Analysis of Antigen Handling by Epidermal Langerhans' Cells During the Early Neonatal Period.

By

M.

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Abstract

Antigen applied to the epidermis of 4-day-old mice results in antigen specific immune suppression and is linked to a decrease in the amount of antigen transported to the draining lymph node by epidermal Langerhans cells (LC). It was therefore proposed that a decrease in receptor-mediated endocytosis was responsible for the reduced antigen transport by the LC. Consequently the aims of this study were to characterise the expression of antigen uptake receptors during development of neonatal mice, evaluate their capacity to internalise antigens via receptor-mediated and fluid phase uptake and to assess antigen proteolysis and MHC-II complexing following exposure to maturation stimuli.

Langerhans cells isolated from 4-day-old epidermis express reduced levels of the C-type lectin receptors DEC-205 and Langerin in comparison to LC isolated from 6-week-old epidermis. Despite this reduction neonatal LC were efficient in the uptake of the mannosylated antigen FITC-dextran. When incubated in the presence of the macropinocytosis inhibitor wortmannin, antigen uptake was reduced to a greater extent in neonatal LC than adult counterparts. As such, neonatal LC preferentially utilise a wortmannin-sensitive fluid phase pathway, such as macropinocytosis, to internalise antigens such as FITC-dextran.

Despite the presence of LAMP-1^{+}MHC-II^{+} vesicles within LC from neonatal epidermis, proteolysed DQ-OVA was localised to a MHC-II^{-} compartment. This suggests that antigen was internalised via macropinocytosis and retained in a LAMP-1^{-} macropinosome. As no differences were observed in DQ-OVA^{+} vesicle localisation, it was evident that neonatal and adult LC were associated with a comparable proteolytic activity. 24 hours following exposure to a maturation-inducing dose of LPS, membrane MHC-II:peptide complexes were evident on adult LC, but not on neonatal LC. Although neonatal LC demonstrated both CD86 up-regulation and MHC-II redistribution following LPS exposure, the transport of MHC-II:peptide complexes to the membrane was diminished. This reduced transport was not simply due to a defect in MHC-II recycling as LPS stimulation of LC caused a redistribution of MHC-II from an intracellular endosome to the cell surface.

Therefore the reduced transport of MHC-II:peptide to the cell surface was most likely a consequence of a failure to associate with MHC-II in the cytoplasm.

The capacity of neonatal LC to sample their external environment without inducing immunity has important biological consequences for the protection against inappropriate responses during the developmental period.

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Table of Contents

Abbreviations

7AAD	7-amino-actinomycin D
APC	Antigen presenting cell
Cat	Cathepsin
CLIP	Class II-associated invariant chain peptides
CTL	Cytotoxic T lymphocyte
°C	Degrees centigrade
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
FCS	Foetal bovine (calf) serum
FITC	Fluorescein isothiocyanate
g	Grams
GM-CSF	Granulocyte/macrophage colony stimulating factor
HEPES	N-2-hydroxyethylpiperizine-N-2-etanesulphonic acid
Ig	Immunoglobin
IFN	Interferon
IL	Interleukin
KC	Keratinocyte
LC	Langerhans cell
2ME	2-mercaptoethanol
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
µg	Micrograms
µL	Microlitre
mg	Milligrams
mL	Millilitre
M	Molar
mM	Millimolar
µL	Micromolar
ng	Nanograms
nM	Nanomolar
LAMP	Lysosome associated membrane protein

LN	Lymph node
LPS	Lipopolysaccharide
OVA	Ovalbumin
PE	Phycoerythrin
PBS	Phosphate buffered saline
RBC	Red blood cell
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
Th1	T helper type one
Th2	T helper type two
TLR	Toll-like receptor
UV	Ultraviolet
U	Units

Publication List

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DEC-205(lo) Langerin(lo) neonatal Langerhans' cells preferentially utilize a wortmannin-sensitive, fluid-phase pathway to internalise exogenous antigen

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Chapter 1
Literature Review

1.1 Langerhans Cells.

Langerhans cells (LC) were first identified in 1868 by Paul Langerhans (Langerhans 1868). They were initially presumed to have a neuronal function due to an association with sensory nerve endings and reactivity with gold salts (Hunter 1985). It is now known that LC are the principle antigen-presenting cell (APC) of the epidermis and as such are critical to the induction of immunity to cutaneous antigen (Cruz and Bergstresser 1990). They are a mobile population of dendritic cell (DC), originating from bone marrow precursors (de Fraissinette et al. 1988; de Fraissinette et al. 1990). Within the epidermis they populate the stratum granulosum and spinosum, constituting approximately 2-4% of epidermal cells (Gielen et al. 1988; Schuler et al. 1991). The integrity of the LC network is maintained by attachment to neighbouring keratinocytes (KC), mediated by adhesion molecule interactions (namely E-cadherin) and Ca^{2+}-dependent mechanisms (Tang et al. 1993). The capacity of LC to survey the extracellular environment surroundings is aided by long membranous extensions called dendrites, enabling LC to form an interconnecting network (Shelley and Juhlin 1976; Shelley and Juhlin 1977). Following antigenic exposure, or receipt of "danger signals" such as lipopolysaccharide (LPS) or epidermal cytokines, LC are mobilised from the epidermis (Cumberbatch et al. 1991; Kimber and Cumberbatch 1992; Cumberbatch et al. 2000). LC then traverse the epidermal-dermal barrier and migrate via the afferent lymphatics to the draining lymph node (LN) (Price et al. 1997; Ratzinger et al. 2002). During transit, LC undergo a process of differentiation, such that they acquire properties of immuno-stimulatory APC, capable of inducing antigen-specific T cell responses (Larsen et al. 1990; Cumberbatch et al. 1996).

During the early neonatal period, antigen treatment of murine epidermis results in a deviation of the immune response from immunity to antigen-specific immune suppression (Dewar et al. 2001). It has been demonstrated that although neonatal LC maintain the capacity to migrate to the draining LN following treatment, they do so in reduced numbers and the amount of antigen they traffic, and thus present to naïve

T cells, is significantly reduced (Dewar et al. 2001). This review will summarise our current understanding of LC function, discussing antigen uptake and processing pathways, the stimuli essential for migration and maturation and finally the signals required for T cell activation and proliferation. The many gaps that remain in our understanding of neonatal immune function will also be discussed.

1.2 Langerhans Cell Phenotype

Langerhans cell phenotype and function is highly dependent on location and maturation status (Larsen et al. 1990; Romani and Schuler 1992; Shibaki et al. 1995). Whilst resident in the epidermis LC are primarily immature, and express low levels of surface molecules required for T cell activation (Witmer-Pack et al. 1988). Such molecules include the antigen presentation molecules, major histocompatibility complex type I and II (MHC-I, MHC-II), and costimulatory molecules CD40, CD80 and CD86 (Schuler et al. 1985; Schuler and Steinman 1985; Witmer-Pack et al. 1987). Although limited in their stimulatory potency, immature LC are adept at antigen uptake and processing, expressing recognition receptors for a range of foreign antigens (Shelley and Juhlin 1977; Katz and Sunshine 1986; Reis et al. 1993). Receipt of maturation stimuli, such as epidermal cytokines or bacterial products, induces dramatic alterations in LC phenotype (Steinman 1988; Geissmann et al. 1999; Cumberbatch et al. 2000). Expression of antigen uptake receptors is down-regulated, whereas expression of MHC-I and II, CD40, CD80 and CD86 becomes more apparent (Singh-Jasuja et al. 2000). Whilst no longer capable of internalising and processing exogenous antigen, mature LC produce biologically dynamic cytokines such as interleukin (IL)-1β, IL-6 and IL-12 and are powerful promoters of MHC-I and II restricted T cell responses (Aiba and Katz 1990; Reis et al. 1993; Jonuleit et al. 1997; Belz et al. 2002b; Sundquist et al. 2003). Aside from producing pro-inflammatory cytokines, maturing LC express a range of chemokine receptors to facilitate their migration from the epidermis and subsequent interactions with lymphocytes in the regional LN (Yanagihara et al. 1998; Wang et al. 1999; Jakob et al. 2001).

LC phenotype has also been shown to alter during *in vitro* culture and this has been proposed to model LC maturation during migration to the draining LN (Larsen et al.

1990). From a model using skin explants, where LC spontaneously migrate out of epidermal sheets, up-regulation of MHC-II expression was observed and compared to *in situ* LC (Larsen et al. 1990). As such, cultured systems such as these have become powerful tools for the study of LC maturation and APC function *in vitro*.

1.3 Langerhans Cell Function

The capacity of LC to induce immunity following internalisation of antigen is well illustrated in the immunological response known as contact hypersensitivity (Cruz 1990; Enk and Katz 1995). Contact hypersensitivity is mediated by T cells, which respond to cutaneous antigens that have been internalised, processed and presented by LC in the draining LN (Wolff and Schreiner 1970; Cumberbatch et al. 1992). Using skin sensitising fluorescent antigens, such as fluorescein isothiocyanate (FITC) it has been demonstrated that topical exposure to antigen induces changes in the microenvironment of the skin (Kripke et al. 1990). Following exposure, LC deplete rapidly from the epidermis, resulting in an accumulation of LC in the regional LN, with a percentage of cells associated with high levels of fluorescent antigen (Kinnaird et al. 1989; Kripke et al. 1990; Cumberbatch et al. 2003). When the antigen bearing cells are isolated from the node they function as APC and are capable of initiating T cell activation *in vitro* (Kripke, 1990). The critical role of LC in contact hypersensitivity has been demonstrated in murine skin deficient in LC (Toews et al. 1980). Following exposure to antigen, these mice consequently fail to demonstrate a hypersensitivity response (Toews et al. 1980; Peeler and Niederkorn 1986).

1.4 Mechanisms of Antigen Uptake

For T cells to respond to exogenous antigen it must be internalised, processed and presented by LC (Taylor et al. 1991; Scheicher et al. 1995; Kato et al. 1998; Stockwin et al. 2000). Antigen uptake can occur via three functionally distinct pathways, accommodating a vast array of possible antigens (Swanson and Watts 1995; Watts 1997). Pinocytosis is the internalisation of non-binding or soluble antigens (Hanau et al. 1985; Swanson and Watts 1995). Receptor-mediated endocytosis and phagocytosis involves the ingestion of small and large particulate antigens following binding of antigen to cell surface uptake receptors (Reis et al. 1993; Sallusto et al. 1995). The ability of LC to internalise and process antigen is

highly dependent on the stage of cell differentiation and receptor expression, limited to epidermal-resident LC as demonstrated in the seminal work of Romani and colleagues (Romani et al. 1989). Using T cell clones, it was demonstrated that the stimulatory capacity of LC varied with maturation (Romani et al. 1989). Whilst freshly isolated epidermal LC actively internalise and process intact myoglobin, they were incapable of presenting the protein in *in vitro* T cell stimulation assays. In contrast, although cultured LC and spleen DC were weakly endocytic, they actively presented the intact protein (Romani et al. 1989). The findings of Romani *et al.* (1989), combined with more recent investigations, have lead to the conclusion that DC function is centred around two distinct phenotypes that develop in succession. Firstly, DC recognise and internalise antigen in peripheral tissues, a prime example being sentinel LC of the epidermis. Following receipt of maturation stimuli, DC acquire T cell stimulatory capacity (Okada et al. 1988; Romani et al. 1989; Lutz et al. 1996a; Lutz et al. 1996b)

1.4.1 Pinocytosis

Fluid phase endocytosis, or pinocytosis, refers to the non-specific bulk uptake of soluble antigen, and non-specialised cells can drink up to their volume in extracellular fluid every day (Swanson and Watts 1995; Amyere et al. 2002). The pinocytic pathway employed is influenced by antigen size (Watts and Marsh 1992). Micropinocytosis is the internalisation of antigen up to 100nm by clathrin-coated pits. In contrast, larger antigens are internalised by macropinocytosis, associated with actin-mediated membrane ruffling (Watts and Marsh 1992).

1.4.1.1 Micropinocytosis

Micropinocytosis, a constitutive process in the majority of DC, is the ingestion of small soluble antigens via clathrin coated pits (Amyere et al. 2002). Within these pits are a variety of receptors, including those specific for carrier proteins and essential nutrients, that enable concentration of antigen (Watts and Marsh 1992; Roberts and Sandra 1994; Amyere et al. 2002). Following internalisation of antigen and ligand release within the appropriate intracellular vesicle, receptors recycle rapidly to the cell surface (Brown and Swank 1983; Ciechanover et al. 1983) Using a novel human B/DC cell line, HBM-Noda, Torii and colleagues investigated the fate of antigen following internalisation by a range of uptake pathways. Using low-

density lipoprotein, LDL, the fate of pinocytosed soluble antigen was traced. It as demonstrated that following binding of LDL to the corresponding receptor, the antigen was internalised with the receptor into endosomes, which later fuse with lysosomes. Following this the receptor was dissociated by an increase in acidity, enabling recycling of the receptor to the plasma membrane (Torii et al. 2001). In so doing the cell is able to perform consecutive rounds of antigen internalisation. As such micropinocytosis plays an important role in both ingestion of antigen for processing and presentation as well as internalisation of nutrients for cell survival.

1.4.1.2 Macropinocytosis

Macropinocytosis is the non-specific, high volume uptake of large soluble antigens via actin-driven membrane ruffling (Swanson and Watts 1995). Following membrane extension the ruffles fuse, enclosing the antigen within large vesicles called macropinosomes (Swanson and Watts 1995). Initiation of macropinosome formation involves several steps (Amyere et al. 2002). Firstly plasma membrane signalling pathways are generated following binding of growth factors such as GM-CSF, or after recruitment of activated oncogenes in transformed cells such as K-ras and v-src to the plasma membrane (Fincham et al. 1996; Veithen et al. 1998; Amyere et al. 2000). The second step involves amplification of these signals by activation of phosphatidylinositol-3-kinase (PI3K) and phosphoinositide-specific phospholipase 3-lipase (PLC) (Amyere et al. 2000). Following these early enzymatic steps is the activation of actin remodelling, induced by calcium release or Rho-GTPases under the influence of PI3K and PLC (Mackay and Hall 1998; Nobes and Hall 1999). Actin remodelling then leads to membrane ruffling and filopodia extension (Amyere et al. 2002). These various processes are abrogated by the irreversible PI3K inhibitor wortmannin, which prevents ruffle fusion (Stein and Waterfield 2000). The kinetics of macropinocytosis varies between cell types, possibly as a result of differences in surface area to volume ratio (Swanson and Watts 1995). Swanson and Watts proposed that cells more capable of forming cytoplasm protrusions are also more likely to membrane ruffle and hence form macropinosomes (Swanson and Watts 1995). Macropinocytosis is associated with the internalisation of large volumes of fluid. To dispose of excess fluid, and thereby concentrate antigen, the macropinosome shrinks through loss of water (Racoosin and Swanson 1993). As the macropinosome is routed through the cell, markers of mature proteolytic

compartments are acquired prior to delivery of degraded antigen to MHC-II processing compartments (Sallusto et al. 1995; Lutz et al. 1997).

Although this is the most documented pathway of macropinocytosis, a divergence has been demonstrated in the macrophage lineage. It has been demonstrated that following macropinosome formation, the vesicle is trafficked through the cell, bypassing intracellular processing pathway (Amyere et al. 2002). The macropinosome is then shuttled to the cell membrane, where the vesicle contents are exocytosed back to the extracellular space (Amyere et al. 2002). Hewlett and colleagues demonstrated a similar pathway in the human cell line A431. Very little interaction of macropinosomes and processing compartments was observed. Rather antigen was retained within the cell before eventually recycling to the cell membrane and exocytosing its content to the extracellular space (Hewlett et al. 1994). Therefore macropinocytosis can be employed by a cell for processing and presentation, or as a scavenging and clearance mechanism.

Macropinocytosis in LC may be related to the expression of aquaporins (de Baey and Lanzavecchia 2000). Aquaporins are a family of water channels involved in the regulation of osmotic pressure and may be required after ingestion of high volumes of water in macropinocytic vacuoles (de Baey and Lanzavecchia 2000). Macropinocytosis is associated with immature DC, as after a few days of culture this activity vanishes as processed peptides are presented (Sallusto et al. 1995).

1.4.2 Receptor mediated endocytosis

Receptor-mediated endocytosis is the internalisation of small particulate antigens following binding to antigen recognition receptors (Reis et al. 1993; Lutz et al. 1997). Following ligation, a cascade of events is induced that leads to the internalisation and subsequent trafficking of antigen to intracellular processing compartments (Sallusto et al. 1995). The efficacy of receptor-mediated endocytosis is influenced by the affinity of the receptor for the corresponding ligand, in addition to the concentration of the ligand-receptor complexes on the plasma membrane (Watts 1997).

1.4.2.1 Lectin Receptors

As professional APC, LC must be able to internalise mannose, as the majority of glycoproteins are mannosylated (Buentke et al. 2000). To enhance the efficacy of binding, LC express members of the C-type lectin receptor family (Kato et al. 1998). Lectin receptors are divided into two broad classes on the basis of their antigen reactivity, type I and type II (Chen 1997).

1.4.2.1.1 DEC-205

DEC-205, an example of a type I receptor, is a 205kD membrane glycoprotein, homologous to the macrophage mannose receptor (Jiang et al. 1995). It has been suggested to initiate uptake and strongly enhance presentation of mannose-conjugated antigen (Kato et al. 1998; Kato et al. 2000). The efficiency of DEC-205 for uptake and antigen-specific T cell activation was illustrated using anti-receptor antibodies (Jiang et al. 1995). LC expressing the receptor demonstrated a 100-fold increase in the presentation of anti-DEC-205 antibody to T cells compared antibody that was not directed to DEC-205 (Jiang et al. 1995).

Following ligand binding, the receptor:antigen complex is internalised and routed to endocytic compartments for processing and presentation (Jiang et al. 1995). Although DEC-205 shares homology with the macrophage mannose receptor, Mahnke and colleagues demonstrated that DEC-205 targets antigen to mature processing vesicles, and is far superior to the macrophage mannose receptor in presenting bound anti-receptor antibodies to T cells (Mahnke et al. 2000).

Kato and co-workers demonstrated that during maturation there are alterations in DEC-205 expression by subtypes of DC (Kato et al. 2000). Analysis of expression by real–time PCR demonstrated that DEC-205 mRNA in immature blood DC, LC and immature monocyte derived DC was low. Subsequent to maturation stimuli DEC-205 expression increased, suggesting that DEC-205 may serve as an indication of maturation.(Kato et al. 2000). These findings were later developed by Ebner *et al* (2004), who furnished evidence for *in situ* alterations in DEC-205 expression by epidermal LC. Whilst resident in the epidermis the level of DEC-205 expression was low. However, following maturation in skin organ explant cultures the level of expression increased (Ebner et al. 2004). In contrast to LC, maturation of blood DC

did not result in no DEC-205 upregulation, suggesting an increase in intracellular localisation. As a consequence of these findings it has been proposed that aside from functioning as an endocytic receptor, DEC-205 may also play a role in antigen processing and presentation (Kato et al. 2000).

1.4.2.1.2 Langerin

Langerin is a type II lectin receptor, expressed in the LC of the skin and epithelia (Valladeau et al. 2003). It is involved in endocytosis of mannose-conjugated antigen and the endocytic function of Langerin agrees with its restricted expression on immature LC (Valladeau et al. 2003). In contrast to the mannose receptor, Langerin does not co-localise with markers of late endosomes and early lysosomes (Valladeau et al. 2003). In addition, internalisation of anti-Langerin antibody does not result in routing into MHC loading compartments. Together these results strongly suggest that the function of Langerin is unrelated to antigen processing within the MHC-II pathway. It has been proposed that Langerin is employed to route extracellular antigen into the MHC-I pathway for the induction of cytotoxic T cell responses (Valladeau et al. 2003). This is plausible as it is known that LC are highly efficient at endocytosis of apoptotic debris and subsequent trafficking of internalised material for presentation on MHC-I molecules (Albert et al. 1998a).

Several lines of evidence indicate that Langerin is down-regulated following LC maturation (Tripp et al. 2004). Firstly, reactivity of anti-Langerin antibody alters following culture of epidermal explants and in maturation stimuli (Bechetoille et al. 2001). Secondly, Langerin antigen has not been detected to a significant degree in secondary lymphoid organs (Valladeau et al. 2002).

1.4.2.2. Fc Receptors

Fc receptors are involved in the recognition of antibody and as such are crucial to the internalisation of opsonised antigen (Esposito Farese et al. 1995). Endocytosis mediated by Fc receptors was illustrated using gold labelled antibodies directed against the outside domain of the receptor (Esposito-Farese et al. 1995). Following internalisation, antibody was localised to a variety of intracellular organelles, including vesicles and endosomes, suggesting that Fc receptors target antigen to processing compartments (Esposito Farese et al. 1995). Fc receptors signal through

tyrosine-based activation motifs and following binding of antigen these motifs become phosphorylated (Lofgren et al. 1999). Phosphorylation recruits the tyrosine kinase syk, whose role is to initiate several intracellular pathways leading to transcriptional activation, cytokine release and rearrangement of the cytoskeleton (Sedlik et al. 2003). Therefore, recognition of antigen via Fc receptors increases the efficiency of presentation by delivering antigen to processing vesicles and inducing the secretion of pro-inflammatory cytokines.

1.4.2.3 Complement Receptors

Complement receptors are involved in the recognition of antigen opsonised with activated complement components, such as C3b (Stingl et al. 1977; Ross and Vetvicka 1993). Akin to expression of lectin and Fc receptors, expression of complement receptors is thought to be restricted to the immature stage of LC differentiation, as expression has been shown to decrease following short-term culture of 1-3 days (Stingl et al. 1977a; Schuler and Steinman 1985). Aside from the significant role in bacterial clearance, the function of complement receptors is varied (Ross and Vetvicka 1993). It has been proposed that complement receptors may allow for physical contact between LC and naïve T cells expressing C3 (Muto et al. 1993).

1.4.2.4 Heat Shock Protein Receptors

Exposure to stress stimuli such as temperature extremes or viruses stimulates the production of pro-inflammatory molecules called heat shock proteins (Schlesinger 1994). Heat shock proteins enhance the immune response via several mechanisms initiated following binding to surface receptors (Born et al. 1990; Hightower 1991; Maytin 1995). Following exposure to heat shock proteins, DC up-regulate molecules associated with professional APC function including CD40, CD83 and CD86, whilst monocytes and macrophages secrete pro-inflammatory cytokines (Matzinger 1994; Kuppner et al. 2001). It has also been postulated that heat shock proteins enhance recognition of malignancies by NK cells, with heat shock proteins located on the surface of some tumour cells, including sarcomas, lung and colon carcinomas (Multhoff et al. 1995; Multhoff et al. 1997; Botsler et al. 1995).

1.4.2.5 CD36

It is essential that apoptotic debris is cleared from the extracellular environment to prevent induction of self-reactive immune responses (Urban et al. 2001). Uptake of apoptotic cells is enhanced via expression of the scavenger receptor CD36 (Ren et al. 1995). The functional significance of apoptotic uptake was elucidated by Urban and colleagues (Urban et al. 2001). It was demonstrated that despite receipt of maturation stimuli, DC remained immature following ingestion of apoptotic cells. In addition, exposure to apoptotic cells resulted in a deviation from the expected secretion of pro-inflammatory cytokines to secretion of anti-inflammatory, tolerance-inducing cytokines such as IL-10 (Urban et al. 2001). As such, prevention of reactivity to self-cells is maintained via expression of CD36.

1.4.2.6 Toll-like receptors

Seminal work by Janeway and colleagues have hypothesised that the host immune system senses invading pathogens via germ line-encoded pattern recognition receptors (Stout and Bottomly 1989; Medzhitov and Janeway 1998). One such group of receptors is the Toll-like receptor (TLR) family. Toll-like receptors are involved the recognition of variety of microbial antigens, including LPS and lipoprotein (Bulut et al. 2001; Werts et al. 2001). Ligand engagement of TLR results in enhancement of protective immune responses. Binding induces activation of the transcription factor NK-κB and leads to the up-regulation of costimulatory molecules and cytokines required for the activation of the adaptive immune response (Bulut et al. 2001).

1.4.3 Phagocytosis

Phagocytosis is the actin-dependent, receptor-mediated ingestion of large particulate antigen (Aderem and Underhill 1999). Through expression of a range of receptors specific for prokaryote membrane antigens and innate opsonins, APC such as macrophages and dendritic cells are equipped with a means by which to selectively bind a broad range of possible microbes, as well as self-antigen derived from dead or dying cells (Reis et al. 1993; Fanger et al. 1996; Albert et al. 1998a; Nauta et al. 2004). Following receptor ligation, actin is recruited to the plasma membrane to drive pseudopodia extension to engulf the antigen (Greenberg et al. 1991). Once pseudopodia fuse, the antigen is drawn into the cell to form a phagosome (Reis et al.

1993). This vesicle matures, acquiring proteolytic markers, before fusing with degradative lysosomes (Rabinowitz et al. 1992; Desjardins et al. 1993; Desjardins et al. 1994; Beron et al. 1995). The mature phagolysosome continues to interact with lysosomes throughout the endocytic pathway, replenishing degradative enzymes to guarantee efficient degradation of phagocytosed antigen (Oh et al. 1996).

1.5 LC Maturation

Whilst LC are resident in the epidermis they are proficient at acquiring antigen, however, their ability to present these antigens to naïve T cells is inept (Mellman et al. 1998). Only following exposure to inflammatory stimuli do APC, such as LC begin a process of maturation involving conversion of the ingested material into immunogenic MHC:peptide complexes, migration to the draining LN and presentation of antigen to naïve T cells (Roake et al. 1995; Tsang et al. 2000). Maturation is associated with a down regulation of antigen uptake receptor expression and macropinocytosis, whilst the expression of molecules required for T cell activation, namely antigen presentation and costimulatory molecules, becomes more evident. Ligation of these two classes of molecules with ligands on the surface of the T cell provides the two signals require for full activation of effector responses. By tying antigen presentation and migration with maturation ensures LC reach full APC potency prior to interaction with naïve T cells (Mellman et al. 1998)

1.6 Antigen Processing

For T cells to respond to antigen it must be processed and presented in association with surface MHC molecules (Watts 1997). Depending on antigen origin, processing can occur via two pathways (Kalish 1995). Intracellular antigen, such as those derived from viral particles or self peptides, are processed via the endogenous pathway (Kalish 1995). Ingested extracellular antigen is in turn processed via the exogenous pathway (Cohen and Katz 1992). Within DC, maturation from the processing to presenting phenotype is accompanied by an up regulation of MHC biosynthesis and export to the cell surface (Cella et al. 1997a; Pierre et al. 1997).

1.6.1. Exogenous pathway

Central to the initiation of an immune response to exogenous antigen is the capacity to convert proteins into peptides within the exogenous pathway (Ziegler and Unanue

1981). Following uptake, antigen-containing vesicles are routed to lysosomal organelles, where exposure to low pH initiates unfolding of the antigen via reduction in disulfide bonds, exposing the substrate to proteolytic enzymes (Collins et al. 1991). One such enzyme is γ-IFN-inducible lysosomal thiol reductase (GILT), which at low pH catalyses the reduction of disulfide bonds (Arunachalam et al. 2000; Lennon-Dumenil et al. 2002c). Complete degradation of antigen however, is not favourable, as MHC-II selects for peptides of a particular length, namely 9-16 residues (Mellman et al. 1998). Thus, for effective antigen processing proteolysis cannot run to completion (Lautwein et al. 2002). How processing is regulated is unknown, but it is conceivable that molecular chaperones, protease inhibitors, cystatins and certain proteases or even specialised intracellular compartments halt proteolysis before the antigen is completely catalysed (Pure et al. 1990; Lennon-Dumenil et al. 2002b).

Occurring simultaneously to antigen proteolysis is the assembly of the α and β MHC-II chains within the endoplasmic reticulum (Cresswell 1994; Cresswell 1996). The linking of the two subunits forms a single peptide-binding groove at the top of the dimer, accounting for the subtle but crucial differences in the range of peptides that can be bound (Mellman et al. 1998). During assembly, MHC-II associates with a chaperon molecule termed the invariant chain (Ii), whose function is to direct class II molecules into the endocytic pathway (Bakke and Dobberstein 1990; Lotteau et al. 1990; Cresswell 1996), and to protect the empty MHC-II from binding endogenous antigen within the endoplasmic reticulum (Roche and Cresswell 1990; Teyton et al. 1990; Cresswell 1994; Cresswell 1996). The requirement of the Ii for the transport of MHC complexes can be demonstrated when unfolded MHC αβ dimers are retained within the endoplasmic reticulum in the absence of Ii (Cresswell 1996).

Following exit from the endoplasmic reticulum and trans-Golgi network, the Ii-MHC-II complex is directed to the late endosomal/pre-lysosomal antigen processing compartments, which are positive for the lysosome-associated membrane protein-1 (LAMP-1) (Kornfeld and Mellman 1989; Guagliardi et al. 1990; Mellman et al. 1998). The first event that occurs within the endosomal pathway is cleavage of the Ii (Roche 1996). Firstly, Ii is cleaved by an aspartyl protease generating an N-terminal

fragment of 10kDa (p10). An internal Ii peptide remains bound to the dimers, termed class II-associated invariant chain peptides (CLIP) (Cresswell 1996). CLIP has two main functions: firstly to maintain the complex of Ii trimer and the αβ dimers (Bijlmakers et al. 1994; Ghosh et al. 1995). The second function is to prevent premature loading of peptides onto the class II dimer, especially peptides originating from endogenous antigen (Cresswell 1996). Via a lysosomal targeting sequence located in the cytoplasmic tail of Ii, the MHC-II:CLIP complex is retained in an intracellular location (Lipp and Dobberstein 1986; Bakke and Dobberstein 1990; Lotteau et al. 1990). Complete degradation of Ii is prevented by cystatin C (Pierre 1998), which inhibits the proteolytic activity of the cysteine protease cathepsin S, responsible for the breakdown of Ii (Riese et al. 1996; Villadangos et al. 1997).

However, following receipt of maturation signals the expression of cystatin C is downregulated (Pierre and Mellman 1998). This allows cathepsin S to be functional (Pierre and Mellman 1998), and when the MHC-II:CLIP complex reaches the appropriate compartment for antigen loading, CLIP is proteolysed by a combination of cathepsin S and acidic pH, thereby releasing the αβ dimers from the nonomer (Roche 1996; Mellman et al. 1998). Some MHC-II molecules bind CLIP very tightly, and an additional MHC-related accessory protein, (H2-M in mouse, or HLA-DM in human), plays an important role (Roche 1995; Denzin et al. 1996). H2-M binds to MHC-II and at low pH catalyses the release of CLIP from the class II peptide-binding groove (Denzin and Cresswell 1995; Sloan et al. 1995; Denzin et al. 1996). Confirmation that cathepsin S is accountable for Ii degradation was furnished by studies utilising leupeptide, a general protease inhibitor, or cathepsin S specific inhibitors, both of which resulted in an accumulation of MHC-II complexes within the cell (Blum and Cresswell 1988; Neefjes and Ploegh 1992; Riese et al. 1996). There are several important consequences of Ii cleavage. Firstly disassociation of CLIP ultimately allows for high-affinity peptides to associate with class II molecules (Mellman et al. 1998). Secondly the removal of the lysosomal targeting sequence, which mediates intracellular retention, permits transport of MHC-II:antigen complexes from lysosomal compartments to the cell membrane (Mellman et al. 1998).

It can be concluded that increased membrane MHC-II expression reflects several intracellular modifications. A major contributing factor is the activation of Cathepsin S, which in turn instigates the post-Golgi degradation of Ii and re-routing of MHC-II to the cell surface (Lipp and Dobberstein 1986; Bakke and Dobberstein 1990a; Lotteau et al. 1990). Secondly, during maturation antigen internalisation is downregulated, possibly due to the diminished expression of surface antigen uptake receptors. As a consequence, surface expression time is increased, thereby amplifying complex half-time and total expression of surface MHC-II complexes (Inaba et al. 2000).

Antigen processing and presentation is regulated at many levels. The effect of inflammatory stimuli on MHC-II:antigen complex expression by DC was demonstrated in the seminal work of Cella and colleagues (Cella et al. 1997a). It was shown that following exposure to maturation stimuli the synthesis of MHC-II:antigen complexes was increased as was the surface half-life. These findings were developed further by the study by Inaba *et al.* (2000). It was found that immature DC were efficient at uptake of hen egg lysosome (HEL) and sequestered the antigen within cytoplasmic vesicles (Inaba et al. 2000). However, the processed peptide was not loaded onto MHC-II until the cell had received a maturation stimulus, with vesicles remaining in a cytoplasmic location for several days until exposed to LPS (Inaba et al. 2000). As such, antigen presentation is not simply a consequence of antigen uptake alone. For maturing DC to load MHC-II with internalised antigen, uptake must be accompanied, or followed by, maturation stimuli. By only permitting complexing of antigen with intracellular MHC-II in the presence of maturation stimuli, ensures immunogenic TCR ligands are formed only under inflammatory, and not steady state, conditions (Pierre et al. 1997; Inaba et al. 2000). Studies by Mellman demonstrated that proteolytic activity is another candidate stage of regulation within the exogenous pathway. Antigen positive vesicles within immature DC were found to contain high levels cystatin C. By regulating the activity of cystatin C, and thereby cathepsin s, the cell has control over invariant chain proteolysis and thus antigen transport to the cell surface (Pierre et al. 1997; Steinman et al. 1999).

1.6.2 Endogenous Pathway

MHC-I, which is expressed on all nucleated cells, presents antigens derived from cytosolic proteins, including peptides from virally infected cells, intracellular bacteria and possibly cancer cells (Watts 2004). The first step in the processing of endogenous antigen is the tagging of protein for destruction by the protein ubiquitin (Yang et al. 1996; Ben-Neriah 2002). Multi-ubiquitinated proteins are then recognised by an intracellular organelle called the proteasome, where antigen is proteolysed into smaller peptide fragments (Ben-Neriah 2002). The majority of antigen is then degraded by cytosolic enzymes. However a few peptides are translocated across the lumen of the endoplasmic reticulum, mediated by the transporter-associated with antigen processing (TAP) (Androlewicz et al. 1994). Within the endoplasmic reticulum antigen binds to newly assembled MHC-I plus microglobulin complex (Motal et al. 1993; Prasanna and Nandi 2004). Binding of antigen stabilises the complex and enables transport to the cell membrane (Motal et al. 1993). The resulting MHC-I:peptide complex is then recognised by $CD8^+$ T cells, inducing antigen-specific cytotoxic effector responses (Huang et al. 1994).

1.6.3 Cross Presentation

The concept of cross-presentation was first proposed by Bevan and colleagues (Bevan 1976a; Bevan 1976b). It was noted that exposure to cells presenting foreign self-minor histocompatibility complex antigen induced $CD8^+$ rather $CD4^+$ T cell priming, as would normally be expected for foreign antigens. These results suggest that host DC had engulfed the cells expressing foreign histocompatibility complex and presented the degraded cells in association with MHC class I (Bevan 1976b; Bevan 1976a). Combined with more recent investigations, it is well accepted that DC possess the unique capacity to present not only intracellular but also extracellular antigen on class I molecules to naïve $CD8^+$ T cells (Brossart and Bevan 1997; Heath and Carbone 1999; Belz et al. 2002a). This occurs, for example, when a virally infected or cancer cell is engulfed by a professional APC and processed via the endogenous, rather than the typical exogenous pathway (Dhodapkar et al. 2002; Furumoto et al. 2004). This distinctive characteristic of APC is not seen in other nucleated cells (Huang et al. 1994; Kurts et al. 1996; Kurts et al. 1997).

Recent studies have proposed that the class of antigen determines the way in which antigen is engulfed for cross-presentation. Firstly microbial associated antigen or peptides from dead or dying cells are phagocytosed and cross-presented by DC (Albert et al. 1998a; Yrlid et al. 2000). In contrast to particulate antigens, concentrations of soluble antigen above physiological concentrations is necessary for cross-presentation following macropinocytosis (Rock et al. 1990; Norbury et al. 1997). Following internalisation by DC, antigen is transferred from endocytic compartments to the cytosol, a feature not demonstrated in other cells (Rock et al. 1990; Norbury et al. 1997; Rodriguez et al. 1999). Within the cytosol, the proteosome degrades the antigen and subsequent peptides are transported across the ER lumen or alternatively into the lumen of the phagosome by TAP. It is here that MHC-I is loaded with the exogenous peptides and transported to the cell surface (Guermonprez et al. 2002; Ackerman et al. 2003; Guermonprez et al. 2003; Houde et al. 2003).

In a similar fashion to the MHC-II processing pathway, cross-presentation is stringently regulated during DC maturation (Gil-Torregrosa et al. 2004). Gil-Torregrosa and colleagues (2004) analysed an array of molecular mechanisms involved in the regulation of FcR-mediated cross-presentation during DC maturation (Gil-Torregrosa et al. 2004). It was demonstrated that cross-presentation of immune complexes by DC was upregulated following LPS treatment. However, this upregulation was short lived, diminishing after several hours as maturation continued (Gil-Torregrosa et al. 2004). The upregulation of cross-presentation in intermediate DC was proposed to involve two mechanisms. Firstly, an increase in endocytosis and transport of ingested antigens into the cytosol. Secondly, and to a lesser extent, an enhanced activity of the proteosome and TAP (Gil-Torregrosa et al. 2004). When fully mature, the cross-presentation pathway in DC is down regulated, possibly as a consequence of a cessation in antigen uptake (Gil-Torregrosa et al. 2004). It can be concluded therefore that cross-presentation is effective for only a short duration and is terminated in fully mature DC. Despite the down regulation of cross-presentation, MHC-I presentation of endogenous antigen remains fully competent. By regulating the presentation of cytosolic antigen, DC can effectively control the presentation of MHC-I restricted presentation of endogenous and exogenous antigens (Gil-Torregrosa et al. 2004). Via cross-presentation LC are equipped to present

exogenous antigen via MHC-I and MHC-II for effective anti-tumour and anti-pathogen responses respectively (Brossart and Bevan 1997).

1.6.4 Extracellular antigen processing

Recent investigations have indicated a significant proportion of DC surface MHC-II is expressed on the plasma membrane in an empty, peptide-receptive formation (Santambrogio et al. 1999). Via expression of HLA-DM on the membrane and exocytosis of proteases, immature DC are equipped with a means by which to process and present antigen in the extracellular space (Carven et al. 2000; Santambrogio et al. 2000a; Santambrogio et al. 2000b). Following ingestion of antigen it is sequestered in endocytic vesicles prior to receipt of maturation stimuli (Turley et al. 2000). As such, for the survival of ingested antigen, it must be able to ensure endocytic conditions prior to loading onto intracellular MHC-II. Labile antigens cannot be retained in an extremely proteolytic endosomal environment (Santambrogio et al. 2000a). Extracellular processing enables such antigens to be presented. Membrane-associated empty class II could also function as an antigen uptake receptor, collecting non-binding peptide that is not internalised by fluid phase uptake (Santambrogio et al. 2000a).

Two hypotheses have been proposed to explain how empty MHC-II is expressed on the DC surface. The first hypothesis states that MHC-II-Ii complexes traffic to the cell surface before fusion with endosomal loading vesicles (Santambrogio et al. 1999). As HLA-DM is found on the plasma membrane, Ii can be degraded and exchanged for extracellular antigen cleaved by secreted proteases. Due to the targeting sequence within Ii, any MHC-II still in association with Ii are endocytosed and trafficked to endosomal compartments. The second hypothesis suggests that empty class II is derived from class II molecules that have not associated with Ii in the endoplasmic reticulum (Stern et al. 2000). By possessing an alternative antigen-processing pathway, such as that described by Santambrogio L et al., DC are able to present a broader array of antigens to naïve T cells (Santambrogio et al. 2000a).

1.7 Langerhans Cell Migration

Naïve T cells do not migrate into peripheral tissue under normal physiological conditions (Alferink et al. 1998; Banchereau and Steinman 1998). Consequently, for

T cells to be activated by antigen, the antigen must be presented by APC in regional LN (Banchereau and Steinman 1998). LC migration has been observed using skin sensitisation models (Enk and Katz 1992a). Following topical application of contact allergens, LC migrate from the epidermis with a subsequent accumulation of antigen-bearing LC in the draining LN (Enk and Katz 1992a). In order to migrate following exposure to antigen, LC must possess the capacity to down-regulate their attachment to the epidermal network and cross the basement membrane (Cumberbatch and Kimber 1992).

LC mobilisation is triggered following exposure to inflammatory stimuli, including the mandatory cytokines TNF-α and IL-1β, and bacterial products such as LPS (Chang et al. 1994; Jakob and Udey 1998). These mediators promote LC competence for migration by inducing specific changes in chemokine receptor expression, LC responsiveness and loss of adhesion to the epidermal network (Cumberbatch et al. 2000).

1.7.1 Tumour Necrosis Factor-α and Interleukin 1-β

Tumour necrosis factor (TNF)-α is secreted by KC and synthesis is rapidly up-regulated following exposure to antigen, LPS and CD40 ligation (Kock et al. 1990; Enk and Katz 1992a; Roake et al. 1995; Moodycliffe et al. 2000). Seminal studies by Cumberbatch and colleagues indicated the critical influence of TNF-α on LC migration (Cumberbatch et al. 1994). Following intradermal exposure to TNF-α LC density was significantly reduced, with a subsequent increase in LN-resident LC (Enk and Katz 1992a; Cumberbatch et al. 1994). Moreover, investigations by Enk and Katz demonstrated that intraperitoneal injection with neutralising anti-TNF-α antibody prior to skin sensitisation with the potent contact allergen oxazolone resulted in substantial inhibition of allergen-induced DC accumulation in the draining LN and a less efficient acquisition of contact sensitisation (Enk and Katz 1992a).

Although TNF-α is essential for LC migration, for effective mobilisation LC require signalling from IL-1β (Enk et al. 1993a). Analogous to TNF-α, intradermal injection with recombinant IL-1β induces LC depletion from the epidermis (Enk et al. 1993b).

In addition, if the availability of this cytokine is compromised by systemic administration of neutralising antibody, migration, and subsequent contact sensitisation, is dramatically reduced (Cumberbatch et al. 1997). In summary, it is believed IL-1β and TNF-α act synergistically to stimulate the dissociation of LC from the epidermal network and migration to the regional LN (Cumberbatch et al. 1997). Firstly, IL-1β stimulates the production of TNF-α by KC. TNF-α and IL-1β then stimulate LC through cytokine specific receptors to down-regulate attachment to neighbouring KC and enable mobilisation (Cumberbatch et al. 1997).

Within the skin are homeostatic mechanisms that guarantee the migratory stimuli provided to LC are stringently regulated. For example, it has been suggested that secretion of IL-10 within the epidermis may inhibit LC migration secondary to an inhibition of TNF-α production and that IL-4 may negatively regulate TNF-α receptor expression (de Waal Malefyt et al. 1991a; de Waal Malefyt et al. 1991b; Hsu et al. 1992; Kutsch et al. 1993). Furthermore, the signal transduction pathways that are activated following IL-1β receptor binding may be controlled by receptor antagonists (Kutsch et al. 1993).

1.7.2 Modulation of Adhesion Molecule Expression

Adhesion molecule interactions, mediated by intracellular adhesion molecules and integrins, are involved in preserving the integrity of the epidermal network (De Panfilis et al. 1991; Tang et al. 1993). However E-cadherin has been postulated to be critical in retaining LC within the epidermis (Jakob et al. 1997). E-cadherin is expressed by LC and KC, mediating the retention of LC within the epidermis (Jakob et al. 1997). Following exposure to pro-inflammatory cytokines, such as TNF-α, E-cadherin expression is down regulated (Jakob and Udey 1998; Jakob et al. 2001). LC are able to dissociate from neighbouring KC and cross the basement membrane via a reduction in E-cadherin mediated adhesion (Jakob and Udey 1998). When resident in the regional LN, epidermal derived LC express little or no E-cadherin (Schwarzenberger and Udey 1996).

Following dissociation from KC, LC migrate through the dermal matrix to the draining lymphatics (Cumberbatch et al. 2000; Romani et al. 2001). This trafficking

requires crossing the basement membrane at the interface between the epidermis and dermis (Weinlich et al. 1998). Interaction with the basement membrane is facilitated by expression of the very late antigen 6 (VLA-6), a membrane complex of α6 and β1 integrins conferring to LC laminin-binding properties (Price et al. 1997). In order to pass through the interface the membrane must be proteolysed. Via possession of the collagenase matrix metalloproteinase-9 (MMP-9) LC are able to overcome the obstacle of the basement matrix and pass through into underlying dermal tissue (Kobayashi et al. 1999; Ratzinger et al. 2002).

1.7.3 Chemokine receptors and ligands

Once dissociated from the epidermal network LC migrate via the lymphatics to the draining LN. The migration of LC is aided by secretion of chemokines, a superfamily of small cytokines which, when secreted, can direct the movement, or chemotaxis, of cells along a concentration gradient (Adams and Lloyd 1997).

It has been suggested that chemokine receptor binding plays a significant role in maintaining LC within the epidermis (Boismenu et al. 1996; Wang et al. 1999; Jakob et al. 2001). With this is in mind, LC migration out of epidermis following receipt of a maturation stimulus must involve down regulation of such receptors, so as not to impede migration from the skin. Aside from altering adhesion molecule expression, cytokines also influence the expression of chemokine receptors and in doing so modulate LC responsiveness (Sallusto et al. 1999). Such receptors include CCR1 and CCR5, with the expression of both becoming less apparent with maturation (Lin et al. 1998; Sallusto et al. 1999)

In order for LC to enter lymphatic vessels, and therefore home to the draining LN, they must up-regulate their expression of various chemokine receptors, most importantly CCR7, and to a lesser extent CCR2 (Lin et al. 1998). It has been shown that following exposure to pro-inflammatory cytokines, LC up-regulate CCR7, enabling responsiveness to the receptor ligands (Dieu et al. 1998). An example of a CCR7 ligand is macrophage inflammatory protein (MIP) 3-β (CCL19), secreted by cells within the node and directs LC localisation to the correct region for interaction with naïve T cells (Boismenu et al. 1996; Dieu et al. 1998; Lin et al. 1998; Sallusto et al. 1999). An additional CCR7 ligand is secondary lymphoid organ chemokine

(SLC/CCL21), expressed constitutively on stromal cells and high endothelial venules in secondary lymphoid organs and endothelium of afferent lymphatics (Luther et al. 2000). The results of Saeki and colleagues have proposed a key role of SLC and CCR7 in the directed movement of DC from peripheral sites to draining LN (Saeki et al. 1999a; Saeki et al. 1999b). Firstly, using confocal microscopy of dual labelled murine dermis, this group were able to demonstrate MHC-II$^+$ cells within SLC$^+$ dermal lymphatic vessels. Secondly using skin organ culture, SLC was shown to increase by 2.5-fold the emigration of MHC-II$^+$ DC compared to control explants. The capacity of mature DC to respond to SLC was demonstrated by real-time PCR analysis of LC, which demonstrated mature, but not resting, LC expressed CCR7. Finally addition of neutralising anti-SLC antibody significantly inhibited the *in vivo* migration of DC from footpad explants, suggesting the potency of SLC as a chemoattractant for mature DC (Saeki et al. 1999a; Saeki et al. 1999b). Combined these results established the key role of CCR7 and the corresponding ligand SLC in the directed migration of mature peripheral DC via draining lymphatics to regional LN (Saeki et al. 1999a; Saeki et al. 1999b). In concert with MIP-3α, these chemokines enhance the likelihood of T cell activation, by inducing the migration DC to T cell rich areas of regional LN.

1.8 Two Signal Model of T Cell Activation

Seminal findings of Lafferty and Cunningham demonstrated that in addition to recognition of antigen presented in association with MHC (Kosugi et al. 1987; Cruz and Bergstresser 1990; Kalergis 2003), naïve T cells require an APC-derived signal for activation, namely the costimulatory signal (Bretscher and Cohn 1970). The two-signal theory of T cell activation was later expanded by Schwartz, Matzinger *et al.* and Jenkins (Schwartz 1990; Jenkins et al. 1991; Schwartz 1992; Matzinger 1994). Provision of signal one alone is insufficient to stimulate T cell activation. Significantly, antigen presentation in the absence of signal 2 can lead to unresponsiveness of the naïve T cell (Gimmi et al. 1993). Signal two is provided by ligation of costimulatory molecules, such as CD40, CD80, CD86 and ICOSL, with complimentary ligands on the surface of the T cell, being CD40L, CD28 and ICOS respectively (Bernard and Bernard 1999; Bocko et al. 2002). Provision of costimulation prevents anergy and promotes the full activation response (Jenkins et

al. 1991; Boussiotis et al. 1993; Ramarathinam et al. 1994; Cerdan et al. 1995; Pericle et al. 1997). Interestingly, activated memory T cells can receive signal two from any cell, not just professional APC (Zinkernagel 2000). However both effector and memory T cells can be rendered anergic if antigen is presented again in the absence of signal two (Zinkernagel 2000). When LC are freshly isolated from the epidermis they express relatively low levels of MHC-II and limited amounts of CD80 and CD86 on the cell membrane (Yokozeki et al. 1996). As these cells mature in culture (paralleling migrating LC *in vivo*) the expression of CD80 and CD86 becomes more apparent corresponding with acquisition of APC function (Weinlich et al. 1998; Aiba et al. 2000).

1.9 Outcome of T Cell Activation

Within two days of antigen presentation, T lymphocytes begin to proliferate, resulting in antigen-specific clones. On recognition of antigenic peptide presented by APC the resulting effector T cell responds in ways that serve to eradicate the infection. Exogenous antigen-specific CD4 cells and endogenous-specific CD8 cells perform separate functions, and their patterns of differentiation are distinct (Mosmann et al. 1986; Mosmann and Coffman 1989; Mosmann and Sad 1996).

1.9.1 CD 4 cells

In 1986, Mossman and colleagues identified two discrete subgroups of CD4 T cells, T helper I (Th1) and T helper 2 (Th2) (Mosmann et al. 1986). The two subsets of clones are classified on the basis of functional characteristics and cytokine secretion (Kim et al. 1985; Mosmann et al. 1986; Stout and Bottomly 1989). CD4 helper cells play a central role in immune regulation as the two panels of cytokines secreted by these subpopulations drive two distinct immune reactions, allowing a specific response against a range of possible antigens (Mosmann et al. 1986; Mosmann and Coffman 1989; Mosmann and Sad 1996). In many situations the progression of disease parallels the balance of Th1 cytokines, which are pro-inflammatory, and non-inflammatory Th2-type cytokines (Kuchroo et al. 1995a; Kuchroo et al. 1995b; Krenger et al. 1996a; Krenger et al. 1996b). Differentiation to either subset is decided during T cell priming, and although many factors influence development, the most discussed is the impact of the cytokine milieu (Paul and Seder 1994; Seder et al. 1994). The key cytokines for the differentiation of Th1 cells are APC secreted IL-

12 and IFN-γ (Swain et al. 1990; Seder et al. 1993), whilst IL-4 has the greatest influence on Th2 differentiation (Swain et al. 1990). A third subset of T helper cell, Th0 has also been identified which secretes a mixture of the two cytokine patterns. Given the broad array of possible cytokines that can be secreted by a T cell it is impossible for there to be a complete dichotomy of Th1 and Th2 cells. Rather it is now well accepted that a gradient exists in the presence of Th1 to Th2 cells (Paliard et al. 1988; Firestein et al. 1989).

1.9.1.1 CD4 T Helper 1 cells

The principle function of Th1 cells is to promote cell-mediated inflammation (Mosmann and Coffman 1989; Romagnani 1992a). One of the potent pro-inflammatory cytokines secreted by Th1 cells is IFN-γ, named due to its interference with viral infection (Cher and Mosmann 1987; Abbas et al. 1991; Abbas et al. 1996). IFN-γ is a potent activator of macrophages and stimulates the production of antibody-isotypes that promote the phagocytosis of microbes (Adams et al. 1986; Reed 1988; Finkelman et al. 1991; Wira et al. 1991; Fanger et al. 1996). These antibodies can either bind directly to Fc receptors on phagocytes or activate complement, generating opsonising products (Koppenheffer 1987; Cutler et al. 1991; Fanger et al. 1996; Devaux et al. 2004). IFN-γ also amplifies the immune response by heightening the potency of APC and the resistance to immune suppression (Gaczynska et al. 1993; Luft et al. 2002). This is demonstrated by the up-regulation of MHC-II and costimulatory molecule expression on APC, and limited proliferation of Th2 in the presence of IFN-γ (Fiorentino et al. 1991; Gaczynska et al. 1993; Noble et al. 1993; Bradley et al. 1995; Lutz et al. 1996a; Luft et al. 2002).

1.9.1.2 CD4 T Helper 2 Cells

Th2 cells are primarily involved in humoral immune responses, providing stimulation to B cells for T-dependent antigen responses (Mosmann and Coffman 1989; Abbas et al. 1996). Th2 cells secrete a particular set of cytokines including IL-4 and IL-5, and whose principle functions are to firstly stimulate IgE production and eosinophil/mast cell-mediated immune reactions and secondly, to down-regulate Th1 responses via production of IL-10 (Mosmann et al. 1986; Haas et al. 1992; Oswald et al. 1992; Romagnani 1992b; Noble et al. 1993; Gajewski et al. 1994; Schwarz et al. 1994; Ullrich 1994).

1.9.2 CD8 cells

CD8 positive T cells are otherwise known as cytotoxic or cytolytic T cells (CTL). Their major effector function is to recognise and kill host cells infected with viruses or other intracellular microbes (Rivas et al. 1989; Gelfanov et al. 1996; Albert et al. 1998b). Infected cells present the intracellular antigen on MHC-I, which is then recognised via the TCR (Albert et al. 1998b). CTL killing of infected cells involves the release of cytoplasmic granules whose contents include membrane pore-forming proteins and enzymes that cause DNA fragmentation and apoptosis (Kagi et al. 1994; Gelfanov et al. 1996).

1.10 Tolerance

A lesser-characterised role of the host immune system is the maintenance of tolerance to self-antigen (Adkins et al. 2004a). Self-tolerance is initiated primarily in the thymus, the organ of T cell development, and is termed central tolerance (Jones et al. 1990; Sprent and Kishimoto 2002). Central tolerance involves the programmed differentiation of immature thymocytes leading to functional, non-self reactive T cells (Jones et al. 1990; Sprent and Kishimoto 2002). During maturation, developing T cells are subject to two selection processes (Jones et al. 1990; Sprent and Kishimoto 2002). T cells fit to recognise foreign antigen presented in the context of MHC are given the signal to survive and undergo further differentiation (positive selection) (Kisielow and von Boehmer 1990; von Boehmer and Kisielow 1990). TCR that do not recognise MHC:foreign antigen complexes are deleted through programmed cell death (Jones et al 1990; vonBoehmer et al. 1990). Secondly, T cells with high affinity for self-antigen are terminated (negative selection) (Sprent and Kishimoto 2002). The clonal deletion of inappropriate T cells through central tolerance is critical for the understanding of self and the avoidance of deleterious autoimmune responses (Jones et al. 1990).

Although central tolerance is stringently regulated, some self-reactive cells do circumvent negative selection within the thymus (Jones et al. 1990). There are two scenarios that result in avoidance of central tolerance. Firstly, T cells that demonstrate low affinity for self-antigen may escape deletion within the thymus, and enter the periphery (Fazekas de St. Groth 2001). Secondly, some self-antigens may

not be expressed in the thymus or secreted in serum and thus incapable of gaining access to the thymus for presentation. Examples of such antigens include harmless environmental antigens and apoptotic debris generated during normal tissue turnover. It is essential that hyper-responsiveness to these antigens is avoided (Fazekas de St. Groth 2001).

It is well documented that DC are key inducers of pro-inflammatory immune responses (Banchereau and Steinman 1998; Banchereau et al. 2000). However, recent investigations have demonstrated the pivotal role DC play in peripheral tolerance under steady state conditions (Steinman et al. 2000). Verification of the tolerising effect of DC came from experiments analysing the outcome of T cells presented with peripheral self-antigen (Steinman et al. 2000). The results demonstrated that under defined circumstances, DC trafficked peripheral self-antigen to the draining LN and activated antigen-specific T cell tolerance (Steinman et al. 2000). What remained unclear however was how DC selected between induction of immunity or tolerance. It has since been proposed that the fate of mature T cells is controlled under normal physiological conditions by the circumstances under which they recognise antigen-MHC and the maturation status of the APC (Schuler and Steinman 1985; Dewar et al. 2001; Woods et al. 2001; Gad et al. 2003; Simpson et al. 2003). Mechanisms that avert self-reactive peripheral T cells inducing autoimmune reactions are termed peripheral tolerance (Walker and Abbas 2002). These mechanisms fall into three mutually exclusive groups: maintenance of immaturity (failure to respond to antigen or presentation of antigen below a measurable response), deletion following antigen presentation and survival/memory responses, which involves T cell deviation and long-term survival following interaction with antigen (Fazekas de St. Groth 2001).It is possible that all three mechanisms can operative simultaneously for a single antigen (Fazekas de St. Groth 2001).This is demonstrated in the phenomenon of antigen dose (Zinkernagel 2000). Firstly, antigen presented in the LN in minimal doses is ignored by the naïve T cell. In contrast, antigen already expressed in the LN, or if expressed in high amounts for long time periods, results in T cell deletion (Zinkernagel 2000). For the purpose of this literature review maintenance of naivety, anti-idiotypic networks and the role of DC in peripheral tolerance in regard to the induction of anergy, regulatory T cells, apoptosis and T cell deviation will be discussed in depth.

1.10.1 Maintenance of a naïve status.

Two mechanisms have been hypothesised to maintain T cells in a naïve phenotype following presentation of antigen to the TCR. Firstly T cells that have low avidity or affinity for self-antigen escape central tolerance, remaining naïve after leaving the thymus (Liu et al. 1995). When self-antigen is presented to the low affinity self-reactive TCR, the cell does not respond and fails to differentiate in an antigen-specific manner (Anderson et al. 1994a; Anderson et al. 1994b; Heath et al. 1998; Girgis et al. 1999; Anderson et al. 2000). In doing so, anti-self responses are not generated (Fazekas de St. Groth 2001). Secondly, peripheral antigen may remain in the tissues, sequestered in a site away from secondary lymphoid organs such as the spleen and LN (Barker and Billingham 1977). If such antigens are incapable of reaching the thymus, even T cells with high affinity for self antigen will remain unresponsive and naïve (Fazekas de St. Groth 2001). A prime example of such an antigen is transgenic antigen expressed by insulin-secreting cells in the pancreas (Ohashi et al. 1991). Peripheral T cells cannot gain access to such tissue, and only become primed to the pancreatic antigen once inflammation is induced (Ohashi et al. 1991). During inflammation local APC transport the self-antigen from the pancreas to the draining LN for presentation to naïve T cells (Ohashi et al. 1991).

1.10.2 Idiotypes and anti-idiotypes

During an immune response to foreign antigen, autoantibodies are generated simultaneously to anti-antigen antibodies (Borghesi and Nicoletti 1996). These autoantibodies can then react to the variable region (idiotype) of the antigen-specific antibody (Borghesi and Nicoletti 1996). As a consequence these autoantibodies must be regulated to avoid anti-self reactions (Borghesi and Nicoletti 1996). The regulation of the idiotypic sequences in immunoglobins, as well as the variable region of the TCR (which in itself is self antigen) (Shoenfeld 1989-90), is involved in both central and peripheral tolerance induction (Durkin and Waksman 2001). Although can be maintained, the precise mechanism is yet to be defined. It has postulated to involve regulatory T cells that interact through idiotypic receptors on the surface of self-reactive T and B cells, leading to their deletion or suppression (Shoenfeld 1989-90). This hypothesis is supported by several studies. Firstly, following exposure to anti-idiotypic antibodies to staphylococcal nuclease, nude

mice were unable to synthesise idiotype-bearing immunoglobin. In contrast, in immune competent mice helper T cells were lysed by complement or anti-idiotype (Miller et al. 1981). In another study, the lysis of idiotype-bearing T suppressor cells by anti-idiotype and complement was also evident (Sercarz and Metzger 1980).

The idiotype network first proposed by Jerne (1974) has been demonstrated to be involved in the regulation of various immune reactions (Jerne 1974b; Jerne 1974a; Jerne 1974-75). Such reactions include B cell tolerance (Brown and Bhoghal 1975), tolerance during ageing (Szewczuk and Campbell 1980), rejection suppression in pregnancy (Suciu-Foca et al. 1985) as well as playing a critical role in various immune disorders.

1.10.3 T cell anergy

For effector T cell activation and differentiation two signals must be provided by the APC (Lafferty and Cunningham 1975; Jenkins et al. 1991). The first signal is generated following ligation of the TCR with MHC:antigen complexes on the APC surface (Kosugi et al. 1987; Cruz and Bergstresser 1990; Kalergis 2003). Signal two is provided by binding of costimulatory molecules, namely CD80 and CD86, with the corresponding T cell receptors (Lafferty and Cunningham 1975; Jenkins et al. 1991). Presentation of antigen alone renders the T cell anergic, refractory to a later encounter with the same antigen, even if costimulation is provided (Burkly et al. 1989; Mueller et al. 1989; Clark et al. 1999). This failure to provide the second signal is brought about by DC immaturity, diminished expression of costimulatory molecules, or the cytokine milieu present during DC:T cell interaction, namely the immunosuppressive cytokines IL-10 and TGF-β (Gad et al. 2003). The influence of cytokines has been demonstrated *in vitro*, where T cells were cultured with supernatants collected from IL-10 treated human DC (Steinbrink et al. 1997b; Steinbrink et al. 1998; Steinbrink et al. 1999; Brossart et al. 2000; Faulkner et al. 2000b). Following exposure there was a significant reduction in the secretion of pro-inflammatory cytokines, such as IL-1β and TNF-α, and a complete absence of IL-12 (Steinbrink et al. 1997; Steinbrink et al. 1998; Steinbrink et al. 1999; Brossart et al. 2000; Faulkner et al. 2000). Following exposure to IL-10, DC are not only inefficient stimulators of Th1 responses but prompt antigen-specific tolerance

through the induction of T cell anergy (Enk et al. 1993c; Enk et al. 1994; Steinbrink et al. 1998; Steinbrink et al. 1999). As opposed to immature DC, mature DC are not responsive to the inhibitory effects of IL-10 (Allavena et al. 1998; Steinbrink et al. 1999; Cavani et al. 2000). As such the function of DC, exposed to immune suppressive cytokines such as IL-10, is modulated such that there is diminished T cell response, characterised by reduced antigen-specific activation and reduced secretion of pro-inflammatory cytokines (Steinbrink et al. 1997; Steinbrink et al. 1999).

1.10.4 T regulatory cells

In some cases auto-reactive T cells are not ignorant or rendered anergic to self-antigen. Such cells can be prevented from reacting to self-antigen by the presence of other T cells, namely suppressor or regulatory T cells (Sakaguchi et al. 1994; Mason and Powrie 1998). In a similar fashion to anergic T cells, the principle inducers of regulatory T cell proliferation and differentiation is immature DC (Shevach 2000). However, semi-mature DC, those that have not fully responded to inflammatory signals, can also activate regulatory T cells (Gad et al. 2003). The mechanism by which DC induce the activation of regulatory and suppressor T cell populations is obscure (Gad et al. 2003), however it has been proposed to be as crucial as anergy in the maintenance of tolerance (Thompson and Thomas 2002; Bilsborough et al. 2003). One subset of $CD4^+$ regulatory T cells, $CD4^+CD25^+$, has been demonstrated to be potent inhibitors of $CD4^+$ and $CD8^+$ T cell activation via an antigen non-specific mechanism (Powrie et al. 1993; Jonuleit et al. 2000; Read et al. 2000; Salomon et al. 2000; Hara et al. 2001). A characteristic of these cells is that they are anergic and inhibit the secretion of IL-2, critical to T cell activation(Gad et al. 2003). Addition of IL-2 breaks the anergic state and suppression induced by the T regulatory cell, highlighting the importance of the cytokine milieu (Takahashi et al. 1998; Thornton and Shevach 1998; Read et al. 2000; Strober et al. 2002). DC possess the exclusive ability to stimulate specific regulatory T cells that protect peripheral tissue from potential autoimmune reactions (Gad et al. 2003). Via the secretion of various cytokines and inhibitory costimulatory molecules, regulatory T cells can further modulate DC function, to either regulate activated T cells or prime new regulatory T cells (Thompson and Thomas 2002).

1.10.5 T cell deletion

Under specific conditions DC are capable of inducing apoptosis, leading to the deletion of self-reactive T cells (Ferguson et al. 2003). One mechanism employed by DC to induce apoptosis is ligation of the death ligand FasL (Singer et al. 1994; Crispe 1994; Nagata and Golstein 1995). It has been demonstrated that FasL-expressing murine spleen DC induce apoptosis in activated $CD4^+$ T cells following binding of Fas on the T cell surface (Suss and Shortman 1996). An additional ligand is TNF-related apoptosis-inducing ligand, TRAIL, whereby expression translates to an ability to kill TRAIL-sensitive targets, including activated T cells (Fanger et al. 1999). TRAIL has been demonstrated to be critical in the induction of tolerance, especially in immune privileged sites (Lee et al. 2002). In the eye anti-self responses, which may be tolerated in larger organs such as the skin, are detrimental to the retina. As such tolerance has become the default pathway, through expression of TRAIL on ocular tissue. Following binding to TRAIL, incoming lymphoid cells are deleted via apoptosis (Lee et al. 2002). In contrast to effector T cells, which are prone to FasL-mediated apoptosis, regulatory T cells appear to be resistant (Banz et al. 2002). Thus in terminating a T cell response, DC and regulatory T cells can function simultaneously (Gad et al. 2003).

Interestingly, the apoptotic T cell inadvertently plays an active role in the maintenance of tolerance (Griffith et al. 1996). It was postulated that within dying cells were molecules capable of initiating tolerance. It was observed that following Fas-mediated apoptosis of T cells was a rapid induction of IL-10 secretion by these cells. Antigen presenting cells that had internalised the dying IL-10-secreting T cells, deviating the outcome of antigen presentation away from Th1 responses and towards Th2 tolerance inducing responses. This response was not observed in IL-$10^{-/-}$ mice, demonstrating the dependence on Il-10 (Gao et al. 1998).

1.10.6 Immune deviation

Immune deviation involves directing the immune response away from the generation of potentially auto-reactive Th1 cell-mediated responses and towards humoral, potentially suppressive Th2-type responses. The ability of DC to deviate the immune response is influenced by the nature of the stimulating antigen and the local microenvironment (Langenkamp et al. 2000; Manickasingham et al. 2003). An

example of the latter is the immunosuppressive cytokine IL-10 (Corinti et al. 2001). When exposed to IL-10 in the periphery, DC migrate to the draining LN in an immature state, associated with a diminished expression of costimulatory molecules and reduced capacity for IL-12 synthesis (Xia and Kao 2003). The level of IL-12 has a direct effect on Th2 polarisation, with diminished IL-12 secretion at the DC:T cell interface resulting in a skewing in the response towards Th2 (Gately et al. 1998). The importance of IL-10 in immune deviation was further supported by studies demonstrating that treatment with neutralising anti-IL-10 antibodies resulted in a pronounced increase in TNF-α and IL-12 secretion and restored the immune response towards Th1 (Corinti et al. 2001). Interestingly, it has also been reported that exposure to antigen during the early neonatal period leads to a bias towards Th2 cells at a second encounter with the same antigen (Adkins et al. 2001). Although self-reactive T cells survive peripheral tolerance during the neonatal period, the memory inherent in T cell deviation ensures that if the same self-antigen is encountered when the animal reaches maturity then self-reactive immune responses will be avoided (Adkins et al. 2001).

1.11 Immune Function During the Early Neonatal Period

Seminal studies by Medawar and colleagues identified the early neonatal period as critical for distinguishing self. If a neonatal rat was injected with allogenic haematopoietic cells, it was be able to successfully accept a tissue transplant from the same donor as an adult (Billingham et al. 1953; Billingham and Medawar 1953; Banz et al. 2002). These findings combined with more recent studies, suggest that neonatal animals are more susceptible to tolerisation (Nossal 1983; Waite et al. 1988; Adorini 1990; Ernst et al. 1993). The precise mechanisms for immune tolerance are unknown but investigations to date have primarily focused on lymphocytes of the neonatal immune system and their functional and phenotypical differences compared to adult lymphocytes (Adkins and Hamilton 1992; Adkins et al. 1993). Two theories for neonatal tolerance were originally proposed. The first theory suggests a passive acquisition of tolerance via negative selection of antigen reactive T cells within the thymus, in a similar fashion as natural self-tolerance (Gammon et al. 1986a; Gammon et al. 1986b; Sercarz et al. 1989; Gammon et al. 1991). Active models propose that mainly suppressive, deviated and anti-idiotypic Th2 immune responses

are generated (Bellgrau et al. 1981; Roser 1989). T cells from both human cord blood and neonatal mice proliferate poorly in response to antigenic stimulation and secrete reduced levels of pro-inflammatory cytokines such as IFN-γ and IL-12 (Petty and Hunt 1998; Dakic et al. 2004). In contrast to adult DC, a study by Langrish and co-workers demonstrated that following exposure to LPS, neonatal DC secrete Th2-promoting cytokines such as IL-10 and little or no IL-12 (Langrish et al. 2002). Rather than a consequence of either passive or active mechanisms, it is now accepted that both models act simultaneously to maintain tolerance induced during the early neonatal period. This is reflected in the tolerance response following presentation of varying doses of antigen (Zinkernagel 2000). While low amounts of antigen presented to the TCR resulted in immunological ignorance, presentation of high levels of antigen resulted in T cell deletion (Zinkernagel 2000). The memory inherent in immune deviation and suppression ensures that if the same antigen is presented again at a later stage, tolerance is maintained.

The recent observation that under certain conditions neonatal T cells can mount effective anti-pathogen responses has prompted several research groups to revisit the paradigm of neonatal tolerance induction. It suggested that as neonatal T cells possess the capacity to respond to antigen exposure, they are not the primary inducer of tolerance (Salio et al. 2003) . As such, studies are now focusing on APC and their capacity to elicit T cell activation through signals provided at the T cell:DC interface during the early neonatal period (Elbe-Burger et al. 2001; Chang-Rodriguez et al. 2004). Recent reports have suggested that activation of regulatory T cells, such as $CD4^+CD25^+$ T cells and γδ T cells is initiated during the interaction of naïve T cells and DC. This is of particular importance as γδ T cells play crucial roles in tumour tolerance, oral tolerance and tolerance induction in immune privilleged sites such as the testes (Mukasa et al. 1995; Seo and Egawa 1995; Ke et al. 1997; Skelsey et al. 2001). Studies utilising contact hypersensitivity as a model of cutaneous immune responses has demonstrated that the reduced capacity of neonatal mice to induce immunity to topically applied antigen was a consequence of inability of neonatal epidermal LC to provide the two signals essential for T cell activation, via a diminished expression of MHC-II and CD40-CD40L costimulatory pathway (Simpson et al. 2003).

The neonatal skin microenvironment is dramatically different in antigen composition compared to adult counterparts (Hoeger and Enzmann 2002). Due to a thickening of the neonatal epidermal barrier, the capacity of environmental antigens to access underlying tissues is less compared to adult counterparts. In contrast, epidermal maturation following birth results in increased cell turnover and death. As a result the relative concentration of self tissue and protein antigen is increased (Hoeger and Enzmann 2002). As hyper-responsiveness to self-tissue and harmless soluble antigen can lead to autoimmune responses and chronic inflammation respectively, a mechanism must be in place to prevent induction of inappropriate immune responses. Research from this department has demonstrated that during the early neonatal period, cutaneous antigen treatment resulted in a deviation in the T cell response from immunity to antigen specific immune suppression (Dewar et al. 2001). The number of LC migrating away from antigen-treated epidermis was reduced and the amount of antigen presented in the context of MHC-II was significantly less compared to adult counterparts (Dewar et al. 2001). Combined with the dramatically reduced expression of CD86, the capacity of neonatal LC to function as potent professional APC is compromised. It was proposed that due to a decreased expression of antigen uptake receptors on LC from neonatal mice that LC were inefficient at the internalisation of antigen via receptor-mediated endocytosis. (Dewar et al. 2001). As the epidermal LC network increased in maturity, the capacity to present processed peptide was acquired. By 20 days following birth LC exhibited morphology and phenotype consistent with adult counterparts and it was at this stage of development that antigen presentation elicited the activation of naïve T cells

During infancy the ability to induce tolerance is an invaluable mechanism to maintain non-reactivity to self-antigen, beneficial skin commensals and harmless protein antigens. Although it is clear that tolerance is a prerequisite to normal skin development the involvement of LC in this process is yet to be fully understood. The primary aim of this study was to characterise the antigen handling capacity of LC during the early developmental period. As the amount of antigen presented in the context of MHC-II can influence the outcome of T cell activation (Sallusto et al. 1995), the divergence in the pathways utilised by neonatal LC to internalise and

process antigen has important implications for the generation of immune suppression. By understanding the underlying mechanisms of cutaneous tolerance during the neonatal period it will be possible to gain more insight to immune function and to possibly utilise such knowledge for developing strategies for either enhancing the immune response for anti-pathogen or cancer protection or suppressing anti-self responses in autoimmune diseases.

Research Proposal

Antigen treatment of 4-day-old epidermis results in a decrease in the amount of antigen transported to the draining lymph node (LN) by epidermal Langerhans cells (LC). It was proposed that a decrease in receptor-mediated endocytosis was responsible for the reduced antigen transport to the LN. The central aim of this study will be to investigate the capacity of LC to handle antigen during the developmental period. To characterise the capacity of LC to internalise antigen during development the following criteria will be assessed:

1. To characterise the ontogeny of antigen uptake receptors during development.
2. To analyse the capacity of LC isolated from neonatal, juvenile and adult epidermis to internalise a variety of antigens via receptor-mediated and fluid phase uptake.
3. To assess antigen proteolysis and MHC-II complexing following exposure to maturation stimuli *in vitro.*

By studying the various aspects of antigen handling. I will be able to determine if the way in which antigen is internalised and presented dictates the direction of T cell activation.

Chapter 2
Materials and Methods

Table 2.1 Commonly used reagents and suppliers.

Reagent	Supplier	Catalogue Number
7-Aminoacetic acid (7AAD)	Sigma	A9400
Acetone	BDH Laboratory Supplies	52090201
Bovine serum albumin (BSA), cell culture grade	CSL Ltd.	52090201
Deoxyribonuclease 1 (DNase)	Invitrogen	18047-019
Dimethyl sulfoxide (DMSO)	Sigma	D-5879
Dispase	Invitrogen	17105-041
Di-sodium hydrogen orthophosphate Anhydrous (NaN_3HPO_4)	AnalaR	010249.0500
Ethylene diamine tetraacetic acid – disodium salt	Serva	39761.02
Fetal calf serum (FCS)	CSL	09702301
Fluorescein Isothiocyanate	Sigma	F-7250
Gentamicin	David Bull Laboratories	2724A
Glucose	Ajax Chemicals	713
L-Glutamine	CSL	09871901
Glycine	BDH (Merck)	0101109.0500
N-2-Hydroxyethylpiperaine-N'-2-ethanesulfonic acid (HEPES)	Sigma	49329
Hanks Balanced Salt Solution (HBSS)	Invitrogen	21250-014
L-lysine (poly-lysine)	Sigma	L-5501
Mannan from *Saccharomyces cerevisiae*	Sigma	M-7504
D(+)-Mannose	Sigma	M-6020
2-Mercaptoethanol (2-ME)	Sigma	M-6250

Paraformaldehyde	BDH	BH151TD
Permafluor	Coulter	0752
Roswell Park Memorial Institute 1640 media (RPMI 1460)	Invitrogen	21875-034
Saponin (Practical Grade)	ICN Biomedicals Inc.	102855.80
Sodium azide (NaN_3)	BDH	30111
Sodium chloride (NaCl)	BDH (Merck)	10241.AP
Sodium chloride anhydrous (NaCl)	Astral	0241
Di-Sodium hydrogen (ortho)phosphate (anhydrous, Na_2HPO_4)	Ajax Chemicals	621
Sodium dihydrogen (ortho0phophate hydrated ($NaH_2PO_4.2H_2O$)	AnalaR	4140
Sodium hydrogen carbonate ($NaHCO_3$)	BDH Chemicals	102247
Sodium Pyruvate	JHR Biosciences	59203-77P
Trypan Blue	Sigma	T-0776
Wortmannin from *Penicillium fumiculosum*	Sigma	W-1628

Table 2.2 Commonly used disposables and suppliers

Reagent	Supplier	Catalogue Number
Acrodisk® PF	Gelman Biosciences	4187
13mm round cover slips	ProSciTech	G402
Cell strainers, 40μm	BD Falcon	352340
15mL polystyrene tubes	Iwaki	2712-002
50mL polystyrene tubes	Iwaki	2340-050
Specimen containers	Techno-Plas	C5744UU
10mL syringes	Terumo	3SS-10ES
20mL syringes	Terumo	3SS-20ES

Table 2.3 Equipment

Equipment	Supplier	Model Number
Flow cytometer	Coulter	EPICS Elite ESP
DMLB2 fluorescent microscope	Leica	-
DM IRB inverted fluorescence microscope	Leica	-
Bench top minifuge	Eppendorf	5415D
Bench top centrifuge	Sorvall®	RT6000D

2.1 Reagents, disposables and equipment

Reagents, disposables and equipment are listed in Tables 2.1, 2.2 and 2.3 respectively. Antibodies for flow cytometry are listed in Table 2.4 and 2.5. Antigens and tracers for analysing *in vitro* antigen uptake and processing are listed in Table 2.6. Primary and secondary antibodies for analysing *in vitro* maturation are listed in Table 2.7.

2.2 Animals

Adult (6-12 weeks old), juvenile (7 and 14 days old) and neonatal (4 day old) male and female BALB/c ($H\text{-}2^d$) mice were obtained from the University of Tasmania Central Animal House. Animals were housed in the Discipline of Pathology animal rooms, and provided with food and water *ad libitum*. Animals were used in accordance with the University of Tasmania Ethics Committee; permit numbers 98013, 5519, A5620 and 6063.

2.3 Solutions

2.3.1 Phosphate Buffered Saline (PBS)

20x PBS was prepared as follows:

NaCl	170g
Na_2HPO_4	21.4g
$NaH_2PO_4.2H_2O$	7.8g

Salts were added to 900mL of Milli-Q® water (Millipore Corporation, USA) and dissolved using a magnetic stirrer. Once dissolved the volume was increased to 1L. Sterile PBS was prepared by filtering the solution through a 0.22μm filter (Millipore Corporation, USA). 20x PBS was diluted 1 in 20 with Milli-Q® water to give a working 1x solution. The solution was stored at room temperature

2.3.2 PBS / 5 %FCS / 0.1 % NaN_3

FCS	50mL
NaN_3	0.1g

FCS and NaN_3 were dissolved in 900mL of PBS using a magnetic stirrer. Once dissolved the volume was increased to 1L with PBS. The solution was stored at 4°C.

2.3.3 PBS / 5% FCS

FCS	50mL

FCS was added to 900mL of PBS and mixed using a magnetic stirrer. Once dissolved the volume was increased to 1L with PBS. The solution was stored at 4°C.

2.3.4 HBSS / 20mM HEPES

HBSS	I sachet (9.5g)
HEPES	4.77g
$NaHCO_3$	0.35g

HBSS (without $CaCl_2$, $MgCl_2$, $MgSO_4$, $NaHCO_3$) was reconstituted using 950mL of Milli-Q® water. HBSS was supplemented with HEPES and $NaCHO_3$ and dissolved using a magnetic stirrer. The pH was adjusted to 7.4 using 1M NaOH or HCl, and the volume increased to 1L using Milli-Q® water. The solution was stored at 4°C.

2.3.5 4% Paraformaldehyde

$NaH_2PO_4.2H_20$	1.56g
Na_2HPO_4 (anhydrous)	5.68g
Paraformaldehyde	20g

Salts were dissolved in 450mL of Milli-Q water using a magnetic stirrer. Paraformaldehyde was added and the solution stirred over gentle heat, without boiling, for 3 to 5 hours. The volume was increased to 500mL with Milli-Q® water. The solution was aliquoted into 50mL polystyrene tubes and stored at –20°C. Once thawed 4% paraformaldehyde was used within one week.

2.3.6 Saponin Buffer

NaN_3	0.10g
saponin	0.10g
EGTA	0.38g
BSA	2.00g

NaN_3, saponin and EGTA were dissolved in 900mL of PBS using a magnetic stirrer. BSA was then added and allowed to settle. Once dissolved the volume was increased to 1L with PBS. The solution was stored at 4°C

2.3.7 FACS Fixative

40% formaldehyde	1mL
D-glucose	10g
15 % NaN_3 in PBS	1.09mL

Formaldehyde, glucose and NaN_3 were dissolved in 450mL of PBS using a magnetic stirrer. Once dissolved the volume was increased to 500mL with PBS. The solution was stored at 4°C

2.3.8 7-amintoactinomycin D (7-AAD)

7AAD was reconstituted to 1 mg/mL with sterile water for injection. Working aliquots were stored at –20°C protected from light.

2.3.9 Trypan Blue

Trypan Blue	0.25g
Milli-Q	100mL

Trypan Blue powder was added to 100mL of Milli-Q water and sterilised through a 0.22μm filter. Aliquots were stored at 4°C.

2.4 Media

2.4.1 RPMI 1460-FCS

FCS	10%
L-glutamine	2mM
Sodium pyruvate	1mM
2-ME	5×10^{-5}M
Gentamicin	30mg/mL

RPMI was supplemented under sterile conditions and stored at 4°C.

2.4.2 IMDM-FCS

FCS	10%
L-glutamine	2mM
Sodium pyruvate	1mM
Gentamicin	30mg/mL

IMDM was supplemented under sterile conditions and stored at 4°C.

2.5 Isolation of Murine Langerhans Cells.

2.5.1 Preparation of epidermal cell suspensions

Adult and juvenile mice were sacrificed by carbon dioxide inhalation. Neonatal mice were anaesthetised by cooling at 4°C followed by decapitation. Fur was removed with clippers and depilatory cream (Veet: Reckitt and Colman). Skin was thoroughly washed with warm tap water, excised and cut into 1 cm^2 sections. Skin sections were incubated in HBSS/0.5% dispase/100U/mL DNase for 2 hours at 37°C,

with slight agitation. Adult and juvenile epidermis were mechanically separated from the dermis by scraping with a small spatula, or for neonatal epidermis, gently peeled with fine forceps. The resulting epidermal scrapings were incubated in HBSS/0.5% dispase/100U/mL DNase for 40 minutes at 37°C, with slight agitation. Following incubation an equal volume of complete RPMI 1460-FCS (supplemented with 10% foetal calf serum, Gentamicin, 2mM L-glutamate, 1mM sodium pyruvate and 5 x 10^{-5} M 2-mercaptoethanol) was added and swirled at room temperature for 5 minutes. To remove particulate matter, the epidermal preparation was filtered through cotton wool and a 40μm cell strainer and centrifuged twice at 400g for 5 minutes with RPMI 1460-FCS. After a final wash with RPMI 1460-FCS the pellet was resuspended in 1mL of RMPI 1640-FCS.

2.5.2 Preparation of spleen cell suspensions.

The spleen was removed from adult mice and placed in a small culture dish containing 5mL of RPMI 1640-FCS. The spleen was then cut with scissors and gently passed through a cell sieve using the plunger of a 10mL syringe. To lyse red blood cells the suspension was incubated for 10 minutes in 10mL/spleen of RBC lysis buffer. Following incubation, the suspension was washed at 800 g for 10 minutes at 4-10°C. Supernatant was aspirated and pellets resuspended in 5mL of RPMI. Cell suspensions were washed a further two times at 800 g, for 5 minutes at 4-10°C with 5mL of RPMI 1640-FCS.

2.6 Flow Cytometric Analysis of Epidermal Langerhans Cells Phenotype During the Early Neonatal Period.

Table 2.4 Primary anti-mouse antibodies and isotype-matched controls

Antibody	Isotype	Concentration (μg/5 x 10^5 cells	Supplier	Catalogue Number
Purified anti- B220	Rat $IgG_{2a,}$ κ	1	PharMingen	553084
Biotin anti- CD11b	Rat $IgG_{2b,}$ κ	1	PharMingen	553309

PE anti-CD11c	Hamster IgG_1, κ	1	PharMingen	553802
FITC anti-CD11c	Hamster IgG_1, κ	0.5	PharMingen	553801
Purified anti-CD14	Rat IgG_1, κ	0.5	PharMingen	553738
Biotin anti-CD18	Rat IgG_{2a}, κ	0.5	PharMingen	557439
Purified anti-CD32/CD16	Rat IgG_{2b}, κ	1	PharMingen	553142
Biotin anti-CD40	Rat IgG_{2a}, κ	0.0625	PharMingen	553789
Biotin anti-CD54	Hamster IgG_1, κ	1	PharMingen	553251
Biotin anti-CD80	Hamster IgG_2, κ	1	PharMingen	553767
Biotin anti-CD86	Rat IgG_{2a}, κ	1	PharMingen	553690
PE anti-I-A/I-E	Rat IgG_{2b}, κ	0.0125	PharMingen	557000
FITC anti-I-A/I-E	Rat IgG_{2a}, κ	1	PharMingen	553623
Biotin anti-B7RP-1 (ICOS-L)	Rat IgG_{2a}, κ	2	eBioscience	13-5985
Biotin anti-DEC-205	Rat IgG_{2a}, κ	1	Serotec	MCA949
Biotin anti-TLR4/MD-2,	Rat IgG_{2a}, κ	2	eBioscience	13-9924
Purified anti-Langerin	Rat IgG_{2a}, κ	1	Gift from Sem Saeland	Clone HD24
Rat IgG_{2a}, κ	-	1	PharMingen	553926
Rat IgG_{2b}, κ	-	1	PharMingen	553987
Hamster IgG_1, κ	-	1	PharMingen	553970

Table 2.5 Secondary fluorescent conjugated antibodies

Antibody	Isotype	Concentration (μg/5 x 10^5 cells	Supplier	Catalogue Number
Streptavidin-APC	-	0.05	PharMingen	554067
Streptavidin-FITC	-	1	PharMingen	554057
Streptavidin-PE	-	1	PharMingen	554061
Anti-rat-FITC	-	1	PharMingen	554016

2.6.1 Single colour staining of epidermal suspensions

Approximately 5 x 10^5 cells/mL were transferred to labelled eppendorf tubes. To inhibit binding of antibody to surface Fc receptors, cells were incubated with purified anti-mouse CD16/32 (FcγIII/II, Fc Block) for 30 minutes at 4°C (excluding those cells that will be stained with goat anti-rat secondary antibody). Following blocking, cells were incubated with primary antibody, as listed in Table 2.4, for 30 minutes at 4°C. Following incubation, cells were washed three times with 1mL of PBS/5% FCS/NaN_3 by pulse spinning at maximum speed for 10 seconds. The final pellet was resuspended in 500μL of PBS/5% FCS/0.1% NaN_3. Cells were incubated with the corresponding fluorescently conjugated secondary antibody as listed in Table 2.5 for 30 minutes at 4°C. Following incubation, cells were washed three times with 1mL of PBS/5% FCS/0.1 NaN_3 by pulse spinning at maximum speed for 10 seconds. Following the final wash, cells were resuspended in 100μL of PBS/5% FCS/0.1% NaN_3 and transferred to labelled tubes in preparation for analysis via flow cytometry. Controls for antibody staining included cells stained with isotype-matched controls, cells incubated with secondary antibody alone and unstained cells.

2.6.2 Multiple colour staining of epidermal suspensions

Approximately 5 x 10^5 epidermal cells were incubated at 4°C with purified anti-mouse CD16/32 (FcγIII/II, Fc block) to block non-specific binding of antibody to Fc receptors. Following incubation cells were incubated for 30 minutes at 4°C with primary antibodies, as listed in Table 2.4. Following incubation cells were washed

three times in PBS/5% FCS/0.1% NaN_3 by pulse spinning at maximum speed for 10 seconds. Following the final wash, cells were resuspended in 500μL and incubated in the corresponding FITC-conjugated secondary antibody, as listed in table 2.5 for 30 minutes at 4°C. After three pulse washes, cells were incubated for 30 minutes at 4°C with phycoerythrin (PE) conjugated rat anti-mouse I-A/I-E and 7-aminoactinymycin D (7AAD) and incubated at 4°C for 30 minutes. After three pulse washes the cells were resuspended in 100μL of PBS/5% FCS/0.1% NaN_3 and transferred to PPN tubes in preparation for analysis via flow cytometry. Controls for antibody staining included cells stained with the isotype-matched control, cells incubated with secondary alone and cells that were unstained.

2.6.3 Analysis

The fluorescence intensity of epidermal cell suspensions was analysed using a Coulter ELITE ESP flow cytometer equipped with a Coherent Innova 90 argon laser. Using CELLQuest™ software version 3.2.1 (Becton Dickinson, San Jose, CA), dead cells ($7AAD^+$) were excluded and LC identified on the basis of MHC-II staining. The measure of receptor expression on MHC-II^+ cells was determined via the intensity of the FITC-conjugated secondary antibody.

2.7 *In Vitro* Assessment of Antigen Uptake by Epidermal Langerhans Cells.

Table 2.6 Fluorescent antigens and tracers

Antigen	Supplier	Catalogue Number
FITC-dextran	ICN Biomedicals	02158062 80
Lucifer Yellow	Sigma	L0259
Zymosan A (from *Saccharomyces cerevisae*) BioParticles® BODIPY® FL conjugate	Molecular Probes	Z2844
Zymosan A BioParticles®, opsonising reagent (purified rabbit polyclonal IgG)	Molecular Probes	Z2850

E.coli (K-12 strain) BioParticles®, FITC conjugate	Molecular Probes	E2861
E.coli BioParticles®, opsonising reagent (purified rabbit polyclonal IgG)	Molecular Probes	E2870

2.7.1 Reconstitution and storage

2.7.1.1 Fluorescein isothiocyanate (FITC)-dextran

FITC dextran was reconstituted to 2mg/mL in complete RPMI 1640-FCS and vortexed gently to dissolve aggregates. FITC-dextran was reconstituted on the day of use and stored at 4°C, protected from light.

2.7.1.2 Lucifer Yellow (LY)

Lucifer Yellow was reconstituted to 2mg/mL in complete RPMI 1640-FCS and vortexed gently to dissolve aggregates. Lucifer Yellow was reconstituted on the day of use and stored at 4°C, protected from light.

2.7.1.3 Zymosan A

Zymosan A from *Saccharomyces cerevisiae* was reconstituted in PBS and then in RPMI 1640-FCS to 20ng/mL and stored in working aliquots at ⁻80°C. When required, aliquots were thawed slowly at room temperature. Aliquots were resuspended in 500μL of PBS and total yeast particles/mL determined with a haemocytometer.

2.7.1.4 Zymosan A polyclonal IgG opsonising reagent

Zymosan opsonising reagent (polyclonal IgG, Molecular Probes) was resuspended in 1mL of tissue culture grade water and stored at 4°C.

2.7.1.5 E.coli

E.coli was reconstituted in PBS and then in RPMI 1640-FCS to 20ng/mL and stored in working aliquots at ⁻80°C. When required, aliquots were thawed slowly at room

temperature. Aliquots were resuspended in 500μL of PBS and total yeast particles/mL determined with a haemocytometer.

2.7.1.6 E.coli A polyclonal IgG opsonising reagent

E.coli opsonising reagent (polyclonal IgG, Molecular Probes) was resuspended in 1mL of tissue culture grade water and stored at 4°C.

2.7.2 *In vitro* antigen uptake assays using FITC-dextran and Lucifer Yellow

Cell suspensions were pulsed with FITC-dextran or Lucifer Yellow (LY) at 37°C and 4°C. The 4°C control was included to determine the degree of non-specific or non-metabolically active binding of antigen to cells. At set time points, cells were removed and uptake ceased by the addition of ice-cold PBC/5% FCS/0.1 % NaN_3. Cells were washed three times by pulse spinning at maximum speed for 10 seconds. Cells were resuspended in 500μL in PBS/5% FCS/0.1% NaN_3.

2.7.2.1 Inhibition of FITC-dextran uptake by mannan and mannose

For blocking of mannose-type receptors, epidermal suspensions were pre-incubated with 10 mg/mL of mannan from *Saccharomyces cerevisiae* and 10mg/mL of D(+)-mannose at 37°C and 4°C for 10 minutes. Cells were transferred to 37°C and incubated with 1 mg/mL of FITC-dextran for 15 minutes in the continuous presence of mannan and mannose.

2.7.2.2 Inhibition of Lucifer Yellow uptake by wortmannin

For the inhibition of macropinocytosis, epidermal suspensions were incubated with 10uM wortmannin, from *Penicillium fumiculosum* for 30 minutes at 37°C. Cells were transferred to 37°C and incubated in 1mg/mL of FITC-dextran or LY for 15 minutes in the continuous presence of wortmannin.

2.7.3 Phagocytosis of Zymosan and *E.coli* by epidermal LC

Cell suspensions were pulsed with Zymosan A and *E.coli* at 37°C and 4°C, at a particle to LC ratio of 20:1. At set time points, cells were removed and uptake ceased by the addition of ice-cold PBC/5% FCS/0.1 % NaN_3. Cells were washed three times by pulse spinning at maximum speed for 10 seconds. Cells were then resuspended in 500µL of PBS/5% FCS/0.1% NaN_3.

2.7.4 Phagocytosis of opsonised Zymosan and *E.coli* by epidermal LC

To opsonise Zymosan and *E.coli*, equal volumes of opsonising reagent were added to particles, vortexed gently and incubated at 37°C for 1 hour. Following incubation, particles were washed three times at 1500 g for 5 minutes to remove excess antibody and NaN_3. Homogeneity was analysed with a light microscope and vortexed if required. Cell suspensions were pulsed with opsonised Zymosan A and *E.coli* at a ratio of 1:20 at 37°C and 4°C. At set time points, cells were removed and uptake ceased by the addition of ice-cold PBC/5% FCS/0.1% NaN_3. Cells washed three times by pulse spinning at maximum speed for 10 seconds. Cells were resuspended in 500µL of PBS/5% FCS/0.1% NaN_3.

2.7.5 Identification of Langerhans cells in whole suspensions.

Following antigen uptake assays approximately 5 x 10^5 epidermal cells were incubated for 30 minutes at 4°C with PE rat anti-mouse I-A/I-E and 7AAD to identify viable LC. Cells were washed three times by pulse spinning at maximum speed for 10 seconds. Cells were resuspended in 100µL of PBS/5% FCS/0.1% NaN_3 and transferred to labelled tubes for analysis via flow cytometry.

2.7.6 Analysis

The fluorescence intensity of epidermal cell suspensions was analysed using a Coulter ELITE ESP flow cytometer equipped with a Coherent Innova 90 argon laser. Using CELLQuest™ software version 3.2.1 (Becton Dickinson, San Jose, CA), dead cells ($7AAD^+$) were excluded and LC identified on the basis of MHC-II staining. To assess the rate of antigen internalisation, the mean fluorescence intensity at 37°C

was divided by the mean fluorescence intensity at 4°C. This normalisation procedure was crucial as it enabled analysis of antigen uptake by neonatal, juvenile and adult LC, which was performed on separate days. Data was analysed via Cricket Graph III, version 3 (Computer Associates).

2.8 *In Vitro* Analysis of Antigen Processing Pathways in Epidermal Langerhans Cells.

Table 2.7 Proteolytic antigens and maturation stimuli

Antigen	**Supplier**	**Catalogue Number**
DQ™ ovalbumin	Molecular Probes	D12-53
Lipopolysaccharides from *Escherichia coli* strain 0111:B4	Sigma	L3012

Table 2.8 Primary anti-mouse antibodies and isotype-matched controls

Antibody	Isotype	Concentration (μg/5 x 10^5 cells	Supplier	Catalogue Number
Biotin- I-A/I-E	Rat IgG_{2a}, κ	1	PharMingen	5553622
FITC-LAMP-1	Rat IgG_1, κ	1	PharMingen	553793
Purified- I-A/I-E (TIB 120)	Rat IgG_{2a}, κ	**1**	American Tissue Culture Collection. Hyridoma clone M5/114.15.2	-
Purified LAMP-1	Rat IgG_1, κ	1	PharMingen	553792
Rat IgG_{2a}, κ	-	1	PharMingen	553926

Table 2.9 Secondary fluorescent conjugated antibodies

Antibody	Isotype	Dilution	Supplier	Catalogue Number
Streptavidin, Alexa Fluor® 488 conjugate	-	1/1000	Molecular Probes	S32354
Streptavidin, Alexa Fluor® 594 conjugate	-	1/2000	Molecular Probes	S32356
Alexa Fluor® 488 goat anti-rat IgG (H+L))	-	1/1000	Molecular Probes	A11006
Alexa Fluor® 594 goat anti-rat IgG (H+L)	-	1/1000	Molecular Probes	A11007
Anti-fluorescein/Oregon Green®, goat IgG fraction, Alexa Fluor®	-	1/5000	Molecular Probes	A11096

2.8.1 Reconstitution and storage

2.8.1.1 DQ-OVA

DQ-OVA was reconstituted to 0.5mg/mL with sterile PBS and vortexed gently to dissolve aggregates. Once reconstituted DQ-OVA was stable for 2 weeks at 4°C, protected from light.

2.8.1.2 Lipopolysaccharide

Lipopolysaccharide (LPS) was reconstituted to 1mg/mL in RPMI 1460-FCS and vortexed gently to dissolve aggregates. Once reconstituted small working aliquots were stored at –20°C. Care was taken to avoid repeated freeze thawing.

2.8.2 Pulse chase antigen processing assays

Cell suspensions were pulsed with DQ-OVA at 37°C and 4°C for 4 hours. The 4°C control was included to determine the degree of non-specific binding of antigen to cells. At 4 hours cells were washed three times with PBS-5% FCS at 800 g for 15 minutes at 4°C to remove free antigen. The suspensions were resuspended in IMDM and returned to 37°C and 4°C and incubated for 24 hours. At set time points, cells were removed and processing ceased by the addition of ice-cold PBC/5% FCS/0.1% NaN_3. Cells were washed three times by pulse spinning at maximum speed for 10 seconds. Cells were resuspended in 500µL of PBS in preparation for adherence to lysine-coated coverslips.

2.8.3 Coating of cover slips with poly-lysine

13mm round glass cover slips were placed in a 24 well plate at 1 slip/well. Slips were coated with 1mL of a 1mg/mL poly-L-lysine/PBS solution for 15 minutes at room temperature. Following incubation lysine was aspirated

2.8.4 Adherence of epidermal LC to lysine coated cover slips

Cell concentration was determined with a haemocytometer and adjusted to between 0.5 and 1 x 10^6 cells/mL in PBS. 500µL of cells were added to each well and allowed to adhere at room temperature for 30 minutes. Following incubation, non-adhered cells were aspirated. 1mL of 4% paraformaldehyde buffer was to each well and incubated for 10 minutes at room temperature. Following incubation paraformaldehyde was aspirated. Coverslips were washed twice in 1mL of saponin buffer for 5 minutes with gentle agitation on a horizontal shaker.

2.8.5 Multiple colour immunofluorescent staining of adhered epidermal cells

Coverslips were incubated at room temperature with the desired rat-anti mouse primary antibody or isotype control, as listed in table 2.8, for 30 minutes with gentle agitation. Following incubation, slips were washed three times in 1mL of saponin buffer for 5 minutes with gentle agitation. Following washing, coverslips were incubated with 500uL of secondary-anti-rat Alexa conjugated antibody, as listed in table 2.9, for 45 minutes at room temperature, protected from light. Coverslips were washed three times with 1mL of saponin buffer for 5 minutes with gentle shaking. Slips were stained with a sequential biotin-conjugated primary antibody for 30 minutes at room temperature with gentle agitation. Following another 3 washes in saponin buffer, cells were stained with 500uL of secondary streptavidin Alexa conjugated antibody for 45 minutes at room temperature, protected from light. Following incubation slips were washed three times in 1mL of saponin for 5 minutes with gentle shaking. Following the final wash, slips were allowed to air dry. Each cover slip was then inverted onto a slide bearing a drop of Permafluor mounting media. The slides were then allowed to set at room temperature before storage at 4°C, protected from light.

2.8.6 Microscopy

The fluorescence intensity and antibody localisation of adhered epidermal cells was analysed using Leica standard and inverted fluorescence microscopes fitted with a digital camera. Using MagnaFire™ software, images were taken in monochrome mode and merged within Adobe PhotoShop™ Version 7 (Adobe Systems Incorporated). Due to the intensity of Alexa 594 staining there was significant background staining. Using Adobe PhotoShop™ the contrast was increased, thereby reducing background level of staining. The reduction of background fluorescence was the only imagine manipulation performed.

2.9 *In Vitro* Analysis of Antigen Trafficking and Maturation by Epidermal Langerhans Cells Following Exposure to LPS.

2.9.1 Pulse chase antigen processing assays in the presence of LPS

DQ-OVA processing assays outlined in section 2.7.1 were repeated in the continuous presence of LPS. Antigen localisation was assessed via fluorescence microscopy as outlined in section 2.7.6.

2.9.2 Detection of intracellular versus extracellular MHC-II:peptide complexes.

24 hours following incubation in the presence or absence of LPS, epidermal suspensions were adhered to coated coverslips and LC identified as outlined in sections 2.7.2 through to section 2.7.4. MHC-II localisation was assessed via fluorescence microscopy as outlined in section 2.7.6.

2.9.3 Analysis of CD86 expression

24 hours following incubation in the presence or absence of LPS, epidermal suspensions were dual stained for expression of CD86 and MHC-II as outlined in section 2.6.2. CD86 and MHC-II expression was assessed via flow cytometry as outlined in section 2.6.3.

Chapter 3

The Ontogeny of Antigen Uptake Receptors on Langerhans Cells During the Early Neonatal Period

3.1 Introduction

Epidermal Langerhans cells (LC), are involved in the uptake and processing of exogenous antigens (Chain et al. 1986; Cella et al. 1997b). The pathways utilised by LC to internalise antigen are influenced by antigen complexity and size (Steinman and Swanson 1995; Watts 1997). Soluble and non-binding antigens can be engulfed via non-specific fluid-phase micro- or macropinocytosis (Watts and Marsh 1992). Small particulate antigens are internalised via receptor-mediated endocytosis, whilst larger particulate antigens, such as microbes, are captured via phagocytosis (Aderem and Underhill 1999; Stepnev and De Camilli 2000). For effective receptor-mediated endocytosis or phagocytosis, it is essential that LC express a diverse range of receptors capable of recognising microbial-associated antigens, immune complexes and activated complement components (Aderem and Underhill 1999; Stepnev and De Camilli 2000). LC possess lectins specific for components of microbial cell walls, including CD14 that recognises LPS (Aderem and Underhill 1999). LC also express members of the C-type lectin family of receptors, including DEC-205 and Langerin, essential to the recognition of terminal mannose residues commonly found on microbial surfaces (Kato et al. 1998; Kato et al. 2000; Valladeau et al. 2000; Valladeau et al. 2003). Antibody-antigen complexes are recognised by Fc receptors, including FcγRII (CD16/32) and FcεRI (Esposito Farese et al. 1995; Fanger et al. 1996), whilst complement complexed antigen is recognised by CD11b/CD18 (CR3) and CD11c/CD18 (CR4) (Okada et al. 1988).

Uptake of mannosylated antigen is a function of endocytic receptor expression, and it has been proposed that an absence of mannose-type receptors negatively impacts on the scavenging capacity of LC (Jiang et al. 1995; Sallusto et al. 1995). During the early neonatal period, the epidermal LC network is phenotypically and functionally immature, with an absence of DEC-205 expression 4 days following birth (Dewar et al. 2001). It was hypothesised that LC isolated from neonatal epidermis had a

diminished capacity to internalise antigen via a receptor-mediated pathway. This hypothesis was further supported when, following application of antigen through 3-day-old epidermis, the amount of antigen transported by LC to the draining LN was less compared with adult counterparts (Dewar et al. 2001). Following presentation of cutaneous antigen by neonatal LC there was a deviation in the T cell response from immunity to antigen-specific immune suppression (Dewar et al. 2001; Simpson et al. 2003). As the amount of antigen presented in the context of MHC-II can influence the outcome of T cell activation (Langenkamp et al. 2002), the divergence in the pathways utilised by neonatal LC to internalise and process antigen has important implications for the generation of suppression. In order to assess the capacity of LC to internalise antigen during development it was first necessary to determine the ability of these cells to recognise exogenous antigen. This chapter examined the phenotype and expression of a range of antigen uptake receptors expressed by LC during the early neonatal period.

3.2 Results

3.2.1 Selecting for Viable MHC-II$^+$ Cells in Epidermal Suspensions.

In order to identify viable MHC-II$^+$ LC in whole epidermal preparations it was necessary to develop an appropriate gating strategy. However, during dispase digestion of excised epidermis a moderate degree of cell death occurs. To assess cell death and to therefore exclude the non-specific autofluorescence associated with dead cells the viability dye 7-AAD was employed and fluorescence analysed using CellQuest™software. Figure 3.1 (a) is a representative of data for adult epidermal suspensions and demonstrates the viability gating strategy. To distinguish viable cells in whole suspensions a gate was drawn around the live, 7-AAD$^-$ population. These viable cells were further gated to identify MHC-II$^+$ cells in a PE-MHC-II fluorescence intensity versus forward scatter dot plot. Gates were drawn to include 98% of MHC-II$^+$ cells. As shown in Figure 3.1 (b) a discrete MHC-II$^+$ population is present. The high level of MHC-II expression suggests that LC are present in epidermal suspensions. This gating strategy was employed for all analyses.

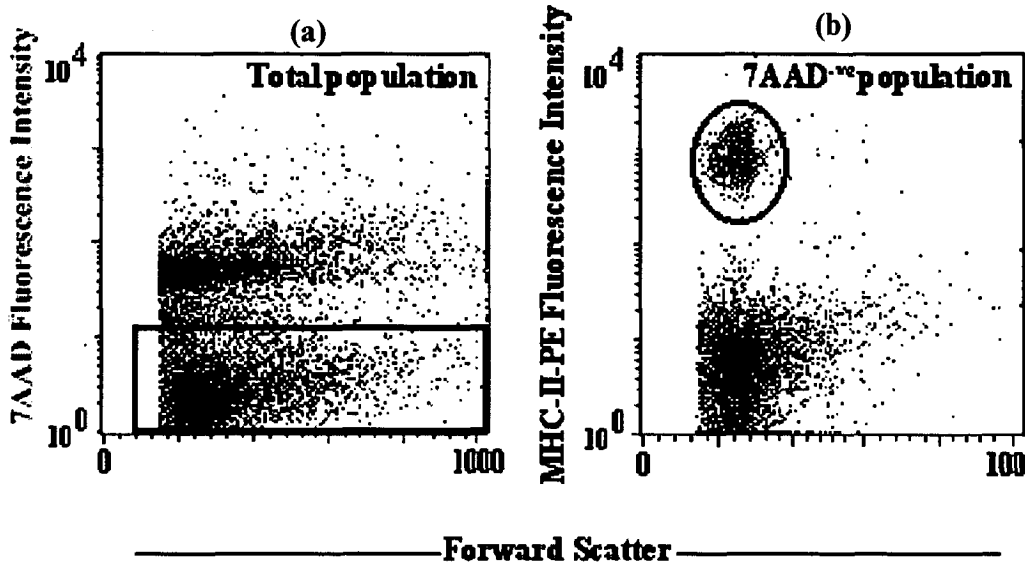

Figure 3.1 Identification of viable MHC-II$^+$ cells in adult epidermal suspensions. A gating strategy is shown for the identification of LC in epidermal suspensions. Epidermal suspensions were prepared from murine adult epidermis 6 weeks following birth by digestion with dispase and stained with anti-major histocompatibility complex class II (MHC-II). To select for viable cells, a gate was drawn around the 7-aminoactinomycin D-negative (7-AAD-) population (a). To select for MHC-II$^+$ cells, a gate was placed to include 98% of MHC-II$^+$ cells. LC are represented by the discrete population of viable MHC-II$^+$ cells (b). Data is representative of three or more experiments.

3.2.2 Phenotype of epidermal cells during the developmental period

Prior to analysis of antigen uptake receptor expression, it was first essential to ascertain the phenotype of MHC-II$^+$ epidermal cells. The primary aim was to confirm that the gating strategy did not contain T and B cells but also to assess if LC express the lineage markers CD4, CD8 and B220 during the developmental period.

3.2.2.1 MHC-II expression on viable LC during the developmental period.

To assess the expression of MHC-II during the developmental period, PE-conjugated anti-MHC-II was used. Figure 3.2 is a representative experiment, where the expression of MHC-II on LC from 4-, 7- and 14-day- and 6-week-old epidermis is shown. It is evident that MHC-II$^+$ cells are identifiable at 4 days following birth and that the level of expression increases with age. The mean fluorescence intensity demonstrated by adult LC is 2-fold greater than that associated with neonatal LC. This suggests that adult LC express more surface MHC-II than neonatal LC.

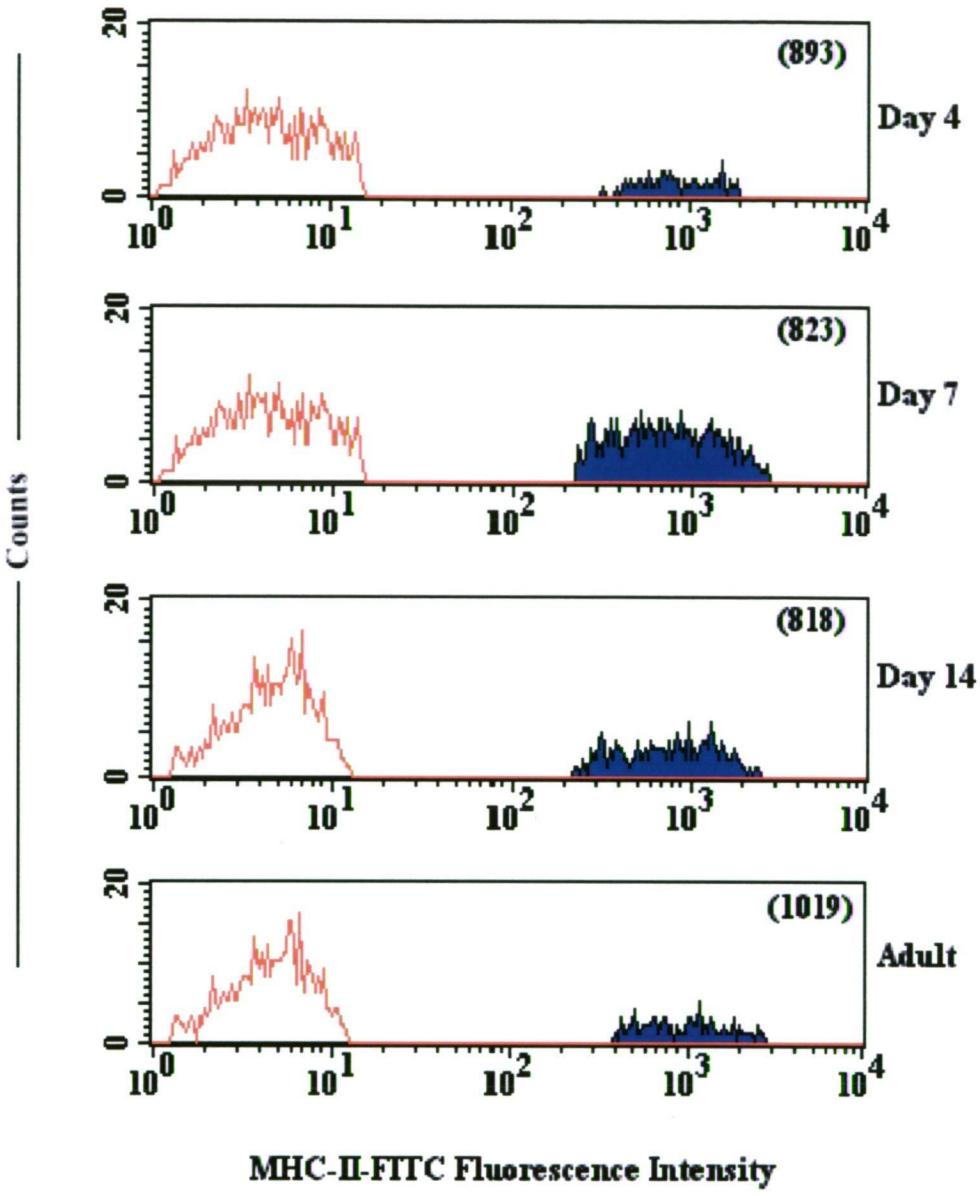

Figure 3.2 MHC-II expression on viable LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of the antigen presentation molecule MHC-II. The filled histogram represents the profile of anti-MHC-II antibody staining. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-MHC-II staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.2.2 Expression of CD4 and CD8 on viable MHC-II$^+$ LC during the developmental period.

To confirm the gating strategy to identify LC amongst the epidermal population did not contain cells with other lineage markers, staining for CD4 and CD8 was undertaken. By carrying out such analyses it was also possible to ascertain if LC express T cell surface molecules during development. It has been previously demonstrated that some subtypes of dendritic cells can express low levels of CD4 or CD8. Figure 3.3 and 3.4 summarises the CD4 and CD8 expression on MHC-II$^+$ cells during development. It is evident that at no point in LC development do they express CD4 or CD8. The absence of these markers strongly argues for the LC phenotype of MHC-II$^+$ and demonstrates that there is no contamination of epidermal preparations with CD4 or CD8 T cells. It also demonstrates that, during the developmental period, Langerhans cells do not display a transient expression of CD4 or CD8.

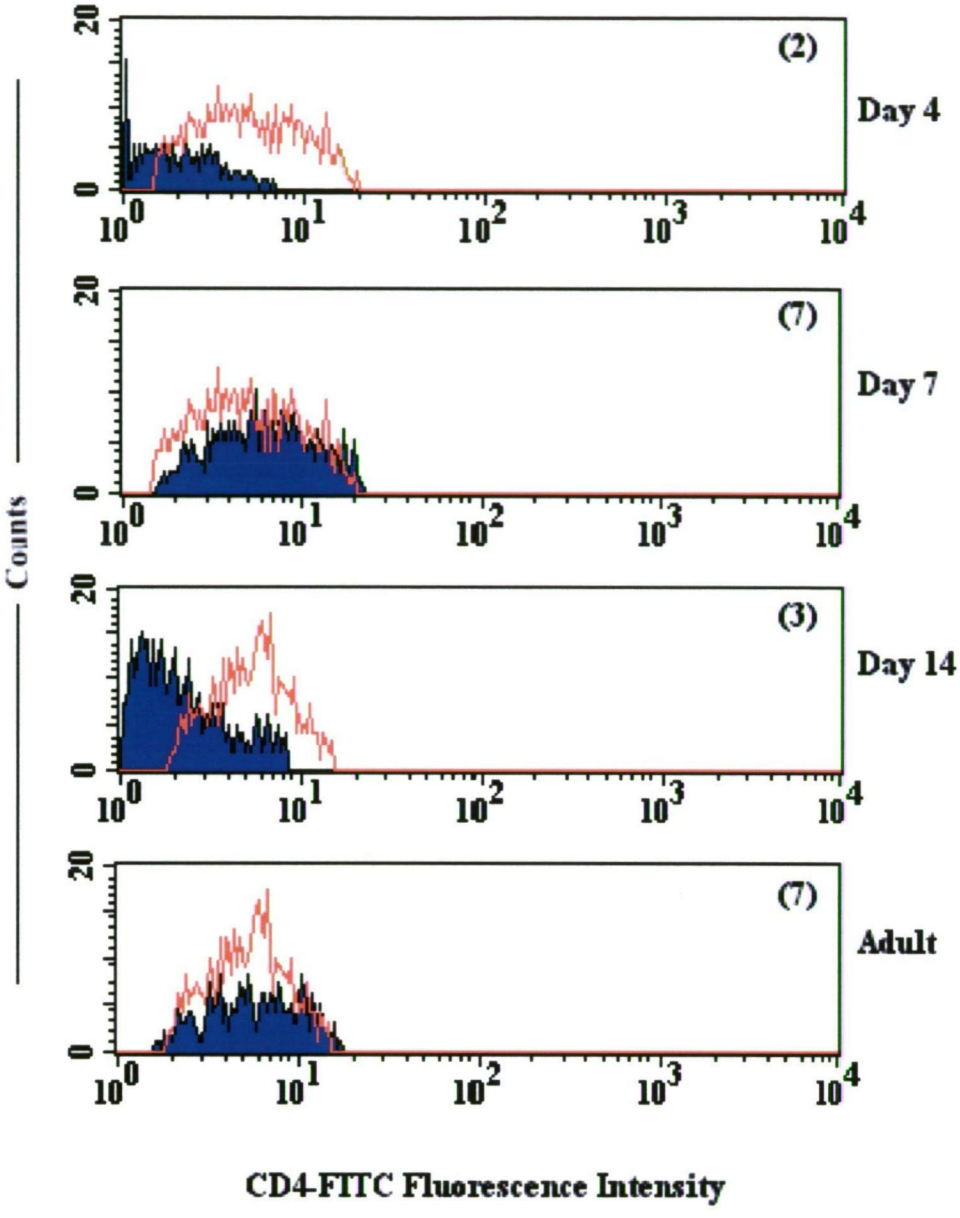

Figure 3.3 CD4 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the T cell marker CD4. The filled histogram represents the profile of anti-CD4 antibody staining. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD4 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

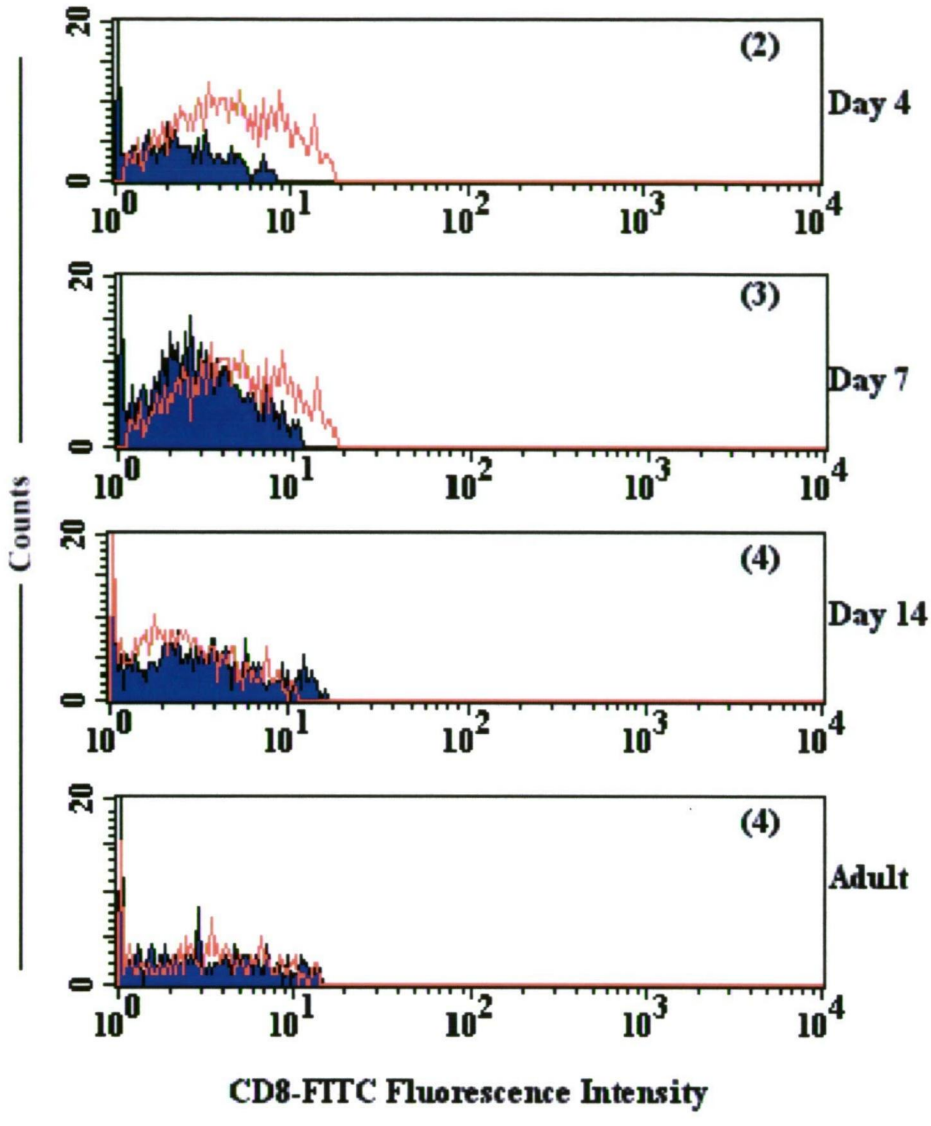

Figure 3.4 CD8 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the T cell marker CD8. The filled histogram represents the profile of anti-CD8 antibody staining. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD8 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.2.3 Expression of B220 on viable MHC-II$^+$ LC during the developmental period

To determine if MHC-II$^+$ contain a population of B cells, analysis of expression of the B cell marker B220 was employed. Figure 3.5 summarises B220 expression during development and demonstrates an absence of contaminating B cells. It is evident that during development MHC-II$^+$ do not express B220.

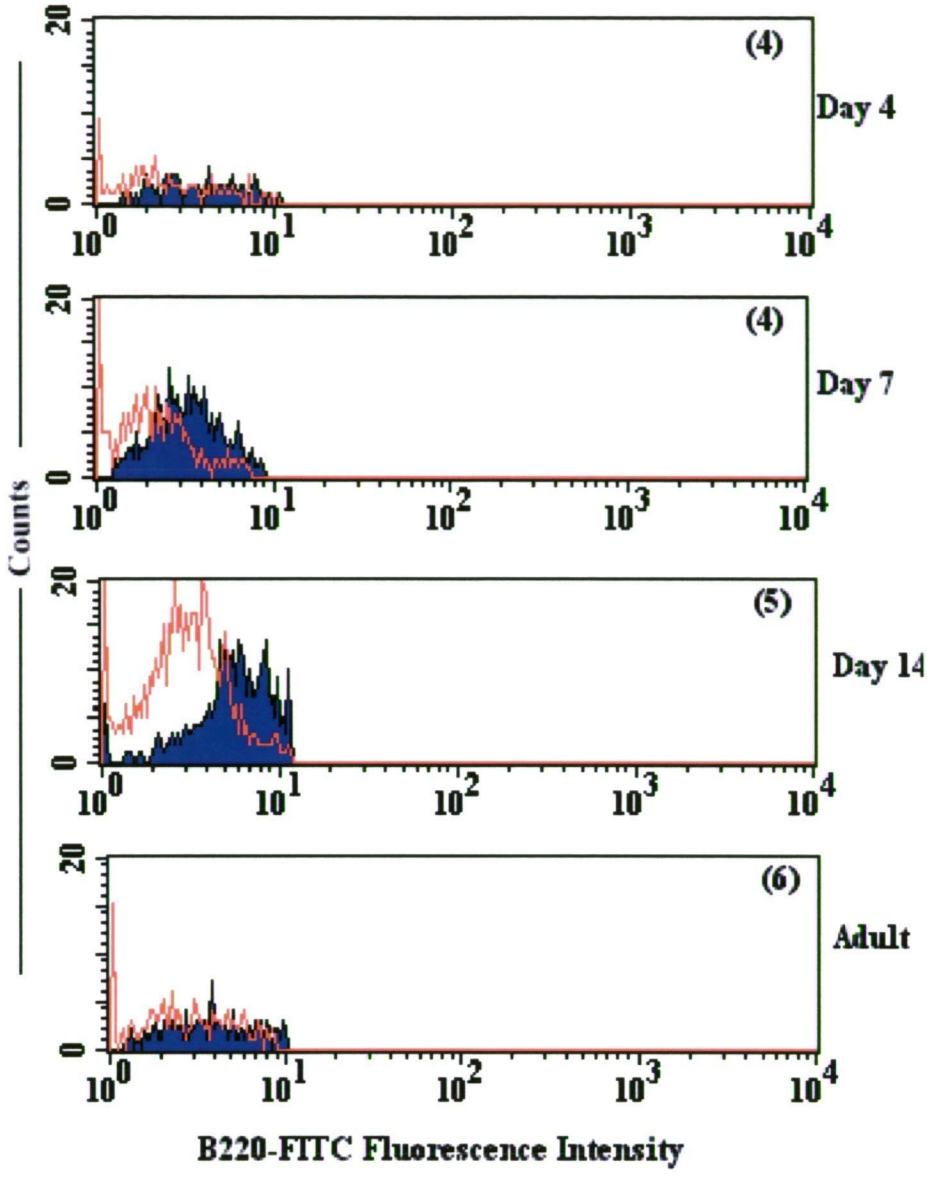

Figure 3.5 B220 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the B cell marker B220. The filled histogram represents the profile of anti-B220 antibody staining. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-B220 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3 The ontogeny of antigen uptake receptors during the developmental period.

The remainder of this chapter will focus on investigating the expression profile of a representative range of antigen uptake receptors essential for LC function. The expression of mannose binding, IgG and complement receptors was assessed in neonatal, juvenile and adult LC.

3.2.3.1 DEC-205 expression on viable MHC-II$^+$ LC during the developmental period

To characterise the capacity of LC during development to bind antigen that contains mannose residues, epidermal suspensions were prepared from 4-, 7- and 14-day- and 6-week-old mice, and stained for expression of the lectin receptor DEC-205. As evident in Figure 3.6, DEC-205 was absent on LC from 4-day-old epidermis. As the epidermal network matured there was a gradual increase in the level of expression, with DEC-205 identified at 7 days following birth. By day 14 the level of expression was comparable with that demonstrated within adult epidermis.

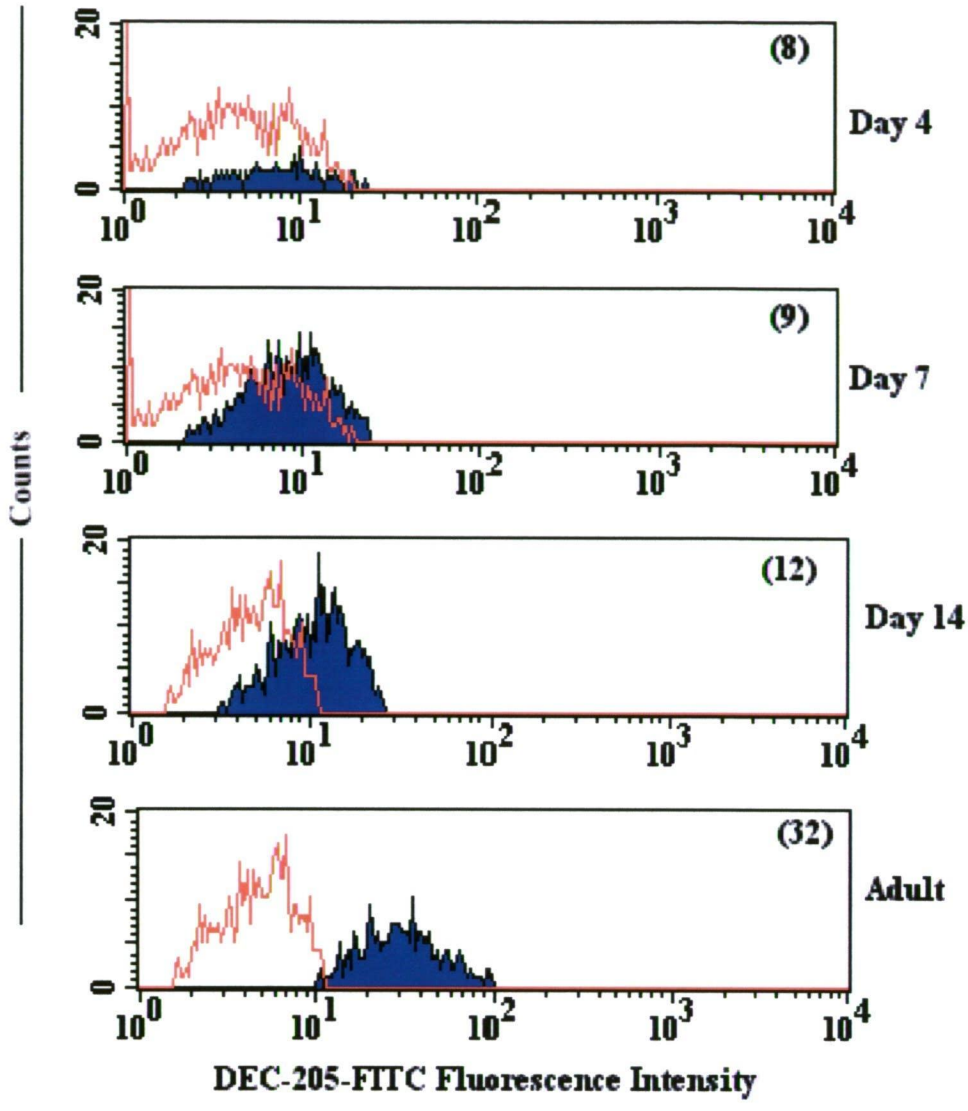

Figure 3.6 DEC-205 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the mannose-specific uptake receptor DEC-205. The filled histogram represents the profile of anti-DEC-205 antibody staining. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-DEC-205 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3.2 Analysis of Langerin expression on permeabilised LC during the developmental period

To further assess expression of mannose-type lectin receptors during development, permeabilised epidermal suspensions were prepared from neonatal, juvenile and adult mice and analysed for expression of lectin-specific receptor Langerin. It is crucial to permeabilise the cells as the anti-mouse Langerin antibody only binds intracellular portions of the molecule. Figure 3.7 demonstrates permeablisation resulted in positive staining for the receptor. Langerin expression was first identified at 4 days following birth, however the level of staining was only slightly higher than the level of binding associated with the isotype-matched control. As such the level of expression at 4 days following birth was low. As the epidermal network matured there was a gradual increase in the level of expression with a two-fold increase in intensity of anti-Langerin staining by day 7 following birth. By day 14 the level of expression had increased and was comparable with staining demonstrated in adult epidermis.

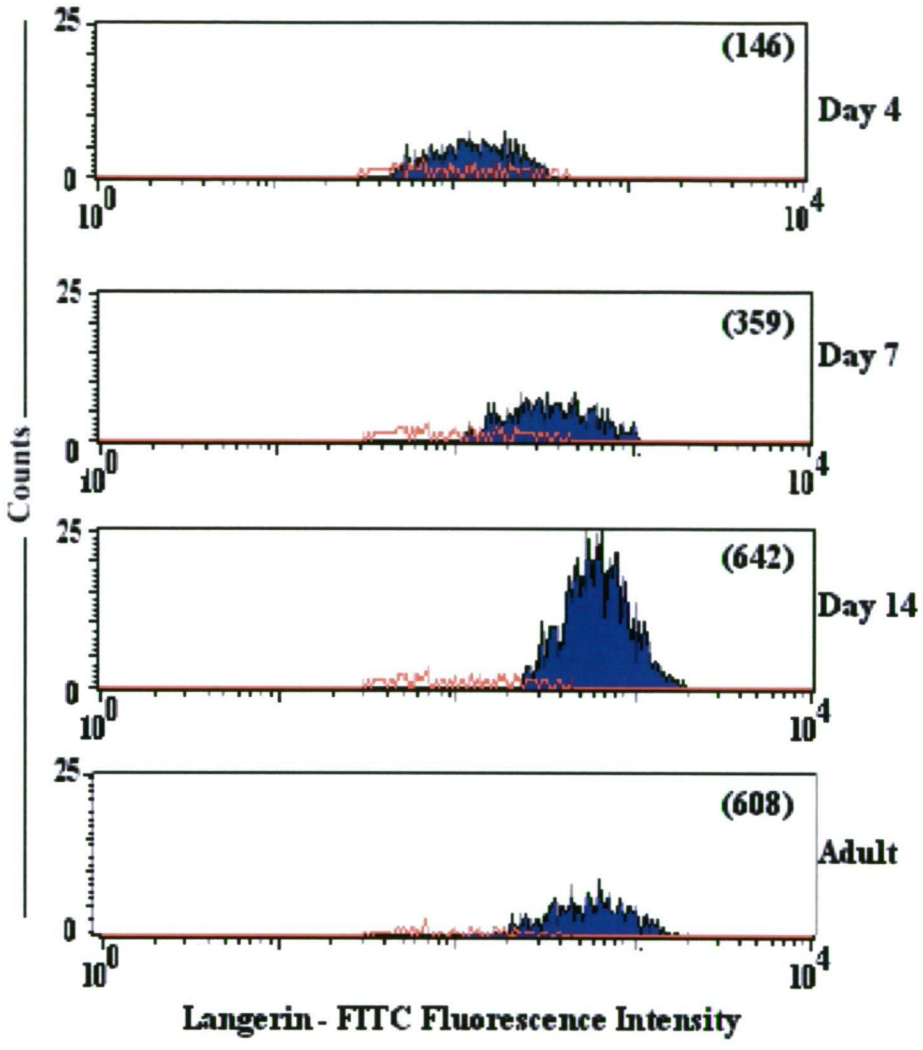

Figure 3.7 Langerin expression on viable, permeabilised MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel), permeabilised and stained for expression of MHC-II and the mannose-specific uptake receptor Langerin. The filled histogram represents the profile of anti-Langerin antibody staining. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-Langerin staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3.3 Analysis of CD16/32 expression of viable MHC-II$^+$ LC during the developmental period.

Opsonisation of particulate antigen, whether through deposition of antibody such as IgG or activated complement components such as C3b, increases the likelihood of uptake and clearance (Esposito Farese et al. 1995; Galon et al. 1997). The potential of LC to bind opsonised antigen was first investigated by assessing the expression of the IgG receptor CD16/32 on LC isolated from neonatal, juvenile and adult mice. Analysis of cell suspensions via flow cytometry demonstrated that CD16/CD32 was present from an early stage in epidermal development, with LC isolated from 4-day-old epidermis associated with a moderate level of expression (Figure 3.8). It is also evident that as the animal matured the level of expression fluctuated, with LC from epidermis of 4-day- and 6-week-old mice associated with greater fluorescence intensity than LC from 7-day- and 14-week-old mice.

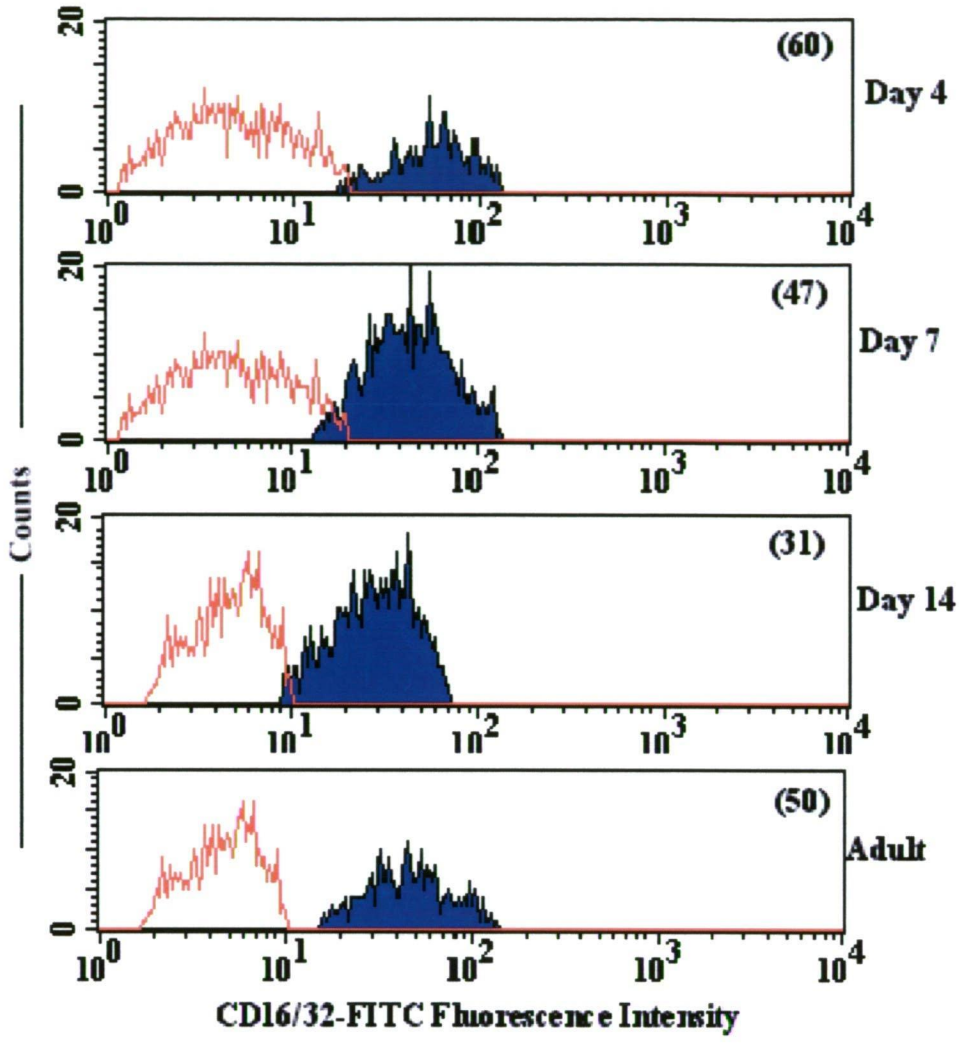

Figure 3.8 CD16/32 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the IgG receptor CD16/32. The filled histogram represents the profile of binding of the anti-CD16/32 antibody. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD16/32 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3.4 Analysis of CD11b expression on viable MHC-II$^+$ LC during the developmental period.

To further assess the expression of opsonin receptors during development, LC isolated from neonatal, juvenile and adult epidermis were analysed for expression of CD11b, a component of complement receptor 3 (CR3) (Okada et al. 1988). Figure 3.9 demonstrates a discrete population of MHC-II$^+$CD11b$^+$ cells isolated from 4-day-old mice. However by day 7 two populations of MHC-II$^+$ were present, MHC-II$^+$CD11b$^-$ and MHC-II$^+$CD11b$^+$. It is also evident that as the epidermal network matured there were fluctuations in the level of expression associated with the MHC-II$^+$CD11b$^+$ population, with the highest level of expression associated with LC isolated from 14-day-old mice. By 6 weeks following birth the level of expression on CD11b^{hi} LC was decreased and similar to that expressed by LC isolated from 4-day-old mice.

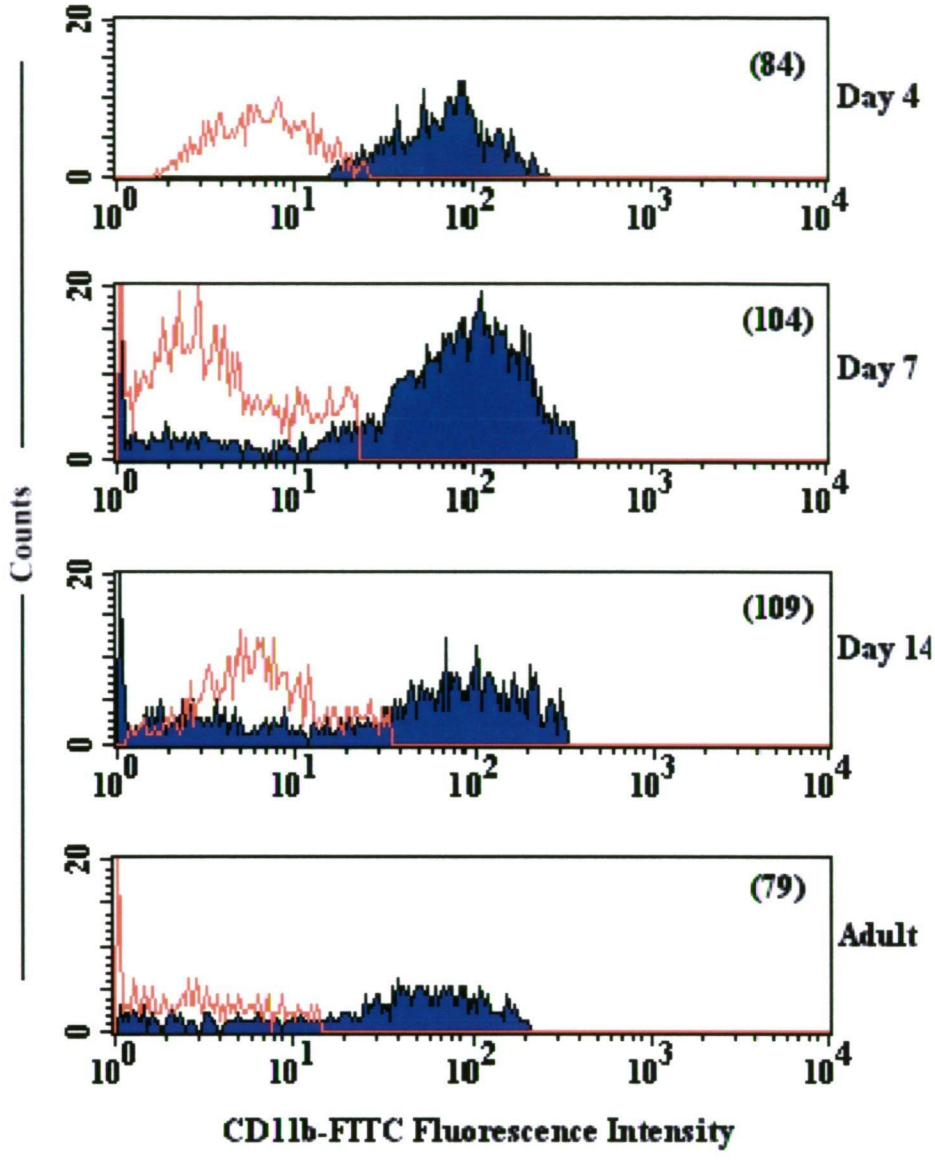

Figure 3.9 CD11b expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the complement receptor CD11b. The filled histogram represents the profile of binding of the anti-CD11b antibody. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD11b staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3.5 Analysis of CD18 expression on viable MHC-II$^+$ LC during the developmental period

To further characterise the expression of the complement receptors during development, LC isolated from neonatal, juvenile and adult epidermis were analysed for expression of CD18, the second component of CR3 (Okada et al. 1988). Figure 3.10 demonstrates a discrete CD18 positive population present at each stage during development. Results also demonstrate that the level of expression increased during development until day 14, when the level of expression was similar to that expressed by LC isolated from 6-week-old mice.

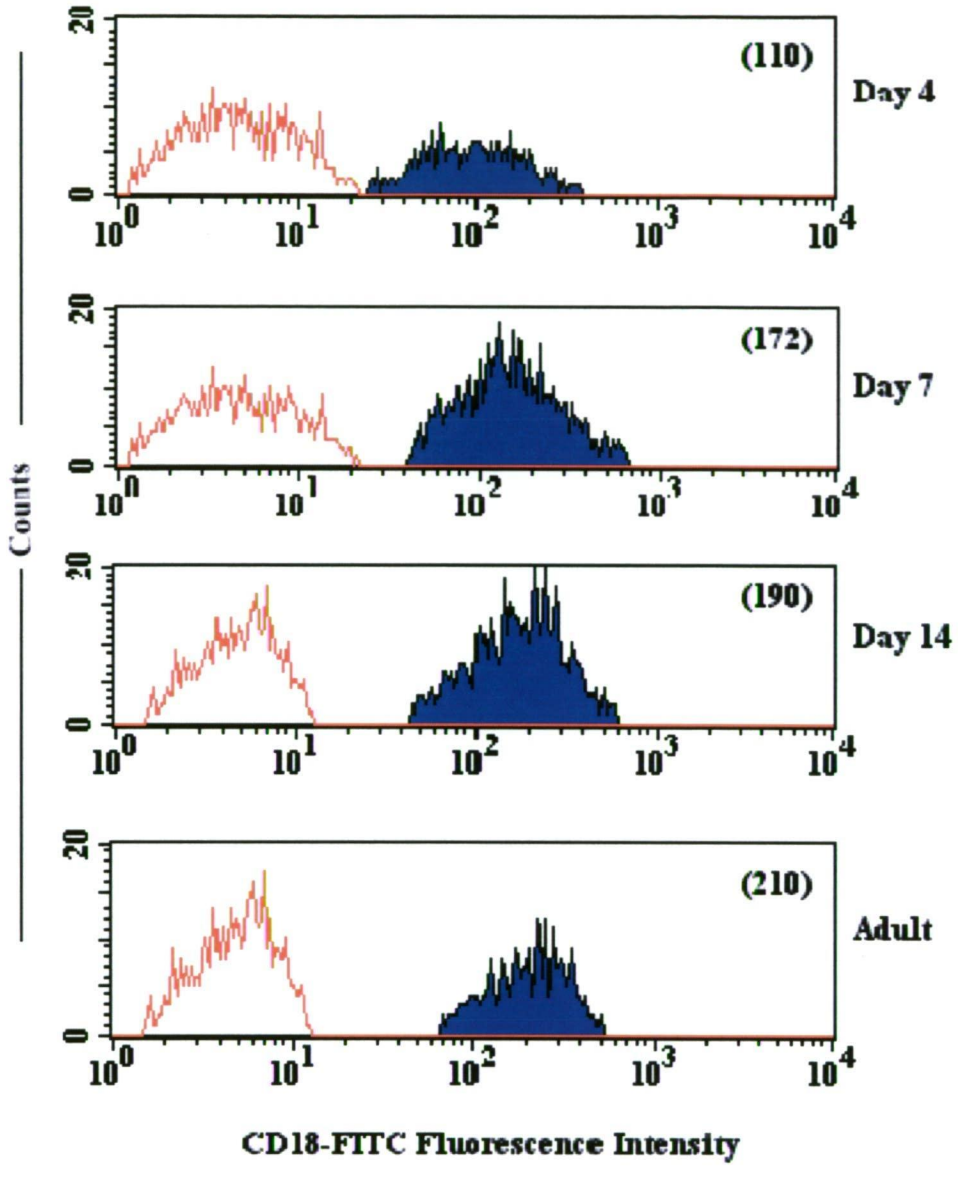

Figure 3.10 CD18 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the integrin CD18. The filled histogram represents the profile of binding of the anti-CD18 antibody. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD18 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3.6 Analysis of CD11c expression on viable MHC-II$^+$ LC during the developmental period

To further assess the expression of opsonin receptors during development, LC isolated from neonatal, juvenile and adult epidermis were analysed for expression of CD11c, a component of CR4. Figure 3.11 demonstrates that at no point during LC development were MHC-II$^+$ cells positive for CD11c.

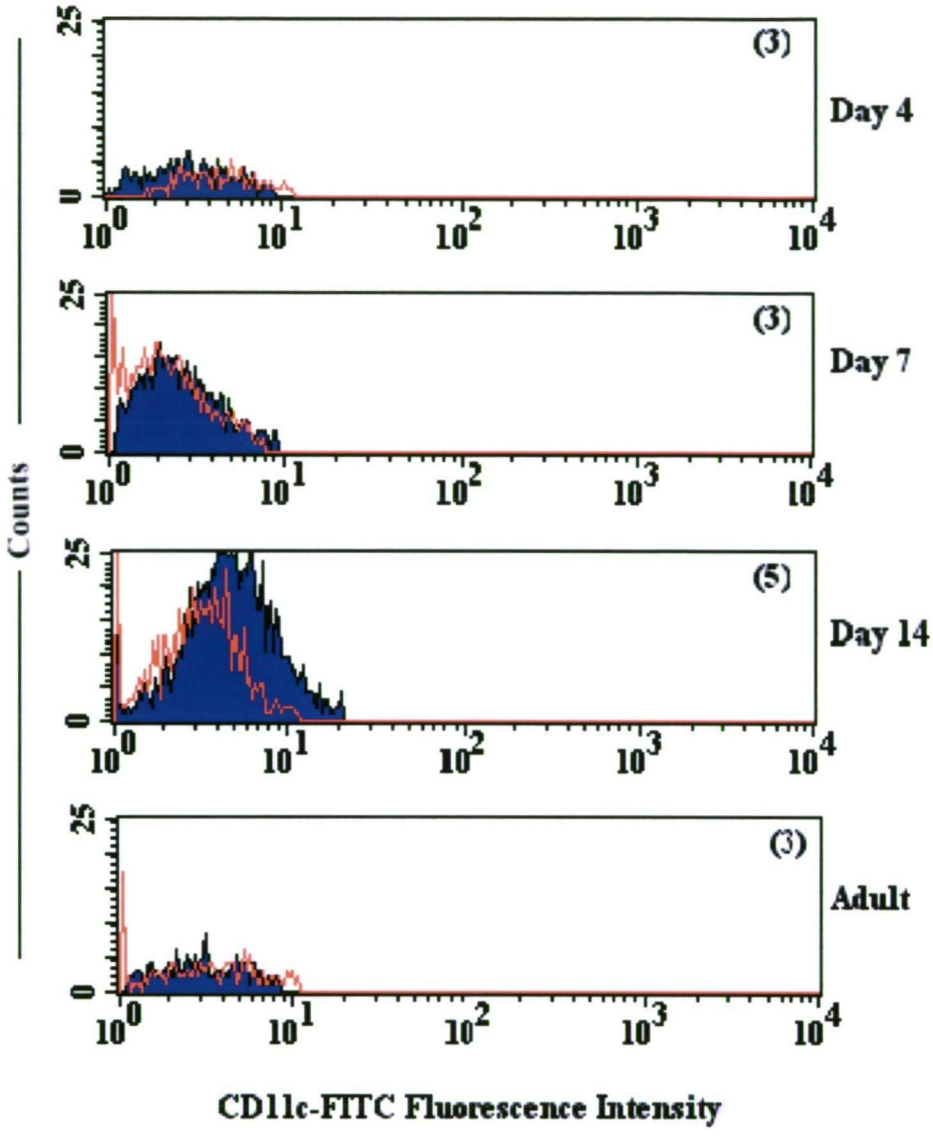

Figure 3.11 CD11c expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the complement receptor CD11c. The filled histogram represents the profile of binding of the anti-CD11c antibody. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD11c staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.2.3.7 Analysis of CD14 expression on viable MHC$^+$ LC during the developmental period.

To characterise the expression of LPS receptors during development, LC isolated from neonatal, juvenile and adult epidermis were analysed for expression of CD14. Figure 3.12 demonstrates LC were negative for the LPS receptor CD14. This suggests that LC do not bind LPS via CD14. It is important to note that dermal DC express CD14, thus this result also demonstrates that contamination with dermal DC did not occur during isolation of epidermal cells.

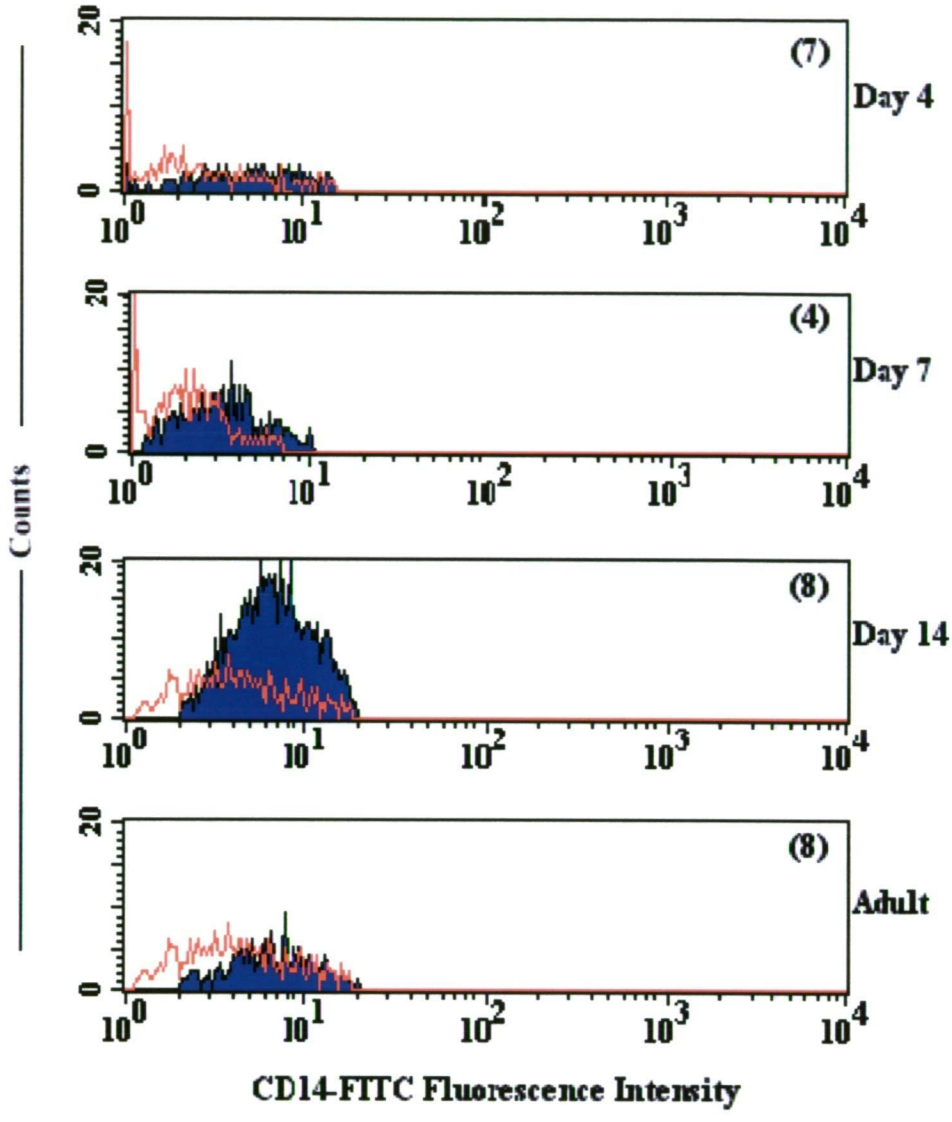

Figure 3.12 CD14 expression on viable MHC-II$^+$ LC during the developmental period. Epidermal suspensions were prepared from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) and stained for expression of MHC-II and the LPS receptorCD14 expression. The filled histogram represents the profile of binding of the anti-CD14 antibody. The open histogram represents binding of the isotype-matched control antibody. The mean fluorescence intensity of anti-CD14 staining is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

3.3 Discussion

Langerhans cells are the resident APC of the epidermis and are critical to the induction of immunity to cutaneous antigen (Shelley and Juhlin 1976; Shelley and Juhlin 1977). Following capture of antigen LC migrate to regional LN where they trigger naïve T cell activation (Kripke et al. 1990). One pathway employed by LC to internalise antigen is receptor-mediated endocytosis, involving the internalisation of antigen following binding of antigenic peptides to surface receptors (Aderem and Underhill 1999; Stepnev and De Camilli 2000). LC express a range of uptake receptors enabling recognition of a vast array of possible antigens, of both extrinsic and intrinsic origin (Aderem and Underhill 1999; Stepnev and De Camilli 2000). LC express CD16/32 that facilitates efficient capture of IgG complexed antigen (Esposito Farese et al. 1995; Fanger et al. 1996). Terminal mannose residues, a common component of microbial membranes, are recognised by the C-type lectin receptors DEC-205 and Langerin (Kato et al. 1998; Kato et al. 2000; Valladeau et al. 2000; Valladeau et al. 2003), whilst complement-opsonised antigens are bound by CD11b/CD18 and CD11c/CD18 (Okada et al. 1988). Following receptor binding, a cascade of events is initiated, leading to the internalisation, processing and subsequent presentation of antigen for T cell activation (Taylor et al. 1991; Peguet-Navarro et al. 1992; Dai et al. 1993). It can be seen that expression of a broad spectrum of antigen-specific receptors is crucial to APC function. The present study sought to investigate the capacity of LC to bind antigen during the developmental period. The receptors analysed in this study were selected to represent the capacity to internalise the major classes of antigens, including antibody and complement-complexed antigens, mannose residues and LPS. By assessing a representative panel of receptors it was possible to investigate the potential of LC to internalise antigen via receptor-mediated endocytosis.

To determine the expression of receptors on LC it was first necessary to identify LC in whole epidermal suspensions. As sorting or density gradient centrifugation can lead to unwanted activation of freshly isolated cells, LC in epidermal cell preparations were identified on the basis of MHC-II expression. Results herein demonstrate that MHC-II$^+$ cells were present at 4 days following birth, with the level

of expression gradually increasing with maturation. This increase in expression may reflect a redistribution of MHC-II from a cytoplasmic location to the cell membrane and dendrites during development. This hypothesis is supported by previous studies that demonstrated that *in situ,* LC exhibit rounded cell morphology and an absence of MHC-II stained dendritic processes (Dewar et al. 2001). These results suggest that during the early stages of development the epidermal network is immature. To determine if this immaturity of the epidermal network impacted on the capacity to bind antigen, LC isolated from neonatal, juvenile and adult epidermis were analysed for expression of a range of uptake receptors.

The ability to bind mannose is of particular importance to APC function, as the majority of glycoproteins derived from bacteria and yeast are mannosylated (Buentke et al. 2000). To facilitate binding, LC express members of the C-type lectin family of receptors, including DEC-205 and Langerin (Kato et al. 1998). DEC-205 has been suggested to initiate uptake and strongly enhance MHC-restricted presentation of antigen, and is up regulated during maturation (Reis et al. 1993; Kato et al. 1998). Analysis of Langerin and DEC-205 expression on LC isolated from 4-day-old epidermis demonstrated a marked decease in receptor expression compared to LC isolated from adult epidermis. Results also demonstrate a gradual increase in receptor expression during development. By day 14 following birth the level of expression was parallel to that observed on LC isolated from adult epidermis. These results are supported by previous studies from this department analysing the expression of lectin receptors on LC *in situ*, utilising epidermal sheets from 4-day and 6-week-old mice. These results suggest that the functional maturity of LC is acquired during development. The factors influencing receptor up-regulation are not fully characterised but it is highly plausible that the external milieu may play an important role (Longoni et al. 1998). The recent finding from our research group that demonstrated the maturation status of the epidermal network can be accelerated by topical application of antigen supports this hypothesis (Wozniak. BSc(Hons) thesis). It is possible that LC resident in neonatal epidermis learn from their environment during antigen capture and up-regulate receptor surface expression accordingly. Aside from antigen exposure, the presence of certain cytokines can elicit alterations in receptor expression (Bieber et al. 1989; Doyle et al. 1994; Brown and Hural 1997). Longoni and colleagues demonstrated a heightened uptake of mannosylated

antigens in monocyte-derived DC exposed to the immuno-suppressive cytokine IL-10 during culture. When added to culture during the last 24 hours of a 7-day culture, IL-10 elicited an increase in the uptake of fluorescently labelled FITC-dextran. This increase in endocytosis was mirrored by a 4-fold increase in expression of the mannose receptor (Longoni et al. 1998). It was postulated that due to the increased endocytic activity of immature DC antigen would be effectively cleared from the extracellular environment, contributing further to the tolerogenic effect of IL-10. It would appear that these findings contradict those of Faulkner and collegues (Faulkner et al. 2000a). Using bone marrow derived DC it was demonstrated that IL-10 did not induce any significant increase in phagocytosis. However this disrepency may be reflected in the two distinct uptake pathways investigated in these two studies. FITC-dextran was utilised in the Longoni study (Longoni et al. 1998) as an indication of receptor-mediated endocytosis of small particulate antigen. Phagocytosis in contrast involves the uptake of large particulate antigen, therefore IL-10 may exert differing effects, depending on the pathway utilised by DC to internalise antigen. By 6 weeks of age the epidermal network has matured to the extent that antigen exposure results in maturation of LC to full APC potency, possibly involving a shift in cytokine secretion to pro-inflammatory cytokines. Although it is highly possible that IL-10 is in the extracellular milieu during development, LC isolated from 4-day-old epidermis do not express DEC-205. Chang-Rodriguez demonstrated that neonatal epidermal leukocytes do not express IL-10 receptor till 5 days following birth (Chang-Rodriguez et al. 2004). As a consequence it is unlikely that LC isolated from mice 4 days following birth will have up-regulated DEC-205 in response to IL-10. However as the epidermal network matures there is an up-regulation of DEC-205, possibly as a result of upregulation of IL-10 receptors. This hypothesis is supported by the finding that DEC-205 is present 7 days following birth. By day 14 following birth the level of DEC-205 expression remains constant. It is possible that secretion of IL-10 is down-regulated at this stage in development, enabling up-regulation of APC potency following exposure to antigen.

The absence of DEC-205 and reduced expression of Langerin suggests that during the early neonatal period LC are significantly reduced in their capacity to internalise mannose-conjugated antigen via a receptor-mediated pathway. In contrast receptors

for opsonised antigen are expressed from an early age. What remains unclear is why mannose specific receptors are absent during early development yet opsonin receptors for IgG and C3b are expressed from an early age. This phenomenon may be explained by discussing the nature of antigen and antibody classes present in the extracellular environment during the early developmental period. IgG is capable of crossing through epithelia and traversing the placenta (Adkins et al. 2004b). As such maternal IgG provides immunity for the first few months of life when the newborn is incapable of producing antibodies (Nelson et al. 1994; Kovarik and Siegrist 1998; Siegrist 2001). These immunoglobins can then pass through the intestinal epithelia of the neonate and into the blood stream. Once in the blood stream and subsequently in the tissues, maternal antigen-specific IgG is available to opsonise foreign antigen. Expression of IgG receptors during the neonatal period would be expected to enable responsiveness to maternal IgG complexed antigen. This study has demonstrated that CD16/32, the receptor responsible for IgG recognition (Espositofarese et al. 1995; Galon et al. 1997; Cohen-Solal et al. 2004), is expressed during the early neonatal period, at a level comparable with LC isolated from adult epidermis. Via expression of CD16/32 neonatal LC retain the capacity to internalise opsonised antigen. It is highly possible that via expression of CD16/32 the neonate is protected at a time when their developing immune system is insufficient. Expression of CD16/32 may also aid in the regulation of immune responses as maternal IgG will preferentially bind foreign antigen and not self tissue (Esposito Farese et al. 1995; Fanger et al. 1996).

In a similar fashion as CD16/32, the C3b receptor CD11b is expressed during the developmental period, and may offer additional protection at a time when the neonatal immune system is developing. Microbial surfaces can activate complement via the alternative pathway without the necessity of bound antibody (Koppenheffer 1987). One can propose that if antigens were to penetrate the neonatal epidermal barrier, LC may use complement receptors to bind microbial antigens until the more specific, carbohydrate recognition receptors such as DEC-205 are expressed. An alternative hypothesis is that neonatal LC may utilise complement receptors to maintain tolerance to self-tissue. One of the antigens that can be opsonised by C3b is apoptotic debris, and it is now accepted that uptake of apoptotic bodies via C3b on DC results in antigen-specific immune suppression (Nauta et al. 2004). This is

essential to ensure non-reactivity to self-antigens and its importance following cutaneous UV exposure is well characterised (Aubin 2003). The transition of neonatal epidermis to mature adult skin involves tissue remodelling and formation of new epidermal layers (Haake and Polakowska 1993). As a consequence of this high cell turnover and death, apoptosis is to be expected (Haake and Cooklis 1997; Haake et al. 1997). Expression of receptors for C3b opsonised apoptotic cells ensures that self-tolerance during infancy is maintained, whilst the absence of receptors for mannosylated antigens and LPS ensures that the reactivity to harmless skin commensals is avoided.

Interestingly, analysis of CD11b expression also demonstrated alterations in the staining profile with maturation of the epidermis. At 4 days following birth a single population of MHC-II^{+}CD11b^{+} cells was identified. By day 7 two populations were present, CD11b^{-} and CD11b^{+}. Little evidence exists in the literature on the expression of CD11b in the developing epidermis, however due to a single population of CD11b positive cells in the epidermis at 4 days following birth it is possible that expression of CD11b may correlate with induction of immune suppression. As the epidermal network matures there is an alteration in the expression profile of both MHC-II and CD11b. It is possible that as the epidermis matures there is a transition from foetal/neonatal LC that emigrate into the epidermis to neonatal/adult LC consisting of both CD11b^{+} and CD11b^{-}. As the ability to induce immunity increases with development these results suggest that the functional maturity of the epidermal network is acquired during development. It is not possible to determine from this study if the staining profile from 7 days following birth represents two separate cell populations or a down regulation of expression on a subset of cells during development. However, it is interesting to speculate that CD11b expression is influenced by antigen encountered in the periphery. During the neonatal period antimicrobial substances present on the skin surface combined with a dramatic thickening of the keratin layer, enhance the physical protection provided by the skin. As a consequence it can be proposed that the proportion of potential pathogens gaining access to the underlying epidermis is less compared to adult counterparts. In contast, during the neonatal period there is a greater proportion of self-antigen than potential pathogens in the extracellular environment due to apoptotic debris generated during postnatal modification of the epidermis (as

reviewed by Adkins et al. 2004). As the antigenic composition of the epidermis shifts to mature adult epidermis, possible receptors for self-antigen, such as CD11b are downregulated on a proportion of cells whilst DEC-205 and Langerin become more aparent.

This chapter has furnished further evidence of a divergence in LC function during the early neonatal period, demonstrated by a decreased capacity to recognise mannosylated antigens. It is likely that the external environment is a key factor that drives immune function. During the early neonatal period the infant is subject to a vast array of possible antigens, ranging from innocuous commensal organisms, and self-antigen to potentially deleterious pathogens that manage to breech the thickened keratin layer. If neonatal LC possessed the ability to respond vigorously to all antigens, this would lead to a constant state of hyper-responsiveness. Such responses could lead to tissue and organ damage through acute inflammation as well as progression of autoimmune disorders. Therefore, neonatal LC regulate such responses by the flexible expression of surface receptors. The absence of mannose specific receptors translates to an ability to regulate and hence avoid hyper-responsiveness to skin commensals. In contrast, by expressing receptors for immunoglobins and opsonins translates to an ability to induce immunity to antigens opsonised by maternal antibody and tolerance to self-tissues respectively. As the neonatal immune system evolves in competence the external environment influences the expression of mannose specific receptors, as reflected in the up-regulation of DEC-205 following exposure to antigen on the day of birth. As a consequence, the immune system can maintain low responsiveness under non-threatening conditions but mature responses when the level of danger is high.

In conclusion, this chapter has shown a reduction in both classes of C-type lectin receptors, DEC-205 and Langerin, during the early neonatal period. As a consequence of reduced receptor expression I hypothesise that neonatal LC are associated with a decreased capacity to internalise antigen via a receptor-mediated pathway. As the amount of antigen presented in the context of MHC-II has important implications on the generation of effective immunity (Langenkamp et al. 2002) the pathways utilised to bind and internalise exogenous antigens has important biological implications on the generation of immune suppression.

Chapter 4

Characterisation of Antigen Uptake by Epidermal Langerhans Cells During the Developmental Period.

4.1 Introduction

Epidermal LC possess distinct endocytic pathways that accommodate a range of possible of antigens, including small and large particulate antigens, as well as soluble peptides (Steinman et al. 1999). Soluble antigen is internalised in the fluid phase by non-specific pinocytosis, which can occur via two distinct mechanisms (Watts and Marsh 1992). Small molecules (~0.1 μm) are internalised via clathrin-coated pits (micropinocytosis). Uptake of larger vesicles (0.5-3 μm) is mediated by cytoskeleton-dependent membrane ruffling (macropinocytosis) (Watts and Marsh 1992). Macropinocytosis enables LC to engulf a large volume of fluid, from which soluble antigens are then concentrated for processing and presentation or exocytosis (Sallusto et al. 1995).

Small particulate antigens can be internalised via receptor-mediated endocytosis, which involves uptake of antigen following recognition by membrane receptors (Slepnev and De Camilli 2000). Motifs that can be recognised by uptake receptors include activated complement components (i.e. CD11b/CD18), IgG complexed antigens (CD16/32) and terminal mannose residues (i.e. Langerin and DEC-205) (Reis et al. 1993; Esposito Farese et al. 1995; Fanger et al. 1996; Kato et al. 1998; Valladeau et al. 2003). Antigens too large to be internalised via receptor-mediated endocytosis can be engulfed via phagocytosis (Reis et al. 1993). Phagocytosis is the ATP-dependent, cytoskeleton-mediated ingestion of antigen following binding of microbial peptides to LC surface receptors (Reis et al. 1993). Phagocytic receptors are unique in their capacity to differentiate between self and prokaryotic antigens, including LPS and peptidoglycan (Aderem and Underhill 1999; Takeuchi et al. 1999; Aderem and Ulevitch 2000; Werts et al. 2001). Following receptor binding and phagocytosis, a cascade of events is initiated that leads to augmentation of the immune response by inducing secretion of pro-inflammatory cytokines and up-regulation of costimulatory molecules (Winzler et al. 1997).

Chapter 3 demonstrated that LC from 4-day-old epidermis lack expression of the mannose-binding receptor DEC-205 and express reduced levels of Langerin when compared to adult counterparts. These results suggest that during the early neonatal period the capacity of LC to uptake antigen via a mannose-type receptor-mediated pathway is diminished. The central aims of this chapter were to assess the acquisition of endocytic function during development and to determine if a correlation exists between expression of antigen uptake receptors and *in vitro* antigen uptake. To do so the uptake of soluble, and small and large particulate antigens was assessed in neonatal, juvenile and adult mice.

4.2 Results

4.2.1 Characterisation of receptor-mediated endocytosis during the developmental period.

Fluorescein isothiocyanate-dextran (FITC-dextran) is a widely used fluorescent antigen for the study of receptor-mediated endocytosis. It is believed that FITC-dextran is internalised via mannose-type receptors as pre-incubation of cells with bacterial monosaccharides such as mannose competitively inhibit uptake (Reis et al. 1993; Lutz et al. 1997). FITC-dextran was employed in this chapter to determine the capacity of LC to internalise antigen via a receptor-mediated pathway during the developmental period.

4.2.1.1 Identification of FITC-dextran uptake by adult MHC-II$^+$ epidermal LC.

In order to evaluate the capacity of LC to internalise antigen via a receptor-mediated pathway it was first necessary to develop an appropriate gating strategy to identify LC in epidermal suspensions. Epidermal suspensions were prepared from 6-week-old epidermis and incubated at 37°C with FITC-dextran. At set time points, a sample of suspension was removed, washed extensively to remove free antigen and LC identified via staining with anti-MHC-II antibody. The fluorescence intensity of epidermal cells was then assessed via flow cytometry.

The strategy for gating viable cells outlined in chapter 3.2.1 was employed to identify viable MHC-II$^+$ in adult epidermal suspensions. Briefly, to select for viable cells, a gate was drawn around the live, 7-AAD$^-$ population on a 7AAD versus cell size dot plot. These viable cells were further gated to identify MHC-II$^+$ cells in a PE-MHC-II fluorescence intensity versus forward scatter dot plot. The viability gate was used to construct a third dot plot of MHC-II-PE versus FITC-dextran fluorescent intensity (Figure 4.1 a). Figure 4.1 (a) demonstrates a single MHC-II$^+$ FITC-dextran$^+$ population present at each time point during incubation. It must also be noted that a population of MHC-II$^-$ cells are also positive for FITC-dextran. This population is most likely to be keratinocytes. As keratinocytes do not express mannose specific-receptors, it is possible that the FITC-dextran fluorescence demonstrated by these cells is either a consequence of antigen associating with the cell membrane in a non-specific manner, or that keratinocytes are able to internalsie FITC-dextran in the fluid phase. To determine the FITC-dextran fluorescence associated with this population a second gate was constructed. This gate was used to create a histogram of cell count versus FITC-dextran fluorescence intensity (Figure 4.1 (b). Using CellQuest™ analysis the mean fluorescence intensity of each peak can be determined, and is shown in parentheses. Figure 4.1 (b) demonstrates a time dependent increase in the FITC-dextran fluorescence associated with MHC-II$^+$ LC. These results show uptake of antigen by LC in a time dependent manner.

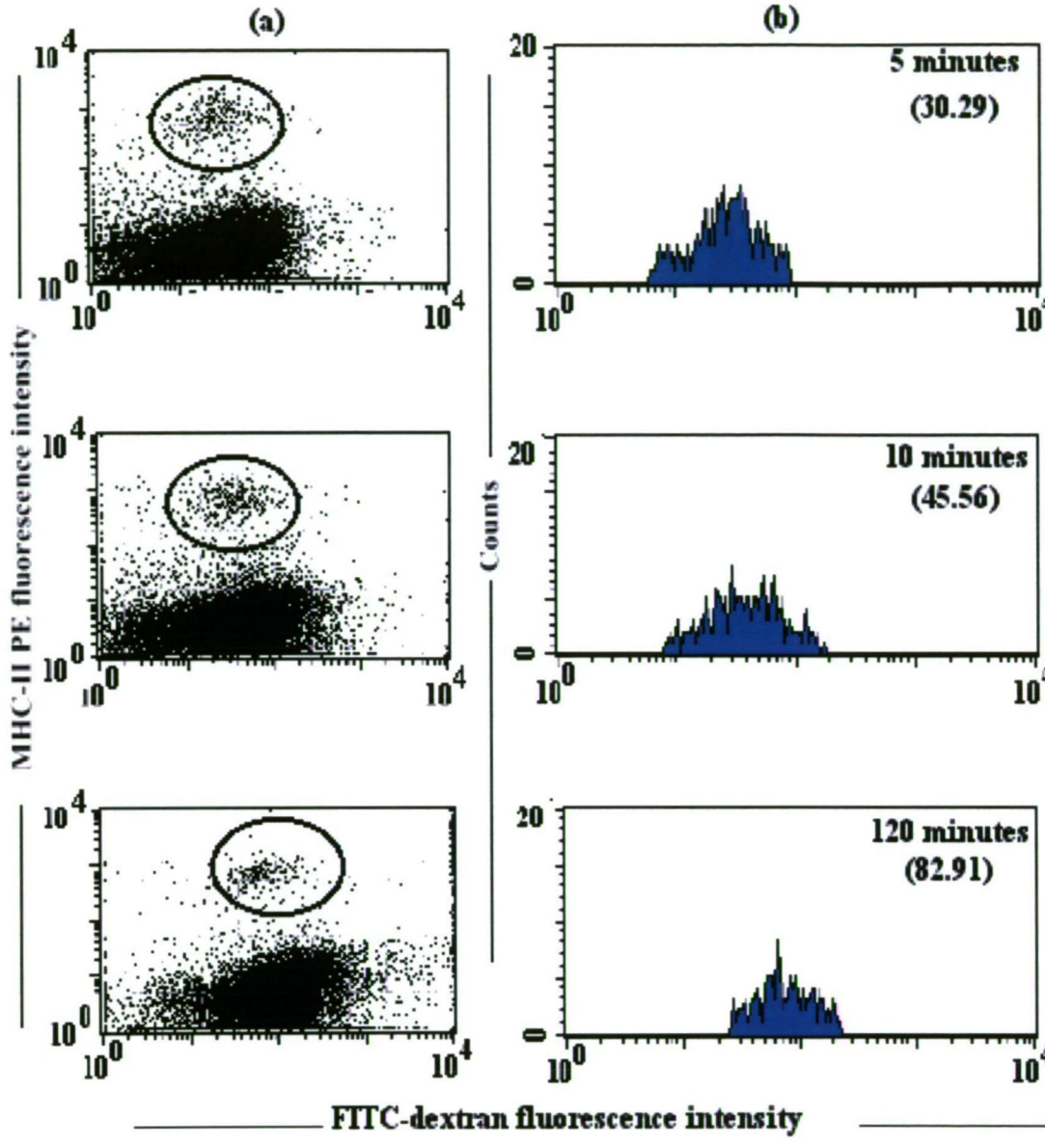

Figure 4.1 Identification of FITC-dextran uptake by MHC-II$^+$ LC in adult epidermal suspensions. Epidermal suspensions were prepared from 6-week-old mice and incubated with FITC-dextran at 37˚C. At 5 minutes (top panel), 10 minutes (middle panel) and 120 minutes (bottom panel) cells were removed, washed and stained for MHC-II expression. Using CellQuest software a gate was drawn around each FITC-dextran$^+$MHC-II$^+$ population (a). This gate was used to construct a histogram of FITC-dextran fluorescence intensity versus cell count (b). FITC-dextran mean fluorescence intensity is shown in parenthesis at the top right hand corner of each histogram. Data is representative of three or more separate experiments.

4.2.1.2 Uptake of FITC-dextran by epidermal MHC-II$^+$ LC during the developmental period

To assess the capacity of LC to internalise antigen via a mannose-type receptor-mediated pathway during the developmental period, epidermal suspensions from 4-, 7- and 14-day- and 6-week-old mice were incubated at 37°C and 4°C with FITC-dextran. The 4°C sample was included as an indication of non-specific or non-metabolically active binding of antigen to cells. Figure 4.2 demonstrates that LC isolated at each age point were capable of accumulating FITC-dextran, demonstrated by a time dependent increase in FITC-dextran fluorescence at 37°C. It was also evident that there were alterations in fluorescence intensity at 37°C with age of the animal. The fluorescence intensity associated with LC isolated from 6-week-old mice was greater than LC from 4-day-old mice, however the intensity at 4°C was also significantly higher (Figure 4.3).

Figure 4.2 Uptake of FITC-dextran by MHC-II$^+$ LC during development. *In vitro* uptake of FITC-dextran at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) following birth. The filled squares represent the fluorescence intensity at 37°C. The open squares represent the fluorescence intensity at 4°C. Data is representative of two or more experiments.

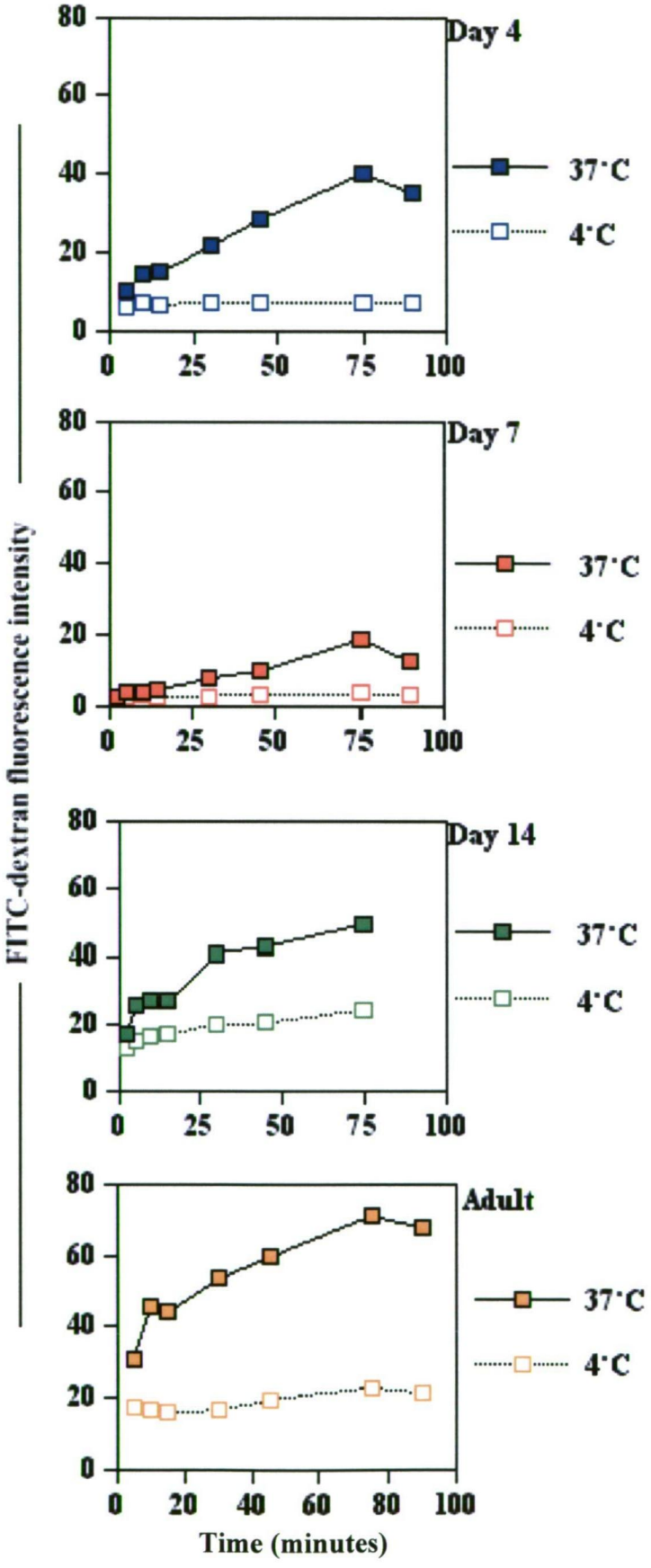

Figure 4.2 Uptake of FITC-dextran by MHC-II$^+$ LC during development

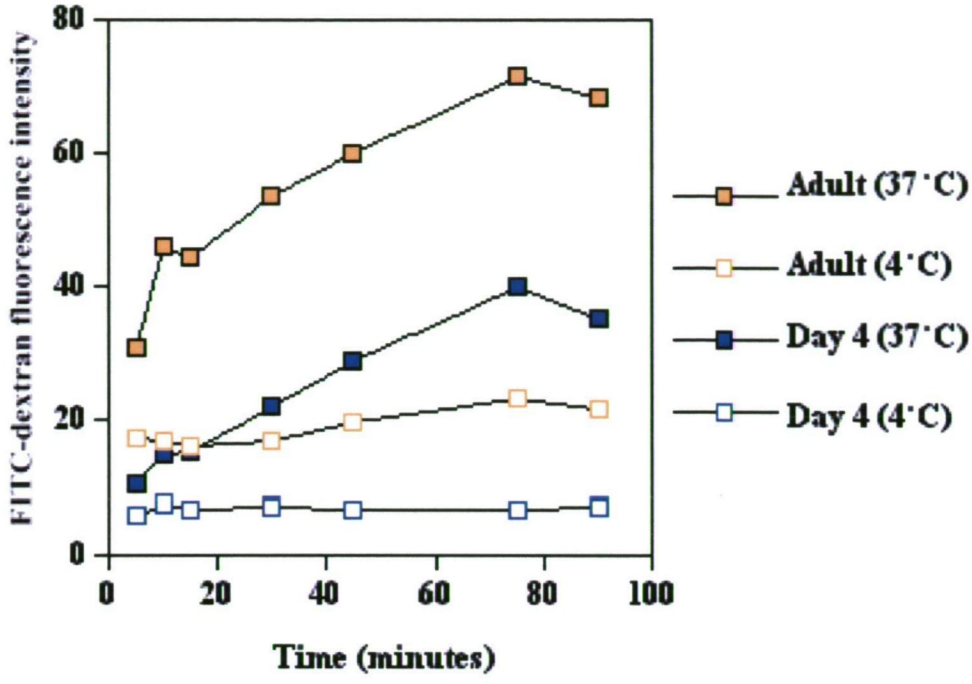

Figure 4.3 Uptake of FITC-dextran by MHC-II$^+$ LC isolated from 4-day and 6-week-old mice. *In vitro* uptake of FITC-dextran via receptor-mediated endocytosis at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. The filled squares represent the fluorescence intensity at 37°C. The open squares represent the fluorescence intensity at 4°C. Data is representative of two or more experiments.

4.2.1.3 Ratio data of FITC-dextran uptake by MHC-II$^+$ epidermal LC during the developmental period.

In order to determine metabolically active uptake of FITC-dextran it was necessary to account for fluctuations in fluorescence intensity at 4°C. To account for these variations, the fluorescence intensity at 37°C was divided by the intensity at 4°C, generating a ratio at each time point (Figure 4.4). When ratio values were calculated it was evident that in comparison to LC isolated from 6-week-old epidermis, LC from 4-day-old epidermis continued to internalise antigen over a longer period of time. It can be seen from the ratio data that the end point of antigen uptake is reduced with age, as LC from 4-day-old mice reached a higher ratio value than 6-week-old mice.

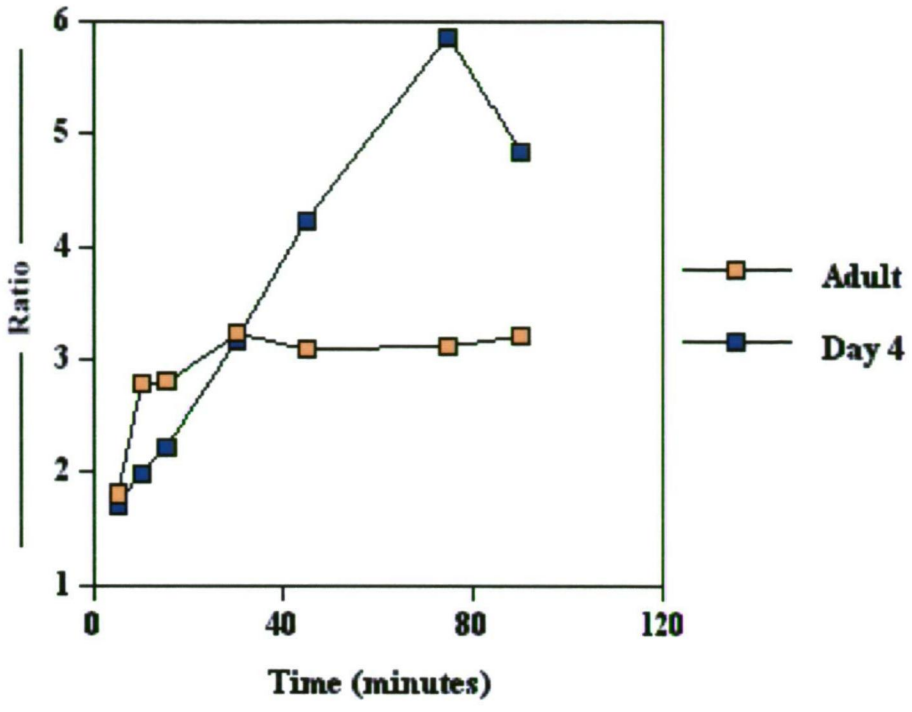

Figure 4.4 Ratio data for uptake of FITC-dextran by MHC-II$^+$ LC during development. *In vitro* uptake of FITC-dextran at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. To account for any variations in non-specific or receptor binding of the antigen at 4°C, the mean fluorescence intensity at 37°C was divided by the mean fluorescence at 4°C, generating a ratio at each time point. To directly compare differences between the different age points, ratio data was calculated for each time point for each age group. As shown, LC from 4-day-old mice took longer to reach maximum uptake compared with adult LC. Each value is representative of three separate experiments.

4.2.2 Characterisation of macropinocytosis during the developmental period.

Lucifer Yellow (LY) was utilised to assess the capacity for macropinocytosis during the developmental period. It has been previously demonstrated that pre-incubation with mannose and mannan does not inhibit uptake of LY, and as such LY is accepted to be a definitive tracer for fluid phase macropinocytosis (Sallusto et al. 1995).

4.2.2.1 Uptake of Lucifer Yellow by epidermal MHC-II$^+$ LC during the developmental period

To assess the capacity of LC to internalise antigen via macropinocytosis during the developmental period, epidermal suspensions from 4-, 7- and 14-day- and 6-week-old mice were incubated with LY at 37°C and 4°C. Using the same gating protocol outlined in 4.1, LY fluorescence intensity was determined at each time point for all age groups. Figure 4.5 demonstrates that LC isolated at each age point were capable of accumulating LY, supported by a time-dependent increase in LY fluorescence at 37°C. It was also evident that there was an increase in fluorescence intensity at 37°C with age of the animal. Figure 4.6 demonstrates that the fluorescence intensity associated with LC isolated from 6-week-old mice was greater than LC from 4-day-old mice, however the intensity at 4°C was also significantly higher.

Figure 4.5 Uptake of Lucifer Yellow by MHC-II$^+$ LC during development. *In vitro* uptake of Lucifer Yellow at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) following birth. The filled squares represent Lucifer Yellow fluorescence intensity at 37°C. The open squares represent the Lucifer Yellow fluorescence intensity at 4°C. Data is representative of two or more experiments.

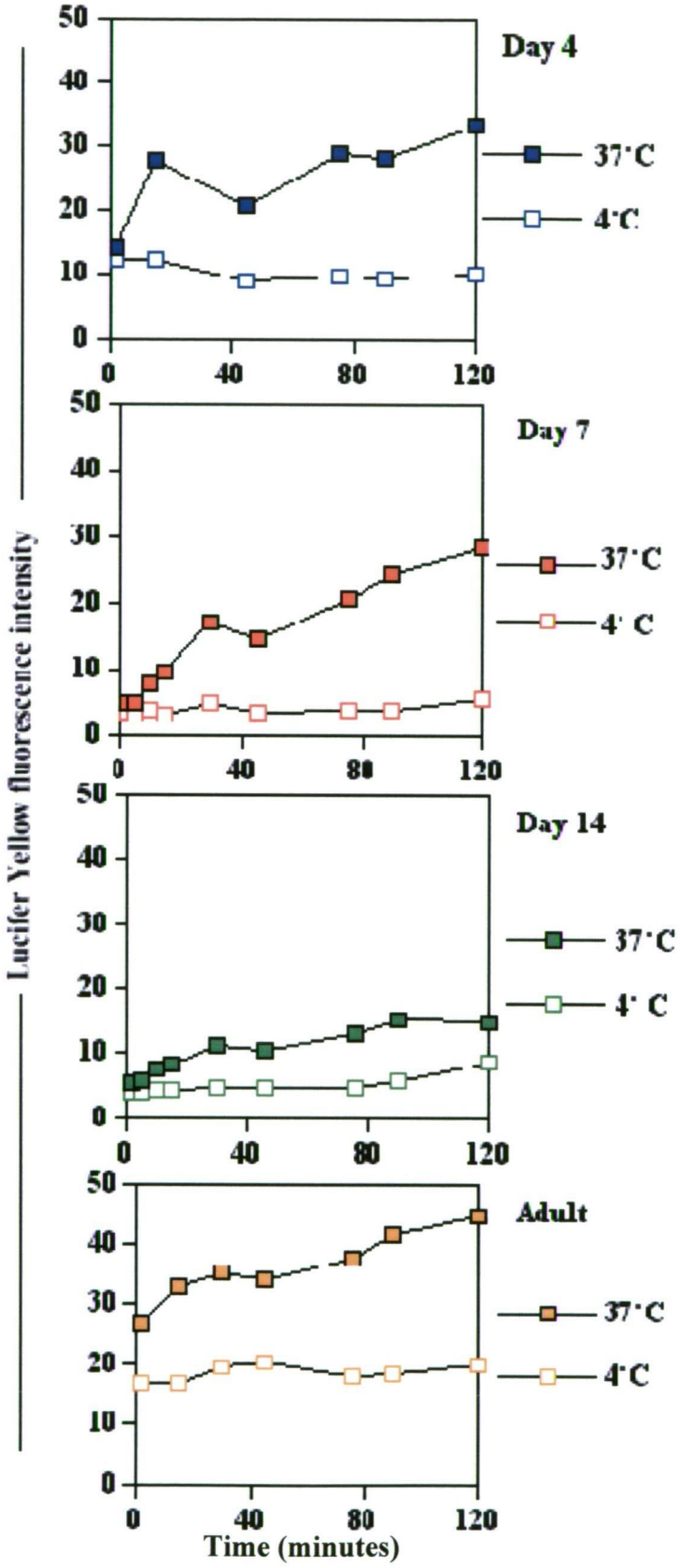

Figure 4.5 Uptake of Lucifer Yellow by MHC-II$^+$ LC during development

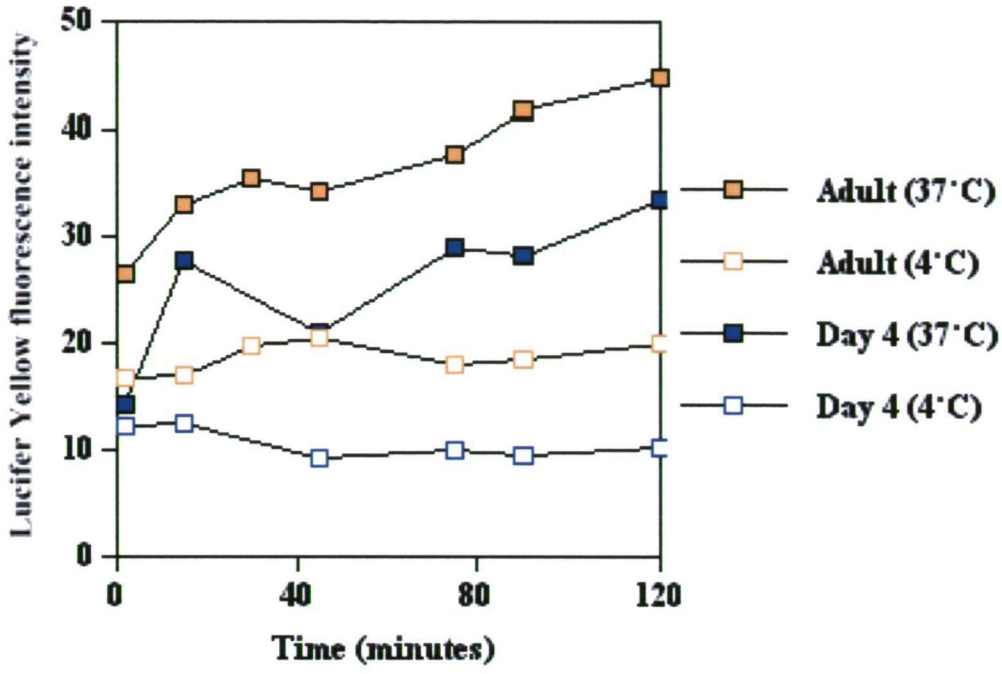

Figure 4.6 Uptake of Lucifer Yellow by MHC-II$^+$ LC isolated from 4-day and 6-week-old mice. *In vitro* uptake of Lucifer Yellow at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. The filled squares represent the fluorescence intensity at 37°C. The open squares represent the fluorescence intensity at 4°C. Data is representative of two or more experiments.

4.2.2.2 Ratio data of Lucifer Yellow uptake by epidermal MHC-II$^+$ LC during the developmental period.

To account for variations in antigen binding at 4°C, LY fluorescence intensity at 37°C was divided by the intensity at 4°C. This generated a ratio value for both age groups throughout the assay (Figure 4.7). When ratio values were calculated it was evident that whilst the initial rate of uptake was comparable between neonatal and adult LC, neonatal LC continued to internalise antigen over a longer period of time. This translated to a higher ratio value at end point compared to adult counterparts, with LC from 4-day-old mice obtaining a higher ratio value than LC from 6-week-old mice.

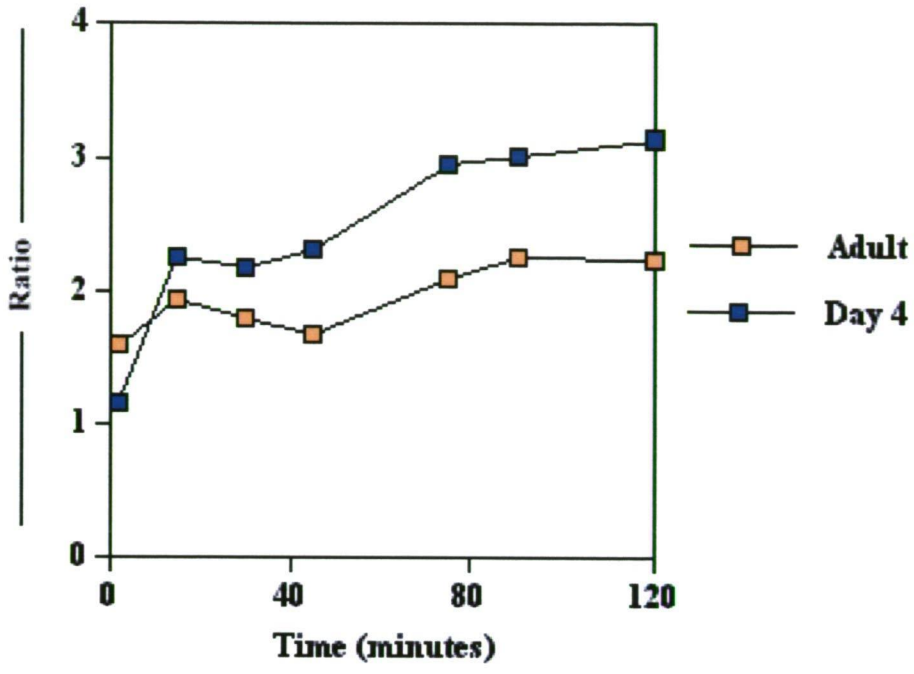

Figure 4.7 Ratio data for uptake of Lucifer Yellow by MHC-II$^+$ LC during development. *In vitro* uptake of Lucifer Yellow via at 37˚C and 4˚C was assessed in epidermal cells isolated from mice at 4 days, 7 days and 6 weeks following birth. To account for any variations in non-specific or receptor binding of the antigen at 4˚C, the mean fluorescence intensity at 37˚C was divided by the mean fluorescence at 4˚C, generating a ratio at each time point. To directly compare differences between the different age points, ratio data was calculated fir each point for each time point, as shown LC from 4-day-old mice took longer to reach maximum uptake compared with LC isolated from 6-week-old mice. Each value is representative of three separate experiments.

4.2.3 Effect of mannan, mannose and wortmannin on the uptake of FITC-dextran by MHC-II$^+$ epidermal LC during the developmental period.

To ascertain the pathway employed to internalise FITC-dextran, FITC-dextran uptake assays were repeated in the presence of inhibitors of receptor-mediated endocytosis and macropinocytosis. To competitively block antigen uptake via mannose-type receptors, LC from 4-day- and 6-week-old mice was pre-incubated with the bacterial monosaccharides mannan and mannose. To inhibit fluid phase uptake, cells were incubated with the phosphatidylinositol 3-kinase inhibitor wortmannin. Figure 4.8 is a representative of the percent decrease in FITC-dextran uptake in various conditions, calculated as a percent when compared to epidermal suspensions incubated with antigen in the absence of inhibitor. When LC were pre-incubated with mannan and mannose it was evident that FITC-dextran uptake by LC from 6-week-old mice was inhibited to a greater extent than LC from 4-day-old mice, with mannan associated with a greater inhibitory effect than mannose. It was also evident that the percent decrease was grater at 37°C than 4°C. In contrast, wortmannin inhibited FITC-dextran internalisation to a greater extent in LC from 4-day-old mice than from 6-week-old mice. Consequently, neonatal LC rely to a greater extent on macropinocytosis than endocytosis. In contrast, adult LC rely on endocytosis to a greater extent than macropinocytosis

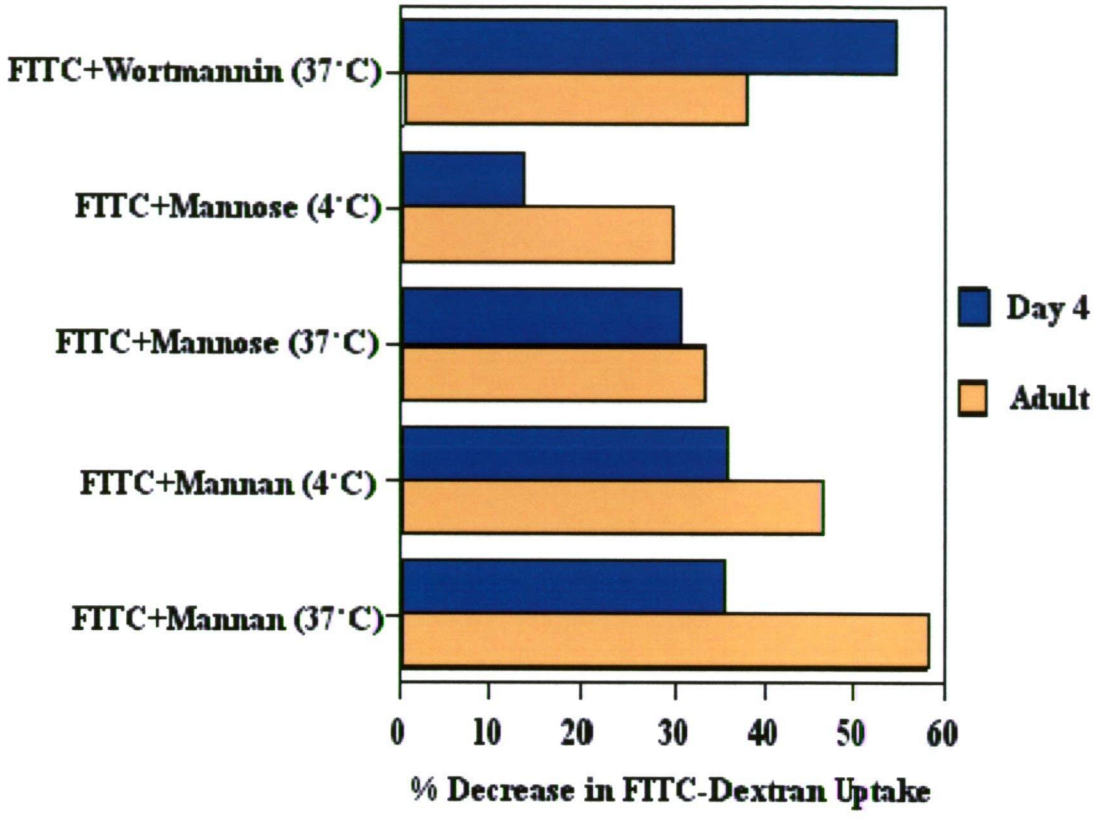

Figure 4.8 The effect of mannan, mannose and wortmannin on the uptake of FITC-dextran by adult and day 4 MHC-II$^+$ LC. The *in vitro* uptake of FITC-dextran in the continuous presence of mannan, mannose and wortmannin was assessed in epidermal cells isolated from mice at 4 days (filled squares) and 6 weeks (open squares) following birth. Cells were pre-incubated with inhibitors at either 37°C or 4°C prior to incubation with FITC-dextran at 37°C. The percent inhibition in uptake was calculated as the percentage decrease of FITC-dextran mean fluorescence intensity at 37°C, in the presence of inhibitors, compared with cells that were not pre-incubated with inhibitors. Data is representative of two separate experiments.

4.2.4 Characterisation of phagocytosis during the developmental period

To assess the potential of LC to phagocytose particulate antigen during the developmental period two fluorescently labelled particulate antigens were used; BODIPY-labelled Zymosan A and FITC-labelled *E.coli*. To investigate the effect of opsonisation on internalisation, particles were incubated with antigen-specific polyclonal IgG prior to incubation with epidermal suspensions.

4.2.4.1 Phagocytosis of Zymosan by MHC-II$^+$ epidermal LC during the developmental period

To characterise the capacity of LC to recognise and phagocytose particulate antigen via mannose-specific receptors epidermal suspensions from 4-, 7-, 14-day- and 6-week-old mice were incubated at 37°C and 4°C with the fluorescently labelled cell wall derivative of *Saccharomyces cerevisiae* (Zymosan A). The 4°C sample was included as an indication of non-specific or non-metabolically active uptake. Figure 4.9 demonstrates that in contrast to receptor-mediated endocytosis, LC internalised very little antigen via phagocytosis, evident as a slight increase in fluorescence intensity with time. Results also indicate that the level of phagocytosis remains constant with development (Figure 4.10). It must also be noted that due to fluctuations in binding of antigen at 4°C the ratio plot appeared variable.

Figure 4.9 Uptake of Zymosan by MHC-II$^+$ LC during development. *In vitro* uptake of Zymosan at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) following birth. The filled squares represent Zymosan-BODIPY fluorescence intensity at 37°C. The open squares represent Zymosan-BODIPY fluorescence intensity at 4°C. Data is representative of two or more experiments.

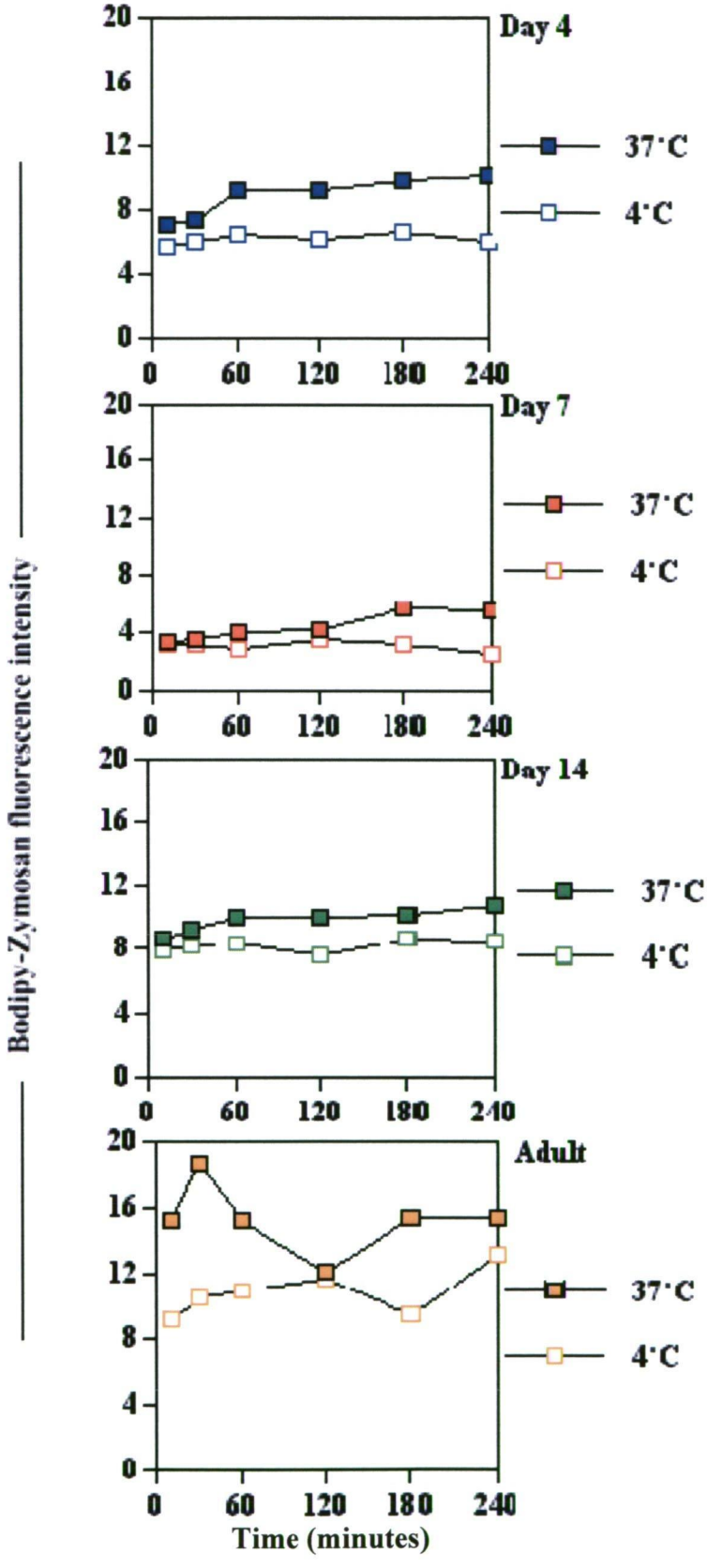

Figure 4.9 Uptake of Zymosan by MHC-II$^+$ LC during development

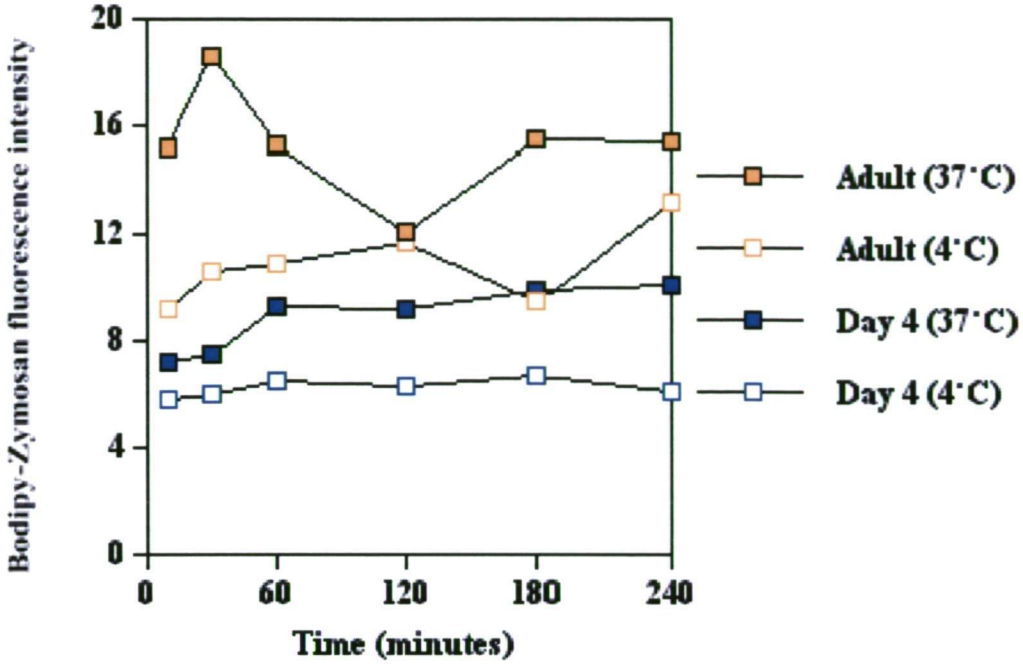

Figure 4.10 Uptake of Zymosan by MHC-II$^+$ LC isolated from 4-day and 6-week-old mice. *In vitro* uptake of Zymosan at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. The filled squares represent the fluorescence intensity at 37°C. The open squares represent the fluorescence intensity at 4°C. Data is representative of two or more experiments.

4.2.4.2 Ratio data of Zymosan uptake by epidermal MHC-II$^+$ LC during the developmental period.

To account for variations in antigen binding at 4°C, BODIPY fluorescence intensity at 37°C was divided by the intensity at 4°C. This generated a ratio value for both age groups throughout the assay (Figure 4.11). When ratio values were calculated it was evident that LC were associated with a low phagocytic capacity, evident as a slight increase in ratio values over the assay.

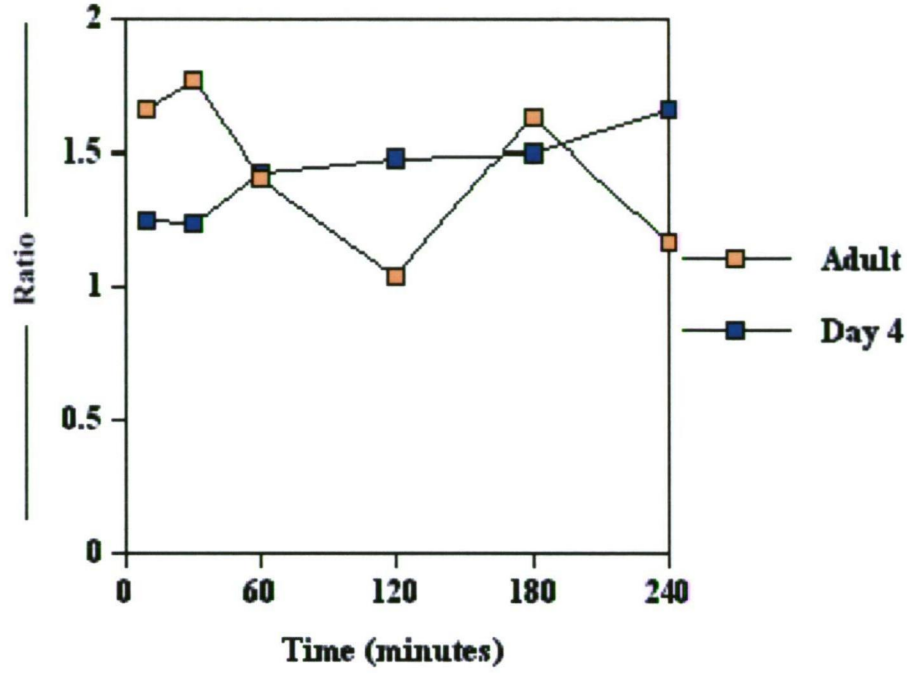

Figure 4.11 Ratio data for uptake of Zymosan by MHC-II$^+$ LC during development. *In vitro* uptake of Zymosan at 37˚C and 4˚C was assessed in epidermal cells isolated from mice at 4 days, 7 days and 6 weeks following birth. To account for any variations in non-specific or receptor binding of the antigen at 4˚C, the mean fluorescence intensity at 37˚C was divided by the mean fluorescence at 4˚C, generating a ratio at each time point. To directly compare differences between the different age points, ratio data was calculated fir each point for each time point, as shown LC from 4-day-old mice took longer to reach maximum uptake compared with adult LC. Each value is representative of three separate experiments.

4.2.4.3 Phagocytosis of opsonised Zymosan by MHC-II$^+$ epidermal LC during the developmental period

To examine whether opsonisation increased the efficiency of phagocytosis, epidermal preparations prepared from 4-, 7-, 14-day and 6-week-old mice were pulsed with IgG-complexed Zymosan *in vitro.* Figure 4.12 demonstrates that there was no difference in the phagocytic capacity with age of the animal. LC isolated from 4-day-old mice were associated with the same profile of uptake as LC isolated from 6-week-old mice, indicating that the phagocytic potential of LC remained constant with age (Figure 4.13). Although LC express moderate levels of the receptor CD16/32, known to recognise IgG, opsonisation did not increase the amount of antigen internalised *in vitro.*

Figure 4.12 Uptake of opsonised Zymosan by MHC-II$^+$ LC during development. *In vitro* uptake of opsonised Zymosan at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days (top panel), 7 days (second top panel), 14 days (second bottom panel) and 6 weeks (bottom panel) following birth. The filled squares represent Zymosan-BODIPY fluorescence intensity at 37°C. The open squares represent Zymosan-BODIPY fluorescence intensity at 4°C. Data is representative of two or more experiments

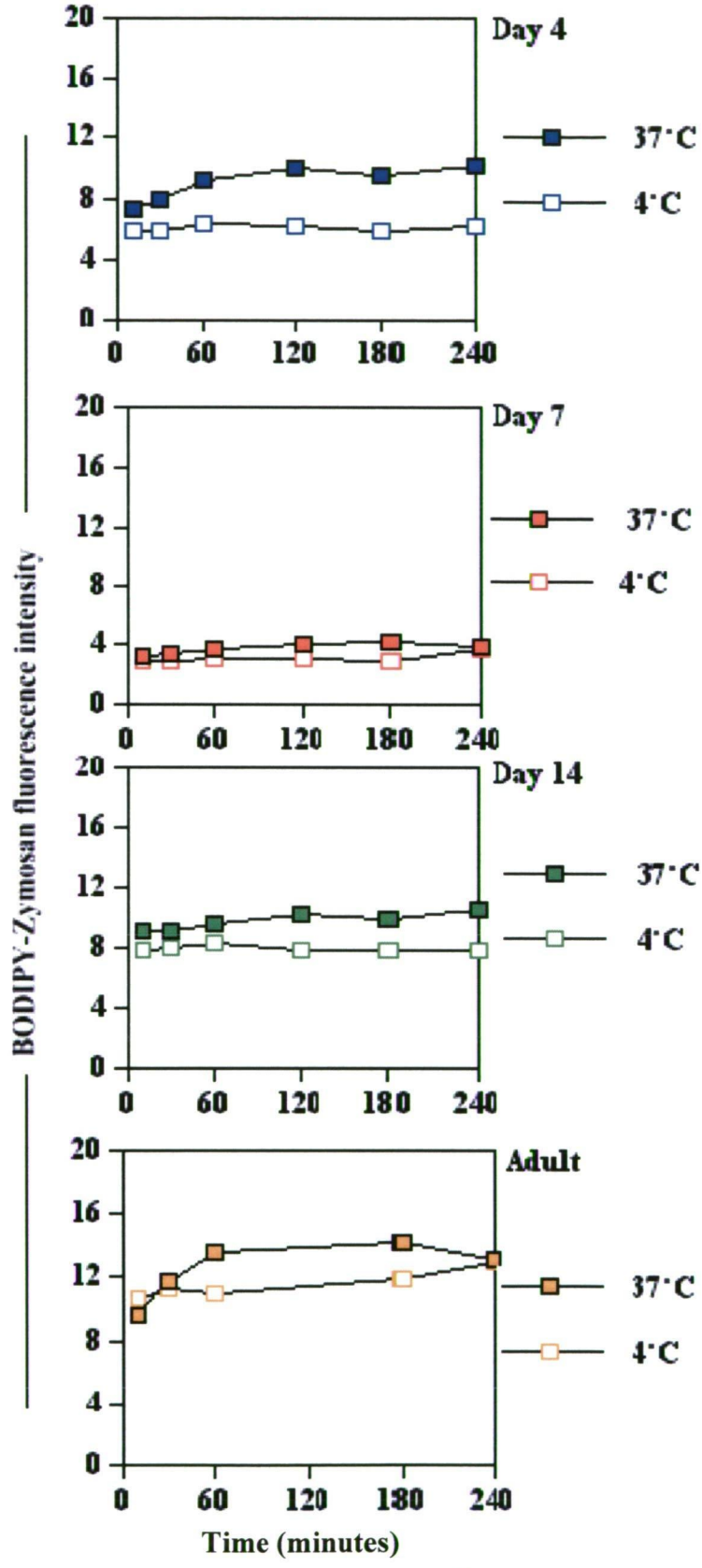

Figure 4.12 Uptake of opsonised Zymosan by MHC-II$^+$ LC during development

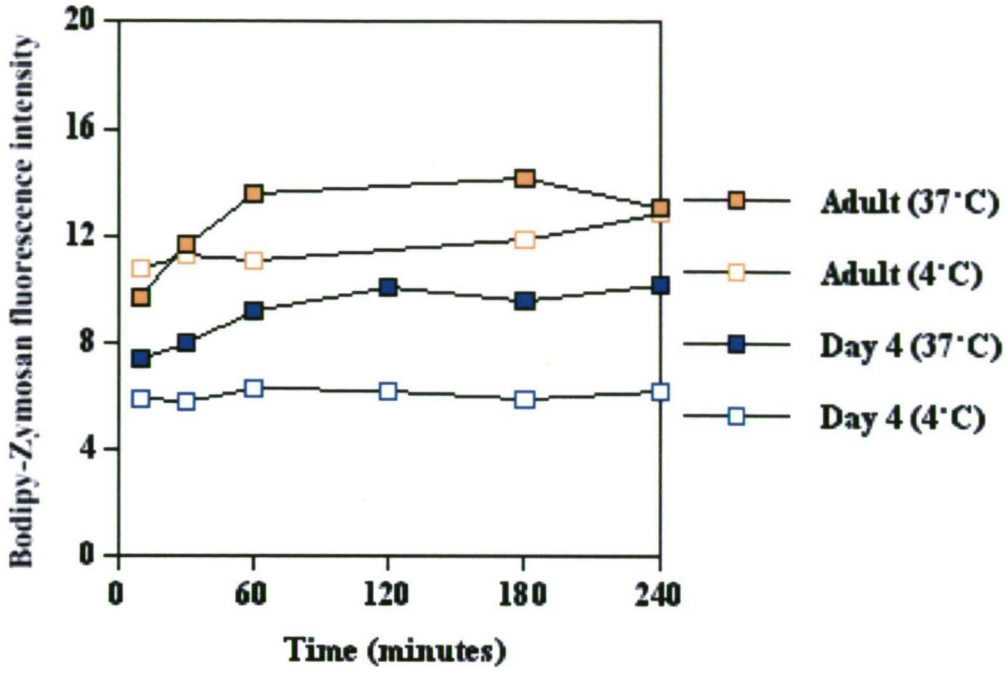

Figure 4.13 Uptake of opsonised Zymosan by MHC-II$^+$ LC isolated from 4-day and 6-week-old mice. *In vitro* uptake of opsonised Zymosan at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. The filled squares represent the fluorescence intensity at 37°C. The open squares represent the fluorescence intensity at 4°C. Data is representative of two or more experiments.

4.2.4.4 Ratio data Zymosan uptake by epidermal MHC-II$^+$ LC during the developmental period.

To account for variations in antigen binding at 4°C, BODIPY fluorescence intensity at 37°C was divided by the intensity at 4°C. This generated a ratio value for both age groups throughout the assay (Figure 4.14). When ratio values were calculated it was evident that LC were associated with a low phagocytic capacity, evident as a slight increase in ratio values over the assay.

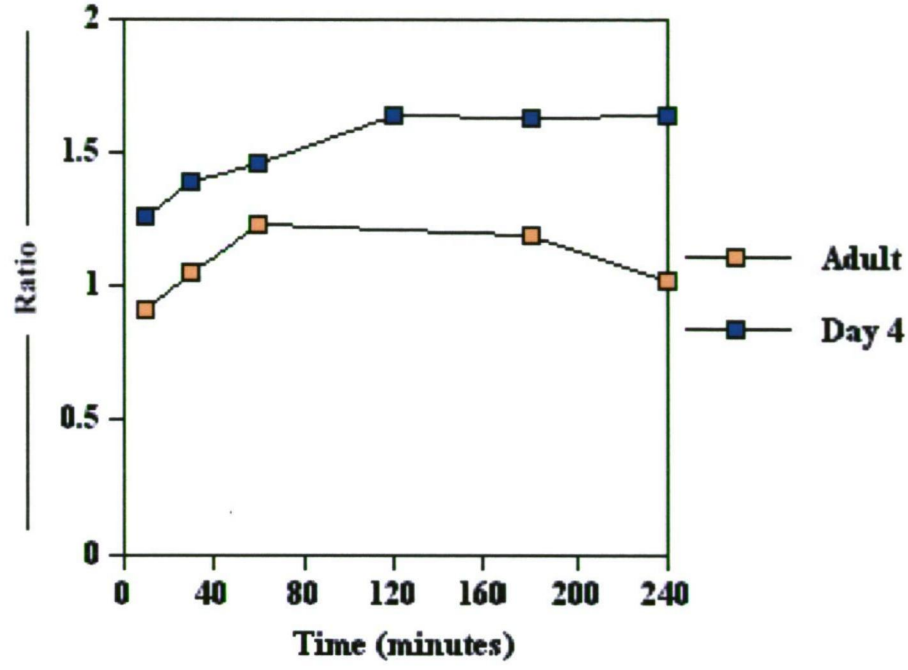

Figure 4.14 Ratio data for uptake of opsonised Zymosan by MHC-II$^+$ LC during development. *In vitro* uptake of opsonised Zymosan at 37˚C and 4˚C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. To account for any variations in non-specific or receptor binding of the antigen at 4˚C, the mean fluorescence intensity at 37˚C was divided by the mean fluorescence at 4˚C, generating a ratio at each time point. To directly compare differences between the different age points, ratio data was calculated fir each point for each time point, as shown LC from 4-day-old mice took longer to reach maximum uptake compared with adult LC. Each value is representative of three separate experiments.

4.2.4.5 Phagocytosis of E. coli by MHC-II$^+$ epidermal LC during the developmental period

To further characterise the potential for phagocytosis, LC isolated from 4-day- and 6-week-old epidermis were first incubated with fluorescently labelled *E. coli.* Results demonstrate only a slight increase in fluorescence intensity with time, suggesting that epidermal LC were weakly phagocytic for uptake of *E.coli* (Figure 4.15 (a). In a similar fashion to Zymosan uptake, fluctuations in binding of antigen at 4°C resulted in variable ratio values over time.

To determine if LC can bind and internalise opsonised *E. coli*, LC were incubated with IgG-coated particles. Figure 4.15 (b) demonstrates little difference in fluorescence intensity with time, suggesting that at each age point LC were weakly phagocytic for opsonised antigen.

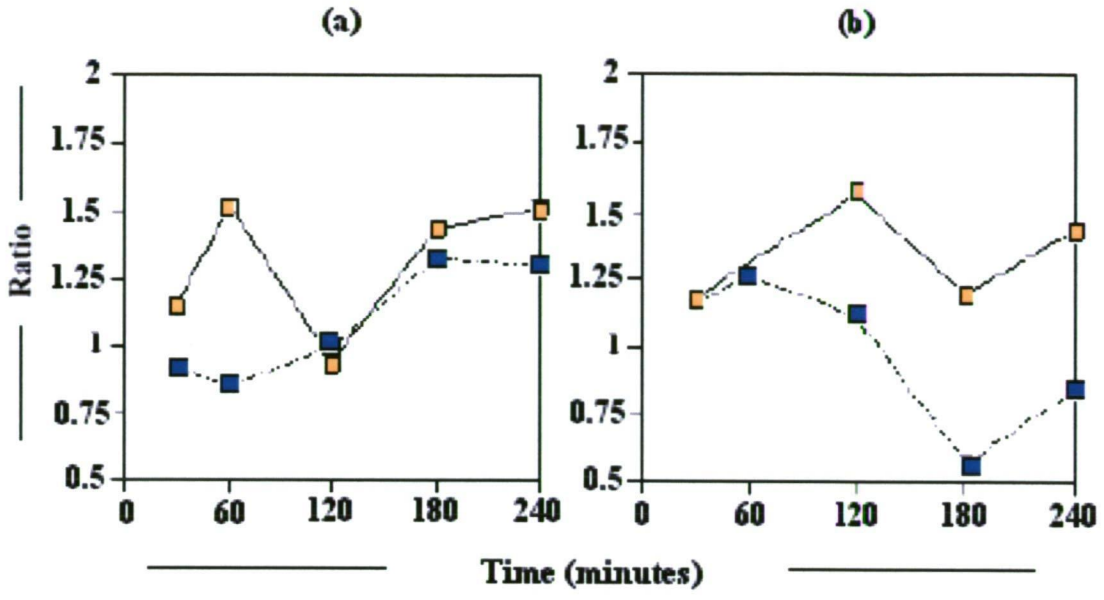

Figure 4.15 Uptake of *E.coli* (a) and opsonised *E.coli* (b) by MHC-II$^+$ LC isolated from 4-day and 6-week-old mice. *In vitro* uptake of *E.coli* at 37°C and 4°C was assessed in epidermal cells isolated from mice at 4 days and 6 weeks following birth. The blue squares represent the fluorescence intensity associated with LC isolated from 4-day-old epidermis. The orange squares represent the fluorescence intensity associated with LC isolated from 6-week-old epidermis. Data is representative of two or more experiments.

4.2.4.6 Phagocytosis of E. coli by adult MHC-II$^+$ spleen cells

To identify phagocytosis, FITC-*E.coli* particles were incubated with whole spleen suspensions at 37°C and 4°C. Within the splenic population are macrophages, previously demonstrated to possess high phagocytic capacity. Figure 4.16 (a) is representative of uptake at 37°C whilst panel (b) demonstrates the degree of binding of antigen at 4°C. It is evident that at 37°C there were two populations of FITC$^+$ cells in spleen suspensions that were absent from the profile of binding at 4°C. The first MHC-II-PE bright population was associated with a low FITC fluorescence intensity and is designated FITC^{lo}MHC-II^{hi}. The second MHC-II-PE bright population was associated with a high FITC fluorescence intensity and is designated FITC^{hi}MHC-II^{hi}. As both populations were absent from the 4°C profile this is suggestive of metabolically active *E. coli* phagocytosis. As there are two populations of FITC$^+$ cells this suggests variable phagocytic activity within the splenic population. Although phagocytosis of *E.coli* or Zymosan by epidermal LC was not evident in 4.2.4.4, the demonstration of internalisation of *E.coli* by spleen cells validates this protocol as a reliable assay of phagocytosis.

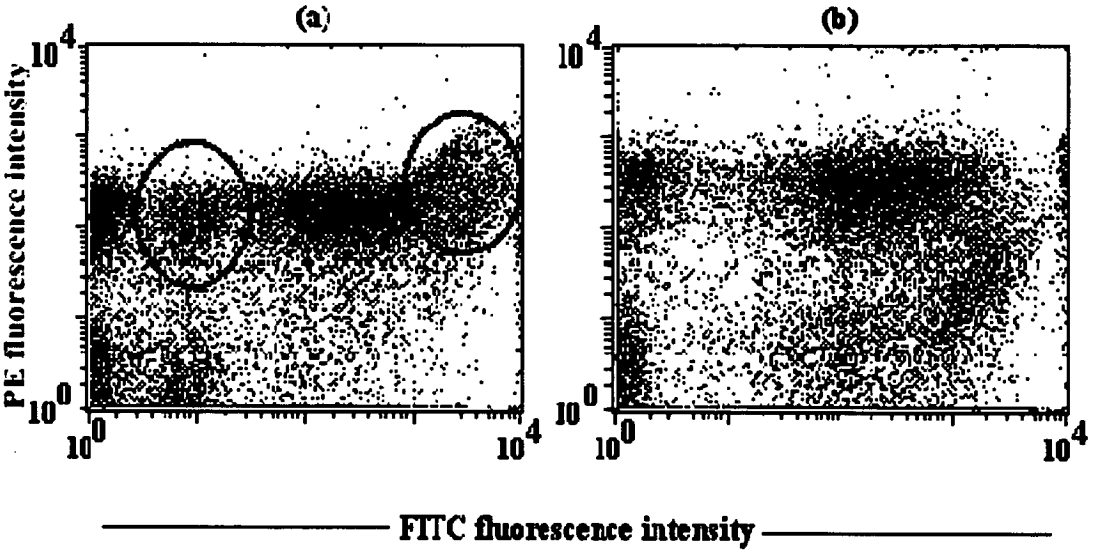

Figure 4.16 Uptake of *E.coli* by adult spleen cells. *In vitro* uptake of *E.coli* at 37°C (a) and 4°C (b) was assessed in spleen cells isolated from mice 6 weeks following birth. Note the FITC-*E.coli*$^+$ population (circled) that is present in (a) and not in (b). This population represents cells that had internalised the antigen.

4.3 Discussion

To enhance the efficacy of mannose recognition and internalisation, LC express members of the C-type lectin family of receptors, including DEC-205 and Langerin (Kato et al. 1998; Kato et al. 2000; Valladeau et al. 2000; Valladeau et al. 2003). DEC-205 has been suggested to initiate uptake and strongly enhance presentation of mannose-conjugated antigens, and an absence of lectin receptors may impact on the ability of LC to scavenge for antigen (Reis et al. 1993; Kato et al. 1998). LC resident in 3-day-old epidermis lack expression of DEC-205 and demonstrate a reduction in MHC-II$^+$ dendritic processes (Dewar et al. 2001). If antigen is applied to neonatal epidermis there is a deviation in the immune response from immunity to antigen-specific immune suppression (Dewar et al. 2001; Simpson et al. 2003). It has been proposed that a contributing factor in the induction of suppression was the decreased ability of epidermal LC to trap and internalise antigen via a receptor-mediated pathway (Dewar et al. 2001). The findings from Chapter 3 furnished evidence supporting this hypothesis, with Langerin expression on LC from 4-day-old epidermis significantly reduced compared to adult LC. A gradual acquisition of receptor expression occurred and, by day 14 following birth, the expression of Langerin was equivalent to that found within adult epidermis. These results indicate that LC resident in neonatal epidermis mature during development.

The delay in surface expression of DEC-205 and Langerin on neonatal LC suggests that these cells would have a reduced capacity to internalise antigen via a receptor-mediated pathway. To address this hypothesis the *in vitro* uptake of fluorescently labelled FITC-dextran was assessed in LC isolated from neonatal, juvenile and adult epidermis. When analysing the fluorescence profile at 4°C it was noted that LC isolated from 6-week-old epidermis were associated with greater fluorescence intensity than LC from 4-day-old epidermis. As metabolism is inhibited at 4°C, the observed increase in fluorescence associated with adult LC was due to either non-specific binding of antigen or binding via specific receptors, rather than by internalised antigen. The latter hypothesis is highly feasible as immuno-phenotyping data presented in Chapter 3 clearly demonstrated that adult LC express significantly

more mannose-type receptors than neonatal LC. At 37°C both adult and neonatal LC were capable of antigen uptake, evident by a time-dependent increase in FITC-dextran fluorescence intensity. It was also clear that the magnitude of fluorescence differed significantly, with LC isolated from adult epidermis associated with greater fluorescence intensity than LC from neonatal epidermis. However, to ascertain metabolically active uptake of antigen, the surface binding of antigen at 4°C must be considered. To account for these fluctuations, the fluorescence intensity at 37°C was divided by the intensity at 4°C, generating a ratio value for each time point. Ratio data demonstrated that, despite the reduced expression of DEC-205 and Langerin, LC from 4-day-old mice were efficient at uptake of FITC-dextran than adult LC, as they displayed a higher ratio value. As neonatal LC continued to internalise antigen over a longer period of time, this suggests that the pathway used to internalise FITC-dextran was more difficult to saturate.

When FITC-dextran uptake assays were repeated in the presence of blocking doses of mannan and mannose, LC from adult epidermis demonstrated a greater percent decrease in uptake compared with LC from neonatal epidermis. This finding strongly suggests that adult LC are more dependent on a mannose-type receptor-mediated pathway than neonatal LC. Due to the decreased dependency on a mannose-type receptor mediated pathway, it is plausible that neonatal LC utilise a receptor-independent pathway, such as macropinocytosis, to internalise antigen. Macropinocytosis provides an efficient method of sampling the extracellular environment for soluble or non-binding antigens (Swanson and Watts 1995). Macropinocytosis utilises the actin cytoskeleton to form macropinosomes, vesicles that arise when surface ruffles fold back against neighbouring ruffles or against the cell wall (Swanson 1989). It has been previously demonstrated that 1mg/mL of FITC-dextran is sufficient to saturate uptake via the mannose receptor, therefore differences in uptake between the age points will primarily reflect changes in macropinocytosis (Sallusto et al. 1995; Buentke et al. 2000). Previous *in vitro* studies have shown that in the presence of mannan, which is the functional equivalent of an absence of specific receptors, LC could still internalise mannosylated antigens in the fluid phase (Kato et al. 2000). As maximum uptake occurred at a lower level in the adult than the neonate, this suggests that adult LC are saturated at a lower concentration. This may be a result of receptor expression, or more likely, that

neonatal LC take up FITC-dextran via a pathway that is more difficult to saturate, such as a receptor-independent pathway. Evidence for an efficient fluid phase pathway was obtained with LY, previously shown to be a definitive marker of macropinocytosis (Piemonti et al. 1999). When incubated with Lucifer Yellow, LC from neonatal epidermis demonstrated a greater ratio value at the end of uptake compared to LC isolated from adult epidermis. These results were further supported by the effective inhibition of FITC-dextran accumulation in neonatal LC by the phosphatidylinositol 3-kinase inhibitor wortmannin, previously demonstrated to prevent closure of membrane ruffles, thereby reducing the pinocytic potential of a cell (Stein and Waterfield 2000). These results strongly indicate that a fully functional macropinocytosis pathway is employed during the early neonatal period to internalise exogenous antigens. I therefore suggest that macropinocytosis is preferentially utilised during the early neonatal period to ensure that the capacity to internalise antigen is not lost.

The epidermal LC network undergoes significant alterations during maturation (Dewar et al. 2001). The decreased density of LC resident in 3-day-old epidermis, combined with the reduction in extensive dendrites, equates to an inability to form a semicontiguous network (Dewar et al. 2001). As an adaptive mechanism, macropinocytosis may be up-regulated to ensure that the capacity to trap antigen is maintained. The lack of dendrites may also indirectly contribute to the increase in pinocytic activity demonstrated *in vitro*. When dendrites are formed the cytoskeleton is extended and anchored firmly to the extracellular matrix (Ross et al. 2000). Adult LC possess a well-developed, anchored dendritic network, with little membrane available for ruffling during macropinocytosis. Conversely, neonatal LC exhibit a more rounded morphology (Dewar et al. 2001), therefore increasing available membrane. As a consequence the magnitude of antigen internalised by macropinocytosis is increased. By day 14 following birth, the dendritic morphology of the LC network is improved (Dewar et al. 2001), and it is at this stage of development that the amount of antigen internalised *in vitro* is reduced and parallel to adult controls.

The results of this study suggest that the capacity to internalise antigen via a receptor-mediated pathway is not acquired until late in development. In contrast the

heightened *in vitro* fluid phase activity of neonatal LC indicates that this pathway is functional from an early age. Despite the efficient internalisation of antigen via macropinocytosis, *in vivo* antigen exposure results in a reduction in the amount of antigen transported to the draining lymph node (Dewar et al. 2001). Although ratio data suggests that, *in vitro*, neonatal LC are more efficient at pinocytosis than adult LC, antigen may still be transported to the draining LN by adult LC via association of antigen to the cell membrane or via non-metabolically active binding of antigen to surface receptors. As a consequence the amount of antigen transported is increased. It is also possible that although neonatal LC are associated with efficient pinocytic pathways, their ability to possess the internalised antigen is inept. The aim of the next and final chapter will be to determine the fate of ingested antigen and whether neonatal LC are capable of responding to maturation stimuli, a crucial event in the acquisition of potent APC function.

It has been demonstrated that a gradual increase in the density of MHC-II$^+$ cells in the epidermis occurs during development, from relatively few positive cells in 3-day-old epidermis increasing to a level which, at day 14, is comparable with that found in adult epidermis (Dewar et al. 2001). There is an increase in MHC-II$^+$ cells during the early neonatal period, yet these cells do not migrate from the epidermis following antigenic challenge. I propose that neonatal LC are actively retained in the epidermis to avoid inducing inappropriate immune response at a time of postnatal epidermal development and maturation. As a result of high cell turnover, one would expect an increase in both harmless soluble antigens as well as self- and protein particulate antigens within the epidermis (Haake and Cooklis 1997). Rather than macropinosomes fusing with degradative lysosomes (Nair et al. 1992), neonatal LC may recycle antigen back to the extracellular space in a clearance and scavenging mechanism (Steinman et al. 1976; Amyere et al. 2002). In such a way neonatal LC may use macropinocytosis to regulate the induction of immune responses to self-peptide and harmless soluble antigens.

In conclusion, this chapter has shown that in the absence of the C-type lectins DEC-205 and Langerin, LC isolated from neonatal epidermis preferentially utilise a wortmannin-sensitive fluid phase pathway, such as macropinocytosis, to internalise small exogenous antigens. The effective pinocytic capacity of neonatal LC is

acquired prior to their ability to initiate effective immunity. The aptitude of neonatal LC to sample their external environment without inducing immunity has important biological consequences for the protection against inappropriate immune responses during the developmental period.

Chapter 5

Characterisation of Antigen Processing and Maturation by Epidermal Langerhans During the Developmental Period

5.1 Introduction

Processing and presentation of internalised antigen is essential to the generation of protective immune responses, and the dynamic life cycle of LC equip them ideally for these physiologically distinct processes (Cella et al. 1997b). During their residence in the epidermis LC possess an array of uptake pathways that accommodate most antigens, including small and large particulate antigens as well as soluble and non-binding peptides (Katz and Sunshine 1986; Swanson and Watts 1995; Goldstein et al. 1996; Watts 1997). Exposure to foreign antigen occurs simultaneously to inflammation or tissue damage and the mediators that are released during these events provide maturation triggers for the LC (Elbe et al. 1989; Girolomoni et al. 1990; Cumberbatch et al. 1991). Following receipt of maturation stimuli, LC down-regulate their attachment to the epidermal network and migrate via the afferent lymphatics to the regional LN (Kimber and Cumberbatch 1992b; Cumberbatch et al. 2000). During transit they undergo a process of differentiation such that they are transformed from a cell primarily involved in antigen uptake to a cell capable of antigen presentation (Cumberbatch et al. 2000). Associated with this change in function is an alteration in phenotype, where the expression of co-stimulatory molecules becomes apparent and newly formed immunogenic MHC-II-peptide complexes are transported to the cell surface (Bigby et al. 1993; Peguet-Navarro et al. 1993; Weinlich et al. 1998b; Stoitzner et al. 2003). It is the binding of these molecules with ligands on the surface of the naive T cell that provides the appropriate signals required for T cell activation (Peguet-Navarro et al. 1993).

Antigen exposure during the neonatal period results in a deviation of the T cell response away from protective immunity and towards immune suppression (Dewar et al. 2001). Contributing factors in the induction of suppression have been proposed to be a decrease in the amount of antigen presented and a reduction in surface expression of the costimulatory molecules CD80 and CD40 (Dewar et al. 2001;

Simpson et al. 2003). It was originally proposed that the reduction in antigen transport was a consequence of a diminished capacity to internalise antigen via a receptor-mediated pathway (Becker et al. 1991; Dewar et al. 2001; Simpson et al. 2003). However, chapter 4 furnished evidence that neonatal LC were able to employ an alternative uptake pathway to offset the reduction in mannose-specific lectin receptors, DEC-205 and Langerin. Due to the inhibition of FITC-dextran internalisation in the presence of the macropinocytosis inhibitor wortmannin, the pathway was proposed to be fluid phase macropinocytosis. Via this pathway neonatal LC were able to efficiently internalise antigen *in vitro* with the same efficiency as adult counterparts.

It is now accepted that the mode of antigen uptake influences the fate of internalised antigen (Steinman et al. 1976). Lutz *et al* demonstrated that antigen internalised via receptor-mediated endocytosis is targeted to lysosome associated membrane protein (LAMP)-1^+ degradative vesicles (Lutz et al. 1997). LAMP-1, is expressed on early endosomes, vesicles that acquire antigen shortly after ingestion as well as early and late lysosomes that contain MHC-II (Lutz et al. 1997). Amyere *et al* (2002) demonstrated that within macrophages, macropinosomes bypass the endocytic pathway and exocytose the digested antigen back to the extracellular space (Amyere et al. 2002). In this situation antigen uptake is utilised as a scavenging and clearance mechanism. The aim of this study was to determine the capacity of LC to process exogenous particulate antigen and to respond to maturation stimuli *in vitro*. To ascertain the processing and maturation capacity during the developmental period, LC were isolated from the epidermis of 4-day- and 6-week-old mice and were analysed for expression of the lysosome-associated membrane protein (LAMP)-1^+ proteolytic vesicles, trafficking of antigen to the cell surface and the up-regulation of CD86 in response to LPS.

5.2 Results

5.2.1 Characterisation of LAMP-1 expression by adult and neonatal MHC-II$^+$ epidermal Langerhans cells.

The endocytic pathway consists of a series of compartments that facilitate antigen proteolysis and MHC-II:peptide complexing (Lutz et al. 1997). These vesicles can be distinguished by expression of LAMP-1 (Lutz et al. 1997). To assess the relative level of endocytic compartments during development, expression of LAMP-1 was assessed in LC isolated from neonatal and adult epidermis. Figure 5.1 is a representative experiment, demonstrating LAMP-1 expression within MHC-II$^+$ LC isolated from the epidermis of 4-day-old and 6-week-old mice. Analysis of LAMP-1 expression within LC from 4-day- and 6-week-old epidermis demonstrated similar distribution of LAMP-1$^+$ vesicles. In both adult and neonatal LC, fluorescently labelled vesicles were found within the cytoplasm, in close proximity to the membrane. It was also evident that a proportion of MHC-II was located in intracellular LAMP-1$^+$ vesicles, demonstrated by double positive staining (Figure 5.1c). Identification of LAMP-1 vesicles within LC from 4-day-old epidermis suggests the presence of endocytic compartments within neonatal LC. Based on this observation, neonatal LC should possess proteolytic activity.

Figure 5.1 Expression of LAMP-1 on viable MHC-II$^+$ LC isolated from 4-day- and 6-week-old epidermis. The expression of the lysosomal marker LAMP-1 was assessed in MHC-II$^+$ cells isolated from 4-day- (a) and 6-week-old (b) epidermis. Using Magnafire caption program single colour images were taken and subsequently merged in Photoshop. Note the co-localisation of MHC-II and LAMP-1 in the merged images. Two representative sets of results are shown for each age point.

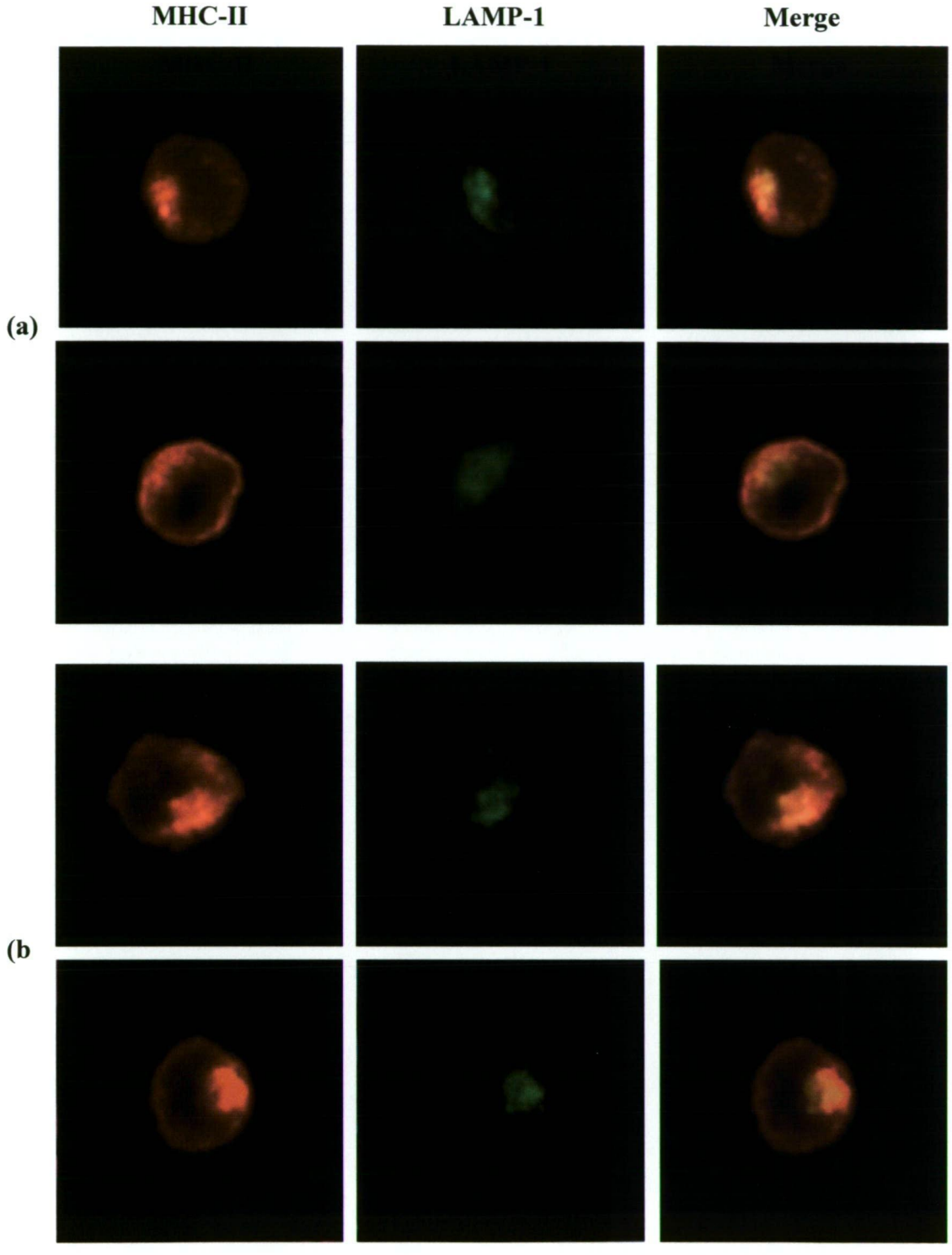

Figure 5.1 Expression of LAMP-1 on viable MHC-II^{+}LC isolated from 4-day- and 6-week-old epidermis

5.2.2 Identification of DQ-OVA proteolysis by adult and neonatal MHC-II$^+$ epidermal LC.

To determine the capacity of LC to proteolyse antigen during development, LC isolated from epidermis of 4-day- and 6-week-old mice were incubated with the fluorescent antigen DQ-Ovalbumin (OVA) for 24 hours at 37°C and 4°C. DQ-OVA emits fluorescence upon proteolysis, and proteolysed antigen is identified as green fluorescent punctate vesicles. In contrast to other proteolytic dyes DQ-OVA is conjugated to the pH insensitive dye BODIPY, translating to stable fluorescence intensity over a broad pH range of 3-9. Another advantage of DQ-OVA is that the level of background fluorescence is more highly quenched, translating to fewer fluorophores required per protein molecule. As such the antigenic epitopes of the protein are preserved. Lutz and colleagues demonstrated that OVA is preferentially internalised via a fluid phase pathway (Lutz et al. 1997), therefore DQ-OVA was chosen as an indication of macropinocytosis. Following internalisation via macropinocytosis, proteolysis can be identified as the appearance of fluorescence positive vesicles. Due to the stablility of the dye at acidic pH it will not be degraded within the endocytic pathway, allowing visualisation of the vesicles as they traffic through the cell. Figure 5.2 is a representative experiment and demonstrates the localisation of DQ-OVA$^+$ vesicles within adult LC 24 hours following incubation with antigen. Following overnight culture at 37°C green fluorescent vesicles were identified within the cytoplasm of MHC-II$^+$ LC. Results also demonstrate that none of the vesicles co-localised with either intracellular or cell surface MHC-II. These results suggest that LC isolated from 6-week-old epidermis were capable of proteolysing antigen. Once proteolysed the antigen-containing vesicles were localised to the cytoplasm. As antigen did not co-localise with intracellular MHC-II, proteolysed DQ-OVA was retained in an MHC-II$^-$ compartment. LAMP-1 and MHC-II have been demonstrated to co-localise therefore the retention compartment was also negative for LAMP-1.

Figure 5.3 is a representative experiment, demonstrating DQ-OVA proteolysis by LC isolated from the epidermis of 4-day-old mice. Following incubation with DQ-OVA, antigen-positive vesicles were identified within the cytoplasm of MHC-II$^+$ LC.

Results also demonstrate that DQ-OVA did not co-localise with MHC-II in the cytoplasm or on the cell surface. These results suggest that, like adult LC, the LC isolated from 4-day-old epidermis were capable of proteolysing antigen, and that proteolysed DQ-OVA was retained in a MHC-II$^-$ compartment. LC from adult epidermis and neonatal epidermis appeared to handle DQ-OVA in a similar manner as there were no differences in either localisation or number of antigen-positive vesicles was observed.

Figure 5.2 Proteolysis of DQ-OVA by MHC-II$^+$ LC isolated from 6-week-old epidermis. The proteolysis of the labeled dye DQ-OVA was assessed in MHC-II$^+$ cells isolated from 6-week-old epidermis at 4°C (a) and 37°C (b). Using Magnafire caption program single colour images were taken and subsequently merged in Photoshop. Note the DQ-OVA$^+$ vesicles when the cells were incubated with antigen at 37°C. Two representative sets of results are shown.

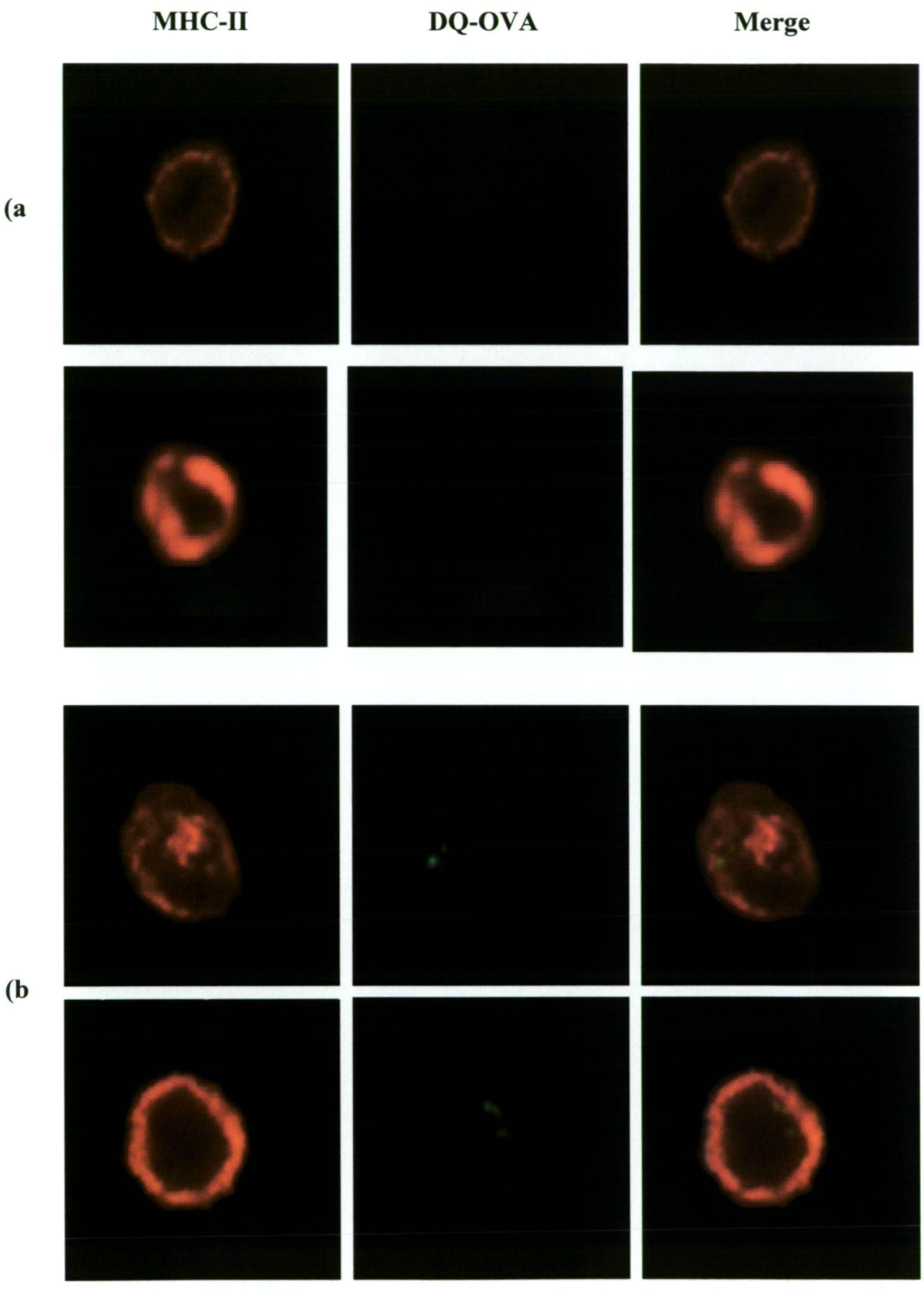

Figure 5.2 Proteolysis of DQ-OVA by MHC-II^{+} LC isolated from adult

Figure 5.3 Proteolysis of DQ-OVA by MHC-II$^+$ LC isolated from 4-day-old epidermis. The proteolysis of the labeled dye DQ-OVA was assessed in MHC-II$^+$ cells isolated from 4-day-old epidermis at 4°C (a) and 37°C (b). Using Magnafire caption program single colour images were taken and subsequently merged in Photoshop. Note the DQ-OVA$^+$ vesicles when the cells were incubated with antigen at 37°C. Two representative sets of results are shown.

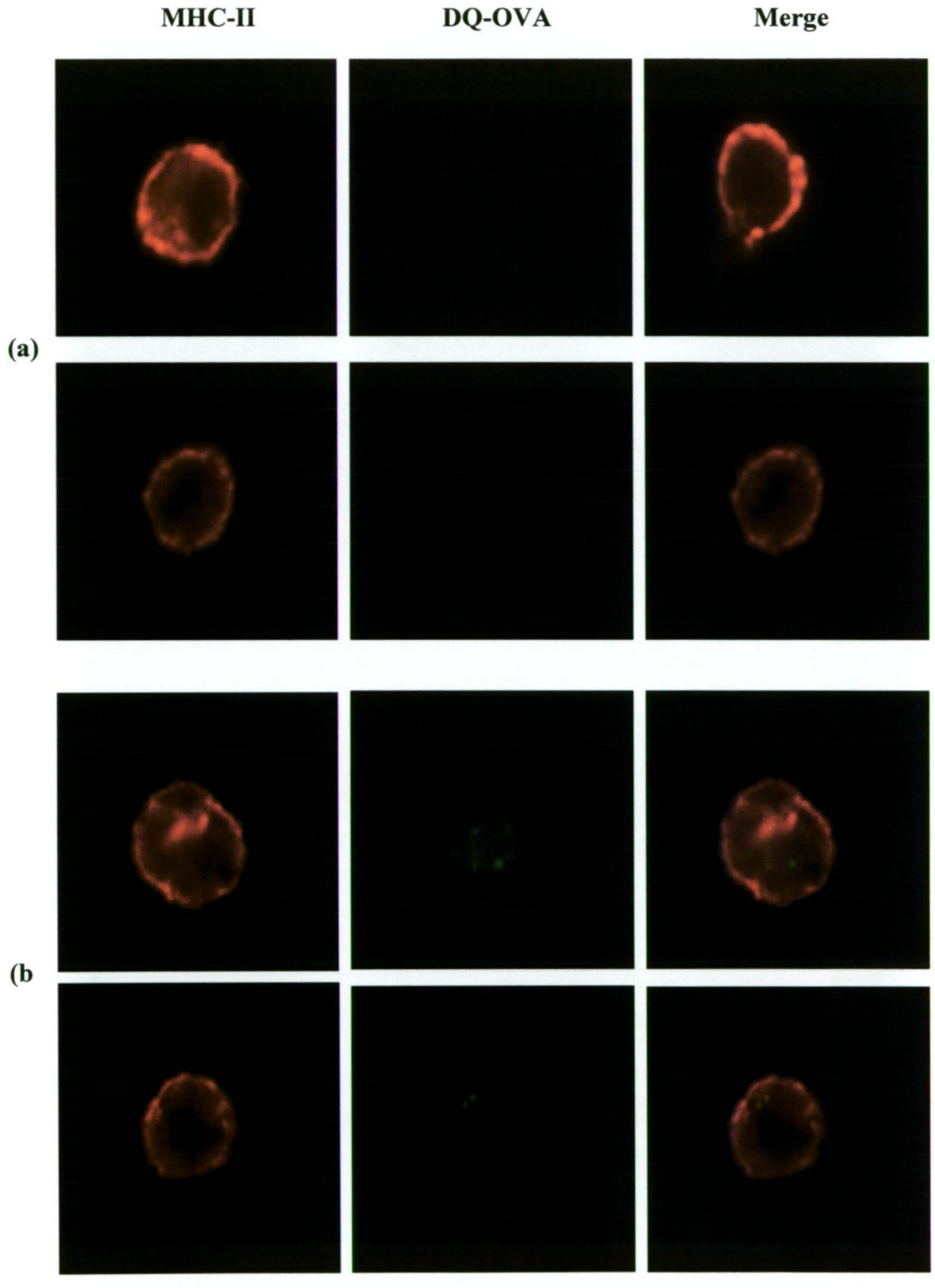

Figure 5.3 Proteolysis of DQ-OVA by MHC-II^{+} LC isolated from 4-day-old epidermis

5.2.3 Trafficking of DQ-OVA to the cell surface by adult and neonatal MHC-II$^+$ epidermal LC

Concomitant with receipt of maturation stimuli is the loading of proteolysed antigen fragments onto newly synthesised intracellular MHC-II. Following loading, the MHC-II:peptide complexes are transported through the cell to the cell membrane for presentation. To assess the capacity of epidermal LC to transport processed peptides to MHC-II$^+$ compartments and to traffic MHC-II:antigen complexes to the cell surface, DQ-OVA proteolysis assays were repeated in the presence of LPS. The concentration of LPS used has been previously demonstrated to induce DC maturation *in* vitro. Figure 5.4 is a representative of two separate experiments and demonstrates the localisation of DQ-OVA$^+$ vesicles in the presence (Figure 5.4a) or absence (Figure 5.4b) of LPS. Vesicles bearing proteolysed antigen were evident as punctate fluorescent green vesicles. If such vesicles were expressed on the MHC-II$^+$ cell membrane (MHC-II staining with red fluorescence), then co localisation was visualised as yellow staining. Figure 5.4 demonstrates that DQ-OVA$^+$ vesicles were evident on the cell membrane, in close association with MHC-II. As DQ-OVA was clearly evident on the cell membrane this suggests that DQ-OVA was able to gain access to MHC-II$^+$ compartments, a site of antigen loading. Results also demonstrate that a small proportion of DQ-OVA$^+$ vesicles were retained within the cell, which was not co-localised to intracellular MHC-II. These vesicles however, were in close proximity to the membrane.

Figure 5.5 is a representative of DQ-OVA proteolysis by LC isolated from 4-day-old epidermis in the presence (Figure 5.5a) or absence (Figure 5.5b) of LPS. Although a small proportion of DQ-OVA$^+$ vesicles were found on the cell membrane, evident as co localisation with surface MHC-II, the majority of antigen-bearing vesicles were retained in a cytosolic MHC-II$^-$ compartment. These results strongly suggest that following exposure to LPS, neonatal LC do not efficiently traffic proteolysed antigen to MHC-II$^+$ compartments.

Figure 5.4 Proteolysis of DQ-OVA by MHC-II$^+$ LC isolated from 4-day-old epidermis. The proteolysis and trafficking of the labeled dye DQ-OVA was assessed in MHC-II$^+$ cells isolated from 6-week-old epidermis in the presence (a) and absence (b) of LPS. Using Magnafire caption program single colour images were taken and subsequently merged in Photoshop. Note the DQ-OVA$^+$ vesicles co-localised with the cell membrane when cells were incubated with antigen in the presence of LPS. Two representative sets of results are shown.

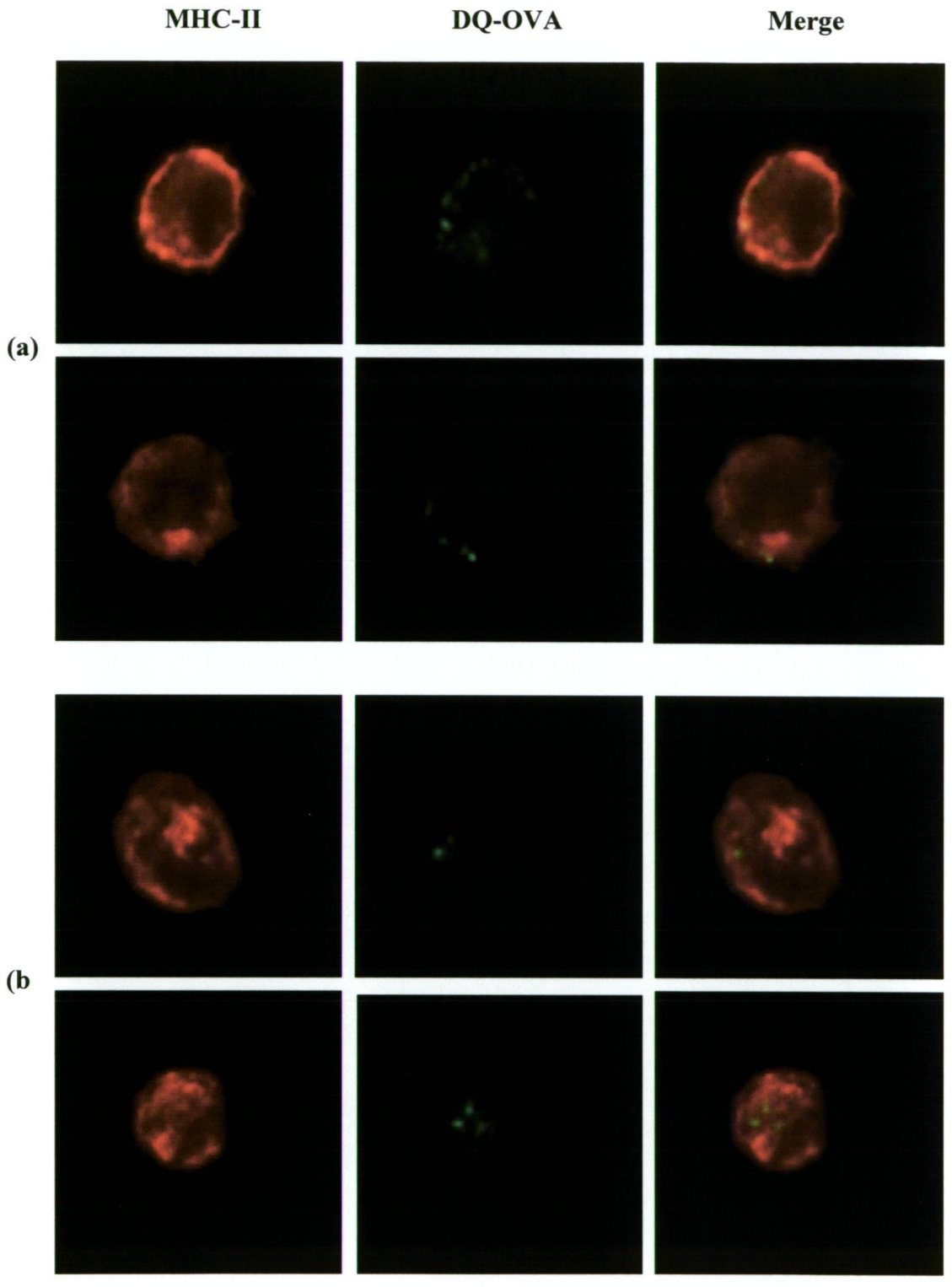

Figure 5.4 Trafficking of DQ-OVA following exposure to LPS by MHC-II^{+} LC isolated from 6-week-old epidermis

Figure 5.5 Trafficking of DQ-OVA following exposure to LPS by MHC-II$^+$ LC isolated from 4-day-old epidermis. The proteolysis and trafficking of the labeled dye DQ-OVA was assessed in MHC-II$^+$ cells isolated from 4-day-old epidermis in the presence (a) and absence (b) of LPS. Using Magnafire caption program single colour images were taken and subsequently merged in Photoshop. Note the DQ-OVA$^+$ vesicles co-localised with the cell membrane when cells were incubated with antigen in the presence of LPS. Two representative sets of results are shown.

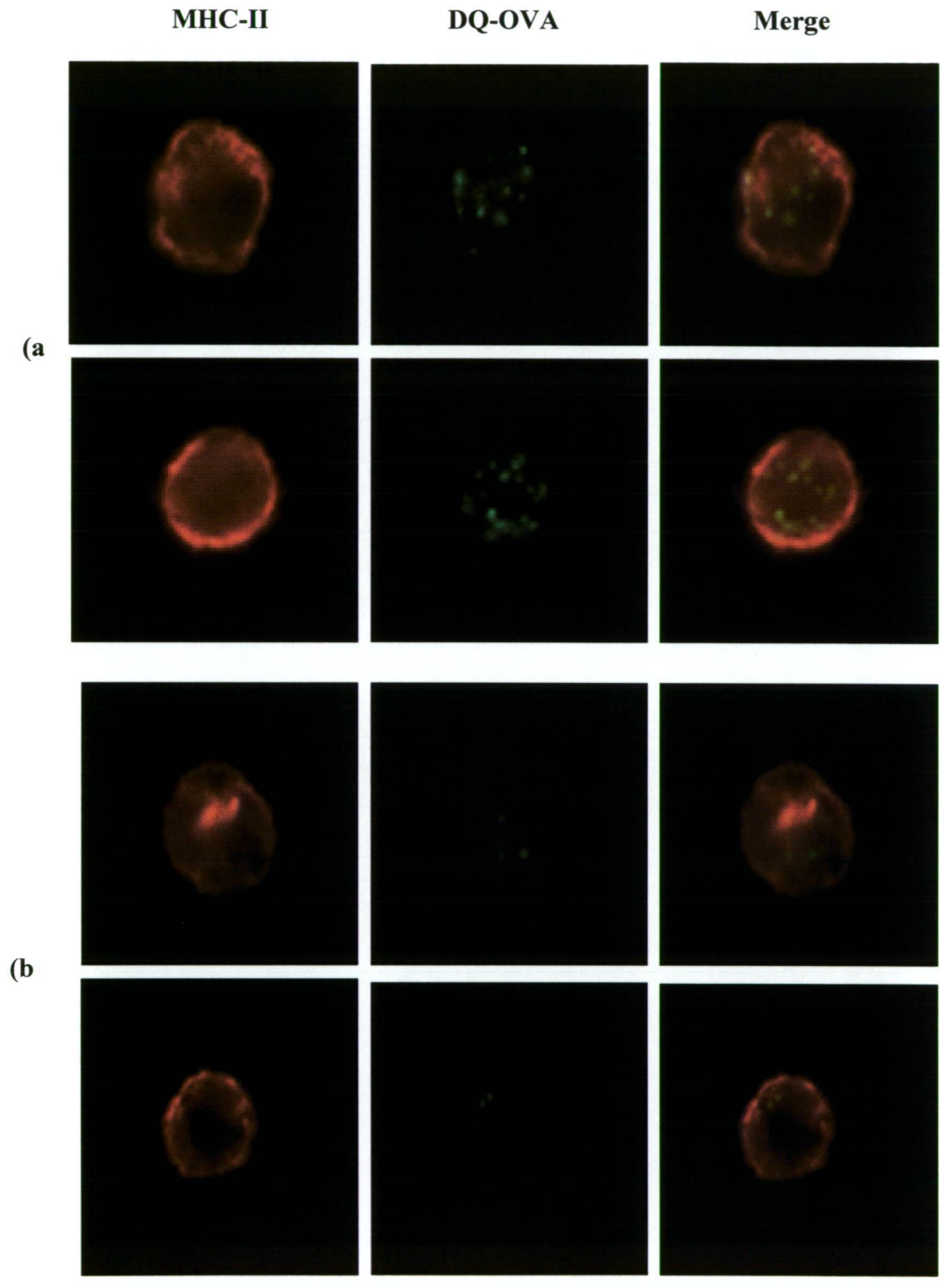

Figure 5.5 Trafficking of DQ-OVA following exposure to LPS by MHC-II^{+} LC isolated from 4-day-old epidermis

5.2.4 Trafficking of MHC-II to the cell surface by adult and neonatal MHC-II$^+$ epidermal LC

Results furnished in this study suggest that although capable of efficiently proteolysing antigen, neonatal LC are inept at the loading and transport of MHC-II:peptide complexes to the cell surface. One step that may account for the reduced expression of membrane complexes, is the trafficking of MHC-II to the cell surface. To characterise MHC-II transport, LC isolated from 4-day- and 6-week-old epidermis were analysed for MHC-II localisation 24 hours following incubation with LPS. Figure 5.6 is a representative experiment and demonstrates MHC-II and LAMP-1 localisation in freshly isolated neonatal (a) and adult (b) LC, and 24 hours following exposure to LPS. Results demonstrate that neonatal and adult LC transported intracellular MHC-II from a LAMP-1$^+$ compartment to the cell membrane. This was evident by an absence of co-localisation of MHC-II and LAMP-1. Consequently, in LC isolated from neonatal and adult epidermis MHC-II was trafficked out of the LAMP-1 compartments to the cell membrane. Results also demonstrate alterations in LAMP-1 localisation following stimulation. In freshly isolated LC, LAMP-1 was localised to a perinuclear location. Following incubation with LPS, LAMP-1 was in a distinct cytoplasmic location. Based on this observation, transport of MHC-II alone was not responsible for the reduced expression of antigen on the cell membrane.

Figure 5.6 Trafficking of MHC-II following exposure to LPS by LC isolated from 4-day- and 6-week-old epidermis. The trafficking of MHC-II to the cell membrane was assessed in MHC-II$^+$ LAMP-1$^+$ cells isolated from 4-day- (a) and 6-week-old (b) epidermis in the presence and absence of LPS. Using Magnafire caption program single colour images were taken and subsequently merged in Photoshop. Note the absence of intracellular MHC-II staining when cells were incubated with antigen in the presence of LPS. Two representative sets of results are shown.

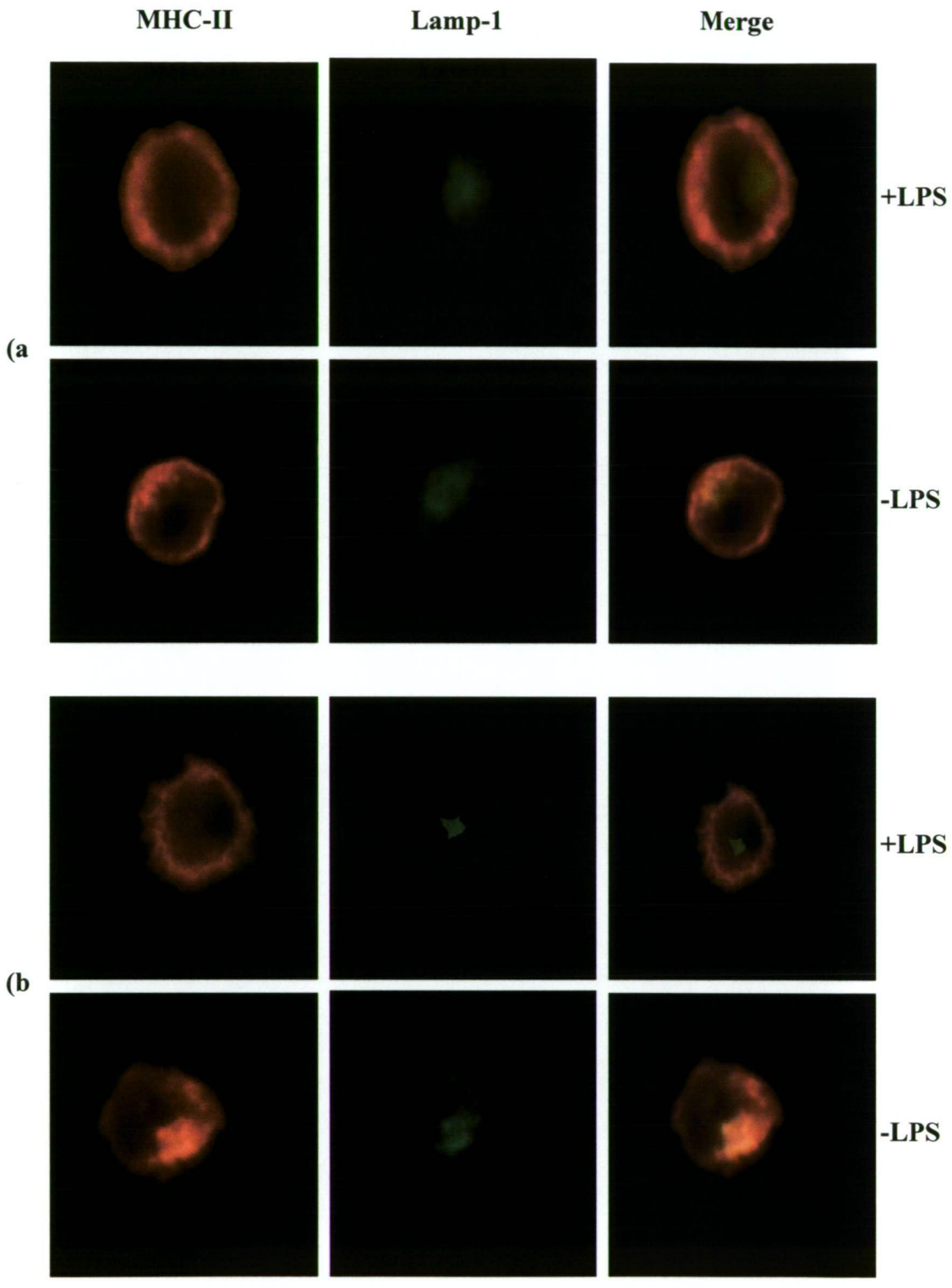

Figure 5.6 Trafficking of MHC-II following exposure to LPS by LC isolated from 4-day- and 6-week-old epidermis

5.2.5 Up-regulation of CD86 by adult and neonatal MHC-II$^+$ epidermal LC

Occurring simultaneously to the transport of MHC-II:peptide complexes to the cell surface is the up-regulation of membrane costimulatory molecules, such as CD86. To ascertain the capacity of LC to up-regulate costimulatory molecule expression following exposure to maturation-inducing stimuli, LC isolated from 4-day- and 6-week-old epidermis were analysed for CD86 expression 24 hours following exposure to LPS *in vitro*. Figure 5.7 is a representative experiment and demonstrates CD86 expression on neonatal and adult LC at 0 and 24 hours following incubation with LPS. Although freshly isolated neonatal LC expressed reduced levels of CD86 compared to adult counterparts, LC from neonatal epidermis up-regulated CD86 expression in response to LPS to the same extent as adult controls. These results suggest that neonatal LC were capable of up-regulating CD86 expression following exposure to LPS.

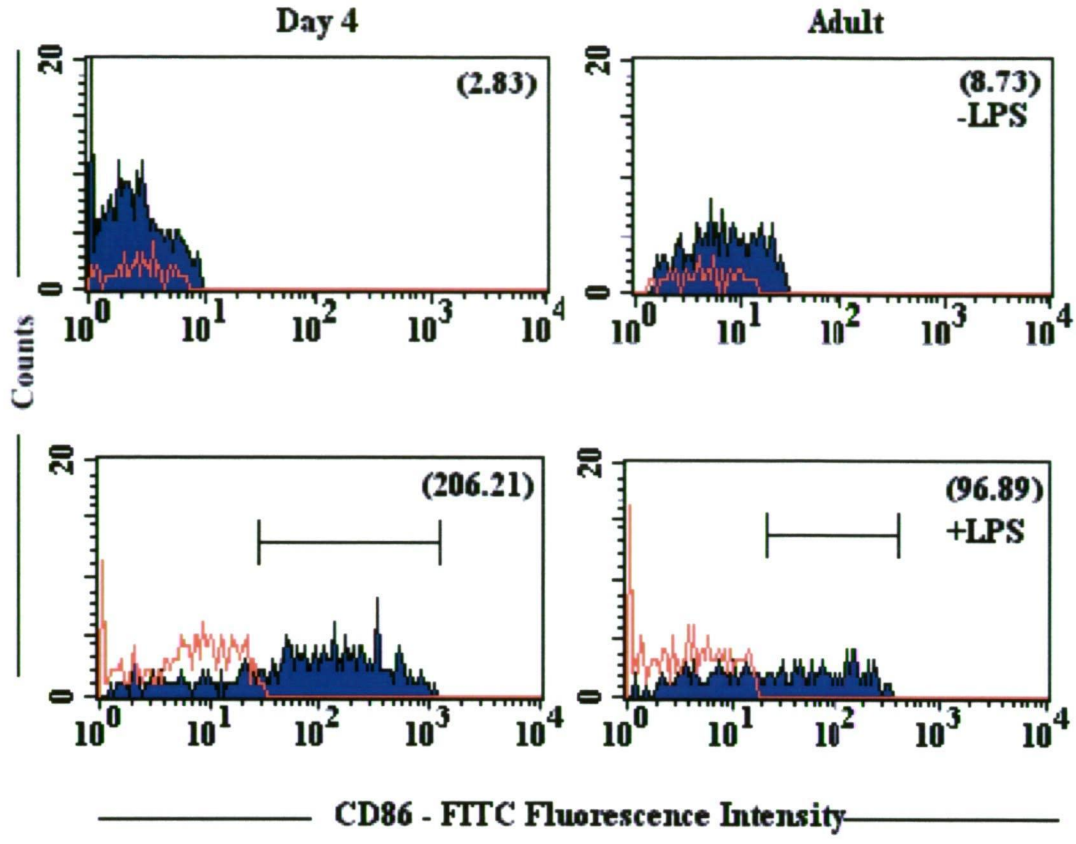

Figure 5.7 CD86 expression on viable MHC-II$^+$ LC isolated from 4-day- and 6-week-old epidermis following exposure to LPS. The expression of CD86 was assessed in MHC-II$^+$ cells isolated from 4-day- and 6-week-old epidermis prior to after 24 hours following incubation with LPS. Results are representative of 2 separate experiments

5.3 Discussion

Results from Chapter 4 indicated that during the early developmental period LC preferentially utilise a fluid phase, wortmannin-sensitive pathway, such as macropinocytosis, to internalise small particulate antigen. By 6-weeks of age the reliance on macropinocytosis is reduced and alternatively cell surface antigen uptake receptors are employed to recognise and bind antigen, and this correlates to successful antigen presentation. As the pathway utilised to internalise antigen has important implications for effective MHC-II:peptide presentation (Langenkamp et al. 2002), it was of great interest to determine the fate of antigen internalised during neonatal development. To characterise the antigen processing potential of LC during the early neonatal period, LC were isolated from 4-day- and 6-week-old epidermis and analysed for expression of proteolytic vesicles, as well as their ability to proteolyse antigen and up-regulate CD86 following *in vitro* exposure to maturation stimuli.

Following receptor-mediated endocytosis, antigens progress through a sequence of maturing proteolytic compartments whose functions are to digest antigen into smaller peptide fragments and to facilitate loading of peptides onto newly synthesised MHC-II (Kleijmeer et al. 1995; Xu and Pierce 1995; Turley et al. 2000). These organelles can be distinguished by expression of LAMP-1, and is expressed in low levels on early endosomes, vesicles that acquire antigen shortly after ingestion as well as early and late lysosomes that contain MHC-II (West et al. 1994; Kleijmeer et al. 1995; Xu and Pierce 1995). In contrast, antigens that gain access to the cell via macropinocytosis are engulfed within macropinosomes that are negative for LAMP-1. It is not until the cell commences maturation that the vesicle acidifies and acquires LAMP-1 expression (Lutz et al. 1997). As OVA is preferentially internalised via macropinocytosis (Lutz et al. 1997), the fluorescent DQ-OVA provides an ideal antigen to follow the fate of antigen following macropinocytosis by both adult and neonatal LC. Despite the observation of LAMP-1 and MHC-II co-localisation in unstimulated LC, proteolysed DQ-OVA did not associate with intracellular MHC-II, and therefore LAMP-1. This suggests that DQ-OVA is not

proteolysed in a vesicle continuing LAMP-1 and MHC-II and that it is retained within a LAMP-1$^-$ compartment. This is consistent with the statement that antigen engulfed by macropinocytosis is targeted to a LAMP-1$^-$ vesicle and provides evidence for a functional macropinocytosis pathway during the early neonatal period. Consequently, neonatal and adult LC target macropinocytosed antigen to a similar LAMP-1$^-$ compartment. But this is where the similarity ends and the proposal is that neonatal LC are unable to transport the antigen to MHC-II$^+$ compartments and subsequently to the cell membrane in association with MHC-II. As the vesicles do not express LAMP-1 this suggests that at this stage the cell has not yet commenced maturation. By coordinating maturation with antigen presentation ensures that the cell has reached full APC potency prior to interaction with naïve T cells.

Under normal situations, exposure to inflammatory mediators signals LC to commence a process of maturation that includes transport of MHC-II into the endocytic pathway (Pierre et al. 1997; Turley et al. 2000). Within the endocytic route, MHC-II$^+$ LAMP$^+$ vesicles fuse with vesicles containing degraded antigen (West et al. 1994; Lutz et al. 1997; Pierre et al. 1997). The peptide fragments are then exchanged for the CLIP fragment occupying the peptide-binding groove of MHC-II, and the resulting complexes shuttled to the cell surface in association with costimulatory molecules (Turley et al. 2000). Turley and colleagues demonstrated that antigen is only loaded onto MHC-II following receipt of maturation stimuli, and that antigen can be retained in vesicles for extended periods of time until the correct signal is received (Turley et al. 2000). It is apparent that neonatal LC do not respond normally to maturation stimuli as DQ-OVA was not directed to an MHC-II$^+$ compartment and therefore it does not become expressed on the cell membrane. Although neonatal LC can transport MHC-II to the membrane in the absence of antigen, they are unable to transport MHC-II:peptide complexes to the cell surface. This strongly suggests that within neonatal LC antigen cannot gain access to intracellular MHC-II, and these findings strongly indicate that the outcome of antigen processing by neonatal LC is different from adult LC.

As there is more than one possible outcome for antigens internalised via macropinocytosis (Watts 1997; Amyere et al. 2002), it is likely that the fate of antigen ingested by neonatal LC will differ from the fate of antigens internalised by

adult LC. A comparison can be made to macrophages that can internalise antigen via fluid phase pinocytosis and this antigen is exocytosed in a clearance mechanism (Amyere et al. 2002). Whilst it is possible that neonatal LC regurgitate some degraded antigen into the extracellular space, the presence of intracellular antigen 24 hours following exposure to maturation stimuli suggests that neonatal LC retain a proportion of antigen within the cell. Consequently an alteration in one of the steps involved in the processing pathway is more likely to be responsible for the reduction in surface complexes

Following dimerisation of α and β subunits of MHC-II is the association of the Ii (Pure et al. 1990; Cresswell 1996). Not only does the Ii bind to the antigen cleft of MHC-II, and hence inhibit loading of endogenous antigen, but it also contains a signalling sequence that directs the MHC-II from the ER, through the Golgi and into the endocytic pathway (Bakke and Dobberstein 1990; Lotteau et al. 1990). If this signalling motif is deficient in neonatal LC then MHC-II will be directed away from the endocytic route, straight to the cell surface. As a consequence antigen will be retained in a MHC-II$^-$ cytoplasmic location, evident 24 hours following incubation with antigen. Cathepsin S may also play a role as this enzyme has been shown to be integrally involved in the exit of MHC molecules from an intracellular compartment where they are held prior to encountering antigen (Nakagawa and Rudensky 1999; Villadangos et al. 1999; Lennon-Dumenil et al. 2002a; Lennon-Dumenil et al. 2002b). If cathepsin S is deficient then MHC-II containing endosomes may be routed directly to the cell surface, and hence bypass interaction with antigen positive vesicles. Not only is cathepsin S involved in transit of MHC-II but also in the degradation of the invariant chain, Ii (Nakagawa and Rudensky 1999; Villadangos and Ploegh 2000). For effective loading of antigen, Ii must be cleaved to free the intracellular antigen-binding site (Alfonso and Karlsson 2000b; Alfonso and Karlsson 2000a). In unstimulated LC cathepsin S is found within late endosomes and is partially inhibited by high levels of cystatin C (Pierre and Mellman 1998). After activation of LC, cystatin C is downregulated allowing cathepsin S to cleave Ii and MHC-II is redistributed to the surface for antigen presentation (Meraner et al. 2000; Fiebiger et al. 2001; Lautwein et al. 2002). If neonatal LC, following receipt of maturation stimuli, do not down-regulate cystatin C activity, cathepsin S-mediated

cleavage of Ii will not occur and MHC-II:peptide complexes will not be exported to the cell surface. This is a particularly exciting possibility as a preliminary proteomics study within our research group has identified an abundance of stefin A (cystatin C) in neonatal skin (Scott. BSc(Hons) thesis). It has been previously demonstrated that antigen that is not bound to MHC-II is quickly degraded into smaller fragments, and this could account for the large number of small antigen containing vesicles evident in neonatal LC following exposure to LPS. As a result divergent cathepsin S activity has important ramifications on both the loading of antigen onto intracellular MHC-II but also the transit of resulting complexes to the cell surface.

An alternative hypothesis for the reduced surface expression of MHC-II:peptide complexes is that, although neonatal LC express MHC-II on the plasma membrane, the complexes are endocytosed prior to interactions with naïve T cells. Wilson and colleagues demonstrated the alterations in the antigen presenting properties during DC maturation (Wilson et al. 2003). DC located in lymphoid organs were found to present mainly self antigens on the cell surface and that these complexes were degraded quickly after their transient expression on the cell surface (Wilson et al. 2003). With this in mind it is possible that via a combination of altered cathepsin S activity and MHC-II endocytosis and degradation the majority of antigen is not loaded onto MHC-II. Those antigens that manage to bind to MHC-II and are subsequently trafficked to the cell membrane are degraded before the antigen can be effectively presented to naïve T cells resident in the draining LN.

A consequence of having two parallel processing steps in the generation of MHC-II:peptide complexes is that by inhibiting either proteolytic step could result in impaired T cell recognition. It is not likely to be exposure to maturation stimuli alone that is responsible for the reduction in surface MHC-II:peptide complexes as neonatal LC are capable of up-regulating CD86 following incubation with LPS, a process that is not dependent on prior exposure to antigen. Rather it is the combination of antigen processing and environmental stimuli that is responsible for the reduced traffic of antigen to the cell surface. Despite the capacity to up-regulate CD86 in response to maturation stimuli *in vitro, in vivo* LC that migrate to the draining LN following antigen exposure to neonatal epidermis express significantly

reduced surface CD86. This is further evidence supporting the impact on the *in vivo* environment on the maturation of epidermal LC. Selective regulation of endosomal proteolysis in neonatal LC could have profound implications for control of immunity against infection, as well as autoimmunity. Neonatal LC continuously sample their environment, one that alters during development period from one with a higher proportion of self-proteins to one of foreign, potentially harmful antigens. As such there is a shift from retaining proteolysed antigen in an immunologically safe intracellular environment to presenting the antigen on the cell surface for T cell recognition.

In conclusion I propose that a divergence exists within the exogenous processing pathway during the developmental period that contributes significantly to the diminished transport of MHC-II:peptide complexes to the cell membrane. This study has been fundamental in demonstrating how processing pathways differ during maturation, however further analysis is required to determine what stage in processing results in the decreased redistribution of MHC-II:peptide complexes to the cell surface. Although neonatal LC are proficient at pinocytosis of exogenous antigen *in vitro* a divergence in the intracellular processing of antigen contributes to the reduced surface MHC-II:peptide complexes. By actively retaining antigen within the cell, neonatal LC may utilise macropinocytosis to avoid presenting antigen at a time at which the immune system is developing. During the early neonatal period danger signals, such as proinflammatory cytokines, are reduced possibly to avoid induction of self-immunity at a time of high cell turnover and tissue remodelling. Rather, following internalisation of antigen, the presence of tolerance inducing cytokines gestures to the LC to maintain an immature state and prevent presentation of antigen by retaining antigen within the cell. Following exposure to high doses of antigen, or maturation of the epidermal cell network with age, the danger signals increase in potency. In response LC up regulate the expression of costimulatory molecules and traffic the antigen to the plasma membrane in association with MHC-II. Presentation of antigen combined with ligation of costimulatory molecules leads to the induction of T cell activation. The flexibility of neonatal LC ensures that during the developmental period tolerance is established and inappropriate immune responses are avoided. This is brought about by an up regulation of scavenging macropinocytosis and down regulation of antigen presentation. However when the

correct danger signals are provided LC increase their APC potency accordingly to enable T cell activation and immunity.

Chapter 6
Discussion

The neonatal skin microenvironment is unique in antigen composition. The extracellular milieu is rich in self and protein antigen, resulting from tissue remodelling and formation of new epidermal layers following birth (Haake and Cooklis 1997; Hoeger and Enzmann 2002). As opposed to adult counterparts, neonatal epidermis is low in particulate antigens as a consequence of epidermal and keratin thickening, increasing the physical barrier of the skin (Scott. BSc(Hons) thesis in preparation). If antigens were to breach the epidermal barrier, they would consist mainly of common environmental antigens and skin commensals. This uniqueness in antigenic composition has important implications for the immune consequence following antigen exposure. As the neonatal immune system has yet to acquire immunological memory (Kovarik and Siegrist 1998; Benschop et al. 2001; Siegrist 2001; Adkins et al. 2004b), it is essential for neonatal LC to be able to establish a tolerant state to environmental and self-antigen (Schurmans et al. 1990). If neonatal LC were to respond strongly to all antigens a chronic state of hyper-responsiveness and inflammation would result (Adkins et al. 2004b). Persistent inflammation is detrimental to skin development due to the negative impact on the establishment of self tolerance as well postnatal maturation of the epidermis (Adkins et al. 2004b). It is essential that immune cells resident in the epidermis are equipped with a means by which to scrutinize their extracellular environment, and according to the antigens encountered, elicit protective or tolerant responses. Exposure to self-antigen will lead to the induction of tolerance (Kurts et al. 1997; Banchereau and Steinman 1998a; Belz et al. 2002a). However when the epidermal barrier is crossed by potential pathogens mechanisms must be in place to induce protective immune responses (Sallusto et al. 1995).

Neonatal mice mount a limited response to infection and the insufficiency of the immune system is evident in the decreased protective response to vaccination during infancy (Siegrist 2001). Studies of the possible mechanisms for immune incompetence have focused primarily on neonatal T cells and their qualitative and quantitative differences compared to immune competent adult counterparts (Adkins and Hamilton 1992; Forsthuber et al. 1996; Adkins and Du 1998a; Adkins et al. 2004b). Although neonatal T cells are uniquely susceptible to tolerisation, recent investigations have highlighted that the maturation state of the APC is a determining factor in the outcome of antigen presentation (Dewar et al. 2001; Woods et al. 2001; Simpson et al. 2003). Following antigenic challenge, neonatal APC are intimately involved in tolerance induction, nevertheless the underlying mechanisms are yet to be fully clarified. It was recently demonstrated by Vollstedt and colleagues that the low number of functionally mature DC in murine lymphoid tissue resulted in an increased susceptibility to microbial infection (Vollstedt et al. 2003). It has also been demonstrated that the way in which antigen is handled and subsequently presented has important implications for the generation of protective or tolerant immune responses (Langenkamp et al. 2002). During infancy the ability to induce tolerance is an invaluable mechanism to maintain non-reactivity to self-antigen, beneficial skin commensals and harmless protein antigens (Min et al. 2001; Adkins et al. 2004b). Understanding the underlying mechanisms of cutaneous tolerance during the neonatal period provides insight into immune function. This knowledge provides a basis for development of strategies to enhance or suppress the immune response for anti-pathogen or cancer protection, or suppressing anti-self responses in autoimmune diseases respectively.

During maturation of the neonatal epidermis there is an associated maturation of the skin immune system, which is linked to the development of LC (Dewar et al. 2001). At 4 days of age, neonatal LC fail to induce an immune response, whereas at 20 days of age the LC are proficient at inducing immunity (Dewar et al. 2001; Simpson et al. 2003). As previous reports have shown that neonatal LC have a reduced expression of antigen uptake receptors it would suggest that during the early neonatal period LC have a reduced ability to internalise antigen (Dewar et al. 2001). This would be a convenient mechanism to prevent induction of an immune response, as diminished antigen uptake would translate to reduced antigen presentation and T cell activation

(Sallusto et al. 1995; Zinkernagel 2000). Chapter 3 furnished evidence to suggest that the ability to bind and internalise mannosylated antigen via a receptor-mediated pathway would be diminished as DEC-205 was absent and Langerin dramatically reduced on LC isolated from 4-day-old epidermis. As the epidermal network matured there was a gradual increase in receptor expression. By day 14 the level of expression had increased and was comparable to adult counterparts. These results strongly suggest that LC acquire functional maturity during development. Early studies have demonstrated that uptake by DEC-205 was as efficient as uptake by the macrophage mannose receptor and that uptake strongly enhanced the presentation of mannose-conjugated antigen (Reis et al. 1993; Kato et al. 1998b). As the majority of microbes possess mannose on the cell surface, an absence of mannose-specific receptors would translate to an inability to scavenge mannose-conjugated particulate antigen via receptor-mediated endocytosis (Jiang et al. 1995; Sallusto et al. 1995; Buentke et al. 2000). This is of particular importance as chapter 4 furnished evidence to suggest that epidermal LC are unable to employ phagocytosis to internalise particulate antigen. It is highly plausible that neonatal LC down-regulate expression of mannose-specific receptors to ensure that responsiveness to skin commensals is avoided and tolerance maintained. This hypothesis was further supported by the finding that neither adult nor neonatal LC possessed the LPS-specific receptor CD14. By limiting the expression of LPS and mannose receptors on LC throughout development the incidence of reactivity to commensals is diminished. Results from our department have demonstrated that application of contact sensitisers on the day of birth accelerates the acquisition of DEC-205 expression (Wonzniak BSc(Hons) thesis). With this in mind it is possible that as the antigen composition of the epidermal environment alters during maturation from soluble self-antigen to particulate antigen that mannose receptors, such DEC-205, are up-regulated accordingly for antigen-specific recognition and internalisation.

Based on the diminished expression of mannose-type receptors it would appear that if pathogens were to breach the neonatal epidermal barrier they would not be internalised by LC. Whilst this is beneficial for avoiding responses to commensal organisms, neonatal LC must maintain the capacity to internalise pathogenic organisms to protect the underlying dermis from attack. To compensate for the diminished expression of mannose-type receptors, LC possess receptors for

opsonised antigen from an early age. This is an interesting dichotomy as, on the one hand receptors capable of binding directly to antigen are reduced but receptors capable of binding opsonised antigens are expressed. Where does the opsonised antigen come from if the neonatal immune system is incapable of synthesising antibody in response to antigen exposure? This incompetence is overcome by maternal antibody. Maternal IgG is capable of crossing through epithelia and placenta, and provides protection whilst the newborn immune system is incapable of producing antibodies (Kovarik and Siegrist 1998; Benschop et al. 2001; Siegrist 2001; Adkins et al. 2004b). Once across the intestinal epithelia and into the blood stream, maternal antigen-specific IgG is available to opsonise foreign antigen. The mother has already been exposed to similar environmental pathogens the neonate may encounter and such immunological knowledge is transferred to the neonate via maternal IgG (Kovarik and Siegrist 1998). Expression of the IgG receptor complex CD16/32 during the neonatal period would be expected to enable responsiveness to antigen opsonised with maternal IgG. The beauty of the neonatal immune system is that under certain stimuli immune responses are avoided to prevent responsiveness to non-threatening environmental antigens. As the mother has initiated tolerance to such antigen, specific antibody will not be transferred to the neonate. However if potential pathogens gain access to the epidermis, antigen specific maternal IgG provides protection at a time at which their developing immune system is insufficient (Kovarik and Siegrist 1998). Interestingly, expression of CD16/32 may also aid further in the regulation of immune responses to self-tissue as maternal IgG only binds foreign antigen and not self-tissue (Esposito Farese et al. 1995; Fanger et al. 1996).

Aside from the impact on primary and recall responses, the inability of the neonate to synthesise antibody would prevent the activation of the complement cascade via the classical pathway. However, microbial surfaces can activate complement via the alternative pathway without the necessity of bound antibody (Koppenheffer 1987). Chapter 3 also demonstrated that the receptor for C3b, CD11b and CD18 (CR3) is expressed on LC isolated from 4-day-old mice. As such neonatal LC may use complement receptors to bind microbial antigens that breach the epidermal barrier until the more specific, carbohydrate-recognition receptors such as DEC-205 are expressed. This further supports the hypothesis that neonatal LC learn and adapt

according to the antigen present in the extracellular environment. An alternative hypothesis is neonatal LC may use complement receptors to further maintain tolerance to self-tissue. Apoptotic debris can be opsonised by C3b and it is well understood that uptake of apoptotic bodies via C3b on DC results in antigen-specific immune suppression (Nauta et al. 2004). This is essential to ensure non-reactivity to self-antigens and its importance following cutaneous UV exposure is well characterised (Aubin 2003). Expression of receptors for C3b ensures that self-tolerance during a time of epidermal development is maintained. . Chapter 3 has furnished strong evidence that suggests, via expression of specific uptake receptors, neonatal LC can modulate the internalisation of antigen for the generation of appropriate immune responses. Through the down-regulation of mannose-specific receptors responsiveness to skin commensals would be avoided. Via thickening of the epidermal barrier, increased keratin and antimicrobial substances provides the neonatal epidermis with an efficient barrier to infection and attack (Marchini et al. 2002). However, if pathogens do cross the epidermal barrier neonatal LC possess receptors such as CD16/32 and CD11b/CD18 that facilitate antigen recognition and internalisation for generation of protective immunity (Okada et al. 1988; Esposito Farese et al. 1996; Fanger et al. 1996).

Based on the dramatic reduction in mannose specific receptors it is logical to propose that neonatal LC are inefficient at the internalisation of the mannose-conjugated antigen FITC-dextran. Despite this reduction, Chapter 4 demonstrated that neonatal LC were efficient at the uptake of small particulate antigens, such as FITC-dextran. However, further analysis demonstrated that if mannose specific receptors were competitively blocked by unlabelled sugar, which is the functional equivalent of a lack of receptor expression, antigen was internalised (Kato et al. 2000). The fact that neonatal LC demonstrated less inhibition in the presence of blocking agents than adult LC suggested that LC from neonatal epidermis are less dependent on receptor-mediated endocytosis to internalise mannosylated antigens. Based on the effective inhibition of uptake by wortmannin it is likely that during the early neonatal period LC preferentially utilise a wortmannin-sensitive fluid phase pathway, such as macropinocytosis, to internalise small particulate and non-binding antigens. As the majority of antigen in the neonatal environment is soluble or non-binding (Adkins et al. 2004b) I propose that neonatal LC employ macropinocytosis to internalise

extracellular antigens, such as apoptotic debris and protein antigen. Given that the way in which antigen is internalised influences the outcome of antigen processing and presentation (Langenkamp et al. 2002), neonatal LC may up-regulate macropinocytosis to direct the pathway utilised to process the internalised antigen.

Chapter 4 has clearly shown that the pathway utilised to internalise antigen alters with acquisition of functional maturity. Ratio data demonstrated that by day 14 the amount of antigen internalised via macropinocytosis is reduced and comparable to that associated with adult LC. Topical application of contact sensitisers results in a decreased amount of antigen transported to the draining LN in association with surface MHC-II compared to adult counterparts (Dewar et al. 2001). As neonatal LC demonstrated an efficient pinocytic activity a divergence in processing must occur to prevent full expression of antigen on the cell membrane. The primary role of receptor-mediated endocytosis is to bind and ingest antigen and to deliver the digested peptides to LAMP-1^{+} vesicles containing MHC-II (Jiang et al. 1995; Lutz et al. 1997). In contrast, studies by Lutz and colleagues indicated that following macropinocytosis of OVA, degraded antigen localised to large vesicles, negative for LAMP-1 (Lutz et al. 1997). Although chapter 5 demonstrated colocalisation of intracellular MHC-II with LAMP-1, the absence of colocalisation of proteolysed antigen with intracellular MHC-II in both neonatal and adult LC suggested that antigen is proteolysed within a LAMP-1^{-} compartment. As it is known that OVA is internalised via macropinocytosis (Lutz et al. 1997), antigen is likely to be proteolysed within immature, LAMP-1^{-}MHC-II^{-} macropinosomes. Very importantly, these results clearly demonstrate that neonatal and adult LC utilise a similar pathway to internalise and proteolyse OVA. However for proteolysed antigen to move out of retention compartments and into MHC-II processing compartments exposure to maturation stimuli is required (Pierre et al. 1997; Turley et al. 2000). Steinman and colleagues have demonstrated that DC are able to retain antigen within the cell for up to several days prior to exposure to maturation stimuli (Turley et al. 2000). Therefore, in order to investigate the relative capacity of neonatal LC to load antigen onto MHC-II and to subsequently traffic these complexes to the cell surfaces LC were exposed to maturation-inducing doses of LPS *in vitro*.

Receipt of maturation stimuli induces the maturation of macropinosomes and the subsequent exchange of Ii for antigens in the peptide-binding groove of MHC-II (Lutz et al. 1997). Although this is the most characterised outcome of maturation, under certain circumstances macropinosomes can bypass the endocytic pathway (Amyere et al. 2002). It has been demonstrated that in B cells and macrophages macropinosomes bypass the endocytic compartment and fuse with the plasma membrane. In doing so degraded antigen is regurgitated back to the extracellular space (Amyere et al. 2002). Rather than utilising macropinocytosis for antigen presentation on MHC-II molecules, it is possible macropinocytosis can be employed as a scavenging and clearance mechanism. Following exposure to LPS surface MHC-II: peptide complexes were evident on the surface of adult LC. This suggests that macropinosomes within adult LC mature in response to LPS and that antigen can be successfully loaded onto MHC-II. In contrast there was a significant reduction in surface MHC-II: peptide complexes on the surface of neonatal LC, with the majority of vesicles retained in a cytoplasmic location distal to the cell membrane. I propose that unlike adult counterparts, macropinosomes within neonatal LC do not mature in response to LPS. As such the machinery required for exchange and loading of digested antigen is not acquired in neonatal LC, thereby reducing the volume of MHC-II: peptide complexes shuttled to the cell surface. As a consequence the relative amount of antigen presented to naive T cells is reduced. By retaining antigen within the cell it cannot be recognised by T cells and as a result effector immune responses are avoided. Therefore despite the high pinocytic activity of neonatal LC they are unable to present antigen to naive T cells effectively. This finding is supported by the recent study of Chang-Rodriguez and colleagues (Chang-Rodriguez et al. 2004). The results suggested that although newborn dendritic epidermal leukocytes were twice as efficient as LC in the uptake of mannosylated antigens, they were incapable of presenting OVA in a MHC-II restricted manner (Chang-Rodriguez et al. 2004). Interestingly when newborn dendritic epidermal leukocytes were enriched to discount for exogenous inhibitory effects the cells maintained the inability to present antigen. However, when the same proportion of cells were isolated from 3-day-old skin the cells presented antigen almost as effectively as LC (Chang-Rodriguez et al. 2004). In comparison to the results of this study where whole preparations were incubated in LPS, it is highly possible that factors secreted by other epidermal cells, such as IL-10, maintain the neonatal LC in

an immature state, rendering them incapable of presenting the OVA efficiently (Enk and Katz 1992b). This is a critical finding as IL-10 inhibits the differentiation, maturation and function of epidermal LC (Willems et al. 1994; Beissert et al. 1995; Peguet-Navarro et al. 1995). In chapter 5 it was suggested that cathepsin S is inactive in neonatal LC, thereby limiting the dissociation of CLIP from the binding groove of MHC-II, thereby limiting the exchange of antigen. Recently it has been demonstrated that IL-10 also inhibits the function of this enzyme (Fiebiger et al. 2001). It was demonstrated that inhibiting cathepsin through IL-10 delayed complexing of MHC-II and digested peptides and subsequently reduced the number of complexes transported to the cell surface (Fiebiger et al. 2001). This finding clearly supports the hypothesis of this study, where although neonatal LC are capable of proteolysing antigen, a combination of heightened cystatin C activity and secreted IL-10, diminishes the capacity of neonatal LC to form immunogenic MHC-II:peptide complexes .

The results furnished from this study have suggested that LC acquire functional maturity during development. As the epidermal network matures there is a subsequent up-regulation of receptor-mediated endocytosis, and application of antigen to this epidermis results in the generation of T cell activation (Dewar et al. 2000). Results from recent studies into the acquisition of functional maturity have demonstrated that the ability to present antigen improved with age (Chang-Rodriguez et al. 2004). When immature APC were isolated at 3 and 6 days post birth the cells demonstrated approximately 47% and 75% of the adult response. Given that at 4-days following birth neonatal LC induce tolerance following cutaneous antigen exposure, the percent function demonstrated by Chang-Rodriguez is insufficient to elicit T cell activation (Chang-Rodriguez et al. 2004). Based on the effective up-regulation of MHC-II and CD86 in response to LPS demonstrated in chapter 5, it is possible that LC resident in 4-day-old epidermis are in a state of transition between functionally inept newborn cells, and fully functional adult LC. These intermediate LC resident in 3-day-old epidermis, although capable of internalising and processing antigen and responding to LPS in culture, are inefficient at the trafficking of MHC-II:peptide complexes to the cell surface. Future studies that investigate antigen processing of epidermal LC throughout the neonatal period is of great interest and worthy of future investigations.

Within the neonatal microenvironment LC endocytose apoptotic debris and present these antigens to induce self-tolerance. Via suppressive cytokines and reduced proinflammatory signals expression of LPS and mannose-specific receptors are maintained at low levels. As a result the internalisation of the majority of environmental and commensal organisms is reduced and the likelihood of excessive responses diminished (Adkins et al. 2004a). However in a stressed microenvironment, such as that which occurs during exposure to potential pathogens and pro-inflammatory cytokines (Stockwin et al. 2000) (Stockwin et al. 2000; Kimber et al. 2002), the antigen is presented in an immunogenic fashion so an anti pathogen response may be elicited (Cruz and Bergstresser 1990). Overall I propose that it is advantageous under most circumstances for neonates to strictly regulate reactions, while retaining the capacity to fully mobilise the adaptive immune system when faced by life threatening or highly infectious agents. Chapter 5 demonstrated that when incubated with high doses of LPS neonatal LC were able to up-regulate CD86 to the same extent as adult LC, supports this hypothesis.

Following application of antigen to the epidermis of neonatal animals the number of skin derived LC resident in the draining LN was significantly reduced (Dewar et al. 2001). I propose that those few neonatal LC that are recruited to the LN do so in an immature state in order to regulate immune responses to innocuous antigens. However the precise mechanism by which neonatal LC regulate tolerance is unknown. It could be hypothesised that immunological tolerance is responsible for the induction and maintenance of neonatal tolerance as the trafficking of cutaneous antigen to the draining LN is significantly reduced compared to adult counterparts (Dewar et al. 2001). However in regards to maintenance of tolerance and the outcome of secondary antigen exposure, there are unfavourable shortcomings associated with this mechanism. The early neonatal period is a time at which the immune response is educated on how to respond appropriately to foreign and self-antigen. If an antigen is ignored, due to incomplete processing and presentation on behalf of the APC, then the immune system cannot distinguish it as self should it be encountered at a later stage. It is then that deleterious autoimmune disorders may be generated. Rather I propose immature neonatal LC actively induce and maintain

peripheral tolerance via a combination of active mechanisms to supplement passive tolerance.

Anergy is a consequence of antigen presentation in the absence of the second, costimulatory signal (Gimmi et al. 1993). This may be applied to neonatal LC, as following exposure to cutaneous antigen, neonatal LC migrate away from the epidermis with reduced expression of CD80 and CD86 (Dewar et al 2001). At the LC:T cell interface insufficient signalling renders the responding T cell anergic. If this antigen is encountered again tolerance will be maintained (Burkly et al. 1989; Mueller et al. 1989). As it has been demonstrated that anergy to a second exposure of the same antigen leads to deletion of the anergic T cell, deletion may also be involved during the neonatal period (Gao et al. 1998; Elhalel et al. 2003; Huang et al. 2003). A possible mechanism of deletion of anergic T cells is through FasL:Fas signalling, inducing apoptosis in selected T cells (Singer et al. 1994; Crispe 1994; Nagata and Golstein 1995). However, as it is possible to break anergy with potent immunogenic signalling (Nossal 1993), additional mechanisms must work in concert with anergy (Takahashi et al. 1998; Thornton and Shevach 1998; Fazekas de St. Groth 2001).

As stated previously, the presence of IL-10 during antigen uptake has significant ramifications for the ability of LC to process and present ingested antigen (Steinbrink et al. 1999; Brossart et al. 2000; Faulkner et al. 2000). The tolerising effect of IL-10 may also extend to the outcome of antigen presentation (Xia and Kao 2003). If exposed to IL-10 at the time of antigen uptake, neonatal LC may migrate away from the epidermis in an immature state, associated with reduced expression of costimulatory molecules and a diminished capacity to synthesis IL-12p70. Following presentation of antigen, these two defects may result in not only anergy (Enk et al. 1993; Enk et al. 1994; Steinbrink et al. 1999), but also a skewing in the Th1-Th2 balance towards tolerance inducing Th2 cells at the second encounter with the same antigen (Adkins et al. 1999). Th2 cells can then act on anti-self effector Th1 cells and down regulate their activity (Mosmann et al. 1986; Haas et al. 1992; Oswald et al. 1992; Romagnani 1992b; Noble et al. 1993; Gajewski et al. 1994; Schwarz et al. 1994; Ullrich 1994). This hypothesis is supported by several studies

by Adkins and colleagues. Firstly, both neonatal mice and humans show a strong bias towards Th2 polarisation (Adkins 1999). Secondly, although capable of eliciting Th1 type immune responses if exposed to strong inflammatory mediators (Adkins et al. 2000; Garcia et al. 2000; Kovarik et al. 2000; Kovarik et al. 2001; Siegrist 2001), Th2 polarisation is evident during primary as well as secondary responses. This contributes to long term self tolerance through the induction and maintenance of memory (Adkins and Du 1998b; Adkins et al. 2001).

Aside from T cell deviation, the cytokine microenvironment may lead to the IL-10-dependent induction of regulatory T cells (Thompson and Thomas 2002). These T cells can then regulate and switch off potentially deleterious responses generated by anti-self effector cells (Mason and Powrie 1998; Sakaguchi 2000). In concert with anergy, immune deviation and regulation will act to induce tolerance as well as maintain memory for further encounters of the same antigen. This is of particular importance as results from two recent studies have demonstrated that the above-mentioned mechanisms of tolerance act together in different ways depending on the type of antigen. T regulatory cells and anergy are involved in tolerance induction to soluble antigens, whereas regulatory T cells and CTLA4 are associated with tissue antigens. Finally, circulating antigens are tolerised via desensitisation of antigen receptors (Abbas et al. 2004). The above model of cutaneous tolerance during the neonatal period is summarised in diagram 6.1.

The hypothesis that neonatal LC actively migrate to the draining LN in order to actively participate in tolerance is supported by the findings of Geissman and colleagues, who suggested that in chronically inflamed skin, LC migrate to the LN without associated maturation. Depending on further signals provided in the LN, these immature LC either induce tolerance or, following receipt of maturation stimuli, induce immunity (Geissmann et al. 2002). As chapter 5 has demonstrated that neonatal LC are fully capable of up-regulating MHC-II and CD86 in response to LPS, it is highly possible *in vivo* neonatal LC are not provided with the correct maturation stimuli when reaching the LN. During the neonatal period the immature LC that do migrate to the draining LN induce tolerance, via a reduction in the two signals required for T cell activation (see diagram 6.1).

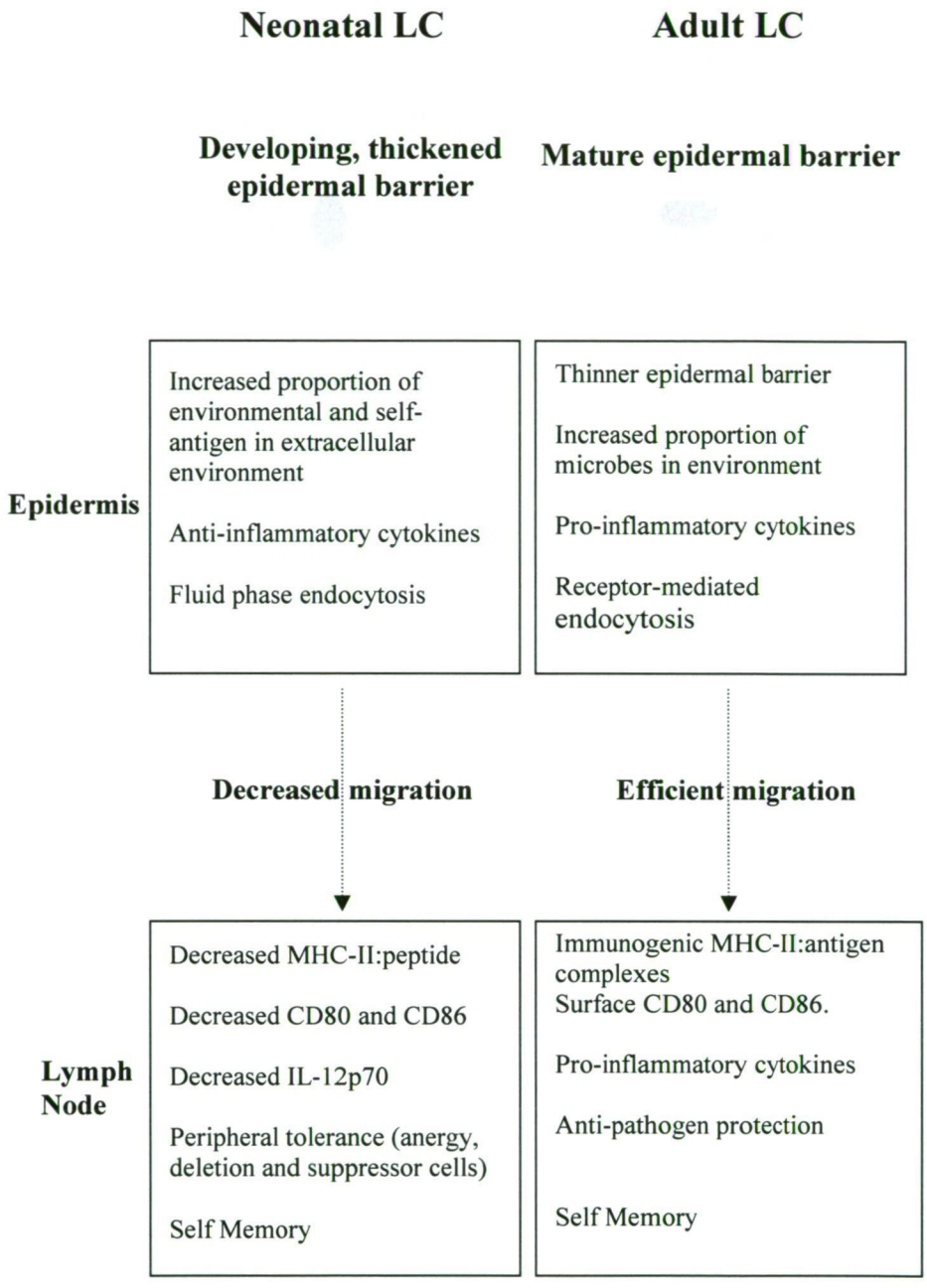

Diagram 6.1 Model of cutaneous tolerance induction during the early neonatal period.

In conclusion I propose that under most circumstances neonates must control inflammatory reactions, while retaining the capacity to fully mobilise components of the adaptive immune system when exposed to highly infectious agents. The aptitude of neonatal LC to sample their external environment without inducing immunity has important biological consequences for the protection against inappropriate responses during the developmental period. Therefore, the elasticity of neonatal immunity might have progressed to avoid inducing inappropriate immune responses under non-threatening conditions through active peripheral tolerance but mature responses when danger is eminent

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