *N. brasiliensis* infection (Finkelman *et al.*, 1988) and during both primary and secondary *H. polygyrus* infections (Urban *et al.*, 1991a), measuring serum IgE levels, both total and *H. polygyrus*-specific, should also be considered for future experiments.

Although Treg marker expression was not affected by re-infection, we did not study Treg cell function. Suppression assays using cells from re-infected animals could shed light on whether Tregs from re-infected animals are as suppressive as those elicited during primary infection.
Experiments should also be set up allowing longer periods between cure and re-infection to determine how long the protection from secondary infection remains. In our experiments, animals were only left for 10 days before re-infection. Could protection still be observed 6 months or 1 year after cure? Experiments such as these could help determine the long term effects of helminths on immune responses.

Re-infections using trickle infections, whereby parasites are inoculated repeatedly over a given period of time, could be studied as well since they more faithfully reflect what occurs in the field. Understanding immune responses to repeated infection by helminths would provide vital information as to the immune status of people exposed to constant transmission. This is especially relevant since we found no significant differences between responses to secondary infections in animals treated with anthelmintic in comparison to animals which were not treated.

Researching re-infection in the context of co-infection would also prove interesting. Indeed, in areas where helminth infection is rife, co-infection levels are also elevated. Would protection from re-infection still occur when other infections interfere with immune responses?
7. CO-INFECTION WITH *H. POLYGYRUS*

7.1. Introduction

In Chapter 5, we showed that helminth infections can strongly affect bystander allergic responses to allergens, even once active infection has been cleared. This raised the possibility that helminth-induced regulation may also impact on concurrent infections. Most helminth-infected individuals live in poverty-stricken areas where disease is rife, and are often harbouring more than one parasite. Modulation of immune responses by helminths may strongly affect responses to other pre-existing or incoming parasites.

Treating human populations with anthelmintics is a complex enterprise and since treatment offers no long-lasting protection, undertaking such projects requires careful consideration. Determining the effects of helminth infections on other parasite infections is important in understanding the appropriate approaches to anthelmintic treatment strategies. It is only by teasing apart the confounding factors which surround co-infection that we will truly understand the extent of the influence of helminths on individuals in areas where multiple infections are common.

Many questions relating to this effect have been raised, such as the relative influence of helminths on Th1- versus Th2-skewing infections, the effects of acute as compared to chronic helminth infections and the influence of pre-existing as opposed
Chapter 7—Co-infection with *H. polygyrus*

to incoming infections. Studies in both animal models and human populations have started to shed light on some of these points.

### 7.1.1. Helminth–virus co-infection

Relatively few studies have been carried out in the human population on helminth-virus co-infection and most of them have been on individuals co-infected with helminths and HIV. In 2000, studies by Lawn and colleagues in Kenya on populations co-infected with schistosomes and HIV revealed that effective treatment of schistosomiasis was not associated with a reduction in plasma HIV viral load. Although, while viral load was not decreased with treatment, the results did not exclude adverse effects of helminth infection on HIV pathogenesis and progression (Lawn *et al.*, 2000). However, work by Elliott and colleagues in Uganda supports that helminth infection does not exacerbate HIV infection. Changes in HIV replication and CD4$^+$ cell counts were not significantly different between helminth-infected and treated individuals, even though the cytokine responses to *Schistosoma mansoni* did vary over the course of HIV infection (Elliott *et al.*, 2003). Later work by the Elliott group in the same country supported these findings. They, however, failed to find a detrimental effect of helminths on HIV progression. When demographic and socioeconomic factors were taken into account, helminth-infected people had no lower CD4$^+$ cells counts or higher mortality rates than helminth-free people. Rather, the decrease in CD4$^+$ cell count was greater in some cases after anthelmintic treatment (Brown *et al.*, 2004), indicating a possible positive effect of helminth infection of concurrent HIV infection/AIDS.
However, the beneficial effect of helminths on HIV progression was not true in all cases (reviewed in Bentwich et al., 1999 and Borkow et al., 2001). More studies on the interactions between chronic viral infections and helminth infections are necessary, especially gut-dwelling helminths as recent findings indicate that the gut can act as a viral reservoir for HIV, even in individuals where anthelmintic treatment was deemed successful (Guadalupe et al., 2006).

7.1.2. Helminth – bacteria co-infection

The data obtained from most research undertaken in the field of helminth-bacteria co-infection have predominantly been from animal models. Studies by Fox and colleagues have demonstrated that concurrent enteric helminth infection can attenuate gastric atrophy in mice initiated by a Helicobacter infection. Co-infection with Helicobacter spp. and H. polygyrus showed that, despite chronic inflammation and high Helicobacter colonisation, the Th2 environment generated by the helminth caused a reduction in Helicobacter-associated gastric atrophy. This correlated with a substantial reduction in mRNA for cytokines and chemokines associated with a gastric inflammatory Th1 response, as well as Helicobacter-specific IgG2a antibodies while helminth-specific antibodies were not affected (Fox et al., 2000). Work by Buendia and colleagues confirmed the beneficial influence of helminths on concurrent bacterial infections. Their findings, using an acute rather than chronic helminth infection model, showed that in animals infected with Chlamydophila abortus and N. brasiliensis, while bacterial replication was increased, morbidity was lowered (Buendia et al., 2002).
Helminth infections also affect pre-existing infection (Sacco et al., 2002). In a model studying the effect of incoming *S. mansoni* on an established *Mycobacterium avium* infection, co-infection led to a shift from the typical Th1 response generated by the bacterium to a helminth-induced Th2 response, as determined by serum antibodies as well as granuloma cell characterisation and cytokine production (Sacco et al., 2002). In terms of pathology, Chen and colleagues demonstrated that pre-existing helminth infections can exacerbate incoming bacterial infections. BALB/c mice co-infected with *Citrobacter rodentium* and *H. polygyrus* displayed a marked increase in morbidity and mortality compared to mice infected with *C. rodentium* alone, which was characterised by both increased bacterial burdens and increased inflammatory cytokine responses (Chen et al., 2005). Helminth-induced Th2 responses were responsible for the impaired host-protective immunity as they led to dysregulated pro-inflammatory Th1 responses in co-infected animals. This detrimental effect was not observed in Stat-6-deficient mice, implicating IL-4 and IL-13 in the processes involved. The Mills group obtained similar results using a different infection model. Th2 responses generated by an infection with the trematode *Fasciola hepatica* suppressed the Th1 responses to *Bordetella pertussis* in co-infected animals, and thus delayed bacterial clearance. This was reliant on IL-4 since it was not observed in IL-4−/+ animals (Brady et al., 1999).

Interestingly, in the study by Chen and colleagues described above, *H. polygyrus* larvae were only administered 7 days prior to bacterial challenge (Chen et al., 2005). Although experiments were run for 37 days, by which time a regulatory network generated by *H. polygyrus* would have been in place (Finney et al., submitted;
Chapter 7 – Co-infection with *H. polygyrus*

Chapter 3), at the time of infection this was not the case. In the study by the Mills group, pathogens were administered concomitantly (Brady *et al.*, 1999). The benefits that occur from increased regulation in helminth-infected animals may therefore only occur if these infections are truly established prior to the bacterial infection.

Studies in co-infection have also yielded some surprising results. The Urban group found that pigs with dual infections of *Trichuris suis* and *Campylobacter jejuni* had much more severe pathology than animals with either infection singly (Mansfield *et al.*, 2003). Administered together, these pathogens caused site-specific disease and pathology, attributed to both mechanical and cytokine-mediated damage (Mansfield *et al.*, 2003). However, again, pathogens were administered simultaneously, not allowing for any regulatory network generated by the helminth pathogen to develop.

7.1.3. Helminth – apicomplexan co-infection

The Apicomplexa are a large group of parasitic protozoa, which include *Plasmodium* species and *Toxoplasma* species, characterised by the presence of an apical complex at some point during their life cycle. Work by numerous groups, in Man and animal models, has found that by skewing immune responses towards a Th2 bias, helminth infections reduce the pathology of incoming apicomplexan parasites.

Human studies by Nacher and colleagues have demonstrated a beneficial role of helminths in the context of cerebral malaria infections. Their study in Thailand found a dose-dependent association between *Ascaris lumbricoides* and protection from
cerebral malaria in humans (Nacher et al., 2000). They postulated that *A. lumbricoides*-infected patients may have had decreased cyto-adherence – a main pathological factor of cerebral malaria – possibly through endothelial cell receptor downregulation and/or decreased splenic clearance leading to the absence of selection of virulent *Plasmodium falciparum* strains. The IgE-αIgE immune complexes resulting from helminth pre-infection may also have had an important role in influencing the clinical presentation of severe malaria, and in establishing malaria tolerance (Nacher et al., 2000).

IgE may also play a role in protection from visceral leishmaniasis (Th1-inducing infection with *Leishmania infantum*) in a murine model of *T. spiralis* and *L. infantum* co-infection. In co-infected animals, both acute and latent leishmaniasis pathologies were reduced (Rousseau et al., 1997). The group postulated that enhanced parasite killing through an IgE/CD23-dependent mechanism may explain the observed reduced disease progression. Indeed, helminth-induced IgE binds CD23 (a low affinity receptor for IgE), ultimately leading to the production of nitric oxide (Dugas et al., 1995; Paul-Eugene et al., 1995). This IgE-mediated nitric oxide production has been implicated in the killing of *Leishmania* parasites (Vouldoukis et al., 1995). *S. mansoni*, however, does not appear to have such a beneficial role on co-infection with *L. major*. Although *L. major* lesions took longer to appear in co-infected individuals, they also took longer to heal in a study by the Pearce group. The results were attributed to altered Th1 responses and impaired macrophage-mediated parasite killing. In this co-infection model, *L. major* did not affect the course of schistosomiasis (La Flamme et al., 2002).
Murine studies by Yan and colleagues confirmed the results from the human studies (Nacher et al., 2000) demonstrating the benefits of pre-existing helminth infection on the outcome of cerebral malaria. In a mouse model of co-infection with *Brugia pahangi* and a lethal strain of *Plasmodium berghei*, co-infected animals were protected against the development of cerebral malaria. They showed prolonged survival (over 3 weeks post-infection) as compared to the animals infected with *P. berghei* alone, which only survived 12 days post-infection. The co-infected animals did not succumb to cerebral malaria as with the singly-infected animals, but rather died of anemia and high parasitaemia. The protection from cerebral malaria was attributed to the filarial-antigen-induced Th2 response in these animals, as defined by peripheral eosinophil counts, total serum IgE levels and cytokine production (Yan et al., 1997). This Th2 response possibly counteracted the malaria-induced and pathology-causing Th1 immunity.

However, work by Helmby and colleagues has demonstrated that, in helminth-malaria co-infected animals, not only is the malaria-specific response affected by the helminth parasite, but the helminth-specific response is also affected by the malaria parasite. In a model of co-infection with Th1-dependent *P. chabaudi* and Th2-dominated *S. mansoni*, the two parasitic infections, when present concomitantly in experimental mice, severely affected the immune response to each other. The helminth did not inhibit or dampen the malaria-induced Th1 response, but rather rendered macrophages unresponsive to stimulation leading to more rapid and elevated levels of parasitaemia. Moreover, the malaria parasite suppressed proliferative and Th2 cytokine responses to *S. mansoni* egg antigen (SEA) (Helmby
et al., 1998). The outcome of co-infection may therefore depend on the intricate relationships between both parasites. However, the Helmbry study does not agree with previous findings by Lewinsohn who found no effect of S. mansoni infection on the parasitaemia of mice infected with P. berghei (Lewinsohn, 1975), but species difference may account for this.

Similarly to the work by Helmbry and colleagues, the Stevenson group observed increased parasitaemia in animals co-infected with P. chabaudi and H. polygyrus. In their studies, H. polygyrus co-infection had a detrimental effect on malaria outcome and was associated with high mortality. Surprisingly, this was not due to increased helminth-specific Th2 cytokines, but rather to increased TGF-β and IL-10 levels which lead to impaired immune protection. These findings imply that the regulatory network induced by H. polygyrus had deleterious effects on the concurrent P. chabaudi malaria infection (Su et al., 2005).

The Allen group also found that malaria infection with P. chabaudi was exacerbated in animals chronically infected with Litomosoides sigmodontis, although the extent of this was dependent upon microfilariae status: the presence of microfilariae appeared to reduce the detrimental effects (Graham et al., 2005).

Some studies show that while helminth infection does not always affect concurrent infection with other pathogens, an apicomplexan parasite infection can affect concurrent helminth infections. Work by the Erb group has demonstrated that infection with N. brasiliensis prior to or simultaneous to Toxoplasma gondii did not
alter the course of *T. gondii* infection. Th1 responses were not altered in co-infected animals, which ultimately died, just as those with single *T. gondii* infections. In co-infected animals, local and systemic *N. brasiliensis* induced Th2 responses however were decreased. Infection with *T. gondii* prior to the helminth also inhibited the Th2 *N. brasiliensis* responses and prolonged egg production in these worms. Thus although the helminth had no effect on *T. gondii*, *T. gondii* had strong effects on *N. brasiliensis* infection (Liesenfeld *et al.*, 2004).

Perhaps the most unpredictable outcome of co-infection is in the arena of pathology. For example, Marshall and colleagues studied co-infection with *S. mansoni* and *T. gondii* to determine the impact of a Th2-skewed environment on the incoming *T. gondii* parasite. In their model, reduced *T. gondii*-induced gut inflammation was observed in co-infected animals, as postulated. However, co-infection also led to high mortality rates due to liver damage attributed to highly dysregulated cytokine responses. Pathology was not associated with increased parasitaemia (Marshall *et al.*, 1999). Studying both parasite infections individually therefore could not have predicted the outcomes observed, and this work highlights the importance of co-infection studies in general.

### 7.1.4. Helminth – helminth co-infection

Few studies have assessed the impact of multiple concurrent helminth infections. Work by the Dunne group has shown that co-infection with *S. mansoni* can render mice, normally susceptible to *T. muris*, resistant. The Th2 response generated
against SEA, characterised by increased Th2 cytokine production and IgE levels as well as decreased levels of IFN-γ, led to the expulsion of *T. muris* in susceptible co-infected animals (Curry *et al.*, 1995). Wescott & Colwell, however, found that *H. polygyrus* infection increased the survival of *N. brasiliensis* in their co-infection model (Wescott & Colwell, 1980). More work is therefore required to fully understand the impact of these helminth parasites on individuals harbouring pre-existing worm infections.

### 7.1.5. Hypotheses and aims

Our study aim was to assess the impact of a pre-existing *H. polygyrus* infection on an incoming *N. brasiliensis* infection. Previous work by Wescott & Colwell has demonstrated that *N. brasiliensis* infection can be prolonged in the presence of chronic *H. polygyrus* infection (Wescott & Colwell, 1980). However, their work was purely parasitological and we wanted to understand the role played by Th2 cytokines as well as Tregs in this setting. We also wished to determine whether the presence of *H. polygyrus* could directly alter responses to *N. brasiliensis*, and if *N. brasiliensis* had any effect on *H. polygyrus* responses. These questions were raised in order to gain some understanding of the impact of the strong regulatory network generated by *H. polygyrus* we characterised in Chapter 3. In Chapter 5 we assessed its impact on bystander allergy responses. Here, we wanted to determine its effects on other concurrent active infections.
7.2. Results

7.2.1. Pre-existing *H. polygyrus* infection delays expulsion of *N. brasiliensis*.

Animals were infected with *H. polygyrus*, *N. brasiliensis* or both parasites. Co-infection lasted either 7, 14 or 42 days (Figure 7.1 A).

*N. brasiliensis*, which causes an acute infection, was expelled from mice prior to d7; no worms were found at any time point in animals which harboured a single infection. This contrasts with other studies where parasites were not expelled this early (Colwell & Wescott, 1973; Wescott & Colwell, 1980). However, improved animal husbandry over the course of the past 30 years and the use of laboratory-adapted strains are thought to account for this.

When mice were co-infected with both *H. polygyrus* and *N. brasiliensis*, *N. brasiliensis* worms were found after 7 days of co-infection, but not at the later time points (d14 and d42) (Figure 7.1 B). *H. polygyrus* infection, which creates a regulatory environment after 28 days (Wilson *et al.*, 2005; Finney *et al.*, submitted; Chapter 3), appears to slow down the expulsion of *N. brasiliensis* but this suppression was not long lived.
As postulated, numbers of *H. polygyrus* did not differ between co-infected and singly-infected animals (Figure 7.1 B). Introduction of *N. brasiliensis* into mice 28 days after *H. polygyrus* infection did not affect *H. polygyrus* numbers suggesting that once a chronic *H. polygyrus* infection was established, parasite numbers were not altered by an incoming acute infection. Interestingly however, *H. polygyrus* numbers were significantly decreased in both singly- and co-infected animals after day 35 of co-infection. *H. polygyrus* infection was not studied in detail between days 28 and 70 in Chapter 3. In the experiment described in this chapter (Figure 7.1) however, it is clear that worm burdens did not slowly decline from d35 to d70, but rather sharply decreased between days 35 and 42, by which time most of the worms had been expelled. Indeed, over the first 35 days of infection (5 weeks), worm burdens decreased from the initial dose (200 infective larvae) to 123 (+/- 9) adult worms. This was followed by a sharp decrease within the following 7 days to 20 worms (+/- 8).

Until d14 post-co-infection, MLN cell numbers were significantly increased in co-infected animals compared to *N. brasiliensis* infected animals but reached the same levels as in *H. polygyrus* singly-infected animals (Figure 7.1 B). However, at d42 post-co-infection, cell numbers were elevated in the co-infected animals as compared to either single infection. Therefore, although MLN cell number appears to have been influenced only by *H. polygyrus* at first, late in co-infection, this was no longer the case.
Figure 7.1 – Pre-existing *H. polygyrus* infection delays expulsion of *N. brasiliensis*.

A: Experimental protocol for co-infection. Mice were infected with 200 *H. polygyrus* L3 larvae on d0, and co-infected with 300 *N. brasiliensis* larvae on d28. Mice were sacrificed on days 35, 42 or 70 of *H. polygyrus* infection, corresponding to days 7, 14 and 42 of co-infection. The experiment was performed once, with a minimum of 4 mice per group.

B: Worm burdens for naïve, *H. polygyrus*-infected, *N. brasiliensis*-infected and co-infected animals (Left). Right, total cell numbers from MLN. Data represent means from groups of a minimum of 4 mice. Data were transformed for statistical analysis; one-way ANOVAs were performed at each time point using all data sets - except for the naïve group - and Bonferroni post-tests compared differences between the single infections and the co-infection (n.s. = no significant difference, * = p<0.05, ** = p<0.01, *** = p<0.001).
Chapter 7 – Co-infection with *H. polygyrus*

**Figure 7.1** – Pre-existing *H. polygyrus* infection delays expulsion of *N. brasiliensis*
7.2.2. Cytokine responsiveness in *H. polygyrus* infection, *N. brasiliensis* infection and co-infection.

Both MLN and spleen cells from naïve and infected mice were cultured with Hp or Nb antigen, and cytokine-release (IL-4, 5, 9, 10, 13) was measured from these cultures after 48 hours. We examined responses to Hp antigen for *H. polygyrus* infected and co-infected animals, as well as responses to Nb antigen for *N. brasiliensis* infected and co-infected animals. A certain degree of cross-reactivity between both antigens was noted but these responses were weak in comparison to the antigen-specific responses obtained.

IL-9 was the only cytokine where no significant differences could be found between single and co-infections in MLN or spleens (Figure 7.2 C). This was surprising as IL-9 has been associated with worm expulsion from the gut (Fallon *et al.*, 2002) and we hypothesised that differences in IL-9 would be found between co-infected animals and *N. brasiliensis* infected animals at d7 post-co-infection, since the latter had expelled all parasites at this time point. Interestingly however, the IL-9 response to Nb antigen in both *N. brasiliensis*-infected and co-infected animals was strongest 35 days post-infection. The explanation for such elevated levels so long after worm expulsion has not yet been determined, although during *H. polygyrus* infection, elevated IL-9 responses are the longest lived of the Th2 cytokines, still present and strongly elevated after 70 days post-infection (Finney *et al.*, submitted, Chapter 3).
For IL-4, no differences were observed in the MLN. However, in the spleen, at d7 of co-infection, both single infections had significantly lower levels than the co-infected animals (Figure 7.2 A). Since IL-4 is characterised as the defining Th2 cytokine, this might explain why changes were observed at the systemic level, rather than the local level.

For IL-5, IL-10 and IL-13, increased levels in the co-infected animals as compared to the single-infected animals were apparent after 7 days of co-infection in both the MLN and the spleen (Figure 7.2 B, D, E). At later time points, these differences were only observed between *N. brasiliensis*-infected and co-infected mice. For these three cytokines, co-infection only had an effect on their production at the start; at later time points during co-infection, responses to *N. brasiliensis* alone were affected. The increases in IL-5 and IL-13 were surprising since both these cytokines are linked to nematode infections and worm expulsion mechanism. However, with single infections of *N. brasiliensis*, worms were already expelled by d7, whilst in the co-infected animals both species of worm were still present and IL-13 production may therefore have continued until expulsion of the remaining worms. The increased survival of worms could also be linked to increases in IL-10, as this cytokine was significantly increased in the spleen and MLN of co-infected animals compared to those infected with *N. brasiliensis* alone (Figure 7.2 E).
Figure 7.2 – Cytokine responsiveness in *H. polygyrus* infection, *N. brasiliensis* infection and co-infection.

Parasite antigen-specific cytokine responses are presented from MLN cells (Left Panels) and spleen cells (Right Panels). Cells were cultured for 48 hours in medium alone, *H. polygyrus* antigen (Hp antigen) or *N. brasiliensis* antigen (Nb antigen). Dotted lines represent the cells from single infections cultured in their respective antigens (O *H. polygyrus*, □ *N. brasiliensis*). The solid lines represent the cells from co-infected animals cultured in Hp antigen (●) and Nb antigen (■). Panels A–E present IL-4, IL-5, IL-9, IL-10 and IL-13. Data represent means and standard errors from groups of a minimum of 4 mice assayed individually. Data were transformed for statistical analysis; one-way ANOVAs were performed for all time points using all data sets - except for the naïve group - and Bonferroni post-tests compared differences between the single infections and the co-infection (n.s. = no significant difference, * = p<0.05, ** = p<0.01, *** = p<0.001). The experiment was performed once.
Chapter 7 - Co-infection with *H. polygyrus*

Figure 7.2 - Cytokine responsiveness in *H. polygyrus* infection, *N. brasiliensis* infection and co-infection
7.2.3. After 7 days, CD25, CD103 and TGF-β are increased in co-infected animals compared to animals with *N. brasiliensis* infection alone.

MLN cells from the different groups of mice were stained for CD25, CD103 and TGF-β. Expression of CD25 was increased at d7 post-co-infection, in MLNC from co-infected animals compared to either single infection (Figure 7.3). However, at later time points (d42 post-co-infection), no differences were apparent with either *H. polygyrus* or *N. brasiliensis*. *N. brasiliensis* itself causes an acute infection and the rise in CD25+ cells seen during a single infection is minimal. Evidently, when in a chronic regulatory environment, where CD25+ cell numbers are already increased, co-infection with *N. brasiliensis* can generate a transitory rise in CD25 expression.

CD103 and TGF-β are two markers involved in helminth regulation (Finney *et al.*, submitted; Chapter 3). Surface expression of these markers is known to be increased in MLN cells from *H. polygyrus*-infected animals after 28 days of infection (Wilson *et al.*, 2005; Finney *et al.*, submitted; Chapter 3). However, they did not rise significantly in animals undergoing an acute *N. brasiliensis* infection (Figure 7.3 B, C). In co-infected animals, both levels and marker intensity of CD103 and TGF-β resemble levels found during single *H. polygyrus* infection, although a slight, but non-significant increase in TGF-β+ cells was noted at d7 post-co-infection in co-infected as compared to *H. polygyrus*-infected animals; *N. brasiliensis* infection did not affect regulatory markers. Hence, *N. brasiliensis* infection does not affect the long standing regulation initiated and maintained by *H. polygyrus* parasites.
Figure 7.3 – After 7 days, CD25, CD103 and TGF-β are increased in co-infected animals compared to animals with a single *N. brasiliensis* infection.

A: Representative plots of bivariate flow cytometry analysis for CD25 and CD103 in the MLN from naïve (Far left), *N. brasiliensis*-infected (Left), *H. polygyrus*-infected (Right) and co-infected (Far right) animals. The experiment was performed once.

B: The difference in CD25 percentages within total CD4$^+$ population (Left) and CD103 percentages within total CD4$^+$CD25$^+$ T cell populations (Centre) were calculated compared to the corresponding naïve group. The percentage change in mean fluorescent intensity of CD103 on CD4$^+$CD25$^+$ T cell populations was also calculated (Right). Dotted lines represent the cells from single infections (Ο *H. polygyrus*, □ *N. brasiliensis*). The solid line represents the cells from co-infected animals (◆). Data represent means and standard errors from groups of a minimum of 4 mice assayed individually. Data were transformed for statistical analysis; one-way ANOVAs were performed for all time points using all data sets - except for the naïve group - and Bonferroni post-tests compared differences between the single infections and the co-infection (n.s. = no significant difference, * = p<0.05, ** = p<0.01, *** = p<0.001).

C: The difference in TGF-β percentages within total CD4$^+$CD25$^+$ T cell populations (Centre) was calculated compared to the corresponding naïve group, as for 7.3 B. The percentage change in mean fluorescent intensity of TGF-β on CD4$^+$CD25$^+$ T cell populations was also calculated (Right) as for 7.3 B.
Figure 7.3 – After 7 days, CD25, CD103 and TGF-b are increased in co-infected animals compared to animals with a single N. brasiliensis infection.
7.2.4. By d42 post-co-infection, there is no parasite-specific IL-4 production by CD4\(^+\) T cells from any of the groups, however in *H. polygyrus*-infected and co-infected animals, *H. polygyrus*-specific IL-10 was produced.

MLN cells were obtained from d14 and d42 co-infected animals, cultured for 3 days with Hp or Nb antigen, and then stained for intracellular IL-4 and IL-10, to determine whether or not co-infection could alter cytokine production specifically by CD4\(^+\) T cells.

MLNC from naïve animals harvested on day 14 and day 42 of co-infection and grown in media for 3 days showed low levels of IL-4- and IL-10-producing cells, in contrast to cells from animals infected with either or both parasites where increased levels of IL-4- and IL-10-producing subsets were observed at both time points (Figure 7.4 B, C). In singly-infected *N. brasiliensis* animals, this increase was observed for both cytokines, at both time points, regardless of culture conditions (media, Nb antigen, Hp antigen), reflecting the high levels of constitutive expression not requiring *in vitro* re-stimulation. Cytokine production by CD4\(^+\) T cells was therefore not considered *N. brasiliensis*-specific in these animals.

Although this was also the case for *H. polygyrus*-specific IL-4 production in animals with single infections of *H. polygyrus*, increases in the percentage of Hp antigen-specific IL-10-producing cells were observed at both time points (significance was only obtained for d42 post-co-infection, Figure 7.4 B, C). Results in co-infected animals mirrored those from the animals singly-infected with *H. polygyrus* (Figure 7.4
B, C). Thus, regardless of the absence/presence of antigen, proportions of IL-4\(^+\) and IL-10\(^+\) cells observed in co-infected animals were similar to those in *H. polygyrus*-infected animals at both time points. Proportions were greater (significantly only at the later time-point) in both co-infected and *H. polygyrus*-infected animals compared to those in *N. brasiliensis* infected animals, indicating that *H. polygyrus* dominates *N. brasiliensis* responses in a co-infection setting.
Figure 7.4 – By d42 post-co-infection, there is no parasite-specific IL-4 production by CD4⁺ T cells from any of the groups, however in *H. polygyrus*-infected and co-infected animals, *H. polygyrus*-specific IL-10 was produced.

Parasite antigen-specific cytokine responses are presented from MLN cells. Cells were cultured for 72 hours in medium alone, or with *H. polygyrus* antigen (Hp antigen) or *N. brasiiliensis* antigen (Nb antigen). The experiment was performed once.

A: Representative plots of bivariate flow cytometry analysis for IL-4 and IL-10 in the MLN from *N. brasiiliensis*-infected (Far left), and d42-co-infected (Left) CD4⁺ cells cultured in Nb antigen, as well as *H. polygyrus*-infected (Right) and d42-co-infected (Far right) CD4⁺ cells cultured in Hp antigen.

B: Percentage of IL-4-producing CD4⁺ cells on d14 of co-infection (Left) and d42 of co-infection (Right) for naïve (empty bars), *N. brasiiliensis*-infected (narrow hatch bars), *H. polygyrus*-infected (solid bars) and co-infected (wide hatch bars). Data were transformed for statistical analysis; a two-way ANOVAs was performed using all data sets (**p < 0.001**).

C: Percentage of IL-10-producing CD4⁺ cells, same as 7.4 B.
Figure 7.4 — By d42 post-co-infection, there is no parasite-specific IL-4 production by CD4+ T cells from any of the groups, however in *H. polygyrus*-infected and co-infected animals, *H. polygyrus*-specific IL-10 was produced.
7.3. Discussion

The results presented in this chapter demonstrate that ongoing chronic infection with one helminth species can dramatically alter responses to other incoming parasite species. In the model tested here, the regulatory environment created by \textit{H. polygyrus} infection was dominant over responses elicited by an acute \textit{N. brasiliensis} infection.

7.3.1. Effect of co-infection on worm burdens

Our results show that a pre-existing \textit{H. polygyrus} infection prolongs \textit{N. brasiliensis} infection and thus confirm previous studies (Colwell & Wescott, 1973; Jenkins, 1975; Della-Bruna & Xenia, 1976; Wescott & Colwell, 1980). However, unlike previous work, we found that on-going infection with \textit{H. polygyrus} could only maintain a \textit{N. brasiliensis} infection for between 7 and 14 days. Wescott & Colwell showed that co-infection with these two parasites could be maintained for up to 90 days with both parasites present (Wescott & Colwell, 1980). In their experiments, delayed rejection of \textit{N. brasiliensis} parasites was initially (days 10 to 18) dependent on \textit{H. polygyrus} dose. By days 60 to 90, the prolonged survival of \textit{N. brasiliensis} was no longer due to \textit{H. polygyrus} dose but rather to the presence of \textit{H. polygyrus} (Wescott & Colwell, 1980). In our experiments this was not the case since at both d14 and d42 post-co-infection, \textit{H. polygyrus} parasites were present with worm burdens of approximately 30 in co-infected animals (initial dose was of 200 larvae).
Using a similar initial dose to our experiments, Wescott & Colwell found just under 30 *N. brasiliensis* parasites after 90 days of co-infection (Wescott & Colwell, 1980). We identified none by 14 days post-co-infection. However, Wescott & Colwell found that single infections with *N. brasiliensis* were not expelled until between 10 and 18 days post-infection. This may help explain the differences with our study since our single infections were expelled much more rapidly (within 7 days). Also, Colwell & Wescott used an outbred strain of mice for their experiments (Colwell & Wescott, 1973) whilst we used BALB/c mice. Differences in strain most likely explain the observed discrepancies.

Our results also show that *H. polygyrus* worm burdens were not affected by co-infection with *N. brasiliensis*, similar to work by the Erb group where the course of *T. gondii* infection was not affected by concurrent *N. brasiliensis* infection (Liesenfeld *et al.*, 1995). Interestingly, in both singly- and co-infected animals, *H. polygyrus* numbers decreased in a relatively stable fashion over the first five weeks of infection, after which a sharp fall in numbers occurred and very few parasites were maintained past this time point. The time course was only performed once, however. Since worm burdens are very variable, a repeat of the experiment is an absolute requirement to ensure results reflect a standard infection. Furthermore, again our results may be mouse strain specific. We worked with BALB/c mice, known as intermediate responders to *H. polygyrus* (Su & Dobson, 1997) in which low-level infections last 10-15 weeks (Robinson *et al.*, 1989); low or high level responders may react differently in our co-infection model.
Finally, significant increases in total MLN cells were observed in co-infected as compared to *N. brasiliensis*-infected animals over the course of infection, even at d42 post-co-infection when co-infected mice were free of *N. brasiliensis*. The prolonged presence of *N. brasiliensis* in co-infected compared to singly-infected mice does therefore not appear to be a result of increased cell trafficking to the MLN. Interestingly, at the first two time points no differences were observed between *H. polygyrus*- and co-infected animals, whilst on d42 post-co-infection, a significant increase in cell numbers was apparent in co-infected animals compared to both single infections. No significant differences in worm burdens were found at this time point for *H. polygyrus*, however this was attributable to variability in the data. Increases in MLN cell numbers in the co-infected group at d42 post-co-infection was therefore considered a consequence of the slightly increased parasite numbers in these animals.
7.3.2. Effect of co-infection on cytokine production

As discussed in the previous chapter (c.f. Chapter 6), IL-4, IL-5, IL-9 and IL-13 have all been implicated in expulsion mechanisms during helminth infections while IL-10 has been associated with regulatory responses. All these cytokines are increased during *H. polygyrus* infection in both MLN and spleen (Svetic *et al.*, 1993; Finney *et al.*, submitted; Chapter 3). We have also found that they were increased after 7 days of single *N. brasiliensis* infection, but to a far lesser extent. Interestingly, our analysis shows that in co-infected animals, the presence of *H. polygyrus* causes an increase in the levels of the Th2 cytokine responses to Nb antigen. Indeed, in a study by Behnke and colleagues where animals were co-infected with *H. polygyrus* and *T. spiralis*, expulsion of the latter was significantly reduced. This was associated with increased levels of IL-3 and IL-4 but reduced levels of IL-9 and IL-10 in the co-infected animals as compared to the *H. polygyrus* single infections. The authors therefore concluded that, in order to facilitate its own survival, *H. polygyrus* selectively modulates specific cytokine secretion by Th2 cells within the MLN during infection (Behnke *et al.*, 1993).

General trends for all the cytokine responses studied in both the MLN and spleen for co-infected animals as well as those with single infections appear to show an increase in levels at d7 post-co-infection, followed by a sharp decrease until d14 post-co-infection, after which levels remain relatively stable until d42 post-co-infection. This trend corresponds to the fluctuations in worm burdens for both parasites, which confirms that cytokine levels are closely linked to parasite load.
Both IL-4 and IL-13 are considered as the two main players in helminth expulsion mechanisms, but their respective roles vary according the parasite involved (reviewed in Else & Finkelman, 1998). Work by the Finkelman group demonstrated that IL-4 is necessary but not sufficient to limit *H. polygyrus* infection, whilst it is sufficient but not uniquely necessary to eradicate *N. brasiliensis* infection (Urban et al., 1995). Indeed, expulsion of *N. brasiliensis* occurs normally in BALB/c mice treated with αIL-4 antibodies that block protective immunity to *H. polygyrus*, as well as in mice that lack a functional IL-4 gene (Madden et al., 1991; Kopf et al., 1993; Urban et al., 1995; reviewed in Finkelman et al., 1997). However, IL-4^−^IL-13^−^ animals display an impaired ability to expel *N. brasiliensis* worms (McKenzie et al., 1999), implying that IL-13 is the major factor required for worm expulsion during *N. brasiliensis* infection.

We found no changes in IL-4 levels in the MLN of co-infected animals as compared to the single infections. Since both parasites are strong Th2 inducers, and generate strong IL-4 responses, it may be that IL-4 levels are at a maximal level before the arrival of a second Th2-inducing parasite. Levels in the spleen however were increased indicating that, although levels may have been saturated at the local level, systemic responses were altered with co-infection. The increases observed in the spleen were not long-lived however, and by d14 post-co-infection, the differences had disappeared, perhaps reflecting that *N. brasiliensis* parasites were no longer present in the co-infected group. Indeed, during *H. polygyrus* infection, cytokine levels in the spleen have been shown to mirror levels in the MLN with a delay.
(Finney et al., submitted; Chapter 3). Experiments should therefore be conducted including earlier time points.

Levels of IL-13 did not follow the same pattern as levels of IL-4. IL-13 levels were higher in co-infected as compared to *N. brasiliensis*-infected animals throughout the time course in both MLN and spleen. Since *N. brasiliensis* parasites were not present at d14 or d42 post-co-infection, increases at these time points must have been influenced by *H. polygyrus* parasites. Increases in IL-13 levels in co-infected animals as compared to *H. polygyrus* infected animals were only observed in the MLN at d7 post-co-infection, and by d42 post-co-infection, levels were significantly higher in the singly-infected rather than the co-infected animals. Thus, *H. polygyrus* infection generates an elevated IL-13 response which is responsible for the increased levels observed in the co-infected as compared to the *N. brasiliensis* infected animals.

Surprisingly, very similar patterns of production were obtained for IL-5 as for IL-13. The role of IL-5 in worm expulsion mechanisms remains controversial (reviewed in Maizels & Balic, 2004). *N. brasiliensis* expulsion is, for example, unaffected in mice deficient in IL-5. However, if the absence of IL-5 is in conjunction with IL-4 and IL-13 deficiency, worm expulsion is significantly delayed, with more worms surviving in the IL-4/5/13−/− animals when compared to IL-4/13+/− mice. Thus, although IL-4 and IL-13 appear to be the primary cytokines involved in worm expulsion, other cytokines, such as IL-5 may also play a role (Fallon et al., 2002).
IL-9 levels were not altered in either MLN or spleen for the co-infected animals compared to the singly-infected animals. Although IL-9 has also been found to contribute to worm expulsion during *T. spiralis* infection (Faulkner *et al.*, 1998), in conjunction with increases in IL-4 and IL-13, the contribution of IL-9 may not be as important in expelling *N. brasiliensis* and *H. polygyrus* (Khan *et al.*, 2003). Indeed, IL-9 has been shown to play a role in *N. brasiliensis* expulsion, but only in the context of quadruple knock-out animals, lacking IL-4, IL-5, IL-9 and IL-13. In these animals, the additional deletion of IL-9 resulted in a further delay in the expulsion of *N. brasiliensis* parasites from the intestine as compared to IL-4/5/13\(^{-/-}\) animals (Fallon *et al.*, 2002).

Finally, levels of IL-10 very much resembled the pattern of IL-5 and IL-13 production: *H. polygyrus* generates high levels of IL-10 in both MLN and spleen (Svetic *et al.*, 1991; Finney *et al.*, submitted; Chapter 3), and increases in IL-10 production in the MLN and spleen of co-infected animals in response to Nb antigen can be attributed to the presence of *H. polygyrus*. Levels peaked strongly at d7 post-co-infection in co-infected animals in response to both parasite antigens, indicating high levels of regulation at this time point. This was also found in a study by Eckwalanga and colleagues, whereby protection from cerebral malaria in animals pre-infected with murine AIDS was linked to increased IL-10 levels (Eckwalanga *et al.*, 1994).
7.3.3. Effect of co-infection on Treg markers

The expression of the Treg markers CD25, CD103 and TGF-β was increased in co-infected animals as compared to *N. brasiliensis* animals, while no changes were observed between co-infected animals and *H. polygyrus* infected animals.

*N. brasiliensis*, like *H. polygyrus* infection, elicits a CD4⁺CD25⁺ cell influx into the MLN. However, at both d7 and d14 post-co-infection, these levels were significantly higher in the co-infected group than in the *N. brasiliensis* infected group. At days 14 and 42 post-co-infection, levels in the co-infected group were similar to those of *H. polygyrus* single infection, so *H. polygyrus* can be deemed responsible for the influx in co-infected animals. Levels of CD103 and TGF-β expression and intensity followed a similar pattern, being higher in the co-infected animals on d7 post-co-infection compared to *N. brasiliensis*-infected mice, but similar to *H. polygyrus* infected mice. The difference between *N. brasiliensis*-infected and co-infected animals was not observed at later time points. Peak levels of regulatory markers were observed when both parasites were present simultaneously, while levels decreased once *N. brasiliensis* was expelled. To some extent this was also seen in animals infected with *H. polygyrus* alone, however, the down-shift in regulatory markers may simply reflect the sharp decrease in *H. polygyrus* parasite numbers between days 35 and 42.

So while the regulatory markers studied were not affected by the incoming *N. brasiliensis* parasites, levels were a function of the regulatory network elicited by the
chronic parasite *H. polygyrus*. However, *N. brasiliensis* is known to generate a strong regulatory response in the lung, to where it migrates before it reaches the gut (reviewed in Olgivie & Jones, 1971). In future experiments, it would be interesting to study whether *H. polygyrus* affects the lung during single infections, and whether co-infection with both parasites alters responses in this organ. Also, experiments whereby *N. brasiliensis* is be inoculated only 7 days after *H. polygyrus* – when regulatory markers are not yet increased (Finney et al., submitted; Chapter 3) – could help elucidate whether the acute infection could affect the generation of a regulatory network by the chronic parasite.

### 7.3.4. Effect of co-infection on CD4$^+$ T cell cytokine production

In our experiments, cytokine production by CD4$^+$ MLN cells was also found to be determined by the *H. polygyrus* rather than the *N. brasiliensis* parasites. At both d14 and d42 post-co-infection, levels of IL-4 and IL-10 produced by CD4$^+$ cells stimulated by Hp antigen were similar in co-infected and *H. polygyrus*-infected animals.

Surprisingly, levels of *N. brasiliensis*-specific cytokine responses from MLN cells from *N. brasiliensis*-infected mice cultured in Nb antigen were never significantly greater than those obtained from animals infected with *H. polygyrus* cultured in the same conditions. At both d14 and d42 post-co-infection, no *N. brasiliensis*-specific responses were detected. Since no parasites were present within the hosts at these time points, repeating the experiment when parasites were present (*i.e.* at d7 post-co-
infection) would indicate whether IL-4- and IL-10- specific responses are generated during active infection, and whether these are increased in co-infected animals.

At d42 post-co-infection, constitutive expression of IL-4 was increased in co-infected animals as compared to *N. brasiliensis*-infected animals. This was also the case for levels of IL-10 for cells cultured with either Nb antigen (not significantly) or Hp antigen (significantly). Thus by d42 post-infection, by which point *N. brasiliensis* had been expelled for over 28 days, cytokine production in response to Nb antigen was decreased in singly-infected as compared to co-infected animals. This was not true at d14 post-co-infection. *H. polygyrus* therefore appears to prolong the Th2-skewed environment in co-infected animals, whilst in *N. brasiliensis* animals, no such milieu exists, explaining why CD4$^+$ cells produce lower levels of Th2 cytokines at this time point.

7.3.5. Concluding remarks and ideas for future research

Here, we have shown that the regulatory environment created during *H. polygyrus* infection is such that it can prolong the survival of an otherwise short-lived acute parasite such as *N. brasiliensis*. *N. brasiliensis* on the other hand does not appear to affect the ongoing chronic *H. polygyrus* infection in our model.

*N. brasiliensis*, just like *H. polygyrus*, is a Th2-skewing parasite. Future experiments, using Th1-skewing acute parasite infections should be conducted so as to determine
whether the regulatory network elicited by *H. polygyrus* infection can also affect parasite infections associated with a different type of immune response.

Also, it would be interesting to determine the effect of *H. polygyrus* infection on pre-established infections, as in the field it is very difficult to determine timing of infections in co-infected individuals and we do not generally know the order in which they are acquired. Since anthelmintics treat current infections but provide no protection to future infections, understanding the impact of helminth infections on pre-existing infections such as malaria, HIV or TB would provide insights into whether or not treating patients of helminths will have a positive long-lasting impact on their immune status.

Finally, experiments whereby animals are cleared of *H. polygyrus* prior to *N. brasiliensis* infection should be performed in order to determine whether active infection is necessary to prolong *N. brasiliensis* survival or that immunity induced by the parasite is sufficient. Results from Chapter 5, where we showed that *H. polygyrus* can affect bystander responses to allergen even after eradication of the parasite strongly suggests this to be the case. It would therefore be extremely interesting to perform such experiments and gain insights into the intricate relationships between parasite infections in the field.
8. CONCLUDING DISCUSSION

Without regulation, the immune system would amount to a set of uncontrolled responses; regulatory processes underlie homeostasis and coordinate immune responses to invading pathogens. However, certain parasites manage to maintain themselves within their hosts by hijacking these very processes. We have shown that Heligmosomoides polygyrus, a murine intestinal parasitic nematode, does just that. It can survive for numerous weeks within its host without generating an immune response strong enough for its removal, by inducing host regulatory cells which dampen effector immune responses to the parasite. These cells were the focus of our study.

8.1. Regulation induced by helminths

Regulation is now understood to be a major facet of parasitic infections (reviewed in Yazdanbakhsh et al., 2001; Yazdanbakhsh et al., 2002; Maizels et al., 2004 and Belkaid & Rouse, 2005). H. polygyrus, as with most helminth parasites, elicits a strong Th2 response. However, unlike acute nematode infections such as those with N. brasiliensis, in order to maintain itself within the host, H. polygyrus also induces a regulatory response. This thesis has concentrated on characterising the CD4^+CD25^+ regulatory T cells involved in the regulatory response.
Nematode parasites cause morbidity rather than mortality. They have evolved with their host so as to achieve a state of homeostasis whereby maximum survival of the parasite is ensured whilst minimal damage to the host ensues during infection. Tregs not only dampen immune responses to the parasite enabling increased survival, but also minimise host-induced pathology. Indeed, in some cases, host-induced pathology, rather the parasite itself, is the underlying cause of disease symptoms.

Numerous markers have been associated with Tregs, however only CD103 and TGF-\( \beta \) expression were increased in our model. By depleting TGF-\( \beta \), we demonstrated that this molecule may play an important role in Treg function. However, CTLA-4, GITR and FoxP3 expression, which have all been strongly associated with the regulatory phenotype and indeed often used to define natural Tregs (reviewed by Belkaid & Rouse, 2005), were not increased by infection in this system. CD62L has also been associated with regulation during graft rejection (Taylor et al., 2004). However, preliminary experiments in our system (data not shown) found no difference in expression between parasite-induced and naïve Tregs.

Although we did not obtain increases in FoxP3 expression within CD4\(^+\)CD25\(^+\) Tregs over the course of infection, there was a marked increase in FoxP3\(^+\) Treg cell numbers due to the general increase in mesenteric lymph node (MLN) and spleen cell numbers over the course of infection in our studies. While this implies FoxP3\(^+\) Treg cells are not being preferentially directed to the lymphoid tissues during infection, there is undoubted expansion of this subset. Moreover, it has not yet been determined how many regulatory cells are required \textit{in vivo} to maintain \textit{H. polygyrus}. 

308
The worm may induce a general influx of cells to the lymphoid tissue, both effector and regulatory. Due to the increased potency of the regulatory cells (c.f. Section 3.2.4), there may be no requirement for preferential influx of these cells at the lymphoid sites, thus masking the potential involvement of FoxP3 in this parasite model.

Much of the work on Tregs has concentrated on their involvement in autoimmunity, the context in which their function and role was first discovered (Sakaguchi et al., 1995). Autoimmune diseases were shown to occur due to dysregulation of the CD4+CD25+ Treg population. The lineage, antigen-specificity, suppressive potential, and characteristic markers of CD4+CD25+ cells have since been intensely studied in the context of autoimmunity (reviewed in Franzke et al., 2006). However, the Tregs induced by nematode infection have yet to be as well described and are likely to have different characteristics due to the exogenous source of antigens and the chronic nature of the infection. For example we showed that Tregs induced by *H. polygyrus* have a stronger suppressive potential than naïve Tregs (c.f. Section 3.2.4), raising the possibility of a different set of mediators involved in the suppressive mechanisms of parasite-induced Tregs.

New specific Treg markers are being implicated in regulatory mechanisms, especially through microarray analysis (Sugimoto et al., 2006). Increased Gpr83, a protein-coupled receptor, expression was determined through such research and further studies have demonstrated that this molecule can be used as a stable, specific
extracellular marker for FoxP3-expressing Tregs (Sugimoto et al., 2006). As more microarray data are analysed, more specific markers involved should come to light.

Parasites could alter the regulation potential of Tregs by affecting the cells which induce them. Dendritic cells (DCs), in their capacity as antigen presenting cells and cytokine producing cells, have the potential to regulate Tregs (reviewed in Powrie & Maloy, 2003 and Belkaid & Rouse, 2005). Current work in our group is studying the effect of *H. polygyrus* excretory/secretory products on both Tregs themselves as well as DCs. Preliminary results have shown that HES (*H. polygyrus* excretory/secretory products) strongly affects both cell types (J. Grainger, unpublished results) and dramatically increases FoxP3 expression within the Treg population. While this appears to contradict our results of a stable proportion of FoxP3^+^ T cells *in vivo*, the *in vitro* stimulation of this subset may reveal a key process leading to Treg dominance in the infection overall.

An important question yet to be resolved is whether Tregs may also accumulate at the site of infection, rather than in the draining lymphoid organs. Unfortunately, in our experiments, attempts at characterising Tregs in the intestinal mucosa were unsuccessful using both cell isolation and staining as well as immunohistochemistry in FoxP3-gfp animals. However, new fluorescence imaging techniques, including multicolour video microscopy, laser scanning cytometry, and multiphoton tissue imaging, have provided methods by which cells can be tracked *in vivo*. It is now possible to follow the migration, activation and cellular interactions of antigen-specific lymphocytes *in situ*, as well as monitor changes in T cell subsets, co-
stimulatory molecules, and chemokine expression within the physiological context of secondary lymphoid organs and also study the antigen presenting cell (APC)-T cell interaction, thus dissecting the role and timing of antigen presentation of particular DC subsets in the initiation of the immune response (reviewed in Adams et al., 2004 and Germain et al., 2006). Therefore these techniques could be used to study and track Treg populations over the course of infection with minimum artifice.

Finally, although CD4^+CD25^+ Tregs have been strongly implicated in regulatory mechanisms, new research has found a role for CD8^+CD25^+ Tregs during infection (Metwali et al., 2006); other regulatory phenotypes have also been described such as Tr1 and Th3 cells (reviewed in Akbari et al., 2003). Therefore, regulatory phenotypes may exist for numerous cell types and characterising the effect on infection on all of these may prove difficult. However, it is vital we understand the differences between naïve and parasite-induced Tregs. Indeed, treatment of humans with eggs from the pig nematode *Trichuris suis* has already been shown to dramatically reduce symptoms of gut inflammation in patients with inflammatory bowel disease (IBD) (Summers et al., 2003). Also, data presented in this thesis have shown that CD4^+CD25^+ cells induced by infection can down-modulate bystander responses and that infection is required to generate but not maintain this regulatory function (c.f. Chapter 5); using parasite-derived products may achieve similar results. Inducing regulation in this way may prove to be an effective method for controlling our increasing susceptibility to allergic responses and chronic inflammation.
8.2. The impact of helminths in the field

This thesis has demonstrated that pre-existing helminth infections have a profound impact on re-infection and co-infection in a mouse model of helminthiasis. However, can such studies ever relate to real situations in the field? Mouse models work with inbred genetic lines of mice infected with mostly laboratory-adapted strains of parasites administered in large single doses according to pre-determined time lines. In the field, human studies have to confront genetically diverse populations infected with numerous parasites, of which the time of infection is hardly ever known. Despite these discrepancies, murine models of disease are important. They provide a tool by which complex immune mechanisms can be studied in a controlled environment. They allow scientists to understand the implications of specific molecules in pre-determined settings, and as such tease out the roles of individual molecules in much more complex situations. However, often data obtained from murine studies is over-interpreted. The studies should be treated as a tool with which we can identify the markers, pathways and general responses of infection, thus targeting what to study in human work. Ideally, scientists working with mouse models should ensure their results are repeatable in more than one strain, thereby indicating that the processes involved are not particular to a single genetic background. However, even then, murine studies should not be used to determine treatment and vaccination strategies in the field without corroborating data from human studies.
Human work, although more relevant than studies in animals, can prove very difficult to interpret. In field studies, genetic ‘noise’ can often hide important findings. Also, due to variations in human populations, large scale studies are required for statistical purposes which can prove very difficult to organise and follow through. This has serious implications for longer term projects, where in a mouse model the experiment could be set up over a few weeks/months, whilst in a human model, years of sampling are required to begin to understand the impact of helminths on communities living in endemic-areas.

Sampling sites may also play a large role in the variation of results between different projects. Numerous findings from human studies have been contradictory, confounding the field of research further (reviewed in Mao et al., 2000). However, each human community studied is unique and for each study, only the parameters of interest are recorded. In a mouse model this has no real impact as the animals are all genetically identical and housed in the same conditions. However, for human work, this not the case and may partly account for the discrepancies in results.

Mouse models are therefore most useful for determining immune parameters involved in disease, whilst human studies show us whether these are relevant in real settings. Helminth infections are a reality in the developing world, and affect host immune systems such that their response to other pathogens or antigens is severely altered. It is only by combining the results of murine and human work that the impact of helminths on vaccination schemes and treatment programs for diseases such as malaria, HIV and TB can be fully assessed. Anthelminthic treatment is currently
underway in many African countries in an attempt by the WHO to control helminth-induced morbidity (Colley et al., 2004). However, as the work described in this thesis shows, the impact of parasitic worms on the immune system can have some benefits to the host. Until these are fully understood, caution should be taken in undertaking such programs. Indeed, clearing parasites, which have evolved to go unnoticed by the host's immune system and cause limited damage, may have unforeseen impacts on other concomitant infections and also facilitate the emergence of resistant parasites.


Chapter 9 - References


325


Chapter 9 – References


10. PUBLISHED RESEARCH
Suppression of allergic airway inflammation by helminth-induced regulatory T cells

Mark S. Wilson, Matthew D. Taylor, Adam Balic, Constance A.M. Finney, Jonathan R. Lamb, and Rick M. Maizels

Allergic diseases mediated by T helper type (Th) 2 cell immune responses are rising dramatically in most developed countries. Exaggerated Th2 cell reactivity could result, for example, from diminished exposure to Th1 cell–inducing microbial infections. Epidemiological studies, however, indicate that Th2 cell–stimulating helminth parasites may also counteract allergies, possibly by generating regulatory T cells which suppress both Th1 and Th2 arms of immunity. We therefore tested the ability of the Th2 cell–inducing gastrointestinal nematode *Heligmosomoides polygyrus* to influence experimentally induced airway allergy to ovalbumin and the house dust mite allergen Der p 1. Inflammatory cell infiltrates in the lung were suppressed in infected mice compared with uninfected controls. Suppression was reversed in mice treated with antibodies to CD25. Most notably, suppression was transferable with mesenteric lymph node cells (MLNC) from infected animals to uninfected sensitized mice, demonstrating that the effector phase was targeted. MLNC from infected animals contained elevated numbers of CD4+CD25+Foxp3+ T cells, higher TGF-β expression, and produced strong interleukin (IL)-10 responses to parasite antigen. However, MLNC from IL-10–deficient animals transferred suppression to sensitized hosts, indicating that IL-10 is not the primary modulator of the allergic response. Suppression was associated with CD4 T cells from MLNC, with the CD4+CD25+ marker defining the most active population. These data support the contention that helminth infections elicit a regulatory T cell population able to down-regulate allergen induced lung pathology in vivo.

INTRODUCTION

The prevalence of allergic diseases, such as asthma and allergic rhinitis, continues to rise in developed countries (1–4). Asthma is a multifarious polygenic disease, in which allergens elicit early- and late-phase airway inflammatory responses culminating in broncho-constriction and airway remodeling. However, sensitization to allergens and disease manifestation is pliant to environmental influences (5–9).

Allergies have traditionally been considered to be Th2 cell–derived immunopathologies, and earlier thinking suggested that declining microbial exposure in Western populations resulted in a weaker Th1 cell responsiveness, and a propensity to develop Th2 cell responses to innocuous allergens (10). However, it is increasingly clear that an imbalance between immunoregulatory and Th2 effector mechanisms can modulate allergy in a critical fashion (2, 3, 11–15).

This conclusion has been supported by studies of Th2 cell–inducing human helminth infections, in which both observational and post-therapy data show an inverse association between chronic infection and overt allergic responsiveness (16, 17). Interestingly, infection primarily regulates late-stage effector phase mechanisms, as proallergic IgE responses remain intact in infected patients (18–23).

Evidence for immune suppression during helminth infections is strong (24–27), and recent studies identified inhibitory mechanisms that dampen allergic and/or autoimmune pathologies (28, 29). Further data now support a role for regulatory T cells (T reg cells) in helminth infection. In human studies, peripheral T cells from infected patients are nonresponsive to parasite antigens, but responses can be restored by antibodies to IL-10 and TGF-β (26). Moreover, T cell clones from *Onchocerca volvulus*–infected patients show antigen-specific IL-10 production and release of TGF-β characteristic of T reg cells (25, 30). Our most recent studies have
Figure 1. Airway cell infiltration and tissue pathology are inhibited in H. polygyrus-infected mice. BALB/c or C57BL/6 mice were sensitized to OVA or Der p 1, respectively, by two i.p. injections of 10 μg of allergen adsorbed to Alum; 14 and 17 d after the second sensitization, mice were infected with the intestinal nematode *Heligmosomoides polygyrus* that elicits a strongly Th2 cell–biased systemic response (39, 40). This parasite has been reported to down-regulate allergies to dietary antigens (28), as well as other intestinal pathologies (41, 42). By studying airway allergy in our experiments, we can exploit the fact that *H. polygyrus* remains entirely within the gastrointestinal tract, and test the immunological intersection between helminth infection and allergic reactivity in two different locales.

Our data show helminth-mediated protection from airway inflammatory responses in both OVA and Der p 1 models. Helminth-driven suppression of effector functions, downstream of allergen sensitization, is responsible for protection from airway inflammation, as down-regulation can be transferred from infected mice to uninfected, presensitized animals and demonstrated the expansion of regulatory phenotype T cells in the mouse model filarial infection *Litomosoides sigmodontis*, and the killing of parasites in animals given antibodies to T reg cell surface marker proteins (31).

New approaches to allergy intervention has focused on restoring regulation. Notably, there is a case building for T reg cells controlling allergic airway inflammation (13, 15, 32–35). For example, T cells that were transfected with IL–10 or TGF–β to confer regulatory function (36, 37) prevent allergic airway inflammation (38). Therefore, the emergence of pathogen-induced T cell regulation together with new concepts of allergy control by T cells provides a feasible model for helminth suppression of allergy.

The data from human studies provide an enticing scenario in which helminth infections keep allergies at bay, but such epidemiological inferences must be tested experimentally if we are to distinguish cause from consequence in these complex immunological settings. Here, we investigate the host immune response at the convergence of these two immunological challenges, which as we show, interact in a dramatic manner. We test allergic airway inflammation, induced by either OVA in the BALB/c strain of mouse or Der p 1 in the C57BL/6 mouse, in mice infected with the intestinal nematode *Heligmosomoides polygyrus* that elicits a strongly Th2 cell–biased systemic response (39, 40). This parasite has been reported to down-regulate allergies to dietary antigens (28), as well as other intestinal pathologies (41, 42). By studying airway allergy in our experiments, we can exploit the fact that *H. polygyrus* remains entirely within the gastrointestinal tract, and test the immunological intersection between helminth infection and allergic reactivity in two different locales.

Our data show helminth-mediated protection from airway inflammatory responses in both OVA and Der p 1 models. Helminth-driven suppression of effector functions, downstream of allergen sensitization, is responsible for protection from airway inflammation, as down-regulation can be transferred from infected mice to uninfected, presensitized animals and demonstrated the expansion of regulatory phenotype T cells in the mouse model filarial infection *Litomosoides sigmodontis*, and the killing of parasites in animals given antibodies to T reg cell surface marker proteins (31).

New approaches to allergy intervention has focused on restoring regulation. Notably, there is a case building for T reg cells controlling allergic airway inflammation (13, 15, 32–35). For example, T cells that were transfected with IL–10 or TGF–β to confer regulatory function (36, 37) prevent allergic airway inflammation (38). Therefore, the emergence of pathogen-induced T cell regulation together with new concepts of allergy control by T cells provides a feasible model for helminth suppression of allergy.

The data from human studies provide an enticing scenario in which helminth infections keep allergies at bay, but such epidemiological inferences must be tested experimentally if we are to distinguish cause from consequence in these complex immunological settings. Here, we investigate the host immune response at the convergence of these two immunological challenges, which as we show, interact in a dramatic manner. We test allergic airway inflammation, induced by either OVA in the BALB/c strain of mouse or Der p 1 in the C57BL/6 mouse, in mice infected with the intestinal nematode *Heligmosomoides polygyrus* that elicits a strongly Th2 cell–biased systemic response (39, 40). This parasite has been reported to down-regulate allergies to dietary antigens (28), as well as other intestinal pathologies (41, 42). By studying airway allergy in our experiments, we can exploit the fact that *H. polygyrus* remains entirely within the gastrointestinal tract, and test the immunological intersection between helminth infection and allergic reactivity in two different locales.

Our data show helminth-mediated protection from airway inflammatory responses in both OVA and Der p 1 models. Helminth-driven suppression of effector functions, downstream of allergen sensitization, is responsible for protection from airway inflammation, as down-regulation can be transferred from infected mice to uninfected, presensitized animals and demonstrated the expansion of regulatory phenotype T cells in the mouse model filarial infection *Litomosoides sigmodontis*, and the killing of parasites in animals given antibodies to T reg cell surface marker proteins (31).

New approaches to allergy intervention has focused on restoring regulation. Notably, there is a case building for T reg cells controlling allergic airway inflammation (13, 15, 32–35). For example, T cells that were transfected with IL–10 or TGF–β to confer regulatory function (36, 37) prevent allergic airway inflammation (38). Therefore, the emergence of pathogen-induced T cell regulation together with new concepts of allergy control by T cells provides a feasible model for helminth suppression of allergy.

The data from human studies provide an enticing scenario in which helminth infections keep allergies at bay, but such epidemiological inferences must be tested experimentally if we are to distinguish cause from consequence in these complex immunological settings. Here, we investigate the host immune response at the convergence of these two immunological challenges, which as we show, interact in a dramatic manner. We test allergic airway inflammation, induced by either OVA in the BALB/c strain of mouse or Der p 1 in the C57BL/6 mouse, in mice infected with the intestinal nematode *Heligmosomoides polygyrus* that elicits a strongly Th2 cell–biased systemic response (39, 40). This parasite has been reported to down-regulate allergies to dietary antigens (28), as well as other intestinal pathologies (41, 42). By studying airway allergy in our experiments, we can exploit the fact that *H. polygyrus* remains entirely within the gastrointestinal tract, and test the immunological intersection between helminth infection and allergic reactivity in two different locales.

Our data show helminth-mediated protection from airway inflammatory responses in both OVA and Der p 1 models. Helminth-driven suppression of effector functions, downstream of allergen sensitization, is responsible for protection from airway inflammation, as down-regulation can be transferred from infected mice to uninfected, presensitized animals.
by mesenteric LN cells (MLNC). Furthermore, protection was most strongly associated with CD4<sup>+</sup>CD25<sup>+</sup> T cells, which is consistent with the hypothesis that parasite-induced regulatory T cells can down-modulate Th2 allergic inflammation.

**RESULTS**

**Significantly reduced airway inflammation in *H. polygyrus*-infected animals**

We first tested allergic reactivity in the airways of animals harboring chronic *H. polygyrus* infections, having established that no changes in airway cell composition or bronchoalveolar lavage fluid (BALF) cytokine secretion in mice occurred as a result of parasite infection per se (unpublished data). Mice were sensitized twice with allergen, at day 28 and 42 of infection, before airway challenge at day 56 and 58. Recovery of airway cellular infiltrates in BALF was performed on day 59, at which time infection status was also evaluated by detection of intestinal adult worms.

Infected mice were found to have significantly reduced airway cellular infiltrates after challenge with OVA (BALB/c, P < 0.001) or Der p 1 (C57BL/6, P < 0.003) (Fig. 1 A). Differential counting of cells recovered revealed a profound reduction of airway eosinophilia (Fig. 1 B, P < 0.0005) and neutrophilia (Table I). Infected BALB/c mice showed 81.6 and 66.7% decreases in airway eosinophils and neutrophils, respectively, 24 h after final challenge (Table I). Similarly, C57BL/6 mice had reduced airway eosinophils (89.0%) and neutrophils (29.0%) 24 h after final challenge with Der p 1, compared with uninfected controls. Macrophage and lymphocyte numbers were also reduced in infected mice after airway challenge (Table I), although the changes did not reach statistical significance.

**Reduced tissue pathology in infected mice**

To determine whether suppressed airway cellular infiltration represented a more general down-modulation of pathology, lung histological sections were compared in allergic and infected-allergic animals. Hematoxylin and eosin staining was used to characterize cellular infiltrates, and Alcian blue-periodic acid Schiff (AB-PAS) to identify mucus-producing goblet cells in the epithelial border. In addition, mast cell degranulation was estimated by measuring levels of β-hexosaminidase, in BALF.

In uninfected, allergen-sensitized mice of both strains, airway challenge leads to a dense peribronchial inflammatory infiltrate of lymphocytes, and mononuclear and polymorphonuclear cells with epithelial shedding and extended columnal cells (Fig. 1 C). Furthermore, an accumulation of mucin-containing goblet cells line the connecting airways, underpinning the overall increase in mucus production. In infected mice, however, tissue inflammation after OVA or Der p 1 challenge was greatly reduced (Fig. 1 C) with significantly less peribronchial and perivascular cellular infiltration and mucin staining. Goblet cell numbers showed a substantial reduction in infected mice (Fig. 1 D, P < 0.0005), whereas the allergen-induced increase in BALF β-hexosaminidase, indicating mast cell mediator release, was also attenuated in infected mice (Fig. 1 E).

To distinguish whether infection interfered with allergen priming or suppressed overt allergic reactions, mice were infected with *H. polygyrus* 14 d after the second sensitization with allergen. As shown in Fig. 1 F, animals infected subsequent to sensitization showed a similar suppression of airway allergy to mice infected at the time of first allergen exposure. Thus, chronic *H. polygyrus* infection protects mice against a range of allergic airway inflammatory pathologies, including fluid and tissue infiltration, mast cell degranulation, and goblet cell proliferation. We selected BAL infiltration as the keynote parameter to dissect the immunological mechanisms leading to this broader diminution of pathology.

**Suppression of local type 2 effector cytokines**

Local cytokine and chemokine levels were measured in the BALF, 24 h after the final allergen challenge in vivo. Infected mice did not show diminished IL-4 responses, while the Th1 cell signature cytokine IFN-γ was undetectable in both infected and uninfected mice (Fig. 2 A). The dominance of Th2 cytokines over Th1 was also observed at equivalent levels in allergen-challenged thoracic LN (TLN) cells from both groups (unpublished data). These data indicate that infection does not alter the highly polarized type 2 cytokine environment that is associated with airway allergy.

The type 2 effector cytokine IL-5, and the chemokine eotaxin, were both elevated in the BALF of infected BALB/c mice after airway challenge (Fig. 2 B). However, IL-5 and eotaxin were significantly diminished by infection (P < 0.008). Similar data were obtained for C57BL/6 mice (IL-5 reduced from 803.6 ± 201.6 to 80.5 ± 29.4 pg/ml, and eotaxin from 434.4 ± 131.1 to 92.1 ± 13.7 pg/ml). Reductions in these two key agents in the mobilization and extravasation of eosinophils provides a mechanistic explanation for the dramatically reduced airway eosinophilia in infected mice. Although IL-13 levels altered less, they were maximal in uninfected allergic mice with the most marked goblet cell hyperplasia.

We also examined the regulatory cytokines IL-10 and TGF-β (Fig. 2 C). The trend for higher IL-10 in infected mice was also not statistically significant in infected mice compared to uninfected controls (P > 0.15), indicating that infection itself does not alter the trend for higher IL-10 in allergic mice. Relevant to this, the trend for lower TGF-β was also not statistically significant in infected mice compared to uninfected controls (P > 0.15), indicating that infection itself does not alter the trend for lower TGF-β in allergic mice.

**Table I.** Total cell counts in BALF

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>0.028 ± 0.014</td>
<td>0.089 ± 0.011</td>
<td>0.648 ± 0.089</td>
<td>0.104 ± 0.025</td>
</tr>
<tr>
<td>Der p 1</td>
<td>1.054 ± 0.175</td>
<td>1.265 ± 0.349</td>
<td>0.909 ± 0.275</td>
<td>0.236 ± 0.044</td>
</tr>
<tr>
<td>Hp:D:D</td>
<td>0.224 ± 0.062</td>
<td>0.421 ± 0.050</td>
<td>0.410 ± 0.053</td>
<td>0.144 ± 0.027</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0.018 ± 0.005</td>
<td>0.073 ± 0.016</td>
<td>0.460 ± 0.026</td>
<td>0.064 ± 0.011</td>
</tr>
<tr>
<td>D:D</td>
<td>1.639 ± 0.312</td>
<td>1.591 ± 0.343</td>
<td>0.394 ± 0.058</td>
<td>0.339 ± 0.059</td>
</tr>
<tr>
<td>Hp:D:D</td>
<td>0.239 ± 0.045</td>
<td>1.129 ± 0.236</td>
<td>0.371 ± 0.041</td>
<td>0.188 ± 0.024</td>
</tr>
</tbody>
</table>

O:D denotes OVA sensitized (day 0 and 14) and challenged (day 28 and 30). D:D denotes Der p 1 sensitized (day 0 and 14) and challenged (day 28 and 30). Cells were harvested at day 31. Hp:O:D and Hp:D:D are mice infected with *H. polygyrus* 28 d before allergen sensitization.

**Downloaded from www.jem.org on November 17, 2005**
Figure 2. Broncho-alveolar lavage cytokine responses. BALF from naive, allergic, and infected-allergic mice assayed for the indicated cytokines and eotaxin. Data are from individual mice, with arithmetic mean points shown in histograms. In cases where responses are similar in BALB/c and C57BL/6 mice, data are shown only from the BALB/c response to OVA. (A) IFN-γ and IL-4, in BALB/c mice; (B) IL-5, IL-13, and eotaxin in BALB/c mice; (C) IL-10 and TGF-β in BALB/c and C57BL/6 mice. (D) Allergen-specific serum IgGl and IgG2a titers 1 d after the final airway challenge in BALB/c mice. (E) Allergen-specific IgE and total IgE levels 1 d after final airway challenge in BALB/c mice.
animals, only reached statistical significance in C57BL/6 mice (P < 0.08). In some individual BALB/c mice, the levels of active TGF-β in BALF were elevated, suggesting that this mediator may play a major role in immune regulation during infection. Overall, the two strains appear to differ in prominence of IL-10 (in C57BL/6) versus TGF-β (in BALB/c), and studies are now under way to evaluate the roles of each cytokine in the two genetic backgrounds for infection and allergy.

**Antibody isotype responses in infection and allergy**

Helminth infections can stimulate a type 2 antibody response (IgG1 and IgE) both to the parasite and to bystander antigens (43, 44). We therefore measured allergen-specific IgG1 and IgG2a isotype responses in BALB/c mice to OVA allergen. It was found that IgG1 increased significantly in infected mice (Fig. 2 D, P < 0.05), which is consistent with the reported high IL-4 environment (45). Less expectedly, IgG2a also rose, although without reaching statistical significance.

We next established that allergen-driven IgE responses were not compromised in helminth-infected mice (Fig. 2 E). This observation, together with the finding that allergy is suppressed in animals infected after sensitization (Fig. 1 F), confirms that Th2 cell priming is not ablated by the parasite infection. In fact, infected animals had greatly elevated polyclonal IgE titers compared with uninfected animals after airway challenge. Elevated polyclonal IgE is symptomatic of most helminth infections, and has been posed as a mechanism of escape from allergy, out-competing parasite-specific IgE for binding sites on mast cell IgE receptors (19). We show, using a transfer experiment (see “Transfer of protection...”), that this hypothesis is not supported in this model system.

**Anti-CD25 antibodies block suppression**

Allergic responses can be down-modulated by the action of T reg cells, through suppressive mediators such as TGF-β (46) or IL-10 (47). Many T reg cell populations constitutively express the IL-2Rα chain, CD25, and such cells can be depleted in vivo using anti-CD25 (PC61) monoclonal antibody (48), with the caveat that activated CD25⁺ effector cells may also be affected. Thus, enhanced responsiveness after CD25⁺ cell depletion is likely to reflect loss of T reg cell activity, whereas diminished responses may result from effector cell depletion. In addition, IL-10 is considered to exert a major influence on airway allergy, and its overexpression can suppress airway inflammation (36). The action of IL-10 family cytokines can be ablated in vivo by injection of antibody to the IL-10R (49).

We administered anti-CD25 antibody to infected and uninfected OVA-sensitized mice 1 d before airway challenge (Fig. 3 A), and found that airway infiltration was restored in infected mice (Fig. 3 B). However, recipients of anti-IL-10R antibody showed unchanged levels of airway infiltration and eosinophilia (Fig. 3 B). Importantly, antibodies to CD25 or IL-10R in uninfected-allergic mice had no significant effects on airway infiltrates. These studies suggest that a cellular population expressing CD25 may be responsible for the suppression of airway infiltration, but that IL-10 signaling during airway provocation is not responsible for the observed protection from allergy.

**Figure 3. Anti-CD25 and anti-IL-10R antibody intervention in infected and allergic mice.** (A) Protocol for treatment with anti-CD25 and anti-IL-10R antibodies. Naive or chronically infected mice were sensitized as previously described in "Significantly reduced airway inflammation...", mice received 1 mg of isotype control, PC61 (anti-CD25), or 1B1.3a (anti-IL-10R) i.p. 1 d before airway challenge on day 56. Anti-IL-10R was also administered on day 58, 1 d before a final airway challenge on day 59. The experiment was terminated on day 60. (B) Total cell counts and eosinophil numbers in BALB/c mice treated with isotype control, and anti-CD25 or anti-IL-10R mAbs. In control Ab-treated animals, both total (P < 0.05) and eosinophil infiltration (P < 0.05) in H. polygyrus-infected mice were significantly reduced, using Student’s t test. In PC61 treated mice, uninfected and infected groups were not statistically different.

**Transfer of protection against allergy with MLNC from infected mice**

To investigate whether a defined cellular population that was generated during chronic infection is capable of suppressing airway inflammation, we adoptively transferred MLNC taken 28 d after H. polygyrus infection (Fig. 4 A). MLNC at d 28 were found to express elevated levels of the...
Figure 4. Transfer of protective effect with MLNC. (A) Protocol for transfer. All MLNC populations were harvested from donor mice 28 d after infection with *H. polygyrus* and transferred to recipients 7 d before airway challenge. (B) TGF-β expression in MLNC from naive and infected mice assayed by flow cytometry at day 14, 21, and 28 after infection with monoclonal anti-TGF-β. Error bars show the mean ± SEM. (C) Expression of Foxp3 transcription factor and IL-4, IFN-γ, and IL-10 by CD25+ and CD25− MLNC from naive and 28-d-infected mice. For intracellular staining of Foxp3 and cytokines, representative individual FACS plots are shown against CD25 staining, together with a summary of percentage of positive
cells taken from groups of five mice. The total number of CD25+ MLNC expressing Foxp3 in naive and infected mice is presented, together with the frequency of these cells within the whole CD4+ T cell population and the proportion of CD25+ cells that express Foxp3. Antigen-specific cytokine release was measured by ELISA from supernatants of 72-h cultured CD25-depleted or enriched MLNC stimulated with medium or antigen extract from H. polygyrus adult parasites. (D) Total cell counts and eosinophil numbers in mice receiving 5 × 10^6 MLNC from naive or infected donors. In the experiment with BALB/c mice, 100 μg of soluble adult parasite antigen was given I d after administration of either naive or infected donor cells. (E) Total cell counts and eosinophil numbers in mice receiving 4 × 10^6 CD4+ or CD4- MLNC from infected donors. Cell fractions were 92.4% (CD4+) and 94.9% (CD4-) pure for BALB/c and 93.1% (CD4+) and 98.1% (CD4-) pure for C57BL/6. Group sizes were 8-10 for BALB/c and 15 for C57BL/6 mice. (F) Total IgE and allergen-specific IgE, measured in IgG-depleted serum, measured in BALB/c mice receiving 4 × 10^6 CD4+ or CD4- MLNC from infected donors. Serum samples were collected from blood I d after final airway challenge. (G) Total cell counts and eosinophil numbers in mice receiving 4 × 10^6 CD4+ or CD4- MLNC from infected donors. Cell fractions were 92.4% (CD4+) and 94.9% (CD4-) pure for BALB/c and 93.1% (CD4+) and 98.1% (CD4-) pure for C57BL/6. Group sizes were 8-10 for BALB/c and 15 for C57BL/6 mice. (H) Total IgE and allergen-specific IgE, measured in IgG-depleted serum, measured in BALB/c mice receiving 4 × 10^6 CD4+ or CD4- MLNC from infected donors. Serum samples were collected from blood I d after final airway challenge.

T reg cell–associated cytokine TGF-β (Fig. 4 B) as well as the transcription factor, Foxp3, and the proportion of CD4+CD25+ MLN T cells from infected mice that were Foxp3+ was consistently >85% (Fig. 4 C). Foxp3 expression was also determined by RT-PCR: CD4+ MLNC from 28-d infected mice contained more than fivefold higher levels of Foxp3 mRNA than similar cells from naive animals. Moreover, within the CD4+ T cell population, levels were maximal in the CD25+ cell fraction (0.88 relative to β-actin), whereas Foxp3 mRNA was below threshold values in the CD25- population. CD4+CD25+ cells contained relatively little IL-4 or IFN-γ by cytoplasmic staining. Both CD25+ and CD25- subsets responded to parasite antigen challenge with IL-10 release, with the CD25- population making the larger contribution (Fig. 4 C). Day 28 MLNC were therefore transferred into the tail veins of uninfected, allergen-sensitized recipient mice. After a further 7 d, recipient mice were given the first of two airway challenges, and airway responses were measured I d after the final challenge (Fig. 4 A).

In the first instance, 5 × 10^7 MLNC from infected or naive donor mice were transferred i.v. into allergen-sensitized BALB/c or C57BL/6 mice. MLNC from infected donors could transfer the protective effect to uninfected sensitized recipients, but naive MLNC induced no marked changes in response. Substantial declines in eosinophil infiltration were observed in mice receiving infected MLNC, compared with those receiving naive MLNC, after allergen challenge. Naive MLNC had no effect on total airway infiltrates or eosinophilia after allergen challenge. Suppression could be observed with as few as 10^7 MLNC from chronically infected donors (data not shown). Transfer of MLNC from chronically infected C57BL/6 mice into Der p 1-sensitized recipients obtained similar results, with significantly reduced total (Fig. 4 D, P < 0.008) and eosinophil (P < 0.02) infiltrates. Der p 1-sensitized C57BL/6 mice showed alleviation of airway inflammation when given MLNC from infected donors, whether or not recipient animals were given 100 μg H. polygyrus antigen 1 d later (unpublished data). From these experiments, MLNC were shown not to require antigen reexposure to transfer the protective effect to allergen-sensitized recipients.

**Transfer of protection with CD4+ T cells**

CD4+ and CD4- cells were isolated from MLNC of d 28 infected animals and transferred into uninfected sensitized recipients. OVA-sensitized BALB/c mice displayed a significant reduction in total airway infiltrates after CD4+ cell transfer and airway challenge (Fig. 4 D). Suppression of airway eosinophilia was observed after the transfer of CD4+ (P < 0.0001) or CD4- (P < 0.025) cells, respectively. These results from the BALB/c model demonstrate that CD4+ T reg cells, engendered during a chronic H. polygyrus infection, can transfer protection against allergy to an allergen–sensitized animal if given before the time of airway challenge.

The regulatory activity may not, however, be limited to a single cellular population. In C57BL/6 mice sensitized to Der p 1, transfer of either CD4+ or CD4- cells resulted in reduced airway infiltrates and eosinophilia, and in some cases, (Fig. 4 E) the CD4+ population showed greater potency. Because the two regulatory populations evident in infected C57BL/6 mice develop in the absence of allergen sensitiza-
JEM

wild-type ('1)4 cells. Transfer of CD4 cells from either IL- and eosinophilia (P < 0.05) compared with imunuls receiving (1)4 cells showed significantly reduced airway infiltration of IL-10 did not compromise the ability of transferred CD4 sensitized wild-type recipients. As shown in Fig. 5, the lack of CD4 activity in this strain relative to BALB/c reflects a genetic difference rather than the choice of allergen used in the two systems.

Unaltered IgE responses accompany suppressed allergy

The transfer of CD4+ cells from infected animals to uninfected, sensitized mice (Fig. 4 F) did not alter total or allergen-specific IgE levels in recipient mice. This finding supports the thesis that a cellular population that was engendered during helminth infection is the major contributor to overall protection from allergic reactivity, and although increased levels of total IgE may play a role in diluting allergen-specific IgE and/or occupy FcεR on mast cells and basophils, it is not the major component of the protective effect observed.

IL-10−/− MLNC transfer protection

CD4+ and CD4− cells were isolated from MLN of chronically infected IL-10−/− mice and transferred into uninfected sensitized wild-type recipients. As shown in Fig. 5, the lack of IL-10 did not compromise the ability of transferred CD4+ cells to suppress allergic reactions. Recipients of IL-10−/− CD4+ cells showed significantly reduced airway infiltration and eosinophilia (P < 0.05) compared with animals receiving wild-type CD4+ cells. Transfer of CD4− cells from either IL-10−/− or wild type showed a diminution of allergic outcome that narrowly failed to show statistical significance. Hence, IL-10 is not an essential component of the pathway by which helminth-induced cells can suppress host allergic reactivity.

CD4+CD25+ T cells transfer protection

To test directly whether infection induces CD25+ T reg cells, day 28 MLNC from infected mice were separated into CD4+CD25− and CD4+CD25+—enriched populations, and transferred into allergen-sensitized recipients 7 d before airway challenge (Fig. 4 A). In both strains of mice, the most significant reduction of airway inflammation occurred with the transfer of CD4+CD25+ cells. CD4+CD25+ cell transfer reduced not only total airway infiltrates (Fig. 6) but also airway eosinophilia (BALB/c, P < 0.02; C57BL/6, P < 0.02), and goblet cell proliferation (BALB/c, P < 0.05). Evidence was also found that the CD4+CD25+ subset also contributes to an abatement of allergic reactivity, but not as consistently as CD4+CD25+ cells. Overall, our data suggest that the CD4+CD25+ phenotype T cell population, at least, can mediate suppression of airway allergy in both strains of mice.

Migration of donor MLNC to the lungs and TLNs

To investigate whether transferred MLNC are capable of relocating to airway-associated tissues in recipient sensitized mice, we followed the fate of transferred cells bearing the Ly5.1 allotypic marker, which permits donor cell identification after transfer into C57BL/6 (Ly5.2) mice. CD4+ and CD4 Ly5.1 MLNC transferred i.v. into sensitized C57BL/6 mice 7 d before airway challenge could be found in the local draining LNs, composing 0.42 ± 0.18% and 0.39 ± 0.07% of the CD4+ and CD4− population, respectively (Fig. 7). Similarly, 0.18 ± 0.10% and 0.30 ± 0.14% of lung CD4+ and CD4− cells were Ly5.1+. These data suggest that donor cells from chronically infected mice migrate to sites of inflammation, either to directly suppress host responses, or to consort resident cells into a regulatory phenotype.

DISCUSSION

Allergies are Th2 cytokine–mediated pathologies, involving IL-4−, IL-5− and IL-13−dependent amplification of innate effector cell populations acting together with antibodies and inflammatory mediators (50). As Th1 and Th2 cell responses are mutually antagonistic, it has been argued that declining microbial stimulation of Th1 cell responses in the developed world has led to over-vigorous allergic Th2 cell reactions (51). However, allergen-specific Th1 cells can in fact exacerbate airway inflammation (52). Moreover, epidemiological reports increasingly link Th2 cell–inducing helminth parasite infections with reduced allergic disease in humans (53), whereas nematode–infected mice display attenuated allergic responses (28, 54, 55). Current theories postulate that pathogen-induced T reg cells control both Th1 and Th2 effector populations (2, 3, 11, 16, 27, 53). The possibility that T reg cells inhibit allergic disease has received growing support from both animal (13, 56) and human (14, 15, 57) studies.
Figure 6 (continued on next page)

Figure 6. Transfer of protective effect with CD4+CD25+ MLNC.
(A) Total cell, eosinophil, and goblet cell numbers in mice receiving 3 × 10⁶ CD4+CD25+ or CD4+CD25− MLNC from infected donors. (B) Purity of CD4+CD25+ and CD4+CD25− populations assayed by flow cytometry.

We now provide direct experimental evidence to support the hypothesis that helminth infection down-regulates allergic reactions through the action of regulatory T cells rather than by altering the Th1–Th2 balance. To do this, we selected the murine intestinal nematode *H. polygyrus* as a model of helminthiasis (28, 58), because it follows a purely enteric infective cycle and establishment; has the ability to establish stable, chronic infections; and evokes a Th2 cell-dominated immune response similar to that observed in general with gastrointestinal nematodes. We chose an allergic
JLM

T reg cell activity may be responsible for this phenomenon sensitized allergy-prone host (Fig. 1 F). The hypothesis that responses in infected and uninfected mice, and the ability of airway inflammation model that permitted us to observe or CD4

therapy demonstrated by the impact of infection on a previously normally is shown by the similar allergen-specific IgE re-

hernihth infection. The fact that Th2 cell priming occurs were suppressed within the context of a Th2 cell—inducing balance. However, the Th2 effector cytokines IL-5 and IL-

suppression, we first established that the Th2-driving cy-

tokine IL-4 was unaffected, as was the overall Thl—Th2

response to infection, or can parasite antigen-specific regulatory

population is self-reactive rather than parasite specific, and is

stimulation was striking. One possibility is that the T reg cell

arise from naive Th(0) precursors? In this respect, the fact

preexisting "natural" T reg phenotype cells that expand in re-

gin and antigen specificity of these cells (65, 66). Are there

related regulatory activity in infection raises the issue of the ori-

iment reversed suppression. However, allergic inflammation was not restored by the administration of anti–IL-10R anti-

body, indicating that T reg cell activity in this setting is not IL-10 dependent.

We then used the adoptive transfer system of MLNC from infected mice into sensitized, but uninfected, hosts to demonstrate that down-regulation is mediated by T cells, primarily those with the CD4\(^+\)CD25\(^+\) phenotype associated with T reg cells (61, 62). Several distinct regulatory pheno-
types of T reg cell cells have been proposed, including "Th3" cells, primarily acting through TGF-B, which are most closely associated with the gut mucosal environment, and "Tr1" cells, which are capable of suppressing airway allergy through the action of IL-10 (13, 56). Interestingly, the H. polygyrus-elicited T reg cell cells are generated in the Th3 environment, but manifest their function in the Tr1 environment.

Although the CD4\(^+\)CD25\(^+\) subset was the most potent at suppressing allergic inflammation, some activity could also be observed in the CD4\(^+\) population, particularly in the C57BL/6 system. Although this may represent a CD8\(^{+}\) regu-

latory T cell (63), we also have evidence that CD19\(^+\) B cell populations are able, on transfer, to exert a dampening effect on immunopathology. Perhaps, in the down-regulatory milieu of a chronic infection, additional non-T reg cell type cells are recruited to curtail pathology more completely.

The cells transferred from infected mice express elevated levels of IL-10 and TGF-\(\beta\), the two principal T reg cell—asso-

ciated down-regulatory cytokines, and contain a significantly higher number of CD4\(^+\)CD25\(^+\) Foxp3\(^+\) cells. However, despite the prominence of IL-10 production by H. polygyrus-exposed T cells, their ability to suppress airway allergy is not mediated by IL-10, as MLNC from infected IL-10\(^-{\text{--}}\) mice can mediate suppression, which is consistent with the failure of anti–IL-10R antibodies to reverse suppression in infected animals. In contrast, TGF-B remains a strong candidate for immune suppression by T reg cells from helminth-infected mice, particularly as this cytokine is known to alleviate experi-

mental airway allergy (37) and has the capacity to instruct peripheral T cells to develop regulatory capacity (46, 64).

CD4\(^+\)CD25\(^+\) T cells are generally associated with self-

reactive regulatory cells that prevent autoimmune reactivities (61, 62). Hence, the expansion of CD4\(^+\)CD25\(^+\) cell—medi-

ated regulatory activity in infection raises the issue of the origin and antigen specificity of these cells (65, 66). Are there preexisting "natural" T reg phenotype cells that expand in re-

sponse to infection, or can parasite antigen-specific regulatory cells arise from naive Th0 precursors? In this respect, the fact that transfer of suppression did not require renewed antigen stimulation was striking. One possibility is that the T reg cell population is self-reactive rather than parasite specific, and is restimulated by ligands in the recipient host. However, it has been shown that T reg cells specific for exogenous antigen (alloantigen or HGG), reactivated in a donor immediately before transfer, suppressed bystander allograft responses in a

Figure 6 (continued from previous page)

(C) AB-PAS staining of formalin-fixed lung tissue, staining mucin-

producing goblet cells, in BALB/c mice receiving 3 \(\times\) 10\(^{6}\) CD4\(^+\)CD25\(^+\) or CD4\(^+\)CD25\(^-\) MLNC from infected donors.
recipient without the need for antigen restimulation in the new host (67). Thus, it is possible that chronic parasite infection maintains a high level of activation in T reg cells, sufficient for their function in our short-term experiments. As with other instances of T reg cell responses to pathogens (66), these issues are currently under active investigation.

The transfer model also allows us to exclude changes in antibody production as a major mechanism for abatement of allergy. For example, the production of allergen-specific-IgE is comparable in mice who have received naive or infected MLNC, which show respectively normal and suppressed allergic reactions. There is also no rise in polyclonal IgE in recipients of infected CD4+ T cells, arguing that changes in either absolute allergen-specific IgE, or in the ratio of nonspecific to specific IgE, are not responsible for the diminution of allergic responses in infected animals. A similar conclusion was drawn from measurements of specific and total IgE in atopic and nonatopic humans harboring chronic schistosome infections (21).

A final intriguing question is why helminth parasites such as H. polygyrus and Schistosoma mansoni (68) induce T reg cells. As argued elsewhere (27), parasites that can exploit host down-regulatory networks are likely to gain advantage in the battle for long-term survival in the host. Much of the pathology encountered in helminth infection is immune mediated (69), and a dampening of responsiveness would not necessarily compromise the host. However, the immune system may have evolved to operate optimally in the regulated environment of infection, and in our more hygienic environment, we are prone to overzealous reactions to innocuous targets, generating the rapidly increasing levels of allergy and autoimmunity being experienced in the developed world.

MATERIALS AND METHODS

Animals. Female BALB/c, C57BL/6, and C57BL/6-Ly5.1 mice, 6–8 wk old, were housed in individually ventilated cages licensed under UK Home Office guidelines. At least five mice were used per experimental group. IL-10-deficient mice were a gift from S. Anderton (University of Edinburgh, Edinburgh, UK).

Parasites. Mice were infected with 200 H. polygyrus L3 larvae (provided in the first instance by J. Behnke, The University of Nottingham, Nottingham, UK) using a gavage tube. Infections were verified by detection of eggs in fecal samples.

Antigens and allergens. For H. polygyrus antigen, a PBS homogenate of adult worms was centrifuged (13,000 g for 10 min), and the supernatant was

---

**Figure 7. Tracking of donor lymphocytes in recipient mice.** 4 × 10^6 CD4+ or CD4- MLNC from infected mice were transferred i.v. into C57BL6/Ly5.2 recipients, 7 d before airway challenge. After the second airway challenge, lungs and thoracic lymph node (TLN) cells were recovered and stained for Ly5.1 and CD4 expression. Error bars show the mean ± SEM.
Allergen-induced airway inflammation. Mice were sensitized i.p. with 10 μg OVA (BALB/c) or Der p 1 (C57BL/6) adsorbed to 9% potassium alum (Sigma A7167), and boosted with the same antigen 14 d later. On day 28 and 31, mice were challenged with 10 μg OVA or Der p 1 in PBS by the intratracheal route. Mice were killed 24 h after final airway challenge to assess airway inflammation. For histopathology, formalin-fixed lungs were embedded in paraffin and sectioned. Hematoxylin and eosin stained sections were analyzed for airway inflammation and pathological changes. Mucus-containing goblet cells were stained with AB-PAS, and the histological mucous index was quantified for goblet cell hyperplasia (71).

BALF cell counts. At 24 h after final challenge, mice were terminally anesthetized, the tracheas were cannulated, and internal airspaces were lavaged with 500 μl PBS, followed by two 350-μl washes. Fluids were centrifuged at 1,200 g, and pellets recovered for cellular analysis. Initial 500-μl BALF samples were stored at −80°C for biochemical analyses. Cytopsin were prepared by spinning 5 × 10^6 cells onto poly-L-lysine-coated slides (BDH) followed by Diff-Quick (Boehringer) staining. Differential cell counts were performed on a minimum of 200 cells at magnification of 1000.

Allergen-specific antibodies and total IgE. Allergen-specific responses were determined by ELISA. Multisorp (Nunc) plates were coated with 4 μg/ml OVA or Der p 1 in 0.05 M carbonate/bicarbonate, overnight at 4°C. Plates were blocked with 5% BSA (fraction V; GIBCO BRL) for 2 h at 37°C. Sera were diluted in TBS, 0.05% Tween (TBS-T) and added to wells overnight at 4°C. Allergen-specific IgG isotypes were detected with HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc.) and anti-IgG2a and ABTS peroxidase substrate (KPL). For allergen-specific IgE assays, proteins G-Sepharose beads were used to remove IgG from sera; biotinylated anti-mouse IgE (BD Biosciences), ExtrAvidin–alkaline phosphatase conjugate (Sigma–Aldrich) and pNPP Substrate (Sigma–Aldrich) were then used. Total IgE was measured with anti-mouse IgE capture (BD Biosciences) and biotinylated anti-mouse IgE detection, using a monoclonal IgE standard curve.

Cytokines and chemokines. Cytokines were measured by ELISA according to suppliers’ guidelines. Capture antibodies for IL-4 (11B11, 4 μg/ml), IL-5 (2 μg/ml), IL-10 (4 μg/ml) and IFN-γ (R46A2, 5 μg/ml) were produced in-house or purchased from BD Biosciences. Capture antibodies for IL-13 (2 μg/ml) and eotaxin (0.4 μg/ml) were obtained from R&D Systems. Biotinylated detection antibodies were purchased from BD Biosciences (5 μg/ml IL-4, 2 μg/ml IL-5, 2 μg/ml IL-10, and 0.5 μg/ml IFN-γ) or R&D Systems (0.1 μg/ml IL-13 and 0.4 μg/ml eotaxin). TGF-β was measured with transfected mink lung epithelial cells expressing luciferase under the plasmidinogen activator inhibitor 1 promoter (72). To assay β-hexosaminidase activity, samples were incubated with 80 μl of substrate solution (1.3 mg/ml p-nitrophenol-B-2-O-2-acetamido-2-oxoylacytopyranoside [Sigma-Aldrich] in 0.1 M citrate, pH 4.5). The reaction was stopped by the addition of 200 μl of 0.2 M glycine, pH 10.7, and ODs were read at 405 nm.

Antibodies to CD25 and IL-10R. Anti-CD25 mAb was PC61 rat anti-mouse IL-2Rα monoclonal, produced in-house from cells provided by P. Prowie (University of Oxford, Oxford, UK), and grown in serum-free media in a cell growth bag (Bio-Vectra). Antibody was purified on protein G-Sepharose, dialyzed against PBS, and given i.p. at 10 mg/ml. Rat anti-mouse CD210 (IL-10R) antibody was IgG1 monoclonal 1B1.3a (BD Biosciences).

Adoptive cell transfer. MLNCs were removed from mice infected 28 d earlier with H. polygyrus. Single-cell suspensions in RPMI-0.5% normal mouse serum were made using a cell strainer. For whole MLNC transfers, 1–5 × 10^7 cells were injected i.v. in PBS. Mice received MLNC 7 d after a second allergen sensitization, which was 7 d before airway challenge.

CD4+ and CD25+ cell enrichment. For CD4+ cell purification, cell suspensions were incubated with CD4 (L3T4) microbeads (Miltenyi Biotech) and separated on MACS LS columns with preseparation filters; 4 × 10^6 CD4+ or CD4− cells were injected i.v. into recipient mice. For CD4+CD25+ cell enrichment, CD4+ cells were first negatively isolated using streptavidin microbeads (Miltenyi Biotec) and biotinylated anti-CD11b (BD Biosciences), anti-CD86 (BD Biosciences), anti-MHC class II (M5114) and anti-Igk (I87.1). Antibody-bound beads and cell solutions were separated on MACS LS columns. CD25+ cells were then positively selected with biotinylated anti-CD25 (BD Biosciences) and streptavidin microbeads. Uninfected, allergen-sensitized mice received 3 × 10^6 CD4+CD25+ or CD4+CD25− cells from infected donors 7 d before the first airway challenge.

Flow cytometry. Antibodies were diluted in PBS, 0.5% BSA (Sigma–Aldrich), 0.05% sodium azide. Cells were stained for 20 min at 4°C. For detection of CD4+CD25+ and CD4+CD25− cells, rat anti-mouse CD4 (L3T4, clone R45-4, IgG2a) and anti-CD25 (clone PC61, IgG1; CALTAG) mAbs were used. For detection of donor CD7167/61.5 (CD41) cells in recipient mice, anti-CD45.1 (clone A20, mouse IgG2a) was used. For staining intracellular cytokines, cells were permeabilized in cytofix/cytoperm, washed in perm/wash buffer (BD Biosciences), and stained with anti–mouse IL-4 (11B11), IFN-γ (XMG1.2), or IL-10 (JES5-16E3) for 30 min. For FcγR3 staining, a kit from eBioscience using mAb FJK–162 was used according to the manufacturer’s instructions. Surface bound TGF-β was detected using rat anti–mouse TGF-B1 (B7-A5–3, IgG2a). The expression of surface markers and intracellular IL-10 was analyzed on a FACSCalibur flow cytometer using FlowJo software (Tree Star). All fluorochrome-labeled antibodies were obtained from BD Biosciences, unless otherwise stated.

Statistics. Student’s t test was used for all statistical comparisons; p-values <0.05 were considered significant.

The authors have no conflicting financial interests.

Received: 17 December 2004
Accepted: 14 September 2005

REFERENCES


HELMINTH DOWN-REGULATION OF ALLERGIC INFLAMMATION | Wilson et al.

1875-1886.


