CHARACTERISATION OF MONONUCLEAR PHAGOCYTES
IN SHEEP

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DECLARATION

I hereby declare that the composition of this thesis and the experiments described are my own work unless specifically stated in the acknowledgements or text. No part of this work has been or will be submitted for any other degree, diploma or qualification.

Vipan Kumar Gupta

September 1994
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ABBREVIATIONS

ADC    afferent dendritic cells
ADCC   antibody dependant cellular cytotoxicity
AM     alveolar macrophages
ANAE   α-naphthylacetate esterase
BCIP   bromo-chloro-indolyl phosphate
BM     bone marrow
BMM    bone marrow macrophages
BNMS   biotinylated normal mouse serum
BSA    bovine serum albumin
CR     complement receptor
C3Rs   complement 3 receptors
CD     cluster of differentiation antigens
DAB    diaminobenzidine-tetrahydrochloride
DC     dendritic cells
DMSO   dimethylsulphoxide
DOC    sodium deoxycholate
DW     distilled water
EC     endothelial cells
E-CFU  erythroid-colony forming unit
EDTA   ethenenediamine tetra acetic acid
ELISA  enzyme-linked immunosorbent assay
FACS   flow cytometry
FCyR   Fc receptor for IgG
FcRs   Fc receptors
FCS    foetal calf serum
FDC    follicular dendritic cells
FITC   fluorescein isothiocyanate
f-met-leu-phe  formyl-methionine-leucine-phenylalanine
FSC    forward angle light scatter
G-CSF  granulocyte colony stimulating factor
GM-CFU granulocyte-macrophage colony forming unit
GM-CSF granulocyte-macrophage colony stimulating factor
GPI    glycosyl phosphatidyl inositol
HAT    hypoxanthine, aminopterin and thymidine
HBSS   Hank's balanced salt solution
HRP    horse radish peroxidase
ICAM   intercellular adhesion molecule
IDC    interdigitating reticulum cell
IFN    interferon
Ig     immunoglobulin
IL     interleukin
IP     intraperitoneally
IPP    ileal Peyers' patch
IV     intravenously
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<td>LFA</td>
<td>lymphocyte functions associated antigen</td>
</tr>
<tr>
<td>LI</td>
<td>leukocyte integrins</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>M-CFU</td>
<td>macrophage-colony forming unit</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MDM</td>
<td>monocyte derived macrophages</td>
</tr>
<tr>
<td>MDMC</td>
<td>monocyte depleted mononuclear cells</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>MEMC</td>
<td>monocyte enriched mononuclear cells</td>
</tr>
<tr>
<td>Mφ</td>
<td>macrophage</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLN</td>
<td>mesenteric lymph node</td>
</tr>
<tr>
<td>MP</td>
<td>mononuclear phagocytes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTC</td>
<td>macrophage tumour cytotoxicity</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro-blue tetrazolium</td>
</tr>
<tr>
<td>NC</td>
<td>nitrocellulose membrane</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NSS</td>
<td>normal sheep serum</td>
</tr>
<tr>
<td>NMS</td>
<td>normal mouse serum</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OPD</td>
<td>O-phenylene diamine</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PALS</td>
<td>periarteriolar lymphoid sheath</td>
</tr>
<tr>
<td>PBG</td>
<td>peripheral blood granulocytes</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood leucocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCV</td>
<td>post capillary venules</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PIPL-C</td>
<td>phosphatidyl inositol phospholipase-C</td>
</tr>
<tr>
<td>PLN</td>
<td>peripheral lymph node</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PMT</td>
<td>photo multiplier tube</td>
</tr>
<tr>
<td>PNAδ</td>
<td>peripheral lymph node addressin</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediates</td>
</tr>
<tr>
<td>SA-PE</td>
<td>streptavidin-phycoerytherin</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDW</td>
<td>sterile distilled water</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SPBS</td>
<td>sterile phosphate buffered saline</td>
</tr>
<tr>
<td>SSC</td>
<td>side angle light scatter</td>
</tr>
<tr>
<td>TD</td>
<td>T cell dependent</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N, 'N'-tetramethylethylene diamine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TI</td>
<td>T cell independent</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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</table>
The mononuclear phagocyte (MP) system is a heterogeneous group of cell populations present in most tissues even in the absence of inflammation. Monocytes and Mø are the major differentiated cells of MP system, have a prominent role in defence against many infectious agents and tumour cells, and are involved in the regulation and induction of immune responses. In addition they secrete a large number of substances which have a role in various physiological and pathological processes.

It is necessary to know the normal distribution and localisation of cell with a particular phenotype in order to understand their involvement in various disease processes. The advent of hybridoma technology has facilitated the dissection of the phenotypic and functional heterogeneity of various cells by producing monoclonal antibodies (mAb) specific to cell surface molecules. Sheep is an important experimental animal model, for the study of the pathogenesis of infectious diseases and little is known about the cell surface determinants of MP in this species.

In this thesis I have characterised MP by developing novel anti-Mø mAb and further characterising anti-cattle mAb which cross react with sheep B2 integrins.

Three mAb (VPM65, 66 and 67) immunoprecipitated cell surface glycoproteins of the same M₉ = 55,000, having approximately 3,000 Da N-linked glycosylation. The antigen is primarily expressed in blood monocytes in addition to its moderate expression in AM and granulocytes but weak expression in different dendritic cells (ADC). They also labelled resident Mø in many different tissues but were non-reactive with lymphocytes. The molecule is anchored to the cell surface via glycosyl-phosphatidylinositol linkage. These mAb recognise the same or overlapping epitopes of the antigen. VPM65/66/67 recognise a homologue of sheep CD14 as determined by antigen preclearing studies and two-colour FACS analysis using human anti-CD14 mAb (TUK4). These mAb also reacted with monocytes and Mø in cattle.

VPM63 recognised a dimer of M₉ = 40,000 and 42,000 having approximately 2,000 Da N-linked carbohydrates. VPM63 reacted with resident Mø in most tissues but was absent from blood monocytes, ADC and any lymphocyte populations. The expression of the antigen is lost within two days in cultured AM and did not appear on in vitro monocyte derived macrophages. Immunoblotting of affinity purified VPM63 antigen with polyclonal rabbit anti-bovine FcγRII synthetic peptide of 15 amino acids (150-168) from the second extra cellular domain, suggests the possibility that VPM63 recognises a Mø specific isoform of sheep FcγRII. However VPM63 failed to block uptake of soluble Ag-Ab complexes by AM. VPM64 immunoprecipitated a heterodisperse antigen of M₉ = 65,000 to 80,000 from surface labelled AM. In addition to AM the antigen is expressed on granulocytes and has low level of expression on monocytes. VPM64 also stained a small population of CD11b⁺, non-T, non-B and non-monocyte (CD14⁺) peripheral blood mononuclear cells, possibly to be 'natural killer' cells. VPM64 labelled resident Mø in spleen, gut and lungs. On the basis of its molecular weight and cellular distribution the possibility of VPM64 recognising an antigen equivalent to the mouse FcγRII is discussed.

A panel of mAb specific for B2 integrins on MP of sheep was characterised by immunoprecipitation, flow cytometry and immunohistochemistry using mAb submitted to the 2nd International Workshop on Ruminant Leukocyte differentiation. Antigens. Immunoprecipitation, immunohistochemical and flow cytometric analysis differentiated these mAb into four distinct groups (Gp). The relationship between these antibodies is shown by sequential immunoprecipitation which showed that the reactivities of antibodies in Gp 1, 2 and 3 were mutually exclusive but that Gp 4 antibodies shared a common specificity with the other three groups. By analogy with the human, mouse and cattle B2 integrin families Gp 1 mAb seem to be specific for CD11a/CD18 (LFA-1); Gp 2 are CD11b/CD18 (CR3 or Mac-1); Gp 3 are CD11c/CD18 (CR4 or p150/95) and Gp 4 are CD14. The differences in the tissue distribution and cellular localisation for the different B2 integrins are described and discussed.

In addition to myeloid cells CD11b and CD11c are also present on a sub-population (30-50%) of resting peripheral blood B cells, which are exclusive to blood and spleen and do not appear to recirculate through peripheral lymph node and do not express L-selectin (DUI.29), a lymph node homing and memory cell marker. In peripheral blood the non-recirculating B-cell population was mutually exclusive to the recirculating B-cell population (Du2.74⁺). Cells resembling non-recirculating B-cells are confined only to marginal zone in the spleen whereas recirculating B-cells are present in the follicles in spleen and ileal Peyrs' patches. These preliminary observations indicate that CD11b⁺ B cells may represent a population of naive B cells and have distinct recirculatory pathways.
CHAPTER ONE

INTRODUCTION
Author's Note

Erratum

The terminology used in this thesis for the leukocyte integrins is misleading in that the \( \alpha \) chains nomenclature (CD11a, CD11b and CD11c) is in places equated with that for the heterodimers (LFA-1, Mac-1 and p150/95). It should thus be made clear that the following correct nomenclature for this family of heterodimers is intended:

<table>
<thead>
<tr>
<th>Leukocyte Integrin</th>
<th>Alternative Name</th>
<th>( \alpha ) Chain</th>
<th>( \beta ) Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1</td>
<td>-</td>
<td>CD11a (=( \alpha L ))</td>
<td>CD18</td>
</tr>
<tr>
<td>Mac-1</td>
<td>CR3</td>
<td>CD11b (=( \alpha M ))</td>
<td>CD18</td>
</tr>
<tr>
<td>p150/95</td>
<td>CR4</td>
<td>CD11c (=( \alpha X ))</td>
<td>CD18</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 MONONUCLEAR PHAGOCYTES

The mononuclear phagocytes (MP) constitute the most primitive natural defence system of the host. The MP system is comprised of bone marrow monoblasts, promonocytes and monocytes, circulating monocytes and resident differentiated tissue macrophages. The monoblast is the least mature cell while the macrophage (Mφ) is the major differentiated cell of the MP system.

The term 'macrophage' was coined by Elie Metchnikoff more than a century ago to describe large mononuclear phagocytic cells in the tissues (Metchnikoff, 1884). The macrophages are widely distributed throughout the body, but are extensively modulated in specialised microenvironments such as the epidermis (Langerhans cells), liver (Kupffer's cells) and nervous system (microglial cells).

MP play a central role in immunoregulatory and immune surveillance functions to protect against adventitious agents and neoplastic disease. They are involved in inflammatory responses to injury, actively clearing damaged cells and immune complexes from the circulation and also take part in tissue resorption and remodelling.

Despite their common bone marrow origin MP show considerable heterogeneity in terms of anatomical localisation, morphology, cell surface biochemistry and function. The heterogeneity of the MP system has been defined using various morphological, functional, cytochemical and immunological techniques. Since the introduction of hybridoma technology (Kohler and Milstein, 1975), monoclonal antibodies (mAb) specific for cell surface antigens have been extremely valuable in dissecting the heterogeneity of these populations. Several myeloid differentiation antigens, some defining clusters of differentiation and other unclustered/unique antigens have been defined by using mAb (Knapp, 1989). In the present study mAb have been developed to characterise MP in sheep.

1.2 ORIGIN OF MONONUCLEAR PHAGOCYTES

Mφ originate from monoblasts in the bone marrow (van Furth, 1989) and represent a particular line of myeloid differentiation known as the mononuclear phagocyte system. They are related to granulocytes through a common progenitor called the granulocyte-macrophage colony forming unit (GM-CFU) (Metcalf, 1971) and to other bone marrow derived cells through a common haemopoietic stem cell.
The GM-CFU differentiate into monoblasts and then to promonocytes which gives rise to monocytes in the bone marrow. Monocytopoiesis is regulated by a number of haemopoietic growth factors, in particular granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage-CSF (M-CSF) which are produced by MΦ themselves (Hamilton, 1993). Fibroblasts, endothelial cells and other non-haemopoietic cells also produce these growth factors under the influence of cytokines (IL-1 and TNF-α) produced by MΦ during an inflammatory response. Monocytopoiesis is also influenced by products of T lymphocytes such as GM-CSF and interleukin (IL)-3 which regulate the production and function of MP during inflammation. A basal level of these, and other, growth factors in the bone marrow maintains normal physiological monocyte production. These monocytes pass through the blood circulation for one to three days and migrate into the tissues where, depending upon the microenvironment of the tissue, they differentiate into mature resident MΦ. The migration of circulating monocytes into extravascular tissue involves transendothelial migration which is mediated by a number of adhesion molecules (described below in section 1.10.7).

More than 95% of tissue MΦ are derived from monocytes and the remaining 5% are contributed by local division of MP in the tissues (van Furth, 1989). Tissue MΦ are constantly being renewed by the influx of fresh monocytes and the ultimate fate of tissue MΦ is uncertain. It is thought that MΦ die in the lymph node after they migrate out of the tissues. For example dendritic cells (DC) enter the lymph node via the afferent lymph but very few accessory cells leave the node in efferent lymph (Hopkins and McConnell, 1993). Less mature cells are recruited to tissues in response to inflammation, infection, degenerative, metabolic and some neoplastic disorders and retain features of the circulating blood monocytes, including an enhanced respiratory burst (van Furth et al., 1973). Recently recruited monocytes account mainly for the contribution of MΦ to host defence and repair. Resident MΦ constitutively present in lymphohaeopoietic and other tissue perform trophic rather than cytotoxic function and thus provide a first line of defence at portals of entry (van Furth, 1989).

Dendritic cells and monocytes are considered to come from a common precursor in the bone marrow because they share some functional and phenotypic properties (Austyn, 1987). This hypothesis is supported by tissue culture studies on human bone marrow where monocytes and DC were identified within mixed colonies (Reid et al., 1990). Recently Inaba et al., (1993) described a common origin for murine phagocytes (granulocytes and MΦ) and DC, by using a GM-CSF semi-solid bioassay.
The DC also express some myeloid markers (CD13, CD33, CD14, CD32 and CD64) though some of them are down-regulated in culture. Altogether this suggests a common myeloid origin for monocytes and dendritic cells.

1.3 CLASSIFICATION AND HETEROGENEITY OF MACROPHAGES
Resident Mφ are widely distributed in all the body tissues in the absence of any inflammatory signal and display regional heterogeneity. The Mφ are heterogeneous in terms of localisation, morphology, phenotype and function (Raff et al., 1980) which may reflect the local environment of the Mφ and involvement in various physiological and pathological processes. Tissue Mφ are heterogeneous in terms of their phenotype and function not only between different anatomical sites but also within the Mφ population of a particular anatomical site (Holt et al., 1982; Gordon, 1993). This may be due to the cells in various stages of differentiation from a common Mφ lineage and/or subsets derived from Mφ precursors of distinct cell lines (Lehnert et al., 1986). Depending upon the maturity and function, tissue Mφ can be classified as resident (unstimulated), elicited (stimulated by inflammatory agents such as thioglycolate) or activated (have enhanced antimicrobial or cytocidal activity after exposure to lymphokines) (Gordon et al., 1986).

Dendritic cells and Mφ are mononuclear phagocytes with different functions and phenotype, nevertheless they are grouped together because they share some of the myeloid antigens (CD13, CD33 & CD14), complement receptors (CD11b & CD11c) and Fc receptors (CD32 & CD64) (Steinman, 1991; Romani et al., 1989; Nestle et al., 1993; Thomas et al., 1993). One of the distinguishing features of DC is high levels of MHC class II as well as MHC class I expression which is consistent with the strong antigen presenting function (Bujdoso et al., 1989; Steinman, 1991; Boog et al., 1988). DC in tissues (dermis & epidermis) or freshly isolated DC (from blood or by cannulation of afferent lymphatics) do express many of these antigens. However the expression of FcyR (CD32, CD64) is down regulated following extensive isolation procedures in humans and mice or when DC are cultured (Steinman, 1991; Romani et al., 1989; Nestle, 1993; Thomas et al., 1993).

Radzun and co-workers proposed to include DC within the MP system, but, subdivided the MP system into a phagocytosing compartment and an immune accessory compartment (Radzun et al., 1988). The cells of the immune accessory compartment, also known as antigen presenting cells, generally include follicular DC (FDC) as B cell antigen presenting cells, and interdigitating reticulum cells (IDC) in
lymphoid interfollicular or other T cell areas as well as Langerhans cells of the epidermis as T cell antigen presenting cells (Tew et al., 1982). The cells which are generally grouped as phagocytic Mφ, in lymphoid tissue include germinal centre tingible body Mφ, starry sky Mφ of the lymphoid follicles, interfollicular and lymph node sinus Mφ. In non lymphoid tissues these include bone marrow Mφ, bone osteoclasts, splenic red pulp Mφ, hepatic Kupffer cells, connective tissue histiocytes, pulmonary alveolar and interstitial Mφ, Mφ of gastrointestinal tract and interstitium of kidney, central nervous system microglial cells, as well as placental Hofbauer cells (Hofman et al., 1984; Franklin et al., 1986; Radzun et al., 1988; Gordon, 1993). Both the accessory and phagocytic Mφ belong to MP system and thus to a common lineage. Their location and specific functional requirement seems to be largely responsible for their morphologic and phenotypic characteristics.

1.4 MORPHOLOGY OF MONONUCLEAR PHAGOCYTES

The morphology and ultrastructure of MP varies considerably depending upon the species, tissue localisation, the stage of development, degree of activation and the procedures employed during their isolation and preparation for microscopy.

There are limitations of light microscopic observations of stained cells for precise study of cytology. Leishman's or Wright-Giemsa stained promonocytes, monocytes and Mφ do show obvious difference in size, in nuclear shape and nucleus to cytoplasmic ratio and in the amount of granularity of the cytoplasm, but little more can be seen using simple staining techniques. Under the light microscope, Mφ are large irregular shaped cells (25-50μm) with a ruffled surface, an eccentrically placed round or kidney shaped nucleus containing fine chromatin and one or two prominent nucleoli. The cytoplasm contains juxtanuclear Golgi complexes, fine and multiple large azurophilic granules, and cytoplasmic vacuoles at the periphery (Douglas and Hassan, 1990).

The monocytes in peripheral blood have characteristics similar to Mφ except for their smaller size (12-15μm diameter) and higher nuclear to cytoplasmic ratio. Promonocytes are slightly larger (12-18μm diameter) than monocytes and are characterised by the presence of several immature and mature azurophilic granules and deeply indented and irregularly shaped nuclei (Douglas and Hassan, 1990).

The electron microscopic method of observation reveals an abundance of ultrastructural features and allows high resolution comparison of the cells (Hirash and
Fedorko, 1969). It is not possible on ultra structural examination alone to recognise distinct subpopulations of monocytes and Mφ but ultrastructural cytochemical/immunochemical studies suggest such subpopulations may exist (Takeya et al., 1989).

1.5 CYTOCHEMISTRY OF MONONUCLEAR PHAGOCYTES

Until the development of mAb histochemistry/cytochemistry was considered as the most reliable technique for the identification of MP. Several hydrolytic enzymes are present in the cytoplasmic granules and plasma membrane of MP including acid phosphatase, β-glucuronidase, N-acetyl-glucosaminidase, lysozyme, α-naphthylbutyrate esterase and peroxidase. After phagocytosis, MP may also contain periodic acid-Schiff and Sudan black positive materials, haemosiderin and other substances.

Lysozyme was the first definitive cytochemical marker for the cells of MP system in paraffin sections (Taylor, 1978). Subsequently Mendelsohn et al., (1980) showed that positive staining for lysozyme is not a feature of all histiocyte populations. Cytochemical observations on the peroxidase content of the MP showed that promonocytes are strongly positive but this decreases with their maturation with resident Mφ being completely devoid of peroxidase (Andreesen et al., 1986). In contrast exudative Mφ showed presence of peroxidase granules (van Furth, 1986).

Non-specific esterase, an enzyme present on the external surface of the plasma membrane has been described as the most commonly used cytochemical marker for MP (Yam et al., 1971). This enzyme is more abundant on most tissue Mφ and is present at a comparatively low level in monocytes and dendritic cells. Traces of this enzyme are also observed on other leukocytes. Acid-phosphatase is another important cytochemical marker for MP. This enzyme is contained in primary lysosomes of MP and several isoenzymes exist in tissue Mφ (Dannberg and Suga, 1981). Attempts have been made to trace the monocytic origin of a particular Mφ population by comparison of the isoelectric focusing pattern of isoenzymes with those of blood monocytes (Radzun et al., 1983). However this has shown that the MP system represents a homogeneous and monocytic derived cell population irrespective of its localisation or features.

1.6 MONONUCLEAR PHAGOCYTE FUNCTIONS

MP provide defence against invasion of the host by microorganisms. This is achieved by phagocytosis, microbial destruction, antigen processing and presentation, antibody
dependent and spontaneous cellular cytotoxicity and the production of various cytokines and enzymes. The MP population or subpopulations exhibit heterogeneity in performing these functions (Bielefeldt-Ohman and Babiuk, 1986). The functions of MP can be dramatically changed by the interaction with products of sensitised T-lymphocytes. Interferon (IFN)-γ produced by sensitised T helper Th-1-lymphocytes can initiate enhanced competence of MΦ to destroy facultative and obligate intracellular parasites and tumour cells. IFN-γ primed MΦ are characterised by increased surface expression of MHC class II, LFA-1 molecules and increased capacity to release reactive oxygen and nitric oxide intermediates (Adams and Hamilton, 1987; Chan et al., 1992). IFN-γ primed MΦ, although not cytolytic become so when pulsed with bacterial lipopolysaccharide (LPS). These cytolytic MΦ, termed as activated MΦ, have the capacity to lyse tumour cells by the production of TNF-α and cytolytic proteases (Adams and Hamilton, 1987).

1.6.1 PHAGOCYTOSIS AND MICROBIICIDAL ACTIVITY

Phagocytosis Phagocytosis is one of the most primitive host defence systems retained by MP. Opsonisation of the microorganism increases the efficiency of phagocytosis. Opsonins are of various types but the most common are IgG and the fragments of the third component of complement. MP have a limited number of receptors which can directly bind to microorganisms, but by means of their Fc receptors they are able to utilise the enormous repertoire of the immunoglobulin recognition system (Bredius et al., 1993). For effective phagocytosis to take place in the tissues, transendothelial migration and rapid accumulation of MP at the sites of injury and infection is necessary as resident MΦ are poorly phagocytic. The molecular events involved in the migration of monocytes and other leukocytes in inflammation are described below in section 1.10.7.

MP internalise the opsonised microorganisms through an appropriate ligand-receptor interaction which starts the process of microbial destruction (Nathan et al., 1980). The ingestion of unopsonised microorganisms via mannose/fucose receptors (Speert et al., 1988) or β2 integrin receptors usually leads to infection (Ross and Vetvicka, 1993). Toxoplasma gondii ingested non-opsonically survives because it fails to trigger an oxidative burst, whereas when opsonised with IgG it is killed by reactive oxygen intermediates (Wilson et al., 1980). Similarly Listeria monocytogenes ingested via CR3 after opsonisation is killed, but non-opsonic CR3 independent ingestion leads to multiplication in mouse MΦ (Drevets et al., 1993). Phagocytosis through opsonic adherence to Fc receptors (FcRs) and complement receptors (C3Rs)
triggers an oxidative burst (Wright and Griffin, 1985), so the fate of the microorganism ingested by MP depends on the mode of entry into the phagocyte.

A number of microorganisms are able to survive within phagocytes by evading different antibacterial strategies. The bacterial organisms such as Chlamydia psittaci (Wyrick and Brownridge, 1978) and Mycobacterium tuberculosis (Armstrong and D'Arcy Hart, 1975) parasitise and multiply in Mφ by interfering in the formation of the phagolysosome. However such bacteria are killed by lysosomal enzymes when they are phagocytosed after opsonisation (Wyrick and Brownridge, 1978; Armstrong and D'Arcy Hart, 1975). In contrast Leishmania and Mycobacterium leprae survive within phagolysosomes. This is due to failure to trigger oxidative burst and resistance of the bacterial wall to the Mφ degradative enzymes (Nathan et al., 1980). Salmonella typhimurium survives in phagolysosomes due to their resistance to the effect of lysosomal enzymes and bactericidal effect of cationic proteins (Groisman and Saier, 1990)

**Microbicidal Mechanism** MP generate superoxide (O$_2^-$) and other reactive oxygen intermediates (ROI) such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH$^-$) following receptor ligand mediated phagocytosis (Adams and Hamilton, 1984). In Mφ the ROI interact, in the presence of iron, to produce toxic hydroxyl radicals (OH) and singlet oxygen. In monocytes the ROI, in the presence of myeloperoxidase, oxidise chloride to produce the hypochlorite anion (OCl$^-$) which is highly bactericidal. This sequence of biochemical events, associated with a sharp increase in oxygen uptake, is referred to as 'oxidative burst' or 'respiratory burst'. Together with lysosomal enzymes these oxidising products serve to kill micro-organisms within the phagosome and in extracellular environment (Pick and Keisari, 1981). Mature Mφ have a weak respiratory burst compared to monocytes (Klebanoff, 1988) and therefore have poor antimicrobial effect. Another consequence of receptor ligand mediated phagocytosis is the fusion of primary lysosome packed with a wide array of antimicrobial substances with the phagosomes (Ganz et al., 1986). Lysosomes contain a variety of digestive enzymes and cationic proteins which exert their microbicidal action mainly by their digestive action and permeabilisation of microbial cell wall respectively (Andrew et al., 1985; Lehrer et al., 1989). The activated Mφ have greater microbicidal capacity through increased respiratory burst and increased elaboration of lysosomal products and secretory activities (Nathan, 1987). The activation of murine Mφ also stimulates inducible nitric oxide synthetase (NOS) for
the production of reactive nitrogen intermediates which are able to kill Mycobacteria (Celada and Nathan, 1994; Chan et al., 1992).

### 1.6.2 CYTOTOXIC FUNCTION OF MONONUCLEAR PHAGOCYTES

The cytotoxic activity of monocytes and Mφ plays a significant role in resistance to infection, tumour rejection and autoimmune reactions. Antibody-dependent cellular cytotoxicity (ADCC) is one of the mechanisms whereby MP readily lyse antibody coated target cells (Adams and Hamilton, 1984). ADCC is mediated by the binding of Fc portion of Ig to Mφ surface receptors. This triggers secretion of lytic mediators especially hydrogen peroxide and cytokines.

Another cytotoxic mechanism exclusive for tumour cell destruction is Mφ mediated tumour cytotoxicity (MTC) (Adams and Hamilton, 1988). The MTC is contact dependent, non-phagocytic and antibody independent process. The Mφ bound to neoplastic cell causes its lysis slowly over a period of 1 to 3 days by the secretion of toxic substances such as TNF-α, serine protease and reactive oxygen intermediates.

### 1.6.3 CYTOKINE PRODUCTION

MP produce a number of cytokines in response to various infectious agents and inflammatory products. MP produce TNF-α, IL-1, IL-6 IL-8, IL-10 and IL-12 in response to LPS and other microbial products and also cytokines (Dentener et al., 1993; Del Prette et al., 1994). The cytokines produced by MP regulate immune response of T and B lymphocytes in the presence of antigen. They are also responsible for effector and regulatory responses of MP as well as the production of leukocytes from bone marrow during microbial infections. Monocytes and Mφ are the main source of TNF-α which is involved in the necrosis of tumour cells, endotoxic shock, and acute phase protein production like IL-1 and IL-6 (Debets et al., 1989; Fletcher Starnes et al., 1990). TNF-α stimulates MP and other cell types to produce cytokines such as IL-1, GM-CSF, IL-6, TNF-α itself and IL-8 (Vassalli, 1992). TNF-α and IL-1 increases the expression and de novo synthesis of adhesion molecules on endothelium (ICAM-1 & E-selectin) (Nicod, 1993) TNF-α has a central role in the induction of inducible NOS (Chan et al., 1992).

Mφ are known to produce IFN-α in response to viruses, bacteria, tumours and foreign cells (Pestka et al., 1987). IFN-α, in addition to its antiviral and antimitotic effect, upregulates MHC class I expression and increases NK cell activity (Pestka et al., 1987). MP together with other cells produce IL-1 and IL-6 which have
costimulatory properties for T cells (Dinarello, 1992). IL-1 is required for the augmentation of immune responses by the Th-2 T cell subset and B cells, reactivity of NK cells and is essential for the activation of T cells depending on the particular T cell population in the presence of antigen (Steinman, 1991). IL-1 has its role in chemotaxis of monocytes, neutrophils and lymphocytes (Dinarello, 1992) and enhances the accessory function of dendritic cells (Koide et al., 1987). IL-1 acts on MP and endothelial cells to increase further synthesis of IL-1 and induce synthesis of IL-6 and IL-8 (Nicod, 1993). MP are the main source of IL-6 which induce the proliferation of T cells as well as expression of IL-2 receptor on these cells (Matsuda et al., 1989), it also acts on B lymphocytes, inducing immunoglobulin (Ig) secretion and regulating B cell growth.

IL-8 expression in monocytes is regulated by LPS, TNF-α, IL-1, IFN-γ and other inflammatory agents. IL-8 stimulates the chemotaxis of both neutrophils and lymphocytes and inhibits IFN-γ release by NK cells in vitro (Lewis et al., 1991). The MP also produce M-CSF, GM-CSF and G-CSF following LPS or IL-1 stimulation (Metcalf, 1987). GM-CSF and M-CSF induce formation of myeloid precursor colonies and also induce production of IL-1, IFN-γ and TNF-α (Clark, 1993). Transforming growth factor-β (TGF-β) is also produced by tissue Mφ and bone marrow derived Mφ along with other cells on activation (Assoian et al., 1987; Adler et al., 1994). TGF-β inhibits all the effects of IL-2 and has a role in fibrosis and wound healing (Chieftez et al., 1987). TGF-β is chemotactic for monocytes and stimulate IL-1 production by monocytes (Hamilton, 1993).

The production of IL-10 and IL-12 by MP plays major role in modulation of Th immune responses (Del Prette et al., 1994). In mice, IL-10 acts as a selective inhibitor of cytokines production by Th-1 cells but stimulates Th-2 activity. In contrast human IL-10 inhibits the proliferation and cytokine production by both Th-1 and Th-2 clones. IL-12 induces Th0 cells to preferentially differentiate into Th-1 phenotype, while neutralisation of IL-12 activity favoured Th-2 differentiation. In general, Th-1 cells expand in response to bacterial and viral antigens and are involved in the induction of delayed type of hypersensitivity. In contrast Th-2 cells expand in response to allergens and parasite antigens and provide excellent help for Ig production (Del Prette et al., 1994)
1.6.4 ANTIGEN PROCESSING AND PRESENTATION

Intracellular and extracellular antigens are generally processed by different pathways for MHC class I and MHC class II restricted presentation respectively. Antigens produced in the cell cytoplasm, such as virus peptides are usually presented to CD8+ T lymphocytes in association with class I MHC molecules which are expressed on most cell types. The expression of MHC class II, required for the presentation of exogenous antigen to CD4+ T lymphocytes, is restricted to a few cell types. MP, expressing both MHC products, can therefore present both intracellular and extracellular antigens. Amongst the MP, dendritic cells express higher level of MHC class II than classical phagocytic Mφ and act as potent antigen presenting cells in both primary and secondary immune responses. An increased level of antigen presentation molecules is also seen on phagocytic Mφ under the influence of inflammatory cytokines.

**Processing for MHC class I restricted antigen presentation** The primary requirement for MHC class I restricted presentation to CD8+ T lymphocytes is the cytoplasmic production of the antigen. Peptides are produced from the antigen by a non-lysosomal proteolytic mechanisms in the cytoplasm. These peptides are then delivered to endoplasmic reticulum by transporter proteins for association with newly synthesised MHC class I molecules. The class I molecule consists of a glycosylated heavy chain with three extracellular domains, non-covalently associated with β2 microglobulin (Bjorkman et al., 1987). The antigen binding groove is formed by the association of the first and second domains of the heavy chain, while the third domain near the membrane interacts with CD8 (Germain and Margulies, 1993). The antigen binding groove of the class I molecule accommodates a peptide of about nine amino acids (Schumacher et al., 1991). Class I molecules with bound peptide are exported to the cell surface via the Golgi. MP, by expressing the antigen with class I molecule, may be targeted by class I-restricted cytotoxic (CD8) T-lymphocyte and destroyed.

**Processing for MHC class II restricted antigen presentation** After internalisation via FcRs or C3Rs, exogenous antigen enters the endocytic vesicle. The endocytic vesicles fuse with lysosomes and the antigen is then cleaved by protease action to small peptides. MHC class II molecules are assembled in endoplasmic reticulum (ER). The class II molecule consists of a non-covalently associated α and β chain, each with two extracellular domains. The invariant chain associates with the α and β chains before export of the class II molecule from ER to endocytic vesicles. This transfer takes place via the Golgi network. It has been proposed that the invariant
chain directs class II molecules to the endocytic route and also prevents the binding of self peptides before MHC class II molecules encounter exogenous peptides in endocytic vesicles (Cresswell, 1994). The class II molecules encounter exogenous peptide in endocytic vesicle and then invariant chain is degraded (Germain and Margulies, 1993) to allow peptide binding to the class II molecule. The size of the peptides eluted from MHC class II molecules have been shown to vary from 12 to more than 20 amino acids in length (Rudensky et al., 1991; Chicz et al., 1992). After peptide binding MHC class II molecules are transported to the cell surface via the Golgi to allow interaction with antigen specific CD4 T lymphocytes.

1.7 MONONUCLEAR PHAGOCYTE SURFACE ANTIGENS
A large number of cell surface molecules present on MP have been identified and characterised by the production of monoclonal antibodies. MP interact with their environment by means of cell surface receptor-ligand interactions. These interactions determine the control of various activities of MP, such as growth, differentiation, activation, recognition, endocytosis, phagocytosis, migration and secretion. The expression of these antigens is controlled by genetic programming and changes during differentiation and activation in response to the extracellular environment. Many of these antigens are predominantly expressed by MP (Table 1.1) while others are shared with many other cell types (Table 1.2). Both categories include recognition or adhesion molecules important in cellular interactions, growth factor receptors and enzymes or receptors for specific ligands important in the role of the MP. The recognised clusters of differentiation antigens present on human MP are listed in Tables 1.1 and 1.2 along with their cellular distributions, molecular weight and assigned function (Knapp et al., 1989; Schlossman et al., 1994). Some of the molecules (CD14, CD16, CD32 and CD11/CD18) are related with the work done in this thesis and are described in detail in subsequent sections.

1.8 CD14
CD14 is a myeloid differentiation antigen expressed primarily on the cell surface of monocytes and Mφ. The CD14 is anchored to the cell surface via a glycosyl phosphatidyl inositol (GPI) linkage (Goyert et al., 1989). The antigen also exists in various soluble forms (Haziot et al., 1988; Durieux et al., 1994). Both the membrane and soluble forms of the antigen bind lipopolysaccharide (LPS) complexed to LPS binding protein (LBP), an acute phase protein present in serum. The binding of LPS-LBP complex to CD14 results in the release of various inflammatory cytokines including TNF-α, leading to the induction of endotoxic shock (Ulevitch, 1993).
TABLE 1.1 Recognised cluster of differentiation antigens predominantly expressed by cells of mononuclear phagocyte series.

<table>
<thead>
<tr>
<th>Cluster Designation</th>
<th>Main Cellular Reactivity</th>
<th>Molecule Weight (kD)</th>
<th>Known or Proposed Function (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
<td>Leukocytes</td>
<td>180</td>
<td>Adhesion; bind to ICAM-1, 2 &amp; 3</td>
</tr>
<tr>
<td>CD11b</td>
<td>G, M, NK</td>
<td>170</td>
<td>Adhesion; receptor for iC3b (CR3)</td>
</tr>
<tr>
<td>CD11c</td>
<td>G, M, NK, B-cell subset</td>
<td>150</td>
<td>Adhesion ? receptor for iC3b (CR4)</td>
</tr>
<tr>
<td>CDw12</td>
<td>M, G</td>
<td>90-120</td>
<td>Phagocytosis and NK cytotoxicity.</td>
</tr>
<tr>
<td>CD13</td>
<td>M, G, M, G, LC</td>
<td>150</td>
<td>Aminopeptidase-N activity</td>
</tr>
<tr>
<td>CD14</td>
<td>M, G, M, G, LC</td>
<td>55</td>
<td>LPS receptor</td>
</tr>
<tr>
<td>CD15</td>
<td>G, M, (GPI-linked)</td>
<td></td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CD16</td>
<td>M, NK, G, G (GPI-linked)</td>
<td>50-65</td>
<td>Low affinity Fc receptor for aggregated IgG (FcγR III) ADCC, phagocytosis, activation of NK cells</td>
</tr>
<tr>
<td>CDw17</td>
<td>G, M, Plat (GPI-linked)</td>
<td></td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CD18</td>
<td>Leukocytes</td>
<td>95</td>
<td>β2 chain of CD11a, 11b, 11c/CD18</td>
</tr>
<tr>
<td>CD31</td>
<td>M, G, B-cell, Endo, Plat</td>
<td>140</td>
<td>Hyaluronate receptor for aggregated IgG (FcγRII), phagocytosis, ADCC</td>
</tr>
<tr>
<td>CD32</td>
<td>M, G, B-cell, M, Endo, Plat</td>
<td>42</td>
<td>Low affinity Fc receptor for aggregated IgG (FcγRII), phagocytosis, ADCC</td>
</tr>
<tr>
<td>CD33</td>
<td>M, Prog, MLC</td>
<td>90</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>CD35</td>
<td>M, G, B-cell, Endo, Plat</td>
<td>160, 190, 220, 250</td>
<td>Platelet adhesion Receptor for thrombospondin and collagen</td>
</tr>
<tr>
<td>CD36</td>
<td>M, M, Plat, Endo</td>
<td>75</td>
<td>High affinity receptor for IgG (FcγRI) ADCC, Phagocytosis</td>
</tr>
<tr>
<td>CD65</td>
<td>M, G</td>
<td>180-200</td>
<td>?</td>
</tr>
<tr>
<td>CD68</td>
<td>M, M, G (intracellular)</td>
<td>110</td>
<td>?</td>
</tr>
<tr>
<td>CD74</td>
<td>M, M, B-cell</td>
<td>33, 35, 41</td>
<td>Associate with newly synthesised class II MHC molecule (invariant chain)</td>
</tr>
<tr>
<td>CD87</td>
<td>G, M, M, Activated T-cells</td>
<td>50-65</td>
<td>Urokinase Plasminogen activator receptor</td>
</tr>
<tr>
<td>CD88</td>
<td>M, Neutrophils, Mast cells, smooth muscle cells</td>
<td>42</td>
<td>Receptor for C5a</td>
</tr>
<tr>
<td>CD89</td>
<td>M, M, Neutrophil, T-cell and B-cell subset</td>
<td>50-70</td>
<td>Fc receptor for IgA</td>
</tr>
<tr>
<td>CD91</td>
<td>M and some non-haemopoietic cell lines</td>
<td>600</td>
<td>Receptor for α2 macroglobulin</td>
</tr>
<tr>
<td>CDw92</td>
<td>M, Neutrophils, Endo, Plat</td>
<td>70</td>
<td>?</td>
</tr>
<tr>
<td>CD93</td>
<td>M, Neutrophils, Endo</td>
<td>70</td>
<td>?</td>
</tr>
<tr>
<td>CDw101</td>
<td>M, M, G</td>
<td>140</td>
<td>?</td>
</tr>
<tr>
<td>CD115</td>
<td>M, M, Placenta</td>
<td>150</td>
<td>Receptor for M-CSF</td>
</tr>
<tr>
<td>CDw116</td>
<td>M, Neutrophils, Eosinophils, Fibroblasts, Endo</td>
<td>75-85</td>
<td>Receptor for GM-CSF</td>
</tr>
</tbody>
</table>

G, granulocytes; M, monocytes; NK, natural killer cells; B-cell, B lymphocytes; MΦ, macrophages; LC, Langerhans cells; Plat, platelets; Endo, endothelial cells; Prog, progenitor cells; MLC, myeloid leukaemic cells; RBC, red blood cells; T-cells, T lymphocytes. (Compiled from Knapp et al., 1989; Schlossman et al., 1994)
TABLE 1.2 Recognised cluster of differentiation antigens shared by cells of mononuclear phagocytes with other cell types.

<table>
<thead>
<tr>
<th>Cluster Designation</th>
<th>Main Cellular Reactivity</th>
<th>Molecular Weight (kD)</th>
<th>Known or Proposed Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>T-helper cells, M, Mφ</td>
<td>56</td>
<td>Adhesion; receptor for MHC class II</td>
</tr>
<tr>
<td>CD9</td>
<td>Pre B-cells, M, Plat</td>
<td>24</td>
<td>Role in platelet activation</td>
</tr>
<tr>
<td>CD21</td>
<td>Mature B-cells, FDC</td>
<td>145</td>
<td>Receptor for C3d (CR2), role in B-cell activation</td>
</tr>
<tr>
<td>CD23</td>
<td>B-cell subset, activated M, Eosinophils</td>
<td>45-50</td>
<td>Low affinity Fc receptor for IgE (FceRII)</td>
</tr>
<tr>
<td>CD25</td>
<td>Activated B and T-cells and activated Mφ</td>
<td>55</td>
<td>Receptor for α chain of IL-2, T-cell growth factor</td>
</tr>
<tr>
<td>CD29</td>
<td>All cells (not RBC)</td>
<td>130</td>
<td>Common integrin β1 chain</td>
</tr>
<tr>
<td>CD43</td>
<td>Leukocytes (not peripheral B-cells)</td>
<td>95</td>
<td>Activation of T &amp; B cells, NK cells and monocytes</td>
</tr>
<tr>
<td>CD44</td>
<td>Leukocytes, RBC, Plat, Brain cells</td>
<td>80-95</td>
<td>Homing receptor for matrix components</td>
</tr>
<tr>
<td>CD45</td>
<td>Pan Leukocyte</td>
<td>180,190, 205, 220</td>
<td>Signal transduction (Tyrosine Phosphate)</td>
</tr>
<tr>
<td>CD46</td>
<td>Leukocytes, epithelial cells Fibroblasts</td>
<td>45-70</td>
<td>Regulation of complement activation</td>
</tr>
<tr>
<td>CD47</td>
<td>Broad distribution</td>
<td>47-52</td>
<td>?</td>
</tr>
<tr>
<td>CD48</td>
<td>Leukocytes</td>
<td>41 (PI-linked)</td>
<td>?</td>
</tr>
<tr>
<td>CD49b</td>
<td>Activated T-cells, M</td>
<td>210</td>
<td>α-1 integrin; adhesion to collagen, laminin</td>
</tr>
<tr>
<td>CD49e</td>
<td>B-cells, M, Plat</td>
<td>160</td>
<td>α-2 integrin; receptor for collagen</td>
</tr>
<tr>
<td>CD49f</td>
<td>Memory T-cells, M, Plat</td>
<td>135/25</td>
<td>α-5 integrin; adhesion to fibronectin</td>
</tr>
<tr>
<td>CD50</td>
<td>Leukocytes</td>
<td>124</td>
<td>α-6 integrin; receptor for Laminin</td>
</tr>
<tr>
<td>CD52</td>
<td>Leukocytes</td>
<td>21-28</td>
<td>Adhesion molecule for CD11a (ICAM3)</td>
</tr>
<tr>
<td>CD53</td>
<td>Leukocytes</td>
<td>32-40</td>
<td>?</td>
</tr>
<tr>
<td>CD54</td>
<td>Endo, many activated cell types</td>
<td>90</td>
<td>Adhesion molecule for CD11a &amp; CD11b (ICAM-1)</td>
</tr>
<tr>
<td>CD55</td>
<td>Broad distribution</td>
<td>70 (PI-linked)</td>
<td>Regulation of complement activation</td>
</tr>
<tr>
<td>CD58</td>
<td>Broad distribution</td>
<td>65-70 (PI-linked)</td>
<td>LFA-3; adhesion ligand for CD2</td>
</tr>
<tr>
<td>CD59</td>
<td>Broad distribution</td>
<td>18-20 (PI-linked)</td>
<td>Regulation of complement action (Lysis)</td>
</tr>
<tr>
<td>CD62 L</td>
<td>B and T-cells, M, NK</td>
<td>150</td>
<td>Adhesion to endothelium</td>
</tr>
<tr>
<td>CD63</td>
<td>M, Mφ, activated Plat</td>
<td>53</td>
<td>?</td>
</tr>
<tr>
<td>CD71</td>
<td>Activated T and B-cells, Mφ proliferating cells</td>
<td>95</td>
<td>Receptor for transferrin</td>
</tr>
<tr>
<td>CD102</td>
<td>Resting lymphocytes, M, Endo</td>
<td>60</td>
<td>Adhesion molecule for CD11a (ICAM2)</td>
</tr>
<tr>
<td>CD117</td>
<td>Progenitor cells</td>
<td>145</td>
<td>Receptor for stem cell factor</td>
</tr>
<tr>
<td>CD119</td>
<td>Mφ, M, B-cells, epithelial cells</td>
<td>90</td>
<td>Receptor for IFN-γ</td>
</tr>
<tr>
<td>CD120a</td>
<td>Most cell types, high on epithelial cells</td>
<td>55</td>
<td>Receptor for TNF</td>
</tr>
<tr>
<td>CD120b</td>
<td>Most cell types, high on myeloid cells</td>
<td>75</td>
<td>Receptor for TNF</td>
</tr>
<tr>
<td>CD121b</td>
<td>B-cells, Mφ, M</td>
<td>68</td>
<td>Type II receptor for IL-1</td>
</tr>
</tbody>
</table>

G, granulocytes; M, monocytes; NK, natural killer cells; B-cell, B lymphocytes; Mφ, macrophages; LC, Langerhans cells; Plat, platelets; Endo, endothelial cells; Prog, progenitor cells; MLC, myeloid leukaemic cells; RBC, red blood cells; T-cells, T lymphocytes; FDC, follicular dendritic cells.
(Compiled from Knapp et al., 1989; Schlossman et al., 1994)
1.8.1 STRUCTURAL FEATURES OF CD14

Human CD14 (HCD14) is a single chain glycoprotein of $M_r = 55,000$. About 20% of its molecular weight is contributed by N-linked carbohydrates (Goyert et al., 1986). The CD14 molecule is composed of 356 amino acids encoded by a gene at q23-q31 bands on the long arm of chromosome 5 in humans. The gene encoding HCD14 is clustered along with the genes encoding several myeloid-specific growth factors and growth factor receptors such as IL-3, GM-CSF, and M-CSF and platelet derived growth factor receptor, $\beta_2$-adrenergic receptor and endothelial cell growth factor receptor (Goyert et al., 1988, Ferrero et al., 1990). The HCD14 protein sequence has features characteristic of a cell surface glycoprotein, has five potential N-glycosylation sites and contains repeated leucine-rich motifs (Goyert et al., 1988, Ferrero et al., 1990; Ferrero and Goyert, 1989). The mouse CD14 (MCD14) and HCD14 genes were found to be highly conserved in their intron-exon organisation and nucleotide sequences. The deduced protein sequence of MCD14 gene shared 66% identity to HCD14. The MCD14 molecule is composed of 366 amino acids and shows high conservation of the leucine rich motifs (Ferrero et al., 1990). Both human and MCD14 lack a characteristic transmembrane region and were later found to be anchored to the cell membrane via GPI linkage (Haziot et al., 1988; Simmons et al., 1989).

HCD14 also exists in two soluble forms, of $M_r = 53,000$ present in urine and of $M_r = 48,000$ present in the serum and culture supernatants of CD14+ cells. These soluble forms are a result of GPI specific enzyme cleavage and the lack of a GPI anchor respectively (Haziot et al., 1988; Bazil et al., 1986). Recently another soluble form of $M_r = 56,000$, higher than that of membrane form of the molecule has been reported from human monocyte-like cell line (Labeta et al., 1993a) and normal human monocytes (Durieux et al., 1994). It has been suggested that this soluble form is directly secreted by the cell after further processing (Durieux et al., 1994). The existence of several soluble forms of CD14 may help to reveal additional functions of these soluble forms in sepsis and other pathological conditions.

1.8.2 DISTRIBUTION OF CD14

Although HCD14 is expressed primarily on monocytes and resident $\text{M}\Phi$, it is also present on granulocytes, IDC and Langerhans cells (Griffin et al., 1981; Goyert et al., 1989). The molecule is absent from stem cells and myeloid cells at an early differentiation stage (Goyert et al., 1988; Gadd, 1989). The expression of MCD14 is also limited to the myeloid lineage as revealed by the reactivity of anti-MCD14
peptide antiserum to mouse monocytes (Ziegler-Heitbrock and Ulevitch, 1993). Recently an anti-MCD14 mAb showed distribution and function of MCD14 similar to HCD14 (Matsura, 1993).

At least three distinct epitopes of HCD14 have been described by epitope mapping studies using competitive binding between $^{125}$I labelled and unlabelled anti-CD14 mAb (Gadd, 1989; Goyert et al., 1989). One of the epitopes is not expressed on Langerhans cells while the other two have normal distribution. This difference in expression, points towards the existence of two different processed forms of the molecule but the evidence for this is lacking. Some of the anti-HCD14 mAb have been found to cross-react with monocytes in primates and pigs (Ziegler-Heitbrock and Ulevitch, 1993) suggesting conservation of some epitopes across species.

A low level of CD14 expression was also seen on non-myeloid cells such as leukemic and normal B-cells, mammary gland cells (Calvo et al., 1987; Ziegler-Heitbrock and Ulevitch, 1993), endothelial cells and kidney tubules (Koller, 1989). The absence of CD14 mRNA in lymphocytes (Goyert et al., 1988) and lack of CD14 expression in any B-cell lines suggest the possibility of cell surface uptake of CD14 on B-cells. The cross reactivity with non myeloid cells may be due to the detection of sCD14 bound to CD14$^+$ cells during LPS activation (Pugin et al., 1993).

HCD14 expression, both on the cell surface and at the mRNA level is negative in granulocyte macrophage colony forming unit (GM-CFU) and cell lines representing promonocytes (Hogg and Horton, 1987). Differential expression was observed in tissue $^{M\phi}$, where peritoneal $^{M\phi}$ stained strongly for CD14 while AM showed weak labelling (Ziegler-Heitbrock and Ulevitch, 1993). Thus CD14 is expressed on mature monocytes and is retained on $^{M\phi}$, with the level of expression dependent on the type of stimulus or tissue of origin.

Using immunohistochemical staining techniques, CD14 has been shown to be expressed by various mononuclear phagocytes (Todd et al., 1981; Hogg et al., 1984; Dimitriu Bona et al., 1983). These include spleen $^{M\phi}$, Kupffer's cells, lymph node sinus $^{M\phi}$, FDC and IDC, thymus $^{M\phi}$ and IDC, Langerhans cells, microglial cells and $^{M\phi}$, epitheloid cells and giant cells in granulomas (Koller, 1989; Franklin et al., 1986; Hancock et al., 1983)
1.8.3 REGULATION OF CD14 EXPRESSION

CD14 can be induced on immature myelomonocytic cells/cell lines and its expression can be modulated on mature cells by cytokines and mitogens stimulation. CD14 is induced on the immature myeloid cells such as HL60 and U937 cell line by IFN-γ, phorbol esters and Vitamin D3 (Rigby et al., 1984; Rigby et al., 1985). This is due to the induction of cell maturation, de novo synthesis of protein and cell surface expression as well as release of sCD14 into culture supernatants. In contrast, mature monocytes or monocyte cell lines showed a decrease in CD14 expression on phorbol esters and IFN-γ stimulation (Wright et al., 1986; Bazil and Strominger, 1991). The phorbol esters stimulated decrease in CD14 expression on blood monocytes is induced by the rapid shedding of the cell surface protein (Bazil and Strominger, 1991). In contrast IFN-γ and IL-4 induced decrease is caused by a direct effect on the synthesis and processing (Lauener et al., 1990).

The synthesis of CD14 and production of TNF-α, IL-1 and IL-6 is induced by LPS stimulation (Marchant et al., 1992). In contrast, Bazil and Strominger (1991), and Wright (1991) reported an opposite effect of LPS treatment and showed a decreased CD14 expression due to loss of surface protein suggesting its role in leukocyte desensitisation to the effect of LPS. The desensitising effects of LPS on repeated stimulation in inducing CD14 specific tolerance have been described in a recent study on a human monocyte cell line (Labeta et al., 1993b). A role for the cytokines, IL-4, IFN-γ, TNF-α is suggested in the decrease or increase in the expression of CD14 on tissue Mφ in the respective microenvironment of the lung (low CD14 on AM) and peritoneal cavity (high CD14 on peritoneal Mφ) (Ziegler-Heitbrock and Ulevitch, 1993).

1.8.4 FUNCTIONS OF CD14

Until the recent discovery that CD14 can function as a receptor for LPS-LBP complex (Wright et al., 1990), it was solely defined as a myeloid differentiation antigen. LBP is a glycoprotein of $M_r = 60,000$, synthesised in liver and is present in normal serum at very low levels ($<0.5\mu\text{gml}^{-1}$ in rabbit and $1-10\mu\text{gml}^{-1}$ in human). After an acute-phase response the LBP concentration can rise to $50\mu\text{gml}^{-1}$ in rabbit and $>300\mu\text{gml}^{-1}$ in human. LBP shows high affinity binding to the lipid A moiety of LPS and opsonises LPS-bearing particles such as intact gram-negative bacteria or LPS coated erythrocytes so mediating their adherence to MP (Schumann et al., 1990; Wright et al., 1990). The membrane receptor for LPS-LBP complexes was found to
be restricted to MP, and was distinct from receptors for other known opsonins such as Ig and complement (Wright et al., 1989b).

Binding of LPS-LBP complexes to monocytes and Mφ induces secretion of TNF-α as well as IL-1, IL-6 and IL-8 (Dentener et al., 1993; Ulevitch, 1993). Inhibition of the LPS-LBP binding by some anti-CD14 mAb also inhibits production of cytokines (Wright et al., 1990; Couturier et al., 1991). Thus a particular site on CD14 is responsible for the binding of LPS.

Some of these studies have shown that there is both LBP dependent and independent binding of LPS to CD14 and that both could induce release of the cytokines (Dentener et al., 1993; Ulevitch, 1993) which were blocked by anti-CD14 mAb. These authors suggested a role for LBP in potentiating the release of TNF-α, IL-1, IL-6 and IL-8 on LPS stimulation of MP. LBP also reduced the threshold stimulatory concentration of LPS by 1000-fold and accelerated the rate of cytokine release by more rapid induction and stabilisation of cytokine mRNA, thereby contributing to enhanced cytokine production (Ulevitch, 1993). Hailman et al., (1994) demonstrated that recombinant LBP did not form detectable ternary complex with recombinant sCD14 and LPS, but instead accelerated their binding so providing the direct evidence for a catalytic role for LBP as a lipid transfer protein in the binding of LPS to soluble and cell surface CD14.

The role of sCD14 in serum in LPS-induced activation of endothelial cells (EC) has been demonstrated by direct binding of LPS to recombinant sCD14, however at low concentrations of LPS this binding was enhanced by LBP (Pugin et al., 1993). Thus suggesting the role of sCD14 as a co-ligand with LPS in the EC-LPS response while the membrane form of CD14 functions as a receptor. The above findings indicate that LPS can directly activate EC by binding through sCD14 however the receptor for LPS-sCD14 complexes is unidentified. The indirect activation of EC is by the action of cytokines such as TNF-α and IL-1 released by binding of LPS to membrane CD14. In a recent study recombinant sCD14 has been shown to bind LPS directly, thus inhibiting LPS induced TNF-α production by monocytes/Mφ (Haziot et al., 1994). This suggests that sCD14 might have a role in preventing endotoxic shock.

These studies have clearly demonstrated the role of membrane CD14 and sCD14 as receptor and co-ligand for LPS in the activation of CD14+ and CD14− cells respectively. Thus CD14 has a role not only in the removal of gram-negative bacteria
but it also increases the sensitivity of immune system by promoting secretory responses of cells to LPS.

CD14 has also been implicated in monocyte function of cytokine activated EC adhesion (Beekhuizen et al., 1991), production of inflammatory mediators (Surette et al., 1993) and intracellular signalling via activation of protein kinases (Lauener et al., 1990).

1.8.5 SIGNAL TRANSDUCTION VIA CD14
It is not clear how the CD14 molecule, as a GPI anchored receptor, transmits a signal that causes such a profound activation. Almost all GPI anchored antigens, including CD14, exist in clusters as membrane sub-domains (Brown and Rose 1992). These sub-domains are enriched with surface glycolipids and cytoplasmic signal transducing molecules such as protein tyrosine kinases (PTK) (Thomas and Somelson, 1992). Several proteins, including those in the membrane sub-domains shows rapid protein tyrosine phosphorylation after Mφ activation by LPS (Weinstein et al., 1992; Ulevitch and Tobias, 1994). Anti-CD14 mAb induced homotypic aggregation of monocytes which was blocked by sphingosine, an inhibitor of protein kinases (Lauener et al., 1990). LPS induced protein tyrosine phosphorylation of a novel protein in a HCD14 cell line was inhibited by anti-CD14 mAb or tyrosine kinase inhibitor herbimycin A (Han et al., 1993). All these findings suggest that signalling by CD14 might occur via protein tyrosine phosphorylation mediated by a closely associated cytoplasmic PTK.

1.9 FC RECEPTORS FOR IgG
The expression of Fc receptors for IgG (FcγR) by MP enables them to phagocytose a variety of antigens in the presence of specific antibody thus enhancing the range of antigens ingested by phagocytes. The FcγR on MP are also responsible for mediating ADCC and stimulating the release of inflammatory and cytotoxic mediators. The MP taking up antigen via FcγR also show enhanced microbicidal activity, antigen processing and presentation.

1.9.1 STRUCTURAL FEATURES OF FcγR
There are three distinct but closely related classes of FcγR in humans and mice; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). FcγRI is a high affinity receptor for monomeric IgG and is saturated with immunoglobulin (Ig) under normal physiological conditions, while FcγRII and FcγRIII are low affinity receptors which bind complexed IgG only (Ravetch and Kinet, 1991). All are members of the immunoglobulin super
family (William and Barclay, 1988) with transmembrane and cytoplasmic regions and a variable number of extracellular domains. FcγRI has three extracellular domains while FcγRII and FcγRIII have only two (Fig. 1.1 ). The first two domains of FcγRI are structurally and functionally related to those of the low affinity receptors. It is thought that the third domain of FcγRI confers its unique affinity for monomeric IgG (Ravetch and Kinet, 1991). The binding of FcγRII with its ligands has been shown to depend on the second extracellular domain (ED2) and more specifically the binding area is identified as residues 146 to 169 of FcγRII (Hulett et al., 1991).

Soluble forms have been demonstrated in human sera for all three classes of FcγR. These molecules lack transmembrane regions and may be released from the cell surface by protease activity (Huizinga et al., 1990), or alternatively FcγR may be synthesised without the necessary transmembrane region and subsequently exported from the cell (Tartour et al., 1993). A role in binding soluble IgG has been suggested for these molecules (van de Winkel and Capel, 1993).

1.9.2 CELLULAR DISTRIBUTION OF FcγR

FcγRI The FcγRI is constitutively expressed by monocytes and Mφ and is inducible on neutrophils and eosinophils (van de Winkel and Capel, 1993). Three highly homologous genes have been identified in humans encoding a receptor of $M_r = 72,000$ (van de Winkel and Anderson, 1991). A single gene has been described in mice encoding a homologous receptor of $M_r = 67,000$ (Sears et al., 1990). Recently, Symons and Clarkson (1992) have cloned and sequenced a FcγRI gene from cattle. Amino acid sequences are conserved between these species, with 58% identity between the human, mice and cattle FcγRI extracellular regions. Association of a γ-γ homodimer with FcγRI on monocytes in human and a promonocyte cell line, has been identified, although it is not required for expression of the receptor (van de Winkel and Capel, 1993; Masuda and Ross, 1993).

FcγRII The FcγRII is expressed by a wide variety of haemopoietic cells and is frequently expressed as the sole one on many cells. It is found on monocytes, Mφ, granulocytes, B-cells, platelets, Langerhans cells and DC but not on NK cells (Thomas et al., 1993). In humans three genes (FcγRIIA, IIB and IIC) have been identified encoding six isoforms of a receptor of $M_r = 40,000$ (Qiu et al., 1990). The different isoforms of the receptor are almost identical in their extracellular sequences but differ in transmembrane and cytoplasmic regions. The products of two genes, FcγRIIA and IIC are expressed on monocytes, neutrophils and Mφ whereas the
FIGURE 1.1 Schematic representation of human Fcγ receptors (FcγRs) showing FcγRI with three extracellular domains and FcγRII and FcγRIII with two extracellular domains. FcγRI may have an associated γ-γ subunit, as shown; however it can also be expressed in the absence of this dimer. FcγRIIIa may have γ-γ, or a ζ-ζ or a γ-ζ dimer associated with it, while the GPI-linked form, FcγRIIIb, has no associated dimers. Mouse FcγRs have a similar structure except for FcγRIII, which exists only as trans-membrane receptor.
product of the third gene (FcγRIIB) is preferentially expressed on B-lymphocytes in addition to its presence on monocytes and Mϕ (Ravetch and Kinet, 1991).

Mouse FcγRII is of Mr = 40,000-60,000 and shows high homology with its human counterpart. In the mouse a single gene encodes two isoforms of FcγRII (b1 and b2) which differ in the length of their cytoplasmic domain (Qiu et al., 1990). FcγRIIb1 is expressed on most haemopoietic cells NK cells whereas FcγRIIb2 is exclusive to Mϕ. Both isoforms have been recognised by the same anti-FcγRII mAb, however their heterogeneity has been demonstrated at the molecular level by the expression of only Mϕ isoform of the receptor (FcγRIIb2) on a mutant cell line (Weinshank et al., 1988). The expression of the Mϕ isoform of the receptor was upregulated on IFN-γ treatment and the cells became competent in the phagocytosis of Ab coated particles. This suggests a role of this isoform in mediating Ab dependent phagocytosis by Mϕ.

Recently Zhang et al., (1994) reported the full length sequence of gene encoding bovine FcγRII. The amino acids sequence of the bovine molecule showed 59% and 55% identity with human and mouse FcγRII respectively. An identity of 47% was observed when the sequences of bovine FcγRI and FcγRII extracellular domains were compared. COS cells transfected with bovine FcγRII could bind complexed cattle IgG1 but not IgG2, thus suggesting the existence of other isoforms of this receptor.

**FcγRIII** In humans and mice, FcγRIII is highly homologous by virtue of sequence similarity, cellular distribution and function. In humans, FcγRIII is encoded by two genes (A and B) giving rise to distinct products. The FcγRIIIA gene encodes a receptor with a conventional transmembrane section and cytoplasmic region. This form is expressed by Mϕ, NK cells and a subpopulation of T-cells and monocytes. The FcγRIIIB gene product is expressed by neutrophils and eosinophils and is anchored to the plasma membrane via a glycosyl phosphatidyl-inositol (GPI) linkage (Van de Winkel and Anderson, 1991). In the mouse only one isoform of the receptor similar to human FcγRIIIA is known which is expressed by Mϕ, NK cells, neutrophils and mast cells (Ravetch and Kinet, 1991). Due to extensive glycosylation FcγRIII in human and mouse has a broad electrophoretic mobility Mr = 50,000-80,000 and 40,000-60,000 respectively.

Human FcγRIIIA is associated with various disulphide-linked subunits as homo- or hetero-dimers (γ-γ or γ-ζ or ζ-ζ) which are necessary for expression of the receptor (Fig. 1.1). These subunits are related to those associated with FcRI for IgE and the T
and B cell antigen receptors and are responsible for signal transduction (Weiss and Littman, 1994). In contrast, FcγRIIIB can be expressed without these subunits (Kurosaki and Ravetch, 1989). The association of 20% of cell surface FcγRII with γ-γ homodimers in cultured human monocytes has been demonstrated recently (Masuda and Roos, 1993). FcγRI is also associated with γ-γ homodimers but can be expressed in the absence of the subunits (van de Winkel and Capel, 1993). These subunits are thought to have a role in signalling on occupancy of the Fc receptor, and different combinations of γ and ζ chains could represent alternative signalling pathways.

1.9.3 FUNCTIONS OF FcγR

The FcγRs are involved in many regulatory and effector mechanisms in immune responses. These include ADCC, promotion of phagocytosis and endocytosis, the release of inflammatory and cytotoxic mediators regulation of Ig production and enhancement of antigen uptake and presentation (Nathan et al., 1980; Fanger et al., 1989; Debets et al., 1990; Klaus et al., 1987). The above functions are executed only after cross linking of Fc receptors; this can lead to stimulatory or inhibitory signals depending on the cell type and the type or isoform of FcγR. For instance, cross linking of FcγRII on Mφ delivers a positive signal to the cell and receptor and ligands are internalised for degradation and subsequent presentation of antigen for mounting an immune response (Macintyre et al., 1988). In contrast, B cell FcγRII-ligand interaction provides an inhibitory signal, the receptor is not internalised and inhibits Ig production (Fridman, 1993). Thus FcγRII on B-cells has a regulatory mechanism for B cell responses.

In mice FcγRIIb2 isoform, found on Mφ, is capable of mediating phagocytosis, while FcγRIIb1 expressed on most haemopoietic cells is not (Miettinen et al., 1989). The affinity of FcγRII on human monocytes for complexed IgG is markedly increased by proteolysis, while the number of receptors on the cell surface remains constant. IgG mediated functions of monocytes such as the release of TNF-α are also increased by proteolysis (Debets et al., 1990). It is therefore possible that proteases released by cells such as granulocytes, activated T cells and Mφ in vivo could increase the affinity of FcγRII especially at the site of inflammation with a corresponding increase in FcR-mediated functions (Tax and van de Winkel, 1990). FcγRIIIA, found on NK cells and Mφ, is capable of mediating ADCC and phagocytosis, while FcγRIIIB on neutrophils, with its GPI linkage is not. However, the latter may act synergistically with other FcR to mediate these functions, or alternatively it could be a means of trapping immune complexes without subsequent cell activation (Selvaraj et al., 1988).
1.9.4 Signal transduction via FcγR

FcγR are signal transducing molecules and cross-linking of FcγR is required before FcγR mediates functions other than uptake. An increase in the intracellular concentration of calcium ions (Ca^{2+}) occurs following FcγR cross linking with immune complexes or anti-FcγR mAb (Young et al., 1984; Van de Winkel et al., 1990). The events involved in signalling following FcγR occupation are not fully understood. Stimulation of FcγRIII on NK cells with ligand results in a rapid rise in Ca^{2+} and hydrolysis of membrane phosphoinositides, resulting in production of inositol triphosphate (IP3) and IP4 (Carasatella et al., 1989). This also results in transcriptional activation of specific cytokines like IFN-γ and TNF-α which is dependent upon influx of extracellular calcium (Anegon et al., 1988).

Signal transduction and internalisation of FcγRIII on different cell types has recently been investigated. The γ and ζ subunits situated with FcγR are associated with a Mr = 70,000 tyrosine phosphoprotein and receptor triggering induces increased association of this phosphoprotein with the ζ subunit in both T and NK cells (Vivier et al., 1993). A similar protein located with the ζ subunit in T cells has been identified as tyrosine kinase, and it has been suggested that the FcR-associated dimers interact with this non-receptor tyrosine kinase in initiation of signalling and cell activation. The requirement of tyrosine phosphorylation in FcR-mediated phagocytosis has been demonstrated in mouse Mφ (Greenberg et al., 1993). FcR mediated phagocytosis by FcγRIII was thought to be due to γ and ζ subunits essential for signal transduction. The internalisation by FcγRII and FcγRI in a human monocytic cell line was also mediated by tyrosine phosphorylation (Ghazizadeh and Fleit, 1994). FcγRII was not tyrosine phosphorylated on FcγRI stimulation, suggesting that these receptors employ distinct signalling pathways. As a consequence of signalling via FcγR, MP release a number of inflammatory mediators such as leukotrienes and prostaglandins, and the inflammatory cytokines IL-1, IL-6 and TNF-α (Krutmann et al., 1990; Debets et al., 1990). Thus FcR signalling and associated secretary activities may regulate the expression of cell surface molecules involved in interaction with other cells and extracellular matrix.

1.9.5 REGULATION OF FcγR EXPRESSION

FcγR expression can be regulated by a number of cytokines. IFN-γ induces expression of FcγRI on neutrophils and eosinophils and FcγRIII on eosinophils, which do not constitutively express these receptors (Fanger et al., 1989; Ravetch and Kinet, 1991). IFN-γ upregulates FcγRI expression on human Mφ, with an enhanced effector
Author's Note

Attention is drawn to the erratum notice on the Title Page of Chapter 1, and in particular to the correct nomenclature for the leukocyte integrins, which is as follows:

<table>
<thead>
<tr>
<th>Leukocyte Integrin</th>
<th>Alternative Name</th>
<th>$\alpha$ Chain</th>
<th>$\beta$ Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1</td>
<td>-</td>
<td>CD11a (=(\alpha L))</td>
<td>CD18</td>
</tr>
<tr>
<td>Mac-1</td>
<td>CR3</td>
<td>CD11b (=(\alpha M))</td>
<td>CD18</td>
</tr>
<tr>
<td>p150/95</td>
<td>CR4</td>
<td>CD11c (=(\alpha X))</td>
<td>CD18</td>
</tr>
</tbody>
</table>
functions such as the oxidative burst while there was decrease in phagocytic activity (Jungi et al., 1989). In contrast IFN-γ does not increase the expression of FcγRII on human Mϕ, although functions are altered as for FcγRI (Jungi et al., 1991). TNF-α and GM-CSF also enhance effector capabilities by increasing the killing capacity of cultured monocytes through FcγRII (Erbe et al., 1990). Weinshank et al., (1988) demonstrated selective upregulation of murine Mϕ isoform of FcγRII in a mutant cell line on stimulation with IFN-γ.

1.9.6 CHARACTERISATION OF FcγR
In humans and mice, FcγRs have been characterised by the generation of several mAb to each of the Fc receptor types, unfortunately none are available for sheep FcγRs. The existence of two distinct FcγR in sheep bone marrow Mϕ similar to human FcγRI and FcγRII has been described using FcR mediated erythrocyte rosetting and its inhibition using different IgG subclasses (Jungi et al., 1992). Functional presence of FcγR on sheep afferent dendritic cells was demonstrated by IgG mediated uptake of antigen (Harkiss et al., 1990). Recently, Coughlan, (1994) identified molecules equivalent to the size of FcγRI and FcγRII from sheep afferent dendritic cells using polyclonal antiserum to peptides of bovine FcγRI, suggesting that both are expressed by these cells.

1.10 LEUKOCYTE INTEGRINS
The leukocyte integrins (β2 integrins, CD11/CD18) are involved in mediating important leukocyte adhesion-dependent interactions, including cell to cell communication thus imparting a significant role in host defence by mediating inflammatory and immune responses. The leukocyte integrins (LI) family is part of the integrin superfamily, members of which are evolutionary, structurally and functionally related to each other (Hynes, 1992). The molecules of LI family are exclusively expressed by leukocytes.

1.10.1 STRUCTURAL FEATURES OF LEUKOCYTE INTEGRINS
The LI family has three structurally related glycoproteins, CD11a (LFA-1), CD11b (Mac-1, CR3) and CD11c (p150,95, CR4). Each of these three glycoproteins exist as an αβ complex in which a unique α subunit is associated with a common β subunit, the β2 (CD18) chain of Mr = 95,000. CD11a possesses the αL chain of Mr = 180,000, CD11b contains αM chain of Mr = 170,000 and the Mr = 150,000 αX chain is associated with CD11c. The CD11a, CD11b, CD11c and CD18 glycoproteins are defined by mAb that are specific for their unique αM, αL, αX and
The α subunits of LI have been shown to be distinct, whereas the β subunits are identical by mAb reactivity and antigen preclearing studies (Trowbridge and Omary, 1981; Sanchez-Madrid et al., 1983). Deglycolysation of LI molecules revealed polypeptide backbones of 149,000 (αL), 137,000 (αM), 132,000 (αX) and 78,000 (β2) (Larson and Springer, 1990). The α subunits, αL, αM and αX, and the common β subunit are synthesised as distinct precursors. The association of α and β subunits is required for further processing in the Golgi apparatus before the mature proteins are transported to the cell surface or intracellular granules (Miller et al., 1987; Bainton et al., 1987). cDNA encoding the common β subunit was isolated and characterised, and the deduced 769 amino acid sequence has the characteristic features of an integral membrane protein (Kishimoto et al., 1987b; Law et al., 1987). The cytoplasmic domain of β subunit has tyrosine, serine and threonine residues as potential phosphorylation sites. The immunochemical analysis of the β subunit protein from purified CD11a, CD11b and CD11c indicates that a single gene encodes the β subunit of all the three leukocyte integrins (Kishimoto et al., 1987b). Subsequently the gene encoding the common β subunit was mapped to the long arm of chromosome 21 (21q 22.3) (Larson and Springer, 1990). The β subunits of the integrin subfamilies share 37-45% amino acid identity in the cytoplasmic and transmembrane domains. The cysteine rich region of the extra cellular domain is highly conserved thus indicating the relationship within integrins as a whole (Kishimoto et al., 1987b).

The cDNA encoding the α subunit of CD11a, CD11b and CD11c and deduced amino acid sequences define homologous integral membrane proteins (Larson and Springer, 1990). Each α subunit contain three homologous repeats that have putative divalent cation binding sites which may account for the Mg$^{2+}$/Ca$^{2+}$ dependency of LI mediated adhesions (Larson et al., 1989). The CD11a, CD11b and CD11c proteins have 1063, 1136-37 and 1144 amino acids with 12, 19 and 10 potential N-glycosylation sites respectively. Thus the differences in the apparent molecular weights of the mature polypeptides are mainly due to the differences in the amount of associated carbohydrates. The αM and αX share 63% amino acid identity with each other but only 35% identity with the αL, with the transmembrane domain and the putative divalent cation binding region being highly conserved, thus reflecting their appropriate
close functional similarity. The cytoplasmic regions of the three \( \alpha \) chains include several serine and threonine residues which may serve as possible targets of phosphorylation (Larson and Springer, 1990). The genes encoding all the three \( \alpha \) subunits of LI were located between bands p11-p13.1 on chromosome 16 (Corbi et al., 1988). Thus the relationship among the \( \alpha \) subunits suggests an evolutionary link between integrin subfamilies to a common origin through \( \alpha \) integrin gene duplication. The \( \alpha \) subunits of LI as well as \( \alpha 1 \) and \( \alpha 2 \) subunits of \( \beta 1 \) integrin contain a segment of around 180 amino acids in the extra cellular region known as insert (I) domain. Recently the I domain of LI has been shown to be important in ligand binding and contain activation epitopes (Diamond et al., 1993).

1.10.2 DISTRIBUTION OF LEUKOCYTE INTEGRINS
The LI are exclusively expressed on leukocytes and their haemopoietic precursors.

**CD11a** CD11a, commonly known as lymphocyte function associated antigen-1 (LFA-1), is expressed on all the leukocytes in human, mouse and other animal species studied with the exception of peritoneal macrophages (Mø) in mouse (Krensky et al., 1983) and rat (Tamatani et al., 1991). Among the LI molecules CD11a/CD18 is the first to be expressed on early progenitors of erythroid burst-forming units, erythroid colony forming units (CFU), granulocyte/macrophage (GM)-CFU and monocyte-CFU. (Todd and Schlossman, 1984).

**CD11b** The CD11b (also known as CR3) was the one of the first defined markers for myeloid cells and is expressed on monocytes, tissue Mø, granulocytes and natural killer (NK) cells (Springer et al., 1979). The CD11b is absent from mixed GM-CFU stem cells and appear later on committed granulocyte and monocyte precursors at myelocytic and monoblastic stages respectively (Todd and Schlossman, 1984). It has also been found to be expressed by immature and CD5+ B-cells in chronic B-cell lymphocytic leukaemia (De La Hera, 1988), and non-specific esterase positive acute myelomonocytic leukaemic cells (Todd et al., 1981). Recently CD11b has been reported to be present on a subpopulation of cytotoxic and helper T-cells in human and these CD11b+ T-cells exhibit more potent cytotoxic and helper functions (Hoshino et al., 1993). In cattle CD11b is expressed by a sub-population of B-cells in addition to its presence on myeloid cells (Splitter and Morrison, 1991). The differential expression of CD11b on B-lymphocytes is discussed in Chapter 7 (see section 7.1).
Sanchez-Madrid et al., (1983) described the existence of a third heterodimeric protein, p150,95 (CD11c). CD11c (commonly known as CR4) has a similar distribution to that of CD11b and is found on monocytes, granulocytes, most tissue Mϕ, NK-cells and acute myeloid and monocytic leukaemic cells (Springer et al., 1986; Miller et al., 1986). CD11c is not expressed on mixed GM-CFU stem cells and like CD11b appears later on myelocytic and monoblastic cells (Todd and Schlossman, 1984). It is absent from normal peripheral blood T and B-cells but is expressed by some B-cell lines such as hairy cell leukaemia and certain cloned cytotoxic T-lymphocytes (Schwarting et al., 1985). In cattle CD11c has been reported to be expressed on granulocytes, monocytes and some activated lymphocytes (Splitter and Morrison, 1991).

The relative abundance of the LI varies depending on the cell type and state of cell activation and differentiation. Lymphocytes constitutively express CD11a. On inflammatory activation of granulocytes and monocytes the cell surface expression of CD11b and CD11c increases by several folds within minutes. This occurs by translocation of intracellular storage granules to the cell surface and fusion of the granule membrane with plasma membrane. This may account for neutrophil polarity in response to exogenous stimuli accompanied by the selective expression of the contents of granules and therefore CD11b and CD11c at the leading edge (Gallin et al., 1982). Like lymphocytes, tissue Mϕ do not contain intracellular pools of these receptors. Peripheral blood monocytes express high levels of CD11b and lower levels of CD11c. However after extravasation and maturation to tissue Mϕ the pattern is reversed with higher level of CD11c being expressed. The relative abundance of LI on resting monocytes is (CD11a > CD11b > CD11c) which is different from that found on tissue Mϕ (CD11c > CD11a > CD11b) (Miller et al., 1986; Fryer et al., 1988; Arnaout et al., 1988).

1.10.3 LIGANDS OF THE LEUKOCYTE INTEGRINS
Several ligands have been found to interact with LI receptor molecules in a divalent-cation dependent manner. Binding of most of the ligands also appears to be critically dependent on the activation state of the receptor. Some of the LI ligands are transmembrane proteins which may also serve signal transduction functions on interaction with their receptors (Larson and Springer, 1990).

CD11a ligands CD11a mediates its activities by binding to three different but homologous counter receptors; the inducible intercellular adhesion molecule
ICAM-1 (CD54) and the constitutively expressed ICAM-2 (CD102) and ICAM-3 (CD50) (Rothlein et al., 1986; Staunton et al., 1989; de Fougerolles and Springer, 1992). These adhesion molecules are members of the immunoglobulin superfamily and have a variable number of Ig like domains. They have a different cellular distribution and thus may be responsible for different functions. A low basic expression of ICAM-1 on endothelium, leukocytes and many epithelial cell types is strongly upregulated in response to several inflammatory mediators like IL-1, TNF-α, IFN-γ or LPS (Springer, 1990). ICAM-2 is constitutively expressed by endothelial cells and other leukocytes. ICAM-2 is the predominant CD11a ligand on endothelial cells and its expression is not affected by cytokines (Staunton et al., 1989). ICAM-3 is not expressed by either resting or stimulated endothelium but has high level of expression on lymphocytes, monocytes and neutrophils (de Fougerolles and Springer, 1992). Both the endothelial ligands, ICAM-1 and ICAM-2 bind to CD11a through the N-terminal domain 1 (Springer, 1990).

**CD11b** CD11b binds fixed C3bi, an inactive complement 3 fragment generated by the cleavage of C3b by factor I and H. Anti-αM subunit specific mAb are able to inhibit this binding (Beller et al., 1982). CD11b on myeloid cells mediates both adherence and phagocytosis of C3bi coated particles (Rothlein and Springer, 1985), and on NK cells CD11b contributes to elevated NK activity against C3bi coated targets (Ramos et al., 1988). Activated CD11b mediates endothelial migration of myeloid cells and binds domain 3 of ICAM-1 at a site distinct from that which binds to CD11a (Diamond et al., 1991). Soluble ligands like Factor X (Altieri and Edington, 1988) and fibrinogen (Altieri et al., 1988; Wright et al., 1988) have been reported to bind activated CD11b. Factor X may provide alternative initiation of the coagulation cascade and along with fibrinogen mediates cell substrate adhesion. In addition, CD11b has been implicated in a number of Mφ-microrganism interaction, including binding to bacterial LPS, yeast β-glucan, and *Leishmania* and *Histoplasma* via recognition of parasite surface phosphosugars (Wright and Jong, 1988; Bullock and Wright, 1987). The binding to phosphosugars and C3bi occurs through distinct binding sites on CD11b (Wright et al., 1989a). The binding of C3bi and fibrinogen on CD11b could be inhibited by a modified Arg-Gly-Asp (RGD) peptide, Lys-X-X-Gly-Asp. Thus CD11b binds to C3bi and fibrinogen on the same or closely associated sites which are different from the binding site for ICAM-1 (Wright et al., 1988). The sites of attachment on CD11b for β-glucan, C3bi and LPS have been shown to be different on the basis of inhibition with mAb and activation of CD11b on binding (Ross and Vetvicka, 1993). Recently four distinct sites for ligand (C3bi, fibrinogen,
ICAM-1 & Factor X) binding have been shown to be located on I domain of αM chain (Diamond et al., 1993).

CD11c A little is known about the ligands that mediate CD11c activity. The CD11c shares with CD11b, C3bi and fibrinogen as its ligands. The binding of C3bi coated particles to monocyte derived Mφ was partially inhibited by anti-CD11b mAb and the remaining activity was blocked by anti-CD11c mAb (Myones et al., 1988). A synergistic role of CD11b and CD11c has been suggested where CD11b mediates adherence of C3bi coated particle and CD11c triggers ingestion (Ross et al., 1991). This suggests that CD11c binds C3bi through a site different from CD11b. CD11c binds fibrinogen through recognition of Gly-Pro-Arg-Pro sequence (Loike et al., 1991), a different site from that which binds CD11b.

1.10.4 FUNCTIONS OF LEUKOCYTE INTEGRINS
All the members of the CD11/CD18 family mediate specific and or overlapping adhesion promoting functions, which are totally dependent on the presence of divalent cations (Mg²⁺/Ca²⁺).

CD11a CD11a is constitutively expressed on most leukocytes including cells of MP system. CD11a mediates adhesive interactions by binding through its ligands ICAM-1, ICAM-2 and ICAM-3 (de Fougerolles and Springer, 1992). The existence of three CD11a ligands with different cellular distribution suggests specialisation for different functional aspects of CD11a dependent leukocyte interactions. Strong induction of ICAM-1 in inflammation and immunity suggests its role in localisation of cells in inflammation and facilitates recognition of specific antigens in the development of immunity (Altman et al., 1989; Dustin et al., 1986; Springer, 1990). Since ICAM-2 is the predominant CD11a ligand on resting endothelium, the CD11a-ICAM-2 pathway of adhesion seems to have an important role in normal recirculation of lymphocytes through tissue endothelium (Mackay et al., 1990; Pals et al., 1988). The finding that adhesion of resting lymphocytes to purified CD11a occurred primarily via ICAM-3, implies its important role in the initiation of immune responses (de Fougerolles and Springer, 1992).

Studies on the inhibitory activities of anti-CD11a mAb revealed its function in mediating cytotoxic activities of T-cell and NK-cell by inhibiting initial conjugate formation (Springer et al., 1987). CD11a mediates a broad range of other lymphocyte functions which include adhesion dependent cell-cell interactions.
Anti-CD11a mAb inhibits T-cell proliferation in response to stimuli that require T-cell-T-cell or T-cell antigen presenting cell adhesion (Hildreth and August, 1985) particularly in primary immune responses. In secondary immune responses however such interactions are largely independent of CD11a because of the increased expression of other adhesion molecules in memory cells (Sanders et al., 1988; Mackay et al., 1992a). B-cell activation and differentiation into Ig secreting cells, and lymphocyte adherence to normal and phorbol ester stimulated human umbilical vein endothelial cells is also inhibited by anti-CD11a mAb. Anti-CD11a mAb can inhibit and reverse in vitro phorbol ester mediated homotypic aggregation of lymphocytes (Rothlein and Springer 1986), and inhibit T-cell dependent but not the T-cell independent antibody responses. Adhesion of T cells to cytokines (IL-1 and TNF-α) or LPS activated endothelium is largely independent of CD11a (Haskard et al., 1986).

In addition to lymphocytes, CD11a has a role in several adhesion dependent functions of phagocytes such as homotypic aggregation of monocytes, ADCC by neutrophils and monocytes, antigen presentation by monocytes and mediates non stimulated neutrophil and monocyte adhesion to endothelium (Springer, 1990; Dustin and Springer, 1991).

CD11b CD11b is restricted in expression to phagocytic cells (granulocytes and monocytes) and NK cells and performs important functions in mediating adhesive interactions in a ligand specific manner as well as been an adhesion promoting receptor. In its resting state CD11b on monocytes and granulocytes binds fixed C3bi on RBC but fails to induce phagocytosis and subsequent cytotoxic events (Beller et al., 1982; Klein et al., 1990). In contrast fixed C3bi on zymosan particles and E. coli bind to resting CD11b and stimulate phagocytosis, degranulation and respiratory burst (Cain et al., 1987; Wright and Jong, 1986). Similarly phorbol esters activated CD11b can mediate phagocytosis and subsequent killing of C3bi-coated particles (Rothlein and Springer 1985) and contributes to elevated NK activity of C3bi coated targets (Ramos et al., 1988). It has been suggested that β-glucan activates CD11b both by protein kinase C and tyrosine kinase associated phosphorylation and exposes new binding sites on CD11b for L-selectin and C3dg (Ross and Vetvicka, 1993). Thus activated CD11b mediates both L-selectin and ICAM-1 dependent neutrophil aggregation (Simon et al., 1992; Anderson et al., 1986) and phagocytosis of C3dg coated particles (Ross and Reed, 1992). Four activation sites which mediate binding of different ligands have been located on domain I of the α-chain of CD11b (Diamond et al., 1993). Multiple forms of CD11b which perform different functions
have been suggested (Graham et al., 1989). The one which is mobile on the cell membrane is proposed to be required for attachment of C3bi opsonised particles and the other less mobile cytoskeleton linked receptor appears to be necessary for initiating phagocytosis. Phorbol esters activated monocytes appear to express the cytoskeleton linked form of the receptor and thus show increased phagocytosis of C3bi opsonised particles (Graham et al., 1989). β-glucan activation greatly enhances CD11b dependent NK cell activity and extends it to C3dg coated targets (Ross and Vetvicka, 1993).

Anti-CD11b mAb inhibit *in vitro* homotypic aggregation of neutrophils, neutrophil and monocyte chemotaxis, and adhesion to endothelium (Anderson et al., 1986; Larson and Springer, 1990). Steady state CD11b and CD11a, are both involved in neutrophil and monocyte binding to endothelium (Smith et al., 1989). However chemotactic stimulation with f-met-leu-phe enhances the attachment of neutrophils to endothelium by a CD11b-ICAM-1 dependent process (Anderson et al., 1986).

In addition to C3bi coated microrganisms, CD11b on monocytes/Mφ is capable of binding a number of microrganisms such as *Leishmania promastigotes* (Russell and Wright, 1988), *E. coli* (Wright and Jong, 1986) and *Histoplasma capsulatum* via recognition of parasite surface phosphosugars (Bullock and Wright, 1987). The binding of microrganisms to CD11b may be important in the uptake and infection of phagocytes (Wright and Jong, 1986). However fixed C3bi on microrganisms bearing the appropriate sugars promotes the avid attachment to CD11b thereby allowing the sugar activation of CD11b followed by phagocytosis and oxidative burst (Ross and Vetvicka, 1993). Uptake via CD11b has been shown to be essential for the killing of *Listeria monocytogenes* whereas CD11b independent uptake leads to multiplication of the organisms in the mouse peritoneal Mφ (Drevets et al., 1993).

Fan and Edgington, (1993) suggested a regulatory role for CD11b on monocytes stimulated by LPS, where engagement of CD11b with mAb or natural endothelial ligand leads to increased TNFα mRNA expression and cytokine secretion by cells. CD11b has been shown to be in near spatial association with FcγRI and FcγRII on monocytes and FcγRI on IFN-γ primed neutrophils (Gadd et al., 1994). This association was demonstrated by the induction of respiratory burst via formation of CD11b mAb-FcR complexes on monocytes and neutrophils. A similar FcR-CD11b association has been described by the ability of anti-CD11b mAb to inhibit
phagocytosis of IgG coated particles (Brown et al., 1988). However the functional significance of such association is not clear.

**CD11c** CD11c also binds to C3bi but this receptor ligand activity is different from that of CD11b (Myones et al., 1988). In contrast to CD11b, the majority of CD11c molecules on tissue Mφ are cytoskeleton linked and are not mobile within the membrane (Ross et al., 1991). It has been suggested that cytoskeleton linked CD11c has its role in mediating phagocytosis of the C3bi opsonised particles The role of CD11c in monocyte chemokinesis and chemotaxis, adherence to endothelium and phagocytosis has been suggested by the inhibitory effect of αX specific mAb (Keizer et al., 1987b). CD11c also mediates some adhesive functions of lymphocytes particularly it has contribution in conjugate formation of some cytotoxic T-cell clones with their targets (Keizer et al., 1987a).

**1.10.5 REGULATION OF FUNCTIONS AND SIGNAL TRANSDUCTION**

The LI mediated immune interactions have been suggested to depend upon the ligand expression and qualitative changes in the receptor rather than quantitative cell surface expression of the receptor (Hynes, 1992; Dransfield, 1991; Springer, 1994). The qualitative changes which increase adhesiveness of the receptor include divalent cation dependent stabilisation, phosphorylation mediated activation and altered membrane distribution of the cell surface receptor. Activation of integrins mediate leukocyte adhesion under various circumstances (Fig. 1.2).

**Divalent cation stabilisation of the receptor** A number of studies have demonstrated the requirement of mM concentrations of divalent cation (preferably Mg$^{2+}$ and or Ca$^{2+}$) by LI for ligand binding. There are three putative cation binding domains in the α subunit of LI suggesting that cations bind directly to the α subunit (Larson and Springer, 1990). Recently an additional binding site for Mg$^{2+}$ and Mn$^{2+}$ but not Ca$^{2+}$ in domain I of the α subunit of LI has been demonstrated to be essential for ligand binding (Michishita et al., 1993). The Mg$^{2+}$ requirement for the stabilisation of the LI receptor has also been suggested by the binding of a mAb only in the presence of Mg$^{2+}$ and such receptor molecules are capable of mediating high affinity ligand binding (Dransfield, 1991). This demonstrates the divalent cation dependence of LI.

**Receptor regulation of cell surface expression** Stimulation of leukocytes with a variety of chemoattractants including f-met-leu-phe, C5a and leukotriene B4 results in
ten fold increase in CD11b and CD11c cell surface expression on monocytes and neutrophils within minutes. The CD11a expression increases only two fold on monocytes and remains unchanged on neutrophils (Larson and Springer, 1990). These stimulated cells showed increased adhesiveness to their respective ligands. The observed increased adherence was found to be due to conformational change in the integrin upon activation rather than increased quantitative expression (Landis et al., 1993; Springer, 1990). Further, the inhibitory effect of activation epitope specific mAb has demonstrated that the ligand binding was mediated by a subpopulation of activated CD11b and CD11a molecules (Diamond and Springer, 1993; Springer, 1994).

**Functionally active state of the receptor** The functional activation of the cell surface LI has been suggested to be mediated from within the cell by protein kinase C dependent phosphorylation of the cytoplasmic tail of the β2 subunit (Valmu et al., 1991). CD11a has also been shown to be activated in response to cross linking of other cell surface receptors such as CD2-CD58, MHC class II-CD4 T cell receptor, CD3-anti-CD3 mAb via intracellular signalling (Dustin and Springer, 1991). CD11b can be activated by chemoattractants and phorbol esters (Valmu et al., 1991). The activation of CD11b upon binding to β-glucan has also been demonstrated by protein kinase C and tyrosine kinase associated phosphorylation of β2 chain (Ross and Vetvicka, 1993). This activation is a reversible event and deactivation has been suggested to be achieved by dephosphorylation of CD11a and CD11b by protein kinase A and phosphatases respectively (Chatila et al., 1989; Valmu et al., 1991). Recently Gadd et al., (1994) presented evidence of signal transduction via the α chain of the leukocyte integrins. This was demonstrated by stimulation of monocyte and neutrophil via the formation of α chain specific CD11b mAb-FcR complex. Such stimulation was not seen when β2 chain specific mAb were used.

**Regulation of ligand cell surface expression** An increase in the cell surface expression of ICAM-1 the ligand for CD11a and CD11b in response to IL-1, IFN-γ, TNF-α and other inflammatory stimuli is well documented. This increase in ICAM-1 expression plays a key role in increased adhesiveness and trans-endothelial migration of leukocytes during an inflammatory response (Dustin et al., 1986; Buckle et al., 1990).

**Altered membrane distribution of receptor** The clustering of CD11b and CD11a within the plane of the membrane has been suggested to increase the avidity of LI
FIGURE 1.2 Diagramatic representation of β2 integrins activation mediated leukocyte adhesion. (a) Two step adhesion. (1) cellular activation originating from a variety of membrane receptors interaction (e.g. MHC clas II-CD4 T cell receptor), promotes an energy dependent, increase of leukocyte integrins avidity for its ligand, thus provide a firm contact during cell-cell interactions; (2) initial adhesion via members of selectin family (L-selectin expressed by leukocytes and P-selectin expressed by endothelial cells) with their ligands results in reduced cell flow and subsequent interaction of low avidity integrins receptor-ligand pairs. This may or may not depend upon activation signals that originate from initial intercellular contact and leads to avidity changes in adhesion receptors. (b) One-step adhesion, under pathological conditions, immediate adhesion that does not require prior cell activation may result from (1) increased receptor and/or ligand density; (2) increased local concentration of divalent cations such as Mn$^{2+}$ that induces conformational changes in adhesion receptors or (3) Chemoattractants mediated activation of leukocyte integrins which also leads to the production of soluble factors further activating integrins functions. (Taken from Pardi et al., 1992)
Increased receptor/ligand density

Co-operative

Increased divalent cation density

Chemoattractant modulating integrin activity

- Binding Integrin Receptor-ligand Pair
- Non-binding Integrin Receptor-ligand Pair
- L-selectin-ligand pair
- P-selectin-ligand pair
receptors for their ligands (Detmers et al., 1987; Fgdor et al., 1990). This could be achieved in response to phosphorylation of the β2 subunit or associated cytoskeletal elements. The clustering of the receptor was suggested by the inhibition of LI function due to disruption of microfilament network with cytochalasins (Wright and Silverstein, 1982; Martz, 1987).

1.10.6 LEUKOCYTE ADHESION DEFICIENCY DISEASE (LAD)
The functional significance of the LI has been elucidated by the discovery and characterisation of leukocyte adhesion deficiency (LAD) disease in humans (Kishimoto et al., 1987a) and more recently in dogs (Trowald-Wigh et al., 1992) and cattle (Shuster et al., 1992). LAD was found to be an autosomal recessive disease characterised by greatly reduced or lack of surface expression of the LI heterodimers. This produced multiple defects in leukocyte functions resulting in frequent and recurrent bacterial and fungal infections (Arnaout, 1990b; Kishimoto, 1989b). LAD is caused by heterogeneous molecular defects in the common β subunit. These range from defective gene expression leading to undetectable mRNA, or aberrant gene splicing to produce an abnormally small β chain, to point mutations in the highly conserved region of the β2 subunit resulting in a superficially normal precursor, which is unable to associate with the α subunit (Dimanche-Boitrel et al., 1988). The amino acid sequence of the β chain of LI in human, mice and cattle have been shown to be >80% identical (Shuster et al., 1992). Amino acid identity was also found in those regions where point mutations were found to cause LAD.

LAD affected individuals showed complete absence of neutrophils in inflamed tissues although lymphocytes, plasma cells and eosinophils are present (Bowen et al., 1982). A similar pathology was observed in LAD cases of cattle and dog where a large number of intravascular neutrophils were present in the inflamed areas (Kehrli et al., 1992; Trowald-Wigh et al., 1992). This occurs due to a lack of adhesiveness of neutrophils to endothelium and subsequent failure in migration to inflamed tissues, despite a persistent peripheral blood neutrophilia (Bowen et al., 1982). In vitro neutrophils and monocytes from such cases failed to demonstrate chemotaxis, aggregation and adherence to plastic and endothelial monolayers in response to chemotactic factors and phorbol esters (Anderson and Springer 1987). These cells were deficient in cell surface expression of the LI and in intracellular storage pools of CD11b and CD11c (Anderson and Springer, 1987). The binding and phagocytosis of C3bi coated particles and subsequent respiratory burst is impaired as is ADCC mediated by neutrophils and monocytes (Kohl et al., 1984; Kehrli et al., 1990;
Trowald-Wigh et al., 1992). In contrast the adherence independent functions of phagocytes such as superoxide generation to soluble stimuli, shape changes and degranulation responses are normal (Kohl et al., 1984). The most common abnormality of LAD lymphocytes is defective T cell cytotoxicity (Krensky et al., 1985). A level of impairment is also seen in helper T cell responses, NK activity, homotypic and heterotypic B and T cell adhesions and antigen dependent proliferative responses (Mazerolles et al., 1988; Krensky et al., 1985). However the adhesion related functions of lymphocytes in secondary responses are normal reflecting the involvement of other adhesive interactions (Springer, 1990).

1.10.7 Role of leukocyte integrins in inflammation
During inflammation, chemoattractants activate LI (CD11b and CD11c) adhesiveness and cytokines (IFN-γ, IL-1 and TNF-α) increase ICAM-1 expression at the site of inflammation (Bevilacqua, 1993). Thus the migration of granulocytes and monocytes from the blood to the site of infection or injury seems to be mediated by molecular changes on the surface of endothelial cells and leukocytes. This migration is regulated by at least three distinct but overlapping molecular signals (Springer, 1994). In the first step, initial tethering of the flowing cells followed by rolling adhesion on endothelium are brought about by the selectin-carbohydrate ligand interactions (Ley et al., 1991; von Andrian et al., 1991). The second step involves the binding of the chemoattractant displayed on or released from endothelium to receptors on granulocytes and monocytes (Snyderman and Uhing, 1992). The chemoattractant receptors are coupled to G protein which activates LI adhesiveness to its endothelial ligands. In the third step, transient firm attachment and spreading of rolling cells on endothelium is mediated via LI endothelial ligand interaction (Larson and Springer, 1990). This is followed by integrin de adhesion, transendothelial migration and entry in to the inflamed tissue along the directional cues from the chemoattractant (Springer, 1994). Thus LI have their role in transient firm attachment before transendothelial migration, an essential step for the migration of leukocytes to the site of inflammation. All three steps are essential for transendothelial migration, as inhibiting any of the steps lead to complete absence of monocytes and neutrophils at the site of inflammation (von Andrian et al., 1991; Springer, 1994). A basal level of similar molecular mechanism is responsible for migration of monocytes in normal physiological conditions.

The adhesiveness of CD11b and CD11a on neutrophils and monocytes is activated by the binding of chemoattractants f-met-leu-phe and IL-8 to their receptors on
phagocytes (Larson and Springer, 1990). The chemoattractant receptor interaction not only activates integrins but also directs migration and stimulate degranulation, shape changes, actin polymerisation and respiratory burst (Snyderman and Uhing, 1992).

However the evidence for a similar chemoattractant mediated activation of CD11a on lymphocytes is missing due to a lack of demonstration of chemoattractant receptors on lymphocytes (Springer, 1994). However the chemoattractants considered to be acting on monocytes also attract lymphocytes (Miller and Krangel, 1992) and may provide the step 2 signal required for integrin mediated emigration of lymphocytes. The partial inhibitory effect of anti-CD11a mAb on lymphocyte accumulation at the site of antigen or IFN-γ or TNF-α injection is suggestive of its role in lymphocyte migration (Scheynius et al., 1993). However the accumulation of lymphocytes at the site of inflammation is almost completely inhibited by a combination of anti-CD11a and antibodies to β1 integrins VLA-4 (Springer, 1994).

The lack of neutrophil infiltration at the site of inflammation in LAD and a similar inhibitory effect of anti-CD11b mAb (Harlan et al., 1992) suggests a major role for CD11b in neutrophil migration in inflammation. The studies on LAD cases (Harlan et al., 1992) and on the inhibitory effect of LI mAb (Arfors et al., 1987) suggest the contribution of both CD11b and CD11a in monocyte migration at the site of inflammation. Recently the role of VLA-4 (β1 integrin), in mediating lymphocyte migration and monocyte migration across the endothelium has been demonstrated (Springer, 1994; Meerschaert and Furie, 1994). The lymphocyte migration in LAD cases is not greatly affected (Bowen et al., 1982) and the memory lymphocytes normally recirculate through tissue endothelium (Mackay et al., 1990). Thus lymphocyte migration across the endothelium is not completely dependent on LI but they seem to follow the same three steps as suggested for phagocytes (Springer, 1994).
AIMS OF THIS THESIS

Little is known about the expression of differentiation antigens on mononuclear phagocytes in sheep. Most of the studies in this species have used monoclonal antibodies to characterise lymphocyte subsets (Hopkins et al., 1993; Mackay, 1988) and MHC molecules (Hopkins and Dutia, 1990; Hopkins et al., 1986; Puri et al., 1987). Only a few monoclonal antibodies which recognise mononuclear phagocytes in sheep are available (Pepin et al., 1992; Gonzalez, 1989). Unfortunately due to their reactivity with other cell types none of them are specific for mononuclear phagocytes. Mononuclear phagocyte specific reagents, are not only required to purify or deplete these cells for various in vitro experiments but also to study the in situ localisation or involvement of different types of these cells (monocytes/macrophages or dendritic cells) under normal and pathological conditions.

The specific aim of this study was to characterise and delineate mononuclear phagocytes in sheep using monoclonal antibodies.

This aim was achieved:

(1) by raising monocyte/macrophage specific monoclonal antibodies by immunising Balb/c mice with sheep alveolar macrophages. The antigens recognised by these monoclonal antibodies were analysed biochemically and their cellular distribution and tissue localisation was determined by flow cytometry and immunohistochemistry.

(2) by identifying and characterising a panel of monoclonal antibodies specific for the ovine homologues of the human β2 (leukocyte) integrins (CD11/CD18).

(3) The specificity of CD11b and CD11c (members of leukocyte integrins family) for myeloid cells was ascertained by analysing non-myeloid leukocytes (lymphocytes) expressing these molecules. This was investigated by phenotyping and localising the CD11b+ lymphocytes in various lymphoid compartments using two-colour flow cytometry and immunohistochemical techniques.

The work done in this thesis characterises new monocyte/Mϕ differentiation antigens in sheep some of which are homologous to the cluster of differentiation (CD) antigens in human thus filling a gap for which nothing was known in sheep.
CHAPTER TWO

MATERIALS AND METHODS
2 MATERIALS AND METHODS

2.1 ANIMALS

2.1.1 SHEEP
Male and female Blackface sheep of 1-2 years of age obtained from the Moredun Research Institute, Edinburgh, were used for the collection of various tissues, blood, lymph and lung washes. Ileal Peyer's patches, thymus and bone marrow were collected from lambs under one year of age.

2.1.2 MICE
BALB/c mice bred in the Department of Veterinary Pathology animal house, University of Edinburgh were used for the production of monoclonal antibody (mAb), ascites and preparation of spleen cells for use as feeder layers in fusion and cloning.

2.2 MEDIA
1. Unless otherwise specified RPMI 1640 (Gibco, Biocult, Uxbridge) containing, 2 mM L-glutamine, 1 mM sodium pyruvate and 25 mM Hepes was used as basic culture medium throughout the work done in this thesis.
2. Fusion was performed in RPMI 1640 containing 15% fetal calf serum (FCS; Gibco) 2 mM L-glutamine and 1 mM sodium pyruvate. Selection was carried out using 2% hypoxanthine, aminopterin and thymidine (HAT) (Sigma, Dorset, Poole England). After the appearance of clones the HAT was gradually replaced with 1% hypoxanthine and thymidine (Sigma).
3. The growing hybridomas were cloned in basic culture medium supplemented with 15% FCS and subsequently maintained in medium containing 10% FCS.
4. Wash medium consisted of basic culture medium supplemented with 1% FCS.
5. Monocytes/macrophage were cultured in basic culture medium supplemented with 10% FCS, 100 U ml⁻¹ benzyl penicillin, 100 U ml⁻¹ streptomycin and 2.5 μg ml⁻¹ fungizone.

2.3 PREPARATION OF CELLS

2.3.1 ALVEOLAR MACROPHAGES (AM)
AM were collected by bronchoalveolar lavage as described by Mayer and Lam (1984). Briefly, trachea, lung and heart were dissected out of the thoracic cavity as a single package and the outer surface was washed with ice-cold sterile phosphate
buffered saline (SPBS). The bronchoalveolar cells were washed out of the lung using ice cold sterile Hank's balanced salt solution (HBSS) in 3-4 washes (1 to 2 L) while the lungs were gently massaged. After removing the floating mucus and cell debris from the top of the cell suspension it was centrifuged at 250 x g for 15 min at 4°C. The pellet was washed once in sterile HBSS and cells harvested at 180 x g for five min at 4°C (unless stated otherwise, similar conditions were used throughout the work done in this thesis when cells were washed and harvested). For hypotonic lysis of contaminating red blood cells (RBC), the cells were resuspended in 10 ml sterile HBSS mixed with 13.5 ml of sterile distilled water (SDW) for 30 seconds and the osmolality was restored by adding 1.5 ml of 10 X SPBS. After washing once in sterile HBSS the cells were resuspended in FCS containing 10% dimethyl sulfoxide (DMSO) at 4°C and cryopreserved at -70°C in aliquots of one ml containing 1 x 10^7 cells. Cytospot of Leishman's stained lung wash cells is shown in Fig. 2.1 and flow cytometry (FACS) scatter profile in Fig. 2.4.

2.3.2 ENRICHMENT OF MONOCYTES, AND MONOCYTE DERIVED MACROPHAGES
Monocyte enriched mononuclear cells (MEMC) were prepared by adhering to gelatine and autologous plasma coated plastic tissue culture flasks by the method of Goddeeris et al., (1986). Briefly blood was collected from the jugular vein into an equal volume of Alsever's solution (Appendix I). Aliquots of 30 ml of diluted blood were layered over 20 ml Lymphoprep (Nygard, Oslo, Norway) at a density of 1.077 g ml^-1 in 50 ml polypropylene conical tubes (Nunclon, Denmark) and centrifuged at 900 x g for 30 min at 20°C. Peripheral blood mononuclear cells (PBMC) were aspirated from the interface, diluted with an equal volume of Alsever's solution, and pelleted by centrifugation at 450 x g for 10 min at 20°C. The PBMC were washed three times in Alsever's solution by repeated suspension and centrifugation at 20°C and resuspended in basic culture medium (section 2.2.1) containing 10% FCS. Tissue culture flasks (75 cm^2; Nunclon, Denmark) were coated with 10 ml of 2% gelatine (BDH, Poole, England) in SDW for 2 hours at 37°C. The gelatine solution was removed and the flasks were dried at 37°C. Autologous plasma was obtained from heparinised blood (10U ml^-1) after centrifugation at 3500 x g for 30 min at 20°C. The plasma was filtered through 0.45µm filters to remove any residual cells, platelets and bacteria. 10 ml of autologous plasma was added to the dry gelatine coated flasks and incubated for one hour at 37°C. The plasma was removed and the flasks rinsed twice with unsupplemented RPMI 1640 medium. 15 ml of cell suspension containing 5 x 10^6 cells ml^-1 of culture medium was incubated in gelatine/autologous plasma coated tissue culture flasks for one hour at 37°C.
After removing the non-adherent cells, the flasks were gently rinsed in culture medium at 37°C and the adherent cells were harvested with calcium and magnesium free HBSS (Appendix I) containing 10 mM disodium ethylenediamine tetra acetic acid (EDTA) by incubating for 5 min at 20°C. The harvested MEMC were washed twice in culture medium (section 2.2.4) at 4°C before use. This cell preparation was usually 60 to 70% enriched for monocytes as determined by cytochemistry and morphology of the cells (Fig. 2.1). The non adherent cells were used as monocyte depleted mononuclear cells. Occasional monocytes were seen in this population. Cytospots prepared from both the populations were stained for non-specific esterase to distinguish monocytes from lymphocytes (Fig. 2.1). FACS scatter profiles of both the populations are shown in Fig. 2.4.

For in vitro monocyte derived macrophages (MDM) the adherent MEMC in tissue culture flasks were incubated in culture medium (section 2.2.5) for 6 days at 37°C in 5% CO2 humidity chambers. The cells were harvested as described for MEMC above.

2.3.3 AFFERENT AND EFFERENT LYMPH CELLS
Efferent and afferent lymph cells were obtained by the chronic cannulation of the prefemoral efferent and pseudoafferent lymphatic ducts (Hopkins et al., 1986). Cells were collected in sterile, siliconised bottles containing 400 U of heparin. All surgery was performed by Dr. J. Hopkins.

2.3.4 ENRICHMENT OF ALVEOLAR MACROPHAGES AND AFFERENT DENDRITIC CELLS
The cell suspensions containing AM and afferent dendritic cells (ADC) were enriched by centrifugation over metrizamide (Nyegaard, Oslo, Norway). Cells were washed in RPMI 1640 containing 10% FCS, harvested and resuspended in the same medium at 4°C. 5 x 10^7 washed cells resuspended in 2 ml were layered onto a 5 ml cushion of 14.5% metrizamide prepared in RPMI 1640 containing 10% FCS and centrifuged at 540 x g for 20 min at 4°C. The cells collected from the interface were washed twice in RPMI 1640. The proportion of AM or ADC was consistently greater than 70% in such cell preparations as determined by cell morphology and cytochemistry (Fig. 2.1).

2.3.5 ISOLATION OF PERIPHERAL BLOOD LEUKOCYTES (PBL)
Ovine peripheral blood obtained from the jugular vein was collected in an equal volume of Alsever's solution. PBL were isolated by hypotonic ammonium chloride lysis (Appendix I) of RBC (Mishell and Shiigi, 1980), harvested by centrifugation at
FIGURE 2.1 Cytospot preparations showing cell morphology:

(a) Sheep lung wash cells, alveolar macrophage (arrow head), lymphocyte (arrow) and granulocyte (double arrow); unfractionated cells (i), metrizamide fractionated alveolar macrophages (ii & iii), stained with Leishman's stain (i & ii) and for non-specific esterase (iii). x 400.

(b) Monocyte enriched (i & ii) and monocyte depleted (iii) peripheral blood mononuclear cells, monocyte (arrow head) and lymphocyte (arrow); stained with Leishman's stain (i) and for non-specific esterase (ii & iii). x 400.

(c) Metrizamide enriched afferent lymph dendritic cell (arrow head), Leishman's stained. x 400.
180 x g for 5 min at 20°C. The cells were washed twice in PBA (PBS, 0.5% bovine serum albumin and 0.05% sodium azide) before cell surface immunolabelling for FACS analysis (scatter profile, Fig. 2.4).

2.3.6 PREPARATION OF SPLEEN, LYMPH NODE AND PEYER’S PATCH LYMPHOCYTES

Cells from lymph node and spleen were separated by teasing and scraping with forceps and scalpel in wash medium (section 2.2.4). For Peyer’s patch cells, 15 cm of terminal ileum was removed, opened along its attached border and cut into 2-3 cm lengths. After extensive washing these were placed in a petri dish containing wash media and the mucosa was gently squeezed using two 19 gauge hypodermic needles bent at right angles to disrupt the follicles underneath (Jones, 1988). After repeated pipetting the large clumps were allowed to settle for 2 min. The resultant cell suspensions were passed through 45μm pore size nylon mesh (Small Parts Inc. USA) and the viable lymphocytes isolated from the supernatant by centrifugation over Lymphoprep at 850 x g for 20 min at 20°C. The lymphocytes were collected from the interface, and washed twice in wash media before using for FACS analysis (scatter profiles, Fig. 2.4).

2.3.7 BONE MARROW CELLS

The cells were prepared from the sternal bone marrow (BM) as described elsewhere (Haig et al., 1991). Briefly the sternum from young sheep was taken and all the fatty tissue was removed. It was then cut longitudinally into two pieces along the midline, exposing the marrow cavity. Marrow fluid from the cut pieces was squeezed out using a vice and collected in plastic universal bottles containing wash medium (section 2.2.4). After repeated pipetting the heavy bony fragments were allowed to settle, while fat accumulated at the top for 2 min. The cells from the middle excluding fat and bony fragments were collected and filtered through lens cleaning tissue before centrifugation at 250 x g for 10 min at 20°C. Cells were washed again and resuspended in wash medium before centrifugation on Lymphoprep at 20°C for 20 min at 900 x g. The cells collected from the interface were washed twice before using as nucleated BM cells for FACS analysis (scatter profile, Fig. 2.4).

2.3.8 SINGLE CELL SUSPENSION OF MOUSE SPLENOCYTES

Mice were killed by cervical dislocation. The spleen was collected under sterile conditions and teased apart in RPMI 1640 as above. Large clumps were allowed to sediment for 2 min and the suspended cells washed three times in RPMI 1640.
2.4 ANTIBODIES AND ANTISERA
The monoclonal antibodies (mAb) used in this thesis, other than those produced by the author (section 2.5), are shown in Table 2.1. This illustrates their isotype, specificity and source. Antiserum to a synthetic peptide of 15 amino acids, 154 to 168 of 2nd extracellular domain of bovine FcγRI (Anti-FcR) was raised in rabbit as described (Coughlan, 1994). The sequence of bovine FcγRI was taken from Symons and Clarkson, 1992. Anti-FcR, and normal serum collected from the same rabbit before immunisation, and affinity purified anti-OVA IgG (Coughlan, 1994) were kind gifts from Dr Susie Coughlan, a recent PhD from the Department of Veterinary Pathology, University of Edinburgh, Edinburgh.

2.5 PRODUCTION OF MONOCLONAL ANTIBODIES
Anti-sheep macrophage mAb were produced by immunising 6-8 wk female BALB/c mice with AM, followed by fusion of mouse splenocytes with NSO non-secreting myeloma cells. After screening, positive lines were cloned by limiting dilution and those growing from a single cell were used as monoclonal cell line for subsequent studies.

2.5.1 IMMUNISATION PROTOCOL
Four BALB/c mice were immunised, each with 2 x 10^6 enriched AM suspended in 0.5 ml SPBS, injected in 3-4 sites sub-cutaneously (SC) and boosted after 2 weeks with a similar injection given SC. A second booster injection of 2 x 10^6 AM in SPBS was given after 4 weeks intra-peritoneally and the mice were test bled 2 weeks later. The blood was allowed to clot for one hour at 20°C. Serum collected by microfugation at 13,000 rpm for 5 min at 20°C was tested for the presence of anti-AM antibodies by FACS analysis. Immunised mice sera reacted positively with AM up to 1:3200, the highest dilution of the serum tested (Fig. 2.2). The last booster injection of 2 x 10^6 AM in sterile PBS was given intra-venously 4 days before fusion.

2.5.2. CELL FUSION.
The single cell suspension of immunised mouse spleen as described above was purified by centrifugation over lymphoprep and the viable cells were collected from the interface. NSO cells in exponential growth phase, and spleen cells were washed twice and resuspended in unsupplemented RPMI 1640. The viable cell count as determined by trypan blue dye exclusion was found to be >95%. NSO and spleen cells were mixed in the ratio of 1:7 and centrifuged at 180 x g for 5 min at 20°C. The
<table>
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<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
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<tr>
<td>86D</td>
<td>Ovγδ TCR</td>
<td>G1</td>
<td>Mackay et al., (1989)</td>
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<td>G2a</td>
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Ov, sheep; Bo, cattle;
*Rat Immunoglobulin.
pellet was gently loosened after removing the supernatant and one ml of 50%polyethylene glycol 4000 (Sigma) in RPMI 1640 containing buffered Tris pH 8.5 (prewarmed to 37°C) was added slowly over a period of 5 to 7 minutes while mixing. The mixture was incubated at 37°C for another two minutes before washing with 20ml of RPMI 1640 at 37°C, by slowly adding first one ml in 3-4 min and the remainder over a period of 5 min. The fused cell suspension was centrifuged at 150 x g for 5 min at 20°C. After washing in RPMI 1640 the pellet was carefully resuspended in 5 ml of HAT medium (RPMI 1640, 15% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 2% HAT). The cells were counted and the concentration of the fused cells was adjusted to 1 x 10^6 ml^-1 with HAT medium. The cell suspension was plated out at 100 µl per well in 96 well flat bottomed microtiter plates (Nunclon, Denmark) containing 100 µl (5 x 10^5 cells well^-1) of irradiated BALB/c mouse spleen cells (section 2.3.8) as a feeder layer and incubated in 5% CO2 humidity chamber at 37°C for one week. Thereafter the wells were scored for the presence of clones of dividing cells and supplemented with 50|il of HAT medium.

2.5.3 SCREENING AND SELECTION OF CLONES
Supernatant from confluent wells was harvested and tested by FACS using AM andPBL (section 2.11). Positive clones were moved to 24 well flat bottom plates (Nunclon, Denmark) and simultaneously an aliquot of cells was cryopreserved in FCS containing 10% DMSO at -70°C before storing in liquid nitrogen. All hybridoma supernatants were stored at 4°C in the presence of 0.01% sodium azide (Fisons, England). The hybridoma cell lines whose supernatant specifically reacted with AM were cloned twice by limiting dilution by plating 0.5, 1.0 and 10 cells per well, and the wells containing only one colony growing from a single cell were selected for further studies and cryopreserved.

2.5.4 PRODUCTION OF SATURATED CULTURE SUPERNATANTS AND ASCITIC FLUID
Cloned cell lines were grown in bulk culture and the cells collected in exponential growth phase were washed twice and resuspended in SPBS. For production of ascitic fluid 10^7 cells were inoculated intraperitoneally to each BALB/c mice injected seven days before with 0.5 ml pristane (Sigma). After 10-14 days ascitic fluid was collected from the peritoneal cavity of the mice in microfuge tubes and centrifuged at 13,000 rpm for 10 min at 20°C. It was stored in one ml aliquots with or without 0.01% sodium azide at -20°C. Saturated culture supernatant was produced by growing the monoclonal cell line to confluency and the cells were allowed to die over a period of 5-7 days. The saturated supernatant rich in monoclonal antibodies was harvested
FIGURE 2.2 Anti-sheep alveolar macrophages (AM) antibody reactivity of immunised Balb/c mice sera as determined by FACS analysis. The graph shows the mean fluorescence intensity of AM stained with various dilutions of serum from two immunised mice used for fusion collected before last intravenous booster injection and one normal mouse serum (NMS) collected before immunisation. The staining was visualised using polyclonal anti-mouse FITC.

FIGURE 2.3 Bio-Rad protein reagent (0.2 ml) mixed with 0.8 ml of various concentrations of protein solution (BSA, 0-100 µg ml⁻¹) was incubated at 20°C for five minutes. The OD₅₉₅ of the reaction mixture is plotted against protein concentration to prepare a standard curve for protein estimation.
FIGURE 2.2

![Graph showing serum dilution and mean fluorescence intensity.](image)

FIGURE 2.3

![Graph showing protein concentration and OD595.](image)
after removing the cell debris at 1500 x g for 15 min and stored at -20°C in the presence of 0.01% sodium azide.

2.6 IMMUNOGLOBULIN ISOTYPE ANALYSIS
Isotype analysis of cloned monoclonal supernatant was performed using a mouse immunoglobulin isotyping kit (ISO-1, Sigma) following the manufacturer’s protocol.

2.7 PURIFICATION OF ASCITIC FLUID
Immunoglobulin (Ig) G from ascites was purified by caprylic acid precipitation. Briefly, ascitic fluid was acidified by the addition of 25 µl ml⁻¹ of glacial acetic acid (BDH, Poole England) and unwanted protein precipitated by the addition of caprylic acid (Octanoic acid; Sigma) at 50 µl ml⁻¹. This was mixed for 30 min at 20°C before removal of precipitates by high speed centrifugation. Solidified caprylic acid was removed from the surface of ascitic fluid after incubation over ice for one hour. The supernatant was dialysed extensively into 0.1 M NaHCO₃, pH 8.4 for 48 hours to remove the remaining acid.

Alternatively Ig was purified by ammonium sulphate precipitation according to the method of Hudson and Hay (1989). Briefly, Ig from ascitic fluid was precipitated with ammonium sulphate at 45% saturation (v/v) for 30 min at 20°C. The precipitates were centrifuged at high speed for 15 min at 4°C and redissolved in SDW. The immunoglobulins were exchanged into 25 mM carbonate buffer pH 9.0 (Appendix I) using Sephadex G 25 (PD10) column (Pharmacia, Sweden). The protein concentration was determined by spectrophotometry OD 280 nm (extinction coefficient OD₂₈₀ x 0.715 = mg ml⁻¹).

2.8 BIOTINYLLATION OF MONOCLONAL ANTIBODIES
Purified Ig at 1 mg ml⁻¹ in 0.1 M NaHCO₃, pH 8.4 was biotinylated by the addition of biotin-amido caproate N-hydroxysuccinimide ester (Biotin; Sigma) in DMSO at 10 mg ml⁻¹ at a biotin to protein ratio of 75 µg : 1 mg. After 4 hours incubation on a rotor at 20°C the reaction was stopped by adding ammonium chloride to 0.1 M. The biotinylated immunoglobulin was dialysed extensively against 0.1 M PBS, 0.01% sodium azide, pH 7.2 for 24 hours. Finally biotinylated antibodies were dialysed in PBS azide containing 20% glycerol for 2 hours at 20°C and stored in aliquots at -70°C.
2.9 FITC LABELLING OF MONOCLONAL ANTIBODIES AND OVALBUMIN.

Purified Ig or ovalbumin (OVA; Sigma) at 20 mg ml⁻¹ in 25mM carbonate buffer pH 9.0 (Appendix I) was conjugated to FITC (Sigma) in DMSO at 10 mg ml⁻¹ at a FITC to protein ratio of 1 mg : 20 mg. This was incubated for 6 hours on a rotor at 20°C before the reaction was stopped by the addition of sodium azide to 0.01M. Free FITC was removed by gel filtration using sephadex G 25 (PD10) column.

2.10 BIO-RAD MINI PROTEIN ASSAY

The assay was used to determine the protein concentration of various preparations. A standard curve was prepared using known concentrations of bovine serum albumin (BSA). Briefly, 0.8 ml of BSA solution (0-100 μg ml⁻¹) was mixed with 0.2 ml of Bio-Rad protein reagent and incubated for 5 min at 20°C. The OD₅₉₅ was measured and plotted against protein concentration in μg ml⁻¹ (Fig. 2.3).

2.11 FLOW CYTOMETRY (FACS)

2.11.1 IMMUNOFLUORESCENCE STAINING OF CELL SURFACE ANTIGENS

The cell preparations were washed twice in PBA buffer (PBS, 0.5% BSA, 0.05% sodium azide, pH 7.2) and cell concentration adjusted to 10⁷ ml⁻¹ for immunolabelling of cell surface antigens.

**Single-Colour Flow Cytometry** Cell surface phenotype was assessed by incubating 50 μl aliquots of cells (5 x 10⁵ cells) with 25 μl of appropriate dilution of primary antibody for 40 min at 4°C. Unbound antibody was removed by washing twice with 900 μl of PBA buffer. The cells were then incubated with 25 μl of appropriate dilution of FITC conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulin (Dakopatts, Denmark) in the dark for 30 min at 4°C. The cells were washed twice as above and resuspended in 500 μl of PBA buffer before analysis. The control samples were treated with culture supernatant of VPM53 (anti-campylobacter mAb, IgG1) or 1:500 normal mouse serum (NMS; Sigma) instead of primary antibody. The comparative histograms of mononuclear cells in peripheral blood (live gate, Fig. 2.4) stained with VPM53 and NMS are shown in Fig. 2.5.

**Two-Colour Flow cytometry** Cells were dispensed as above and incubated simultaneously with 25 μl each of the non-biotinylated/FITC labelled and biotinylated mAb as appropriate. They were incubated and washed twice in PBA as above. Two colour immunofluorescence staining requires that each mAb be detected by a different
fluorochrome. The biotinylated primary mAb was detected by the addition of appropriate dilution of streptavidin conjugated to phycoerytherin (SA-PE) (Amersham International, UK). The other primary antibody was either FITC labelled or detected with an isotype specific FITC-conjugated second antibody (sheep anti-mouse IgG1-FITC, IgG2a-FITC, IgM-FITC, The Binding Site, Birmingham, UK, and goat anti-mouse IgG2b-FITC, Sera Lab, Sussex, UK). 25 µl each of the appropriately diluted SA-PE and isotype specific FITC-conjugated second antibodies were again simultaneously added for visualisation of the red and green fluorescence respectively. They were then incubated at 4°C in dark for 30 min washed twice as above and resuspended in 500 µl of PBA buffer before analysis. Negative controls were stained with 1:500 biotinylated NMS (BNMS) and saturated supernatant of VPM53 as first antibodies (Fig. 2.5).

Double staining with two mAb of the same isotype was done by separately staining with each antibody. First with the antibody to be labelled with anti-isotype FITC conjugate. After incubation the cells were washed twice and free sites of FITC conjugate blocked by incubating with 15 µl of the same primary antibody for 30 minutes at 4°C. Cells were then stained with second biotinylated primary antibody prior to labelling with SA-PE. Subsequently, the cells were washed as above and analysed.

2.11.2 FLOW CYTOFLUORIMETRY AND ANALYSIS OF CYTOMETRIC DATA

The immunofluorescence studies described were carried out using a fluorescence-activated cell analyser, FACSCAN cytometer (Becton Dickenson, Mountain View, CA, USA). Data was analysed using Becton Dickenson 'Consort 30' and 'Lysis' programmes. In each case the data was derived by analysing 10,000 cells (or events) per sample. After immunolabelling the fluorescence measurements were made on homogenous populations of cells by setting electronic (live) gates on a dot plot (Fig. 2.4). Different cell populations used for FACS were distinguished by live gating using appropriate linear photo multiplier tube (PMT) voltage amplifications for forward (FSC) and side (SSC) scatter parameters excluding unwanted cells. These FSC and SSC parameters define cell size and complexity respectively. Alveolar macrophages (AM) and afferent lymph dendritic cells (ADC) both have high FSC and high SSC, peripheral blood granulocytes (PBG) have low FSC and high SSC, peripheral blood monocytes (PBM) have high FSC and medium to low SSC and lymphocytes have low to medium FSC and low SSC (Fig. 2.4). However the monocyte population overlaps
FIGURE 2.4 Flow cytometry display for 'live gating' using forward and side angle light scatter (FSC and SSC), profiles. The PMT voltage of both FSC and SSC was optimised for each cell population.

(a) Lung wash cells, alveolar macrophages (R1) and lymphocytes (R2).
(b) Unlysed peripheral blood, red blood cells (R2) and platelets (R1).
(c) Bone marrow cells, erythroid and lymphoid cells (R1); lymphoid and myeloid cells (R2) and granulocytes (R3).
(d) Peripheral blood leukocytes (lysed RBC), granulocytes (R1); small and large mononuclear cells (R2) and small mononuclear cells (R3).
(e) Monocyte enriched mononuclear cells (R1).
(f) Monocyte depleted mononuclear cells (R1).
(g) Afferent lymph, non-fractionated cells (i), metrizamide fractionated cells (ii), dendritic cells (R1) and lymphocytes (R2).
(h) Efferent lymph lymphocytes (R).
(i) Peripheral (prefemoral) lymph node small mononuclear cells (R).
(j) Spleen small mononuclear cells (R).
(k) Ileal Peyer's patch small mononuclear cells (R).
the lymphocyte and could not be distinguished on the basis of live gating of the whole population of peripheral blood mononuclear cells (PBMC).

For single-colour parameters the fluorescence profiles of a gated population of cells are displayed as a histogram of $\log_{10}$ relative fluorescence intensity versus cell number. The histogram of negative control (cells stained with 1:500 NMS or saturated supernatant of VPM53) was moved to the left hand corner using appropriate PMT voltage for FITC (Fig. 2.5). Positive fluorescence was established in comparison to the negative control. A marker was set to the right hand side of the negative control fluorescence peak, and any fluorescence of greater intensity than this marker (i.e. to the right of the marker) was considered as positive and due to the binding of monoclonal antibody to its specific cell surface antigen (Fig. 2.5).

For two-colour parameters, the negative control lymphocytes (stained with 1:500 BNMS or saturated supernatant of VPM53) inside the monitor gate (in the FL1 vs FL2 parameter option) were adjusted to the lower left corner of the box (Fig 2.5) using optimum FITC and SA-PE voltage levels. These optimal PMT voltages were found to be 625 mV and 574 mV for FITC and SA-PE respectively and both FL1 (FITC) and FL2 (SA-PE) were in logarithmic amplifications. To overcome the unwanted spectral overlap of emitted light from the FITC and SA-PE fluorochromes used, the appropriate %FL1-FL2 and %FL2-FL1 PMT voltage compensation was used. The data is presented as contour plots showing contour lines (at 10, 20 30 and 40%) representing the cell density distribution over this area. For statistical data analysis, the contour plots were set into calculation regions defined by setting quadrant markers. Quadrant 1 represents PE (biotinylated mAb) positives only, quadrant 2 represents dual (FITC/PE) positive, quadrant 3 represents negative population for both the mAb while quadrant 4 is FITC positive only (Fig. 2.5).

2.11.3 OPTIMISATION OF MONOCLONAL ANTIBODIES AND CONJUGATE DILUTIONS

All mAb and conjugates were titrated for optimal working dilutions. Sheep PBL and AM were stained for flow cytometry with doubling dilutions of mAb as well as NMS. Red and green fluorescence was visualised using ten fold dilutions of SA-PE and FITC conjugates. The optimal working dilution of each of the antibodies chosen was that which gave the strongest fluorescence intensity and clear distinction between the positive and negative populations within the normal range. The optimal working dilutions of the various conjugates was determined using different dilutions against the
FIGURE 2.5 (a) Single-colour FACS histogram display of the peripheral blood mononuclear cells, negative control stained with normal mouse serum, 1:500 (---) and VPM53 (anti-campylobacter, IgG1) saturated supernatant (----) adjusted for optimal FITC (FL1) voltage level. A marker was set as shown for the analysis of cells.

(b) Two-colour contour plot of the peripheral blood mononuclear cells negative control stained with biotinylated normal mouse serum 1:500 (FL2) and VPM53 saturated supernatant (FL1) adjusted for optimal FITC (FL1) and PE (FL2) voltage levels. A quadrant was set as shown to analyse the cells. Quadrant 1, 2, 3 and 4 will contain FL1-FL2+, FL1+FL2+, FL1+FL2- and FL1-FL2- cells respectively.
optimal dilutions of the mAb. The dilution giving strongest specific fluorescence and lowest background staining of the negative control cells was chosen.

2.12 PHOSPHATIDYL INOSITOL PHOSPHOLIPASE-C (PIPL-C) TREATMENT OF CELLS
In order to determine if antigen is linked to the cell surface by a glycosyl phosphatidyl-inositol (GPI) linkage, cells were treated with PIPL-C (Boehringer-Mannheim, Germany) (Simmons et al., 1989). The cell suspensions (2 x 10^7 cells ml^-1) in RPMI 1640 containing 1% BSA, 0.01% sodium azide and 2 mM PMSF, were incubated in the presence or absence of 10 U PIPL-C for 60 min at 37°C. The reaction was stopped by addition of cold PBA buffer and the cells were stained as usual for FACS analysis.

2.13 PROTEIN IMMUNOCHEMISTRY

2.13.1 IMMUNOPRECIPITATION
This technique allows the isolation and identification of small amounts of protein antigens precipitated from radiolabelled cell lysate by specific binding to a mAb bound to protein-A-sepharose beads or ELISA plate. After disassociation of the immune complexes the free isolated antigen is fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography to detect the radiolabelled antigen and determine its molecular weight.

Radio-iodination and preparation of cell lysate Cells were radio-iodinated after treatment with Wood's reagent as described previously (Wall and Fitch, 1985). Briefly, after washing twice in SPBS, 2 x 10^7 cells were resuspended in 20 ml SPBS pH 8.3 containing 1 mM methyl-p-hydroxy benzimidate (Wood's reagent, Sigma) and incubated at 37°C for 30 min to enhance iodine labelling of the cell surface. Cells were washed three times in SPBS and finally resuspended in 1 ml of ice cold SPBS pH 7.2. Cells (2 x 10^7 ml^-1) in ice cold SPBS were surface labelled for 18 min with one mCi ^125I (Amersham International, UK); in the presence of 10 µg (1 mg ml^-1) of lactoperoxidase (Sigma), 40 µg (1 mg ml^-1) of glucose oxidase (Sigma) and 1.44 mg (36 mg ml^-1) of D-glucose (Sigma) added at time 0, 6 and 12 min. The reaction was terminated by washing the cells twice with ice cold SPBS and 2 x 10^7 cells were lysed in one ml of TNT lysis buffer pH 8.0 (20 mM Tris HCl, 0.15M NaCl, 1.0% Triton x 100) containing 2 mM PMSF (Sigma), on ice for 30 min. For precipitation of GPI linked antigen the cells were lysed at 37°C for 10 min (Brown and Rose, 1992) in the
presence of 5 μg aprotinin ml$^{-1}$ (Sigma) in addition to 2 mM PMSF as protease inhibitors. Lysate was centrifuged at 13,000 rpm for 10 min at 4°C to remove the cell debris. Supernatant was cleared of free $^{125}$I by gel filtration using Sephadex G 25 column (PD-10), equilibrated with TNT lysis buffer pH 8.0.

**Precipitation of antigen using Protein-A-Sepharose** Protein-A-Sepharose CL 4 B (Fermentech, Scotland) was linked to mouse Ig (mAb) through rabbit anti-mouse Ig (Serotec, England) bridge, for immunoprecipitation of radio-labelled antigen. For each precipitation, 100 μl suspension of Protein-A-Sepharose beads was washed twice in SPBS and then incubated with 200 μg of rabbit anti-mouse Ig overnight at 4°C. This complex was incubated with an equal volume of non-iodinated cell lysate overnight at 4°C to prevent non-specific binding. The complex (protein-A-sepharose–rabbit anti-mouse Ig) was washed three times in ice cold SPBS before linking with mouse Ig (100 μg of mAb) incubated for 4 hour at 4°C. The radioiodinated lysate was also cleared of non-specific binding proteins before immunoprecipitation by incubating with an equal volume of protein-A-sepharose–rabbit anti-mouse Ig complex over night at 4°C. The specific antigen was immunoprecipitated from pre-cleared $^{125}$I labelled cell lysate using mouse mAb linked to protein-A-sepharose–rabbit anti-mouse Ig complex, by incubating for 4 hours at 4°C. The beads were washed four times in buffer containing 0.5% sodium deoxycholate (DOC), 150 mM lithium chloride, 0.1 M Tris pH 8.0, with an intermediate wash in buffer containing 0.5% DOC, 0.5 M NaCl, 0.1 M Tris HCl pH 8.0. The bound antigen was eluted by boiling the immune precipitates in 50 μl SDS sample buffer (125 mM Tris HCl, 10% sodium dodecyl sulphate, 20% glycerol, 0.01% bromophenol blue) with or without 10% (v/v) β-mercaptoethanol under reducing and non-reducing conditions respectively.

**Precipitation of antigen by ELISA plate method** ELISA plates (Dynatech, Sussex, England) were coated with 100 μl per well of goat anti-mouse Ig (Sigma) at 10 μg ml$^{-1}$ in borate buffered saline pH 8.2 (Appendix I) and incubated overnight at 4°C. The plates were washed five times in PBS/0.01% Tween 20 before incubation with appropriate dilution of mAb (100 μl per well) for 1 hour at 20°C. The plates were washed as above and incubated for 3 hour with $^{125}$I-labelled AM lysate (100 μl per well) at 4°C. After washing as above, bound antigen was eluted by boiling for 3 min in 50 μl of SDS sample buffer as described above.
2.13.2 N-GLYCOSIDASE F TREATMENT OF ANTIGEN
The amount of carbohydrate associated with the protein antigen was ascertained by digestion of immunoprecipitated antigen with the enzyme N-glycosidase F. Briefly after immunoprecipitation using protein-A-sepharose the washed beads (section 2.13.1) were boiled with 20 µl of 0.75% SDS for 3 min followed by addition of 80 µl of reaction mixture (0.01 M EDTA, 1% Triton X 100 in SDW) and 1 U ml⁻¹ of the enzyme N-glycosidase F (Boehringer Mannheim, Germany). The contents were mixed and incubated for 16 hours at 37°C. The antigen was eluted by boiling in an equal volume of SDS sample buffer for 3 min and fractionated by SDS-PAGE.

2.13.3 SDS-PAGE
Proteins were fractionated by discontinuous polyacrylamide gel electrophoresis through Tris-glycine buffer containing SDS (384 mM, glycine, 1.0% SDS, 50 mM Tris HCl). Gels were either single concentration or 5-20% linear gradient acrylamide gels (30% acrylamide : 0.8% bisacrylamide) in 375 mM Tris HCl, pH 8.7, 0.1% SDS buffer (Appendix I). The stacking gel consisted of 3% acrylamide, 0.08% bisacrylamide in 125 mM Tris HCl pH 6.8, 0.1% SDS buffer. Protein samples were boiled in SDS sample buffer before electrophoresis through vertical slab gels using Bio-Rad Mini Protean II gel equipment. Gels were run at 200 Volts constant current for 30-45 min until the dye front reached the bottom of the gel. Bio-Rad Electrophoresis Calibration Kit markers (molecular weight range 14.4-97.0 kDa) or Rainbow markers (molecular weight range 14-200 kDa) were electrophoresed on every gel.

2.13.4 STAINING OF SDS-PAGE GELS
SDS-PAGE gels were either stained with coomassie blue or silver nitrate.

Coomassie blue staining The gels were incubated with 0.025% Coomassie Brilliant Blue R in 20% v/v methanol, 5% v/v acetic acid for 30 min on a shaker. The gels were then briefly washed in DW and then destained in several changes of 20% v/v methanol, 5% v/v acetic acid until a clear background was obtained. After staining gels were transferred onto 3 mm wet filter paper and dried for 3 hours at 80°C under vacuum on a Bio-Rad Laboratories gel drier, Model 583.

Silver staining Gels were fixed in 50% v/v methanol, 10% v/v acetic acid for 15 min followed by incubation in 5% v/v methanol, 7.5% v/v acetic acid and then 10% v/v gluteraldehyde for 20 min each with constant agitation. After washing in several
changes of DW the gels were placed in 0.1% w/v solution of silver nitrate for 15 min. The gels were washed again and then developed in 3% w/v solution of Na<sub>2</sub>CO<sub>3</sub> containing 0.02% v/v formaldehyde. The reaction was stopped by adding solid citric acid and gels washed in several changes of DW. Gels were fixed in 10% Ilfofix (Ilford, England) for 1 min, washed in DW and dried as above.

2.13.5 AUTORADIOGRAPHY
For autoradiography gels were dried and set in an autoradiographic cassette superimposed with a X-ray film (X-Omat TR, Kodak). The film was exposed at -70°C before being developed.

2.13.6 WESTERN BLOT ANALYSIS
After electrophoretic separation on SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond-C, 0.45μm, Amersham) using a semi-dry electroblotter (Trans-Blot SD, Bio-Rad). The transfer was performed in 25 mM Tris HCl, 20% v/v methanol for 1 hour at a constant current of 120 mA. After blotting, the nitrocellulose membrane was blocked using 5% w/v fat free dried milk (Sainsburys, UK) in PBS for one hour before overnight incubation in primary antibody diluted in PBS, 1% w/v dried milk. Blots were washed in several changes of washing buffer (PBS, 1% w/v dried milk, 0.1% v/v Tween 20) for 3 min at 20°C. Bound antibody was detected by incubating for 1 hour at 20°C with either rabbit anti-mouse (Serotec) or sheep anti-rabbit Ig-biotin conjugate (1/1000) (Sigma) followed by 30 min incubation with biotin-alkaline phosphatase bridged to streptavidin (1/1000) (Boehringer Mannheim). Biotin-AP–streptavidin bridge was prepared by incubating 20U of biotin-AP conjugate (2U μl<sup>-1</sup>) with 40 μg of streptavidin (1 mg ml<sup>-1</sup>) in 5 ml of TBS (20 mM Tris HCl, 150 mM NaCl pH 8.0) 1% w/v BSA for 30 min at 20°C and diluted 1:10 in wash buffer before use. Excess reagents were removed by washing in several changes of wash buffer over 30 min at 20°C. The immunoblots were developed with 20 μg ml<sup>-1</sup> nitro-blue tetrazolium (NBT) (Sigma) and 100 μg ml<sup>-1</sup> 5-bromo-4-chloro-3 indolyl phosphate (BCIP) (Sigma) in 0.1 M Tris HCl pH 9.5, 5mM MgCl<sub>2</sub>, 0.1 M NaCl after washing once in 0.1 M Tris HCl pH 9.5 as described by Pluzek and Ramlau (1988). The reaction was stopped by washing in DW.

2.13.7 PREPARATION OF CELL LYSATE FOR ANTIGEN PURIFICATION
AM prepared in section 2.3.4 were used for preparing cell lysate. Peripheral blood granulocytes (PBG) were prepared from the pellet obtained after lymphoprep separation of PBMC in section 2.3.2, by hypotonic ammonium chloride lysis of RBC.
(Appendix I). Cells were washed twice in sterile PBS and harvested by centrifugation at 630 x g for 15 min at 4°C. The pellet was resuspended (2 x 10^7 cells ml^{-1}) in ice cold TNT cell lysis buffer pH 8.0, containing 0.2 mM PMSF incubated at 4°C for 30 min. Cell debris was removed by centrifugation at 630 x g for 20 min, followed by centrifugation at 12,000 x g for 2 hour at 4°C. Residual cell debris was removed by filtering through Millipore prefilters. The filtered lysate was used fresh or stored at -70°C after addition of 0.05% sodium azide.

2.13.8 IMMUNOPURIFICATION OF THE ANTIGEN
The purified mAb (section 2.7) was coupled to Affigel-10 (Bio-Rad) according to the manufacturer's protocol. The required amount of Affigel-10 (2/3 v/v suspension in isopropanol) was washed twice in ice cold SDW in a sintered glass funnel after removing the isopropanol. The washed affigel was transferred into a glass universal containing immunoglobulins (1-3 mg ml^{-1}) in 0.1 M phosphate buffer pH 7.2 and left rotating overnight at 4°C. After removing the supernatant the remaining binding activity of the affigel was blocked with 1 M triethanolamine (TEA) pH 8.0 (0.1 ml per ml of affigel) and 1 ml PBS, 0.05% sodium azide for one hour at room temperature. The affigel was washed extensively with PBS, 0.05% sodium azide and stored at 4°C. The amount of immunoglobulin bound was consistently greater than 80% as ascertained by measuring the protein contents in immunoglobulin solution before and after binding to affigel using the Bio-Rad mini protein assay. Non-specifically bound material was removed by washing the affigel column for 30 min with wash buffer (15 mM TEA, 0.5% DOC, pH 8.0) followed by elution buffer (15 mM TEA, 0.5% DOC, 0.5 M NaCl, pH 11.3) for not more than 30 min. The affigel column was washed with SPBS pH 7.2 containing 0.05% sodium azide, followed by TNT lysis buffer pH 8.0, before applying the cell lysate at a rate of 10 ml hour^{-1} recirculating for 48-72 hours. A NMS affigel column was prepared as above and used as a pre-column. After applying the cell lysate the column was washed extensively with TNT lysis buffer pH 8.0 followed by wash buffer until no protein was detectable in the flow through. The bound material was eluted with elution buffer. 2 ml fractions were collected into 0.5 M TEA pH 7.9 (0.3 ml per ml of elute) to neutralise the pH of the eluted material. All the fractions were tested using ELISA and silver stained SDS-PAGE. ELISA positive fractions showing specific protein band in SDS-PAGE were pooled for subsequent use.
2.13.9 ACETONE PRECIPITATION OF PROTEINS

For SDS-PAGE analysis proteins were concentrated, by acetone precipitation. After addition of four volumes of ice cold acetone, samples were left at -20°C for at least 2 hours. The precipitates were pelleted by centrifugation at 13,000 rpm for 10 min at 4°C and resuspended as required.

2.13.10 ELISA

100 µl of antigen (samples in 0.5% DOC were diluted 1:10 in SPBS pH 7.2, 0.01% sodium azide) was put into wells of a 96-well flat bottomed ELISA plate (Immulon, Nunclon, Denmark) and incubated overnight at 4°C. The wells were blocked with 200 µl of 2% BSA (Sigma) in PBS for 60 min at 20°C after removing the antigen. Plates were washed three times with PBST (PBS pH 7.2, 0.01% Tween-80) buffer.

75 µl of primary antibody was added to each well and plates were incubated for 30 min at 20°C. After washing three times in PBST buffer, 75 µl of anti-species Ig horseradish peroxidase (HRP) conjugate (SAPU, Scotland) 1:1000 diluted in 1% BSA in PBS, was added and incubated for 30 min at 20°C. After washing the plates were developed by adding 75 µl of ELISA substrate (0.08% O-phenylene diamine in 0.1 M Na₂HPO₄, 0.05 M citric acid containing 0.0016 % v/v H₂O₂) in the dark for a maximum of 20 min. The reaction was stopped with 50 µl of 2 M H₂SO₄. The optical density was measured at 492 nm using a blank plate as a background control, on a Dynatech MR3000 micro ELISA plate reader.

2.14 CYTOCHEMISTRY

2.14.1 PREPARATION OF CYTOSPOTS

Cytological studies onfractionated and unfractionated cells were carried out by preparing cytospots of single cell suspensions. 100 µl of cell suspension (5 x 10⁵ cells ml⁻¹) was spun on to a cytospot at 1000 rpm for 3 min using a Cytospin-2 (Shandon). Cytospots were fixed in ice cold acetone for 5 min before staining/storing at -70°C.

2.14.2 LEISHMAN'S STAINING

Air dried cytospots were stained by covering with concentrated Leishman's stain (BDH, Poole, England). After 2-3 min it was diluted with equal volumes of buffered water pH 6.8 (BDH, Poole England). After staining for 8-10 min smears were washed, dried and covered with a cover-glass using DPX (Lamb, R.A., England) as mounting medium.
2.14.3 STAINING FOR NON-SPECIFIC ESTERASE
The presence of α-naphthyl acetate esterase (ANAE) activity was used as a means of identifying monocytes and macrophages (Hudson and Hay, 1989). Cytospots were fixed for 30 seconds at 4°C in 30 ml of 0.1 M phosphate buffer pH 6.6 mixed with 45 ml of acetone and 25 ml of formaldehyde solution, washed three times in water and air dried. The smears were incubated in the freshly prepared staining solution (6 ml of 4% w/v sodium nitrite mixed with equal volume of 40% pararosaniline solution prepared in 2 M HCl, diluted to 200 ml with 0.067 M phosphate buffer pH 5.0, pH adjusted to 5.8 with 0.1 M NaOH and finally 50 mg of α-naphthyl acetate dissolved in 2 ml acetone was added) for 45 min at 37°C. Smears were rinsed in water and counter stained in 0.4 % aqueous solution of methyl green (BDH, Poole England) for 5 min, washed in water, dried and mounted with DPX mounting medium.

2.15 IMMUNOHISTOCHEMISTRY
The tissues were collected soon after slaughtering and mounted in OCT embedding compound (Tissue Tek; Miles, USA) on cork stubs and snap frozen in liquid nitrogen before storing at -70°C. 6-8 μm thick sections were cut on a cryotome (SLEE, London), placed on Vectabond (Vector lab. UK) treated slides, air dried overnight at 20°C and fixed in ice-cold acetone for 5 min before staining or stored at -70°C for subsequent use.

2.15.1 INDIRECT IMMUNOPEROXIDASE STAINING USING ANTI-MOUSE HRP CONJUGATE
After thawing the dried cryostat sections and cytospots of cell suspensions were refixed in ice-cold acetone for 3 min before being rehydrated for 15 min in ice-cold PBS (all the washings were done in ice-cold buffers). The endogenous peroxidase activity was blocked in two changes of 15 min each in PBS pH 7.2, 0.01% sodium azide containing 1% hydrogen peroxide (BDH, Poole England). Intestine sections were blocked overnight at 4°C. Tissue sections were washed once in PBS for 10 min and twice for 10 min in PBNT buffer (PBS, 0.1% BSA, 2% normal sheep serum, 0.08% Tween 80) pH 7.4. Sections were incubated with saturated culture supernatant or appropriate dilution of mAb for 1-2 hours at 20°C. The excess mAb was rinsed of and slides washed twice in PBNT buffer. Bound primary antibody was detected by incubating with 1:50 HRP conjugated sheep anti-mouse Ig (SAPU, Scotland) for 60 min at 20°C. After washing with PBNT followed by PBS for ten min each, the immune complexes were visualised by applying a filtered solution of diamino-benzidine tetrahydrochloride (DAB, Sigma) as substrate (10 mg DAB in 20
ml PBS, 0.02% H₂O₂) for five min. After rinsing in water the staining was enhanced using 1% w/v copper sulphate solution in water for 5 min. The slides were rinsed in water, counter-stained briefly with hematoxylin, washed with excess water and dehydrated through the alcohols, cleared using histoclear and mounted in DPX.

2.15.2 SINGLE AND DOUBLE IMMUNOHISTOCHEMICAL STAINING USING VECTASTAIN ABC KIT.

Staining using the Vectastain ABC (Vector Lab. Peterborough, UK) was performed following manufacturer's protocol. Briefly, the tissues sections stained with primary antibody as above were incubated with anti-mouse Ig–biotin (1:200) diluted in PBNT buffer for 30 min at 20°C. After washing twice with PBNT buffer for 10 min the sections were incubation with avidin–biotin–HRP complex (1:200) diluted in PBNT buffer for 30 min at 20°C. Thereafter the complexes were visualised, enhanced, sections counter stained and mounted as described in section 2.15.1. For double staining, the tissue sections were stained with one primary antibody as usual and processed up to visualisation using DAB as described above. After two washes in PBNT buffer for 10 min the tissues were again incubated with a second primary antibody for 60 min at 20°C, followed by incubation with anti-mouse Ig–biotin as above. The sections were incubated for 30 min at 20°C with avidin-biotin-alkaline phosphatase complex after washing twice with PBNT buffer for 10 min. The sections were washed in PBNT buffer followed by TBS (0.1 M Tris HCl, 0.15 M NaCl) pH 8.6 before being visualised with red chromogen (Vector Red) diluted in TBS pH 8.6 containing 1mM levamisole (Sigma). The slides were mounted in DPX as described in section 2.15.1 without any counter-stain.
CHAPTER THREE

CHARACTERISATION OF THE OVINE CD14 HOMOLOGUE
3.1 INTRODUCTION

Human CD14 (HCD14) is a myeloid differentiation antigen expressed predominantly on the surface of monocytes and Mφ (Goyert et al., 1989). It functions as high affinity receptor for LPS-LBP complex and upon binding stimulates mononuclear phagocytes (MP) to synthesise and secrete immunoregulatory and inflammatory molecules such as TNF-α, IL-1, IL-6 and IL-8 (Wright et al., 1990; Ulevitch, 1993). The molecule is expressed at low levels by granulocytes and is absent from stem cells and early differentiation states of myeloid cells (Hogg and Horton, 1987; Gadd, 1989). It is a cell surface glycoprotein of Mr = 55,000, anchored to the cell surface via a GPI linkage (Haziot et al., 1988). In addition it exists in at least 3 soluble forms of Mr = 53,000, present in urine of nephrotic patients and of Mr = 48,000 and Mr = 56,000 present in the serum of normal individuals and culture supernatants of CD14+ cells (Haziot et al., 1988; Bazil et al., 1986; Labeta et al., 1993a). These soluble forms also bind LPS and inhibit LPS induced TNF-α production by monocytes/Mφ (Haziot et al., 1994). The sCD14 bound to LPS was able to stimulate endothelial and epithelial cells by binding through an unknown receptor which was associated with secretion of cytokines and upregulation of adhesion molecules by these cells (Pugin et al., 1993).

At least three distinct epitopes on HCD14 antigen have been identified (Goyert et al., 1989). One of the epitopes is not expressed by Langerhans cells and has a low level of expression on granulocytes. The other two epitopes have normal distribution on Langerhans cells, granulocytes, monocytes and Mφ. Such difference in the expression suggests post-translational modification of the antigen or that one of the epitopes is lacking on the antigen expressed by some cells. Reactivity of some of the anti-HCD14 mAb in primates and pigs (Ziegler-Heitbrock and Ulevitch, 1993) suggests conservation of the antigen and some of the epitopes across species.

The mouse and HCD14 genes were found to be highly conserved in their intron-exon organisation and nucleotide sequences (Ferrero et al., 1990). Both human and mouse CD14 lack a characteristic transmembrane region and were found to be anchored to the cell membrane via a GPI linkage (Haziot et al., 1988 and Simmons et al., 1989). Similar expression and function of mouse CD14 (MCD14) have been described (Matsura, 1993). The structure, distribution and functions of this antigen are discussed in detail in section 1.8.
In this chapter, the phenotypic and immunochemical characteristics, of the ovine homologue of CD14 recognised by three murine mAb is described.

3.2 RESULTS
The monoclonal antibodies to sheep AM were produced with the aim of developing reagents for monocyte/Mφ differentiation antigens.

3.2.1 PRODUCTION OF MONOCLONAL ANTIBODIES
The fusion of spleen cells of BALB/c female mice immunised using sheep AM, with NSO myeloma cells was performed as described earlier (section 2.5). One hundred and nineteen hybridomas obtained from one fusion were screened for the presence of specific antibodies by FACS analysis of AM. The supernatants from eight clones showing reactivity with AM were further analysed for the staining of peripheral blood leukocytes (PBL), and could be differentiated into four groups (Table 3.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Clone Number</th>
<th>Cells Staining</th>
<th>Designated Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1A11</td>
<td>AM +, PBMC -, PBG -</td>
<td>IgG₁, VPM63</td>
</tr>
<tr>
<td>B</td>
<td>2C4</td>
<td>AM +, PBMC low + (sub), PBG +</td>
<td>IgG₁, VPM64</td>
</tr>
<tr>
<td>C</td>
<td>2G5</td>
<td>AM +, PBMC high + (sub), PBG +</td>
<td>IgG₁, VPM65</td>
</tr>
<tr>
<td>C</td>
<td>5F6</td>
<td>AM +, PBMC high + (sub), PBG +</td>
<td>IgG₁, VPM66</td>
</tr>
<tr>
<td>C</td>
<td>6C10</td>
<td>AM +, PBMC high + (sub), PBG +</td>
<td>IgG₁, VPM67</td>
</tr>
<tr>
<td>D</td>
<td>2D2</td>
<td>AM +, PBMC +, PBG +</td>
<td>ND, ND</td>
</tr>
<tr>
<td>D</td>
<td>2F2</td>
<td>AM +, PBMC +, PBG +</td>
<td>ND, ND</td>
</tr>
<tr>
<td>D</td>
<td>5D2</td>
<td>AM +, PBMC +, PBG +</td>
<td>ND, ND</td>
</tr>
</tbody>
</table>

AM, alveolar macrophages; PBMC, peripheral blood mononuclear cells; PBG, peripheral blood granulocytes; high, high intensity of staining; low, low intensity of staining; (sub), subpopulation of cells; +, staining; -, not staining; ND, not done.
The hybridomas in group A, B and C were cloned twice by limiting dilution (section 2.5) and these cloned cell lines were grown in bulk cultures for production of saturated supernatant and ascitic fluid for further characterisation. The mAb produced by the clones, 1A11, 2C4, 2G5, 5F6 and 6C10 were designated as VPM63, VPM64, VPM65, VPM66 and VPM67 respectively and will be referred to by these names in rest of the thesis (Table 3.1). The results of the characterisation of VPM63 and VPM64 are described in Chapter 4. Three mAb, namely VPM65, VPM66 and VPM67 behaved identically and are described in this chapter.

3.2.2 IMMUNOGLOBULIN ISOTYPE ANALYSIS

Isotype analysis of cloned monoclonal supernatants was performed (section 2.6) and all the five selected clones were found to produce the IgG1 isotype of mouse immunoglobulin.

3.2.3 FLOW CYTOMETRIC ANALYSIS

The reactivity of VPM65, VPM66 and VPM67 on individual leukocyte (AM, ADC, PBL, afferent and efferent lymphocytes) populations was investigated by FACS using indirect immunofluorescence (section 2.11). The mAb VPM18 (anti-CD45), VPM46 (anti-MHC class II), and BAQ30A (anti-CD18) were used as positive controls and VPM53 (IgG1, anti-campylobacter) and normal mouse serum 1:500 (NMS) were used as negative controls. Different cell types were distinguished by live gating using FSC and SSC parameters and dead cells were excluded as described earlier (section 2.11.2; Fig. 2.4). The results are expressed as peak (mode) fluorescence intensity where 100% of the cells are stained, and as percent (%) positive where a subpopulation is stained (Table 3.2). The reactivity with isotype matched negative control (VPM53, IgG1) is also given. The results obtained with VPM65 were identical to those by VPM66 and VPM67. RBC and platelets were not stained by any of these mAb (results not shown).

Alveolar macrophages (AM) VPM65 stained all the AM (live gate, Fig. 2.4) and the results are shown in Fig. (3.1) including a negative control.

Peripheral blood mononuclear cells (PBMC) A high intensity staining of a sub population (8-15%) of PBMC was observed (Fig. 3.1) when monocytes and lymphocytes were analysed together. Analysis of cells staining in relation to FSC (cell size) by the 'Paint-a gate' programme (Becton and Dickenson) illustrated that the intensely stained cells were qualitatively larger than the unstained cells indicating that
they are blood monocytes. Thus suggesting a mixed scatter profile of monocytes and lymphocytes.

Monocyte enriched mononuclear cells (MEMC) MEMC (section 2.3.2) were analysed by setting a gate on large cells as shown earlier (Fig. 2.4). VPM65 showed a high intensity staining of the majority (90%) of these cells. The overlay histograms, including negative control, are shown in Fig. (3.1). The small population of cells amongst MEMC not stained by these mAb was mainly T or B lymphocytes (Fig 3.2).

Peripheral blood granulocytes (PBG) PBG were analysed by staining PBL (section 2.3.5) and setting a live gate as shown earlier (Fig. 2.4). VPM65 showed moderate intensity staining of all the PBG (Fig. 3.1).

Afferent lymph dendritic cells (ADC) Metrizamide enriched ADC were analysed after live gating as shown earlier (Fig. 2.4). VPM65 showed a low intensity staining of ADC (Fig 3.1).

Lymphocytes in peripheral blood, afferent and efferent lymph. Blood lymphocytes were analysed after staining monocyte depleted mononuclear cells (MDMC, section 2.3.2) (live gate, Fig. 2.4). Afferent and efferent lymphocytes were analysed after setting a live gate as shown earlier (Fig. 2.4). VPM65 did not stain any lymphocyte in blood (Fig. 3.1), afferent and efferent lymph (data not shown). During analysis of cells after setting a small gate on peripheral blood mononuclear cells (Fig. 2.4), a few cells were found positive, but in monocyte depleted mononuclear cells (MDMC) such staining was only occasionally observed. This is possibly due to the presence of a small number of monocytes in the lymphocyte gate and was confirmed by two-colour FACS analysis in the experiment described below.

Double staining of PBMC with VPM65 for co-expression on B and T lymphocytes In order to find co-expression of VPM65 antigen on B and T lymphocytes, two-colour FACS analysis of PBMC (live gate, Fig. 2.5) was performed after simultaneous staining with VPM65 and VPM30 (pan B-cell mAb) or SBUT1 (anti-CD5, a T-cell marker). These results showed that VPM65 antigen was not present on any of the T and B lymphocyte populations (Fig. 3.2).
TABLE 3.2 Mean (Range) of Peak Fluorescence Intensity/Percent (%) cells Reacting with VPM65, VPM66 and VPM67.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Peak Fluorescence Intensity</th>
<th>% Cells Reacting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM (n = 6)1</td>
<td>MEMC (n = 3)</td>
</tr>
<tr>
<td>VPM65</td>
<td>7.2 (6-8)3</td>
<td>46.4 (20-76)</td>
</tr>
<tr>
<td>VPM66</td>
<td>7.2 (6-9)</td>
<td>39.6 (22-51)</td>
</tr>
<tr>
<td>VPM67</td>
<td>7.4 (6-10)</td>
<td>34.8 (19-45)</td>
</tr>
<tr>
<td>VPM532</td>
<td>2.6 (2-3)</td>
<td>4.1 (3-5)</td>
</tr>
</tbody>
</table>

AM, alveolar macrophages; MEMC, monocyte enriched mononuclear cells; PBG, peripheral blood granulocytes; ADC, afferent dendritic cells; PBMC, peripheral blood mononuclear cells; MDMC, monocyte depleted mononuclear cells.

1 number of samples examined.
2 VPM53 (isotype control), showing background reactivity.
3 (range of reactivity)
FIGURE 3.1  Frequency histograms of: (a) Peripheral blood mononuclear cells (PBMC), (b) Monocyte enriched mononuclear cells (MEMC), (c) Alveolar macrophages (AM), (d) Peripheral blood granulocytes, (e) Afferent lymph dendritic cells (ADC) and (f) Monocyte depleted peripheral blood mononuclear cells (MDMC) mainly lymphocytes, stained with VPM65 (——) and VPM53 (-----) an irrelevant IgG1 isotype control. Similar results were obtained with VPM66 and 67 (not shown).
FIGURE 3.2 Contour plots of two-colour staining of monocyte enriched mononuclear cells (MEMC) and peripheral blood mononuclear cells (PBMC) showing VPM65 staining on FL2, and VPM30 (pan B-cell) (A) and SBUT1 (CD5, T-cell) (B) staining on FL1. Similar results were obtained with VPM 66 and 67 (not shown).
Bone marrow cells The bone marrow (BM) cells were analysed after setting a live gate excluding dead cells and most of RBC. Three analytical gates were chosen to define bone marrow cell subpopulations (Haig et al., 1991), R1 (37-41% of BM cells): predominantly contain small nucleated erythroid series cells, RBC and a few small lymphocytes, R2 (15-21% of BM cells): monocyte/Mφ series cells, myeloid blast cells and some medium-large lymphocytes, and R3 (31-36% of BM cells): granulocytes at various stages of maturation (>90% neutrophils and eosinophils) (Fig. 3.3). VPM65 stained a small proportion (1 to 2%) of total nucleated cells of bone marrow which mainly constitute 11-12% of the cells gated in R2 (monocyte/macrophage and large lymphoid series cell) and 3-4% in R3 (granulocyte series cells). Cells gated in R1 were not stained by VPM65. Analytical gating of bone marrow cells (R1, R2 and R3) and the results of staining with VPM65 are shown in Fig. 3.3.

3.2.4 IMMUNOCHEMICAL ANALYSIS
The biochemical characteristics of VPM65, 66 and 67 antigens were investigated by immunoprecipitations of proteins and deglycosylation of the antigens as well as by immuno-blotting as described earlier (section 2.13).

Immunoprecipitation All three mAb immunoprecipitated a single polypeptide chain which has an apparent Mr = 55,000 from AM under both reducing and non-reducing conditions. The results of immunoprecipitations using VPM65, 66 and 67 along with VPM53 (negative control) are shown in Fig. 3.4. None of the three mAb recognised this antigen on western blot analysis (data not shown).

Deglycosylation In order to determine the amount of N-linked carbohydrate associated with the molecule the immunoprecipitates were treated with N-glycosidase F as described earlier (section 2.13.2). After deglycosylation the Mr of the antigen recognised by each of these mAb was reduced by approximately 3000 Da (Fig. 3.5).

3.2.5 GLYCOSYL PHOSPHATIDYL INOSITOL (GPI) LINKAGE OF ANTIGEN
In order to investigate if the antigen recognised by VPM65/66/67 is associated with the cell surface via a GPI linkage, cells were treated with enzyme phosphatidyl inositol phospholipase-C (PIPL-C) and then analysed by FACS (section 2.12). This enzyme digests the GPI linkage and releases the antigen molecule from the cell surface. The peak fluorescence intensity of AM and blood monocytes stained with VPM65 after PIPL-C treatment was reduced by >90% (Fig. 3.6). Similar treatment of granulocytes
FIGURE 3.3 (a) Forward and side angle scatter profile of bone marrow (BM) cells showing analytical gates R1, R2 and R3.
Frequency histograms of all the bone marrow cells (b), cells in R1 (c), R2 (d), and R3 (e) stained with VPM65 (——) and VPM53 (······) an isotype control.
FIGURE 3.4 Autoradiograph of $^{125}$I labelled cell surface antigens immunoprecipitated from alveolar macrophage detergent lysate using VPM65, VPM66 and VPM67 by ELISA plate method. The samples were fractionated on 5-20% gradient SDS-PAGE under non-reducing condition (a), and reducing condition (b). VPM53 was used as isotype negative control (a & b) and BAQ30A (anti-CD18) as positive control (a).
FIGURE 3.5 Autoradiograph of $^{125}$I labelled cell surface antigens immunoprecipitated from alveolar macrophage detergent lysate using VPM65, VPM66 and VPM67 by protein-A sepharose method. The eluted antigens were fractionated on 12.5% linear SDS-PAGE after N-glycosidase F treatment as described earlier. As a control VPM65 antigen was also fractionated without N-glycosidase F.
resulted in 100% loss of VPM65/66/67 staining (Fig. 3.6). Identical results were obtained with VPM66 and VPM67 (data not shown). The peak fluorescence intensity of the PIPL-C treated or untreated cells stained with BAQ30A (anti-CD18) mAb remained unchanged (Fig. 3.6). This demonstrates that VPM65/66/67 antigen is GPI linked.

3.2.6 DIFFERENTIATION OF THE EPITOPE RECOGNISED BY VPM65, 66 AND 67
The results shown above suggest that VPM65, 66 and 67 all recognise the same or closely related GPI-anchored cell surface antigen(s) of apparent Mr = 55,000. In order to determine the relationship between the antigens/epitopes recognised by the three mAb, AM were incubated with saturating concentrations of VPM65, 66 and 67 for 60 min. VPM65 completely blocked the subsequent binding of biotinylated VPM65, VPM66 and VPM67 as visualised using SA-PE by FACS analysis. A similar blocking effect was observed with VPM66 and 67. The binding of unrelated mAb VPM46 (anti-MHC class II) remained unaffected (Fig. 3.7). This shows that VPM65, 66 and 67 recognise the same or overlapping epitopes on the same molecule.

3.2.7 REACTIVITY OF HUMAN CD14 (HCD14) mAb IN SHEEP
Recently an anti-HCD14 mAb TUK4 (Dako, Denmark) of mouse origin (IgG2a isotype) was reported to be reacting in cattle (Alder et al., 1994). In humans TUK4 recognises a Mr = 55,000 single chain GPI-anchored cell membrane glycoprotein. The antigen is primarily expressed on monocytes and Mφ but also on granulocytes, dendritic reticulum cells and Langerhans cells (Goyert et al., 1989). The mAb also reacted in sheep, where it stained a subpopulation of PBMC and showed moderate reactivity with AM but did not stain PBG (Fig. 3.8). The reactivity of TUK4 could not be blocked with VPM65/66/67 separately or when used together (Results not shown). Double staining of PBMC with VPM65 and TUK4 not only revealed double labelling of all the same cells but produced a diagonal contour plot (Fig. 3.9), characteristic of labelling of same or very closely associated cell surface molecule(s) with two fluorochromes (FITC and PE). As a control, similar diagonal contour plot produced by double staining of cell surface immunoglobulin with anti-Ig light chain (VPM8) and anti-IgM heavy chain (VPM13) mAb is shown (Fig. 3.9). These results suggest that VPM65/66/67 and TUK4 recognise two different epitopes of the same antigen or very closely related antigens on the surface of monocytes. But the VPM65/66/67 epitope is present on sheep granulocytes whereas TUK4 epitope is absent.
FIGURE 3.6 Frequency histograms of, monocyte enriched mononuclear cells (a), alveolar macrophages (b) and blood granulocytes (c). Fluorescence (—) of PIPL-C treated (+) or untreated control (-) cells stained with VPM65 and BAQ30A (anti-CD18). Fluorescence (----) of parallel cell samples stained with VPM53 (isotype negative control). The peak fluorescence staining intensity of monocytes and alveolar macrophages, and granulocytes labelled with VPM65 after PIPL-C treatment was reduced by 94% and 100% respectively. The staining of anti-CD18 was not affected by PIPL-C treatment. Similar results were obtained using VPM66 and VPM67 (not shown).
FIGURE 3.7 Showing differentiation of epitopes recognised by VPM65/66/67. Frequency histograms of alveolar macrophages (AM) incubated for 60 min with VPM53 (IgG1, isotype negative control) before staining with biotinylated antibody (——) and biotinylated normal mouse serum (· · · ·) showing normal staining. AM blocked by incubating with saturating concentration of VPM65 before staining with biotinylated antibody (-----) VPM65 (a), VPM66 (b), VPM 67 (c) and VPM46 (anti-MHC class II) an unrelated antibody (d). Similar results were obtained when AM were blocked with saturating concentration of VPM66 and VPM67 (not shown).
FIGURE 3.8 Frequency histograms of peripheral blood mononuclear cells (a), alveolar macrophages (b), and peripheral blood granulocytes (c), stained with anti-HCD14 monoclonal antibody TUK4 (---) and normal mouse serum, negative control (· · ·). Staining of peripheral blood granulocytes with VPM65 (-----) is also shown (c) for comparison.
FIGURE 3.9 Two-colour contour plots of peripheral blood mononuclear cells. VPM65 and TUK4 (anti-HCD14) double stained cells produced a diagonal contour plot (a), similar to the contour plot produced by double labelling of Ig light chain (VPM8) and μ heavy chain (VPM13) of cell surface IgM (b), in contrast μ heavy chain (VPM13) and pan B-cell (VPM30) double labelling of different cell surface molecules produced normal contour plot (c).
3.2.8 ANTIGEN PRECLEARING STUDIES

To investigate further if the mAb VPM65 and TUK4 recognise the same cell surface antigen, sequential immunoprecipitations were performed. Both the mAb precipitated the antigen of the same $M_r = 55,000$ from surface radioiodinated AM lysate (Fig. 3.10). However the band of the antigen precipitated by TUK4 was weaker in intensity as compared to that of VPM65. For sequential precipitations the VPM65 antigen was removed from $^{125}$I labelled cell lysate by five sequential cycles with VPM65 mAb linked to protein A-sepharose as described earlier (section 2.13.1). Subsequent precipitation using TUK4 failed to precipitate any antigen from the VPM65 precleared lysate, however an unrelated molecule using VPM63 mAb could be precipitated from VPM65 precleared lysate (Fig. 3.10). The positive precipitation of an antigen of $M_r = 55,000$ with TUK4 mAb before but not after preclearing with VPM65, and all preceding data indicate that these mAb are specific for sheep CD14.

3.2.9 IMMUNOHISTOCHEMISTRY

In order to determine the tissue distribution, frozen sections of various lymphoid (thymus, spleen and peripheral lymph node) and non lymphoid organs (lung, liver, kidney, intestine, skin, brain and cotyledon) were stained with VPM65, VPM66 and VPM67 by indirect immunoperoxidase technique (section 2.15.1). Routine formalin fixed, paraffin embedded sections did not reveal any reactivity using a similar staining technique. Negative controls were stained with VPM53 saturated supernatant or normal mouse serum (1:200) instead of primary antibody. The results of immunohistochemical distribution of VPM65 were identical to those of VPM66 and VPM67, except for some differences observed in the staining of intestine sections. The tissue distribution of MP stained with these mAb is summarised in Table 3.3.

Peripheral lymph node The reactivity of VPM65 in prefemoral lymph node was restricted to mononuclear phagocytes (MP). In the cortex, $\Phi$ and interdigitating reticulum cells (IDC) were stained in the interfollicular T-cell area whereas no staining was observed in the follicles (Fig. 3.11a). The $\Phi$ in the medullary (Fig. 3.11b) and subcapsular sinuses were also stained.

Thymus VPM65 stained $\Phi$ with cytoplasmic processes throughout the thymus. These cells were comparatively strongly labelled and more abundant in the cortex, than in the medulla (Fig. 3.11c). The mAb did not react with other thymic structures.
FIGURE 3.10 Auto-radiograph of sequential immunoprecipitations of $^{125}$I labelled cell surface antigen from alveolar macrophage detergent lysate using VPM65 and TUK4 (anti-HCD14) and VPM53 (isotype negative control) by protein-A-sepharose method. Control precipitations, VPM53 (lane 1), TUK4 (lane 2) and VPM65 (lane 3); sequential depletion of antigen using VPM65 first cycle (lane 3) and last cycle (lane 4); followed by precipitations using TUK4 (lane 5) and VPM63 (lane 6) a unrelated positive control antibody.
Spleen  In the spleen, VPM65 weakly labelled elongated cells resembling cordal Mφ running along the red pulp sinuses. A few Mφ in the periarteriolar lymphoid sheath and marginal zone were also stained (Fig. 3.11d).

Lung  In the lung sections VPM65 weakly stained both AM and interstitial macrophages present in the airspaces and interstitial tissue respectively (Fig. 3.12a).

Liver  VPM65 weakly stained a few Kupffer cells both in the hepatic sinusoids (Fig. 3.12b) and in the portal area. But the majority of Kupffer cells remained negative.

Kidney  In kidney, glomerular cells were stained with VPM65. Interstitial cells which have cytoplasmic processes were also stained at the junction of the tubules (Fig. 3.12c).

Skin  VPM65 stained dermal Mφ but epidermal Langerhans cells could not be stained even with Vectastain ABC method (Fig. 3.12d).

Intestine  VPM65, 66 and 67 could be discriminated on immunohistochemical staining of jejunal Peyer's patches. The three mAb were similar in staining, interfollicular T-cell area Mφ and weakly stained lamina propria Mφ (Fig. 3.13a). In addition to this, VPM65 and VPM67 weakly stained few Mφ in jejunal Peyer's patch follicles whereas VPM66 failed to stain follicular Mφ (Fig. 3.13b). Only VPM65 showed cross reactivity with mucosal lining of the intestinal crypts (Fig. 3.13a).

Placental cotyledon  VPM65 stained lamina propria and perivascular Mφ in the sections of full term cotyledons (Fig. 3.13c).

Brain  VPM65 stained some elongated microglial like cells in the sections prepared from cerebrum (Fig. 3.13d).

3.2.10 REACTIVITY WITH CELLS OF OTHER SPECIES
VPM65, 66 and 67 reactivity was observed with cattle peripheral blood leukocytes and frozen sections of the thymus. These mAb stained a subpopulation of PBMC as seen in sheep but failed to stain any granulocytes in cattle blood. Immunohistochemically the distribution of staining of thymus macrophages was the same as observed in sheep where both cortical and medullary Mφ with cytoplasmic
### TABLE 3.3 Immunohistochemical Distribution of Mφ Stained with VPM65 in Frozen Sections of Various Lymphoid and Non-Lymphoid Organs.

<table>
<thead>
<tr>
<th>Tissue (n = 4)*</th>
<th>VPM65$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LYMPH NODE</strong></td>
<td>Subcapsular &amp; Medullary sinus</td>
</tr>
<tr>
<td></td>
<td>Interfollicular area</td>
</tr>
<tr>
<td></td>
<td>Follicular dendritic cells</td>
</tr>
<tr>
<td><strong>THYMUS</strong></td>
<td>Cortex</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
</tr>
<tr>
<td><strong>SPLEEN</strong></td>
<td>Red pulp</td>
</tr>
<tr>
<td></td>
<td>Marginal zone &amp; PALS</td>
</tr>
<tr>
<td><strong>JEJUNUM</strong></td>
<td>Peyer's patch follicles</td>
</tr>
<tr>
<td></td>
<td>Interfollicular area</td>
</tr>
<tr>
<td></td>
<td>Lamina propria</td>
</tr>
<tr>
<td></td>
<td>Glandular mucosa</td>
</tr>
<tr>
<td><strong>LUNG</strong></td>
<td>Alveoli</td>
</tr>
<tr>
<td></td>
<td>Interstitium</td>
</tr>
<tr>
<td><strong>LIVER</strong></td>
<td>Kupffer's cells</td>
</tr>
<tr>
<td><strong>KIDNEY</strong></td>
<td>Glomerulus</td>
</tr>
<tr>
<td></td>
<td>Interstitium</td>
</tr>
<tr>
<td><strong>SKIN</strong></td>
<td>Epi. Langerhans cells</td>
</tr>
<tr>
<td></td>
<td>Dermis</td>
</tr>
<tr>
<td><strong>PL. COTYLEDONS</strong></td>
<td>Perivascular area</td>
</tr>
<tr>
<td></td>
<td>Lamina propria</td>
</tr>
<tr>
<td><strong>BRAIN</strong></td>
<td>Microglial cells</td>
</tr>
</tbody>
</table>

* number of samples examined.
+ positive staining; - no staining; ± weakly staining few cells, PALS, periarteriolar lymphoid sheath.
$^1$ Staining with VPM66 and VPM67 was similar to VPM65 except
$^2$ Follicular Mφ were not stained by VPM66 and
$^3$ Glandular mucosa was not stained by VPM66 and VPM67.
FIGURE 3.11 Indirect immunoperoxidase staining of VPM65 in frozen sections of:

(a) Prefemoral lymph node cortex, showing staining of M\(\Phi\) (arrow) and interdigitating reticulum cells (arrow head) in the interfollicular area. No staining of follicular (F) cells was observed. x 100.

(b) Prefemoral lymph node medulla, showing staining of M\(\Phi\) (arrow) lining medullary sinuses. x 100.

(c) Thymus, showing staining of M\(\Phi\) in the cortex (C) and weak labelling of M\(\Phi\) in the medulla (M). x 100. Inset, cortical M\(\Phi\) in higher magnification showing dendritic morphology. (x 250).

(d) Spleen, showing staining of elongated cordal M\(\Phi\) running along the red pulp (RP) sinuses, few M\(\Phi\) in the periarteriolar lymphoid sheath (PALS) and marginal zone area (MZ). x 250.
FIGURE 3.12 Indirect immunoperoxidase staining of VPM65 on frozen sections of:

(a) Lung, showing staining of alveolar MΦ (arrow) and interstitial MΦ (arrow head). x 250.

(b) Liver, showing weak staining of isolated Kupffer cells in the hepatic sinusoids (arrow). x 250.

(c) Kidney, showing staining of glomerular cells (arrow) and interstitial MΦ (arrow head) at the junction of renal tubules. x 250.

(d) Skin, showing staining of dermal MΦ. x 250.
FIGURE 3.13 Indirect immunoperoxidase staining of frozen sections of:

(a) Jejunum with VPM65, showing staining of interfollicular (IF) Mφ and a weak labelling of Mφ in the follicle (F, arrow) and lamina propria (LP, arrow). VPM65 also cross reacted with the mucosal lining (arrow head) of the crypts. x 100.

(b) Jejunum with VPM66, showing staining of Mφ in the inter follicular space (IF, arrow head) and lamina propria (LP, arrow). x 100.

(c) Full term placental cotyledon with VPM65, showing staining of perivascular Mφ (arrow) in the sub mucosal area and Mφ in the lamina propria (arrow head). x 100.

(d) Brain (cerebrum) with VPM65, showing staining of irregular elongated microglial like cells (arrow) in the parenchyma. x 250.
processes were stained. These mAb did not recognise any human antigen when tested on PBL.

3.3. DISCUSSION
This study describes the production and characterisation of three mAb, VPM65, VPM66 and VPM67 which originated from three distinct clones and were directed against the cell surface antigens of monocytes and Mφ. In addition some reactivity on granulocytes and ADC was also observed. These mAb immunoprecipitated a single band of apparent Mr = 55,000 from cell surface antigens derived from alveolar macrophages. A number of murine mAb have been reported which recognise HCD14, a glycoprotein of Mr = 55,000 expressed on monocyte, macrophage, granulocytes and mature myeloid cell lines (Gadd, 1989; Goyert et al., 1989). Antigen preclearing studies and two colour flow cytometry using VPM65 and TUK4 (mouse anti-HCD14 mAb) revealed that both the mAb reacted with the same (Mr = 55,000) antigen in sheep.

In sheep the antigen recognised by VPM65, 66 and 67 is anchored to the cell surface via a GPI linkage as revealed by phosphatidyl inositol phospholipase-C (PIPL-C) enzyme digestion. This is in agreement with the earlier observations in humans where cell surface expression of CD14 antigen on human monocytes and HCD14 transfected cell lines was considerably reduced on PIPL-C treatment (Haziot et al., 1988). And complete loss of CD14 antigen was observed on PIPL-C treatment of CD14 transfected COS cells (Simmons et al., 1989). Both groups showed lack of reactivity of anti-HCD14 mAb with monocytes obtained from paroxysmal nocturnal hemoglobinuria (PNH) patients having a genetic defect in GPI linkage. The deduced protein sequences of both human and mouse CD14 revealed lack of characteristic transmembrane region (Haziot et al., 1988; Simmons et al., 1989). This indicates that CD14 exists only in a GPI-linked form on the cell surface and the feature is conserved across species.

The sheep antigen recognised by VPM65/66/67 is a glycoprotein having approximately 3000 Da (about 5%) N-linked carbohydrate as revealed by N-glycosidase F digestion. In contrast HCD14 has been shown to consist of about 20% N-linked carbohydrate as revealed by treatment with endoglycosidase (Maliszewski et al., 1985; Goyert et al., 1986). Such variations in the level of glycosylation may be attributed to the different species involved. One such example of difference in glycosylation between species is that sheep TNF-α is glycosylated...
(Green et al., 1993) in contrast human TNF-α is not glycosylated (Pennica et al., 1984).

Lack of VPM65/66/67 reactivity with lymphocytes in sheep is similar to the observations in humans where lymphocytes were found to be deficient in cell surface expression of CD14 (Hancock et al., 1983; Franklin et al., 1986) and CD14 specific mRNA (Goyert et al., 1988). This suggests a similar myeloid restricted expression of the antigen in sheep.

Reactivity of these mAb with a small proportion of BM cells in sheep (R2, Fig. 3.3) is similar to CD14 expression on mature monocytes in BM in humans (Loken et al., 1987). Presence of CD14 specific mRNA in human monocytes and granulocytes but not in immature myeloid cells (Goyert et al., 1988) is suggestive of its expression in mature myeloid cells.

The tissue localisation of HCD14 antigen has been described in various lymphoid and non lymphoid organs (Franklin et al., 1986; Hancock et al., 1983; Koller 1989). This distribution seems to be quite similar to the present observations in sheep except for some minor differences. The localisation of the antigen recognised by VPM65/66/67 on MP was similar to that of HCD14 in thymus (Franklin et al., 1986), lung, liver, skin (Franklin et al., 1986; Hancock 1983; Koller, 1989), and placenta (Bulmer and Johnson, 1984; Sutton et al., 1986). On the basis of epidermal Langerhans cells staining, anti-HCD14 mAb have been divided into two groups, where one group of mAb stained these cells while the other failed (Franklin 1986; Koller 1989; Goyert et al., 1989). The antigen polymorphism has not been reported so far in humans. In the present study Langerhans cells were not stained by immunohistochemical techniques used though afferent lymph dendritic cells were weakly labelled as determined by FACS analysis. Goyert et al., (1989) reported lack of expression of one of the CD14 epitopes on Langerhans cells which was associated with low levels of expression on granulocytes. Thus the epitope recognised by VPM65/66/67 mAb in sheep seems to be similar to the one which is absent on Langerhans cells in humans.

In lymph node and spleen the staining of VPM65/66/67 was restricted to MP and was similar to the earlier observations in human (Franklin et al., 1986; Hancock et al., 1983) except for the lack of staining of follicular dendritic cells (FDC) in sheep. In kidney, staining of interstitial cells in sheep is similar to the earlier observations in human, whereas the staining of all glomerular cells in sheep is in contrast to the
isolated glomerular cell staining in human (Franklin et al., 1986). Staining of a few microglial cells in sections of brain tissue in sheep is similar to human (Franklin et al., 1986) in contrast perivascular Mφ were not stained in sheep. The competitive binding assay of these mAb (VPM65, 66 and 67) revealed their binding to the same or very closely related epitopes but the minor differences observed in the staining of Peyer's patch might be attributed to differences in the binding affinity of the three mAb.

Species cross reactivity of VPM65, 66 and 67 with bovine monocytes and Mφ but not granulocytes, and anti-HCD14 mAb (TUK4) with sheep monocytes and AM but not granulocytes suggests that some of the CD14 epitopes are conserved across species. However anti-sheep mAb VPM65, 66 and 67 failed to react with the epitope present on human monocytes and granulocytes indicating some species variations in the antigen.

These observations confirm the reactivity of VPM65, VPM66 and VPM67 with the ovine homologue of human CD14. Besides minor differences CD14 seems to be fairly conserved across species, thus one can speculate similar functional significance of the molecule as a receptor for LPS in sheep. An experiment to study whether sheep CD14 has a similar functional activity in binding LPS-LBP complexes leading to the secretion of TNF-α was planned. Due to non availability of a quantitative TNF-α assay and shortage of time to establish such assay, the functional significance of this antigen in sheep remains to be established.

3.4 CONCLUSION
From the observations made above it is concluded that mAb VPM65, VPM66 and VPM67 recognise sheep homology of the human monocyte antigen CD14. This conclusion is based on their reactivity with a GPI anchored cell surface glycoprotein of Mr = 55,000 which is primarily expressed by monocytes and macrophages, and that VPM65 and anti-HCD14 (TUK4) recognise the same antigen as revealed by antigen pre-clearing studies and two-colour flow cytometry.
CHAPTER FOUR

CHARACTERISATION OF ANTI-Fcγ RECEPTOR MONOCLONAL ANTIBODIES
4 CHARACTERISATION OF ANTI-Feγ RECEPTOR MONOCLONAL ANTIBODIES

4.1 INTRODUCTION
Receptors for the Fc domain of IgG (FeγR) play a central role in the immune responses by providing a bridge between the humoral and cellular branches of the immune system. Interaction of FeγR with its ligand (IgG), triggers a variety of biological activities. These include antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, endocytosis, stimulation of degranulation releasing inflammatory and cytotoxic mediators, and enhancement of antigen uptake processing and presentation (Nathan et al., 1980; Fanger et al., 1989; Van de Winkel and Capel, 1993). FeγR on lymphocytes has regulatory functions, such as lymphocyte proliferation, cytokine and antibody production (Sandor and Lynch, 1993; Fridman, 1993).

There are three distinct but closely related classes of FeγR; FeγRI (CD64) has high affinity for monomeric IgG, while FeγRII (CD32) and FeγRIII (CD16) are low affinity receptors for complexed IgG (Van de Winkel and Capel, 1993; Ravetch and Kinet, 1991; Unkeless et al., 1988). FeγR are members of the immunoglobulin (Ig) superfamily with a variable number of extracellular domains. FeγRI has three Ig like extracellular domains. The first two domains are homologous to the two extra cellular domains of FeγRII and III while the third domain is unique for FeγRI (Ravetch and Kinet, 1991). FeγR are predominantly expressed in mononuclear phagocytes and neutrophils although some are also present on natural killer (NK) cells (FeγRIII), B lymphocytes (FeγRII) and activated T lymphocyte subpopulations (FeγRII and III ) (Ravetch and Kinet, 1991; Sandor and Lynch, 1993).

FeγRI, a glycoprotein of Mr = 72,000, is constitutively expressed by monocytes and Mφ, and is inducible in neutrophils and eosinophils (Van de Winkel and Capel, 1993). FeγRII, a glycoprotein of Mr = 40,000, is expressed by mononuclear phagocytes and a variety of other haemopoietic cells except NK cells. In humans this receptor has six isoforms encoded by three genes, FeγRIIA, B and C (Qiu et al., 1990). The products of FeγRIIB are preferentially expressed in lymphocytes, whereas IIA and IIC are preferentially expressed in neutrophils. Monocyte/Mφ express all three gene products. The extracellular and transmembrane domains of the different subclasses of human FeγRII are highly identical while the cytoplasmic domains are divergent (Brooks et al., 1989; Qiu et al., 1990). In mice, two isoforms of FeγRII, FeγRIIB1 and FeγRIIB2, are generated as a result of alternative splicing of sequences coding for
47 amino acid residues of the cytoplasmic domain (Ravetch et al., 1986; Miettinen et al., 1989). The FcγRIIb1 isoform which is mainly expressed on B and T lymphocytes has an 47-amino acid insertion in the cytoplasmic domain and is inefficient in mediating endocytosis. The FcγRIIb2 isoform which lack cytoplasmic insertion is found on Mφ and mediates endocytosis and delivery into lysosomes. Reactivity with mAb could not discriminate between these isoforms due to their identical extracellular domains (Ravetch et al., 1986).

Human and mouse FcγRIII are highly homologous to each other and immunoprecipitate as a broad band between Mr = 50,000 to 80,000 due to extensive glycosylation (Ravetch et al., 1986). In humans FcγRIII is encoded by two genes (A and B) giving rise to distinct products. The FcγRIIIA gene product is a conventional transmembrane receptor, expressed on macrophages, NK cells, some lymphocytes and monocytes. The FcγRIIIB gene product is linked to the plasma membrane via a glycosyl phosphatidyl-inositol (GPI) anchor and is selectively expressed on neutrophils and eosinophils (Ravetch and Perussia, 1989). The structures and functions of FcγRs are described in Chapter 1 (section 1.9).

Functional evidence for membrane FcγRs on monocyte/Mφ have been demonstrated in many animal species such as dog (Lucas et al., 1980), pig (Chrley and Frenove, 1980), horse (Dyer and Leid, 1983), cattle (Howard et al., 1980) and sheep (Harkiss et al., 1990; Jungi et al., 1992). Due to the lack of mAb specific for FcγR in cattle and sheep, the exact cellular and tissue distribution of the receptors is not available. Evidence for FcγRI in cattle has recently been demonstrated at the molecular level by sequencing extracellular domain exons (Symons and Clarkson, 1992). This study revealed 58% identity with the amino acid sequence of human and mouse FcγRI. More recently Zhang et al., (1994) reported a full length sequence of the gene encoding bovine FcγRII and showed 59% and 55% amino acids identity with human and mouse FcγRII respectively. An identity of 47% was shown when the sequence was compared with cattle FcγRII extracellular domains.

Gonzalez (1989) described three mouse anti-sheep macrophage mAb which recognise a molecule of Mr = 67,000 on immunoprecipitation and showed broad electrophoretic mobility on immunoblotting. These mAb were able to inhibit binding to sheep macrophages of erythrocytes coated with rabbit antibody. They recognised heterogenous populations of resident Mφ and also labelled some dendritic cells and lymphocytes. It has been suggested that these mAb recognise heterogenous epitopes.
of an antigen similar to human FcγRIII, but no confirmatory evidence is yet available. FcγR mediated erythrocyte rosetting and its inhibition using different IgG subclasses on sheep bone marrow macrophages (BMM) suggested the existence of two distinct FcγR similar to human FcγRI and II (Jungi et al., 1992). The molecules equivalent to the size of FcγRI and FcγRII were also identified from afferent dendritic cells in sheep using polyclonal antiserum to synthetic peptides of cattle FcγRI (Coughlan, 1994). The above findings in ruminants are suggestive of the presence of three different classes of FcγR similar to those present in man and mouse.

In this chapter the immunophenotypic and biochemical characteristics of two anti-sheep Mφ mAb VPM63 and VPM64 which recognise molecules equivalent to FcγRII and possibly FcγRIII respectively are described. VPM63 recognises a differentiation antigen exclusively expressed by resident tissue Mφ whereas VPM64 recognises an antigen expressed on a subpopulation of mononuclear cells, some tissue Mφ and granulocytes.

4.2 RESULTS

4.2.1 PRODUCTION OF MONOCLONAL ANTIBODIES AND ISOTYPE ANALYSIS
mAb were produced by immunisation of BALB/c mice with sheep AM as described (section 3.2.1). The immunoglobulin isotype of both mAb VPM63 and VPM64 was found to be IgG1 (section 3.2.2).

4.2.2 FLOW CYTOMETRIC ANALYSIS
Leukocyte populations (AM, ADC, PBL, afferent and efferent lymph cells) and bone marrow cells were stained with VPM63 and VPM64 for FACS analysis as described earlier (section 2.11). mAb VPM53 (anti-campylobacter) an irrelevant IgG1 isotype was used as negative control. VPM19 (anti-MHC class I) and BAQ30A (anti-CD18) were used as positive controls where appropriate. FACS data was analysed using programmes 'Consort-30' (Becton Dickenson). The results are expressed in terms of peak (mode) fluorescence intensity where 100% of the cell population is positive, or percent positive where a subpopulation is stained, the background negative control values are also shown (Table 4.1). The reactivity of VPM63 and VPM64 on RBC and platelets was negative (results not shown).

Alveolar Macrophages All the AM live gated as large complex cells (Fig. 2.4) were stained with both the mAb, VPM63 (Fig. 4.1) and VPM64 (Fig. 4.2).
Peripheral blood mononuclear cells (PBMC)  Peripheral blood leukocytes (PBL) stained using VPM63 and VPM64 were analysed by FACS after setting a live gate which included all the small and large mononuclear cells (Fig. 2.4). VPM64 stained a subpopulation of PBMC at low intensity (Fig. 4.2) whereas no staining was achieved with VPM63 (Fig 4.1). Two-colour FACS analysis of PBMC stained with VPM64, and VPM65 (anti-CD14 a monocyte marker, Chapter 3) divided VPM64+ cells into two subpopulations of VPM64+VPM65+ and VPM64+VPM65− cells (Fig. 4.3). However double labelling of PBMC with VPM64, and CC125 (anti-CD11b, Chapter 5) revealed co-expression of CD11b (a monocyte, NK cell and B cell subpopulation marker) on all VPM64+ cells. Double staining of PBMC using mAb VPM30 (pan B-cell marker) and SBUT1 (CD5, T cell marker) did not reveal any double positive cells with VPM64 (Fig. 4.3). VPM64 was also not expressed on individual subpopulations of T cells (SBUT4, CD4; SBUT8, CD8 and 86D, γδ T cells) (results not shown). These results show that the antigen recognised by VPM64 is not expressed on any B-cell and T-cell lineages in the blood. VPM64 is expressed on VPM65+ cells (monocytes) and a small subpopulation of VPM65− but CD11b+ cells possibly natural killer (NK) cells. The proportion of VPM65−VPM64+ PBMC varied from 2.6 to 3.5% as revealed by the results of two independent experiments, the results of one such experiment are shown in Fig. 4.3.

Monocyte enriched mononuclear cells  FACS analysis of monocyte enriched mononuclear cells (MEMC) stained with VPM64 indicated a low level of expression (Fig. 4.2) whereas VPM63 failed to stain MEMC (Fig. 4.5 la).

Peripheral blood granulocytes (PBG)  FACS analysis of PBG ( live gate, Fig. 2.4) showed no reactivity with VPM63 (Fig 4.1), whereas all the granulocytes were stained with VPM64 (Fig 4.2).

Afferent lymph dendritic cells (ADC)  Metrizamide enriched ADC were stained and analysed after setting a live gate as shown (Fig. 2.4). VPM64 stained a variable number (0.5-19%) of ADC with low intensity (Fig 4.2) whereas no positive staining was observed with VPM63 (Fig 4.1).

Lymphocytes in peripheral blood, afferent and efferent lymph  Peripheral blood monocyte depleted mononuclear cells (mainly lymphocyte), afferent and efferent
TABLE 4.1  Mean (Range) of Peak Fluorescence Intensity/Percent (%) Cells Reacting with VPM63 and VPM64.

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Peak Fluorescence Intensity</th>
<th>% Cells Reacting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM (n = 6)</td>
<td>MEMC (n = 3)</td>
</tr>
<tr>
<td>VPM63</td>
<td>7.8(^3) (6-11)(^4)</td>
<td>4.2 (2-6)</td>
</tr>
<tr>
<td>VPM64</td>
<td>8.8 (7-10)</td>
<td>6.0 (4-8)</td>
</tr>
<tr>
<td>VPM53(^2)</td>
<td>2.6 (2-3)</td>
<td>4.1 (2-4)</td>
</tr>
</tbody>
</table>

AM, alveolar macrophages; MEMC, monocyte enriched mononuclear cells; PBG, peripheral blood granulocytes; ADC, afferent lymph dendritic cells; PBMC, peripheral blood mononuclear cells; MDMC, monocyte depleted mononuclear cells (lymphocytes).

\(^1\)Number of samples examined.

\(^2\)VPM53 (IgG1, isotype control), showing background reactivity.

\(^3\)Mean value

\(^4\)(range of sample values)
FIGURE 4.1 Frequency histograms of Alveolar macrophages (AM), Peripheral blood mononuclear cells (PBMC), Afferent lymph dendritic cells (ADC) and Peripheral blood granulocytes (PBG), stained with VPM63 (---), and VPM53 (-----) an irrelevant IgG1 isotype control and BAQ30A (anti-CD18, ·····) a positive control.
FIGURE 4.2 Frequency histograms of Alveolar macrophages (AM), Peripheral blood mononuclear cells (PBMC), monocyte enriched mononuclear cells (MEMC), monocyte depleted mononuclear cells (MDMC), Afferent lymph dendritic cells (ADC) and Peripheral blood granulocytes (PBG), stained with VPM64 (---), and VPM53 (-----) an irrelevant IgG1 isotype control.
FIGURE 4.3  Contour plots of two-colour staining of peripheral blood mononuclear cells.  
(a) double negative control, normal mouse serum (FL1) and biotinylated NMS (FL2), (b) 
VPM64 (FL1) and VPM65 (FL2), (c) VPM64 (FL1) and VPM30 anti-B cell (FL2), (d) 
CD11b (FL1) and VPM64 (FL2), and (e) SBUT1 anti-T cell (CD5, FL1) and VPM64 
(FL2).
lymph cells stained using VPM63 and VPM64 were analysed after setting a live gate on lymphocytes excluding large complex and dead cells (Fig. 2.4). These mAb failed to stain lymphocytes in any of these compartments. A representative profile of monocyte depleted mononuclear cells stained with VPM64 is shown (Fig. 4.2).

**Bone marrow cells** The bone marrow (BM) cells were analysed after live gate exclusion of dead cells and most of RBC on the basis of FSC and SSC. Three analytical gates (R1, R2 an R3) were chosen (Fig. 4.4a) to define bone marrow subpopulations (see section 3.2.3). BM cells prepared from two sheep were analysed. VPM63 stained 3-7% of BM cells only in R2 (Fig. 4.4d) whereas VPM64 stained all the cells in R3 (Fig. 4.4e) in addition to staining a subpopulation (16-17%) of cells in R2 (Fig. 4.4b). Non-specific staining due to binding of mAb to FcR was ruled out using the same isotype negative control (VPM53). These results suggest that VPM63 might recognise a small proportion of BM cells (R2) as resident Mφ population. The 16-17% of the cells in R2 stained by VPM64 is the proportion of BM cells which would be expected to be monocytes/Mφ (Haig et al., 1991). In addition, VPM64 stained granulocytes at various stages of maturation. Further investigations are required to determine the exact cell type in BM recognised by these mAb.

**Antigen expression on monocyte derived macrophages (MDM) and alveolar macrophages in culture** *In vitro* culture of MEMC for six days which gives rise to MDM resulted in loss of expression of the antigen recognised by VPM64. It did not result in induction of the antigen recognised by VPM63 as determined by FACS analysis. A representative of three such experiments is shown (Fig. 4.5). MDM remained VPM63 negative even after two weeks in culture (results not shown). AM, maintained in culture, lost the antigens recognised by VPM63 by day 2 (Fig. 4.6 I) and VPM64 by day 6 (Fig. 4.6 II). VPM 19 (anti-MHC class I mAb) was used as positive control, and the stained cells were analysed by FACS. A representative of four such experiments is shown in Fig. (4.6).

### 4.2.3 IMMUNOCHEMICAL ANALYSIS

**Immunoprecipitation** Antigens recognised by VPM63 and VPM64 were immunoprecipitated from surface 125I labelled alveolar macrophage lysate by the ELISA plate method (section 2.13.1). VPM63 immunoprecipitated a smearing doublet of apparent Mr = 40,000 to 42,000 and VPM64 immunoprecipitated a wide band of Mr = 65,000-80,000 in non-reducing conditions (Fig 4.7a & b). Similar
FIGURE 4.4 (a) Forward and side angle scatter profile of bone marrow (BM) cells showing analytical gates R1, R2 and R3. Frequency histograms of all bone marrow cells (b), cells in R1 (c), R2 (d), and R3 (e), stained with VPM63 or VPM64 (——) and VPM53 (·····), an irrelevant IgG1 isotype control.
FIGURE 4.5 Frequency histograms of monocyte enriched mononuclear cells day 0 (la & IIa), and monocyte derived macrophages day 6 (lb & IIb), stained with VPM63 (I) or VPM64 (II) (—), and VPM53 (-----) an irrelevant IgG1 isotype control. A positive control, VPM65 (····) staining monocyte derived macrophages at day 6 is shown in lb & IIb.
FIGURE 4.6 Frequency histograms of alveolar macrophages (AM) day 0 (la & IIa), day 1 in culture (lb), day 2 in culture (lc) and day 6 in culture (ld) stained with VPM63 (la, b & c), VPM64 (IIa & b) (—), VPM53 (---) an irrelevant IgG1 isotype control and VPM19 (anti-MHC class I, ------) as positive control.
molecular weight antigens were immunoprecipitated under reducing conditions (Fig. 4.7c). Both mAb failed to recognise any antigen from AM lysate by immunoblotting.

**N-glycosidase F treatment of antigen**  N-linked carbohydrate on the VPM63 antigen was determined by digestion of $^{125}$I labelled immunoprecipitated antigen with N-glycosidase F (section 2.13.2). A decrease of approximately 2000 Dalton in $M_r$ of the VPM63 antigen was observed (Fig. 4.7d).

**PIPL-C treatment of cell surface antigens**  In order to determine if the antigen is anchored to cell surface via a glycosyl phosphatidyl inositol (GPI) linkage, cells were treated with PIPL-C before FACS analysis (section 2.12). None of the antigens recognised by VPM63 (on AM) and VPM64 (on AM and PBG) were found to be GPI linked (results not shown).

**Affinity purification of antigens**  The antigens recognised by VPM63 and VPM64 were affinity purified from the AM and PBG lysates respectively (section 2.13.8). The antigens purified using VPM63 ($M_r = 40,000-42,000$; Fig. 4.8a I) and VPM64 ($M_r = 80,000$; Fig. 4.8b I) were fractionated on a 12.5% linear SDS-PAGE and stained with silver nitrate (section 2.13.4). An additional band of $M_r = 34,000$ observed in VPM64 purified antigen (Fig. 4.8b I) may be due to the recognition of additional antigen or precursor molecule on granulocytes. Affinity purified antigens showed specific reactivity with VPM63 and VPM64 mAb by ELISA as described earlier (section 2.13.10).

**Western blotting with anti-FcγR peptide antiserum**  Affinity purified VPM63 and VPM64 antigens, and crude AM and PBG lysates were fractionated on SDS-PAGE, electrophoretically transferred to nitrocellulose paper and immunoblotted (section 2.13.6) using rabbit anti-FcR peptide antiserum and normal rabbit serum (section 2.5). A region of peptide synthesised from second extracellular domain of cattle FcγRI (Symons and Clarkson, 1992) is compared with human and murine FcγRs sequences in the region corresponding to the peptide (Fig. 4.9). Specific blotting of VPM63 affinity purified antigen of apparent Mr = 40,000-42,000 by anti-FcR peptide antiserum is shown in Fig. (4.8a II). The reactivity of anti-FcR peptide antiserum with proteins of apparent $M_r = 40,000$ and $M_r = 72,000$ from alveolar macrophage lysate was also observed which is consistent with FcγRII and FcγRI respectively. This suggests that the antigen recognised by VPM63 is an FcγRII. The anti-FcR
FIGURE 4.7 Auto radiograph of $^{125}$I labelled cell surface antigens immunoprecipitated from alveolar macrophage detergent lysate, using VPM63, VPM64, VPM53 (isotype negative control) and BAQ30A (anti-CD18) as positive control by ELISA plate method (a, b & c) and by protein-A-sepharose (d).

(a) The samples were fractionated on 5-20% gradient SDS-PAGE under non-reducing condition.

(b) VPM63 immunoprecipitated antigen fractionated on 12.5% linear SDS-PAGE under non-reducing condition is showing smearing doublet of the antigen.

(c). The samples were fractionated on 5-20% gradient SDS-PAGE under reducing condition. VPM63 in lane 1 is showing smearing doublet after short time exposure of the autoradiograph as compared to thick band observed in lane 2 after longer exposure. Some of the background bands observed in negative control (lane NEG.) are also present in VPM64 and VPM63 lane 2 and are comparatively faint in VPM63 lane 1.

(d) VPM63 immunoprecipitated antigen was fractionated on 12.5% linear SDS-PAGE after treatment with N-Glycosidase F (+) or without (-) treatment. A slight decrease (approximately of 2,000 Da) in the size of VPM63 antigen after N-glycosidase F treatment was observed.
FIGURE 4.8  Affinity purification of VPM63 and VPM64 antigens and Western blot analysis using rabbit anti-FcγR peptide anti-serum.

a(I) VPM63 affinity purified antigen (+) from alveolar macrophage and pre-elute (-) used as negative control were fractionated on 12.5 % linear SDS-PAGE. The gel was stained with silver nitrate as described earlier.

a(II) Western blot analysis of AM lysate and VPM63 (V63) purified antigen fractionated on 12.5 % linear SDS-PAGE. Rabbit anti-FcγR peptide anti-serum (+) and the control pre-inoculation serum (-) were used at 1/100 dilution. Rabbit antibodies were detected using anti-rabbit biotin-alkaline phosphatase-streptavidin bridge as described and blots were developed with NBT and BCIP.

b(I) VPM64 affinity purified antigen (+) from peripheral blood granulocyte lysate and pre-elute (-) used as negative control were fractionated on 12.5 % linear SDS-PAGE. The gel was stained with silver nitrate as described earlier.

b(II) Western blot analysis of granulocyte lysate (Gr) and VPM64 (V64) purified antigen fractionated on 12.5 % linear SDS-PAGE. Rabbit anti-FcγR peptide anti-serum (+) and the control pre-inoculation serum (-) were used at 1/100 dilution. Rabbit antibodies were detected using anti-rabbit biotin-alkaline phosphatase-streptavidin bridge as described and blots were developed with NBT and BCIP.
peptide antiserum failed to react with affinity purified VPM64 antigen \((M_r = 80,000)\) whereas it showed positive reactivity with bands of \(M_r = 72,000\) and \(40,000\) from the granulocyte lysate (Fig. 4.8b II) which is consistent with FcγRI and II respectively. It also recognised faint bands of the size between \(M_r = 50,000-70,000\) in both granulocyte and AM lysates (Fig. 4.8) which could be FcγRIII.

### 4.2.4 FUNCTIONAL STUDIES

**Surface FcγR mediated uptake of immune complexes by alveolar macrophages**

The above findings suggest that VPM63 and possibly VPM64 recognise low affinity FcγRs, FcγRII and FcγRIII respectively. These receptors are known to function by binding to antigen-antibody (Ag-Ab) complexes. This experiment was planned to investigate if VPM63 or VPM64 can inhibit the functional binding of these receptors. The surface uptake of soluble antigen-antibody (Ag-Ab) complexes through FcγR on AM was determined by FACS analysis of AM incubated with FITC labelled OVA (section 2.9) and affinity purified sheep anti-OVA IgG (Coughlan, 1994).

#### Optimisation of OVA-FITC and anti-OVA IgG

The amount of OVA-FITC giving minimum background uptake by AM was initially determined by incubating \(10^5\) AM suspended in 50\(\mu\)l of PBA buffer with 50\(\mu\)l of OVA-FITC (0-250\(\mu\)g ml\(^{-1}\) final concentration) for 30 min at 20°C. The cells were washed three times in PBA before FACS analysis. The mean fluorescence intensity (MFI i.e. mean channel number) of the cells as determined by programme 'Lysis' (Becton Dickenson) is plotted against OVA-FITC concentration (Fig. 4.10a). Subsequently sheep anti-OVA IgG (0-225\(\mu\)g ml\(^{-1}\) final concentration) was titrated, using 30 \(\mu\)g ml\(^{-1}\) final concentration of OVA-FITC which gave minimum background staining, to determine optimal proportions of Ag and Ab. 50\(\mu\)l suspension of AM (\(10^5\) cells) was incubated with 25\(\mu\)l of OVA-FITC (30\(\mu\)g ml\(^{-1}\) final concentration) and 25\(\mu\)l of anti-OVA IgG (0-225\(\mu\)g ml\(^{-1}\) final concentration) for 30 min at 20°C. The cells were analysed by FACS after three washings in PBA buffer. The MFI is plotted against anti-OVA IgG concentrations (Fig. 4.10b). The Ag:Ab ratio of 1:5 (30\(\mu\)g OVA-FITC:150\(\mu\)g anti-OVA IgG) on the linear part of the curve (Fig. 4.10b), producing maximum fluorescence intensity was considered optimum and was used in subsequent blocking assays.

#### Blocking of FcγR mediated Ag-Ab complex uptake by AM with VPM63 and VPM64

The above assay of Ag-Ab complex uptake via FcγR on the surface of AM was used to determine the blocking effect of mAb VPM63 and VPM64. Alveolar macrophages were pre-incubated with a saturating amount i.e. 100\(\mu\)g ml\(^{-1}\) (10 times
(a) Amino Acid Sequence of Bovine FcγRI (Second extracellular Domain) (Source: Symons and Clarkson, 1992), with region of peptide boxed.

```
D W L L L Q V T S R V F T E G D P L A L R
C H A W K N M P V Y K M L F Y K D G K P F
R F S S Q D S E F T I L Q T N L S H N G I
Y H C S G E R R R R Y T S A G G S I T I K
```

(b) Comparison of amino acid sequences of Murine and Human FcγRs in the Region corresponding to the PEPTIDE above. The sequences were obtained from Swissport database, and the percentages of identity (I) and similarity (:) were calculated using the programme "Gap".

(i) Murine

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<th>FcγRII vs FcγRIII</th>
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<td>CSGT.GRHRYTSAGVS</td>
<td>CSGT.GRHRYTSAGVS</td>
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<td>CKGSGLSTQHQSKPVT</td>
<td>CKGSGLGRTLHQSKPVT</td>
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<tr>
<td>% identity</td>
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<tr>
<td>% similarity</td>
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(ii) Human

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<th>FcγRII vs FcγRIII</th>
<th>FcγRII vs FcγRIII</th>
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<td>CSG.MGKHRYTSAGIS</td>
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<td></td>
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<td>CTGNIGYTLFSSKPVT</td>
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<tr>
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<table>
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<th>FcγRII vs FcγRIII</th>
<th>FcγRII vs FcγRIII</th>
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</thead>
<tbody>
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<td>CSGT.GRHRYTSAGIS</td>
<td>CSGT.GRHRYTSAGIS</td>
<td>CSGT.GRHRYTSAGIS</td>
</tr>
<tr>
<td></td>
<td>CTGNIGYTLFSSKPVT</td>
<td>CTGNIGYTLFSSKPVT</td>
<td>CTGNIGYTLFSSKPVT</td>
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<tr>
<td>% similarity</td>
<td>43.8</td>
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</table>
FIGURE 4.10 Antigen-antibody immune complex uptake by alveolar macrophages and its blocking using VPM63 and VPM64

(a) Mean fluorescence intensity (MFI) of alveolar macrophages (AM) incubated with various dilutions of OVA-FITC for 30 min at 20°C and analysed by FACS after three washings.

(b) Mean fluorescence intensity (MFI) of AM incubated in the presence of fixed concentration (30μgml⁻¹) of OVA-FITC with various concentration of anti-OVA IgG for 30 min at 20°C. The cells were analysed by FACS after three washings.

(c) Mean fluorescence intensity of AM (minus background MFI of AM incubated with OVA-FITC alone) pre-treated with wash buffer (PBA, untreated control) or saturating concentration of VPM53 (V53, IgG1 isotype control) or VPM63 (V63) or VPM64 (V64) for 60 min at 20°C, subsequently incubated in 30μgml⁻¹ OVA-FITC and 150 μgml⁻¹ anti-OVA IgG for 30 min at 20°C in the presence of blocking antibody. The results of two independent experiments are shown as mean of triplicate samples with standard error (error bars). Difference between the MFI of test (VPM63 or VPM64) and negative controls (VPM53 or PBA buffer) was not significant (P>0.05).
optimal staining concentration) of VPM63 or VPM64 or an equivalent protein concentration of VPM53 (an irrelevant IgG1 isotype control mAb), and PBA (no antibody) for one hour at 20°C, before using for Ag-Ab complex uptake assay. The inhibitory effect of these mAb (VPM63 and VPM64) was absolute. This was determined by FACS analysis of VPM63 and VPM64 blocked cells after staining with biotinylated VPM63 and VMP64 respectively and was detected with SA-PE conjugate on FL2. The antigen (OVA-FITC, 30µg ml⁻¹) and antibody (anti-OVA IgG, 150µg ml⁻¹) were incubated with AM in the presence of blocking antibody in triplicates for 30 min at 20°C. The cells were washed 3 times in PBA before FACS analysis. The mean of MFI of triplicate samples, after subtracting the background fluorescence of OVA-FITC alone, of VPM63 or VPM64 treated AM is compared with VPM53 (isotype control) and PBA buffer treated cells (Fig. 4.10c). The results of two independent experiments showed no significant difference (P>0.05) in immune complex uptake by AM between treated (test mAb) and control or untreated cells. Unfortunately there was no positive control for this experiment, anti-FcR peptide antiserum could not be used as positive control because the synthetic peptides were conjugated to OVA for raising anti-peptide antibodies. The affinity purified anti-peptide antibodies still had a reactivity with OVA (Coughlan, 1994).

4.2.5 IMMUNOHISTOCHEMISTRY

Immunohistochemical staining using VPM63 and VPM64 in lymphoid (lymph node, spleen and thymus) and non lymphoid organs (lung, liver, kidney, intestine, skin, brain and cotyledons) was determined by indirect immunoperoxidase technique on frozen sections as described earlier (section 2.15). The tissue localisation of VPM63 and VPM64 positive Mφ is listed in Table 4.2a & b.

Lymph node In prefemoral lymph node sections VPM63 stained most Mφ populations including those in the subcapsular sinus, interfollicular area, paracortical area and medullary sinuses. In addition VPM63 stained interdigitating reticulim cells (IDC) in the T-cell area between follicles. Some follicular dendritic cells were weakly stained with VPM63 (Fig. 4.11). VPM64 weakly stained a few isolated Mφ in cortex and medulla but failed to stain any other cell in the lymph node (Fig. 4.11).

Thymus The antigen recognised by VPM63 was expressed by cortical Mφ with long cytoplasmic processes. A low intensity staining of medullary Mφ was also seen (Fig. 4.11). In contrast VPM64 failed to produce any specific staining in thymus.
Spleen  Both VPM63 and VPM64 stained Mφ in the red pulp sinuses and a few marginal zone Mφ with moderate to weak intensity (Fig 4.12). A few PALS Mφ were stained with VPM63 but not with VPM64.

Lung  In lung sections alveolar macrophages were stained by both VPM63 and VPM64. VPM63 also stained a few interstitial macrophages (Fig. 4.12).

Liver  VPM63 stained Kupffer's cells in the hepatic sinusoids and portal area (Fig. 4.13). Only isolated cells in the hepatic sinusoids and portal area were stained with VPM64 (Fig. 4.13).

Kidney  VPM63 stained most of the glomerular cells and weakly stained a few Mφ in the interstitium (Fig. 4.13). VPM64 failed to stain cells in kidney.

Placental Cotyledon  VPM63 stained perivascular and lamina propria Mφ (Fig. 4.13) in frozen sections of full term placental cotyledons. VPM64 failed to stain any cell in the sections of cotyledon.

Skin  Frozen sections of skin were stained using Vectastain ABC method. Both VPM63 and VPM64 stained dermal Mφ, but some of the epidermal Langerhans cells were weakly stained with VPM64 only (Fig. 4.14).

Brain  VPM63 (Fig. 4.14) and VPM64 (Fig. 4.14) both stained elongated microglial like cells in the brain parenchyma but the staining with VPM64 was very weak.

Jejunal Peyer's Patch  VPM63 stained interfollicular Mφ, and some lamina propria Mφ with weak intensity in addition to its reactivity with the mucosa of the glands in the lamina propria (Fig. 4.15). VPM64 stained Mφ like cells in the lamina propria and isolated Mφ in the interfollicular area (Fig. 4.15). Both mAb failed to stain follicular cells.

4.2.6 REACTIVITY WITH BOVINE CELLS
Cross reactivity of these mAb in cattle was examined by staining tissue sections and blood leukocytes. VPM63 stained Mφ in frozen sections of cattle thymus and revealed a similar pattern of staining of thymus Mφ as was seen in sheep. Neither VPM64 nor VPM63 reacted with cattle blood leukocytes.
TABLE 4.2(a) Immunohistochemical Distribution of Mφ Stained with VPM63 and VPM64 in Frozen Sections of Lymph Node, Thymus, Spleen and Jejunum.

<table>
<thead>
<tr>
<th>Tissue (n = 4)*</th>
<th>VPM63</th>
<th>VPM64</th>
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</thead>
<tbody>
<tr>
<td>LYMPH NODE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcapsular sinus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Interfollicular area</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Follicular dendritic cells</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Medullary sinus</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>THYMUS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Medulla</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SPLEEN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red pulp</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Marginal zone</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Periarteriolar lymphoid sheath</td>
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<td>-</td>
</tr>
<tr>
<td>JEJUNUM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peyer's Patch follicles</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Interfollicular area</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>±</td>
<td>+</td>
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<tr>
<td>Glandular mucosa</td>
<td>+</td>
<td>-</td>
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</table>

* number of samples examined.
+ , positive staining; - , no staining; ±, weakly staining few cells.
TABLE 4.2 (b) Immunohistochemical Distribution of Mφ Stained with VPM63 and VPM64 in Frozen Sections of Parenchymatous Organs.

<table>
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<th>VPM64</th>
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<td></td>
</tr>
<tr>
<td>Alveoli</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Interstitium</td>
<td>±</td>
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</tr>
<tr>
<td>LIVER</td>
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</tr>
<tr>
<td>Kupffer's cells</td>
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</tr>
<tr>
<td>KIDNEY</td>
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<td></td>
</tr>
<tr>
<td>Glomerular</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Interstitial</td>
<td>±</td>
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<td></td>
</tr>
<tr>
<td>Epidermal Langerhans cells</td>
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<td>Lamina propria</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BRAIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microglial cells</td>
<td>+</td>
<td>±</td>
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</table>

* number of samples examined.

+, positive staining; -, no staining; ±, weakly staining few cells.
FIGURE 4.11 Indirect immunoperoxidase staining of frozen sections:

(a) Prefemoral lymph node with VPM63, showing staining of Mφ in the subcapsular (SC) area, and Mφ (arrow) and interdigitating reticulum cells (arrow head) in the interfollicular (IF) area. A weak labelling of follicular (F) dendritic cells (arrow head) was also observed. x 100.

(b) Prefemoral lymph node with VPM63, showing staining of Mφ in the medullary sinuses (arrow). x 250.

(c) Prefemoral lymph node with VPM64, showing weak labelling of isolated Mφ in the cortico-medullary area (arrow). x100.

(d) Thymus with VPM63, showing staining of Mφ in the cortex (C) and weak labelling of Mφ in the medulla (M). x 100. Inset, cortical Mφ in higher magnification (x 250).
FIGURE 4.12 Indirect immunoperoxidase staining of frozen sections:

(a) Spleen with VPM63, showing staining of Mφ in the red pulp (RP) sinuses, few Mφ in the marginal zone (MZ) and periarteriolar lymphoid sheath (PALS) area x 100.

(b) Spleen with VPM64, showing staining of Mφ in the red pulp (RP) and few Mφ in the marginal zone (MZ) area. x 250.

(c) Lung with VPM63, showing staining of alveolar (arrow) and a few interstitial Mφ (arrow head). x250.

(d) Lung with VPM64, showing staining of alveolar Mφ (arrow). x250.
FIGURE 4.13 Indirect immunoperoxidase staining of frozen sections:

(a) Liver with VPM63, showing staining of Kupffer's cells with long cytoplasmic processes (arrow) in the hepatic sinusoids. x 100.

(b) Liver with VPM64, showing staining of isolated Kupffer's cells in the hepatic sinusoids (arrow). x 100.

(c) Kidney with VPM63, showing staining of glomerular cells (arrow) and weakly labelled interstitial Mø (arrow head) at the junction of renal tubules. x 100.

(d) Full term placental cotyledon with VPM63, showing staining of perivascular Mø (arrow) in the sub mucosal area. x 100.
FIGURE 4.14 Immunoperoxidase staining of frozen sections, of skin using Vectastain ABC method, and brain using Indirect immunoperoxidase method:

(a) Skin with VPM63, showing staining of dermal Mφ (arrow). x 250.

(b) Skin with VPM64, showing weak staining of some epidermal Langerhans cells (arrow) and few dermal Mφ (arrow head). x 250.

(c) Brain (cerebrum) with VPM63, showing staining of irregular elongated microglial like cells (arrow) in the parenchyma. x 250.

(d) Brain (cerebrum) with VPM64, showing weak staining of a few irregular elongated microglial like cells (arrow) in the parenchyma. x 250.
FIGURE 4.15 Indirect immunoperoxidase staining of frozen sections:

(a) Jejunum with VPM63, showing staining of inter follicular Mφ (IF, arrow) and weak labelling of Mφ in the lamina propria (LP, arrow). VPM63 also cross reacted with the mucosal lining of the sub mucosal crypts (arrow head). x 100.

(b) Jejunum with VPM64, showing staining of Mφ like cells in the lamina propria (arrow). x 100.
4.3 DISCUSSION

The results of the immunophenotypic and biochemical characterisation of mAb VPM63 and VPM64 described in this chapter indicate that these two mAb recognise distinct antigens expressed by sheep myeloid cells. In the majority of cell populations their expression is mutually exclusive, being co-expressed only on AM and a few other tissue Mφ.

The antigen recognised by VPM63 was widely distributed on resident tissue Mφ, but was absent from any leukocyte populations in the blood, afferent or efferent lymph and most bone marrow (BM) cells. In BM the low level of VPM63 staining of a very small proportion of large complex cells (3-7% in R2) is suggestive of its expression on resident Mφ. Thus VPM63, appears to recognise a differentiation antigen present on most tissue Mφ. Both cellular distribution and Mr distinguished the VPM63 antigen from previously defined myeloid antigens (Knapp, 1989).

Several mAb which recognise antigens restricted in expression on mature macrophages have been described (Zwaldo et al., 1985, 1986; Andreeson et al., 1986; Radzun et al., 1988; Nash et al., 1989; Micklem et al., 1989). Only mAb L9, specific to human placental Mφ (Nash et al., 1989), recognised an antigen of Mr = 40,000-43,000 and showed characteristics similar to VPM63. Loss of VPM63 reactivity on AM in culture demonstrate in vitro phenotypic modulation. The antigen is either shed or undergoes proteolytic cleavage from cell surface in cultured AM (Astier et al., 1994; Sautes et al., 1991). Other Mφ surface antigens, expression of which decreases in culture include MHC class II (Beller and Unanue, 1981), F4/80 (Schuler and Steinman, 1985) and murine FcγRII (Sautes et al., 1991). FcγRII expression on monocytes and Langerhans cells in humans is also reduced in culture, which is associated with the release of soluble forms of the receptor (Clarkson and Ory, 1988; Astier et al., 1994).

The reactivity of VPM63 with glycoproteins of Mr = 40,000/42,000 is consistent with FcγRII (Ravetch and Kinet, 1991). VPM63 antigen affinity purified from AM was recognised by an anti-FcγR peptide anti-serum on immunoblotting. Anti-FcγR peptide anti-serum also reacted with two bands from AM lysate of Mr = 40,000 and 72,000 which correspond to the predicted sizes for FcγRII and I respectively. AM are known to express FcγRI and FcγRII in mice and humans (Ravetch and Kinet, 1991). The reactivity of this anti-FcγR peptide anti-serum in sheep with bands equivalent to FcγRI (Mr = 72,000) in AM and dendritic cells, and FcγRII (Mr = 40,000) in AM,
dendritic cells and lymphocytes have been demonstrated (Coughlan, 1994). The antiserum was raised in rabbit against a peptide sequence (Coughlan, 1994) taken from second extracellular domain of cattle FcγRI (Symons and Clarkson, 1992). This antiserum was expected to cross react with sheep FcγRI as there is a high sequence homology between mice, humans and cattle FcγRI (Symons and Clarkson, 1992). The first two extracellular domains of FcγRI show 40% identity with the extracellular domains of FcγRII and FcγRIII in mice (Hulett et al., 1991) and 50% in humans (Allen and Seed, 1989). The corresponding region of peptide sequence show considerably high amino acid identity and similarity between three FcγRs in mice but much less in humans (Fig. 4.9). If the sheep FcγRs have a high amino acid identity in the peptide region similar to that of murine FcγRs, then the anti-FcγR anti-serum may account for its cross reactivity with different classes of FcγRs in sheep. Thus the recognition of VPM63 antigen by anti-FcγR peptide anti-serum provides evidence that this mAb recognises an epitope on an isoform of FcγRII. However the functional activity of this antigen as FcγRII could not be unambiguously determined as VPM63 failed to block the uptake by AM of soluble Ag-Ab immune complexes. This could be due to the binding of VPM63 to an epitope distant from the functional binding site (Hulett et al., 1991).

A number of mAb which recognise FcγRII showed wide distribution on monocytes, macrophages, granulocytes, platelets and B-cells have been described (Schmidt, 1989; Vaughn et al., 1985; Antoun et al., 1988). None of these have been shown to recognise a Mφ specific FcγRII (Looney et al., 1986; Zipf et al., 1989; Pulford et al., 1986). Koller (1989) described much similar tissue distribution of Mφ (besides other cells) stained with various anti-FcγRII mAb in humans as observed with VPM63 in sheep. VPM63 staining of Mφ in placental cotyledons was similar to some of the anti-FcγRII mAb staining of placental Mφ in human (Schmidt, 1989). However the molecular characterisation of FcγRII gene in mice revealed a Mφ specific isoform (FcγRIIb2) (Miettinen et al., 1989). This Mφ specific receptor (FcγRIIb2) is capable of localising at clathrin coated pits and is involved in rapid receptor mediated endocytosis of bound ligand, while the B-cell isoform (FcγRIIb1) is not (Weinshank et al., 1988; Miettinen et al., 1989). Though human FcγRII-C is highly homologous to the mice Mφ isoform, its distribution is not restricted to Mφ (Stuart et al., 1989). Specific expression of VPM63 on tissue Mφ but not on epidermal Langerhans cells and afferent lymph dendritic cells is suggestive of a role in phagocytosis/endocytosis similar to that of the mice Mφ isoform (Miettinen et al., 1989). The expression of FcγRII on human and mouse Langerhans cells (Hong, 1984; Astier et al., 1994), and
evidence of FcγRII in sheep ADC (Coughlan, 1994) has been reported. However if VPM63 is anti-FcγRII it recognises an isoform absent on DC.

The possibility that VPM63 recognises a conformational epitope is suggested by its failure to immunoblot denatured antigen. Otherwise VPM63, which recognises a cell surface antigen, was expected to react with all the isoforms of the FcγRII because of the high homology of the extracellular domains in other species (Ravetch et al., 1986; Qiu et al., 1990).

VPM63 failed to react with COS-7 cells transfected with cattle FcγRII (personal communication Dr. C. J. Howard, IAH, Compton) though it stains cattle Mφ. It is possible that the epitope recognised by VPM63 is not present on the particular transfected FcγRII isoform (Zhang et al., 1994).

VPM63 cross reactivity with the epithelium of the intestinal lamina propria gland is similar to the reactivity of some anti-FcγRII mAb with transporting epithelium of placenta, liver and intestine in human (Stuart et al., 1989; Schmidt, 1989).

Molecular weight, cellular distribution and cross reactivity with anti-FcγR peptide anti-serum is consistent with VPM63 antigen being one of the Mφ specific isoforms of FcγRII. This suggests the existence of another FcγRII isoform in sheep present on monocytes, dendritic cells etc, because of functional and other evidences of the receptor on these cells. However amino acid sequencing of the antigen or reactivity of mAb VPM63 with transfectants is required to obtain unambiguous confirmation of its identity.

VPM64 recognised a wide band of apparent Mr = 65,000 to 80,000 from alveolar macrophages. The cellular and tissue distribution of mAb VPM64 indicate that it recognises an antigen shared by granulocytes and monocytes in blood, Mφ in lung, spleen and dermis, and some epidermal Langerhans cells and a subpopulation of dendritic cells in afferent lymph. In addition to monocytes (VPM65+VPM64+) VPM64 also recognised a small subpopulation of PBMC which was CD11b+CD14+. This suggests the existence of either CD14+ monocytes in sheep (not reported in human) or VPM64+CD14+ but CD11b+ subpopulation of NK cells. VPM64, though not highly expressed in other cells except granulocytes, was also detected on bone marrow cells of all the granulocyte lineage (mature and immature) as well as a
sub-population (16-17%) of BM cells in region containing monocytes/macrophages series cells, myeloid blasts and some medium large lymphocytes (Haig et al., 1991). The proportion of BM cells positive for VPM64 was quite similar to that of CD11b (Haig et al., 1991; Chapter 5). In human, CD11b has been shown to be expressed from monoblasts and myelocyte BM precursors of monocyte and neutrophil respectively (Rosenthal et al., 1983; Fleit et al., 1984)).

A number of mAb which recognise antigens expressed by different cells of myeloid lineage and NK cells have been described (Hogg and Horton, 1987; Schmidt, 1989; Knapp, 1989). Of these antigens only FcyRIII (CD16), an antigen of M_r = 50,000-80,000 (Fleit et al., 1989), is widely distributed on granulocytes, Mφ and NK cells. The various characteristics of VPM64 antigen seems to be somewhat similar to FcyRIII. In humans FcyRIII exists in two distinct forms (A and B), IIIB is exclusively expressed on neutrophils and is anchored to the cell surface via GPI linkage; IIIA is expressed in NK cells and Mφ as a transmembrane molecule (Ravetch and Perussia, 1989). In mouse only one form of FcyRIII, similar to hFcyRIIIA, is expressed as a transmembrane molecule on neutrophils, Mφ and NK cells (Weinshank et al., 1988). VPM64 precipitated an antigen of M_r = 65,000-80,000 from AM which is similar to FcyRIII in humans and mouse, whereas the antigen purified from granulocytes revealed a sharp band of M_r = 80,000. Such antigenic polymorphism may be due to different levels of glycosylation following post-translational modification of the products of the same gene. Unlike the case in humans the VPM64 antigen on granulocytes was not GPI-linked, suggesting its resemblance to mouse FcyRIII. There are conflicting reports regarding the expression of CD16 on resting monocytes (Unkeless et al., 1988; Fleit et al., 1982). In humans blood monocytes express low levels of FcyRIII and the expression is upregulated on monocyte derived Mφ (Vaughn et al., 1985; Fleit et al., 1984), whereas in the present study the low level of expression of VPM64 is lost on monocyte derived Mφ. VPM64 stained some Langerhans cells and a subpopulation of ADC, in contrast Langerhans cells were not stained using various anti-CD16 mAb (Koller, 1989). VPM64 recognised all the granulocyte series cells in the BM whereas in human low affinity FcyRs are expressed from metamyelocyte stage (ie late differentiation stage of neutrophils) onwards (Fleit et al., 1984).

Anti-FcyR peptide anti-serum failed to recognise VPM64 antigen (M_r = 80,000) purified from granulocytes. It is possible that either the sequence of the Fc peptide is not conserved in VPM64 antigen or mAb VPM64 recognises a different antigen.
VPM64 failed to block soluble Ag-Ab complex uptake by AM. However this is not universal as many anti-mouse and anti-human FcγRIII mAb also fail to inhibit immune complex uptake because they recognise an epitope distant from the functional binding site (Perussia et al., 1984).

Thus from the present analysis of mAb VPM64, it remains inconclusive whether it recognises sheep FcγRIII (CD16) or a new antigen. To elucidate the precise nature of this antigen, further biochemical and functional characterisation and the reactivity of the mAb with suitable transfectant is required.

4.4 CONCLUSIONS

The mAb VPM63 described in this chapter appears to recognise a differentiation antigen on mature tissue Mφ since it does not react with blood leukocyte (including monocytes) or with afferent and efferent lymph cells (including dendritic cells). This mAb will be useful in studying Mφ populations in various tissues under normal and diseased conditions.

VPM63 immunoprecipitated an antigen of Mr = 40,000-42,000 and the affinity purified antigen was recognised by anti-bovine FcγRI peptide anti-serum raised against a synthetic peptide from second extracellular domain. However, VPM63 failed to inhibit functional binding of the receptor, thus suggesting that the mAb recognises an epitope distant from the functional binding site on sheep FcγR homologous to an isoform of the murine/human FcγRII (CD32) restricted to Mφ.

VPM64 recognises an epitope on antigen shared by various cells of the myeloid lineage (blood granulocytes and monocytes, some tissue macrophages, few Langerhans cells, a subpopulation of dendritic cells in the afferent lymph) and possibly NK-cells in blood. The size of the antigen recognised by VPM64 and its distribution on a wide variety of cells suggests its possible identity as sheep FcγRIII.
CHAPTER FIVE

CHARACTERISATION OF SHEEP β2 (LEUKOCYTE) INTEGRINS
**Author's Note**

Attention is drawn to the erratum notice on the Title Page of Chapter 1, and in particular to the correct nomenclature for the leukocyte integrins, which is as follows:

<table>
<thead>
<tr>
<th>Leukocyte Integrin</th>
<th>Alternative Name</th>
<th>(\alpha) Chain</th>
<th>(\beta) Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1</td>
<td>-</td>
<td>CD11a (=(\alpha L))</td>
<td>CD18</td>
</tr>
<tr>
<td>Mac-1</td>
<td>CR3</td>
<td>CD11b (=(\alpha M))</td>
<td>CD18</td>
</tr>
<tr>
<td>p150/95</td>
<td>CR4</td>
<td>CD11c (=(\alpha X))</td>
<td>CD18</td>
</tr>
</tbody>
</table>
5 CHARACTERISATION OF SHEEP β2 (LEUKOCYTE) INTEGRINS

5.1 INTRODUCTION
The term integrin was proposed by Hynes, (1987) to describe a family of integral membrane adhesion promoting receptors, which integrate the cytoskeleton of one cell with that of the other cells or with the extra cellular matrix. These adhesive interactions are crucial to the regulation of homeostasis and diverse cellular functions including tissue organisation, lymphocyte recirculation, and immune and inflammatory responses of leukocytes (Kishimoto et al., 1989a; Hogg, 1989; Yong and Khwaja, 1990; Hynes, 1992). The integrin superfamily is comprised of evolutionary related heterodimers each composed of non-covalently associated α and β transmembrane subunits, which vary in size between $M_r = 120,000$ to $180,000$ and $90,000$ to $110,000$ respectively. Several integrin families have been defined on the basis of a common β subunit. There are at least 8 β and 14 α subunits in which each β chain is shared by one or more α chains. Some of the recently identified α subunits (α4, α6, αV and perhaps others) can associate with more than one β subunit thus giving rise to at least 20 different heterodimers (Hynes, 1992). These molecules engage in heterophilic interactions with both cell surface ligands and extra cellular matrix components.

The β2 integrin family (also known as leukocyte integrins), has three members which are exclusively expressed on leukocytes, and play a critical role in mediating adhesive interactions during inflammation and the immune response (Springer 1990; Larson and Springer, 1990). The members of β2 integrin family differ from each other by having distinct α chains each associated with a common β chain (CD18) of $M_r = 95,000$. CD11α (LFA-1) possesses the αL chain of $M_r = 180,000$, CD11b (CR3 or Mac-1) contains the αM chain of $M_r = 170,000$ and the αX chain associated with CD11c (CR4 or p150/95) has a $M_r = 150,000$. The αM and αX subunits of the leukocyte integrins (LI) are more homologous to each other than to αL chain, suggestive of their close functional similarity (Arnaout, 1990a). The α and β subunits of LI are synthesised as distinct precursors but their association is required for further processing before the mature proteins are transported to the cell surface or intracellular granules (Todd et al., 1984; Bainton et al., 1987). The characteristics of the LI are conserved across species and mAb specific for murine antigens have been used to characterise human leukocyte integrins (Larson and Springer, 1990; Kishimoto et al., 1989a). In the present study one of the potential anti-bovine CD18 mAb (BAQ30A) was found to cross react with human leukocytes in addition to its reactivity in sheep.
CD11a is expressed by all leukocytes in most animal species studied with the exception of peritoneal Mφ in mouse (Krensky et al., 1983) and rat (Tamatani et al., 1991). CD11a is primarily involved in leukocyte-leukocyte or leukocyte-endothelium interaction through its ligands, intercellular adhesion molecule (ICAM)-1, -2 and -3 (Springer, 1990; de Fougerolles and Springer, 1992). In bone marrow CD11a is expressed on early erythroid progenitors, GM-CFU and M-CFU in mouse and man (Todd and Schlossman, 1984). A similar heterodimeric antigen (CD11a) which is widely distributed on mature leukocytes has been reported in cattle (O'Reilly et al., 1991; flail et al., 1993) and sheep (Mackay et al., 1990). Unlike human and mouse, erythroid progenitors in cattle (Muiya et al., 1993) and sheep (Haig et al., 1991) do not express CD11a.

In humans and mice CD11b and CD11c are expressed on committed neutrophil and monocyte precursors at the myelocytic and monoblastic stages respectively (Todd and Schlossman, 1984). In mature cells, CD11b and CD11c are expressed on monocytes/macrophages, granulocytes and NK cells (Kishimoto et al., 1989a). In addition, CD11b is expressed on a subpopulation of cytotoxic and helper T cells (Hoshino et al., 1993) and CD11c on a subpopulation of B cells (Wormsley et al., 1990) in normal human peripheral blood. In humans CD11b and CD11c are also expressed on a number of lymphocytic and myeloid malignancies (Todd et al., 1981, De La Hera, 1988). The expression of CD11b and CD11c on monocytes, granulocytes and macrophages has been described in cattle (Splitter and Morrison, 1991; Hall et al., 1993). In addition, CD11b was also found on a subpopulation of B-cells in cattle (Naessens and Williams, 1992) while CD11c was absent on resting lymphocytes (Splitter and Morrison, 1991). Unlike CD11a, CD11b and CD11c in addition to their cell surface expression are also present in intracellular storage granules in granulocytes and monocytes (Gallin et al, 1982). In common with lymphocytes, tissue Mφ do not contain intracellular pools of these receptors. Peripheral blood monocytes express high levels of CD11b and lower levels of CD11c. However after extravasation and maturation to tissue Mφ the pattern is reversed with higher level of CD11c (Miller et al, 1986; Fryer et al, 1988). In addition to the interaction of CD11b with ICAM-1 (CD54), CD11b and CD11c also bind extracellular matrix components and the complement component C3bi. It is through their binding to C3bi they function as receptors for opsonisation (Kishimoto et al., 1989a).
Although leukocytes express a number of other adhesion molecules (e.g. CD2, L-selectin) it is the β2 integrins which mediate high affinity adhesion. The importance of this is evident from the discovery of cases (humans, cattle and dogs) which suffer from recurrent life-threatening bacterial infections due to the lack all the three β2 integrins on their leukocytes (Kishimoto et al., 1987a; Shuster et al., 1992; Trowald-Wigh et al., 1992). The clinical syndrome, termed leukocyte adhesion deficiency (LAD) is caused by heterogeneous defects in the common β subunit due to point mutations in the highly conserved region which leads to a total absence of or defective biosynthesis of the common β subunit CD18.

The interactions of β2 integrins with their ligands are controlled through the phosphorylation of the integrin β chain rendering the molecules adhesive for their ligands (Valmu et al., 1991). Integrins with unphosphorylated β chains are non-functional. Activation of the integrins is triggered through a number of receptor ligand interactions such as the T cell receptors and their co-receptor molecules (CD4 and CD8) or via the interaction of particular cytokines (e.g. endothelial derived IL-8 and platelet activating factor) or chemoattractants (e.g. f-met-lue-phe) with their receptors (Nathan et al., 1989; Dustin and Springer, 1991; Springer, 1994). The structure and functions of Leukocyte integrins are discussed in detail in Chapter 1 (section 1.10).

5.2 OBJECTIVE OF THIS STUDY
The objectives of this study were 1) To identify and characterise anti-sheep leukocyte integrins monoclonal antibodies, and 2) To investigate their distribution with the main aim to delineate mononuclear phagocytes by their differential expression of leukocyte integrins.

In order to meet these objectives the putative CD11/CD18 and monocyte/Mφ specific mAb of the Second International Workshop on Ruminant Leukocyte Differentiation Antigens were screened for their reactivity with sheep alveolar macrophages and blood leukocytes by flow cytometry (FACS), and resident Mφ by immunohistochemistry (lung, liver and skin). The mAb specifically reacting with Mφ and other leukocytes and showing no reactivity with tissue parenchyma, were selected (Table 5.1).

In this chapter characterisation of these mAb, for their immunochemical properties by immunoprecipitations and western blotting, is described in an attempt to define ovine
homologues of leukocyte integrins. Their cellular distribution and tissue localisation on mononuclear phagocytes was determined by FACS and immunohistochemistry respectively

5.3 RESULTS

5.3.1 IMMUNOCHEMICAL ANALYSIS
The molecular weight of the antigens recognised by these mAb was investigated by immunoprecipitation and western blotting. By antigen preclearing studies the α chains were found to be unique for each member of LI whereas the β chain was common. Immunochemical analysis showed that the panel of mAb used could be differentiated into four groups (Table 5.1).

Immunoprecipitation The mAb listed in Table 5.1 were used to immunoprecipitate antigens from surface radio-iodinated alveolar macrophage lysate by the ELISA plate method as described previously (section 2.13.1). The results are shown in Fig. 5.1. The mAb could be divided into 4 distinct groups on the basis of immunoprecipitation analysis:- Group 1 mAb MD2B7, MUC76A, F10-150 and 72-87, each precipitated a heterodimer of apparent $M_r = 95,000$ and 180,000 corresponding to the β2 and αL chain of the LI. Group 2 mAb CC125 and IL-A15 precipitated a heterodimer of $M_r = 95,000$ and 170,000 which corresponds to the β2 and αM chains of LI. Group 3 mAb OM1 precipitated bands of $M_r = 95,000$ and 150,000 similar to the β2 and αX chains of LI. Group 4 mAb BAQ30A and MF14B4 each precipitated $M_r = 95,000$ β chain along with other α chains of apparent $M_r = 150,000$ to 180,000. Immunoprecipitations could not be achieved with mAb CC126 and BAQ153A. In all groups similar molecular weight antigens were obtained both under reducing (Fig. 5.1a) and non-reducing (Fig. 5.1b) conditions.

Western Blotting Unlabelled AM lysate was fractionated on 5-20 % gradient SDS-PAGE and the proteins were transferred to nitrocellulose membrane using a semi-dry electroblotter as described previously (section 2.13.6). After incubation with specific mAb followed by anti-mouse biotin and streptavidin-biotin alkaline phosphatase the blots were developed using NBT and BCIP. mAb BAQ30A and MF14B4 (Group 4) recognised an antigen of apparent $M_r = 95,000$ corresponding to β2 chain of LI (Fig. 5.1c). No specific blotting was obtained with the other mAb in the panel.
TABLE 5.1 Immunochemical Analysis of Monoclonal Antibodies and Proposed Specificities (CD11/CD18) in Sheep.

<table>
<thead>
<tr>
<th>Group</th>
<th>Monoclonal Antibodies</th>
<th>Molecular Weight of Antigen recognised</th>
<th>Proposed Specificity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><strong>MUC76A, 72-87.</strong> F10-150, MD2B7.</td>
<td>180,000/95,000</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td><strong>CC125, IL-A15, CC126</strong>*</td>
<td>170,000/95,000</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td><strong>OM1, BAQ153A</strong>*</td>
<td>150,000/95,000</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td><strong>BAQ30A, MF14B4.</strong></td>
<td>95,000/150,000/170,000/180,000</td>
<td>95,000</td>
</tr>
</tbody>
</table>

mAb shown in bold are representing the group.

* mAb failed to immunoprecipitate.
- , mAb in the group failed to immunoblot.

IP = Immunoprecipitation, WB = western blotting, OvCD = ovine cluster of differentiation.
FIGURE 5.1 Autoradiograph of antigens immunoprecipitated from $^{125}$I surface labelled sheep alveolar macrophage lysate under reducing (a) and non-reducing (b) conditions, and western blot analysis (c).

(a) Group 1 (72-87, F10-150, MD2B7 & MUC76A), Group 2 (CC125 & IL-A15), Group 3 (OM1) and Group 4 (MF14B4 & BAQ30A) monoclonal antibodies. VPM53 (anti-campylobacter mAb) as negative control. The samples were fractionated on 5-20% gradient SDS-PAGE under reducing conditions.

(b) Group 1 (MUC76A), Group 2 (CC125), Group 3 (OM1) and Group 4 (BAQ30A) monoclonal antibodies. VPM53 (anti-campylobacter mAb) as negative control. The samples were fractionated on 5-20% gradient SDS-PAGE under non-reducing conditions.

(c) AM lysate, fractionated on 5-20% gradient SDS-PAGE under reducing condition, electrophoretically transferred on to nitrocellulose membrane was analysed by western bloting with Group 4 mAb MF14B4 (lane 1) BAQ30A (lane 2), and VPM53 (negative control, lane 3). Antibodies were detected using anti-mouse biotin-alkaline phosphatase streptavidin bridge as described and blots were developed with NBT and BCIP.
(a)

(b)

(c)
Sequential immunoprecipitation  Immunoprecipitation and immunoblotting results could discriminate the mAb listed in Table 5.1 into four distinct groups each recognising a specific α or β chain of LI as evidenced by the Mr of the antigen. The relationship between these different groups of mAb was investigated by sequential immunoprecipitation.

β chain sequential immunoprecipitation  Removal of all the reactivity to the Group 4 mAb BAQ30A from 200μl of radio-labelled AM lysate in seven sequential changes through protein-A-sepharose coupled to mAb, removed all reactivity to the anti-α chain mAb MUC76A (Group 1), CC125 (Group 2) and OM1 (Group 3) (Fig. 5.2a). This proves formally that the β (Mr = 95,000) chain precipitated by anti-α chain mAb (Group 1, 2 & 3) are the same.

α chain sequential immunoprecipitation  To demonstrate the independent existence of three different α chains associated with a common β chain, sequential immunoprecipitations were performed by initial clearing of the αL chain in 200μl of radio-labelled AM lysate in five sequential changes through protein-A-sepharose coupled to mAb MUC76A (Group 1). This was followed by clearing of αM using three sequential changes of the lysate above using mAb CC125 (Group 2) and finally removal of αX in two changes using mAb OM1 (Group 3) coupled to protein-A-sepharose. Fig. 5.2b shows that CC125 immunoprecipitated a heterodimer of Mr = 95,000/170,000 from lysate cleared of all reactivity to MUC76A (Mr = 95,000/180,000 heterodimer). Similarly OM1 precipitated a heterodimer of Mr = 95,000/150,000 from lysate cleared of all reactivity to MUC76A and CC125. After removing three distinct higher Mr α chains, BAQ30A could still precipitate a faint band of Mr = 95,000 β chain not associated with any apparent α chains. This confirms that the mAb MUC76A, CC125 and OM1 are specific for distinct higher molecular weight α chains associated with a common β chain recognised by BAQ30A.

The division of putative LI mAb into four distinct groups on the basis of immunochemical analysis as well as their relationship with each other was confirmed by antigen preclearing studies. These results formally prove the proposed specificities to each group of mAb as given in Table 5.1. So, from now onwards these mAb will be referred to by their specificities as CD11a (Group 1), CD11b (Group 2), CD11c (Group 3) and CD18 (Group 4) instead of groups.
FIGURE 5.2 Autoradiographs showing sequential immunoprecipitations of β chain (a) and α chain (b) from $^{125}$I surface labelled alveolar macrophage lysate, using mAb MUC76A (Group 1), CC125 (Group 2), OM1 (Group 3) and BAQ30A (Group 4), and VPM53 (anti-campylobacter, negative control). The samples were fractionated under reducing conditions.

(a) Showing depletion of antigen (β chain) recognised by BAQ30A (lanes 2 to 4), followed by failure of precipitations by MUC76A (lane 5), CC125 (lane 6) and OM1 (lane 7). VPM53 negative control (lane 1).

(b) Showing depletion of antigens (α chains) recognised by MUC76A (lanes 1 to 5), followed by CC125 (lanes 6 to 8) and then by OM1 (lanes 9 & 10), finally precipitation by BAQ30A (lane 11). VPM53 negative control (lane 12).
5.3.2 FLOW CYTOMETRIC ANALYSIS
FACS analysis was performed on various leukocyte populations stained with the mAb listed in Table 5.1 as described earlier (section 2.11). Individual cell populations within peripheral blood, afferent and efferent lymph, lung wash and bone marrow were delineated by live gating on the basis of cell size (FSC) and cell complexity (SSC) (section 2.11.2; Fig. 2.4). Reactivity of the mAb within each group was essentially the same. The results of one mAb representing each group are shown in terms of percent positive cells, after subtracting the values of background negative control (Table 5.2). The reactivity of the mAb with various leukocyte populations is also summarised in Table 5.3. These mAb did not show any reactivity with RBC and platelets in peripheral blood (results not shown).

Reactivity of anti-CD11a and -CD18 monoclonal antibodies The distribution of anti-CD11a (MUC76A) and -CD18 (BAQ30A) mAb in leukocyte populations was identical as determined by FACS analysis. These mAb (MUC76A and BAQ30A) stained all the leukocyte populations that were analysed. This included alveolar macrophages (AM), afferent dendritic cells (ADC), monocyte enriched mononuclear cells (MEMC), peripheral blood granulocytes (PBG), PB mononuclear cells (PBMC) and, afferent and efferent lymphocytes. Representative FACS profiles with relevant negative controls are shown in Fig. 5.3a, b & c. The bone marrow (BM) cells were analysed after live gate exclusion of dead cells and most of the RBC on the basis of FSC and SSC. Three analytical gates (R1, R2 and R3) were chosen (Fig. 5.4I) to define bone marrow subpopulations (see section 3.2.3). BM cells prepared from two animals were analysed. Anti-CD11a stained 35-48% of all the gated BM cells, this include 73-87% of cells in R3 (granulocyte series), 39-47% of the cells in R2 (monocyte/Mφ series and large lymphocytes) and 8% of cells in R1 (nucleated erythroid series and small lymphocytes) (Fig. 5.4I). Anti-CD18 stained 40-56% of all the gated BM cells which include 88-95% of cells in R3, 48-51% of the cells in R2 and 10-12% of cells in R1 (Fig. 5.4I).

Reactivity of anti-CD11b monoclonal antibodies Anti-CD11b (CC125) mAb reacted with most AM, MEMC (monocytes), PBG, and a subpopulation of PBMC and peripheral blood small lymphocytes (Table 5.2; Fig. 5.3a, b & c). In contrast only a few lymphocytes (1-2%) in the afferent and efferent lymph were stained (Fig. 5.3c). A few isolated ADC cells stained with anti-CD11b mAb showed Mφ like (large rounded cells) morphology by indirect immunoperoxidase staining of cytopsots (Fig. 5.5). Anti-CD11b mAb stained 27-46% of all the gated BM cells, this includes
77-93% cells in R3 (granulocyte series), 15-19% of the cells in R2 (monocyte/Mφ series and large lymphocytes) and less than 1% cells from R1 (nucleated erythroid series and small lymphocytes) (Fig. 5.4II).

Reactivity of anti-CD11c monoclonal antibodies  Anti-CD11c (OM1) mAb, stained most AM, ADC and a small subpopulation of PBG (2-7%) but staining of MEMC (monocytes) was very weak (Table 5.2; Fig. 5.3a & b). Anti-CD11c, like anti-CD11b mAb, stained a subpopulation of peripheral blood small lymphocytes, and only a few afferent and efferent lymphocytes (Fig. 5.3c; Table 5.2). Anti-CD11c mAb stained 7-10% of all the gated BM cells, which include a subpopulation (23-27%) of cells in R3 (granulocyte series) and 4-6% cells from R2 (monocyte/macrophage & large lymphocyte) and less than 1% cells from R1 containing erythroid series and small lymphocytes (Fig. 5.4II).

5.3.3 IMMUNOHISTOCHEMISTRY  
The immunohistochemical distribution within each group remained fairly consistent as revealed by preliminary tissue staining (lung, liver and skin) with all the mAb (Table 5.1). The only difference observed was the cross reactivity of mAb MF14B4 (CD18) with non-leukocyte cells, such as hepatocytes, pulmonary epithelium, vascular endothelium, inner epidermis, hair follicle and mucosa of the dermal glands (Gupta et al., 1993). For the purpose of detailed immunohistochemical studies each group is represented by one mAb, such as MUC76A (CD11a), CC125 (CD11b), OM1 (CD11c) and BAQ30A (CD18). The reactivity of these mAb with mononuclear phagocyte (MP) populations in lymphoid organs (Lymph node, spleen, and thymus; Table 5.4) and in non-lymphoid organs (lung, liver, kidney, intestine, skin and brain; Table 5.5) was determined by immunoperoxidase staining on frozen sections. Staining could not be achieved in formalin fixed paraffin embedded sections using similar indirect immunoperoxidase technique. All the tissues were stained by the indirect immunoperoxidase method (section 2.15.1) except for intestine, lymph node and skin sections which were stained by Vectastain ABC method as described earlier (section 2.15.2). The parallel sections were also stained with saturated supernatant of VPM53 (anti-campylobacter mAb) or NMS (1:200) as negative controls. No background staining was observed with negative controls in any of the tissues stained.

Table 5.4 summarises the reactivity of mAb CC125 (anti-CD11b) and OM1 (anti-CD11c) with MP in lymphoid tissues. All tissue leukocytes in these organs were
**TABLE 5.2 Mean (Range) of Percent Cells Staining with Leukocyte Integrins (CD11/CD18) Monoclonal Antibodies**

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>MUC76A (CD11a)</th>
<th>CC125 (CD11b)</th>
<th>OM1 (CD11c)</th>
<th>BAQ30A (CD18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar Macrophages (n=4)</td>
<td>79(^1)</td>
<td>68</td>
<td>74</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>(66-88)(^2)</td>
<td>(45-82)</td>
<td>(54-90)</td>
<td>(76-89)</td>
</tr>
<tr>
<td>Peripheral Blood Granulocytes (n=6)</td>
<td>94</td>
<td>92</td>
<td>6</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>(91-97)</td>
<td>(87-96)</td>
<td>(2-7)</td>
<td>(85-97)</td>
</tr>
<tr>
<td>Peripheral Blood Mononuclear Cells (n=4)</td>
<td>79</td>
<td>28</td>
<td>26</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>(60-91)</td>
<td>(27-28)</td>
<td>(18-35)</td>
<td>(69-91)</td>
</tr>
<tr>
<td>Afferent Dendritic Cells (n=4)</td>
<td>58</td>
<td>6</td>
<td>76</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>(37-76)</td>
<td>(4-9)</td>
<td>(68-85)</td>
<td>(81-96)</td>
</tr>
<tr>
<td>Monocyte Enriched Mononuclear Cells (n=3)</td>
<td>73</td>
<td>75</td>
<td>36(^{Low})</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>(48-92)</td>
<td>(70-85)</td>
<td>(27-45)</td>
<td>(63-92)</td>
</tr>
<tr>
<td>Peripheral Blood Small Lymphocytes (n=5)</td>
<td>83</td>
<td>25</td>
<td>26</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>(63-94)</td>
<td>(14-32)</td>
<td>(18-40)</td>
<td>(59-99)</td>
</tr>
<tr>
<td>Afferent Lymphocytes (n=4)</td>
<td>91</td>
<td>2</td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>(88-95)</td>
<td>(1-3)</td>
<td>(1-3)</td>
<td>(87-99)</td>
</tr>
<tr>
<td>Efferent Lymphocytes (n=4)</td>
<td>93</td>
<td>1</td>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>(90-95)</td>
<td>(0.5-2)</td>
<td>(1-2)</td>
<td>(93-98)</td>
</tr>
</tbody>
</table>

\(^*\)number of samples analysed.

\(^1\)percent cell staining.

\(^2\)percent cell staining range of samples.

\(^{Low}\)Low intensity of staining.
FIGURE 5.3 (a) Frequency histograms showing staining of alveolar macrophages, peripheral blood monocytes (monocyte enriched mononuclear cells) and afferent dendritic cells with MUC76A (CD11a), CC125 (CD11b), OM1 (CD11c) and BAQ30A (CD18) mAb (---), and VPM53 negative control (--.--).
FIGURE 5.3 (b) Frequency histograms showing staining of peripheral blood mononuclear cells (PBMC) and PB Granulocytes (PBG) with MUC76A (CD11a), CC125 (CD11b), OM1 (CD11c) and BAQ30A (CD18) mAb (-----), and VPM53 negative control (-----).
Frequency histograms showing staining of peripheral blood small lymphocytes, afferent and efferent lymphocytes with MUC76A (CD11a), CC125 (CD11b), OM1 (CD11c) and BAQ30A (CD18) mAb (—), and VPM53 negative control (……).
**TABLE 5.3** Profile of Cellular Distribution of Leukocyte Integrins (CD11/CD18) as Determined by FACS.

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>CD11a &amp; CD18</th>
<th>CD11b</th>
<th>CD11c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar Macrophages</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Afferent Dendritic Cells</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Peripheral Blood Granulocytes</td>
<td>+</td>
<td>+</td>
<td>Sub.</td>
</tr>
<tr>
<td>Monocyte Enriched Mononuclear Cells</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Peripheral Blood Small Lymphocytes</td>
<td>+</td>
<td>Sub</td>
<td>Sub.</td>
</tr>
<tr>
<td>Afferent Lymphocytes</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Efferent Lymphocytes</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+, positive staining; -, no staining; ±, weak staining; sub, staining a subpopulation.
FIGURE 5.4(I) (a) Forward and side angle scatter profile of bone marrow (BM) cells showing analytical gates R1, R2 and R3.
Frequency histograms showing staining of bone marrow cells with MUC76A (CD11a) and BAQ30A (CD18) mAb (——), and VPM53 negative control (⋯⋯). All bone marrow cells (b), cells in R1 (c), R2 (d), and R3 (e).
FIGURE 5.4II Frequency histograms showing staining of bone marrow cells with CC125 (CD11b) and OM1 (CD11c) mAb (-----), and VPM53 negative control (------). All bone marrow cells (a), cells in R1 (b), R2 (c), and R3 (d).
FIGURE 5.5 Cytospots of fractionated afferent dendritic cells stained by indirect immunoperoxidase method with (a) VPM53 negative control, (b) CC125 (CD11b) showing staining of comparatively large round morphology Mφ like cell (arrow) and (c) OM1 (CD11c) staining most dendritic cells (arrow). x 400
stained with MUC76A (anti-CD11a) and BAQ30A (anti-CD18). The distribution of MP stained with all the four groups in non-lymphoid organs is given in Table 5.5.

**Lymph node** In prefemoral lymph node sections anti-CD11a and -CD18 mAb stained all leukocytes (Fig. 5.6). Anti-CD11b and -CD11c mAb stained subcapsular, interfollicular and medullary sinus large Mφ like cells, and stained a few Mφ in the follicle and the surrounding mantle. Interdigitating reticulum cells (IDC) in the cortex and medulla were stained by both the mAb. Anti-CD11c mAb could be discriminated by staining of IDC in the periphery of follicle/follicular mantle and comparatively more IDC in the interfollicular area (Fig. 5.6).

**Spleen** Anti-CD11a and anti-CD18 mAb, stained most leukocyte populations of spleen though with low intensity in the follicles (Fig. 5.7). Anti-CD11b produced characteristic staining of splenic marginal zone (MZ) cells. Some scattered Mφ in the red pulp, follicles and perivascular lymphoid sheath (PALS) were also stained (Fig. 5.7). Anti-CD11c mAb produced comparatively low intensity staining of MZ cells but staining of large Mφ like cells was more prominent (Fig. 5.7). As compared to CD11b, anti-CD11c stained more Mφ in the red pulp but fewer Mφ in the follicles and PALS were stained.

**Thymus** Anti-CD11a and anti-CD18 mAb, stained all the leukocytes in frozen sections of thymus (Fig. 5.8). Anti-CD11b and anti-CD11c mAb stained cortical Mφ, and most interlobular septal and perivascular Mφ. Anti-CD11b stained fewer Mφ and interdigitating cells in the medulla which is in contrast to anti-CD11c staining majority of Mφ and interdigitating cells in the medulla (Fig. 5.8).

**Lung** Anti-CD11a and anti-CD18 mAb, stained both, AM and interstitial Mφ populations in the lung sections (Fig. 5.9). Anti-CD11b and -CD11c mAb the stained majority of AM but fewer interstitial Mφ were stained (Fig. 5.9).

**Liver** Anti-CD18 mAb, stained majority of Kupffer's cells (Fig. 5.10). A lesser number of Kupffer's cells were stained with anti-CD11a mAb in parallel liver sections (Fig. 5.10a). In contrast anti-CD11b and -CD11c mAb stained few isolated Kupffer's cells in the liver sections (Fig. 5.10).
TABLE 5.4 Tissue Distribution of β2 (Leukocyte) Integrins on Mononuclear Phagocytes in Lymphoid Organs (prefemoral lymph node, thymus and spleen).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Monoclonal Antibody* (specificity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC125 (CD11b)</td>
</tr>
<tr>
<td></td>
<td>OM1 (CD11c)</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td>Subcapsular Sinus</td>
<td>Mφ</td>
</tr>
<tr>
<td>Follicle</td>
<td>Few Mφ</td>
</tr>
<tr>
<td>Perifollicular area</td>
<td>Few Mφ</td>
</tr>
<tr>
<td>Interfollicular area</td>
<td>Mφ &amp; IDC</td>
</tr>
<tr>
<td>Medulla</td>
<td>Mφ &amp; IDC</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>Mφ</td>
</tr>
<tr>
<td>Medulla</td>
<td>Few Mφ &amp; IDC</td>
</tr>
<tr>
<td>Interlobular Septae</td>
<td>Mφ</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>PALS and Follicle</td>
<td>Few Mφ</td>
</tr>
<tr>
<td>MZ</td>
<td>All cells</td>
</tr>
<tr>
<td>Red Pulp</td>
<td>Mφ</td>
</tr>
</tbody>
</table>

The distribution is based upon immunoperoxidase staining of frozen tissue sections. *MUC76A and BAQ30A mAb stained all the leukocyte populations. Mφ = Cell morphology identified as macrophages; IDC = Cell morphology identified as interdigitating reticulum cells. PALS = Periarteriolar lymphoid sheath; MZ = Marginal zone;
FIGURE 5.6 Vectastain ABC immunoperoxidase staining of frozen sections of prefemoral lymph node with (a) MUC76A (CD11a) and (d) BAQ30A (CD18) showing staining of all the leukocytes including subcapsular sinus Mφ (arrow); (b) CC125 (CD11b) staining Mφ in the follicular (F), perifollicular (PF) and subcapsular (SC) areas; and (c) OM1 (CD11c) showing staining of FDC and Mφ in the follicles (F), and IDC and Mφ in the perifollicular (PF) area. x100.
FIGURE 5.7 Indirect immunoperoxidase staining of frozen sections of spleen with (a) MUC76A (CD11a) and (d) BAQ30A (CD18) showing staining of most splenic leukocytes in red pulp (RP), marginal zone (MZ) and follicle (F); (b) CC125 (CD11b) and (c) OM1 (CD11c) showing staining of marginal zone (MZ) cells, follicular (F) Mφ and dendritic cells, and some red pulp (RP) cells. x100.
FIGURE 5.8 Indirect immunoperoxidase staining of frozen sections of thymus with (a) MUC76A (CD11a) and (d) BAQ30A (CD18) showing staining of all the leukocytes in the thymus; (b) CC125 (CD11b) and (c) OM1 (CD11c) showing staining of cortical (C) Mφ (arrow) and medullary (M) Mφ (arrow). Staining of interdigitating reticulum cells (arrow head) in the medulla was also observed with OM1 (CD11C). x250 (a,b & c), x100 (d).
Kidney Anti CD11a and CD18, stained a few cells in the glomerulus and tubular interstitium. Only isolated cells were stained in the glomerulus by anti-CD11b mAb and in the interstitium by anti-CD11b and -CD11c mAb (results not shown).

Intestine Peyer’s Patches All the four groups of mAb stained Mφ in lamina propria and dome regions of the jejunal and ileal Peyer’s patches (PP) except for weak staining of anti-CD11a (MUC76A) in both the PP. In the follicles of both the PP cells resembling Mφ/dendritic cells were stained by the mAb of all the four specificities except anti-CD11a in ileal PP and anti-CD11c (OM1) in jejunal PP. However the intensity of staining with anti-CD11b was weak in jejunal PP and of anti-CD11c was weak in ileal PP (Fig. 5.11 & 5.12). Anti-CD11a and anti-CD18 (BAQ30A) mAb stained follicular lymphocytes in jejunal PP (Fig. 5.11) but not in ileal PP (Fig. 5.12), in contrast follicular lymphocytes were not stained by anti-CD11b and -CD11c. In jejunal PP interfollicular irregular Mφ like cells in T-dependent area were stained by all the four groups of mAb. Staining of cells in the follicle associated epithelium (FAE) was observed with anti-CD11b, -CD11c and -CD18 mAb in ileal PP (5.12) and a weak staining was observed only with anti-CD18 mAb in jejunal PP. Anti-CD11a (MUC76A) mAb did not stain any cell in FAE of both the PP.

Skin Skin collected from the groin, was used for immunohistochemical analysis because of the lack of wool in this region. Epidermal Langerhans cells were stained by anti-CD18 and -CD11c, and comparatively a lesser number were stained with anti-CD11a mAb but none with anti-CD11b (Fig. 5.13). Dermal Mφ/dendritic cells were stained by all the four groups, comparatively a lesser number was stained with anti-CD11b and -CD11c mAb (Fig. 5.13).

Brain Anti-CD11a, -CD11b and -CD18 mAb stained a population of irregular elongated cells indistinguishable from microglial cells in the brain (cerebrum) parenchyma (Fig. 5.14). Comparatively a lesser number of microglial cells were weakly labelled with anti-CD11c mAb (Fig. 5.14c). The morphology of the cells recognised by all the groups remained the same.
TABLE 5.5 Tissue Distribution of β2 (Leukocyte) Integrins on Mononuclear Phagocytes in Non-Lymphoid Organs.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Monoclonal Antibody (Specificity)</th>
<th>MUC76A (CD11a)</th>
<th>CC125 (CD11b)</th>
<th>OM1 (CD11c)</th>
<th>BAQ30A (CD18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung:</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Alveolar Mφ</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Interstitial Mφ</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Liver:</td>
<td></td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Kupffer’s Cells</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Kidney:</td>
<td></td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Glomerular Cells</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Interstitial Mφ</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Skin:</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Dermal Mφ</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Langerhans Cells</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Jejunum:</td>
<td></td>
<td>+*</td>
<td>±</td>
<td>-</td>
<td>+*</td>
</tr>
<tr>
<td></td>
<td>PP Follicle Mφ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Inter-follicular Mφ</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lamina Propria and Dome Mφ</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FAE cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Ileum:</td>
<td></td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PP Follicle Mφ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lamina Propria and Dome Mφ</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FAE cells</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brain:</td>
<td></td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Microglial Cells</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

The distribution is based upon immunoperoxidase staining of frozen tissue sections.
Mφ, macrophages; PP, Peyer’s patch; FAE, follicle associated epithelium.
+ , Positive staining; -, no staining; ±, few weak staining cells.

1number of samples examined.

*Follicular lymphocytes were also stained with weak to moderate intensity.
FIGURE 5.9 Indirect immunoperoxidase staining of frozen sections of lung with (a) MUC76A (CD11a) and (d) BAQ30A (CD18) showing staining of interstitial (arrow head) and alveolar (arrow) Mφ; (b) CC125 (CD11b) and (c) OM1 (CD11c) showing staining of most alveolar Mφ (arrow) and few interstitial Mφ (arrow head). x250 (a,b & c), x400 (d).
FIGURE 5.10  Indirect immunoperoxidase staining of frozen sections of liver with (a) MUC76A (CD11a) and (d) BAQ30A (CD18) showing staining of most Kupffer's cells (arrow) in the hepatic sinusoids; b) CC125 (CD11b) and (c) OM1 (CD11c) showing staining of few isolated Kupffer's cells (arrow). x100.
FIGURE 5.11 Vectastain ABC immunoperoxidase staining of frozen sections of jejunal Peyer's patch with (a) MUC76A (CD11a) and (d) BAQ30A (CD18) showing staining of follicular (F) and lamina propria (LP) cells; (b) CC125 (CD11b) and (c) OM1 (CD11c) showing staining follicular (F) and lamina propria (LP) cells. x100.
FIGURE 5.12 Vectastain ABC immunoperoxidase staining of frozen sections of ileal Peyer's patch with (a) MUC76A (CD11a) showing a weak staining of lamina propria (LP) and dome (DE) cells; cells in follicle (F) and follicle associated epithelium (FAE, arrow) are not stained; (b) CC125 (CD11b) staining large MΦ like cells in lamina propria (LP), dome (DE), follicle (F) and FAE (arrow); (c) OM1 (CD11c) showing staining of lamina propria (LP) and follicular (F) large MΦ like cells; and (d) BAQ30A (CD18) staining large MΦ like cells in follicle (F), lamina propria (LP), dome (DE) as well as FAE (arrow). x100 (a, b & d), x250 (c).
FIGURE 5.13 Vectastain ABC immunoperoxidase staining of frozen sections of skin with (a) MUC76A (CD11a) showing staining of dermal Mφ (arrow) and epidermal Langerhans cells (arrow head); (b) CC125 (CD11b) staining dermal Mφ (arrow); (c) OM1 (CD11c) and (d) BAQ30A (CD18) showing staining of dermal Mφ (arrow) and epidermal Langerhans cells (arrow head).  x250.
FIGURE 5.14 Indirect immunoperoxidase staining of frozen sections of brain cerebrum with (a) MUC76A (CD11a); (b) CC125 (CD11b) and (d) BAQ30A (CD18) showing staining of irregular elongated microglial like cells (arrow) in the brain parenchyma; and (c) OM1 (CD11c) showing weak staining of a few microglial like cells (arrow). x400.
5.4 DISCUSSION

The aim of this study was achieved by defining a panel of mAb specific for the ovine β2 integrins (OvCD11/CD18). The antigens recognised by these mAb were characterised for their immunochemical characteristics, cellular distribution and tissue localisation.

On the basis of immunoprecipitation analysis the panel of mAb could be differentiated into four groups. All the mAb tested except CC126 and BAQ153A precipitate a heterodimer including a β chain of Mr = 95,000 associated with α chains of Mr = 150,000 or 170,000 or 180,000. The sequential immunoprecipitation experiments prove that the β chain is common and is associated with one of the distinct α chains.

The anti-CD11a and -CD18 mAb had very similar cellular and tissue distribution staining most of the leukocyte populations. A distinguishing feature is that anti-CD11a antibodies reacted with just one α chain of Mr = 180,000 and are therefore specific for the αL chain of CD11a. Similar characteristics of OvCD11a (Mackay et al., 1990) and BoCD11a (O'Reilly et al., 1991) have been defined earlier. Anti-CD18 mAb precipitated α chains of Mr = 150,000 to 180,000 but was specific for the common β chain (CD18). The specificity of anti-CD18 mAb to Mr = 95,000 β2 subunit of LI was revealed by western blot analysis. This specificity of anti-CD18 mAb for the common β subunit was further confirmed by antigen preclearing studies, in which no α subunit of LI could be precipitated from the lysate precleared of all the β subunit molecules using anti-CD18 mAb. This confirmed that the β chain of Mr = 95,000 is common to all the three members of LI family. The specificity of anti-CD11a to αL and anti-CD18 to the β chain was further confirmed by α chain sequential immunoprecipitation. The existence of remaining β chain after removal of all three α chains suggests the existence of free β chain not associated with α chains. The α and β subunits of LI have been reported to exist only as dimers on the cell surface (Todd et al., 1984). The existence of free β chains, suggests the possibility of detection of externally acquired β-chains in a ligand receptor interaction, by cell surface labelling (Trowbridge and Omary, 1981). However, the absence of any α chains after this protocol argues against the existence of a fourth, unidentified, β2 integrin molecule. MF14B4 mAb had been shown to be specific for the β2 (BoCD18) subunit of cattle leukocyte integrins (Letesson and Delcommenne, 1993) thus confirming the present finding in sheep.
Anti-CD11b mAb labelled all the AM, monocytes and granulocytes but did not stain ADC. These mAb precipitated αM chain of \( M_r = 170,000 \) suggesting their specificity for ovine CD11b. Similar characteristics of BoCD11b have been reported earlier (Splitter and Morrison 1991).

Anti-CD11c mAb immunoprecipitated α chain of \( M_r = 150,000 \) suggesting specificity for the αX chain of CD11c. This antibody stained all AM and ADC, weak on monocytes but, unlike anti-CD11b mAb, did not react with neutrophils. Its reactivity on granulocytes is explained by its reactivity on eosinophils (Pepin et al., 1992). This is in contrast to cattle (Splitter and Morrison 1991), human (Fryer et al., 1988) and mouse (Kishimoto et al., 1989a) where all the granulocytes express CD11c. The staining of epidermal Langerhans cells by OM1 is in contrast to a previous report (Pepin et al., 1992) and might be attributed to different staining techniques used. A similar CD11c+ and CD11b− phenotype of dendritic cells has been described in human (Steinman et al., 1991) and Langerahns cells in cattle (Hall et al., 1993).

Anti-CD11b and -CD11c mAb could be discriminated by the difference in the α chains of \( M_r = 170,000 \) and 150,000 respectively associated with a common β2 chain of \( M_r = 95,000 \). The uniqueness of the α chains, recognised by anti-CD11a, -CD11b and -CD11c was determined by sequential depletion of αL followed by αM and finally precipitation of αX. This confirms that the mAb representing each group recognise distinct cell surface molecules, which are related to each other by virtue of their association with common β2 subunit indicating their relationship within the leukocyte integrins family.

The results of immunochemical analysis indicate that, like human (Kishimoto et al., 1989a), mouse (Pont et al., 1986) and cattle (Howard and Naessens 1993), the sheep β2 integrins consist of three closely related heterodimeric antigen molecules.

Lymphocytes from peripheral blood, afferent and efferent lymph showed identical reactivity with anti-CD11b and -CD11c mAb. Both recognised a substantial subpopulation of resting lymphocytes in blood (14-32%) but a small proportion (1-3%) of lymphocytes were stained in afferent and efferent lymph. In cattle, CD11b is present on a subpopulation of B lymphocytes (Naessens and William, 1992), but CD11c is absent from normal lymphocytes in peripheral blood (Splitter and Morrison, 1991). In human CD11b is present on a small subpopulation of T lymphocytes (Hoshino et al., 1993) but is not expressed on B lymphocytes (De La Hera et al., 1988). CD11c is present both on normal B cell subset (Wormsley et al., 1990) and
activated B-cells (Postigo et al., 1991) in peripheral blood. CD11b and CD11c have also been found on a number of lymphoid and myeloid malignancies in human (Todd et al., 1981; De La Hera et al., 1988). The subpopulation of lymphocytes expressing CD11b and CD11c is described in Chapter 6.

In bone marrow (BM), lack of expression of CD11a and CD18 on some cells is similar to earlier findings in sheep (Haig et al., 1991) and cattle (Muiya et al., 1993). It has been suggested that CD11a is not expressed in erythroid progenitors and may be lacking in very early stages of myeloid progenitors in sheep and cattle. This is in contrast to human where all erythroid and myeloid progenitors express CD11a and CD18 (Todd and Schlossman, 1984). CD11b expression on BM cells is similar to those reported earlier in sheep (Haig et al., 1991). In humans and mice both CD11b and CD11c have been detected from committed neutrophil and monocyte precursors at myelocytic and monoblastic stages respectively (Todd and Schlossman, 1984). Thus in sheep, CD11b expression on BM cells appears similar to that in other species, but CD11c differs due to lack of expression on some of the granulocytes and myeloid cells.

CD11a and CD18 were expressed by most tissue leukocytes as revealed by immunohistochemical staining. The difference observed was lack of or low level of CD11a expression by some cells as compared to CD18. This difference was more prominent in Kupffer's cells in liver and epidermal Langerhans cells. The expression of CD11a and CD18 on most leukocyte populations and tissue leukocytes in sheep is consistent with earlier observations in humans (Kishimoto et al., 1989a; Yong and Khwaja 1990), mouse (Pont et al., 1986) and cattle (Howard and Naessens 1993; Hall et al., 1993). However in mice (Krensky et al., 1983) and rats (Tamatani et al., 1991) peritoneal macrophages are devoid of CD11a. The difference in expression of CD11a and CD18 is obvious because different populations of cells may be stained by mAb to CD11a, CD11b and CD11c but CD18 being common to all will stain all the cells.

The expression of CD11b and CD11c in tissues remained restricted to resident mononuclear phagocytes except in spleen where marginal zone lymphocytes are also positive for CD11b and weakly positive for CD11c. The tissue distribution of CD11b and CD11c was quite similar except that CD11c was more common on cells with dendritic morphology such as Langerhans cells, IDC in lymph node etc. as compared to CD11b. Thus on the basis of tissue localisation both CD11b and CD11c can be
regarded as myeloid differentiation antigens except in spleen. Similar tissue
distribution on MP with some variations among different mAb of the same specificity
have been described in human (Franklin et al., 1986; Koller, 1989), mouse (Kurzinger
et al., 1981; Krensky et al., 1983) and cattle (Splitter and Morrison, 1991; Hall et al.,
1993).

5.5 CONCLUSIONS.
The following conclusions can be drawn from the present study:

1. Monoclonal antibodies MUC76A, MD2B7, 72-87 and F10-150, are specific to αL
chain and thus define the ovine homologue of CD11a, which is expressed on most
leukocytes.

2. Monoclonal antibodies CC125 and IL-A15 specifically recognise the αM chain and
thus define the ovine homologue of CD11b. The molecule is expressed predominantly
on MP but absent in ADC and epidermal Langerhans cells. Interestingly it was found
to be present on a subpopulation of peripheral blood lymphocytes and MZ
lymphocytes in spleen (described in Chapter 6).

3. Monoclonal antibody, OM1 recognised an epitope present on the αX chain of LI
thus defining ovine homologue of CD11c. It is predominantly expressed on MP
including ADC and Langerhans cells. Lack of CD11c on neutrophils is unique to
sheep. Its expression on lymphocytes is similar to CD11b.

4. Monoclonal antibodies, BAQ30A and MF14B4 specifically reacted with epitope(s)
expressed on β2 chain common to all the three members of LI thus defining the ovine
homologue of CD18. Like CD11a, it is widely distributed on leukocyte populations.

This work identifies, unambiguously, a panel of mAb against the β2 (leukocyte)
integrins in sheep. These are now available for basic cell biological and
immunological studies in this species as well as investigations into disease
pathogenesis in sheep.
CHAPTER SIX

CHARACTERISATION OF CD11b+ B LYMPHOCYTES IN VARIOUS LYMPHOID COMPARTMENTS
6 CHARACTERISATION OF CD11b+ B LYMPHOCYTES IN VARIOUS LYMPHOID COMPARTMENTS

6.1 INTRODUCTION
The data presented in Chapter 5 shows that CD11b (CR3) is predominantly expressed by myeloid cells. Expression of this molecule was also observed on a substantial proportion (14 to 36%) of small lymphocytes in peripheral blood and on all the marginal zone lymphocytes in spleen. By contrast, CD11b is expressed on only a negligible proportion (0.5 to 2%) of lymphocytes in afferent and efferent lymph. The pattern of CD11c (CR4) expression on lymphocytes is essentially identical to that of CD11b. This differential expression of these molecules prompted an investigation to define the lymphocyte subset(s) expressing CD11b.

Preliminary investigations revealed that the CD11b+ lymphocytes are B lymphocytes. Therefore the present work was undertaken to define the phenotype and distribution of CD11b+ and CD11b− B cells in different lymphoid compartments. Their relationship with peripheral lymph node homing receptor (L-selectin) was also investigated in order to define the difference in the anatomical distribution. An attempt was made to find out whether these populations are inter-convertible on activation or represent different cell lineages. Before presenting the results, I will briefly introduce the origin of B lymphocytes, their relation with CD11b or related antigens in other species, phenotype of naive and memory B cells and physiology of peripheral lymph node recirculation.

6.1.1 ORIGIN OF B LYMPHOCYTES
Broadly, B cell production can be divided into antigen-independent and antigen-dependent stages. The antigen-independent development occurs in a variety of primary lymphoid organs depending upon the stage of the foetal development and species involved. It results in the generation of mature B cells which enter the peripheral lymphoid pool. This antigen independent phase lasts throughout the life time of the individual. In man and mice, the bone marrow is the primary lymphoid organ of B cell production, whereas in ruminants, the ileal Peyer's patch (IPP) also makes a large contribution to B cell production (Reynaud et al., 1991; Motyka and Reynolds, 1991; Reynolds et al., 1991; Griebel et al., 1992).

In the mouse, early B cell production occurs in the liver and later in the bone marrow. B cells are derived from pluripotent stem cells which do not express surface
immunoglobulin (Ig). The pre-B lymphocyte is the earliest cell type that synthesizes a detectable Ig gene product in the form of cytoplasmic μIg heavy chains composed of variable (V) and constant (C) regions. As pre-B lymphocytes do not make light chains, they are incapable of expressing membrane Ig on their cell surface. At the next stage in B cell development, membrane IgM is expressed on immature B lymphocytes following synthesis of κ or λ light chains. The B cell at this stage is sensitive to inactivation (tolerance) by antigen recognition, which leads to deletion of self reactive B cells from the antibody repertoire (Nossal, 1983). These B cells then co-express membrane IgD and become mature B lymphocytes. Both classes of membrane Ig have the same V region associated with the same κ or λ light chain and hence the same antigen specificity. The B cells are capable of binding antigen to its membrane antigen receptor and generate a positive response by Ig production. After acquiring complete Ig, mature B cells migrate out of the bone marrow and can be found in the peripheral circulation and lymphoid tissues. Differentiation to the mature B lymphocyte stage is antigen independent. The mechanism that controls the exit of recently mature B cells from their site of production is poorly understood, although Osmond, (1986) postulated that murine B cells receive a final maturation signal within the sinusoids of the bone marrow.

In lambs, the development and involution of the ileal Peyer's patch (IPP) has many characteristics of the bursa of Fabricius in birds. The IPP develops in the terminal ileum from approximately 60 days of gestation onwards. It consists of tightly packed follicles with little interfollicular tissue and it is histologically mature well before birth. After birth, the IPP begins to involute and is virtually absent by 18 months of age. The removal of the IPP before or shortly after birth results in a severe deficiency of Ig bearing cells in the blood and lymph (Gerber et al., 1986). However these lambs have serum Ig levels within normal range which is in contrast to bursectomised chicken. In addition these animals are able to respond to Salmonella muenchen in a similar fashion to intact animals (Gerber et al., 1985). The prenatal exit of B cells from the IPP may account for the presence of B cells and therefore antibodies in ilectomised animals. This situation appears to be analogous to the chicken embryo when bursectomy is performed at 16 days of incubation. Alternatively the existence of normal immune responses and the level of Ig in ilectomised lambs may be due to the presence of another population of B cells as described in the spleen of foetal lambs (Press et al., 1993). Production of B cells within the IPP follicles is characterised by rapid proliferation and extensive cell death by apoptosis. Less than 5% of B cells emigrate from the follicles (Motyka and Reynolds, 1991) and perfusion studies have
shown that the cells exiting the IPP colonise secondary lymphoid organs (Reynolds et al., 1991). Further, the generation of antibody diversity in IPP through high rate somatic hypermutation in the V genes of the Ig light chain, strongly supports the hypothesis that sheep IPP functions as a bursa equivalent and a primary B cell organ (Reynaud et al., 1991).

6.1.2 DEVELOPMENT AND DISTRIBUTION OF CD5+ B CELLS

CD5+ B cells differ from conventional B cells by a number of criteria including their surface phenotype, tissue distribution and antibody specificity. According to a recent B cell nomenclature (Kantor, 1991), B-1 cells represent Ly-1+ or CD5+ B cells which arise early in ontogeny both in mice and humans. In mice B-1 cells have a characteristic phenotype (IgM<sup>hi</sup> IgD<sup>low</sup> CD11b<sup>+</sup> CD45<sup>low</sup>). The self-replenishing murine B-1 cells are further subdivided into two independent populations: B-1a cells which express CD5 and constitute the majority of B-1 cells in normal animals and B-1b cells which share phenotype and functional characteristics with B-1a cells but do not express detectable levels of CD5. B-2 cells represent conventional B cells (IgM<sup>low</sup> IgD<sup>high</sup> CD5<sup>-</sup>) which arise late in ontogeny both in mice and humans. B-1b cells are preferentially located in the marginal zone and not in the follicles of the murine spleen (Kroemer et al., 1993). In contrast B-1a cells predominate in the peritoneal and pleural cavities, are rare in the spleen and undetectable in blood and lymph nodes (Hayakawa et al., 1983; Hayakawa et al., 1986). In humans CD5+ B cells constitute up to 25% of the B cells in the adult peripheral blood and spleen (Casali and Notkins, 1989). However CD5+ B cells are a predominant component of B cells in human foetal spleen and blood (Hayakawa and Hardy, 1988). It has not yet been established whether they constitute a different lineage with different precursors (Hayakawa et al., 1985), or whether they represent a distinct maturational stage because human CD5<sup>-</sup> B cells express CD5 following phorbol ester treatment (Freedman et al., 1989). There are different opinions about the origin of CD5+ B cells. The strongest claim for a separate CD5+ B cell lineage has been made in the mouse, where it is derived from early progenitors that have both heavy and light chain genes in germ line configuration (Palacios et al., 1987; Katoh et al., 1990). These progenitors of the CD5+ lineage differentiate to either pre-B or myeloid cells depending upon the environment of stromal cells and cytokines. IL-5 induces pre-B cell production while GM-CSF induces myeloid cell differentiation (Katoh et al., 1990).
According to the Herzenberg model of a layered immune system (Herzenberg and Herzenberg, 1989) based upon ontogeny, B-1a cells associated with the earliest evolutionary layers would be expected to respond very rapidly to invading pathogens. They have a relatively restricted repertoire that is rarely extended by somatic mutation (Herzenberg et al., 1986). The next lineage gives rise to the B-1b cells and appears to be at a somewhat more advanced functional level. The third lineage generates the conventional B-2 cells that constitute the majority of lymphocytes present in adult spleen, peripheral blood and lymph node and are responsible for most of the primary and secondary antibody responses. The B-2 cells are generated throughout life from their bone marrow progenitors and unless they encounter antigen and differentiate into memory cells they tend to be short lived (Linton et al., 1989). The B-2 cells have a virtually unlimited, highly mutated repertoire which comes into play mainly when the immune system is severely challenged or antigen is met in the secondary lymphoid organs.

In contrast to the Herzenberg model, a common origin of both the B cell lineages with the option to give rise to a B-2 or, after activation to the B-1 phenotype has also been suggested (Kroemer et al., 1993), the commitment to the B-1 lineage being irreversible. This model explains the expression of CD5 on CD5⁻ cells following activation (Freedman et al., 1989)

CD5⁺ B cells are responsible for the production of most of the normal serum Ig. They produce polyreactive antibodies and antibodies with specificity for autologous antigens (Forster and Rajewsky 1987). They do not appear to be involved in secondary antigen specific responses and their primary role seems to be in homeostasis of the humoral immune system and as a first line of defence when a high affinity antibody system is not yet available (Casali and Notkins, 1989).

The CD5 molecule is well conserved in sheep and its distribution in sheep is similar to that reported for other species (Mackay et al., 1985; Beya and Miyasaka, 1986). CD5 is expressed on all T lymphocytes and a small proportion of adult spleen B cells weakly express this molecule. CD5⁺ B cells are not detectable within lymph nodes or efferent lymph, indicating that these cells may be non-recirculating (Mackay et al., 1985; Mackay, 1988). The proportion of CD5⁺ B cells remained below 13% of the B cells in peripheral blood of sheep (Onah, 1992). CD5⁺ B cells in cattle represent a quarter of the B cells in peripheral blood and 3-9% in spleen (Naessens and Williams, 1992). They represented a negligible proportion in lymph node and were absent in
Peyer's patches. There is a sharp increase in CD5+ B cells following Trypanosome infection in both sheep and cattle, which was related to high surface and serum IgM levels (Naessens and Williams, 1992; Onah, 1992). Most of the B cells from bovine leukemia virus (BLV) infected sheep also express CD5 molecules (Letesson et al., 1990). A small proportion of CD5+ B cells in post natal life and foetal lambs suggests predominance of B-1b (CD5−) cells rather than B-1a (CD5+) in normal sheep (Press et al., 1993). The increase in number of CD5+ B cells following Trypanosome and BLV infection in ruminants seems to be analogous to human where B cells express CD5 following in vitro activation and lymphocytic leukaemias (Freedman et al., 1989; De La Hera et al., 1988).

6.1.3 EXPRESSION OF CD11b ON B CELLS AND ITS RELATIONSHIP WITH CD5+ B CELLS
As described above CD11b (Mac-1) has been associated with B-1 cells in mice (Herzenberg et al., 1987). Davidson et al., (1988) and Katoh et al., (1990) suggested a close relationship between the myeloid (CD11b) and the pre-B lymphoid (B-1a) pathways of differentiation in mice. They suggested that progenitors of the B-1a lineage differentiate to either pre-B or myeloid cells depending upon the treatment of cultured cells. Such a relationship of CD11b with CD5+ B cells has not been reported in normal humans and rats. However CD5+ B cells from chronic lymphocytic leukaemia patients have been shown to co-express CD11b, whereas in normal individuals the proportion of such cells is of the order of ~1% of the B cells (De La Hera et al., 1988). CD11c has been shown to be a marker for leukemic B cells where all the CD11c+ B cells co-express CD5 and a small proportion of such cells exists in normal humans (Wormsley et al., 1990). CD11b and CD11c expressed on activated or leukemic B cells in humans retain their role in B cell activation and adhesion function (Postigo et al., 1991; De La Hera et al., 1988). Co-expression of CD11b on CD5+ B cells has recently been demonstrated in normal and Trypanosome infected cattle where the majority of CD11b+ B cells co-express CD5 but a small proportion remain CD5−CD11b+ (Naessens and Williams, 1992).

6.1.4 ANATOMICAL LOCALISATION OF IgM+ IgD− B CELLS
B cells with the surface phenotype of IgM+IgD− represent B-1 cells irrespective of the expression of CD5 as described above. In mice, IgM+IgD− B cells are preferentially located in the marginal zone (MZ) but not in the follicles of spleen and constitute <15% of the splenic B cells. Cells with a similar phenotype are virtually absent in lymph nodes and peripheral blood (Kroemer et al., 1993).
In both rat and man the cells with the above phenotype are predominant in the MZ of spleen though a small number are present in the peripheral blood and lymph node (Gray et al., 1982; Timens, 1991). Cells with a similar phenotype have been described forming a band between the follicular mantle and dome epithelium in Peyer’s patches (Spencer et al., 1985). Isolated collections of such cells are present under the subcapsular sinus of lymph nodes (Liu et al., 1989). These MZ B cells are sessile and remain in the spleen (Kumararatne et al., 1981; Kumararatne and MacLennan, 1981). The specific localisation of IgM+IgD" B cells to the MZ in spleen in diverse species is suggestive of a specific function. The MZ in rats and human are perfused with blood sinusoids which makes them well placed to respond to antigens in the blood. The MZ B cells are able to respond to all three classes of antigens, protein based T cell dependent (TD) antigens, LPS based T cell independent (TI)-1 antigens and polysaccharide based TI-2 antigens (Liu et al., 1988). MZ B cells are of particular importance because of their ability to respond to TI-2 antigens as other B cell subsets lack this capacity. MZ B cells remain within the MZ without dividing, unless they are induced by antigens to migrate to sites where they proliferate and differentiate. They migrate to follicles and or periarteriolar T cell areas, and this migration is important for the transfer of antigens to FDC, IDC and T cells (MacLennan and Liu, 1991).

6.1.5 PHYSIOLOGY OF LYMPHOCYTE MIGRATION AND RECIRCULATION

Newly formed lymphocytes are exported from the primary lymphoid organs (bone marrow and thymus) to secondary lymphoid organs mainly via blood circulation. Peyer’s patch lymphocytes migrate to mesenteric lymph node via lymphatics. Secondary lymphoid organs dispersed all over the body, are connected by the blood vessels and the lymphatic ducts which allow the lymphocytes to recirculate throughout the body. Lymphocyte recirculation is a normal physiological process independent of prior stimulation with external antigens as it takes place in the ovine foetus, which develops in an environment devoid of extrinsic antigens (Pearson et al., 1976).

In post natal life, lymphocyte recirculation allows effective uptake and presentation of antigens to a small set of antigen specific lymphocytes in a larger pool of cells (Hopkins et al., 1981; Hopkins et al., 1989). It disseminates the effector and memory cells to facilitate rapid secondary responses throughout the body (Hopkins and McConnell, 1993; Bujdoso et al., 1989) and allows effector molecules and cells to localise rapidly to the site of infection.
The physiology of lymphocyte recirculation through the peripheral lymph node has been extensively studied in sheep using the isolated lymph node model (McConnell and Hopkins, 1981; Hopkins and McConnell, 1993; Miyasaka and Trnka, 1986; Bujdoso et al., 1989; Morris and Courtice, 1977). 90% of the lymphocytes which exit the lymph node in the efferent lymph enter from the blood across the specialised endothelium of the post capillary venules (PCV) (Hall and Morris, 1965; Gowans and Knight, 1964). These endothelial cells lining the PCV are cuboidal/columnar shaped rather than the normal flattened endothelium of the blood vessels. This region of the PCV is known as high endothelial venule (HEV) (Gowans and Knight, 1964). Less than 10% of the efferent lymphocytes are contributed by the afferent lymph flowing in to the lymph node and less than 2% are generated by cell division within the lymph node itself. Lymphocytes leaving the node in the efferent lymph are responsible for the establishment of immunological memory and dissemination of the immune response to other lymphoid organs.

**Adhesion molecules involved in lymphocyte migration** The migration of lymphocytes between blood and lymph is non-random (Mackay et al., 1990; Bujdoso et al., 1989). The different patterns of lymphocyte traffic in vivo are critically dependent upon cell-cell and cell matrix receptor ligand interactions occurring between recirculating lymphocytes and endothelial cell/extracellular matrix (Stoolman, 1989; Osborn, 1990; Dustin and Springer, 1991). The extravasation in the lymph node is initially mediated by homing receptor molecules on the lymphocyte cell surface that interact with vascular addressins expressed on HEV (Stoolman, 1989). Lymphocyte-HEV interaction was first demonstrated in in vitro studies by Stamper and Woodruff (1976 & 1977). The development of HEV is influenced by IL-1, IFN-γ and TNF-α released by activated lymphocytes (Cavender et al., 1987). In man and mouse three families of lymphocytes homing receptors have been identified, the selectins, CD44 and the integrins (β1 and β2) (Kimpton et al., 1994). These receptor molecules associate with their endothelial ligands, the addressins, molecules of the Ig superfamily and the cell matrix (Fig. 6.1).

L-selectin is the best documented peripheral lymph node homing receptor on lymphocytes in sheep (Mackay et al., 1992a) and cattle (Howard et al., 1992). L-selectin interacts with an oligosaccharide ligand expressed on HEV which is associated with the peripheral lymph node addressin (PNAd) (Mackay et al., 1992a). The majority of efferent lymph cells are L-selectin+. Although L-selectin is important in mediating lymphocyte entry to peripheral lymph nodes, there are different
FIGURE 6.1 Schematic presentation of possible lymphocyte-endothelial interactions which may regulate the homing of lymphocytes to normal and inflamed skin tissue and peripheral lymph node. PNAd, peripheral lymph node addressin; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; LFA-1, lymphocyte function associated antigen-1 (αLβ2 integrin); VLA-4, α4β1 integrin. (taken from Kimpton et al., 1994) Some of the adhesion molecules are known to regulate the homing of T lymphocytes but their role in B cell homing is largely unknown.
NORMAL LYMPH NODE

Lymphocyte

- L-Selectin
- Possible T Cell or B Cell subset adhesion molecule
- LFA-1

Endothelial Cell

- PNAd
- Unknown
- ICAM-1
- ICAM-2

STIMULATED LYMPH NODE

Lymphocyte

- L-Selectin
- Possible T Cell or B Cell subset adhesion molecule
- ICAM-1
- ICAM-2
- LFA-1

Endothelial cell

- PNAd
- VLA-4
- Unknown
- ICAM-1
- ICAM-2

NORMAL SKIN

Lymphocyte

- Skin homing receptor unknown
- Possible T Cell or B Cell subset adhesion molecule
- LFA-1

Endothelial Cell

- Skin addressin unknown
- Unknown
- ICAM-1
- ICAM-2

STIMULATED SKIN

Lymphocyte

- Skin homing receptor unknown
- Possible T Cell or B Cell subset adhesion molecule
- L-Selectin
- LFA-1

Endothelial Cell

- Skin addressin unknown
- VCAM-1
- Unknown
- PNAd
- ICAM-1
- ICAM-2
proportions of T lymphocyte subsets in blood and efferent lymph suggesting that additional subset linked selection mechanisms may be operating at the HEV. T lymphocytes localised in the skin express a carbohydrate known as cutaneous lymphocyte associated antigen that can bind to E-selectin expressed on dermal endothelial cells (Springer, 1994). Skin homing T cells in afferent lymph have been shown to display memory phenotype as compared to the naive phenotype of the peripheral lymph node homing cells which enter via HEV (Mackay et al., 1992a). It is not known whether such B cell subsets show any preferential homing to skin and lymph node.

6.1.7 NAIVE AND MEMORY B LYMPHOCYTES
Immunohistochemical studies of spleen for antigen specific antibody forming cells and their isotypes has lead to a generalised hypothesis for the migration of B lymphocytes. Naive B cells enter the MZ of the spleen and after antigen stimulation these cells migrate through the periarteriolar lymphoid sheath (PALS) where they contact antigen presenting cells and T cells. After traffic through MZ bridging channels they accumulate as mature plasma cells around the terminal arteriole of PALS in the red pulp of spleen (Von Rooijen et al., 1986). In contrast recirculating memory cells preferentially migrate into germinal centres (Vonderheide and Hunt, 1990).

There are no specific markers for naive and memory B cells, however they can be distinguished to some extent by their surface immunoglobulin. Cells bearing surface IgM and IgD present in the peripheral recirculating pool, in spleen and bone marrow are considered naive. This designation was based upon the repertoire of V region gene expressions in IgM+IgD+ B cells in different compartments in mice (Gu et al., 1991). The main characteristics of memory B cells are that they have undergone clonal expansion and somatic mutation in their V region gene and are no longer in cell cycle. The predominant Ig isotype on memory cells is IgG (Coffman and Cohn, 1977) however these cells can express IgM (Abney et al., 1976) and may still secrete IgM (Lafrenz et al., 1986). An increased affinity of the surface Ig for a particular antigen has also been used as a marker for memory cells (Yefenof et al., 1986). It has been proposed that in the absence of further stimulation, a memory B cell may revert to its naive phenotype (Gray, 1993).

Non-Ig surface markers differentially expressed on virgin and memory B cells include peanut-agglutinin binding molecules (PNABM), MEL-14 (L-selectin) and a heat stable antigen recognised by mAb J11D (Bruce et al., 1981; Kraal et al., 1988; Coico
et al., 1983). MEL-14 and PNABM distinguish naive and memory cells at a very specific point in their development, namely in germinal centres. As they begin to proliferate in germinal centres B cells become PNABM+ and MEL-14+; after this phase the selected population that rejoins the recirculating pool as memory cells becomes PNABM- and MEL-14+ (Kraal et al., 1988; Coico et al., 1983). The mAb JD11 has been shown to distinguish naive B cells from memory B cells (Bruce et al., 1981). In subsequent experiments JD11hi cells were found to take part in primary antibody responses and JD11low cells only in secondary antibody responses. Also the somatic mutations were found only in the Ig V region in J11Dlow cells and the same cells give rise to germinal centres thus fulfilling the criteria of memory B cells (Linton et al., 1989; Linton et al., 1992).

6.2 AIMS OF THIS CHAPTER
The work in this chapter is the continuation of Chapter 5 where CD11b(CR3) a predominant myeloid antigen was found on a substantial proportion of lymphocytes in peripheral blood but not in afferent and efferent lymph. CD11b+ lymphocytes were found to be B lymphocytes. Therefore this work was undertaken with the specific aims:

1) To define the phenotype and distribution of CD11b+ and CD11b- B lymphocytes in various lymphoid compartments.

2) To investigate the relationship of CD11b+ B cells with L-selectin a peripheral lymph node homing receptor.

3) To investigate the inter-conversion of CD11b+ and CD11b- B lymphocytes upon activation.
6.3 RESULTS

Cells from various lymphoid compartments (peripheral blood, afferent and efferent lymph, lymph node, spleen, ileal Peyer’s patch and bone marrow) were prepared as described earlier (Section 2.3). Two-colour FACS analysis of cells was performed (section 2.11) after setting a live gate on small mononuclear cells to exclude large complex and dead cells (Fig. 2.4). All the mAb used in this work have defined specificity (Table 2.1) except mAb Du2.74. Du2.74 recognises a non-Ig determinant present only on a subset of surface Ig+ cells in sheep blood. It stains all the B cells in the blood of foetuses and young post-natal lambs. However in older animals it stains a subset of peripheral blood B cells which recirculate through lymph nodes and enter efferent lymphatics. The target molecule(s) recognised by the mAb is not known (Dr. W. R. Hein, Basal Institute of Immunology, Basal, Switzerland, personal communication). VPM30 a pan-B cell marker in cattle (Naessens and Howard, 1991) was used in this study. However, two-colour FACS analysis of VPM30 with T lymphocytes subsets (CD4, CD8 and γδ) revealed a low level of VPM30 expression on a small proportion of CD4 cells (Fig. 6.2). Therefore for the analysis of B lymphocytes stained with VPM30, the quadrant excluding low expressing VPM30 cells was set as shown in Fig. 6.2.

6.3.1 DISTRIBUTION OF CD11b+ B LYMPHOCYTES

Lymphocytes in various lymphoid compartments were analysed for the expression of CD11b on VPM30+ B lymphocytes by two-colour FACS analysis (Table 6.1; Fig. 6.3). CD11b was not found to be expressed on any of the T lymphocyte subsets (CD4, CD8 and γδ) in peripheral blood (Fig. 6.2). Similar results were obtained in other compartments (results not shown).

A mean of 38% of the lymphocytes in peripheral blood are B lymphocytes and CD11b expression was seen on about half of the B lymphocytes (Table 6.1; Fig. 6.3). Afferent and efferent lymph both contain a mean of 18% of the lymphocytes as B lymphocytes and very few of the B lymphocytes (<5%) express CD11b.

The situation in peripheral (prefemoral) lymph node (PLN) as well as mesenteric lymph node (MLN) was the same as in afferent and efferent lymph (Table 6.1; Fig. 6.3). In both PLN and MLN, only a small proportion of B cells (2-3%) express CD11b (Fig. 6.3). These CD11b+ cells are not confined to any particular area in the lymph node and are uniformly scattered in the paracortex and medulla as observed by immunohistochemistry (Fig. 6.4).
FIGURE 6.2 Two-colour contour plots of peripheral blood small mononuclear cells stained on FL1 with VPM30 (Pan B lymphocyte; a, b & c), CC125 (CD11b; d, e & f); and on FL2 with SBUT4 (CD4; a & d), SBUT8 (CD8; b & e) and 86D (γδT cell receptor, γδTCR; c & f).
In spleen a mean of 70% of the lymphocytes are B cells and CD11b is expressed on about a quarter (27%) of the B lymphocytes (Fig. 6.3; Table 6.1). All the CD11b+ cells in spleen are confined to marginal zone, except a few isolated cells scattered in the red pulp (Fig. 6.5). CD11b+ lymphocytes are absent in ileal Peyer's patches (IPP) as revealed by FACS analysis (Fig. 6.3; Table 6.1). Anti-CD11b (CC125) stained large Mø like cells in the follicles, follicle associated epithelium, dome and villi lamina propria in frozen sections of the same IPP but lymphocytes are not stained (Fig. 6.4). In bone marrow (BM) VPM30 stained about 3% of the cells in lymphoid and myeloid cell region (R1+R2, regions shown in Fig. 2.4) and these are CD11b+ cells (Fig. 6.3). None of the BM cells expressed surface IgM (results not shown).

Thus these results indicate that CD11b is expressed on a substantial proportion of B cells in peripheral blood and spleen. In contrast a negligible proportion of afferent and efferent lymph as well as PLN and MLN B cells express CD11b, implying that these cells do not enter lymph nodes. CD11b+ B cells are absent in IPP a primary B cell lymphoid organ.

The distribution of CD11c+ cells was more or less similar to CD11b particularly its staining of peripheral blood lymphocytes, marginal zone in spleen and non-staining of lymphocytes in any other lymphoid organs (Chapter 5). The majority of CD11b+ cells co-express CD11c (Fig. 6.7III), thus suggesting similar characteristics of CD11c expressing B cells.

6.3.2 RELATIONSHIP BETWEEN CD11b+ CELLS AND DU2.74+ B CELLS
To investigate the relationship between CD11b+ B cells and recirculating B cells two-colour FACS analysis was performed using Du2.74 which stains recirculating B cells (a gift from Dr W. R. Hein, Basal, Switzerland) and anti-CD11b mAb. In peripheral blood, Du2.74 stains a mean of 45% of the B lymphocytes and only a small proportion (<8%) of the B cells co-express Du2.74 and CD11b (Table 6.1; Fig. 6.6). In afferent and efferent lymph, Du2.74 stains the majority of B cells and <3% of the B cells (i.e. about half of the CD11b+ B cells) co-express Du2.74 and CD11b (Table 6.1; Fig. 6.6).

As in afferent and efferent lymph, Du2.74 stains the majority of the B cells obtained from PLN. Du2.74 is co-expressed with CD11b on about 3% of the B cells (i.e. almost all of the CD11b+ B cells) in PLN. Similar results were obtained with MLN cells (Fig. 6.6; Table 6.1). Immunohistochemical staining of lymph node revealed the
<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>PBL (n=10)</th>
<th>AFF (n=5)</th>
<th>EFF (n=10)</th>
<th>PLN (n=2)</th>
<th>MLN (n=3)</th>
<th>SPLEEN (n=3)</th>
<th>IPP (n=3)</th>
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</thead>
<tbody>
<tr>
<td>VPM30⁺ (B Cells)</td>
<td>38² (32-43)</td>
<td>18 (12-25)</td>
<td>18 (14-34)</td>
<td>66 (63-69)</td>
<td>55 (44-62)</td>
<td>70 (69-71)</td>
<td>80* (63-97)</td>
</tr>
<tr>
<td>CD11b⁺ (B cells)</td>
<td>53³ 20²(13-28)</td>
<td>5 (0.5-2)</td>
<td>5 (0-2)</td>
<td>3 (2-2)</td>
<td>3 (1-2)</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Du2.74⁺ (B cells)</td>
<td>45³ 17²(12-21)</td>
<td>94 (11-24)</td>
<td>89 (12-32)</td>
<td>92 (55-66)</td>
<td>93 (42-54)</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td>CD11b⁺ Du2.74⁺</td>
<td>8³ 3²(2-5)</td>
<td>3 (0.5-1.5)</td>
<td>3 (0.5-1)</td>
<td>3 (1.5-2.5)</td>
<td>1 (0.5-1)</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

Small mononuclear cells in, peripheral blood (PBL), afferent lymph (AFF), efferent lymph (EFF), prefemoral lymph node (PLN), mesenteric lymph node (MLN) and ileal Peyer's patch (IPP).

¹number of samples analysed.
²mean percent (range) of the total number of small mononuclear cells showing positive staining.
³percent of the total number of B lymphocytes (VPM30⁺)

*percent of total B lymphocytes in IPP = total IPP lymphocytes - CD5⁺ cells, because in IPP all the B cell antigens are expressed at low intensities proportion of CD5⁺ lymphocytes varied from 3-37% and there was no CD5⁺ B lymphocyte.

-, no cell staining or background staining.
FIGURE 6.3 Two-colour contour plots of small mononuclear cells in peripheral blood (PBL; a), afferent lymph (AFF; b), efferent lymph (EFF; c), ileal Peyer's patch (IPP; d), Spleen (e), prefemoral lymph node (PLN; f), mesenteric lymph node (MLN; g) and bone marrow (BM; h) stained with CC125 (CD11b) on FL1 (a to g), FL2 (h) and VPM30 (Pan B Lymphocyte) on FL2 (a to g), FL1 (h).
FIGURE 6.4 Vectastain ABC immunoperoxidase stained frozen sections of prefemoral lymph node showing follicle (F), cortex (C) and medulla (M) (a & b), and ileal Peyer's patch showing follicle (F), follicle associated epithelium (arrow), dome (DE) and lamina propria (LP) (c & d). The sections were stained with mAb CC125 (CD11b; a & c) and Du2.74 (b & d).
FIGURE 6.5 Vectastain ABC stained frozen sections of spleen showing red pulp (RP), marginal zone (MZ), follicle (F) and periarteriolar lymphoid sheath (PALS) areas (a & b). (a) Two-colour staining with mAb CC125 (CD11b; red, MZ) and Du2.74 (brown, F); (b) parallel section of spleen stained with SBUT4 (CD4; brown PALS).
FIGURE 6.6 Two-colour contour plots of small mononuclear cells in peripheral blood (PBL; a & g), afferent lymph (AFF; b & h), efferent lymph (EFF; c & i), prefemoral lymph node (PLN; d & j), spleen (e & k) and ileal Peyer's patch (IPP; f & l) stained with VPM30 (Pan B Lymphocyte) on FL2 and Du2.74 on FL1 (a to f); CC125 (CD11b) on FL2 and Du2.74 on FL1 (g to l).
distribution of Du2.74 in the follicles and along medullary sinuses (Fig. 6.4). In
spleen a mean of 70% of the B cells express Du2.74 and only about 4% of the B cells
co-express CD11b and Du2.74 (Fig. 6.6; Table 6.1). Du2.74 cells in spleen are
divided into two subpopulations, of high and low expressing cells. Two colour
immunohistochemistry in frozen sections of spleen stained with Du2.74 and anti-
CD11b revealed that Du2.74+ cells are mainly confined to the follicles and CD11b+ cells
are confined to the MZ (Fig. 6.5). The periarteriolar lymphoid sheath area in the
white pulp, not stained by anti-CD11b/Du2.74, is shown to be occupied by CD4+ T
lymphocytes in parallel sections of the spleen (Fig. 6.5).

In IPP, a low intensity Du2.74 staining was observed on about 30% of the
lymphocytes. Some of the Du2.74+ cells co-express VPM30 (Fig. 6.6; Table 6.1),
and slgM (Table 6.3). CD11b+ B cells were undetectable in the lymphocytes
prepared from IPP (Fig. 6.6). Immunohistochemical staining of IPP revealed that
most of the follicle cells are positive for Du2.74 and only medium to large MΦ like
cells are stained by anti-CD11b (Fig 6.4).

Thus both the populations of B cells (Du2.74+ and CD11b+) seems to be mutually
exclusive, except for an overlap of a small fraction of cells which express both the
molecules.

6.3.3 CO-EXPRESSSION OF L-SELECTIN (Du1.29) ON CD11b+ AND DU2.74+ CELLS

This experiment was performed to investigate if the distinct distributions of the two
populations (suggested by the results above) are related to the expression of L-
selectin. In peripheral blood a mean of 37% of the small lymphocytes are L-selectin+
of which only about 7% are B cells, of the B cells about 18% are L-selectin+ (Table
6.2). Two-colour analysis using anti-L-selectin (Du1.29) and Du2.74 or anti-CD11b
shows that most Du2.74+ cells are low L-selectin+ (Fig. 6.7I & II). Low intensity
of L-selectin expression on Du2.74+ cells is also shown in overlay histograms by gating
on Du2.74+ cells (Fig. 6.7II). L-selectin is co-expressed with CD11b on about 4% of
the gated cells. Between 2-3% of the cells in this gate are monocytes (VPM65+) and
all these monocytes are L-selectin+ (Fig 6.7III). Consequently most of the CD11b+L-
selectin+ cells are monocytes but for a negligible proportion (<2%) of B cells that may
be CD11b+L-selectin+. This could have been confirmed by 3 colour FACS analysis.
The attempts to achieve successful thre-colour FACS analysis failed. This was due to
the inability of obtaining appropriate compensation (FL3-FL1) on the FACS analyser
used and the small number of cells being analysed. These results indicate that L-selectin is expressed on majority of Du2.74+ cells and CD11b+ B cells are L-selectin-.

In afferent and efferent lymph a mean of 43% and 62% of the lymphocytes respectively express L-selectin (Table 6.2). In both afferent and efferent lymph most of the B cells and thus Du2.74+ cells express a low level of L-selectin (Fig. 6.7I & II). An earlier study has also shown a significant level of L-selectin expression on Du2.74+ cells (Dr W. R. Hein, personal communication). But there are few CD11b+ B cells (<5%) in afferent and efferent lymph and about 2% of the B cells are CD11b+L-selectin+ (Table 6.2; Fig. 6.7I).

L-selectin is expressed only on a minor subset of lymphocytes (<6%) in PLN, MLN, IPP and spleen. The vast majority of these cells are T cells as only 3-5% of the B lymphocytes express L-selectin (Fig 6.7I).

6.3.4 SURFACE IgM EXPRESSION ON CD11b+ AND DU2.74+ CELLS

The above findings suggest that CD11b+ B cells in sheep may be homologous to the MZ B cells in other species. The MZ B cells in humans and rodents have phenotype of IgMhighIgDlow when compared to conventional B cells (IgMlowIgDhigh) (Kroemer et al., 1993). To address this hypothesis an experiment was performed to quantitate surface IgM (sIgM) on CD11b and Du2.74 B cells. sIgM expression was detected by two-colour FACS analysis using mAb VPM13 specific to μ heavy chain and anti-CD11b or Du2.74 mAb (Table 6.3; Fig. 6.8I). Fig. 6.8I & II shows that CD11b+ B cells express a higher level of sIgM as compared to Du2.74+ cells in most lymphoid compartments analysed (Table 6.3). The quantitative difference in sIgM expression on these two populations is shown by comparing the mean fluorescence intensity (MFI) of CD11b+IgM+ with Du2.74+IgM+ cells in peripheral blood and spleen using 'one tailed' student T-test. In peripheral blood the MFI of CD11b+IgM+ cells (186 ±26) was significantly (P<0.01) higher than Du2.74+IgM+ cells (114 ±5). Likewise in spleen the MFI of CD11b+IgM+ cells (129 ±32) was significantly higher (P<0.05) than Du2.74+IgM+ cells (71 ±31) though the difference was less significant than in peripheral blood. Thus CD11b+ B cells express significantly higher levels of sIgM as compared to Du2.74+ cells.

6.3.5 CD5 EXPRESSION ON CD11b+ AND DU2.74+ CELLS

CD11b+ B cells in cattle and mice have been shown to co-express CD5 (Herzenberg et al., 1987; Naessens and Williams, 1992). In order to investigate if similar
TABLE 6.2 Percent B cells expressing L-selectin in various lymphoid compartments.

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>PBL (n=10)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AFF (n=5)</td>
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<tr>
<td></td>
<td>EFF (n=10)</td>
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<tr>
<td></td>
<td>PLN (n=2)</td>
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<tr>
<td></td>
<td>MLN (n=3)</td>
</tr>
<tr>
<td></td>
<td>SPLEEN (n=3)</td>
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<tr>
<td>L-Select+ Lymphocytes</td>
<td>37^2</td>
</tr>
<tr>
<td></td>
<td>(28-47)</td>
</tr>
<tr>
<td></td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>(37-51)</td>
</tr>
<tr>
<td></td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>(50-74)</td>
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<tr>
<td></td>
<td>5.5</td>
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<td>6.5</td>
</tr>
<tr>
<td></td>
<td>(6-7)</td>
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<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(2-3)</td>
</tr>
<tr>
<td>L-Select+ B cells</td>
<td>18^3</td>
</tr>
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<td></td>
<td>*27</td>
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<td></td>
<td>*44</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>L-Select+ Du2.74^+</td>
<td>16^3</td>
</tr>
<tr>
<td></td>
<td>*27</td>
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<td></td>
<td>*44</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>L-Select+ CD11b^+</td>
<td>&lt;2^#</td>
</tr>
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<td>&lt;1</td>
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</table>

Small mononuclear cells in, peripheral blood (PBL), afferent lymph (AFF), efferent lymph (EFF), prefemoral lymph node (PLN) and mesenteric lymph node (MLN). ND, not determined.

1 number of samples analysed.

2 mean percent (range) of the total number of small mononuclear cells showing positive staining.

3 percent of the total number of B lymphocytes (VPM30^+).

#L-selectin+VPM65^+ (monocyte) an average of 3% was deducted from L-selectin+CD11b^+ cells to determine the proportion of B cells expressing CD11b and L-selectin (see Fig. 6.7III).

*all the B cells show low level of L-selectin expression (see Fig. 6.7II)
FIGURE 6.71  Two-colour contour plots of small mononuclear cells in peripheral blood (PBL; a, d & g) afferent lymph (AFF; b, e & h), efferent lymph (EFF; c, f & i), prefemoral lymph node (PLN; j), mesenteric lymph node (MLN; k) and ileal Peyer's patch (IPP; l) stained on FL2 with Du1.29 (L-selectin; a to l) and on FL1 with VPM30 (Pan B lymphocyte; a, b, c, j & k), Du2.74 (d, e & f), CC125 (CD11b; g, h & i), normal mouse serum (NMS; l).
FIGURE 6.7II Frequency histograms showing L-selectin expression on Du2.74+ cells (---) in peripheral blood (PBL; a), afferent lymph (AFF; b) and efferent lymph (EFF; c). Du2.74+ cells in quadrant 2+4 as shown in Fig. 6.7I (d, e & f) were gated for this analysis. The negative control of single stained Du2.74+ cells (........) is shown for comparison.
FIGURE 6.7III Two-colour contour plots of small mononuclear cells in peripheral blood (PBL; a, b & c). (a) Stained with Du1.29 (L-selectin; FL2) and VPM65 (monocytes; FL1). (b) Stained with VPM65 (monocytes; FL2) and CC125 (CD11b; FL1). (c) Stained with CC125 (CD11b; FL1) and OM1 (CD11c; FL2).
TABLE 6.3 Percent B cells expressing cell surface IgM in various lymphoid compartments

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<thead>
<tr>
<th>Cell Phenotype</th>
<th>PBL (n=10)</th>
<th>AFF (n=5)</th>
<th>EFF (n=10)</th>
<th>PLN (n=2)</th>
<th>MLN (n=3)</th>
<th>SPLEEN (n=3)</th>
<th>IPP (n=3)</th>
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<tbody>
<tr>
<td>sIgM+ B-cells</td>
<td>82^3</td>
<td>89</td>
<td>ND</td>
<td>63</td>
<td>77</td>
<td>*64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31(23-29)^2</td>
<td>16(13-24)</td>
<td>ND</td>
<td>34(23-45)</td>
<td>54(47-64)</td>
<td>51*(31-73)</td>
<td></td>
</tr>
<tr>
<td>sIgM+ Du2.74^+</td>
<td>32^3</td>
<td>72</td>
<td>ND</td>
<td>36</td>
<td>47</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12(9-18)^2</td>
<td>13(8-23)</td>
<td>ND</td>
<td>20(17-23)</td>
<td>33(30-36)</td>
<td>19(13-28)</td>
<td></td>
</tr>
<tr>
<td>sIgM+ CD11b^+</td>
<td>47^3</td>
<td>5</td>
<td>ND</td>
<td>&lt;2</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18(12-25)^2</td>
<td>1(0.5-1)</td>
<td>ND</td>
<td>1</td>
<td>12(11-14)</td>
<td></td>
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Small mononuclear cells in, peripheral blood (PBL), afferent lymph (AFF), efferent lymph (EFF), prefemoral lymph node (PLN), mesenteric lymph node (MLN) and ileal Peyer's patch (IPP). ND, not determined.

1number of samples analysed.

2mean percent (range) of the total number of small mononuclear cells showing positive staining.

3percent of the total number of B lymphocytes (VPM30^+).

*all cells show low intensity staining.

-, no staining or background staining.
FIGURE 6.81 Two-colour contour plots of small mononuclear cells in peripheral blood (PBL; a & b), spleen (c & d), efferent lymph (EFF; e & f) and mesenteric lymph node (MLN; g & h), stained on FL2 with VPM13 (IgM; a to h) and on FL1 with CC125 (CD11b; a, c, e & g), Du2.74 (b, d, f & h).
FIGURE 6.8II Frequency histograms showing surface IgM expression on CD11b+ (a & c) and Du2.74+ (b & d) cells in peripheral blood (PBL; a & b) and spleen (c & d). CD11b+ or Du2.74+ cells in quadrant 2+4 as shown in Fig. 6.8I (a, b, c & d) were gated for this analysis. The negative control of single stained Du2.74+ or CD11b+ cells (.........) is shown for comparison.
co-expression exists in sheep, two-colour FACS analysis was performed using anti-
CD5 and anti-CD11b or Du2.74 mAb. In peripheral blood a mean of 59% of the 
lymphocytes are CD5+ and of the B cells a mean of 13% express a very low level of 
CD5 (Fig. 6.9I; Table 6.4). All these B cells are CD11b+ none co-express Du2.74 
(Fig. 6.9I; Table 6.4). In spleen a mean of 20% of the lymphocytes are CD5+ and of 
the B cells a mean of 5% express a low level of CD5. Again these cells co-express 
CD11b but not Du2.74 (Fig. 6.9I; Table 6.4). In afferent and efferent lymph, PLN, 
MLN and IPP, the proportion of CD5+ B cells remains negligible (Fig. 6.9II; Table 
6.4). Thus, a low level of CD5 expression remained restricted to CD11b+ cells in 
peripheral blood and spleen.

6.3.6 CD11b AND Du2.74 EXPRESSION IN EFFERENT LYMPH AND BLOOD IN OVA 
STIMULATED SHEEP

This experiment was performed to investigate whether CD11b+ B cells migrate across 
the endothelium of a stimulated lymph node and co-express Du2.74. Lymphocytes in 
efferent lymph and mononuclear cells in peripheral blood collected before and after 
secondary OVA stimulation in the area of draining lymph node were analysed. Both 
small lymphocytes and large lymphocytes were included in the analysis. The sheep 
was previously primed with OVA (1mg s/c) and subsequently stimulated locally with 
OVA (100μg s/c) after cannulation. There was an increase in percent B lymphocytes 
both in efferent lymph and peripheral blood after secondary OVA stimulation (Table 
6.5). The increase in B cell proportion in peripheral blood was due to the increase in 
CD11b+VPM30+ cells whereas in efferent lymph it was due to the increase in 
Du2.74+VPM30+ cells. The percentage of Du2.74+VPM30+ cells in peripheral blood 
and CD11b+VPM30+ cells in efferent lymph remained unchanged. Overall the 
proportion of L-selectin+ cells was not affected in peripheral blood but a decrease in 
L-selectin+ cells was observed in efferent lymph following stimulation. In addition 
there was an increase in CD11b and Du2.74 double positive cells from < 1% to over 
3% in peripheral blood but no such change was observed in efferent lymph since there 
was no increase of CD11b+ cells in this compartment.

These preliminary observations from the studies on one animal data indicate that 
CD11b+ B cells do not migrate across the secondary OVA stimulated lymph node. 
However there was a slight increase CD11b+Du2.74+ cells in the peripheral blood in 
response to the stimulation.
TABLE 6.4 Percent B cells expressing CD5 in various lymphoid compartments.

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>PBL (n=10)</th>
<th>AFF (n=5)</th>
<th>EFF (n=10)</th>
<th>PLN (n=2)</th>
<th>MLN (n=3)</th>
<th>SPLEEN (n=3)</th>
<th>IPP (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5+ Lymphocytes</td>
<td>59(^2) (45-80)</td>
<td>72 (60-80)</td>
<td>74 (56-85)</td>
<td>28 (24-32)</td>
<td>35 (28-46)</td>
<td>20 (16-24)</td>
<td>19 (3-37)</td>
</tr>
<tr>
<td>CD5+ VPM30+</td>
<td>13(^3) (3-13)</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD5+ CD11b+</td>
<td>13(^3) (2-12)</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>CD5+ Du2.74+</td>
<td>&lt;1(^3) (0-2)</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
</tr>
</tbody>
</table>

Small mononuclear cells in peripheral blood (PBL), afferent lymph (AFF), efferent lymph (EFF), prefemoral lymph node (PLN), mesenteric lymph node (MLN) and ileal Peyer's patch (IPP). ND, not determined.

1 number of samples analysed.

2 mean percent (range) of the total number of small mononuclear cells showing positive staining.

3 percent of the total number of B lymphocytes (VPM30+).

*all cells show low intensity staining.

-, no staining or background staining.
FIGURE 6.91 Two-colour contour plots of small mononuclear cells in peripheral blood (PBL; a, b & c), spleen (d, e & f), ileal Peyer's patch (IPP; g, h & i) stained with SBUT1 (CD5) on FL1 (a, b & e), FL2 (c, d, f, g, h & i); and with VPM30 on FL2 (a), FL1 (d & g); CC125 (CD11b) on FL2 (b & e); Du2.74 on FL1 (c, f & i), VPM13 (IgM) on FL1 (h).
FIGURE 6.9II Two-colour contour plots of small mononuclear cells in afferent lymph (a, b & c), efferent lymph (d, e & f), prefemoral lymph node (PLN; g & h) and mesenteric lymph node (MLN; i) stained with SBUT1 (CD5) on FL2 (a, c, f & i), FL1 (b, d, e, g and h) and with VPM30 on FL1 (a & g), FL2 (d); CC125 (CD11b) on FL2 (b, e and h), Du2.74 on FL1 (c, f and i).
**TABLE 6.5** Percent of PBMC\(^1\) and Efferent Lymphocytes\(^1\) in *in vivo* secondary OVA stimulated sheep in the area of draining lymph node.

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>PBMC</th>
<th></th>
<th>EFFERENT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D2</td>
<td>D4</td>
</tr>
<tr>
<td>VPM30(^+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B Cells)</td>
<td>33</td>
<td>34</td>
<td>40</td>
</tr>
<tr>
<td>VPM30(^+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b(^+)</td>
<td>13</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>VPM30(^+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Du2.74(^+)</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>L-Selectin(^+)</td>
<td>28</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Du2.74(^+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Selectin(^+)</td>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CD11b(^+)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>L-Selectin(^+)</td>
<td>&lt;1</td>
<td></td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD11b(^+)</td>
<td>&lt;1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*\(^1\)cells analysed including both small and blast cells gated together.*

PBMC, Peripheral blood mononuclear cells; EFF, Efferent lymphocytes.

D0, on the day before secondary OVA stimulation; D2, D3, D4, D6, days post secondary OVA stimulation.

*\(^*\)values are the percent of the total cells analysed.*
6.3.7 IN VITRO STIMULATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND EFFEERENT LYMPH CELLS WITH LPS

The increase in CD11b+Du2.74+ cells in peripheral blood following secondary OVA stimulation of the lymph node suggested that peripheral blood B cells may co-express these molecules after activation. To confirm this hypothesis peripheral blood mononuclear cells and efferent lymph cells were stimulated with LPS (Salmonella abortus equi, Sigma) a poly-reactive antigen in an in vitro assay. Peripheral blood mononuclear cells (PBMC) were separated from defibrinated blood (using sterile glass beads) after centrifugation over lymphoprep. The PBMC and efferent lymphocytes were washed twice in culture media (section 2.2.5) and adjusted to 2 x 10^6 cells ml^-1. The cells were prepared and cultured under sterile conditions. The cells were incubated in aliquots of one ml with 50µg LPS ml^-1 at 37°C in humid chamber in 5% CO₂.

The following observations were made on the cells collected from one animal. Upon LPS activation the majority of B cells in PBMC showed blast formation. On day 1 out of the 71% of B cell blasts about 63% were CD11b+VPM30+ and about 37% were Du2.74+VPM30+ (Table 6.6; Fig. 6.10). About 2/3rd of the Du2.74+ B cell blasts co-expressed CD11b. From day 2 most of the LPS stimulated B cell blast started losing both CD11b and Du2.74 expression (Table 6.6; Fig. 6.10). After in vitro LPS activation of efferent lymph cells, a small number of B cells showed blast formation (Table 6.6; Fig. 6.11). Du2.74 expression on B cells was lost from day 1 onwards and the proportion of CD11b+ B cells never showed any increase.

Thus after LPS stimulation the majority of peripheral blood B cell blasts expressed CD11b and only a third B cell blasts co-expressed CD11b and Du2.74. In contrast efferent lymph B cells lost Du2.74 expression but did not express CD11b. Putting these results together, it appears that the Du2.74+CD11b- B cell population (efferent B cells) does not change to Du2.74+CD11b+ upon LPS activation thus it is the CD11b+ B cells in peripheral blood which co-express Du2.74 on some of the B cells upon activation. In addition efferent lymphocytes (Du2.74+ B cells) showed slow reactivity to LPS as compared to peripheral blood lymphocytes (CD11b+ and Du2.74+ B cells). This was evident from the low proportion of blast lymphocytes in efferent lymph (D0 3%, D1 5% & D2 7%) when compared to that in peripheral blood (D0 3%, D1 16%, D2 22% & D3 26%).
<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>PBMC</th>
<th>EFF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0 AL SL BL D1 SL BL D2 SL BL D3 SL BL</td>
<td>D0 AL SL BL D1 SL BL D2 SL BL</td>
</tr>
<tr>
<td>VPM30+ (B cells)</td>
<td>35* 23 71 17 75 26 78</td>
<td>21 5 16 2.5 12</td>
</tr>
<tr>
<td>CD11b+ VPM30+</td>
<td>21 9 63 4 30 5 10</td>
<td>1 0.5 0.5 0.5 0</td>
</tr>
<tr>
<td>VPM30+ Du2.74+</td>
<td>14 14 37 6 15 6 10</td>
<td>20 0.5 2 1 1</td>
</tr>
<tr>
<td>CD11b+ Du2.74+</td>
<td>4 2 23 2 10 1 2</td>
<td>0.5 0 0 0 0</td>
</tr>
</tbody>
</table>

PBMC, Peripheral blood mononuclear cells; EFF, Efferent lymphocytes.
D, Day; AL, All PBMC; SL, Small lymphocytes; BL, Blast cells.
*values are the percent of the total cells analysed e.g. in small lymphocytes (SL) as percent of total SL analysed, likewise in blast cells (BL) as percent of total blast cells.
FIGURE 6.10  *In vitro* LPS stimulated peripheral blood mononuclear cells (PBMC). FSC/SSC scatter profile of live gated PBMC Day0 (a), stained on FL2 with VPM30 (Pan B Lymphocyte; b & c), CC125 (CD11b; d), and on FL1 with CC125 (CD11b; b), Du2.74 (c & d). FSC/SSC scatter profile showing analytical gate on blast PBMC Day1 post LPS stimulation (e), stained on FL2 with VPM30 (Pan B Lymphocyte; f & g), CC125 (CD11b; h), and on FL1 with CC125 (CD11b; f), Du2.74 (g & h).
FIGURE 6.11 *In vitro* LPS stimulated efferent lymphocytes (EFF). FSC/SSC scatter profile of live gated EFF Day0 (a), stained on FL2 with VPM30 (Pan B Lymphocyte; b & c), CC125 (CD11b; d), and on FL1 with CC125 (CD11b; c), Du2.74 (b & d). FSC/SSC scatter profile showing analytical gate on blast AFF Day1 post LPS stimulation (e), stained on FL2 with VPM30 (Pan B Lymphocyte; f & g), CC125 (CD11b; h), and on FL1 with CC125 (CD11b; g), Du2.74 (f & h).
6.4 DISCUSSION

The results in this chapter describe the existence of at least two distinct subpopulations of B lymphocytes in adult sheep. One, which expresses CD11b (CR3) on its surface, represents about half of the B cells in peripheral blood and about a quarter of the B cells in spleen specifically located in the marginal zone (MZ). B cells expressing CD11b are negligible in both afferent and efferent lymph, lymph nodes (both peripheral and mesenteric) and totally absent in ileal Peyer's patch (Fig. 6.3). A small proportion of these (CD11b+) cells weakly express CD5 in peripheral blood and spleen but most are CD11b+CD5− (Fig. 6.9) The CD5+ B cells have been shown to represent a small population in foetal lambs and adult sheep (Press et al., 1993). This confirms that sheep predominantly possess B-1b (CD5−) like cells rather than B-1a (CD5+) like cells. The majority of CD11b+ B cells express significantly higher levels of surface IgM as compared to CD11b− B cells (Fig. 6.8) suggesting a naive phenotype. Their MZ localisation in the spleen also suggests a naive phenotype as the B-1b cells in other species localise at this site (Strober and Dilley, 1973a & b; Abney et al., 1978; Kumararatne et al., 1981). Another characteristic of MZ B cells in human and rats is a strong expression of CD21 (CR2) (Timens et al., 1989; Kumararatne et al., 1981). In mice the MZ B cells also express CD11b although they represent <15% of spleen B cells (Kroemer, et al., 1993). Thus MZ B cells in sheep resemble those in mice although they are present at a higher proportion (>25%). The phenotype of MZ B cells in rats and humans cannot be equated with sheep as such because the expression of IgD (Jones, 1988) and CD21 has not yet been defined in sheep. The cells in these species with MZ phenotype are also present in a small proportion in peripheral blood lymph nodes and IPP (Liu et al., 1989; Gray et al., 1982; Timens, 1991).

The other B cell population in peripheral blood, recognised by mAb Du2.74, is essentially mutually exclusive of CD11b+ B cells. These B cells represent about half of the peripheral blood B cells and are the major B cell population of lymph (afferent and efferent) and lymph nodes (Fig. 6.6). The Du2.74 B cells mainly occupy the follicles in the spleen, lymph node and IPP (Fig. 6.4). These B cells show significantly lower levels of sIgM and do not express CD5 as compared to CD11b+ B cells (Fig. 6.8). These characteristics of Du2.74 B cells are suggestive of conventional (B-2) B cells. Du2.74 has been shown to recognise all the B cells in fetal lambs and young post natal lambs. However, it recognises only the recirculating B cell subset in older animals (W. R. Hein, personal communication). So one possibility of the origin of CD11b B cells is from a common B cell pool following exposure to environmental...
antigens after birth, as the foetus in sheep is protected from external antigens. Alternatively, CD11b B cells could expand after birth from a minor subset of CD5+ B cells present in fetal lambs (Press et al., 1993). This speculation is further supported by the observed increase in the proportion of CD11b+CD5+ B cells in cattle following Trypanosome infection (Naessens and Williams, 1992). This seems to be due to non-specific (polyclonal) activation as reported in other species (Casali and Notkins, 1989) and may represents a 'primitive' level of immunity. The ontogeny of the B-1 population of cells in sheep remains to be investigated, though it has characteristics similar to that of mice.

The absence of CD11b+ B cells in both afferent and efferent lymph and peripheral lymph node suggests that these cells do not recirculate. In contrast Du2.74+ B cells are present in afferent and efferent lymph and peripheral lymph node and represent the recirculating B cell population. This is supported by restricted expression of L-selectin on Du2.74+ B cells (Dr W. R Hein, personal communications) and its absence on CD11b+ B cells. The expression of L-selectin on B cells has also been described as a marker for memory B cells (Kraal et al., 1988; Coico et al., 1983). The characteristics of Du2.74+ B cells suggests that they are conventional B-2 cells and represent a memory B cell pool. The MZ localisation of CD11b+ B cells as well as their phenotype resembling B-1 cells suggests that they represent a naive B cell population (Kroemer et al., 1993; Strober and Dilly, 1973b).

Mackay showed distinct pathways of memory and naive T cell recirculation through peripheral lymph node and tissues in sheep (Mackay et al., 1990; Mackay et al., 1992a). The naive T cells recirculate through peripheral lymph nodes, while memory T cells home preferentially to skin and peripheral tissue where they may have an increased likelihood of interacting with previously encountered antigen. A similar model does not exist for B lymphocytes. The proportion of recirculating B cells in sheep has been shown to be much less than that in peripheral blood (Kimpton et al., 1989; Washington et al., 1988). If Du2.74+CD11b+ B cells are memory cells then this is analogous to memory T cells interacting at skin endothelium but these do not interact with lymph node endothelium. However the migration across the lymph node endothelium of B cells with similar phenotype (Du2.74+CD11b+) but not of CD11b+Du2.74- B cells goes against the T cell model. The migration across the lymph node endothelium may be accounted for by the expression of L-selectin on Du2.74+ B cells and not on CD11b+ B cells. This suggests that it is the memory B cells which migrate across the endothelium both in peripheral tissue and peripheral
lymph node. Alternatively there are two subpopulations within Du2.74+ B cells, ones which migrate through skin endothelium representing memory cells and others which migrate through lymph node endothelium representing naive cells. This is contrary to the earlier observations of Kraal et al., (1988) who showed L-selectin as a marker for memory B cells. It is possible that a B cell subset specific homing receptor is controlling the selective migration of a particular set of B lymphocytes across the endothelium perhaps in association with L-selectin at the lymph node and independent of L-selectin in the peripheral tissue. In this context it is possible that the non-Ig cell surface antigen recognised by Du2.74 is a likely candidate for a B lymphocyte subset adhesion receptor. 

The lack of recirculation of CD11b+ B cells suggests that these cells do not take part in the generation of memory responses because of their early origin in ontogeny (Herzenberg and Herzenberg, 1989) and therefore do not recirculate through lymph node in search of previously encountered antigen. The cells with MZ phenotype have been shown to be largely responsible for immediate primary immune responses to all the three classes of antigens present in blood. These cells are specific for T independent type-2 antigens which never produce memory responses (Casali and Notkins, 1989; Forster and Rajewsky, 1987). The presence of such a large proportion of CD11b+ B cells in peripheral blood resembling MZ B cells in other species is so far, unique to sheep. B cells with MZ phenotype do not circulate even between spleen and blood in rodents (Kroemer et al., 1993; Kumararatne et al., 1981; Kumararatne and MacLennan, 1981).

In vivo secondary responses of the draining lymph node has no effect on the endothelial migration of the CD11b+ B cells. This suggests that these cells do not take part in lymph node memory responses. By contrast the increase in Du2.74+ B cells in efferent lymph suggests their increased migration across lymph node endothelium and their role in memory response. The increase of CD11b+ B cells in peripheral blood may be a result of non-specific response as suggested for similar phenotype cells in other species (Casali and Notkins, 1989; Forster and Rajewsky, 1987).

The in vitro LPS activation of peripheral blood mononuclear cells and efferent lymphocytes showed rapid response of CD11b+ B cells whereas Du2.74+ B cell response was slow. There was no conversion of Du2.74+ cells to CD11b+ cells as seen by the LPS activation of efferent cells. The loss of Du2.74 antigen following LPS activation is similar to the earlier observation that the antigen is not expressed by dividing cells or cells in cycle (W. R. Hein, personal communication). Following LPS activation of PBMC the majority of B cell blasts were CD11b+ and only a third
coexpressed Du2.74. A negligible proportion of B blasts are Du2.74+CD11b-.

Both the antigens are lost from the 2nd day of LPS activation. This experiment shows a lack of conversion of CD11b+Du2.74- to CD11b-Du2.74+ and vice versa. These findings suggest that these two populations may be different and not different maturational stages of B lymphocytes. A small proportion of B cells normally coexpress CD11b and Du2.74. An increase in proportion of this population after LPS activation of PBMC suggests a very low level of conversion from CD11b+ B cells to CD11b+Du2.74+ cells. A very slow rate of conversion from recirculating B cells to MZ B cells in rats has been suggested by studies on regeneration of MZ B cells after adoptive transfer of recirculating B cells (Kumararatne and MacLennan, 1981). This seems to be unlikely in the sheep as recirculating efferent lymph B cells (Du2.74+) did not change to CD11b+ phenotype after activation.

Further studies on depletion and repopulation of MZ cells in sheep may help to prove the relationship between these two populations. The rapid activation of CD11b+ B cells in response to LPS seems to be similar to the responses of B cells with MZ phenotype in other species. CD21 (CR2), in close linkage with slgM on human MZ B cells has been shown to be involved in rapid B cell activation (Timens et al., 1989; Kay et al., 1987). In sheep CD11b (CR3) as well as CD11c (CR4) may have a similar role in rapid activation of B cells. CD11b and CD11c, expressed on leukemic B cells or induced upon activation on human B cells, have been shown to retain their adhesion and activation functions (De La Hera et al., 1988; Postigo et al., 1991). CD11b and CD11c on myeloid cells bind LPS without producing any secretary response (Wright and Jong, 1986). Thus it is possible that CD11b and CD11c on sheep B cells might have a role in binding LPS and thus helping in producing rapid B cell response.

6.5 CONCLUSIONS

The following conclusions can be drawn from the work presented in this chapter:

1. There are at least two distinct B cell populations in sheep:

   (a) CD11b+L-selectin-/slgM^high (Du2.74^-) - represent naive B cells.

   (b) CD11b+L-selectin+/slgM^low (Du2.74^-) - represent memory B cells.

2. In the peripheral blood of adult sheep these are present in approximately equal proportions. In spleen the CD11b+ population represent a quarter of the B cell population but this population is absent from lymph and lymph nodes. The distribution of CD11b+ cells is summarised in Fig. 6.12.
FIGURE 6.12 Diagram showing the distribution and proportion of CD11b+ B lymphocytes in various lymphoid compartments in adult sheep.
3. The CD11b+ B cells are present in the marginal zone of spleen. By their tissue distribution and phenotype they resemble the B-1b B cells of rodents and man.

4. CD11b+ B cells are absent in ileal Peyer's patch (primary B lymphoid organ). There does not seem to be a conversion of CD11b+ to CD11b-(Du2.74+) by activation. Therefore it is unlikely that they represent different maturational stages.

5. CD11b+ B cells respond rapidly to LPS. Du2.74+ cells respond very slowly and Du2.74 antigen is rapidly lost upon LPS activation.
CHAPTER SEVEN

SUMMARY AND CONCLUSIONS
7 SUMMARY AND CONCLUSIONS

The aims of the thesis were achieved by developing monoclonal antibodies specific for sheep monocytes/macrophages and leukocyte integrins to characterise mononuclear phagocytes. The results are discussed in the respective chapters. The delineation of mononuclear phagocytes recognised by these mAb is summarised in Table 7.1.

7.1 ANTI-SHEEP MONOCYTE/MACROPHAGE MONOCLONAL ANTIBODIES

Monoclonal antibodies were raised by immunising Balb/c mice using sheep alveolar macrophages. Five mAb specifically reacting with sheep alveolar macrophages/peripheral blood monocytes were selected for subsequent characterisation. All these mAb were of mouse IgG1 isotype.

7.1.1 VPM65, VPM66 AND VPM67 Monoclonal Antibodies

VPM65, VPM66 and VPM67 originated from three different clones. They recognised the same or very closely related epitopes on the same antigen. The antigen recognised by these mAb can be described as a GPI linked cell surface glycoprotein of apparent Mr = 55,000. The antigen was predominantly expressed by peripheral blood monocytes and tissue macrophages. A low level of expression was also observed on peripheral blood granulocytes and afferent dendritic cells. This antigen was not found to be expressed on T and B lymphocytes. The antigen preclearing studies and two-colour FACS analysis using anti-human CD14 mAb (TUK4) and other characteristics revealed this antigen as sheep homologue of human CD14. However the functional role of this antigen, in binding LPS which leads to the production of TNF-α and other cytokines in humans (Ulevitch, 1993; Wright et al., 1990) remains to be investigated.

7.1.2 VPM63 Monoclonal Antibody

VPM63 was unique in its specific recognition of tissue macrophages. It failed to stain bone marrow precursors, monocytes in peripheral blood and dendritic cells in afferent lymph. This mAb did not react with any granulocytes or lymphocytes so far tested. Thus this mAb can be regarded as a mature tissue macrophage differentiation marker. VPM63 immunoprecipitated a smearing doublet of Mr = 40,000-42,000 under reducing and non reducing conditions. Affinity purified VPM63 antigen was recognised by anti-FcR peptide antiserum by Western blot analysis. However this mAb failed to demonstrate any inhibitory effect on immune complex uptake by alveolar macrophages. The molecular weight of the antigen and its reactivity with
anti-FcR peptide antiserum are strongly suggestive of it being sheep homologue to a macrophage isoform of murine/human FcyRII (CD32). Further characterisation, such as NH2-terminal sequencing of the antigen and/or reactivity of VPM63 with suitable transfectant would help establish its identity.

7.1.3 VPM64 Monoclonal Antibody
VPM64 immunoprecipitated a smearing band of Mr = 65,000-80,000 from alveolar macrophages. The antigen is widely distributed on a variety of cells of the myeloid lineage. VPM64 stains cells of granulocyte lineage in bone marrow as well as mature granulocytes in the peripheral blood. A low level of expression was seen on mature peripheral blood monocytes. VPM64 stains most of the Mφ in lung alveoli and in spleen. VPM64 stains a few Mφ in lymph node and dermis, some epidermal Langerhans cells and up to 19% of afferent dendritic cells. VPM64 also reacted with a small population of mononuclear cells, possibly NK cells, which are CD14−, CD11b+ and are not T or B lymphocytes. The molecular weight of the antigen and its distribution on a wide variety of leukocytes suggests its possible identity as sheep FcyRIII (CD16). Further biochemical analysis including NH2-terminal sequencing, and/or reactivity with a suitable transfectant would reveal its true identity.

7.2 ANTI-SHEEP LEUKOCYTE (β2) INTEGRINS MONOCLONAL ANTIBODIES
A panel of mAb specific for three α chains and a common β chain of leukocyte integrins was identified and characterised (Table 5.1). Tissue localisation and cellular distribution of leukocyte integrins was also investigated.

7.2.1 Anti-CD11a
Monoclonal antibodies MUC76A, 72-87, F10-150 and MD2B7 each immunoprecipitated a heterodimer of Mr = 180,000/95,000. Sequential immunoprecipitation using MUC76A proved that it was specific for the αL chain (CD11a). The molecule is expressed by the majority of leukocyte populations analysed so far.

7.2.2 Anti-CD11b
Monoclonal antibodies CC125 and IL-A15 each immunoprecipitated a heterodimer of Mr = 170,000/95,000. Their specificity to the αM chain (CD11b) was confirmed by sequential immunoprecipitation experiments. The molecule is expressed by the cells of myeloid lineage including blood monocytes and granulocytes, and tissue
macrophages. The characteristic exclusions were epidermal Langerhans cells and afferent dendritic cells.

The expression of CD11b on a substantial proportion (about 50%) of peripheral blood B lymphocytes was unique to sheep. CD11b expression was seen on more than a quarter of splenic B lymphocytes which are specifically located in the marginal zone in spleen. CD11b expressing B lymphocytes were negligible in other secondary lymphoid organs (peripheral and mesenteric lymph node) and were absent in ileal Peyer's patch, a primary B lymphocyte organ in ruminants. The CD11b+ B lymphocytes were negligible (<5% of B cells) in afferent and efferent lymph implying that these cells are a non-recirculating type of B lymphocyte. CD11b+ lymphocytes showed a significantly high level of surface IgM as compared to CD11b− B (Du2.74+) lymphocytes. A small proportion of CD5+ B lymphocytes present in peripheral blood (13%) and spleen (4%) coexpress CD11b. CD11b+ B cells in peripheral blood are L-selectin+. These characteristics of CD11b+ B lymphocytes are suggestive of a naive phenotype (IgMhigh, L-selectin−). CD11b+ B cells appear to belong to B-1 (particularly B-1b) type whereas CD11b− (Du2.74+) B cells are of conventional (B-2) type as described in rodents and humans (Kantor, 1991).

Further studies on foetal/neonatal lambs would be useful to investigate the origin of CD11b+ B cells. The investigation of the function of these B cells in specifically mediating T-independent type 2 responses as reported in rodents and humans (Casali and Notkins, 1989; Forester and Rajewsky, 1987) would be another area of interest. Also it would be interesting to know whether the marginal zone B cells (CD11b+) in sheep circulate in blood or remain restricted to spleen as observed in rodents (Gray et al., 1982; Timens, 1991).

7.2.3 Anti-CD11c
Monoclonal antibody OM1 immunoprecipitated a heterodimer of Mr = 150,000/95,000. The specificity of this mAb to the αX chain (CD11c) was confirmed by sequential immunoprecipitation. The cellular distribution and tissue localisation of this molecule was almost similar to CD11b (CR3) with some characteristic differences. CD11c is expressed on epidermal Langerhans cells and afferent dendritic cells whereas CD11b was not expressed on these cells. Lack of CD11c expression on neutrophils is unique to sheep and its expression on granulocytes has been demonstrated only on eosinophils (Pepin et al., 1992). In
tissue sections CD11c was expressed on a higher number of Mφ as compared to CD11b.

7.2.4 Anti-CD18

Monoclonal antibodies, BAQ30A and MF14B4, immunoprecipitated all the α chains of $M_r = 180,000$ to 150,000 along with a β chain of $M_r = 95,000$. The specificity of these mAb to the β chain ($M_r = 95,000$) was confirmed by Western blot analysis. Sequential immunoprecipitations not only confirmed their specificity to the β chain but also proved that this β chain is common to all the three members (CD11a, CD11b and CD11c) of β2 (leukocyte) integrin. Like CD11a this molecule was expressed by all the leukocyte populations analysed so far.
### TABLE 7.1
Summary of delineation of various mononuclear phagocyte populations with anti-
- Deep monococyte/macrophage and Fc (IgG) receptor monoclonal antibodies.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>BM, All Bone Marrow Cells</th>
<th>Mo, Monocytes</th>
<th>M&lt; &gt;, Macrophages</th>
<th>LC, Langerhans Cells</th>
<th>Tissue</th>
<th>BM, Mo, M&lt; &gt;, TC, ADC, FDC, PGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD18</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td></td>
<td>qns+++</td>
</tr>
<tr>
<td>CD11e</td>
<td>qns++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>qns+</td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>qns++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>qns++</td>
<td></td>
</tr>
<tr>
<td>CD11a</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>VpM65/66/67</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>qns+</td>
</tr>
<tr>
<td>VpM64</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VpM63</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BM, All Bone Marrow Cells; Mo, Monocytes; M< >, Macrophages; LC, Langerhans Cells; Tissue, Various Tissue; BM, Mo, M< >, TC, ADC, FDC, PGC, Peripheral Blood Granulocytes; I< >, Immunophocytes, id, Staining subpopulation. +++, strong expression; ++, moderate expression; +, weak expression; qns, weak expression on a few cells; - no staining.
REFERENCES
REFERENCES


SANDERS, M.E., MAKGObA, M.W., SHARROW, S.O., STEPHANY, D., SPRINGER, T.A., YOUNG, H.O. & SHAW, S. (1988) Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2 and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-production. J. Immunol. 140, 1401.


BUFFERS AND REAGENTS

1. Alsever's solution
   Glucose - 20.5 g
   Sodium Citrate - 8.0 g
   Sodium Chloride - 4.2 g
   Adjust pH to 6.1 with 10% citric acid solution
   and make with distilled water to - 1000 ml

2. Borate buffer saline pH 8.2
   Boric acid (100 mM) - 6.20 g
   Disodium tetraborate (25 mM) - 9.54 g
   Sodium Chloride (75 mM) - 4.40 g
   Dissolve in distilled water adjust pH to 8.2
   with 1M HCl or 1M NaOH and make to - 1000 ml

3. Carbonate-bicarbonate buffer pH 9.0
   Sodium bicarbonate - 4.2 g
   dissolved in 250 ml distilled water, pH to 9.0
   with 0.2 M solution of anhydrous sodium carbonate
   and make in distilled water to make - 1000 ml

4. Hank's balanced salt solution (HBSS) Ca2+, Mn2+ free.
   Potassium Chloride (KCl) - 0.4 g
   Potassium Dihydrogen Phosphate (KH2PO4) - 0.06 g
   Sodium Chloride (NaCl) - 8.0 g
   Sodium Bicarbonate (NaHCO3) - 0.35 g
   Disodium Hydrogen Phosphate (Na2HPO4) - 0.048 g
   D-Glucose - 1.0 g
   Phenol Red - 0.01 g
   Adjust pH to 7.2 with 0.1 M NaOH and
   dissolve in distilled water to 1000 ml

5. Hypotonic RBC lysis buffer (Tris-NH4Cl)
   (a) Ammonium Chloride (0.16 M) - 8.3 g
       Distilled water - 1000 ml
   (b) Tris (Hydroxymethyl) aminomethane (0.17 M) 20.6 g
       Distilled water - 1000 ml

To prepare lysis buffer add 10 ml of (a) to 90 ml of (b) and adjust pH to 7.2
using 1M HCl. 35 ml of this solution warmed to 37°C is sufficient to lyse 5 ml
of blood collected in equal volume of Alsever's solution or 10 ml blood as
such.
6. **Phosphate buffered saline (PBS, pH 7.2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>8.00 g</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>0.20 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
<td>1.15 g</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate (KH₂PO₄)</td>
<td>0.20 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water adjust pH to 7.2 with 1M NaOH or 1M HCl and make to 1000 ml.

7. **PBA Buffer for FACS analysis**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin (0.5%)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium azide (0.05%)</td>
<td>0.5 g</td>
</tr>
</tbody>
</table>

Make up in PBS pH 7.2 to 1000 ml.

8. **Tris HCl buffered saline**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (Hydroxymethyl) aminomethane (0.1 M)</td>
<td>12.1 g</td>
</tr>
<tr>
<td>Sodium Chloride (0.15 M)</td>
<td>8.75 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water pH to 8.4 and make to 1000 ml.

9. **SDS-PAGE**

   (a) **Separating gel composition for two mini-gels**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final polyacrylamide concentration: 5%</td>
<td>7.5%</td>
</tr>
<tr>
<td>30% w/v Acrylamide, 0.8% w/v Acrylamide</td>
<td></td>
</tr>
<tr>
<td>N,N methylene-bis-acrylamide</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>1.0 M Tris HCl pH 8.7</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.5 ml</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulphate</td>
<td>100µl</td>
</tr>
<tr>
<td>10% w/v Ammonium Persulphate</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
</tr>
</tbody>
</table>

(b) **Stacking gel composition sufficient for two mini-gels**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% w/v Acrylamide,</td>
<td></td>
</tr>
<tr>
<td>0.8% w/v NN methylene-bis-acrylamide</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>1 M Tris HCl pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulphate</td>
<td>100µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.6 ml</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
Some aspects of this work were published and presented in scientific meetings as follows:


6.5 Reactivity of the CD11/CD18 workshop monoclonal antibodies in the sheep

V.K. Gupta*, I. McConnell, J. Hopkins

Department of Veterinary Pathology, University of Edinburgh, Edinburgh, EH9 1QH, UK

Abstract

The anti-CD11/CD18 monoclonal antibodies (mAbs) submitted in the Second International Workshop on Ruminant Leukocyte Differentiation Antigens, were analysed for their reactivity with the ovine homologue of CD11/CD18. Their reactivity was tested on healthy sheep tissues, and alveolar macrophages, afferent dendritic cells, peripheral blood granulocytes and monocytes. The CD11a/CD18 mAbs found positive in the sheep were reactive with all the cell populations tested. The CD11b mAbs reacted with all the cells except afferent dendritic cells, whereas CD11c were non-reactive to blood granulocytes. This is in contrast to humans and cattle where blood granulocytes express CD11c. The mAbs 72-87, F10-150, MD2B7 and MUC76A were found to be homologous to CD11a whereas BAQ30A seemed to be homologous to CD18, instead of proposed initial specificity to CD11a. CC125 and IL-A15 mAbs were found to be homologous to CD11b. OM1, which clustered with a recognized CD1 mAb in the first cluster analysis, precipitated a heterodimer of molecular weight 95 000/150 000. We propose that OM1 reacts with sheep CD11c. The mAb MF14B4 was found to react with sheep CD18.

Introduction

The human CD11/CD18 leukocyte surface adhesion molecules are commonly known as leukocyte integrins or the β2-integrin family. The leukocyte integrin family comprises three α/β heterodimeric transmembrane glycoproteins which share a common β chain, designated CD18 of molecular weight (MW) 95 000. The α subunit of CD11a (αL) has a MW of 180 000 and the dimer is commonly known as lymphocyte function associated antigen-1 (LFA-1). CD11b α chain (αM) of 170 000 MW combines with CD18 the β chain to produce the molecule macrophage antigen-1 (Mac-1) or complement receptor 3 (CR3). Likewise CD11c α chain (αX) of MW 150 000 with its β chain produce the molecule p150,95. These molecules are intimately associated with cell–cell or cell–matrix adhesion. CD11a is primarily involved in lymphocyte–lymphocyte or lymphocyte–macrophage interaction and associates with its ligands, intercellular adhesion molecule-1 (ICAM-1) and ICAM-
Table 1
Workshop monoclonal antibodies used in the present studies in the sheep

<table>
<thead>
<tr>
<th>Workshop number</th>
<th>Clone number</th>
<th>Isotype</th>
<th>Laboratory</th>
<th>Putative specificity</th>
<th>Temporary cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>72-87</td>
<td>G2a</td>
<td>UMELB</td>
<td>CD11a</td>
<td>8</td>
</tr>
<tr>
<td>85</td>
<td>IL-A99</td>
<td>G2a</td>
<td>ILRAD</td>
<td>CD11a</td>
<td>8</td>
</tr>
<tr>
<td>94</td>
<td>F10-150</td>
<td>G1</td>
<td>BII</td>
<td>CD11a</td>
<td>30</td>
</tr>
<tr>
<td>100</td>
<td>MD2B7</td>
<td>G1</td>
<td>FUNDIP</td>
<td>CD11a</td>
<td>8</td>
</tr>
<tr>
<td>176</td>
<td>MUC76A</td>
<td>G2a</td>
<td>WSU</td>
<td>CD11a</td>
<td>4</td>
</tr>
<tr>
<td>178</td>
<td>BAQ30A</td>
<td>G1</td>
<td>WSU</td>
<td>CD11a</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>CC126</td>
<td>G2b</td>
<td>IAH</td>
<td>CD11b</td>
<td>20</td>
</tr>
<tr>
<td>23</td>
<td>CC125</td>
<td>G1</td>
<td>IAH</td>
<td>CD11b</td>
<td>20</td>
</tr>
<tr>
<td>62</td>
<td>IL-A15</td>
<td>G1</td>
<td>ILRAD</td>
<td>CD11b</td>
<td>20</td>
</tr>
<tr>
<td>180</td>
<td>BAQ153A</td>
<td>M</td>
<td>WSU</td>
<td>CD11c</td>
<td>16</td>
</tr>
<tr>
<td>105</td>
<td>OMI</td>
<td>G1</td>
<td>INRA</td>
<td>Monocyte, macrophage</td>
<td>19</td>
</tr>
<tr>
<td>102</td>
<td>MF13F5</td>
<td>G1</td>
<td>FUNDIP</td>
<td>CD18</td>
<td>26</td>
</tr>
<tr>
<td>103</td>
<td>MF14B4</td>
<td>G1</td>
<td>FUNDIP</td>
<td>CD18</td>
<td>8</td>
</tr>
</tbody>
</table>

The other CD11/CD18 mAbs including those from temporary clusters TC8, TC20 and TC16 were tested and found to be unreactive.

2. CD11b and CD11c both have a role in leukocyte–endothelial interaction where their ligand is one of the cell–matrix molecule such as vitronectin, fibrinogen and collagen. They can also function as receptor for the complement component iC3b, where they contribute to opsonization (Hogg, 1989; Kashimoto et al., 1989; Larson and Springer, 1990).

The purpose of this study was to identify the ovine equivalent of CD11/CD18 monoclonal antibodies (mAbs). On initial screening (by fluorescence activated cell sorting (FACS) analysis) of Second International Ruminant Workshop mAbs (CD11/CD18, monocyte, macrophage specificities, and temporary clusters 8, 16 and 20) with sheep alveolar macrophages (AM), peripheral blood leukocytes and afferent lymph cells, a panel of CD11/CD18 mAbs was identified (Table 1). Some of these were further characterized, for distribution and size of the molecule recognized, by, fluorescence activated cell sorter (FACS) analysis, immunohistochemistry and immunoprecipitation.

Materials and methods

Cells and tissues

Alveolar macrophages were obtained by bronchoalveolar lavage of healthy sheep with ice-cold Hank's balanced salt solution immediately after slaugh-
tering, as described earlier (Mayer and Lam, 1984). Peripheral blood leukocytes (PBL) were isolated from heparinized venous blood by ammonium chloride lysis (Mishell and Shiigi, 1980). Peripheral blood monocytes (PBM) were enriched by adherence to autologous plasma coated gelatine plates as described earlier (Goddeeris et al., 1986). Afferent dendritic cells (ADC) were obtained by cannulation of pseudoafferent lymphatics as previously described (Hopkins et al., 1986). The tissues were collected from normal adult sheep soon after slaughtering, and snap frozen in liquid nitrogen before storing at $-70^\circ\text{C}$.

**Monoclonal antibodies (mAbs)**

The mAbs used were those submitted to the Second International Workshop on Leukocyte Differentiation Antigens of Ruminants, for which there was published or preliminary evidence that they are directed against the homologue of the human CD11/CD18 antigens. In addition, mAbs reacting to sheep macrophage populations were also tested for $\beta_2$-integrin specificity.

**Flow cytometric analysis**

The mAbs were analysed for their reactivity with AM, ADC, PBM, and peripheral blood granulocytes (PBG) by flow cytometry (FACS). In brief, $5 \times 10^5$ cells of each population were incubated with $50 \mu l$ of the appropriate dilution of mAbs diluted in phosphate buffer saline (PBS), 0.1% bovine serum albumin, 0.01 M sodium azide (PBA). After 60 min incubation at $4^\circ\text{C}$ the cells were washed twice in PBA and incubated for 60 min with antimouse fluorescein isothiocyanate (The Binding Site, UK) before final washing and analysis; $10^4$ cells were analysed by flow cytometry using a Becton Dickenson FACScan Analyser (Mountain View, CA). Live gating using forward scatter (FSC) and side scatter (SSC) parameters was used to distinguish different cell populations. AM and ADC have high FSC/high SSC, PBG have low FSC/high SSC, and PBM have high FSC/medium to low SSC.

**Immunohistochemistry**

The reactivity of mAbs was characterized by indirect immunoperoxidase staining of serial sections of healthy sheep tissues such as skin, lung, liver, kidney, lymph node, thymus, spleen, brain and Peyer's patches in small intestine. Frozen sections of 5–7 μm thick were cut by cryotome (SLEE, London), placed on Vectabond (Vector Labs, UK) treated slides, air-dried overnight at room temperature and fixed in ice cold acetone for 5 min. Slides were wrapped in aluminium foil and stored at $-70^\circ\text{C}$ before use. After thawing the sections were rehydrated for 15 min in cold PBS. The endogenous peroxidase activity
was blocked in two changes of 15 min each in PBS pH 7.2, 0.01% sodium azide and 1% hydrogen peroxide (BDH, Poole, England) at 4°C. The intestine sections were blocked overnight. The sections were stained by standard immunohistology as described (Taylor, 1978).

**Immunoprecipitation**

The AM obtained by bronchoalveolar lavage were surface labelled with $^{125}$I (Amersham International) using the glucose oxidase, lactoperoxidase method after treatment with Wood's reagent as described (Wall and Fitch, 1985). The cells were washed three times in ice-cold PBS/azide and lysed in 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% Triton X-100, containing 2 mM phenylmethyl sulphonyl fluoride. After 30 min on ice the nuclei and debris were removed by spinning in a microcentrifuge (Micro Centaur MSE) at high speed for 30 min at 4°C. The lysate was precleared using Sephadex G25 (Column PD-10; Pharmacia, Uppsala, Sweden). The immunoprecipitation of mAbs was done by enzyme-linked immunosorbent assay (ELISA) plate method. The ELISA plates (Dynatech) were coated with goat antimouse Ig (Sigma) at 10 µg ml$^{-1}$ in borate buffered saline (100 µl per well) incubated overnight at 4°C. The plates were washed five times in PBS/0.1% Tween 20 before incubation with appropriate dilution of mAbs (100 µl per well) for 1 h at room temperature, washed and incubated for 3 h with $^{125}$I-labelled AM lysate (100 µl per well). Bound antigen was eluted by boiling in SDS polyacrylamide gel sample buffer and antigens were fractionated on 5–20% gradient gels (Laemmli, 1970), and visualized by autoradiography.

**Results**

**FACS analysis**

Table 2 shows the proportions of the different cell populations reacting with the anti-CD11/CD18 workshop mAbs. All mAbs reacted with the majority of AM. The CD11a mAbs interact with most of the other cell populations tested. The CD11b mAbs react with AM, PBM, PBG but not ADC. The CD11c mAbs, in contrast, stain all cells tested but the PBG. CD18 mAbs have a similar distribution to the CD11a mAbs. Representative FACS profiles of different cell populations are given in Fig. 1.

**Immunohistochemistry**

Table 3 shows the tissue distribution of CD11/CD18 antigens on macrophages in different tissues. For immunohistochemistry two CD11a (MD2B7 and MUC76A), three CD11b (CC126, CC125 and IL-A15), two CD11c
Table 2
Reactivity of CD11/CD18 workshop monoclonal antibodies with different cell types in sheep and proposed specificities

<table>
<thead>
<tr>
<th>Workshop number</th>
<th>Clone number</th>
<th>Percentage of positive cells</th>
<th>Molecule immunoprecipitated</th>
<th>Proposed specificity in sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alveolar macrophages</td>
<td>Afferent dendritic cells</td>
<td>Peripheral blood granulocytes</td>
</tr>
<tr>
<td>44</td>
<td>72-87</td>
<td>82-87</td>
<td>82-99</td>
<td>86-90</td>
</tr>
<tr>
<td>85</td>
<td>IL-A99</td>
<td>70-73</td>
<td>57-69</td>
<td>80-85</td>
</tr>
<tr>
<td>94</td>
<td>F10-150</td>
<td>69-71</td>
<td>71-83</td>
<td>83-85</td>
</tr>
<tr>
<td>100</td>
<td>MD2B7</td>
<td>81-86</td>
<td>46-75</td>
<td>91-97</td>
</tr>
<tr>
<td>176</td>
<td>MUC76A</td>
<td>75-85</td>
<td>37-71</td>
<td>20-97</td>
</tr>
<tr>
<td>178</td>
<td>BAQ30A</td>
<td>81-89</td>
<td>82-96</td>
<td>85-97</td>
</tr>
<tr>
<td>11</td>
<td>CC126</td>
<td>79-82</td>
<td>5-17</td>
<td>89-97</td>
</tr>
<tr>
<td>23</td>
<td>CC125</td>
<td>78-82</td>
<td>4-9</td>
<td>87-96</td>
</tr>
<tr>
<td>62</td>
<td>IL-A15</td>
<td>79-83</td>
<td>6-14</td>
<td>87-96</td>
</tr>
<tr>
<td>105</td>
<td>OM1</td>
<td>78-82</td>
<td>68-85</td>
<td>6-7</td>
</tr>
<tr>
<td>180</td>
<td>BAQ153A</td>
<td>80-85</td>
<td>51-64</td>
<td>0'-7</td>
</tr>
<tr>
<td>102</td>
<td>MF13F5</td>
<td>68-70</td>
<td>22-74</td>
<td>77-79</td>
</tr>
<tr>
<td>103</td>
<td>MF14B4</td>
<td>83-84</td>
<td>60-94</td>
<td>91-97</td>
</tr>
</tbody>
</table>

*Range of two animals.

*Range of three animals.

*Range of five animals.

*Single animal value.

*Background (control) response.

*Precipitations could not be achieved.

*Not determined.

(OM1 and BAQ153A) and two CD18 (MF14B4 and BAQ30A) mAbs were used. The CD11a and CD18 distribution was identical, except for cross-reactivity of MF14B4 (a CD18 mAb) with some non-macrophage cell populations (hepatocytes, pulmonary mucosa, vascular endothelium, inner epidermis, hair follicle and mucosa of dermal glands) besides staining of lymphocytes by both CD11 and CD18 mAbs. The discrimination between CD11a, CD11b and CD11c was most obvious in the spleen, intestine, lung, liver and kidney. In spleen the red pulp and marginal zone areas were stained uniformly by CD11a/CD18, but CD11b gave intense staining of marginal zone cells and only few cells were stained in the red pulp area, whereas CD11c stained only few cells in the marginal zone and red pulp areas (Fig. 2). In the intestine CD11a/CD18 gave intense staining of villi macrophages and few interdigitating cells in the Peyer's patch follicle, whereas CD11b and c mAbs stained fewer villi macrophages with low intensity besides staining a small number of interdigitating cells in the follicle. All the AM are stained by CD11/CD18 but more tissue macrophages are stained by CD11a/CD18 than by
CD11b and c mAbs. Liver showed strong staining of most Kupffer cells by CD11a/CD18, but few cells were stained strongly by CD11b and moderately by CD11c mAbs (Fig. 2). In thymus most interlobular septal macrophages were stained by CD11b, fewer cells by CD11a/CD18 and very few cells were stained by CD11c. Lymph node showed intense staining of large numbers of macrophages in the paracortex and medulla by CD11a/CD18, whereas small numbers of cells were stained by CD11b and c mAbs. In kidney moderate numbers of cells were stained in glomerulus and interstitium by CD11a/CD18, but few cells were stained in glomerulus by CD11b and isolated cells in the interstitium by CD11b and c mAbs. In the skin more dermal macrophages were stained by CD11a/CD18 than by CD11b, and few cells were stained by CD11c mAbs. In brain a moderate number of microglial cells were stained by CD11a,b/CD18, and few cells by CD11c mAbs.
Table 3
Tissue distribution of CD11/CD18 antigens on macrophages recognized by workshop monoclonal antibodies in the normal sheep

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CD11a&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD11b</th>
<th>CD11c</th>
<th>CD18&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medulla</td>
<td>+++ (s)</td>
<td>+ (s)</td>
<td>+(m)</td>
<td>+++ (s)</td>
</tr>
<tr>
<td>Paracortex</td>
<td>+ + (s)</td>
<td>+ (s)</td>
<td>±(w)</td>
<td>+ + (s)</td>
</tr>
<tr>
<td>Follicle</td>
<td>+ (w)</td>
<td>+ (w)</td>
<td></td>
<td>+ (w)</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red pulp</td>
<td>+++ (s)</td>
<td>+ (s)</td>
<td>+(m)</td>
<td>+++ (m)</td>
</tr>
<tr>
<td>Marginal zone</td>
<td>+++ (s)</td>
<td>+++ (s)</td>
<td>+(m)</td>
<td>+++ (m)</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>+ (s)</td>
<td>+ (s)</td>
<td>+(m)</td>
<td>+ + (m)</td>
</tr>
<tr>
<td>Medulla</td>
<td>+++ (s)</td>
<td>+++ (s)</td>
<td>+(m)</td>
<td>+++ (m)</td>
</tr>
<tr>
<td>Interlobular septa</td>
<td>+ (s)</td>
<td>+++ (s)</td>
<td>+(m)</td>
<td>+ + (m)</td>
</tr>
<tr>
<td>Intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peyer's patch follicle</td>
<td>+ + (s)</td>
<td>++ (w)</td>
<td>±(w)</td>
<td>++ (m)</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>+++ (s)</td>
<td>+ (s)</td>
<td>+(m)</td>
<td>++ + (s)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>+++ (s)</td>
<td>+++ (m)</td>
<td>+++ (m)</td>
<td>+++ (s)</td>
</tr>
<tr>
<td>Tissue macrophages</td>
<td>+++ (s)</td>
<td>+ (w)</td>
<td>+ (w)</td>
<td>+++ (s)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>+++ (s)</td>
<td>+ (s)</td>
<td>+(m)</td>
<td>++ + (s)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerulus</td>
<td>+ (s)</td>
<td>+(m)</td>
<td></td>
<td>+ + (m)</td>
</tr>
<tr>
<td>Interstitium</td>
<td>+ (s)</td>
<td>±(w)</td>
<td>±(w)</td>
<td>+ + (m)</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermis</td>
<td>+++ (s)</td>
<td>+ (m)</td>
<td>+ (w)</td>
<td>++ + (s)</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microglial cells</td>
<td>+ (s)</td>
<td>+ (m)</td>
<td>+ (w)</td>
<td>+ + (s)</td>
</tr>
</tbody>
</table>

Number of macrophages staining positive: —, none, +, few; ++++, many.
Staining intensity: w, weak; m, medium; s, strong.
<sup>a</sup>Stained lymphocytes in lymphoid organs besides macrophages.

**Immunoprecipitations**

Antigens were immunoprecipitated from <sup>125</sup>I-labelled AM by the ELISA plate method. Fig. 3 shows antigens precipitated by four CD11a mAbs (MD2B7, MUC76A, F10-150 and 72-87) detecting molecules of 95 000 and 180 000 MW, by two CD11b mAbs (CC125 and IL-A15) of MW 95 000 and 170 000, by mAb OM1 of MW 95 000 and 150 000, and by two mAbs
Fig. 2. Immunohistochemistry of (A, C and E) spleen and (B, D and F) liver sections stained with (A and B) BAQ30A (CD18), (C and D) IL-A15 (CD11b) and (E and F) BAQ153A (CD11c) mAbs. mz, marginal zone; rp, red pulp; Kupffer cell (arrow).

Fig. 3. Autoradiograph of antigens precipitated from $^{125}$I surface labelled sheep alveolar macrophages lysate by mAbs. The samples were fractionated on 5–20% gradient SDS–PAGE.
(BAQ30A and MF14B4) of MW 95,000/150,000/180,000, when precipitates were run on SDS-PAGE under reducing conditions. Antigens could not be precipitated with CC126 and BAQ153A mAbs. Identical results were obtained on non-reducing gels.

Discussion and conclusion

All the CD11a mAbs showed high FACS reactivity with all the cell types tested, except for MUC76A which showed low reactivity with PBG and ADC from one animal each, suggesting some antigenic polymorphism. There was no difference in tissue distribution among CD11a mAbs (MD2B7 and MUC76A). The four mAbs used precipitated identical molecular weight antigens, suggesting the antigen identified by 72-87, F10-150, MD2B7 and MUC76A as the ovine homologue to CD11a. The antigen identified by BAQ30A showed its homology to CD18 instead of the initially proposed CD11a specificity. The mAb IL-A99 may be specific for OvCD11a, but it was not used for tissue staining and immunoprecipitation.

The three CD11b mAbs (CC126, CC125 and IL-A15) showed high FACS reactivity with AM, PBM and PBG but no reactivity with ADC (Steinman, 1991). The tissue distribution of these three mAbs was identical. CC125 and IL-A15 mAbs precipitated identical molecular weight antigens, thus suggesting their ovine homology of CD11b. Precipitations with CC126 could not be achieved but it may be specific for CD11b. The mAbs BAQ153A and OM1 showed high FACS reactivity with AM and AD low with PBM, but the reactivity with PBG varied from 0% to 7%, which is in contrast to high reactivity in humans (De La Hera et al., 1988) and cattle (Splitter and Morrison, 1991). The OM1 mAb has been reported to react only with eosinophils amongst granulocytes (Pepin et al., 1992). OM1 precipitated an antigen of MW 95,000/150,000, suggesting it reacted with the ovine homologue of CD11c. BAQ153A may also be specific for CD11c considering its resemblance to OM1, but its failure to precipitate the antigen, may be due to its IgM isotype.

BAQ30A, MF13F5 and MF14B4 showed similar FACS reactivity and tissue staining to that of CD11a mAbs. However, when the mAbs BAQ30A and MF14B4 were used for precipitation, three polypeptide chains were identified suggesting that they react with the ovine homologue of CD18. Unambiguous assignment of specificities of these monoclonal antibodies awaits CD11/CD18 transfections.

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References


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A new ovine monocyte / macrophage cell surface antigen was recognised by three mouse monoclonal antibodies (mAb) VPM65, 66 and 67. These antibodies also reacted with bovine cells. The three mAb immunoprecipitated a single 55,000 m wt glycoprotein which, when deglycosilated with N-glycosidase F was reduced to 53,000. They reacted strongly with peripheral blood monocytes, moderately with alveolar macrophages (AM) and peripheral blood granulocytes, and weakly with afferent lymph dendritic cells (ADC). They also reacted with macrophages in many different tissues but were non reactive with lymphocytes. The molecule is associated with the cell surface, via a phosphatidylinositol linkage. Affinity purified antigen is biochemically characterised. These data demonstrate that the mAb react with the ovine homologue of CD14.
W18. FC RECEPTORS

15.

A MONOCLONAL ANTIBODY RECOGNISING DIFFERENTIATION ANTIGEN ON RESIDENT TISSUE MACROPHAGES IN SHEEP - A POTENTIAL FCyRII

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Purpose of this study was to produce and characterise monoclonal antibodies (mAb) recognising differentiation antigen on mononuclear phagocytes (MPh) in sheep. A mAb VPM63 was raised against sheep alveolar macrophages (AM), by fusion of spleen cells of the immunised BALB/c mice with NSO myeloma cells. Immunohistochemically VPM63 recognised most tissue macrophages in lymphoreticular organs such as lymph node, thymus and spleen. Kupffer's cells in the liver, interstitial and glomerular macrophages of the kidney, perivascular and villi macrophages of the full term cordedons, macrophages in lamina propria and interfollicular space of ileum, dermal and brain macrophages were also labelled. VPM63 did not react with any peripheral blood, afferent and efferent lymph cells including monocytes and dendritic cells. With time in culture, the expression of the antigen is lost from alveolar macrophages and did not appear on monocytes even after five days in culture. Immunoprecipitation and SDS-PAGE analysis indicated that two proteins of molecular weight 40,000 and 43,000 were recognised by VPM63 under reducing and non-reducing conditions. The antigen was found to be a glycoprotein having 2,000-3,000 D N-linked glycosylation. The antigen was not linked to the cell surface via phosphatidyl inositol linkage. The western blotting of VPM63 affinitypurified antigen from alveolar macrophages, with rabbit anti-bovine FcγRI synthetic peptide of 15 amino acids from second extracellular domain, suggest the possibility of VPM63 recognising an isoform of sheep FcγRII, present only on resident tissue macrophages. The antigen recognised by VPM63 is apparently distinct from the previously defined myeloid antigens in sheep. However, a similar mAb L9 specific for macrophages in human placenta has been reported (Nash et al. 1989, Immunology 68, 332-340).