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Title

Cellular and structural factors influencing the induction of Th1 mucosal responses against an enteric pathogen

by Sue fen Kwa

**Thesis submitted for the degree of Doctor of Philosophy
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Abstract

Secondary lymphoid structures are organised networks which support the generation of efficient immune responses by facilitating antigen presentation between an antigen presenting cell (APC) and an antigen-specific naïve T cell. Professional APC such as dendritic cells (DC) are potent primers of T cells and have the capacity to determine the T-helper type 1 or 2 direction of the immune response. Lymphotoxin (LT) is a cytokine of the TNF family which has diverse biological roles, including one as a cytotoxic mediator of immunity. Moreover LT-mediated signals are required for the organogenesis and maintenance of lymphoid structures. The role of various lymphoid structures may have profound effects on the co-ordination of primary immune responses and LT-disruption has been used to examine these requirements. To date, most studies have focussed on systemic infection and here, the roles of LT and various gut-associated lymphoid tissues (GALT) were addressed in the context of enteric infection with the gut-tropic apicomplexan parasite, *Eimeria vermiformis*. Immunity to infection and the induction of protective gut Th1 responses were dependent upon the rapid recruitment of DC to lymphoid structures. Deficiency in lymphoid structures affected the induction of protective immunity and increased susceptibility to infection. Despite the lack of infection in the PP (*E. vermiformis* targets crypt enterocytes), a role for PP was established in the rapid induction of immunity. The anti-parasite response required a co-operative interaction between PP and mesenteric lymph nodes (MLN) with the PP influencing the time of induction of responses in the MLN. Examination of DC numbers and phenotypes revealed a delay in accumulation of DC subsets in PP-deficient mice and supports the role for DC traffic between these lymphoid structures in the induction of rapid gut immune responses.

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Sf Kwa, P Beverley and AL Smith (2006) Peyer's patches are required for the induction of rapid Th1 responses in the gut and mesenteric lymph nodes during an enteric infection. *The Journal of Immunology, 2006, 176: 7533-7541.*

Sf Kwa, P Hesketh, A Archer and AL Smith. Dose-dependent influence of Th1 kinetics during infection with *E. vermiformis.* (manuscript in preparation).

Abbreviations

2-ME	2-mercapethanol
β2M	*β-2-microglobulin*
APC	Antigen presenting cells
BM	*Bone marrow*
BMDC	*Bone marrow-derived dendritic cells*
BrdU	*5-bromo-2'-deoxyuridine*
CP	*Cryptopatches*
DC	*Dendritic cell*
DPI	*Day post-infection*
DN	*Double negative for CD8 and CD4*
DNA	*Deoxynucleotide acid*
E	*Embryonic day*
FACS	*Flow analysis cytometry*
FAE	*Follicle-associated epithelium*
FCS	*Foetal calf serum*
GALT	*Gut-asscociated lymphoid tissues*
GI	*Gastrointestinal*
HEV	*High endothelial venules*
IEL	*Intraepithelial ymphocytes*
IFN	*Interferon*
IFR	*Interfollicular region*
IL	*Interleukin*
ILF	*Isolated lymphoid follicles*
Ig	*Immunoglobulin*
LPL	*Lamina propria lymphocytes*
LP	*Lamina propria*
LPS	*Lipopolysaccharide*
LT	*Lymphotoxin*
LN	*Lymph node*
MAdCAM	*Mucosal vascular addressin cellular adhesion molecule*
MHC	*Major histocompatibility complex*
MLN	*Mesenteric lymph nodes*
M cell	*Microfold cell*
NALT	*Nasopharyxn-asscociated lymphoid tissues*
NK cell	*Natural killer cell*
ODN	*Oligodeoxynucleotide*
OVA	*Ovalbumin*
PAMP	*Pathogen-associated molecular pattern*
PBS	*Phosphate buffered solution*
PP	*Peyer's patches*
PRR	*Pattern recognition receptors*
RNA	*Ribonuecleic acid*
RPMI	*Rosswell park memorial institute*
SD	*Standard deviation*

SED	*Subepithelial dome*
SEM	***Standard error of the mean***
TCR	*T cell receptor*
TGFβ	*Tumour growth factor-β*
Th	*T-helper*
TLR	*Toll-like receptor*
TNFα	*Tumour necrosis factor-α*
VCAM	*Vascular cellular adhesion molecule*

Acknowledgements

1 Introduction

1.1 General introduction

Mammalian adaptive immune systems are complex and built upon a physical infrastructure consisting of primary (thymus, bone marrow), secondary (spleen, lymph nodes, Peyer's patches) and tertiary (eg. isolated lymphoid follicles and lymphoid aggregates induced during chronic inflammation) lymphoid tissues connected by blood and lymphatic vasculatures to non-lymphoid tissues (Figure 1.1). The mucosal immune system provides the first line of host defence against pathogen intrusion through the oral, respiratory or urogenital routes and is a sophisticated network of compartments containing diverse populations of immune cells. With the gastrointestinal (GI) mucosal immune system, it is an immense challenge fine-tuning responses to differentiate between pathogenic and harmless antigenic derivatives from the constant exposure to food and microbes in the GI lumen. This demands the presence of specialized immune cells, cellular organisation and compartmentalisation to ensure that pro-inflammatory responses occur only when and where appropriate (ie. during infection), and when such immune responses are warranted, they are carried out with uncompromising efficacy. In order to fully comprehend the complexity of mechanisms present in the GI immune system, there must be a basic understanding of the immune anatomy and the interactions that are associated with the GI tract. This introduction begins with a description of the cellular organisation within immune compartments and the development of such compartments, reviewing the roles of different cell populations in the maintenance of oral tolerance and/or control of enteric infections. Finally, the background and rationale for the experimentation presented in this thesis are provided.

Systemic circulation

Left subclavian vein

Thoraic duct

Heart

Spleen

Skin

Epidermis

cutaneous-draining lymph node

Small intestine

Intestinal villus

Peyer's patch

mesenteric lymph nodes

Legend

Lymphatic vessel and direction of lymph flow

Blood vessel and direction of blood flow
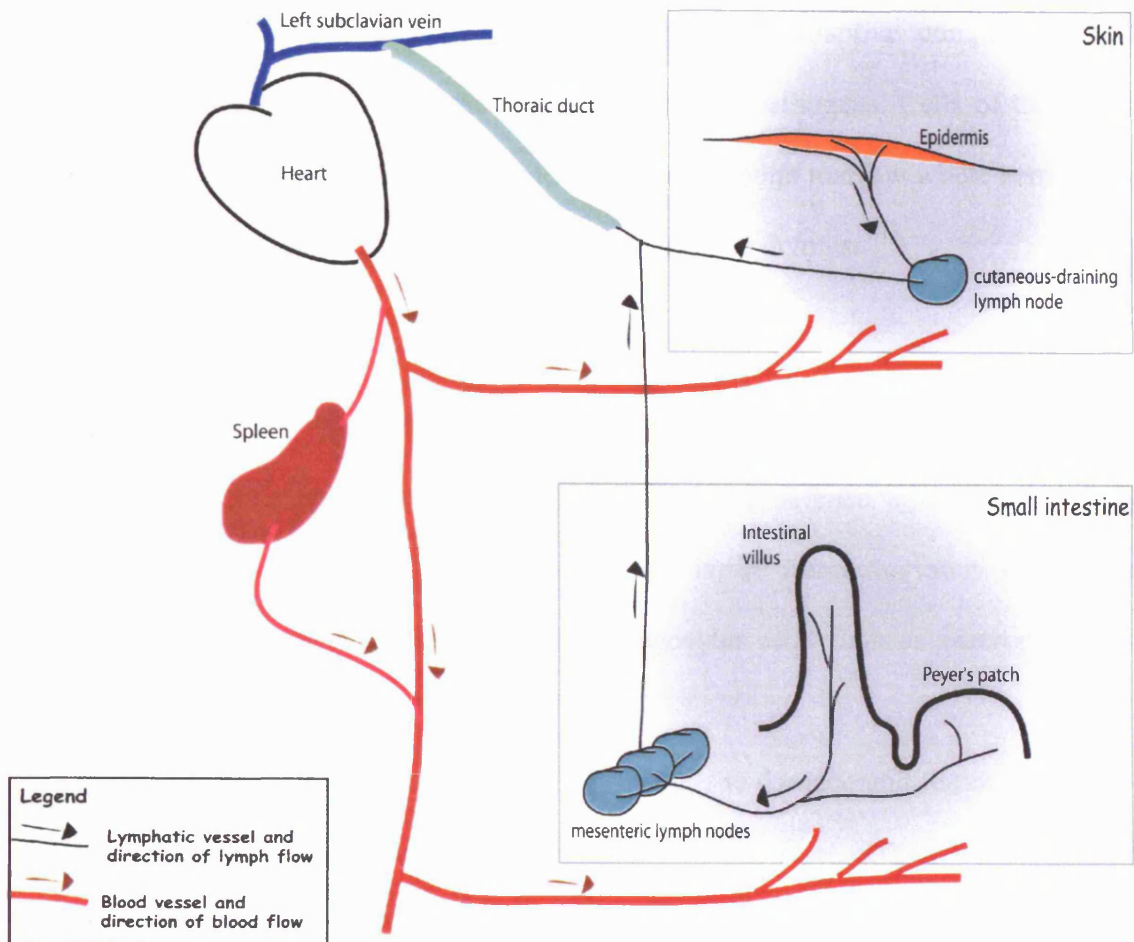
Figure 1.1   General illustration of the lymphatic and blood vasculatures linking non-lymphoid and lymphoid tissues.

The skin epidermis is drained by afferent lymphatics into the cutaneous lymph node while the small intestinal areas (eg. villus and Peyer's patches) are drained by afferent lymphatics into the mesenteric lymph nodes. Efferent lymphatics exit both tissue-draining lymph nodes into the thoracic duct, which empties into the left subclavian vein and connects the lymphatic system to the blood circulation. Blood filters through the spleen and re-enters non-lymphoid tissues during circulation.

1.2 Basic overview of the mammalian immune system

The mammalian immune system consists of innate and adaptive components which function cooperatively to protect the host from invading pathogens. Cells of the immune system are derived from hematopoietic stem cells in the bone marrow where some mature and others migrate out to further develop and mature in the thymus.

1.2.1 The innate immune system

The innate immune system serves as the first level of defence against infection and responds in a generic manner. Components of the innate immune system include anti-microbial peptides, the complement system, phagocytic cells such as macrophages and neutrophils, and natural killer (NK) cells.

Anti-microbial peptides contribute to bacterial membrane damage and the opsonisation of bacteria which facilitates phagocytosis (Krisanaprakornkit et al., 2000; Ouellette, 1999). The complement system is made up of a large number of distinct plasma proteins which opsonise pathogens, cause damage by inducing pores into bacterial membrane and induce inflammatory responses against infection (Frank and Fries, 1991). Opsonised pathogens are engulfed by macrophages and neutrophils which recognise, ingest and destroy pathogens without help from the adaptive immune system. Cells of the innate system discriminate pathogens through pattern recognition receptors (PRR) such as Toll-like receptors (TLR), mannose receptors, scavenger receptors and CD14 (bacterial LPS receptor) which recognise pathogen-associated molecular patterns (PAMPs)(Kopp and Medzhitov, 1999; Medzhitov and Janeway, 2000). NK cells do not require prior activation and through the recognition of changing levels of self molecules (eg. MHC class I), kill infected cells by

releasing cytotoxic granules into their target cells. NK cells are important contributors during the early phases of infection with intracellular pathogens such as herpes virus, trypanosomes and *Listeria monocytogenes* (Albright et al., 1997; Biron et al., 1999; Dunn and North, 1991). There are two types of receptor that regulate NK cell cytotoxicity, one is an activating receptor which triggers killing while the other inhibits the killing of normal host cells (Lanier, 1998).

1.2.2 The adaptive immune system

The adaptive immune system serves as a second level of defence against infection and is distinguished from the innate immunity by its specific response and ability to develop into immunological memory. The main components of the adaptive immune system are T cells and B cells which both express antigen-specific receptors. B cells recognise antigen through the B cell receptor in the form of immunoglobulins. Immunoglobulins of the same antigen-specificity can also be secreted as antibodies which bind to pathogens, neutralising them or marking them to be destroyed by phagocytes and complement. T cells express T cell receptors (TCR) which recognise specific antigens presented to them on a major histocompatibility complex (MHC) expressed by professional antigen-presenting cells (APC) which include dendritic cells (DC), B cells and macrophages.

The T cells express TCRαβ or TCRγδ chains, which recognise antigen presented in the context of classical (class I and II) or non-classical MHC (eg. CD1) molecules respectively. Two basic types of TCRαβ+ T cells can be further distinguished by the expression of CD8 or CD4. Naïve T cells need to be activated (presented antigen by DC) before they

differentiate to become effector T cells. CD8+ T cells recognise antigen in the context of MHC class I presentation and can be activated to acquire cytotoxic effector function which is important during anti-viral responses. CD4+ T cells are restricted by MHC class II and differentiate into T helper (Th) effector cells. There are two main Th effector cells which are important in immunity against infections. The Th1 CD4+ T cell is characterised by the higher levels of IFNγ, IL2 and TNF produced while the Th2 CD4+ T cell produces higher levels of IL4, IL5 and IL10 (Mosmann et al., 1986; Swain et al., 1988). Broadly, Th1 CD4+ T cells activate macrophages and also activate B cells to secrete certain classes of antibodies. Th2 CD4+ T cells help initiate B cell proliferation and differentiation, and the production of immunoglobulins. Generally, the B cell response constitutes the humoral immune response.

The cellular basis of memory in both T and B cells is the result of changes in the frequency of responding cells and in the activation requirements for previously-stimulated cells. The response from immunological memory is more rapid and effective against a recurring infection with the same pathogen and both T and B cells contribute to immunological memory. T cells develop into central and effector memory T cell (Sallusto et al., 1999) where the former circulates through lymphoid organs and the latter is found in non-lymphoid tissues. B cells differentiate to form plasma cells which produce long-lasting antibodies and memory B cells which have higher immunoglobulin affinity (McHeyzer-Williams, 2003).

When considering immunity, the division of these components is an over-generalisation of the complex system involved. The innate and adaptive immune systems interact extensively and share common components and characteristics. Dendritic cells (further discussed later in section 1.6) contribute to both innate and adaptive immunity. They are central to the development of adaptive immunity as they are able to stimulate naïve T cells (Banchereau and Steinman, 1998). They also activate NK cells through IL12 production (Fernandez et al., 1999). Furthermore, there are T cells with NK-like characteristics (NKT cells) which are important for early responses to *Listeria* infection (Ranson et al., 2005). Recently, DC with NK-like characteristics have been described and shown to produce high levels of IFNγ (a trait characteristic of NK cells) and to present antigen (Pillarisetty et al., 2005). Moreover, NK cells have been shown to display some level of immunological 'memory' (O'Leary J et al., 2006), suggesting that NK cells may contribute more to adaptive immunity than previously thought.

1.2.3　The general immune response during infection

The immune response may be divided into the immediate (0-4hr), early (4-96hr) and late (>96hr) phases. Innate immunity plays an important role during the initial phases of infection. The immediate phase is carried out by phagocytes (mainly macrophages) surveying the area of tissue that has been breached by an invading pathogen. During the early phase, increased inflammatory cytokines secreted by the innate immune system helps recruit more immune cells to the site of infection. At the same time, DC which are also constantly surveying tissues, uptake pathogen-derived material and process it for presentation to antigen-specific naïve T cells in secondary lymphoid organs. When antigen

presentation occurs, the adaptive immune response is initiated. It takes at least around four days for adaptive immunity to appear as time is required for B or T cells to locate their specific antigen, proliferate and differentiate. The effector T cells or antibodies produced are then dispersed to the site of infection for elimination of the pathogen. A successful adaptive immune response results in the generation of immunological memory which responds with greater efficiency when a second infection with the same pathogen occurs, providing protection to the host.

1.2.4   Secondary lymphoid compartmentalisation

The encounter between an APC and naïve T cell is limited by low frequencies of antigen-specific T cells (~1 in 10000) (Blattman et al., 2002) and therefore the presence of organised secondary lymphoid tissues serves as a point of convergence where both immune cells and antigens can interact more efficiently. Secondary lymphoid tissues comprise of the spleen, lymph nodes (LN), mucosal-associated lymphoid tissues (MALT), Peyer's patches (PP) and other organised lymphoid clusters associated with gastrointestinal, genitourinary and respiratory tracts (although some of these clusters may be classified as 'tertiary'). The MALT include tonsils, nasalpharynx-associated lymphoid tissues (NALT) and gut-associated lymphoid tissues (GALT). Secondary lymphoid tissues such as LN and PP are equipped with lymphatic and vascular systems which drain from specific tissues and provide access to the blood circulation through high endothelial venules (HEV) respectively. Common features of secondary lymphoid tissues are the organisation of T and B cell zones, germinal centers, vasculature for cell traffic, all of which provide strategic meeting points for effective and efficient immune interactions.

In a LN, the outer cortex is made of primary B cell follicles, some of which differentiate to become secondary follicles which develop into germinal centers (Figure 1.2). The paracortical region contains T cells and DC, and the medullary cord contains macrophages and highly-differentiated B cells (plasma cells). Naïve T cells constantly pass through the LN via HEV found in the T cell-rich paracortical area, where they scan the surface of antigen-presenting DC for specific peptide-MHC complexes. The DC carrying antigen uptaken from non-lymphoid sites, enter the LN through the afferent lymphatics. If antigen-recognition does not occur, the T cells exit the LN via the efferent lymphatic vessel and back into the blood circulation through the thoracic duct. Re-circulating B cells enter the LN through the HEV and some become trapped in the T cell zone where they are stimulated to proliferate (Liu et al., 1991). Some B cells will migrate towards the primary follicle area to develop into germinal centers or the medullary cords to develop into effector and memory B cells (Garside et al., 1998).
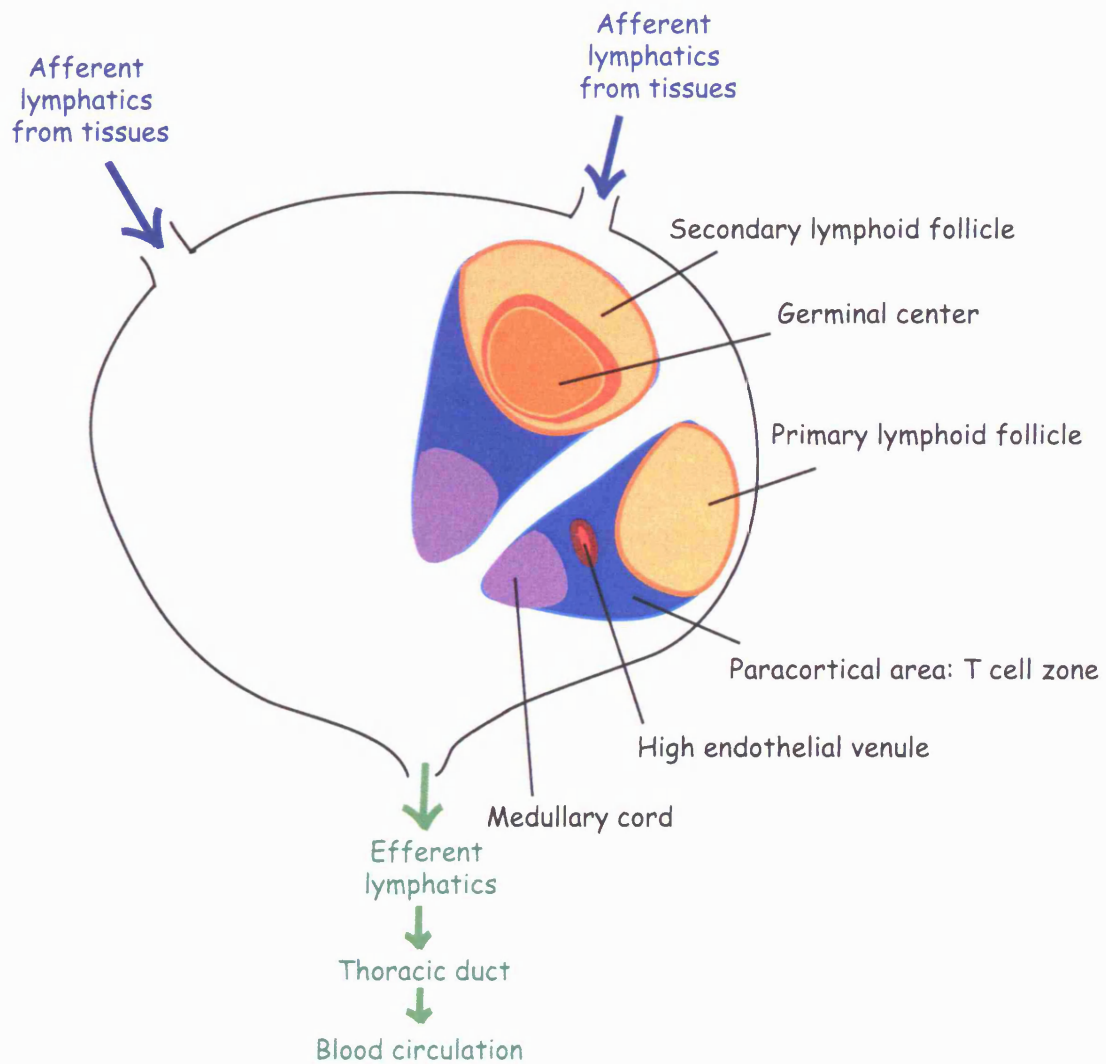
Figure 1.2 Schematic of a lymph node organisation.

Afferent lymphatics carry lymph containing various cells (eg. DC) from the peripheral tissues into the lymph node (LN). The outer region of the LN is composed of B cell lymphoid follicles and an inner region contains paracortical areas comprised of mainly T cells and DC. Secondary lymphoid follicles contain germinal centers where active B cell proliferation occurs. The medullary cord contains macrophages and plasma cells which secrete antibody. Specialized high endothelial venules (HEV) enable naïve lymphocytes cells to enter the LN. The T cells which enter the LN and are activated with a specific antigen presented by DC, remain to proliferate while non-activated T cells exit the LN through the efferent lymphatics to recirculate the body.

1.3 Immunology of the intestinal mucosa

The GI tract is an organ for nutrient absorption and for this purpose, contains millions of intestinal villi which provide an immensely large surface area ($\sim$400m^2 in humans) that is highly exposed to antigen from commensal microbes, pathogens and food (MacDonald and Gordon, 2005). This complex, compartmentalised organ has important roles not only in digestion and nutrient absorption, but also in host defence against infections. The epithelial layer of the GI tract acts as a barrier against pathogens invading through the oral route and the GI tract is organised into inductor and effector sites equipped with a diverse range of immune cells for protection against infection from across the intestinal lumen. The inductor and effector sites include the intraepithelial (IE) and lamina propria (LP) compartments and the GALT such as Peyer's patches (PP), isolated lymphoid follicles (ILF) and mesenteric lymph nodes (MLN) (Figure 1.3A). These sites are essential for immune responses against infections but at the same time, regulatory mechanisms are in place to allow oral tolerance and prevent damaging inflammations in the absence of infection.

1.4 Intraepithelial and lamina propria compartments

Intraepithelial lymphocytes

The IE and LP compartments of the GI tract contain subsets of T lymphocytes that are unique from 'conventional' systemic T cells circulating the periphery. The IE compartment contains lymphocytes (IEL) which are found in the IE area above the basement membrane and below the tight junctions of epithelial cells (Goodman and Lefrancois, 1989) (Figure 1.3A). Approximately 10-20 IEL are present for every 100 villus enterocytes lining the

A

Intestinal lumen

Villus

IE compartment

Basement membrane

IEL

Epithelial cell

Isolated lymphoid foliicle

FAE

SED

lf

LP compartment

Crypt

Peyer's patch

FAE

SED

lf

IFR

lf

Not drawn to scale

B

FAE

SED

lf

IFR

lf

Peyer's patches
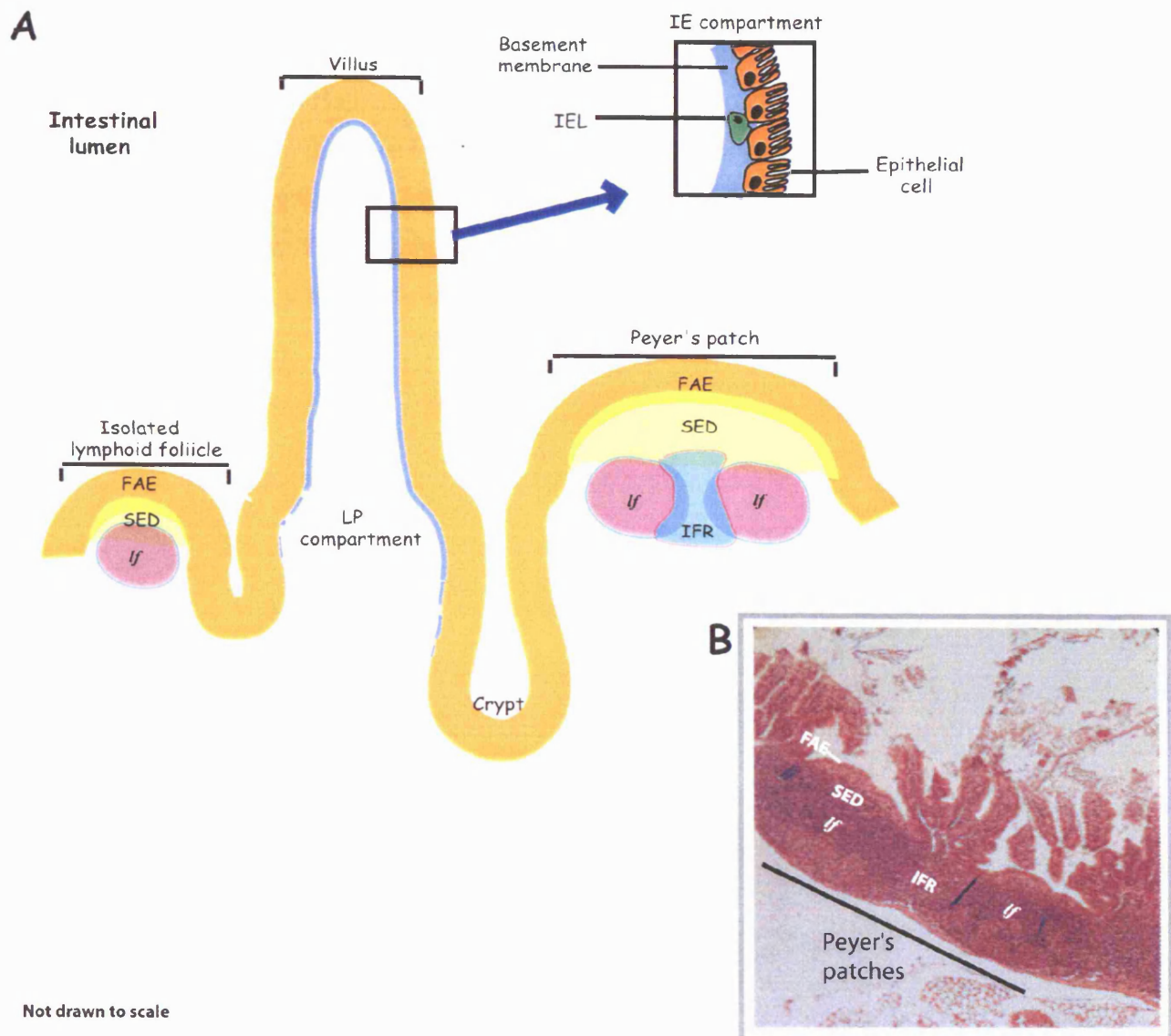
Figure 1.3 Location of compartments and lymphoid structures in the small intestine.
A) The villus is an intestinal feature which protrudes outwards into the lumen. The intestinal IE compartment is lined by a single layer of epithelial cells and lies above the LP compartment, separated by a basement membrane. IEL are found between the epithelial layer and basement membrane. The crypt is a region of proliferating epithelial cells which differentiate as they move up towards the tip of the villus or in the opposite direction to form the FAE of PP. The PP and ILF are lymphoid structures found interspersed along the

small intestine. Both PP and mature ILF contain a FAE layer which lies above the SED and *If*. The ILF are not known to have IFR unlike PP. Immature ILF have smaller *If* and do not have a FAE layer. B) Hematoxylin and eosin-stained section of PP from a naïve B6 mouse with the various compartments labelled. Abbreviations: IE, intraepithelial; IEL, intraepithelial lymphocytes LP, lamina propria; PP, Peyer's patches; ILF, isolated lymphoid follicles; FAE, follicle-associated epithelium; SED, subepithelial dome; *If*, lymphoid follicles; IFR, interfollicular region.

small intestine of humans (Ferguson, 1977). The majority of IEL in the small intestine are CD8+ T cells and differ from the CD8:CD4 ratio (2:3) seen with systemic T cells although in the large intestine, there is a higher proportion of CD4+ IEL (Camerini et al., 1993). The IEL are further divided into CD8αβ+, CD8αα+, CD4+CD8αα+ and CD4-CD8- double negative (DN) subsets (Figure 1.4A), all of which, except the CD8αβ+ subset, are rare or absent from systemic circulation. Around 10-40% of the small intestinal IEL population express TCRγδ compared with the spleen where less than 5% are TCRγδ+ (Goodman and Lefrancois, 1988). Where most 'conventional' peripheral T cells are TCRαβ+, the IEL have a substantial population of both TCRαβ+ and TCRγδ+ T cells. The TCRγδ+ IEL are mainly CD8αα+ or DN while TCRαβ+ IEL are CD8αβ+ or CD8αα+.

A simple classification suggests the grouping of IEL according to MHC class restriction (Hayday et al., 2001) (Figure 1.4B). The first group (type a) represents TCRαβ+ IEL which recognise antigens presented by conventional MHC class I and II. The second group (type b) includes TCRαβ+ CD8αα+ and TCRγδ+ IEL which recognise antigen presented in the context of non-classical MHC molecules. Conventional MHC class I and II-restricted IEL (type a) bear similar characteristics to the systemic T cells, being able to respond through cytokines and mediate cytotoxicity for protection against cytomegalovirus, rotavirus, *Toxoplasma gondii* and other pathogens (Dharakul et al., 1990; Lepage et al., 1998; Muller et al., 2000). These classical 'T cell–like' IEL are primed in the gut lymphoid tissues and drain into the efferent lymphatics to the thoracic duct. After which they enter the blood, home back to the lamina propria and then to the epithelium of the small and large intestine (Arstila et al., 2000; Hayday et al., 2001).
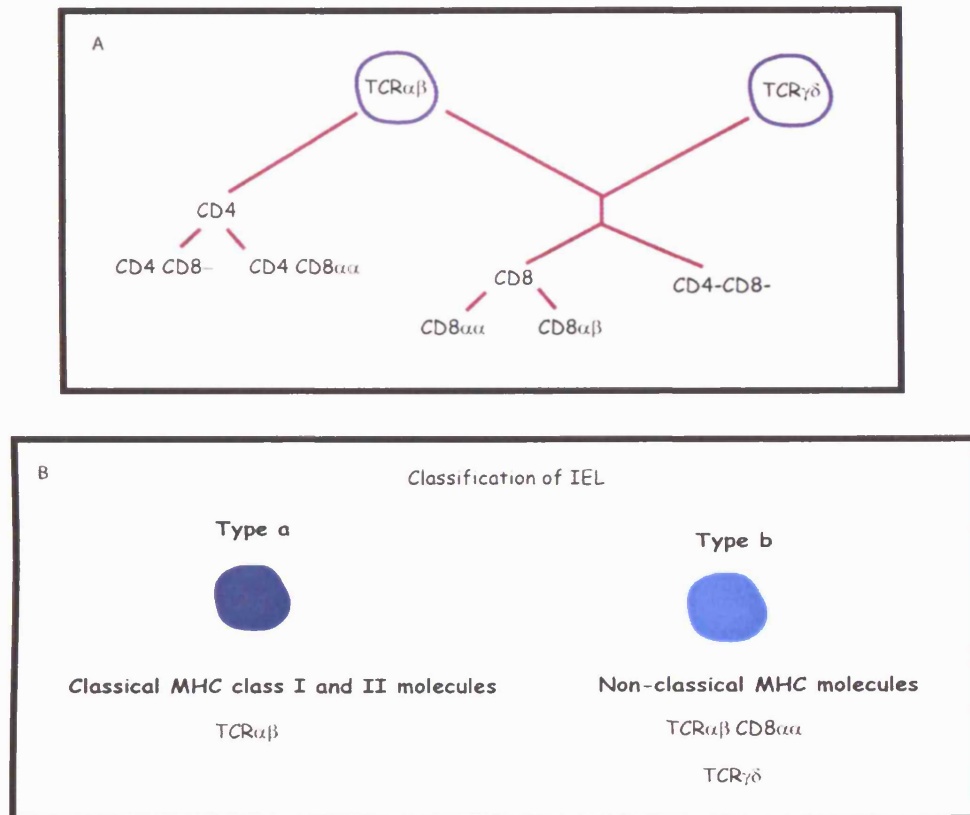
Figure 1.4 IEL subsets and classification.

A) The major IEL subsets described using CD8, CD4 and TCR markers. B) Categorisation of IEL by classical and non-classical MHC class restriction into type a or type b IEL (Hayday et al., 2001).

Antigen-primed IEL that have been adoptively transferred, provide protection and traffic to the intestine of *Toxoplasma gondii* infected mice (Buzoni-Gatel et al., 1999; Buzoni-Gatel et al., 1997).

Type b IEL are considered to play an immunoregulatory role in modulating pro-inflammation since their proportions seem to correlate inversely with disease symptoms (Hayday et al., 2001) and they may be involved in maintenance of intestinal epithelial cell proliferation (Boismenu and Havran, 1994; Yang et al., 2004). The type b IEL which are restricted in the context of non-classical molecules and selected by self-antigens in the gut, show poor responses towards stimulation. For example, CD8αα+ IEL do not exhibit the classical antigen-specific cytotoxic T cell activity and they proliferate poorly in response to mitogens, superantigens or anti-TCRαβ antibody (Poussier et al., 1992; Poussier et al., 1993; Sydora et al., 1993). Further examples are self-specific TCRαβ+ CD8αα+ CD4- IEL which protect against colitis (Poussier et al., 2002), TCRγδ+ IEL which inhibit CD4+ T-helper and CD8+ CTL responses when transferred into naïve hosts (Kapp et al., 2004) and the aboragation of oral tolerance after treatment with anti-TCRγδ antibody (Ke et al., 1997). Generally, type b IEL have hyporesponsiveness and immunoregulatory properties although there are exceptions. TCRγδ+ IEL are shown to contribute to protection against enteric infections of very young mice with *Eimeria vermiformis, Cryptosporidium parvum* (Ramsburg et al., 2003; Waters and Harp, 1996), indicating that type b IEL also participate in the defence against invading pathogens.

Lamina propria lymphocytes

The intestinal LP is mainly made up of loose connective tissue enriched with cells (eg. fibroblasts, lymphocytes, plasma cells, macrophages, eosinophilic leukocytes, mast cells) and is found beneath the basement membrane which supports the overlying layer of intestinal epithelial cells (Lefrancois and Puddington, 2006). Cells from both innate and adaptive immunity such as DC, macrophages, T cells, B cells and IgA-producing plasma cells are present in the LP. The LPL share more similarities with peripheral T cells as they have a CD4:CD8 T cell ratio of 2:1 and a smaller proportion of TCRγδ+ T cells. Many of the lymphocytes in the LP are activated or memory T cells passing through the gut from the periphery although many are known to be terminally differentiated (Sprent, 1976). A large proportion of memory T cells is known to be retained in the LP after infection with pathogens such as *Listeria monocytogenes* which spreads systemically (Pope et al., 2001).

1.4.1 Organised gut-associated lymphoid tissues

Organised GALT structures include PP, ILF, cryptopatches (CP) and MLN. The LP and IE compartments are also considered effector sites associated with the GALT but do not have the same level of organisation seen in secondary lymphoid organs. The ILF are a recent addition to this group and the role of these structures is less studied compared to PP and MLN which are both considered fundamental inductor and effector sites of intestinally-derived immune responses.

Peyer's patches

Peyer's patches are macroscopic aggregates of lymphoid cells found along the anti-mesenteric border of the small intestine and contain organised T cell and B cell zones, sharing similarities with LN organisation (Figure 1.2 and 1.3). There are variations in PP structures across vertebrate species: In mice and humans, PP are found as macroscopic aggregates of lymphoid cells scattered along the small intestine where as many as 300 PP can be found in man while the numbers are much smaller in mice depending on the strain (4-9 average) (Cornes, 1965). The PP of sheep, cattle and pigs occur as a continuous ridge-like structure along the ileum region of the small intestine, extending 1-2 metres in length (Griebel and Hein, 1996; Yasuda et al., 2006).

Peyer's patches have an overlying follicle-associated epithelium (FAE) which is made up of a layer of cuboidal enterocytes with brush borders thicker than those of villus enterocytes and has no Paneth cells or goblet cells (Kraehenbuhl and Neutra, 2000). Luminal antigen sampling is carried out through endocysis and phagocysis by specialized microfold cells (M cells) in the FAE. A pocket of space exists basolaterally beneath the M cell which is accessible to antigen presenting cells (APC) and lymphocytes (Figure 1.5). Unlike LN, PP do not have afferent lymphatics although this is debatable at present as some report the presence of lymphatics draining the intercellular spaces of the intestinal epithelium in pigs (Lowden and Heath, 1994). However, PP are exposed to antigens from the lumen and most antigens in the PP are obtained through M cell sampling of the lumen. Immediately beneath the FAE is the subepithelial dome (SED) which overlies the PP lymphoid follicle (Figure 1.3). There are germinal centers and a network of follicular DC in

the B cell lymphoid follicles which lie below the SED. The interfollicular region (IFR) is mainly a T cell zone and contains HEV which provide lymphocyte access from the blood circulation. Antigens and microorganisms transported across M cells are believed to be taken up by DC which form the vast majority of APC in the SED (Kelsall and Strober, 1996). The DC which have uptaken antigen, either migrate to the adjacent T cell zone within the PP (Shreedhar et al., 2003) or migrate to the MLN via lymphatic vessels draining the area (Macpherson and Uhr, 2004; Pron et al., 2001) to present to T cells.
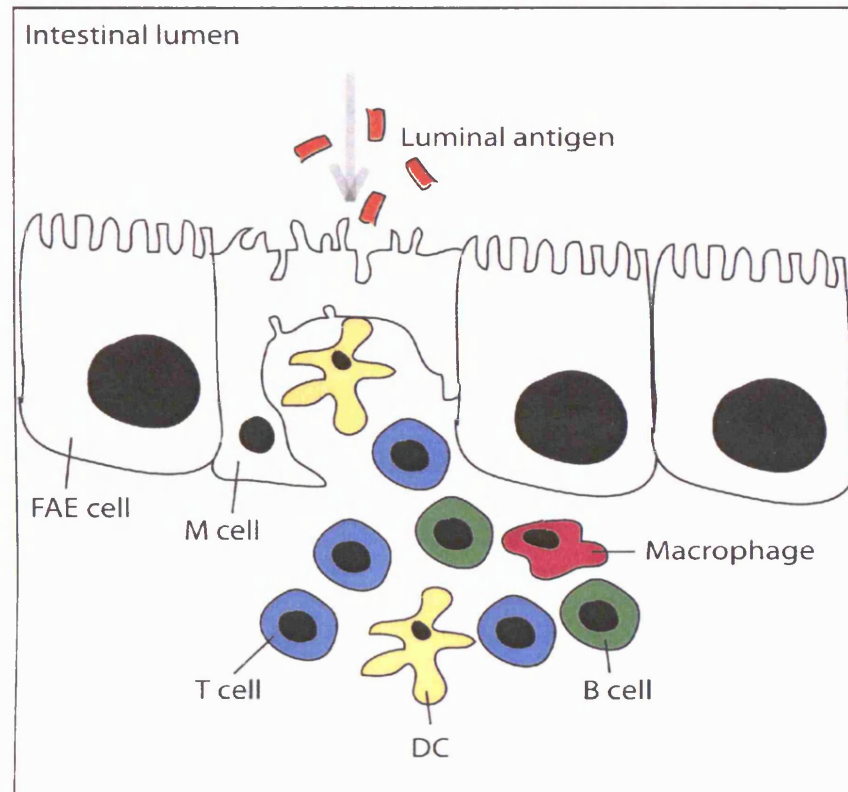
Figure 1.5 Illustration of the microfold cell location.

The M cell lies in the FAE layer between epithelial cells and has a basolateral pocket which is accessible by antigen-presenting cells (DC, macrophages) and lymphocytes (T and B cells). The microvilli of FAE cells have a shorter brush border than villus epithelial cells. Antigen-sampling occurs through endocytic and phagocytic processes which transfer luminal Ag across the M cell to local antigen-presenting cells. Abbreviations: FAE, follicle-associated epithelium; M cell, microfold cell.

Possessing a network of cells from both innate (macrophages, NK cells) and adaptive immunity (DC, T cells, B cells) and the ability to sample luminal antigen, the PP become important inductive and effector sites of mucosal immunity. T cell proliferation and Th1-type cytokine responses are generated in the PP during the course of infection with some pathogens (Fan et al., 1998; McSorley et al., 2002). A large amount of immunoglobulin A (IgA) production occurs in the PP (although PP are not required for IgA production, (Chin et al., 2003). IgA is an important element of the mucosal humoral immune response and serves as a defense mechanism against pathogens on the mucosal surface (Burrows and Cooper, 1997; Craig and Cebra, 1971; Fagarasan et al., 2001; Macpherson et al., 2000). During 'steady state' conditions, PP T cells are biased towards a typical Th2 range of cytokines, producing more IL4, IL5, IL10 than IFNγ and such an environment may be influenced by B cells (Iwasaki and Kelsall, 1999; Kellermann and McEvoy, 2001). This Th2 bias may exist to support the predominance of IgA+ B cells in the PP and also contribute to oral tolerance (Newberry and Lorenz, 2005). Cell populations such as PP-DC and PP-regulatory T cells which produce IL10 and TGFβ may drive the Th2-type environment as IL10-neutralising antibody in cultures resulted in increases in IFNγ expression (Iwasaki and Kelsall, 1999). The maintenance of such a Th2-type microenvironment serves to modulate immune responses (oral tolerance) and avoid unnecessary and potentially detrimental pro-inflammatory responses against antigens derived from food and commensal microbes.

There is some level of participation by PP in the induction of oral tolerance, although the MLN may be more important as oral tolerance can be induced in PP-deficient mice (Kunkel

et al., 2003; Spahn et al., 2002). Recent evidence shows that the major contributors of oral tolerance are LP-DC which express CCR7 and present antigen in the MLN (Jang et al., 2006; Worbs et al., 2006). However, the requirement for PP in the induction of oral tolerance may depend on the type and dose of oral antigen (Kunisawa et al., 2002; Kunkel et al., 2003; Spahn et al., 2002; Yamamoto et al., 2000). Alternatively, other gut-associated lymphoid structures such as ILF may contribute to oral tolerance, a factor that has not been considered in experiments using PP-deficient mice (Newberry and Lorenz, 2005).

Isolated lymphoid follicles

The ILF are small lymphoid aggregates found along the anti-mesentery of the small intestine and bear similar features to PP (a FAE with M cells, SED and a single follicle) (Hamada et al., 2002)(Figure 1.3A). However, there are no defined T cell zones (unlike the IFR in PP) and the formation of this tertiary lymphoid organ can be induced during adulthood (Lorenz et al., 2003). The development of ILF is influenced by adaptive immune responses and immature ILF have a smaller lymphoid follicle but no FAE unlike mature ILF. As with PP, ILF are sites for IgA class switching and the priming of T cell immune responses against orally administered antigen (Lorenz and Newberry, 2004; Shikina et al., 2004).

Mesenteric lymph nodes

The MLN form the largest set of LN in the body and consist of a chain-like structure of lymph nodes with blood vasculature and lymphatics draining from different regions of the gut. This structure has an important role in the induction of oral tolerance and immune responses. The MLN have the general characteristics of LN with demarcated T and B cell

zones, germinal centres, HEV, afferent and efferent lymphatics. Naïve T cells from the systemic circulation enter the MLN through HEV while gut-derived cells (eg. MLN-DC, T cells) and antigens enter the MLN through afferent lymphatics that drain the gut (Azzali, 2003; Liu and MacPherson, 1993). The MLN can be described as a 'gateway' between mucosal and systemic immune systems where systemically-derived cells from the circulation may encounter intestinally-derived cells. The high load of food antigens and commensal microbes found in the intestine is kept 'compartmentalised' away from the systemic immune system which does not have the same mechanisms of tolerisation as the GI mucosal immune system. This function supports the role for the MLN as a gatekeeper, preventing unwanted systemic responses to microbes that are non-pathogenic (Macpherson and Smith, 2006).

Cryptopatches

Cryptopatches (CP) are clusters of cells found at the base of intestinal crypts, in the lamina propria area (Kanamori et al., 1996). In mice, CP consist of approximately 1000 cells and do not have a fixed pattern of distribution along the small intestine or colon. There are three main cell types found in CP: lineage marker (lin)- c-kit+ cells, CD11c+ cells and VCAM1+ stromal cells. In contrast to PP, T cells and B cells are absent or scare (~<2%) in the CP. The majority of CP cells are lin- c-kit+ cells which may be similar to the inducer cells which are involved in the development of PP. CD11c+ cells form the second major cell population.

There is conflicting evidence for and against the CP as sites of extrathymic development of IEL. Direct evidence for the extrathymic development of IEL from CP is seen in athymic

mice reconstituted with wild-type bone marrow and T-cell deficient mice grafted with tissues (Saito et al., 1998; Suzuki et al., 2000). However, IEL are present in lymphotoxin-deficient mice which do not have CP (Pabst et al., 2005). Perhaps CP function as sites containing progenitor cells that have the potential to develop T cells but are not entirely necessary for the development of IEL. The CP are also thought to act as ILF precursors because immature ILF bear resemblance to CP containing B cells (Newberry and Lorenz, 2005; Pabst et al., 2005).

1.5   Lymphotoxin

Lymphotoxin (LT) (also known as TNFβ) is a member of the TNF superfamily, a family of receptors defined by a cysteine-rich ectodomain which is involved in controlling cell death, survival and differentiation. There are two structural forms of LT: LTα and LTβ, whose encoding genes lie separately adjacent to TNFα on mouse chromosome 17 (chromosome 6 in humans) which also encodes the MHC genes (Nedospasov et al., 1986). Two functional forms of LT predominate: a soluble LTα_3 homotrimer and a membrane-bound LT$\alpha_1\beta_2$ heterotrimer (Androlewicz et al., 1992). A minor LT$\alpha_2\beta_1$ heterodimeric form exists but at present, it has no defined role and is expressed at very low levels by T cells (Ware, 2005). LTα_3 binds the same receptors as TNFα (TNFR1 and TNFR2). LT$\alpha_1\beta_2$ binds LTβR which is encoded on chromosome 6 of the mouse (chromosome 12 in humans) and shares this receptor with LIGHT, another LT-related molecule. LIGHT also binds the herpesvirus entry mediator which is an entry route utilised by herpes simplex virus (Montgomery et al., 1996).

LTα₃ and LTα₁β₂ are expressed by haematopoietic cells such as T cells, NK cells, B cells and DC (Androlewicz et al., 1992; Browning et al., 1997). LTβR is expressed by stromal cells such as follicular DC and fibroblasts but not on lymphocytes (Murphy et al., 1998). Lymphotoxin has cytotoxic properties (Browning et al., 1996; Mackay et al., 1996) and is associated with T cell activation and Th1 polarisation as recently activated naïve CD4+ T cells and Th1 effector cells express LTα₁β₂, and IL4 downregulates LT during Th2 polarisation (Gramaglia et al., 1999). During embryonic development, LT expressed by bone marrow-derived inducer cells is critical in the generation of lymphoid organs (discussed below). This dynamic process involves the mobilisation of inducer cells responding to the appropriate organiser cells (stroma) and is aided by chemokines and adhesion molecules.

1.5.1 Lymphotoxin and the development of secondary lymphoid structures

Lymphotoxin is closely linked with lymphoid organogenesis and LT-deficient mice have various defects in secondary lymphoid organisation (Table1.1). The variable phenotype of LT-deficient mice indicates that several LT-associated pathways are involved in the development of different lymphoid structures. The LTα-/- mice are deficient in both LTα₃ and LTα₁β₂ signalling, LTβ-/- mice lack LTα₁β₂ signalling while LTβR-/- mice exhibit losses in LTα₁β₂ and LIGHT signalling. LTα-/- and LTβR-/- mice lack all LN and PP while LTβ-/- mice possess a similar phenotype with the exception of a residual MLN and cervical LN being present (Banks et al., 1995; De Togni et al., 1994; Koni et al., 1997; Rennert et al., 1996). The LTβR-/-, LTα-/- and LTβ-/- mice have disorganised spleens and NALT although the splenic defects in LTβ-/- mice may be less severe (Alimzhanov et al., 1997; Drayton et

al., 2006; Futterer et al., 1998; Ying et al., 2005). The MLN in LTβ-/- mice contain germinal

centers but lack follicular DC (Koni et al., 1997). The proinflammatory cytokine TNFα,

which is closely related to LT, is also involved in lymphoid organisation as TNF-/-and

TNFR-1-/- mice have disorganised spleens (absent B cell follicles, germinal centers,

follicular DC) and disorganised PP (Korner et al., 1997; Pasparakis et al., 1997). However

these mice retain LN and PP unlike LTα-/- mice. LIGHT-/- mice have no phenotypic defects

associated with secondary lymphoid structures (Scheu et al., 2002). In addition,

alymphoplasia (*aly*) mice which are natural mutants with abnormal lymphoid development

have a similar phenotype with LTα-/- mice. Although the defect in *aly* mice is attributed to a

stromal factor (whereas the defect in LTα-/- mice is bone marrow-dependent), there is

evidence that LTβR-signalling is also disrupted (Matsumoto et al., 1999).

Transgenic mice	Lymph nodes	Peyer's patches	Splenic architecture		
			Germinal centers	*T and B cell segregated zones*	*Follicular DC network*
LTα-/-	Absent	Absent	Absent	Disorganised	Absent
LTβ-/-	Residual MLN and cervical LN	Absent	Absent	Disorganised	Absent
LTβR-/-	Absent	Absent	Absent	Disorganised	Absent
LTα+/-β+/-	Present	Absent	Present	Organised	Present
TNF-/- or TNFR1-/-	Present	Disorganised	Absent	Organised	Absent
LTβ-/-TNFR1-/-	Absent	Absent	Absent	Disorganised	Absent
TNFR2-/-	Present	Present	Present	Organised	Present
TRANCE-/- or TRANCER-/-	Absent	Present	Present	Organised	Present
LIGHT-/-	Present	Present	Present	Organised	Present
LIGHT-/- LTβ-/-	All LN absent in 75% of mice and 25% retain MLN	Absent	Absent	Organised	Absent

Table 1.1 Defects in the lymphoid structures of transgenic mice.

1.5.1.1   Lymph node development

Lymph sac formation precedes the development of LN and occurs around embryonic day (E) 10.5. The first anlagen are formed when endothelial cells bud from larger veins during embryogenesis and connective tissues protrude into lymph sacs (Mebius, 2003). Lymphatic vasculature starts to develop and the network is completed by E15.5 (Mebius, 2003; Wigle and Oliver, 1999). Clusters of CD4+CD3- inducer cells of haematopoietic origin which are detected at E12.5-13.5, co-localize with VCAM+ stromal cells (also known as organiser cells) (Mebius, 2003). The earliest LT-signalling in lymphoid organogenesis occurs when $LT\alpha_1\beta_2$ expressed by inducer cells interacts with $LT\beta R$ on stromal cells. The downstream events involve a combination of chemokines and adhesion molecules and are better defined in PP development (discussed later) where similar but separate clusters of inducer cells are involved (Cupedo et al., 2004). A series of studies blocking $LT\beta R$ signalling by treating mice *in utero* with $LT\beta R$-Ig fusion protein or restoring LT-signalling through the use of agonistic $LT\beta R$ antibodies injected *in utero* at different time-points helped determine a timeline for the development of different secondary lymphoid structures (Mebius, 2003; Rennert et al., 1997; Rennert et al., 1998) (Figure 1.6). Injection of the $LT\beta R$-Ig fusion protein after E17.5 did not hinder the development of any LN however injection at around E9, arrested the development of all LN except the MLN, sacral LN and cervical LN. This indicated that the MLN, sacral LN and cervical LN are the earliest LN to develop and the temporal requirements differ between LN structures (Figure 1.6).
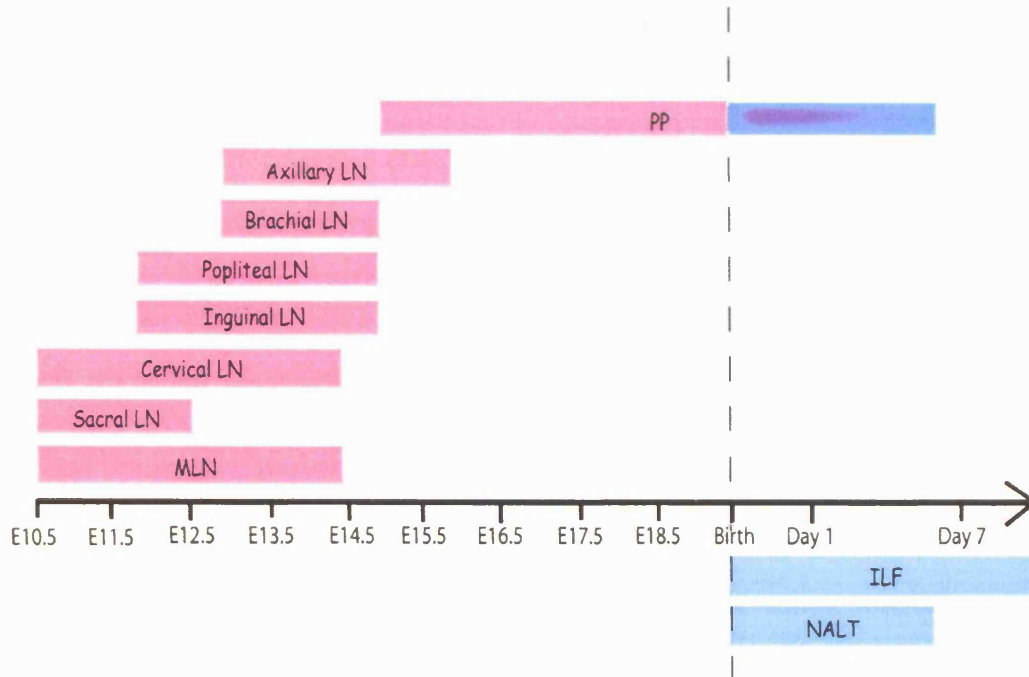
Figure 1.6 Time-line for the development of lymphoid structures in the mouse.

Embryonic days (E) during which individual LN, PP, ILF and NALT are developed. The cervical, sacral and mesenteric LN are the earliest LN to develop. Peyer's patches develop from E15 onwards but their development is only blocked by anti-LTβR-Ig fusion protein during a narrow period between E12 and E14. The vertical dashed line separates the period before and after birth when NALT and ILF are formed. (Information for the time-line was derived from the following references: (Kiyono and Fukuyama, 2004; Mebius, 2003; Nishikawa et al., 2003; Rennert et al., 1997; Rennert et al., 1998; Tumanov et al., 2003). Abbreviations: LN, lymph node; PP, Peyer's patches; NALT, nasopharynx-associated lymphoid tissues; ILF, isolated lymphoid follicles.

1.5.1.2 Mesenteric lymph node development

The MLN develop between E10.5-E15.5 (Figure 1.6). Organogenesis of MLN occurs independently of TNF, TNFR2 and $LT\alpha_1\beta_2$ but depends on LTβR-signalling involving $LT\alpha_3$ and LIGHT (Cuff et al., 1999; Koni et al., 1997; Scheu et al., 2002). There are redundancies in the signalling pathways involved with MLN organogenesis. TNFR1-/- and LTβ-/- mice both possess MLN but not LTβ-/- TNFR1-/- double knockout mice. In addition to $LT\alpha_3$ which signals through TNFR1, LIGHT may be involved in MLN organogenesis as ~75% of LTβ-/- LIGHT-/- double knockout mice have no MLN (Scheu et al., 2002) despite the presence of MLN in LIGHT-/- mice. Alternatively, an undefined ligand which associates with LTα and LTβR may explain the 'redundancy' seen in the LTβR-signalling pathways involved with MLN development.

A difference between PP and MLN organogenesis is signalling by TNF-related activation-induced cytokine (TRANCE) and TRANCE-receptor which are both expressed by haematopoietic cells. TRANCE-/- or TRANCE-R-/- mice possess PP but have a loss of all LN (except the cervical LN which is retained in 30% of mice) (Cupedo et al., 2004; Kim et al., 2000).

1.5.1.3 Peyer's patch development

The development of PP involves a range of cellular signalling, one of which is the interaction between LTβR and $LT\alpha_1\beta_2$ as LTα-/-, LTβ-/- and LTβR-/- mice are deficient in PP. IL7Rα+CD4+CD3- inducer cells recruited from the fetal liver, migrate to the embryonic intestine and express $LT\alpha_1\beta_2$ upon ligation of IL7Rα (Finke and Kraehenbuhl, 2001; Mebius,

2003; Yoshida et al., 1999). Organiser stromal cells express LTβR and other molecules complementary to those expressed on the inducer cells (for example, CXCL13 which binds CXCR5 and MAdCAM1 which binds $\alpha4\beta7$ (Honda et al., 2001)), thereby stimulating downstream events that lead to PP formation (Cupedo et al., 2002; Nishikawa et al., 2003). In the development of PP in mice, IL7Rα signalling is independent of IL7 expression as IL7-/- mice possess PP (Nishikawa et al., 2003). The development of PP can be blocked irreversibly using anti-IL7Rα monoclonal antibody but only between 12-14 days of gestation (Yoshida et al., 1999). Downstream events include nuclear factor-κB which stimulates the early expression of adhesion molecules and chemokines by PP organiser cells (Weih and Caamano, 2003). Mice develop PP postnatally at E15.5 while humans develop PP at 14 weeks of gestation onwards (Spencer et al., 1986; Yoshida et al., 1999).

The PP in the mouse are distributed along the small intestine in a non-defined manner and there is a variable number of PP (within a range averaging from 4 to 9) in different strains. The scattered occurrence of PP at intervals along the intestine may be due to a feedback loop mechanism involving IL7Rα and $LT\alpha_1\beta_2$ expression by inducer and organiser cells (Nishikawa et al., 2003). Interestingly, LTα+/-β+/- mice which are the F1 progeny of LTα-/- and LTβ-/- parents, are deficient in PP while LT single heterozygote mice (LTα+/- and LTβ+/- mice) possess PP (Koni and Flavell, 1998). This may indicate a LT-gene dose effect in the development of PP.

LN and PP formation are developmentally-fixed as intact wildtype-donor BM cannot restore LN and PP despite reorganisation of the spleen in LTα-/- mice (Mariathasan et al., 1995).

Continual signalling between LT and LTβR is required for the maintenance of organised architecture in LN.

1.5.1.4 Isolated lymphoid follicle development

The development of ILF depends on LTβR-signalling between B cells and stromal cells, and is inducible during adulthood after the reconstitution of LTα-/- recipient mice with LT-intact bone marrow (Lorenz et al., 2003). There are similarities with PP development where IL7Rα and LTβR are required (Hamada et al., 2002). However there are differences which distinguish between the two structures. ILF formation is inducible once LT-LTβR signalling is restored whereas PP formation is developmentally fixed within 12-14 days of gestation. Additionally, ILF formation also requires TNFR1 while PP formation does not (Lorenz et al., 2003).

1.5.1.5 Cryptopatch development

Cryptopatches are detected 14-17 days after birth and CP development depends upon the expression of retinoid acid-related orphan receptor γt isoform and IL7R (Ehlers et al., 2003; Taylor et al., 2004). The requirements for LT in CP formation are similar to those of LN, PP and ILF where lin- c-kit+ cells in the CP express $LT\alpha_1\beta_2$ which interacts with LTβR on VCAM1+ stromal cells. In CP formation, the LT-LTβR interaction elicits CCL25 production by CD11c+ cells recruited to the region. CCL25 attracts more lin- c-kit+ cells for further clustering. Like ILF, CP development is inducible during adulthood (Taylor et al., 2004).

1.5.1.6 Spleen development

The spleen is morphologically separated into a red and white pulp. The red pulp filters out old or damaged erythrocytes from the blood circulation and consists of a reticular network of stromal cells and macrophages, plasma cells. The white pulp is the main site of lymphoid activity where antigen-dependent responses of T and B cells occur. There is no lymphatic vasculature in the spleen but antigen and immune cells gain entry from the circulation via the splenic artery. The white pulp is organised into T and B cell compartments with branching arterioles and closely resembles the organisation in LN. The splenic artery branch out with arterioles into the T cell zone (also known as the periarteriolar lymphoid sheath) which contains antigen-presenting interdigitating DC (Fu and Chaplin, 1999). In the T cell zone, T cells, DC and passing B cells interact and in the B cell zones, B cell clonal expansion, isotype switching and somatic hypermutation occurs.

The spleen is derived from the mesoderm and develops at E12, from the splanchnic medodermal plate (SMP) which forms the anlagen (Mebius, 2003). Mice defective in *homeobox 11* (HOX11), do not develop spleens (Roberts et al., 1994). On E13.5, CD4+CD3-CD45+ cells inducer cells similar to those that initiate the development of LN and PP appear in the spleen however the molecular interactions involved in the formation of the spleen are different (Mebius, 2003). In LTα-/- mice, the architecture of the spleen can be reorganised by the reconstitution of LT-intact cells (Fu and Chaplin, 1999).

1.5.2 The influence of lymphotoxin on cellular organisation

Interactions between LT and LTβR are required for maintenance of the lymphoid microenvironment where LN, PP, spleen and NALT organisation are affected (Gommerman and Browning, 2003; Ware, 2005). Signalling by $LT\alpha_3$ regulates the expression of adhesion molecules such as VCAM, ICAM, E-selectin, MAdCAM expression. $LT\alpha_1\beta_2$ regulates expression of the same adhesion molecules including the peripheral node addressin (Cuff et al., 1999; Drayton et al., 2006) and chemokines such as CCL21, RANTES, MCP1 (Cuff et al., 1999; Lo et al., 2003). These chemokines and cytokines are essential for the trafficking of cells involved in lymphoid organogenesis. The downstream effect of blocking LT-signals includes reduced cellularity in the PP and LN of mice (Dohi et al., 2001). The HEV present in LN and PP, have a specialized vascular endothelium which facilitates the entry of naïve lymphocytes into lymphoid structures. When LTβR-signalling is disrupted, HEV differentiation is affected due to decreased levels of peripheral lymph node addressin and MAdcam expression in the HEV, thereby reducing the cellularity of LN and PP and affecting the trafficking of cells (Browning et al., 2005).

The lymphoid architecture is held by a network of reticular scaffolding which provide anchorage and positioning for various cell populations (Gommerman and Browning, 2003). Follicular DC which contribute to reticular networks require maintenance through continual LT-signalling by B cells (Fu and Chaplin, 1999) without which, causes a collapse of the reticular network which in turn results in the loss of marginal zones, T cell zones in the spleen (Mackay and Browning, 1998) and loss of follicles and T cell zones in the PP (Tumanov et al., 2004). Further demonstration of the function of LT in lymphoid organisation is seen in the

development of ectopic and neogenic tertiary lymphoid organs (Drayton et al., 2006). LT expression regulated by the rat insulin promoter resulted in the ectopic development of lymphoid aggregates in non-lymphoid tissues (for example the pancreas and liver). These tertiary lymphoid structures contain vasculature, T and B cell compartments similar to LN, and the capacity to induce humoral immune responses (Drayton et al., 2006). Neogenic tertiary lymphoid organs usually arise through chronic inflammations and the constitutive signalling of LT.

1.5.3 The requirement for lymphotoxin in host defence

During an infection, the requirements for LT may be associated with lymphoid development and organisation or cytokine expression by immune cells. Numerous studies using bacterial, viral and parasitic models of infection have been carried out examining the role of LT, where most underline a role for LT-expression during the immune response against pathogens.

Bacterial infections

The control of infection with intracellular bacteria such as *Mycobacterium tuberculosis* requires LTβR signalling as infection in LTβR-/-, LTα-/- and LTβ-/- mice is exacerbated (Ehlers et al., 2003). Bone marrow chimeric mice (RAG1-/- recipients) reconstituted with LTα-/- BM cells but not LTβ-/- BM cells are susceptible to *M. tuberculosis* infection (Roach et al., 2001), indicating a role for B cell-derived LTα$_3$. There is no indication of how LTβR is specifically involved since LIGHT, another ligand of LTβR, is not required. This is because LIGHT-/- mice show no difference in phenotype with *M. tuberculosis* infection to wild-type

mice. Instead, LTβR may be involved in the organisation of lymphoid structures as T cells are unable to co-locate with macrophages in granulomas during mycobacterial infection.

With *Salmonella enterica* serovar Typhimurium infection, LTβR-/- mice are no more susceptible than B6 mice (Barthel et al., 2003). Infection in LTα/TNFα-/- double knockout mice is lethal due to reduced neutrophil recruitment and killing by granulocytes (Dharmana et al., 2002), and indicates a role for LTα$_3$ during infection. *Citrobacter rodentium* bacterial loads are higher in LTα-/- mice compared to wild-type mice which do not suffer from epithelial ulcers and LP bacterial abscesses, and carry the bacteria only in the intestinal lumen (Spahn et al., 2004). In this bacterial infection, LTα$_1$β$_2$ interaction with LTβR is essential for immunity.

Parasitic infections

In parasitic infections with *Toxoplasma gondii*, both LTα and TNFα produced by BM-derived cells are required for control of infection (Schluter et al., 2003). LTα-/-, TNFα-/- and TNF/LTα-/- double knockout mice fail to control infection by *T. gondii* due to decreased expression of inducible nitric oxide synthetase and splenic nitric oxide levels. In *Leishmania donovani* infections, LT production in the liver and by T cells is required for leukocyte migration and the control of parasite infection respectively (Engwerda et al., 2004). Despite a genetically-resistant B6 background, LTβ-/- mice succumb to *Leishmania major* infections. In this circumstance, lymphoid structures (dependent on the host genotype) influence the timing of cellular and humoral immune responses as LTβ-/- mice reconstituted with wild-type bone marrow remain susceptible compared with wild-type mice reconstituted with LTβ-/- bone marrow (Wilhelm et al., 2002).

Viral infections

LTα-/- mice are highly susceptible when infected with herpes simplex virus (HSV), failing to mount CD8+ cytotoxic T lymphocyte (CTL) responses (Kumaraguru et al., 2001) and TNF/LTα-/- double knockout mice express reduced anti-viral CTL responses to vaccinia virus and lymphocytic choriomeningitis virus (LCMV). Similarly reduced anti-viral CTL responses toward LCMV infection are observed in LTβ-/- and LTα-/- mice (Eugster et al., 1996). Further studies using bone marrow chimeric mice (LTβ-/- bone marrow cells into wild-type recipient) or adoptively transferred cells (TNF/LTα-/- B cells into RAG1-/- recipient), show that splenic organisation is required in order to mount anti-viral CTL responses against LCMV (Berger et al., 1999; Muller et al., 2002; Suresh et al., 2002). In LTα-/- mice, effective anti-viral CTL responses can be generated against acute LCMV and influenza virus A infections although responses are delayed as a result of disorganised lymphoid organs (Lund et al., 2002).

The above examples of the phenotypes displayed by LT-deficient mice toward infection by bacterial, viral or parasitic pathogens demonstrate the influence of LT on essential immune cell functions and/or on lymphoid structural organisation (spleen or local LN draining a site of infection) which consequently affects the ability of the host to mount efficient and effective immune responses.

Positive effects in the absence of LT during infection

Contrary, defective LT expression can reduce immune-mediated pathology as TNFα-/--LTα-/- double knockout mice are resistant to LPS-induced systemic shock from *Escherichia coli* and *Klebsiella pneumoniae* which is lethal in wild-type mice (Dharmana et al., 2002; Eugster et al., 1996). LTα-/- mice infected with the parasite *Trypanosoma brucei*, are able to control disease

as they have increased levels of anti-parasite antibody which prolong survival (Magez et al., 2002). In oral prion infection, LT-deficient or PP-deficient mice are very resistant to challenge compared to intact wildtype mice due to the lack of PP structures which are thought to be entry points for neuroinvasion (Prinz et al., 2003). These examples further highlight the potent effects of LT-expression during inflammation and how pathogens exploit the efficient network of cells within lymphoid structures for survival within the host.

1.6   Immunobiology of dendritic cells

Dendritic cells are unique professional APC with the ability to stimulate naïve T cells and induce the generation of immunological memory (Banchereau and Steinman, 1998). This DC function requires the aid of costimulatiory signals between CD80/86 and CD28, and TCR-MHC ligation. The DC are well-distributed throughout various tissues ranging from lymphoid organs, mucosa-associated systems (eg. gastrointestinal, respiratory) to skin and muscle (Anjuere et al., 1999; Chirdo et al., 2005; Flores-Langarica et al., 2005; Sung et al., 2006; Vremec et al., 2000).

The heterogeneity of DC populations in different tissues can be seen with various surface markers (CD8α, B220, CD11b etc) (see Table 1.2), some of which help to define myeloid and lymphoid DC lineages. Cells of myeloid lineage come from bone marrow precursors which give rise to megakaryocyte-erythrocyte or granulocyte-macrophage progenitors and have no lymphoid differentiation potential. Cells of lymphoid lineage come from bone marrow precursors which generate T cells, B cells and NK cells and have no myeloid differentiation potential (Ardavin, 2003). These two developmental pathways of DC used to be loosely differentiated by the marker CD8α. For example, CD8α helps to differentiate between

lymphoid (CD8α+) and myeloid lineages (CD8α-) (Vremec et al., 1992; Wu et al., 1995).

However CD8+ DC of the myelod lineage can also be generated (Traver et al., 2000).

The bone marrow is the site of origin for DC haematopoietic precursors. In the mouse bone marrow, DC can be generated using granulocyte-macrophage colony stimulating factor (GM-CSF) and the cells produced display morphological characteristics typical of DC such as MHC class II expression and the ability to home to draining lymph nodes (Inaba et al., 1993). Monocytes derived from the bone marrow, can migrate into inflamed tissues and develop into macrophages or monocyte-derived DC which participate in the uptake and presentation of antigen in the lymph node (Lutz and Schuler, 2002; Randolph et al., 1999).

Table 1.2 Main subsets of DC identified in the mouse

DC subsets defined in the mouse	Thymus	Spleen	LN (pLN, MLN)	Skin	PP	LP
CD8α+ CD11b-	+	+	+	-	+	+
CD8α- CD11b+	+[a]	+	+	-	+	+
CD8α- CD11b-	ND	ND	+ (MLN), ND (pLN)	-	+	+
CD8α^{int}	-	-	+	-	+	ND
B220+ (plasmacytoid DC)	+	+	+	+	+	+
Langerhans cell (CD8α- CD11b+)	-	-	-	+	-	-
Dermal DC (CD8α- CD11b+)	-	-	-	+	-	-
References	1,9	1,6-8	2, 3, 6-8	2,10	2,4, 8	5

[a] DC proportions found are very small. Abbreviations: ND, not defined. pLN, peripheral LN. CD8α^{int}, intermediate expression of CD8α. References: 1, (Vremec et al., 2000). 2, (Anjuere et al., 1999). 3, (Henri et al., 2001). 4, (Kelsall and Strober, 1996). 5, (Chirdo et al., 2005). 6, (Asselin-Paturel et al., 2003). 7, (Martin et al., 2002). 8, (Bilsborough et al., 2003). 9, (Okada et al., 2003). 10. (Palamara et al., 2004)

1.6.1 Dendritic cell function

Dendritic cells are highly mobile and strategically positioned in non-lymphoid tissues as sentinels where they can encounter foreign antigens and readily locate to lymphoid organs to interact with a specific naïve or memory T cell. Antigen is captured, processed and displayed as MHC-peptide complexes on the surfaces of DC which also upregulate costimulatory molecules such as CD80 and CD86. Upon antigen-uptake and activation, DC mature while migrating to lymphoid organs where they also secrete chemokines which attracts T cells to them (Adema et al., 1997; Banchereau and Steinman, 1998; Steinman, 1991). Despite their relatively lower numbers, DC are potent inducers of the adaptive immune response. The unique ability of DC to present exogenous antigen to naïve T cells is not limited to MHC class II-

restricted CD4+ T cells as cross presentation on MHC class I allows them to efficiently induce strong CTL responses from naïve CD8+ T cells (Inaba et al., 1987; Young and Steinman, 1990). DC are also essential for the survival of naïve CD4 T cells and immune T cell memory (Brocker, 1997; Zammit and Lefrancois, 2006). The determination of Th1/Th2 responses is influenced by DC where for example, the production of IL12 and IL18 by DC elicits Th1 responses (Macatonia et al., 1995; Okamura et al., 1995) while the IL10 and specific microbial influence (acted through PRR) of DC may drive the development of Th2-type responses (Manickasingham et al., 2003).

1.6.2 Dendritic cell maturation

Most DC in tissues are immature and are highly phagocytic and endocytic for the purpose of efficiently capturing antigens (Sallusto et al., 1995). DC maturation occurs after the uptake of antigen and exposure to pathogen-associated molecular patterns (PAMP) expressed by microbial organisms and the mature DC is identified by the high levels of MHC class II and costimulatory molecules expressed. The maturation process also involves the loss of endocytic and phagocytic receptors, the extension of dendrites to increase the surface area for T cell interaction and a change in chemokine receptor expression to promote DC migration to lymphoid tissues (Banchereau et al., 2000; Winzler et al., 1997).

The maturation state of DC is determined by signals stimulated by microbial products which are recognised by PRR such as mannose receptors, C-type lectins, TLRs. Dendritic cells express PRR which enable them to recognise PAMP to differentiate between commensal and pathogenic organisms and hence respond accordingly. The PAMP consist of conserved

molecular structures shared among pathogens and include polysaccharides, lipids, proteins, ribonucleic acids, nuclei acid motifs, all of which are recognised by TLRs. The activation of TLRs induces cell migration to sites of inflammation by influencing the expression of selectins, chemokines and their receptors (Huang et al., 2001). Maturation can also occur during inflammation in the presence of TNFα, type I IFN, intracellular compounds such as uric acid and during CD40-CD40L ligation (Banchereau et al., 2000; Kapsenberg, 2003; Reis e Sousa et al., 2003).

The maturation state and type of DC influence the type of T cell response. Mature DC activate and drive clonal expansion of T cells, usually in a pro-inflammatory response, and also influence CD8+ CTL to proliferate vigorously (Bhardwaj et al., 1994). Immature DC which are unstimulated, express low levels of MHC class II and costimulatory molecules, and where antigen presentation occurs in the absence of costimulation, T cell anergy results (Lutz and Schuler, 2002; Schwartz et al., 1989). However, some tolergonic DC are required to express costimulatory molecules to induce tolerance (Reis e Sousa, C 2006). Under non-inflammatory conditions, there is a steady-state migration of tissue-derived DC from non-lymphoid sites such as the intestine via lymph to draining lymphoid sites such as the MLN (Huang et al., 2000). These steady-state DC which are thought to be immature or partially activated, continuously present self antigens from dying apoptotic cells for the induction and maintenance of self tolerance (Huang et al., 2000; Inaba et al., 1998; Steinman, 2003). Immature DC residing in lymphoid tissues, also process antigens that enter blood systemically and cross-present self antigens to T cells (Belz et al., 2002; Larsson et al., 2003).

In the event of pathogenic or inflammatory stimulation, immature DC become activated and migrate to draining lymphoid tissues or T cell zones (in the case of those already residing in lymphoid tissues) to prime the adaptive immune response instead. Migration by DC into lymphoid tissues is shown by the increasing turnover rate of DC in tissues and draining lymph nodes in the presence of inflammatory cytokines such as TNFα.

1.6.3   The plastic and intrinsic properties of dendritic cells

In the past, when individual DC subsets were discovered, their functional properties were distinct enough to differentiate DC into groups that were either Th1-inducing or Th2-inducing through the use of the CD8α marker, also previously used to differentiate between DC of myeloid and lymphoid lineages. Splenic DC differentiated by CD8 expression were intrinsically-biased towards either Th1 (CD8+ DC) or Th2 (CD8-DC) responses (Maldonado-Lopez et al., 1999; Pulendran et al., 1999). In the spleen and PP, CD8- DC are found present in the antigen uptake areas (marginal zone and SED respectively) whereas CD8+ DC are found mostly in the T cell zone (Iwasaki and Kelsall, 1999; Leenen et al., 1998). Studies on CD8- and CD8+ DC showed some different properties between the two subsets. For example, CD8- DC have higher endocytic and phagocytic capacities than CD8+ DC (Kamath et al., 2002; Leenen et al., 1998) although only CD8+ DC can phagocytose apoptotic cells (Iyoda T 2002). CD8+ DC can activate T cells through cross presentation and CD8- DC are able to cross-prime only after Fc receptor-mediated activation (den Haan and Bevan, 2002; den Haan et al., 2000).

Despite some intrinsic features exclusive to specific subsets, some DC functional plasticity exists and the current consensus favours more DC plasticity within a framework of different DC subsets. For example, Th1 cyokines can be induced by CD8- DC with CD40 ligation or in

the absence of IL10 while CD8+ DC induce Th2 cytokines at low doses of antigen. The conversion of Th1-favouring DC into Th2-inducing DC is possible with the treatment of cells with IL10, TGFβ, steroids, prostaglandin E2 (Banchereau et al., 2000). Generally, DC plasticity can be influenced by factors such as activation state, antigen type, antigen concentration, PRR and cytokines present in the microenvironment (Boonstra et al., 2003; Fallarino et al., 2002; Maldonado-Lopez and Moser, 2001; Manickasingham et al., 2003). It is also clear that the DC draining different tissues are influenced by their tissue site of origin. Intrinsic properties are found not only within DC subsets but also within tissue types. For example, mucosal DC seem to produce more Th2-type cytokines (discussed later). However, some properties may not be fixed and may be influenced by signals in the tissue microenvironment. A clear example is shown where gut-derived DC transplanted in a different tissue environment lose their ability to induce gut-homing adhesion molecules (this DC property is discussed later) (Dudda et al., 2005).

1.6.4 Tissue-specific dendritic cell subsets

Multiple DC subsets have been described in different tissue types and in human and mice (Anjuere et al., 1999; Ardavin, 2003; Shortman and Liu, 2002). A summary of murine DC subsets is found in Table 1.2. In the adult thymus, the majority of thymic DC are CD8+ CD4-CD11b- (Vremec et al., 2000). In the murine spleen, there are three main DC subsets expressing CD11c+CD11b+ which are subdivided into CD4-CD8-(DN), CD4+CD8- and CD11c+CD11b-CD4-CD8+ DC. In the peripheral LN of mice, there are two similar subsets (CD11b-CD8+; CD11b+CD8-) as the spleen and another DC subset displaying $CD8^{lo}$ or $CD8^{int}$ expression. Both murine MLN and PP DC contain similar subsets of DC: CD11c+ CD11b-

CD8+, CD11c+CD11b+DN, CD11c+CD11b-DN (Anjuere et al., 1999) which are further described later.

Plasmacytoid DC are characterised by their ability to produce large amounts of type 1 IFN which makes them important during anti-viral responses (Colonna et al., 2004). Plasmacytoid DC are found in lymphoid (thymus, spleen, LN, PP) and non-lymphoid tissues (skin, intestinal LP), and in the blood (Anjuere et al., 1999; Asselin-Paturel et al., 2003; Bilsborough et al., 2003; Martin et al., 2002; Nakano et al., 2001; Okada et al., 2003; Palamara et al., 2004). Langerhan cells are a distinct DC subset which expresses langerin. They are exclusive to the skin and skin-draining LN, and mature Langerhan cells also express similar maturation markers as mature DC (high levels of MHC molecules, CD40, CD80/86 expressed) (Henri et al., 2001; Lenz et al., 1989; Romani and Schuler, 1989).

In humans, CD11c is not the definitive marker for DC and instead the human leukocyte antigen (HLA)-DR is used to characterise DC, mostly circulating DC from peripheral blood in a majority of studies (Kelsall and Leon, 2005). Additionally, CD4 and not CD8 is found on human DC (Shortman and Liu, 2002). The human Langerhans cell is also distinguished by the expression of langerin and CD1a (Shortman and Liu, 2002).

The functional differences between DC isolated from specific tissues may be indicative of their present state that is influenced by the tissue microevironment. For example, resident PP-DC induce T cells to stimulate IL4 and IL10 compared to splenic DC which stimulate IFNγ from T cells instead. However when IL10 and TGFβ are neutralised, IFNγ production is enhanced in PP cultures instead (Iwasaki and Kelsall, 1999). Another important tissue-specific trait of gut-

derived DC (from PP, MLN, LP) is their ability to upregulate the gut-homing molecules, $\alpha4\beta7$ and CCR9 on T cells more efficiently than splenic DC (Johansson-Lindbom et al., 2005; Mora et al., 2003; Stagg et al., 2002). A similar induction of tissue-specific homing molecules is seen in skin-derived DC which are better at inducing skin homing molecules (eg. E-selectin, P-selectin, CCR4, CCR10) on T cells (Dudda et al., 2005).

DC trafficking

Trafficking of DC occurs through afferent lymphatic vessels where they pass through to enter lymphoid organs. Lymph flows unidirectionally from the periphery to the heart: lymph absorbing interstitial fluid from tissues which is recirculated back to the blood via the thoracic duct. While doing so, lymph passes through lymphoid organs. Lymphatic vessels have thin walls and a wide lumen, surrounded by endothelial cells which have little or no basement membrane and are highly endocytic and permeable to proteins (Leak, 1971). The translocation of DC into the LN requires molecular interaction (possibly by ICAM and JAM1) to occur between DC and the lymphatic endothelium (Cera et al., 2004; Xu et al., 2001).

Under steady-state conditions, mouse DC express CCR7 to migrate to lymph nodes though peripheral lymphatic vessels (Forster et al., 1999; MartIn-Fontecha et al., 2003). CCR7+ DC respond to the chemotactic gradient of chemokines CCL19 and CCL21 that originate from the lymphatic vessel (Randolph et al., 2005). The *plt* (paucity of lymph node T cells) mice which lack CCL19 and a non-functional form of CCL21 (Luther et al., 2000), have impaired DC migration (Gunn et al., 1999; Qu et al., 2004; Yoshino et al., 2003). CCL21 is also required for DC migration to the LN (Saeki et al., 1999). The migration of DC to the LN is also mediated by integrin LFA1 ($\alpha L\beta2$).

1.6.5 Intestinal DC

In the intestine, the IE layers holding enterocytes cover the mucosal surface of the gastrointestinal tract and this large area requires surveillance by DC. The DC play pivotal roles in the regulation of tolerance and induction of active immunity to commensal microbes in the gut and are clearly important in inflammatory diseases such as Crohn's disease, ulcerative colitis (Annacker et al., 2005; Drakes et al., 2005; Krajina et al., 2003). Intestinal DC constantly survey luminal contents which include commensal microbes and pathogens and help to regulate immune responses towards the vast array of potential stimuli.

Peyer's patch dendritic cells

In the PP, Kelsall and Iwasaki (1999) have identified specific populations residing in the SED and IFR regions. There are five DC subsets present in the murine PP. Three populations are $CD11c^{hi}$ and differentiated into CD8α+ CD11b-, DN CD11b+ and DN CD11b- subsets (Iwasaki and Kelsall, 2001). The levels of CD11b and CD8α expressed are fixed upon activation and serves as appropriate markers for differentiating between subsets (Kelsall and Leon, 2005). $CD11c^{hi}$ CD11b+ DC are found in the SED, CD8α+ DC are found in the IFR and CD11b- DN DC are found in both regions. CD11b+ CD8α- DC which form a majority of DC in the SED, are required in the induction of PP immune responses towards oral antigen and enteropathic rotavirus (Cook et al., 2000). The plasmacytoid $CD11c^{int}$ B220+ DC which produce high levels of type I IFN, are also found in the PP (Asselin-Paturel et al., 2003).

The properties of PP-DC are intrinsically different from that of splenic DC. PP-DC are relatively immature in phenotype but can mature fully upon activation, expressing higher levels

of MHC class II than splenic DC (Kelsall and Strober, 1996). T cells primed by PP-DC produce high levels of IL4, IL6 and IL10 compared to the high levels of IFNγ produced by T cells primed by splenic DC (Everson et al., 1998; Iwasaki and Kelsall, 1999; Sato et al., 2003). Moreover, PP-DC produce IL10 when stimulated with the costimulatory molecule RANK (receptor activator of nuclear factor κB) unlike splenic DC which produced IL12 when activated similarly (Williamson et al., 2002). These properties of PP-DC are similar to DC found in the lungs where T cells are driven toward IL10 production (Upham, 2003) and may be characteristic of mucosal DC functions under steady state conditions. The PP is a major site for IgA production which is encouraged by CD11b+ PP-DC which secrete IL6, an important cytokine for the development of IgA-producing B cells (Sato et al., 2003).

Intestinal DC efficiently upregulate gut-homing adhesion molecules (α4β7 and CCR9) on T cells (Mora et al., 2003; Stagg et al., 2002). This ability to imprint gut tropic properties has been attributed to the conversion of retinal by retinoic acid dehydrogenases which are expressed by both PP and MLN DC (Iwata et al., 2004).

Mesenteric lymph node dendritic cells

The MLN-DC share similar characteristics with PP-DC. The murine MLN have five major populations of DC that are similar to those found in the PP. Located in the MLN T cell zones are CD8+, CD11b-DN and plasmacytoid DC while CD11b+ DC reside outside the T cell zones (Asselin-Paturel et al., 2003; Iwasaki and Kelsall, 2000). MLN-DC from antigen fed mice also stimulate CD4+ T cells to preferentially produce IL4, IL10 and TGFβ (Alpan et al., 2001). In the rat, analysis of DC collected from the small intestine after removal of MLN and thoracic

cannulation showed that intestinal DC are able to uptake orally-administered antigen and migrate towards the MLN (Liu and MacPherson, 1993). The steady state traffic of DC from the intestine into the MLN included DC carrying enterocyte-derived apoptotic DNA and esterase-derivatives typically found in enterocytes (Huang et al., 2000). This data also demonstrated that all DC examined in the MLN are not necessarily MLN-residents and may originate from the PP or LP. This explains why DC subsets from the LP, PP and MLN share relatively similar phenotypes.

Lamina propria dendritic cells

Subsets of DC found in the LP regions of the small intestine and colon have been described (Chirdo et al., 2005; Johansson-Lindbom et al., 2005; Krajina et al., 2003). Small intestinal LP-DC are mainly immunomodulatory during steady state and are known to carry intestinal antigens for induction of tolerance in the MLN (Chirdo et al., 2005; Huang et al., 2000; Worbs et al., 2006). The majority of small intestinal LP-DC are CD11b+CD8- (60%) followed by CD11b-CD8+ and CD11b-CD8- DC. Small intestinal LP-DC can also be defined by the intermediate expression of CD11c (CD11c^{int}) and these DC are mainly immature (MHC class II ^{lo}) and B220-Ly6C/G- (30% are B220+Ly6C/G+). The B220+Ly6C/G+ DC subset resembles the murine equivalent of human plasmacytoid DC (Chirdo et al., 2005). When compared with splenic DC, small intestinal LP-DC express similar levels of IL10 mRNA, lower levels of IL12p40 (low levels that are similar to PP-DC) and higher levels of IFNβ. Colonic LP-DC are mostly CD11b+ apart from a small proportion (1-10% of LP-DC) which are CD11b- CD8+. Similarly with small intestinal LP-DC, colonic LP-DC secrete lower levels of IL12p40 than splenic DC upon CD40 ligation (Krajina et al., 2003). LP-DC also express CD103 and

upregulate α4β7 and CCR9 on T cells more efficiently than MLN and PP DC (Johansson-Lindbom et al., 2005).

Intestinal antigen sampling and presentation

The generation of antigen-specific responses after the oral administration of antigen, confirm the uptake of antigen and presentation by DC from the intestine. Antigen sampling can occur through many routes:

Microfold cells present in the FAE of PP are specialized in efficiently sampling antigens from the lumen and have a basolateral pocket that contains APC such as DC, allowing the easy transfer of antigens. In addition, M cells found in the epithelial layer of the intestinal villi show similar antigen-uptake properties as those in the PP (Jang et al., 2004). Tertiary lymphoid structures such as ILF may also act as sites for antigen sampling as they also contain M cells (Hamada et al., 2002). Sampling of the lumen can also be carried out directly by intestinal DC expressing tight junction proteins which allow them to extend their dendrites between enterocytes and sample from the lumen (Rescigno et al., 2001). This method may be employed by perhaps CX3CR1+ CD11c+ CD11b+ LP-DC, found predominantly in the terminal ileum, which can extend their transepithelial dendrites into the luminal areas to sample antigens (Niess et al., 2005). CX3CR1 is also expressed on PP-DC and may suggest that M cell-independent sampling by PP-DC can occur.

After antigen uptake

Due to the enormous antigen load contributed by food and commensal microbes, intestinal DC have the difficult task of inducing tolerogenic responses to harmless antigens (including self-

antigens) and inflammatory responses when appropriate. When antigen is uptaken by intestinal DC, some of these cells migrate through lymphatic vessels to the draining MLN to present to a specific naïve T cell. PP-DC which obtain antigen may either migrate to T cell zones in the IFR of the PP (Iwasaki and Kelsall, 2000; Shreedhar et al., 2003) or to the draining MLN (Huang et al., 2000; Macpherson and Uhr, 2004). Soluble antigens may be carried by lymph and trapped in the MLN where they are taken up by local DC to be presented.

1.7   Infection models of immunology

The understanding of immunological processes has been helped by studies utilizing infections by pathogens that include bacteria (eg. *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes, M. tuberculosis*), viruses (eg. Influenza virus, LCMV, RSV, herpes simplex virus) and parasites (eg. *Toxoplasma gondii, Plasmodium* spp, *Cryptosporidium parvum, Nippostrongylus brasiliensis, Schistosoma mansoni, Eimeria* spp.). Most experimental models are designed to emulate infections causing health problems in humans while some models are designed to study direct problems associated with commercial livestock. For example, S. Typhi causes typhoid fever in humans which is similar to the symptoms caused by S. Typhimurium in mice (Santos et al., 2001); *Citrobacter rodentium* infection of mice is a model for human infection with enteropathogenic *E. coli* (Levine, 1987); *L. major* causes similar clinical symptoms of cutaneous leishmaniasis in both humans and specific mice strains (Gumy et al., 2004). Host-pathogen studies and the use of susceptible and resistant mouse strains have been useful in deciphering the different characteristics and mechanisms of immune responses against invasion, and help to define the genetics affecting the immune response.

The polarisation of Th responses is demonstrated in a number of infections. Typically, Th1-dominated immune responses are important against intracellular pathogens (eg. S. Typhimurium, *L. monocytogenes*) while Th2-dominated responses are important against extracellular pathogens (eg. *N. brasiliensis*, *Brugia malayi*) (Liu et al., 2005; Tawill et al., 2004). Th1 responses are characterized by the expression of IFNγ, IL2, TNFα and LT while Th2 responses are characterized by IL4, IL5, IL0 expression (Mosmann et al., 1986). Cytokines such as IL12, IL18, IL23 or IL4, IL10, TGFβ help determine the balance between Th1 and Th2 responses. Th-polarisation depends on the type of APC present (Moser and Murphy, 2000), the nature of costimulatory signals (Kuchroo et al., 1995), TCR engagement (Pfeiffer et al., 1995), antigen dose (Hosken et al., 1995), antigen route of administration (Guery et al., 1996), T cell division (Bird et al., 1998), the cytokines present and secreted during infection and PRR-stimulation by microbial signals (Boonstra et al., 2003; Napolitani et al., 2005).

The importance of IFNγ in the Th1 response against pathogens is demonstrated in IFNγ-/- mice are susceptible to intracellular pathogens like *M. tuberculosis* and S. Typhimurium (Bao et al., 2000; Flynn et al., 1993; Mastroeni et al., 1999; Wang et al., 1994) and in the administration of anti-IFNγ antibody which incapacitates the ability of mice to resolve infection by *L. major* (Belosevic et al., 1989; Scott, 1991). IFNγ, a major product of Th1 cells, activates NK cell, macrophages and promotes adaptive immunity by increasing antigen processing, MHC antigen presentation, costimulatory molecules on APC (Schroder et al., 2004). Anti-microbial mechanisms dependent on IFNγ include macrophage production of reactive oxygen intermediates and hydrogen peroxide which help in killing of intracellular parasites such as *T. gondii* (Flesch and Kaufmann, 1991; Murray et al., 1985; Schroder et al., 2004). Catabolic

enzymes such as indoleamine 2,3 dioxygenase are induced by IFNγ and has been implicated in the killing of intracellular pathogens such as *T. gondii, Chlamydia trachomatis* and *C. psitacci* (Taylor and Feng, 1991). The anti-viral properties of IFNγ involve synergy with type I IFN which are essential during the immediate response against infection. The T cell production of IFNγ is influenced by the expression of Tbet and STAT4 activation (Szabo et al., 2000).

1.7.1 *Eimeria vermiformis* infection

1.7.1.1 Life history

Eimeria species are monozenous, completing their entire life cycle within a single host. The life history of *Eimeria* spp. can be divided into sporogony, schizogony and gametogony stages. Unsporulated oocysts previously released in the excrement, into the environment by an infected animal, undergo sporulation which is influenced by oxygen levels, humidity, temperature in the environment (Levine, 1982). Meiotic, mitotic nuclear divisions and cytoplasmic differentiation occurs during sporulation. Four sporocysts each containing two sporozoites are contained within a sporulated oocyst which is potentially infective once ingested by another host.

Upon ingestion, the oocyst is mechanically and enzymatically disrupted by processes within the gastrointestinal tract (Blagburn et al., 1982). Sporozoite stages of *Eimeria* spp. which infect chicken (eg. *E.tenalla, E.maxima, E. necatrix, E.acervulina*) are known to be transported in intraepithelial lymphocytes to the intestinal villus crypts where they invade enterocytes (Al-Attar and Fernando, 1987; Fernando et al., 1987; Riley and Fernando, 1988). The sporozoite invades the enterocyte and develops into a trophozoite. After several nuclear divisions (asexual

reproduction), the trophozoite develops further into a first generation schizont which matures to produce merozoites. The schizont ruptures, releasing merozoites into the gut lumen which enter another enterocyte to develop into the next generation of schizonts. Schizogony occurs around 3-4 generations depending on the *Eimeria* spp. The final generation of merozoites develops into gametocytes. Microgametocytes generate large numbers of microgametes while macrogametocytes further mature into macrogametes. The biflagellated microgametes are released from microgametocytes to enter macrogametes, resulting in fertilization and the formation of a zygote. The zygote further develops to form a resistant wall and is shed as a oocyst via the faecal route.

Eimeria spp. are host-specific unlike other apicomplexan parasites such as *Crytosporidium* spp. and *Toxoplasma gondii,* and can invade a wide range of vertebrates (Levine, 1982) (Levine, 1982). Most *Eimeria* spp. parasitise the intestinal epithelium but the site specificity of infection depends on the species. Respectively, *E. maxima* and *E. tenalla* invade the mid-region of the small intestine and caecum of the chicken while *E.vermiformis* and *E. pragensis* invade the two-third distal region of the small intestine and large intestine of the mouse (Todd and Lepp, 1971).

1.7.1.2   Immunity to *E.vermiformis* infection

Eimeria vermiformis is a close relative of human pathogens such as *Cryptosporidium* spp., *Toxoplasma gondii* and *Sarcocystis* spp., and invades the host mouse though the gastrointestinal route (Current, 1990). Intestinal epithelial cells are specifically targeted by *E. vermiformis* which invades these cells to undergo four generations of asexual reproduction

(schizogony) followed by sexual reproduction (gametogony) and finally the oocyst stage which exits the host through the faecal route (Figure 1.7). Although infection with *E. vermiformis* is limited by the host's immune response, the infection is also self-limiting with the parasite clearing immunodeficient hosts within 3-4 weeks (Roberts et al., 1996; Rose).

Control of primary infection with E. vermiformis

The immune responses generated during primary infection with *E. vermiformis* limit the duration and magnitude of infection (Rose et al. 1984). Moreover, since Eimeria *spp.* are self-limiting, highly susceptible hosts are free from infection within 2-3 weeks. A summary of the essential immune components against *E. vermiformis* infection is given in Table 1.3. A T cell-dependent immune response is necessary for the control of *E. vermiformis* infection as mice with a *scid* mutation or *nude* mutation are highly susceptible to infection and do not display any immunity to re-infection (Rose and Millard, 1985; Rose et al., 1984). The *scid* mice suffer severe combined immune deficiency, lacking T and B cells due to the inability to rearrange immunoglobulin and T cell receptor) and *nude* mice have a defective development of the thymic epithelium which results in deficient T cell development (Bosma et al., 1983; Carroll and Bosma, 1991). There is a minor role for B cells as mice deficient in B cells (μMT-/-) produce slightly higher numbers of oocysts compared with intact B6 mice (Smith and Hayday, 2000). The T cell-dependent immunity developed in intact mice is transferable and is more effective with MLN cells than splenic cells (Rose et al., 1988b). A role for CD4+ T cells is seen where MLN cells depleted of CD4+ T cells do not confer immunity while depletion with CD8+ T cells has no effect on immunity (Rose et al., 1988a).

The Th1 immune responses in the form of MHC class II-restricted CD4+ TCRαβ+ cells are essential during primary infection (Roberts et al., 1996; Rose et al., 1988a; Smith and Hayday, 2000). Mice deficient in MHC class II (IA-β-/-) or TCRαβ+ T cells (TCRα-/-, TCRβ-/- ,TCRβxδ-/-) are highly susceptible to infection with *E.vermiformis,* indicating a role for a MHC class II-restricted TCRαβ+ cell, likely to be a CD4+ T cell as seen from the data on the depletion of CD4+ T cells. Both TAP1 and β_2m are involved in MHC class I processing but only β_2m-/- mice and not TAP1-/- mice, are slightly more susceptible than intact mice (Smith and Hayday, 2000). This suggests a non-classical MHC pathway rather than a classical MHC class I pathway is involved.
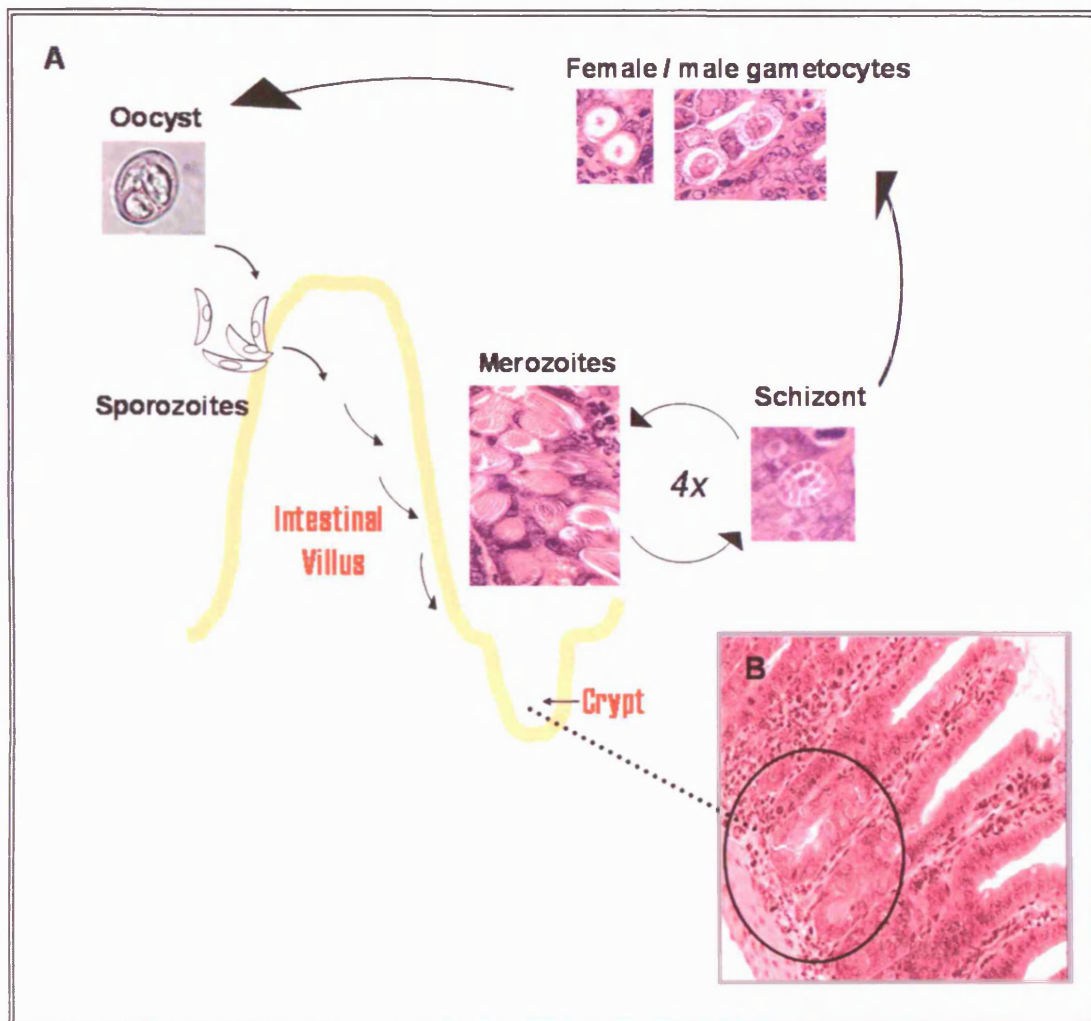
Figure 1.7 General life history of *E.vermiformis* in the murine small intestine.
A) Schematic showing the different parasite stages, starting from the oocyst which has been ingested by the host. Sporozoites break out from the oocyst and move downwards to the crypt region where they invade enterocytes to replicate. Asexual reproduction occurs and a schizont stage which contains merozoites develops. Merozoites break out from host cells and enter more cells to repeat replication for four generations. The male and female gametocytes develop to carry out sexual reproduction and produce the oocysts which are expelled through the faecal route into the external environment.
B) Hematoxylin and eosin-stained section of intestinal crypts filled with numerous parasites from an infected mouse.

IFNγ is an essential component during primary infection as IFNγ-/- mice and mice treated with IFNγ neutralising antibody are highly susceptible unlike IL4-/-, FasL-deficient and perforin-/- mice which are as resistant as intact B6 mice (Rose et al., 1989; Smith and Hayday, 2000).

There is no evidence of a requirement for NK cells in the innate immune response against *E.vermiformis*. Although NK cell activity was influenced during primary infection with *E. acervulina*, *E. maxima* (Lillehoj, 1989) and *E. vermiformis* (Smith et al., 1994), the depletion of NK cells (through the use of anti-Asialo GM1 antibody and the infection of *beige* mice which have defects in cytotoxic NK function) shows that NK cells do not play a major role during infection (Rose et al., 1995; Smith et al., 1994). There is the exception where a NK cell-dependent production of IFNγ production was required during *E. papillata* infections in mice (Schito and Barta, 1997). It is likely that IFNγ is mainly produced by T cells, particularly CD4+ T cells which are essential in the immune response against *E.vermiformis*.

Table 1.3

Essential components of the primary immune response against *E. vermiformis* infection

Immune requirements in the primary response against *E.vermiformis* infection	Supporting evidence
■ T cell-dependent immunity	TCRα-/-, TCRβ-/-, TCRβxδ-/-, *scid* and *nude* mice are highly susceptible while μMT-/- mice are not.
	Depletion of NK cells using anti-asialo GS1 showed no effect on outcome of infection; SCID *beige* mice, deficient in NK cell function, are not susceptible.
■ CD4+ T cells	Adoptive transfer of MLN CD4+ T cells conferred protection contrary to CD8+ T cells.
	IAβ-/- mice are highly susceptible.
■ MHC class II presentation ■ Non-classcial MHC class presentation dependent on β₂m	IA-β-/- and β₂m-/- mice are highly susceptible (higher susceptibility is seen in IA-β-/-) while TAP1-/- mice are not susceptible.
■ IFNγ	Mice given neutralizing anti-IFNγ antibody and IFNγ-/- mice are highly susceptible.
■ Th1 immune response	IL4-/-, perforin-/-, FasL-/-, mice are not susceptible and evidence from above.
■ Early timing of immune response	MLN cellularity, *ex vivo* stimulated cell proliferation and IFNγ production increased or peaked earlier in relatively resistant BALB/c strains compared with relatively susceptible B6 strains.

Control of secondary infection with E.vermiformis

All intact mouse strains develop complete immunity to secondary challenge with *E.vermiformis* evident by the lack of oocyst production. Immune protection is conferred by TCRαβ+ T cells as TCRα-/-, TCRβ-/- and TCRβxδ-/- mice continue to produce oocysts when rechallenged while TCRδ-/- mice are protected when rechallenged (Roberts et al., 1996; Smith and Hayday, 2000). While β2m and IFNγ are required during the primary infection, they are not required during the secondary infection as β2m-/- and IFNγ-/- mice are protected when rechallenged ((Smith and Hayday, 2000) and Marguerite IA munscript submitted). However, protection in IFNγ-/- mice is dependent on the dose of oocysts used during primary and secondary challenge. The requirement for MHC class II during secondary infection is less stringent as IA-β-/- mice display some degree of immunity when rechallenged (Smith and Hayday, 2000).

1.7.1.3   Host genetics influences resistance to *E. vermiformis* infection

When challenged with 1000 oocysts, BALB/c mice are relatively resistant to *E. vermiformis*, producing up to 100 times fewer oocysts than susceptible C57BL/6 (B6) mice (Rose and Hesketh, 1986) (Figure 1.8). A comparison of the immune response between strains through the measurement of MLN cellularity, *ex vivo* proliferation, *ex vivo* cytokine production, revealed differences (Rose et al., 1990; Wakelin et al., 1993)). MLN celullarity in resistant BALB/c increased earlier than B6 mice and the proliferation of MLN cells stimulated *ex vivo*, peaked around 5 DPI for BALB/c mice and 8 DPI in B6 mice. *Ex vivo* antigen-stimulated production of IFNγ correlated with the peak of proliferation and reflected similar differences

between strains where production of IFNγ peaked earlier in BALB/c mice. The effect of earlier immune responses in BALB/c mice can be seen in the overall reduction of oocyt output and earlier cessation of oocyst production by around 4-5 days whereas that in B6 mice is around 7-8 days (Rose and Hesketh, 1986). Therefore the ability to control primary infection with *E. vermiformis* correlates with the timing of a Th1 immune response.

1.7.1.4 Parasite dose influences host resistance to *E. vermiformis* infection

The timing of Th1 responses which is influenced by genetic factors as observed by the phenotypes displayed by different strains, is also affected by the size of the infecting dose of parasite. With a dose of 1000 oocysts, there is a large difference in oocyst output between BALB/c and B6 mice whereas at smaller doses, different results are obtained. Work carried out by P, Hesketh and A, Archer shows that BALB/c mice produce more oocysts with doses of 25, 50 or 100 oocysts compared with 1000 oocysts (Figure 1.9). This relative increase in the susceptibility of BALB/c mice is not reflected in B6 mice which show no increase in oocyst production with lower doses. Furthermore at a dose of 25 parasites, there is no difference in oocyst output between each strain.

Preliminary analysis of the immune response with relation to parasite dose showed a delay in the timing of Th1 responses in BALB/c mice infected with 25 or 1000 oocysts. The MLN cellularity, *ex vivo* proliferation and *ex vivo* IFNγ production in BALB/c mice were all affected by lower parasite doses (work by P, Hesketh and A, Archer). Therefore, parasite dose can influence the timing of Th1 responses rendering a resistant strain relatively more susceptible with a lower dose of oocysts.

Immune response influenced by genetics
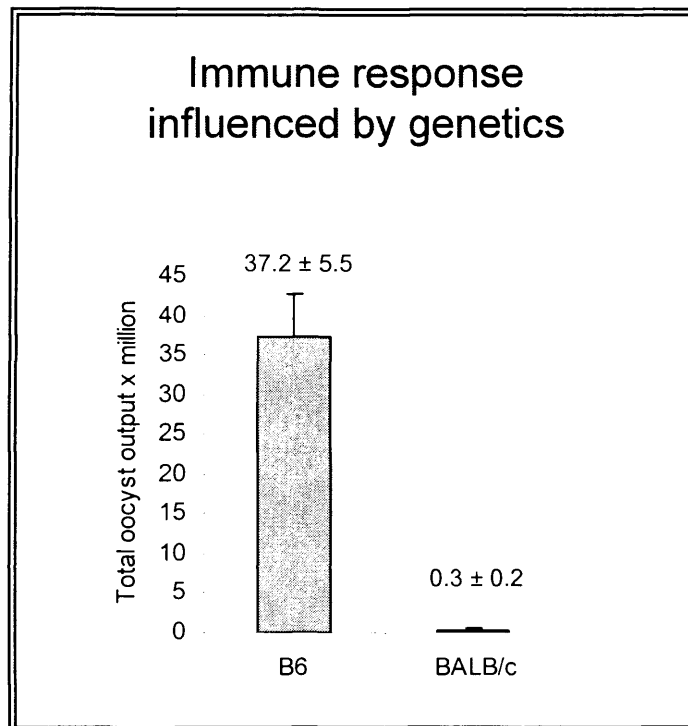
Figure 1.8 The influence of host genetics on resistance against *E. vermiformis*.

B6 mice are relatively more susceptible than BALB/c mice, producing approximately 100 times more oocysts. The data reflects the total oocyst output during primary infection in B6 and BALB/c mice challenged with 1000 oocysts. This figure represents work carried out by P, Hesketh and A, Archer, and is used for reference only.

Immune response
influenced by parasite dose
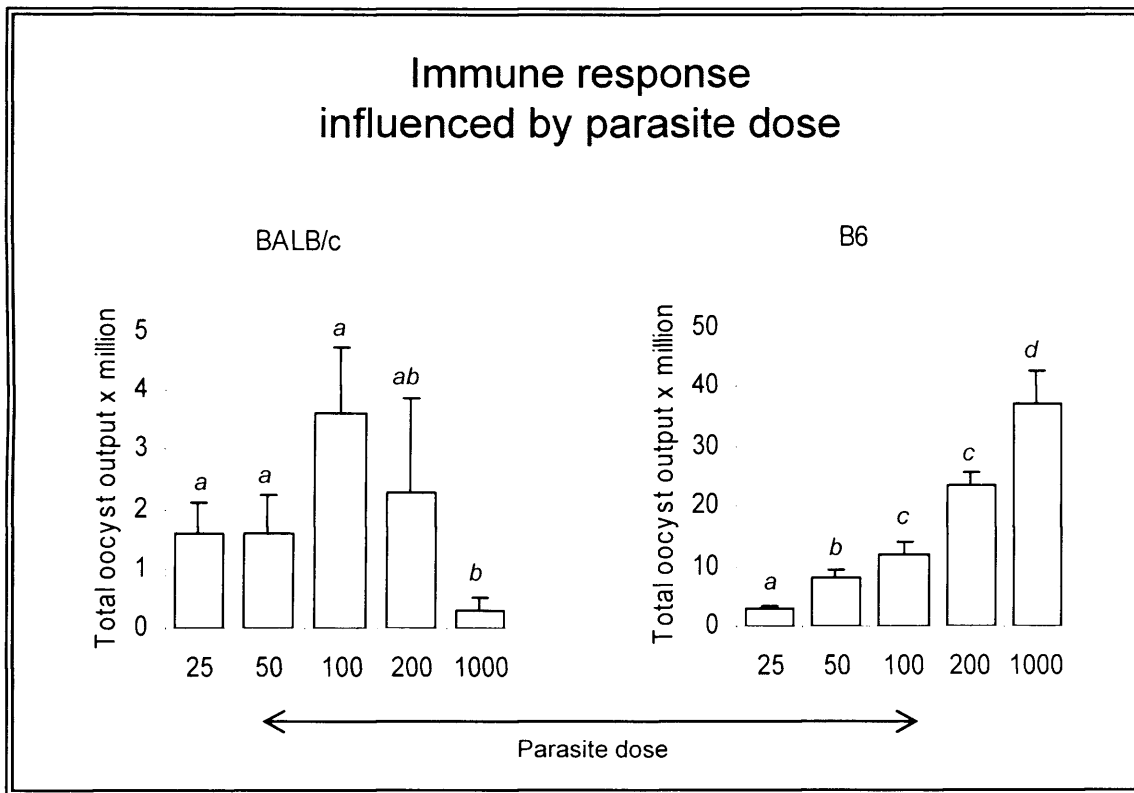
Figure 1.9 The influence of parasite dose on host resistance against *E. vermiformis*.

The influence of parasite dose on the outcome of infection is different between BALB/c and B6 mice. The relative resistance of BALB/c mice decreases with lower parasite doses compared with B6 mice which display improved resistance with lower parasite doses. The data reflects the total oocyst output during primary infection in B6 and BALB/c mice challenged with 25, 50, 100, 200 or 1000 oocysts. Groups with different letters annotated are significantly different from other groups (P<0.05) while groups with the same letters are not significantly different. This figure represents work carried out by P, Hesketh and A, Archer, and is used for reference only.

1.8   Rationale

Many models of oral infection such as S.typhimurium and *L. monocytogenes,* result in the rapid dissemination of pathogens from the mucosal site of invasion to the systemic sites, making it difficult to examine entirely local mucosal responses towards infection. Numerous studies have provided useful information on the characteristics of systemic immune responses against such infections but few studies have addressed specifically mucosal-derived responses due to a lack of appropriate infection models. Most GALT-associated responses studied are related to oral immunisation and oral tolerance in the absence of inflammatory-stimuli and active infections by gut-pathogens. Although some studies do utilise gut infections to study IEL and LPL responses (eg.(Buzoni-Gatel et al., 1999; Little et al., 2005), there are none at present that address the issues related to how GALT structures and the associated cellular factors are involved and necessary for the induction of immune responses against a solely enteric infection.

There is a substantial amount of work carried out to understand Th1 responses toward *E. vermiformis* in mice and the main components for effective primary immunity have been elucidated (Table 1.3). Therefore, this enteric-residing pathogen is ideal for examining factors which influence the induction of responses in the gut. An outline of the areas of investigation in this thesis is depicted in Figure 1.10. There are four chapters examining the influence of different factors (host genetics, parasite dose, secondary lymphoid organisation influenced by LT, LT expression during the immune response, influence of LT on DC, influence of GALT structures) on the immune response against *E. vermiformis.*
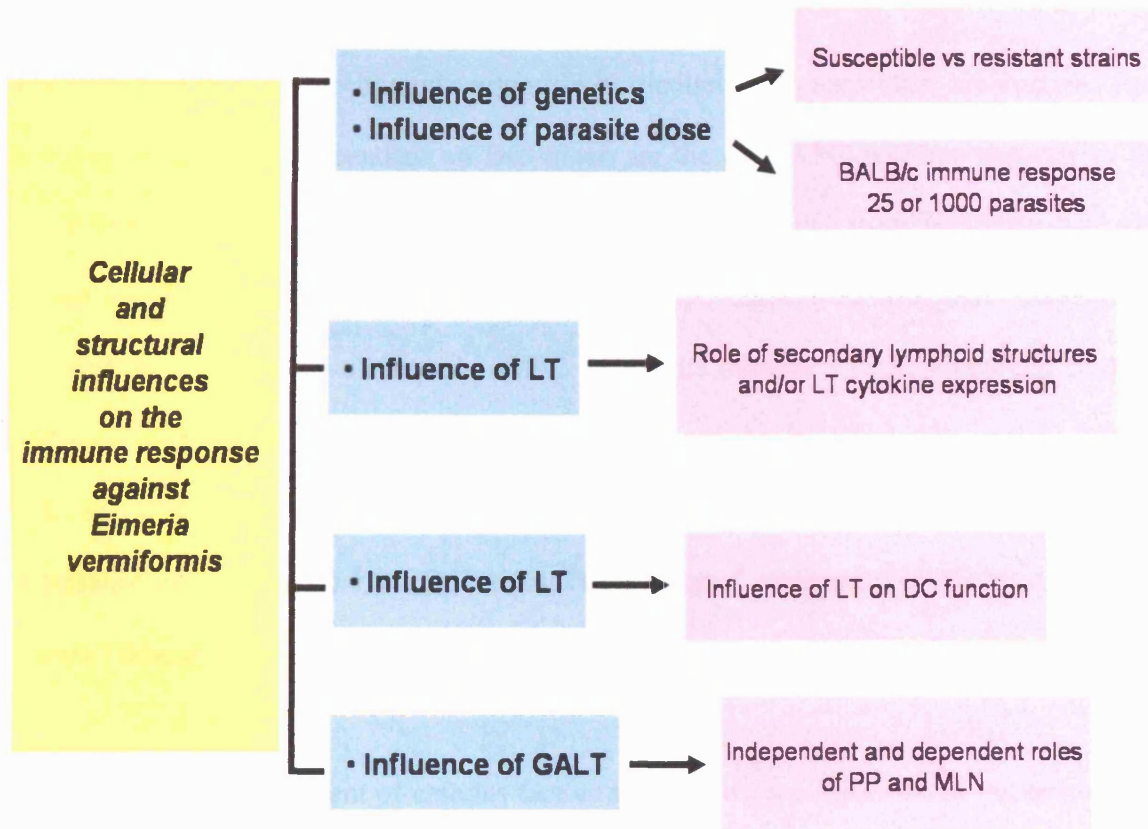
Figure 1.10 **Schematic outline of the main areas of investigation carried out in the thesis.**

The induction of adaptive immune responses particularly Th1 responses, are required against *E.vermiformis*, and is dependent on DC which are the only APC with the capacity to prime naïve T cells and induce T cell memory. An important finding in a study by Jorsey *et al.* (1988) is that immune resistance is dependent upon a bone marrow-derived cell. Therefore, this initiated the examination of the role of DC during the immune response between susceptible and resistant mice (chapter three). The role of DC could also be affected by various influences such as parasite dose as seen from the differential response to dose by the different strains mentioned earlier (Figure 1.9). Of interest is the unusual response of BALB/c mice to lower parasite dose and the effect of parasite dose on DC responses with respect to those of T cells.

Apart from the involvement of cellular factors such as DC, the induction of responses depends on the microenvironment where cell interactions occur. Importantly, organised lymphoid organs play an essential role in facilitating efficient cell interactions especially when antigen or antigen-specific T cells are rare. The availability of LT-deficient mice which have absent or defective secondary lymphoid organs enables the examination of the requirements for different lymphoid structures during a gut infection, particularly the GALT structures (PP, MLN) (chapter four). The PP and MLN are well-studied in terms of their roles in oral tolerance. There are some studies which describe the types of immune responses occurring within PP and MLN but none which addresses the role played by either tissue independently or dependently of each other. Therefore the aims for this study are further justified by the current lack of understanding of specific PP and MLN roles and a suitable gut-infection model. The investigation of the role of GALT structures also extends to their influence on DC induction of the immune response (chapter six) and any influence of the absence of LT on DC functions in

LT-deficient mice (chapter five). Intestinal DC are described in the MLN, LP and PP, and their role in immune surveillance makes them important inducers of the immune response. This is why chapter six focuses on the individual roles of MLN and PP as instructional microenvironments and how DC responding to infection are affected by these related and physically connected lymphoid structures.

The aims here are to achieve a comprehensive understanding of the immune mechanisms occurring within different lymphoid compartments of the GI immune system and how each mechanism influences one another and/or are influenced by the tissue microenvironment in the efficient induction of effective immune response against a rapidly replicating enteric pathogen.

2   Materials and methods

2.1 Materials used

Biochemical Reagents	Supplier	Catalogue No.
2-Mercapethanol (2-ME) - molecular biology grade	Sigma-Aldrich	M-3148
Albumin (chicken)	Sigma-Aldrich	A-5378
Brefeldin A	Sigma-Aldrich	B-6542
5-bromo-2'-deoxyuridine (BrdU)	Sigma-Aldrich	B-5002
Calcium chloride	Sigma-Aldrich	C-4901
Collagenase III	Worthington	4183
Collagenase VIII	Sigma-Aldrich	C-2139
Ditherthythiol (DTE)	Sigma-Aldrich	D-8161
DNase I	Sigma-Aldrich	DN-25
EDTA	Sigma-Aldrich	E-7889
Ethanol	BDH AnalaR	200-578-6
Fluoresbrite^{TM} plain YG microspheres (0.2 microns)	Polysciences	9003-53-6
Haematoxylin QS	Vector Labs	H3404
Hydrochloric acid	BDH AnalaR	130721
Hydrogen peroxide	Sigma-Aldrich	H-1009
Isopentane	Fluka	59080
Magnesium chloride	Sigma-Aldrich	102380
Neomycin sulphate	Sigma-Aldrich	H-6386
Nycoprep (1.077A)	Axis-Shield	NYC 12023
O.C.T^{TM} compound, Tissue-tek	Sakura	4583
Paraformaldehyde	Sigma-Aldrich	P-6148
Percoll	Amersham Biosciences	170891-01
Saponin	Sigma-Aldrich	S-4521
Sodium Nitrite	Sigma-Aldrich	S-3421
Sodium chloride	BDH AnalaR	10245K
Trypan blue	Sigma-Aldrich	T-8154
Water (molecular biology grade)	Eppendorf	955155033
Water (cell culture)	Sigma-Aldrich	W3500
Xylene	BDH AnalaR	10293 6H
Methyl-^{3}H-Thymidine	Amersham Biosciences	TRA120

Media and other cell culture reagents	Supplier	Catalogue No.
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich	H-6648
Rosswell Park Memorial Institute (RPMI) 1640	Gibco-Invitrogen	21875-034
10X phosphate buffer solution (PBS)	Gibco-Invitrogen	70013-016
1X PBS	Gibco-Invitrogen	20012-019
HEPES	Gibco-Invitrogen	15630-056
Foetal calf serum (FCS)	PAA labs	Batch no. 083 and 910003
2-ME (cell culture)	Sigma-Aldrich	M-7522
Penicillin and streptomycin	Sigma-Aldrich	P-3539
Recombinant murine (rm) GM-CSF	R&D systems	415-ML
rm CCL21	R&D systems	457-6C
rm flt3-ligand	Insight Biotech	IB-1234

Toll-like receptor agonists	Supplier	Catalogue No.
ODN 1826 5'-CCATGACGTTCCTGACGTT-3'	InvivoGen	Tlrl-modn
Pam3Cys	InvivoGen	Tlrl-pmc
R848	InvivoGen	Tlrl-r848
Flagellin (S.Typhimurium)	InvivoGen	Tlrl-stfla
LPS (S.Typhimurium)	Sigma-Aldrich	L-6143

Magnetic beads for cell sorting	Supplier	Catalogue No
Anti-mouse CD11c microbeads	Miltenyi Biotech	130-052-001
Anti-mouse CD90 microbeads	Miltenyi Biotech	130-049-101
Anti-mouse CD45R/B220 microbeads	Miltenyi Biotech	130-049-501
Anti-mouse MHC class II microbeads	Miltenyi Biotech	130-052-401
Anti-mouse CD11b microbeads	Miltenyi Biotech	130-049-601
Anti-mouse CD4 particles	BD^{TM} IMag	551539

Miscellaneous Kits	Supplier	Catalogue No.
RNeasy mini kit	Qiagen	74104
DNeasy tissue kit	Qiagen	69504
BD™ Cytometric bead array (CBA) mouse inflammation kit	BD biosciences	552364
BD™ CBA mouse Th1/Th2 cytokine kit	BD biosciences	551287
BrdU-*in situ* detection kit	BD biosciences	551321
Eurogentec RT qPCR mastermix plus	Eurogentec	RT-QPRT-032X
Eurogentec qPCR mastermix plus	Eurogentec	RT-QP2X-03-075X

Plastics and other hardware	Supplier	Catalogue No.
Nylon cell strainers (40um and 70um)	BD Falcon™	2340/2350
96-well plates (U-bottom well) Nunclon™ delta surface	Nunc™	163320
24-well plates Nunclon™ delta surface	Nunc™	142485
15 ml polypropylene conical tube (17 x 120mm)	Falcon	352096
50ml polypropylene conical tube (30 x 115mm)	Falcon	352070
5ml polystyrene round-bottom tube (12 x 75mm)	Falcon	352054
14ml polystyrene round-bottom tube (17 x 100mm	Falcon	352057
8 ml polystyrene round-bottom tube (13 x 100mm)	Falcon	352027
Tips (filtered and non-filtered, 1000µl, 200 µl and 10 µl)	Rainin	RT10F, RT20F, RT200F, RT1000F, GPS250
Needles (27G 1/2") 0.4 x 13 mm	BD Microlance™	300635
Syringes (1ml, 2ml, 5ml, 10ml)	Terumo^R	BS01T, BS025, BS05S, BS10ES
Transwell (3.0µm pore, 6.5mm diameter)	Corning Costar	345
1.0mm Glass beads	Biospec	11079110
Glass balls (up to 0.05mm diameter)	Jencons	136-008
10ml sterile pipette	Corning	4101
5ml sterile pipette	Corning	4051
25ml sterile pipette	Fisher	FB51887
Sterile pastettes	Scientific Lab	PIP4208
Glass slides for microscopy (superfrost-plus)	Menzel-Glaser	MNJ-700-010N

Antibody	Conjugation	Clone	Supplier	Catalogue No.
Anti-mouse CD3e	FITC	145-2C11	Pharmingen	553061
Anti-mouse CD3ε	PE	145-2C11	Pharmingen	553063
Anti-mouse CD3ε	APC	145-2C11	Pharmingen	553066
Anti-mouse CD4	FITC	GK1.5	Pharmingen	553729
Anti-mouse CD4	PE	RM4-5	Pharmingen	553730
Anti-mouse CD4	Cy-Chrome	RM4-5	Pharmingen	553050
Anti-mouse CD4	APC	RM4-5	Pharmingen	553051
Anti-mouse CD8α	Cy-Chrome	53-6.7	Pharmingen	553034
Anti-mouse CD8β.2	FITC	53-5.8	Pharmingen	01054D
Anti-mouse CD8β.2	PE	53-5.8	Pharmingen	01055A
Anti-mouse TCRβ chain	PE	H57-597	Pharmingen	01305A
Anti-mouse CD45.1	PE	A20	Pharmingen	553376
Anti-mouse CD45.1	fitc	A20	Pharmingen	553375
Anti-mouse CD11c	APC	HL3	Pharmingen	550261
Anti-mouse CD11c	fitc	HL3	Pharmingen	557400
Anti-mouse CD45R/B220	PE	RA3-6B2	Pharmingen	553090
Anti-mouse CD11b	PE	M1/70	Pharmingen	557317
Anti-mouse CD11b	fitc		Pharmingen	557396
Anti-mouse CD25	PE	PC61	Pharmingen	557192
Anti-mouse CD69	FITC		Pharmingen	557392
Anti-mouse CD44	Cy-Chrome	1M7	Pharmingen	553135
Anti-mouse CD11a	Biotin		Pharmingen	557365
Anti-mouse CD40	Fitc	3/23	Pharmingen	553790
Anti-mouse CD80	PE	16-10A1	Pharmingen	553769
Anti-mouse Gr1/Ly6G	Fitc	RB6-8C5	Pharmingen	553126
Anti-mouse α4β7	PE	DATK 32	Pharmingen	553811
Anti-mouse CCR7	Pe	4BI-2	eBioscience	12-1971
Anti-mouse CCR9	Pe	242503	R&D systems	FAB2160P
Anti-mouse IFNγ	FITC	-	Pharmingen	554411
Anti-mouse IFNγ	Pe	-	Pharmingen	554412
Anti-mouse IL-4	Pe		Pharmingen	554435
Anti-mouse IL12p40/p70	APC	-	Pharmingen	554480
Anti-mouse TNFα	Pe		Pharmingen	55419
Strepavidin	APC	-	Pharmingen	554067
Anti-BrdU	Fitc	B44	BD Biosciences	347583
Rat anti-mouse IgG$_{2a}$ κ	Pe	-	Pharmingen	553930
Rat anti-mouse IgG1	APC	-	Pharmingen	554686

2.2   Sequences of primers, probes and peptide used

Primer, probe and peptide sequences	
(Fahlen et al., 2005) T-bet sense	5'-CAACAACCCCTTTGCCAAAG-3'
T-bet anti-sense	5'-TCCCCCAAGCAGTTGACAGT-3'
T-bet probe	FAM-5'-CCGGGAGAACTTTGAGTCCATGTACGC-3'-TAMRA
(Overbergh et al., 2003) IFNγ sense	5'-TCAAGTGGCATAGATGTGGAAGAA -3'
IFNγ anti-sense	5'-TGGCTCTGCAGGATTTTCATG-3'
IFNγ probe	FAM-5'-TCACCATCCTTTTGCCAGTTCCTCCAG-3'-TAMRA
(Overbergh et al., 2003) IL12p40 sense	5'- GGAAGCACGGCAGCAGAATA-3'
IL12p40 anti-sense	5'-AACTTGAGGGAGAAGTAGGAATGG-3'
IL12p40 probe	FAM-5'-CATCATCAAACCAGACCCGCCCAA-3'- TAMRA
(Overbergh et al., 2003) IL18 sense	5'- CAGGCCTGACATCTTCTGCAA-3'
IL18 anti-sense	5'- TCTGACATGGCAGCCATTGT-3'
IL18 probe	FAM-5'-CTCCAGCATCAGGACAAAGAAAGCCG-3'-TAMRA
(Luther et al., 2000) HPRT sense	5'-AGGTTGCAAGCTTGCTGGT-3'
HPRT anti-sense	5'-TGAAGTACTCATTATAGTCAAGGGCA-3'
HPRT probe	FAM- 5'- TGTTGGATACAGGCCAGACTTTGTTGGAT-3'-TAMRA

Primer, probe and peptide sequences (continued)	
(Fahlen et al., 2005) CD3γ sense	5'-CACCAAGAGCAAGGAAGAAGATG-3'
CD3γ anti-sense	5'-TTACAGAATGTGTGAAAACTGCATTG-3'
CD3γ probe	5'-FAM-ACATAGGCACCATATCCGGCTTTATCTTCG-TAMRA-3'
(Blake et al., 2006) *E. vermiformis* 5S rRNA sense	5'-GCCGTCATCACCCAAAGGGAT-3'
E. vermiformis 5S rRNA anti-sense	5'-TTCATACTGCGTCTAATGCAC-3'
OVA (323-339) peptide	H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-OH

2.3 Mice used

BALB/c, C57BL/6 (B6), Lymphotoxin (LT) α-/-, LTβ-/-, LTα+/-β+/-, LTα+/-, LTβ+/-, TCRβxδ-/- and OT-II TCR transgenic (RAG1-/- B6 background) mice were bred in the specific pathogen facility at the Institute for Animal Health (Compton, UK) or purchased from Harlan (UK). All mice used (except BALB/c mice) were on a B6 background and 7-14 weeks of age unless indicated otherwise. LTα-/- mice were originally obtained from Nancy Ruddle (Yale University, USA), LTβ-/- mice from Pandelakis Koni (Georgia University, USA) and Richard Flavell (Yale University, USA), OT-II TCR transgenic mice from Scripps (San Diego, USA) through David Tough (GSK, UK), TCRβxδ-/- mice from Charles River. All animal experiments were performed according to the United Kingdom Animals (Scientific Procedures) Act of 1986.

2.4 Isolation of cells

2.4.1 Isolation of lymphocytes

Cells were isolated from lymph nodes, Peyer's patches or spleen by mechanical disruption using a cell strainer (40µm; Falcon) and plastic syringe pestle or by enzymatic digestion using 1mg/ml of collagenase III (Worthington) (as described below).

2.4.2 Isolation of dendritic cells

DC were isolated as using a method previously described (Kamath et al., 2005). MLN were cut into several small pieces and digested with 1mg/ml type III collagenase (Worthington) and 0.5mg/ml DNAse I (Sigma-Aldrich) in RPMI 1640 (Gibco) (supplemented with 5% foetal calf serum (FCS) (PAA labs), 2mM EDTA (Sigma-Aldrich) and 25 mM HEPES (Sigma-Aldrich)) at 37°C for 10 minutes and at room temperature (RT) for 15 minutes. Following this, cell suspensions were treated with 0.079M of EDTA to disrupt DC-T cell complexes. Total cell numbers were evaluated and stained for CD11c and all remaining cells were enriched in a Nycoprep 1.077A (Axis-Shield) gradient centrifuged at 600 x g for 25 min at RT. Further identification of DC subsets was carried out using antibodies to CD11b, CD8α, B220, CD80 and CD40. Total CD11c+ cell numbers were calculated by multiplying the total cell count by the proportion of CD11c+ cells obtained by FACS analysis. The total CD11c+ numbers were used to calculate numbers within DC subsets with reference to the proportions (minimum of 1500 CD11c+ events collected) obtained by FACS analysis after Nycoprep enrichment.

2.4.3 Isolation of intraepithelial and lamina propria lymphocytes

IEL and LPL were isolated using a modified protocol as described previously (Laky et al., 1997). Briefly, the small intestine was excised of all Peyer's patches, cut into 1cm pieces and washed before incubation in HBSS (Sigma-Aldrich) supplemented with 10% FCS, 1mM HEPE and 1mM DTE (Sigma-Aldrich) at 37°C in a gently shaking water bath for 20 min. After 10 seconds of vigorous vortexing, the supernatant was collected and cell pellet washed. IEL were purified using a gradient consisting of 44% and 67.5% Percoll (Amersham Biosciences). Gradients were centrifuged at 600 x g, for 25 minutes at RT and IEL were collected at the 44%/67.5% interphase and analyzed. For LPL isolation, the remaining intestine pieces after incubation with DTE were incubated in HBSS containing 1.3mM EDTA at 37°C in a gently shaking water bath for 30 min and another 30 min at RT in RPMI 1640. The pieces were digested for 1hr at 37°C in a vigorously shaking water bath with collagenase VII (100units/ml) (Sigma-Aldrich) in RPMI 1640 supplemented with 1mM CaCl$_2$ (Sigma-Aldrich), 1mM MgCl$_2$ (Sigma-Aldrich) and 5 % FCS. The supernatant was collected and cell pellet washed. LPL were purified through the Percoll gradient, using the same procedure as described for IEL.

Where mentioned, CD4+ IEL were further enriched through magnetic sorting using anti-mouse CD4 particles (BD ^{TM}IMag) according to manufacturer's instructions. The enriched fraction was later used for mRNA analysis.

2.5 Methods applied for *in vivo* experiments

2.5.1 Infection of mice and enumeration of oocyst numbers

Mice were infected orally with sporulated oocysts and individual faecal samples from day 7 post-infection were collected daily for the enumeration of parasite oocysts. All mice were infected with 100 oocysts unless mentioned otherwise (in chapter three and four) where doses of 1000, 50 or 25 oocysts were used. Counts were taken on a daily basis until no more oocysts were produced. Oocyst output was determined as described previously (Rose et al., 1984). Faeces were soaked in water overnight and homogenised using a vortex. The homogenised samples were diluted in saturated salt and oocysts were enumerated microscopically using a McMaster parasite egg counting chamber. Enumeration of oocysts is calculated using the following formula: N x chamber volume x dilution factor (where N refers to the number of oocysts counted in the 10 lanes found in one chamber).

2.5.2 Generation of bone marrow chimeric mice

Recipient mice were lethally irradiated at 900 rads and injected intravenously with 3.5×10^{6} bone marrow cells in 200µl of RPMI supplemented with 100units/ml of penicillin and streptomycin. Mice were provided 2mg/ml of neomycin sulphate (Sigma-Aldrich) in their drinking water for 2 weeks. All bone marrow chimeric mice were kept for 4 months to allow reconstitution before infection. Reconstitution was checked using the Ly5 marker by FACS where more than 90% reconstitution was obtained in all recipients. Experiments were also repeated using syngenic B6 donor mice.

2.5.3 T cell and tissue-specific cell adoptive transfer

The T cells from spleen and MLN were sorted using anti-CD90 microbeads (Miltenyi Biotech) according to the manufacturer's instructions. A total of 1 x 10^7 CD90+ cells were injected intraperitoneally into recipient mice which were left for 7 days before infection. Purity of sorted cells was >90%. For tissue cell transfer, 2 x 10^7 MLN cells or 2 x 10^6 PP cells from day 8 post-infected B6 mice were injected intraperitoneally into recipient mice which were kept for 7 days before challenge.

2.5.4 Dendritic cell adoptive transfer

The DC were isolated from the MLN as described but without a Nycoprep gradient step. Enrichment of DC was carried out by depleting B and T cell populations, using anti-CD45R/B220 (Miltenyi Biotech) and anti-CD90 microbeads according to the manufacturer's instructions. Further segregation of DC subsets was done by positive selection using anti-CD11b microbeads (Miltenyi Biotech). A total of 1 x 10^5 cells from the enriched CD11b+ or CD11b- fractions were injected intraperitoneally into LTα+/- β+/- recipients which were infected 4 days later.

2.5.5 *In vivo* expansion of DC using Fms-like tyrosine kinase 3 (Flt3) ligand for the *in vitro* analysis of DC function

Mice were injected intraperitoneally every 2 days with 5mg of recombinant murine Flt3-ligand (Insight Biotech). After 6 days, DC were isolated from their spleens, MLN and PP and used for *in vitro* analysis of DC antigen presentation to OT-II T cells. The DC were purified using anti-CD11c+ microbeads (Miltenyi Biotech). A total of 1 x 10^4 DC were added to wells with or without 2 x 10^5 OT-II T cells (enriched by depleting MHC class II+ cells using

microbeads (Miltenyi Biotech)) in the absence or presence of S.Typhimurium flagellin (50ng/ml, Invivogen) or Pam3Cys (200ng/ml, Invivogen). Finally, OVA protein (50μg per well, Sigma-Aldrich) was added to the cultures which were incubated for 72 hr. During the last 18 hr of incubation, 1μCi of ^{3}H-thymidine (Amersham Biosciences) per well was added. Cultures were harvested and assayed using a beta counter.

2.6 Methods applied for *ex vivo* and *in vitro* experiments

2.6.1 Preparation of oocyst lysate

Oocysts were washed free from potassium dichromate and cleaned in 10% chloros for 10 min on ice. Four washes in distilled water followed by one wash in PBS (pH 7.2) were carried out to remove any chloros. Sporulated oocysts were resuspended at a concentration of 1.2 x 10^7 oocysts/ml and cracked using glass beads (up to 0.05mm diameter, Jencons) on a vortex in a 50 ml tube. Each 2 min-long burst was followed by incubation on ice. Cracking of oocysts was checked under a microscope. After cracking, oocysts were sonicated twice for 30 seconds while on ice. Finally, the sonicated oocysts were put through a freeze-thaw cycle, 3 times in liquid nitrogen and protein concentrations were measured using a spectrophotometer.

2.6.2 FACS staining and analysis

Cells were stained for 10 minutes at RT and washed twice with PBS containing 1% FCS and 0.1% sodium azide (Sigma-Aldrich) before analysis using the FACScaliber instrument (BD Biosciences). On most occasions, cells were fixed in 1% paraformaldehyde (Sigma-Aldrich) prior to FACS analysis.

2.6.3 Generation of bone marrow-derived dendritic cells

Muscle tissues were removed from tibias and femurs of mice and cut at the epiphyses. Bone marrow was flushed out of bones using RPMI 1640 (supplemented with 100units/ml penicillin-streptomycin) and a 27G ½ needle. The cell suspension was collected and strained to remove any debris before being washed twice and spun at 400 x g, for 8 min at 4°C. Cells were grown in Falcon 1029 petri dishes (2 x 10^6 cells per dish) in 10ml of R10 (RPMI 1640, 10% FCS, 100 units/ml of penicillin-streptomycin, 50nM 2-ME) supplemented with 10ml of 20ng/ml of recombinant (rm) GM-CSF (R&D) and incubated at 37°C, 5% CO2 for 8 days. On day 3, cultures were replenished with 10ml of 20ng/ml of rmGM-CSF in R10. On day 6, 10 ml of media were withdrawn from each dish and spun down to retrieve any cells lost. A further 10ml of R10 supplemented with 10ng/ml of rmGM-CSF was added. On day 8, partially adherent cells were loosen by thorough pipetting and collected. The BMDC collected were used with stimulation assays. On every occasion, BMDC purity was checked using the marker CD11c through FACS analysis. Where required, BMDC were positively sorted using anti-CD11c microbeads.

2.6.4 Cytokine assays

A total of 1 x 10^6 cells from the PP, MLN or spleen of infected and uninfected mice were incubated at 37°C, 5% CO_2 in 200µl of R10 with or without oocyst lysate for 24 hr. Where required, supernatants were taken from 24 hr cultures and analyzed using the Cytometric Bead Array kit (BD Biosciences). Alternatively, cells were analyzed for the production of intracellular cytokines in the method described below.

Intracellular cytokine staining

Cells from infected or uninfected mice were cultured with or without oocyst lysate for 24 hr as described for the cytokine assays. During the last 6 hr of incubation, $10\mu g/ml$ of Brefeldin A (Sigma-Aldrich) was added to inhibit cytokine export from the cell. After washing, cells were surface-stained for CD4, CD8α and/or CD3ε before washing and vigorously resuspended in $100\mu l$ of brefeldin A (0.1 $\mu g/ml$) and $100\mu l$ of 4% paraformaldehyde (Sigma-Aldrich). After 20 min of incubation at RT, cells were washed twice before permeabilization in 0.5% saponin (Sigma-Aldrich) for 10 min at RT. Cells were washed, resuspended and stained for intracellular IFNγ in 0.5% saponin for 30 min at RT. Two washes in 0.5% saponin and two washes in FACS wash were carried out to remove excess antibody and avoid non-specific staining. Cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry

BMDC stimulation assays

Between 2 x 10^4 and 5 x 10^4 BMDC (depending on experiments, are CD11c+ sorted or not) were cultured with TLR agonists for 24hr and supernatants were taken from the cultures and analyzed using the CBA kit. The TLR agonists used are as follows: S.Typhimurium LPS ($5\mu g$ /ml); S.Typhimurium flagellin (50ng/ml); R848 ($1\mu g$ /ml); CpG ($1\mu g$ /ml); Pam3cys ($1\mu g/ml$); ODN 1826 (500nM); *E.vermiformis* oocyst lysate ($10\mu g/ml$ or $50\mu g$ /ml).

Intracellular cytokine staining of IL12p40/p70

Alternatively, intracellular cytokine staining of IL12p40/p70 was carried out using the method already described. A total 5 x 10^5 BMDC were cultured with or without stimuli for

24hr and brefeldin A was added in the last 6 hr to stop cytokine export from the cells. The

TLR agonists used were S.Typhimurium LPS (5μg /ml), S.Typhimurium flagellin (50ng/ml),

Pam3cys (1μg/ml); ODN 1826 (500nM), *E.vermiformis* oocyst lysate (10μg/ml or 50μg /ml)

and anti-mouse CD40 (5μg/ml) was used where indicated.

2.6.5 OVA peptide presentation by BMDC

On day 8 of BMDC culture, BMDC were sorted for CD11c+ cells and matured overnight

with or without S. Typhimurium LPS (1μg/ml, Invivogen) or ODN 1826 (2.5μM, Invivogen)

in 24-well plates at a cell density of 1.5 x10^6 cells /ml (total volume of 2 ml per well). The

BMDC were pulsed with 10μg/ml OVA 323-339peptide (Anaspec Inc) for 4 hr and washed

twice before plating cells in U-bottom 96 well plates. The OT-II T cells were enriched by

depletion using anti-MHC class II microbeads and 2 x10^5 OT-II T cells were cultured with 2

x 10^5, 2 x 10^4, 2 x 10^3 or 2 x10^2 BMDC in 200μl of R10 media for 72hr at 37°C, 5% CO_2.

During the last 18 hr of incubation, 1μCi of ^{3}H-thymidine (Amersham Biosciences) per well

was added. Cultures were harvested and assayed using a beta counter.

2.6.6 Chemotaxis Assay

A total of 2 x10^5 DC were resuspended in 100μl of R10 and plated into the upper transwell

(3.0μm pore, Corning Costar). 1μg/ml CCL21 (R&D) in 600μl of media was added to the

lower well and the plate was incubated at 37 °C, 5% CO2 for 4 hr. The upper transwell was

removed and all cells in the lower well were counted using a haemocytometer. EDTA (2mM)

was added to ensure the removal of all cells. Cells were counted and plotted as a percentage

of migrated cells. The percentage of migrated cells was calculated as: Total number of cells

migrated / $2 \times 10^5 \times 100\%$.

2.6.7 Staining of bromodeoxyuridine (BrdU) -labelled cells

Fresh changes of drinking water with BrdU (0.8mg/ml) added were given to mice daily. Following removal of tissues and isolation of cells for analysis, cells were stained for extracellular surface markers as described previously and washed. A total of 1×10^6 cells were resuspended in 0.5ml of ice-cold 0.15M NaCl (AnalaR) and kept on ice. 1.2ml of ice-cold 95% ethanol was added drop-wise onto cells while vortexing and cells were kept on ice for 30 min. Cells were washed with 2 ml of PBS and fixed in 1ml of PBS with 1% paraformaldehyde and 0.01% Tween-20 (Sigma-Aldrich) for 30 min at RT. After spinning down cells, DNA denaturation was carried out by adding 1ml of 50 Kunitz units/ml DNase I, 0.15M NaCl, 4.2mM $MgCl_2$ and 10μM HCL (Sigma-Aldrich) in PBS and incubating cells for 10 min at RT. Cells were washed with 2 ml of PBS and stained with 10μl of fitc-conjugated anti-BrdU (25μg/ml, BD Biosciences) for 30 min at RT. Finally cells were washed with 2ml of PBS and analyzed by flow cytometry.

2.6.8 *Ex vivo* assays examining CD11b+ and CD11b- dendritic cell function

The DC were isolated from the MLN of B6 mice at day 6 post-infection and enriched by depleting T and B cells with anti-CD90 and anti-CD45R/B220 microbeads. The T and B cell-depleted fraction was further separated using anti-CD11b microbeads. The CD11b+ and CD11b- fractions were used without further fractionation in cytokine or proliferation assays using OT-II T cells. The OT-II T cells were obtained from the spleen and MLN and enriched by depletion using anti-MHC class II microbeads.

The functional properties of CD11b+ and CD11b- DC isolated from the MLN at day 6 post-infection were examined in cultures with OT-II T cells. The CD11b+ or CD11b- DC were first pulsed with OVA 323-339 peptide ($10\mu g/ml$) at a concentration 1×10^6 cells /ml for 2 hr at 37°C, 5% CO_2 and washed prior to incubation for 48 hr with 2×10^5 OT-II T cells in quadruplet wells. To measure cytokine production, supernatants from the cultures were analyzed using the BD^{TM} CBA kit.

Alternatively, infected B6 mice were given OVA protein (0.04mg/ml, Sigma-Aldrich) in their drinking water from day 3 post-infection onwards. At day 6 post-infection, DC were isolated from the MLN as described above. The presentation of OVA obtained *in vivo* was measured where 1×10^4 CD11b+ or CD11b- DC were cultured with 2×10^5 OT-II T cells in quadruplet wells. During the last 18 hr of incubation, $1\mu Ci$ of ^{3}H-thymidine per well was added. Cultures were harvested and assayed using a beta counter.

2.7 Molecular techniques

2.7.1 RNA isolation and quantitative RT-PCR analysis

Cell samples were counted and RNA was isolated using the QIAGEN RNeasy kit according to manufacturer's instructions. Tissue samples were stored in RNAlater (Qiagen) before homogenisation in lysis buffer using 1.0mm glass beads (Biospec) and a bead beater. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on mRNA using a qRT-PCR mastermix (Eurogentec) and mRNA quantities were analyzed

using the ABI Prism 7700 Sequence BioDetector instrument (PE Applied Biosystems, Foster City, CA).

A final reaction volume of 25µl consisting of primers (sense and anti-sense, 100nM), probe (2.5nM), EuroScript+ reverse transcriptase (0.25U/µl), RNAse inhibitor (0.1U/µl), master mix reaction buffer (1x concentration) and RNA sample was added to each well. Samples were analyzed in duplicate wells. Wells containing the reaction buffer without RNA samples were included as non-template controls to monitor background levels. 5 serial 10-fold dilutions of standards were used to ensure >95% PCR efficiency.

The cycling conditions consisted of an initial cycle of 2 min at 50°C, 30 min at 60°C and 5 min at 95°C followed by 40 cycles of 20 sec at 94°C and 60 sec at 59°C. Each cytokine of interest was normalised either levels of CD3γ or HPRT mRNA. The results represented relative fold differences between uninfected and infected samples and were calculated using the ΔΔ CT (Cycling threshold) method where ΔΔ CT = (CT of gene of interest) - (CT of HPRT or CD3γ RNA). Fold difference = $2^{-(\Delta\Delta\ CT\ of\ infected\ sample)\ -\ (mean\ of\ \Delta\Delta\ CT\ of\ uninfected\ samples)}$

2.7.2 DNA isolation and detection of *E. vermiformis* DNA

A total of 2×10^5 cells were counted and digested with proteinase K, and DNA were isolated using the Qiagen DNeasy kit according to manufacturer's instructions. Detection of *E. vermiformis* DNA was carried out as described previously (Blake et al., 2006). DNA samples isolated from selected cells were amplified in duplicate wells in a reaction (total of 50µl) containing 25µl of master mix buffer, primers (sense and anti-sense, 300µM) and a probe (200µM). The cycling conditions consisted of an initial cycle of 2 min at 50°C, 10 min at

95°C followed by 40 cycles of 15 sec at 94°C and 1 min at 60°C. The aim here was only to detect the presence of parasite material in specific cells and therefore only 40-CT values were compared without normalization.

2.8 Statistical analysis

The level of statistical significance was determined using one-way ANOVA and where appropriate, post tests using Student's t-test or Tukey test were carried out. Generally the Student's t-test was applied as follows: 1) determining the earliest time-point when a significant difference is observed between uninfected (day 0 post-infection) groups and infected groups 2) determining significant differences between two groups.

The one-way ANOVA analysis and the Tukey test were applied to determine the overall significant differences between multiple groups and in experiments where the Student's t-test was inappropriate.

Chapter Three

Factors influencing the induction kinetics of Th1 immune responses against *E.vermiformis*

3.1. Introduction

Primary infection with *E.vermiformis* is controlled by MHC class II-restricted CD4+ TCRαβ+ cells (Rose et al., 2000; Rose et al., 1988a; Rose et al., 1988b; Smith and Hayday, 2000). IFNγ plays a very important role in the control of infection with *E.vermiformis* (Rose et al., 1989; Rose et al., 1991; Smith and Hayday, 2000) as with a range of intracellular pathogens such as *M. tuberculosis*, *L. major* and *L. monocytogenes* (Belosevic et al., 1988; Flynn et al., 1993; Kiderlen et al., 1984). Compared with IFNγ-/- mice which are highly susceptible during primary infection, IL4-/-, perforin -/- and Fas ligand-deficient mice remain relatively resistant to infection (Smith and Hayday, 2000). Collectively, these studies indicate an essential role for a Th1-driven immunity for resistance during primary infection with *E.vermiformis*.

As highlighted in chapter one (Figure 1.8 and 1.9), the level of resistance against *E. vermiformis* is influenced by factors such as host genetics and parasite dose. The difference in genetic susceptibility to *E. vermiformis* between BALB/c and B6 mouse strains, is evident by the greater oocyst output in B6 mice (up to 100 times more than BALB/c mice). Resistant BALB/c mice display earlier immune responses to infection (Rose et al., 1984; Rose et al., 1985), evident by the earlier increases in MLN cellularity and *ex vivo* antigen-specific production of IFNγ (Roberts et al., 1996; Rose et al., 1988a; Rose et al., 1988b). Such a

'resistant' phenotype depended upon the genotype of bone marrow-derived cell populations (Joysey et al., 1988).

Previous studies on genetic resistance were performed with modulating doses of 1000 parasites however, recent work in the present laboratory also indicated that host resistance is dependent on the inoculating dose (ALS personal communication). At low doses, there is little difference in oocyst output between BALB/c and B6 mice whereas at higher doses, B6 mice are more susceptible to infection. Preliminary data also suggests that the timing of host responses may be different between these strains at different doses.

3.2. Rationale and experimental approach

The aim of this chapter is to examine the influence of host genetics and parasite dose on the induction kinetics of Th1 responses. Here, the timing of T cell responses was closely analysed in detail with reference to the suggestion that earlier induction of the immune response contributes to greater resistance to infection (Rose et al., 1985). The DC are important and potent primers of the adaptive immune system and help shape the Th1/Th2 direction of the immune response. Therefore, it was important to determine the kinetics of DC recruitment into the MLN as this may influence the timing of immune induction. The work presented here, tests the hypothesis that early T cell responses are influenced by DC recruitment into induction sites and are required for more effective host resistance during infection with E. vermiformis.

The MLN and PP form the GALT and are essential sites for the induction of immune responses against gut infections. The MLN is the major lymphoid structure draining the gut and therefore is representative of the immune responses that are induced during an enteric infection. The

kinetics of MLN cellularity between resistant and susceptible strains of mice during infection with *E.vermiformis* has been demonstrated by Rose *et al.* (1985). With a dose of 1000 oocysts, BALB/c mice are more resistant than B6 mice, producing up to 100 times fewer oocysts during infection (Figure 1.8, Chapter one; work by P, Hesketh and A, Archer). With doses of 25, 50 and 100 oocysts, BALB/c mice produced more oocysts than with a dose of 1000 oocysts (Figure 1.9, Chapter one; work by P, Hesketh and A, Archer). There was no significant difference between oocyst output between BALB/c mice challenged with doses of 25, 50 or 100 but there were significant differences in oocyst output for B6 mice challenged with the same range of parasite doses.

Here, two main factors of influence over the outcome of infection are investigated: Firstly, the difference in oocyst output between strains indicates an influence from host genetics and secondly, the different response between strains to various parasite doses suggests that the influence of dose on the outcome of infection, also depended on host genetics.

In order to examine the influence of genetics on immune responses, age and sex-matched BALB/c and B6 mice were infected with 1000 oocysts. During time-points over a period of 15 days post-infection (DPI), MLN and PP were isolated to examine DC, T cell and cytokine production. The same approach was applied when examining the influence of parasite dose. Only BALB/c mice were used and infected with 1000 or 25 parasites since BALB/c mice respond more dynamically to different doses.

3.3. Results

3.3.1. Influence of genetics during infection with *E.vermiformis*

Resistant BALB/c mice display earlier T cell responses

In view of the preliminary studies carried out, a further examination of the T cell response occurring in the MLN of infected mice was done. MLN cellularity in BALB/c mice increased significantly from 5 day post-infection (DPI) onwards while that in B6 increased from 6 DPI onwards (Figure 3.1A). It was mostly CD4+ T cells which contributed to the increase in MLN cellularity while CD8+ T cells remained the minority and no significant difference in CD8+ T cell kinetics was observed between the two strains. CD4+T cell kinetics corresponded generally to increasing MLN kinetics as CD4+ T cells from BALB/c mice also increased earlier at 3 DPI and 5 DPI onwards, compared with B6 mice at 8 DPI. On closer examination, CD4+ and CD8+ T cells numbers do not completely account for the overall MLN cell number at each time-point and may indicate the presence of non-T cells such as B cells, NK cells, macrophages, DC.

CD4+ T cells were also analyzed using the activation markers, CD69 and CD25, and in both BALB/c and B6 mice, the number of CD69+ CD4+ T cells increased significantly at 4 DPI although the levels of cellular increment were greater in BALB/c mice and no significant difference could be seen in B6 mice at 5 DPI (Figure 3.1B). CD25+ CD4+ T cells peaked 2-fold at 6 DPI in BALB/c mice (P<0.001) while B6 mice showed a significant increase only at 8 DPI (P<0.05). CD25 expression on CD8+ T cells was low and remained unchanged (data not shown) while no significant difference was observed in CD69+ CD8+ T cell kinetics between BALB/c and B6 mice (P=0.46). However, despite the lack of increase in CD8+ T cells, there were increases in CD69+ CD8+ T cell numbers in BALB/c from 4 DPI onwards and in B6 mice from 6 DPI onwards.

A

MLN cells

CD4+ T cells

CD8α+ T cells

B

CD4+ CD69+ T cells

CD4+ CD25+ T cells

CD8α+ CD69+ T cells

BALB/c

B6
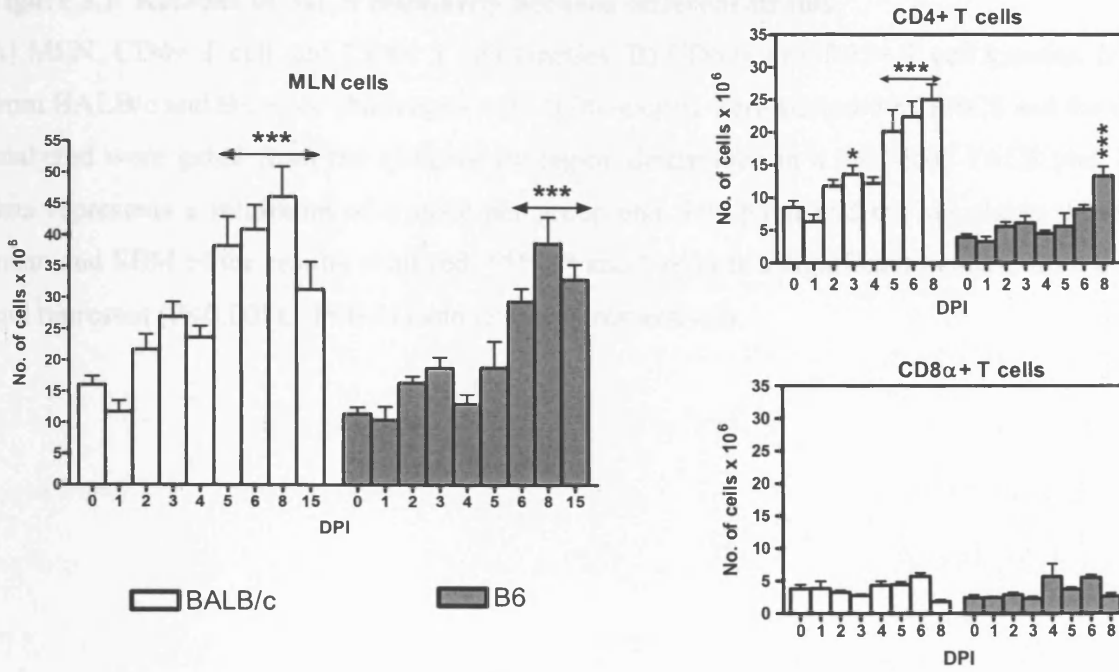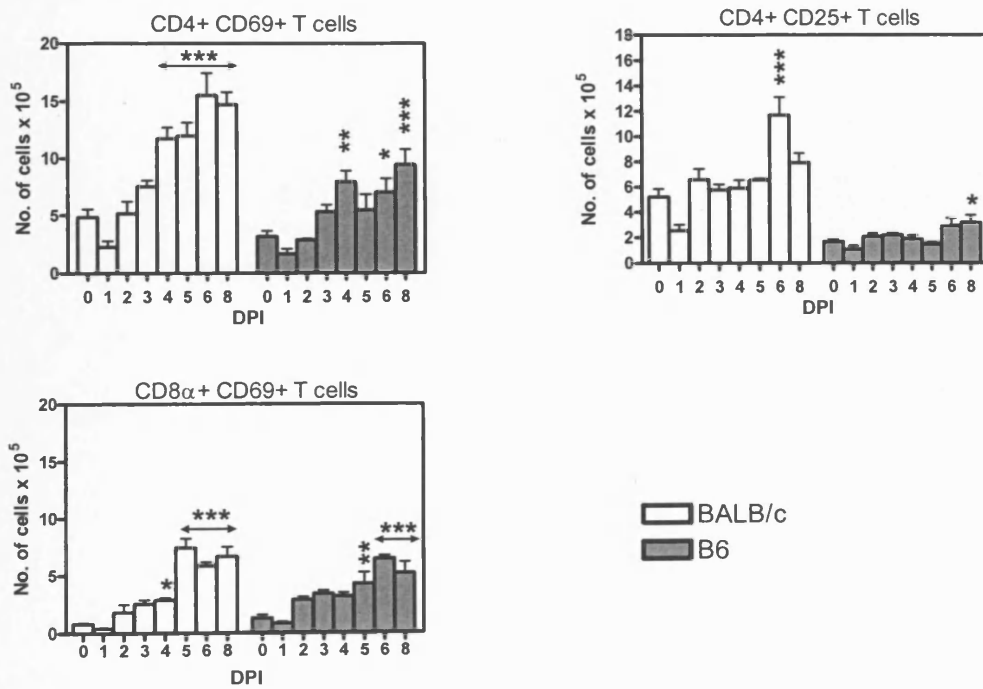
Figure 3.1 Kinetics of MLN cellularity between different strains.

A) MLN, CD4+ T cell and CD8+ T cell kinetics. B) CD69+ or CD25+ T cell kinetics. MLN from BALB/c and B6 mice challenged with 1000 oocysts were analysed by FACS and the cells analyzed were gated from the lymphocyte region determined in a FSC-SSC FACS plot. The data represents a minimum of 4 mice per group and time-point and the bar charts show the mean and SEM of the results achieved. ***, ** and * refer to a significant increase from 0 DPI and represent (P<0.001), (P<0.01) and (P<0.05) respectively.

The PP are located along the small intestine and contain M cells which continuously sample the intestinal lumen for antigens and transfer them to the APC below in the M cells' basolateral pocket. *E.vermiformis* is not known to invade the PP unlike pathogens such as S. Typhimurium and *L. monocytogenes*. However, PP are inductor and effector sites of gut-associated immune responses, and are strategically placed to facilitate local DC and T cells interactions. Therefore, PP immune responses between strains were compared where it was observed that naïve BALB/c mice possessed higher numbers of PP cells than naïve B6 mice (Figure 3.2A). A significant increase in PP cells occurred at 5 DPI in BALB/c mice while in B6 mice, PP cells increased at 8 DPI. The majority of T cells in the PP were CD4+ T cells (in both strains) which rose significantly at 5 DPI in BALB/c mice but showed no significant changes in B6 mice. CD8+ T cells in the PP from BALB/c mice also increased slightly at 5 and 6 DPI while that in B6 mice increased only at 8 DPI. In BALB/c mice, a significant increase in CD69+ CD4+ T cells was seen only at 5 DPI (P<0.01) and corresponded with CD4+ CD25+ T cells which increased at 5 and 6 DPI (Figure 3.2B). There were increases in the number of CD69+ CD4+ T cells in B6 mice at 3, 5 and 8 DPI although none were seen at 4 DPI and 6 DPI. CD25+ CD4+ T cell levels remained low in B6 mice and only significantly increased at 8 DPI. In both strains, there were no difference in CD69+ CD8+ T cells and CD8+ T cells expressed low or negligible levels of CD25 (data not shown).
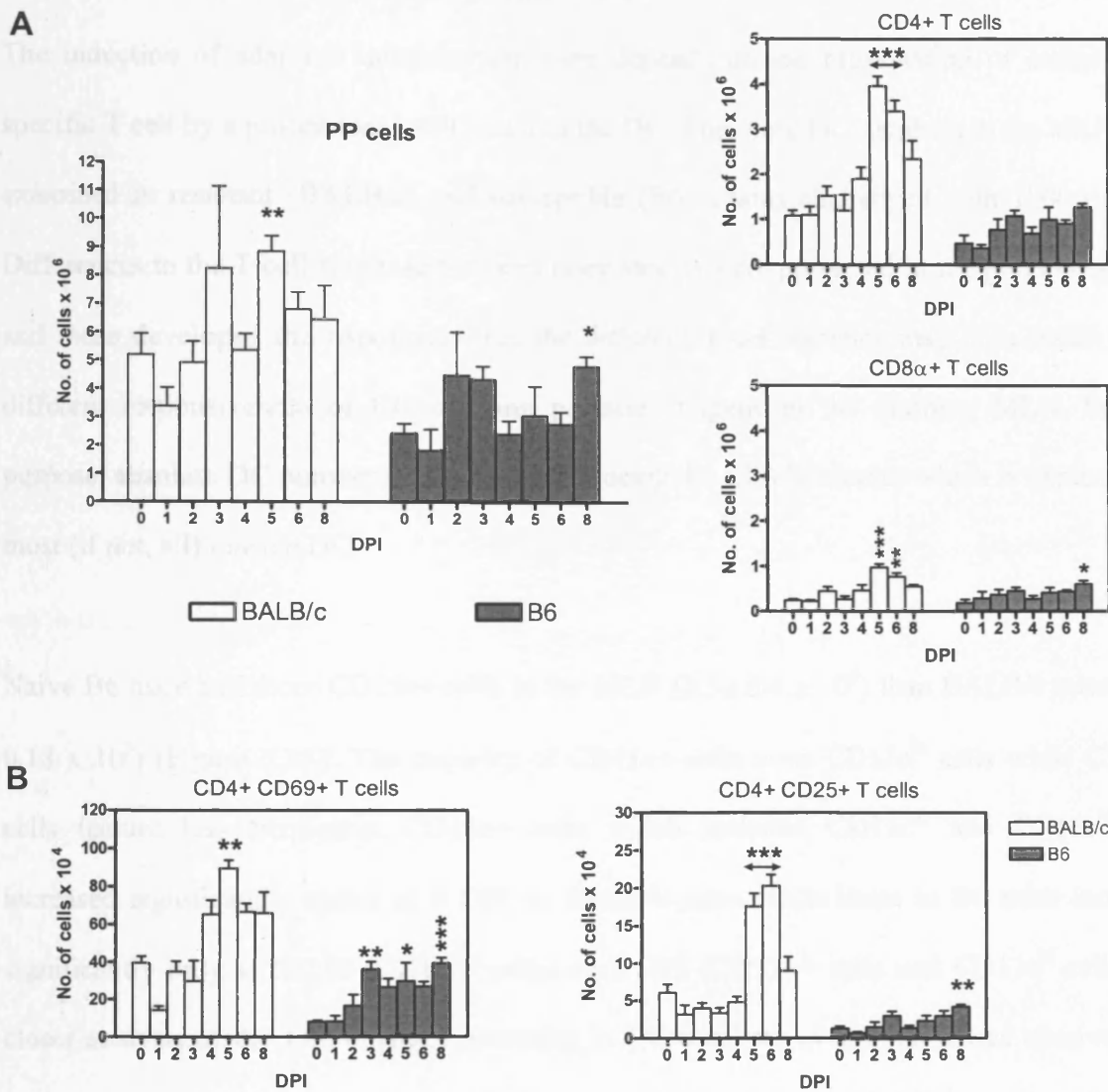
Figure 3.2 Kinetics of PP cellularity between different strains.

A) PP, CD4+ T cell and CD8+ T cell kinetics. B) CD69+ or CD25+ T cell kinetics. The PP from BALB/c and B6 mice challenged with 1000 oocysts were analysed by FACS and the cells analyzed were gated from the lymphocyte region determined in a FSC-SSC FACS plot. The data represents a minimum of 4 mice per group and time-point and the bar charts show the mean and SEM of the results achieved. ***, ** and * refers to a significant increase from 0 DPI and represent (P<0.001), (P<0.01) and (P<0.05) respectively.

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Earlier DC recruitment in the MLN of BALB/c mice

The induction of adaptive immune responses depends on the presentation of antigen to a specific T cell by a professional APC such as the DC. Therefore DC numbers in the MLN were examined in resistant (BALB/c) and susceptible (B6) strains challenged with 1000 oocysts. Differences in the T cell response between mice strains were presented in the previous section and these developed the hypothesis that the different T cell kinetics may be a result of the different responsiveness of DC carrying parasite antigens to the draining MLN. For this purpose, absolute DC numbers were quantified using the CD11c marker which is expressed by most (if not, all) murine DC.

Naive B6 mice had more CD11c+ cells in the MLN ($3.3 \pm 0.4 \times 10^5$) than BALB/c mice ($2.2 \pm 0.18 \times 10^5$) (Figure 3.3A). The majority of CD11c+ cells were CD11c^{lo} cells while CD11c^{hi} cells feature less frequently. CD11c+ cells which included CD11c^{lo} and CD11c^{hi} cells increased significantly earlier at 4 DPI in BALB/c mice while those in B6 mice increased significantly only at 5 DPI (CD11c^{lo} cells) or 6 DPI (CD11c+ cells and CD11c^{hi} cells). On closer analysis of the fold changes occurring in DC numbers over time, it was observed that fold increases in CD11c+ cells remained similar in both strains (at 3 DPI) and earlier fold increases were seen at 2 DPI in both CD11c^{lo} and CD11c^{hi} cells from BALB/c mice compared to B6 mice at 3 DPI (Figure 3.3B).

Analysis of DC subsets show that CD8α-CD4- (DN) DC were the largest subset (Figure 3.4). There were earlier increases in CD11c+ DN DC at 5 DPI in BALB/c mice compared to B6

mice at 6 DPI. Smaller but significant increases in CD11c+ CD8α+ DC were seen at 3 DPI in BALB/c mice compared to B6 mice at 6 DPI.

In the PP, there were significant increases in CD11c+ cells from BALB/c mice at 4, 5 and 8 DPI while CD11c+ cells in B6 mice increased only at 3 DPI (Figure 3.5A). The earliest fold increase in CD11c+ cells occurred at 4 DPI in BALB/c mice and at 2 DPI in B6 mice (Figure 3.5B). There was some correlation (similar significant increases occurring at 5 DPI) between CD11c+ cell and CD4+ T cell (including CD69+ CD4+ and CD25+ CD4+ T cells) kinetics in the PP of BALB/c mice. In the PP of B6 mice, there was a correlation between CD11c+ cell and only CD69+ CD4+ T cells at 3 DPI.

3.3.2. Influence of parasite dose during infection with *E.vermiformis*

Slower T cell immune responses in BALB/c mice infected with a lower parasite dose

Resistant BALB/c mice produce up to 100 times fewer oocysts than susceptible B6 mice when challenged with 1000 oocysts. This is associated with an earlier immune response in BALB/c mice as demonstrated in the previous section. However, when challenged with a lower dose of oocysts (eg. 25 oocysts), there is no difference in the relative efficiency of parasite killing between BALB/c and B6 mice (Figure 1.9, chapter one). It was demonstrated by members of the lab that the *ex vivo* antigen-specific T cell immune response in BALB/c mice was slower with a dose of 25 than a dose of 1000 oocysts (data not shown). Hence, a more detailed examination of the cellular response was carried out to determine T cell kinetics in relation to parasite dose. With a dose of 25 oocysts, there was a delayed increase in MLN cellularity occurring at 6 DPI compared to 4 DPI in mice challenged with 1000 oocysts (Figure 3.6A).

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A

CD11c+

CD11c^{lo}

CD11c^{hi}

B

CD11c+

CD11c^{lo}

CD11c^{hi}

☐ BALB/c
■ B6

Gated on R1
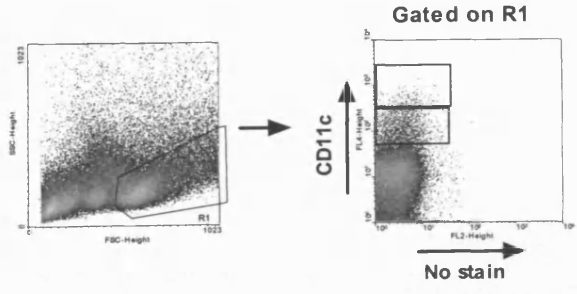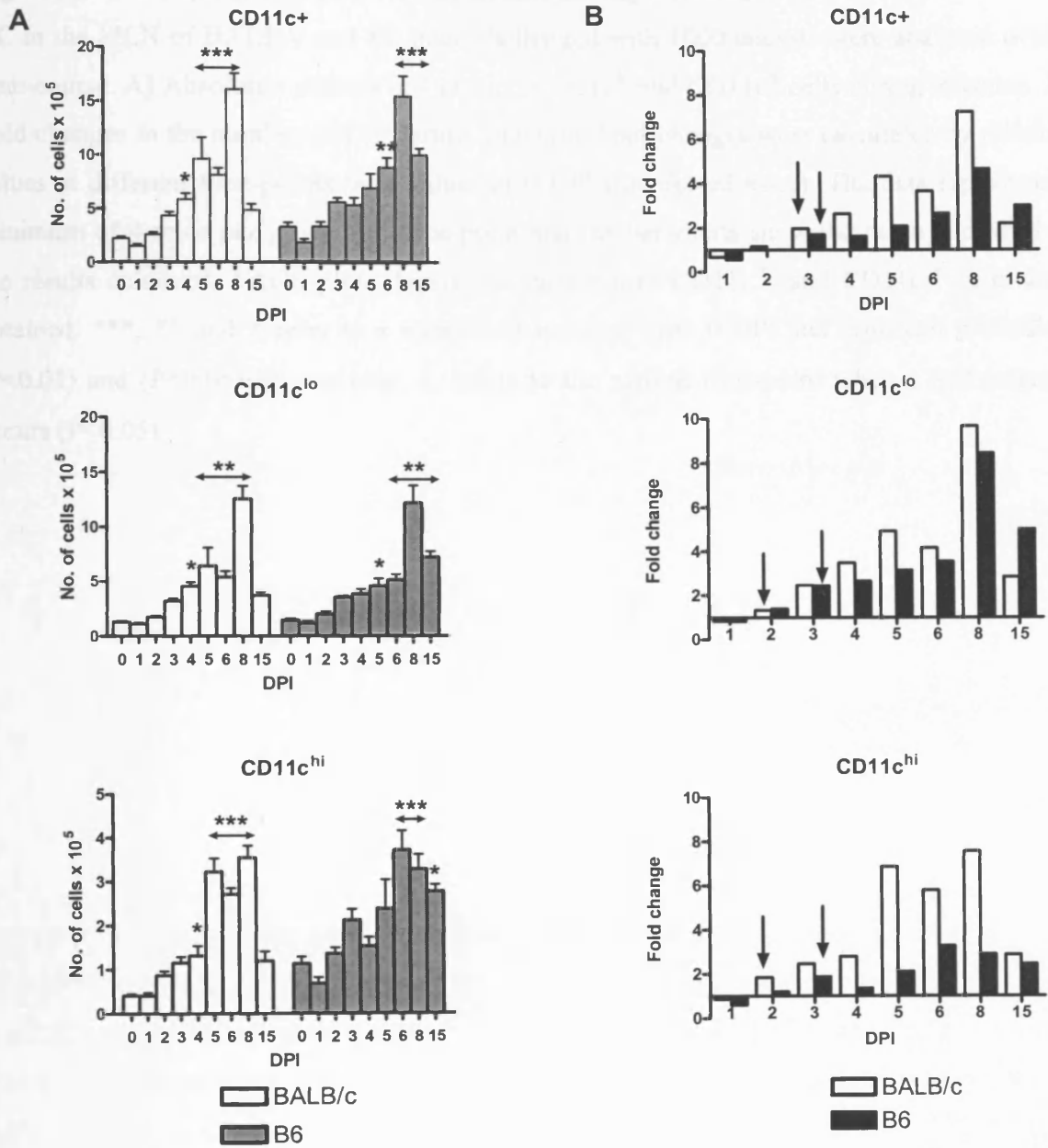
Figure 3.3 DC recruitment in different strains during infection.

DC in the MLN of BALB/c and B6 mice challenged with 1000 oocysts were analyzed over a time-course. A) Absolute numbers of CD11c+, CD11c^{lo} and CD11c^{hi} cells during infection. B) Fold changes in the number of DC during infection. Fold changes were calculated by dividing values at different time-points over values at 0 DPI (uninfected mice). The data represents a minimum of 4 mice per group and time-point and the bar charts show the mean and SEM of the results achieved. FACS plots depict the gates where CD11c^{hi} and CD11c^{lo} cells were obtained. ***, ** and * refer to a significant increase from 0 DPI and represent ($P<0.001$), ($P<0.01$) and ($P<0.05$) respectively. ↓, refers to the earliest time-point when a fold increase occurs ($P<0.05$).
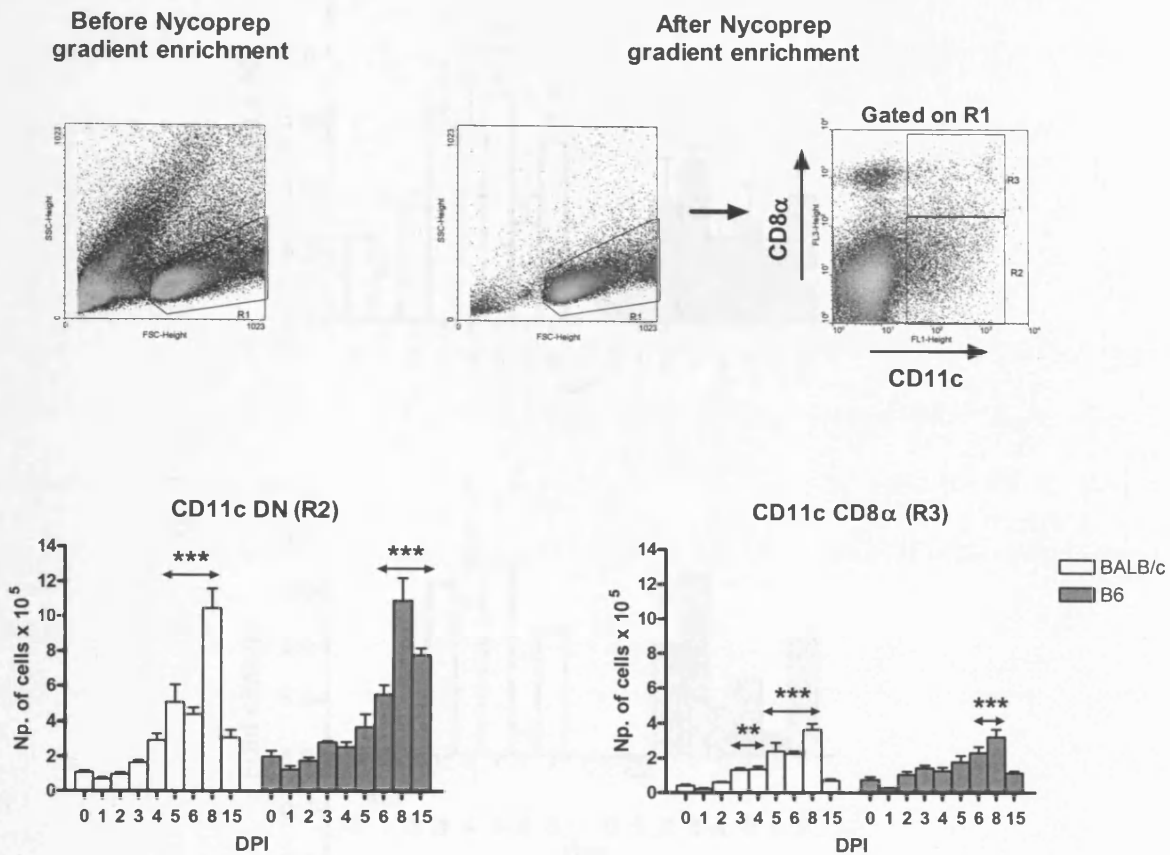
Figure 3.4 DC subsets in different strains during infection.

CD11c+CD4-CD8α- (DN) and CD11c+ CD8α+ subsets were determined after Nycoprep enrichment and calculated as described in chapter two. FACS plots depict gates on which subset proportions were obtained. The data represents a minimum of 4 mice per group per time-point and the bar charts show the mean and SEM of the results achieved. ***, ** and * refer to significant increase from 0 DPI and represent (P<0.001), (P<0.01) and (P<0.05) respectively.
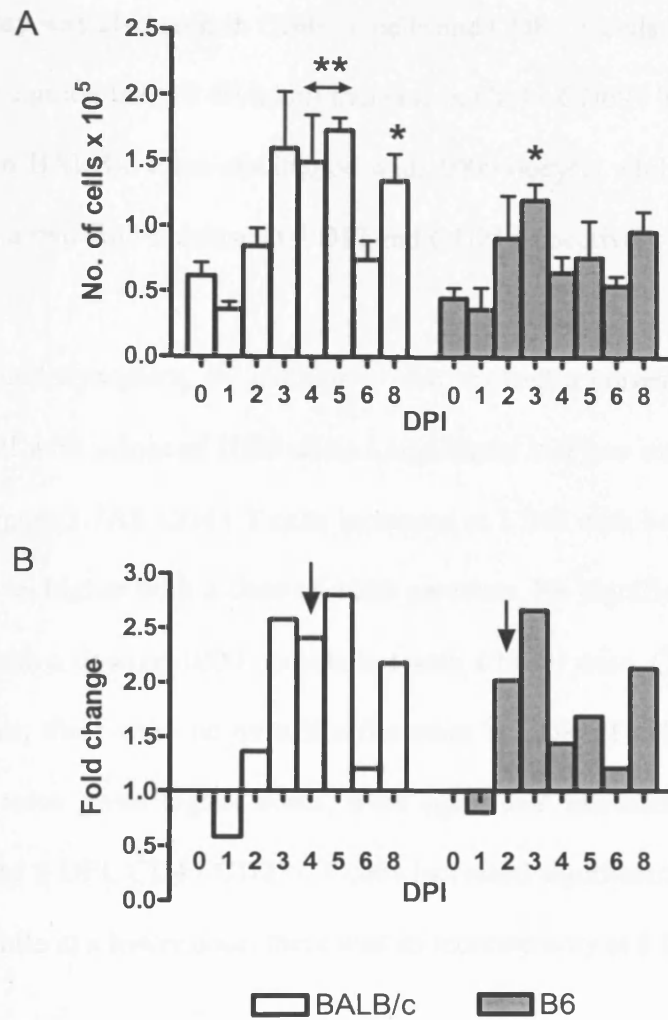
Figure 3.5 DC numbers in the PP of different strains.

A) Absolute numbers of CD11c+ cells and B) fold changes in CD11c+ cell numbers in the PP of BALB/c and B6 mice during infection. Fold changes were calculated by dividing values at different time-points over values at 0 DPI (uninfected mice). The data represents a minimum of 4 mice per group per time-point and the bar charts show the mean and SEM, or fold changes of the results achieved. ** and * refer to significant increase from 0 DPI and represent (P<0.01) and (P<0.05) respectively. ↓, refers to the earliest time-point when a fold increase occurs (P<0.05).

This similar delay was also seen in CD4+ T cells and CD8+ T cells. There was a two-fold (this value is used as a guide for cell division) increase in CD4+ CD69+ T cells and CD4+ CD25+ T cells at 5 DPI in BALB/c mice challenged with 1000 oocysts while mice challenged with 25 oocysts showed a two-fold increase at 7 DPI and 6 DPI respectively (Figure 3.6B).

As with MLN cell dynamics, PP cellularity also showed a correspondingly early pattern of increase at 6 DPI with a dose of 1000 while a significant increase was not seen until 8 DPI with a dose of 25 (Figure 3.7A). CD4+ T cells increased at 7 DPI with both doses but the number of CD4+ T cells was higher with a dose of 1000 parasites. No significant increases were seen in CD8+ T cells with a dose of 1000 oocysts but with a lower dose, CD8+ T cells increased at 7 DPI. Despite this, there were no overall differences in CD8+ T cell kinetics between the two doses. Only in mice given higher doses, were significant increases in CD4+ CD69+ T cells seen at 1 DPI and 8 DPI. CD4+ CD25+ T cells increased significantly at 6 DPI and 7 DPI with a higher dose while at a lower dose, there was an increase only at 8 DPI (Figure 3.8B).

Parasite dose affects DC recruitment to the MLN

DC recruitment was associated with the rate of immune response induction during infection of BALB/c and B6 mice with 1000 oocysts. Since the size of parasite dose also influences the rate of MLN immune responses in BALB/c mice, it was important to test the influence on DC recruitment to the MLN. There was no noticeable difference in the timing of CD11c+ cell (including CD11c^{hi} and CD11c^{lo} cells) increase between both doses (Figure 3.8A). However, at
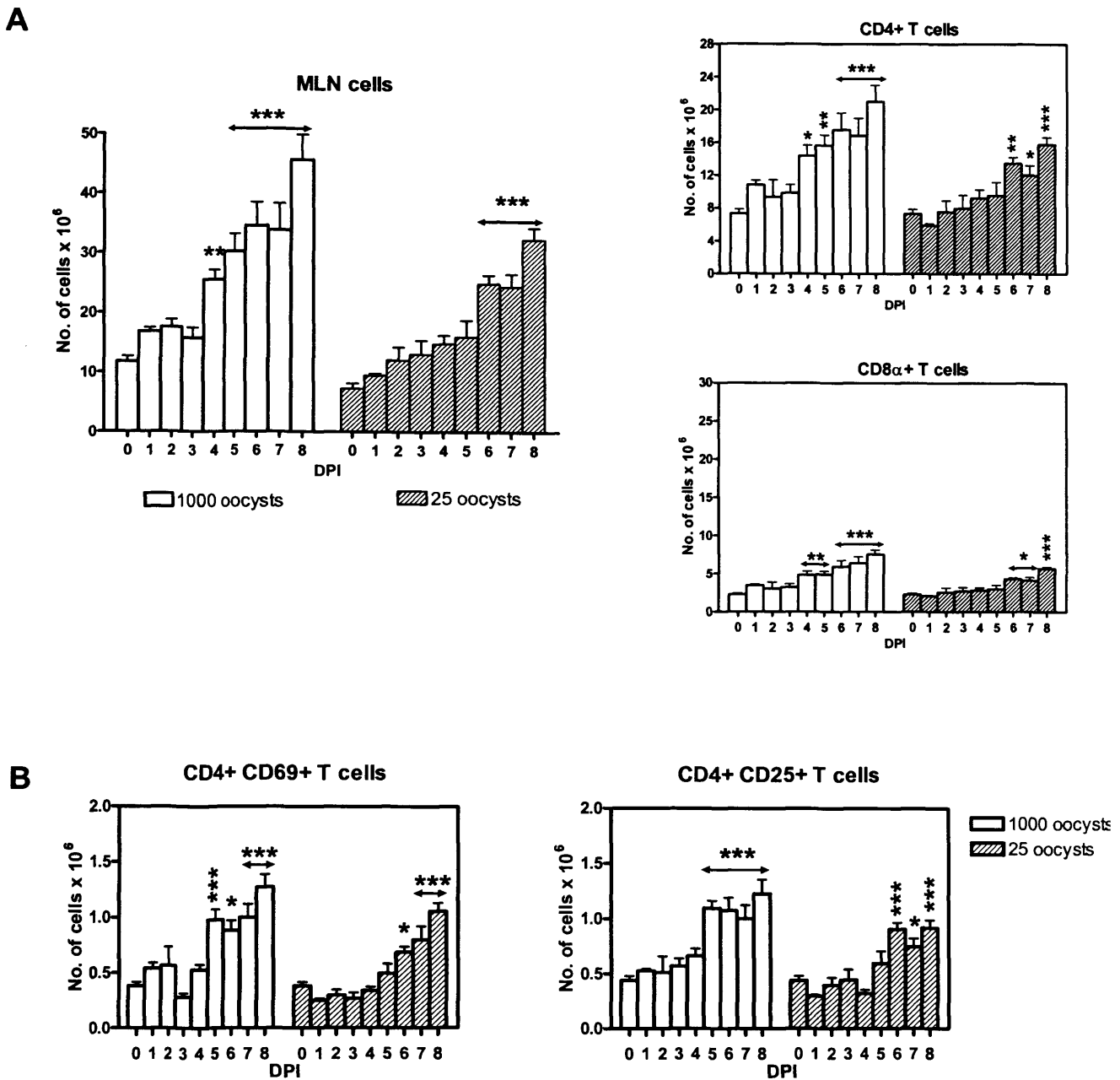
Figure 3.6 Influence of parasite dose on the kinetics of MLN cellularity in BALB/c mice.

A) MLN, CD4 + T cell and CD8+ T cell kinetics. B) CD69+ or CD25+ T cell kinetics. MLN from BALB/c mice challenged with either 1000 or 25 oocysts were analyzed using FACS and the cells analyzed were gated from the lymphocyte region determined in a FSC-SSC FACS plot. The data represents a minimum of 4 mice per group per time-point and the bar charts show the mean and SEM of the results achieved. ***, ** and * refer to a significant increase from 0 DPI and represent (P<0.001), (P<0.01) and (P<0.05) respectively.

A

PP cells

CD4+ T Cells

CD8+ T cells

B

CD4+ CD69+ T cells

CD4+ CD25+ T cells
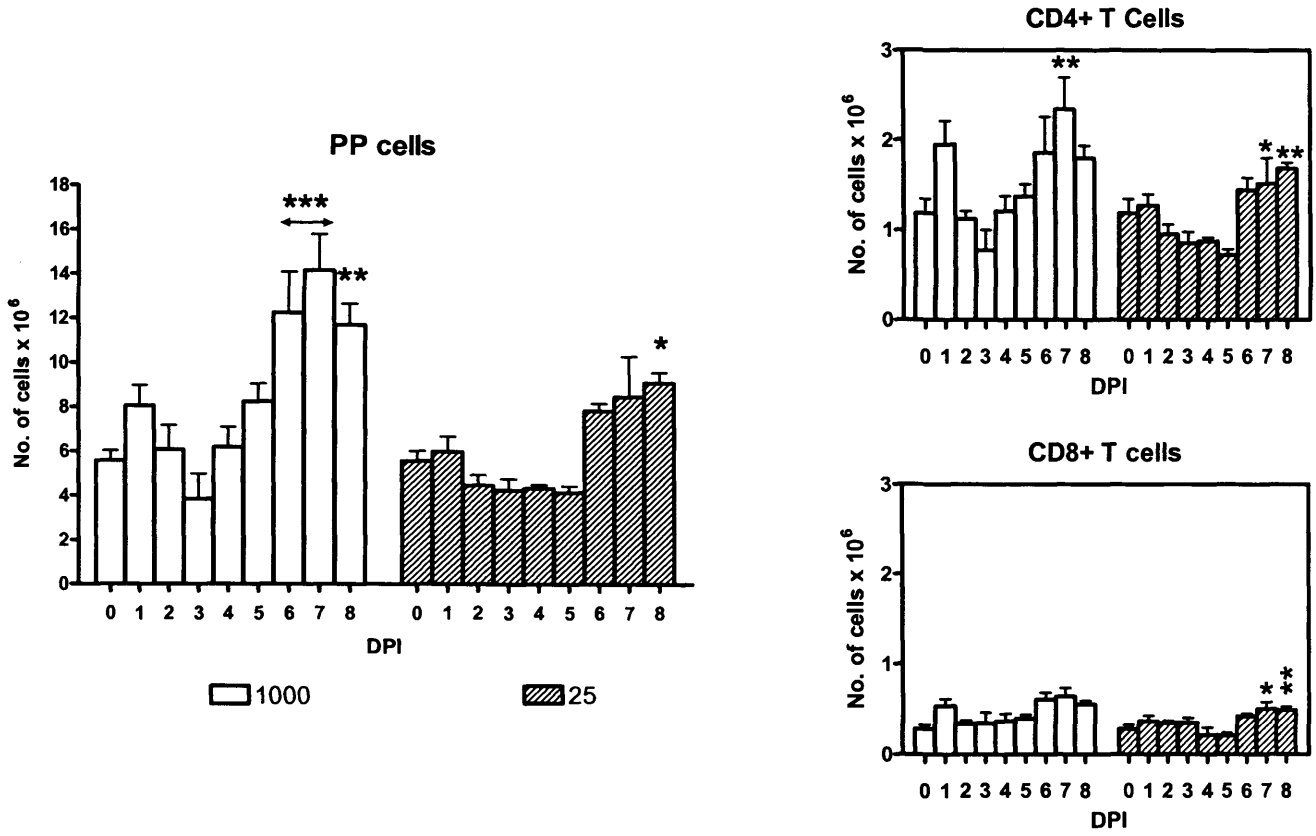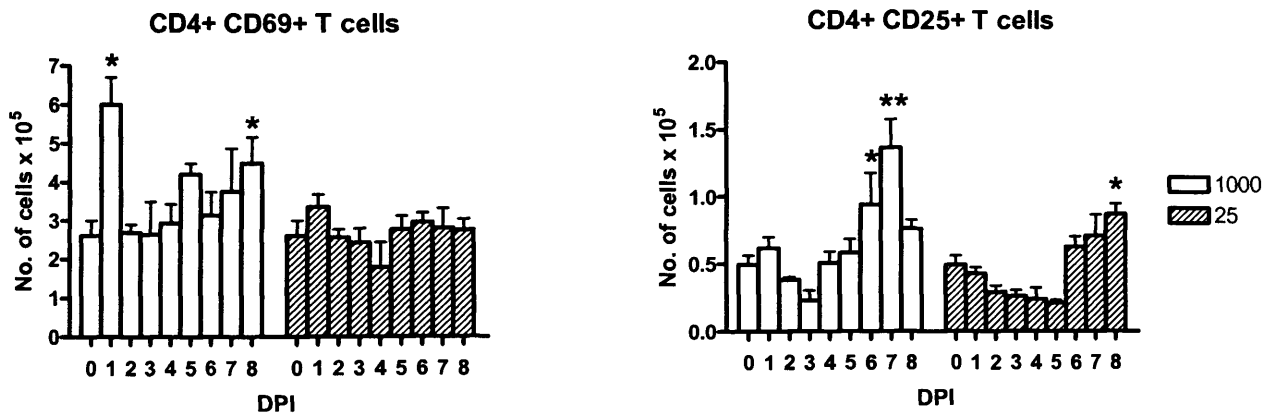
Figure 3.7 Influence of parasite dose on the kinetics of PP cellularity in BALB/c mice.

A) PP, CD4 and CD8 T cell kinetics. B) Activated CD4 T cell kinetics. PP from BALB/c mice challenged with either 1000 or 25 oocysts were analyzed and the cells analyzed were gated from the lymphocyte region determined in a FSC-SSC FACS plot. The data represents a minimum of 4 mice per group per time-point and the bar charts show the mean and SEM of the results achieved. ***, ** and * refer to a significant increase from 0 DPI and represent ($P<0.001$), ($P<0.01$) and ($P<0.05$) respectively.

a higher parasite dose (1000 oocysts), earlier fold increases were seen in CD11c+, CD11c^{hi} and CD11c^{lo} cells at 4 DPI (Figure 3.8B). Fold increases of CD11c+, CD11c^{hi} and CD11c^{lo} cells in mice given 25 oocysts were significant only at 6 DPI. In mice challenged with 1000 oocysts, fold increase of CD11c^{hi} cells were noticeably higher (up to 14 times at 8 DPI) than CD11c^{lo} cells (up to 5 times at 8 DPI).

The increase in DC numbers also reflected increases in DC expressing maturation markers. The majority of DC was CD11c+ CD8α- and those expressing CD40 and/or CD80 increased earlier in mice infected with 1000 oocysts at 4 DPI (Figure 3.9). CD40+ DC in mice challenged with either doses increased significantly at 4 DPI. Interestingly, the increase CD40+ and/or CD80+ DC in mice infected with 25 oocysts did not reach the same levels as DC numbers in mice infected with 1000 oocysts. This was similar to the higher fold increases seen in MLN CD11c^{hi} DC of mice challenged with a higher dose.

In the PP, the overall CD11c+ cell kinetics between mice given 1000 or 25 oocysts were significantly different (P<0.001) (Figure 3.10) and the earliest fold increase in CD11c+ cell numbers were seen at 6 DPI in mice given 1000 oocysts while no fold increase was seen at all in mice given 25 oocysts.
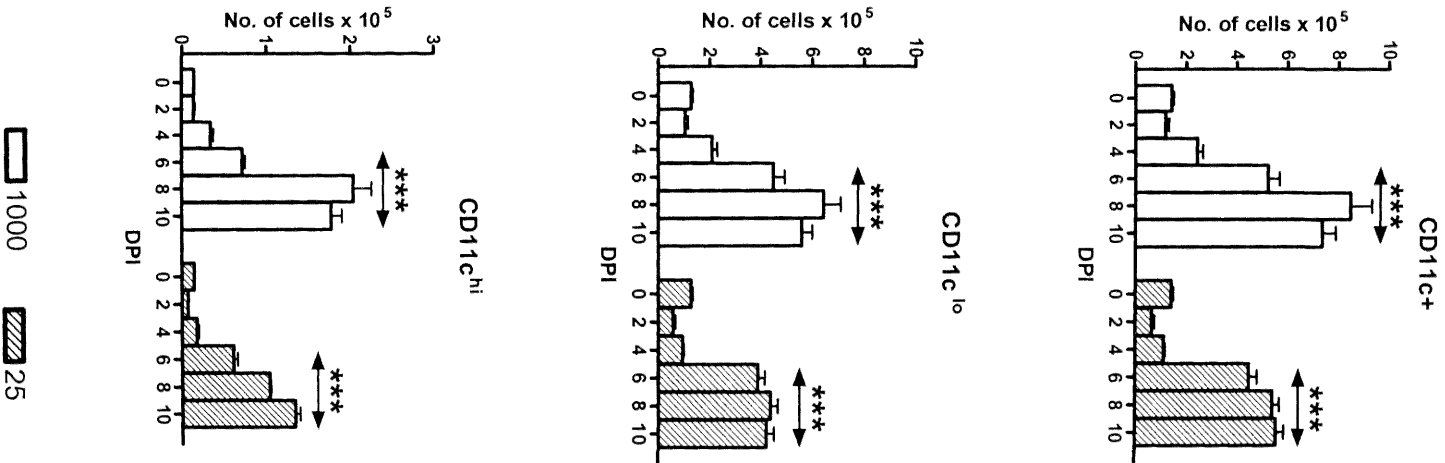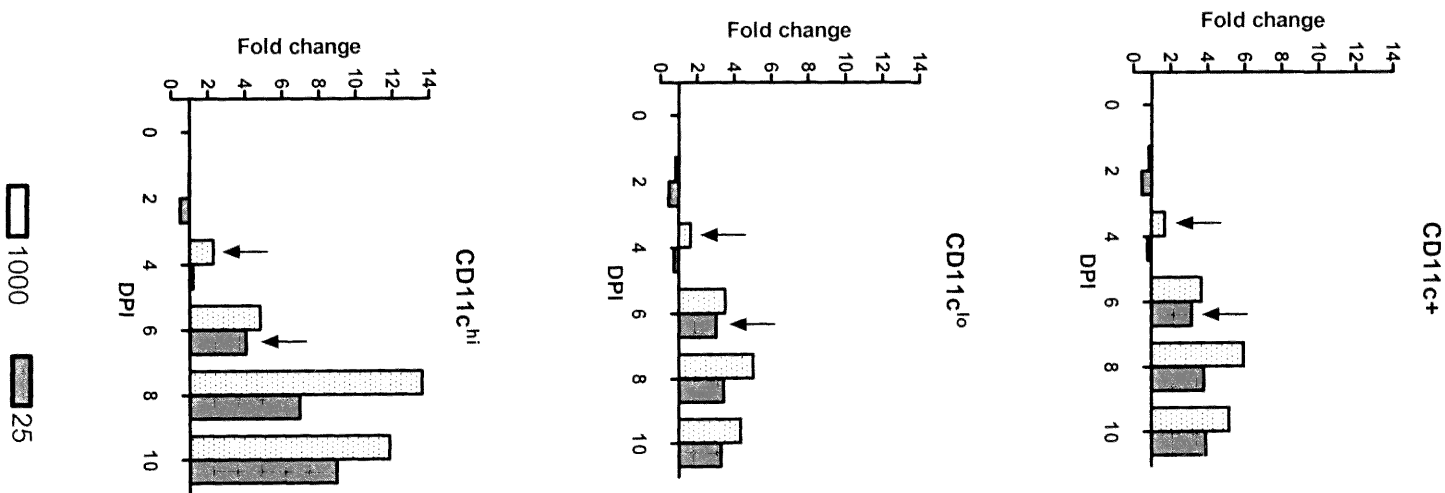
Figure 3.8 Influence of parasite dose in DC recruitment in the MLN of BALB/c mice.

A) Absolute numbers of CD11c+, CD11c^{lo} and CD11c^{hi} cells during infection. B) Fold changes in the number of DC during infection. CD11c+ cell numbers from the MLN of BALB/c challenged with 1000 or 25 oocysts were analyzed over a time-course. Fold changes were calculated by dividing values at post-infection time-points over values at 0 DPI (uninfected mice). The data represents a minimum of 4 mice per group and time-point and the bar charts show the mean and SEM, or fold changes of the results achieved. ↓, refers to the earliest time-point when a significant fold increase occurs (P<0.05).
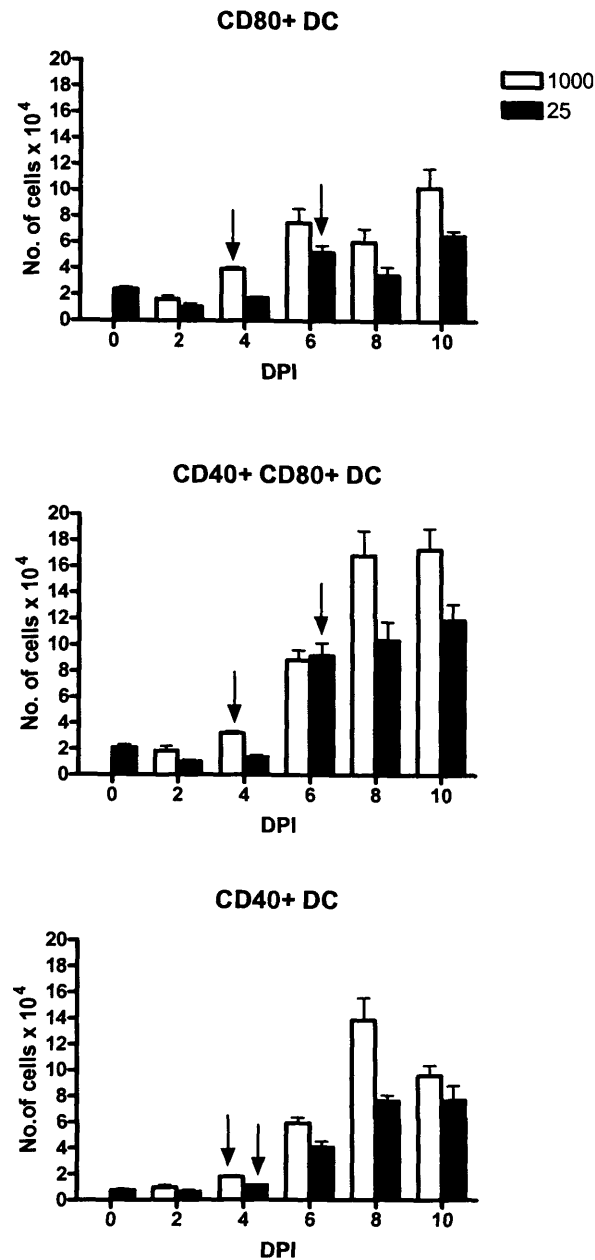
Figure 3.9 Influence of parasite dose on mature DC during infection.

Absolute numbers of CD80+CD40-CD11c+CD8α- cells, CD40+CD80+CD11c+ CD8α- cells and CD40+CD80-CD11c+ CD8α- cells from the MLN of BALB/c mice challenged with 1000 or 25 oocysts were examined during infection. The data represents a minimum of 4 mice per group and time-point and the bar charts show the mean and SEM of the results achieved. ↓, refers to the earliest time-point when a significant fold increase occurs (P<0.05).
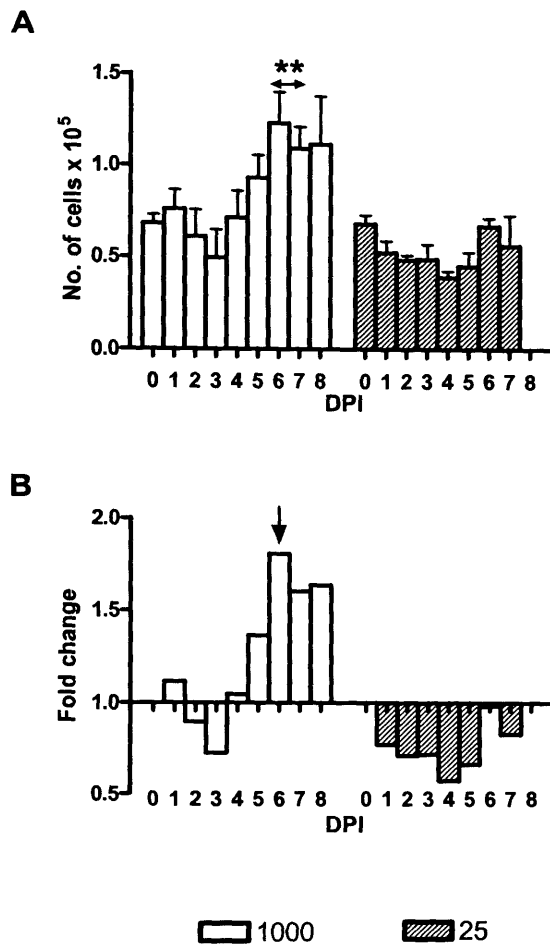
Figure 3.10 Influence of parasite dose on DC numbers in the PP.

A) Absolute numbers of CD11c+ cells and B) fold changes in CD11c+ cell numbers in the PP

of BALB/c mice infected with 1000 or 25 oocysts. Fold changes are calculated by dividing

values at post-infection time-points over values at 0 DPI (uninfected mice). The data represents

a minimum of 4 mice per group per time-point and the bar charts show the mean and SEM, or

fold changes of the results achieved. **, significant increase from 0 DPI (P<0.02). ↓, refers to

the earliest time-point when a significant fold increase occurs (P<0.05).

Responses in the MLN and PP to parasite dose reflect responses in the small intestine

Although the immune responses in the MLN and PP were examined, it was also important to examine the response occurring at the site of infection. IFNγ is required for control of primary infection as demonstrated by the high level of susceptibility in IFNγ-/- mice (Smith and Hayday, 2000) and the effects of anti-IFNγ treatment (Rose et al., 1991). Therefore, IFNγ mRNA was measured in the ileum of BALB/c mice given 1000 or 25 parasites. As with MLN and PP responses, there was a delayed upregulation of IFNγ mRNA in mice given 25 oocysts. Mice challenged with 1000 oocysts displayed upregulation at 4 DPI unlike mice challenged with 25 oocysts which showed upregulation of IFNγ at 8 DPI (Figure 3.11). The level of increase in IFNγ mRNA at 8 DPI was similar in BALB/c mice given 1000 or 25 oocysts (approximately 2-fold) although the fold change levels peaked nearly 9 times with the larger dose at 4 DPI.

25 oocysts
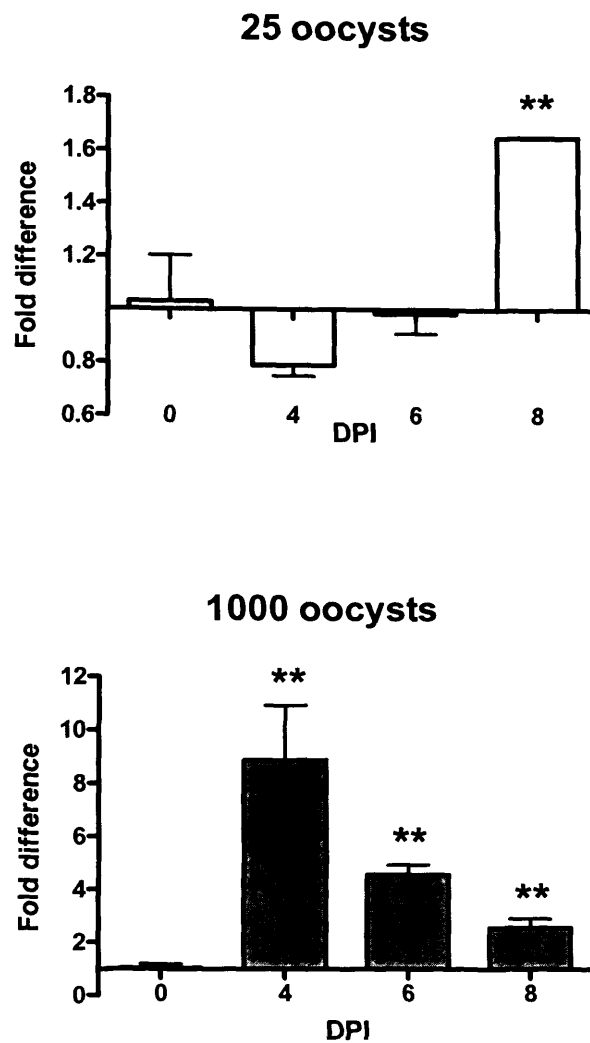
1000 oocysts

Figure 3.11 Influence of parasite dose on IFNγ expression in the small intestine during infection.

mRNA was isolated from the ileum of BALB/c mice challenged with 1000 or 25 oocysts and measured for IFNγ. Relative mRNA levels were measured as mentioned in materials and methods and normalized to CD3γ levels and plotted as fold differences in the bar charts. The data represents a minimum of 4 mice per group per time-point and the bar charts show the mean and SEM of the results achieved. **, significant increase from 0 DPI (P<0.02).

3.4. Discussion

E.vermiformis is an apicomplexan parasite which resides in the small intestine of mice and completes its life cycle in approximately eight days. Based on the knowledge that the parasite undergoes a localised replication cycle within the host small intestine, the oocyst output per mouse is well-suited for the measure of mucosal immune responses against an enteric infection. With this model of infection, the objectives for experimentation in this chapter are to examine the dynamics of immune responses associated with the gut and GALT (MLN, PP) in relation to host susceptibility influenced by host genetics and parasite doses. A summary of the earliest time-points where a significant increase occurred can be found in Tables 3.1, 3.2, 3.3 and 3.4. Overall, the data presented shows earlier DC and T cell responses in genetically resistant BALB/c mice compared with B6 mice challenged with 1000 oocysts and BALB/c mice challenged with a lower dose (25 oocysts).

Resistant BALB/c mice induced a more rapid immune response against infection with 1000 oocysts than B6 mice. In both strains, CD4+ T numbers dominated the increase in MLN cellularity compared with CD8+ T cells. The difference in susceptibility was a matter of timing rather than a difference in Th1 or Th2 responses. This was evident with the earlier increase in CD4+ T cell numbers in the MLN and PP and the observation that 'activated' populations of CD4+ T cells expressing either CD25 or CD69 also increased earlier in BALB/c mice and work demonstrating that IFNγ was upregulated in both BALB/c and B6 mice, although later in the latter (Rose et al., 1985). The MLN cellular dynamics was more clearly-defined than in the PP which contained fewer cells and displayed more subtle increases in CD4+CD25+ and CD4+ CD69+ T cells which were detectable at 5 DPI in

BALB/c mice. B6 mice have fewer PP cells than BALB/c mice and despite displaying an increase in the number of CD4+ CD69+ T cells at 3 DPI onwards, this was much lower than that seen with BALB/c mice.

The increase in MLN cellularity included non-T cells such as B cells, macrophages and NK cells. These cells are not essential during primary infection with *E. vermiformis*. This is because mice deficient in NK cytoxicity (*beige/beige* mice or *in vivo* depletion of NK cell activity) or in B cells (μMT-/- mice) were not compromised in their ability to control infection ((Rose et al., 1995; Smith et al., 1994) and Marguerite IA manuscript submitted). Stimulation or depletion of macrophages also showed no effect on infections with *E. maxima* (Ovington et al., 1995).

Primary T cell responses are initiated by DC which migrate from tissues to lymphoid structures such as the MLN. Earlier increases in CD11c+ cells were detected with infected BALB/c mice compared with B6 mice, supporting the hypothesis that DC recruitment to the MLN played a role in inducing a more rapid response. Greater fold differences were seen in CD11c [hi] subset compared to CD11c [lo] subset which may suggest that CD11c [hi] DC are important during the early induction of immune response. Interestingly, naive B6 mice have a higher number of CD11c+ cells in the MLN than BALB/c mice while in the PP, BALB/c mice have slightly higher numbers of CD11c+ cells. Hence, higher 'resting' DC numbers in the MLN do not necessarily indicate a greater capacity to induce earlier immune responses in the case of B6 mice. More likely, it is the response of DC carrying antigens (most likely from the gut and PP) to the appropriate site of presentation that determines speed of response.

Table 3.1 **Influence of host genetics on T cells in mice challenged with 1000 oocysts.**

	Day showing the first significant increase	
	BALB/c	B6
MLN		
MLN cells	5	6
CD4+ T cells	5	8
CD4+ CD69+ T cells	4	4
CD4+ CD25+ T cells	6	8
CD8+ T cells	none	none
CD8+ CD69+ T cells	4	5
PP		
PP cells	5	8
CD4+ T cells	5	none
CD4+ CD69+ T cells	5	3
CD4+ CD25+ T cells	5	8
CD8+ T cells	5	8

Table 3.2 **Influence of host genetics on DC in mice challenged with 1000 oocysts.**

	Day showing the first significant increase	
	BALB/c	B6
MLN		
Fold increase in CD11c+ cells	3	3
Fold increase in CD11c^{lo} cells	2	3
Fold increase in CD11c^{hi} cells	2	3
CD11c+ CD8-CD4- DC	5	6
CD11c+ CD8+	3	6
PP		
Fold increase in CD11c+ cells	4	2

Table 3.3
Influence of parasite dose on T cells in BALB/c mice challenged with 1000 or 25 oocysts.

	Day showing the first significant increase	
	1000 oocysts	25 oocysts
MLN		
MLN cells	4	6
CD4+ T cells	4	6
CD4+ CD69+ T cells	5	6
CD4+ CD25+ T cells	5	6
CD8+ T cells	4	6
PP		
PP cells	6	8
CD4+ T cells	7	7
CD4+ CD69+ T cells	1	none
CD4+ CD25+ T cells	6	8
CD8+ T cells	none	7

Table 3.4
Influence of parasite dose on DC in BALB/c mice challenged with 1000 or 25 oocysts.

	Day showing the first significant increase	
	1000 oocysts	25 oocysts
MLN		
Fold increase in CD11c+ cells	4	6
Fold increase in CD11c^{lo} cells	4	6
Fold increase in CD11c^{hi} cells	4	6
CD80+ DC	4	6
CD80+ CD40+ DC	4	6
CD40+ DC	4	4
PP		
Fold increase in CD11c+ cells	6	none

This also highlights the ability of a single DC to prime up to 20 naïve T cells (Langenkamp et al., 2002) and therefore effective stimulation can be easily achieved even with tiny increments in DC numbers as seen in infected BALB/c mice. Moreover, the role of responding DC from the gut is further enforced by the finding that BALB/c mice intraperitoneally injected with poly I.C displayed enhanced susceptibility to *E. vermiformis* infection (Rose et al., 1995) (in this paper, the authors' aim was to stimulate NK cells and they did not examine DC responses). The injection of a TLR agonist such as poly I.C was likely to have caused the emigration of intestinal DC away from the small intestine prior to infection, an effect similar to the treatment of TLR4, 7 and 8 agonists in rats (Turnbull et al., 2005; Yrlid et al., 2006). Hence the lack of DC in the small intestine during infection may have explained the higher oocyst output in the poly I.C treated BALB/c mice.

The kinetics of DC in the MLN were more defined than DC in the PP, the former showing clearly an increase in DC numbers during infection. This may be attributable to the greater changing dynamics of DC movement within, in and out of the PP. Although there were some increases in CD11c+ cell numbers, there could have been possible decreases in CD11c+ cells which were masked by incoming DC into the PP. However, there were increases in 'activated' CD4+CD25+ T cells around the time-points that fit the development of adaptive responses, indicative of a local immune response. The hypothesis that the different level of resistance between strains is associated with differential kinetics of responding DC is further supported by work done with bone marrow chimeric mice. Resistance to infection with *E. vermiformis* is dependent on bone marrow-derived cells and not on stromal cells (Joysey et al., 1988). The study involved, utilized bone marrow chimeric mice (BALB/c or B6) reconstituted with bone marrow from MHC-compatible BALB/b (H-2^b) and C57BL/10 (H-2^d) mice where regardless

of the H-2 haplotype, the ability to confer resistance depended upon donor bone marrow cells and not the recipient's genotype.

There is the question of why DC are quicker to respond in BALB/c mice and there may be some factors involved: Different distribution of DC subsets at the site of infection may activate different DC subsets which may differ in their capacity to respond; Different DC subsets may express different levels of PRR, chemokine receptors or adhesion molecules, which may influence their activated status and their rates of migration to the draining lymph node. There were earlier increases in the overall DC population in the MLN of BALB/c mice but there was also a timing difference in the arrival of DC subsets (CD11c+ DN at 5 DPI compared with CD11c+ CD8+ DC at 3 DPI) (Figure 3.5). Moreover, anatomical factors (influenced by host genetics) such as a larger PP structure (in BALB/c mice) with perhaps greater resources available (such as more microfold cells, DC, T cells) for Ag-sampling and immune induction, enable the intestinal immune system to respond more quickly to infection compared with B6 mice which have relatively smaller PP in general.

Different doses used in various infections have been shown to influence the immune response (Compton and Farrell, 2002; Menon and Bretscher, 1998; Taylor-Robinson and Phillips, 1998) usually by influencing the balance between Th1 and Th2 responses. In models of infection such as *L. major* and *P. chabaudi*, changes in parasite dose were shown to influence the Th1 or Th2 direction of the immune response (Menon and Bretscher, 1998; Taylor-Robinson and Phillips, 1998). With *L. major* infections, a susceptible strain is capable of controlling infection when inoculated with low doses of parasites by promoting a Th1 response (Bretscher et al.,

1992) while increasing infectious doses of *P. chabaudi* helps augment Th1 responses required against infection.

With *E.vermiformis* infections, parasite dose influenced BALB/c mice more than B6 mice but in a manner that was related to the timing of Th1 responses rather than a shift between Th1 and Th2 responses. The increased susceptibility of BALB/c mice to 25 oocysts was associated with a slower T cell response in the MLN, PP and small intestine. Analysis of DC subsets in the MLN demonstrated delayed recruitment of DC with a lower dose where a delay of around two days was sufficient to render BALB/c more susceptible to infection. The increase in all CD11c+ DC (CD11c^{lo} and CD11c^{hi} DC) from mice given 25 parasites, was not as high as those given 1000 oocysts and perhaps are a reflection of the amount of parasite material available for DC activation (eg. PRR signals). This was the same with mature DC expressing CD40 and CD80. From the data presented, it is only shown that the timing of DC recruitment is influenced by dose while there is no indication that specific DC subsets are differentially-influenced by dose. The data emphasises the importance of DC response towards a rapidly growing infection where delayed responses are too late to control the infection effectively. Relative fold changes in CD11c^{hi} cells were greater than CD11c^{lo} cells despite the lower CD11c^{hi} cell number, suggesting a greater influence of parasite dose on the dynamics of CD11c^{hi} cells recruited into the MLN.

The magnitude of gut immune responses correlates with the amount of parasite material where ileal expression of IFNγ increased 9-fold at 4 DPI in mice challenged with 1000 oocysts while none was seen at the same time-point in mice given 25 oocysts. Furthermore, in mice given 25 oocysts, IFNγ mRNA only increased 1.6 fold at 8 DPI (Figure 3.11). This suggests that

BALB/c mice are less sensitive with lower doses and their level of immune sensitivity is restricted by the numbers of infecting parasites. This immune sensitivity relates to the number of 'injections' or immunizations given to the host by means of parasite penetration into each host cell. A smaller number or area of 'injections' by fewer parasites, may suggest slower or lack of DC detection, leading to less robust responses. However, given the right dose of oocysts or 'immunizations', resistant BALB/c mice can deliver a rapid immune response in the gut as seen by the rapid increase in IFNγ expressed. Furthermore, if structures such as larger PP in BALB/c mice contribute to greater resistance compared to B6 mice, then the amount of parasite material available in the lumen will be a limiting factor for antigen sampling. Therefore, the lack of luminal parasite antigen may have resulted in the delayed responses seen in BALB/c mice given smaller doses. The ability to control infection is dependent on the timing of immune responses and because B6 mice fail to induce rapid responses, fewer parasite injections may not influence their already slow immune response during infection. The hypothesis that PP play an important role during *E. vermiformis* infection is later tested using mice with lymphoid structural deficiencies (chapter four).

Overall, the data presented, demonstrates that DC recruitment (to the MLN) which is influenced by host genetics, the amount of parasite material available and number of parasite injections, plays an important role in determining host resistance by the induction of a rapid immune response.

Chapter Four

The influence of lymphoid structures on the kinetics of Th1 immune response

4.1. Introduction

The GI tract is a complex, compartmentalised organ equipped to protect against infections by pathogens invading the host via the oral route. Within the GI tract exists an organisation consisting of IE and LP compartments, and the GALT which include PP and MLN. These organised compartments contain immune cell populations which contribute to the active immune response against infection and the maintenance of oral tolerance towards gut commensal microbes and food antigens. The MLN form a chain-like structure of lymph nodes draining the GI tract via lymphatics and are the largest lymph nodes in the body. The PP are macroscopic lymphoid aggregates distributed along the small intestine.

The organogenesis of PP and MLN depends on LT-signalling but the requirements for LT are different. As a cytokine, LT exists functionally as a soluble homotrimer ($LT\alpha_3$) and a membrane-bound heterotrimer ($LT\alpha_1\beta_2$) which are expressed by most haematopoietic cells (Androlewicz et al., 1992; Browning et al., 1997). MLN organogenesis is independent of TNF, TNFR2 and $LT\alpha_1\beta_2$ but is dependent on LTβR-signalling involving $LT\alpha_3$ and LIGHT (Cuff et al., 1999; Debard et al., 2001; Scheu et al., 2002). The PP are formed at E15.5 and depend on the expression of $LT\alpha_1\beta_2$ by CD4+ CD3- progenitor cells and IL7Rα-signalling (Finke and Kraehenbuhl, 2001; Mowat, 2003; Yoshida et al., 1999). For these reasons, deficient LT signalling is associated with structural defects in various knockout mice: LTα-/- mice have disorganised splenic architecture, no lymph nodes and PP (De Togni et al., 1994). LTβ-/- mice have a similar phenotype but possess residual cervical LN and MLN structures (Koni et al.,

1997). The F1 progeny (LTα+/-β +/- double heterozygote) of LTα-/- and LTβ-/- parents are

intact in all secondary lymphoid organs except PP which suggests a gene-dose influence on the

formation of PP (Koni and Flavell, 1998 and Figure 4.2). Unlike LTα+/-β+/- mice, LTα+/- and

LTβ+/- single heterozygotes retain PP structures ((Koni and Flavell, 1998) and Figure 4.2).

Both PP and MLN are considered important sites for the induction of immune responses

against invading pathogens in the gut. These lymphoid structures share characteristics such as

B cell follicles and T cell zones (Witmer and Steinman, 1984), similar to peripheral lymph

nodes although PP lack the afferent lymphatics associated with MLN. Importantly, the

structural organisation in PP and MLN enables efficient cellular interaction and co-ordinates

APC interaction with naïve antigen-specific T cells.

Studies on infection models (eg. *L. monocytogenes, T. gondii, Influenza virus, M. tuberculosis*)

using LT-deficient mice describe requirements for cytokine expression or structural

requirements but many of these infections result in systemic pathogenesis (Ehlers et al., 2003;

Lund et al., 2002; Roach et al., 2005; Schluter et al., 2003) To date, few studies have

addressed the absolute requirements for PP and MLN and their independent contributions

towards the development of primary immune responses and protective immunity against gut

infections. *E.vermiformis* infection is confined to the vicinity of the small intestine which

makes it ideal for studying direct gut immune responses. Where the immune components

essential against the *E.vermiformis* have been described (Rose et al., 1984; Rose et al., 1985;

Smith and Hayday, 2000 and chapter three), this chapter is an extended investigation as to

how the timing of immune responses which are critical in providing effective control of

infection, is limited by requirements for GALT structures. Many studies describe immune

responses occurring in the PP during infection (Bumann, 2001; Fan et al., 1998; McSorley et

al., 2002) and seem to suggest an accessory (non-essential) role for PP. In this chapter, it is demonstrated here that PP influence the efficiency of Th1 induction in the MLN and that coordinated PP- and MLN-mediated immune responses are required to provide rapid and effective immunity against gut infection.

4.2. Experimental approach

Various LT-deficient mice (LTα-/-, LTβ-/-, LTα+/-β+/-, LTα+/-, LTβ+/-) and B6 mice of 8-14 weeks were infected with sporulated *E.vermiformis* oocysts and some were rechallenged after 30 days post-infection. All mice were challenged with 100 oocysts except where indicated otherwise. The experimental approach was to use T cell adoptive transfers and bone marrow (BM) chimeric mice to determine the requirements for LT-expression by non-stromal cells and/or for lymphoid structures during infection by measuring oocyst output per mouse. To assess the kinetics of the immune response during infection, immune cells from various infected LT-deficient mice were isolated and examined for cytokine production, expression of activation markers, gut-homing adhesion molecules and chemokine receptors.

4.3. Results

4.3.1. Specific LT-deficiencies exacerbate infection with *E.vermiformis*

In order to examine the requirement for LT expression and/or secondary lymphoid structures in the immune control of the enteric parasite, *E.vermiformis*, we measured infection in unmanipulated LTα-/-, LTβ-/-, LTα+/-β+/-and B6 mice. Intact B6 mice were relatively resistant to infection when compared with LTα-/- and LTβ-/- mice, both of which were highly susceptible, producing ~35 million oocysts (Figure 4.1A). LTα+/-β+/- mice displayed an intermediate level of susceptibility and produced ~20 million oocysts per mouse. In a separate

experiment, LTα+/- and LTβ+/- mice challenged with 100 oocysts were more resistant to infection, producing fewer oocysts than LTα+/-β+/- mice. LTα+/- and LTβ+/-mice seem to be marginally more susceptible than B6 mice although the levels of oocysts produced were much lower than LTα+/-β+/- mice (Figure 4.1B). These single LT heterozygote mice were intact for PP unlike LTα+/-β+/- (Figure 4.2 and (Koni and Flavell, 1998)) which suggests an association between PP and the control of infection. There was no difference in the patent period (duration for which parasites were produced) between LTα+/-, LTβ+/-, LTα+/-β+/- and B6, which was around 8 days while in both LTα-/- and LTβ-/- mice, the patent period was longer, for around 10 days (Table 4.1).

The level of susceptibility in LTα-/- and LTβ-/- mice was comparable to that seen in T-cell deficient TCRβxδ-/- mice (Smith and Hayday, 2000). One month after primary infection, complete immunity (evident by the absence of oocysts) was observed in all LT-deficient mice rechallenged with *E. vermiformis* and this also indicated that primary immune responses had occurred in all mice (Figure 4.1C).

A

B

C
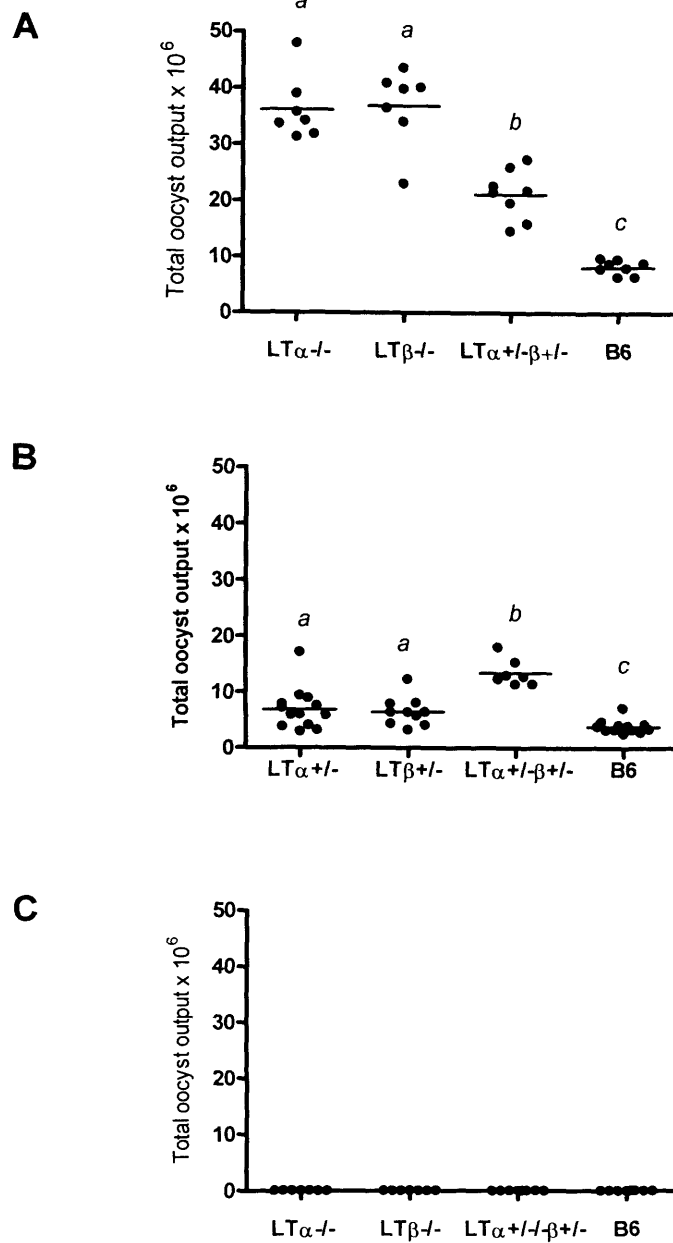
Figure 4.1 Specific LT-deficiencies exacerbate infection with *E.vermiformis*.

(A) (B) Total oocyst output in mice during primary infection. (C) Total oocyst output per mouse during secondary infection. Results represent a minimum of 7 mice per group in 2-3 experiments. *a,b,c*, Groups annotated with the same letter are not significantly different while those labeled with different letters are significantly different as follows A) and C) ($P<0.01$), B) ($P<0.05$).
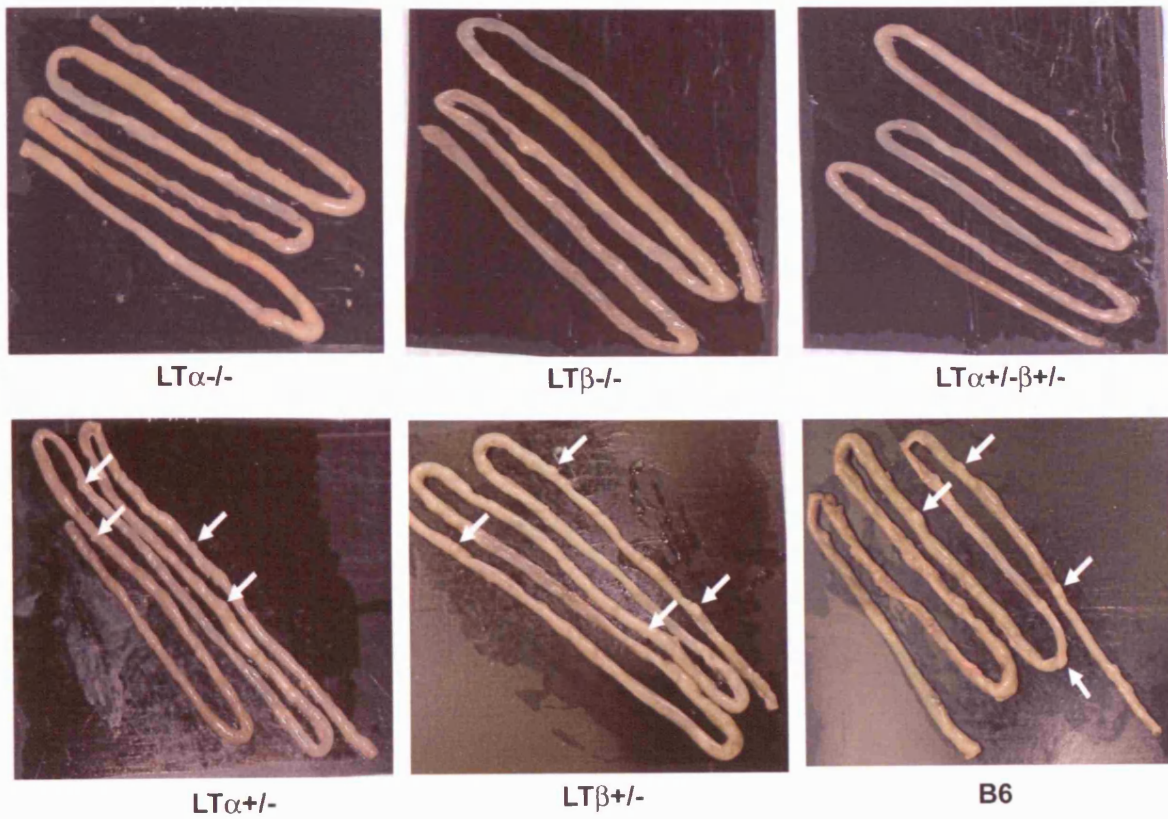
Figure 4.2 Small intestines from different LT-deficient mice.

Arrows point to some of the PP found along the small intestine. Pictures without arrows indicate absence of all PP.

Table 4.1. Length of patent period in mice challenged with 100 oocysts

Mouse Strain [a]	Patent period, days (Mean ± SEM)	Significant difference [b] (P<0.05)
LTα-/-	9.7 ± 0.3	*a*
LTβ-/-	10.4 ± 0.5	*a*
LTα+/-β+/-	8.3 ± 0.2	*b*
LTα+/-	8.0 ± 0.2	*b*
LTβ+/-	8.1 ± 0.3	*b*
B6	8.3 ± 0.2	*b*

[a] 7 or more mice were examined. [b] Groups annotated with the same letter are not significantly different while those annotated with different letters are significantly different (P<0.05).

4.3.2. The MLN and PP are both required for the control of infection

The susceptible phenotype of specific LT-deficient mice may be attributed to the defects in T cell production of LT or the developmental effects on secondary lymphoid structures. To examine these possibilities, T cells from LTα-/- or B6 donors were adoptively transferred into TCRβxδ-/- recipients which were subsequently infected. Since the expression of LTα_3 and LT$\alpha_1\beta_2$ required the *LTα* gene, only LTα-/- T cells were examined. There was no difference in oocyst production between TCRβxδ-/- recipients given T cells from either LTα-/- or B6 donors (Figure 4.3). TCRβxδ-/- mice given no cells remained highly susceptible to infection underlining the T cell-dependent nature of the immune response. Protection against secondary infection was seen in all mice (evident by the absence of oocysts) except unmanipulated TCRβxδ-/- recipients, when rechallenged with *E. vermiformis*, indicating that LT is not required for the development of memory T cells.

Since LT expression by T cells was not required to control infection, susceptibility in LT-deficient mice may instead be influenced by secondary lymphoid structures and/or LT expression by non-T cells. LT is required for the organogenesis and organisation of lymphoid structures and although reconstitution of LT-deficient hosts with LT-intact cells does not rescue missing lymphoid structures, it does result in reorganisation of splenic tissues (Fu and Chaplin, 1999; Wilhelm et al., 2002). Bone marrow chimeric mice were generated using LT-deficient and B6 mice as donors and recipients. This approach produced mice with or without LT-expression in the absence or presence of lymphoid structures. Reconstitution was determined using congenic CD45.1 mice which could be differentiated through FACS analysis. Two sets of BM chimeric mice were made using BM from congenic CD45.1 mice

and normal B6 mice to ensure BM reconstitution was not influenced by a difference in the CD45 allele. All chimeras were kept for four months prior to infection and to ensure more than 90% reconstitution with donor cells. In all cases, susceptibility to infection was associated with the genotype of the recipient. Recipients retaining lymphoid structural integrity (ie. B6 recipients), remained resistant to infection regardless of the origin of donor BM cells. Chimerism of LTα-/- and LTβ-/- recipients with B6 bone marrow did not rescue their phenotype and these mice remained highly susceptible, producing higher numbers of oocysts compared with B6 recipients (Figure 4.4A and B). Both LTα+/-β+/- recipients given either B6 or LTα+/-β+/- BM cells remained equally susceptible and produced more oocysts than B6 recipients (Figure 4.4C). Interestingly, B6 recipients reconstituted with B6 BM were marginally more resistant than B6 recipients reconstituted with LTα+/-β+/- BM.

Although MLN are present in LTβ-/- mice, these were physically smaller and shorter in chain length compared to age-matched intact B6 (Figure 4.5). In some cases, viable lymphocyte retrieval from the MLN was low and the CD4:CD8 lymphocyte ratio was lower compared to B6 mice (Figure 4.6). However this may be due to low MLN cell numbers as the CD4:CD8 ratio in the LTβ-/- spleen is comparable to the B6 spleen (data not shown and (Alimzhanov et al., 1997)). However, although BM chimeras were kept for four months, MLN reconstitution only occurred partially as the MLN structure in LTβ-/- recipients given B6 cells contained fewer cells than B6 recipient chimeras (Figure 4.7A) despite the presence of a MLN structure (lymph node and vessel present where much of it is encased by fatty tissue) (Figure 4.7B). This suggests that LTβ-/- BM chimeric recipients remained highly susceptible to infection due to the unsuccessful reconstitution of the MLN. Despite reorganisation in the spleen of LTα-/- and LTβ-/- mice given B6 BM (Fu et al., 1998;

Wilhelm et al., 2002 and Figure 4.8), these recipients remained susceptible, indicating that splenic reorganisation was inadequate to effectively induce immunity against infection. Unmanipulated and BM chimeric LTα+/-β+/- mice both retain splenic organisation (Figure 4.9) and MLN (Figure 4.5), and responded better than LTα-/- and LTβ-/- mice but remained more susceptible than B6 mice. This suggests a role for PP which are absent in these mice. An issue with regards to a role for ILF was raised as they are absent in LTα-/- and LTβR-/- mice (Lorenz et al., 2003). However, ILF are present in LTα+/-β+/-, LTα+/- and LTβ+/- mice (Figure 4.10) and any defects would have been corrected by reconstitution with LT-intact BM cells since ILF development is inducible during adulthood (Lorenz et al., 2003).

Once again, all BM chimeras regardless of recipient or donor origin were protected (evident by the lack of oocysts) against secondary infection with *E.vermiformis* (data not shown), indicating that GALT structures are dispensable for the development of protective memory. Collectively, the data presented above indicates no requirement for LT-expression by a BM-derived cell during infection and is consistent with a phenotype influenced by the presence or absence of MLN and PP.

4.3.3. Delayed kinetics of Th1 immune responses during infection

Immunity to primary infection with *E.vermiformis* depends on the timing of Th1 immune responses (Rose et al., 1984) and the protective immunity developed in LT-deficient mice against secondary infections indicated that a primary immune response had been induced. Hence, it is possible that the susceptibility of LT-deficient mice seen during primary infection may be a result of delayed Th1 immune responses. To test this hypothesis, the
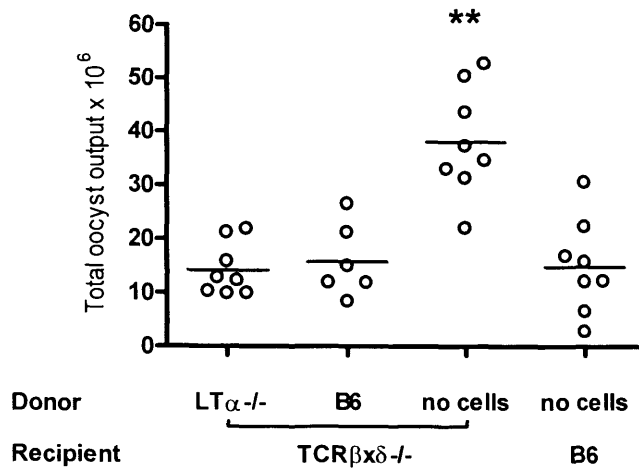
Figure 4.3 Expression of LT by T cells is not required during infection.

A total of 1 x 10^7 CD90+ sorted T cells from naive LTα-/- or B6 donors were adoptively transferred into TCRβxδ-/- recipients which were subsequently infected. Results show the total oocyst output of individual mice from each group. **, indicates significant difference (P<0.002) from all other groups.
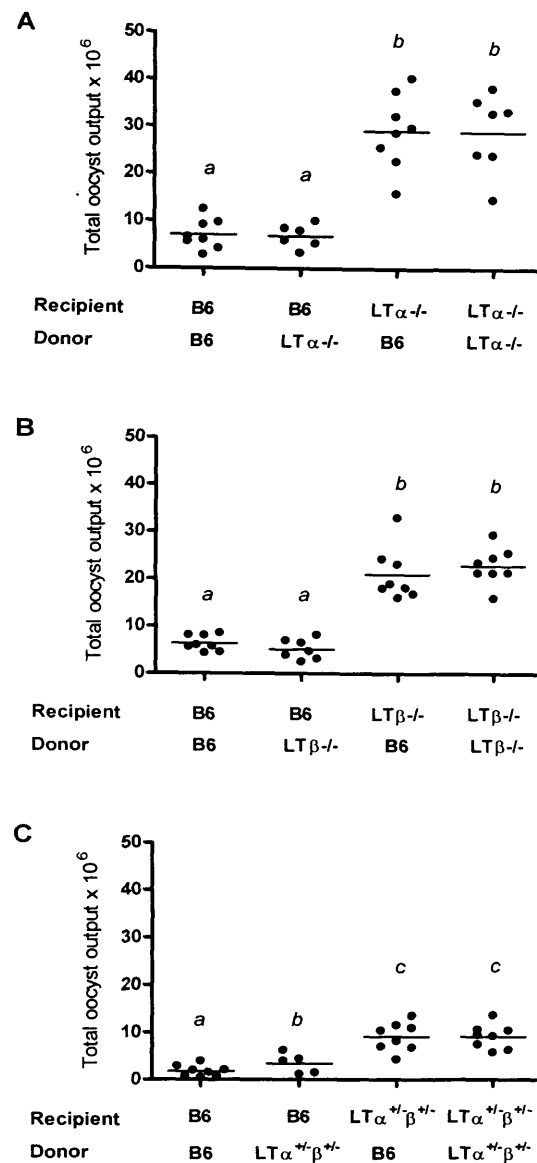
Figure 4.4 Requirement for LT in structural organization.

Bone marrow chimeric mice were made using LT-deficient and B6 mice as donors or recipients. Results show total oocyst output of individual mice from infected (A) LTα-/- (B) LTβ-/- (C) LTα+/-β+/- BM chimeric mice. 5-8 mice per group were used and the data represents 1 of 2 experiments. *a,b,c*, Groups annotated with the same italic letter are not significantly different while those annotated with different letters are significantly different as follows A) and B) (P<0.01), C) (P<0.05).

LTα-/-   LTβ-/-   LTα+/-β+/-

LTα+/-   LTβ+/-   B6
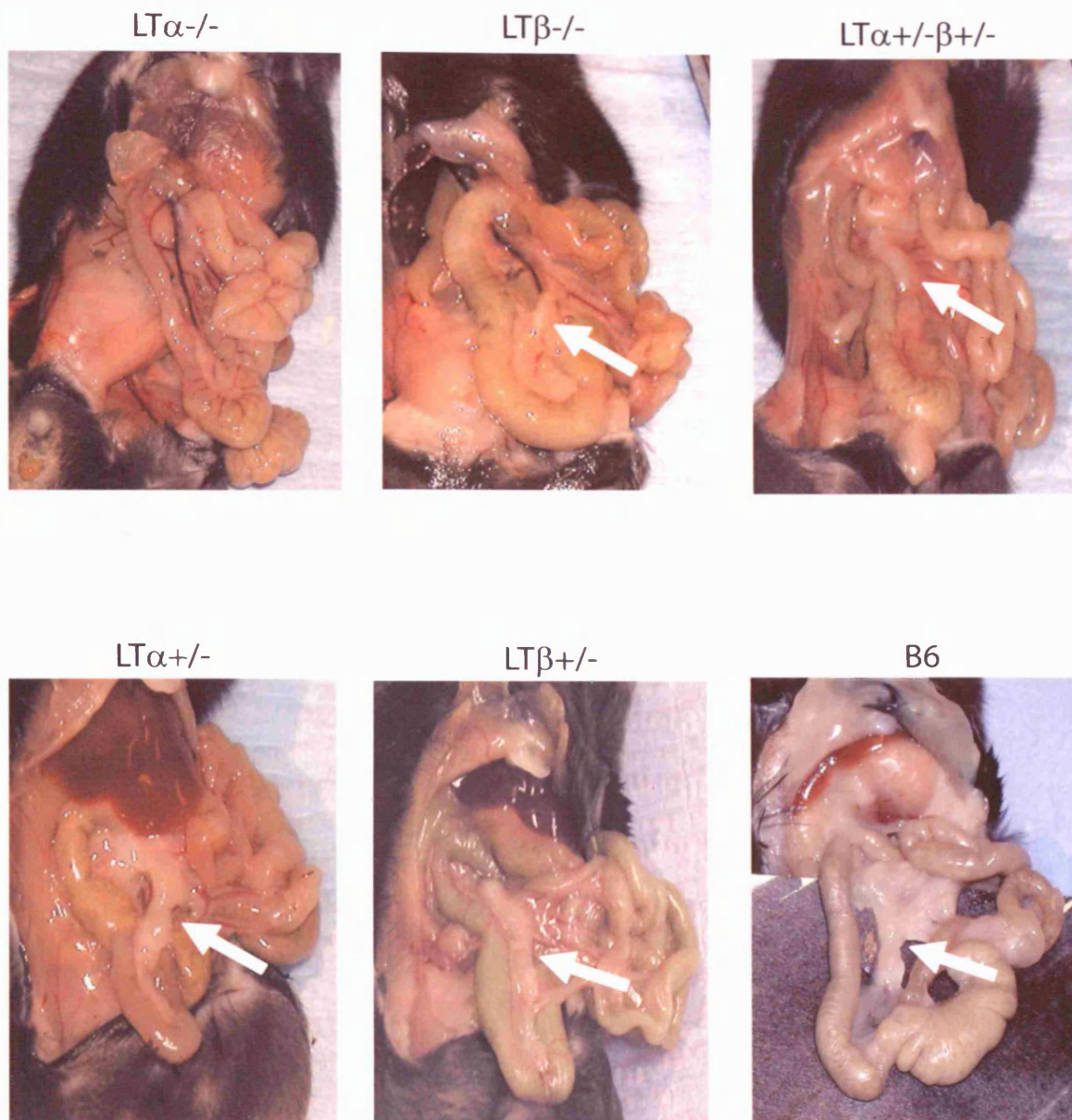
Figure 4.5 Mesenteric lymph nodes from LT-deficient and B6 mice.

White arrows point to the MLN structure (if present). Note the smaller MLN structure seen

in LTβ-/- mice.

Gated on R1

LTβ-/- MLN
example #1

20.5 | 0.2
42.4 | 36.3

LTβ-/- MLN
example #2

negligible

CD8α

B6 MLN

35.5 | 0.2
21.8 | 42.6

CD4
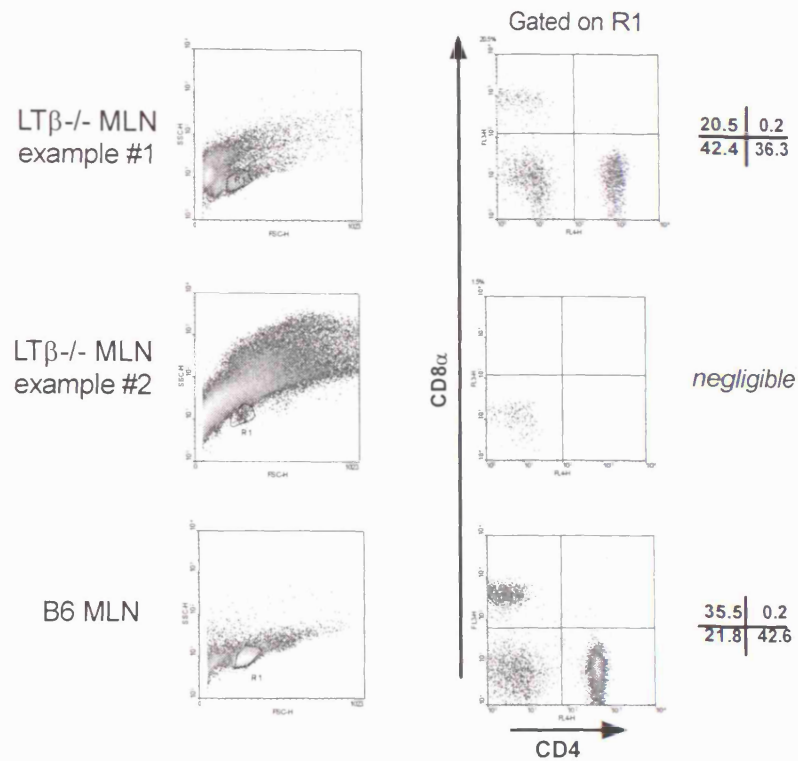
Figure 4.6 The MLN in naïve LTβ-/- mice.

FACS analysis of CD4+ and CD8+ cell proportions in the MLN of individual LTβ-/- and B6 mice. Proportions are shown (where possible) in the quadrants to the right of the FACS plots. FACS plots are individual examples of the variation seen in the MLN of LTβ-/- mice compared with a B6 MLN.

A

MLN example #1

Lymphocyte numbers here are negligible

MLN example #2

Gated on R1

CD3ε

no stain

Gated on R1 and CD3ε+ cells

CD4

CD8α

	46.2	0.3
	28.7	24.8

MLN viable cell count	
Example #1	4.2×10^5
Example #2	3.2×10^5

B

Spleen

LTβ-/- BM cells
→ LTβ-/- recipient

B6 BM cells
→ LTβ-/- recipient

Gated on R1 and CD3ε+ cells

CD8α

23.3

53.4

27.6

52.2

CD4
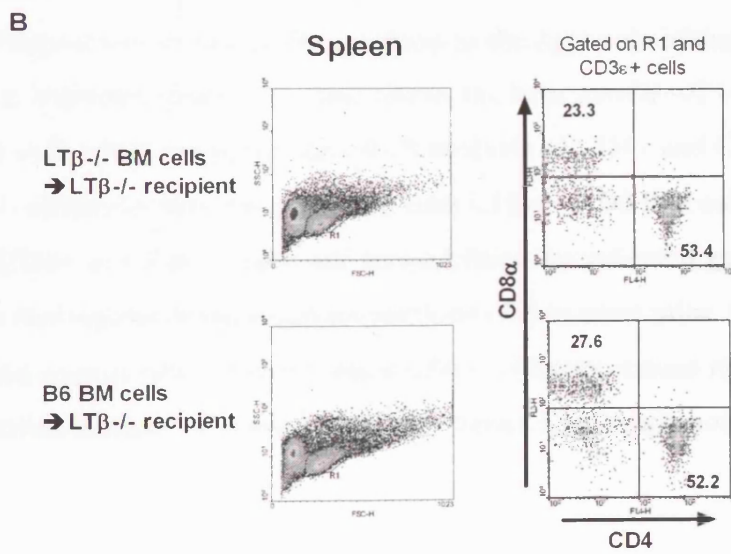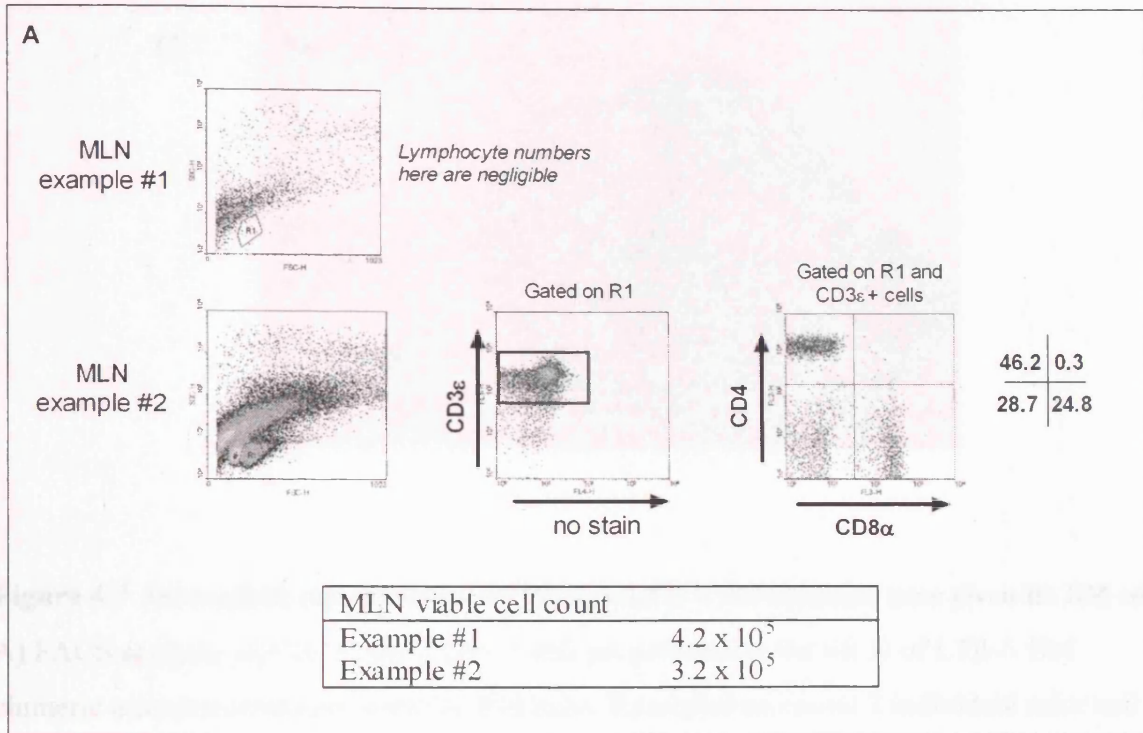
Figure 4.7 (see next page)

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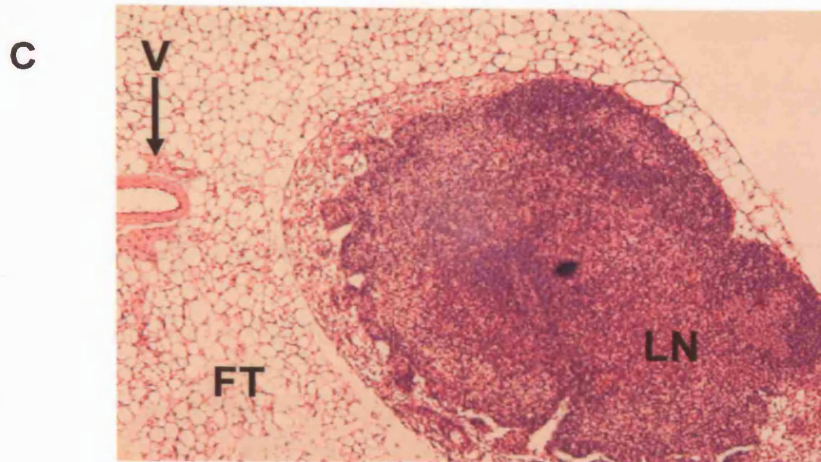
Figure 4.7 Incomplete reconstitution of MLN in LTβ-/- BM chimeric mice given B6 BM cells.
A) FACS analysis of CD4+ and CD8+ T cell proportions in the MLN of LTβ-/- BM
chimeric mice reconstituted with B6 BM cells. Examples represent 2 individual mice and
are used as a general representation of LTβ-/- BM chimeric mice reconstituted with B6
bone marrow. Proportions shown in the quadrant to the right side of the FACS plots are
derived from the indicated gates. The table shows the total number of viable cells from
the MLN of the individual examples. B) FACS analysis of CD4+ and CD8+ T cells in the
spleen of LTβ-/- chimeric mice reconstituted with LTβ-/- or B6 BM cells. The
proportions of CD4+ or CD8+ T cells are derived from the indicated gates, displayed on
the FACS plots and represent the mean proportions of 6 or more mice. C) Hematoxylin
and eosin-stained section of the MLN from a LTβ-/- chimeric mouse reconstituted with
B6 BM cells. Abbreviations: V, vessel; FT, fatty tissue; LN, lymph node.

timing of IFNγ mRNA upregulation was assessed in the small intestine and where possible, in the MLN of infected mice. In B6 mice, increased levels of IFNγ mRNA in the small intestine were detected at 6 days post-infection (DPI) onwards whereas with LTα-/- and LTβ-/- mice, no response was detected until 10 DPI (Figure 4.11A). In LTα+/-β+/- mice, which were intermediately susceptible, IFNγ mRNA in the small intestine was upregulated at 8 DPI. In the MLN of LTα+/-β+/- mice, IFNγ mRNA was upregulated at 6 DPI while that in B6 mice was upregulated earlier at 4 DPI. To substantiate the view that the delayed immune responses towards infection were Th1-mediated, intracellular cytokine staining on MLN cells from LTα+/-β+/- and B6 mice during infection was carried out. There was a greater proportion of CD4+ IFNγ+ T cells than CD8α+ IFNγ+ T cells in the MLN (Figure 4.11B). B6 mice had a higher proportion of IFNγ+ CD4+ T cells in the MLN at 6 DPI than LTα+/-β+/- mice (Figure 4.11C). Proportions of IFNγ+ CD4+ T cells were similar in both LTα+/-β+/- and B6 mice by 8 DPI (data not shown). In the small intestine, CD4+ IEL were sorted at 8 DPI from LTα-/- and B6 mice and analyzed for IFNγ mRNA levels. Only the CD4+ IEL from B6 mice showed upregulation of IFNγ at 8 DPI, showing that the delayed IFNγ expression in the upper ileum of LTα-/- was related to the lack of expression by CD4+ IEL (Figure 4.12). In addition, upregulation of IFNγ mRNA was noted in the CD4- IEL sorted fraction (data not shown)
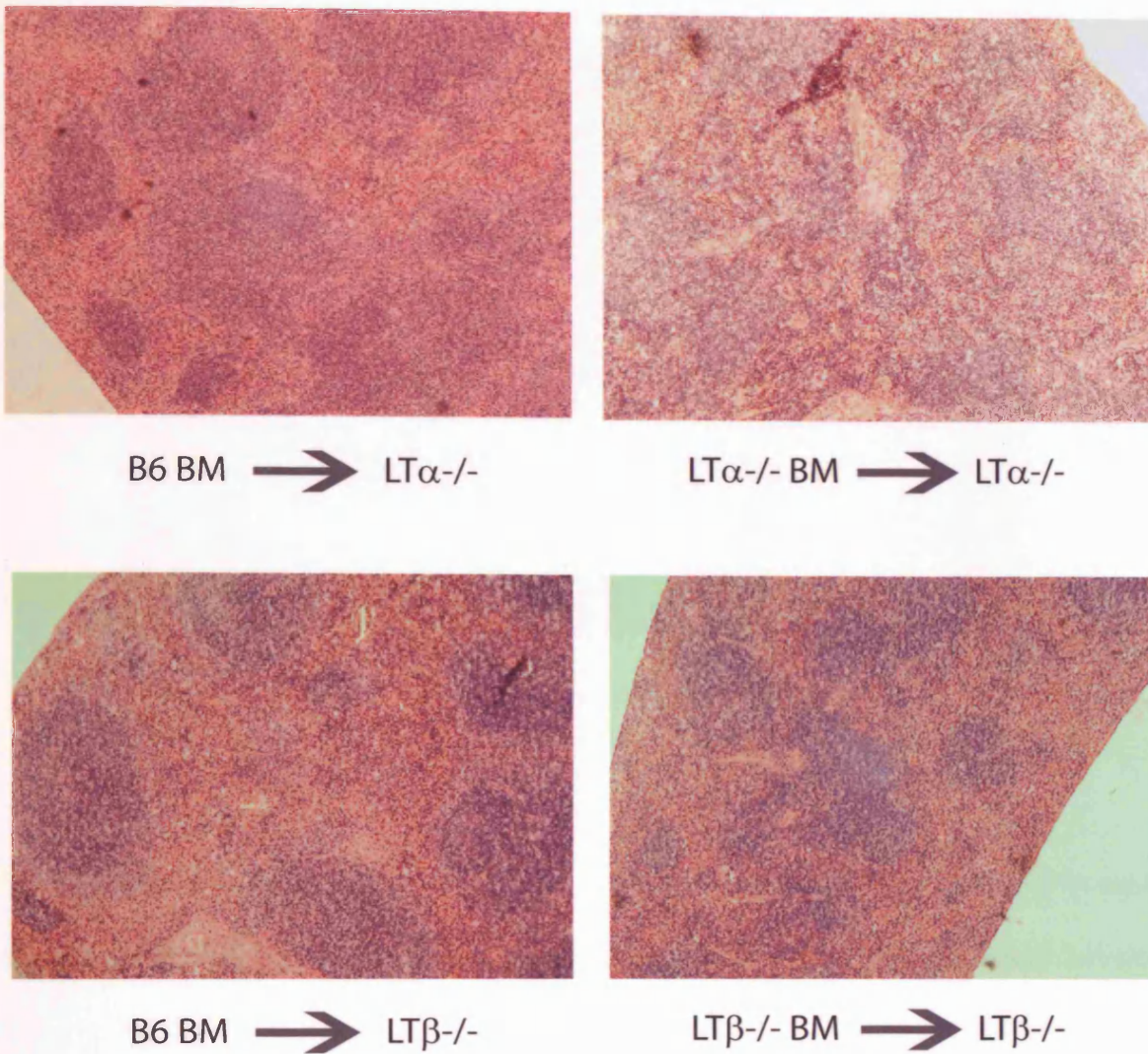
Figure 4.8 Splenic organisation in LT-deficient BM chimeric mice.

Hematoxylin and eosin-stained sections of spleens from LTα-/- or LTβ-/- recipients reconstituted with either LT-deficient or LT-intact BM. Distinct organised lymphoid follicles can be seen in the spleens of recipients which received B6 bone marrow while those which received LT-deficient bone marrow retained splenic disorganisation. A minimum of 4 mice per group was examined. Arrows point to the recipient strain receiving donor BM.

LTα+/-β+/-
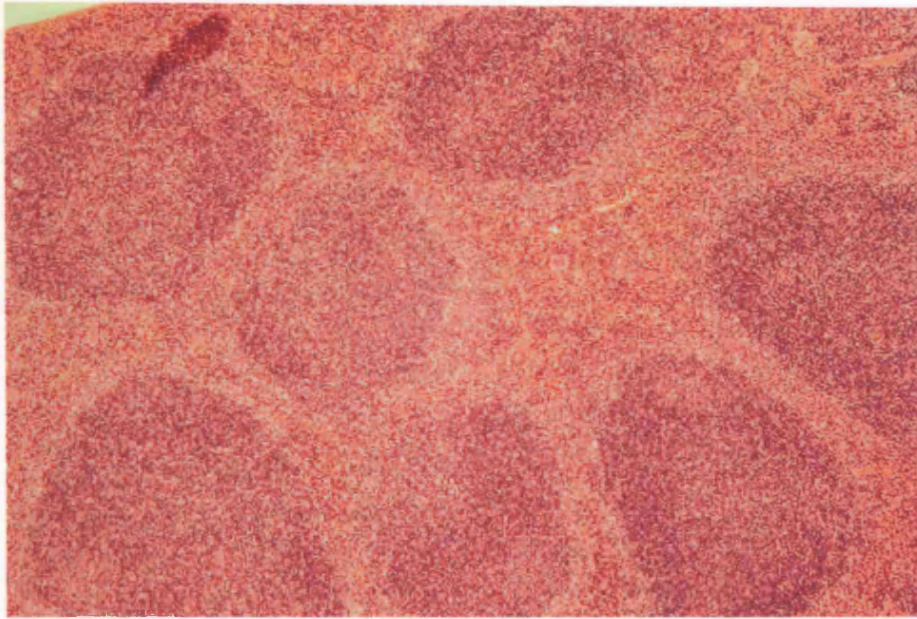
Figure 4.9 Hematoxylin and eosin-stained section of a spleen from a LTα+/-β+/- mouse.

Splenic organisation is present as determined by the presence of distinct lymphoid follicles.
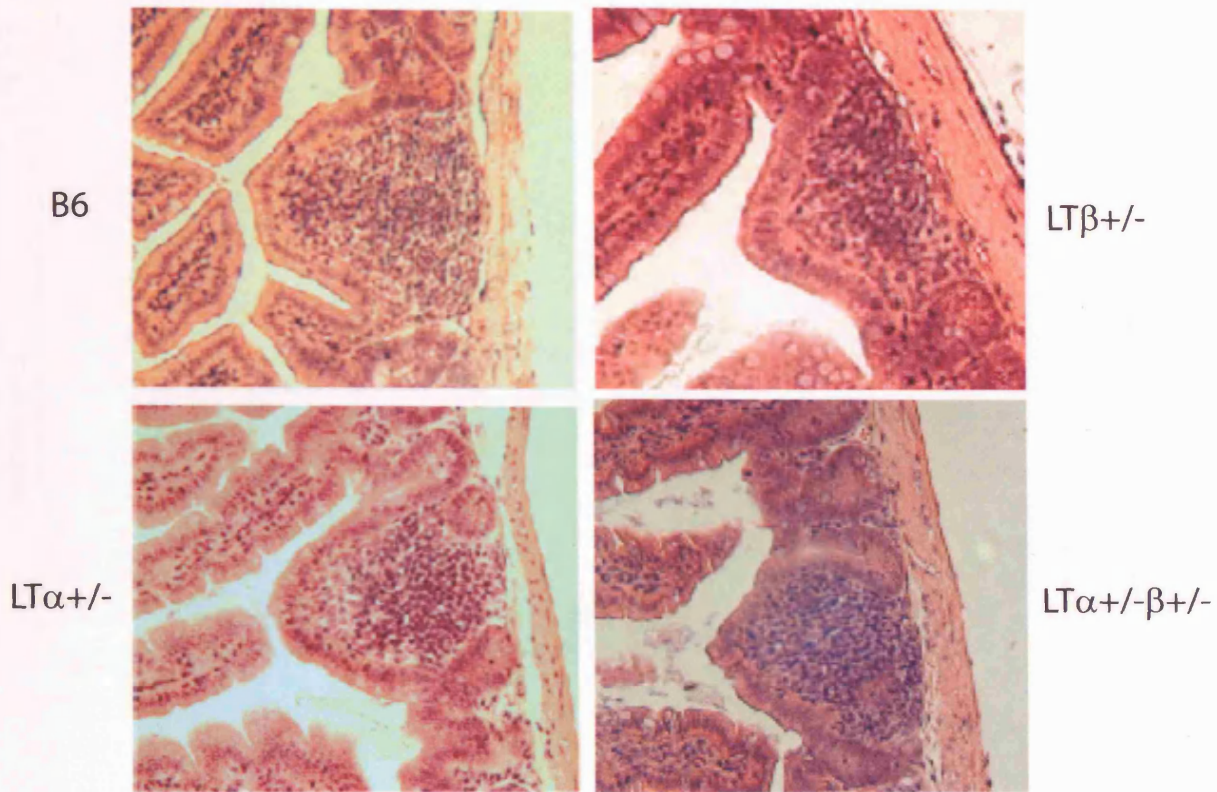
Figure 4.10 Isolated lymphoid follicles in LT single and double heterozygote mice.
Hematoxylin and eosin-stained sections of ILF from the small intestine of different strains of mice. 3 mice per group were examined, all of which possessed ILF.
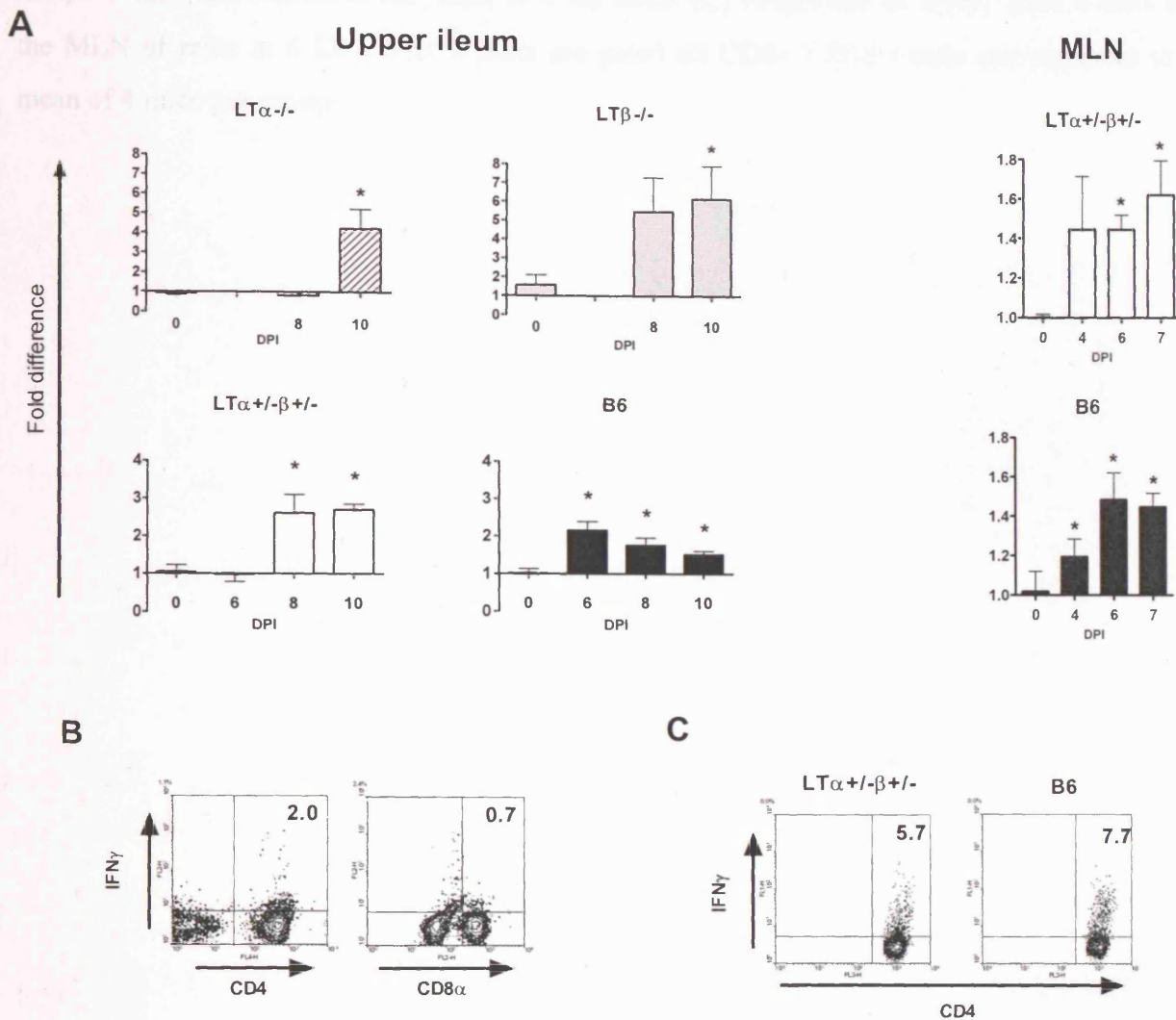
Figure 4.11 Delayed IFNγ expression in the small intestine and MLN of LT-deficient mice.

(A) mRNA was isolated from the ileum and MLN. Quantitative RT-PCR was carried out and relative levels of IFNγ mRNA were normalised against CD3γ mRNA. Bar charts show the fold difference in the levels of IFNγ mRNA expressed. Fold differences are calculated against uninfected controls as mentioned in materials and methods. Each bar corresponds to the mean and SEM of 3 or more mice per time-point and results are representative of 2-3 experiments. *, Significant difference (P<0.05) when compared to uninfected controls. (B) The majority of IFNγ+ cells are CD4 T cells. FACS plots are gated on CD3ε + cells, show the proportion of

IFNγ+ T cells and represent the mean of 4 B6 mice. (C) Proportion of IFNγ+ CD4 T cells in the MLN of mice at 6 DPI. FACS plots are gated on CD4+ CD3ε + cells and represent the mean of 4 mice per group.
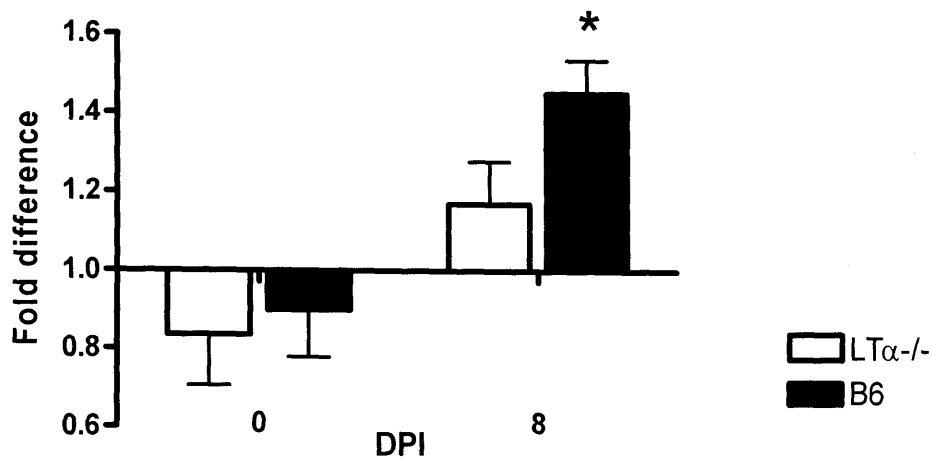
Figure 4.12 Expression of IFNγ by CD4+ IEL.

CD4+ IEL from infected LTα-/- and B6 mice were enriched using magnetic beads and analysed for IFNγ mRNA expression. The data represents the fold difference in IFNγ mRNA expressed (calculated as described in chapter two). The bar chart represents the mean and SEM of 4 mice per group per time-point. IFNγ mRNA was normalised to CD3γ mRNA levels. *, represents significant increase from 0 DPI (P<0.05).

4.3.4. Delayed increase in gut-homing CD4 T cells in the small intestine

Stimulation of T cells with gut-derived DC leads to upregulation of $\alpha 4\beta 7$ (Mora et al., 2003; Stagg et al., 2002), a gut-homing integrin, which interacts with the mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) expressed by high endothelial venules and flat-walled venules in the gut (Berlin et al., 1993; Butcher et al., 1999). In B6 mice, increased proportions of $\alpha 4\beta 7$ + CD4+ T cells were detected in the IE compartment from 8 DPI onwards while proportions in LTα-/- mice increased only at 14 DPI onwards (Figure 4.13A). Similar patterns of delayed increase in proportions of $\alpha 4\beta 7$+ CD4+ IEL were seen in LTβ-/- mice (data not shown). At present there were no detectable differences in $\alpha 4\beta 7$+ CD4 IEL proportions between LTα+/-β+/- and B6 mice. Two subsets of CD4+ IEL, the CD4+CD8$\alpha\alpha$+ and the CD4+ CD8- subsets, are present in the small intestine and further analysis showed both subsets had similar proportions of cells expressing $\alpha 4\beta 7$ (data not shown). Earlier increases of $\alpha 4\beta 7$ + CD4+ T cells were found in the MLN of B6 mice at 6 DPI compared with LTα+/-β+/- mice at 7 DPI (Figure 4.13B). Gut-tropic T cells also deploy the chemokine receptor, CCR9, which recognizes TECK (CCL25) expressed by small intestinal epithelial cells (Kunkel et al., 2000). At 8 DPI, the proportion of CCR9+ CD4 IEL increased in B6 mice but only increased at 10 DPI in LTα-/- mice (Figure 4.14).

In order to determine if the delayed presence of $\alpha 4\beta 7$+ CD4+ IEL in LTα-/- and LTβ-/- mice were not a result of an existing defect in subset proportions, IEL subsets were examined in naïve LTα-/- and LTβ-/- mice. All IEL express CD3 and no differences were seen in the proportions of CD8α- CD3ϵ + and CD8α + CD3ϵ+ cells in LTα -/- and B6 mice (Table 4.2). However, there was a higher proportion of TCR$\alpha\beta$+ cells in LTα -/- and LTβ-/- mice

compared with B6 mice and the proportion of TCRγδ+ cells were lower in LTα-/- mice. CD4+ IEL are usually TCRαβ+ and further dissection of the TCRαβ+ CD4+ IEL subsets (CD4+ CD8αα+ and CD4+ CD8α-) showed no diminished numbers in LTα-/- and LTβ-/- mice which both had slightly higher proportions than B6 mice. B6 mice seem to have a higher proportion of CD8αβ+ CD4- TCRαβ+ cells than CD8αα+ TCRαβ+ cells while it is the opposite in LTα-/- and LTβ-/- mice. However, CD8+ T cells do not play an essential role during primary infection with *E.vermiformis* (Rose et al., 1988; Smith and Hayday, 2000).

In addition, the kinetics of IEL subsets were examined in LTα-/- mice during infection. CD11a, an activation marker upregulated on activated T cells, was used to examine activated IEL. CD11a was expressed mainly on CD8αβ+ and CD8α- CD8β- IEL and not CD8αα+ IEL (Figure 4.15). The proportions of CD11a+ IEL and CD11a+ CD8α- CD8β- IEL were significantly higher earlier at 11 DPI and 8 DPI respectively in B6 mice while proportions in LTα-/- mice only increased at 20 DPI and 15 DPI respectively.
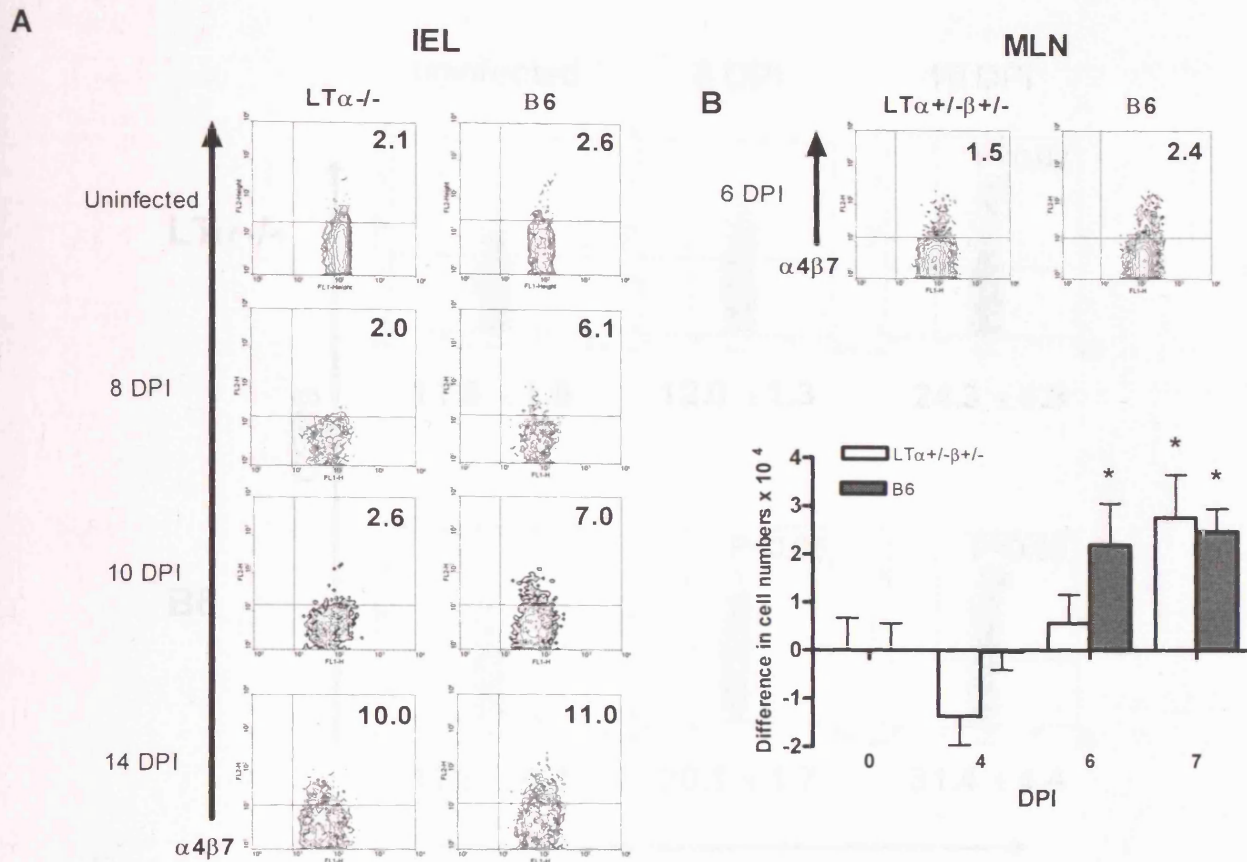
Figure 4.13 Delayed presence of α4β7+ CD4+ T cells in LT- deficient mice during infection. (A) α4β7+ CD4+ T cell in the small intestinal IEL compartment of LTα-/- and B6 mice. (B) α4β7+ CD4+ T cells in the MLN of LTα+/-β+/- and B6 mice. FACS plots show the proportion of CD4+ T cells expressing α4β7 and represent 1 of 4 mice per time-point in 2 experiments. All plots represent cells gated on CD4 and CD3ε. The bar chart depicts the changing numbers of α4β7+ CD4+T cell in the MLN which are calculated from differences in α4β7+ CD4+T cell numbers between infected and uninfected mice. Bars correspond to the mean and SEM of 4 mice per time-point. *, indicates significant difference (P<0.05) when compared to uninfected controls.
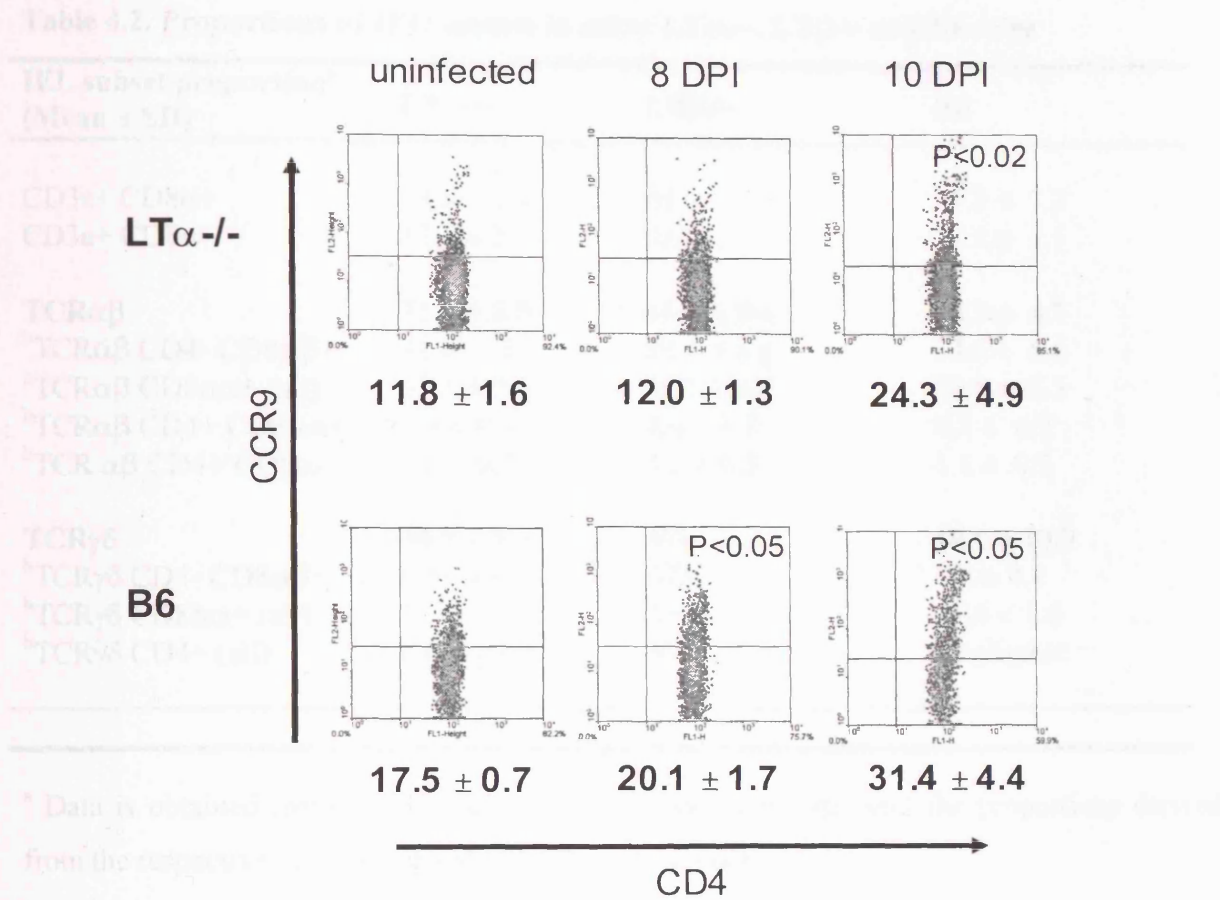
Figure 4.14 Delayed presence of CCR9+ CD4+ IEL in LTα-/- mice during infection. Proportions of CCR9+ CD4+ T cells in the small intestinal IE compartment of LTα-/- and B6 mice. FACS plots are representative of 1 of 4 mice per group per time-point. All plots are gated on CD4+CD3ε+ IEL. Values show the mean and SEM proportion of CCR9+ CD4+ IEL per group.

Table 4.2. Proportions of IEL subsets in naïve LTα-/-, LTβ-/- and B6 mice

IEL subset proportion[a] (Mean ± SD)	LTα-/-	LTβ-/-	B6
CD3ε+ CD8α+	83.8 ± 2.4	*ND*	77.3 ± 7.2
CD3ε+ CD8α-	12.3 ± 2	*ND*	15.4 ± 3.1
TCRαβ	**71.0 ± 8.9**	**66.5 ± 8.4**	**33.3 ± 4.7**
[b]TCRαβ CD4- CD8αβ+	31.6 ± 8.3	23.5 ± 6.4	53.8 ± 6.5
[b]TCRαβ CD8αα+ (all)	58.1 ± 9.3	33.1 ± 9.2	29.8 ± 3.3
[b]TCRαβ CD4+ CD8αα+	3.8 ± 0.9	4.0 ± 3.3	0.3 ± 0.2
[b]TCR αβ CD4+ CD8α-	3.8 ± 0.7	3.2 ± 0.3	1.1 ± 0.5
TCRγδ	**38.9 ± 9.6**	*ND*	**54.5 ± 10.9**
[b]TCRγδ CD4- CD8αβ+	0.69 ± 0.2	*ND*	3.8 ± 0.8
[b]TCRγδ CD8αα+ (all)	31.2 ± 1.6	*ND*	41.6 ± 1.6
[b]TCRγδ CD4+ (all)	Negligible	*ND*	Negligible

[a] Data is obtained from 4 mice per group. [b] Values here represent the proportions derived from the respective cell population in bold. *ND*, not done.
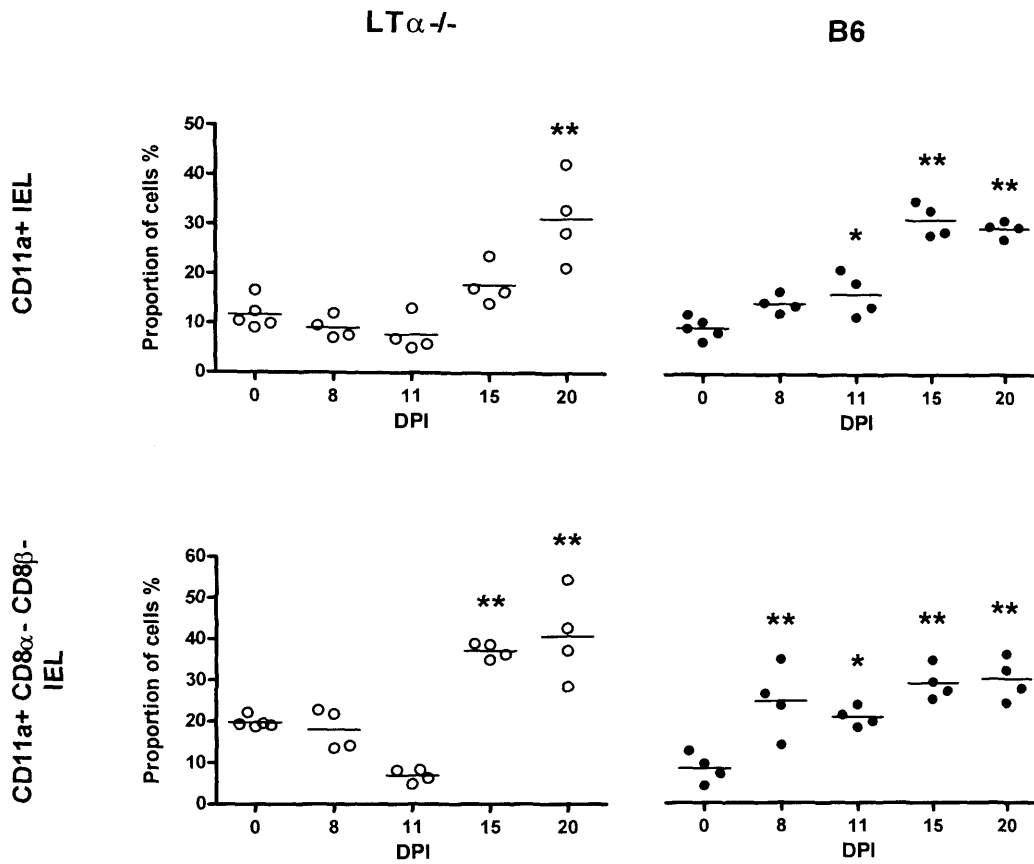
Figure 4.15 Delayed increase in activated IEL during infection in LTα-/- mice.

IEL from infected LTα-/- and B6 mice were isolated over a time-course and examined for CD11a expression. Data represents 4 or 5 mice per time-point per strain. * and **, indicates (P<0.05) and (P<0.02) respectively, and significant difference from uninfected controls at 0 DPI.

4.3.5. Influence of parasite dose on LT-deficient mice

In chapter three, parasite dose was demonstrated to influence the pattern of immune response and susceptibility between BALB/c and B6 mice. It is possible that PP which are located closely to the sites of infection in the gut, may be essential for the detection of low levels of parasite invasion and induction of rapid immune responses. Hence LTα-/-, LTβ-/- and LTα+/-β+/- mice were challenged with 50 and 100 parasites and oocyst output was assessed. It was hypothesized that the lack of PP in LTα+/-β+/- mice would render them more susceptible to a lower parasite dose (than with a dose of 100 oocysts) since fewer parasites may mean less efficient immune detection by the host and/or fewer DC activated by parasite injections. However, LTα+/-β+/- mice remained intermediately susceptible to infection when compared to LTα-/- or LTβ-/- mice at doses of 50 or 100 oocysts (Figure 4.16A). The fecundity of *E.vermiformis* was measured by dividing total oocyst output by the starting dose of parasite and provides a measure of the parasite's replicative potential. Parasite fecundity was higher in LTβ-/- mice given 50 oocysts than LTβ-/- mice given 100 oocysts (P<0.01) (Figure 4.16B). There were approximately 6 x 10^5 parasites produced per oocyst in LTβ-/- mice challenged with 50 oocysts compared with 4 x 10^5 parasites produced per oocyst in LTβ-/- mice challenged with 100 oocysts. All other mice showed no increase in parasite fecundity at a lower dose of 50. The B6 mice produced between 1-2 x 10^5 parasites produced per oocyst with either doses of 100 or 50. The LTα-/- mice produced around 4 x 10^5 parasites produced per oocyst with either doses of 100 or 50 while LTα+/-β+/- produced around 3 x 10^5 parasites produced per oocyst with either doses of 100 or 50.

4.3.6. Antigen-specific responses in LTα-/- mice

At 8 DPI, spleens from infected LTα-/- and B6 mice were stimulated *ex vivo* with oocyst lysate. Higher levels of antigen-specific IFNγ TNF and IL2 were produced in the supernatants of LTα-/- and B6 splenic cells stimulated with oocyst lysate compared with unstimulatd splenic cells (Figure 4.17). The stimulated splenic cells from infected LTα-/- mice also produced higher levels of antigen-specific IFNγ and TNF than B6 splenic cells stimulated with oocyst lysate.

4.3.7. Long term protection to secondary infection

All LT-deficient mice were protected against secondary infection as previously shown however it was unknown if this level of protection could be sustained for a longer term. Therefore LTα-/-, LTβ-/- and B6 mice were kept more than eight months after primary infection with 100 oocysts before rechallenge with the same number of oocysts (Table 4.3). All B6 mice were protected, producing no oocysts. However, three out of seven LTα-/- mice and four out of five LTβ-/- mice produced a small amount of oocysts (the number of oocysts was not entirely quantified but was much lower than the levels produced on the day of peak oocyst production during primary infection). LTβ-/- mice also produced more oocysts than LTα-/- mice on the same day of peak oocyst production (data not shown).
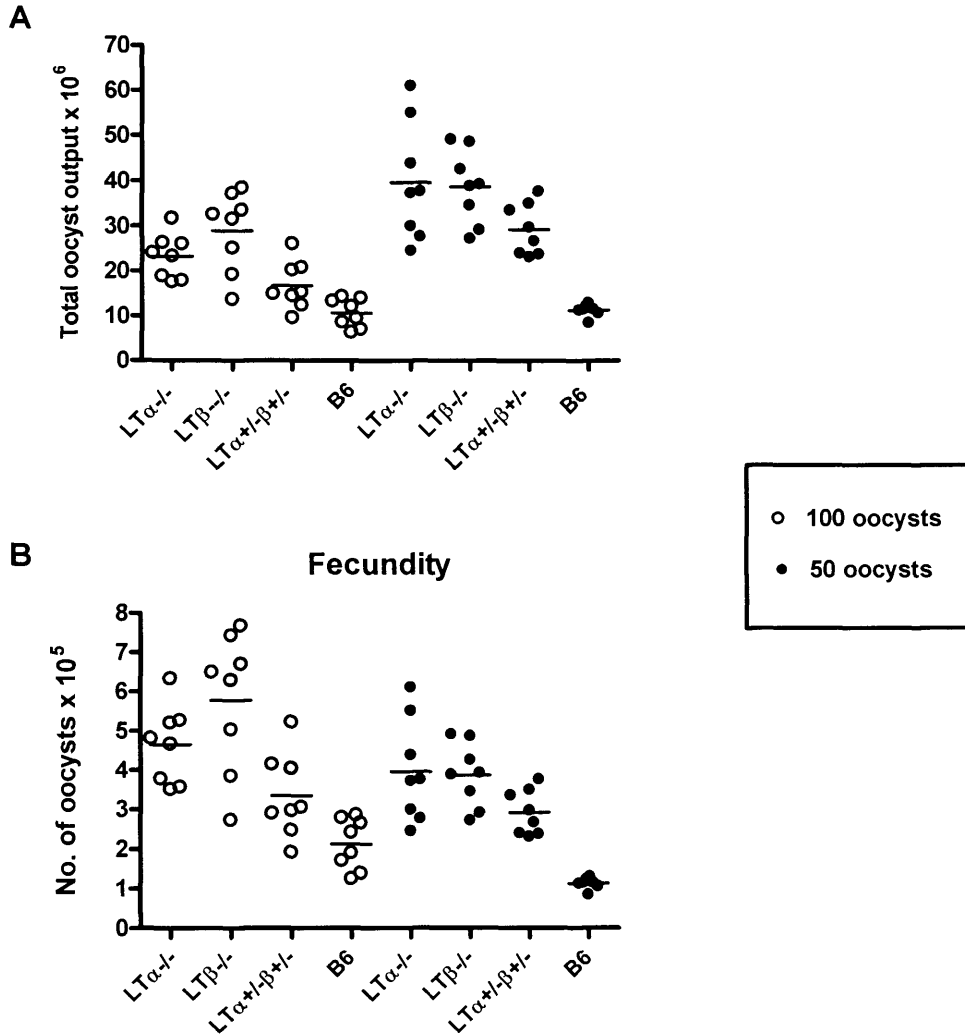
Figure 4.16 Influence of parasite dose on LT-deficient mice.

Mice were challenged with 100 or 50 oocysts and data represents the A) total oocyst output

and B) fecundity (total oocyst output divided by parasite dose). The data represents a

minimum of 7 mice per group.
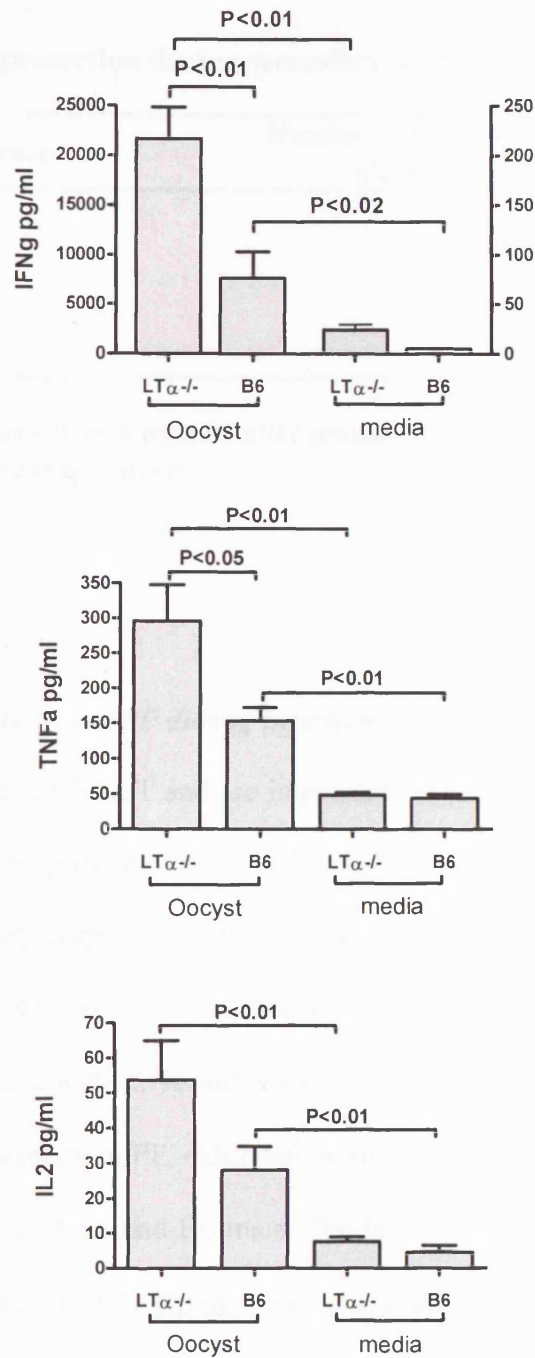
Figure 4.17 Antigen-specific immune responses in the spleen.
Splenic cells from day 8 post-infected- mice were stimulated *ex vivo* with oocyst lysate and measured for IFNγ, TNFα and IL2 production. Bar charts represent the mean and SEM of 4 mice per group.

Table 4.3 Long term protection during secondary infection with *E. vermiformis*.

[a] Strain of mice	Number of mice producing oocysts after re-infection/ Total mice examined
LTα-/-	3/7
LTβ-/-	4/5
B6	0/7

[a] Mice were kept for more than 8 months after primary infection with 100 oocysts before re-infection with the same parasite dose.

4.4. Discussion

The requirement for MLN and PP during infection

The MLN and PP form the GALT and are important lymphoid structures that accommodate and coordinate antigen presentation for the induction of T-cell mediated responses, particularly with primary responses from naïve T cells. Requirements for GALT structures during infection have not been well-addressed and therefore were investigated by challenging various LT-deficient mice with *E. vermiformis*. LTα-/-, LTβ-/- and LTα+/-β+/- mice which have in common a deficiency in PP, exhibited increased susceptibility to infection compared with PP-intact LTα+/-, LTβ+/- and B6 mice. The level of susceptibility seen in LTα-/- and LTβ-/- mice resembled that of TCRβxδ-/- mice suggesting that the induction of effective T cell responses has to occur in a localised and organised manner, a purpose facilitated by the presence of secondary lymphoid structures. With *E. vermiformis* infection, both PP and MLN structures are required for resistance against the enteric pathogen. The use of T cell adoptive transfer and BM chimeric mice further substantiated the necessity for organised MLN and PP rather than immune cell-derived LT expression during infection. LT-deficient T cells were

sufficient to confer protection in TCRβxδ-/- mice and capable of developing into memory T cells sufficient for protection against a secondary infection. The same was seen with BM chimeric mice, where there was no role for LT in BM-derived cells and it was the genotype and existing lymphoid structures in recipient chimeric mice that were required for efficient responses against infection. Moreover, LT-deficient BM cells were capable of developing into memory cells which were able to protect against secondary infections. This data supports earlier studies that secondary lymphoid organs are dispensable for the generation of immune memory (Chalasani et al., 2002; Davis et al., 1998).

Follicular DC are stromal cells and part of the reticular network which is responsible for lymphoid organisation within the lymphoid organ. Their presence is maintained by LT-signalling and although they are absent in LTα-/- or LTβ-/- mice, they do not play a role during infection since they are restored in LT-/- chimeric mice reconstituted with LT-intact B6 BM cells (Fu et al., 1997; Matsumoto et al., 1997).

The PP may become more important with lower parasite doses due to their antigen-sampling capacity. The level of susceptibility may be influenced in a similar manner to that seen in chapter three where BALB/c mice were more susceptible to lower doses. Therefore it was hypothesized that LTα+/-β+/- mice may be more susceptible with a lower parasite dose while LTα-/- and LTβ-/- mice were not expected to be more susceptible than they were already. However, this was not evident as the parasite's replicative potential (ie. fecundity) was different only between LTβ-/- mice given 50 and 100 oocysts. This suggested that there were subtle differences in LTβ-/- mice which permitted a better parasite yield than the other LT-deficient mice. These differences could be due to minor influences of LTα1β2-signalling

on specific cell types (eg. DC), compromising immune detection and allowing parasite replication to proceed to a greater extent.

The structural integrity of MLN is required while ILF and CP are non-essential

Initially, LTβ-/- mice were expected to have a more resistant phenotype than LTα-/- mice since they possess MLN. However this was not the case as the LTβ-/- MLN structure was smaller and could not be reconstituted in BM chimeric mice to recover full functionality despite reconstitution in the spleen (Figure 4.5, 4.6 and 4.7). This suggests that the residual MLN structure in LTβ-/- mice may be defective, disallowing full cellular reconstitution by LT-intact cells. The presence of a functionally intact MLN in LTα+/-β+/- mice provided an alternative test for the capacity of MLN to mediate protection against *E.vermiformis* infection but the lack of PP did not help in mediating the same level of resistance seen in B6 and LT single heterozygote mice. The intermediate resistance of LTα+/-β+/- mice indicated that an organised MLN can confer a degree of protection against infection but the presence of PP is required in order to deliver the same level of resistance as B6 mice,

There were considerations that ILF and crytopatches (CP) may be involved in influencing susceptibility since they were absent in LTα-/- and LTβ-/- mice (Hamada et al., 2002; Lorenz et al., 2003; Pabst et al., 2005; Taylor et al., 2004). The ILF which have similar morphological features to PP, such as a FAE layer and germinal centers, redevelop in LT-deficient recipients reconstituted with B6 bone marrow (Lorenz et al., 2003). It was thought that ILF may also have contributed to the intermediate phenotype of LTα+/-β+/- mice which possess ILF (Figure 4.10). However, LTα-/- and LTβ-/- recipient chimeric mice

(reconstituted with B6 BM) which redevelop iLF do not display any improved resistance to infection. With similar characteristics with PP, it is unclear as to why ILF are less critical during infection with *E.vermiformis* but there are questions which will need to be addressed: For example, are there similarly efficient networks of efferent lymphatics draining the PP into the MLN, found in the ILF? Nonetheless, in this infection model, the data presented provides a strong argument that PP and functional MLN are the major requirements for the induction of rapid Th1 responses against a gut infection.

Cryptopatches are also sites of lymphoid aggregation and are potential sites for the extrathymic development of IEL (Kanamori et al., 1996; Lambolez et al., 2002; Rocha et al., 1994; Saito et al., 1998) hence their absence may disrupt the IEL populations in LT-deficient mice. However, this factor may be excluded since IEL development can proceed independently of CP and iLF (Pabst et al., 2005) and IEL populations were checked in other studies (Gabor et al., 2001; Pabst et al., 2005) and supported by the data presented here where the general consensus was that TCR$\alpha\beta$+ cell populations were increased and there may also be an increased ratio of CD8$\alpha\alpha$:CD8$\alpha\beta$ IEL. The view here is that conventional T cells will suffice for the role of immune protection against primary infection with *E.vermiformis* since TCRβxδ-/- mice which do not have IEL can be rescued through the adoptive transfer of splenic or MLN T cells (Figure 4.3). The IEL examined during the course of infection may consist of conventional T cells (probably migrated from the MLN/PP) moving into the IE compartment which when retrieved, would have included any lymphocytes that were present in this compartment. The role for CD4+ T cells is further demonstrated by the increased proportion of activated CD8α-CD8β- IEL(although this may

include CD4-CD8- IEL, it seems unlikely as seen in the data on gut-homing IEL which is discussed below).

Although LTα+/- and LTβ+/- mice were more resistant than LTα+/-β+/- and LTα-/- mice, they seemed to be slightly less resistant than B6 mice. This phenotype may be a result of lower expression of MAdCAM-1 levels (Browning et al., 2005; Cuff et al., 1999) which may in turn affect the homing of cells expressing α4β7 which binds to MAdCAM-1. In highly susceptible LTα-/- and LTβ-/- mice, the partial defect in MAdCAM-1 expression does not contribute hugely to their susceptible phenotype since LTα+/- and LTβ+/- mice (which were also reported to have relatively reduced MAdCAM expression) were relatively more resistant to infection with *E.vermiformis*. Furthermore, gut-homing molecules also include CCR9 and there are no reported deficiencies in CCL25 levels in LT-deficient mice, indicating no defect in homing by T cells expressing CCR9. In addition, gut-homing T cells are known to express CCR9 or α4β7 separately (Stenstad et al., 2006).

The timing of responses against E. vermiformis is associated with the MLN and PP

It is believed that increased susceptibility to primary infection with *E. vermiformis* in LT-deficient mice was a result of the delayed development of Th1-type immune responses. Peak *E. vermiformis* replication in the small intestine occurs around 7-8 DPI and IFNγ, a key component in the control of infection (Smith and Hayday, 2000), was upregulated too late in the small intestine of LTα-/- mice. IFNγ mRNA expression was upregulated in CD4+ IEL from B6 at 8 DPI, indicating their contributory role in IFNγ production, although expression was not restricted to these cells since CD4- IEL sorted fractions also show upregulation of

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IFNγ. There was no indication of a permanent defect in IEL since the proportion of CD11a+ IEL eventually increases as with $\alpha 4\beta 7+$ CD4+ IEL and CCR9+ CD4+ IEL in LTα-/- mice. The higher proportion of CD4+ CD8$\alpha\alpha$+ subset in LTα-/- mice may dispute the hypothesis for a delayed immune response (by skewing proportions) but delays in $\alpha 4\beta 7$ and CCR9 expression were also seen in CD4+ CD8- IEL (note that there is no differentiation here between IEL residents and T cells migrated into the IE compartment). Delays in immune responses have also been observed in LTα-/- mice infected with influenza virus (Lund et al., 2002).

The timing of responses in various immune compartments of infected B6 mice corresponded to events that follow immune induction: The earliest increase in IFNγ expression and $\alpha 4\beta 7+$ CD4+ T cells in the MLN occurred at 4 DPI and 6 DPI respectively, followed by increased IFNγ expression and increased proportion of $\alpha 4\beta 7+$ CD4 IEL in the small intestine from 6 DPI onwards. Therefore, the data suggests that the MLN is a major inductive site and a good indicator of the timing of gut-related immune responses, and therefore a predictable measure of resistance to *E. vermiformis*. Importantly, the results demonstrate that the timing of Th1 immune responses in the small intestine plays an important role in the effective control of *E. vermiformis* where a delay of two days in the expression of Th1 immune responses results in approximately three-fold difference in oocyst output between B6 and LTα+/-β+/- mice.

Development of T cell memory in GALT-deficient mice

All LT-deficient mice and bone marrow chimeras developed a high level of protection against re-infection indicating that primary immune responses had been initiated in all LT-

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deficient strains and demonstrating that protective immunity against a gut infection can be generated in the absence of organised GALT structures. This brought about the question of where immunity could be initiated in mice deficient for GALT structures. Parasite antigens may have entered the bloodstream and been taken up by blood-derived DC, and/or reached the spleen and presented to T cells by local DC. Greater levels of antigen-specific IFNγ production were seen in the spleen of LTα-/- mice than B6 mice at 8 DPI (Figure 4.16) and may indicate greater priming activity due to a larger amount of parasite material draining into the spleen in the absence of GALT structures and a greater infection occurring in highly susceptible mice. Although the spleen itself is a candidate location for initiating the primary immune response in LTα-/- mice, the increased proportion of α4β7+ or CCR9+ CD4 IEL in LTα-/- mice also suggests stimulation by gut-derived DC (although this was too late to confer immunity). It is possible that gut-derived DC may migrate to unusual sites to present in the absence of MLN (eg. spleen or bone marrow) or initiate adaptive immunity in the small intestine, although these are clearly inefficient processes. Nonetheless, the spleen is a site for blood draining the gut as oral infection with *S.typhimurium* elicits splenic bacterial-specific T cell proliferation at 3 days post-infection whereas no proliferation was seen in other lymph nodes such as the inguinal, brachial and auxillary LN (McSorley et al., 2002).

All LT-deficient and intact mice were protected during secondary infection, one month after primary infection. Furthermore, reinfected B6 mice retained the same level of protection after more than eight months. However this was not the case for LTα-/- and LTβ-/- mice which showed evidence of oocyst production although the amount produced was much fewer (up to ~10,000 times) than that produced during on a peak day of oocyst production. There may be some differences in the way protective memory is developed between B6 and LT-deficient

mice. Possible reasons may be due to inefficient T cell priming in the absence of organised lymphoid structures leading the generation of a memory T cell pool that is not sustained for as long in LT-deficient mice. Recently, it was demonstrated that the differentiation of effector CD4 T cells into memory T cells was affected in the absence of lymphoid organs (Obhrai et al., 2006). However, further experimentation is necessary before any conclusions can be made although later in this thesis, DC antigen presentation in LT-deficient mice is further analyzed (chapter five).

In summary, resistance to infection increased with the presence of an organised MLN as seen in LTα+/-β+/- mice but their lack of PP rendered them more susceptible than PP-intact LTα+/-, LTβ+/- and B6 mice. Therefore in this infection, organised GALT structures are critical in influencing the rapid induction of protective responses, possibly by the organisation of DC-T cell interactions. Most certainly, there is an essential role for PP in the rapid induction of gut Th1-responses and evidence for a cooperative role with the MLN in the development of effective immunity in the gut.

Chapter Five

Investigating the influence of lymphotoxin on DC function

5.1.   Introduction

Dendritic cells are central to the development of adaptive immune responses and the efficacy of the DC response affects the ability to respond effectively against infections as demonstrated in chapter three and four. Factors which influence the type of immune response include DC numbers, DC subsets, the site of origin of DC, antigen dose and the different nature of microbial stimulation acting through different TLRs (Langenkamp et al., 2000; Manickasingham et al., 2003; Napolitani et al., 2005; Pulendran, 2005; Vieira et al., 2000; Whelan et al., 2000). Microbial stimuli are sensed by pathogen recognition receptors such as TLRs, C-type lectins and mannose receptors which in turn influence the activation and phenotype of DC. Where a proinflammatory response is required, different microbial stimuli instruct the induction of distinct Th responses (Agrawal et al., 2003). Furthermore, the suppression of T cell activation mediated by regulatory T cells (Piccirillo and Shevach, 2001), is inhibited by TLR-induced cytokine production by DC (Pasare and Medzhitov, 2003).

The importance of the contribution of DC to the induction of Th1 responses is demonstrated in genetically-different BALB/c and B6 strains (chapter three). In chapter four, LT-deficient mice and BM chimeric mice with a LT-deficient genotype, displayed susceptible phenotypes during infection with *E.vermiformis*. Although the protection against infection in bone marrow chimeric mice (wild-type recipient only) show no

requirement for LT-expression by BM-derived cells (which include DC), there may still be a microenvironmental influence dependent upon the host genotype, that is involved. For example, the development of myeloid DC is influenced by microenvironmental signals such as GM-CSF and IL4 which are both utilized in the *in vitro* generation of BM-derived DC (Inaba et al., 1993). Another component involved in DC expansion, the Fms-like tyrosine kinase 3 (Flt3) ligand, (Maraskovsky et al., 1996), generates DC of different lineages from those generated using GM-CSF (Boonstra et al., 2003; Gilliet et al., 2002). Therefore, DC development in the absence of LT-signalling between stroma and non-stromal cells may result in the defective primary immune response to *E. vermiformis* infection in LT-/- recipients which remained susceptible despite reconstitution with LT-intact B6 BM cells. A role for T cells is excluded, as the adoptive transfer of LTα-/- T cells into TCRβxδ-/- recipients showed no role for T cell-dependent LT-expression nor is T cell function affected by the lack of LT-signalling during development, as LTα-/- T cells transferred into TCRβxδ-/- protected as effectively as naïve B6 mice (chapter four). Defects in DC development or function arising as a result of deficient LT-signalling within the microenvironment may explain the defects in LT-deficient mice and hence should be examined. This chapter investigates whether there are differences between DC from LT-deficient and normal mice that might further support the hypothesis that lymphoid structural effects arising from LT-deficiencies play a major role in influencing susceptibility to *E.vermiformis* infection.

5.2. Experimental approach

Mice deficient in specific TLRs succumb to parasitic infections such as *T. gondii, L. major, Plasmodium* spp., *C. parvum* (Drennan et al., 2005; Kropf et al., 2004; Mun et al., 2003). The GPI anchors of protozoan parasites act as agonists for TLR2 signaling, inducing a MyD88-dependent signalling cascade (Campos et al., 2001; Nebl et al., 2005). TLR2 may be important as TLR2-/- mice are susceptible to *T. gondii*, an apicomplexan relative of *Eimeria*, (Campos et al., 2001) and *Eimeria* parasites have surface GPI-anchored proteins (Tabares et al., 2004) which are recognised by TLR2. Others such as TLR9, TLR4 are important in *Trypanosome brucei, L. major* parasite infections repectively (Drennan et al., 2005; Kropf et al., 2004). Therefore, a range of TLR agonists such as Pam3Cys (TLR2), LPS (TLR4), flagellin (TLR5), R848 (TLR7), CpG motifs (TLR9) and lysates of parasite oocyst were used to examine DC responses in the presence or absence of microbial stimuli. The concentrations of TLR agonist selected were based on information from published papers, manufacturers' recommended levels or dose titrations. The responses of DC from LT-deficient mice to multiple microbial stimuli were assayed to measure any altered functions in for example cytokine production, CD80 expression and antigen presentation.

5.3. Results

5.3.1. Cytokine and chemokine production

A preliminary experiment was carried out using unsorted LTα+/-β+/- and B6 BM-derived

DC stimulated with agonists for TLR 4 (LPS from S.Typhimurium), TLR 7 (R848) or TLR 9

(CpG) and analyzed for cytokine (TNFα, IL6, IL10) and chemokine (MCP-1) production. In

unstimulated cultures, the levels of cytokines/chemokine produced were very low or

negligible (data not shown). In cultures stimulated with LPS, LTα+/-β+/- BMDC produced

significantly higher levels of TNFα, IL6 and MCP-1 than B6 cultures (Figure 5.1). No

difference in IL10 levels was seen. In cultures stimulated with R848 and CpG, no differences

were seen in TNFα, IL6, MCP-1 and IL10. MCP-1 was not detected in cultures stimulated

with CpG. The levels of TNFα and IL6 in both B6 and LTα+/-β+/- cultures stimulated with

CpG were relatively lower than cultures stimulated with LPS or R848.

With cultures stimulated with oocyst lysate, cytokine levels were lower than those cultures

stimulated with LPS or R848. There were higher TNFα levels in B6 cultures stimulated with

oocyst lysate compared to LTα+/-β+/- cultures while no difference was observed with IL6 and

MCP-1 levels, and IL10 was not detected in both cultures. The differences in cytokines and

chemokine produced by LTα+/-β+/- and B6 cultures (with LPS or R848) could be explained

by different proportions of CD11c+ BMDC between cultures (LTα+/-β+/- BMDC were 69.1%

CD11c+ while B6 were 35.8% CD11c+).
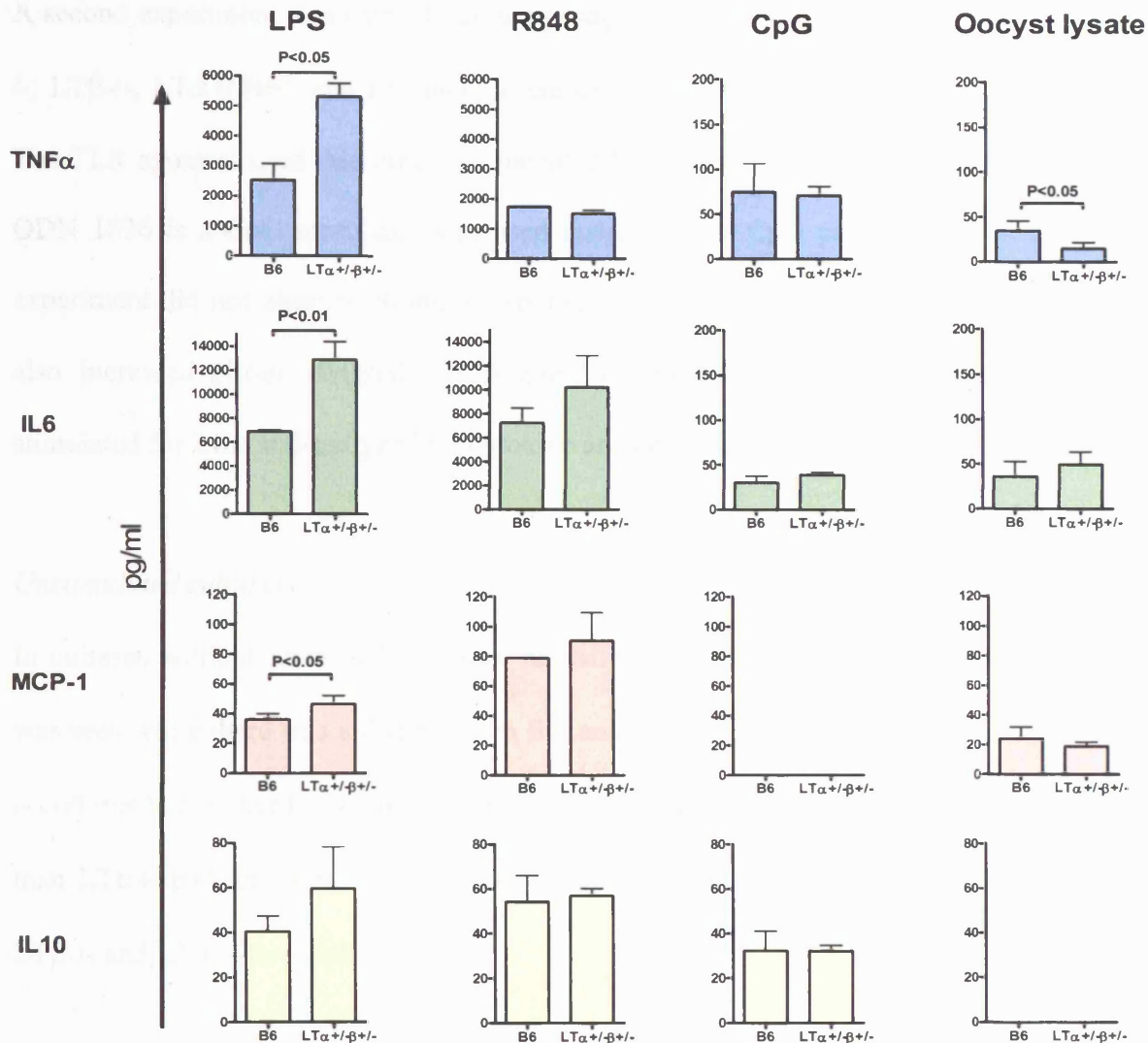
Figure 5.1 Influence of LT on BMDC cytokine production in response to microbial-stimulation.

A total of 2×10^4 BMDC from LTα+/-β+/- and B6 mice were cultured for 24 hr with or without the following TLR agonists in 200μl of R10 media: LPS (5μg/ml); R848 (1μg/ml); CpG (1μg/ml) and *E.vermiformis* oocyst lysate (10μg/ml). Supernatants from cultures were analyzed for TNFα, IL6, MCP1, IL10 using the CBA assay. Bar charts represent the mean and SD levels of cytokine produced in triplicate wells.

A second experiment was carried out using magnetically-sorted CD11c+ BMDC from LTα-/-, LTβ-/-, LTα+/-β+/- and B6 mice, to ensure uniformity in the number of CD11c+ cells. The TLR agonists used this time also included Pam3Cys (TLR 2) and ODN 1826 (TLR 9). ODN 1826 is a CpG motif and was used instead as the CpG previously used in the first experiment did not elicit as strong a response as expected. Oocyst lysate concentration was also increased (from 10μg/ml to 50μg/ml) to elicit stronger responses. Cultures were stimulated for 24hr and analyzed for cytokine and chemokine production.

Unstimulated cultures

In cultures without microbial stimulus, no difference in TNFα, IL10 levels between groups was seen while there was a difference in IL6 and MCP-1 levels (Figure 5.2). LTα-/- and LTβ-/- cultures had higher levels of IL6 than B6 cultures. LTβ-/- BMDC also had higher IL6 levels than LTα+/-β+/- cultures. MCP-1 levels were higher in both LTα-/- and B6 cultures than LTβ-/- and LTα+/-β+/- cultures.

Cultures with Pam3Cys

There was no difference in TNFα and IL6 levels when comparing stimulated and unstimulated cultures. IL10 levels were higher than cultures without stimulus. No difference in MCP-1 levels was seen among the different groups except in LTα-/- cultures where MCP-1 levels were very much higher.

Cultures with LPS

In cultures stimulated with LPS, LTα-/- and B6 cultures produced less TNFα than LTβ-/- while no difference with LTα+/-β+/- was noted. IL6 levels in both LTα+/-β+/– and LTβ-/- cultures were higher than LTα-/- and B6 cultures. The opposite was seen with MCP-1 levels which were higher in LTα-/- and B6 cultures than LTα+/-β+/– and LTβ-/- cultures. IL10 levels increased in all cultures but no significant difference was seen among the different groups.

Cultures with R848

In cultures stimulated with R848, TNFα levels in all groups were higher than LTα-/- cultures. IL6 and IL10 levels were higher in LTβ-/- and LTα+/-β+/- cultures than both LTα-/- and B6 cultures. No changes in MCP-1 levels were seen, as levels in all cultures remain similar to those in unstimulated cultures

Cultures with ODN 1826

With ODN 1826, TNFα levels increased in all groups but were highest in LTβ-/- mice. IL6 levels increased in all groups with the highest seen in LTα+/-β+/- cultures. No difference was observed with MCP1 in all cultures compared with cultures without stimulus. IL10 levels increased but were highest in both LTβ-/- and LTα+/-β+/- cultures.

Cultures with oocyst lysate

In cultures stimulated with oocyst lysate, there was no significant difference in TNFα, IL6, MCP1 and IL10 levels in all groups when compared to cultures given no stimulus.
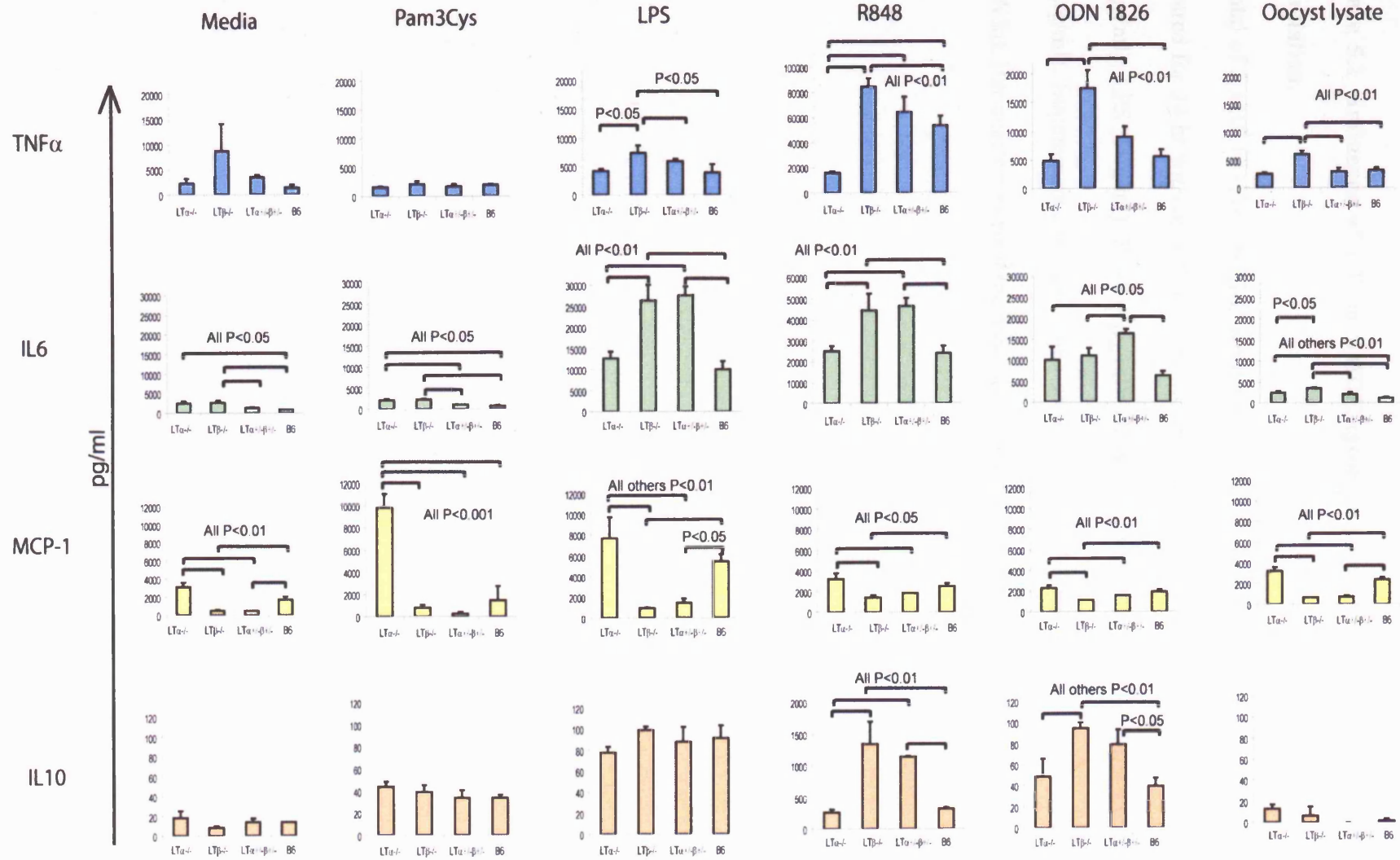
Figure 5.2 Influence of LT on BMDC cytokine production in response to microbial-stimulation.

A total of 5 x10^4 CD11c+ BMDC were magnetically purified using anti-CD11c microbeads and cultured for 24 hr with or without the following TLR agonists in 200μl of R10 media: Pam3Cys (1μg /ml); LPS (5μg/ml); R848 (1μg/ml); ODN 1826 (500nM) and *E.vermiformis* oocyst lysate (50μg/ml). Supernatants from cultures were analyzed for TNFα, IL6, MCP1, IL10 using the CBA kit. Bar charts represent the mean and SD levels of cytokine produced in triplicate wells.

DC expression of IL12 in response to stimuli

IL12 is an important component of the Th1-response and was examined through intracellular cytokine staining carried out on BMDC stimulated with various TLR agonists. Stimulation with LPS, flagellin, Pam3Cys, ODN 1826 and oocyst lysate with or without CD40-ligation was carried out on BMDC cultures. CD40 ligation increases the antigen presentation and costimulatory capacity of DC (Caux et al., 1994; Delamarre et al., 2003) and is required for induction of adaptive immunity by DC (Fujii et al., 2004). When CD40 interacts with CD40L on T cells, the high levels of bioactive IL12 produced together with the upregulation of CD80 or CD86 on DC results in T cell proliferation and IFNγ production (Cella et al., 1996).

There was no difference in the proportions of IL12p40/p70+ CD11c+ cells in unstimulated BMDC of mice of all genotypes (medium) (Figure 5.3). LPS stimulated the highest proportion of IL12p40/p70+CD11c+ cells in all cultures but a higher proportion of IL12p40/p70+ CD11c+ cells was observed in LTα-/- and LTα+/-β+/- cultures compared with LTβ-/- cultures (P<0.05). Only LTα-/- cultures had a significantly higher proportion of IL12p40/p70+ CD11c+ cells than B6 cultures stimulated with LPS. There was a higher proportion of IL12p40/p70+ CD11c+ cells in B6 cultures stimulated with flagellin compared with LTβ-/- cultures (P<0.05) (LTα-/- is not compared here because there is only one well). There were no significant differences in the proportion of IL12p40/p70+ CD11c+ cells in all cultures stimulated with Pam3Cys compared with unstimulated cultures. Oocyst lysate stimulated increased proportions of IL12p40/p70+ CD11c+ cells in all groups although the increase seen in B6 cultures was marginal. LTα-/- cultures showed the highest proportion of IL12p40/p70+ CD11c+ cells and were significantly higher than all others (P<0.05). The

proportions in both LTβ-/- and LTα+/-β+/- were significantly higher than B6 cultures (P<0.05). Upon CD40 ligation and stimulation with oocyst lysate, a higher proportion of IL12p40/p70+CD11c+ cells was seen in all cultures compared with cultures stimulated with oocyst lysate. Proportions were highest in LTα-/- and LTα+/-β+/- cultures followed by LTβ-/- culures and proportions in all three cultures were significantly higher then B6 cultures (P<0.01). However the greatest increase of 1.6% was seen in LTα+/-β+/- cultures compared to cultures stimulated with oocyst lysate only.

5.3.2. CD80 upregulation in response to microbial stimuli.

DC maturation is associated with an increased ability to activate T cells during inflammatory responses while immature DC are closely-associated with T cell anergy (Banchereau and Steinman, 1998; Lutz et al., 2000; Steinbrink et al., 1997). Therefore any maturation defect may influence downstream immune responses. There was no apparent defect in CD40 signalling as the data presented above show that the proportion of IL12-expressing BMDC from LT-deficient mice increased in response to CD40 ligation. However, CD40 is not the only marker for maturation as the costimulatory molecule markers, CD80 and CD86 are also upregulated during DC maturation (Banchereau and Steinman, 1998). Therefore, a preliminary experiment was carried out using LTα+/-β+/- and B6 BMDC to examine CD80 expression following stimulation with LPS, R848 or CpG. Upregulation of CD80 was gauged by the ΔMFI level which was obtained by subtracting the mean MFI of control cultures (medium) from the mean MFI of stimulated cultures. There was upregulation of CD80 in cultures stimulated with either LPS or R848 (Figure 5.4). Little upregulation of CD80 was

Figure 5.3   Influence of LT on IL12 production by BMDC in response to microbial-stimulation.

Intracellular cytokine staining was carried out on 5 x10^5 BMDC which were cultured for 24 hr with or without the following TLR agonists in 200μl of R10 medium: Pam3Cys (1μg/ml); S. typhimurium LPS (5μg/ml); S. typhimurium flagellin (50ng/ml), ODN 1826 (500nM), E.vermiformis oocyst lysate (50μg/ml) and where appropriate, anti-mouse CD40 (5μg/ml). In the last 6 hr, Brefeldin A was added to stop cytokine export from cells. FACS plots are gated on CD11c+ cells and the values represent the mean and SEM of the proportion of CD11c+ IL12p40/p70+ cells from triplicate wells. *, LTα-/- sample had only 1 well.

seen in cultures stimulated with CpG. In cultures stimulated with R848, CD80 upregulation was higher in B6 cultures than LTα+/-β+/- cultures. This gave rise to the hypothesis that a difference in maturation between DC from LT-deficient mice may affect their response during infection. Therefore further testing of BMDC from LTα-/-, LTβ-/- and LTα+/-β+/- mice was carried out.

In order to ensure uniformity, BMDC were magnetically-sorted for CD11c+ cells and these were subjected to stimulation with LPS, ODN 1826, Pam3Cys, R848 and oocyst lysate, and analyzed for CD80 upregulation. In unstimulated cultures (medium), CD80 MFI levels were higher in LTα-/- and B6 cultures than in LTα+/-β+/-and LTβ-/- cultures (Figure 5.5). CD80 expression in LTα-/- cultures stimulated with Pam3Cys was slightly lower than other cultures, which remained relatively unchanged.

ΔMFI levels of CD80 expression in LTα-/- and B6 cultures stimulated with ODN 1826 was higher than LTβ-/- cultures. CD80 expression in LTα-/- cultures was also higher than LTα+/-β+/- cultures. In cultures stimulated with LPS, LTα-/- and B6 cultures were significantly higher than LTβ-/- cultures. With R848 stimulation, the increase in CD80 expression in LTα-/-, LTβ-/- and LTα+/-β+/- cultures was similar but lower than B6 cultures. With cultures stimulated with oocyst lysate, CD80 expression was highest in LTα-/- and B6 cultures.

When comparing the preliminary experiment (Figure 5.4) with the experiment above (Figure 5.5), it was confirmed that no differences were seen between LTα+/-β+/- and B6 BMDC

stimulated with LPS while differences were seen between LTα+/-β+/- and B6 BMDC

stimulated with R848. In the second experiment, there was a difference in CD80 upregulation

in LTβ-/- cultures stimulated with LPS, ODN 1826 and oocyst lysate (when compared with

B6 and LTα-/-). LTα-/- BMDC showed similar CD80 ΔMFI levels to B6 in cultures

stimulated with LPS, ODN 1826 and oocyst lysate, and do not display the same difference

seen in LTβ-/- cultures. However it would be necessary to repeat the experiment to confirm

such observations.

5.3.3. Induction of T cell proliferation

The presentation of antigen by mature DC to T cells is essential to the development of T cell-

dependent immunity against *E. vermiformis* infections. Hence, the ability of mature BMDC

to present antigen to T cells was examined. Eight day-cultured and CD11c+ BMDC sorted

from LT-deficient or B6 mice were subjected to overnight stimulation with TLR agonists and

pulsed with OVA 323-339 peptide to examine their ability to present antigen to OT-II TCR

transgenic T cells. No difference in T cell proliferation was seen in wells containing $2x10^2$ or

$2x10^3$ DC from LTα-/-, LTβ-/-, LTα+/-β+/- and B6 mice (Figure 5.6). T cell proliferation in

wells containing of $2x10^5$ BMDC was lower than wells containing $2x10^4$ DC and may have

been a result of T cell proliferation occurring prior to addition of ^{3}H-thymidine (data not

shown).

Small differences were observed in wells containing $2x10^4$ BMDC. In wells containing $2x10^4$

DC pulsed with OVA 323-339 only (without a TLR agonist), LTβ-/- BMDC induced the

lowest levels of proliferation (P<0.05) while levels of proliferation were similar with LTα-/-,

LTα+/-β+/- and B6 BMDC (Figure 5.6A). Maturation of BMDC with ODN 1826 or LPS
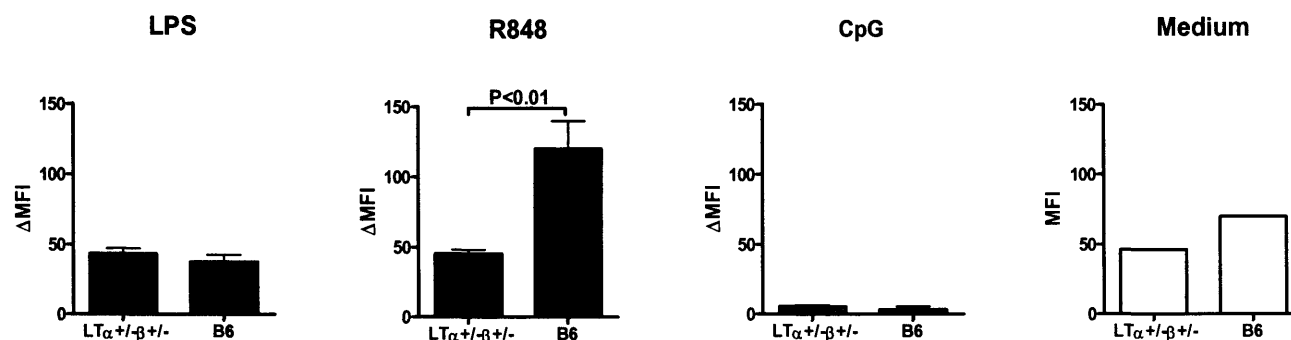
Figure 5.4 Influence of LT on CD80 expressed by BMDC during microbial-stimulation.

A total of 2×10^4 BMDC from LTα+/-β+/- and B6 mice were cultured for 24 hr with or without the following TLR agonists in 200μl of R10 medium: LPS (5μg/ml); R848 (1μg/ml); CpG (1μg/ml). BMDC were gated on CD11c+ cells and analyzed for CD80 expression by FACS. ΔMFI represents the MFI of stimulated cultures corrected with the MFI of unstimulated cultures (medium). Bar charts represent the mean and SEM of triplicate wells.
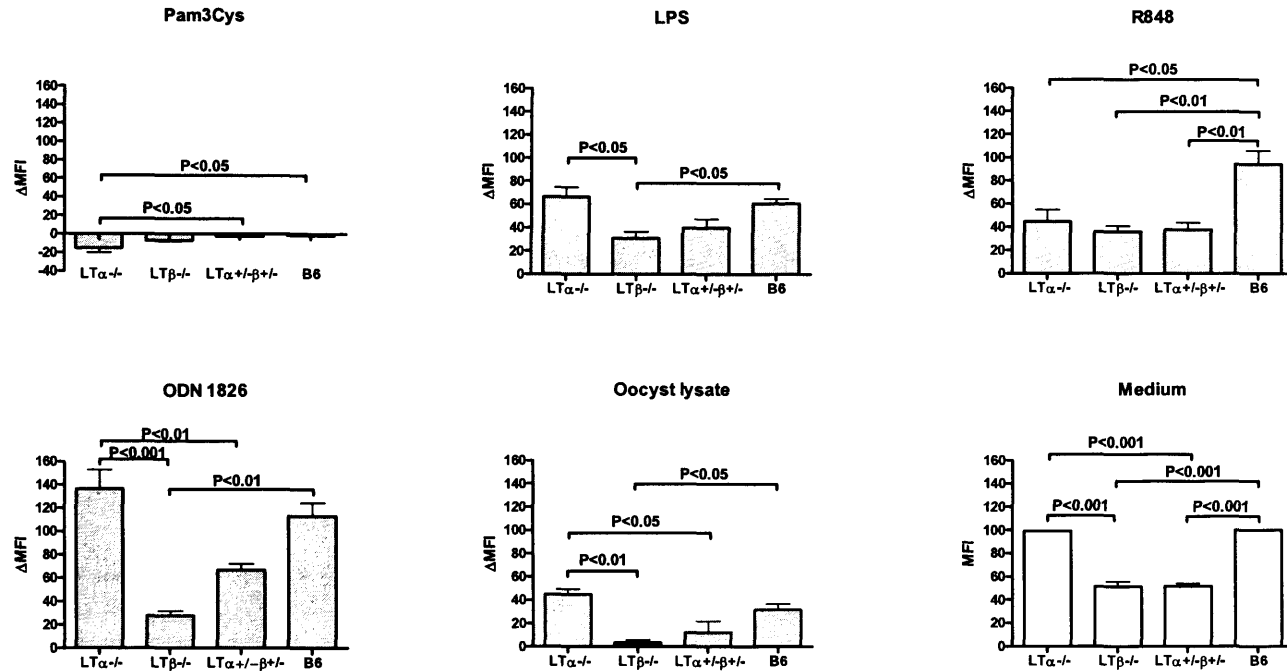
Figure 5.5 Influence of LT on CD80 expressed by BMDC during microbial-stimulation.

A total of 5×10^4 CD11c+ BMDC were magnetically-purified using anti-CD11c microbeads and cultured for 24 hr with or without the following TLR agonists in 200μl of R10 medium: Pam3Cys (1μg/ml); LPS (5μg/ml); R848 (1μg/ml); ODN 1826 (500nM) and *E.vermiformis* oocyst lysate (50μg/ml). CD11c+ BMDC were analyzed for CD80 expression by FACS. ΔMFI represents the MFI of stimulated cultures corrected with the MFI of unstimulated cultures (medium). Bar charts represent the mean and SEM of triplicate wells.

showed increased T cell proliferation in wells containing 2×10^4 BMDC from LTα-/- and B6

mice. There was a small increase in T cell proliferation only in wells containing 2×10^4 LTβ-/-

BMDC stimulated with ODN1826 but not LPS. No difference in T cell proliferation was seen

in wells containing 2×10^4 LTα+/-β+/- BMDC stimulated with ODN 1826 or LPS compared to

wells without stimulus. In all wells containing 2×10^4 LTβ-/- BMDC with or without stimulus,

T cell proliferation levels were lowest and significantly different to LTα-/- and B6 wells

($P < 0.05$). The lack of change in response to treatments with ODN 1826 or LPS in LTα+/-β+/-

or LTβ-/- mice may indicate possible contamination (perhaps LPS) in unstimulated cultures or

that there is in fact a difference in the response of LTβ-/- or LTα+/-β+/- BMDC to microbial

stimuli.

5.3.4. MyD88 mRNA levels in response to microbial stimulation

MyD88-dependent pathways are required for resistance against *T. gondii* (Scanga et al., 2002),

C. parvum (Rogers et al., 2006) and *T. brucei* (Drennan et al., 2005). TLR 2, 4 and 9 have

MyD88-dependent pathways and because responses to microbial stimuli may be different in

LT-deficient BMDC, MyD88 mRNA expression was analyzed in BMDC stimulated with LPS,

ODN 1826 and oocyst lysate. Although MyD88 is not known to be transcriptionally regulated,

its levels are elevated in the presence of LPS and IFNγ (Tamai R et al., 2002; Tamai R et al.,

2003). Hence, a comparison of MyD88 mRNA levels between LT-deficient and intact mice

would suggest differences in the response to TLR agonists and perhaps indicate that there were

indeed differences in maturation in LTβ-/- BMDC.

A comparison was made between samples with and without stimuli. There was no upregulation of MyD88 mRNA in response to oocyst lysate while with ODN 1826, there was no change in mRNA levels in LTα-/- BMDC but downregulation was seen in B6 and LTβ-/- BMDC (Figure 5.7). The application of LPS stimulated the upregulation of MyD88 mRNA in all cultures.

5.3.5.   BMDC migration

The chemokine receptor for CCL21 and CCL19, CCR7 is upregulated by maturing DC, allowing them to migrate to lymphoid tissues. Hence, BMDC from LT-deficient mice were tested for their chemotactic response to CCL21, expressed by endothelial cells in lymphoid tissues. A higher percentage of cells from LTα-/- cultures than LTβ-/- cultures migrated without CCL21 (Figure 5.8A). Although there were differences in the migration of cells from LTα-/-, LTβ-/- and B6 cultures stimulated with CCL21, the differences were not significant when the levels of background migration (ie. values derived from wells without CCL21) were taken into consideration and subtracted. In a separate experiment, the migration of cells from LTα+/–β+/- and B6 cultures with or without CCL21 was similar (Figure 5.8B).
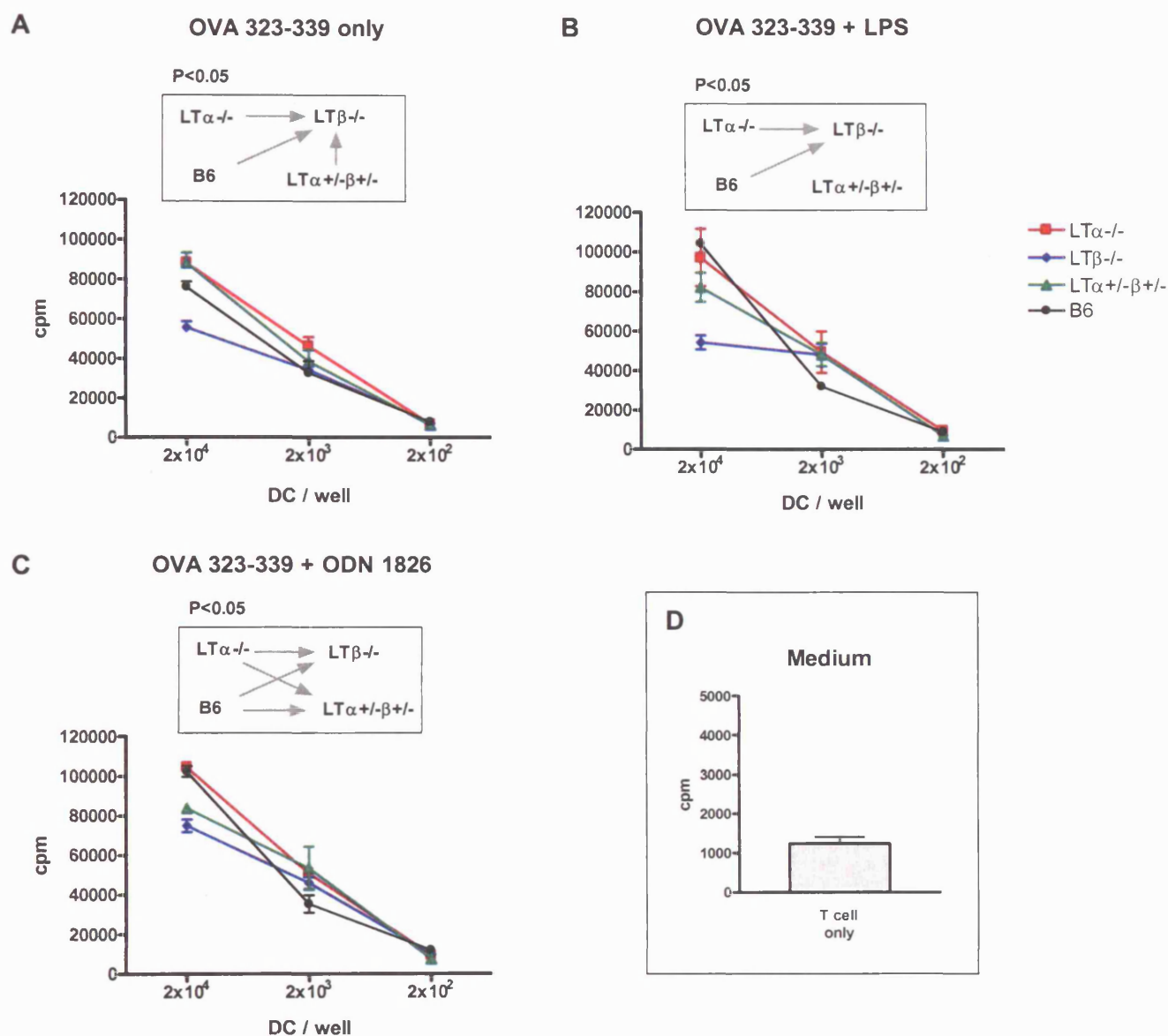
Figure 5.6 Analysis of peptide presentation by stimulated LT-deficient BMDC.

8 day old cultured CD11c+ BMDC from LT-deficient and B6 mice were magnetically-purified and cultured overnight A) without stimulus or with B) LPS (1μg/ml) or C) ODN 1826 (500nM) and pulsed for 4hr with 10μg/ml of OVA 323-339 peptide the following day. D) Wells containing T cells only in medium. After washing, titrated numbers of CD11c+ BMDC were cultured with 2×10^5 OT-II T cells (depleted of MHC class II+ cells using

magnetic microbeads) for 72 hr and analyzed for ^{3}H-thymidine incorporation. Results represent the mean and SEM of triplicate wells. $\rightarrow$ indicates significantly higher levels (P<0.05) than the group to which the arrow points to and refers to only wells containing 2×10^4 DC.
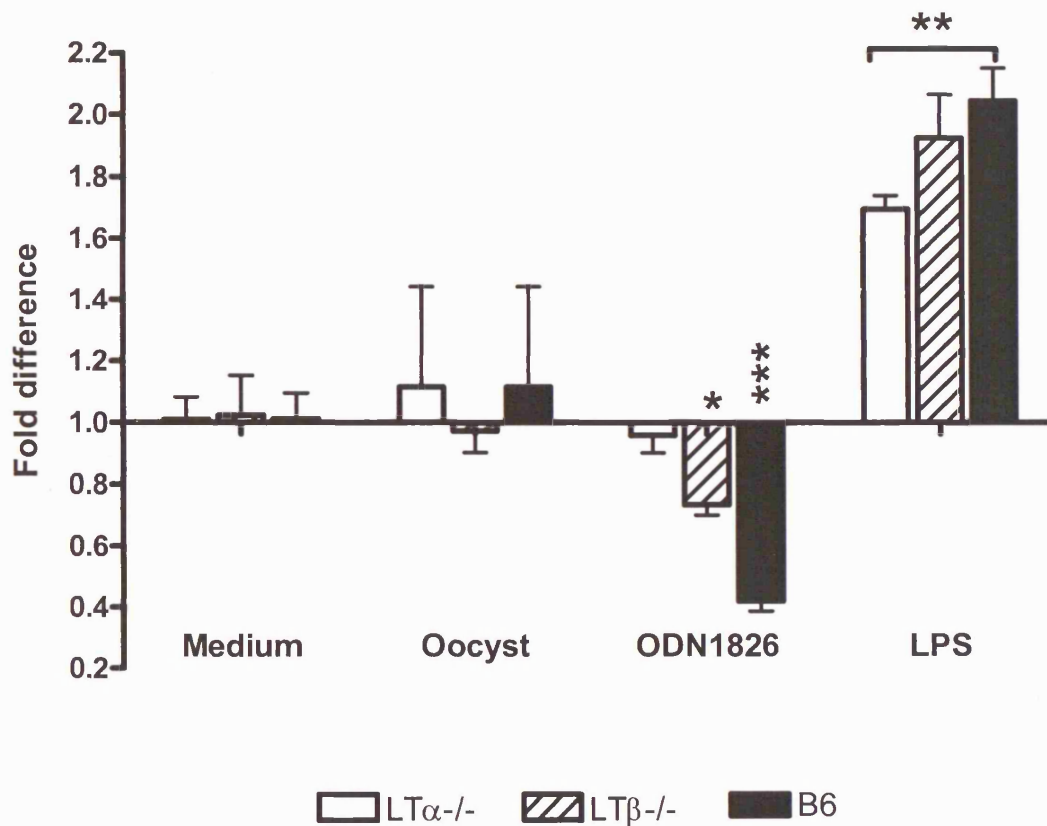
Figure 5.7 Influence of LT on MyD88 expression during microbial stimulation.

MyD88 mRNA was analyzed in LTα-/-, LTβ-/- and B6 BMDC cultures stimulated with TLR agonists. 5×10^4 CD11c+ BMDC (magnetically sorted) were cultured for 24 hr with or without the following TLR agonists in 200μl of R10 medium: Pam3Cys (1μg/ml); LPS (5μg/ml); ODN 1826 (500nM) and *E.vermiformis* oocyst lysate (50μg/ml). The data represents the mean and SEM of quadruplicate wells. MyD88 mRNA was normalised to HPRT mRNA levels and depicted as fold differences in MyD88 mRNA expression (calculated as described in chapter two). *, ** and *** refer to significant difference to wells without stimulus (medium) and represent P<0.05, P<0.02 and P<0.001 respectively. All BMDC used were >95% CD11c+.
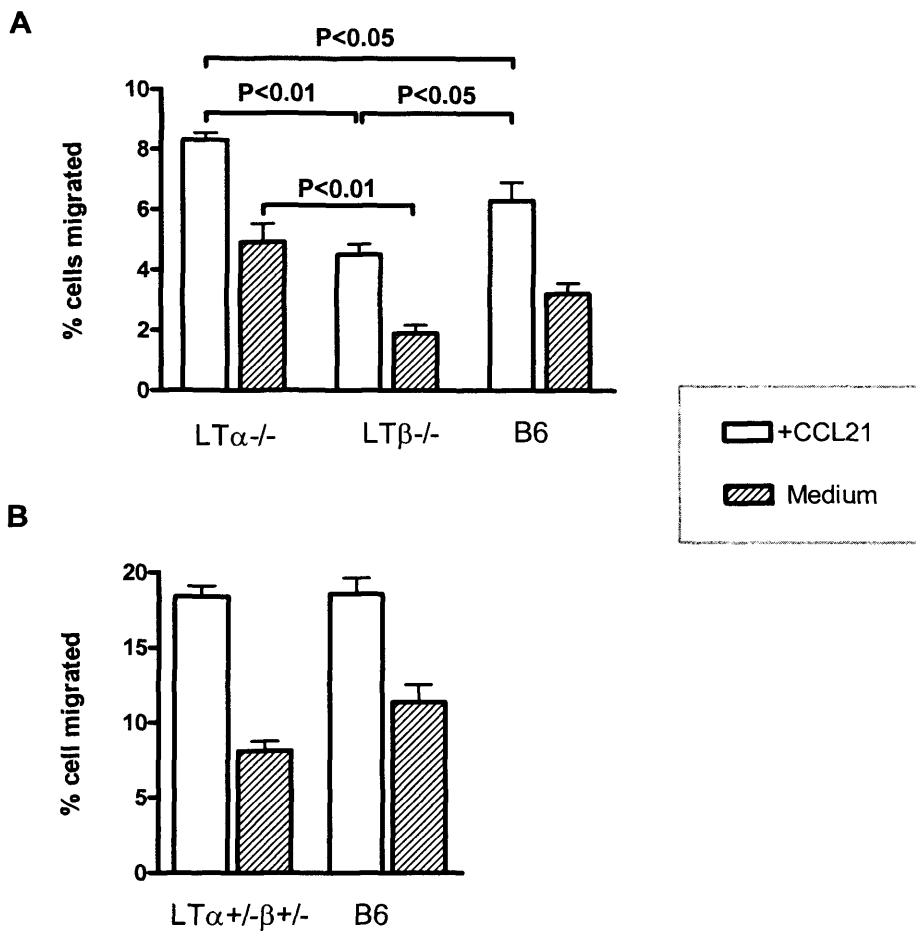
Figure 5.8 Influence of LT on BMDC chemotaxis towards CCL21.

A total of 2x10⁵ BMDC (either magnetically sorted for CD11c or unsorted) were cultured in transwell inserts (Pore size: 3.0μM) in 700μl of R10 medium with or without CCL21 (1μg/ml) for 4 hr in 24-well plates. All BMDC if magnetically sorted were >95% CD11c+. The percentage of cells migrating across the transwell membrane was calculated as: Total number of cells migrated / 2x10⁵ x 100%. A) Migration of CD11c+ sorted BMDC from LTα-/-, LTβ-/- and B6 mice. B) Migration of unsorted BMDC from LTα+/-β+/- and B6 mice. Results represent the mean and SEM of 4-5 wells from each mouse strain.

5.4 Discussion

The priming of naïve T cells is a primary role of DC which are capable of stimulation at a ratio of 1 DC: 10 T cells (Hopken et al., 2005). The outcome of the adaptive immune response is shaped by the type of presenting DC which in turn is influenced by PRR-stimulation by microbial stimuli. Pathogen recognition receptors such as TLRs trigger DC maturation and the secretion of a milieu of cytokines and chemokines (Reis e Sousa et al., 1999) which influence the type of Th response developed: IL12 is usually associated with Th1 responses (Park et al., 2000; Skeiky et al., 1995); IL4 and IL5 favour Th2 responses (Swain et al., 1988); IL10 promotes Treg function (De Smedt et al., 1997; Rutella et al., 2004). Other factors influencing DC maturation include CD40 engagement and TNF (Banchereau and Steinman, 1998; Cella et al., 1996). The type of Th responses is important in the eradication of different pathogens where Th1-mediated responses and Th2-mediated responses effectively control intracellular pathogens and extracellular pathogens respectively. Hence, turning on the correct type of T cell response is essential in ensuring effective immunity against an infection.

The work presented here addressed the possibility that defects in BMDC attributable to a LT-deficient microenvironment, may contribute to the high susceptibility displayed by LT-deficient mice during infection with *E. vermiformis*. There is no major role for LT production by BM-derived cells (as demonstrated in chapter four) where the infection of BM chimeric mice showed that resistance to *E.vermiformis* was influenced by existing lymphoid structures. However, it is possible that a LT-deficient microenvironment (eg. stroma) may influence DC development and compromise their ability to induce effective adaptive

immunity. Such a defect may explain the inability of LTα-/- and LTβ-/- mice to maintain long term memory responses as these mice showed signs of parasite reproduction (very small levels of oocysts produced) during re-infection in contrast to B6 mice which remained protected (chapter four) when rechallenged after more than eight months. There may be a minor requirement for LT as BM chimeric B6 mice reconstituted with LTα+/-β+/- BM were also marginally less resistant (P<0.05) than B6 mice reconstituted with B6 BM (chapter four) (although both remained more resistant than LTα+/-β+/- BM recipients). These minor lapses in immunity hinted at a subtle influence of LT on the induction of adaptive immunity and may be reflected in a defect in DC maturation.

Unfortunately, it is difficult to draw any conclusions from the cytokine experiments (Figure 5.1 and 5.2) as the two experiments are incomparable and require repeating due to the *in vitro* nature of the experiments (repeated experiments were regrettably not done due to time constraints). The proportions of CD11c+ cells differed between B6 and LTα+/-β+/- BMDC cultures in the first experiment from (Figure 5.1) which makes the data non-comparable to the second experiment (Figure 5.2). Despite the magnetic-sorting of CD11c+ cells, no clear conclusion can be derived from the *in vitro* analysis of cytokines. The cytokine responses in LT-deficient and B6 BMDC were varied, and it was difficult to spot any obvious defects of biological significance despite some hints of differences in cytokine production. All BMDC from LT-deficient mice have the capacity to produce increased levels of TNFα, IL6 and IL10 in response to different TLR agonists (ie. LPS, R848, ODN 1826) although TNFα levels in LTα-/- cultures do not seem to reach the high levels seen in B6 cultures. MCP-1 levels were lower in both LTβ-/- and LTα+/-β+/- cultures stimulated with LPS and the reduced MCP-1

levels seem to correspond with CD80 levels in LTβ-/- cultures stimulated with LPS, ODN

1826 and oocyst (but not LTα+/-β+/- cultures) (Figure 5.5). Mature DC are known to

produce MCP-1 *in vitro* (Sallusto et al., 1998; Zhou and Tedder, 1995) and perhaps the less

mature state of LTβ-/- BMDC may explain the lower production of MCP-1. However this

was not reflected in cultures stimulated with ODN 1826 where although CD80 levels in LTβ-

/- were lower than LTα-/- and B6 cultures, there were no differences in the MCP-1 levels

produced, compared to cultures without stimulus. Therefore it is not possible to relate CD80

upregulation with cytokine production without further experimentation. Variability has been

observed between different studies, all of which report conflicting results and may be a

product of different isolation methods and culture conditions (Koch et al., 2005), suggesting

caution when dealing with *in vitro* work.

CD40L-/- mice showed impaired protective T cell responses (Grewal et al., 1995) and

defective CD40-CD40L interaction may explain the lack of long-term memory seen in

rechallenged LTα-/- and LTβ-/- mice. However, stimulation through CD40-CD40L

interaction enhanced IL12 production in all cultures (Figure 5.3). LT-deficient BMDC have

the potential to induce Th1 responses through IL12 as IL12+ CD11c+ cells were present in

all cultures stimulated with LPS, flagellin and oocyst lysate. The proportions of IL12+

CD11c+ cells differ but such differences were small and may not have any biological

significance although experiments have to be repeated to confirm this.

In the literature, splenic DC and BMDC from LTα-/- mice maintain normal T cell

stimulatory functions and comparable maturation phenotypes (measured by MHC class II

and CD86 expression) to wild-type B6 mice (Koch et al., 2005; Ritter et al., 2003). The differences in T cell proliferation levels observed in LTβ-/- or LTα+/-β+/- cultures when compared with LTα-/- and B6 cultures were less than a differential factor of 2 which is commonly viewed as a gauge for significant differences. Furthermore, no differences were seen in wells containing 2×10^3 or 2×10^2 DC from all LT-deficient and B6 groups. Ideally, carrying out a similar experiment using DC in antigen presentation but over variable time-points, would help support any differences even if the differential factor was less than 2. Therefore here, no difference can be found between LT-deficient and B6 BMDC in their antigen presentation function. The maturation of DC is associated with an increased ability to stimulate T cell proliferation however the inability to enhance T cell proliferation after microbial stimulation cannot be concluded without a repeat and there may also be a problem associated with LPS-contamination of samples which may mask any potential defects if any.

MyD88-/- DC fail to induce antigen-specific Th1 responses and do not mature due to deficient TLR-signalling (Schnare et al., 2001). However, MyD88 mRNA levels in LT-deficient BMDC do not differ from intact B6 BMDC in cultures stimulated with LPS and oocyst lysate although LTα-/- BMDC show no changes in response to ODN 1826. Any possible difference in maturation response to LPS in LTβ-/- BMDC is not influenced by MyD88 since LPS stimulates cytokine production from DC in a MyD88-dependent manner and DC-maturation depends on TLR4 and not MyD88 (maturation is seen in MyD88-/- but not TLR4-/- mice) (Kaisho and Akira, 2001). Maturation by CpG (TLR9 agonist) is dependent on MyD88 (eg. (Edwards et al., 2002) but MyD88 mRNA levels from LTβ-/- BMDC in response to ODN 1826, were downregulated as with B6 BMDC. Therefore any

maturation defect (if any) in LTβ-/- BMDC, is not MyD88-related. No significant differences in terms of antigen presentation (T cell proliferation assay) and DC maturation (CD80 expression) were seen in LTα-/- BMDC compared to B6 BMDC despite a slightly greater downregulation of MyD88 mRNA in response to ODN 1826.

All LT-deficient BMDC showed no differences when migrating in response to CCL21. Moreover, comparable expression of CCR7 can be found between matured BMDC of LTα-/- mice and B6 mice (Ritter et al., 2003). Therefore delayed responses seen in LT-deficient mice during infection with *E. vermiformis* are not the result of defective DC migration (this is further examined in chapter six).

Overall, only the following can be concluded:

1. The *in vitro* BMDC culture system variably generates CD11c+ cells therefore purification of CD11c+ cells is necessary.

2. LT-deficient DC are able to produce TNFα, IL6, IL10, IL12, MCP1 in response to stimuli, including the oocyst lysate which can be considered a DC stimulus.

3. There is interaction between CD40 and CD40L in LT-deficient DC.

4. LT-deficient DC migration in response to CCL21 is intact.

5. There is no evidence for a major difference between LT-deficient and wild-type DC.

6. Finally, all the experiments presented here have to be repeated.

Chapter Six

Investigating the influence of PP on the induction of Th1 immune responses during infection

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6.1 Introduction

Previous data demonstrated that absence of PP attributed to the slower induction of immune responses in both the MLN and small intestine (chapter four). The LTα+/-β+/-mice displayed an intermediate level of susceptibility to *E.vermiformis* infection compared with mice bearing more severe lymphoid tissue deficiencies (LTα-/-, LTβ-/-) and mice deficient in T cells (TCRβxδ-/-). The strategic location and lymphoid organisation of PP make them important sites for the induction of adaptive immunity against gut infections. At the same time, regulatory mechanisms are well-placed to ensure that pro-inflammatory responses are tightly controlled in the PP environment which is exposed to an immense load of antigen-stimuli derived from commensal microbes and ingested food in the intestinal lumen.

Many studies on immune responses associated with GALT structures have been carried out in the context of oral tolerance and some show conflicting requirements for PP in the induction of oral tolerance (Fujihashi et al., 2001; Spahn et al., 2002). The induction of responses in the PP during enteric infections has been described (eg. (Djamiatun and Faubert, 1998; Fan et al., 1998; McSorley et al., 2002)) but few studies emphasise any requirements for PP, suggesting PP to play more of an accessory role (Mowat, 2003). The relative contributions against an enteric infection, made separately by PP and MLN remain poorly defined. The work presented here forms an extension from chapter four, examining the differential requirements for PP and MLN and the cellular changes associated with each tissue during *E.vermiformis* infection.

6.2   Experimental approach

A series of *in vivo* and *in vitro* experiments were carried out to examine MLN and PP

responses from either LTα+/-β+/- or B6 mice. The responses (eg. T cell proliferation,

cytokine expression) occurring in MLN and PP, are compared by analyzing T cell

proliferation using BrdU-incorporation assays and cytokine expression at the RNA and

protein levels. The adoptive transfer of cells was carried out to examine the roles of

tissue-specific cells (PP and MLN from naïve or un-infected donors) and DC subsets

during infection. *Ex vivo* studies such as ^{3}H-thymidine proliferation assays and cytokine

analysis at RNA and protein levels were carried out at specific time-points to examine

the antigen presentation properties and cytokine responses of different DC subsets. The

use of OT-II TCR transgenic mice also permitted the examination of cellular function

more specifically in the absence of a TCR-transgenic mouse specific for any

E.vermiformis component. OVA protein or MHC class II-restricted OVA (323-339)

peptide was used *in vivo* or *in vitro* to examine antigen presentation by DC to OT-II T

cells. Mice were given OVA protein in their drinking water during infection or DC were

pulsed with OVA (protein or peptide) for the experiments undertaken.

6.3   Results

6.3.1   The absence of PP results in delayed T cell responses

Delayed Th1 responses in PP-deficient LTα+/-β+/- mice were previously defined in

chapter four where a delay of approximately two days in the upregulation of IFNγ and

increase in gut-homing CD4+ T cells was observed in the MLN. This delay was also

reflected in the upregulation of IFNγ in the ileum of LTα+/-β+/- mice. Here, the *in situ*

levels of T cell proliferation were examined in the MLN of LTα+/-β+/- and B6 mice

which were given a nucleotide analogue, BrdU, in their drinking water. The incorporation of BrdU occurs in dividing cells and is tracked by a fitc-conjugated antibody to BrdU. It was clear that the proliferating MLN immune response from both LTα+/-β+/- and B6 mice consisted mainly of CD4+ cells which represented a higher proportion of BrdU+ cells than CD8+ cells (Figure 6.1A). The majority of CD4+ and CD8+ cells shown here are likely to be T cells despite the lack of CD3ε staining due to limited flow cytometry channels available. At 4 DPI, the proportion of BrdU+ CD4+ cells was higher in B6 mice than LTα+/-β+/- mice (P<0.05) (Figure 6.1A) despite close differences in values (see also histogram). However, by 6 DPI, both mice show similar proportions of BrdU+ CD4+ cells. While the proportion of BrdU+ CD4+ cells remained constant throughout from 6 DPI onwards in B6 mice, there was a steady increase in BrdU+ CD4+ cell proportions in LTα+/-β+/- mice. There were small increases in BrdU+ CD8+ cell proportions in both B6 and LTα+/-β+/- mice and apart from 4 DPI when there was a slightly higher proportion of BrdU+ CD8+ cells in B6 mice (P<0.05), overall, there were no significant differences between the two strains.

In the PP, the proportion of BrdU+ CD4+ cells was higher than BrdU+ CD8+ cells (Figure 6.1B). A significant increase was seen from 4 DPI onwards in both CD4+ and CD8+ cells (P<0.05) although there was a slight drop in BrdU+CD4+ cell proportions at 8 DPI.

During steady state, the cytokines expressed in the PP microenvironment are more Th2-like but may differ during the course of infection. The Th1-inducing signals include IL12, IL18 which are produced by DC (Okamura et al., 1995; Stoll et al., 1998; Trinchieri, 1993) and T-bet which induces the polarization of Th1 CD4 responses and is

expressed in parallel with IFNγ (Szabo et al., 2000). In the PP of infected B6 mice, the levels of IL12p40 and Tbet mRNA increased at 6 and 8 DPI while no increase in IL18 mRNA was detected (Figure 6.2). The levels of IFNγ mRNA increased at 4 DPI (Figure 6.3A) and *ex vivo*-stimulated PP cells showed increased levels of IFNγ and TNFα at 6 DPI and 8 DPI in the supernatants of stimulated cultures (Figure 6.3B). The levels of IL4 remained relatively low and unchanged. Overall, the PP microenvironment during *E. vermiformis* infection showed a trend towards Th1-responses.

6.3.2 Independent development of immunity in PP and MLN

Both PP and MLN are considered functionally as inductor and effector sites and it was important to examine the extent of immunity developed at these sites independently. A total of 2 x 10^6 PP cells and 2 x 10^7 MLN cells were isolated from day 8 post-infected B6 mice and adoptively transferred by intraperitoneal injection into naïve TCRβxδ-/- mice. The number of PP and MLN cells selected was based on the approximate numbers normally present in intact B6 mice. Both MLN and PP cells conferred similar levels of protection in the respective TCRβxδ-/- recipients and greater reduction of oocyst output than TCRβxδ-/- mice given no cells (Figure 6.4). Moreover, oocyst output was reduced to a greater degree in TCRβxδ-/- recipients given PP or MLN cells when compared with unmanipulated B6 mice. A delay in the MLN immune response was evident in LTα+/-β+/- mice but it was possible that the quality of this immune response was less effective than that induced in the B6 MLN. In order to test the efficiency of induced responses in LTα+/-β+/- mice, 2 x 10^7 MLN cells from LTα+/-β+/- mice (8 days post-infected) were adoptively transferred into TCRβxδ-/- mice which were infected alongside TCRβxδ-/- mice which received MLN or PP cells from

A
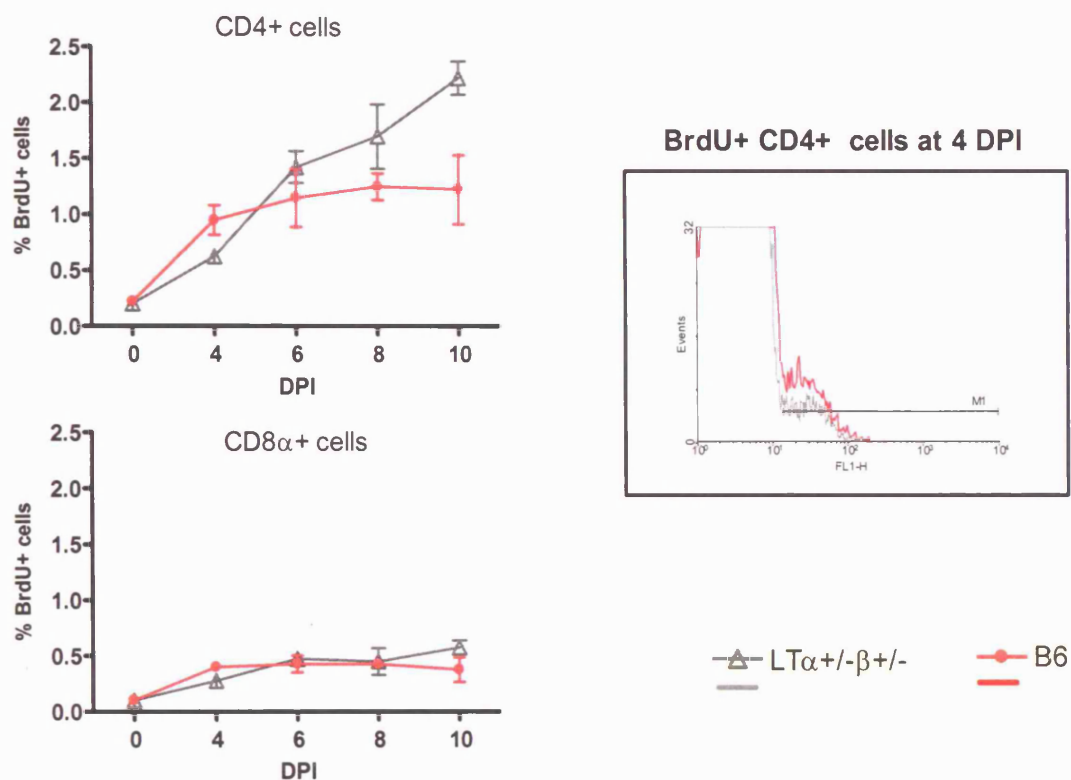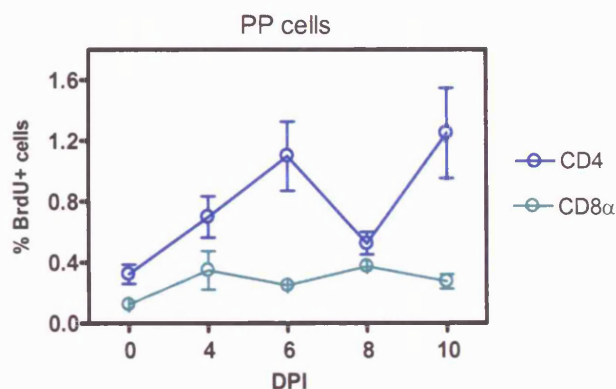
Figure 6.1 BrdU-incorporation by CD4+ or CD8α+ cells

BrdU+ CD4+ or BrdU+ CD8α+ cells in the A) MLN and B) PP of infected mice. BrdU was added to the drinking water of infected mice. MLN (LTα+/-β+/- and B6 mice) and PP (B6 mice only) cells were isolated and stained for BrdU incorporation. Plots show the proportion of BrdU+ CD4+ or BrdU+ CD8+ cells and represent the mean and SEM of 4 mice per group per time-point. The histogram depicted shows the difference in the number of BrdU+ CD4+ events between LTα+/-β+/- and B6 mice in the MLN at 4 DPI.
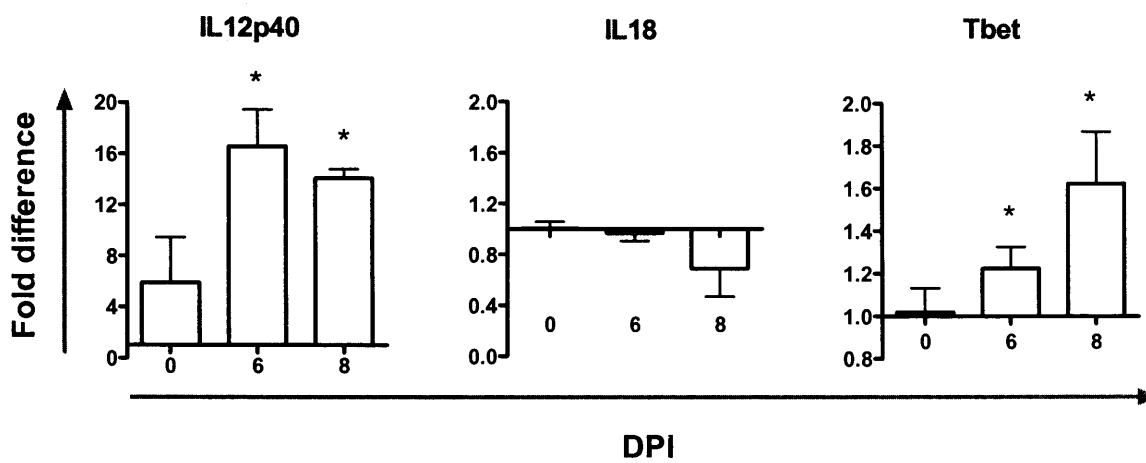
Figure 6.2 Th1-inducing signals in the PP during infection.

IL12p40, IL18 and Tbet mRNA levels were analyzed in the PP of infected B6 mice. The mRNA levels are expressed as fold differences and calculated as described in chapter two. Bar charts represent the mean and SEM of 4 mice per time-point. *, indicates significant difference ($P<0.05$) to samples at 0 DPI.
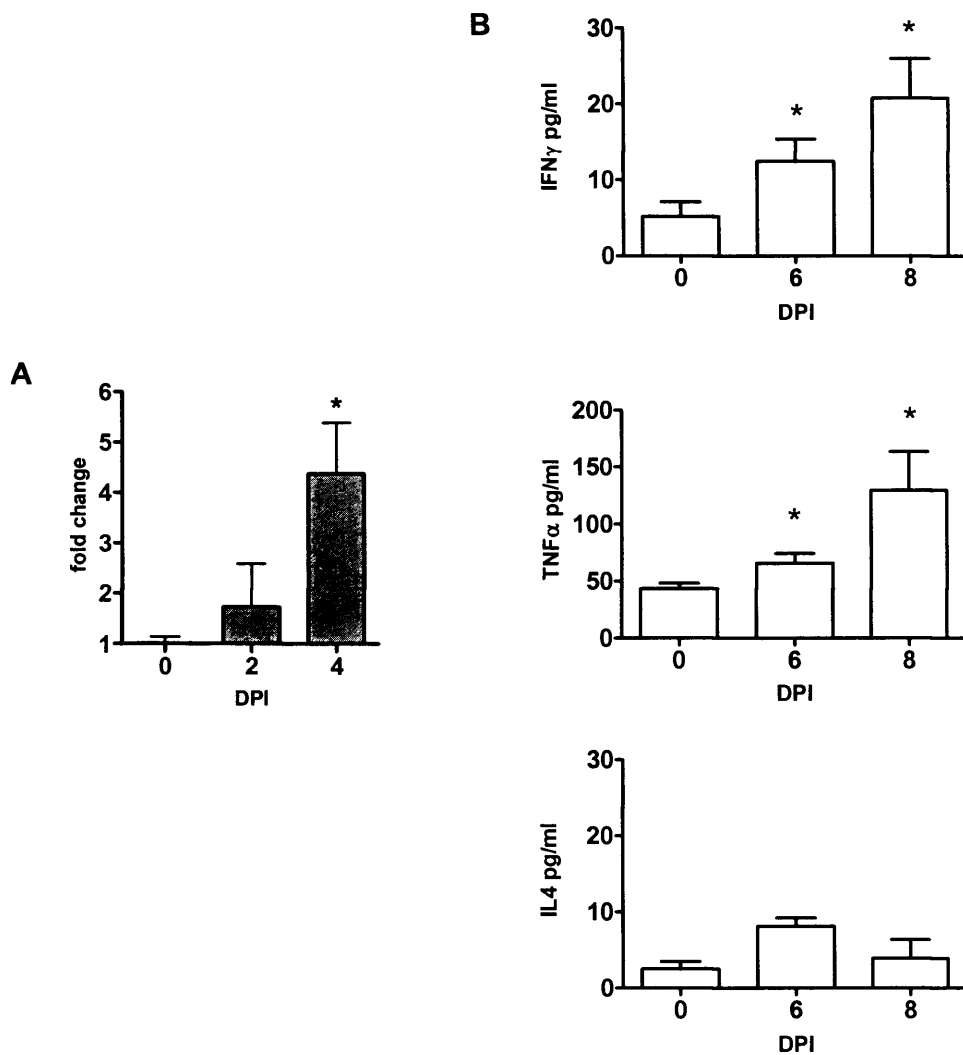
Figure 6.3 Antigen-stimulated Th1-type cytokine expression in the PP during infection with *E. vermiformis*.

Levels of A) IFNγ mRNA and B) IFNγ, TNFα, IL4 cytokines from the PP of infected mice. A total of 1 x 10^6 PP cells were stimulated *ex vivo* with oocyst lysate (50μg/ml) for 24 hr prior to measurement of and cytokines from the supernatants of cultures. Bar charts represent the mean and SEM of 4 mice per time-point. *, indicates significant increase (P<0.05) when compared to samples at 0 DPI.

B6 donors (8 days post-infected). The level of oocyst reduction was similar to TCRβxδ-/- recipients which received PP or MLN cells from infected B6 donors (8 days post-infected), indicating that immunity induced in the MLN of LTα+/-β+/- mice was transferable and as effective as B6 MLN cells.

The MLN cells from naïve B6 mice were able to confer resistance in TCRβxδ-/- recipients, suggesting the presence of T cell clones capable of effective responses against *E.vermiformis*. However it was unclear if PP from naïve mice contained a similar population of T cell clones since PP have relatively fewer lymphocytes than MLN. TCRβxδ-/-recipients which received 2 x10^6 PP cells from naïve B6 donors produced as many oocysts as unmanipulated B6 donors while TCRβxδ-/- recipient which received PP cells from infected B6 mice produced fewer oocysts than unmanipulated B6 mice (Figure 6.5A). These differences were more distinct when examining the pattern of daily oocyst output where the peak oocyst output (at 11 DPI) in unmanipulated B6 mice was higher than TCRβxδ-/- recipients which have received PP cells (Figure 6.5B). The general pattern of output was also different between TCRβxδ-/- recipients which received naïve or infected PP cells and unmanipulated B6 mice. Resolution of oocyst output was slower in TCRβxδ-/- recipients of naïve PP cells compared with the faster reduction of oocysts in TCRβxδ-/- recipients of PP cells from infected B6 donors and in unmanipulated B6 mice.

After one month, the mice from the above experiment were rechallenged with *E.vermiformis*. All mice were protected and produced no oocysts, indicating that both PP- and MLN-derived cells were capable of developing into protective memory responses (data not shown).

It was also observed in the experiment described above that all TCRβxδ-/- recipients of PP cells from infected B6 donors, developed prolapses after more than seven weeks (these mice were culled by schedule one) contrary to TCRβxδ-/- recipients of PP cells from naïve B6 donors.

6.3.3 Delayed recruitment of DC subsets to the MLN in the absence of PP

A difference in the timing of immune responses correlated with the level of susceptibility (as measured by oocyst output) seen with different strains of mice. This delay in immune responses also correlated with a slower DC response in the MLN of susceptible strain B6 mice given 1000 oocysts (compared with BALB/c mice) and BALB/c mice given lower doses of parasites (compared with BALB/c mice given higher doses). Susceptibility in LT-deficient mice strongly correlated with delayed immune responses occurring in the MLN and small intestine, a likely consequence of the inability to coordinate immune responses in the absence of organised structures. LTα+/-β+/- mice are intermediately susceptible compared with highly susceptible LTα-/- and LTβ-/- mice, indicating that the presence of an organised MLN was insufficient to generate the rapid Th1 responses associated with resistance to infection with *E. vermiformis*.
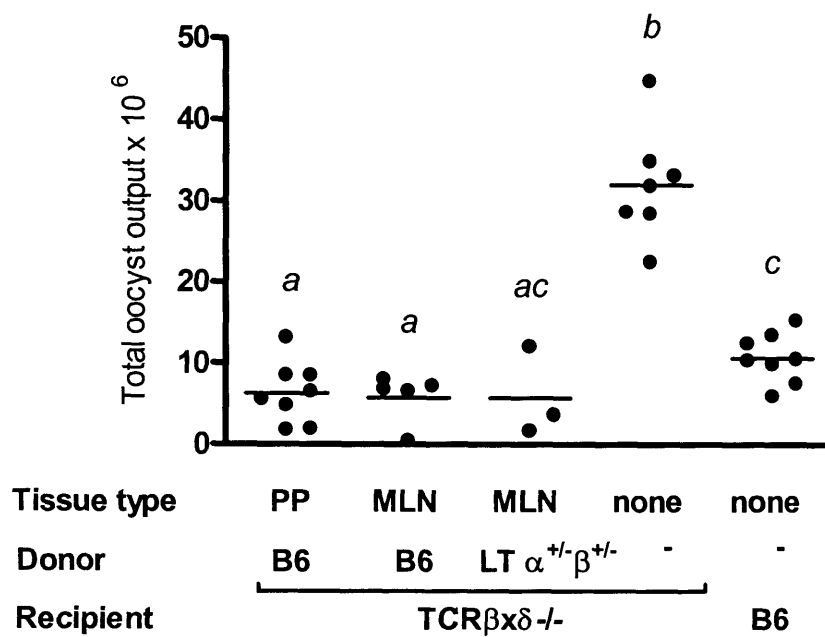
Figure 6.4 Peyer's patch- and MLN-derived cells independently confer immunity.

A total of 2 x 10^6 PP or 2 x 10^7 MLN cells from day 8 post-infected B6 and

LTα+/-β+/- mice (MLN only) were adoptively transferred into TCRβxδ-/- recipients which were infected with *E.vermiformis* 7 days later. The data represents the total oocyst output per mouse and 3-7 mice were used per group. Groups with different letters annotated are significantly different (P<0.05) from each other while groups with the same letters are not significantly different.
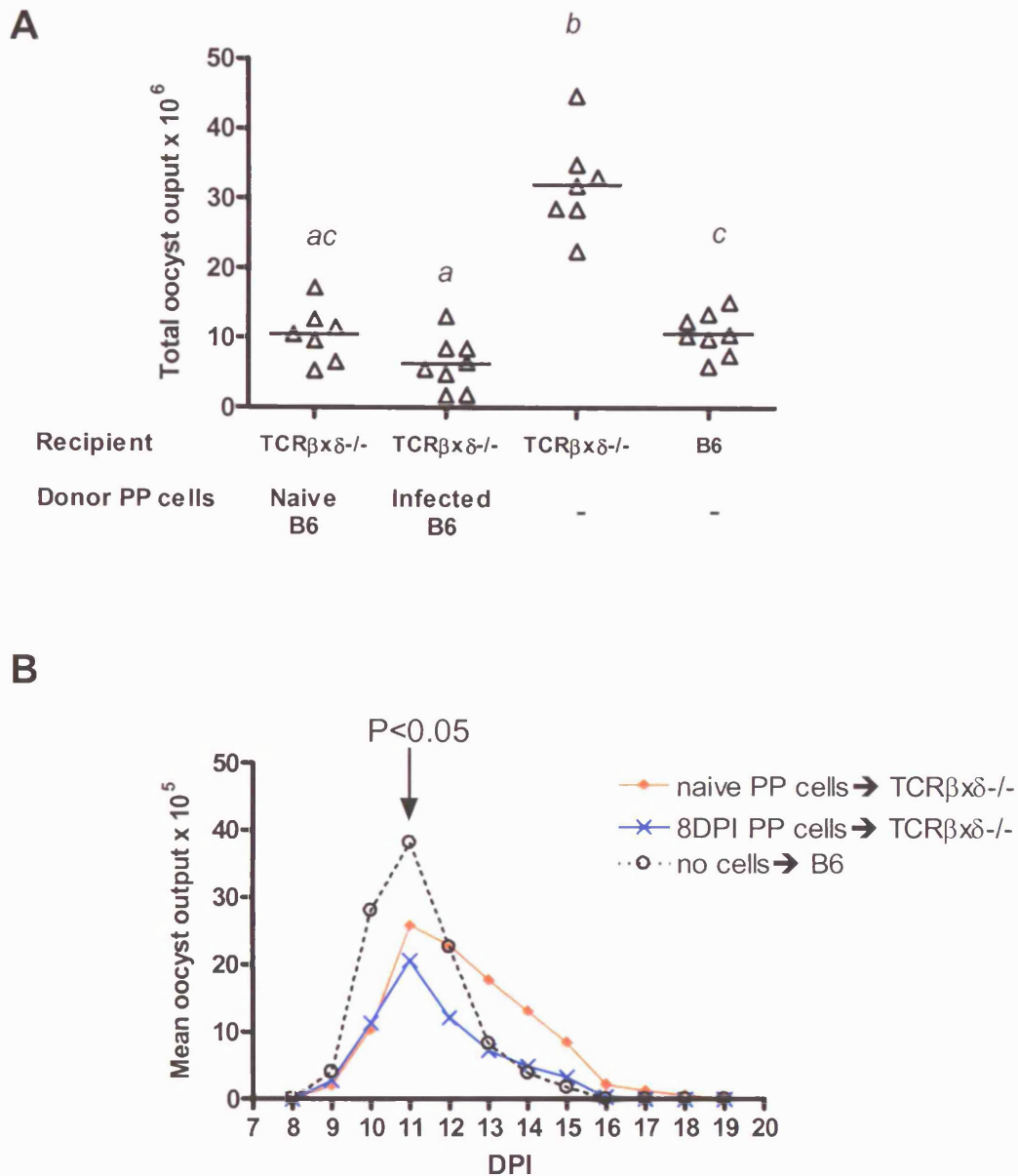
Figure 6.5 Different levels of immunity conferred by PP cells from infected or naïve donors.

PP cells from naïve and 8 day post-infected B6 mice were adoptively transferred into TCRβxδ-/- recipients which were infected 7 days later. A) Total oocyst output per mouse during primary infection. Different letters in italics annotate significant difference from other groups ($P<0.05$). B) Mean daily oocyst output per group. The data represents a minimum of 6-7 mice were used per group. Here, ($P<0.05$) refers to the significant difference at 11 DPI.

The inability of LTα+/-β+/- mice to match the relatively faster response seen in B6 mice may be attributable to delayed DC responses, a hypothesis which is tested here. The numbers of CD11c+ DC were examined and it was observed that LTα+/-β+/- mice displayed delayed recruitment of DC to the MLN compared with intact B6 mice (Figure 6.6A). The CD8α- DC increased at 6 DPI in LTα+/-β+/- mice and earlier at 4 DPI in B6 mice. The number of CD8α+ DC in LTα+/-β+/- and B6 mice increased over time although the overall numbers were lower than those of CD8α- DC. There was a constant increase over time in LTα+/-β+/- mice while in B6 mice, there were increases at 4 DPI and 10 DPI but decreases at 6 and 7 DPI. There were significant increases in mature DC expressing CD40 and/or CD80 from 4 DPI in the MLN of B6 mice while similar subsets increased later from 7 DPI in LTα+/-β+/- mice (Figure 6.6B).

The subsets included a range defined by the markers B220, CD11b and CD8α. The majority of DC was CD11c+ CD8α- which formed the focus of this study. There was no delay in the arrival of CD11b- B220+ DC in the MLN (Figure 6.7). In contrast, there was a delay in the arrival of CD11b-B220- and CD11b+B220- DC subsets in the MLN of LTα+/-β+/- mice during infection. In addition, a CD11b^{hi} B220- DC population was noticeably reduced in the MLN of LTα+/-β+/- mice during infection (Figure 6.8A). The CD11b^{hi} B220- DC proportions increased to ~18.7% in B6 mice at 6 DPI while in LTα+/-β+/- mice, the proportions rose to ~7.2% and remained around this level from 6 DPI to 10 DPI. At 7 DPI, the proportion of CD11b^{hi} B220- DC in B6 mice increased further to ~24% but later decreased at 10 DPI. Interestingly in the PP of B6 mice, the decreased number of CD11b^{hi} B220- DC at 6 DPI inversely corresponds to the increase seen in the MLN (Figure 6.8B). Despite the initial lack of CD11b^{hi} B220- DC, there

were increases in the overall population of CD11b+ B220- (includes CD11b^{hi} and CD11b^{lo}) DC in the MLN of LTα+/-β+/- mice (although occurring later at 7 DPI) suggesting accumulation of similar populations which were derived from non-PP regions of the gut (eg. LP).

There was evidence of parasite DNA in the enriched DC fraction isolated from the MLN of B6 mice at 6 DPI which may suggest that DC recruited to the MLN carried parasite material and contributed to the induction of MLN-mediated anti-parasite responses (Table 6.1). The parasite DNA detected was based on the 5S rRNA sequence that is conserved across *Eimeria* spp (Blake et al., 2006).

From all the data presented so far, it is clear that PP influence the rate of DC accumulation in the MLN and may be the initial source of CD11b^{hi} B220- DC which were mobilized to the MLN of B6 mice during the early immune response against *E.vermiformis*.

Table 6.1 Detection of parasite DNA in enriched DC fractions.

[a] 40-CT	Mean	SD
[b] sample #1	17.4	0.23
sample #2	21.9	2.71
sample #3	14.5	2.57
sample #4	25.6	0.35
sample #5	15.3	2.21
sample #6	25.6	0.35
gut tissue sample from an infected mouse (positive control)	9.2	1.14
gut tissue sample from an uninfected mouse (negative control)	0.0	0.00
No template control	0.0	0.00

[a] 40-CT values are from enriched DC fractions (samples #1-6 each consisting of 2×10^5 cells), gut samples and control, and represent the relative levels of *E. vermiformis* DNA present compared to the uninfected sample or non-template control. [b] Samples #1-6 were taken from the MLN of day 6 post-infected B6 mice and gut samples were from infected or uninfected B6 mice. The methods used are described in chapter two.

A

B
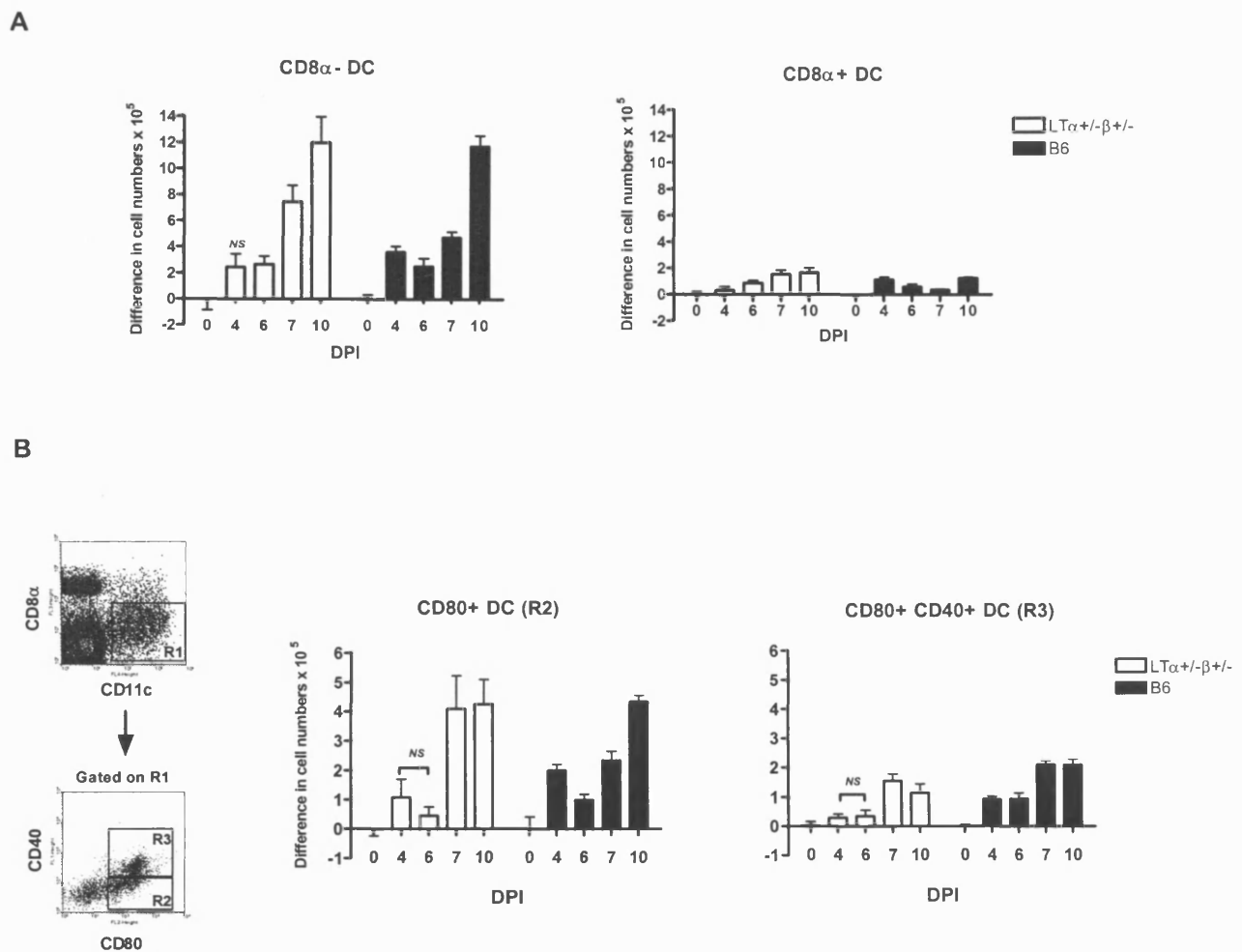
Figure 6.6 Delayed recruitment of DC into the MLN during infection.

A) CD8α- and CD8α+ DC, B) CD40+ and/or CD80+ CD8α- DC from the MLN of infected LTα+/-β+/- and B6 mice were isolated and quantitated. Bar charts show the changing number of cells calculated by subtracting cell numbers in uninfected mice from infected mice and represent the mean and SEM of 4 mice per group per time-point. The data is representative of 2 experiments. FACS plots depict the gates where CD11c+ cells expressing CD40 and/or CD80 are analyzed. A minimum of 1500 CD11c+ gated events were collected. *NS*, indicates no significant difference (P>0.05) to samples at 0 DPI. All other samples not annotated are significantly higher than samples at 0 DPI (P<0.05).
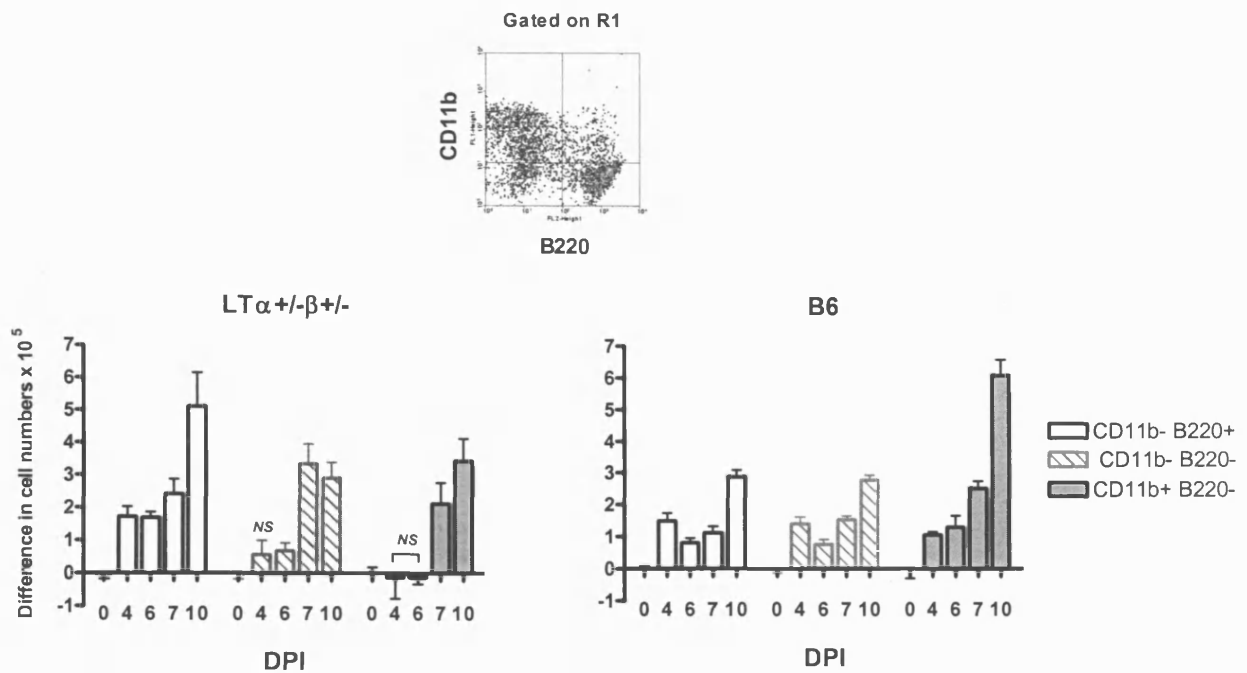
Figure 6.7 Delayed recruitment of DC subsets expressing CD11b and/or B220 into the MLN during infection.

DC from the MLN of infected LTα+/-β+/- and B6 mice were isolated and quantified. Bar charts show the changing number of cells calculated by subtracting cell numbers in uninfected mice from infected mice and represent the mean and SEM of 4 mice per group per time-point. The data is representative of 2 experiments. The FACS plot depicts the gate where cells expressing CD11b and/or B220 are analyzed from where R1 is the same region seen in figure 6.6. A minimum of 1500 CD11c+ gated events were collected. *NS*, indicates no significant difference (P>0.05) to samples at 0 DPI. All other samples not annotated are significantly higher than samples at 0 DPI (P<0.05).

A

CD8α

CD11c

R2

R1

CD11b

uninfected

4 DPI

6 DPI

7 DPI

10 DPI

B220

LTα+/-β+/-

4.7±1.0

3.1±0.6

7.2±0.7

6.3±0.6

6.1±0.6

B6

7.4±0.3

6.3±0.6

18.7±1.3

24.0±1.2

12.0±0.7

B

Total cell numbers x 10^4

DPI

P<0.05

P<0.002

PP

MLN
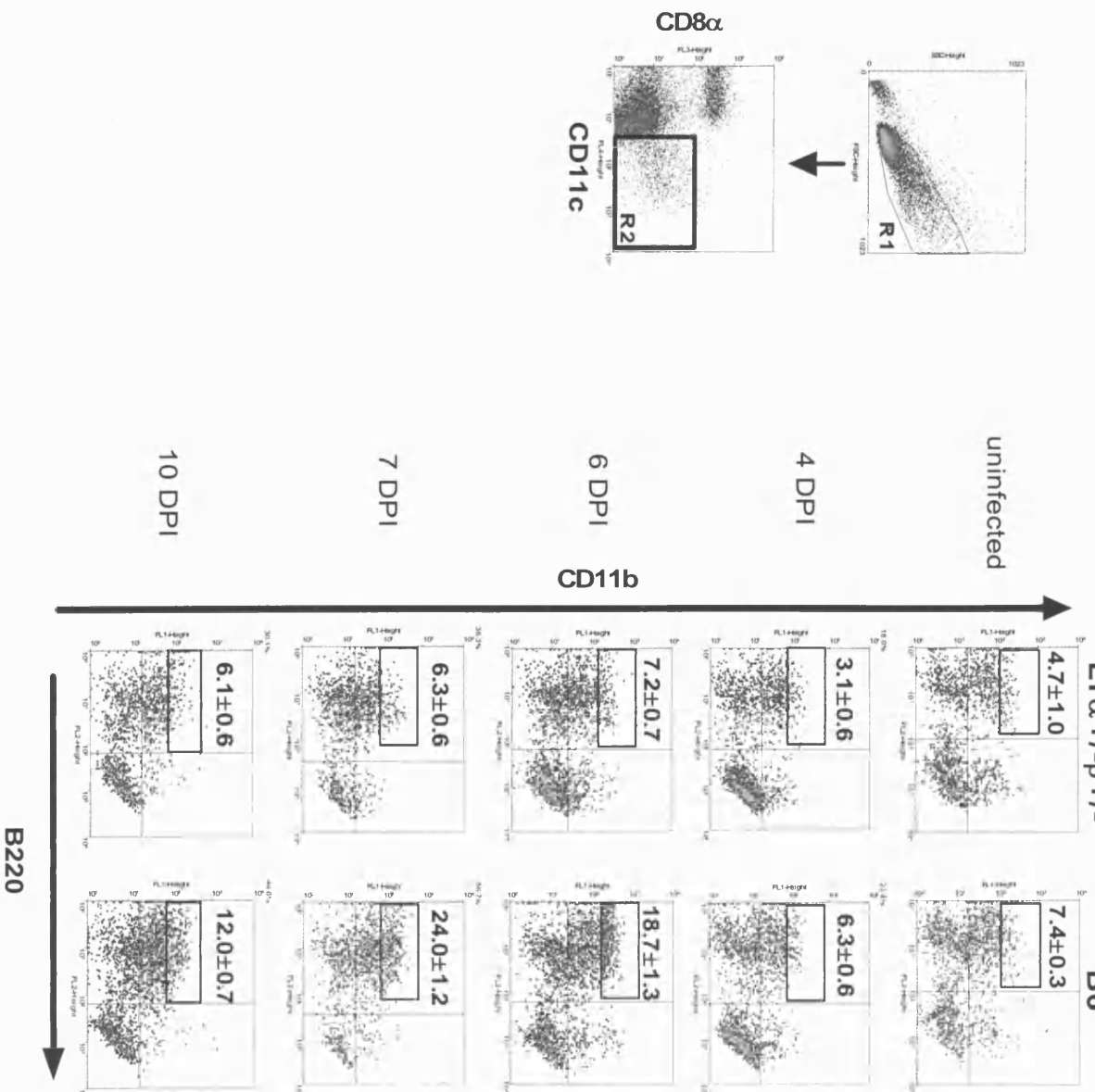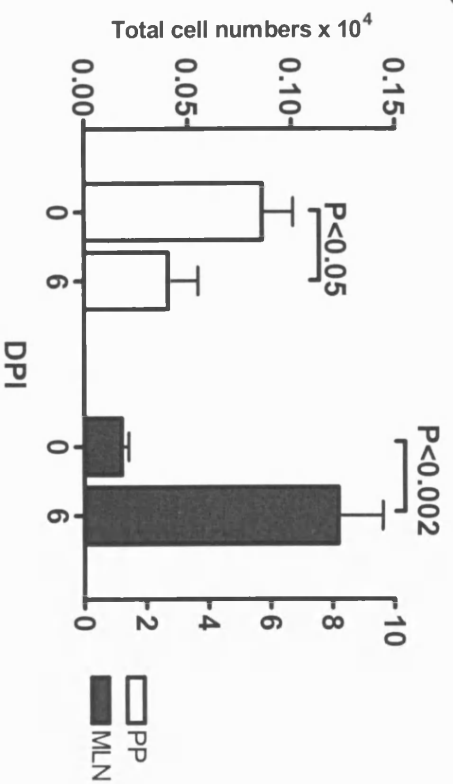
Figure 6.8 Reduced numbers of CD11b^{hi} B220- DC in the MLN of PP-deficient mice.

A) FACS plots show a distinct lack of CD11b^{hi} B220- DC subset in the MLN of LTα+/-β+/- mice. FACS plots are gated on R1 and R2 regions and values represent the mean and SEM proportions of D11b^{hi} B220- DC of 4 mice per group per time-point. Values are derived from the region depicted. The data here is representative of 2 experiments. B) The numbers of CD11b^{hi} B220- DC in the PP and MLN of B6 mice. The bar chart represents the mean and SEM of 4 or more mice per time-point and the data represents 1 out of 3 experiments.

6.3.4 CD11b^{hi} B220- DC function (determined through CD11b+ B220- DC analysis)

The CD11b+ B220- DC were subjected to a series of *in vitro* and *in vivo* analyses. Since CD11b^{hi} B220- DC were associated with differences between LTα+/-β+/- and B6 mice during the early phase of infection, CD11b+ B220- DC from day 6 post-infected B6 mice were enriched and adoptively transferred by intraperitoneal injection into LTα+/-β+/- mice. The oocyst output obtained with CD11b+ B220- or CD11b- B220- DC transfers remained greater than that seen in unmanipulated B6 mice. Neither LTα+/-β+/- mice given CD11b+ B220- or CD11b- B220- DC were significantly different from LTα+/-β+/- mice given no cells (Figure 6.9). However, LTα+/-β+/- mice given CD11b+ B220- cells produced slightly fewer oocysts (~2.2 million) than LTα+/-β+/- mice given CD11b- B220- cells (~2.5 million) (P<0.05).

In order to determine if the CD11b+ B220- DC subset was carrying antigen from the gut, B6 mice were challenged with *E.vermiformis* and provided with OVA protein in their drinking water. At 6 DPI, CD11b+ B220- and CD11b- B220- DC were isolated from the MLN and cultured with OT-II T cells enriched by depleting MHC class II+ cells. This assay compared the two DC subsets, examining which subset had captured and processed luminal OVA-antigens for presentation in the MLN. There was slightly higher ^{3}H-thymidine incorporation by OT-II T cells cultured with CD11b+ B220- DC than OT-II T cells cultured without DC or with CD11b- B220- DC (Figure 6.10). Some modulation of ^{3}H-thymidine incorporation may have occurred in OT-II T cells cultured with CD11b- B220- DC as the incorporated cpm levels were lower than cultures of OT-II T cells without DC. However, differences in T cell proliferation levels may or may

not be reflected as the difference is less than a factor of 2, an arbitrary factor considered for significance.

In addition, CD11b+ B220- DC and CD11b- B220- DC enriched from the MLN of day 6 post-infected B6 mice were analyzed for IL12 and IL18 mRNA levels. There was no detectable IL12p40 mRNA in either subset but IL18 mRNA was expressed in both subsets and was significantly higher in the enriched CD11b+ B220- fraction (Figure 6.11A). In addition, the CD11b+ B220- DC and CD11b- B220- DC enriched subsets were pulsed with a MHC class II-restricted OVA 323-339 peptide to assess their capacity to stimulate OT-II T cell differentiation into Th1 cells. Cytokine production in the supernatants of DC and T cell cultures was analyzed after 48 hours. The CD11b+ B220- DC fraction and not CD11b- B220- DC fraction induced high levels of IFNγ but only when pulsed with OVA peptide and cultured with OT-II T cells (Figure 6.11B). Levels of TNFα were higher in OT-II T cell cultures containing CD11b+ B220- DC but this was due to higher constituitive levels produced by CD11b+ B220- DC alone. The levels of IL2, IL4 and IL5 were negligible in all CD11b+ B220- and CD11b- B220- DC cultures with or without OT-II T cells.

The CD11b^{hi} B220- DC subset may play a prominent role early during immune induction since it is diminished in the MLN of LTα+/-β+/- mice which are more susceptible than intact B6 mice which possess this subset. To further validate the association of this subset with the PP and its functional importance during infection, CD11b^{hi} B220- DC were examined in the MLN of different mouse strains which possess PP and display greater resistance to infection than PP-deficient mice. Firstly, there was no lack of this subset in PP-intact LT single heterozygotes (LTα+/-, LTβ+/- mice) unlike LTα+/-β+/- mice. Increases in CD11b^{hi} B220- DC in the MLN of LT single heterozygotes occurred during infection (Figure 6.12) as observed with B6 mice.

It was not possible to compare the kinetics of CD11b^{hi} B220- DC in the MLN between LT single heterozygotes and B6 mice as there was no B6 group examined within the same experiment. However, both LT single heterozygote mice and B6 mice seem to have comparable increases of CD11b^{hi} B220- DC during infection. Secondly, BALB/c mice which showed greater control of infection than B6 mice during infection with 1000 oocysts, possess CD11b^{hi} B220 DC in the MLN (Figure 6.13). With lower parasite doses, BALB/c mice become more susceptible to infection as a result of delayed DC responses. A correlation between DC responses and susceptibility was seen in CD11b^{hi} B220 DC during infection as there were earlier increases in this DC subset at 2 DPI in BALB/c mice given a dose of 1000 oocysts than 25 oocysts. Increased proportions of CD11b^{hi} B220 DC occurred later at 6 DPI in BALB/c mice given 25 oocysts.

6.3.5 Regulatory effect of PP cells on different mice strains

The PP are regarded as important inductive sites for gut-associated responses because of their strategic location in the GI tract and lymphoid organisation. The PP-deficient LTα+/-β+/- mice lack a population of CD11b^{hi} B220- DC which are suggested (from the above mentioned data) to originate from the PP and may be essential for inducing early Th1 responses in the MLN. Crosstalk between MLN and PP may exist and be relayed by cells such as DC or T cells (Huang et al., 2000; Kobayashi et al., 2004; Macpherson and Uhr, 2004; Mowat, 2003). Perhaps the inability of LTα+/-β+/- mice to develop similarly rapid responses as intact B6 mice is due to a lack of PP-derived cells. In order to test this hypothesis, 5 x 10^5 PP cells from naïve B6 donors were adoptively transferred by intraperitoneal injection into LTα+/-β+/- recipients which were subsequently infected with *E.vermiformis* after four days. Unexpectedly, LTα+/-β+/- recipients which received PP cells fared worst than those which received no cells and
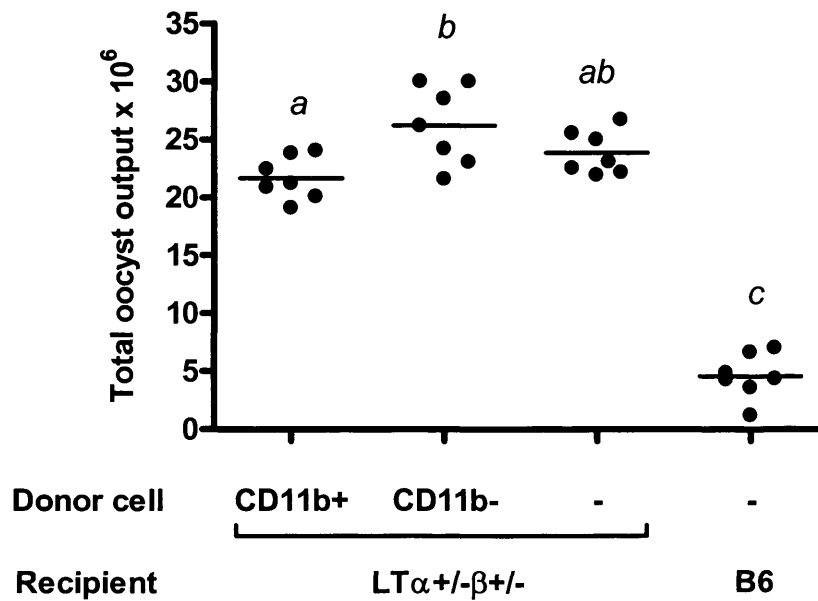
Figure 6.9 *In vivo* **transfer of DC subsets into LTα+/-β+/- mice.**

CD11b+B220- or CD11b-B220-DC were enriched from the MLN of day 6 post-infected B6 mice and adoptively transferred by intraperitoneal injection into LTα+/-β+/- mice which were subsequently infected 4 days later. The data represents total oocyst output from individual mice and 7 mice per group were used. Groups annotated with different letters indicate a significant difference from other groups (P<0.05) while groups with the same letters are not significantly different.
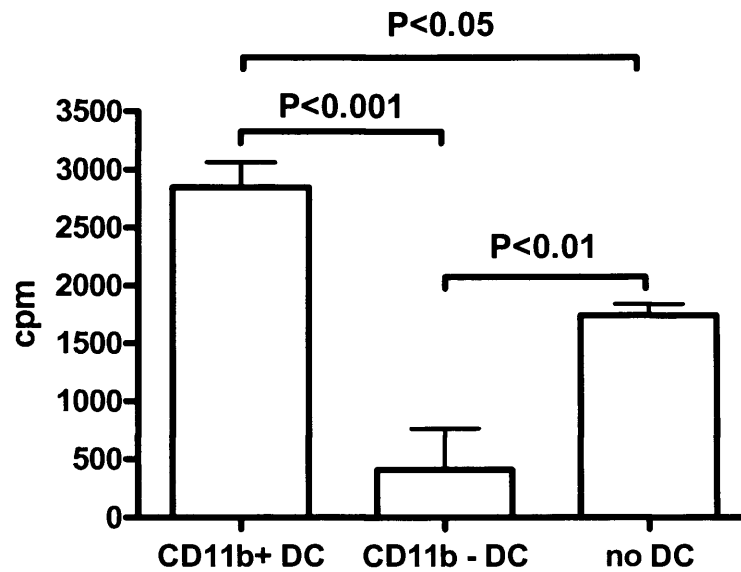
Figure 6.10 OVA-specific antigen-presenting ability of *in vivo*-pulsed CD11b+ B220- DC.

CD11b+B220- or CD11b-B220- DC were enriched from the MLN of day 6 post-infected B6 mice which were also given OVA protein (0.04mg/ml) in their drinking water from 3 DPI onwards. 1×10^4 DC were cultured with 1×10^5 OT-II T cells enriched by MHC class II depletion for 72 hr before analysis for ^{3}H thymidine uptake. Bar charts represent quadruplicate wells of OT-II T cells cultured with or without DC.
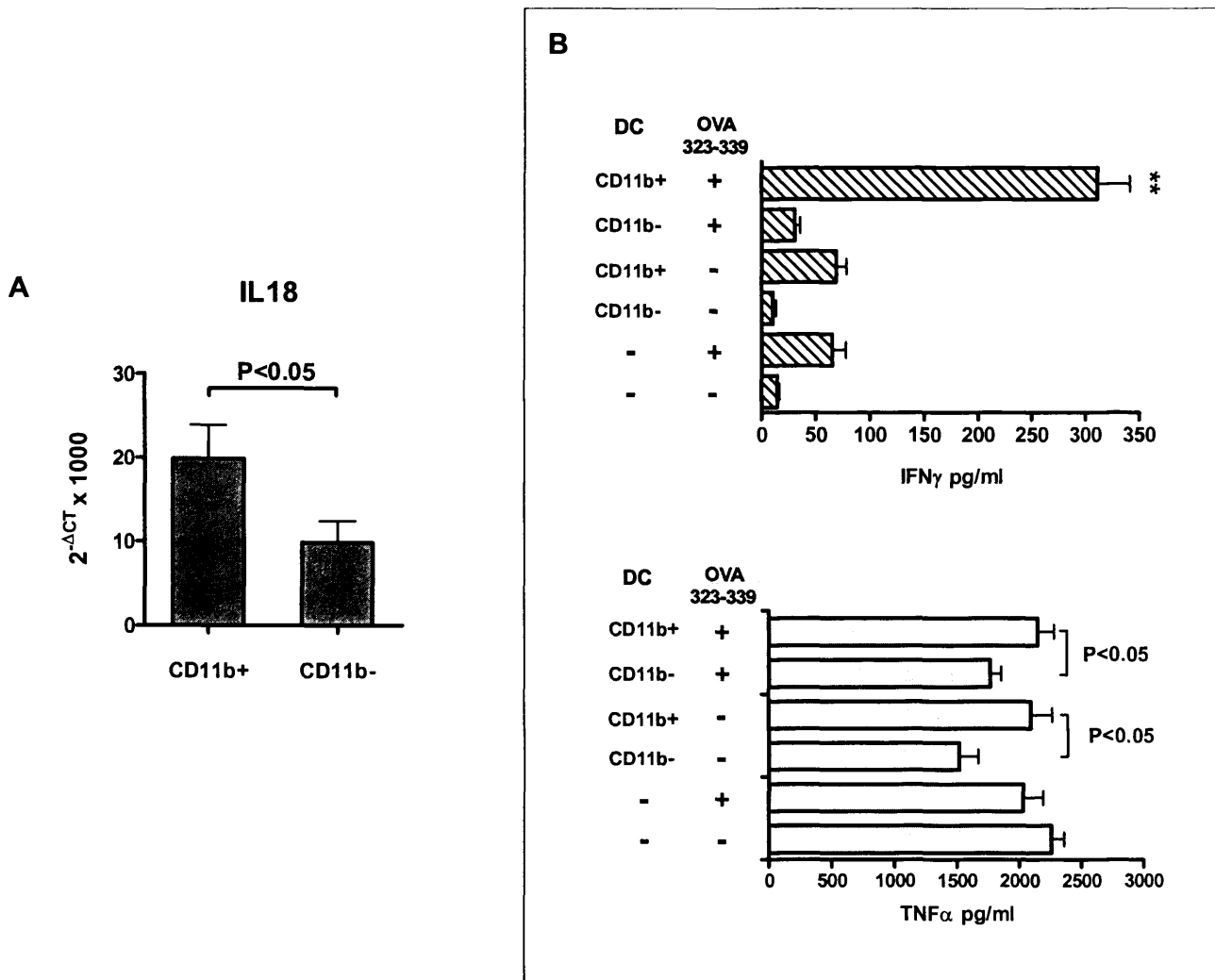
Figure 6.11   The Th1-inducing properties of CD11b+B220- DC.

A) CD11b+B220- DC were enriched from the MLN of day 6 post-infected B6 mice and analyzed for IL12p40 and IL18 mRNA however only relative changes in IL18 were detected (data for IL12p40 is not shown). Data is plotted as fold changes in IL18 mRNA expression and represents 6 samples each derived from a pool of 2 mice. B) CD11b+ B220- DC were pulsed with OVA 323-339 peptide (10µg/ml) before being cultured with OT-II T cells for 48hr. Supernatants from cultures were taken and examined for cytokine production. DC were isolated from a pool of 11 mice and the bar charts represent quadruplicate wells. **, indicates significant difference from all cultures (P<0.02).
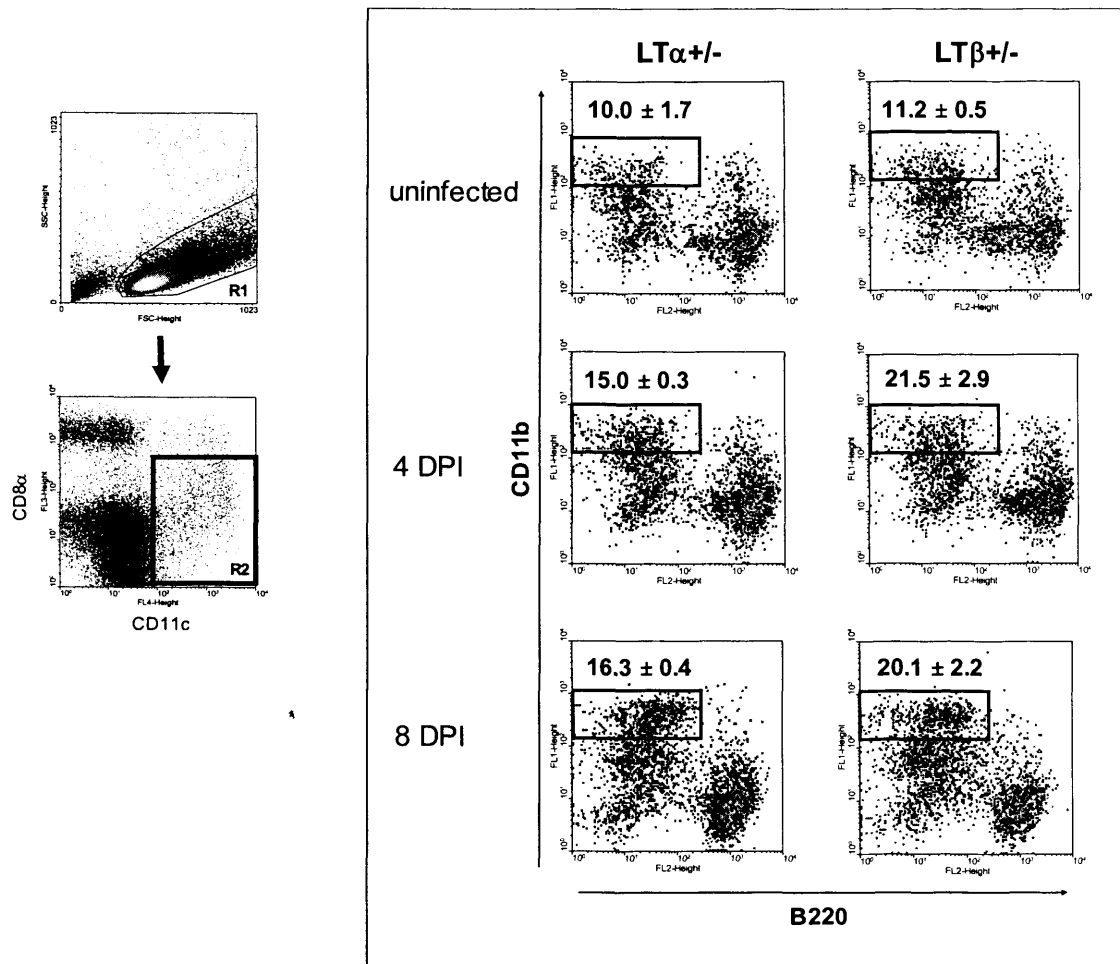
Figure 6.12 Analysis of CD11b^{hi} B220- DC in the MLN of LT single heterozygote mice during infection.

FACS plots are gated on R1 and R2 regions and values represent the mean and SEM proportion of CD11b^{hi} B220+ DC in 3-4 mice per group per time-point. Values are derived from the region depicted.
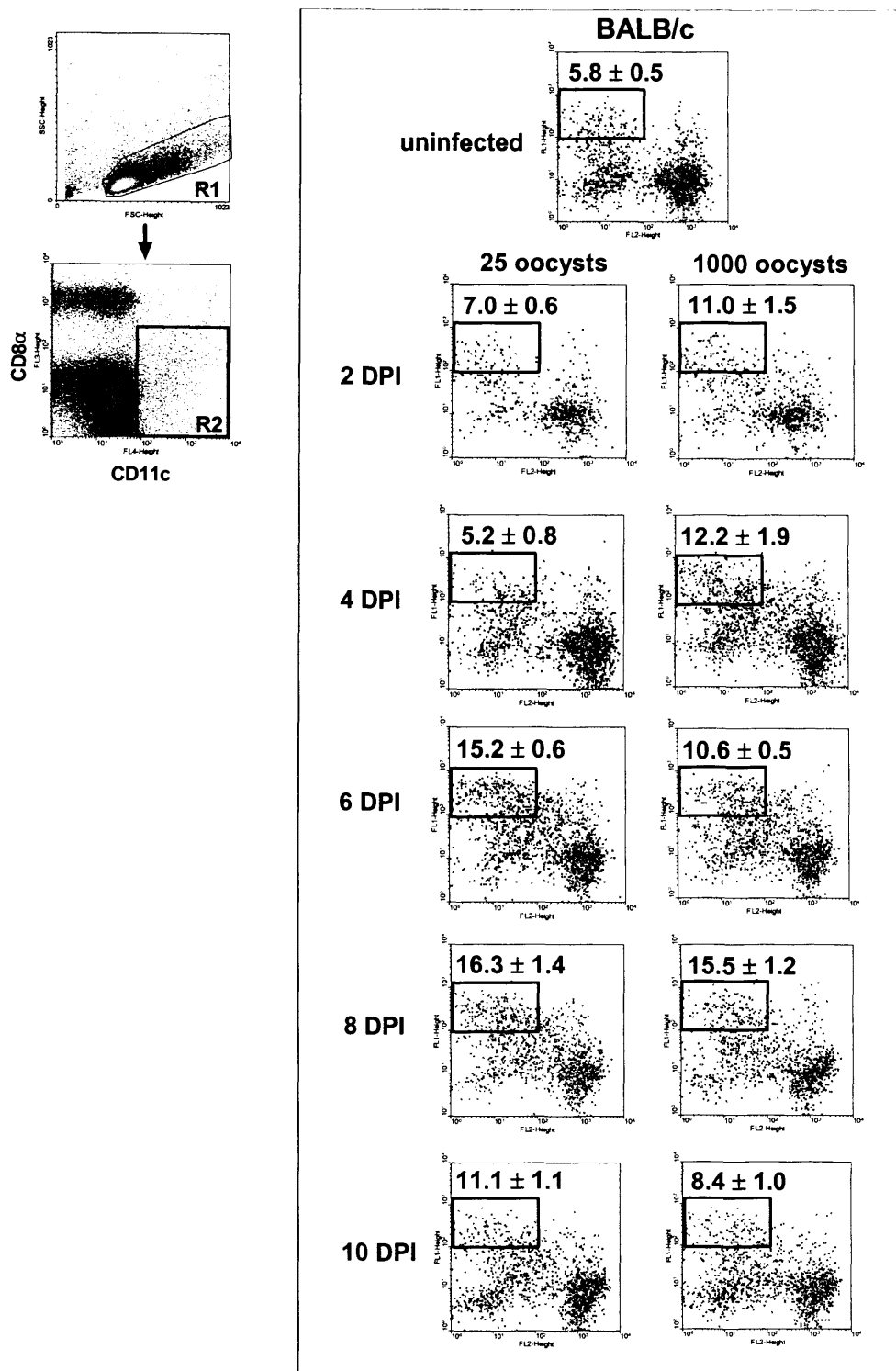
Figure 6.13 Analysis of CD11b^{hi} B220- DC in the MLN of BALB/c mice infected with 1000 or 25 oocysts.

FACS plots are gated on R1 and R2 regions and values represent the mean and SEM proportion of CD11b^{hi} B220- DC in 4 or more mice per group per time-point. Values are derived from the region depicted.

produced slightly more oocysts (Figure 6.14). It was unknown whether other PP-deficient mice, eg. LTβ-/- mice may be affected in a similar manner. However, LTβ-/-recipients which received naïve PP cells produced similar numbers of oocysts as LTβ-/-recipients which received no cells (Figure 6.15). In contrast, B6 recipients which received PP cells from naïve B6 donors, were slightly more resistant, producing fewer oocysts than B6 recipients which received no cells ($P<0.05$) (Figure 6.14). These results accentuate the importance of the tissue microenvironment where depending on the anatomical availability of lymphoid structures in LT-deficient or B6 mice, the same donor cell-type may not function in the same way.

A difficulty in working with PP-DC is their low numbers and therefore it was decided that Fms-like tyrosine kinase 3 (Flt3)-ligand administered to B6 mice might be useful in generating sufficient DC for an *ex-vivo* analysis since Flt3-ligand has been shown to expand DC populations (Maraskovsky et al., 1996). CD11c+ DC were sorted from the PP, MLN and spleen of flt3-ligand-treated B6 mice and examined for their ability to induce T cell proliferation and cytokine production (using OVA 323-339 peptide and OT-II T cells). Microbial stimuli are important in influencing the maturation and functional status of BMDC (as seen in chapter five). Therefore PP, MLN and splenic DC were matured with TLR agonists (Pam3cys or flagellin) to measure any differences in their inductive functions. DC plasticity was not seen as expected as the PP, MLN and splenic DC stimulated with TLR agonists were not different in their ability to induce T cell proliferation when compared to unstimulated DC (Figure 6.16). Splenic DC induced the highest levels of T cell proliferation in OT-II T cells (~27000 cpm) followed by MLN DC (~18000 cpm) while the lowest level of T cell proliferation was seen in cultures containing PP DC (~300cpm).
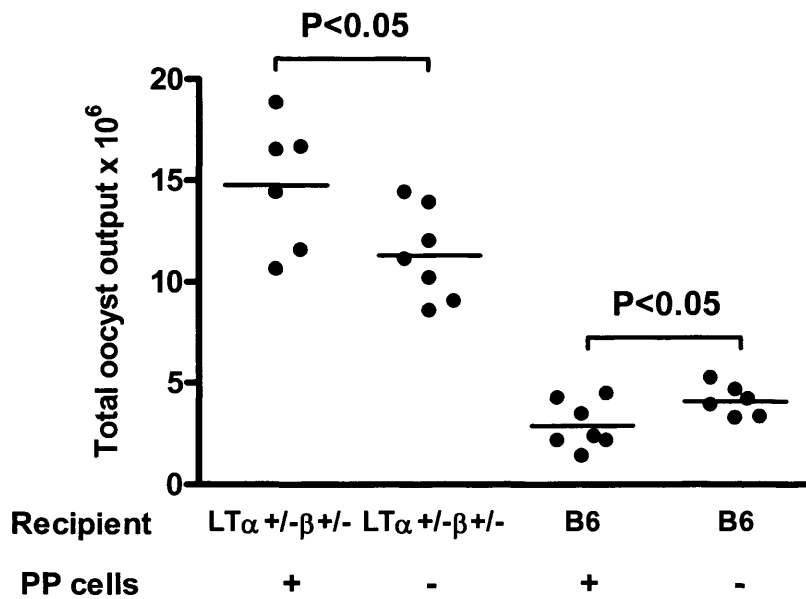
Figure 6.14 Influence of the adoptively transferred PP cells on PP-deficient LTα+/-β+/- mice during infection.

A total of 5 x 10^5 PP cells from naïve B6 donors were injected intraperitoneally into either LTα+/-β+/- or B6 recipients 4 days prior to infection. The data represents the total oocyst output per mouse and 6 or more mice were used per group.
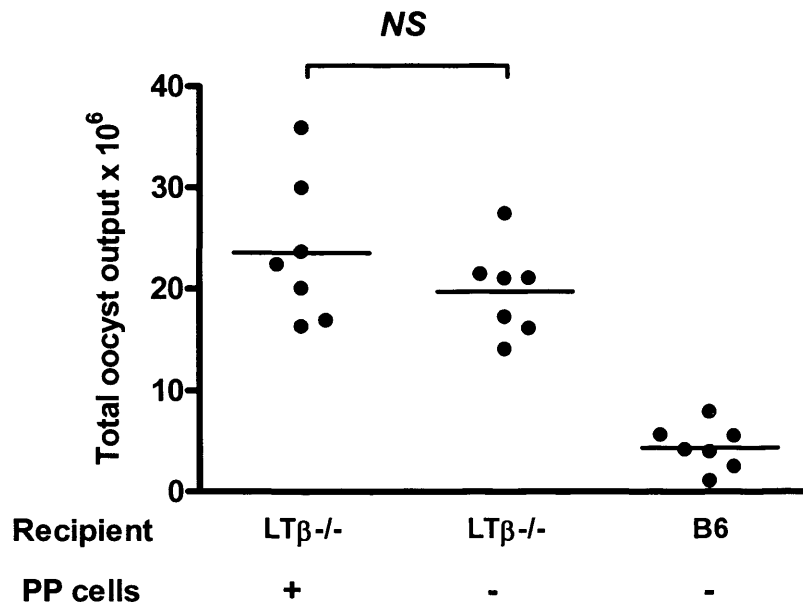
Figure 6.15 Influence of adoptively transferred PP cells on PP-deficient LTβ-/-mice during infection.

A total of 5 x 10^5 PP cells from naïve B6 donors were injected intraperitoneally into LTβ-/- recipients 4 days prior to infection. Data represents the total oocyst output per mouse and 6-7 mice were used per group.
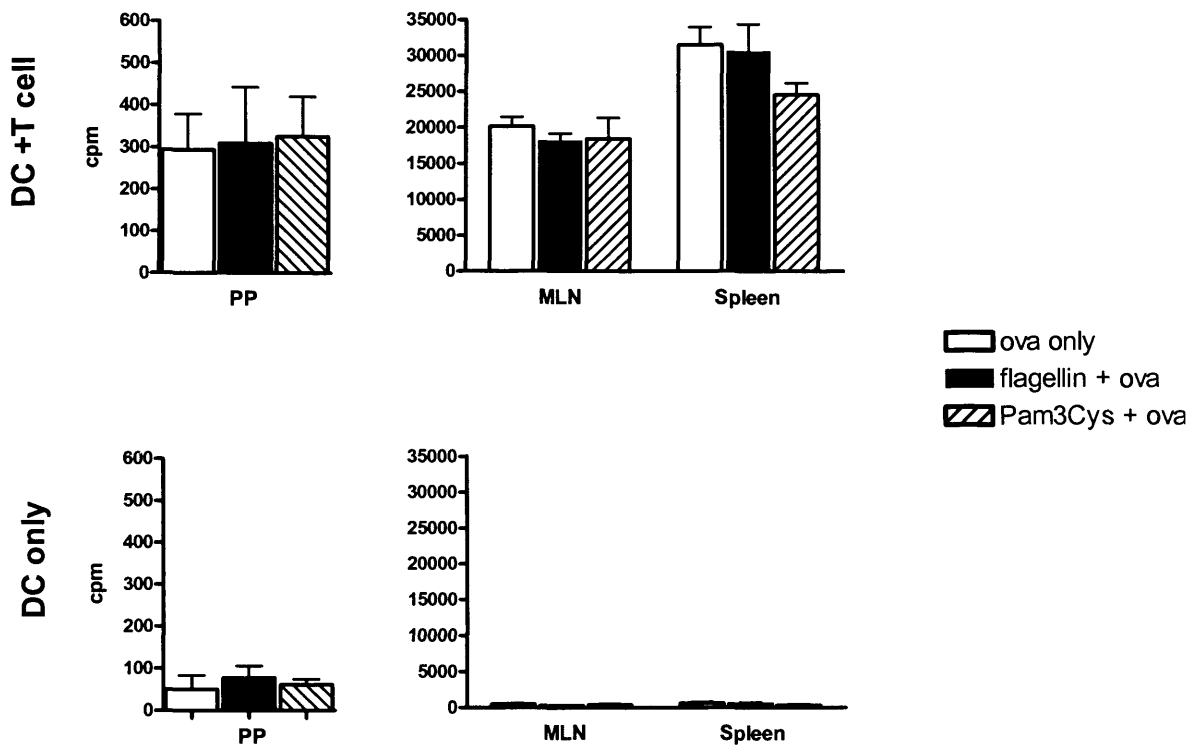
Figure 6.16 Analysis of the inductive properties of DC from different tissues.

PP, MLN and splenic DC were isolated from flt3-ligand treated mice which were injected intraperitoneally with 5 mg of flt3-ligand every alternative day over 6 days. The DC were magnetically sorted with CD11c microbeads and cultured with OT-II T cells (depleted of MHC class II+ cells by magnetic sort) in the presence or absence of either S. typhimurium flagellin (50ng/ml) or Pam3Cys (200ng/ml). OVA protein (50µg per well) was added to the cultures which were left incubated for 72 hr and analyzed for ^{3}H-thymidine uptake.

6.4   Discussion

The role of MLN and PP in the immune response against the enteric pathogen, *E.vermiformis* was highlighted in susceptible PP-deficient LTα-/-, LTβ-/- and LTα+/-β+/- mice in chapter four. Although the absence of intact MLN in LTα-/- and LTβ-/- mice indicated a MLN-requirement during infection, the infection of LTα+/-β+/- mice showed that the MLN requires coordinated help from the PP as PP-deficient LTα+/-β+/- mice were more susceptible than B6 mice. As shown in chapter four, the kinetics of Th1 responses (IFNγ expression, gut-homing CD4+ T cells) against *E.vermiformis* infection in the MLN of PP-deficient mice were delayed and work in this chapter identified delays in the antigen-specific proliferation of MLN CD4+ T cells (Figure 6.1A). In both LTα+/-β+/- and B6 mice, the response was dominated by proliferating CD4+ T cells revealed by BrdU-staining. Immune responses in intact B6 mice included PP-mediated responses which constituted mainly of proliferating CD4+ T cells. There are two mechanisms which may underpin the role for PP during *E. vermiformis* infection:

1) The recruitment of PP-DC to the MLN and 2) PP-derived T cells that directly contribute to immunity.

The role for Peyer's patch dendritic cells in the induction of responses

The DC recruited to the MLN during infection contained parasite DNA which may indicate carriage of parasite material by DC for priming in the MLN. A range of DC subsets arrived later in the MLN of LTα+/-β+/- mice and were likely to have originated from all areas of the gut (eg. LP). No delays were seen in CD11b- B220+ DC from LTα+/-β+/- mice and this may indicate their LP (or other non-PP site) origin although it does not necessarily suggest that all the other DC subsets in B6 mice came from the PP.

There are two possibilities for the delayed response in LTα+/-β+/- mice where one is a lack of PP-DC (eg. CD11b^{hi} B220- DC) and the other is the less efficient uptake of antigen in the absence of PP resulting in delayed DC responses from non-PP regions of the gut. The CD11b^{hi} B220- DC which are reduced in LTα+/-β+/- mice were most likely to have come from the PP where this subset was first described (Kelsall and Strober, 1996). This subset was found in the MLN of all PP-intact mice examined (LT single heterozygotes, B6 and BALB/c mice) and increased in numbers during infection in the MLN (Figure 6.8, 6.12 and 6.13).

The importance of CD11b^{hi} B220- DC in the induction of Th1 responses is also supported by the higher expression of IL18 mRNA and ability to induce IFNγ when presenting OVA peptide to OT-II T cells, in contrast to CD11b- B220- DC. In a separate experiment, CD11b- B220- DC seemed to exert some form of immunomodulation as OT-II T cell proliferation was subdued compared to proliferation induced by CD11b+ B220- DC however this experiments needs to be repeated (Figure 6.10). CD11b+ B220- DC adoptively transferred into LTα+/-β+/- mice did not completely rescue their phenotype but conferred a low (but statistically significant) degree of immunity when compared with CD11b- B220- DC (Figure 6.9). It is possible that at the time of DC isolation, the CD11b+ B220- DC transferred may contain a greater amount of parasite antigen which may immunize the recipients prior to infection. However, there was no statistical difference with LTα+/-β+/- mice given no cells and may require additional testing. Despite this, it is likely that CD11b^{hi} B220- DC are important early during induction of Th1 responses during infection with *E. vermiformis* since they may be the one of the first DC subset to be exposed to parasite material due to their location in the PP where active antigen sampling occurs.

When compared with naïve PP-intact mice, LTα+/-β+/- mice are deficient in CD11b^{hi} B220- DC however the proportions of similar subsets increased slightly in the MLN of LTα+/-β+/- mice over time later during the course of infection (although proportions were still lower than B6 mice). Therefore this subset is not necessarily confined to the PP but may also be present in the LP. The presence of CD11b^{hi} B220- DC in the MLN of naïve mice does suggest some DC subset migration to the MLN during steady state and may indicate other roles for this DC subset in the absence of infections. BALB/c mice which are more resistant than B6 mice have CD11b^{hi} B220- DC. Furthermore, the earlier increases in the numbers of CD11b^{hi} B220- DC from BALB/c mice given doses of 1000 oocysts (when compared with mice given 25 oocysts) reflected the contribution by this subset in inducing earlier MLN immune responses from BALB/c mice. Thus indicating a significant role played by this subset in the induction of rapid immune responses. It further highlights the effect DC responses have on the outcome of infection.

An important general observation is that the timing in which the number of CD11b^{hi} B220- DC increases correlates with the rapid development of Th1 responses in different circumstances. These circumstances included the presence or absence of PP (LTα+/-β+/- vs B6 mice), host strain-dependent resistance (BALB/c vs B6) and parasite dose in one strain of mouse (BALB/c mice infected with 1000 or 25 parasites). Although no data is available here to show an association with host-strain dependent resistance (comparing B6 and BALB/c mice), the evidence that a BM-derived cell is involved in determining host resistance (Joysey et al., 1988) and that DC recruitment to the MLN is slower in B6 mice compared to BALB/c mice (chapter three), supports such a correlation. Collectively, these data strongly support the important role for CD11b^{hi}

B220- DC in the induction of anti-parasite responses under three independent circumstances.

The role for lamina propria dendritic cells

A substantial amount of attention has been paid to LP DC function where most studies suggest an immunomodulatory role during steady state (Chirdo FG 2005; Jang MH 2006). However it is likely with *E. vermiformis* infection and possibly other enteric infections that LP-DC contribute to the induction of immune responses since the increased numbers of MLN-DC were too large to have been derived entirely from the PP. Furthermore, the DC accumulation in the MLN most likely represents recruitment of DC from the gut (Angeli et al., 2006) and is not a result of proliferating resident MLN-DC proliferating. At non-PP sites, some LP-DC express CX3CR1 (Niess et al., 2005) and extend their processes between intestinal villus epithelial cells to sample luminal antigens, some may acquire antigen from villus M cells and others may acquire antigen direct from the site of infection where epithelial cells have been invaded by the parasites. It is also suggested that LP-DC may be more important in sampling antigens within the surface mucus layer while luminal antigens are largely sampled by PP-DC (Macpherson and Uhr, 2004). However, *E.vermiformis* invades the villus epithelial cells and replicates mainly in the crypts of intestinal villi (Todd and Lepp, 1971). It is not known to reside on the mucus layer unlike pathogens such as *Helicobacter pylori*, *Nippostrongylus brasiliensis, Giardia intestinalis* (Roxstrom-Lindquist et al., 2006; Sutton, 2001). Therefore, the contributions of PP-DC and LP-DC and their effects on the outcome of infection in intact mice are different. Perhaps future experiments may examine the kinetics of florescent bead uptake by different DC subsets and their

movement to the MLN in both PP-deficient and PP-intact mice during a concurrent infection with *E. vermiformis*.

PP-derived immunity contributes independently to parasite reduction

Clearly, PP are required for the induction of rapid MLN responses with the notion that PP-DC contribute to the early phase of immune induction whereas LP-DC may contribute but are slower to respond. This early response produced a difference of two days between B6 and LTα+/-β+/- MLN responses, which was sufficient to reduce parasite numbers by around three-fold in B6 mice. Moreover, during *E. vermiformis* infection, local T cell immune responses are induced in the PP (as evident by the adoptive transfer experiments) and it has been observed that PP T cells exit faster than those in LN (Kellermann and McEvoy, 2001). Such a shorter retention time in the PP means faster T cell recruitment to site of infection, adding further to the incentives of having PP.

Both PP and MLN cells independently conferred a degree of immunity in highly susceptible TCRβxδ-/- recipients and this supports the hypothesis that the lack of PP-immunity was responsible for the intermediate susceptibility seen in LTα+/-β+/- mice. The number of PP or MLN cells transferred was different (10-fold fewer PP cells) yet the level of protection conferred was similar with both tissue types. This is possibly due to the expansion of T cells in the lymphopenic environment of TCRβxδ-/- mice during the seven days prior to infection. The expansion of T cells from either PP or MLN occurred to a similar extent such that there were sufficient T cell clones present to respond against primary infection and provide protection.

PP cells exert different influences in different recipient strains

The PP have been characterised as a site that expresses typically Th2-type signals during steady-state due to the presence of T regulatory cells (Jump and Levine, 2002) and IL10-producing DC subsets (Everson et al., 1998). Such an environment exerts a modulatory influence on T cell responses, rendering them less responsive to chemokines and stimuli (Kellermann and McEvoy, 2001; Tsuji et al., 2001). The adoptive transfer of PP cells from naïve donors into recipient mice either with deficient or intact lymphoid structures demonstrated the influence of different tissue microenvironments on cell functions. The lymphopenic environment of TCRβxδ-/- mice resulted in the expansion of naïve PP cells which provided a level of resistance comparable to naïve B6 mice. However, it was different in LTα+/-β+/- mice which retained an intact T cell immune system which possibly competed with donor PP cells and limited their expansion. Contrary to the TCRβxδ-/- recipients, LTα+/-β+/- recipients which received the same cells became slightly more susceptible than unmanipulated LTα+/-β+/- mice (Figure 6.5 and Figure 6.14). The differences mentioned above are likely to be the result of differential cell proliferation in a T cell-deficient (TCRβxδ-/- mice) or T cell-intact (LTα+/-β+/- mice) environment. It is also suggested that the same PP cells were subjected to an environment with or without a PP location where these adoptively transferred cells can home to. Usually, where PP are present, PP cells may be subjected to regulation by mechanisms present in the PP site. However, in the absence of PP, donor cells may home to other sites (eg. MLN) and modulate immune responses (eg. in LTα+/-β+/- recipients) in a way that increases the recipient's susceptibility to infection. Alternatively, the tissue environment in LTα+/-β+/- mice may lack sufficient LT expression to modulate responses, allowing donor PP cells to negatively influence the immunity against *E. vermiformis* infection. In contrast, intact B6 recipients given donor

PP cells improved their resistance against *E. vermiformis* infection and this may be a result of transferring more protective T cell clones which contributed to parasite reduction and the intact microenvironment of B6 recipients which helps to modulate donor cells. PP-deficient LTβ-/- recipients given naïve PP cells were no different to LTβ-/- recipients given no cells and perhaps it is because LTβ-/- mice were already highly susceptible to *E. vermiformis* infection and any influences from PP cells (if present) would be masked. In retrospect, the reconstitution of donor cells in the recipients should have been examined for any differences seen with specific cell subsets (eg. CD4+CD25+ T cells). For future experiments, it would be appropriate to sort PP-cells into DC, T or B cells for adoptive transfers to determine which cell populations are likely to influence the immune responses in LTα+/-β+/- recipients.

Despite the protection conferred by effector cells from the PP of infected donors, the absence of regulatory cells resulted in the formation of prolapses in TCRβxδ-/- recipients. This may be a consequence of changes in the balance of 'activated' and 'regulatory' T cell subsets in the PP of infected donors and more donor effector cells than regulatory cells may have been transferred, resulting in the pathology observed. On the contrary, TCRβxδ-/- mice which received naïve PP cells did not develop prolapses and this suggests that sufficient regulatory cells were transferred along with naive T cells.

Intrinsic properties of tissue-specific DC may also influence the development of immune responses. For example, a greater level of T cell proliferation was induced in MLN and splenic DC cultures compared with PP-DC cultures. The addition of TLR agonists did not elicit different responses in DC and may be due to pre-exposure to

LPS-contamination. Even after TLR agonist-induced stimulation, the phenotype seen with PP-DC suggests a regulatory role in the immune response. Perhaps this demonstrates the plasticity of PP-DC to regulate and also contribute to the immune response as shown with the work presented on CD11b^{hi} B220- DC. However, greater stimulation (through ongoing infection) may be required in order to activate the adaptive immune response against pathogens.

In summary, the PP is a complex environment which is well-adapted to effectively execute pro-inflammatory or immunomodulatory responses when required. The DC present in PP may mediate immunomodulatory responses during steady state but can be altered to induce protective T cell responses against *E. vermiformis* infection. PP-DC seem to operate both locally and in the MLN to generate effective Th1 responses. Such versatility accounts for the influence of PP on MLN immune responses mediated by PP-DC such as CD11b^{hi} B220- DC which are important in initiating early immune responses in the MLN. This chapter also underlines the different contributions between DC from PP and non-PP sites.

Chapter Seven

Final discussion

The mammalian immune system is equipped with elaborate innate and adaptive mechanisms which function in concert to respond against infections. The demand for efficient and effective adaptive immune mechanisms requires order which is provided through the structural compartmentalisation and organisation of lymphoid tissues. Complex lymphoid structures have evolved to facilitate the co-localisation and optimal interaction between various immune cell types. One of the most important interactions is perhaps that between the naïve T cell and DC where the organised architecture present in lymphoid structures is crucial in coordinating interactions between rare antigen-specific T cells and DC carrying the specific antigen.

The role for secondary lymphoid organs can be studied using LT-deficient mice which possess phenotypic defects in lympho-organogenesis and organisation. The spleen, LN and PP are examples of secondary lymphoid structures that facilitate efficient DC-T cell interactions, enabling the generation of efficient adaptive immune responses. In the control of *E. vermiformis* infection, the timing of the response is of great importance and the presence of lymphoid structures plays a key role in contributing to the rapid induction of Th1 responses. In this thesis, the main focus is on primary immune responses induced in the small intestine and GALT, and how various factors influence the induction of rapid Th1 responses in general, more specifically those protective against *E. vermiformis*. The different chapters (three to six) have connected the role of DC during infection with the

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influence of anatomical structures and explain how the outcome of infection is affected overall.

The findings presented in the thesis relate to five areas of general discussion:

1) The timing of T cell responses is influenced by DC recruitment to the MLN

2) DC responses in the MLN are influenced by upstream factors

3) The MLN is an important induction site for gut-associated immune responses

4) How does the role of Peyer's patches compare with that of the MLN?

5) The influence of tissue-specific mediators and microenvironment

The timing of T cell responses is influenced by DC recruitment to the MLN

The Th1-type CD4+ T cell dependence of protective immune responses against *E. vermiformis* has been well-documented (Rose et al., 1988a; Rose et al., 1984; Rose et al., 1985; Rose et al., 1989; Smith and Hayday, 2000; Wakelin et al., 1993). In chapter three, the temporal patterns of T cell activity in gut-associated tissue compartments and DC mobilization were examined in the context of host genetics and parasite dose (both of which influence the induction of adaptive immunity in the MLN of infected mice). The data documents the temporal pattern of DC recruitment to the MLN and correlates this with differences in the timing of primary immune responses in the MLN. The different timing in DC response between susceptible and resistant strains may form the basis of susceptibility associated with host genotype which has been shown to depend on BM-derived cells (Joysey et al., 1988). The timing of DC response in the MLN has an impact on the downstream generation of CD4+ T cell immune response in the MLN, demonstrated by delayed kinetics of 'activated' CD4+ T cells (chapter three), CD4+ T cell proliferation (chapter six), delayed IFNγ expression (chapter three and four). These differences in the

timing of DC influence the eventual outcome of infection as indicated by the higher susceptibility of B6 mice compared with BALB/c mice, LTα+/-β+/- mice compared with B6 mice (chapter four) and efficacy of parasite killing in BALB/c mice given lower parasite doses (chapter three). The quantification of DC showed differences in 'resting' DC numbers between naïve strains (B6 vs BALB/c) however it was the number of the responding DC that was crucial in determining outcome of infection. Being potent inductors of adaptive immunity, it is no surprise that small differences in DC numbers could have a dramatic effect on the outcome of infection.

The timing of the immune response is important during *E. vermiformis* infection because of the parasite's ability to replicate rapidly. When ingested by a host, excystation, sporozoite invasion of intestinal epithelial cells and the development of first generation schizonts occur within 2-3 days, followed by three further rounds of schizogony (Rose et al., 1992). The asexual stages of reproduction are followed by sexual reproduction and the life cycle is completed within eight days. The early phase of development clearly drives DC responses which were significantly increased in BALB/c and B6 mice challenged with 1000 oocysts (the former being quicker by one day). Generally, parasite replication occurs in localised areas and not along the entire length of the intestine or in PP. Therefore the host has the difficult task of detecting the parasite regardless of where replication occurs. This may be where the PP play an important role as they have access to luminal contents where parasite material may be present (particularly from the sporozoite stages). A functional role for the PP is clearly demonstrated in infected PP-deficient mice. In addition, the slower the host detects parasite invasion, the more time is available for greater parasite replication. In BALB/c mice challenged with 1000 oocysts, the earlier immune responses seen in the MLN and PP, resulted in the huge reduction in oocyst output compared with B6 mice.

Hence, the difference in host-strain responses may be attributed to faster DC mobilization into the MLN, helped by larger PP structures found in BALB/c mice (see below).

Dendritic cell responses in the MLN are influenced by upstream factors

The importance of DC responses in the control of *E. vermiformis* is also shown by the impact that upstream factors affecting DC responses have on the outcome of infection. Parasite doses effectively reflect the amount of stimulation (ie. immunizing injections by invading parasites in the gut) received by DC and when reduced, results in delayed DC mobilization to the MLN. In BALB/c mice challenged with low doses, the DC mobilization to the MLN picks up after a few days delay but the overall DC accumulation did not match the DC numbers accumulated in mice infected with 40 times more oocysts. Therefore DC responses are limited by the amount of initial stimulation at the site of infection. Furthermore, the host genotype influence on DC responses may be related to anatomical differences in PP size. The larger PP in BALB/c mice may indicate a greater capacity to induce faster responses to gut infection. Although no extensive histological analysis of PP-cells was carried out to determine the numbers of M cells, locations of DC and T cells in the SED and IFR, at steady state the number of DC (only marginally higher) and T cells is higher in BALB/c than B6 mice. This aspect of a requirement for PP during the immune response may be unexpected considering that the parasite does not target the PP. However with the results obtained from LT single heterozygote mice, it is suggested that differences in the efficiency of sampling in the PP may be a feature of host genotype-dependent resistance against *E. vermiformis*.

The MLN is an important induction site for gut-associated immune responses

In oral infections, the recruitment of DC into the MLN occurs and this has been demonstrated using *Trichuris* and S. Typhimurium (Koyama, 2005; Sundquist and Wick, 2005). Another study demonstrated that the initial mucosal immune responses induced are critical for the early development of salmonella-specific T cells despite the systemic dissemination of S. Typhimurium, (McSorley et al., 2002). Furthermore with *E. vermiformis*, the adoptive transfer of MLN cells, conferred greater protection than splenic cells, during infection (Rose et al., 1988b). These data demonstrate that the MLN is an important site for the generation of adaptive immunity directed against gut infections and is highlighted during the infection of MLN-defective LTα-/- and LTβ-/- mice which were highly susceptible (chapter four).

The possibility that DC proliferation was responsible for the observed changes can be excluded since the lifespan of lymphoid tissue DC is short and DC turnover rates in the lymph nodes are between 1.5 and 3 days (Kamath 2002; Ruedl C 2000), both of which do not explain how so many DC (eg. In BALB/c mice: from ~2 x 10^5 to ~1.6 x 10^6) can accumulate in the MLN over a week during infection with *E. vermiformis*. Steady state or resident lymphoid DC have a basal level of proliferation (Angeli V 2006; Kabashima K 2005) but migrating DC from peripheral tissues to the LN during inflammation (induced by immunization) did not incorporate BrdU (Angeli V 2006) further emphasizing that the majority of DC accumulation in the MLN is due to migrating DC.

E. vermiformis does not replicate in the MLN and whole parasites are not found in the MLN unlike *E. coecicola* in rabbits (Pakandl et al., 2006). Therefore parasite material must have been carried there by intestinal DC as parasite DNA was found in DC from the

MLN (chapter six). There is a possibility that soluble or particulate parasite material was carried in the lymph to the MLN but this is unlikely to occur during the early stages of infection with the low levels of antigen available. It has to be considered that parasite material has to 'diffuse' towards openings of lymphatic vessels as opposed to being carried by a DC with chemotactic prowess. Essentially, the data shown demonstrates how the MLN are a destination for intestinal DC carrying antigenic material from gut infections and is concordant with other studies demonstrating the migration of intestinal DC from LP and PP into the MLN (Huang et al., 2000; Jang et al., 2006; Macpherson and Uhr, 2004).

How does the role of Peyer's patches compare with that of the MLN?

Clearly, the PP and MLN are essential components of primary immunity against *E. vermiformis* as seen in mice with defective or deficient for MLN and/or PP. It is important to consider how DC responses are affected at the site of infection, in the small intestine. The observation that naïve age-matched BALB/c mice had larger PP than B6 mice and the infection of PP-deficient LTα+/-β+/- provided the hypothesis that PP-cooperation was required alongside the MLN. Indeed, a lack of PP resulted in delayed DC recruitment and deficient numbers of 'early-responding' CD11b^{hi} B220- DC in the MLN of LTα+/-β+/- mice. This influence exerted by PP over the MLN suggests that a level of cooperation or crosstalk exists between the two GALT structures. Crosstalk may be carried out in the form of cellular messengers carrying antigenic messages to the MLN for induction of immunity (eg. PP-DC such as CD11b ^{hi} B220- DC). The lymphatic network in PP is described to be like a basket wrapped round lymphoid follicles and conveyed into lymph collectors before draining away from the PP (Azzali, 2003). In guinea pig, rabbit and humans, the lymphatic network in the PP seems to be separate from the lymphatics that drain the areas of the gut

which have no lymphoid tissues. Hence, it is possible that the PP are well-equipped with more efficient lymphatic drainage than the other areas of the gut and may explain why the PP plays a more important role than ILF. At present, there is no data indicating that the lymphatic network present in ILF is as extensive or functions as efficiently as the network in the PP. The ILF are inducible during adulthood and the establishment of lymphatics especially those that drain efficiently into the MLN, seems unlikely because PP are developed pre-natally (Yoshida et al., 1999). Vasculature that may develop to drain ILF are possibly blood vessels since HEV development is inducible during lymphoid neogensis (Drayton et al., 2006) while the relevant lymphatic development remains unclear.

The other role for PP is to serve as a distinct site for the generation of an independent, locally acting T cell-mediated protective immunity. Both MLN and PP sites provide independent sources of immunity despite the differences in cell numbers (MLN are larger by 10-fold). The adoptive transfer of fewer PP cells (2×10^6) compared with MLN cells (2×10^7) into TCRβxδ-/- mice (both similarly protected) showed that the generation of appropriate T cell effector clones (Both sites contained protective T cell clones which provided effector protection against primary infection) may be more important than T cell numbers (although in both cases, T cells expansion occurs in the lymphopenic environment of TCRβxδ-/- mice). Ideally, if MLN-deficient mice with PP (eg. TRANCE-/- mice (Kim et al., 2000)) were available, it will help to examine whether the PP can function alone and generate sufficient immunity against *E. vermiformis* infection, despite the capacity of MLN to develop greater volumes of cellularity. Recent oral tolerance experiments suggest a more important role for MLN in the induction of oral tolerance (Fujihashi et al., 2001; Spahn et al., 2002; Yamamoto et al., 2000) however experiments were done in PP-deficient mice

(with MLN) only. In order to define the relevant roles of PP and MLN, it would be important to examine MLN-deficient mice (with PP). It is reasonable to suggest that both sites play redundant roles in inducing pro-inflammatory responses and oral tolerance but with regard to timing of rapid responses, the PP role becomes more critical (through PP-derived immunity and the influence of PP on MLN).

Perhaps, the contributions of the MLN and PP may be better discussed in the context of infection occurring in the host (Figure 7.1):

In a B6 mouse infected orally with 100 oocysts, the oocysts are pushed along the GI tract along by peristalsis. By the time the oocysts reach the small intestine, their outer shell would have been broken down by trypsin and bile in the stomach. Sporozoites break out of the oocysts and invade epithelial cells of the intestinal villi within a few hours. This is followed by a 48 hour- development of the first generation schizont. By this time, it will be around 2-3 DPI, a critical period of time for host detection. There will be cell debris and parasite material drifting about in the intestinal lumen due to the occurrence of some cell apoptosis occurring during the course of parasite invasion. The parasite material (antigen) is sampled by PP- M cells which transfer parasite material on to local PP-DC. PP-DC may either move into the T cell zone of the PP to present to local T cells or migrate towards the MLN. DC are known to reach draining lymph nodes in around 24 hr (Mempel et al., 2004; Miller et al., 2004) and the earliest increase in MLN- DC numbers observed at 4 DPI, fits this frame of time. Adaptive immunity is initiated by migrant DC from both PP and non-PP sites but the earliest DC may come from the PP. In the MLN, IFNγ is upregulated and CD4+ T cell proliferation occurs at 4 DPI onwards, resulting in the increased numbers of gut-homing T cells (6 DPI onwards). Meanwhile in the PP, a local immune response is also

initiated evident by the upregulation of Th1-type signals (Tbet, IFNγ, IL12, TNFα) and CD4+ T cell proliferation at 4 DPI onwards. Due to the proximity of PP-T cells to the sites of infection, their input to the immune response (perhaps 5-6 DPI considering the time it takes for the adaptive immunity to develop) is critical during the time it takes for MLN-T cells to reach the site of infection (perhaps 7 DPI onwards based upon the increase in α4β7+ CD4+ T cells in the MLN at 6 DPI). It is not thought that PP-mediated responses alone are sufficient as the MLN are the major source of proliferating CD4+ effector T cells. Therefore, the roles of PP and MLN seem to be more of a cooperative endeavour where neither can be exclusively attributed with the generation of protective immunity.
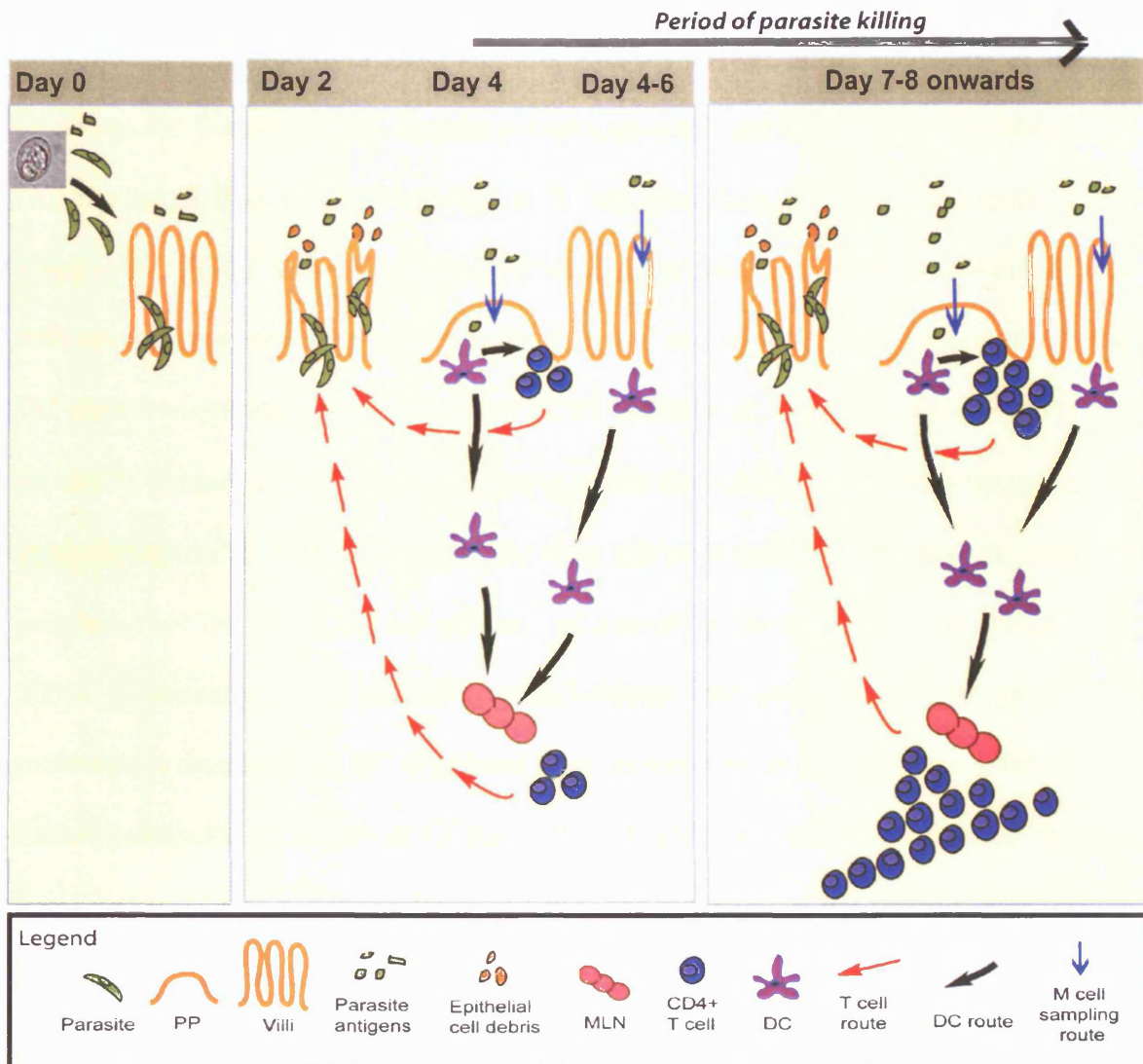
Figure 7.1 Schematic time-line for the induction of immune responses in the small intestine of a B6 mouse during infection.

PP-DC have earlier access to parasite antigens and respond by initiating local PP adaptive response and/or migrating to the MLN to initiate MLN adaptive responses. DC in the intestinal villi may also obtain antigen through villus M cells or direct sampling. Upon uptake of antigen, the intestinal villi DC migrate to the MLN to present. A higher proportion of CD4+ T cells proliferate in the MLN compared to the PP however, this requires time during which the PP-mediated response helps in providing early responses against the parasites. Parasite killing occurs around the time taken for the appearance of T cell-dependent immunity.

The influence of tissue-specific mediators and microenvironment

Dendritic cells derived from different tissue sites are distinct in the instruction of homing directions for T cells: gut-DC imprint gut-homing $\alpha 4\beta 7$ and CCR9 on T cells while skin-DC imprint CCR10, E- and P-selectin on T cells (Dudda et al., 2005; Mora et al., 2003; Stagg et al., 2002). Recent data also further differentiates the DC subsets within tissues with specific properties, for example CD103+ DC and retinal dehydrogenase-expressing DC are the major inducers of $\alpha 4\beta 7$ and CCR9 (Iwata et al., 2004; Johansson-Lindbom et al., 2005). Retinol is converted by dehydrogenases into retinoic acid which induces $\alpha 4\beta 7$ upregulation on T cells and these enzymes are expressed by intestinal DC but not peripheral LN DC which are not efficient inducers of gut-homing molecules (Iwata et al., 2004). Importantly, these studies provide evidence that the imprinting of gut-homing molecules is dependent on DC originated from the intestine. In *E.vermiformis* infection, the timed appearance of $\alpha 4\beta 7$+ or CCR9+ CD4+ T cells in the MLN and small intestine correlated with host resistance (chapter four). The induction of gut-homing molecules on MLN CD4+ T cells would have depended on intestinal DC mobilization to the MLN.

Interestingly, DC upregulate CCR7 to home to the draining lymph node and both intestinal and skin DC upregulate CCR7 (Jang et al., 2006; Ohl et al., 2004). Yet the DC-mediated imprinting of homing molecules on T cells differs. However, when intestinal DC are exported to the skin, they lose the ability to induce gut-homing molecules on T cells which indicates a level of tissue microenvironmental influence over DC function (Dudda et al., 2005). Hence, the factors that influence the behavioural pattern of DC in the microenvironment are important and possible candidates are epithelial cells of the skin or gut. One example of an influence exerted by epithelial cells is through their expression of thymic stromal lymphopoietin (TSLP) which temporally modulates DC function (Rimoldi

et al., 2005). Newly recruited DC from the blood enter inflamed intestinal tissues and are

activated to induce Th1 responses. At the same time, prolonged exposure to epithelial cells

-produced TSLP modulates DC towards a Th2-type behaviour, similar to the DC residing

in the gut. This is an example of how DC responses are controlled in the gut and may

represent the modulation of the balance between pro- and anti-inflammatory responses by

the gut microenvironment. In the case of imprinting tissue-specific molecules, the

environmental presence of dietary vitamin A (a precursor of retinol) may distinguish the

gut from the skin. In fact, retinoic acid inhibits the expression of skin-homing molecules,

E-selectin ligand, P-selectin ligand and CCR4 on T cells and peripheral lymph node DC do

not express as much retinal dehydrogenases as gut-associated DC. The mechanism which

skin-DC imprint skin-homing molecules on T cells, has not been elucidated.

Physiological factors such as vitamin A and signals expressed by epithelial cells and other

stromal cells in the tissue microenvironment play a role in influencing DC response. An

example of the influence of microenvironments can be seen in the PP-cell adoptive transfer

experiments. Naïve PP cells function in a different way when transferred into TCRβxδ-/-

mice and LTα+/-β+/- mice as demonstrated by the difference in susceptibility. There is no

doubt the absence of PP in LTα+/-β+/- mice contributed to susceptibility but this was

exacerbated in LTα+/-β+/- mice given PP cells which may suggest that regulatory elements

from PP are absent in LTα+/-β+/- mice or donor cells (which have no PP to home to) may

interfere with immune responses in the MLN. This is in contrast with TCRβxδ-/- recipient

mice, PP are present for PP cells to home back to where they may be modulated by the PP

environment and the lymphopenic environment of TCRβxδ-/- mice permits the expansion

of T cells essential against infection. The evidence for regulatory elements is seen in TCRβxδ-/- mice given PP cells from infected donors, which developed prolapses (a sign of an inflammatory bowel condition) and in mice given naïve PP cells, indicating a lack of regulatory elements (perhaps regulatory T cells). The presence of anatomical structures is clearly important for the containment of regulatory and inflammatory cells which when left to 'wander' into the wrong tissues, may result in an imbalance in the immune system.

Conclusions

In summary, the work presented defines the separate requirements for PP and MLN during infection and how the MLN responses depend on PP-mediated responses, and is summarised in Figure 7.2. Control of primary infection depends on the timing of DC responses which is dependent on both MLN and PP. Both LTα-/- and LTβ-/- mice have absent or defective MLN and PP and are more susceptible than LTα+/-β+/- mice which have the advantage of a functional MLN. The inducible reconstitution of ILF in the appropriate BM chimeric mice cannot rescue the susceptible phenotype. Resistance is dependent on the presence of intact MLN and PP as seen in B6 and BALB/c mice. However factors such as genetically-influenced anatomical variables (PP size and cellularity) and parasite doses influence the rate of induction and therefore the level of resistance. The efficacy and rate of primary responses depend on PP and MLN but requirements differ in the immune induction of secondary responses which can occur in the absence of GALT (MLN, PP, ILF, CP). However, the deficiency of LT has implications in the long term where immune memory does not last for as long in LT-deficient mice compared with intact mice. This may be related to the lack of a robust immune response in the absence of lymphoid organisation which results in a less-sustained T cell memory pool.

No doubt, GALT structures are not essential in the induction of memory and it is unknown where the site of initial induction is although possible sites may include the spleen, bone marrow or intestine where CD70+ stromal cells have antigen-presenting capacity to induce proliferation (Laouar et al., 2005). However, the inefficiency of these sites to induce primary gut-associated responses is a reason why GALT structures are present.

Strain	LTα-/-	LTβ-/-	LTα+/-β+/-	[a]B6 [b]LTα+/- [b]LTβ+/-	BALB/c
Susceptibility to 1° Infection	++++	++++	+++	[c]++	+
Structural Factors					
Cellular Factors	→ Slow T cell responses → Absent MLN and PP responses	→ Slow T cell responses → Absent MLN and PP responses	→ Slow T cell responses → Absent PP responses but MLN responses present	→ Fast DC and T cell responses → MLN and PP responses present	→ Faster DC and T cell responses than B6 → More PP cells than B6
2° Infection					
Immunity when rechallenged 1 mth later	Protected	Protected	Protected	Protected	Protected
Immunity when rechallenged 8 mth later	Unprotected	Unprotected	ND	[a]Protected [b]ND	ND

LEGEND: Villi | ILF | PP | Partial MLN | MLN | Disorganized Spleen | Organized Spleen | Lymphatics | Blood circulation

Figure 7.2 (see next page)

Figure 7.2 Summary of the structural and cellular factors influencing the outcome of primary and secondary infections.

The presence of an intact MLN and PP helps mediate resistance to *E. vermiformis* primary infection. The role of ILF is not essential as demonstrated from the data with BM chimeric mice. The ILF may not have the same lymphatics draining into the MLN as the PP and therefore do not play an essential role during infection. In LTα-/- mice, the absence of a MLN leads to draining of antigen to sites such as the spleen although it is not known what the direct route taken is. During secondary infection, protective immunity is conferred in the absence of GALT. However, differences can be seen in the long term where LTα-/- and LTβ-/- mice fail to mount protective memory responses, indicating possible defects in the generation of long term sustainable T cell memory. +, indicates the level of susceptibility where ++++ refers to highest susceptibility and + refers to lowest susceptibility. [a] refers to intact B6 mice; [b] refers to LTα+/- and LTβ+/- mice; [c] B6, LTα+/- and LTβ+/- mice are more resistant to infection compared to LTα-/-, LTβ-/- and LTα+/-β+/- mice. However there may be some evidence that B6 mice show slightly higher resistance than LTα+/- and LTβ+/- mice. *ND*, not done.

Future considerations and applications

For future considerations in continuing this work presented, there are many different areas of interest that can be further examined. For example, the instructional events (if any) which determine PP-DC migration to the MLN are unknown. Also, when DC upregulate CCR7 to home to the MLN, the actual mechanisms occurring in the lymphatic vessels, are unclear. We know about rolling and adhesion movements between endothelial walls by migrating cells but what are the factors regulating lymph pressure and flow change during infection which affects DC mobilization to the MLN? The availability of scanning two-photon lasers has helped redefine our perspective of immune interactions in a three-dimensional way (von Andrian, 1996). The compartments seen on cross-sections of tissues, are whole and perfused with interconnecting vessels which permit entry/exit into or from the LN. It will be exciting to identify DC subset movements within the lymph nodes, their interactions with gut-homing T cells and to plot entirely the routes through which DC travel from the gut to the MLN and T cells from the MLN to the gut. Further areas of interest include whether the CD11b^{hi} B220- subset in LTα+/-β+/- mice, is developed specifically in the PP (ie. further differentiation after migration from the bone marrow). If so, how does the PP-environment instruct DC differentiation or other lymphoid organs instruct the differentiation of other DC subsets?

These questions may involve the application of transcriptome (eg. microarray technology or SAGE) and proteomic analysis (eg. mass spectrometry) to analyze intrinsic DC differences, determine any difference or influence of particular signalling pathways at RNA or protein levels respectively. Further determination of any particular gene of interest found in different DC subsets, may be used in RNA silencing or knockout/knockin transgenic technology to generate transgenic mice which may provide further *in vivo* analysis of the

gene's function. The use of *E. vermiformis* has its advantages for the study of infections confined to the small intestine such as its predictability and reliability in providing a consistent measure of immune responses. Such a model will better utilised if a suitable *E. vermiformis*-specific TCR transgenic T cell is available as many other infections with a specific TCR model, have been useful in studying immune responses against infections. Alternatively the development of a stable transfection of OVA into *E. vermiformis* can be accommodated as OVA-specific TCR transgenic mice are available. The genetic mapping of BALB/c and B6 strains through quantitative trait loci analysis, can help identify a candidate susceptibility gene that may be used in transgenic mutant mice or RNA silencing, to study the gene's functional role during infection.

The influence of PP over MLN responses may have implications in the design of vaccines. Clearly, the MLN has great potential in generating massive responses and this should be exploited for therapies against enteric infections and inflammatory bowel diseases. Since PP are potentially accessible from the lumen (through M cells), it is ideal to exploit this route for immunizations using DC as carriers. We know more about how intracellular signalling by G-proteins and MAPK family members regulate DC chemotaxis and migratory speed to lymphoid organs (Riol-Blanco et al., 2005) but can we harness this knowledge as a means to specify instructions to DC? The lymphatics that link PP to the MLN may be exploited for the transport of immunogens (carried by DC) and one thing that is lacking in many mucosal-vaccination strategies is the ability to direct DC movements. The route of injection helps determine DC migration (Dudda et al., 2004) however, it is possible to refine such strategies to produce more specific immune responses. By understanding DC migration, one may be able to direct specific responses eg. induce

plasmacytoid DC migration to the MLN to initiate anti-viral immune responses by orally feeding TLR7/8 agonists (Yrlid et al., 2006) or targeting CD103+ DC which may support regulatory T cells and help prevent colitis (Annacker et al., 2005). No doubt, this is not as simple as it seems bearing in mind that a balance is required to ensure no overt immune responses (pro or anti-inflammation) occur unnecessarily. Therefore a combination of strategies involving DC and regulatory elements such as T regulatory cells, may be considered in the development of mucosal vaccines. Invasive pathogens have been used as immunogen/antigen carriers, for example in cancer immunotherapy where attenuated but invasive S. Typhimurium is used (Avogadri et al., 2005). Perhaps parasites with a self-limiting replication (eg. An *E. vermiformis* equivalent in humans), expressing an appropriate immunogen (eg. cancer cell antigen, antigen for eliciting mucosal responses against infections) may also be considered.

To date, not much is known about the human PP. Many studies are based on mice bred in specific pathogen-free units which may explain the differences in the Th-bias of the murine and human PP (the former seems Th2-biased while the latter, Th1-biased). Such studies may have to take into account the immune events occurring when the host is exposed to 'normal' environment. Furthermore, what more do we know about the lymph nodes draining the human gut? Are they influenced by the DC responses in the PP? There is a great deal of work to be done to understand the human gut (of course human experimentation has its limits) as much as we do in the mouse. On a final note, there is not a good reason to ignore the ~300 PP sites found along in the human gastrointestinal tract, which form an excellent vaccination route through targeting M cell-specific receptors, for eliciting effective mucosal immune responses.

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Appendix

Peyer's Patches Are Required for the Induction of Rapid Th1 Responses in the Gut and Mesenteric Lymph Nodes during an Enteric Infection[1]

Sue-fen Kwa,*[†] Peter Beverley,[†] and Adrian L. Smith[2]*