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Dendritic cell mediated mucosal cross talk.



A thesis submitted to
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For the degree of
Doctor of Philosophy

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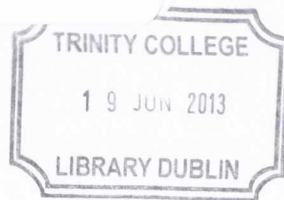
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Declaration of Authorship

This thesis is the sole work of the author and has not been submitted in whole or in part to this or any other university for any other degree of doctor of philosophy.



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DECLARATION

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Finally, I thank my parents, Pauline and John, and dedicate this thesis to them, for encouraging and inspiring me in life and in science.

Abstract

Abstract: Developing efficacious vaccines against enteric diseases is a global challenge and priority. The rational design of such vaccines requires that novel pathways of cellular recruitment to the gastrointestinal (GI) tract be explored. The current paradigm of T cell homing to the GI tract involves the induction of $\alpha 4\beta 7$ and CCR9 by Peyer's patch (PP) and mesenteric lymph node (MLN) dendritic cells (DC) in a retinoic acid (RA) dependent manner. To examine alternative pathways of cellular migration to the GI tract, we have examined the ability of lung DC (LDC) to recruit lymphocytes to the GI tract. Using a DC:OT-II co-culture system, we have identified that LDC (both CD103⁻ and CD103⁺) up regulate gut homing integrin $\alpha 4\beta 7$ *in vitro* and induce T cell migration to the GI tract *in vivo*. LDC mediated $\alpha 4\beta 7$ induction required retinoic acid and TGF- β signaling. Significantly, targeting of LDC by intranasal immunization induced protective immunity against enteric challenge with a highly pathogenic strain of *Salmonella typhimurium*. The present report demonstrates the presence of significant mucosal cross talk mediated by DCs. Additionally; we define a novel pathway of lymphocyte migration to the GI tract, which potentially paves the way for rational design of vaccines against mucosal pathogens

Publications

Pantel A, Cheong C, Dandamudi D, Shrestha E, Mehandru S, Brane L, **Ruane D**, Teixeira A, Bozzacco L, Steinman RM, Longhi MP. (2012) A new synthetic TLR4 agonist, GLA, allows dendritic cells targeted with antigen to elicit Th1 T-cell immunity in vivo. *Eur J Immunol*. 2012 Jan;42(1):101-9. doi: 10.1002/eji.201141855. Epub 2011 Dec 12 (See Appendix I).

Ruane D, Lavelle EC. (2011) The role of CD103⁺ dendritic cells in the intestinal mucosal immune system. *Front. Immun.* 2:25. doi: 10.3389/fimmu.2011.00025 (See Appendix I).

Abbreviations

Ag: Antigen
APAF-1: Apoptosis protease activating factor 1
APC: Antigen presenting cell
ASC: Apoptosis associated speck-like protein
BAL: Bronchoalveolar Lavage
BCL6: B cell lymphoma 6
BCR: B cell receptor
BMDC: Bone marrow derived dendritic cell
CAPS: Cryopyrin associated periodic syndrome
CCR9: C-C chemokine receptor type 9
cDC: Conventional dendritic cell
CDP: Common dendritic cell precursor
CFA: Complete freunds adjuvant
CIITA: Class II transactivator
CpG: Cytosine-phosphate-guanine
CT: Cholera Toxin
CTB: Cholera toxin B subunit alone
CTL: Cytotoxic T lymphocyte
CTLA: CTL-associated protein 4
DAMP: Danger-associated molecular pattern
DC: Dendritic cell
DLL: Delta like ligand
DNA: Deoxyribonucleic acid
EAE: Experimental allergic encephalomyelitis
ELISA: Enzyme-linked immunoadsorbant assay
ER: Endoplasmic reticulum
ETEC: Enterotoxigenic *E. Coli*
FCS: Fetal calf serum
FOXP: Forkhead box containing protein
GALT: Gut associated lymphoid tissue
GATA: GATA- binding protein
GLA: Glucopyranosyl lipid A
GM-CSF: Granulocyte monocyte colony stimulating factor
ICAM-1: Inter-cellular adhesion molecules 1
ICOS: Inducible co-stimulator
iDC: Inflammatory dendritic cell
IFA: Incomplete freunds adjuvant
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
iNKT: Invariant natural killer T cell

IPAF: ICE-protease activating factor
 IPS-1: Interferon- β promoter stimulator 1
 IRAK: IL-1 receptor associated kinase
 ITAM: Immunoreceptor tyrosine based activation motif
 iTreg: Inducible regulatory T cell
 LC: Langerhans cell
 LDC: Lung dendritic cell
 LPS: Lipopolysaccharide
 LRR: Leucine rich region
 mAb: Monoclonal antibody
 MAPK: MAP kinase
 MDA-5: Melanoma differentiation associated gene 5
 MDP: Monocyte/macrophage/dendritic cell precursor
 MHC: Major histocompatibility complex
 MLN: Mesenteric lymph node
 MPL: Monophosphoryl lipid A
 NALT: Nucleotide-binding oligomerization domain receptors
 NKT: Natural killer T cells
 PD-1: Programmed cell death 1
 pDC: Plasmacytoid dendritic cell
 PDI: Protein disulfide isomerase
 PP: Peyer's patch
 PMA: Phorbol myristate acetate
 Poly I:C: Polyinosine-deoxycytidylic acid
 PP: Peyer's patch
 RA: Retinoic acid
 RAR: Retinoic acid receptor
 RIG: Retinoic acid inducible gene
 RNA: Ribonucleic acid
 ROR γ t: Retinoid-related orphan receptor γ
 ROS: Reactive oxygen species
 RT-PCR: Real time polymerase chain reaction
 SDS: Sodium dodecyl sulphate
 SILP: Small intestinal lamina propria
 STAT: Signal transducer and activator of transcription
 TAP: Transporters associated with antigen presentation
 T-Bet: T-box expressed in T cells
 TCR: T cell receptor
 TGF- β : Transforming growth factor β
 Th: Helper T cell
 TipDC: TNF/inducible nitric oxide synthase producing dendritic cell
 TLR: Toll-like receptor
 nTreg: Natural T regulator cell
 TNF: Tumour necrosis factor
 Treg: Regulatory T cell
 TSLP: Thymic stromal lymphopoietin

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Chapter 1

Introduction

1.1 Introduction

“The circassians [a middle European people] perceived that of a thousand persons hardly one was attacked twice by full blown smallpox; that in truth one sees three or four mild cases but never two that are serious and dangerous; that in a word one never truly has that illness twice in life.” – Voltaire, “On Variolation” Philosophical Letters, 1743.

Infectious diseases are the leading cause of death worldwide. The two most important contributions to improving public health over the past 100 years have been the introduction of sanitation and vaccination. Together they have dramatically reduced the number of deaths resulting from infection. “Vaccines can be described as formulations that induce specific, non-toxic, and long-lasting immune responses to prevent or treat diseases” (Steinman 2008).

Vaccination probably originated in homeopathic beliefs regarding small doses of diseased tissue, providing protection against severe infection. By the beginning of the 11th century Chinese literature revealed the use of variola scabs insufflated into the nose to immunize against small pox (Plotkin 2005). Although the Chinese are generally credited with the introduction of variolation, the other candidate region as the origin of variolation is India, where a scarification procedure was either invented or imported from China (Plotkin 2005). From these humble beginnings variolation began to spread throughout the Middle East and Africa, Turkey, Great Britain and Europe. Variolation was so successful that during the American revolution British troops were exposed to variola scabs to prevent small pox outbreaks, a measure which was eventually adopted by George Washington and the US army (Fenn, E.A. Pox Americana: The Great Smallpox Epidemic of 1775-82. 1 (Hill and Wang, New York, 2001).

Despite its success, variolation was associated with significant and even fatal reactions, which resulted in its eventual cessation. No doubt this was the impetus for Edward Jenner (Plotkin 2005) who first established the conceptual framework for the development of a

vaccine in the late 18th century. Jenner had made the important observation that milkmaids who contracted the cowpox virus failed to develop fatal smallpox. On May 14th 1796, Jenner inoculated a small boy, James Phillips, with a live cowpox virus that had been removed from the hand of a milkmaid, Sarah Nelmes. The procedure he termed “vaccination” resulted in the boy becoming immune to the small poxvirus (Hill, Shirley et al. 2009).

This experiment would not only lay down the foundation for the eventual eradication of smallpox but also lead to the development of modern immunology (Steinman 2008). Progress in the vaccine science field continued as Louis Pasteur developed a vaccine against chicken cholera in 1879 and human rabies in 1885 (Halsted 1895). Pasteur’s work revealed that attenuated microorganisms could induce strong long-term protection against infection resulting from virulent or infectious organisms.

The 19th century saw the wide scale introduction of vaccination and as a result greatly reduced the number of deaths due to infectious diseases. By the end of the 19th century vaccines against small pox, rabies, typhoid, cholera and plague were in use. During the 20th century, more than 40 vaccines were introduced for human use (Poland, Ovsyannikova et al. 2007). All of these vaccines have been generated based on traditional approaches, first developed by Pasteur, including the isolation and inactivation of infectious pathogens or their toxins.

However, over recent decades, safety issues and advances in molecular biology and immunology have lead to the development of subunit vaccines, which comprise purified components of organisms (Lavelle 2006). These subunits may be peptide or protein antigens, polysaccharides or DNA (Chang, Huang et al. 2010). Moreover advances in genomics and bioinformatics have greatly improved the potential for developing new vaccines. The increased safety profile, which is associated with these subunit vaccines, comes with the cost of reduced immunogenicity, directly affecting vaccine efficacy. This is due to the absence of pattern recognition patterns (PAMPs) such as flagellin, which are highly immunostimulatory.

1.2 Overview of the immune system

The immune system is divided into two distinct arms, innate and adaptive. The innate immune system is composed of two elements, firstly the cellular components such as dendritic cells (DCs), macrophages, natural killer cells, neutrophils, and secondly soluble components such as cytokines, and complement proteins. These cells play a vital role in the early sensing of pathogenic material and react quickly to mount an effective immune response. In contrast, the adaptive immune system is composed of T and B-lymphocytes, which respond more slowly, however unlike the innate immune response, which is non-specific, the adaptive immune generates an immune response targeted to a particular epitope.

1.3 Innate Immune System

1.3.1 Dendritic Cells

The first observation of Dendritic cells (DCs) was made in the 1800s by Paul Langerhans who identified what are now known as Langerhans cells in skin sections (Steinman and Banchereau 2007). However, it was not until 1973 that DCs were finally described. During their initial studies attempting to determine “the capacity of an antigen to provoke an immune response” (Sela 1969) Steinman and Cohn identified an “unusual cell that did not look like any other white blood cells that had been seen before” (Steinman and Cohn 1973; Steinman 2007).

These cells had a prominent stellate shape and continually extended their processes or dendrites. These cells also lacked the typical features of lymphocytes and macrophages (Steinman and Cohn 1974; Steinman, Lustig et al. 1974). These novel cells were termed dendritic cells for “treelike”, from the Greek “dendron” or “dendreon”. The immunogenic role of DCs was initially noted in the context of transplantation, in a tissue culture model for graft rejection entitled the mixed leukocyte reaction (MLR) (Steinman

and Witmer 1978). The MLR detects genetic incompatibility in the MHC between a donor and a recipient during organ transplants.

Steinman and colleagues made the initial discovery that DCs were 100 times more potent at inducing MLR than total spleen cells, which contained only 1% DCs (Steinman and Witmer 1978). Similar observations were described by Don Mason and Wesley Van Voorhis, investigating rat afferent lymph and human blood (Mason, Pugh et al. 1981; Van Voorhis, Hair et al. 1982). In contrast, macrophages and B cells were poor inducers of the MLR. Microscopy revealed the clustering of T cells around the DC which lead to the hypothesis that “DC [would] prove to be a critical accessory cell required in the generation of many immune responses” (Steinman and Witmer 1978). Despite the ability of MHC II⁺ B cells and macrophages to perform secondary T cell MLR recall, only DCs could initiate MLR with naïve T cells. (Inaba and Steinman 1984). These early experiments lead to the establishment of a new field in Immunology, dendritic cell science.

DCs are enormously plastic in nature, owing to various micro-environmental adaptations and influences. The DC population can be further subdivided based on their location. DCs can be found at sites including the skin, lung and intestine, while other DCs are constantly circulating. DC subpopulations also differ in their migrating pathways, immunological function and the types of stimuli required for their activation and/or generation. Despite their heterogeneity (Shortman and Liu 2002), DCs share functional properties that distinguish them as a pivotal link between the innate and adaptive immune system (del Rio, Bernhardt et al. 2010).

All DCs are capable of antigen uptake, processing and presentation to naïve T cells. The various DC subtypes differ depending on their location, migratory pathways and dependence on infections or inflammatory stimuli for their generation. “DCs are a uniquely positioned [and] prime target for disease-relevant stimuli” (Steinman 2007). In addition to their role in the induction of immune responses, DCs also have a role in maintaining tolerance during the steady state. Following the deletion of DCs, it was

observed that mice suffered from the rapid onset of inflammation, induction of auto-reactive CD4⁺ T cells and increased IFN- γ and IL-17 producing cells, all associated with severe autoimmunity (Ohnmacht, Pullner et al. 2009). Coquerelle *et al.*, (2010) have suggested that the tolerogenic phenotype observed may be due to environmental conditioning and unique maturation signals (Coquerelle and Moser 2010)

1.3.2 Dendritic cell subtypes

DCs can be classified into three main groupings; conventional DCs (cDC), inflammatory DCs and pre-DCs (Inaba, Witmer et al. 1984) (Mucosal specific DCs are discussed in section 1.10). cDCs is a term used to describe DCs which already have a dendritic form and exhibit DC function in the steady-state. Pre-DCs are DC precursors, which require further development to acquire a dendritic form and complete DC function. During their development pre-DCs become terminally differentiated and undergo the last developmental precursor stage *en route* to cDC development. Moreover little or no cell division is required for their complete development into cDCs. Different types of pre-DCs produce different types of DCs, with many requiring microbial or inflammatory stimulation to complete their developmental process (Grouard, Rissoan et al. 1997).

In fact plasmacytoid DCs are thought to respond to viral infection via the expression of TLR7 and TLR9, therefore detecting viral nucleic acids (Coquerelle and Moser 2010), however plasmacytoid DCs are less effective than cDCs at presenting antigenic material due to the low expression of MHC Class II molecules. A proportion of plasmacytoid DCs also carry characteristic immunoglobulin rearrangements (Corcoran, Ferrero et al. 2003). Monocytes (precursors of macrophages) can also serve as pre-DCs in response to microbial stimulation (Cheong, Matos et al. 2010). These cells have been termed monocyte derived DCs (Mo-DCs) and are recruited from blood monocytes into lymph nodes by lipopolysaccharide and live or dead gram-negative bacteria. Mobilization (in response to these stimuli) requires TLR4 and its CD14 co-receptor and Trif. When tested for antigen-presenting function, Mo-DCs are as active as classical DCs, including cross-presentation of proteins derived from live gram-negative bacteria on MHC I *in vivo*. Fully

differentiated Mo-DCs acquire DC morphology and localize to T cell areas via L-selectin and CCR7. Thus the blood monocyte reservoir becomes the dominant presenting cell in response to select microbes, yielding DC-SIGN⁺ cells with critical functions of DCs (Cheong, Matos et al. 2010).

cDCs can be further subdivided into two distinct lineages, migratory and lymphoid tissue resident. Both lineages are generally short-lived, with lymphoid tissue cDCs replaced by blood-borne precursors (Liu, Victora et al. 2009). The migratory cDC population “have the classic text-book DC life history” (Bell, Young et al. 1999), serving as antigen-sampling cells. Migratory cDCs are found in peripheral tissue; there they capture antigens prior to migrating to local draining lymph nodes. One such example of a migrating cDCs population is the epidermal Langerin positive cDC, these cells are easily distinguished from the dermal derived DCs due to their high expression of Langerin (CD207) a type II lectin with mannose specificity found in the epidermis, in addition to other sites (Henri, Vremec et al. 2001).

These cells capture antigenic material in the skin before migrating to local skin draining lymph nodes and presenting antigens in the T and B cell zones. In contrast, lymphoid resident cDCs do not migrate into lymphoid organs from the lymphatics; rather, they collect and present antigens in the lymphoid organ itself. Lymphoid resident cDCs can be found in both the thymus (Ardavin 1997) and spleen (Vremec, Pooley et al. 2000), with splenic DCs being further subdivided into CD8 α ⁺ and CD8 α ⁻. These cells differ in both their immune function and cytokine profiles (Hochrein, Shortman et al. 2001). CD8 α ⁺ cDCs are capable of cross priming, a method of presenting exogenous peptides in complex with the MHC class I complex (den Haan, Lehar et al. 2000), while CD8 α ⁻ lack this ability. CD8 α ⁻ cDCs can be further subdivided depending on their CD4 expression pattern (CD4⁺CD8⁻ and CD4⁻CD8⁻) (Vremec, Pooley et al. 2000). Further complexity amongst the CD8 α ⁻ cDCs subset has been revealed in recent studies investigating the expression pattern of the cell surface integrins CD103 and CD11b (discussed in detail in section 1.10.1.2). Taken together cDCs can be divided based on their expression of CD4, CD8, CD11b and CD103, which is related to their effector functions and thus highlights

the heterogeneity of cDC subsets. Unlike migratory cDCs, which have a mature phenotype upon reaching the lymph nodes, lymphoid-tissue-resident cDCs only become active following antigen uptake and processing (Wilson, El-Sukkari et al. 2003; Wilson, El-Sukkari et al. 2004). The majority of cDCs found in the lymphoid organs during steady state conditions are monocyte-independent, not requiring the growth factor macrophage colony stimulating factor (M-CSF) for their development, instead favouring fms-like transcript 3 ligand (Flt3) (**Fig 1.1**) (Helft, Ginhoux et al. 2010; Liu and Nussenzweig 2010). Although present in the human circulation, cDCs are rare in mouse blood.

During steady state conditions, when a healthy mammal is not exposed to infectious or inflammatory stimuli only pre-DCs and cDCs are present. However during times of inflammation or infection, inflammatory DCs are normally induced. One such example of an inflammatory DC found *in vivo* is induced in response to influenza virus. Exposure of pre-DCs to the influenza viral particles (O'Keeffe, Hochrein et al. 2002), induced the development of inflammatory DCs. Another example is the inflammatory DCs induced following infection with *Listeria monocytogenes* (Serbina, Salazar-Mather et al. 2003), termed Tip DCs as they produce considerable amounts of tumour-necrosis factor (TNF) and inducible nitric-oxide synthase. These Tip DCs were found in the spleens of *L. monocytogenes*-infected mice. The absence of Tip DCs results in profound TNF and iNOS deficiencies and an inability to clear primary bacterial infection. However, CD8 and CD4 T cell responses to *L. monocytogenes* antigens were preserved in CCR2-deficient mice, indicating that Tip-DCs are not essential for T cell priming. Tip DCs, as the predominant source of TNF and iNOS during *L. monocytogenes* infection, orchestrate and mediate innate immune defence against this intracellular bacterial pathogen (Serbina, Salazar-Mather et al. 2003). Kool *et al.*, (2008) have also revealed that inflammatory DCs are recruited to the site of injection following immunization with alum-adjuvanted ova in what appears to be a uric-acid dependent mechanism (Kool, Soullie et al. 2008).

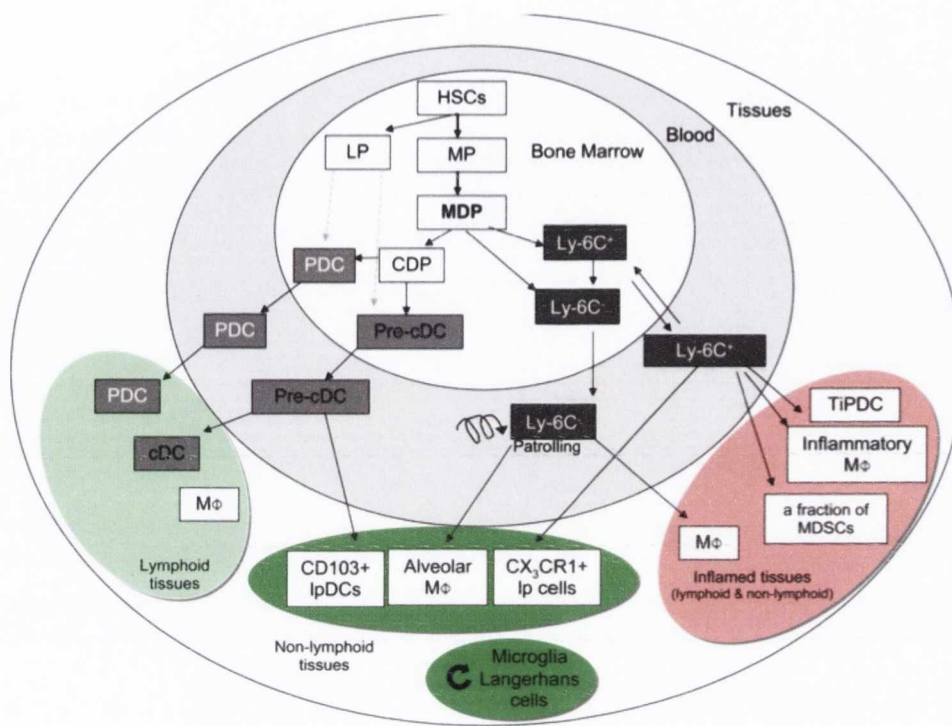


Fig. 1.1 Differentiation of DCs and macrophages in mice.

In the bone marrow, hematopoietic stem cells (HSC) produce myeloid (MP) and lymphoid (LP) committed precursors. MP give rise to monocyte/macrophages and DC precursors (MDP). MDP give rise to monocytes, and to a common DC precursor (CDP). Two monocyte subsets, Ly-6C⁺ and Ly-6C⁻ leave the bone marrow to enter the blood. CDP give rise to pre-classical dendritic cells (pre-cDC) and plasmacytoid dendritic cells (PDC). Pre-cDC circulate in blood and enter lymphoid tissue, where they give rise to CD8 α ⁺ and CD8 α ⁻ cDCs, and non-lymphoid tissues, where they may give rise to CD103⁺ lamina propria DC (IpDC). Under homeostatic conditions, Ly-6C⁻ monocytes may contribute to alveolar macrophages (M Φ) and Ly-6C⁺ monocytes can become CX3CR1⁺ IpDCs in non-lymphoid tissues. During inflammation, Ly-6C⁺ monocytes give rise to monocyte-derived DCs, e.g. TNF and iNOS-producing dendritic cells (TipDC), inflammatory macrophages, and may contribute to myeloid-derived suppressor cells (MDSC) associated with tumors. They are also suspected to contribute to microglia and Langerhans cells in selected experimental conditions. Microglia and Langerhans cells can renew independently from the bone marrow (curved arrow). HSC can also leave their bone marrow niche and enter peripheral tissues, where they differentiate to myeloid cells during inflammation. It is unclear at this time if the LP contributes significantly to PDC and cDCs (dashed arrow). (Frederic Geissmann *et al.*, 2010)

1.3.3 Antigen presentation by Dendritic Cells

The most important feature of the DC is the ability to induce priming, differentiation and activation of naïve T cells, all of which occurs in the lymph node. Three signals are required for the complete activation of naïve T cells. The first signal is the antigen-specific signal resulting from the T cell receptor (TCR) recognition of peptide loaded MHC molecules on the DC. Upon engulfing antigenic material, DCs can process and present small antigen complexes via MHC Class I, MHC Class II or CD1 molecules.

1.3.3.1 The MHC class I presentation pathway:

The major histocompatibility complex (MHC) class I molecules are composed of MHC-encoded heavy chains (HC)[human leukocyte antigen (HLA)-A,-B, or -C] and the invariant subunit β_2 -microglobulin (β_2m) (Peaper and Cresswell 2008). Presented on the cellular surface, antigen-specific cytotoxic CD8⁺ T cells recognise peptides of eight to ten amino acids in length. These amino acids are found in a cleft region associated with the MHC class I/ β_2m complexes. In addition to DCs the MHC Class I pathway is also found on almost all cell types, this allows for the effective presentation of endogenous peptides on the cell surface, which is important during viral infections. Peptides presented by the MHC Class I complex are processed in the cytosol by the proteasome allowing CD8⁺ T cells to respond to peptides derived from intracellular pathogens. Initial folding and oxidation of MHC class HC and assembly with β_2m occurs in the endoplasmic reticulum (ER), these early events are mediated by the chaperone calnexin. Calnexin is responsible for the stable folding and assembly of the α subunit with β_2m . Following association a partially folded complex entitled $\alpha:\beta_2m$ forms and immediately dissociates from calnexin. This partially folded intermediate interacts with additional soluble chaperones including calreticulin, ERp57, tapasin and TAP. Proteins destined for degradation in the proteasome are initially conjugated to ubiquitin. These labelled proteins are transported to the proteasome where they are degraded and translocated to the ER via transporters associated with antigen processing (TAP) 1 and 2 (**Fig 1.2**).

Now entitled the MHC class I loading complex this large complex of proteins facilitates the final assembly with peptides, delivery into the ER by TAP and “provide[s] quality control for exported peptide-MHC complexes” (Elliott and Williams 2005). Loading of peptide fragments into the MHC class I loading complex occurs during the assembly within the ER (Elliott and Williams 2005). During peptide loading an additional exchange entitled peptide editing takes place. Tapasin has been postulated to have a key role in peptide editing, however many components of the peptide-loading complex may be actively involved.

ERp57 is a thiol oxidoreductase which forms a disulfide bond with tapasin (Dick, Bangia et al. 2002). Due to its ability to maintain proper disulfide structure within the MHC complex, ERp57 may regulate peptide loading via disulfide exchange (Dick, Bangia et al. 2002). In fact a highly conserved disulfide bond in the $\alpha 2$ domain is critical for stabilizing the conformation of the peptide-binding domain. It has also been revealed that ERp57 is important in recruiting and retaining MHC molecules in the peptide-loading complex. Moreover ERp57 has been shown to be required for optimal expression of stable MHC-peptide complexes on the cell surface (Garbi, Tanaka et al. 2006).

Protein disulfide isomerase (PDI) is an enzyme found within the ER whose primary function is the generation of disulfide bonds during protein folding. In 2006, Park *et al* revealed an additional function for PDI and identified its role in the peptide-loading complex (Park, Lee et al. 2006). The disulfide bond within the peptide-binding cleft is in a dynamic state, constantly changing from an oxidised state to a reduced state. The regulation of the redox state of the disulfide bond in the MHC class I $\alpha 2$ domain is essential for peptide-loading, PDI and other members of the peptide-loading complex now appear to be crucial for this regulation (Park, Lee et al. 2006). In addition to catalytic domains, PDI has a domain for binding small peptides and substitutions in the PDI peptide domain were found to impair MHC Class I binding (**Fig 1.3**).

The peptides that enter the MHC Class I pathway for presentation are generated from a wide variety of sources including misfolded proteins. In fact, due to the error prone nature of the ribosomal translation process, it was proposed that these biologically irrelevant proteins appeared to be the main antigen entering the MHC Class I pathway (Schubert, Anton et al. 2000). However, this hypothesis has recently been called into question and new evidence suggests that newly synthesised proteins are protected from degradation during and after translation in the ribosome and “that pre-existing proteins represent the main proteasome substrates” (Vabulas and Hartl 2005).

In addition to the presentation of endogenous peptides via MHC Class I, DCs and macrophages have the ability to present exogenous antigens; this is mediated by the internalization of antigens through the endocytic pathway to CD8⁺ T cells (Watts 2004). This pathway is essential for the initiation of CD8⁺ T cell responses to antigens that would otherwise fail to gain access to the MHC Class I presentation pathway in DCs. Within the spleen there appears to be a “division of labour” between the CD8⁺ and CD8⁻ DCs. Here the CD8⁺ population is efficient at cross presenting antigens, in contrast the CD8⁻ DC population has a superior ability to induce antigen specific CD4⁺ T cells (den Haan, Lehar et al. 2000; Dudziak, Kamphorst et al. 2007). There are a number of pathways mediating cross-presentation, including TAP-dependent and TAP-independent pathways. The TAP independent pathway (also known as the vacuolar pathway) involves peptide exchange in recycling endosomes or at the cellular surface, with the lysosomal protease cathepsin S predominantly responsible for generating peptide fragments for presentation (Shen, Sigal et al. 2004).

TAP-dependent pathways seem to predominate and are thought to involve the transfer of antigens from the endosome to the cytoplasm. Once in the cytoplasm, these antigens directly enter the MHC Class I presentation pathway as substrates for the proteasome. New observations have suggested that the mechanism of antigen internalization may influence the ability of said antigens to under go cross-presentation to CD8⁺ T cells (Burgdorf, Kautz et al. 2007). For example antigen uptake by mannose receptor mediated endocytosis results in the preferential cross-presentation to CD8⁺ T cells. In contrast

antigens taken up via scavenger receptors or pinocytosis are targeted to lysosomes and as a result are presented to CD4⁺ T cells, not CD8⁺ T cells. This model supports the hypothesis that individual phagosomes are self-sufficient for antigen cross presentation and antigen processing (Gagnon, Duclos et al. 2002; Houde, Bertholet et al. 2003)

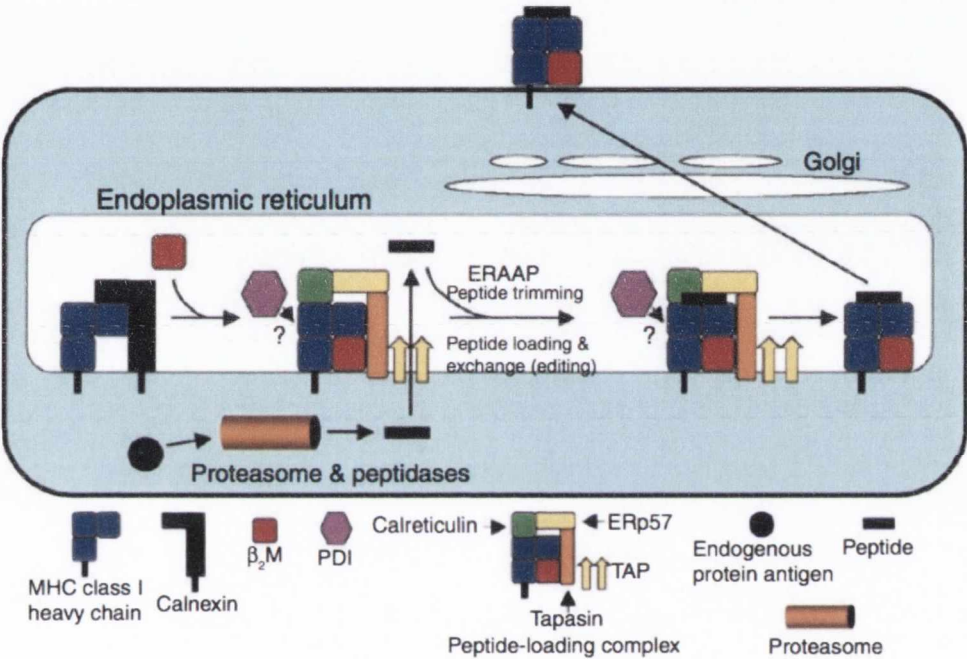


Figure 1.2 : The direct MHC Class I processing pathway.

The MHC Class I processing pathway is found in almost all cell types, thereby allowing the display of endogenous peptides on the cell surface during viral infection. The initial stages of the pathway involve degradation of cytosolic proteins. MHC Class I heavy chains assemble with β₂-microglobulin followed by the recruitment of the peptide-loading complex in the ER.

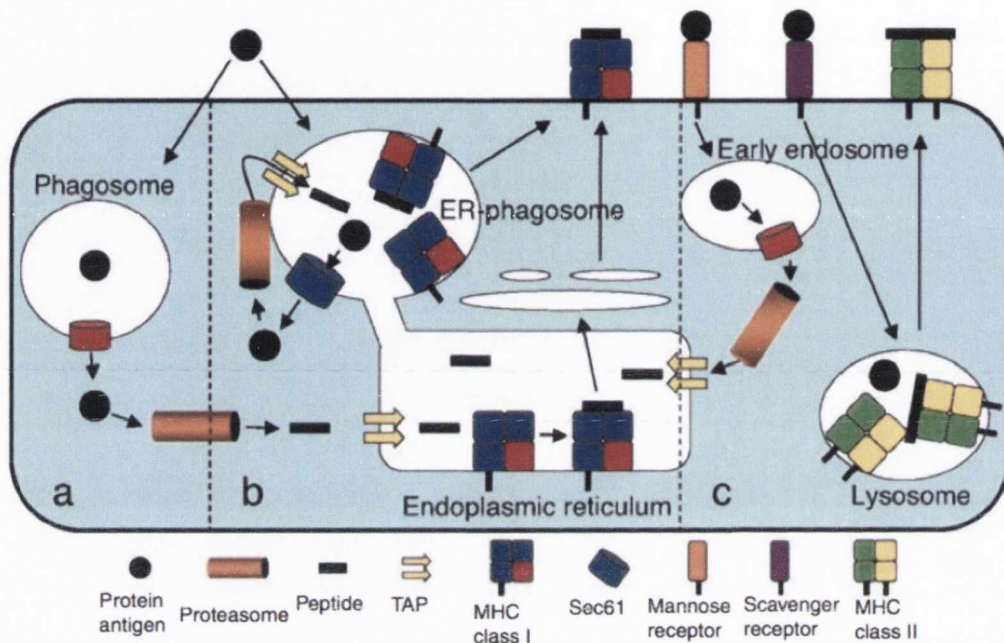


Figure 1.3: Proposed pathways for cross-presentation by MHC Class I molecules.

(a) In the phagosome-to- cytoplasm pathway, particle- or cell-associated proteins or peptides are transported from the phagosome to the cytoplasm, where they enter the direct MHC class I pathway as substrates for proteasomes. The export mechanism is unknown. (b) The endoplasmic reticulum (ER)-phagosome represents an essentially autonomous compartment for generating peptide-MHC class I complexes from exogenous antigens. Components of the endoplasmic reticulum are incorporated into phagosomes that contain internalized antigens. Proteins are exported to the cytoplasm by the endoplasmic reticulum-derived Sec61 translocation complex, where they become substrates for locally associated proteasomes. The resulting peptides are transported back into the endoplasmic reticulum-phagosome by TAP, followed by binding to MHC class I molecules and transport to the cell surface. (c) Soluble proteins can be targeted to different processing pathways after internalization through receptor-mediated endocytosis.

1.3.3.2 MHC Class II

This pathway is only found in professional antigen presenting cells (APCs) such as DCs, macrophages and B cells. MHC Class II is essential for the presentation of exogenous antigens captured by APCs. Once captured these antigen can be subsequently presented to CD4⁺ T cells. DCs express high levels of MHC Class II molecules and as a result are effective at stimulating CD4⁺ T cell responses (Metlay, Witmer-Pack et al. 1990; Steinman 1991). Antigen captured by DCs, are directed towards lysosome-related intracellular compartments containing MHC class II molecules, known as MHC Class II-rich compartments or MIIC (Kleijmeer, Ossevoort et al. 1995). During transportation to the MIIC these antigens are degraded to small peptides typically 13- to 25- amino acids long. Fusion of lysosomes with the phagosome is essential for complete degradation of the internalized proteins. These newly formed phagolysosomes contain a wide variety of lytic enzymes such as cathepsins, which subsequently become active due to the acidifying conditions within the phagolysosome (Deguchi, Yamamoto et al. 1994; Trombetta, Ebersold et al. 2003).

MHC Class II is a heterodimer complex consisting of an α and β chain that are assembled in the ER. In order to facilitate binding of peptides only generated in the endosomal-lysosomal system, the newly synthesized $\alpha\beta$ dimers are associated with a type II chaperone protein entitled the invariant (ii) chain. This complex contains three class II $\alpha\beta$ dimers associated to a common Ii chain trimer. This Ii chain associates directly with the peptide binding groove preventing misfolding and promotes stable assembly (Cresswell 1996). Following transport of the $\alpha\beta$ Ii complex through the Golgi apparatus, targeting signals (protein addressins) result in shuttling of the complex into the endosomal-lysosomal pathway. Once the $\alpha\beta$ Ii complex has gained entry into this pathway the Ii chain is proteolytically degraded by Cathepsin S, a cysteine endoproteases which resides within the endosomal-lysosomal pathway and is responsible for the cleavage of the cytoplasmic domain of the Ii chain leaving a smaller peptide fragment,

the class II-associated Ii chain peptide or CLIP (Cresswell 1996). Cleavage of the Ii chain to CLIP results in the removal of the ER retention signal (**Fig 1.4**) (Jensen 2007). Following DC maturation, the relative levels of cathepsin S increase, resulting in the promotion of peptide presentation (Pierre and Mellman 1998). Interaction of $\alpha\beta$ CLIP with another class II-related $\alpha\beta$ dimer, called HLA-DM (in humans) and H2-M (in mice) results in the release of the small CLIP fragment. HLA-DM or H2-M found within the MIIC, facilitates the stabilization of the MHC Class II dimer and also catalyzes the removal of CLIP, leading to the exposure of the antigen binding groove and enhances the binding of antigen peptides (Cresswell 1996). New evidence has suggested that HLA-DM may facilitate CLIP removal by binding to the MHC Class II complex and weakening the covalent bonding interactions between CLIP and the MHC binding groove (Carven and Stern 2005).

Association with a peptide fragment is essential for the stable expression of the MHC Class II complex on the cell surface; otherwise the MHC complex is rapidly endocytosed and degraded. Antigens for presentation arise from a pool of proteins that gain access to the endosomal pathway and peptides are generated by the actions of endosomal proteases (Jensen 2007). Upon binding of a peptide to the peptide binding groove in the MHC complex, the now stabilized peptide:MHC II complex is transported to and expressed on the cell surface. Surface expression and turnover rates of MHC Class II molecules are regulated by cytoplasmic domain ubiquitination in DCs and B cells (Ohmura-Hoshino, Matsuki et al. 2006; Matsuki, Ohmura-Hoshino et al. 2007). Blander *et al.*, (2004) revealed that ubiquitination of MHC II molecules ceased in mature DCs, resulting in the accumulation of peptide:MHC II complexes on the cell surface (Blander and Medzhitov 2006). TLR signalling may also play a role in phagosome maturation since local TLR signalling can also affect antigen processing in individual membrane-bound compartments (Blander and Medzhitov 2004).

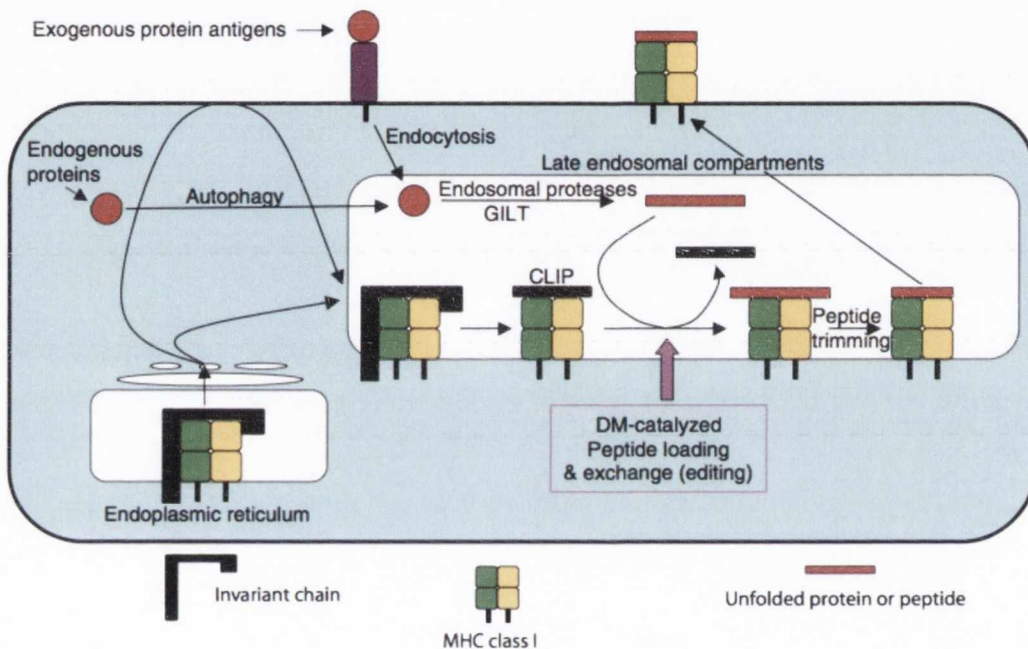


Figure 1.4: MHC Class II presentation pathway.

This pathway allows exogenous antigens to be actively presented by APCs to $CD4^+$ T cells initiating an antigen specific immune response. Exogenous antigens are captured by DCs and directed towards lysosome-related intracellular compartments containing MHC class II molecules. CLIP, is a peptide that remains in the antigen-binding groove until HLA-DM catalyzes its removal facilitating antigen presentation.

1.3.4 Dendritic cell maturation

DCs found circulating in the periphery exist in a state designated as immature, these immature DCs are specialised in antigen capture via non-specific uptake mediated by macropinocytosis, receptor mediated endocytosis via cell surface C-type lectins such as CD205 or CD64 and phagocytosis (Trombetta and Mellman 2005). Once antigen capture has occurred, several changes are triggered in the DC, which converts it into a potent APC.

Following stimulation mediated by PAMP-PRR ligation and uptake, these immature DCs undergo a maturation process during their migration to the lymph nodes (Reis e Sousa 2006). The theory that DCs have two distinct functional states was first proposed following studies by Schuler and Steinman who discovered that although freshly isolated Langerhans cells (LCs), a type of DC found in the epidermis and mucosa, expressed high levels of MHC class II molecules, they were poor stimulators of T-cell proliferation. By contrast, LCs purified from epidermal cell suspensions that were cultured for 2 days *in vitro* were exceedingly potent T-cell stimulators in the same assays. Subsequent work by Romani *et al.*, (1989) showed that cultured LCs were less efficient than freshly isolated LCs at processing intact antigens, formally separating T-cell stimulation from antigen acquisition and processing. This was further confirmed showing that cultured LCs could stimulate T-cell proliferation more efficiently than freshly isolated LCs, even when freshly isolated LCs presented an excess of CD3- specific antibody. Overall, these observations led to the concept that antigen- presenting DCs can exist in two states, 'off' (immature) or 'on' (mature), with only mature DCs able to drive T cell clonal expansion and prime immune responses (Romani, Koide et al. 1989).

Upon stimulation, a number of distinct events are involved in maturation including, acidification of the lysosomal compartment for antigen processing, loss of endocytic receptors, redirection of MHC Class II molecules from late endocytic compartments to the plasma membrane and up regulation of co-stimulatory molecules including CD40,

CD80 and CD86 and increased expression (10 fold) of MHC class II molecules at the cell surface (Reis e Sousa 2006). Co-stimulatory molecules can also be up regulated on the DC surface following exposure to type I interferons. Other cell surface maturation changes include the switching of CCR6 to CCR7, thereby facilitating the migration of the mature DC to the lymph nodes (Sallusto, Mackay et al. 2000). These phenotypic changes are essential for T cell interaction, conferring the ability of the DC to prime and activate T cells. Following complete maturation, endocytosis is down regulated and the ability of the DC to capture antigens is impaired. New evidence by Platt *et al.*, (2010) has revealed the ability of DCs to exclusively down regulate macropinocytosis, however these mature DCs still retain the ability to capture, process and present antigens (Platt, Ma et al. 2010). They revealed that mature DCs continue to accumulate antigens especially by receptor mediated endocytosis and phagocytosis. These internalized antigens were transported to the late endosomes and lysosomes prior to loading onto the MHC Class II complex. These antigens were subsequently presented efficiently to T cells, despite the fact that maturation results in the general depletion of MHC Class II from late endocytic compartments (Platt, Ma et al. 2010). A number of specific models of DC maturation have been proposed in order to explain how DCs have the ability to induce several different effector responses to various activating signals received (**Fig. 1.5**). These models attempt to explain the ability of DCs to direct distinct T helper cell responses. The first model (**Fig. 1.5 (a)**) suggests that distinct DC subsets are onto-genetically programmed to differentiate into mature Th1 and Th2 cell inducing DCs in response to environmental signals. This theory is linear and therefore true to the original model of DC maturation (Liu 2001). An alternative model (**Fig. 1.5 (b)**) has reported that DCs maturation is more flexible and occurs sequentially; first into a mature Th1 cell inducing DC which produces IL-12 and then into a mature Th2-cell inducing DC incapable of producing IL-12. This model is referred to as the temporal model (Langenkamp, Messi et al. 2000). The final model (**Fig. 1.5 (c)**) entitled the flexible model, states that single DC subsets can give rise to distinct types of mature DCs that favour Th1 or Th2 cell differentiation (Kalinski, Hilkens et al. 1999).

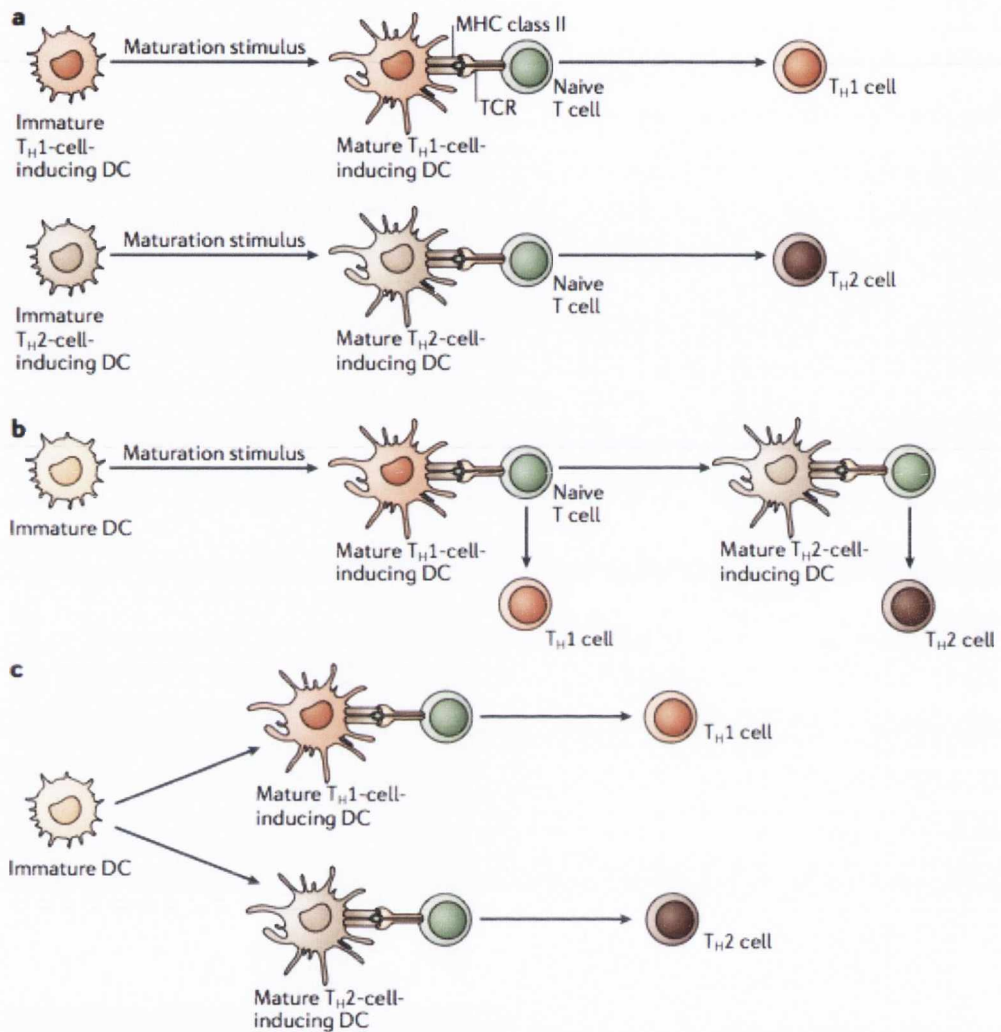


Figure 1.5 : Models of dendritic-cell maturation and the induction of different T-helper-cell fates.

At least three models have been proposed to explain the ability of dendritic cells (DCs) to direct distinct T helper (TH)-cell responses. a | In the first model, distinct DC subsets are ontogenetically programmed to differentiate into mature TH1-cell- or TH2-cell-inducing DCs in response to environmental signals. b | In the second model, DCs mature sequentially: first into a mature TH1-cell-inducing DC and then into a mature TH2-cell-inducing DC. c | In the third model, a single DC subset can give rise to distinct types of mature DC that favor TH1-cell or TH2-cell differentiation. The quality of the maturational signal dictates which type of mature DC is generated. There is evidence in favor of all three models and many investigators believe that a combination of all three might regulate CD4⁺ T-cell immunity. (Reis e Sousa, C. 2008)

1.4 DEC-205 receptor

Several cell surface receptors deliver ligands to the endocytic system resulting in extensive intracellular digestion with lysosomes (Mahnke, Guo et al. 2000). A number of these receptors have specific endocytosis functions, such as the low-density lipoprotein receptor (LDLR) (Brown, Anderson et al. 1983) and the asialoglycoprotein receptor. These receptors typically localize to clathrin-coated pits, increasing uptake via coated vesicles. Upon ligand release these receptors recycle rapidly to the cell surface where they are once again poised to respond to external stimuli. By actively avoiding proteolysis during recycling these receptors are useful for nutrient delivery and scavenging, for example cholesterol, altered glycoproteins and iron. In contrast other receptors have signalling functions in addition to mediating absorptive uptake of their corresponding ligands. These receptors are typically broken down within the lysosome rather than being actively recycled, following ligation. Adsorptive endocytosis receptors such as the macrophage mannose receptor (MMR), FcR, and B cell antigen receptor (BCR), are used by the immune system to enhance antigen capture and presentation of peptides to T cells (Engering, Cella et al. 1997). Expressed on the surface of DCs and thymic epithelial cells, DEC-205 is one such example of an endocytic receptor (Haymann 1995; Jiang, Swiggard et al. 1995)



Figure 1.6: Schematic representation of DEC-205.

DCs also have a number of other receptors for adsorptive uptake of antigens. Some are shared with other cells, however others are more restricted e.g. Langerin or CD207, DC-

SIGN or CD209 and BDCA-2 to name a few. Despite the many DC cell surface receptors with the potential to enhance antigen uptake and processing, only DEC-205 has been visualised on most DCs in the T cell areas of human lymphoid organs (Lozach, Burleigh et al. 2005). DEC-205 is a 205-kD protein, which contains 10 external, contiguous, C-type lectin domains and as described by Jiang *et al.*, (1995), via sequence analysis, is a homologue of the MMR (Jiang, Swiggard et al. 1995). The amino termini of both MMR and DEC-205 have two domains, one cysteine rich and the next fibronectin like, followed by the 10 external contiguous carbohydrate recognition domains (**Fig 1.6**). Both MMR and DEC-205 mediate adsorptive uptake and both have cytoplasmic domains with requisite coated pit localization sequences (Jiang, Swiggard et al. 1995). Unlike MMR, which recycles through the early endosomal compartments and presents bound antigens, DEC-205 targets to late endosomes or lysosomes in developing DCs. Distinct distal regions with an acidic EDE triad facilitate late endosomal targeting and this was shown to result in more efficient antigen presentation on MHC class II products relative to ligands for MMR. This distal region and its acidic sequence are required for recycling beyond the early endosomes. Moreover DEC-205 is far superior at presenting bound antigens to T cells than the MMR (Chesnut and Grey 1981).

Mouse DEC-205 is expressed on DCs in the T cells areas, whereas human CD205 is expressed on a diverse number of immune cells in addition to DCs (Kato, McDonald et al. 2006). CD205 is highly expressed on CD8⁺ DCs that have been shown to have an important role in cross presentation (Schnorrer, Behrens et al. 2006). In both humans and mice, DEC-205 is also expressed on T cells, B cells and CD14⁺ cells albeit at a much lower level of expression. Thus, DEC-205 is widely expressed amongst hematopoietic subsets, but higher levels tend to be detected on cells with antigen presenting capability. As it is an antigen uptake receptor, monocytes and immature DC possessed extensive intracellular compartments containing DEC-205. However, an analysis of mature DC reveals DEC-205 staining to be predominantly at the cell surface with little intracellular staining. This appears to indicate that, although mature DC has increased expression of DEC-205, there is a down regulation of DEC-205 mediated endocytosis.

The natural ligands for DEC-205 are currently unknown, but antibodies have been used as surrogate ligands. A number of studies, predominantly within the lab of Ralph Steinman, have revealed that proteins can be targeted specifically to mouse DCs *in vivo* upon incorporation into a monoclonal antibody (mAb) specific for a DEC-205 (Hawiger, Inaba et al. 2001; Bonifaz, Bonnyay et al. 2002). Targeting of DCs with DEC-205 fusion protein increases the efficiency of antigen presentation by MHC Class I and II by approximately 100 fold (**Fig. 1.7**) (Bonifaz, Bonnyay et al. 2004; Trumpfheller, Finke et al. 2006; Bozzacco, Trumpfheller et al. 2007; Soares, Waechter et al. 2007), in addition to the induction of robust, protective T cell immunity (Trumpfheller, Finke et al. 2006). In one of the first studies, ovalbumin (ova) protein was linked to inter-heavy chain thiol groups on a DEC205 monoclonal antibody generated by mild reduction conditions. After determining that the antibody was still functional, it was shown that ova was presented by CD11c⁺ lymph node DCs to CD4⁺ and CD8⁺ T cells.

Presentation was at least 400 times more efficient than in the case of unconjugated ova. Furthermore, targeting anti-DEC205-ova to DCs in the steady state initially induced 4-7 cycles of T cell division, but the T cells were then deleted and the mice became specifically unresponsive to re-challenge with ova in complete Freund's adjuvant. Thus, the anti-DEC205-ova antibody induced tolerance when given in the steady state. In contrast, when anti-DEC205-ova antibody was simultaneously delivered with a DC maturation stimulus, strong immunity was induced. The CD8⁺ T cells responding to the antigen produced large amounts of IL-2 and IFN- γ , acquired cytolytic function *in vivo*, and responded vigorously to ova re-challenge.

After the initial success of antigen delivery to DCs via the DEC205 receptor, other antigens have been linked to the DEC205 antibody. To induce tolerance, superagonist peptide MimA2 (an insulin peptide that is recognized by diabetogenic CD8⁺ T cells) was linked to the DEC205 antibody. The anti-DEC205-MimA2 antibody delivers MimA2 to DCs for presentation on class I MHC both *in vivo* and *in vitro*. By selectively removing DCs from a transgenic non-obese diabetic (NOD) mouse using diphtheria toxin, the group was also able to show that DCs were required for this presentation. Next, it was

shown that the transferred cell-autoreactive CD8⁺ T cells were eventually deleted in response to the delivery of the MimA2 peptide. Finally, it was shown that the anti-DEC205-MimA2 antibody induced peripheral CD8⁺ T cell tolerance in the NOD mice.

Research by Trumpfheller *et al.*, (2007) revealed that a single vaccination administered subcutaneously (s.c) or intraperitoneally (i.p) with the DEC-205 targeted antibody conjugated to the HIV fusion protein gag p24 elicited strong CD4⁺IFN- γ ⁺IL-2⁺ T cells. Moreover long-term memory responses were observed compared with HIV gag DNA or a recombinant Adenovirus-gag (**Fig 1.8**) (Trumpfheller, Finke et al. 2006). This DEC-205: antigen construct required the addition of the TLR 3 adjuvant poly IC and anti-CD 40 mAb for the activation of DCs and this approach was effective even under dose sparing conditions. New evidence has now revealed that DEC-205 targeting with poly IC alone is sufficient to induce durable long-term immunity (Trumpfheller, Caskey et al. 2008). CD8⁺ T cell responses were comparable for p24 Gag DNA and the DEC-205:p24 fusion protein immunization however they were significantly lower than immunization with a recombinant Adenovirus-Gag vaccine. In addition to the induction of antigen specific T cells, DEC-205 fusion antibodies also induced an increase in antibody titres, owing to better CD4⁺ T cell help (Boscardin, Hafalla et al. 2006).

The discovery that DEC-205 is expressed on the surface of human DCs has ignited the need for translational approaches to be adopted. In order to extend the concept of protein targeting for human therapies Cheong *et al.*, (2010) have developed human IgG mAbs to human DEC-205 (hDEC-205). In their study they revealed that a human anti- hDEC-205-HIV Gag p24 fusion mAb can enhance humoral and cellular immunity *in vivo* when poly IC is co administered as an adjuvant (Cheong, Choi et al. 2010). Most recently, the human cancer antigen, mesothelin, was linked to the DEC205 antibody. Mesothelin, which is expressed on normal mesothelial cells, is overexpressed in several cancers, including mesothelioma, ovarian cancer, pancreatic adenocarcinoma, lung adenocarcinoma, uterine serous carcinoma, and acute myeloid leukemia. Targeting human mesothelin to the DEC205 receptor, in the presence of an adjuvant, induced stronger CD4⁺ T-cell responses compared to high doses of mesothelin protein. CD4⁺ T

cells were primed to produce IFN- γ , tumor necrosis factor (TNF)- α , and IL-2. Targeting also resulted in cross-presentation to CD8⁺ T cells. Thus, anti-DEC205-mesothelin antibody could be valuable for enhancing immunity to mesothelin in cancers where this nonmutated protein is expressed.

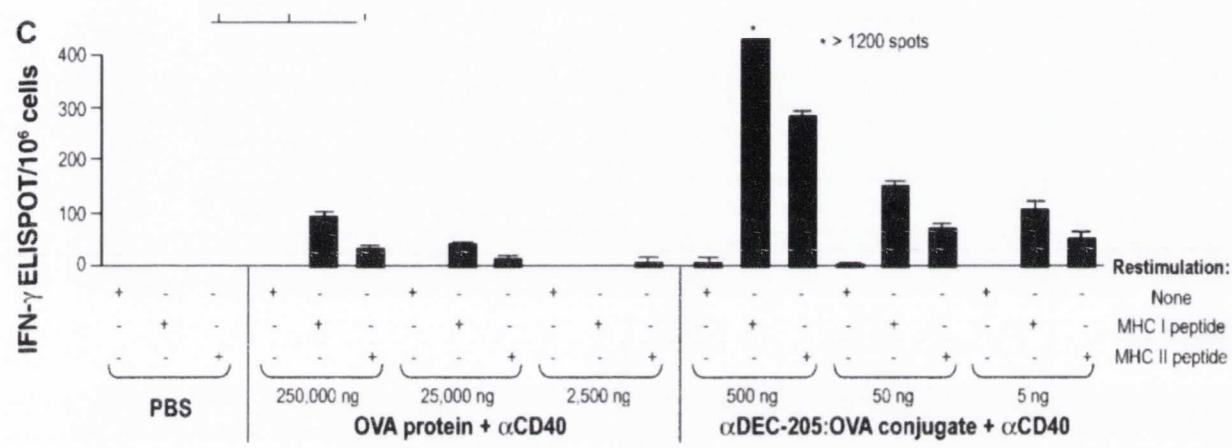


Figure 1.7: *In vivo* targeting of antigens to maturing DCs via the DEC-205 receptor improves T cell vaccination using OVA (Bonifaz, Bonnyay et al. 2004).

Mice were immunized with grade doses of ova as a soluble protein or conjugated to DEC-205. IFN- γ secretion was evaluated after 7 d in the lymph nodes and spleen. Representative of at least two experiments.

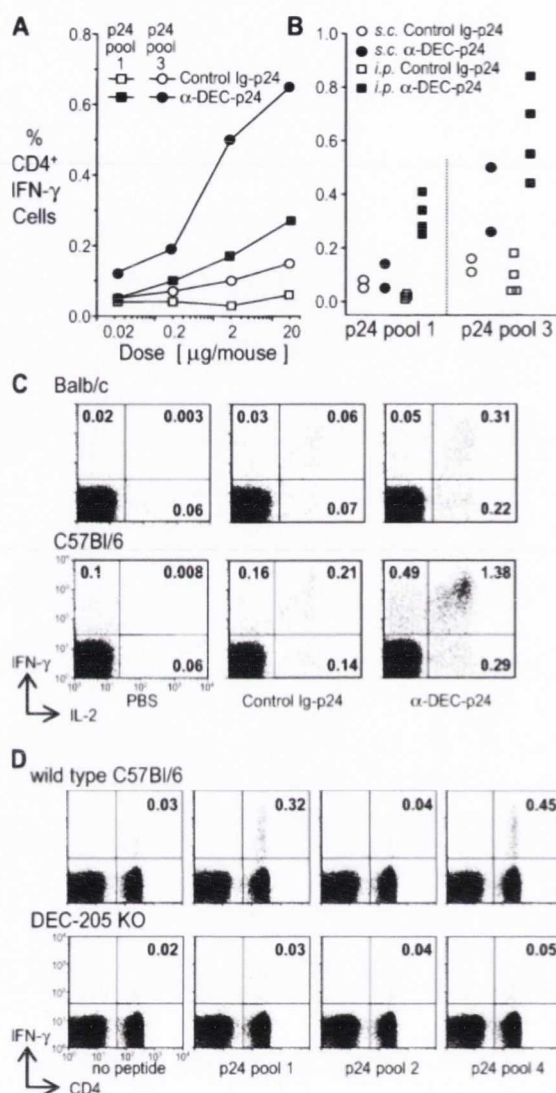


Figure 1.8: Strong CD4⁺ T cell responses to a single dose of anti-DEC-p24 fusion mAb vaccine (Trumpfheller, Finke et al. 2006).

BALB/c mice were vaccinated s.c. with graded doses of anti-DEC-p24 or control Ig-p24 mAbs and maturation stimulus, 19 days post vaccination IFN- γ ⁺CD4⁺ T cells were assessed in gated CD3⁺ spleen T cells using gag p24 peptide pools.

1.5 Adaptive Immune System

The initial line of defence against a pathogen is mediated by the non-specific properties of the innate immune system, with its ability to detect conserved molecular patterns and danger signals produced by dying/infected cells. DCs are the pivotal link between the innate and adaptive immune response. The adaptive immune system is comprised of two main cells, T cells and B cells and the adaptive immune system is responsible for the development of cell-mediated and humoral immunity. Activation of T cells generates cell-mediated immunity, while B cells develop into antibody-producing plasma cells, resulting in humoral immune responses.

1.6 T lymphocytes

T cells are a particular type of lymphocyte and predominant effector cell in the adaptive immune response. Characterized based on their development in the thymus and the presence of a cell surface heterodimeric $\alpha\beta$ receptor known as the T cell receptor (TCR) expressed in association with CD3 molecules, these cells exert several effector functions. The majority of T cells are classified as conventional as they express the TCR $\alpha\beta$ and TCR associated molecules CD4 or CD8.

T cells can be broadly classified based on their co-receptor expression and specific effector functions. Helper T cells (Th) express CD4 on their cell surface, which acts as a co-receptor for MHC Class II. Naïve $CD4^+$ T cells reside in the lymph nodes and after antigen-specific stimulation by APCs expand and develop into effector cells. $CD4^+$ T cell functions include activation of macrophages to kill intracellular bacteria, activation of B cells and the production of cytokines and chemokines that mediate recruitment of immune cells to the site of infection and orchestrate the immune response. $CD4^+$ T cells have the ability to further differentiate into one of several lineages, including Th1, Th2, Th17, regulatory T cells and follicular T-helper cells (Tfh). These cells differ in terms of their cytokine response and effector function.

Cytotoxic T cells (CTL) express the MHC Class I co-receptor CD8 on their surface and their primary function is to kill virally infected cells. A smaller subpopulation of T cells known as non-conventional T cells also exists, these cells are characterised based on the expression of the $\gamma\delta$ TCR. The $\gamma\delta$ TCR displays less diversity than the $\alpha\beta$ counterpart. They reside principally below epithelial surfaces but $\gamma\delta$ T cells can also be found in the spleen, liver and peritoneum albeit at a lower frequency. $\gamma\delta$ T cells recognise non-classical MHC molecules and fail to express CD4 or CD8; these cells are believed to play a role in immunoregulation.

1.6.1 Th1 cells

Early studies by Mossman and Coffman in 1986 lead to the subdivision of $CD4^+$ T cells into Th1 and Th2 cells based on their cytokine profile (Mosmann, Cherwinski et al. 1986). However the existence of these $CD4^+$ T cell subpopulations was not confirmed in humans until 1990, when samples from patients with allergic or infectious diseases were investigated (Wierenga, Snoek et al. 1990). These effector cells “underlie successful adaptive immune responses aimed at distinct categories of pathogens” (Murphy and Stockinger 2010). Activated $CD4^+$ T cells isolated from immunized mice which secreted IFN- γ , IL-2, IL-3 and GM-CSF were classified as Th1 cells, these cells also assist B cell Isotype switching to IgG2a in response in Con A or antigen (Mosmann, Cherwinski et al. 1986).

Activated Th1 cells play a key role in immune response against intracellular bacteria (such as *Mycobacterium tuberculosis*) and viruses and are responsible for delayed-type hypersensitivity. These cells are also implicated in the development of autoimmune disorders producing large amounts of pro-inflammatory cytokines. IL-12 and the cell surface molecule CD40, the main sources of which are DCs and macrophages promote Th1 responses (Cella, Scheidegger et al. 1996). DCs and macrophages up-regulate these Th1 polarizing factors upon encounter with microbial products such as LPS and other TLR ligands which signal through cell surface TLRs. Natural Killer (NK) cells have been

identified as an early source of IFN- γ , which is further reinforced by IL-12 from DCs acting in a feed forward mechanism (Martin-Fontecha, Thomsen et al. 2004). Additional factors such as IL-27 produced by DCs are also involved in Th1 cell differentiation.

Initially described by Szabo *et al.*, (2000), IL-12R ligation on the surface of the T cell results in the downstream activation of STAT-4 and T-bet (T-box expressed in T cells), the initiation of the resulting signal transduction pathway results in the activation of NF κ B and NFAT (nuclear factor of activating T cells) (**Fig 1.9**) (Szabo, Kim et al. 2000). NF κ B and NFAT activation results in the up regulation of IFN- γ and a decrease in IL-4 (Szabo, Kim et al. 2000), polarizing the CD4⁺ T cell towards the Th1 differentiation pathway (Mullen, High et al. 2001; Afkarian, Sedy et al. 2002).

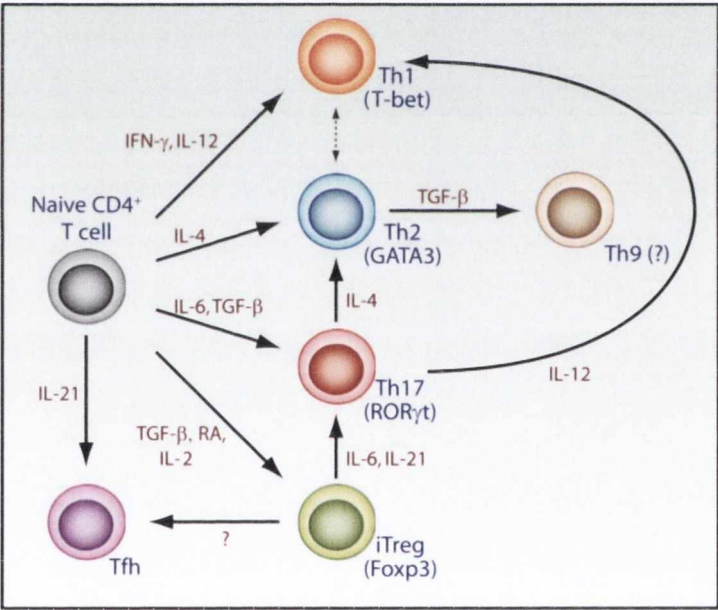


Figure 1.9: The cytokine milieu determines CD4⁺ T cell differentiation and conversion

Upon encountering foreign antigens presented by antigen-presenting cells, naive CD4⁺ T cells can differentiate into Th1, Th2, Th17, iTreg, and Tfh cells. These differentiation programs are controlled by cytokines produced by innate immune cells, such as IL-12 and IFN- γ , which are important for Th1 cell differentiation, and IL-4, which is crucial for Th2 cell differentiation (Zhou et al., 2009)

Th1 and Th2 cells may represent distinct terminally differentiated lineages as these phenotypes could not be reversed even when transferred into new polarizing conditions (Murphy, Shibuya et al. 1996). Moreover under polarizing conditions, stable cytokine production was only observed after a set number of cell divisions; thereby inferring a requirement for the development of a stable transcriptional program (Bird, Brown et al. 1998; Grogan, Mohrs et al. 2001). In addition to distinct “master regulators”, crosstalk also exists during Th1 and Th2 development. Th1 lineage commitment is associated with the expression of the $\beta 2$ chain of the IL-12R complex, thus inferring IL-12 responsiveness. Meanwhile IL-4 appears to repress IL-12 signalling via down regulation of the IL-12R $\beta 2$ (Szabo, Dighe et al. 1997). IFN- γ induces T-bet, which induces Runx3 expression, enhancing the ability of T-bet to further promote IFN- γ production while silencing the *Il4* gene in Th1 cells by binding to the *Ifng* promoter and the *Il4* silencer, respectively (Djuretic, Levanon et al. 2007). Runx3 is a member of the runt domain transcription factors, which function mainly in cell-specific lineage decisions. T-bet and Runx3 binding to the *Ifng* promoter enhances a positive feed back loop through the secretion of IFN- γ (Djuretic, Levanon et al. 2007; Hu, Djuretic et al. 2007; Naoe, Setoguchi et al. 2007). Kano *et al.*, (2008), have also suggested a role for the transcription factor IRF-1 (interferon response factor-1) during Th1 cell differentiation. They demonstrated the ability of IRF-1 to regulate the expression of the IL-12R $\beta 1$ subunit and the p40 and p35 subunits of IL-12, mediating the enhancement of the both the secretion of IL-12 and the ability of T cells to respond to IL-12 (Kano, Sato et al. 2008).

Despite the important role of IL-12 in Th1 cell differentiation research has revealed that Th1 responses *in vivo* to certain antigens can occur in an IL-12 independent manner. For example mice, which have a targeted genetic mutation in the IL-12 p40 subunit, retained the ability to generate strong Th1 responses, when infected with mouse hepatitis virus (Schijns, Haagmans et al. 1998). These studies and others have revealed that IL-12 may not be the primary inducer of Th1 response, but may in-fact act as an enhancer of effector cells already committed to the Th1 differentiation pathway (Trembleau, Penna et al. 1999; Amsen, Antov et al. 2009).

1.6.2 Th2 cells

The second of the CD4⁺ T cell subsets identified by Mossman *et al.*, are entitled Th2. Pathogenic insult by extracellular pathogens such as *Schistosoma japonicum* and allergic immune responses are controlled by Th2 cells (Mosmann, Cherwinski *et al.* 1986). Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. Although IL-4 is a signature cytokine of Th2 cells, it is also essential for Th2 cell development acting as a positive feedback signal. Th2 cells are also responsible for enhanced B cell production of both IgE and IgG1 (Mosmann, Cherwinski *et al.* 1986).

Despite being essential for Th2 cell differentiation the source of IL-4 has still yet to be definitively determined, as DCs do not produce this cytokine (Le Gros, Ben-Sasson *et al.* 1990). It has been postulated that DCs present antigen via the MHC Class II pathway and that an accessory cell provides a source of IL-4, thereby polarizing the T cell response. Eosinophils, mast cells, basophils and NK cells have also been proposed as this external source of IL-4 (Seder, Boulay *et al.* 1992; Seder, Paul *et al.* 1992; Brunner, Heusser *et al.* 1993; Moqbel, Ying *et al.* 1995; Ying, Humbert *et al.* 1997). A number of recent elegant publications have provided evidence that basophils may be inducing the development of Th2 cells *in vivo*, especially during allergy. Basophils have the capability to actively home to the lymph nodes (Sokol, Chu *et al.* 2009) and are required for the *in vivo* induction of Th2 responses to protease allergens. Medzhitov's group revealed that although DCs were dispensable for allergen-induced activation of Th2 responses both *in vitro* and *in vivo*, antigen presentation by basophils was necessary and sufficient for this. Other studies by Perrigoue *et al.*, (2009) and Yoshimoto *et al.*, (2009) have revealed the role for basophils during helminths infections and antigen-IgE complexes *in vivo* respectively. Evidence has also revealed the fundamental importance of DCs for Th2 induction (Phythian-Adams, Cook *et al.* 2010)

Research by Ouyang *et al.*, (2000) has revealed that Th2 cell differentiation can occur in the absence of IL-4 (Ouyang, Lohning *et al.* 2000). In their seminal study they revealed

that Stat-6 independent GATA 3 auto-activation directs IL-4 independent Th2 development and commitment (Ouyang, Lohning et al. 2000). Evidence has revealed that STAT-6 deficient mice infected with parasitic helminths had the same abundance of Th2 cells than their wild type counterparts (Jankovic, Kullberg et al. 2000). Similar to IL-12, it has been proposed that IL-4 is necessary for the enhancement of already differentiated Th2 cells (Amsen, Antov et al. 2009). IL25 (or IL-17E) has been proposed to have a role in Th2 development, as it can enhance production of IL-4, IL-5 and IL-13 via signalling through the IL-17RB receptor (Angkasekwinai, Park et al. 2007; Wang, Angkasekwinai et al. 2007). Additional polarising factors for Th2 development include monocyte chemotactic protein 1 (MCP1), OX40 ligand (OX40L) and thymic stromal lymphopoietin (TSLP) (Zhou, Comeau et al. 2005).

Binding of IL-4 to its corresponding cell surface receptor results in the activation of the transcription factor STAT-6, inducing the expression of GATA-3 and c-Maf, directly up regulating IL-4 and IL5 and suppressing IFN- γ secretion (Zheng and Flavell 1997). GATA-3 is a zinc finger domain containing protein that was originally described to bind to the TCR α gene enhancer element, however, it is principally known for its role as the master regulator of Th2 cell differentiation. GATA-3 directly inhibits Th1 cell differentiation by preventing the increase in expression of the IL-12 β chain, resulting in the cells unresponsiveness to IL-12 and enhanced sensitivity to local IL-4 levels (Ansel, Djuretic et al. 2006). Additional transcriptional factors implicated in Th2 differentiation include STAT5, interferon regulatory factor (IRF) 4 and Ikaros (Quirion, Gregory et al. 2009).

Th2 cells are responsible for protection against helminths, facilitating B cell antibody switching and mediating the functions of eosinophils, mast cells and basophils. Th2 cells also control responses in the lungs and intestinal compartment, with IL-4, IL-9 and IL-13 enhancing mucus producing in these regions.

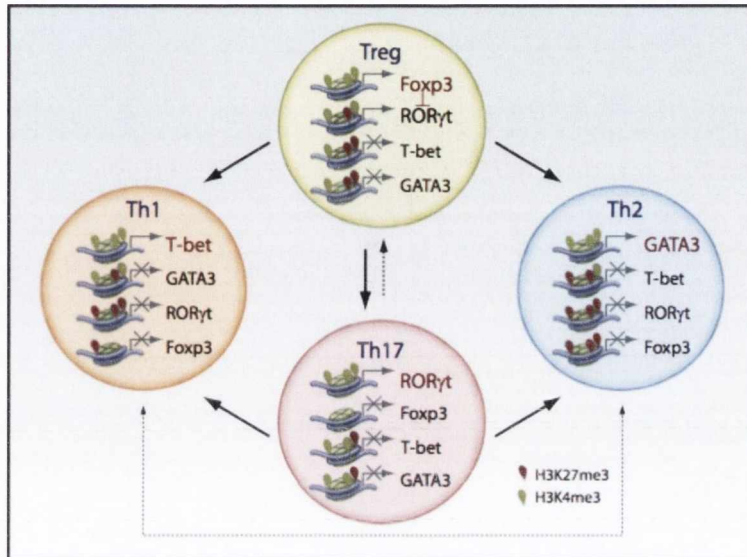


Figure 1.10: Epigenetic status of lineage-specific transcriptional regulator gene loci in CD4⁺ T cells.

Recent findings suggest that T helper cell differentiation is more plastic than previously appreciated. Each CD4⁺ T cell subset can adopt alternate cytokine profiles in response to cytokine environmental changes. Among four subsets of T cells, Treg cells and Th17 cells display the highest propensity to switch to other phenotype. (Zhou et al., 2009)

1.6.3 Regulatory T cells.

A number of sophisticated regulatory mechanisms are employed by the immune system to maintain local immune homeostasis, prevent autoimmunity and control inflammation in response to pathogens and environmental antigens (Mills 2004; Vignali, Collison et al. 2008). One of the key cells of the immune response whose primary role is regulation is the regulatory T (Treg) cell. Treg cells are now regarded as the primary mediator of peripheral tolerance and are vital in the prevention of chronic inflammatory conditions. Treg cells have also been implicated in blocking beneficial responses by preventing sterilizing immunity to certain pathogens and limiting auto tumour immunity.

It is now firmly established that there are two groups of regulatory T (Treg) cells. These include natural (or constitutive) and inducible (or adaptive) Treg cells. These cells are believed to have overlapping and complementary functions in the control of the host immune response. Natural regulatory T (nTreg) cells are characterised by the expression of CD4 and CD25, these cells are naturally occurring and functionally mature upon leaving the thymus. Initially described by Sakaguchi and colleagues (Sakaguchi, Sakaguchi et al. 1995; Sakaguchi, Sakaguchi et al. 2011), these cells leave the thymus and enter the periphery where they mediate peripheral tolerance by suppressing self-reactive T cells (Bluestone and Abbas 2003; Cozzo, Larkin et al. 2003; Cassis, Aiello et al. 2005).

Natural CD4⁺CD25⁺ Treg cells were first defined by Sakaguchi *et al.*, (1995), here they demonstrate that the transfer of CD4⁺ lacking the alpha-chain of the IL-2 receptor (i.e. CD25) resulted in the spontaneous development of T cell mediated autoimmune diseases (Bluestone and Abbas 2003). Moreover, T cell mediated autoimmunity was alleviated following reconstitution with CD4⁺CD25⁺ Treg cells (Mills 2004).

Murine CD4⁺CD25⁺ T cells were shown to directly suppress CD4⁺CD25⁻ T cells in a cell-contact dependent manner *in vitro* (Bluestone and Abbas 2003). This suppression

involves the inhibition of IL-2 production by these CD4⁺CD25⁻ cells. Together with work on a CD45RB^{low} population of T cells by Fiona Powrie and colleagues, these studies began to challenge the traditional theory of central tolerance via clonal deletion as being the only mechanism of self-tolerance in the host. These CD4⁺CD25⁺ Treg cells constitute around 5-10% of peripheral T cells in both mice and humans. Although initially defined by the expression of the cell surface marker CD25, natural Treg cells are also defined by the expression of the transcription factor, forkhead box transcription factor (FoxP3), which is the most definitive marker for Treg cells in both humans and mice (Fontenot, Rasmussen et al. 2005). Scurfy, an X-linked syndrome in male mice is linked to specific mutations in FoxP3 and results in the hyper proliferation of CD4⁺ T cells. Moreover, mice with the Scurfy phenotype could avoid disease when CD4⁺CD25⁺ Tregs were reconstituted or when the same mice expressed the *FoxP3* transgene (Kim and Rudensky 2006). Transfection of CD4⁺CD25⁻FoxP3⁻ T cells with the transcription factor FoxP3 confers them with regulatory activity (Fontenot, Rasmussen et al. 2005).

1.6.4 Adaptive Regulatory T cells.

In contrast to nTreg cells, which develop in the thymus, adaptive Treg cells are derived from mature CD4⁺ T cells found in the periphery. These mature CD4⁺ T cells develop into adaptive Treg cells following antigenic stimulus and/or co stimulation from APCs. Moreover the presence of exogenous cytokines may also play a role in the development of these cells. The various subsets of adaptive Treg cells are characterised by the expression of cytokines. Unlike nTreg cells, they are specific for antigens not encountered within the thymus during the process of central tolerance. These cells are characterised as Tr1, Th3 and CD8⁺ regulatory T cells. Due to their importance in maintaining immune homeostasis, inducible antigen specific Treg cells are now being employed in clinical trials in order to treat a number of autoimmune diseases where the causative auto antigen is known.

1.6.4.1 Tr1 Cells.

Initially describe by Groux *et al.*, (1997), these IL-10 producing cells were first demonstrated to induce tolerance in mice following antigenic stimulation (to a self antigen) in the presence of IL-10 (Groux, O'Garra et al. 1997). These cells are hypo-proliferative following antigen stimulation and despite being negative for the transcription factor FoxP3, they are functionally similar to natural Treg cells. McGuirk *et al.*, (2002) demonstrated the generation of these cells from CD4⁺ T cells by repeated TCR stimulation by immature DCs in the presence of IL-10. Furthermore these cells can also be generated in culture with vitamin D3 and dexamethasone. In addition to their role in immune regulation and the prevention of autoimmune disease, a number of pathogens such as Epstein-Barr virus have been reported to induce the production of these IL-10 producing Th1 cells. This is advantageous for the pathogen as it acts as a mechanism of immune evasion

1.6.4.2 Th3 Cells

In 1994, Weiner and colleagues demonstrated the induction of oral tolerance following the administration of self-antigens (Chen, Kuchroo et al. 1994). Oral tolerance was

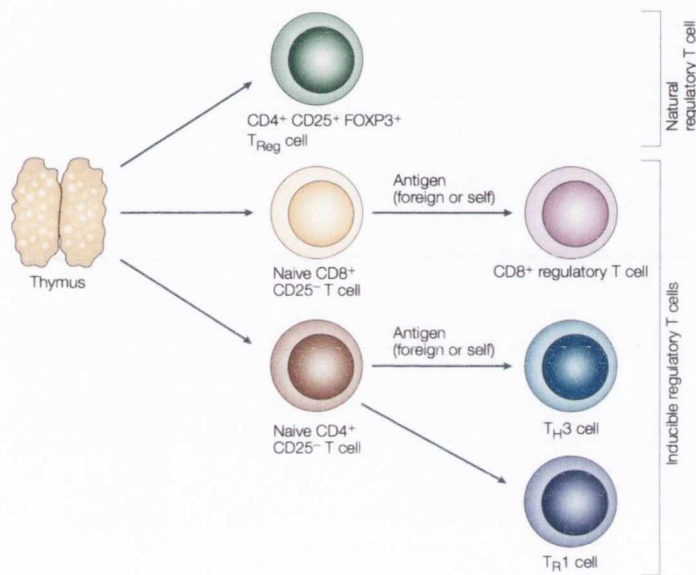


Figure 1.11: Natural and inducible regulatory T cells (Mills 04).

Natural regulatory T cells express the cell-surface marker CD25 and the transcriptional repressor FoxP3 (forkhead box P3). These cells mature and migrate from the thymus and constitute 5–10% of peripheral T cells in normal mice. Other populations of antigen-specific regulatory T cells can be induced from naive CD4⁺CD25⁻ or CD8⁺CD25⁻ T cells in the periphery under the influence of semi-mature dendritic cells, IL-10, TGF-β and IFN-α. The inducible populations of regulatory T cells include distinct subtypes of CD4⁺ T cell: Tr1 cells, which secrete high levels of IL-10, no IL-4 and no or low levels of IFN-γ; and Th3 cells, which secrete high levels of TGF-β. Although CD8⁺ T cells are normally associated with cytotoxic T-lymphocyte function and IFN-γ production, these cells or a subtype of these cells can secrete IL-10 and have been called CD8⁺ regulatory T cells. (Mills 04)

induced and Th1 mediated autoimmunity prevented by a group of TGF-β secreting cells in the gut (Chen, Kuchroo et al. 1994). These cells were classified as Th3 cells and are characterised by the expression of the cytokine TGF-β. These Th3 cells are believed to be induced upon TGF-β stimulation (**Fig 1.11**) (Chen, Kuchroo et al. 1994). These cells are

distinct from other T cell subtypes such as Th2 and Tr1, as they secrete little or no IL-4 or IL-10. Following adoptive transfer these cells are capable of inducing tolerance *in vivo* and similar results have been observed *in vitro* (Miller et al., 1992).

In addition to its role in suppressing the immune system, the inhibitory cytokine TGF- β , also plays a role in the induction of Treg cells in the periphery. Fiona Powries group have identified a novel sub-population of CD103-expressing mesenteric lymph node dendritic cells. These cells induce the development of FoxP3⁺ Treg cells in the periphery. The promotion of Treg cell development by these CD103⁺ DCs is dependent on TGF- β and the dietary metabolite, retinoic acid (RA) (Xiao, Jin et al. 2008). These Treg cells represent a population of adaptive FoxP3 positive cells in the intestine and may have an important role in mediating oral tolerance (see section 1.10.1.1).

1.7 Adjuvants

Vaccination remains the most valuable tool for preventing infectious diseases. The use of well-defined antigens for the generation of subunit vaccines has led to products with an improved safety profile. However, purified antigens are usually poorly immunogenic, making essential the use of adjuvants (Steinman and Pope 2002). However, only a handful of vaccine adjuvants are licensed for human use. Thus, the development of new adjuvants, which are able to promote broad and sustained immune responses, remains a major goal in vaccinology (Steinman and Pope 2002).

For more than 70 years, alum (aluminum salts) has been licensed as a vaccine adjuvant for prophylaxis in humans. Today, alum is the most widely used adjuvant and is found in numerous vaccines, including HAV, HBV, HPV, Diphtheria and Tetanus (DT), *Haemophilus influenzae* type B (HIB), and pneumococcal conjugate vaccines (Ebensen and Guzman 2008; Ebensen and Guzman 2009). Alum appears to work by increasing antigen uptake and stability at the site of injection (Mbow, De Gregorio et al. 2010). In addition, alum induces a local pro-inflammatory reaction that can increase immunogenicity (Goto and Akama 1982; Morefield, Sokolovska et al. 2005). Several

independent studies have shown that alum also activates NLRP3, a component of the inflammasome complex, implicated in the induction of pro-inflammatory cytokines (Goto, Kato et al. 1997; Eisenbarth, Colegio et al. 2008). However, it has also been demonstrated that this pathway is dispensable for alum-mediated adjuvant activity *in vivo* (Li, Willingham et al. 2008). Thus, it appears that redundant pathways modulate the activity of alum in the host. Although alum is the most widely used adjuvant, pre-clinical and clinical studies have shown that alum is often less potent than other adjuvants. In addition, alum is a poor inducer of protective TH-1 associated immune responses, characterized by the production of cytotoxic T-lymphocytes, macrophages, NK cells and cytokines, which are crucial for the development of vaccines against intracellular pathogens (Kool, Petrilli et al. 2008). This is in contrast to Th-2 response, which involves the production of antibody molecules in response to antigen and is mediated by B-lymphocytes. The squalene-based oil-water emulsion, MF59, has been licensed in Europe for adjuvanted Flu vaccines since 1997. This emulsion has been shown to increase hemagglutination inhibition (HI) titers and cross protection in both the elderly and the young. In 2009, a clinical trial demonstrated that Avian H5 pandemic flu vaccines containing MF59 were superior to alum-adjuvanted and non-adjuvanted vaccine; MF59 containing vaccine resulted in increased seroconversion and cross-presentation, promoted CD4⁺ T cell responses that predict long term persistence of protective antibodies, and allowed for better recall responses in individuals boosted many years after their primary vaccination (Podda 2001). MF59 induced a local immunostimulatory environment at the injection site characterized by up-regulation of cytokines, chemokines, and other innate immunity genes (Clark, Pareek et al. 2009; Vigano, Giacomet et al. 2011). MF59 is also known to enhance antigen uptake by dendritic cells (Mosca, Tritto et al. 2008).

More recently, a new class of vaccine adjuvants have been designed that target the Toll-Like-Receptor (TLR) pathways. TLRs are single, membrane-spanning receptors that recognize structurally conserved molecules derived from microbes. Activation of TLRs leads to the transcriptional activation of genes encoding pro-inflammatory cytokines, chemokines, and co-stimulatory molecules, which subsequently control the activation of immune responses (van Kooyk 2008). Table 1 summarizes the main ligands for the TLRs

as well as some other important immune sensors.

The TLR4 agonist, 3-O-desacyl-4'-monophosphoryl lipid A (MPL), is currently the only adjuvant of this type to be licensed; it has been approved as a component of the adjuvant system ASO4 for use against human papilloma virus and hepatitis B (Dupuis, Murphy et al. 1998). Studies indicated that MPL produced a transient and local activation of NF- κ B activity and cytokine production, thus providing an innate immune signal for activation of antigen presenting cells (Harper 2009). MPL is adsorbed to alum, yet no synergistic effect between the two compounds was noted. It appears, however, that the alum prolongs the cytokine response induced by MPL at the injection site.

There are numerous TLR agonists in pre-clinical and clinical trials for use as vaccine adjuvants. For example, the TLR9 agonist, 1018 ISS, has been effective in boosting responses to recombinant hepatitis B surface antigen vaccine (Didierlaurent, Morel et al. 2009). Another TLR9 agonist based adjuvant, IC31, consists of a cationic peptide KLKL(5)KLK vehicle and the immunostimulatory oligodeoxynucleotide ODN1a sequence. The complex between the bacterial peptide and ODN results in prolonged retention of antigen-adjuvant formulation and subsequent amplification of immune response. This adjuvant is currently undergoing human clinical trials in a vaccine against TB (Agger, Rosenkrands et al. 2006; Barry and Cooper 2007; Cooper and Mackie 2011). The small molecules imiquimod (TLR7 agonist) and resiquimod (TLR 7/8 agonist) were shown to improve the immunogenicity of a variety of vaccine adjuvants if adequately formulated or directly conjugated to protein antigens (Aagaard, Hoang et al. 2009).

In addition to the TLR pathway, there are other innate pathways that could be exploited to design effective and potentially safe vaccine adjuvants. The intracellular innate receptor, retinoic acid inducible gene I (RIG-I), is one possible candidate. This receptor recognizes double-stranded RNA and initiates signaling cascades that lead to the activation of the protein kinases IKK- α β , TBK1, and IKK- γ and subsequent activation of the transcription factors NF- κ B and IRF3 (Wille-Reece, Wu et al. 2005). The activation of these transcription factors leads to the production of interferon alpha/beta and TNF- α .

RIG-I is known to strongly bind poly I:C, poly A:U, and 5' and 3' untranslated regions of hepatitis C virus genomic RNA. It does not bind dsDNA, poly(rA), or yeast tRNA (Sen and Sarkar 2005). Recently, Ablasser *et. al.*, described a novel pathway where poly dA:dT activated RIG-I *in vitro*. In one key experiment, it was shown that knockdown of RIG-I significantly decreased the production of IFN- α in human monocyte derived dendritic cells (MoDCs) when lipofectamine-transfected with poly dA:dT (Yoneyama and Fujita 2007). Furthermore, it was determined that RNA polymerase III was also essential for cytokine production in this assay. It was concluded that RNA polymerase III transcribes poly dA:dT to poly (rA:rU), which then activates RIG-I (**Fig 1.12**) (Yoneyama and Fujita 2007; Chiu, Macmillan et al. 2009). This activation was abolished when RNA 5'-polyphosphatase was present, indicating the importance of 5'-triphosphorylation. Of note, the pathway appears to be different in humans and mice; knockdown of RIG-I does not significantly reduce the amount of IFN- α produced in mouse DC. (Yoneyama and Fujita 2007). Figure 1.12 depicts the pathway described above/

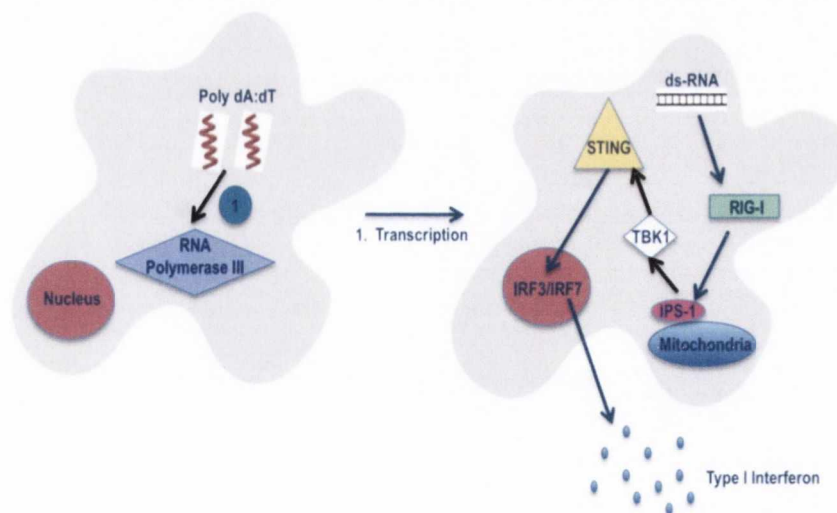


Figure 1.12 Poly dA:dT induced RIG I Activation Pathway.

Poly dA:dT enters into the cytoplasm of a cell with the help of lipofectamine. It is transcribed into poly (rA:rU) with 5' triphosphorylation by RNA polymerase III. The ds-RNA activates RIG-I, which in turn,

activates IPS-1 on the mitochondria. This activates the molecule TANK-binding kinase 1 (TBK1) which interacts with STING to lead to gene transcription of IRF3 and IRF7 in the nucleus. Type I interferon is produced and secreted from the cell.

PRRs	Localization	Ligand	Origin of Ligand
TLR			
TLR1	Plasma Membrane	Triacyl Lipoprotein	Bacteria
TLR2	Plasma Membrane	Lipoprotein	Bacteria, virus, parasite, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma Membrane	LPS	Bacteria, virus, self
TLR5	Plasma Membrane	Flagellin	Bacteria
TLR6	Plasma Membrane	Diacyl Lipoprotein	Bacteria, virus
TLR7	Endolysosome	ssRNA	Virus, bacteria, self
TLR8	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA virus, DNA virus
MDA-5	Cytoplasm	Long dsRNA	RNA virus
CLR			
Dectin-1	Plasma Membrane	β-Glucan	Fungi
Dectin-2	Plasma Membrane	β-Glucan	Fungi
DEC205	Plasma Membrane	Unknown	Unknown

Table 1: PRRs and their ligands.

Pattern recognition receptors (PRRs) are proteins expressed by cells to identify pathogen-associated molecular patterns (PAMPs). The table lists three types of PRRs: Toll-Like Receptors (TLRs), Retinoic acid-inducible (RIG-I)-like receptors (RLRs), and C-type lectin receptors (CLRs).

1.8 HIV-1

1.8.1 HIV-1 Background

Kaposi Sarcoma (KS) is a rare form of a relatively benign cancer, which tends to affect older individuals. However, in March 1981, at least eight cases of a more aggressive form of KS was identified amongst young gay men in New York (Hymes, Cheung et al. 1981). Moreover, during April of the same year, the centre for disease control (CDC) in Atlanta noticed an increase in the number of cases of a rare lung infection, *Pneumocystis carinii* pneumonia (PCP). An increase in PCP infection rates was noticed by the high number of requests for the drug pentamidine, used for treating the disease.

“A doctor was treating a gay man in his 20s who had pneumonia. Two weeks later, he called to ask for a refill of a rare drug that I handled. This was unusual – nobody ever asked for a refill. Patients usually were cured in one 10-day treatment or they died” – Sandra Ford (CDC drug technician) Newsweek (Daniel McGinn, 'MSNBC: AIDS at 20: Anatomy of a Plague; an Oral History', Newsweek Web Exclusive)

In June, the CDC published a report about the occurrence, without an identifiable cause for the PCP in five men in Los Angeles (MMWR Weekly (1981) 'Pneumocystis Pneumonia- Los Angeles', June 5, 30 (21); 1-3). This report is commonly referred to as the “beginning” of AIDS. The disease still did not have a name, with different groups referring to it in different ways, for example some still linked the disease to gay men, with a letter in The Lancet calling it “gay compromise syndrome” (Brennan and Durack 1981; Hymes, Cheung et al. 1981). Others called it GRID (gay-related immune deficiency), “gay cancer” or “community-acquired immune dysfunction” (Altman, L.K. (1982); The Washington Blade (1982) 'Gay cancer focus of hearing', April 16).

In June, a media report suggested that an infectious agent spread by sexual transmission might cause the disease. Later that month the first reports of the disease were reported in a Haitian population, as well as haemophiliacs (MMWR Weekly

(1982) 'Opportunistic infections and Kaposi's Sarcoma among Haitians in the United States', July 9,31 (26); 353-4,360-1 and MMWR Weekly (1982) 'Epidemiologic notes and reports *Pneumocystis carinii*, Pneumonia among persons with hemophilia A', July 16, 31(27); 365-7). The identification of the disease within the non-homosexual population meant that such names as GRID were now redundant. The acronym AIDS was adopted by the CDC in September of that year.

In May 1983, researchers at the Institute Pasteur in France, reported on the identification of a new virus, when they suggested was linked to AIDS (Barre-Sinoussi, Chermann et al. 1983). In May 1986, the virus responsible for AIDS was finally given a name, the human immunodeficiency virus (HIV) (Coffin, Haase et al. 1986). Since the declaration of the pandemic, HIV has been responsible for the deaths of over 25 million people (WHO. HIV and AIDS estimated data). Today, more than 33 million people are living with the virus; with nearly 8,500 deaths being attributed to AIDS daily. The global distribution of HIV infection is equally as alarming.

Certain regions of the globe are more affected than others; in Sub Saharan Africa approximately 7.4% of young adults are infected. This number increases to 25% in some countries such as Botswana and Zimbabwe. Elsewhere there are growing numbers of infections in China and India, where some surveys have shown 1 – 2% prevalence of HIV infection in pregnant women. At its current rate, HIV is spreading faster across the earth than any known chronic infectious agent in the last half-century. The recent clinical emergency is likely the consequence of a number of political, economic, and societal changes, including the breakdown of national borders, economic distress with the migration of large populations, and the ease and frequency of travel throughout the world.

1.8.2 HIV-1 The Lentivirus Genus

HIV is a member of the Lentivirus genus, which is part of the Retroviridae family. Of the Lentiviruses, equine infectious anemia virus was one of the first viruses to be identified in nature. The Retroviridae are a family of viruses characterised by the presence of the enzyme reverse transcriptase, which is responsible for the transcription of RNA into complementary DNA (cDNA) for integration into the host genome.

The term Lentivirus originates from the Latin “Lentus” meaning slow, this characterises the gradual course of disease progression caused by these viruses. There are a number of defining characteristics of this family, including the following, they are typically transmitted by exchange of bodily fluids, they persist indefinitely within their host (although virus titres may be low), they have a high mutation rate with different mutants being selected under different conditions and viral infection progresses through a number of specific stages. Lentiviruses can be clustered into five groups depending on the hosts that they infect.

Table 2: Representative members of the Lentivirus genus		
Origin of Isolates	Virus	Diseases
<u>Humans</u>	HIV-1 (SIVcpz) HIV-2 (SIVsm)	AIDS (Acquired Immune Deficiency Syndrome)
<u>Nonhuman Primates</u>		
Chimpanzee	SIVcpz	Simian AIDS
Sooty Mangabey	SIVsm	
Macques	SIVmac	
African Green Monkey	SIVagm	
Sykes Monkey	SIVsyk	
Mandrill	SIVmnd	
L’Hoest Monkeys	SIVlhoest	
<u>Nonprimates</u>		
Cat	Feline Immunodeficiency virus	Feline AIDS

Cow	Bovine Immunodeficiency virus	
Sheep	Visnae, maedi	Lung, CNS disease
Horse	Equine Infectious Anaemia	Anaemia
Goat	Caprine Arthritis, encephalitis	Arthritis, encephalitis

There are two main types of HIV: HIV-1 and HIV-2, which are closely related. HIV-2 is the most prevalent form found in West Africa, with an increasing abundance being reported in India. However HIV-1 is much more virulent and is responsible for much of the global cases of AIDS.

Both forms of the virus are believed to have spread from primate species to humans. The primate reservoir of HIV-2 has been identified as the sooty mangabey (*Cerocebus atys*) while the origin of HIV-1 appears to be the *Pan troglodytes* chimpanzee (Gao, Bailes et al. 1999). Initially described by Francois Clavel, Montagnier and coworkers, HIV-2 is distinct from the original type (HIV-1). Individuals infected with HIV-2 also develop AIDS, but have a longer clinical latency period and lower morbidity. Most individual infected with the virus progress to AIDS in 9 to 10 years, as with HIV-1; others are described as long-term nonprogressors.

Nucleotide sequence comparisons from many independent isolates of both HIV-1 and HIV-2 have revealed two major groups among HIV-1 isolates. These include group M and group O. Group M includes most HIV-1 isolates while group O represents what appear to be relatively rare outliers. Group M isolates can be further subdivided into seven subtypes called clades, each of which is prevalent in a distinct geographical area. North America and Europe have a distinct predominant subtype; clade B. HIV-2 can also be subdivided into five clades (**Fig 1.13**) (Simon, Mauciere et al. 1998).

The earliest record of HIV-1 infection was described in a serum sample obtained in 1959 from a Bantu male in the city of Kinshasa, in the Democratic republic of Congo.

Phylogenetic analysis verify that this is the oldest known HIV-1 infection and place the viral sequence (ZR59) near the ancestral node of clades B and D, these studies published in *Nature Medicine* suggest that two subtypes, and perhaps all of group M, evolved from a single trans-species transmission into the African population in the 1940s or early 1950s (Simon, Mauclore et al. 1998).

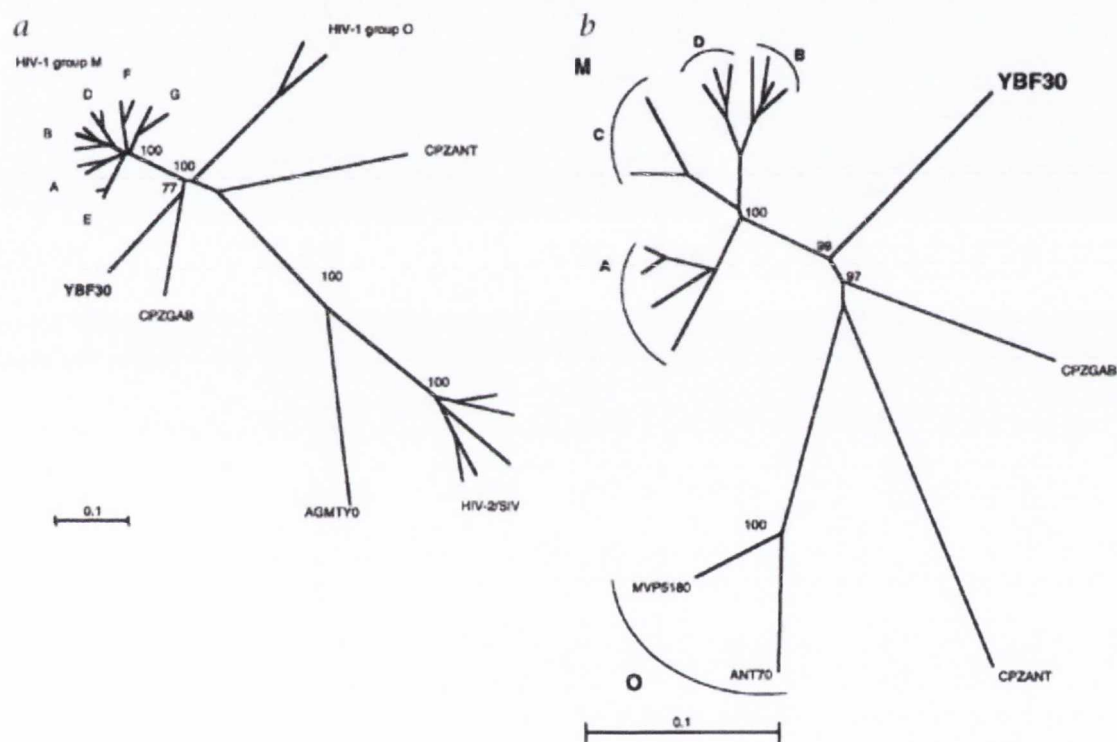


Figure 1.13: Phylogenetic analysis of the ZR59 sequence.

A weighted-parsimony tree shows that the virus ZR59 is closely related to the common ancestor of modern HIV-1 subtypes B, D and F and that virus YBF30 defines a new group, N-, that lies phylogenetically between groups M and O (Simon, Mauclore et al. 1998).

1.8.3 HIV infection

The vast majority of HIV cases are the result of infection due to sexual exposure through the genital tract or rectal mucosa. The initial stages of infection in humans are almost impossible to study *in vivo*, however utilising mucosal tissue explants, valuable information has been gathered regarding early transmission (Hu, Frank et al. 2004; Veazey, Klasse et al. 2005; Veazey, Springer et al. 2005). Furthermore Li *et al* (2009) have enhanced our understanding of the early stages of transmission using macaques inoculated with SIV (Li, Skinner et al. 2009). Despite these advances questions remain regarding early stage transmission, such as is HIV-1 transmitted in a free or cell-bound form. The mechanism utilized by HIV to pass across the mucosal epithelium is also unclear, as diffusion is slowed due to cervico-vaginal mucus (Lai, Hida et al. 2009).

1.8.3.1 Eclipse phase.

Following the initial transmission of the virus, there is an initial period of approximately 10 days, known as the eclipse phase. During this period no viral RNA is detectable in the plasma. A single virus has been shown to be sufficient to infect ~80% of mucosal cells (Keele, Giorgi et al. 2008). In their study Keele *et al.*, (2008) used single-genome amplification of viral RNA obtained from patients with acute HIV-1 infection, here they revealed that ~80% of infections arise from a single transmitted or founder virus.

1.8.3.2 Peak viraemia

Following the completion of the eclipse phase, the virus and/or virus-infected cells reach the local draining lymph nodes; here they come into contact with CD4⁺CCR5⁺ T cells, the primary target for further infection. The process of CD4⁺CCR5⁺ T cell infection is further amplified due to the ability of DCs to bind and internalize HIV through the DC-specific ICAM-3-grabbing non-integrin (DC-SIGN/CD209) (**Fig 1.14**) (Geijtenbeek, Kwon et al. 2000).

Following the infection of the local DC population, the virus spreads rapidly to other lymphoid tissues, particularly the gut associated lymphoid tissue (GALT) where a large reservoir of activated memory $CD4^+CCR5^+$ T cells resides (Brenchley, Schacker et al. 2004). Within the first three weeks of infection ~80% of all $CD4^+$ T cells in the GALT are depleted (Veazey and Lackner 1998; Veazey, Klasse et al. 2005). This includes ~60% of uninfected $CD4^+$ T cells which become activated and die of apoptosis (Gasper-Smith, Crossman et al. 2008) and ~20% of $CD4^+$ T cells which become infected with the virus.

During this rapid replication period in the GALT and regional lymphoid tissue, the level of plasma viraemia increases, usually peaking between day 21 and 28 in humans, the level of $CD4^+$ T cells eventually return to normal levels in the blood, however they fail to recover in the GALT (**Fig 1.14**) (Brenchley, Schacker et al. 2004; Mattapallil, Douek et al. 2005; Wang, Rasmussen et al. 2007).

1.8.3.3 Viral set point

Following the completion of the acute phase of the infection, the viral load decreases over a 12-20 week period. The viral load reaches a more stable level known as the viral set point (Schacker, Hughes et al. 1998). In the absence of pharmaceutical anti-viral intervention, in the form of ART therapy, the viral set point is maintained by a balance between virus turnover and the host immune response.

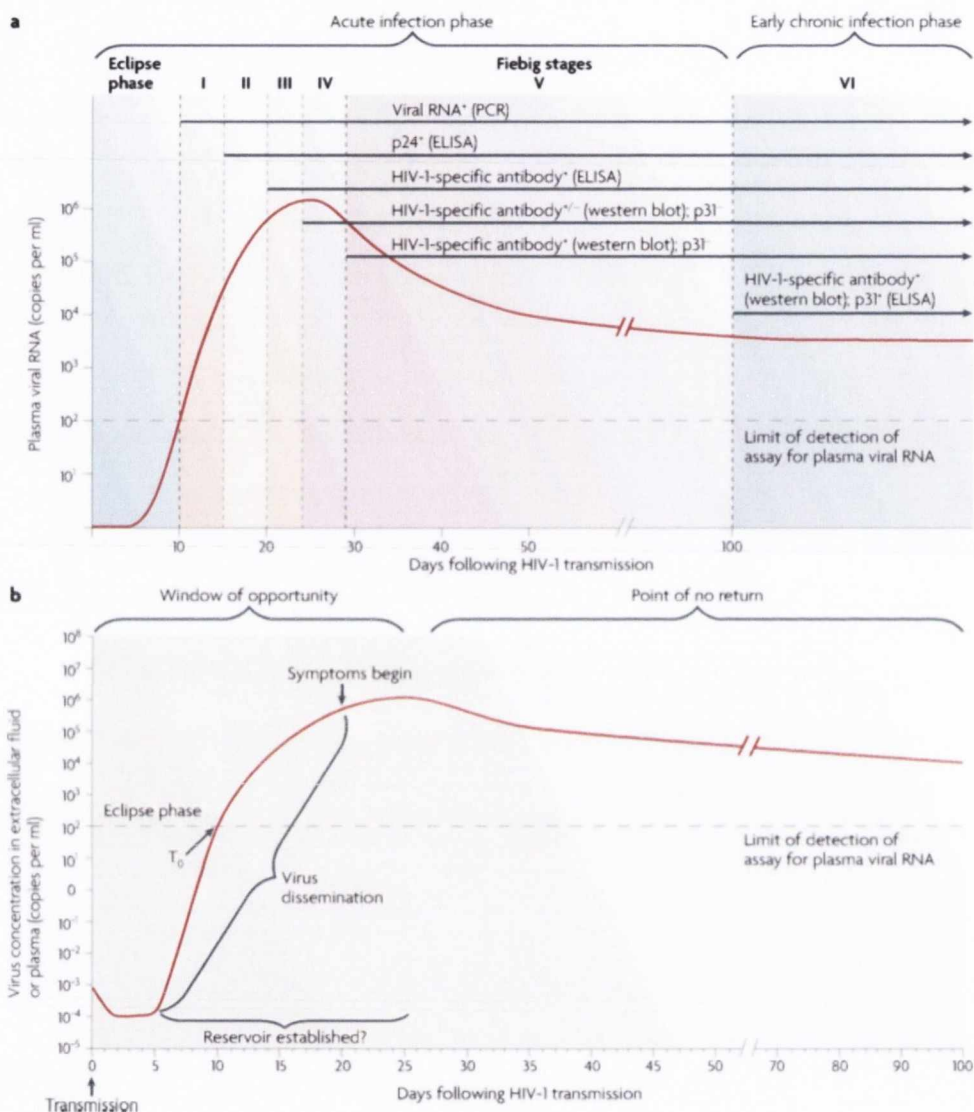


Figure 1.14: Illustration of acute HIV-1 infection.

A | Recent analysis of samples from individuals early after infection with HIV-1 has revealed that the first weeks following infection can be divided into clinical stages that are defined by a stepwise gain in positivity for the detection of HIV-1 antigens and HIV-1-specific antibodies in diagnostic assays (in brackets). Patients can be categorized into Fiebig stages I–VI, which are based on a sequential gain in positive HIV-1 clinical diagnostic assays (viral RNA measured by PCR, p24 and p31 viral antigens measured by enzyme-linked immunosorbent assay (ELISA), HIV-1-specific antibody detected by ELISA and HIV-1-specific antibodies detected by western blot). Patients progress from acute infection through to the early chronic stage of infection at the end of Fiebig stage V, approximately 100 days following infection, as the plasma viral load begins to plateau. **B** | Fundamental events in acute HIV-1 infection. Following HIV-1 infection, the virus first replicates locally in the mucosa and is then transported to draining lymph nodes, where further amplification occurs. This initial phase of infection, until systemic

viral dissemination begins, constitutes the eclipse phase. The time when virus is first detected in the blood is referred to as T0; after this there is an exponential increase in plasma viraemia to a peak 21–28 days after infection. By this time, significant depletion of mucosal CD4⁺ T cells has already occurred. Around the time of peak viraemia, patients may become symptomatic and reservoirs of latent virus are established in cells that have a slower rate of decay than CD4⁺ T cells. The ‘window of opportunity’ between transmission and peak viraemia, prior to massive CD4⁺ T cell destruction and the establishment of viral reservoirs, is the narrow but crucial period in which an HIV-1 vaccine must control viral replication, prevent extensive CD4⁺ T cell depletion and curb generalized immune activation. (McMichael et al. 2010)

1.8.4 HIV infections and the gastrointestinal immune system

In addition to its devastating systemic effects, HIV also directly affects tissues at a local level. In 1984, Kotler and colleagues, determined that HIV-infected patients had histologic abnormalities of the GI mucosa, lymphocyte depletion and malabsorption, noting “The histologic findings suggest that a specific pathological process occurs in the lamina propria of the small intestine and colon in some patients with the syndrome” (Kotler, Gaetz et al. 1984). The enteropathy that afflicts HIV-infected patients can occur during the acute phase of infection through the advanced stages of the disease. It includes diarrhoea (Kapembwa, Fleming et al. 1991), increased GI inflammation, increased intestinal permeability and malabsorption of bile acid and vitamin B12 (Bjarnason, Sharpstone et al. 1996; Sharpstone, Neild et al. 1999). At a physiological level, enteropathy is associated with inflammatory infiltrates of lymphocytes and damage to the GI epithelial layer, including villous atrophy, crypt hyperplasia, and villous blunting (Batman, Miller et al. 1989; Heise, Miller et al. 1994; Batman, Kotler et al. 2007). These changes occurs in the absence of detectable bacterial, fungal or viral enteropathogens that are often associated with enteropathy (Batman, Miller et al. 1989).

HIV has been associated with the development of structural abnormalities within the GI tract. Although the exact mechanisms behind these abnormalities have yet to be fully determined, a number of studies have begun to shed light on the mechanism, with some researchers suggested it may be the “virotoxic” effects of HIV itself on enterocytes (Heise, Dandekar et al. 1991). Canani *et al.*, (2006), have revealed the ability of the HIV

accessory protein Tat to directly inhibit the ability of enterocytes to take up glucose (Canani, De Marco et al. 2006). Furthermore, HIV gp120 can also directly effect tubulin depolymerization, which results in increased concentrations of calcium in enterocytes, disrupting the ability of enterocytes to maintain ionic balance (Maresca, Mahfoud et al. 2003). HIV has also been shown to directly increase the proliferation of enterocytes, in addition to affecting their differentiation (Heise, Dandekar et al. 1991; Batman, Fleming et al. 1994). These results are supported by evidence that antiretroviral therapy ameliorates GI symptoms (Kotler, Shimada et al. 1998).

1.8.5 HIV and gastrointestinal CD4 T-cell depletion.

As early as 1992, researchers began to turn their attention to the mucosal immune system and its role during HIV infection. Initially, immunohistochemistry studies revealed the infiltration of proinflammatory lymphocytes into the GI tract, however these studies also revealed the absence of CD4⁺ T cells (Rodgers, Fassett et al. 1986; Clayton, Reka et al. 1992). Studies investigating the small and large intestine revealed the striking absence of CD4⁺ T cells post HIV infection (Kotler, Reka et al. 1993; Clayton, Snow et al. 1997), revealing that the deleterious effects of HIV-1 involved the entire bowel. Despite these advances, all the initial studies were carried out with patients who were chronically infected with HIV or were in the later stages of AIDS.

Utilising rhesus macaque models of SIV infection, Veazey *et al.*, (1998) revealed the dramatic depletion of intestinal CD4⁺ T-cells within days of infection (Veazey et al. 1998). These studies have dramatically impacted HIV research to date, as they revealed the majority of the hosts CD4⁺ T cells are depleted within the very early stages of acute infection, this is in contrast to traditional research approaches measuring peripheral blood and lymph nodes.

More detailed studies began to examine CD4/CD8 ratios in both the duodenum and peripheral blood; these revealed that HIV preferentially depletes CD4⁺ T cells in the GI tract (Shacklett, Cox et al. 2003). Complete depletion of CCR5⁺CD4⁺ T cells from

mucosal sites of HIV-infected humans and SIV-infected macaques was also observed (Shacklett, Cox et al. 2003; Mehandru, Poles et al. 2004). These cells were preferential targeted by HIV-1, Mehandru *et al.*, (2004) revealed extensive depletion of these cells within the lamina propria as compared to other immune inductive sites such as the Peyer's patches. T cells that reside within the GI tract are infected by HIV 10-fold more frequently than those found in peripheral blood (Mehandru, Poles et al. 2007). Moreover *in vivo* findings have also shown that up to 60% of the resident CD4⁺ T cells can be infected with the virus and as the majority of T-cells reside within the gut, this has revealed a much larger viral reservoir than previously anticipated.

1.9 The mucosal immune system

“The mammalian mucosal immune system is an integrated network of tissues, lymphoid, effector cells and molecules which protect the host from infection of the mucous membrane surfaces” (Gill et al. 2010)

Infections caused by many established and emerging pathogens such as *Mycobacterium tuberculosis*, *Aspergillus fumigatus* and HIV, pose very serious health problems for the global population and great challenges to the realization of Millennium Development Goals outlined in the United Nations Millennium Declaration (Chen and Cerutti 2010). These health problems are particularly serious for persons living in developing countries where poor hygiene compromises immunity. A common feature of infections caused by these diverse types of pathogens, whether they are helminths or viral infection, is that they usually occur or initiate at mucosal surfaces. Although experimental evidence has revealed that systemic vaccination is adequate to offer protection against selective pathogens, such as polio and influenza (Haan, Verweij et al. 2001), an increasing number of studies has shown the need for the induction of immunity at mucosal surfaces for effective protection against other important pathogens, such as HIV, *Vibrio cholera* and *Mycobacterium* species (Chen, Wang et al. 2004; Wang, Thorson et al. 2004)

The immune system can be distinguished into a series of anatomically distinct compartments, each of which is specifically adapted to generate a response to antigens encountered in a particular set of tissues. In addition to the adaptive immune response, which is initiated in the peripheral lymph nodes and spleen as a result of antigen exposure, there is an additional compartment of the adaptive immune system of even greater surface area. This compartment is located near surfaces exposed to antigenic stimulus and is a common site of pathogen entry. This is the mucosal immune system.

The epithelial surfaces of the body are constantly exposed to a wide variety of antigenic material, ranging from ubiquitous non-pathogenic material such as food antigens to bacteria, viruses and allergens. These antigens are separated from the delicate underlying body surfaces by a thin layer of cells, the epithelium. These tissues are essential for life and as a result require constant protection against pathogenic organisms (Holmgren and Czerkinsky 2005; Brandtzaeg 2007).

The mucosal immune system comprises the gastrointestinal tract, the upper and lower respiratory tracts and the urogenital tract. It also includes the exocrine glands associated with these organs, such as the pancreas, the conjunctive and lachrymal glands of the eye, the salivary glands, and the lactating breast. The mucosal surfaces represent an enormous area to be protected. One striking example of the size of the mucosal surface comes from the small intestine; here the surface area is almost 400m^2 , which is 200 times that of the skin. Moreover, the lungs have a surface area of over 150m^2 ; within which a volume of 350 litres per hour of environmental air is ventilated at rest (Lavelle 2005).

To facilitate important physiological functions such as gas exchange (lungs), nutrient absorption (gut) and reproduction (uterus and vagina), the mucosal surfaces found in these regions are thin, delicate and permeable. Given their functional importance to life it is critical to have effective defence mechanisms in place to protect them from invasion, however their fragility and permeability creates an obvious vulnerability to infection and it is not surprising that the vast majority of pathogens invade the human body by these routes.

A second important consideration for the immunology of mucosal surfaces is that they are also portals of entry for a wide array of foreign antigens that are not pathogenic. This is best seen in the intestine, a site where there is continuous antigenic challenge from birth to death in the form of food antigens. It is estimated that the intestine is exposed to between 10-15kg of food proteins per year per person. However, the intestine is also home to an incredibly complex microbiota especially within the distal ileum and colon. This region provides an abundant source of potentially detrimental organisms, ligands and antigens that could activate pathogenic innate and adaptive immune responses in addition to the normal bacterial flora.

Many of the anatomical and immunological principles underlying the mucosal immune system apply to all of its constituent tissues. The mucosal-associated lymphoid tissue (MALT) is the most extensive compartment of human lymphoid tissue. Mucosal inductive sites of the gastrointestinal tract include the Peyer's patches (PP), the appendix and solitary lymphoid nodules, which collectively comprise the gut-associated lymphoreticular tissues (GALT), the tonsils and adenoids, or nasal-associated lymphoreticular tissues (NALT), serve as the inductive sites for the upper respiratory tract and nasal/oral cavity.

Meanwhile the immunity in the upper and lower airways is maintained by the bronchus-associated lymphoid tissue (BALT). Humans generally do not have an anatomically well-defined NALT, except during early ages, however they do possess oropharyngeal lymphoid tissues such as pharyngeal, tubal, palatine and lingual tonsils (Kunisawa, Nochi et al. 2008). This set of lymphatic tissues is called Waldeyers ring and may constitute the human equivalent of mouse NALT (Kunisawa, Nochi et al. 2008). All these MALT structures comprise anatomically and functionally distinct inductive and effector sites. Inductive sites include mucosa-associated follicles, such as intestinal PPs, IFLs and mesenteric lymph nodes; here antigen specific B and T cells undergo maturation, expansion and differentiation into effector cells. These cells subsequently migrate to and from the priming sites to the effector sites to carry out their effector functions. Effector

sites are present in all mucosal zones as non-organized lymphoid tissues distributed through the lamina propria (Kiyono and Fukuyama 2004).

Originally described by Johan Peyer over 300 years ago, Peyer's patches are extremely important sites for the initiation of the immune responses in the gut. PP populations within the gut vary with respect to species, anatomical location, with age/developmental stage and also due to exogenous factors. Typically in a healthy human the number of PP along the length of the GI tract is ~300, however this number declines after puberty. The largest density of PP can be found in the ileum where they comprise 10-1000 individual follicles organized into discrete lymphoid structures overlaid by follicle-associated epithelium (FAE). PP are large, rounded, elongate structures which are visible to the naked eye on the luminal surface of the intestine. Their average size ranges from an eighth of a centimetre in mice, to one centimetre in diameter in humans. In ruminants this diameter can increase to ten centimetres. PP can also be found in lower parts of the GI tract, such as the colon. However their exact function within this region remains unclear.

In 1922, rabbit PPs were identified as site of antigen uptake (*Mycobacterium tuberculosis* uptake), however this was dismissed as being a non-specific process of little importance. The FAE appears to be specifically designed to allow access of antigens and microorganisms to the local epithelial surface and to promote their uptake by transepithelial transport. The principal cell responsible for antigen uptake is the M cell. The M cell like other IECs is connected to its neighbours by tight junctions maintaining barrier function; however unlike IEC it provides functional openings in the epithelial barrier through which antigen transport can occur.

1.9.1 M cells as gateways to the Mucosal Immune System

Like other M-cell containing GALT such as the tonsils and appendix, at one time the PPs were considered as vestigial organs. However due to recent developments this view has changed. FAE-located M cells sample particulates from the luminal side of the

gastrointestinal tract and actively present them to the lamina propria. Located within the lamina propria are a wide variety of immunologically relevant cells including macrophages and dendritic cells and dense populations of lymphocytes. These cells were identified in the rabbit appendix by J.F. Schmedtje (Owen & Jones, 1974) and were initially called lymphoepithelial cells but were later renamed M cells. Owen and Jones (1974) utilized the electron microscope to study human PP and found the presence of “microfolds” on the apical surface of these specialised cells.

Keljo *et al.*, (1983) were the first group to highlight the ability of the PP to absorb proteins (Keljo and Hamilton 1983). It was later shown that the PP also has the capability to absorb antigens (Kim 2002). The ability of the M cell to absorb whole antigens is unique to the gut as the epithelium is considered an impermeable barrier to the entry of non-nutritional molecules from the lumen. Although the relative number of M cells was believed to be related to bacterial challenge, there are a number of studies which have found an abundant number of PP-like follicles in the small intestine of germ free neonatal ruminants and swine. (Savidge, Smith et al. 1991)

The epithelium overlying the PP, more specifically the M cell, appears to contribute to antigen trafficking across the epithelium. Therefore, in the case of neonatal large farm animals, the rate of uptake by the FAE could be of a magnitude that is sufficient to facilitate passive immunity via the transfer of maternal immunoglobulin molecules. However the overall contribution to the function of the immune system remains to be full determined. As a portal of entry M cells represent a generic antigen-sampling site. Their main functional role is the delivery of exogenous material to the subepithelial compartment(s).

Although M cell trafficking of antigenic material across the epithelium may appear as a breach in the innate immune system, it may be advantageous to the host, if such antigen delivery is coupled with immune protection and the induction of immunological tolerance to food antigens. From studies with IgA knockout mice including that of Silvey *et al.*, (2001), it has been revealed that the production of secretory IgA in response to specific

viral pathogens is an effective way to prevent viral attachment to the PP mucosa and this protects against oral challenge (Silvey, Hutchings et al. 2001).

1.10 Innate cells of the mucosal immune system – Dendritic Cells.

1.10.1 Intestinal Dendritic Cells

The intestinal mucosa is home to many cells of the mononuclear leukocyte family, including macrophages and DCs which are strategically positioned for antigen capture and it is now well established that they play a vital role in orchestrating intestinal immune responses (Kelsall 2008). DCs are potent antigen-presenting cells (APC) and are key modulators of the immune response, due to their ability to process and present antigens to T cells. Intestinal DCs are essential for the activation of inflammatory pathways and maintaining local immunological tolerance. An increasing number of DC subtypes have been identified and characterized.

DCs are enormously plastic in nature, owing to various microenvironmental adaptations and influences. Despite their heterogeneity, these cells share functional properties that distinguish them as a pivotal link between the innate and adaptive immune system (del Rio, Bernhardt et al. 2010). DCs are located throughout the intestine, including the lamina propria (LP) of both the small and large intestine, Peyer's patches (PP), draining mesenteric lymph nodes (MLNs) and at isolated lymphoid follicles (Rescigno and Di Sabatino 2009). Recent work by Miriam Merad and colleagues has revealed the presence of CD11c^{high}MHC class II^{high} DCs within both the intestinal epithelial cell (IEC) fraction and LP, meanwhile these cells were absent from both the serosal and muscularis layers. These cells were further characterised into two distinct populations, CD11c^{high}MHC class II^{high}CD103⁺CD11b⁺ and CD11c^{high}MHC class II^{high}CD103⁻CD11b⁺.

The majority of CD11c^{high}MHC class II^{high} DCs in the small intestinal LP (SILP) express the CD103 integrin and have distinct functional properties (del Rio, Rodriguez-Barbosa et al. 2008). These cells are also found within the PPs (Jaensson, Uronen-Hansson et al.

2008), colonic LP and mesenteric LNs (MLN) (Johansson-Lindbom, Svensson et al. 2005). Immunofluorescence studies have revealed the presence of CD103⁺CD11c⁺ DCs in the LP and within the intraepithelial space of the apical villi, in contrast CD103⁻CD11b⁺ DCs accumulated mainly in the LP (Bogunovic, Ginhoux et al. 2009).

The T-epithelial cell associated integrin CD103, also known as the α E β 7 integrin was discovered in the late 1980s as human mucosal lymphocyte antigen HML-1 (Cerf-Bensussan, Jarry et al. 1987) and its functional role was described in 1994 (Cepek, Shaw et al. 1994). Integrins are a large group of transmembrane $\alpha\beta$ heterodimers that mediate cell to cell binding and cell to extracellular matrix interactions and are vital for T cell homing, cell signalling, adhesion and migration (Dustin, Garcia-Aguilar et al. 1989; Springer 1994; Springer 1995). The CD103 integrin is expressed as a heterodimer with its β 7 chain partner (Kilshaw and Murant 1990).

In addition to its expression on mucosal CD11c^{high}MHCII^{high} DCs, CD103 is also expressed on a subset of effector memory CD8⁺ T cells in addition to subsets of CD4⁺ and CD8⁺ regulatory (Treg) T cells (Lehmann, Huehn et al. 2002; Uss, Rowshani et al. 2006). The principal ligand for CD103 is E-cadherin, expression of which is confined to the basolateral surface of epithelial cells and is not detected on endothelial cells. CD103 has also been shown to be responsible for influencing cellular shape and motility in a ligand-dependent fashion (Schlickum, Sennefelder et al. 2008).

1.10.1.1 Origins and development of the intestinal CD103⁺ DC population

DCs within the intestinal compartments have been extensively studied and much is now known regarding their phenotype. CD11c⁺ MHC class II⁺ DCs have been shown to be present in the LP and interepithelial cell (IEC) fraction and were absent from the muscularis and serosa. The LP is home to two distinct DC subsets; CD11c^{high}MHC class II^{high}CD103⁺CD11b⁺CX₃CR1⁻M-CSF^{low} (CD103⁺CD11b⁺) and CD11c^{high}MHC class II^{high}CD103⁻CD11b⁺CX₃CR1⁺M-CSF^{high} (CD103⁻CD11b⁺) DCs. Meanwhile the PPs are home to CD11c^{high}MHC class II^{high}CD103⁺CD11b⁻CX₃CR1⁻ (CD103⁺CD11b⁻) DCs.

CD11c^{low}MHC class II^{high}CD103⁻CD11b⁺ DCs are found throughout the muscular and serosal layers of the gut, with equivalent populations found in the large bowel (**Fig 1.15**) (Bogunovic, Ginhoux et al. 2009).

Granulocyte-macrophage-colony-stimulating factor (GM-CSF) and Fms-like tyrosine kinase 3 (Flt3) ligand are two important cytokines involved in DC development (Merad and Manz 2009). In contrast, the development of macrophage populations in addition to epidermal Langerhans cells requires ligands for the macrophage colony stimulating factor receptor (M-CSFR) (Ginhoux, Tacke et al. 2006). Given the key role of M-CSFR ligands in the development of these populations, there may also be a role for these factors in the development of intestinal DCs. In fact, the development of CD103⁻CD11b⁺ LP DCs was dramatically affected following the selective deletion of M-CSFR (*Csf1r*^{-/-}), whereas the CD103⁺CD11b⁺ population was unaffected. Flt3 is required for the development of both populations of LP-DCs, with a stronger requirement in the case of the CD103⁺CD11b⁺ cells. CD103⁺CD11b⁺ DCs also require GM-CSF for their development unlike the CD103⁻CD11b⁺ DC population.

Common DC precursor (CDP) and pre-DC do not contribute substantially to the CD103⁻CD11b⁺ LP-DC population under steady state conditions while monocytes actively differentiate into CD103⁻CD11b⁺ LP-DCs. However, Bougouniv *et al.* (2009) revealed that the GM-CSFR is dispensable for CD103⁻ DC differentiation but is required for the differentiation of CD103⁺CD11b⁺ and CD103⁺CD11b⁻ DCs (Bogunovic, Ginhoux et al. 2009). Research also suggests that although the CD103⁺ population expresses CCR7 and actively migrate during steady state conditions, the CD103⁻ DCs appear to be a resident non-migrating population in the MLN. In fact, only approximately 20% of the MLN CD103⁻ DCs express CX₃CR1, suggesting a different developmental origin than LP CD103⁻ DCs, which express CX₃CR1.

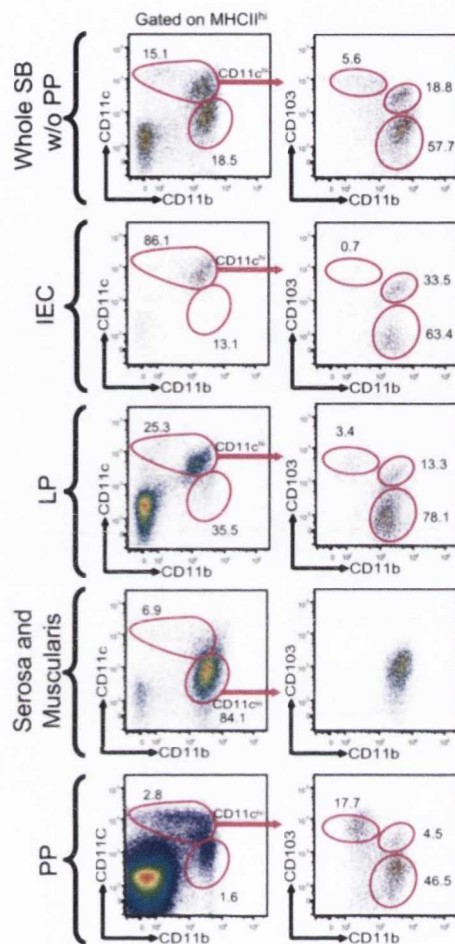


Figure 1.15: Characterization of CD103⁺ DC subsets in the small bowel (Bogunovic, Ginhoux et al. 2009).

Single cell suspensions were prepared from various anatomical locations found throughout the small bowel, these included the IEC compartment, lamina propria (LP), serosa with the intestinal muscularis and PP. The populations were gated on DAPI negative, CD45 positive, MHC Class II high and CD11c^{high}. The CD11c^{high} population was further characterised on the basis of CD103 and CD11b expression.

1.10.1.2 CD103⁺ DCs promote Intestinal T cell homing.

CD103⁺ DCs in the MLN have the unique ability to induce gut-homing phenotypic changes (Annacker, Coombes et al. 2005; Johansson-Lindbom, Svensson et al. 2005). These cells are responsible for the up regulation of C-C chemokine receptor type 9 (CCR9) and $\alpha 4\beta 7$ expression on CD8⁺ T cells. These cell surface markers are essential for the active migration of cells to peripheral non-lymphoid tissues. Induction of CCR9 and $\alpha 4\beta 7$ expression was initially observed following the co-culture of MLN CD103⁺ DCs from the MLN with T cells after oral antigen administration. In contrast, these cells failed to induce CCR9 and $\alpha 4\beta 7$ expression on T cells following intraperitoneal (I.P) antigen injection. Agace and colleagues transferred TCR transgenic ovalbumin (Ova) specific OT-I cells into recipient mice and the expression of CCR9 and $\alpha 4\beta 7$ in the MLNs was examined 3 days following I.P or oral antigen administration. CCR9 and $\alpha 4\beta 7$ endowed the newly activated T cell with the capacity to migrate to the small intestine and exert effector functions (Annacker, Coombes et al. 2005; Johansson-Lindbom, Svensson et al. 2005).

The vitamin A metabolite, Retinoic acid (RA), has been shown to be responsible for the induction of CCR9 and $\alpha 4\beta 7$ expression on T cells (Iwata, Hirakiyama et al. 2004). RA conversion depends on Retinaldehyde dehydrogenases (RALDH), which are expressed on MLN DCs as well as stromal cells. Despite expression of RALDH2 by both the CD103⁺ and CD103⁻ MLN DCs, MLN and LP CD103⁺ DCs express much higher levels of the enzyme than their CD103⁻ counterparts (Coombes, Siddiqui et al. 2007). While CD103⁻ DC in the MLN retain the capacity to prime CD4 and CD8 responses, these cells lacked the ability to induce gut-homing signatures on naïve T cells (Annacker, Coombes et al. 2005; Johansson-Lindbom, Svensson et al. 2005) highlighting the unique role of the CD103⁺ DC subset.

A recent exciting development has linked bile retinoids to the imprinting of LP CD103⁺ DCs in the small intestine, which have the ability to generate gut-homing T cells. Agace and colleagues demonstrated that the imprinting of CD103⁺ DCs is itself dependent on

vitamin A and occurs locally within the small intestine, with high levels of retinol being detected within the bile (Jaensson-Gyllenback, Kotarsky et al. 2011). The authors found that RA induced retinol-metabolizing activity in DCs both *in vivo* and *in vitro*. CD103⁺ DCs in the small intestinal LP constantly receive RA signalling *in vivo* at much higher levels than colonic CD103⁺ DCs. In a series of elegant experiments it was revealed that small intestinal CD103⁺ DCs remained imprinted in mice following the depletion of dietary but not systemic retinol (Jaensson-Gyllenback, Kotarsky et al. 2011).

1.10.1.3 Regulation of immune responses by CD103⁺ DC

A number of regulatory mechanisms are employed by the immune system to maintain local immune homeostasis, prevent autoimmunity and control inflammation (Mills 2004). Treg cells are regarded as the primary mediators of peripheral tolerance and are vital in the prevention of chronic inflammatory conditions.

The gastrointestinal tract is constantly exposed to a wide variety of antigenic material and maintenance of the local homeostasis in the gut stretches the discriminatory powers of the immune system to its limits (Wright 2005). Although the vast majority of incoming antigens are harmless, some are highly immunogenic and have a propensity to induce inflammation, highlighting the importance of regulatory mechanisms in the gut. The GI mucosal immune system must carry out the difficult task of discriminating between the background antigenic noise and the much rarer signals transmitted by pathogens and their associated antigens. One strategy to prevent inflammation is the positioning of DCs in these compartments to capture and process antigens, and induce tolerance. Iliev *et al.* (2009) revealed that CD103⁺ DCs induce tolerance and gut homing, protecting against experimentally induced colitis in mice. CD103⁺ DCs exerting tolerogenic effects has been further suggested by the recent finding the CD11c⁺CD103⁺ DCs are decreased in the celiac lesion during celiac disease (Beitnes, Raki et al. 2011).

MLN CD103⁺ DCs mediate the conversion of naïve T cells to FoxP3-expressing T cells by producing TGF- β and RA (Coombes, Siddiqui et al. 2007). When MLN DCs loaded with ova were cultured in the absence of TGF- β , CD103⁺ DCs induced the development of antigen specific iTreg cells. Furthermore, CD103⁻ DCs failed to induce Treg development (del Rio, Bernhardt et al. 2010) and it was suggested that “the special capacity of MLN-derived CD103⁺ DCs to drive iTreg cell differentiation may relate to the abundant expression of tissue plasminogen activator, TGF β 2, and latent TGF- β binding protein 3”. Both TGF β 2 and latent TGF- β binding protein 3 are required for the activation and active secretion of TGF- β . Upon addition of TGF- β , much higher levels of iTregs were induced by the CD103⁺ DC than the CD103⁻ population.

In addition to TGF- β , RA produced by CD103⁺ DCs is an essential cofactor for increasing the rate of iTreg cell induction. In fact, preincubation of DCs with a synthetic RA inhibitor prevented the induction of iTreg cells (Coombes, Siddiqui et al. 2007). iTreg cells induced via the co-culture of CD103⁺ and CD4⁺ T cells were capable of directly suppressing the proliferation of CD4⁺ T cells under *in vitro* culture conditions (Annacker, Coombes et al. 2005). Recent reports have also suggested a role for indoleamine 2,3-dioxygenase (IDO) in CD103⁺ DC-mediated iTreg cell induction. It was shown that CD103⁺ DCs but not CD103⁻ DCs expressed IDO whose inhibition resulted in reduced CD4⁺FoxP3⁺ Treg cell conversion and enhanced T cell proliferation (Matteoli, Mazzini et al. 2010). Genetic deletion of IDO resulted in an increase in proinflammatory Th1 and Th17 cells and moreover IDO depletion results in the blockage of Treg cell induction and exacerbation of colitis in mice (Matteoli, Mazzini et al. 2010).

In contrast to the suppressive role of CD103⁺ DCs, CD103⁻ DCs isolated from the MLN are associated with the production of proinflammatory cytokines. Upon stimulation with LPS or R848, CD103⁻ DCs produced significant concentrations of TNF- α and IL-6 (Coombes, Siddiqui et al. 2007). The CD103⁻ population may be derived from a recruited blood-borne precursor and as a result do not undergo conditioning by the immune suppressive gut microenvironment. In fact it has been shown that intestinal epithelial

cells induce the expression of CD103 on the surface of DCs (Iliev, Mileti et al. 2009) and thereby promote their development.

New evidence has also revealed that inflammation directly dampens the tolerogenic ability of the MLN CD103⁺ DC population. This effect on the MLN CD103⁺ DC population was mediated by a down-regulation of the *tgfb2* and *aldh1a2* genes in the DC. MLN CD103⁺ DCs isolated from colitic mice had an impaired ability to induce FoxP3⁺ Treg cells; in contrast these cells had an enhanced ability to prime CD4⁺ T IFN- γ producing T cells (Laffont, Siddiqui et al. 2010). Laffont *et al.*, (2010) also revealed that CD103⁺ cells from naïve mice and mice with induced colitis share the same developmental pathway. Furthermore, it has also been revealed that CD103 expression is lost from the surface of gut resident DCs during colitis (Strauch, Grunwald et al. 2010). These findings highlight the role of environmental influences in directing the tolerogenic properties of these cells.

Under normal physiological conditions, the intestinal immune system is tolerant to food antigens as well as to local commensal bacteria; break down of this tolerance can result in severe inflammatory reactions. It is therefore assumed that CD103⁻ DCs are poised to respond to pathogenic insult, while CD103⁺ DCs are responsible for the induction of Treg cells and hence the maintenance of intestinal homeostasis.

1.10.1.4 The migratory ability of CD103⁺ DC

In 2001, Rescigno and colleagues proposed an M cell-independent pathway for antigen uptake (Rescigno, Urbano et al. 2001). Previous studies had revealed that *Salmonella typhimurium*, lacking genes encoded on the pathogenicity island 1 (SPI1) that are necessary for invasion, were observed within the spleen following oral administration (Galan and Curtiss 1989). Rescigno *et al.*, (2001) revealed the ability of resident intestinal DCs to open tight junctions between epithelial cells and extend their dendrites into the epithelium and directly sample bacteria. Interestingly, because DCs express the tight junction proteins zonula, occludin and claudin I, the integrity of the epithelial barrier is preserved, preventing the development of overt inflammatory reactions to intestinal bacteria (Rescigno, Urbano et al. 2001). Further work utilising transgenic mice which express GFP under the control of CX₃CR1, demonstrated that this chemokine receptor was vital for the development of transepithelial dendrites, enabling these cells to directly sample antigens from the lumen (Niess, Brand et al. 2005).

Regarding the CD103⁺ DC population, it now appears that these cells are distinct from the CX₃CR1-expressing population within the LP. Despite the fact the CX₃CR1 DC population outnumbers the CD103⁺ DCs, CD103⁺ DCs selectively localise close to the epithelium (Schulz, Jaensson et al. 2009). These cells also differ in their ability to respond to growth factors and have different turnover rates (Schulz, Jaensson et al. 2009), suggesting that they are derived from very distinct precursors. Schulz *et al.*, (2009) elegantly revealed that in mice lacking CX₃CR1, the LP CD103⁺ DC population are the main migratory population moving to the MLNs. Meanwhile the CX₃CR1^{high} LP cells may represent a non-migratory tissue resident population (**Fig 1.16**) (Schulz, Jaensson et al. 2009). Evidence also suggests that the CD103⁺ DCs are much more potent at inducing intestinal T cell responses compared to the CX₃CR1^{int} and CX₃CR1^{high} populations, highlighting their importance. These populations also differed in their ability to generate RA. CX₃CR1⁺ DCs expressed much lower levels of *aldh1a2* and as a result were inefficient at inducing the up regulation of CCR9 on T cells. It has been postulated that perhaps CX₃CR1⁺ LP DCs engulf luminal bacteria (Niess, Brand et al. 2005) and take up

orally administered antigens (Schulz, Jaensson et al. 2009) before directly passing these antigens to CD103⁺ LP DCs, which migrate to the MLNs where they mediate tolerogenic immune responses. However this has yet to be observed experimentally. Impairment of DC trafficking to the MLNs in mice lacking the CCR7 receptor results in defective induction of tolerance to oral antigens (Worbs, Bode et al. 2006), therefore establishing the importance of continual antigen transport to the MLNs in maintaining normal mucosal integrity. This was further highlighted by Varol *et al.* (2009) they determined that when mice display only CD103⁻CX₃CR1⁺ DCs, they are more susceptible to dextran sodium sulphate (DSS)-induced colitis (Varol, Vallon-Eberhard et al. 2009).

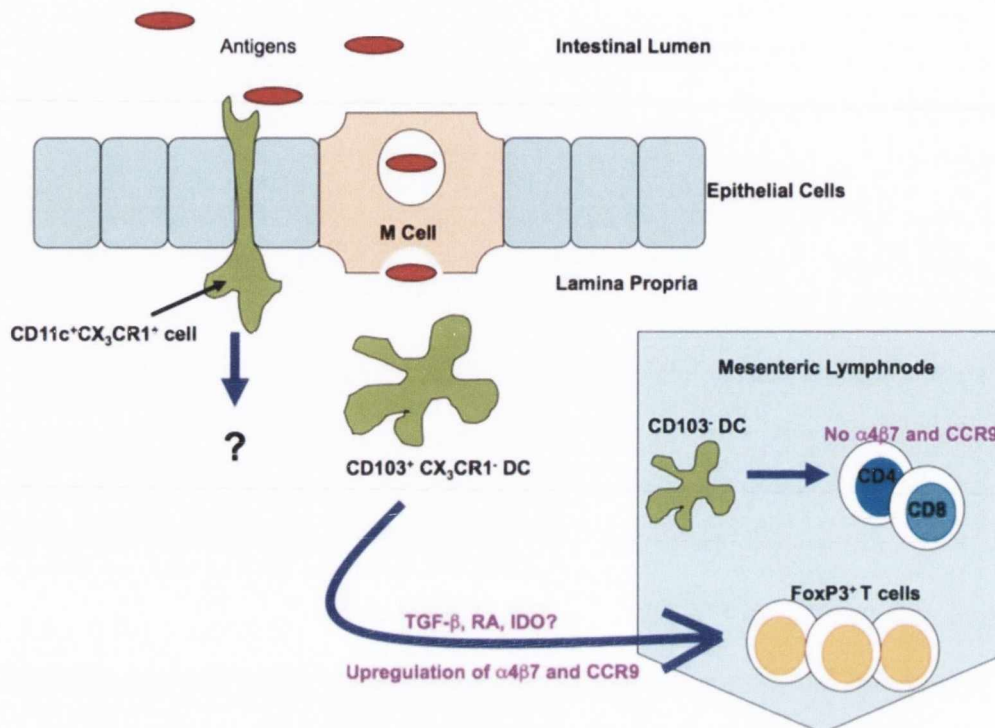


Figure 1.16: Mechanisms of antigen sampling by lamina propria DC populations under steady state conditions.

DCs can capture antigenic material (including bacteria and other antigens) from the luminal compartment via direct transport through the M cell. CX3CR1⁺ cells can sample antigens directly by extending their dendrites into the luminal compartment. This is achieved due to the ability of the CX3CR1⁺ cell population to open the tight junctions between epithelial cells. CD103⁺ CX3CR1⁻ DCs actively migrate to the mesenteric lymph nodes under steady state conditions; these cells are responsible for the induction of antigen specific Treg cells. Moreover these cells are also responsible for the induction of gut homing phenotypic changes, such as up regulation of CCR9 and α4β7 on T cells. Antigens can also be captured by the underlying immune cells via breaches in epithelial integrity (not shown) and by direct transport via the fetal Fc receptor (not shown).

1.10.2 Dendritic cells of the upper and lower airways

1.10.2.1 Organization of the immune system in the lungs

The lung can be divided into two compartments; these regions are distinct due to their anatomical and immunological differences. (1) The conducting airway is overlaid by mucosal tissue while (2) the lung parenchyma is specialized for gas exchange.

1.10.2.2 Conducting Airway

The conducting airway contains goblet and ciliate cells, which make up the “mucociliary escalator” (Langenback, Bergofsky et al. 1990; Langenback, Bergofsky et al. 1990). The secretion of mucus by goblet cells provides a physical barrier, which traps antigens that gain entry to the airway epithelium during respiration. The ciliated cells facilitate mucus migration to the epiglottis where it can be swallowed and neutralised in the stomach. Cells of the conducting airway also secrete IgA, which allows for the effective clearance of pathogens. This system is so efficient that >95% of all inhaled antigens will be removed within the conducting airway.

A dense network of DCs and macrophages are also found within the mucosa, which develops early in life. Although both myeloid DCs and plasmacytoid DCs are found in this region, myeloid DCs predominate. Another interesting immune cell, which is strategically positioned in the conducting airway for antigen capture is the airway mucosal dendritic cell (AMDC). Although AMDCs can take up antigenic material, they do not present antigen as efficiently as normal DCs found at other mucosal sites. AMDCs have also been shown to directly sample material from the lumen of the conducting airway by extending protrusions through the airway epithelium.

1.10.2.3 Lung Parenchyma

The parenchymal lung includes the bronchioles and the alveolar ducts that give rise to blind-ended alveolar sacs. This region is functionally important to the lung, as it is here that gas-exchange will take place. The alveoli are separated by a thin wall of interstitium, which contains pulmonary capillaries that are in close contact with the alveolar space. The immune cells that reside within the lung parenchyma, are found on both the alveolar epithelium located in the terminal airways and in the underlying parenchyma. Under normal steady-state conditions, the main leukocyte population within the alveolar space is the alveolar macrophage, in-fact the alveolar macrophage accounts for >90% of the total immune cell population in this region. The remaining 10% include “immature” DCs and T cells. Unlike the conducting airway no plasma cells are found within the lung parenchyma.

Following inhalation of airborne antigens, it has been postulated that the primary immune responses are initiated in the draining lymph nodes of the lung. Bice *et al* (1988) also believe that memory T cells will undergo homing and will localise to the lung tissue where they initiate a secondary immune response to their specific antigen (Bice and Muggenburg 1988).

T-cells within the lung parenchyma display cell surface markers, which would correlate with a memory /activated phenotype and Marathias *et al.*, (1991) revealed that the activation of these cells appears to be a relatively recent event (Marathias, Preffer *et al.* 1991). Such alterations in surface markers include increases in human lymphocyte antigen HLA-DR and a reduction in CD3 expression (in comparison to T cells derived from peripheral blood) (Becker, Harris *et al.* 1990; Nylén, Becker *et al.* 1990; Yamaguchi, Okazaki *et al.* 1990). Furthermore Holt *et al.*, (2008) have revealed the expression of the memory marker CD45RO on both CD4⁺ and CD8⁺ human T cells within the lung parenchyma (Wright 2005). Similar to other mucosal sites in the body such as the gut, the relative abundance of these CD4⁺ and CD8⁺ T cells differ depending

on their location. Abundant levels of CD4⁺ T cells are found within the lamina propria while high numbers of CD8⁺ T cells are confined to the intraepithelial region.

1.10.2.4 Immune induction in the lung: cellular dynamics.

Due to their biological function and anatomical positioning the respiratory mucosal surfaces, particularly the upper conducting airways, are constantly exposed to a wide range of non-pathogenic environmental antigens. Protection of these vital mucosal surfaces from the harmful effects of chronic inflammation is due in part to the local “default” immune response. This “default” immune response protects the integrity of this region via the induction of a low-level Th2 response (Stumbles, Upham et al. 2003). Moreover T cell mediated immunological tolerance has also been suggested (Holt, Reid et al. 1987; Wright 2005; Umetsu and Dekruyff 2006) similar to that found within the chronically stimulated gastrointestinal mucosa (Weiner, Friedman et al. 1994).

These mechanisms, which are underlying the protective “default”, are not yet fully understood. However evidence has suggested a role for DC subsets in controlling the local lung dynamics and the anti-inflammatory state (de Heer, Hammad et al. 2004) The lung alveolar macrophage (AM), which represents the other major antigen-presenting cell in the lung, also offers another level of control. They too can regulate memory T cell responses, which evade this protective Th2 default. AMs are of particular importance in the lung parenchyma where they provide the last line of defence against T cell mediated inflammation in the alveolar spaces (Lambrecht 2006).

Pattern recognition receptors (PRRs) such as Toll-like receptors are expressed by the AMDCs and AMs, which can circumvent the lungs default Th2 response. This results in the generation of a Th1 cell-mediated immune response, which is important for the clearance of pathogens via the generation of effector T cells (Wright 2005).

1.10.2.5 CD103⁺ DCs in the lung

Immature MHCII⁺ CD11c⁺ DCs are found distributed throughout the lung and are at the focal control point determining the induction of pulmonary immunity and tolerance (Akbari, DeKruyff et al. 2001). These DCs are strategically positioned to capture inhaled antigens and perform a unique sentinel function in the pulmonary immune response.

The lung is home to four major DC subsets CD11c^{high}CD11b^{low}MHCII^{high}CD207⁺ CD103⁺ DCs, CD11c^{int}CD11b^{high}MHCII^{high}CD103⁻DCs, CD103⁻MHCII^{neg-med}CD11^bhigh monocyte DCs, and a small subset of B220⁺Ly6C⁺MHCII^{low} pDCs (**Fig 1.17**)(Sung, Fu et al. 2006; del Rio, Rodriguez-Barbosa et al. 2007; Kim and Braciale 2009). These cells are subdivided based on their origin, anatomical location and function.

During steady-state conditions, the conducting airways are lined with an intraepithelial highly dendritic network of MHCII^{high}CD11c^{high} cells that are mostly CD11b⁻ and in the mouse express high levels of CD103, with a small subpopulation of CD103⁺ DCs extending their dendrites into the airway lumen through the tight junctions of the bronchial epithelial cells (von Garnier, Filgueira et al. 2005; Wikstrom and Stumbles 2007). Intercalation between CD103⁺ DCs and bronchial epithelial cells is facilitated by the expression of claudin-1, claudin-7 and ZO-2 on the DC cell surface (Sung, Fu et al. 2006). During steady state condition approximately 40 to 60% of the resident DCs express CD103 and belong to those lung-derived DCs that migrate to the bronchial LNs (brLN). Lung-derived DCs migrate in a CCR7-dependent manner and cross present to CD8⁺ T cells in the brLNs (Hintzen, Ohl et al. 2006). Kim et al (2009) have also revealed that these resident CD103⁺ DCs are mobilized and migrate in response to influenza viral infection, inducing the development of effector CD8⁺ T cells (Kim and Braciale 2009).

Immediately below the conducting airway lies the lamina propria which contains MHCII^{high}CD11c^{high} highly expressing CD11b and are a rich source of inflammatory chemokines such as CCL17 and CCL22 (van Rijt, Jung et al. 2005; Beaty, Rose et al. 2007).

Lung CD103⁺ DCs and CD11b⁺ DCs express distinct toll-like, cytokine and chemokines receptor profiles during the steady states (Sung, Fu et al. 2006; Beaty, Rose et al. 2007). High levels of secreted cytokines and cytokine specific mRNA have been observed in the CD11b⁺ lung DC subset during the steady state, meanwhile the CD103⁺ subset produce very low levels of chemokines, with the exception of CCL22. CCL22 is an important chemokine in the recruitment of Th2 and regulatory T cells during steady state conditions (Beaty, Rose et al. 2007). This is consistent with the premise that during steady state conditions the lung is a predominant Th2 polarizing environment.

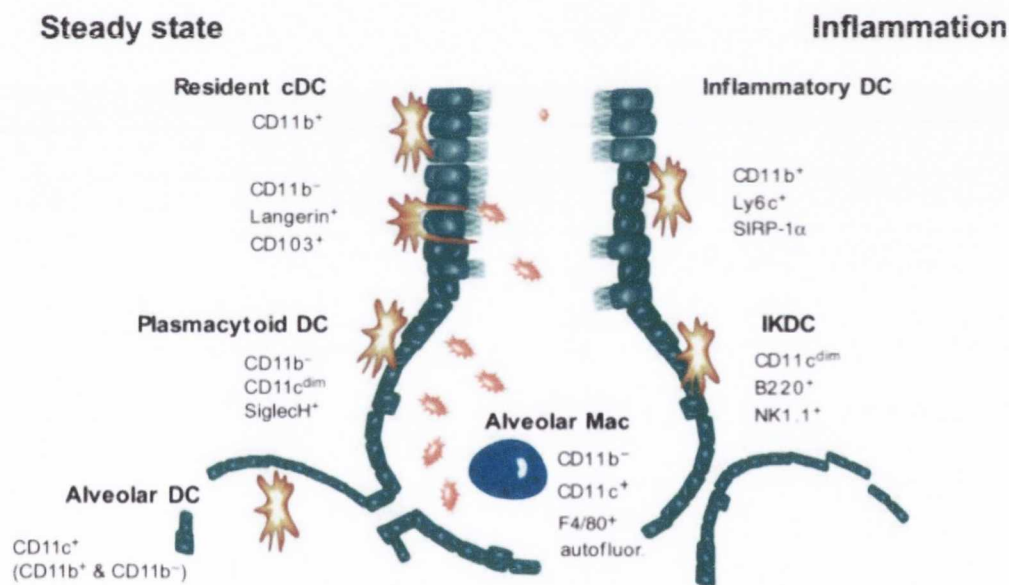


Figure 1.17: Dendritic cell subsets in the lung under steady state and inflammatory conditions. (Lambrecht and Hammad 2010).

Del Rio *et al* (2007) revealed that lung CD103⁺ DCs have a lower capacity to capture soluble antigens compared with lung CD11b⁺ DCs (del Rio, Rodriguez-Barbosa et al. 2007). Although lung CD103⁺ DCs have a lowered capability to phagocytose antigens,

they are much more efficient at presenting antigens to OT-I and OT-II cells, whereas CD11b⁺ DCs presented antigens to OT-II cells but not OT-I cells (del Rio, Rodriguez-Barbosa et al. 2007). From a developmental stand point CD103⁺ DCs are related to the CD8 α ⁺ DC family known for their cross presentation abilities. CD103⁺ express high levels of CLEC9A, which has been shown to be required for the cross-presentation of necrotic cell-associated antigens to CD8⁺ T cells (Sancho, Joffre et al. 2009). CLEC9A has also been shown to be preferentially expressed on the surface of CD8 α ⁺ DCs and not CD4⁺ DCs.

During lung infection with influenza or HSV virus, the CD103⁺ DC population actively migrated to the lung-draining LNs and were more efficient at priming CD8⁺ T cells, but not memory CD8⁺ T cells (Belz, Smith et al. 2004), whereas both populations were effective at inducing antigen specific CD4⁺ T cells (**Fig 1.18**) (Kim and Braciale 2009).

The utilization of transgenic mice expressing the DTR under the mouse Langerin promoter has revealed that following Langerin depletion the lung CD103⁺ population was eliminated while the CD11b⁺ population remained unaffected (GeurtsvanKessel, Willart et al. 2008). The severity of infection with influenza virus was increased following the addition of DT, compared to control mice (GeurtsvanKessel, Willart et al. 2008). However Merad and colleagues have revealed that LN CD8 α ⁺ DCs also express Langerin, therefore the exact role of CD103⁺ DCs during viral infection *in vivo* remains to be fully determined.

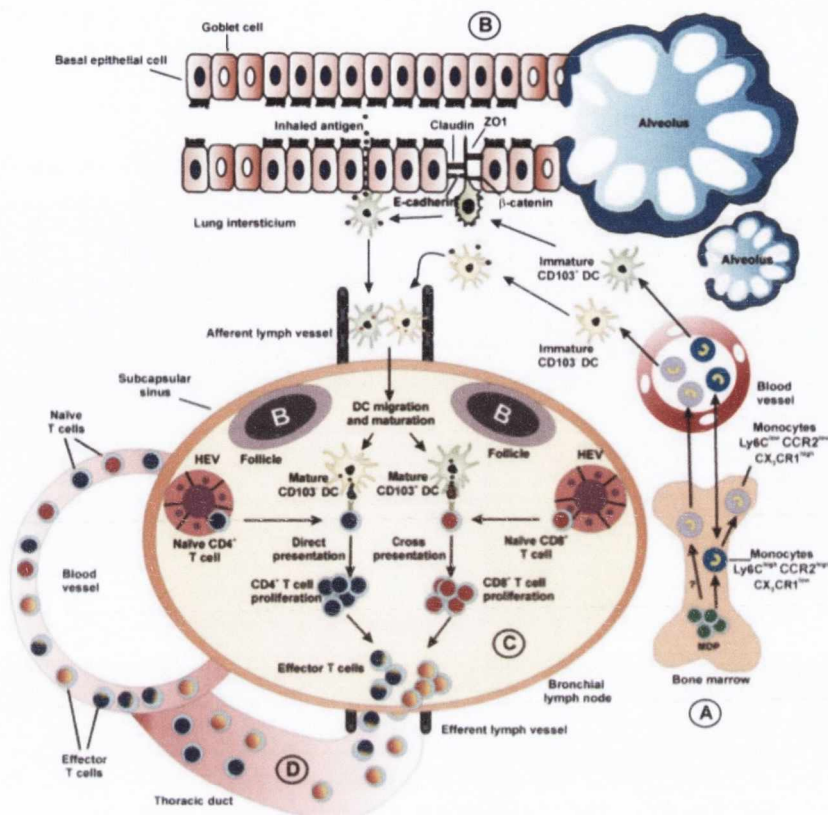


Fig. 1.18. Development and functional specialization of lung-migrating CD103⁺ dendritic cells (DCs) at cross-presenting innocuous inhaled antigen in the lung-draining lymph node.

The binding of CD103 integrin to its ligand E-cadherin, which is expressed on the basolateral side of epithelial cells, allows CD103⁺ DCs attach to lung epithelium, also causing clustering β -catenin to the cell surface. Subsequently, lung CD103⁺ DCs expressing claudin and zonula occudens (ZO1) may establish tight junctions with epithelial cells. Lung CD103⁺ DCs can sample antigens from the airway lumen extending their dendrites between epithelial cells, whereas CD103⁻ DCs are located in the submucosa of the conducting airways. Following uptake of antigens, DCs migrate to the bronchial lymph node (brLN) presenting processed antigen to $CD4^{+}$ and $CD8^{+}$ naive T cells in the T zone (light yellow; B-cell follicle: purple). Naive T cells enter the brLN via high endothelial venules. Lung-derived CD103⁺ DCs preferentially cross- present innocuous antigen to $CD8^{+}$ T cells, whereas CD103⁻ DCs are specialized at presenting antigen to $CD4^{+}$ T cells (C). Once antigen is presented or cross-presented by CD103⁻ and CD103⁺ DCs to $CD4^{+}$ and $CD8^{+}$ T cells, respectively, effector T cells leave the brLN through efferent lymph vessels and enter circulation (D). (del Rio, Bernhardt et al. 2010)

A successful mucosal vaccine should therefore be able to penetrate the mucosal barrier at the right mucosal district in a “controlled manner and induce both innate and adaptive immune responses, such as the activation of resident DCs, macrophages and epithelial cells of the innate immune system, as well as antigen-specific effector and memory T and B cells of the adaptive immune system, which cooperate with one another to achieve optimal potency and duration of protection”(Chen and Cerutti 2010)

1.11 Mucosal vaccination

1.11.1 Intranasal Vaccination

Currently, most commonly used vaccines are administered by needle principally via intramuscular vaccination. However in the developed world adults and children have an aversion to injections. Therefore the development of new vaccines, which do not require needles, may be advantageous in increasing vaccine compliance rates. The role of needles and syringes in immunization practice is much more problematic in developing countries where major efforts are under way to increase immunization coverage and to introduce new vaccines. Except for the long-standing use of the oral polio vaccine and the new rotavirus vaccine used in some countries, all other vaccines recommended by the expanded program on immunization (EPI) are administered using needles. However, in many developing countries, injection safety is notoriously unsatisfactory (Aylward, Lloyd et al. 1995; Simonsen, Kane et al. 1999).

Needle free vaccination strategies are becoming increasingly popular for many reasons. 1) They are easy to administer, not requiring medically trained personnel, 2) Depending on the route of administration they are much more effective at inducing local mucosal immune responses. Moreover in many developing countries injection safety is not satisfactory and as a result improper practices using non-sterile needles and syringes can lead to the transmission of hepatitis B, C and HIV (Simonsen, Kane et al. 1999).

Mucosal immunization has been reported to induce both mucosal and systemic immune responses unlike conventional injections (Jabbal-Gill 2010). Mucosal vaccination can be achieved via a number of routes including oral, rectal, pulmonary, vaginal, or intranasal. Despite multiple approaches to induce robust mucosal immune responses, not all are equally practical. For example, although highly efficient at eliciting immune responses in adults, rectal administration faces many cultural obstacles and is unpopular in many countries (Katz, Kempe et al. 1960), furthermore defecation by infants shortly after rectal delivery might also pose a problem and reduce vaccine immunogenicity (Forrest, Shearman et al. 1990)

The nose offers an excellent route of vaccine administration and is more advantageous in terms of lower antigen doses required and the lack of exposure to extreme pH or digestive enzymes (Jabbal-Gill 2010). The nasal cavity has a large surface area with local draining lymph nodes lying under the respiratory epithelium. Moreover due to the highly vascularized nature of the surrounding microenvironment, antigens can easily enter the circulation actively avoiding first pass metabolism. Nasal delivery could therefore potentially provide a more acceptable, safe, and noninvasive needle free option for vaccination.

In order for a vaccine to be effective following intranasal delivery, the formulation should “ensure stability of the antigen, reside long enough time for the antigen to interact with the lymphatic system, stimulate both the innate and cellular systems with or without the use of safe efficacious adjuvants, by targeting specific parts (such as DCs) of the immune cells, and provide long-term immunity against the pathogen” (Jabbal-Gill 2010).

In recent years, considerable advances have been made in the field of intranasal vaccination, including the development of a live-cold adapted trivalent influenza vaccine (FluMist). FluMist is administered using a single-use spray device that delivers large-droplet aerosol vaccine into the nasal mucosa. This intranasal vaccine has been officially licensed by the US FDA (King, Stoddard et al. 2006) and has proved effective in post-licensure assessments.

1.12 Hypothesis: Dendritic cells at non-GI sites have the capacity to induce gut homing.

1.13 Aims:

Aim 1: Determine if non-GALT associated DCs induce the expression of gut-homing markers on T cells.

Aim 2: Investigate if externally primed T cells traffic to the gastrointestinal tract.

Aim 3: Study the physiological relevance of such immune trafficking

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Tissue culture reagent

2.1.1.1 Ammonium Chloride Red Blood Cell Lysis Solution

0.77g Ammonium Chloride (NH₄CL) dissolved in 100ml dH₂O and filter sterilized

2.1.1.2 Cell Culture Medium

Rosewell Park Memorial Institute (RPMI)-1640 medium (Biosera) was supplemented with 8% heat inactivated (56°C for 30 mins) foetal calf serum (FCS, Biosera), 100mM L-Glutamine (Gibco) and 100µg/ml penicillin streptomycin (Gibco). Complete RPMI (cRPMI) was used to culture all cells *in vitro*.

Note: See Appendix I for details on all other reagents and materials used.

2.2 Methods

2.2.1 Animals.

Pathogen free female BALB/c and C57BL/6 mice were purchased from Jackson Labs and Taconic Farms (NJ, USA). All mice were housed and maintained at the comparative bioscience center (CBC) at the Rockefeller University. All animals were maintained according to the regulations and guidelines of the National Institute of Health and the guidelines of the European Union. All experiments were conducted under university ethical approval and under licence from the National Institute of Health. Mice were 6 to 10 weeks old at the initiation of each experiment. C57BL/6 mice (B6), C57BL/6-Tg(TcraTcrb)425Cbn/J (OTII), C57BL/6-Tg(Tcra Tcrb)1100MjbJ (OTI) were purchased from Taconic Labs or bred at Rockefeller University. CD11c-DTR (B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J) mice, 6–8 wk old, were purchased from The Jackson Laboratory. TGF-βR^{fl/fl} OTII mice and the dnRAR-OTII mice were generated in the Mucida Laboratory (Rockefeller University). Briefly, CD4^{CRE} mice were crossed with

with TGF- β R^{fl/fl} mice to specifically deplete TGF- β R from CD4 T cells. To avoid autoimmune disease and premature death in CD4 TGF- β R^{fl/fl} mice, these animals were back-crossed to OTII Rag^{-/-} animals to obtain TGF- β R^{def} OTII mice. To analyze RA signaling in CD4T cells, CD4^{CRE} mice were crossed with dnRAR^{fl/fl} mice^{16,17} to specifically express the dominant negative form of RAR in CD4⁺T cells. To address the importance of RA for mucosal T-cell migration after specific T-cell activation, these animals were back crossed to an OTII Rag^{-/-} background. MDA-5^{-/-}/TLR3^{-/-} mice¹⁸ were kindly provided by M. Colonna (Washington University, St. Louis, MO).

Bone marrow chimeras were created by transferring bone marrow from CD11c-DTR mice into lethally irradiated WT C57BL/6 mice to avoid lethality associated with DT treatments of CD11c-DTR mice^{12,13}. Briefly, C57BL/6 hosts underwent two treatments of 500 rads in an animal γ -irradiator, and 2.5×10^5 bone marrow cells from a CD11c-DTR donor were transferred i.v. The resulting mice (referred to herein as CD11c-DTR) were housed under immunocompromised mouse conditions and treated regularly with 2 mg/ml neomycin from Sigma-Aldrich (St. Louis, MO) in their drinking water. The CD11c-DTR were fully reconstituted and ready for experimental use after 6 wk.

2.2.2 Cell counts.

Cell counting was performed by diluting 10 μ l of cells in 10 μ l of Ethidium Bromide-Acridine Orange (EBAO). A 10 μ l volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). The number of viable cells (green) and dead cells (orange) were then counted using a fluorescent microscope. The number of cells per ml was calculated according to the manufactures protocol by multiplying the cell number in 9 squares, within 1 large square, by 10^4 , by the dilution factor.

$$\text{Number of cells/ml} = \text{cell count} \times \text{dilution factor} \times 10^4$$

2.2.3. Cell Culture

Cells were cultured at 37°C with an atmosphere maintained at 95% humidity and 5% Carbon Dioxide.

2.2.4 Isolation of lamina propria immune cells.

C57BL/6 mice were purchased from Taconic Farms, maintained under specific pathogen-free conditions and used at 5 – 7 weeks with institutional animal care and use guidelines. All animals were given food and water *ab libitum*. Mice were euthanized by carbon dioxide, using established, IACUC approved protocols. A small subcutaneous incision on the ventral side of the animal was performed and the abdominal cavity opened exposing the intestines. The entire small intestine was removed and placed in CMF in a petri dish. The small intestines were initially removed by severing their connection point to the stomach (on the distal side of the ligature, past the pyloric sphincter). As the small intestine is being extracted all visible mesentery was removed. The removal of mesentery and local adipose tissue deposits are essential for improved cell viability. The intestine was removed as individual small pieces until the caecum was reached. Once the entire small intestine has been removed it was placed in CMF for no longer than 15 minutes.

The intestine was positioned longitudinally and the Peyer's patches identified, these appear as discrete raised structures on the anti-mesenteric border. The Peyer's patches and mesentery were completely removed. The intestines are opened by making a longitudinal incision and rinsed thoroughly with CMF. All faecal material was removed during this stage. The intestines (now open) were placed back into CMF and gently washed for 3 minutes using a cell shaker. This procedure was repeated three times, to completely remove faecal material. The tissue was transferred to a clean petri dish and chopped into fine pieces using a scissors.

The intestine was then placed into a 50ml falcon tube with ~15ml of CMF. The tissue was once again washed three times by inverting the tube and discarding the supernatant.

Once the supernatant has been removed, 25mls of EDTA/PBS (130 μ l of 0.5M EDTA/50mls of PBS) was added. The lamina propria cell suspension was incubated on a cell shaker for 60 minutes at 37°C.

Upon completion of the EDTA/PBS incubation, the cell suspension was vortexed at high speed for fifteen seconds. The tissue was allowed to settle before removing the supernatant. The tissue was then washed with 20 – 25mls of RPMI three times. For the first of these washes, the cell suspension was simply allowed to settle, on subsequent washes a centrifuge was used for fifteen – twenty seconds at 1200rpm. 10 – 15mls of collagenase (0.5mg/ml) solution was added and incubated on a rocker for 60 minutes at 37°C.

Using a 10ml syringe and 18G needle, the cell suspension was aspirated ten - fifteen times to disrupt the remaining large fragments. The contents were aspirated and filtered through a cell strainer. Using RPMI the syringe and falcon tube were cleaned to remove all remaining cell fragments. The now filtered cell suspension was centrifuged at 1500 rpm for 8 minutes, the supernatant was subsequently discarded and pellet washed in 15mls of RPMI.

1ml of 44% percoll was added to reconstitute the pellet and transferred to a 15ml tube, which had been pre-coated with FCS. Another 2-3mls of 44% percoll was added to the same 50 ml falcon tube to remove all the remaining cells and transferred to the 15ml tube. The final volume of 44% percoll was 8mls. The 44% percoll:cell suspension was under laid using 66% percoll. Taking a glass Pasteur pipette (without cotton plug) 2mls of 80% percoll was under laid. The 44%/80% percoll solution was centrifuged at 2800rpm for 20 minutes with the breaks off.

Following centrifugation the cellular interface was removed and added to a 15ml falcon tube and washed twice in 10ml of RPMI. The lamina propria cells were again centrifuge at 1500 rpm for an additional 15 minutes. The supernatant was discarded and pellet re-

suspended in 3ml of RPMI. The cells were counted with a hemocytometer as described on 2.2.2. Yield is typically $2-6 \times 10^6$ /mouse.

2.2.6 Isolation of Vaginal cells

Mice were euthanized by carbon dioxide and the vaginal T cell isolation performed. A small subcutaneous incision on the ventral side of the animal was performed and the abdominal cavity opened exposing the intestines and vagina. The entire small intestine was removed and discarded. The reproductive tract was initially removed by severing its connection points with the abdomen. The entire female reproductive tract was removed and all visible mesentery removed. The removal of mesentery and local adipose tissue deposits are essential for improved cell viability. Once the reproductive tract had been removed it was placed in CMF for no longer than 15 minutes. The vagina was transferred to a clean petri dish and chopped into fine pieces using a scissors.

The vagina was then placed into a 50ml falcon tube with ~15ml of CMF. The tissue was once again washed three times by inverting the tube and then discarding the supernatant. Once the supernatant has been removed, 25mls of EDTA/PBS (130 μ l of 0.5M EDTA/50mls of PBS) was added. The vaginal cell suspension was incubated on a cell shaker for 60 minutes at 37°C.

Upon completion of the EDTA/PBS incubation, the cell suspension was vortexed at high speed for fifteen seconds. The tissue was then allowed to settle before removing the supernatant. The tissue was then washed with 20 – 25mls of RPMI three times. For the first of these washes, the cell suspension was simply allowed to settle, on subsequent washes a centrifuge was used for fifteen – twenty seconds at 1200rpm. 10 – 15mls of collagenase (0.5mg/ml) solution is added and incubated on a rocker for 60 minutes at 37°C.

Using a 10ml syringe and 18G needle, the cell suspension was aspirated ten - fifteen times to disrupt the remaining large fragments. The contents were aspirated and filtered through a cell strainer. Using RPMI the syringe and falcon tube are cleaned to remove all

remaining cell fragments. The now filtered cell suspension was centrifuged at 1500 rpm for eight minutes, the supernatant was subsequently discarded and pellet washed in 15mls of RPMI, before three more additional centrifugation steps.

1ml of 44% percoll was added to reconstitute the pellet and transferred to a 15ml tube, which had been pre-coated with FCS. Another 2-3mls of 44% percoll was added to the same 50 ml falcon tube to remove all the remaining cells and transferred to the 15ml tube. The final volume of 44% percoll was 8mls. The 44% percoll:cell suspension was under laid using 66% percoll. Taking a glass Pasteur pipette (without cotton plug) 5mls of 66% percoll was under laid. The 44%/66% percoll solution was centrifuged at 2800rpm for 20 minutes with the breaks off.

Following centrifugation the cellular interface was removed and added to a 15ml falcon tube and washed twice in 10ml of RPMI. The vaginal cells were again centrifuge at 1500 rpm for an additional 15 minutes. The supernatant was discarded and pellet re-suspended in 3ml of RPMI. The cells were counted with a hemocytometer as described on 2.2.2.

2.2.7 Isolation of Splenic T cells.

Mice were sacrificed by carbon dioxide and the whole spleen removed by dissection under sterile conditions. Isolated organs were transferred to a sterile petri dish, suspended in complete RPMI (see Materials 2.1). Unless stated otherwise, all cell cultures were performed with sterile complete RPMI. Splenocytes were obtained by disruption and homogenisation of the dissected organ, and passed through a 70mM cell strainer. Cell suspensions were spun down by centrifugation (1300 rpm for 5 minutes) and supernatants were discarded. Erythrocytes were lysed by resuspension of the pellet in 1ml of freshly prepared lysis buffer (see Material 2.1) and incubated on ice for 5 minutes. Lysis was terminated by the addition of 15ml RPMI. Cells were spun down again at 1300rpm for 5 minutes and resuspended in an appropriate volume of medium for counting. An aliquot by taken for cell counting using Trypan Blue (20ml cells in 180ml Trypan blue). The suspension was readjusted to the appropriate viable number of cells per ml.

2.2.8 Cell Sorting

In order to isolate lung and mediastinal CD45⁺MHCII^{high}CD11c^{high} > CD103⁺CD11b⁻, CD103⁻CD11b⁺, CD103⁻CD11b⁻ DC subsets, the cells were isolated according to specific protocols and centrifuged at 1600rpm for five minutes to generate a pellet. The cell pellet was resuspended at a concentration of 50*10⁶ cells/ml in cRPMI. The cells were subsequently stained with anti-mouse CD45 – PerCP (eBioscience), MHC Class II – A700 (eBioscience), CD11c – PeCy7 (eBioscience), CD103 – PE (eBioscience) and CD11b – PB (eBioscience). The cells were subsequently sorted (see gating attached) using a BD FACSARIATM IIa, Special order system (kindly donated by the Howard Hughes Medical Institute).

2.2.9 Flow cytometry analysis for T cell and DC surface markers

Cells were isolated as described above. Prior to staining, cells were washed and resuspended in staining buffer containing 1x PBS, 2% BSA, 10 mM EDTA and 0.01%NaN₃. To block non-specific staining, the 2.4G2 anti-CD16/32 antibody was added. Antibodies for cell surface markers were added and cells were incubated for 25 min at 4°C. Following the staining, the cells were washed twice and analyzed real time or fixed in PBS containing 1% paraformaldehyde and 0.01% NaN₃ and analyzed later on an LSR II (Becton Dickinson) using multiparameter flow cytometry.

For intracellular cytokine staining, post surface staining, cells were resuspended in Fixation/Permeabilization solution (BD Cytofix/Cytoperm kit-BD PharMingen, USA), and intracellular cytokine staining was performed according to the manufacturer's protocol. Flow cytometric data was analyzed with FlowJo software (TreeStar). For analysis of the adoptively transferred OT-I and OT-II populations, the transferred cells were identified by forward and side scatter characteristics, dead cells were excluded using Live-dead fixable Aqua (Invitrogen) followed by sequential gating on CD45.1⁺, CD3⁺, CD4⁺Va2⁺ double positive populations followed by the expression of αβ7 on CFSE^{lo} cells.

2.2.10 *In vivo* diphtheria toxin treatment

Diphtheria toxin (DT) purchased from Sigma–Aldrich was prepared in a sterile solution of PBS at a concentration of 1 mg/ml. Transient DC depletion was effected in CD11c-DTR chimeras by a single i.p. injection of 200 ng DT.

2.2.11 ELISA Protocol - Enzyme Linked Immunosorbent Assay.

An important immunological technique used was Enzyme Linked Immunosorbent Assay (ELISA). ELISA is a procedure used to detect small amounts of specific proteins. The assay is based on the binding of antigens to antibodies to form specific antigen-antibody complexes. These complexes can subsequently be detected by the catalytic action of an enzyme chemically linked to the antibody. The activity of the enzyme is used to determine the level of the substrate present and therefore the amount of antibody present.

There are six main steps in the production of an ELISA. (1) Coating, (2) Blocking, (3) Addition of standards and samples to plates, (4) Addition of secondary antibody, (5) Addition of enzyme conjugate and (6) Developing.

96 well flat bottom plates were coated with capture antibody and incubated overnight at 4°C. Following the incubation the plates were washed with PBS-Tween and then blocked for two hours using blocking solution at room temperature. Samples were then added to the plates and left overnight at 4°C. 50µl of each respective detection antibody was added and the plates were left to incubate for two hours. 50µl of Horse- radish peroxidase (HRP) conjugated to streptavidin was added to the plates and incubated at room temperature for half an hour. Following a wash with PBS-Tween the plates were coated with OPD substrate. This resulted in a colour reaction that was allowed to develop for 10 minutes. Before the plates were read the reaction was stopped using H₂SO₄. The plates

were read at an absorbance of 492nm using an ELISA plate reader and the results were processed using Softmax Pro software.

2.2.12 Western Blotting

A western blot is a method used to detect a specific protein in a given sample. The first process in the development of a western blot is gel electrophoresis. Gel electrophoresis is used to separate out proteins depending on size.

15% SDS-PAGE gels were used and they were produced using the method attached (see appendix I). Once produced N,N,N,N tetramethylethylenediamine (TEMED) was added to the gel solution and the gel was poured into the plates. The 15% running gel was allowed to set for 20 minutes. 5% Stacking gel was placed on top of the running gel. A 1X-running buffer with a pH of 8.3 was added to the electrophoresis tank.

Following electrophoresis the proteins were transferred to a nitrocellulose membrane using a semi-dry transfer for 1 hour and 30 minutes at 30mA.

When the semi-dry transfer was complete the nitrocellulose membrane was removed from the gel and placed in a blocking solution for 1 hour. After the blocking period, the blocking agent was removed and the nitrocellulose was washed for 30 minutes in PBS-Tween. The nitrocellulose membrane was washed for six five-minute periods. The nitrocellulose membrane was incubated with the primary antibody in a 3% Marvel solution overnight at 4°C.

Post primary incubation the blot was washed in PBS-Tween. Then the nitrocellulose was incubated with the secondary antibody for one hour. When this incubation was complete the nitrocellulose was washed with PBS-Tween for 30 minutes. Following the wash the detection agent was added. This detection agent was left to incubate for 2 minutes. The nitrocellulose was then developed using developing film.

2.2.13 DEC-205 Fusion mAb production

➤ Isolation of plasmid DNA from transformed E.Coli

Transformed *E.Coli* was grown on LB medium. 250ml of the transformed *E.Coli* was pelleted in a large V-bottomed flask. 10mls of resuspension buffer (R3) containing RNase A was used to resuspend the cell pellet and mixed until the solution was homogenous.

10mls of Lysis buffer (L7) was added to the solution and mixed by inverting the cells five times before a five-minute incubation at room temperature. After which 10mls of precipitation buffer (N3) was added and mixed to generate a homogenous mixture. The cells and solutions were subsequently centrifuged for 30 minutes at 13,000rpm at room temperature. The supernatant was removed and placed onto an equilibrated column; column equilibration was achieved by placing 30mls of equilibration buffer onto a HiPure

Maxi Column. The column was washed once with 60mls of wash buffer and the solution allowed to elute by gravity. 15mls of elution buffer (E4) was added to the column to elute the DNA. 10.5mls of isopropanol was added to the elution tube to precipitate the plasmid DNA.

The elute was centrifuged at 15,000rpm for 30 minutes at 4°C, and the supernatant discarded. 5mls of 70% ethanol was added to the pellet and centrifuged for a further 30 minutes at 4000rpm. The supernatant was removed and the pellet was allowed to air-dry for 10 minutes. The pellet was dissolved in 200-500µl of LPS free water and the plasmid DNA stored at -20°C.

➤ Calcium phosphate transfection

8x10⁶ 293 T cells were split into 15-cm tissue culture flasks the day prior to the transfection. 30ug of heavy chain DNA, 30ug of light chain DNA, 5µg of helper DNA, and 10µl of 100mM Chloroquine were placed into 2.7ml of HyClone endotoxin free

water and 300ul of 2.5M CaCl_2 added. An additional 3mls of 2X HBS was added to the solution and the solution vortexed. The solution was allowed to precipitate at room temperature for 15 minutes.

Following the formation of precipitate the calcium phosphate-DNA solution was added to the tissue culture flasks containing the 293 T cells. 6mls of calcium phosphate-DNA solution was used for each 15-cm dish (20mls of sterile medium was also added). The cells were incubated for 6 hrs in this calcium phosphate-DNA solution-medium solution at 37°C. After this period the medium was removed and the cells washed with 10ml of sterile PBS. The cells were suspended in 40ml of DMEM containing Nystatin and the cell supernatant harvested 4 to 5 days after transfection.

➤ **Harvesting supernatant containing DEC205 fusion Ab.**

After 5 days the supernatant was removed from the 293 T cells and placed in large V bottom flasks for centrifugation. Eight cell culture flasks were incorporated into each V bottom flask. The supernatant was centrifuged at 2500rpm for 25 minutes the cell pellet was transferred through a filter to remove fragments. 60% volume of ammonium sulphate was added to the beaker containing the filtered cell supernatant and allowed to dissolve overnight at 4°C.

Following the overnight incubation the solution was centrifuged at 3500rpm for 30 minutes at 4°C, with the brakes released. The supernatant was removed and discarded. The pellet was resuspended in PBS and transferred to a 50ml beaker. Equal volumes of saturated ammonium sulphate solution were added and the solution was allowed to homogenise for 30 minutes at 4°C before being centrifuged again at 3500rpm for 20 minutes. The supernatant was removed and stored and the pellet was resuspended in exactly 2.5ml of PBS. A PD-10 column was washed with 25mls of PBS and the 2.5ml sample allowed to elute by gravity. An additional 3.5ml of PBS was added and the elution collected. This solution was then stored at RT for analysis. 20ul of the solution was removed for SDS-pages analysis and western blotting. Moreover additional LPS tests were conducted to confirm the DEC-205 fusion Ab was free of LPS.

2.2.14 Antibody ELISA

IgA

Fecal pellet and mucosal antigen-specific antibodies were analysed using commercially available antibodies (Pharmingen). Medium binding plates (Thermo Scientific) were coated (50µl/well) with 50 micrograms of CTB or antigen of interest in carbonated buffer overnight at 4°C. Plates were then washed in PBS-Tween before addition the blocking agent. 100µl of 3% BSA in PBS was added to each well, the plates were included at room temperature for two hours. Plates were washed three times with PBS-Tween before the addition of mucosal samples. 100µl of diluted fecal pellet/mucosal wash were added to the plate and incubated at 4°C over night. After sample incubation, plates were washed and 50µl anti-mouse (BD Pharmingen) at a concentration of 0.5µg/ml in PBS added for 1 hour and incubated at 37C.

Plates were again washed and 50µl of extravidin-peroxidase dilutes in PBS (Sigma) added to the each well for 20 minutes at room temperature. Finally the plates were washed and 50 µl of OPD substrate in phosphate citrate buffer (0.4mg/ml) was added. The enzyme reaction was quenched by adding 25µl of 1M H₂SO₄.

The OD values were determined by measuring absorbance at 492nm using a microtitre plate reader. Antibody concentrations are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal samples levels to a cut off point of two standard deviations.

2.2.15 CFSE labelling OT-II T cells

Mice were sacrificed by carbon dioxide and the whole spleen removed by dissection under sterile conditions. Isolated organs were transferred to a sterile petri dish, suspended in cRPMI (see Materials 2.1). Unless stated otherwise, all cell cultures were performed with sterile cRPMI. 4.5ml of RPMI. Following red blood cell lysis, the splenic cellular suspension was placed into hybridoma supernatant containing MGCII, B cell, F4/80 and

MHCII for one hour on ice. Following incubation, magnetic beads were added to the cells containing the hydridoma supernatant and incubated at 4°C for 45 minutes on a cell shaker. In order to separate the cells, the cellular suspension was placed on a magnet and the cell supernatant extracted and washed in cRPMI. This supernatant contains the CD4 T cells. For CFSE labelling, the cells were washed one time in 10ml of PBS and resuspended in PBS containing 0.1% BSA. 5mM of CFSE was added to the cells and incubated at exactly 37 for 10 minutes. After 10 minutes the reaction was quenched by adding cRMPI containing FBS. The cells were centrifuged at 1600rpm for 7 minutes and resuspended in 10ml of cRPMI for counting.

2.2.16 Isolation of Dendritic cells

Single cell suspensions were isolated from the murine lung, spleen, mesenteric, mediastinal and skin draining lymph nodes as described above. CD11c⁺ cells were isolated as previously described using CD11c magnetic beads (Miltenyi). Briefly, the cells were washed in 1ml of MACS buffer (Miltenyi), prior to 30 minutes incubation on ice with CD11c⁺ beads. The cellular suspensions were washed twice in MACS buffer and passed through a magnetic column, CD11c⁺ cells were isolated by positive selection, washed, resuspended in cRPMI and counted prior to co-culture with T cells as described below.

Lung DC subset isolation: Lung mononuclear cells were isolated as described above. Using magnetic beads, CD11c⁺ cells were enriched and sorted on FACS Aria (BD biosciences) as B220⁻MHC II⁺CD11c^{hi}CD103⁺CD11b⁻ and B220⁻MHC II⁺CD11c^{hi}CD103⁻CD11b⁺ subsets.

2.2.17 Detection of aldehyde dehydrogenase

ALDEFLUORTM (Aldagen) was used to identify, evaluate and isolate CD103⁺CD11b⁻, CD103⁻CD11b⁺, CD103⁻CD11b⁻ DC populations that expressed high levels of aldehyde dehydrogenase (ALDH). The fluorescent ALDEFLUOR reagent freely defuses into cells and is a non-toxic substrate for ALDH. The amount of fluorescent ALDH reaction

product that accumulates in cells directly correlates to the ALDH activity. Following cell isolation, one sample was labelled as “test” and the other “control”. 5ul of DEAD solution was added to the “control” tube. The tube was recapped and vortexed. The DEAD solution was provided in 95% ethanol. 5ul of activated

ALDEFLUOR substrate was added per millilitre of samples to the tube labelled “test”. This tube was also recapped and vortexed. After 3 minutes the active ALDEFLUOR substrate was added to the “control” tube and the samples placed at 37C for 30 minutes. After incubation the cells were surface stained and analysed. Using this protocol the cells cannot be fixed prior to analysis.

2.2.18 Histology

The histology and immunohistology work in this project was carried out in collaboration with Dr. Hung Fa (Clinical Pathologist) at Mt. Sinai Hospital. The experimental set up and section isolation was carried out at the Rockefeller University while the embedding, sectioning and staining was carried out at Mt. Sinai under supervision.

For the *in vivo* histological samples (Salmonella challenge experiments), various intestinal, liver and spleen samples were removed and preserved in 4% Formalin (Sigma) for further histological examination. Preserved samples were then trimmed into the appropriate size and shape and placed in embedding cassettes. The cassettes were then paraffin embedded using the following protocol.

1. 70% Ethanol, two changes, 1 hr each
2. 80% Ethanol, two changes, 1 hr each
3. 95% Ethanol, two changes, 1 hr each
4. 100% Ethanol, two changes, 1 hr each
5. Xylene, three changes, 1 hr each
6. Paraffin wax (56C – 58C), two changes, 1hr 30 min each
7. Embedding tissues into paraffin blocks (Histocentre 3 Thermoshandon)

Paraffin blocks were then trimmed and cut into sections of 3µm (Leica RM2135 Microtome). The paraffin ribbon obtained was placed into a water bath (40C) and mounted onto a glass slide. The slides were allowed to air dry for 2 hrs. The paraffin was then removed from the sections by reversing the schedule outlines above with the addition of a rinse in distilled water to finish. Sections were then ready for staining and immunohistochemistry. Slides were then stained with haemotoxylin and eosin (Thermo).

2.2.19 *In vitro* DC: T cell cocultures.

The culture medium used for DC-T cell cultures was RPMI (Gibco, USA) supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 mM β-mercaptoethanol (all Sigma, USA). Allogeneic T cells were isolated from spleens of C57BL/6 mice by excluding B220⁺, F4/80⁺, CD49b⁺, and I-Ab⁺ cells with anti-rat IgG Dynalbeads (Invitrogen). T cells were added to round-bottom microtest wells at 1×10^5 /well and mixed with isolated DCs at a DC:T cell ratio of 1:2 and 1:5 and 1:10 in the presence of OVA (25µg/ml final). After 5 days, the expression of α4β7 on proliferating T cells was evaluated by FACS. For the retinoic acid inhibition assays, the DC:T co-cultures were incubated with 1µM of retinoid acid receptor antagonist LE540 (Wako, Japan, dissolved in DMSO at a stock concentration of 1 mM) and added to cultures at final concentration of 1 µM

2.2.20 *Salmonella* Challenge:

Recipients of adoptively transferred OT-II cells, mice immunized i.n. or s.c. with OVA and mice immunized with heat inactivated Paraformaldehyde fixed *Salmonella* (1×10^6 CFU) were inoculated orally by gavage with wild-type *Salmonella* -OVA (1×10^6 CFU/Animal). Mice were monitored with daily weights. Stool culture was performed by culturing pre-weighed stool pellets on days 2, 4, 8, 14 and 40 post challenge (day 40 stool cultures were performed in the long term survivors to detect for persistence of *Salmonella* in the stool) and expressed as *Salmonella* CFU/gm of stool tissue. Blood cultures were

performed by obtaining blood in a heparinized syringe by submandibular phlebotomy on days 3, 5 and 8 post challenge. Kaplan Meier curves were used to determine the frequency of survival among the challenged mice

2.2.21 Immunization:

CFSE-labeled OT-I or OT-II were adoptively transferred into syngeneic age, gender matched wild-type mice and controls. Two hours later, the recipient mice were immunized i.n. or s.c. with 100 µg OVA (LPS-free, Seikagaku Corp.) and 50µg of poly ICLC (Oncovir, Inc) using isoflurane as short acting inhalant anesthetic. All animals were given food and water *ad libitum*.

2.2.22 Adoptive Cell transfer:

OT-I or OT-II mice were used as cell donors for adoptive transfer into syngeneic recipient animals. Lymphocytes were isolated from the spleen and labeled with 5 µM CFSE (Invitrogen) for 10 minutes at 37°C. After washing twice with PBS containing 3% FCS, 7×10^6 cells per mouse were injected into the retro orbital venous plexus.

2.2.23 General Antibodies:

The following reagents were from BD biosciences: anti-CD3 (500A2), anti-CD3 (145-2011), anti-CD103 (M290), anti-CD4 (RM4-5), and anti-CD8 (53-6.9). From eBioscience: anti-MHCII (M5/114.15.2), anti-IFN-g (XMG1.2), anti-CD4 (RM4-5), anti-CD8 (53-6.9). Antibodies from Biolegend were: anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 anti-CD11c (N418), anti-CD11b (M1/70), anti-IFN-g (XMG1.2), anti-CD103 (M290), anti-LPAM-1 (DATK32), and anti-TCRVa2 (B20.1). AQUA (L34957) was from Invitrogen and CFSE was from Sigma. Anti-CD205 (NLDCC) was produced locally as described¹⁹. The anti-CD205 mAbs were characterized by SDS/PAGE and Western blotting using HRP sheep anti-mouse IgG (GE Healthcare,

Buckinghamshire, U.K.) or HRP-anti-gag p24 (ImmunoDiagnostics, Woburn, MA). mAb binding was verified on CHO cells stably transfected with mouse DEC-205 by FACS using PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Anti-CD205 mAbs were tested to be endotoxin-free in Limulus Amebocyte Lysate assay, QCL-1000 (Cambrex, Walkersville, MD)

2.2.24 Vaccination Protocol

Age, gender matched control and experimental mice were vaccinated with α CD205-gag p24 or α CD205-OVA delivered intranasally. Boost vaccination was administered exactly 4 weeks later. For i.n. priming to α CD205-gag p24, mice in all groups were sacrificed 7 days post-immunization. Immune cells isolated from mice vaccinated to α CD205-gag were restimulated with p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining and 2 μ g/mL α CD28 antibody was added.

2.2.25 Statistical Analysis

Statistical analysis was performed using the computer based mathematical package Prism 5. A statistical difference in mean cytokine values between different experimental groups was determined using the students unpaired t test. P values of less than 0.05 were considered significant.

Chapter 3

Dendritic cell mediated cross talk between two mucosal
compartments.

3.1: Introduction

John Bienenstock first formulated the concept of a “*common mucosal immunological system*” nearly 40 years ago. He proposed this theory following his group’s examination of bronchus-associated lymphoid tissue and its similarity to those in the gastrointestinal tract (Bienenstock and McDermott 1978). Since then, the mucosal immune system has received a great deal of attention and is now described as an integrated network of tissue cells and effector molecules that protect the host from infection and environmental insult at the mucosal surface.

The mucosal surfaces of the body are immunologically unique, acting as the primary interface between the host and the external physical environment, yet they also have key barrier functions. The majority of research undertaken to date, on the mucosal immune system, has focused on specific individual components of the system. For example, a large amount of data has been gathered on the mechanism responsible for the induction of immune responses to viruses and bacteria in the GI tract. Moreover, the means by which the respiratory tract deals with pathogenic challenges has also been well established. However, the mucosal immune system has yet to be examined from a holistic viewpoint as a global organ (Figure 3.1).

It has been suggested by many that “[future] research should examine how the different components [of the mucosal immune system] affect each other and how crosstalk is achieved between individual [compartments]” (Gill et al, 2009). More importantly, future research should also investigate cellular trafficking between distinct mucosal sites (Gill et al, 2009). Given the anatomical similarities shared by distal mucosal compartments, mucosal priming at one site may induce antigen specific T cell responses at another mucosal location.

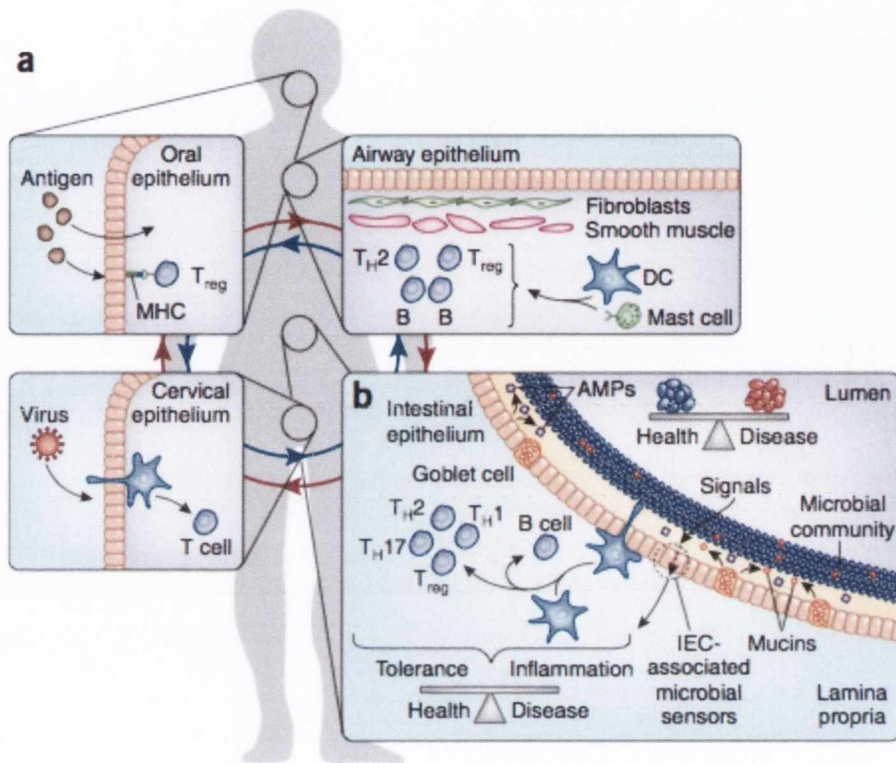


Figure 3.1: The mucosal immune system.

The mucosal immunological system. (a) Recent advances suggest that mucosal sites function together as a system-wide organ. Various mucosal sites throughout the body act as an interface between the physical environment (food, airborne, viral and commensal antigens) and host mucosal defenses. (b) The intestinal mucosal interface, a complex system that must integrate interactions among the microbiota, mucus layer, associated protective compounds, epithelial cells and underlying immune cells of the lamina propria. Notably, it has become clear that both the state of the microbial community and underlying immune cells contribute to the health or disease of the host. T, T cell; Treg, regulatory T cell; MHC, major histocompatibility complex; B, B cell; DC, dendritic cell; IEC, intestinal epithelial cell; AMP, antimicrobial peptide (Gill et al, 2010)

Systemic vaccination is associated with the induction of T cells with multiple homing capabilities (Masopust, Vezys et al. 2001; Reinhardt, Khoruts et al. 2001; Reboldi, Coisne et al. 2009), the site of antigen entry determines, at least in part, the adhesion receptors that T cells acquire (Mora 2008). T cell homing to the small intestinal lamina propria (SILP) is dependent on the expression of the cell surface molecules $\alpha 4\beta 7$ (integrin) and CCR9 (chemokine receptor). The ligand for the $\alpha 4\beta 7$ integrin, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is expressed on the intestinal endothelium. CCL25 (thymus-expressed chemokine), the CCR9 ligand; is expressed by epithelial cells in the small intestine, particularly those in the crypt region most closely associated with MAdCAM-1-positive vessels, and therefore recruits $\alpha 4\beta 7^+ \text{CCR9}^+$ lymphocyte to the small intestine.

The administration of vaccines orally induces the preferential expression of $\alpha 4\beta 7$ on antigen specific T cells, however parenteral vaccination does not induce this gut-homing integrin to a significant level (Mora and von Andrian 2006). The current paradigm of T cell homing to the gastrointestinal tract involves the induction of $\alpha 4\beta 7$ and CCR9 by MLN and PP DCs (Mora et al., 2003); meanwhile those T cells activated in the peripheral lymph nodes acquire the expression of E- and P-Ligand, but not gut-homing receptors (Campbell and Butcher 2002; Mora 2008). Further evidence has revealed that lymphoid microenvironment DCs and stromal cells are sufficient to imprint tissue-specific tropism on T cells upon *ex vivo* activation. Only DCs from the MLN, PP and SILP, but not DCs from peripheral tissues are capable of inducing $\alpha 4\beta 7$ and CCR9 expression on T and B cells (Johansson-Lindbom, Svensson et al. 2003; Mora, Bono et al. 2003; Ahrendt, Hammerschmidt et al. 2008; Jaensson, Uronen-Hansson et al. 2008; Mora 2008; Molenaar, Greuter et al. 2009).

This gut-specific imprinting property is dependent on the selective capacity of DCs to metabolize vitamin A (retinol) into all-*trans* retinoic acid (RA) (Iwata, Hirakiyama et al. 2004). RA is necessary and sufficient to induce gut-tropism both *in vivo* and *in vitro*. RA not only induces gut-tropic T cells, but also prevents the generation of skin-homing

receptors by blocking the induction of FucT-VII and P- and E-Lig on T cells (Iwata, Hirakiyama et al. 2004)). However, this does not explain the well-documented recruitment of antigen specific cells to the GI tract following non-GI mucosal antigen exposure.

A number of studies have determined that intranasal immunization with herpes simplex virus type-2, results in vaginal protection against genital infection following challenge (Wizel et al., 2012). In addition, Artenstein *et al* (1997), revealed that patients infected with HIV have higher concentrations of virus specific IgA in a variety of mucosal secretions including vaginal secretions, nasal washes, saliva and endocervical secretions. More recently, studies from Masopust *et al.*, (2010) have revealed that mice infected intranasally with influenza have flu specific CD8⁺T cells expressing $\alpha 4\beta 7$ in the lung. Flu specific T cells were detected in the small intestinal lamina propria but not the PP or MLN (Masopust et al., 2010). Similarly, intranasal challenge with H1N1 influenza results in the accumulation of antigen specific Th17 cells in the small intestinal lamina propria (Esplugeus et al., 2011). Evidence also suggests that the use of antibiotics in neonates is associated with an increased risk of developing asthma (Sobko et al., 2010), suggesting that alterations in the microflora of the gut can affect the lungs and thereby suggests a link between these compartments.

Collectively, such studies suggest that the mucosal immune system is actually one large interconnected network and that individual components are very efficient at sharing information distally.

Key objectives of Chapter 3:

- **Determine if non-GALT associated DCs induce the expression of gut-homing markers on T cells.**
- **Investigate if externally primed T cells traffic to the gastrointestinal tract.**
- **Study the physiological relevance of such immune trafficking.**

3.2 Results

3.2.1 Murine lung dendritic cells induce the expression of $\alpha 4\beta 7$ *in vitro*.

Following studies by Mora *et al.*, (2003) and Agace *et al.*, (2003) a considerable amount of evidence has revealed that DCs in the PP and local gut draining lymph nodes have the capacity to induce the expression of the integrin $\alpha 4\beta 7$ and chemokine receptor CCR9 on T cells (Berlin *et al.*, 1996; Zabel *et al.*, 1999). Thus orally administered antigens can induce effector/memory cells that express these essential receptors for intestinal homing. Stimulation of CD8⁺-expressing T cells by DCs from PPs, peripheral lymph nodes and spleen induce equivalent activation markers and effector activity in T cells, but only PP and MLN DCs induce high levels of $\alpha 4\beta 7$, responsiveness to TECK and the ability to home to the small intestinal lamina propria. PP and MLN DCs imprint gut homing and thus license effector/memory cells to access anatomical sites most likely to contain their cognate antigen. This paradigm however, is difficult to reconcile with reports of GI T cell responses after i.n. delivery of antigens that do not directly target the GI lymphoid tissue (*as previously discussed*). Therefore understanding the communication between mucosal sites is fundamental to the next phase of disease characterization and vaccine development. To explore alternative pathways of cellular migration, the ability of DCs from mucosal and non-mucosal tissues to recruit lymphocytes to the GI tract was investigated.

The initial objective was to determine if DCs at non-GI sites have the capacity to induce the expression of gut homing markers on T cells thereby supporting the classical theory of a “*common mucosal immunological system*”. In order to determine whether DCs at non-GI sites could induce gut-homing markers on antigen specific T cells, a screen was conducted to examine the ability of mucosal and non-mucosal DCs to induce gut homing integrin- $\alpha 4\beta 7$ expression. CD11c⁺ DCs were magnetically isolated from the spleen, lung, mediastinal lymph nodes and MLN. These DCs were cultured *in vitro* for 5 days with

splenic CFSE labelled OT-II cells at various ratios (1:2, 1:4, 1:10). T cells from the spleen of ova reactive OT-II mice were isolated by magnetic depletion of non-T cells.

On day 0, the co-culture was pulsed with 5 μ g of Ova protein. **Figure 3.1** illustrates a representative experiment. After 5 days the cells were stained with fluorochromes conjugated to Aqua (dead/live), CD3, CD4, TCR V α 2 and α 4 β 7 (**Fig 3.1a**). Firstly, all cells were selected using forward by side scatter, followed by the isolation of live cells only (Aqua), T cells were isolated using CD3 and the transgenic OT-II cells identified using CD4 versus TCR V α 2. Following the isolation of the OT-II cells only the proliferating cells were examined using CFSE dilution and the induction of α 4 β 7 was subsequently investigated.

Unexpectedly, we observed that the expression of α 4 β 7 was significantly up-regulated on OT-II cells by lung DCs, similar to the level induced by MLN DCs, but not splenic derived DCs or skin draining lymph node DCs, with α 4 β 7 up-regulation most prominent on proliferating (CFSE^{low}) cells (**Fig. 3.1b**). Although all stimulated T cell populations expressed higher levels of α 4 β 7 than naïve T cells, there was a significant increase in the number (**Fig. 3.2**) and percentage (**Fig. 3.1b**) of α 4 β 7⁺ cells induced by lung DC and MLN DC compared to splenic DC or skin draining lymph node DC.

As a positive control the induction of α 4 β 7 by MLN DCs was examined, in line with the current literature (Mora et al., 2003; Mora et al., 2005), DCs from this compartment were capable of inducing significant levels of α 4 β 7 on OT-II cells, with ~68% of OT-II cells expressing α 4 β 7.

Overall, these results confirm that non-GI DCs, in this case lung DCs, have the capacity to induce the expression of α 4 β 7 on CD4⁺ T cells.

3.2.2 Enhanced levels of $\alpha 4\beta 7$ are detected *in vivo* on antigen specific T cells post intranasal but not subcutaneous immunization.

Following on from the initial observation that lung DCs induce the expression of $\alpha 4\beta 7$ on T cells *in vitro*, we sought to determine if lung DCs had the capacity to up-regulate $\alpha 4\beta 7$ *in vivo* following intranasal vaccination. In order to determine the *in vivo* induction of gut-homing receptors, a TCR transgenic OT-II system was utilized. Specifically, 7×10^6 congenic CD45.1⁺V α 2⁺CD4⁺OT-II cells were fluorescently labelled with CFSE and adoptively transferred i.v. into C57BL/6 CD45.2⁺ wild-type recipients. One-hour post adoptive transfer, the recipient animals were vaccinated with a single dose of 100 μ g ova and 50 μ g of poly ICLC. Ova of greater than 99% purity was used for all vaccinations, which was LPS free, thereby not containing immunologically relevant amounts of endotoxins that may serve as adjuvants in this experimental set up.

In order to determine the level of $\alpha 4\beta 7$ expression on the adoptively transferred congenic cells, a specific gating strategy was developed to identify the transferred OT-II cells *in vivo* (Fig. 3.3). Upon isolation of immune cells from multiple compartments, cells were stained with fluorochromes conjugated to various markers; all cells were gated, followed by live cells only (Aqua). CD4⁺ cells were isolated using a CD4/CD3 gate; and finally the transferred cells were isolated using a CD45.1 and TCR V α 2 gate.

Recipient C57BL/6 animals were vaccinated either i.n. or s.c., to compare vaccination by mucosal and non-mucosal routes. As described, CFSE labelled CD45.1⁺V α 2⁺CD4⁺OT-II cells were adoptively transferred into CD45.2⁺ recipients. One hour post adoptive transfer the recipient animals received a single s.c. injection of 100 μ g of ova and 50 μ g of poly ICLC into their footpad or received a single i.n. vaccination with the same vaccine.

On day 4 post immunization the number and percentage of CD45.1⁺V α 2⁺CD4⁺CFSE^{low} cells expressing $\alpha 4\beta 7$ was determined in the draining lymph nodes, lung, spleen and

MLN. As shown in **Figs. 3.4** and **3.5**, a significantly higher percentage of CFSE^{low} $\alpha 4\beta 7^+$ cells were induced in the lung and mediastinal LN following i.n. immunization than in the popliteal, skin draining LN following s.c. immunization

In order to determine the kinetics of $\alpha 4\beta 7$ induction and the frequency of adoptively transferred T cell migration to the small intestinal lamina propria, colon and MLN, a time course experiment was conducted. CFSE labeled CD45.1⁺ OT-II cells were adoptively transferred to naïve CD45.2⁺ recipients and immunized after one hour with ova (100 μ g) protein and poly ICLC (50 μ g) delivered i.n. as described above. Recipient animals were sacrificed on Day 1, 2, 3, 4 and 7.

As expected, the transferred cells failed to proliferate on day 1, however proliferation was observed in the mediastinal LN within 48 hours of vaccination. By day 3, robust proliferation was observed in the mediastinal LN and lung compartment (**Fig. 3.6a**), in addition, a sharp rise in the expression of integrin $\alpha 4\beta 7$ on the transferred cells in the mediastinal LNs and the lungs was observed. Induction of $\alpha 4\beta 7$ peaked at day 4 and regressed by day 7.

The frequency of transferred CD45.1⁺V $\alpha 2^+$ CD4⁺ T cells showed a striking rise on day 4 in the GI tissues (SILP, Colon and MLN) (**Fig. 3.6b, 3.7b**) corresponding to the peak expression of integrin $\alpha 4\beta 7$ on lung and mediastinal LN resident transferred cells

3.2.3 Both lung-resident dendritic cell populations induce the expression of $\alpha 4\beta 7$ on T cells *in vitro*.

DCs within the lung were initially described as a single population of dendritic-shaped cells with a high expression of CD11c and MHC Class II, however it is now clear that multiple subpopulations of DCs can be found in the lung and lung draining LNs (Lambrecht and Hammad. 2012). These subsets are either resident in the mediastinal lymph nodes or present at this site as a result of DC migration from the lung or the periphery.

These DCs can be identified by an array of cell surface markers. Among the LN resident DCs are subsets, which express CD8 α or CD4 or are double negative for CD8 α and CD4. These subsets can be distinguished by their capacity to elicit T cell responses (Section 1.10.2.1). In addition to lymph node resident DCs, lung-resident DCs that have the ability to migrate to the local draining LNs following infection, express the cell surface integrin CD103. In contrast, CD103⁻CD11b⁺ DCs are restricted to the lung-parenchyma. Studies have revealed that CD103⁺ DCs are the most efficient activators of virus-specific CD8⁺ T cells. These cells are also efficient at capturing antigens and migrating to the mediastinal and other local lymph nodes. Both the CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DCs express high levels of CD11c and account for >90% of the lung resident DCs, with pDCs forming a smaller population whose location within the lung has yet to be fully determined. The alveolar space is also home to CD103⁺ DCs.

To date several functional properties have been ascribed to murine intestinal DCs. These include the enhanced ability to promote FoxP3⁺ Treg cell development, IgA secreting B cell differentiation and induction of $\alpha 4\beta 7$ and CCR9 on responding B and T cells. These diverse functions appear, at least in part, to be driven through intestinal DC-mediated retinoic acid receptor (RAR) signalling. Thus, the vitamin A metabolite retinoic acid (RA) induces the expression of gut-homing receptors and enhances FoxP3⁺ T cell and IgA⁺ B cell differentiation, whereas RAR antagonists block the ability of intestinal DCs to generate these populations.

Only a subset of DCs residing in the MLN, which express CD103, are efficient at inducing gut-homing receptors on responding T cells and mediating FoxP3⁺ T cell differentiation *in vitro*, the CD103⁺CD11b⁺ DCs lack this capability. CD103⁺ MLN DCs express higher levels of *aldh1a*, the gene encoding RALDH2, which is a key enzyme involved in metabolizing retinal to RA, in comparison their CD103⁺CD11b⁺ MLN counterparts lack this enzyme. Consistent with this, CD103⁺ MLN DCs induce enhanced RAR dependent signalling in responding T cells, compared with splenic or CD103⁺CD11b⁺ MLN DCs. Following the initial observation that lung CD11c⁺ DCs could induce $\alpha 4\beta 7$ *in vitro* and *in vivo*, the next question addressed was whether both the lung resident CD103⁺CD11b⁺ and CD103⁺CD11b⁺ were equally competent at inducing gut homing or whether a “division of labor” exists, as seen in the gastrointestinal tract. Therefore, an examination into the ability of various lung DC subsets to induce $\alpha 4\beta 7$ expression on T cells was conducted.

CD11c⁺ DCs were isolated from the lung and MLN of naïve C57BL/6 mice and subsequently sorted into their respective CD103⁺ and CD11b⁺ subpopulations (gating strategy shown in **Fig. 3.8**). These DCs were cultured in the presence of CFSE labelled OT-II cells and pulsed with 5 μ g of Ova *ex vivo*. On day 5 the induction of $\alpha 4\beta 7$ was investigated on the proliferating CFSE^{low} OT-II cells.

Consistent with the current literature, MLN CD103⁺ CD11b⁺ DCs were proficient at inducing $\alpha 4\beta 7$ on the OT-II cells (Mora et al. 2003), while the CD103⁺CD11b⁺ population lacked the capacity to induce the expression of this integrin, despite retaining the capacity to induce proliferation of these cells (**Fig. 3.9**). Unexpectedly, both CD103⁺CD11b⁺ and CD11b⁺CD103⁺ lung resident DC subsets induced $\alpha 4\beta 7$, with expression of $\alpha 4\beta 7$ being even higher in the CD103⁺CD11b⁺ population (**Fig. 3.9, 3.10**).

Despite differences in terms of their anatomical location, immune function and migratory potential, the ability of lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ to induce α 4 β 7 was comparable.

3.2.4 CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DCs isolated from the murine lung induce the expression of Foxp3 on T cells *in vitro*.

Powrie and colleagues had previously demonstrated that expression of CD103 on DCs is required for the CD4⁺CD25⁺ T-reg cell-mediated control of experimental colitis (Coombes, Siddiqui et al. 2007) suggesting that CD103⁺CD11b⁻ DCs may be functionally specialised to drive T reg cell responses. Coombes *et al.*, (2007) compared the ability of CD103⁺ and CD103⁻ DCs isolated from the MLNs of steady state mice to induce the expression of FoxP3 on splenic CD4⁺ T cells. Despite the ability of both CD103⁺ and CD103⁻ MLN DC populations to induce proliferation, only the CD103⁺ DC population had the ability to induce the expression of FoxP3 on T cells. The ability of the individual DC subtypes in the lung to induce FoxP3 on CD4⁺ T cells in an antigen specific manner was subsequently investigated.

Prior to cell sorting, into their respective CD103⁺ and CD11b⁺ subtypes (gating strategy shown in **Fig. 3.8**), CD11c⁺ DCs were isolated from the lung and MLN. These DCs were cultured *in vitro* for 5 days with splenic CFSE labelled OT-II cells. On day 0, the co-culture was pulsed with 5 μ g of ova protein. **Fig. 3.12**, illustrates a representative experiment. After 5 days the cells were stained with fluorochromes conjugated antibodies against, CD3, CD4, TCR V α 2, FoxP3 and α 4 β 7, Aqua (dead/live) was used to assess viable cells. Following the isolation of the OT-II cells only the proliferating cells were examined using CFSE dilution and the induction of FoxP3 was subsequently investigated. Similar to the α 4 β 7 and CCR9 data, both lung resident populations induced FoxP3 expression to levels that were comparable to MLN CD103⁺ DCs (**Fig. 3.11**). However, lung CD103⁺CD11b⁻ DCs were much more proficient at inducing FoxP3 than CD11b⁺ DC, further confirming their role as mediators of tolerance.

3.2.5 Enhanced levels of $\alpha 4\beta 7$ are detected *in vivo* on OT-I cells post intranasal but not subcutaneous immunization.

To date these studies focused on the ability of lung DCs to induce $\alpha 4\beta 7$ expression on CD4⁺ T cells using an OT-II transfer system. MLN and PP DCs also have the capacity to induce $\alpha 4\beta 7$ on CD8⁺ T cells both *in vitro* and *in vivo* (Eksteen B et al., 2009); therefore the ability of lung DCs to imprint CD8⁺ T cells with $\alpha 4\beta 7$ was investigated. 7×10^6 congenic CD45.1⁺V $\alpha 2$ ⁺CD8⁺OT-I cells were fluorescently labelled with CFSE and adoptively transferred into C57BL/6, CD45.2⁺ wild-type recipients. One hour post adoptive transfer these recipient animals received a single s.c. injection of 100 μ g of ova and 50 μ g of poly ICLC into their footpad or received a single i.n. vaccination with the same vaccine.

4 days post vaccination the animals were sacrificed and various immune compartments isolated, including the gut draining LNs (MLN), lung, spleen, blood and lymph node draining the site of vaccine delivery. For the s.c. vaccination group the popliteal lymph nodes were removed and for the i.n. group; the mediastinal LN was isolated.

Following a single s.c. immunization, CD45.1⁺V $\alpha 2$ ⁺CD8⁺OT-I cells, proliferated in the local draining lymph node and could be detected in a variety of systemic compartments, including the blood, spleen and lung. In accordance with the current literature, s.c. immunization did not induce $\alpha 4\beta 7$ expression to a significant level (**Fig.3.12, 3.13**). In contrast, following i.n. immunization CD45.1⁺V $\alpha 2$ ⁺CD8⁺OT-I cells primed in the lung and mediastinal LN readily up-regulated the $\alpha 4\beta 7$ -integrin. Similar results were obtained in the blood, spleen and MLN of i.n. immunized mice, where a four fold higher level of $\alpha 4\beta 7$ induction was observed on these antigen specific CD8⁺ cells in the MLN compared with s.c. immunization (**Fig 3.13**). This thereby confirmed the ability of lung DCs to induce gut-homing phenotypic changes on both CD4⁺ and CD8⁺ transgenic T cells.

3.2.6 Lung and Mediastinal LN DCs primed with poly ICLC *in vivo* retain the capacity to induce $\alpha 4\beta 7$ *in vitro*.

50 μ g of poly ICLC was delivered i.n. to wild type C57BL/6 mice and 24 hours later CD11c⁺ DCs were magnetically isolated from the spleen, lung, mediastinal lymph nodes and MLN. These DCs were cultured *in vitro* for 5 days with splenic CFSE-labelled OT-II cells.

On day 0, the co-culture was pulsed with 5 μ g of Ova protein. Following the isolation of the OT-II cells only the proliferating cells were examined using CFSE dilution and the induction of $\alpha 4\beta 7$ was subsequently investigated. Expression of the integrin $\alpha 4\beta 7$ was significantly up regulated on OT-II cells by lung and mediastinal LN DCs, but not splenic derived DCs or skin draining lymph node DCs, with $\alpha 4\beta 7$ up-regulation most prominent on proliferating (CFSE^{low}) cells (**Fig. 3.14**).

3.2.7 Lung Dendritic cells licence antigen-specific T cells to migrate to the gastrointestinal tract.

As $\alpha 4\beta 7$ and CCR9 are essential intestinal homing receptors we postulated that adoptively transferred OT-II cells would home to the GI tract more effectively following i.n. vaccination than after s.c. immunization. In order to address this issue, 7 $\times 10^6$ congenic CD45.1⁺V α 2⁺CD4⁺OT-II cells were fluorescently labelled with CFSE and adoptively transferred into C57BL/6 CD45.2⁺ wild-type recipients. One hour post adoptive transfer these recipient animals received a single s.c. injection of 100 μ g of ova and 50 μ g of poly ICLC into their footpad or a single i.n. vaccination with the same vaccine. 7 days post vaccine delivery, the animals were sacrificed and gastrointestinal immune inductive and effector sites, including the small intestinal lamina propria, colon and MLN were isolated. The transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells were identified using the gating strategy outlined in **Fig. 3.3**. We compared the frequencies of adoptively

transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells in these compartments to determine the effects of i.n. and s.c. immunization on GI homing potential.

The frequency of CD45.1⁺V α 2⁺ cells in the small intestines and colon was 3-10 fold higher following i.n. immunization than following s.c. immunization demonstrating that lung DC-primed congenic OT-II cells migrated to the lamina propria with a significantly greater efficiency than skin DC-primed OT-II cells (**Fig 3.15**)

3.2.8 Enhanced migration of antigen specific T cells to the gastrointestinal tract post intranasal vaccination is dependent on α 4 β 7

Having established that lung DCs can induce α 4 β 7 expression on T cells *in vitro* and that i.n. vaccination results in enhanced migration of antigen-specific T cells to the gastrointestinal tract, we sought to examine the migratory ability of lung DC primed α 4 β 7 expressing cells to the GI tract and the role of α 4 β 7.

Lymphocytes move to the GALT by means of differential expression and activation of adhesion molecules. Emigration of lymphocytes from the vasculature into tissues requires them to roll along the endothelial cells, arrest and firmly adhere to endothelial cells, and migrate across the endothelium. Localization of lymphocytes to the colon differs in some ways from migration to the small bowel—it requires either α 4 β 7 or α 4 β 1, but not CCR9. The ligand for α 4 β 7, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is expressed in the small bowel and colon, whereas CCL25 is expressed in the small bowel only. Additionally, CCR9 is required for T-cell migration and pathogenicity primarily in the small intestine, whereas α 4 β 7 is required for T-cell migration and pathogenicity in the small bowel and colon.

In order to assess the role-played by α 4 β 7, in terms of enhanced homing to the gastrointestinal tract post i.n. immunization; a blocking antibody against α 4 β 7 was utilized. Splenic CD45.1⁺V α 2⁺CD4⁺OT-II cells were isolated as previously described and

i.v. transferred (7×10^6 cells/animal) into CD45.2⁺ C57BL/6 recipients. The animals were subsequently immunized with 100µg of ova and 50µg of poly ICLC on day 0 via i.n. delivery. 100µg/mouse of anti- $\alpha 4\beta 7$ (BD Pharmingen) was administered i.p on day -1 and day 2. Seven days post transfer the recipient mice were sacrificed. Utilizing the gating strategy adopted in **Fig. 3.3**, the frequency of transferred CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells was analyzed in a variety of systemic compartments (lung, mediastinal LN, spleen and blood) and in gastrointestinal induction and effector sites (small intestinal lamina propria, colon, mesenteric LN and intraepithelial lymphocytes).

Again, this data revealed that i.n vaccination, was more effective at recruiting antigen specific cells to the gastrointestinal tract (*s.c. comparison not shown*), with significant numbers of CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells homing to the colon, small intestinal lamina propria and intraepithelial cell (IEL) compartment. CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells were also present in systemic compartments (**Fig. 3.16, 3.17**). As shown in **Fig 3.16**, animals that had been administered 100µg of anti- $\alpha 4\beta 7$ on day -1 and 2 prior to i.n. vaccination had a significant reduction in the numbers of CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells that homed to the gastrointestinal tract. A significant reduction in CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cell homing was observed in the colon, intraepithelial compartment and small intestinal lamina propria, all sites that require $\alpha 4\beta 7$ for migration and trafficking. In contrast, no differences were observed in systemic compartments, with CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells proficient in their ability to traffic to the spleen, mediastinal LN, lung and blood. In fact, increased numbers of CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells were observed within systemic compartments of animals given anti- $\alpha 4\beta 7$, due to the inability of these cells to traffic to the gastrointestinal tract post i.n. vaccination (**Fig. 3.17b**).

This demonstrates that lung DC-induced OT-II cell migration to the GI tract is mediated by $\alpha 4\beta 7$.

3.2.9 Lung DC stimulated antigen specific T cells have a 5 fold higher propensity to migrate to the gastrointestinal tract compared to splenic stimulated T cells

In order to determine the homing capacity of OT-II cells following *in vitro* culture with spleen and lung DCs, the homing protocol devised by Mora *et al* (2005) was utilized (**Fig. 3.18**). As described previously, CD11c⁺ DCs were isolated from the lung and spleen and cultured in the presence of 5µg of ova with OT-II cells. After 5 days, the cells were isolated and α4β7 induction confirmed by flow cytometry. The OT-II cells cultured in the presence of lung DCs were labelled with CFSE, while splenic DC stimulated OT-II cells were labelled with cell tracker (CT). The cells were admixed (in a 1:1 ratio) and injected i.v. into naïve C57BL/6 mice (**Fig. 3.18**). 16 hours post i.v. transfer the recipients were sacrificed and the lungs, small intestinal lamina propria, spleen and MLN isolated.

Following the determination of the input ratio, the homing index was calculated using the homing index equation (**Fig. 3.18d**). Our competitive homing experiments with differentially labelled OT-II cells revealed that lung DC stimulated T cell homing was 5 times higher in the small intestinal lamina propria compared to splenic DC stimulated T cells (**Fig. 3.19a**). Comparably pronounced differences were observed once all the data had been collated (**Fig. 3.19b**). Lung DC stimulated OT-II cells homed with higher efficiency to the MLN and lung than splenic DC stimulated OT-II cells, but these differences were comparatively modest, with homing indices of 1 and 1.5 respectively.

3.2.10 Lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ dendritic cells express high levels of RALDH.

Findings from this study have demonstrated that lung DCs induce $\alpha 4\beta 7$, which results in increased trafficking of antigen-specific T cells to the gastrointestinal tract, in a $\alpha 4\beta 7$ dependent manner. Following on from this, the underlying mechanism mediating $\alpha 4\beta 7$ induction by lung DC was investigated.

It has been shown that the vitamin A (retinol) metabolite all-trans retinoic acid (RA) is necessary and sufficient to induce gut-tropism *in vitro* and *in vivo* in murine and human T and B cells. RA not only induces the generation of gut-tropic T cells, but also prevents the generation of skin-homing receptors by blocking the induction of FucT-VII and P- and E-lig on T cells. To date, it has been reported in the literature that gut-associated but not extra-intestinal DC, express high levels of retinal dehydrogenase (RALDH 1 and RALDH 2 isoforms), critical enzymes responsible for the synthesis of RA from vitamin A. Moreover, among MLN-DC, only CD103⁺CD11b⁻ DC, which arrive from the small intestinal lamina propria, express RALDH2 and imprint gut-tropic lymphocytes, whereas CD103⁻CD11b⁺ DCs do not exhibit gut-imprinting properties. However given the ability of lung DC to induce $\alpha 4\beta 7$ *in vitro* and *in vivo*, the presence of RALDH-1 and 2 within murine lung DCs was determined.

To investigate the presence of RALDH within lung DCs, a standardized ALDEFLUOR® (StemCell Technologies) fluorescent reagent system was utilized. This data revealed the presence of RALDH in both the lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DC populations. In fact levels of RALDH were comparable to the positive control, MLN CD103⁺ DCs. In contrast, splenic CD8 α ⁺ DCs failed to express RALDH, as previously described (**Fig. 3.20**).

This is in concurrence with a similar report by Guillems *et al* (Guillems et al., 2010) although in that study, the ability of lung DCs to induce integrin $\alpha 4\beta 7$ was not examined.

In order to determine the effect of adjuvant administration on RALDH levels, C57BL/6 mice were immunized with 50µg of poly ICLC i.n. and 24 hours later lung DCs were isolated and analysed. The data revealed no significant change in RALDH levels post i.n. adjuvant delivery (**Fig. 3.21**).

3.2.11 $\alpha 4\beta 7$ induction by lung resident dendritic cells is dependent on retinoic acid signalling.

Given the ability of lung CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DCs to express the enzyme RALDH and induce the expression of $\alpha 4\beta 7$ *in vitro* on antigen-specific T cells, the relationship between lung DC mediated $\alpha 4\beta 7$ induction and retinoic acid signalling was determined.

RA exerts its gut-homing imprinting effects on lymphocytes by acting via RA receptors of the RAR family. RAR can heterodimerize to nuclear receptors of the RXR family, which can also homodimerize and heterodimerize with other nuclear receptors, such as PPAR γ . RA signalling results in up-regulation of CCR9 and $\alpha 4\beta 7$, accordingly RA deficiency causes a reduction in $\alpha 4\beta 7^{+}$ memory/activated T cells in lymphoid organs and a depletion of T cells from the intestinal lamina propria. Therefore, the impact of inhibiting retinoic acid receptor (RAR) signaling on $\alpha 4\beta 7$ induction on T cells by lung DCs was determined. .

CD11c⁺ DCs were isolated from the lung and MLN by magnetic separation. These DCs were cultured *in vitro* for 5 days with splenic OT-II cells. T cells from the spleen of ova reactive OT-II mice were isolated by magnetic depletion of non-T cells. On day 0, all the co-culture was pulsed with 5µg of ova protein and half with 1mM of LE540. LE540, is a retinoid antagonist, developed on the basis of the ligand superfamily concept, others include LE135, LE511 and LE550. LE540 is an effective RAR antagonist, whereas LE511 selectively induces RAR β transcriptional activity. LE540 selectively inhibits RA-

induced transcriptional activity of RAR β , but not RAR α , RAR γ or retinoid X receptor α (RXR α).

Fig. 3.22, illustrates a representative experiment. Five days post the commencement of the co-culture, the expression of $\alpha 4\beta 7$ on T cells was investigated. Consistent with the previous data, $\alpha 4\beta 7$ was significantly up regulated on OT-II cells by lung DCs and MLN DC as compared to splenic DCs (**Fig. 3.22a,b**). Culture of MLN DCs with OT-II cells in the presence of LE540, resulted in a statistically significant decrease in $\alpha 4\beta 7$ on the T-cells, consistent with reports from Iwata *et al.*, (2004) (Iwata *et al.*, 2004). Similarly, incubation with 1 μ M LE540 resulted in a statistically significant decrease in the induction of $\alpha 4\beta 7$ on OT-II cells induced by lung DCs (**Fig. 3.22c**). This therefore confirms that RA is essential for the induction of $\alpha 4\beta 7$ on T cells by lung DCs *in vitro*.

3.2.12 $\alpha 4\beta 7$ induction by lung resident dendritic cells is dependent on retinoic acid and TGF- β signalling *in vivo*

Having shown that RA signalling is essential for the induction of $\alpha 4\beta 7$ on T cells by lung DCs, the next key question was whether such signalling pathways were required *in vivo*.

In addition to RA, which is necessary and sufficient to enhance $\alpha 4\beta 7$ expression and suppressed E-lig on CD4 and CD8 T cells upon activation (previously described Section 3.2.6), the importance of TGF- β in $\alpha 4\beta 7$ induction has also recently been demonstrated (Kang *et al.*, 2011). Kang *et al.*, (2011), found that TGF- β signaling is required for the up-regulation of Itg- $\beta 7$ in primary CD4⁺ T cells utilizing T cells isolated from transgenic mice expressing a dominant negative form of TGF β RII (dnTGF β RII mice). In their study, naïve T cells from the dnTGF β RII mice were inefficient in surface expression of Itg- $\beta 7$ in response to RA, while expression of Itg- $\alpha 4$ was induced normally (Kang *et al.*, 2011).

In order to investigate the role of RAR and TGF- β signaling *in vivo* a dominant negative RAR (dnRAR) mouse crossed to OT- II on a RAG background (Pino-Lagos et al., 2011; Rajaii et al., 2008) as well as a TGF- β R^{def} OT-II mouse were used, respectively.

Congenic WT OT-II cells, dnRAR-OT-II cells or TGF- β R^{def} OT-II cells (7×10^6) were transferred into recipient mice and after one hour, each animal was vaccinated i.n. with 100 μ g of ova and 50 μ g of poly ICLC. Four days post vaccination the local lymph nodes draining the sites of vaccine delivery, in addition to other immune compartments, were isolated and analysed. Confirming the *in vitro* data, it was demonstrated that $\alpha 4\beta 7$ induction was attenuated on TGF- β R^{def} OT- II cells and virtually absent on dnRAR-OT-II cells suggesting important roles for TGF- β and RAR signaling in lung DC-mediated $\alpha 4\beta 7$ induction (Fig. 3.23, 3.24, 3.25).

3.3 Discussion

Since efficient trafficking of immune cells to the GI tract is critical for host defense against infection, studying cellular recruitment pathways to the GI tract is important for future development of novel vaccines against mucosally transmitted diseases including HIV-1 infection.

Naïve T cells acquire the capacity to migrate to extra-lymphoid tissues once activated by their cognate antigen (Butcher et al., 1999; von Andrian and Mackay, 2000). These antigen-experienced effector T cells migrate preferentially to the tissues where they first encountered the antigen (Campbell and Butcher, 2002; Kantele et al., 1999). For example, early observations demonstrated that cells activated in the GI tract home back to intestinal effector sites (Cahill et al., 1977; Hall et al., 1977). Integrin $\alpha 4\beta 7$ and chemokine receptor 9 (CCR9) are among the best-studied gut-specific “homing” molecules (Berlin et al., 1995; Zabel et al., 1999). The $\alpha 4\beta 7$ ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1) mediates recruitment of T cells to the intestinal lamina propria (Berlin et al., 1995) and the CCR9 ligand TECK, expressed by small intestinal epithelial cells recruits T cells to the small bowel (Zabel et al., 1999).

Dendritic cells (DC) are well recognized as the initiators of the adaptive immune response (Steinman and Cohn, 1973) as well as mediators of tolerance to self-antigens in steady state conditions (Hawiger et al., 2001). Additionally, there is increasing evidence for DC as conductors of immunological traffic to the skin and the GI tract (Johansson-Lindbom et al., 2005; Johansson-Lindbom et al., 2003; Mora et al., 2003; Mora et al., 2005; Sigmundsdottir et al., 2007). DCs can imprint on T cells the capacity to migrate to the tissue in which the T cells were originally activated. For example, gut-associated DCs induce expression of the gut-homing receptors $\alpha 4\beta 7$ and CCR9 on T cells upon activation (Johansson-Lindbom et al., 2003; Mora et al., 2003; Stagg et al., 2002).

RA is necessary and sufficient for DC to induce gut homing receptors on T cells (Iwata et al., 2004). The main pathway of RA biosynthesis *in vivo* is dependent on the intracellular

oxidative metabolism of retinol (Duester, 2000; Napoli, 1999), catalyzed by a family of alcohol dehydrogenases including RALDH, a class I aldehyde dehydrogenase which mediates the irreversible oxidation of retinal to RA. RA in turn is thought to induce RALDH-2 in a positive feedback loop (Hammerschmidt et al., 2011; Villablanca et al., 2011; Yokota et al., 2009) and RA levels correlate with the ability of the intestinal DCs to induce gut-tropic T cells. Vitamin A is introduced via dietary or biliary sources (Jaensson-Gyllenback et al., 2011). Among the cellular sources of RA in the intestinal mucosa are DCs (Iwata et al., 2004), stromal cells (Hammerschmidt et al., 2008; Molenaar et al., 2009), intestinal epithelial cells (Bhat, 1998; Lampen et al., 2000) and intestinal macrophages (Denning et al., 2007) with the DCs likely playing a key role in the induction of gut homing phenotype on T cells. Among the intestinal DCs, the CD103⁺ DC subset expresses high levels of RALDH-2 and is capable of generating high levels of RA (Coombes et al., 2007; Jaensson et al., 2008; Johansson-Lindbom et al., 2005; Sun et al., 2007). In contrast, the CD103⁻CD11b⁺CX3CR1⁺ macrophage-like population in the intestinal lamina propria expresses RALDH-1 and not RALDH-2 and exhibits a lower RA producing capacity (Denning et al., 2011; Schulz et al., 2009) and therefore a decreased capacity to induce gut homing potential on T cells (Jaensson et al., 2008).

Combined, a paradigm has emerged wherein only the intestinal CD103⁺ DCs, capable of metabolizing vitamin A, can induce GI specific homing on T cells (Jaensson et al., 2008). This paradigm however, is difficult to reconcile with reports of GI T cell responses after i.n. delivery of antigens that do not directly target the GI lymphoid tissue. For example, mice infected intranasally with influenza virus did not contain activated virus-specific CD8⁺ T cells in the MLN or PP, yet flu-specific CD8⁺ cells within the lung associated tissues express $\alpha 4\beta 7$ and memory CD8⁺ T cells are established within the small intestinal epithelium (Masopust et al., 2010). Similarly, a recent report demonstrates that i.n. challenge with H1N1 influenza results in the accumulation of Th17 cells within the small intestinal lamina propria (Esplugues et al., 2011). A number of association studies between COPD and IBD have suggested that these represent systemic inflammatory diseases with peripheral lymphocyte activity contributing to pathogenesis. During inflammation, the bronchus associated lymphoid tissue regulates lymphocyte trafficking

from the lung through the general circulation. This mirrors the role of gut-associated lymphocytoid tissue and both lung and intestinal lymphocytes migrate to other mucosal sites as part of the common mucosal immune system. Indeed, there is increasing appreciation of the mucosal immune system as an integrated network of tissues, cells and effector molecules, although the cellular factors that link different mucosal compartments are not well understood (Gill et al., 2010).

The lungs harbor prominent extrahepatic stores of Vitamin A (Dirami et al., 2004; Okabe et al., 1984), whose metabolite, RA plays an important role in pulmonary alveolar development (Dirami et al., 2004) and has a putative therapeutic role in emphysema (Massaro and Massaro, 1997). Additionally, although RA production has been considered to be the forte of gut resident DCs, other DC populations also express RALDH, particularly lung resident DCs that express RALDH-2 (Guilliams et al., 2010). In short, Vitamin A, a critical factor in the induction of $\alpha 4\beta 7$ expression is stored in the lung where it plays an important role in lung physiology and lung DCs contain the enzymatic machinery required metabolize Vitamin A into RA.

The present study identifies multiple lines of evidence to prove lung-DC mediated gut migration of T cells. Ova-specific OT-II cells co-cultured with lung DCs in the presence of ova express integrin $\alpha 4\beta 7$ at levels comparable to those induced by MLN CD103⁺ DCs. Second, integrin $\alpha 4\beta 7$ is induced on adoptively transferred OT-II cells following i.n. immunization. Third, T cells can be tracked to the GI lamina propria following i.n. immunization.

Neutralizing antibodies against either $\alpha 4\beta 7$ or MAdCAM-1 attenuated inflammation in experimental models of colitis or ileitis. Furthermore a humanized antibody against $\alpha 4\beta 7$ was found to induce clinical and endoscopic remission in patients with active CD, with similar results being obtained in knock out mice. For example, a study by Lefrancois *et al* (1999), demonstrated that following injection of mice with recombinant ova-expressing vesicular stomatitis virus, Ova-specific $\beta 7$ deficient CD8⁺ T cells were compromised in their ability to enter the small intestinal and colonic mucosa compared to their $\beta 7$

sufficient counterparts. These results provide strong evidence that $\alpha 4\beta 7$ plays a role in mediating effector T cell migration to the intestinal mucosa. Therefore, experiments were conducted to determine if the enhanced T cell migration to the GI tract post i.n. vaccination was the result of enhanced $\alpha 4\beta 7$ induction on antigen specific T cells *in vivo*. Consistent with previous data revealing the important role of $\alpha 4\beta 7$ in GI homing, these findings confirmed that $\alpha 4\beta 7$ was essential for mediating homing post i.n. vaccination. Further, T cell migration can be blocked by anti- $\alpha 4\beta 7$ antibody, causing the transferred T cells to accumulate in systemic compartments.

The conducting airway and lung parenchyma are home to two predominant classes of CD11c⁺ DCs, CD103⁺CD11b⁻ and CD103⁻CD11b⁺. Although both populations are present in the mediastinal LNs draining the pulmonary compartment during times of inflammation and steady state and thus participate in the initiation of adaptive immune responses in the draining lymph nodes, these subsets differ regarding their location within the lung, as well as in the transcriptional factors required for their development. CD103⁺CD11b⁻ lung DCs are primarily found in the respiratory lamina propria and are capable of extending their dendrites into the airway lumen and capturing antigens. Meanwhile, the CD103⁻CD11b⁺ DCs are restricted to the basal lamina. In addition to distinct locational differences; these cell also differ in terms of their function. Using fluorescent lipophilic dye-labeled influenza virus, Ho *et al* (2011) demonstrate that CD103⁺CD11b⁻ DCs are the dominant lung DC population transporting influenza virus to the posterior mediastinal lymph node as early as 20 h post-infection (Ho et al., 2011).

By contrast, CD103⁻CD11b⁺ DC although more efficient at taking up the virus within the lung, migrate poorly to the draining lymph node and remain in the lung where they produce proinflammatory cytokines instead. CD103⁺CD11b⁻ DCs efficiently load viral peptide onto MHC class I complexes and therefore uniquely possess the capacity to potently induce proliferation of naive CD8 T cells, thereby revealing a distinct “division of labor’ between these two lung resident DCs.

Prior reports have shown a significant difference between CD103⁺CD11b⁻ and CD103⁻

CD11b⁺ MLN DC in terms of $\alpha 4\beta 7$ induction with CD103⁺CD11b⁻ DCs capable of inducing significantly higher levels of $\alpha 4\beta 7$ and CCR9 compared to CD103⁻CD11b⁺ DCs (Agace 2005). To assess whether similar differences existed within the lung DC subsets, lung cell suspensions were magnetically enriched for CD11c⁺ cells. MHC-II^{high}CD11c⁺ lung DC were FACS-sorted into CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DC populations and co-cultured with CFSE labelled OT-II cells. Unexpectedly, both CD103⁺CD11b⁻ and CD103⁻CD11b⁺ lung DC subsets induced $\alpha 4\beta 7$, with expression of $\alpha 4\beta 7$ on T cells being even higher in the case of the CD103⁻CD11b⁺ population. To serve as a positive control, MLN DC subsets were examined simultaneously and as previously described, CD103⁺CD11b⁻ DCs induced high levels of $\alpha 4\beta 7$ whereas CD103⁻CD11b⁺ DCs did not.

Dietary vitamin A is an essential precursor of tissue retinol, which participates in a variety of biological processes including vision, reproduction, bone development and immunity. RA directly and indirectly regulates cellular functions including activation-induced apoptosis and the differentiation of Th1 and Th2 cells. RAs bind to two families of nuclear receptors, the RAR isotypes (α , β and γ) and the RXR isotypes. 9-*cis*-RA binds to both RAR and RXR, whereas the major physiological form of RA, all-*trans*-RA, binds to RAR and may bind to RXR at high concentrations. These receptors function as ligand-inducible transcription factors mainly as RXR/RAR heterodimers. A major characteristic of small intestinal CD103⁺ DCs is their enhanced ability to metabolise vitamin A and generate RA. Murine small intestinal lamina propria DCs and MLN CD103⁺CD11b⁻ DCs express high levels of *Aldh1a2* mRNA, display increased ALDH activity, and exhibit enhanced RA signalling in responding T cells *in vitro* compared with DCs in other compartments. The ability of intestinal CD103⁺CD11b⁻ DC to generate RA appears to underlie many of their unique properties including the capacity to induce gut homing receptors. Both small intestinal lamina propria and MLN DC subsets display enhanced vitamin A metabolising activity. RALDH expression was believed to be limited to certain cell types and despite the widespread availability of retinol, only cells expressing one of the RALDHs can oxidize retinaldehyde to RA. Given the essential role played by RA in gut immunity and homing of T cells to the immune inductive sites, the role played by RA produced by lung DC-derived RA was investigated.

By combining multiparameter flow cytometry and staining with ALDEFLUOR, a fluorescent substrate allowing for detection of ALDH activity at a single cell level, we aimed at identifying cells within the lung endowed with RA-producing capacity. Among DCs found within the lung, the CD103⁺CD11b⁻ and CD103⁻CD11b⁺ both expressed high levels of ALDH activity, consistent with the presence of RALDH. This confirms the data recently published by Guilliams *et al.*, (2011), who determined the expression of RALDH in both lung resident populations.

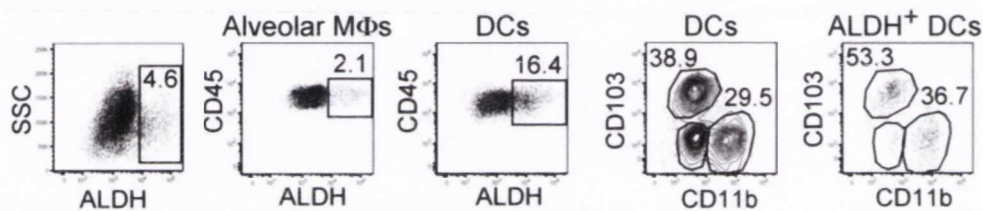


Figure 3.3 : Identification of ALDH⁺ DCs in the lung. Light density cells isolated from the lung were incubated with ALDEFLUOR and analysed by flow cytometry for expression of CD11c, MHCII, CD11b, CD103, CD45 and ALDH activity. (Guilliams *et al.*, 2011)

In accordance with Guilliams *et al* (2011), the relative level of expression was higher within the lung resident CD103⁺CD11b⁻ population, however the ALDH activity was similar between the two subpopulations. In fact ALDH activity was comparable to that of MLN CD103⁺CD11b⁻ DCs. Moreover, poly ICLC administration did not affect the overall ALDH activity in either of the two DC subsets.

Concomitant *in vitro* experiments showed that the vitamin A metabolite RA is sufficient to induce $\alpha 4\beta 7$ and CCR9 expression on activated T cells even in the absence of DC. Moreover, RA actively blocks the default up-regulation of E- and P-ligands on T cells similar to the case of T cells activated in the presence of GALT-DC, which express RALDH. In agreement with the role played by RA in the imprinting of gut-homing capacity, blocking RA receptors (of the RAR family) significantly decreases the

induction of $\alpha 4\beta 7$ -mediated by GALT-DC. In addition to GALT-DC, it is also possible that other sources of RA may contribute to $\alpha 4\beta 7$ induction *in vivo*. IEC express RALDH and an IEC line *in vitro* can produce RA. Intraperitoneal immunization also results in the induction of gut-homing T cells and some non intestinal viral infections induce $\alpha 4\beta 7$ expression on T cells, thus it is possible that gut-homing T cells can be imprinted in regions others than the GALT.

The mechanisms underlying $\alpha 4\beta 7$ induction appear to be conserved between lung and GI resident DCs. For example, consistent with studies into the induction of $\alpha 4\beta 7$ by gut DCs (Iwata et al., 2004; Kang et al., 2011), our results show that RA and TGF- β are required in the lung DC-mediated induction of $\alpha 4\beta 7$. Using an *in vitro* culture system, it was determined that the retinoic acid receptor- β inhibitor- LE540, blocked the induction of $\alpha 4\beta 7$ by both lung and MLN DCs. To study the role of RA *in-vivo*, a novel dominant negative RAR-OTII Rag^{-/-} system was used, revealing an almost complete inhibition of $\alpha 4\beta 7$ expression on T cells suggesting that RA was a critical component of this pathway.

Additionally, using a novel system of TGF- β receptor^{fl/fl}-CD4^{CRE} back-crossed to OT-II Rag^{-/-} mice, the role of TGF- β we interrogated and attenuated expression of $\alpha 4\beta 7$ was found in the absence of TGF- β signaling on CD4⁺ T cells. Notably, the effect of RA was much more pronounced than that of TGF- β in the induction of $\alpha 4\beta 7$.

In summary, the current data identify a novel pathway of T cell homing to the GI tract where lung DCs induce the expression of the gut homing molecule $\alpha 4\beta 7$ in a retinoic acid dependent manner. This opens up new avenues of directing lymphocyte traffic to the GI tract to enhance immunity against pathogens or potentially to generate tolerogenic cells directed against GI inflammation. While lung DC induced T cell homing to the GI tract was the subject of the current study, similar pathways may also exist for lung DC mediated B cell homing. Finally, elucidation of lung DC mediated gut homing provides a mechanistic understanding of how i.n. immunization induces GI directed immune

responses and supports the notion of a common mucosal immunological organ proposed more than 40 years ago.

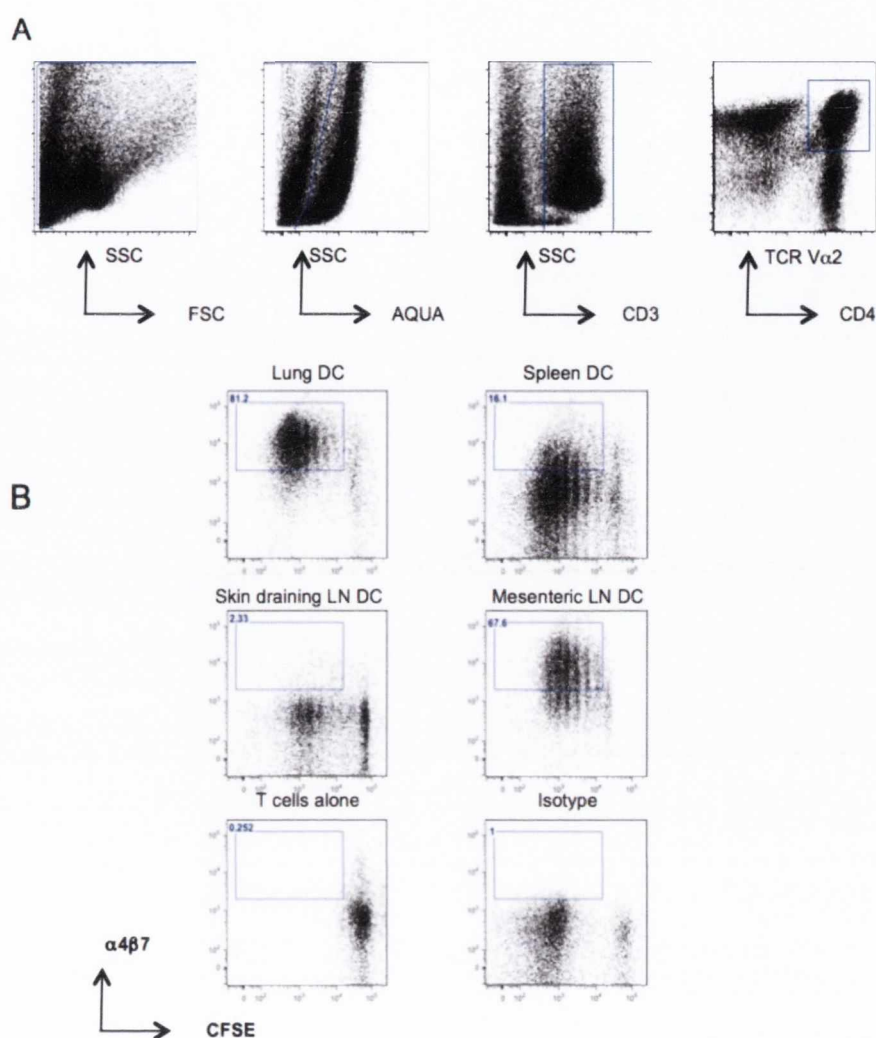


Figure 3.1: Murine lung DCs induce the expression of $\alpha 4\beta 7$ on T cells *in vitro*. Representative flow cytometry plots demonstrating the *in vitro* induction of integrin $\alpha 4\beta 7$ (y-axis) on proliferating OVA transgenic OT-II cells (CFSE^{low} , x-axis) by lung, spleen, skin draining LN and mesenteric LN derived DCs (DC:T cell ratio- 1:2). C57BL/6 animals were sacrificed and the spleen, mesenteric LNs, lungs and skin draining LNs removed (see methods). CD11c^+ DCs were isolated using MACS bead sorting and pulsed with $5\mu\text{g}$ of ova *ex vivo* before being cultured at a 1:2 ratio with CFSE labeled OT-II cells *in vitro*. 5 days after co-culture the T cells were isolated (a) utilizing the above gating strategy and (b) $\alpha 4\beta 7$ expression determined by flow cytometry.

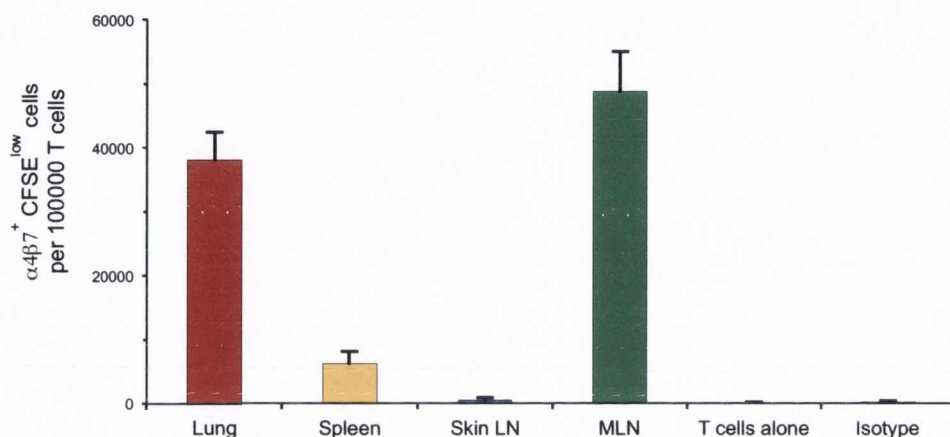


Figure 3.2: Murine lung DCs induce the expression of $\alpha 4\beta 7$ on T cells *in vitro*.

Cumulative data from 3 individual experiments comparing $\text{CFSE}^{\text{low}} \alpha 4\beta 7^{\text{high}}$ cells per 100,000 T cells. C57BL/6 animals were sacrificed and the spleen, mesenteric LNs, lungs and skin draining LNs removed (see methods). CD11c^+ DCs were isolated using MACS bead sorting and pulsed with $5\mu\text{g}$ of ova *ex vivo* before being cultured at a 1:2, 1:4 and 1:10 ratio with CFSE labeled OT-II cells *in vitro*. 5 days after co-culture the T cells were isolated and $\alpha 4\beta 7$ expression determined by flow cytometry.

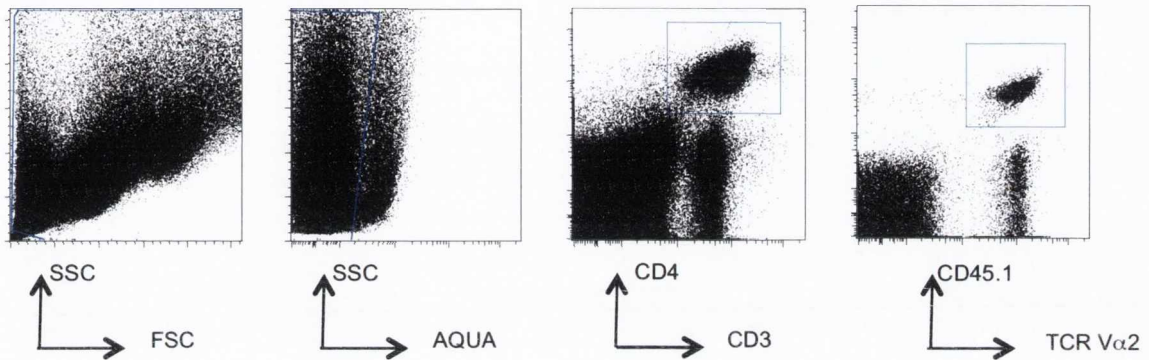


Figure 3.3: Gating strategy utilized to detect transferred congenic OT-II cells *in vivo*. 7×10^6 congenic CD45.1⁺ CFSE labeled OT-II cells were transferred i.v. into each C57BL/6 animal. 1-hour post OT-II cell transfer, each animal was vaccinated with 100 μ g of LPS-free ova and 50 μ g of poly ICLC via either the intranasal or subcutaneous route of vaccine delivery. 4 days post vaccination the local lymph nodes draining the site of vaccine delivery, in addition to other immune compartments, were isolated and analyzed.

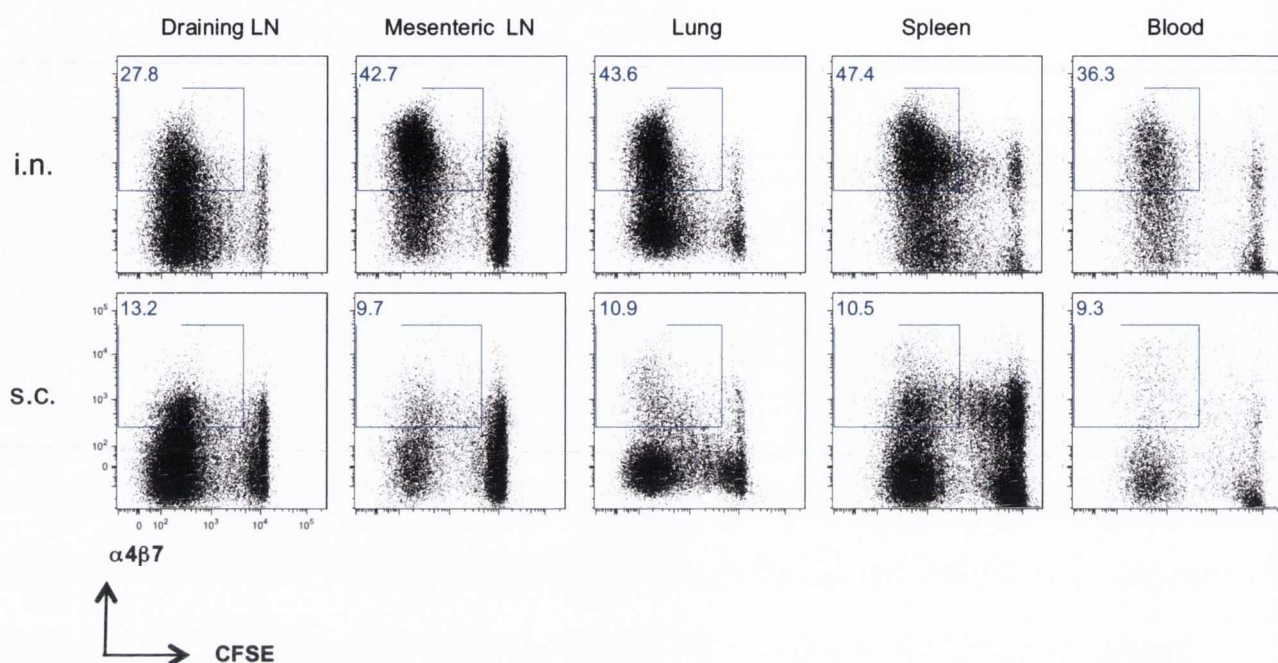


Figure 3.4: Enhanced levels of $\alpha 4\beta 7$ are detected *in vivo* on antigen-specific T cells post intranasal but not subcutaneous immunization. Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells. 7×10^6 congenic CD45.1⁺ CFSE labeled OT-II cells were transferred i.v. into each C57BL/6 animal. 1-hour post OT-II cell transfer, each animal was vaccinated with 100 μ g of LPS-free ova and 50 μ g of poly ICLC via either the intranasal or subcutaneous route of vaccine delivery. Four days following i.n. (top panels) or s.c. (bottom panels) immunization, the transferred cells were examined for the up-regulation of $\alpha 4\beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the draining LN (mediastinal and inguinal LN respectively), mesenteric LN, lung, spleen and blood. Figure 3.4 illustrates a representative experiment.

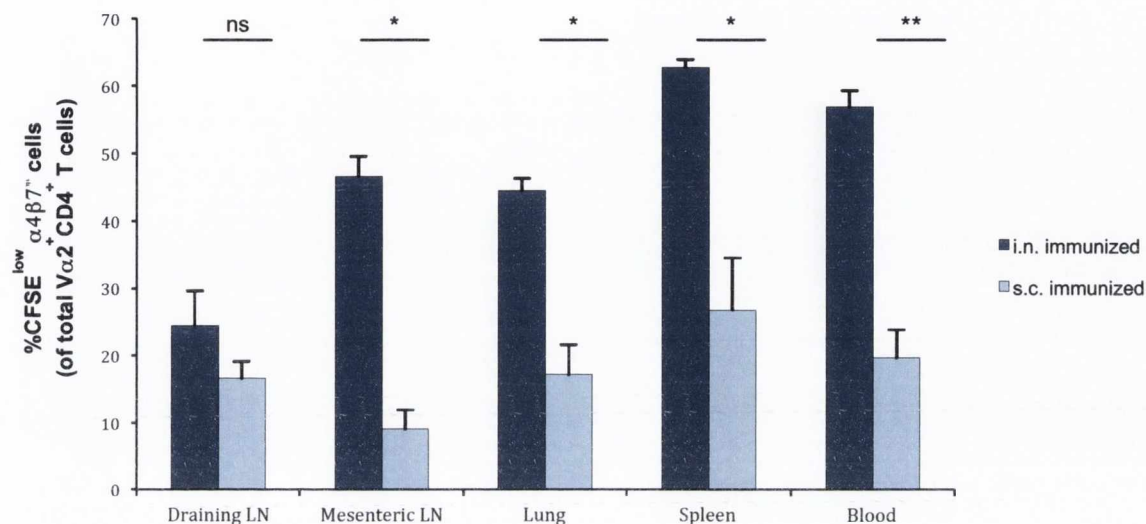


Figure 3.5: Enhanced levels of $\alpha 4\beta 7$ are detected on antigen specific T cells *in vivo* post intranasal but not subcutaneous immunization. Cumulative data from 3 experiments comparing the induction of $\alpha 4\beta 7$ on CD45.1⁺V α 2⁺CD4⁺OT-II cells (y- axis) between mice immunized i.n. (dark blue) and s.c. (light blue). 10x10⁶ congenic CD45.1⁺ CFSE labeled OT-II cells were transferred i.v. into each C57BL/6 animal. 1-hour post OT-II cell transfer, each animal was vaccinated with 100 μ g of LPS-free ova and 50 μ g of poly ICLC via either the intranasal or subcutaneous route of vaccine delivery. 4 days post vaccination the local lymph nodes draining the site of vaccine delivery were isolated and analyzed, in addition to other immune compartments. *p<0.05, **<p<0.01, ***p<0.001.

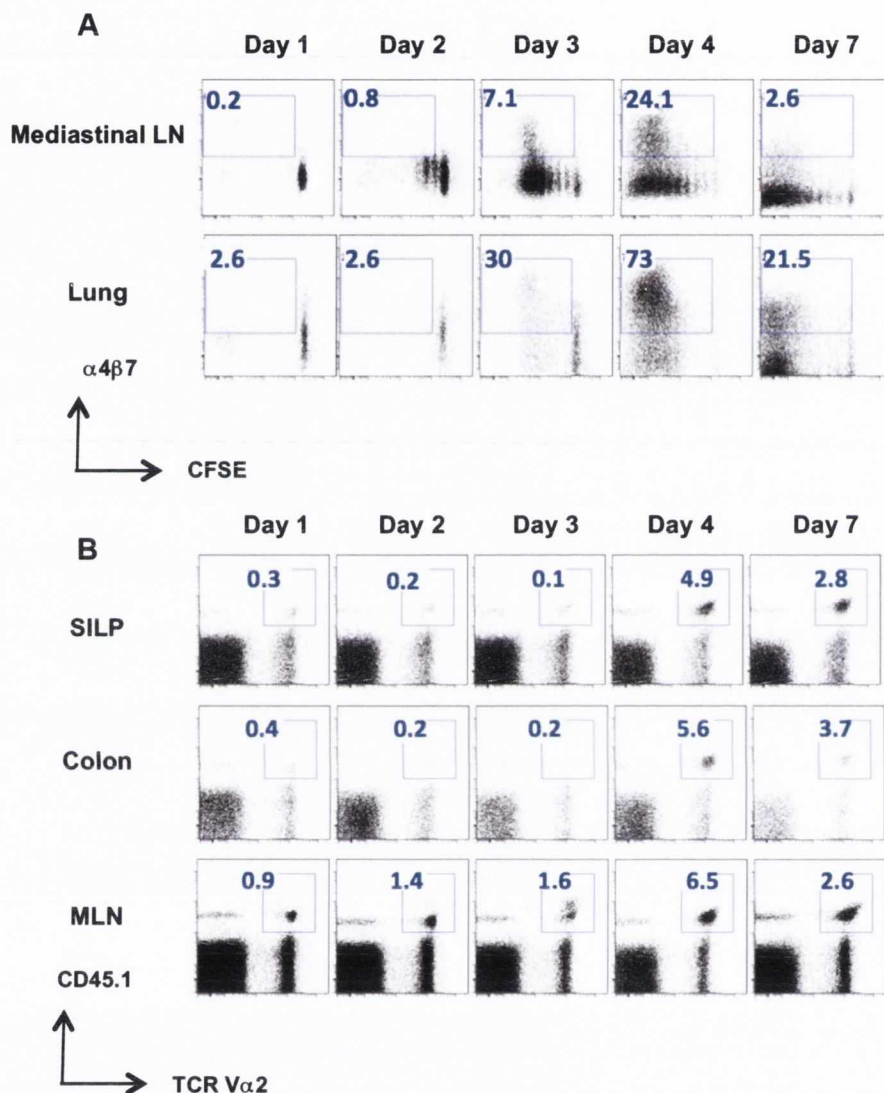


Figure 3.6: $\alpha 4\beta 7$ induction is detected *in vivo* on antigen-specific T cells 3 days post intranasal vaccination correlating with the appearance of transferred cells in the GI mucosa. (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells, following i.n. immunization with OVA (100 μ g) and polyICLC (50 μ g). The transferred cells were examined for the up-regulation of $\alpha 4\beta 7$ (y-axis) on proliferating (CFSE^{lo}, x-axis) cells in the draining LN (mediastinal LN) and lung. (b) Representative flow cytometry plots data revealing the migration of adoptively transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells to

the small intestinal lamina propria (SILP), colon and mesenteric LN following i.n immunization with OVA (100µg) and polyICLC (50µg) in a time course fashion.

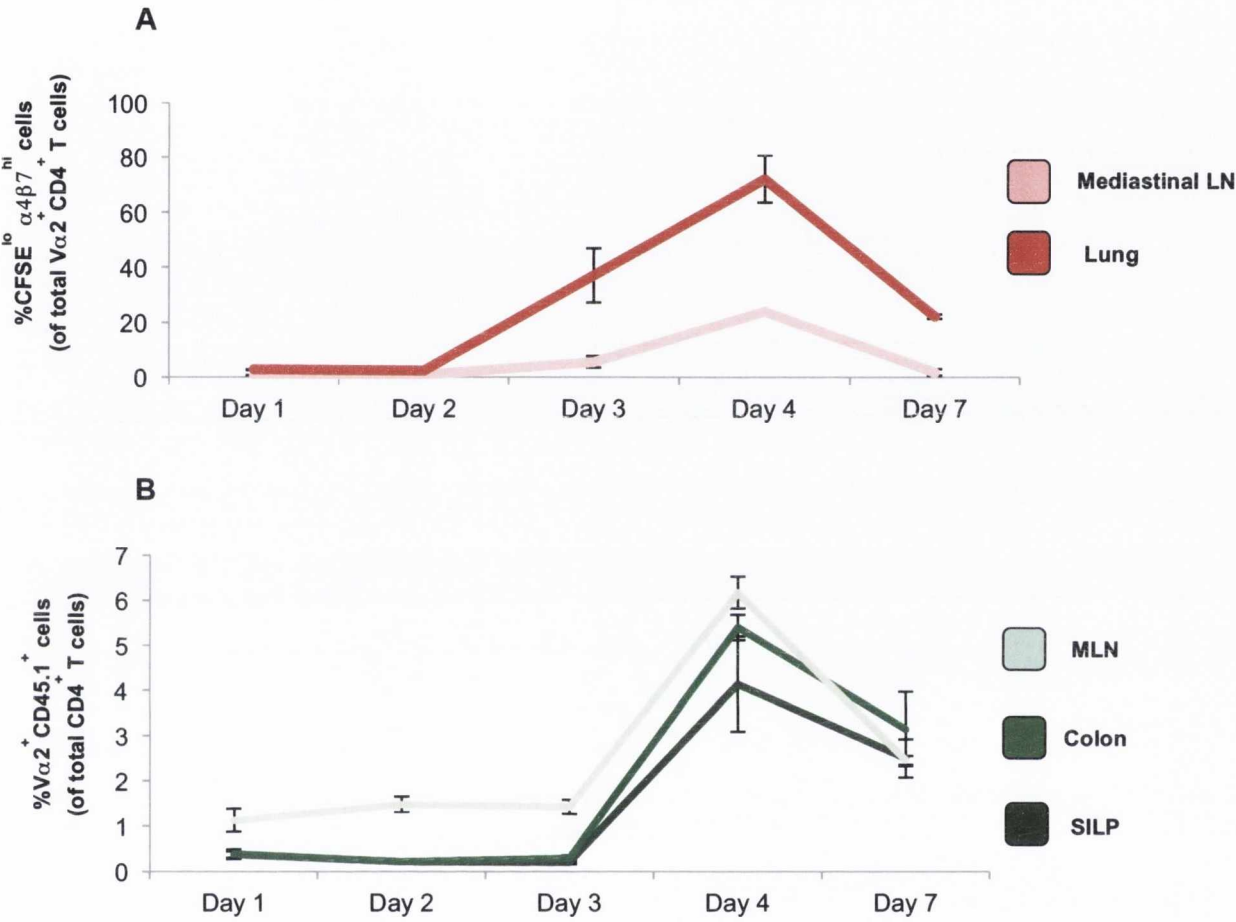


Figure 3.7: α4β7 induction is detected *in vivo* on antigen-specific T cells 3 days post intranasal vaccination correlating with the appearance of transferred cells in the GI mucosa. (a) Cumulative data showing the *in vivo* induction of integrin α4β7 on adoptively transferred CD45.1⁺Vα2⁺CD4⁺OT-II cells, following i.n. immunization with OVA (100µg) and polyICLC (50µg), the transferred cells were examined for the up-regulation of α4β7 (y-axis) on proliferating (CFSE^{lo}, x-axis) cells in the draining LN (mediastinal LN) and lung. (b) Cumulative data revealing the migration of adoptively transferred CD45.1⁺Vα2⁺CD4⁺OT-II cells to the small intestinal lamina propria (SILP), colon and mesenteric LN following i.n immunization with OVA (100µg) and polyICLC (50µg).

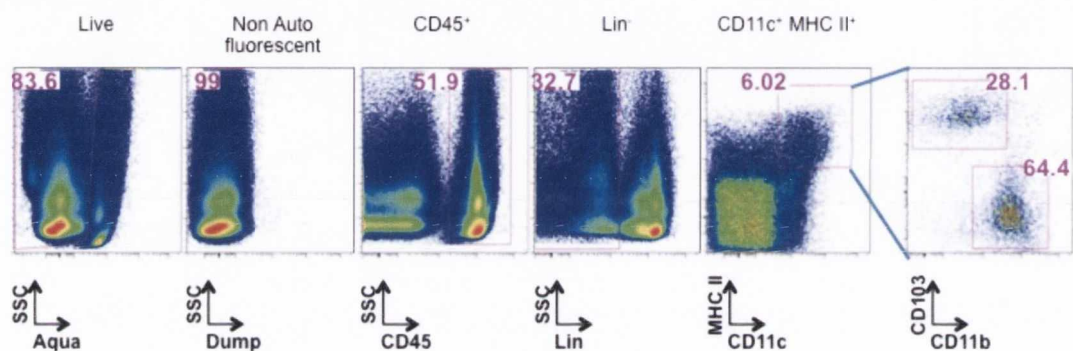


Figure 3.8: Gating strategy to sort lung Dendritic cells. Cells were identified on the basis of forward and side scatter. Dead cells and auto fluorescent cells were excluded. Hematopoietic (CD45⁺), lineage negative (CD3⁻B220⁻NK1.1⁻) cells were examined for the expression of CD11c and MHCII. CD11c^{high}MHCII⁺ cells were classified into CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DC subsets.

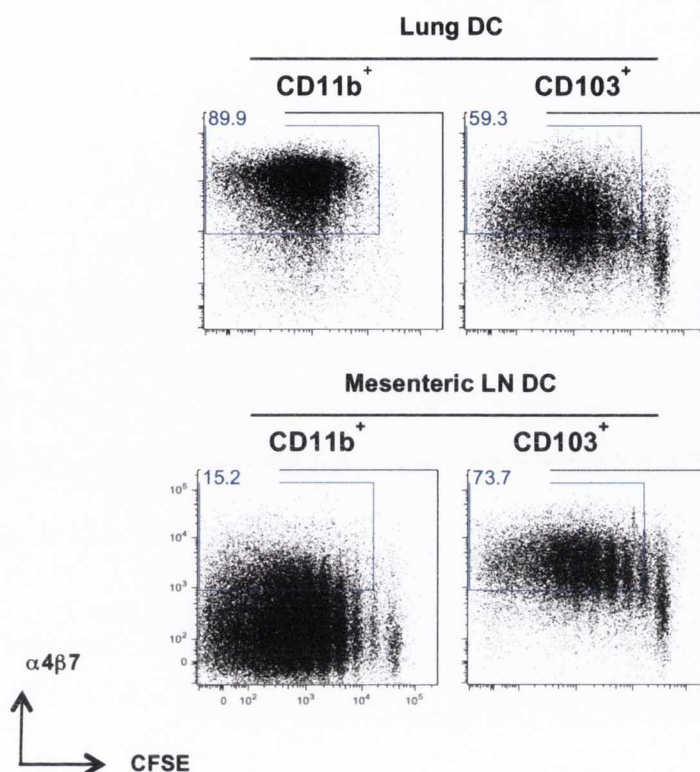


Figure 3.9: Murine lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ dendritic cell populations induce the expression of $\alpha 4\beta 7$ in an antigen-specific manner. Representative flow cytometry plots demonstrating the induction of integrin $\alpha 4\beta 7$ by lung derived MHCII⁺CD11c^{high}CD11b⁺CD103⁻ DCs (top left panel) and MHCII⁺CD11c^{high}CD11b⁻CD103⁺ DCs (top right panel). These individual DC populations were pulsed with 5 μ g of ova *ex vivo* before being cultured at a 1:2 ratio with CFSE labeled OT-II cells *in vitro*. 5 days after co-culture the T cells were isolated and $\alpha 4\beta 7$ expression was determined by flow cytometry. Figure 3.9 illustrates a representative experiment.

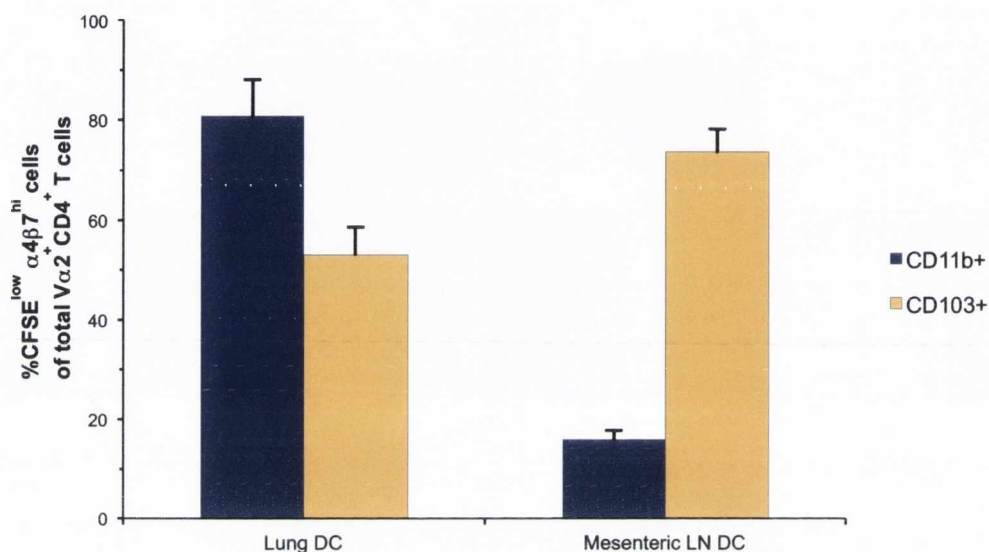


Figure 3.10: Murine lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ Dendritic Cell populations induce the expression of α4β7 in an antigen-specific manner. Cumulative data from 3 experiments comparing the percentage of CFSE^{low}α4β7^{high} cells induced by lung and mesenteric LN derived MHCII⁺CD11c^{high}CD11b⁺CD103⁻ (blue) and MHCII⁺CD11c^{high}CD11b⁻CD103⁺ (yellow) DC subsets. C57BL/6 mice were sacrificed and the mesenteric LNs and lungs removed (see methods). CD11c⁺ DCs were isolated using magnetic isolation (MACS beads sorting). The CD11c⁺ cells were subsequently sorted into CD103⁺CD11b⁻ and CD103⁻CD11b⁺ populations using the LSR II (2) Aria. These individual DC populations were pulsed with 5μg of ova *ex vivo* before being cultured at a 1:2 ratio with CFSE labeled OT-II cells *in vitro*. 5 days after co-culture the T cells were isolated and α4β7 expression determined by flow cytometry.

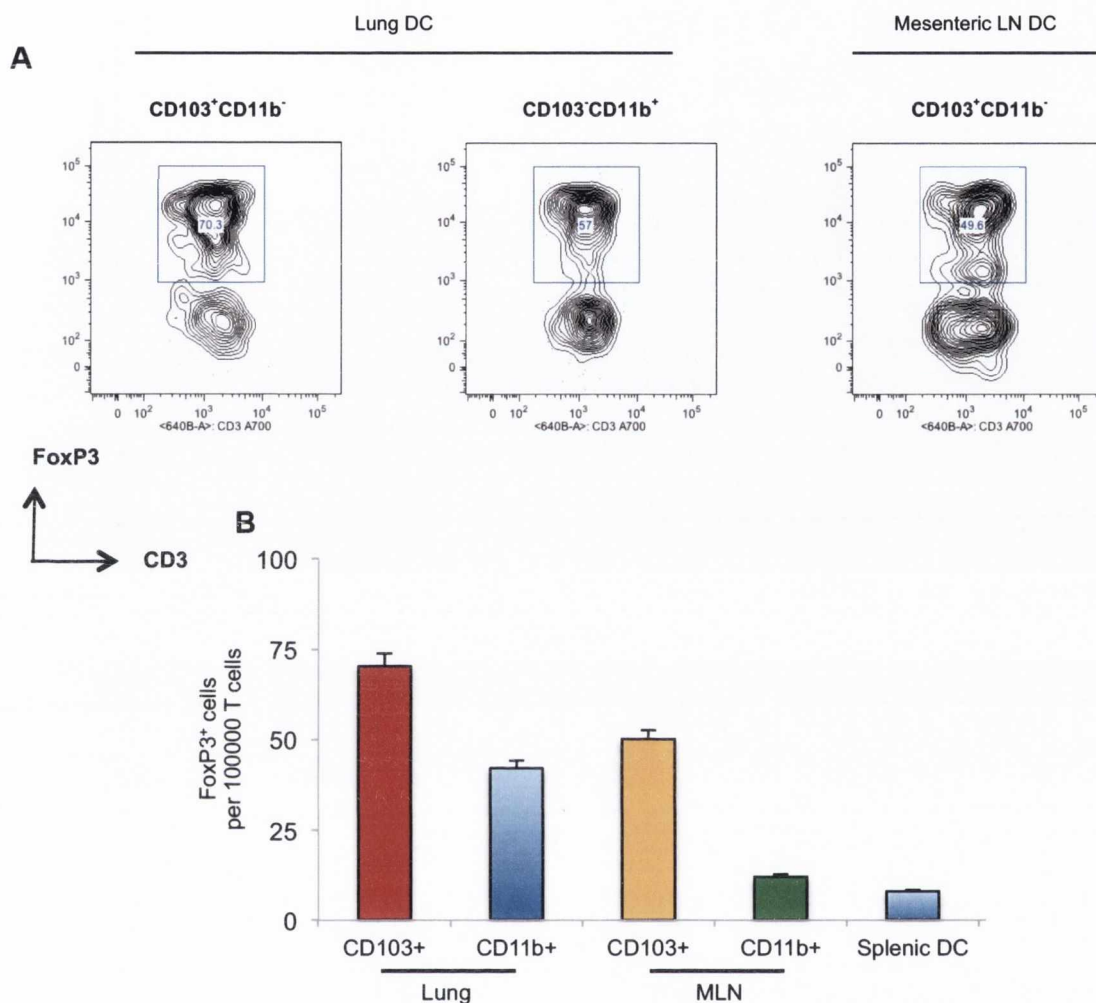


Figure 3.11: Both lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ dendritic cells induce the expression of Foxp3 in an antigen specific manner *in vitro*. C57BL/6 mice were sacrificed and the mesenteric LNs and lungs removed (see methods). CD11c⁺ DCs were isolated using MACS bead sorting. The CD11c⁺ cells were subsequently sorted into CD103⁺CD11b⁻ and CD103⁻CD11b⁺ populations using the LSR II (2) Aria. These individual DC populations were pulsed with 5μg of ova *ex vivo* before being cultured in a 1:2, 1:4 and 1:10 ratio with CFSE-labeled OT-II cells *in vitro*. 5 days after co-culture the T cells were isolated and Foxp3 expression determined by flow cytometry. Figure 3.11 (a) illustrates a representative experiment (b) cumulative data.

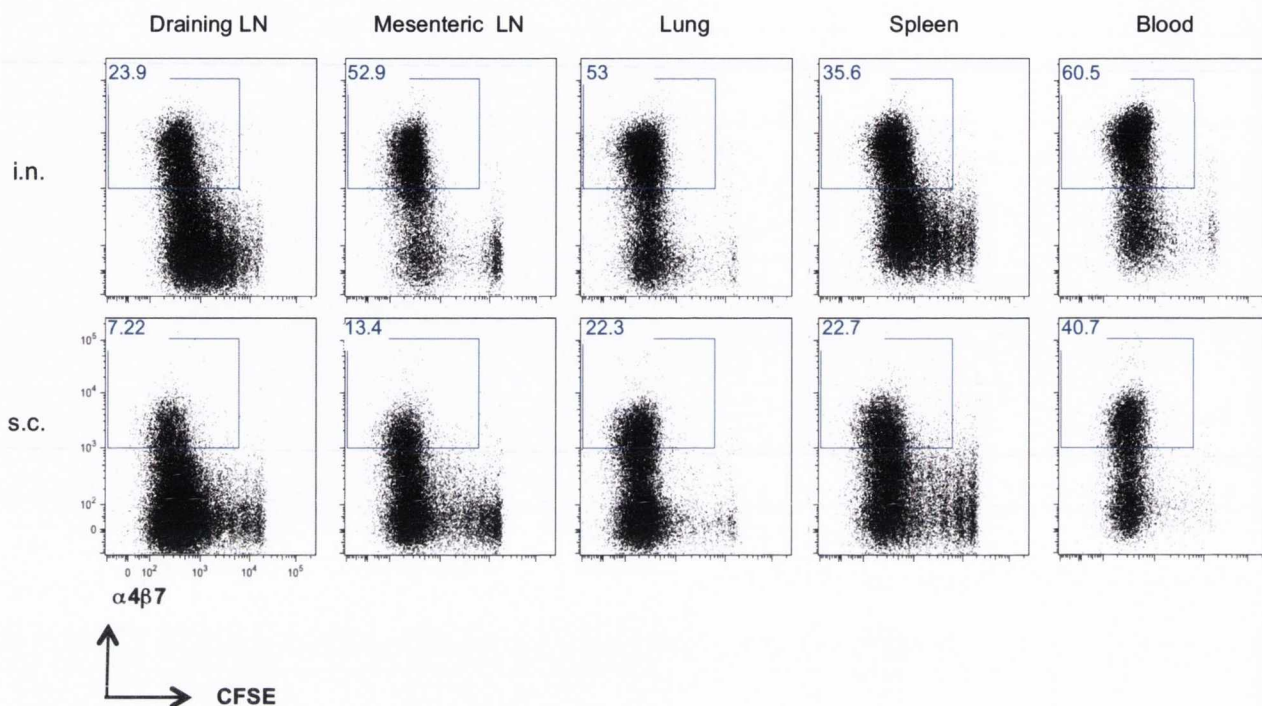


Figure 3.12: Enhanced levels of $\alpha 4 \beta 7$ are detected on OT-I antigen-specific T cells post intranasal but not subcutaneous immunization. Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4 \beta 7$ on adoptively transferred CD45.1⁺V α 2⁺CD8⁺OT-I cells. 10×10^6 congenic CD45.1⁺ CFSE labeled OT-I cells were transferred i.v. into each C57BL/6 mouse. 1-hour post OT-I cell transfer, each animal was vaccinated with 100 μ g of LPS-free ova and 50 μ g of poly ICLC via either the intranasal or subcutaneous route of vaccine delivery. Four days following i.n. (top panels) or s.c. (bottom panels) immunization, the transferred cells were examined for the up regulation of $\alpha 4 \beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the draining LN (mediastinal and inguinal LN respectively), mesenteric LN, lung, spleen and blood.

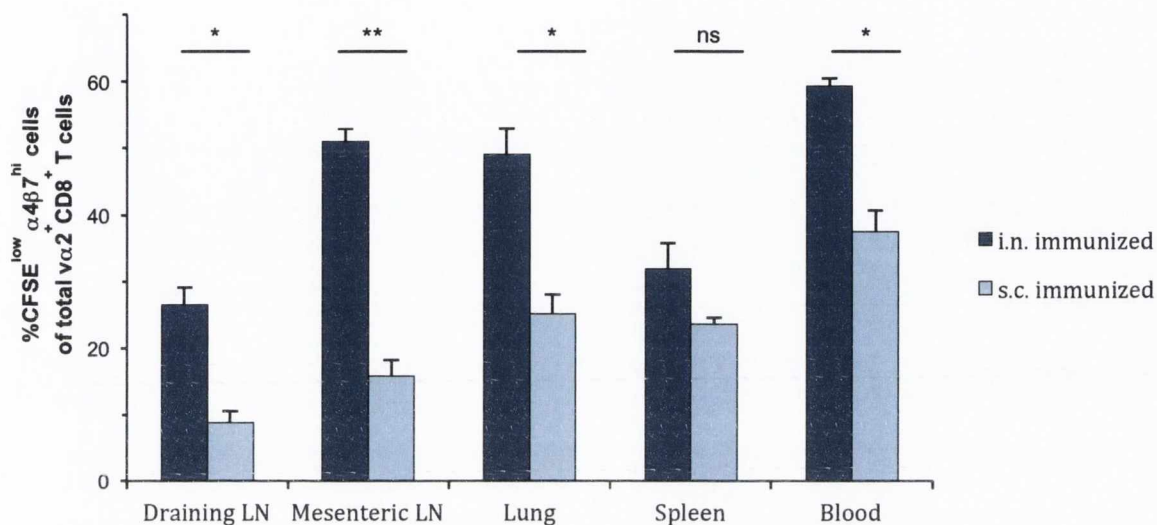


Figure 3.13: Enhanced levels of $\alpha 4\beta 7$ are detected on OT-I T cells post intranasal but not subcutaneous immunization. Cumulative data from 3 experiments comparing the induction of $\alpha 4\beta 7$ on $CD45.1^+V\alpha 2^+CD8^+OT-I$ cells (y- axis) between mice immunized i.n. (dark blue) and s.c. (light blue). 10×10^6 congenic $CD45.1^+$ CFSE labeled OT-I cells were transferred i.v. into each C57BL/6 animal. 1 hour post OT-I cell transfer, each animal was vaccinated with $100\mu g$ of LPS-free OVA and $50\mu g$ of poly ICLC via either the intranasal or subcutaneous route of vaccine delivery. 4 days post vaccination the local lymph nodes draining the sites of vaccine delivery, in addition to other immune compartments, were isolated and analysed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

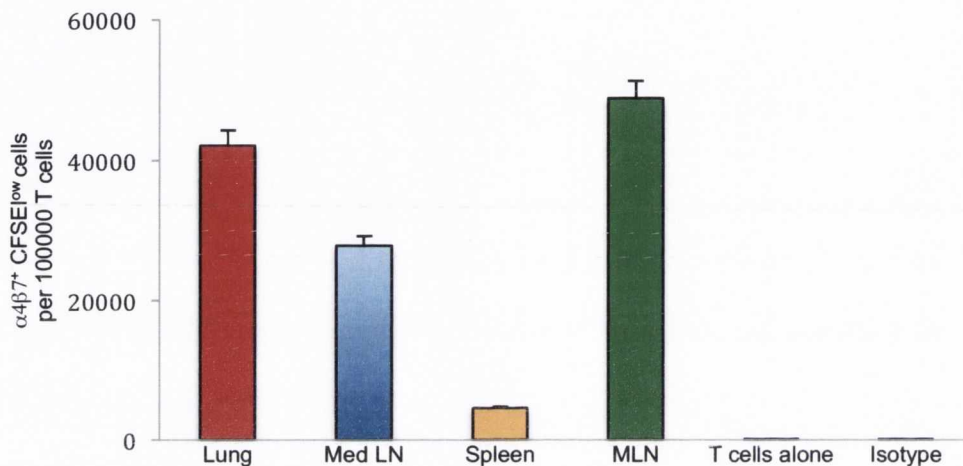


Figure 3.14: poly ICLC stimulated lung DCs induce the expression of $\alpha 4\beta 7$ on T cells *in vitro*.

Cumulative data from 3 individual experiments comparing CFSE^{low} $\alpha 4\beta 7^{\text{high}}$ cells per 100,000 T cells. C57BL/6 animals were immunized with Poly ICLC (50 μ g) and 24 hours later sacrificed. The spleen, lung, mediastinal LN and mesenteric LNs isolated (see methods). CD11c⁺ DCs were isolated using MACS bead sorting and pulsed with 5 μ g of ova *ex vivo* before being cultured at a 1:2, 1:4 and 1:10 ratio with CFSE labeled OT-II cells *in vitro*. 5 days after co-culture the T cells were isolated and $\alpha 4\beta 7$ expression determined by flow cytometry

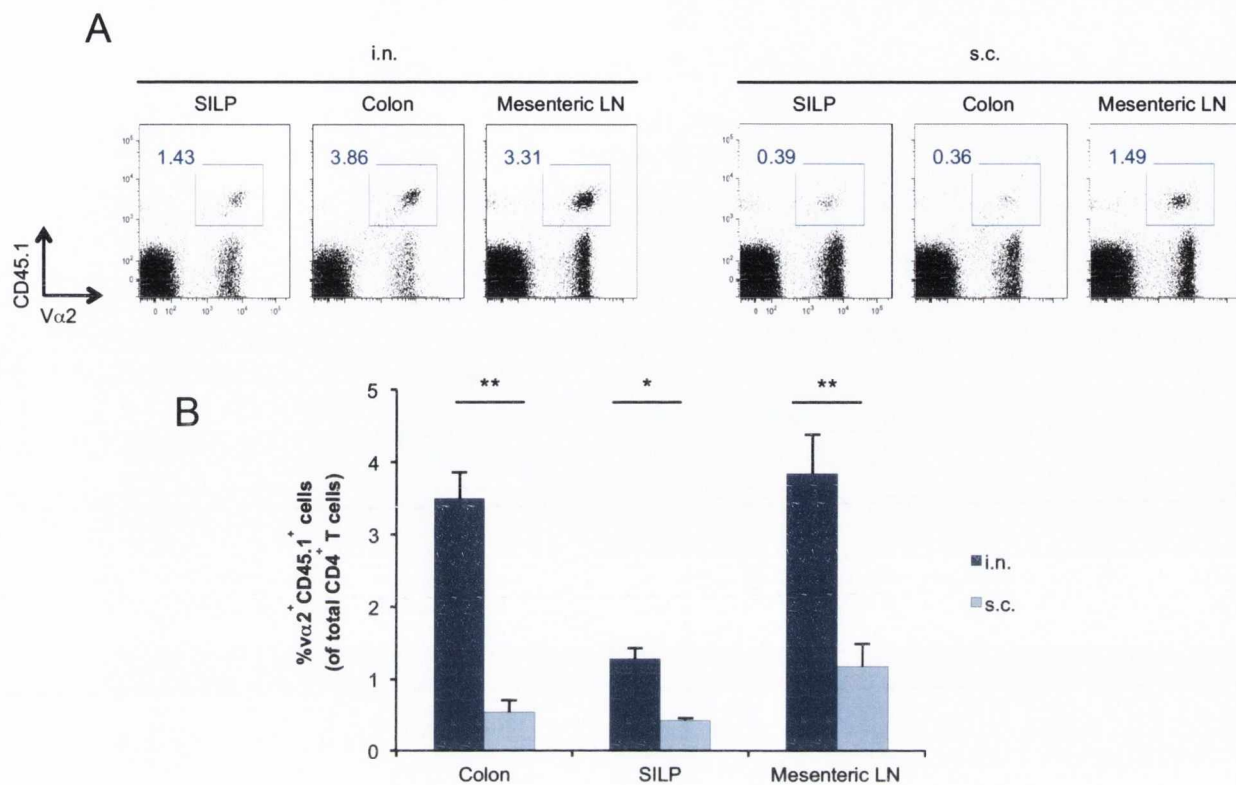


Figure 3.15: Intranasal vaccination results in enhanced migration of antigen specific T cells to the gastrointestinal tract. (a) Representative flow cytometry plots comparing the migration of adoptively transferred CD45.1⁺Vα2⁺CD4⁺OT-II cells to the small intestinal lamina propria (SILP), colon and mesenteric LN following i.n. (left three panels) and s.c. immunization (right three panels). (b) Cumulative data comparing the migration of CD45.1⁺Vα2⁺CD4⁺OT-II cells to the intestinal tissues following i.n. (dark blue) and s.c. (light blue) immunization. 7x10⁶ congenic CD45.1⁺ CFSE labeled OT-II cells were transferred i.v. into each C57BL/6 mouse. 1 hour post OT-II cell transfer, each animal was vaccinated with 100μg of LPS-free ova and 50μg of poly ICLC via either the i.n. or s.c. route of vaccine delivery. 7 days post vaccination the gastrointestinal immune inductive and effector sites were analyzed. *p<0.05, **<p<0.01, ***p<0.001.

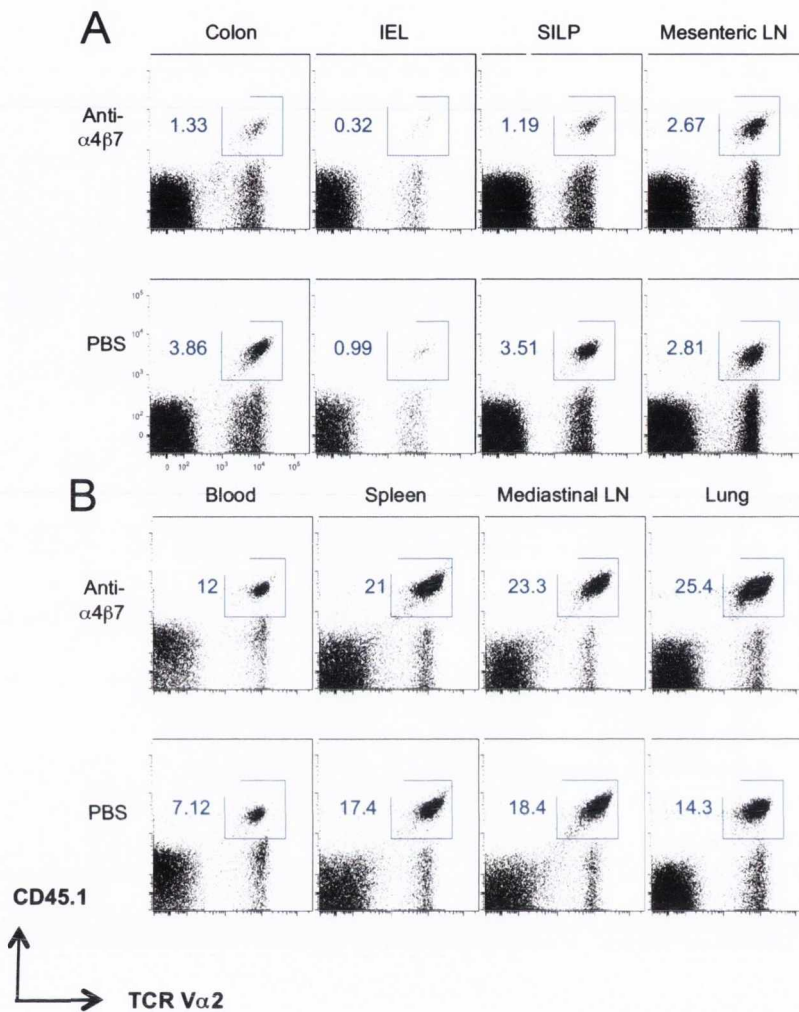


Figure 3.16: Enhanced migration of antigen-specific T cells to the gastrointestinal tract post intranasal vaccination is dependent on $\alpha 4\beta 7$. (a) Intestinal Compartments (b) Systemic Compartments. 100 μ g/mouse of anti- $\alpha 4\beta 7$ or PBS (BD Pharmingen) was administered i.p to naïve C57BL/6 on day -1 and day 2. On day 0 7×10^6 congenic CD45.1 $^+$ V $\alpha 2^+$ CD4 $^+$ OT-II CFSE-labeled cells were transferred i.v. into each C57BL/6 animal. 1-hour post OT-II cell transfer, each animal was i.n. vaccinated with 100 μ g of LPS-free ova and 50 μ g of poly ICLC. 7 days post vaccination the gastrointestinal and systemic immune inductive and effector sites were analyzed. Figure 3.16 illustrates a representative experiment.

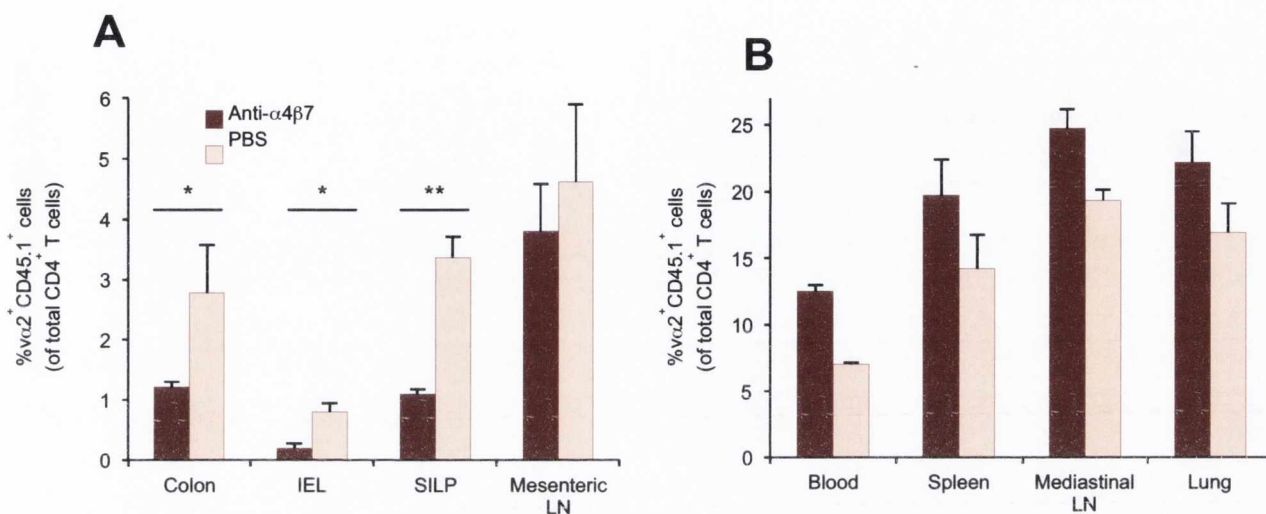


Figure 3.17: Enhanced migration of antigen specific T cells to the gastrointestinal tract post intranasal vaccination is dependent on $\alpha 4\beta 7$. Cumulative data from 3 experiments comparing mice where anti- $\alpha 4\beta 7$ antibody (dark brown) or PBS (light brown) was administered following i.n. immunization with OVA (100 μ g) plus polyICLC (50 μ g). The percentage of the transferred V $\alpha 2^+$ CD45.1⁺ cells (of total CD4⁺T cells), y-axis is compared between different tissues (x-axis). 100 μ g/mouse of anti- $\alpha 4\beta 7$ or PBS (BD Pharmingen) was administered i.p to naïve C57BL/6 on day -1 and 2. 7 days post vaccination the (a) gastrointestinal and (b) systemic immune inductive and effector sites were analysed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

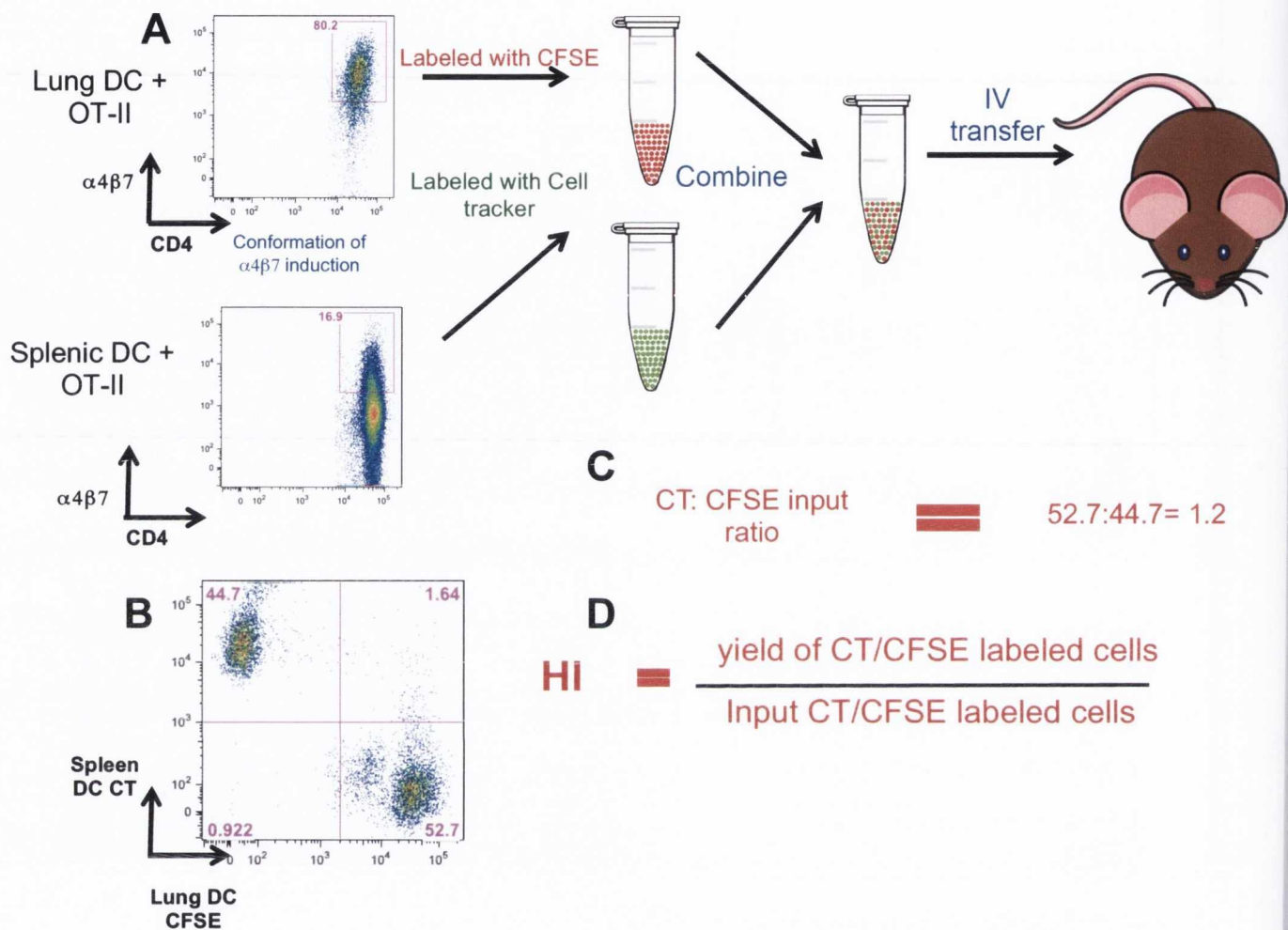


Figure 3.18: Strategy for homing index calculations. C57BL/6 were sacrificed and the spleen and lung removed (see methods). CD11c⁺ DCs were isolated via MACS beads sorting. (a) Lungs and splenic DCs were isolated and pulsed with ova *ex vivo* before being cultured with OTI-II cells in vitro. 7 days after co-culture the T cells were isolated and $\alpha 4\beta 7$ induction determined. Lung stimulated OT-II cells were labelled with CFSE, while splenic DC stimulated OT-II cells were labelled with cell tracker (CT)(Invitrogen). Equal ratios of Lung-simulated and splenic-stimulated OT-II cells were admixed and i.v. injected into naïve mice (b). A small sample of labelled OT-II cells were isolated prior to i.v. transfer and the input ratio determined by flow cytometry. (c) The CT:CFSE ratio was determined. (d) Equation utilized to calculate the homing index post transfer.

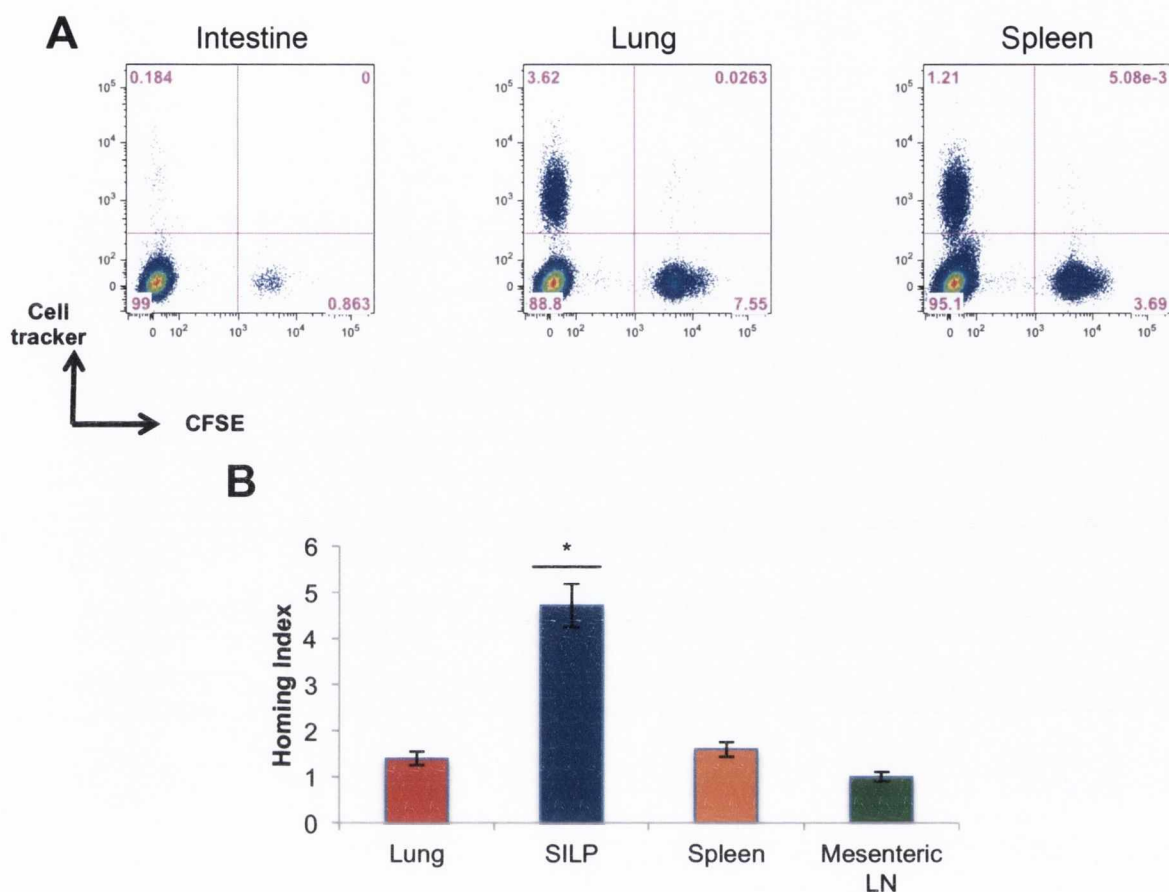


Figure 3.19: Lung DC stimulated transgenic T cells have a 5 fold higher propensity to migrate to the gastrointestinal tract compared to splenic DC stimulated T cells. C57BL/6 mice were sacrificed and the spleens and lungs were removed (see methods). CD11c⁺ DCs were isolated via MACs bead sorting. (a) Lung and splenic DCs were isolated and pulsed with Ova *ex vivo* before being cultured with OT-II cells *in vitro*. 7 days post co-culture commencement the T cells were isolated, subsequently labelled and $\alpha 4\beta 7$ induction determined. Lung DC stimulated OT-II cells were labelled with CFSE, while splenic DC stimulated OT-II cells were labelled with cell tracker (CT)(Invitrogen). Equal ratios of lung DC- and Splenic DC- stimulated OT-II cells were admixed and i.v. injected into naïve mice. 16 hour post transfer, the spleen, lung, SILP and MLN were analysed. (b) Cumulative data from two experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

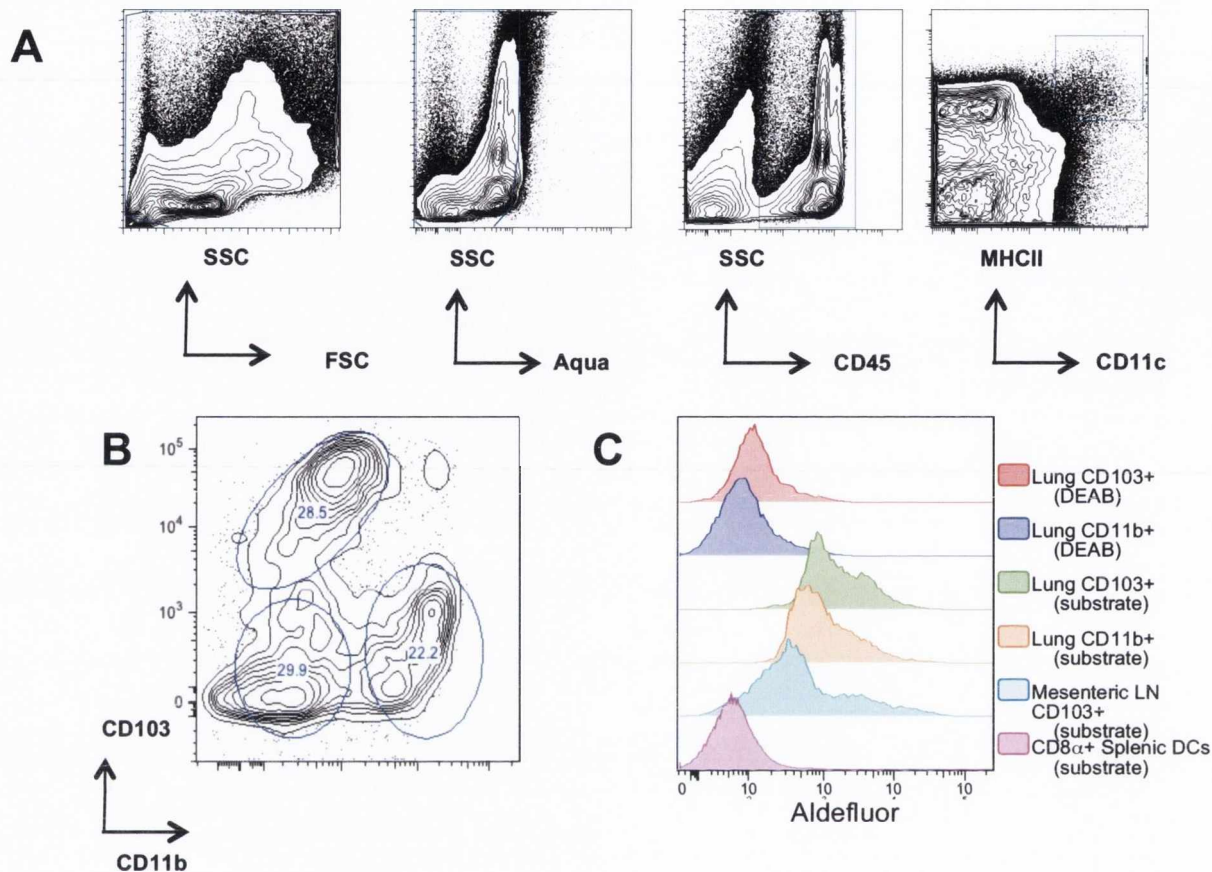


Figure 3.20: Identification of ALDH activity in lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ dendritic cells. Demonstration of aldehyde dehydrogenase (ALDH) activity in lung-derived DCs using flow cytometry. The MFI of a fluorescent ALDH substrate was quantified for lung DCs in the presence (top two curves) or absence (middle two curves) of the ALDH inhibitor-DEAB. Mesenteric LN CD103⁺ DCs and splenic CD8 α ⁺ DCs served as the positive and negative controls respectively (bottom two curves). C57BL/6 animals were sacrificed and the mesenteric LNs, spleen and lungs removed (see methods). CD11c⁺ DCs were isolated via MACS beads sorting. (a) In order to identify the resident lung DCs, the gating strategy above was adopted based on lineage positive cells (CD45), MHCII high cells, CD11c high and (b) CD103 and CD11b expression. (v) The cells were incubated with ALDEFLUOR and analyzed by flow cytometry.

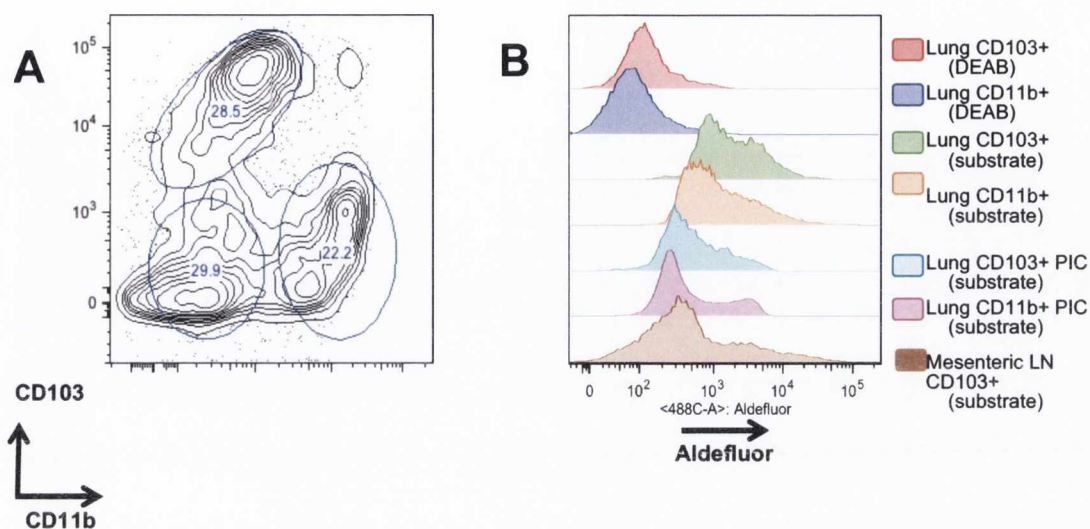


Figure 3.21: Identification of ALDH activity in lung resident CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DCs post poly IC administration.

Demonstration of aldehyde dehydrogenase (ALDH) activity in lung derived DCs following adjuvant administration using flow cytometry. The MFI of a fluorescent ALDH substrate was quantified for lung DCs in the presence (top two curves) or absence (middle two curves) of the ALDH inhibitor-DEAB. Mesenteric LN CD103⁺ DCs served as the positive controls (bottom curve). C57BL/6 animals were immunized i.n. with poly ICLC (50µg/ml), 24hrs later they were sacrificed and the mesenteric LNs and lungs removed (see methods). CD11c⁺ DCs were isolated via MACS beads sorting. In order to identify the resident lung DCs, the gating strategy above was adopted based on lineage positive cells (CD45), MHCII high cells, CD11c high (see Figure 3.15A) and (a) CD103 and CD11b expression. (b) The cells were incubated with ALDEFLUOR and analyzed by flow cytometry.

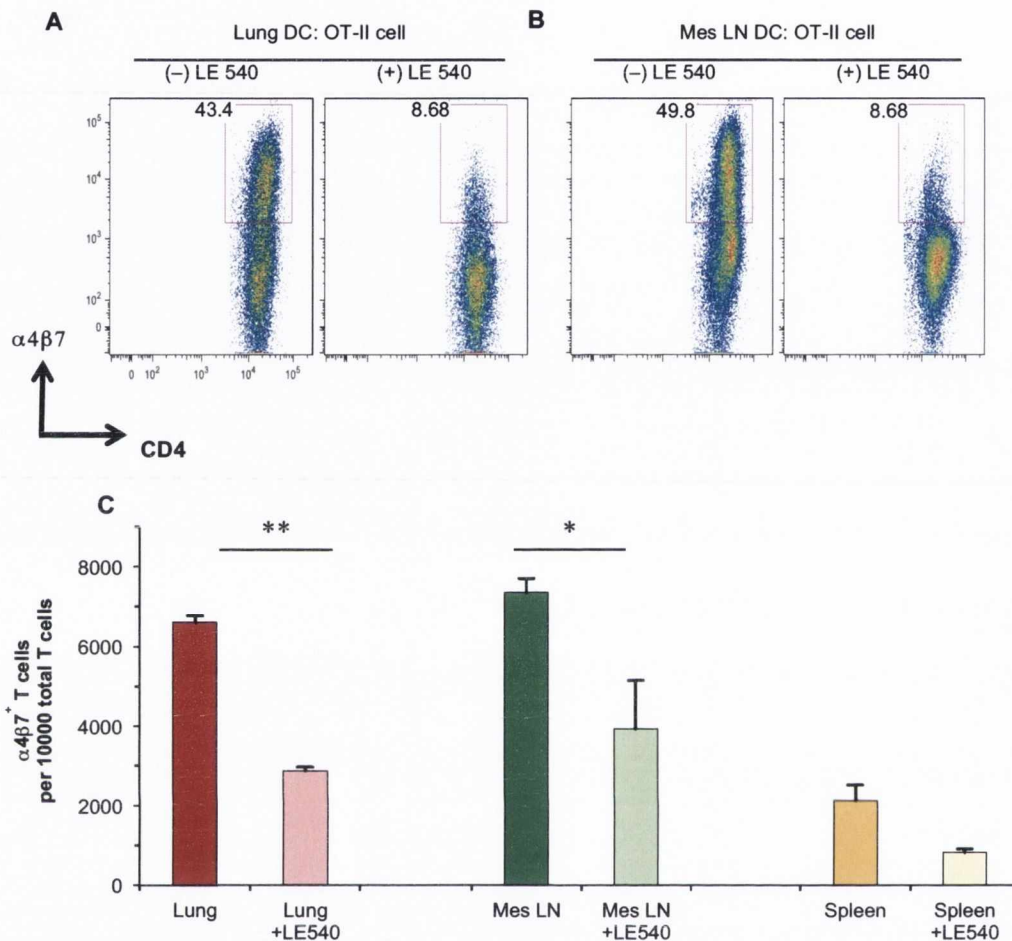


Figure 3.22: $\alpha 4\beta 7$ induction by lung dendritic cells is dependent on retinoic acid signaling. (a,b) Representative flow cytometry plots and (c) cumulative data from three experiments, demonstrating the inhibition of lung DC mediated $\alpha 4\beta 7$ induction on OT-II cells by the RAR- β antagonist LE-540 (1 μ M) (DC:T cell ratio- 1:2). Mesenteric LN derived DCs served as the positive control (DC:T cell ratio- 1:2). C57BL/6 mice were sacrificed and their mesenteric LNs, spleens and lungs removed (see methods). CD11c⁺ DCs were isolated by MACS bead sorting. (a) Lung DCs and (b) Mesenteric LN DCs were pulsed with 5 μ g of ova *ex vivo* prior to being cultured at a 1:2 ratio with CFSE-labeled OT-II cells in vitro. 5 days after co-culture the T cells were isolated and $\alpha 4\beta 7$ expression determined by flow cytometry. *p<0.05, **p<0.01, ***p<0.001.

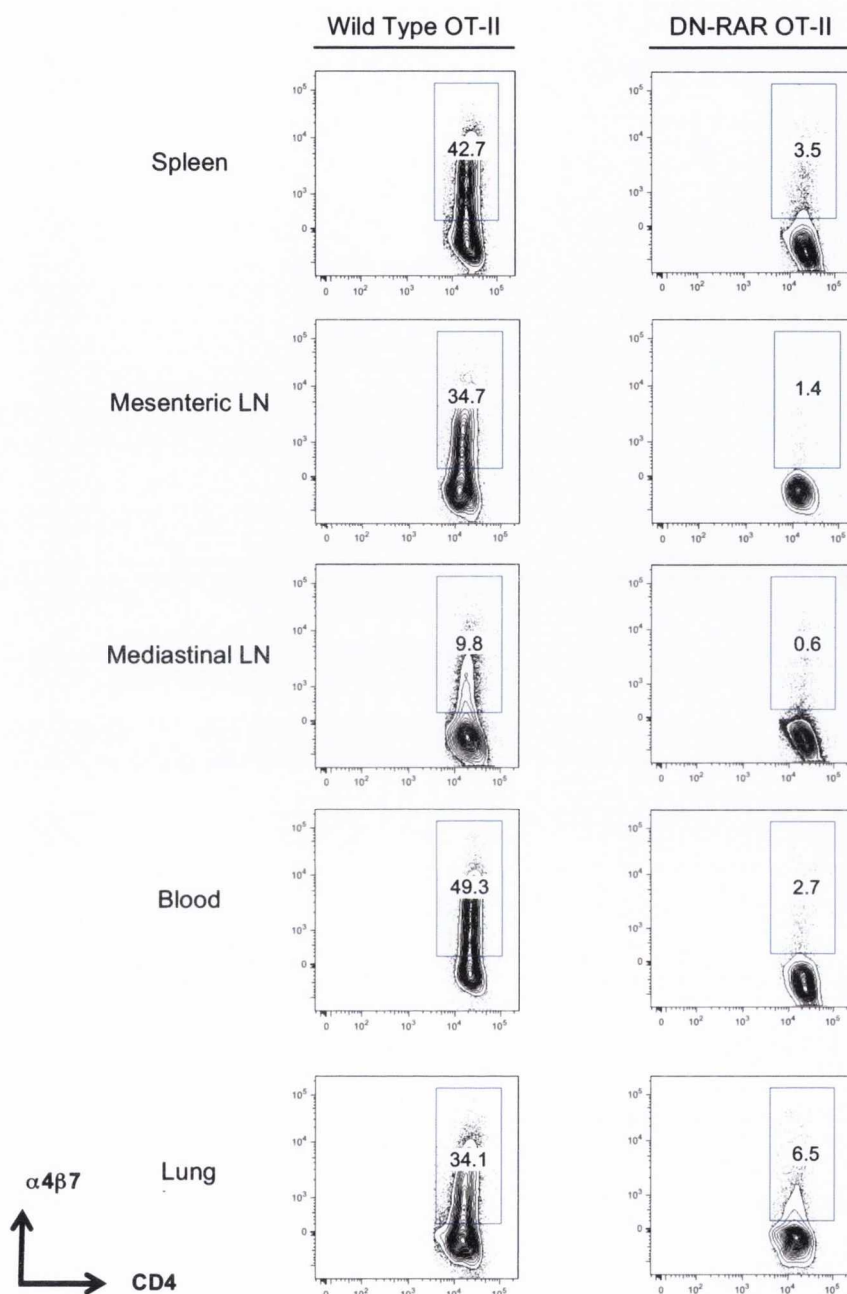


Figure 3.23: $\alpha 4\beta 7$ induction by lung resident dendritic cells is dependent on retinoic acid signalling *in vivo*. Representative flow plots demonstrating the induction of $\alpha 4\beta 7$ on WT $V\alpha 2^+CD4^+$ OT-II cells or $dnRAR^+V\alpha 2^+CD4^+$ OT-II cells following i.n. vaccination. 10×10^6 congenic $CD45.1^+$ OT-II and DN-RAR OT-II cells were transferred i.v. into each C57BL/6 mouse. 1-hour post OT-II cell transfer, each animal was vaccinated with $100\mu g$ of LPS-free ova and $50\mu g$ of poly ICLC i.n. 4 days post

vaccination the local lymph nodes draining the sites of vaccine delivery were isolated and analysed were isolated and analysed.

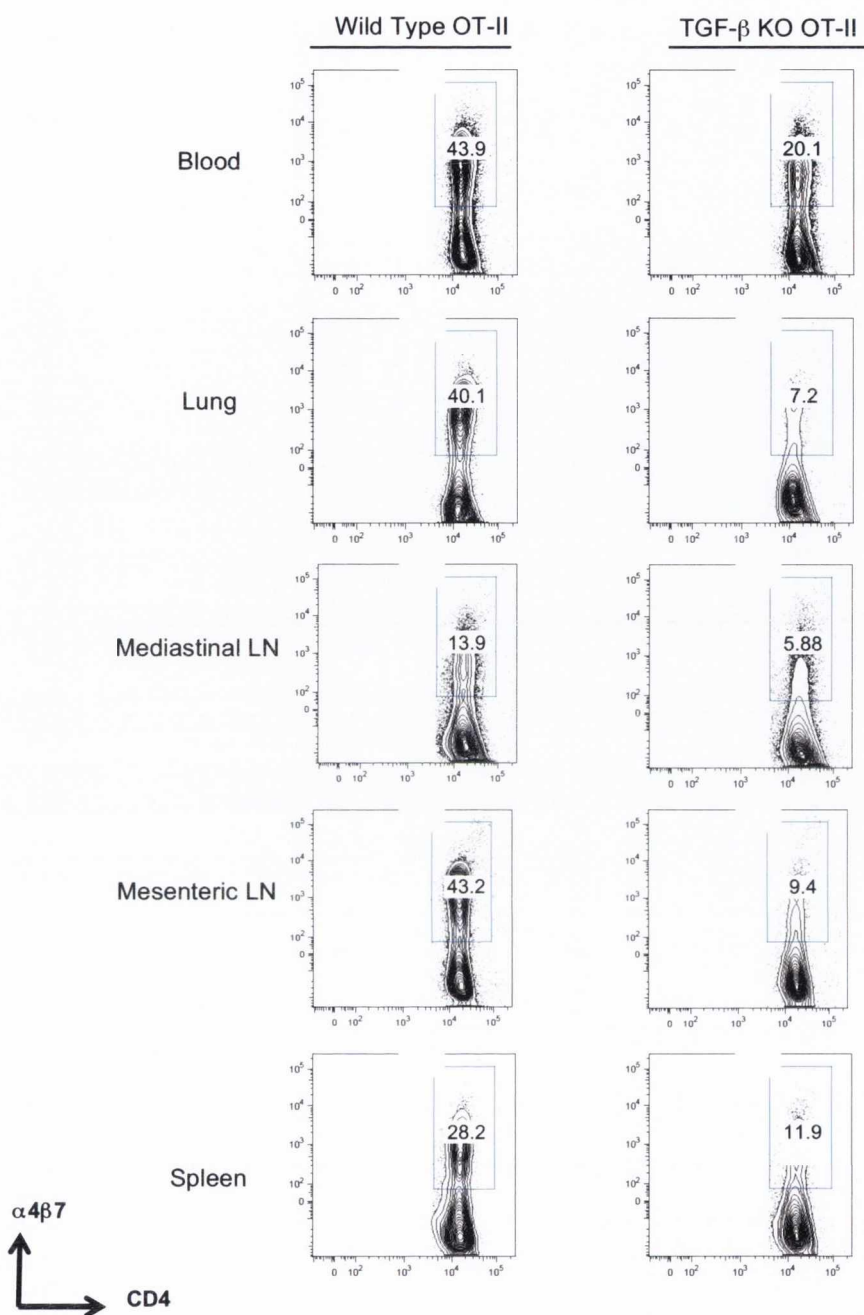


Figure 3.24: $\alpha 4\beta 7$ induction by lung resident dendritic cells is dependent on TGF- β signalling *in vivo*. Representative flow plots demonstrating the induction of $\alpha 4\beta 7$ induction on WT $V\alpha 2^+CD4^+$ OT-II cells or TGF- $\beta^{P_{def}}$ $V\alpha 2^+CD4^+$ OT-II cells following i.n. vaccination 10×10^6 congenic $CD45.1^+$ OT-II and TGF- β KO cells were transferred i.v. into each C57BL/6 animal. 1-hour post OT-II cell transfer, each animal was vaccinated with $100\mu g$ of LPS-free ova and $50\mu g$ of poly ICLC via either the intranasal or subcutaneous route of vaccine delivery. 4 days post vaccination the local lymph nodes

draining the sites of vaccine delivery, in addition to other immune compartments were isolated and analysed.

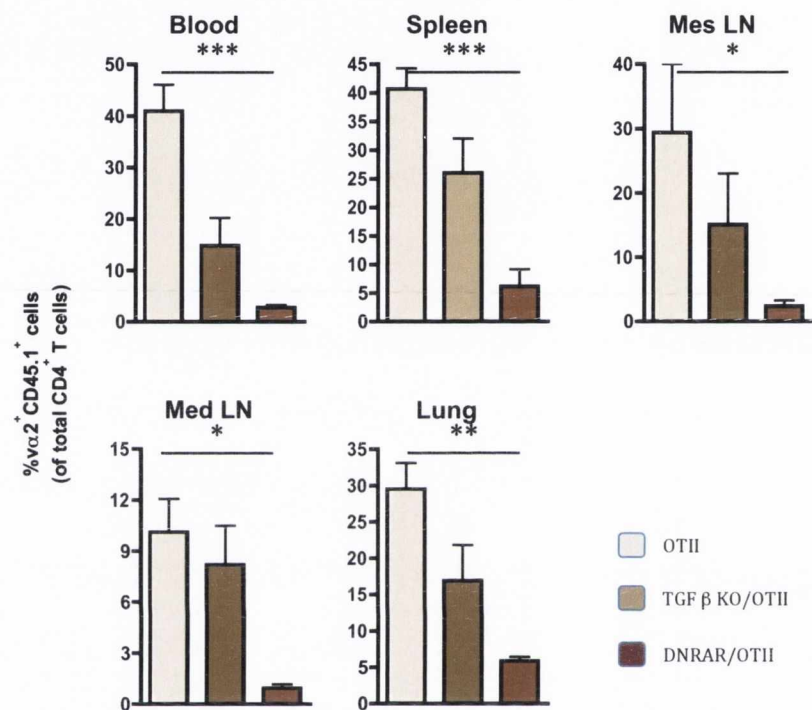


Figure 3.25: $\alpha 4\beta 7$ induction by lung resident dendritic cells is dependent on retinoic acid and TGF- β signalling *in vivo*. Cumulative data from three individual experiments revealing the percentage of CFSE^{low} $\alpha 4\beta 7^{\text{high}}$ cells (y-axis) is compared between mice that were immunized i.n. with ova (100 μ g) plus polyICLC (50 μ g) following the adoptive transfer of WT V $\alpha 2^+$ CD4⁺OT-II cells or, TGF- β P^{def}V $\alpha 2^+$ CD4⁺OT-IIcells or, dnRAR⁺V $\alpha 2^+$ CD4⁺OT-II cells into WT congenic hosts. 10x10⁶ congenic CD45.1⁺ OT-II or DN-RAR OT-II or TGF- β KO OT-II cells were transferred i.v. into each C57BL/6 animal. 1-hour post OT-II cell transfer, each animal was vaccinated with the i.n. (ova (100 μ g) plus polyICLC (50 μ g)) 4 days post vaccination the local lymph nodes in addition to other immune compartments were isolated analysed. *p<0.05, **<p<0.01, ***p<0.001

Chapter 4

Protective, gut homing cells induced by intranasal immunization
and the role of CD11b⁺ Lung Dendritic Cells.

4.1 Introduction:

The epithelial lining of mucous membranes covers an area of 200 square meters in an adult, or 100 times the area of the skin (Hall, Clare et al. 2010). These mucosal surfaces are found throughout the gastrointestinal, respiratory and urogenital tracts. In the skin, several layers of cells, including stratified epidermis, and dermis, generate a physical barrier that separates the internal components of the body from the outside world (Reis and Mucida 2012). However, in the intestine and other mucosal sites, a single layer of absorptive epithelial cells forms an interface between the lumen and the lamina propria, these surfaces are protected from external pathogenic insult by physicochemical defence strategies and innate and adaptive defences (Reis and Mucida 2012).

Effective immune responses generated at mucosal surfaces can range from - antibody-mediated pathogen exclusion or neutralization, to complex antibody and cellular interactions. While there are shared similarities with the systemic immune system, a particular hallmark of immune responses at the luminal mucosal surface is the production and secretion of IgA (Brandtzaeg P. 1997). The fact that mucosal immunization or infection at one tissue site can provide secretory protective IgA production at a distant mucosal surface provided the foundation for the concept of an interconnected “common mucosal immune system” (Mestecky J. 1987). More recently, this has been refined to a “compartmentalized” or “integrated” mucosal immune system, as not all mucosal surfaces seemed to be interconnected (Brandtzaeg P. 1997). It had been postulated that some of these connections might be related to the anatomical differences between the mucosal surfaces. While the mucosa-specific migration patterns (previously discussed in **Chapter 3**) are well established for mucosal tissues of the GI tract, they are less well defined for other mucosal sites that often show mixed migration patterns (Pedersen and Cox 2012).

Since its introduction in the 19th century, vaccination has had a profound effect on mortality and morbidity rates (Steinman 2008). Vaccination designs have progressed from the use of killed whole cell vaccines and live attenuated organisms to specific

antigenic components of viruses and bacteria (Steinman 2008). Despite the effective utilization of vaccination against a number of pathogens including Polio and *Bordetella pertussis*, effective vaccines against other highly infectious agents such as HIV and malaria have yet to be developed.

Mucosal vaccination offers many attractive features including the ease of administration, potential for mass immunization, reduced cost of production, storage and delivery (Fujikuyama, Tokuhara et al. 2012). Nevertheless, clinical vaccine research has largely been based on systemic administration of antigens with only a few mucosal vaccines approved for human use (Meeusen E 2011). The reasons for this apparent disconnect between the potential benefits of mucosal vaccination and the few available vaccines are many-fold, the major one being the induction of oral tolerance (the active suppression of immune responses to antigens delivered via the oral route) (Fujikuyama, Tokuhara et al. 2012), as well as the fact that optimal doses of mucosally delivered antigens are difficult to establish; proteolytic enzymes in digestive secretions necessitate the need to protect antigens administered via a mucosal route; and the acceptability of reactogenicity of potent adjuvants critical to mounting a mucosal immune response, is also a limiting factor (Fujikuyama, Tokuhara et al. 2012).

Various routes of inoculation have been employed to induce mucosal immune response, including oral (Masuet Aumatell, Ramon Torrell et al. 2011), intranasal (Dormitzer, Tsai et al. 2012), sublingual (Czerkinsky et al. 2011), intrarectal (Bolton, Song et al. 2012), intravaginal (Li, Lu et al. 2012) transcutaneous (Lawson, Clements et al. 2012) and the targeting iliac lymph nodes (Lehner, Wang et al. 1996). A study by Belyakov *et al*, highlighted the benefits of mucosal vaccination when demonstrating that mucosally (but not systemically) immunized mice were protected against infection after mucosal challenge with a recombinant vaccinia virus expressing HIV glycoprotein 160 (gp160) (Belyakov and Ahlers 2012) (Belyakov, Ahlers et al. 2008). In another study by the same group, robust CD8⁺ T cell responses were detected in the mucosal and systemic compartments in mice that received the vaccine intra-rectally. In contrast, systemic vaccine administration generated systemic immune responses but did not produce

protective CD8⁺ T cell responses in the mucosa. Mucoadhesive formulations, liposomes and selectively engineered micro/nanoparticles have been proposed to serve as delivery vehicles for oral vaccination. Translocation across the epithelial layer can also be effectively achieved when antigens are co-administered or linked to bacterial enterotoxins or incorporated into live mucosal vectors (Pasetti MF, 2011). While these strategies have been effective for oral delivery of subunit vaccines in small animal models, most have not been translated into clinical practice due predominantly to safety concerns. Oral delivery of vaccine antigens in large animal species and man also poses enormous limitations with respect to vaccine stability and dilution through the gastrointestinal tract. To date, the only killed oral vaccine on the market for humans (Dukoral ®) is a whole-cell/recombinant CTB subunit vaccine against cholera (Wiedermann U, 2006). A significant obstacle is that a clear understanding of the priming of antigen-specific T cell responses in the gastrointestinal tract is lacking.

Phobia of needles is often cited as an obstacle to vaccination in both infants and adults. Therefore less invasive routes of vaccine administration may increase vaccine acceptance and compliance. FluMist, an intranasally delivered live-attenuated form of the influenza virus, is the only intranasally administered vaccine on the market. Apart from providing a less invasive method of vaccination, intranasal vaccines offer additional immunological and potentially protective advantages. Intranasal immunization has a long history, having been used as a route of variolation in ancient times. It has attracted increasing attention because of a reported higher efficacy in inducing mucosal immune responses than systemic vaccination (Berzofsky, Ahlers et al. 2004). Although effective immunization of rodents by the i.n. route was considered to be the result of stimulating of NALT, data supporting these findings is limited (Wu et al. 1993). This is a favored route of mucosal immunization as other routes such as intrarectal or intravaginal would be impractical in humans.

Results from Chapter 3 have shown that lung resident DCs can recruit T cells to the GI tract, challenging the current dogma that only gut CD103⁺ DCs have this capacity. Rather, evidence was provided showing that lung DC, targeted by i.n. immunization, are

capable of inducing robust expression of gut homing integrin $\alpha 4\beta 7$, and licensing cells to migrate to the GI tract. This reveals that pathways for recruiting of antigen-specific immune cells to the gut are more promiscuous than previously appreciated. Therefore, since it was conceivable that lung DC could mediate immunity against enteric diseases, in this chapter the physiological relevance of such immunologic trafficking from the lung was investigated.

Key objectives of Chapter 4:

- **To determine the protective efficacy of intranasal vaccination against enteric infection**
- **To study the physiological relevance of immune trafficking following intranasal vaccination.**
- **Determine the lung DC subset responsible for imprinting gut homing properties on T cells *in vivo*.**

4.2.1 Intranasal administration of fluorescently labeled antibodies reveals APC localization postnasal immunization.

The ability of nasal immunization to induce primed T cells, not only in the draining lymph nodes but also distal lymphoid organs, could be due to dissemination of antigen bearing DCs, which leads to local proliferation of resident naïve T cells or a redistribution of primed T cells from the lymphoid compartment draining the immunization site. To determine the fate of antigen bearing DCs after mucosal administration, CD11c-Alexa fluor 647, was delivered with poly ICLC (50µg) by the nasal route, and the dissemination of these DCs was studied at different time points, in draining lymph nodes and distal lymph nodes, small intestinal lamina propria and spleen (Fig. 4.1 – 4.4).

Following nasal administration of CD11c-A647, DCs were found in the mediastinal LN 24 hours post administration of poly ICLC (Fig. 4.1) Within the mediastinal LN the dominant targeted DC population was the lung migratory CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DC, consistent with previous literature (Lambrecht and Hammad 2010). Nasal CD103⁺ DCs were also labeled in the NALT 6hrs post delivery (*data not shown*). In distant sites, such as the spleen, MLN and small intestinal lamina propria, labeled DCs were not detected at any time point, revealing that following nasal immunization antigen-loaded DCs are localized to the mediastinal lymph node and lung.

4.2.2 Administration of FTY-720 leads to accumulation of transferred CD45.2⁺Vα2⁺CD4⁺OT-II cells in the mediastinal lymph nodes but not in the mesenteric lymph nodes following i.n. vaccination

Next, it was important to confirm that the transferred CD45.1⁺Vα2⁺CD4⁺ T cells detected in the GI tissues post i.n. immunization (Fig 3.4) were being generated in the lung draining lymph nodes and not gut draining lymph nodes due to inadvertent swallowing of intranasally delivered antigen. We used FTY-720, an inhibitor of lymphocyte egress from lymph nodes (Fig. 4.5) whereby CFSE labeled CD45.1⁺ OT-II cells were adoptively transferred to naïve CD45.2 recipients that were immunized after 2 hours with OVA protein and polyICLC delivered i.n. On days 0- 3, recipient mice were administered FTY-720 (1µg/g mouse). The mice were sacrificed on day 4 and the frequency of transferred

CD45.1⁺V α 2⁺CD4⁺ T cells was quantified in the mediastinal lymph node, mesenteric lymph node, SILP and colon. An increase in the frequency of transferred cells was noted in the mediastinal lymph node. In contrast, we noted a decrease in the frequency of transferred cells in the mesenteric lymph nodes suggesting that antigen exposure and expansion of the transferred OT-II cells was occurring in the mediastinal lymph nodes and not mesenteric lymph nodes. As expected, a significant decrease in the frequency of transferred cells was noted in SILP and colon due to their arrest within the mediastinal lymph node. Thus, based on the time course and FTY experiments we were able to confirm that following i.n. delivery of antigen, integrin α 4 β 7 was induced on transferred T cells within the mediastinal lymph node and lungs.

4.2.3 The adjuvant poly ICLC alone is not responsible for α 4 β 7 induction post intranasal administration.

Following on from these investigations into the role of lung DC-mediated induction of α 4 β 7 and CCR9, the effects of adjuvant administration were determined. This was essential since low levels of integrin α 4 β 7 can be induced by cellular activation.

Synthetic double-stranded RNA. poly ICLC (Longhi et al., 2009, Stahl-Hennig et al., 2009). This double stranded RNA is a pathogen-associated molecular pattern that activates innate immunity. Both poly IC and poly ICLC are recognized by the cytosolic RNA helicase MDA-5 and by endosomal TLR3. A major mechanism underlying the strong adjuvant function of poly IC in mice is that it promotes systemic type I IFN, which has many immune stimulatory roles for both T and B cells and DCs. Following on from their 2011 paper, Caskey *et al.*, (2011) gained a systems-wide appreciation of the innate immune responses induced in the blood of healthy volunteers after s.c. injection of poly ICLC, and found that poly ICLC is a potent inducer of many arms of innate immunity in humans and that many of the triggered pathways mimic what is seen with YF17D, a successful live attenuated viral vaccine (Longhi, Trumpfheller et al. 2009; Caskey, Lefebvre et al. 2011).

In order to test for involvement of polyICLC-induced, RIG-I-and TLR3-mediated DC maturation in α 4 β 7 induction, CD45.1⁺OT-II cells were transferred into wild type, MDA-

5^{-/-}TLR3^{-/-} and MyD88^{-/-}TRIF^{-/-} hosts (McCartney et al., 2009). No significant difference was noted between the MDA-5^{-/-}TLR3^{-/-} and WT mice (Fig. 4.6 – 4.9). Additionally, in order to rule out a unique role for the adjuvant, CD45.1⁺OT-II cells were transferred into CD45.2⁺ hosts, the recipient animals were immunized i.n. with ova alone (100µg), ova/LPS (100µg/5µg), ova/polyICLC (100µg/50µg) or polyICLC alone (50µg). 4 days post vaccination the animals were sacrificed and various immune compartments isolated, including the systemic, gut and local lymph nodes draining the site of vaccine delivery. Administration of poly ICLC alone did not enhance α4β7 expression whereas ova alone did, demonstrating the need for antigen presentation. Additionally, ova/LPS also induced α4β7 suggesting that α4β7 induction was not adjuvant specific (Fig. 4.10 – 4.11). Thus it was determined that expression of α4β7 on T cells following i.n. immunization is not induced in a non-specific fashion by polyICLC alone.

4.2.4 Antigen-specific, OT-II cells, expand in the gastrointestinal effector sites post intranasal administration of vaccine.

Having established the role-played by lung DCs at inducing T cell homing to the gastrointestinal tract, the relationship between i.n. vaccination and effective mucosal protective immunity was investigated. The overall aim of our study was to induce GI immune responses to specific HIV antigens *in vivo*, however given the current challenges in generating an effective mouse model for HIV, other challenge models were adopted. We focused our initial protection studies on the well-described enteric pathogen *Salmonella typhimurium*. *Salmonella enterica* are Gram-negative bacteria that infect humans and animals, causing a spectrum of disease ranging from systemic infection to gastroenteritis, depending on the particular bacterial serovar and the host species infected. The *Salmonella enterica* serovar *typhimurium* infection of susceptible mouse strains causes an invasive systemic disease that is similar to typhoid fever (Watson and Holden 2010).

Host defense against *Salmonella typhimurium* infection requires significant contributions from both the innate and adaptive immune system. The initial stages of infection are

characterized by an innate immune response, triggered by conserved pathogen-associated molecular patterns such as LPS and lipoproteins (Carter and Collins, 1974).

Salmonella typhimurium infection also induces antigen-specific CD4, CD8 T cell and B cell responses, all of which contribute to protective immunity. Immunodeficient mouse strains are unable to control the *in vivo* replication of attenuated *Salmonella* strains, providing a reasonable model for determining the contribution of different cell types to the primary immune responses. For example, nude, TCR receptor $\alpha\beta$ and MHC II deficient mice succumb to infection with strains of attenuated *Salmonella* that are normally successfully cleared in wild type mice (Ravindran and McSorley 2005).

Mice lacking MHC Class I restricted T cells, display no difference, or only mild defects in the resolution of primary infection with attenuated *Salmonella*. Several groups have reported that mice lacking B cells are able to control primary infection with *Salmonella* in a manner similar to wild type controls, these studies therefore suggest that CD4 T cells are critical for resistance to *Salmonella* (Hess, Ladel et al. 1996; Sinha, Mastroeni et al. 1997; Weintraub, Eckmann et al. 1997), and that other lymphocyte populations play a more minor role in controlling primary infection (Ravindran and McSorley 2005).

In order to determine protective efficiency in the GI tract following i.n. immunization a TCR transgenic adoptive transfer system was initially adopted. Chen *et al* (1999) and others believe that such systems are arguably the best immunological tools for detailed analysis of naïve T cell activation *in vivo*.

In order to achieve this, CD45.1⁺OT-II cells were i.v. injected into naïve recipient mice and immunized i.n. or s.c. (100µg of Ova and 50µg of poly ICLC) with unimmunized mice serving as controls. 7 days post vaccination, the recipient animals were gavaged with pathogenic *Salmonella typhimurium* expressing ova (1×10^6).

4 days post *Salmonella* administration, the GI immune inductive and effector sites in addition to systemic sites were isolated and analyzed. Interestingly, transferred V α 2⁺CD45.1⁺CD4⁺ T cells were enriched in the small and large intestinal lamina propria

and intraepithelial compartments in the i.n. immunized mice compared to the s.c. immunized animals (Fig. 4.12).

Following *Salmonella-ova* challenge all the unimmunized and s.c. immunized mice died between days 6-9. In contrast, survival in the i.n. immunized mice was prolonged by 2-3 days (Fig. 4.13). Having observed that i.n. immunization extended the survival of *Salmonella* challenged animals in the transgenic ova TCR transfer model, the effects of i.n. vaccination on bacterial egress into systemic compartments was investigated.

4.2.5 Intranasal vaccination, significantly reduces *Salmonella-ova* infiltration into the Liver.

Although *Salmonella*-specific CD4⁺ T cells localize to the gastrointestinal mucosal lymphoid tissue, bacteria are able to escape this lymphoid compartment and penetrate to systemic sites, most notably the spleen and liver (Watson and Holden 2010). In order to determine protection, in terms of reduced bacterial infiltration into the liver using the transgenic T cell approach, CD45.1⁺ OT-II cells were transferred i.v. into wild type recipients and vaccinated i.n. or s.c. Seven days post vaccination; the vaccinated and naïve animals were challenged with 1×10^6 *Salmonella-Ova* by gavage. Six days post *Salmonella* administration; the left lobe of the liver was removed and re-suspended in LB broth. The suspension was subsequently plated on Macconkey agar, in serial dilutions (1:10). Significantly lower levels of *Salmonella* colony-forming units were observed in the liver of i.n. immunized mice compared to unimmunized mice (Fig. 4.14).

These results, in addition to results from Section 4.2.4, suggest that in addition to enhanced T cell migration and expansion in the gastrointestinal tract, i.n. vaccination was more effective at protection against an enteric pathogen, as confirmed by decreased *Salmonella* infiltration into the liver post challenge, using this TCR transgenic transfer model.

4.2.6 Intranasal vaccination, with heat inactivated *Salmonella* and poly ICLC, results in a significant decrease in bacterial infiltration into the spleen and liver post challenge.

Having established that i.n. vaccination, results in a modest increase in survival in mice following *Salmonella* challenge and reduced infiltration of *Salmonella* into the liver, it was necessary to further investigate if protective immune responses could be generated from the endogenous immune repertoire. The effects of prime and prime boost vaccination regimes on protection were subsequently investigated. Animals were primed i.n. or s.c. with 1×10^6 heat inactivated *Salmonella* and 50µg of poly ICLC.

Initially, a single prime vaccination regime was adopted; the animals were vaccinated on day zero and challenged with 1×10^6 *Salmonella* by gavage on day fourteen. In addition to the vaccinated groups, unvaccinated mice were also challenged orally. All experimental groups were sacrificed six days post challenge. This time point was chosen for sacrifice, as this is one day prior to lethality in the wild type unvaccinated group (Section 4.2.5). The spleens were isolated from all groups and processed into a single cell suspension prior to being re-suspended in LB broth and plated on Macconkey agar.

Consistent with our previous protection data (Fig. 4.14), using the TCR transgenic transfer system, i.n. vaccination resulted in a significant reduction in *Salmonella* infiltration into the spleen (Fig. 4.15), as determined by lower *Salmonella* colony forming units, compared to wild type controls.

4.2.7 Intranasal vaccination, results in increased survival in mice following *Salmonella* challenge

Having established that i.n. vaccination results in decreased infiltration of pathogenic *Salmonella* into both the liver and spleen six days post challenge, further studies were carried out to investigate the effects of i.n. immunization on long term survival post *Salmonella* challenge. In a similar manner to previous experiments, animals were primed with 100µg of ova and 50 µg of poly ICLC either s.c. or i.n. Three weeks post initial priming; the animals were boosted. One-week post boost all groups, including the unvaccinated group, were challenged with 1×10^6 *Salmonella*-Ova by gavage.

Utilizing a Kaplan Meier survival curve the effects of vaccination via either the i.n. or s.c. route could be determined. Similar to the previous TCR transgenic T cell transfer model (Figure 4.13) unvaccinated animals succumbed to *Salmonella* infection prior to day nine, with all the unvaccinated animals reaching the experimental end point within nine days.

Similarly, 70% of animals that received the vaccine s.c. also succumbed to *Salmonella-Ova* infection prior to day eleven, with 25% of s.c. immunized animals surviving until day twenty two (**Fig. 4.16**).

Interestingly 40% of the i.n. immunized animals survived until day forty. Day forty was chosen as the end point for this experimental set up and as a result all remaining mice were sacrificed, despite lacking any symptoms of *Salmonella* infection. In-fact the remaining i.n. vaccinated mice maintained weight throughout the entire challenge experiment, unlike all animals in the s.c. group, which gradually lost weight due to *Salmonella* infection and the subsequent gastroenteritis (*data not shown*). Survival was significantly greater in the i.n. immunized mice compared to the unimmunized group. Stool cultures revealed a significantly higher level of *Salmonella* in the stool of i.n. immunized mice on days 2 and 4 post challenge (**Fig. 4.17**), perhaps due to luminal sequestration of the bacteria. Interestingly, the i.n. immunized mice showing long term survival continued to demonstrate detectable levels of *Salmonella* in the stool even at day 40 (*data not shown*). Taken together, these results demonstrate that i.n. vaccination targeting lung DCs, induce protective immunity in the GI tract.

4.2.8 Intranasal vaccination, with heat inactivated *Salmonella* and poly ICLC, results in decreased pathological damage in the MLN, Liver and Spleen.

Having established that i.n. vaccination results in long term survival following challenge and decreased infiltration of pathogenic *Salmonella* into the spleen six days post challenge, the gross pathology of the spleen, liver and mesenteric lymph nodes was investigated. Following prime boost vaccination and challenge (as described in **Section 4.2.8**), the liver, spleen and mesenteric lymph nodes were isolated from naïve (unchallenged mice), wildtype (unvaccinated challenge mice) and the i.n. vaccinated (challenged) group. These organs were cut and mounted on pathological slides, H&E stained and preserved for histological examination by a board certified pathologist. Firstly, examination of the liver in the unvaccinated group challenged with 1×10^6 *Salmonella-Ova* revealed a scattered mixed lymphoplasmacytic and neutrophilic infiltrate in the portal and lobules with central venulitis and fibrin thrombi formation (x200,

original magnification) (**Fig. 4.18**). Portal and periportal regions contained a mixed inflammatory infiltrate with endotheliitis and fibrin thrombi. The central venular area shows venulitis and occlusion by fibrin thrombi. These are all hallmarks of severe inflammation and disseminated intravascular coagulation due to bacterial infiltration (**Fig. 4.18 – 4.21**). Within the mesenteric lymph nodes of unvaccinated, challenged mice the overall architecture was distorted by a mixed inflammatory infiltrate with vasculitis and fibrin thrombi (**Fig. 4.20**). Similarly, the normal spleen architecture is partially effaced by mixed inflammatory infiltrate and high power revealed vasculitis with fibrin thrombi formation (**Fig. 4.21**). In contrast no pathology correlating with severe inflammation or pathogenic insult was observed in the spleen, liver or mesenteric lymph nodes of mice vaccinated i.n. In-fact, the overall pathology of these tissues was indistinguishable from the unchallenged wild type controls.

Thus, using three different models of protection against pathogenic *Salmonella*, i.n. immunization extends survival, and is associated with a striking absence of pathology in the liver, spleen and MLN in the immunized compared to unimmunized mice.

4.2.9 Following i.n. immunization, induction of integrin $\alpha 4\beta 7$ is mediated by DCs

Lung DCs are an essential bridge between innate and adaptive immunity, and depending on the context also induce the development of Th1, Th2 or Th17 responses to efficiently clear infectious threats. As previously discussed, the lung parenchyma and conducting airway are home to a dense network of DCs, that can be subdivided into four distinct subsets, the most simple discrimination being at the level of expression of CD11c and CD11b. Conventional DCs (cDC) express a high level of CD11c compared with plasmacytoid DCs which are CD11c^{dim}. A highly developed network of CD11b⁺ cDCs are found in the epithelial layer of the conducting airways, and they form long cellular extensions, these DCs express high levels of the E-Cadherin binding integrin CD103 and Langerin (Lambrecht and Hammad 2012). To test for the role of DCs in the induction of $\alpha 4\beta 7$ post i.n. immunization, two DC depletion systems were adopted.

CD11c-DTR mice (Jung et al., 2002) and zDC-DTR mice (Meredith et al., 2012a).

CD11c-DTR and zDC-DTR chimeras (CD11c-DTR bone marrow into WT mice) were generated to avoid the lethality of DT treatment in DTR mice (Zammit et al., 2005). Bone marrow chimeras were created by transferring bone marrow from CD11c-DTR and zDC-DTR mice into lethally irradiated WT C57BL/6 mice (Jung et al., 2002; Zammit et al., 2005) or zDC-DTR (Meredith et al., 2012a). Briefly, C57BL/6 hosts underwent two treatments of 500 rads in an animal γ - irradiator 3 hours apart, and 2.5×10^6 bone marrow cells from a CD11c-DTR and zDC- DTR donor respectively were transferred i.v. The resulting mice (referred to herein as CD11c-DTR and zDC-DTR) were housed under immunocompromised mouse conditions and treated regularly with 2 mg/ml neomycin from Sigma-Aldrich (St. Louis, MO) in their drinking water. The chimeric mice were fully reconstituted and ready for experimental use after 6 weeks.

Twenty-four hours post diphtheria toxin (DT) administration, CD45.1⁺OT-II cells were transferred into the DTR recipients and mice were immunized with ova and polyICLC. CD11c-DTR mice administered PBS served as controls. Significantly lower levels of $\alpha 4\beta 7$ were induced on the transferred V $\alpha 2^+$ CD45.1⁺CD4⁺CFSE^{low} cells following DT mediated ablation of DCs (**Fig. 4.22**). CD11c-DTR mice were homozygous for the transgene and upon DT treatment, all CD8⁺ and CD8⁻ DCs were selectively ablated. However since CD11c is also expressed on various cells including activated monocytes, macrophages and plasmacytoid DCs (pDCs), the CD11c-DTR model cannot definitively distinguish the role of classical DCs (cDC) from activated monocytes and macrophages (Bennett and Clausen, 2007; Probst et al., 2005; Zammit et al., 2005) in $\alpha 4\beta 7$ induction. To discern the role of lung cDCs in $\alpha 4\beta 7$ induction, the recently described zDC-DTR mice were adopted (Meredith et al., 2012a; Meredith et al., 2012b).

In these z-DTR mice, a zinc finger transcription factor, *Zbtb46*, which is specific to cDCs is conditionally ablated, thus distinguishing the role of cDC from other cells types expressing CD11c. Again, zDC-DTR chimeras (zDC-DTR bone marrow into WT mice) were generated to avoid the lethality of DT treatment in zDC-DTR mice (Meredith et al., 2012a). CD45.1⁺OT-II cells were transferred into zDC-DTR chimeras 24 hours post DT ablation and the mice were immunized with ova and polyICLC delivered i.n. zDC-DTR

mice not administered DT served as controls. Lung DC depletion following DT administration was confirmed (*data not shown*). Significantly lower levels of $\alpha 4\beta 7$ were induced on the transferred $V\alpha 2^+CD45.1^+CD4^+CFSE^{low}$ cells following DT mediated ablation of cDCs (**Figure 4.23**). Thus, using two different methods of DC depletion, it was confirmed that lung DCs mediated the induction of integrin $\alpha 4\beta 7$ *in vivo*.

4.2.10 Following i.n. immunization, induction of integrin $\alpha 4\beta 7$ is mediated by lung resident DCs.

Results from the previous experiment have demonstrated the role of lung DCs in terms of $\alpha 4\beta 7$ induction *in vivo*. Following on from this, the role of lung resident DCs in $\alpha 4\beta 7$ induction was investigated. In a similar manner to the previous studies, $CD45.1^+OT-II$ cells were transferred into recipient animals, which were immunized with ova and polyICLC. In addition to wild type recipients, $CCR7^{-/-}$ animals were also utilized (Foster et al., 1999). Since these mice lack the CCR7 receptor, preventing the entry of lymphocytes and DCs into secondary lymphocyte compartments, thereby preventing egress of DCs from the lung and draining lymph node compartments. 4 days post vaccination the groups were sacrificed and the $\alpha 4\beta 7$ expression on transferred $V\alpha 2^+CD45.1^+CD4^+CFSE^{low}$ cells was investigated. Interestingly, the induction of $\alpha 4\beta 7$ was significantly higher on the $V\alpha 2^+CD45.1^+CD4^+CFSE^{low}$ cells in all compartments, including the blood, lung, mediastinal lymph node and mesenteric lymph node, in the $CCR7^{-/-}$ mice compared to wild type group (**Fig. 4.24**). The induction of $\alpha 4\beta 7$ in the wild type group was comparable to previous *in vivo* transfer experiments.

4.2.11 Following i.n. immunization, induction of integrin $\alpha 4\beta 7$ is mediated by $CD11b^+$ DCs.

Contrary to the MLN where only $CD103^+$ DCs (and not $CD11b^+$ DCs) up regulate gut homing phenotypic markers (Johansson-Lindbom et al., 2005) it was found (**Fig. 3.19**) that both $CD103^+$ and $CD11b^+$ lung DC subsets express ALDH and both lung DC subsets up regulated $\alpha 4\beta 7$ *in vitro* (**Fig. 3.9**). Having discerned the role of cDC in the lung, the effect of ablating specific lung DC populations on the induction of $\alpha 4\beta 7$ *in vivo* was

investigated. To deplete CD11b⁺ lung DCs, CD11b-DTR mice were utilized (Duffield et al., 2005). CD11b-DTR chimeras were created (CD11b-DTR bone marrow into WT mice) (**Fig. 4.27**). On day 2 post-DT, CD45.1⁺OT-II cells were adoptively transferred and the mice were immunized with ova and polyICLC. Four days later the V α 2⁺CD45.1⁺CD4⁺ transferred cells were examined for α 4 β 7 induction. CD11b-DTR chimeras that received PBS instead of DT served as controls. As shown in **Figure 4.26**, the α 4 β 7 level on transferred V α 2⁺CD45.1⁺CD4⁺CFSE^{low} cells in the blood, lung and mediastinal LN were significantly lower in the DT injected mice compared to mice that received PBS. Additionally, the transferred CD45.1⁺ T cells in the spleen and MLN of recipient mice were assessed and a similar attenuation of α 4 β 7 induction was observed. Multiple doses of DT were tested and it was found that two doses of 25 μ g/g, 2 days apart were optimal in effecting depletion of CD11b⁺ lung DCs and mediastinal LN DCs (**Fig. 4.27**). One dose of DT resulted in monocyte depletion in the blood but not in lung tissue and three doses of DT were lethal following i.n. administration of PolyICLC (*data not shown*).

4.2.12 Following i.n. immunization, induction of integrin α 4 β 7 is not mediated by Langerin⁺ CD103⁺ DCs.

In addition to the lung lamina propria resident CD11b⁺ DCs, a distinct population of CD103⁺ DCs are found in the epithelial layer, which have the capacity to sample antigenic material from the conducting airways. These DCs are CD103⁺, Langerin⁺ and express high levels of XCR1, a lymphotactin receptor. To examine the effect of depleting CD103⁺ lung DCs, two systems were employed- temporal (*Langerin DTR* (Kissenpfennig et al., 2005)) and constitutive (*Batf3*^{-/-} (Hildner et al., 2008)). In the *Langerin DTR* mice, one day following administration of DT (Ablation data **Fig. 4.29**), CD45.1⁺OT-II cells were adoptively transferred and the mice were immunized with ova and polyICLC. Four days later, the transferred cells were investigated for α 4 β 7 induction. *Langerin DTR* mice administered PBS served as controls. There was no difference in the levels of α 4 β 7 between PBS- and DT-treated mice (**Fig. 4.28**).

The CD103⁺CD11b⁻ lung cDC do not develop in mice lacking the transcription factors *ORF8*, *BATF3*, or *ID2*, placing them in the same lineage as CD8 α ⁺ cDC of the central lymphoid organs that develop from a common DC progenitor. Data from the Immegen consortium (<http://www.immgen.org>) (Lambrecht and Hammad, 2012) also support the idea that the expression of many genes is shared between CD103⁺CD11b⁻ DCs and CD8 α ⁺ DCs, with both of these DC subsets absent in mice lacking Flt3L (Ginhoux et al., 2009). In order to confirm the role of CD103⁺ DCs a constitutive knock out system was utilized. For this purpose responses in *Batf3*^{-/-} and WT mice were compared. Again, following adoptive transfer of congenic OT-II cells and immunization with ova and PolyICLC, no difference was observed between the levels of α 4 β 7 induced on the adoptively transferred cells (**Fig. 4.30**). Together, these data suggest that *in vivo*, lung CD11b⁺ DC subsets are more proficient in inducing α 4 β 7 than the CD103⁺ DCs.

4.3 Discussion:

The majority of “*research undertaken in the past several decades on the mucosal immune system has focused on specific individual components of the system*” (Finlay et al. 2011), however the mucosal immune system has yet to be examined as a global organ. As many features are shared between individual mucosal sites, it is likely that these aspects may have compelling roles in the mucosal immune response, and there could be unappreciated levels of communication between various mucosal compartments (Gill, Wlodarska et al. 2010). To date, there has been ample evidence suggesting that mucosal immunity is executed by a system wide organ (Gill, Wlodarska et al. 2010), with seminal studies revealing that intranasal immunization generates vaginal IgA and protection against genital herpes. Many reports including Finley et al, have suggested that understanding communication between mucosal sites is fundamental to the next phase of disease characterization and vaccine development, particularly against enteric pathogenic challenge. Appreciation of the mucosal immune system as a global organ will involve determining what factors link one area of the mucosal immune system to another “and the intricacies of this communication” (Finley et al. 2011). Following on from the initial studies, described in Chapter 3, examining the ability of lung DCs to induce the expression of gut homing receptors on T cells, thereby enhancing the migration of transferred cells to home efficiently to the GI tract, whether this cross talk between mucosal sites was sufficient to mediate protection against enteric pathogenic challenge was investigated.

It may be argued that i.n. immunization may lead to inadvertent swallowing of antigen and targeting of GI resident DCs that are known to induce migration of T and B cells to the gut. To exclude this possibility, a time course experiment was adopted to identify the populations targeted by i.n. delivery of fluorescent, DC-specific antibodies. Fluorescent antibodies were readily detected, specifically targeting the mediastinal lymph node and lung resident DCs but not GI resident DCs. Since this does not exclude transport of antigen to the GI tract, specific experiments were performed to block the egress of T cell from lymph nodes using FTY-720, to identify the site of T cell priming post i.n.

vaccination. FTY720 does not impair T- and B-cell activation, proliferation or effector functions, but interferes with cell traffic between lymphoid organs and blood. The molecular basis for this effect has only recently been established. FTY720, after phosphorylation, acts as a high-affinity agonist at the G protein-coupled sphingosine 1-phosphate receptor-1 (S1P(1)) on thymocytes and lymphocytes, thereby inducing aberrant internalization of the receptor. This renders the cells unresponsive to the serum lipid sphingosine 1-phosphate (S1P), depriving them from an obligatory signal to egress from lymphoid organs. As a consequence, lymphocytes are unable to recirculate to peripheral inflammatory tissues and graft sites but remain functional in the lymphoid compartment (Thangada et al., 2010).

Utilizing FTY-720 the transferred cells were observed accumulating in the mediastinal lymph nodes and in contrast, a drop in transferred cell frequency was seen in the mesenteric lymph nodes, blood (*data not shown*), spleen (*data not shown*), SILP and colonic lamina propria in mice given FTY-720, thereby confirming that i.n. immunization-mediated expansion of transferred cells was occurring in the former and not the latter lymphoid sites. Finally, proliferation of transferred cells within the mediastinal lymph nodes was observed early (day 2) as was the appearance of integrin $\alpha 4\beta 7$ on mediastinal lymph node and lung resident cells (day 3), followed by the migration of transferred cells to GI compartments (day 4), which could be blocked with an anti- $\alpha 4\beta 7$ antibody (Chapter 3). These data extend the work of Ciabattini *et al* who have recently demonstrated that following i.n. immunization, antigen-specific T cells trafficked to distal skin draining lymph nodes in a CD62L dependent fashion and to the mesenteric lymph nodes in an $\alpha 4\beta 7$ dependent fashion (Ciabattini et al., 2011). However, unlike the present study, the mechanisms underlying $\alpha 4\beta 7$ induction i.e. the role of lung DCs or the physiological relevance of these pathways was not explored by Ciabattini *et al*. Integrin expression may be induced via non-specific stimuli such as cellular activation (Laudanna et al., 2002). Since the *in vivo* experimental system involved the use of the TLR-3 agonist, polyICLC, the effects of the adjuvant on induction of integrin $\alpha 4\beta 7$ were determined, in order to rule out a unique role of the adjuvant. Initially, polyICLC was administered alone (without antigen), however, in this case, no significant induction of

$\alpha 4\beta 7$ was observed, whereas immunization with ova alone induced $\alpha 4\beta 7$ on transferred OT-II cells. Further, by using the MDA5^{-/-}TLR3^{-/-} mice, it was possible to confirm that i.n. immunization induced $\alpha 4\beta 7$ was not a non-specific effect triggered by the adjuvant. Interestingly, enhanced levels of $\alpha 4\beta 7$ were observed on the transferred cells in the MDA5^{-/-}TLR3^{-/-} mice. These findings were not statically significant, however in addition to $\alpha 4\beta 7$ we will further examine if in the absence of MDA5 and TLR3, the expression of CCR9 is also increased. In addition to i.n. vaccination, we will examine the effects of MDA5 and TLR3 signalling during oral vaccination, in order to determine if these findings are a feature of the route of vaccination or if the $\alpha 4\beta 7$ increase is a unique feature in these KO animals. Additionally, different adjuvants were investigated for their ability to induce $\alpha 4\beta 7$ following i.n. immunization comparable results were found. Combined, it can be speculated that in the optimal cellular context, i.e. local availability of RA and TGF- β , the use of an adjuvant amplifies the induction of $\alpha 4\beta 7$ due to DC and T cell activation as well as DC migration.

Having shown that lung DCs induce $\alpha 4\beta 7$ expression and induce cellular migration to the GI tract, the physiological consequences of such cellular trafficking were investigated, specifically whether pathogen-specific intestinal immunity could be induced by targeting lung DCs. *Salmonella typhimurium* was chosen as the model system as it is a pathogen of global significance (Levine, 2006) that is transmitted across the intestinal mucosa and causes a spectrum of diseases ranging from localized intestinal infection to severe systemic illness (Griffin and McSorley, 2011). To confirm protection against enteric challenge with *Salmonella*, three different experimental systems were used: 1) Passive immunization using transferred OT-II cells followed by i.n. immunization where a modest increase in survival was demonstrated. 2) Active prime-boost immunization with ova showing a significantly increased survival and lack of systemic pathology in the i.n. immunized mice, and 3) Active Prime-boost immunization using inactivated *Salmonella* showing reduced systemic burden in the i.n. immunized mice. Initially, to examine whether i.n. immunization conferred protection against enteric pathogens, CD45.1⁺OT-II cells were transferred into naïve mice which were immunized i.n. or s.c. with unimmunized mice serving as controls. Following gavage of pathogenic *Salmonella*

typhimurium expressing ova, all the unimmunized or s.c. immunized mice died between days 6-9. In contrast, survival in the i.n. immunized mice was prolonged by 2-3 days. Transferred V α 2⁺CD45.1⁺CD4⁺ T cells were enriched in the small and large intestinal lamina propria and intraepithelial compartments in the i.n. immunized mice compared to the s.c. immunized animals. Having observed that i.n. immunization extended the survival of *Salmonella* challenged animals, whether a protective immune response could be generated from the endogenous immune repertoire was determined. WT mice were immunized with ova/polyICLC delivered i.n. or s.c. in a prime-boost fashion (a booster dose of the vaccine was given 4 weeks after priming) and mice were challenged orally with *Salmonella*-Ova. Survival was significantly greater in the i.n. immunized mice compared to unimmunized with 40% of the mice demonstrating long term survival (>40 days, $p < 0.05$). Histological examination of the liver, spleen and MLN demonstrated significant disruption of hepatic, splenic and lymph node architecture, vasculitis, coagulative necrosis and intravascular thrombi in the challenged, unimmunized mice compared to WT mice similar to published reports. In contrast, the liver, spleen and MLN of i.n. immunized mice were indistinguishable from WT mice. Stool cultures showed a significantly higher *Salmonella* CFUs in the stool of i.n. immunized mice on days 2 and 4 post challenge, perhaps due to luminal sequestration of the bacteria and subsequent shedding in the faecal pellets. Interestingly, the i.n. immunized mice showing long term survival continued to demonstrate low levels of *Salmonella* in the stool even at day 40 (*data not shown*). Finally, naïve C57BL/6 mice were immunized with heat and paraformaldehyde inactivated *Salmonella*, delivered i.n. or s.c. Significantly lower numbers of *Salmonella* CFUs were observed in the spleen of i.n. immunized mice compared to unimmunized mice. Thus, using three different models of protection against pathogenic *Salmonella*, it was demonstrated that i.n. immunization extends the survival, and is associated with a striking absence of pathology in the liver and spleen in the immunized compared to unimmunized mice. Notably, in mice that received a s.c. vaccination, a non-significant increase in survival was also observed. It is hypothesized that this may be due to the systemic immunity generated by the s.c. vaccine. Thus, using multiple experimental systems, this study reveals that lung DCs targeted by i.n. immunization are able to induce protective immunity within the GI tract.

Having established the potential effects of i.n. vaccination to protect mice against enteric pathogenic challenge, it was subsequently necessary to analyze the distinct role played by lung DCs in $\alpha 4\beta 7$ induction post i.n. vaccination. It was shown in Chapter 3 that *in vitro*, lung-resident DCs had the capacity to induce $\alpha 4\beta 7$, moreover following i.n. delivery of fluorescent DC specific antibodies, i.n. vaccination targets both CD103⁺ and CD11b⁺ DCs in the lung. To examine the role of lung DCs in the *in vivo* induction of $\alpha 4\beta 7$, two different conditional knockout models were used. Use of the CD11c DTR leads to the depletion of 85-90% of CD11c⁺ DCs (Bennett and Clausen. 2007). However, since this model cannot distinguish between classical DCs, activated monocytes and alveolar macrophages, all of which express CD11c and are therefore depleted (Probst et al., 2005; Zammit et al., 2005), the newly described zDC-DTR model (Meredith et al., 2012a; Meredith et al., 2012b) was also used.

Significantly attenuated numbers of transferred congenic cells expressing $\alpha 4\beta 7$ were seen in the mucosal and systemic sites in both models, demonstrating that lung DCs induced integrin $\alpha 4\beta 7$ on CD4⁺ T cells post i.n. vaccination. These results need to be interpreted with the caveat that both DT induced depletion of DCs, and nasal administration of polyICLC is likely associated with lung inflammation (Tittel et al., 2012). Despite ablation of lung DCs, low levels of $\alpha 4\beta 7$ induction were observed on the transferred cells, it is speculated that local inflammation is responsible for the variability observed in the levels of $\alpha 4\beta 7$ expressed on transferred cells isolated from various compartments, the expression being high on cells isolated from the lungs compared to the cells isolated from the mediastinal lymph node, blood or from the spleen and MLN where a more profound attenuation of $\alpha 4\beta 7$ expression was seen. In order to examine the *in vivo* role of lung DCs, CCR7^{-/-} animals were also utilized (Foster et al., 1999). These mice lack the CCR7 receptor, preventing the entry of lymphocytes and DCs into secondary lymphocyte compartments, thereby preventing egress of DCs from the lung and draining lymph node compartments. Interestingly, the induction of $\alpha 4\beta 7$ was significantly higher on the V $\alpha 2$ ⁺CD45.1⁺CD4⁺CFSE^{low} cells in all compartments, including the blood, lung, mediastinal lymph node and mesenteric lymph node, in the

CCR7^{-/-} mice compared to wild type group. This could be due to a number of distinct factors, primarily the relative availability of lung DCs during antigen capture, increased levels of locally derived RA and TGF- β in addition to the formation of isolated lymphoid-like structures in the murine lung of CCR7^{-/-} animals (Fainaru et al. 2005).

These data generated using a number of different experimental approaches demonstrate that following i.n. immunization, lung and mediastinal LN resident DCs are targeted which in turn induce the expression of integrin α 4 β 7 on T cells, resulting in their localization to the GI tract. This provides a mechanistic insight into the observation of T cell responses in the GI lamina propria following i.n. challenge (Esplugues et al., 2011; Masopust et al., 2010).

Although the mechanisms underlying α 4 β 7 induction appear to be conserved between lung and GI resident DCs, the DC subsets involved show-striking differences. For example, consistent with studies of α 4 β 7 induction by gut DCs (Iwata et al., 2004; Kang et al., 2011), the results presented in Chapter 3 revealed that RA and TGF- β are required in the lung DC mediated induction of α 4 β 7. In addition to an *in vitro* culture system, dominant negative RAR-OTII Rag^{-/-} mice and TGF- β receptor ^{η/η} -CD4^{CRE} OT-II Rag^{-/-} mice we utilized to answer these questions. Splenic OT-II cells from the dominant negative RAR-OTII Rag^{-/-} mice and TGF- β receptor ^{η/η} -CD4^{CRE} OT-II Rag^{-/-} mice were used as donor cells, so local, lung DC subsets in these mice are not relevant here

In contrast to the existing literature showing that CD103⁺ MLN DCs induce integrin α 4 β 7 whereas the CD11b⁺ MLN DCs do not, here it is demonstrated that both CD103⁺ and CD11b⁺ lung DC populations induced integrin α 4 β 7, the expression being higher in the lung CD11b⁺ DC induced co-cultures *in-vitro*. Since these differences were novel and unexpected, further experiments were conducted to determine the roles of specific DC subsets using *in-vivo* DC depletion models. To deplete the CD103⁺ DC population, *Langerin DTR* mice were used (Bennett et al., 2005), following selective ablation of this DC subset in the lung, there was no detectable significant differences, in the level of

$\alpha 4\beta 7$ induction between the Langerin depleted and replete mice. To further confirm this *Batf3*^{-/-} mice where deletion of the transcription factor *Batf3* results in ablation of the cross presenting, CD103⁺ tissue DCs were used (Hildner et al., 2008). No significant differences in the induction of $\alpha 4\beta 7$ were observed between WT and *Batf3*^{-/-} animals. Finally, to deplete the CD11b⁺ lung DCs, CD11b DTR mice were used (Both CD11b expressing alveolar macrophages and DCs are depleted in this model, however, the use of zDC-DTR demonstrates the role of classical DCs). Attenuated levels of $\alpha 4\beta 7$ were observed in the mice where CD11b⁺ expressing cells were depleted. Therefore, in contrast of MLN DCs, both CD103⁺ and CD11b⁺ lung DC populations induced integrin $\alpha 4\beta 7$ *in vitro*, although *in vivo*, the CD11b⁺ populations appeared to have a more dominant effect on the induction of $\alpha 4\beta 7$. Thus, the predominant role of CD103⁺ DC in mediating gut homing in MLN is not observed in the case of lung DC. Following on from this work, it is speculated that while the migratory gut CD103⁺ DCs use dietary and biliary sources of vitamin A to generate RA (Jaensson-Gyllenback et al., 2011), the lung DCs either use haematogenous Vitamin A, released from hepatic stores or local stores of vitamin A (Dirami et al., 2004; Okabe et al., 1984). Thus, CD103⁺ DCs may not be pre-conditioned to metabolize vitamin A but rather, they may acquire this property based on local environmental factors like the availability of Vitamin A, TLR ligands or other inflammatory stimuli. Detailed mechanistic studies are underway to further address this question including a study of the relative expression of RALDH and the effect of adjuvants on RALDH expression by lung DC subsets. To conclude, in this chapter, the current paradigm that only CD103⁺ gut resident DCs can recruit T cells to the GI tract has been challenged. Rather, this study provides compelling evidence that lung DCs, targeted by i.n. immunization, are capable of inducing robust expression of gut homing integrin $\alpha 4\beta 7$, and licensing T cells to migrate to the GI tract mediating protection against enteric challenge. Pathways of recruitment of antigen specific immune cells to the gut are therefore much more promiscuous than previously appreciated. There appears to be considerable, albeit hitherto unrecognized, DC orchestrated, mucosal cross talk which can inform the rational design of novel vaccines.

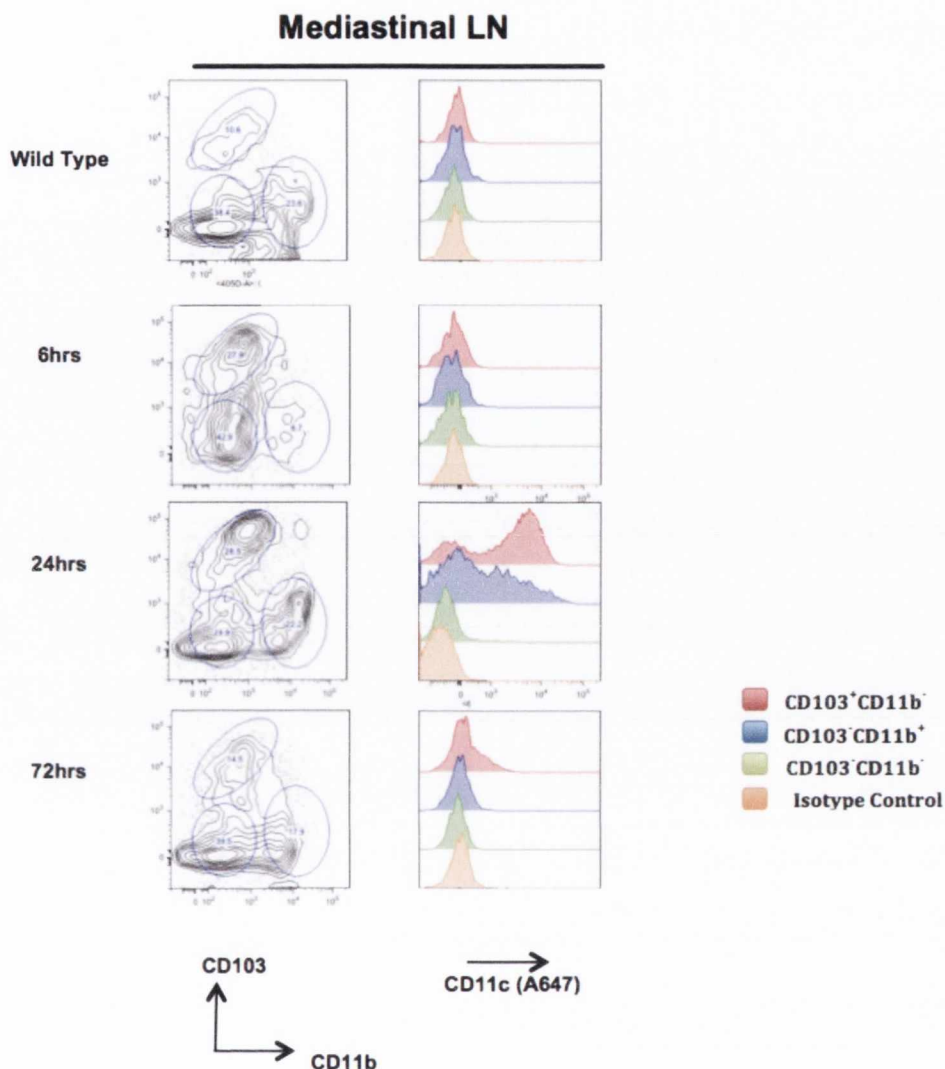


Figure 4.1: I.N administration of fluorescent anti-CD11c antibodies target lung draining lymph node DCs. Representative flow plots showing DC subsets targeted by anti-CD11c. C57BL/6 mice were immunized with poly ICLC (50µg) and fluorescently labelled anti-CD11c (5µg) delivered i.n. The mediastinal LN was examined at baseline, 6hrs, 24hrs and 72 hours post antibody administration. MHCII⁺CD11c^{hi} gated CD103⁺CD11b⁻, CD103⁻CD11b⁺ and CD103⁻CD11b⁻ lung DC subsets targeted with anti-CD11c A647 were examined by flow cytometry as shown in a representative experiment. Figure 4.1 illustrates a representative of three experiments

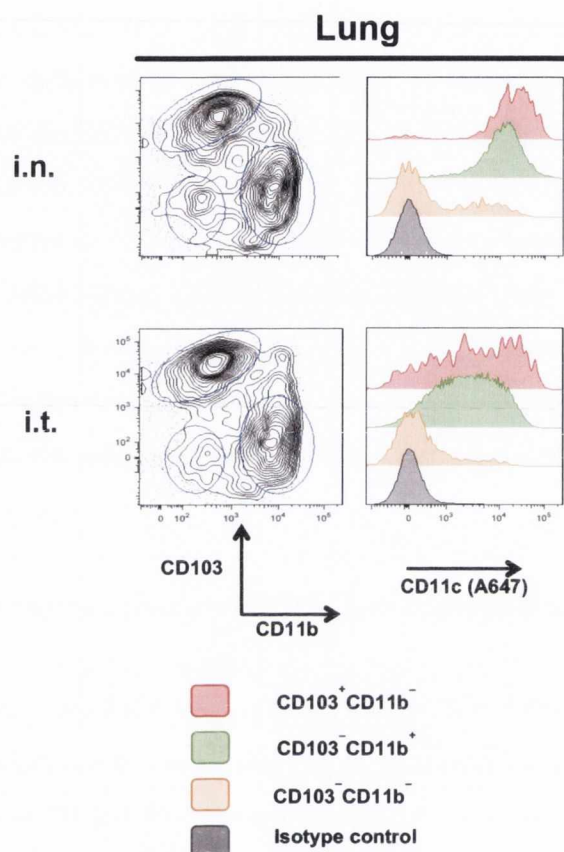


Figure 4.2: I.N and I.T. administration of fluorescent anti-CD11c antibodies target lung resident DCs. Representative flow plots showing DC subsets targeted by anti-CD11c. C57BL/6 mice were immunized with poly ICLC (50 μ g) and fluorescently labelled anti-CD11c (5 μ g) delivered i.n. (top panels) or i.t. (bottom panels). The lung was examined 24 hours post antibody administration. MHCII⁺CD11c^{hi} gated CD103⁺CD11b⁻, CD103⁻CD11b⁺ and CD103⁻CD11b⁻ lung DC subsets targeted with anti-CD11c A647 were examined by flow cytometry as shown in a representative experiment. Figure 4.2 illustrates a representative of three experiments.

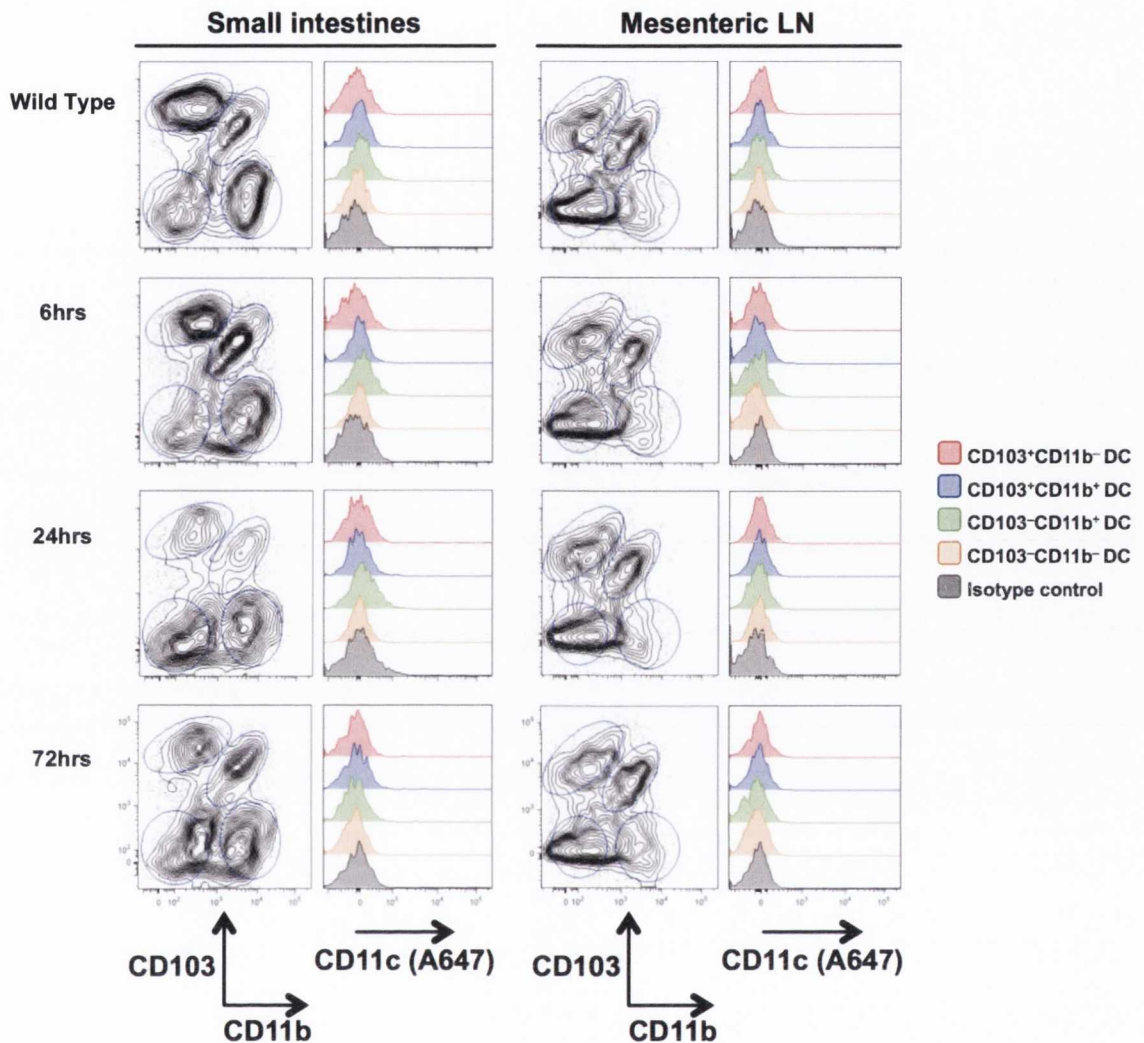


Figure 4.3: I.N administration of fluorescent anti-CD11c antibodies does not target small intestinal lamina propria or intestinal draining lymph node DCs. Representative flow plots showing DC subsets targeted by anti-CD11c. C57BL/6 mice were immunized i.n. with poly ICLC (50 μ g) and fluorescently labelled anti-CD11c (5 μ g). The SILP and MLN were examined at baseline, 6hrs, 24hrs and 72 hours post antibody administration. MHCII⁺CD11c^{hi} gated CD103⁺CD11b⁻, CD103⁺CD11b⁺, CD103⁻CD11b⁺ and CD103⁻CD11b⁻ gut DC subsets targeted with anti-CD11c A647 were examined by flow cytometry as shown in a representative experiment. Figure 4.3 illustrates a representative of three experiments.

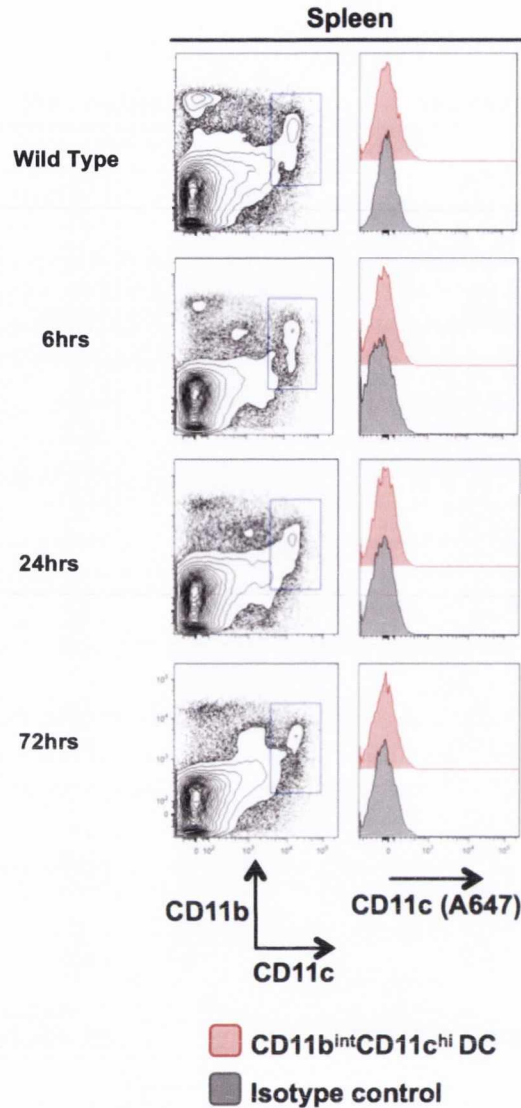


Figure 4.4: I.N administration of fluorescent anti-CD11c antibodies does not target splenic DCs. Representative flow plots showing DC subsets targeted by anti-CD11c. C57BL/6 mice were immunized i.n. with poly ICLC (50 μ g) and fluorescently labelled anti-CD11c (5 μ g). The spleen was examined at baseline, 6hrs, 24hrs and 72 hours post antibody administration. MHCII⁺CD11c^{hi} gated CD11b⁺CD11c⁺, splenic DC subsets targeted with anti-CD11c A647 were examined by flow cytometry as shown in a representative experiment. Figure 4.4 illustrates a representative of three experiments.

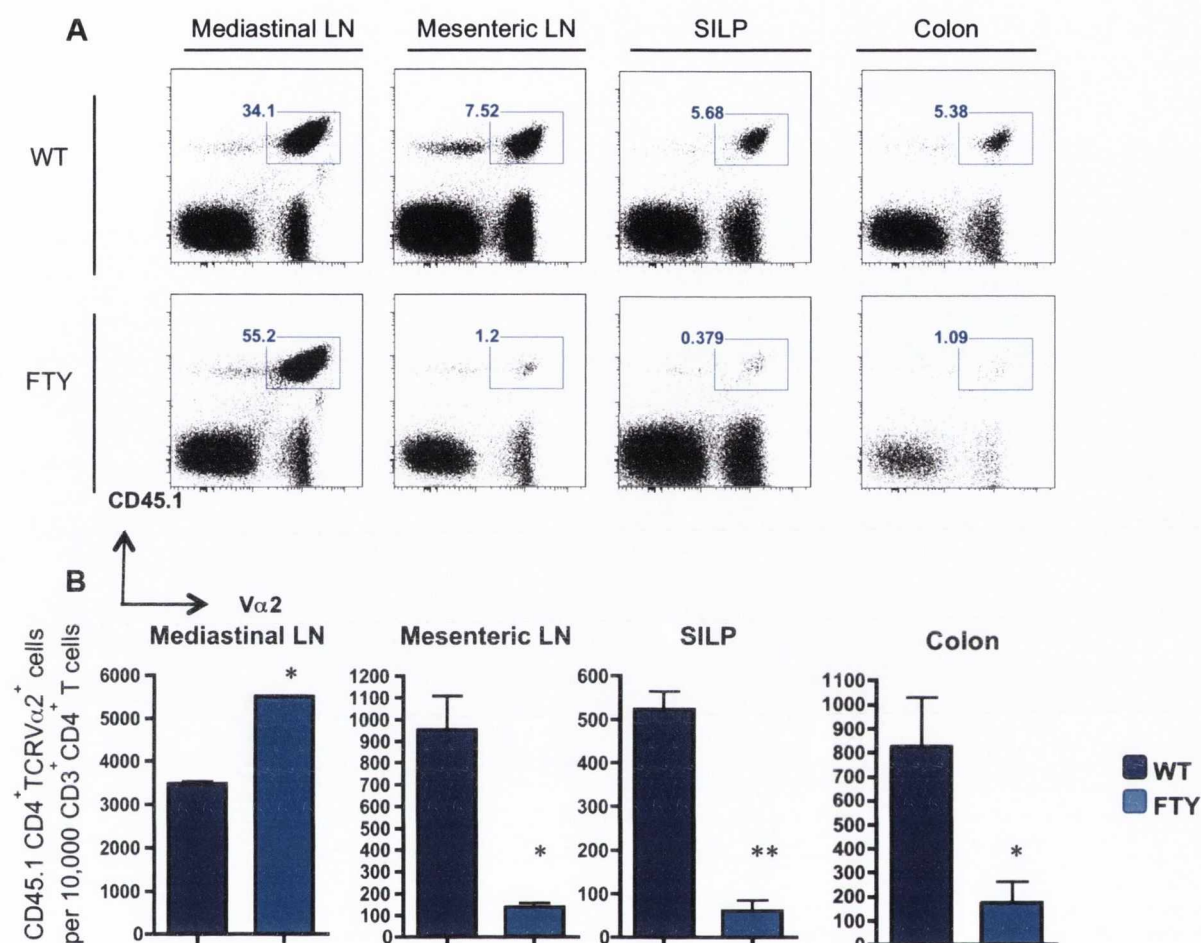


Figure 4.5 Administration of FTY-720 leads to accumulation of transferred CD45.1⁺Vα2⁺CD4⁺OT-II cells in the mediastinal lymph nodes but not in the mesenteric lymph nodes following i.n. vaccination. (a) Representative flow cytometry plot comparing the frequency of adoptively transferred CD45.1⁺Vα2⁺CD4⁺OT-II cells in the mediastinal lymph nodes, mesenteric lymph nodes, SILP and colon between untreated mice (top panels) and mice administered FTY-720 (bottom panels) following i.n. immunization. (b) Cumulative data from three individual experiments comparing the migration of CD45.1⁺Vα2⁺CD4⁺OT-II cells to mediastinal lymph nodes, mesenteric lymph nodes, SILP and colon in WT (dark blue) and FTY-720 administered mice (light blue).

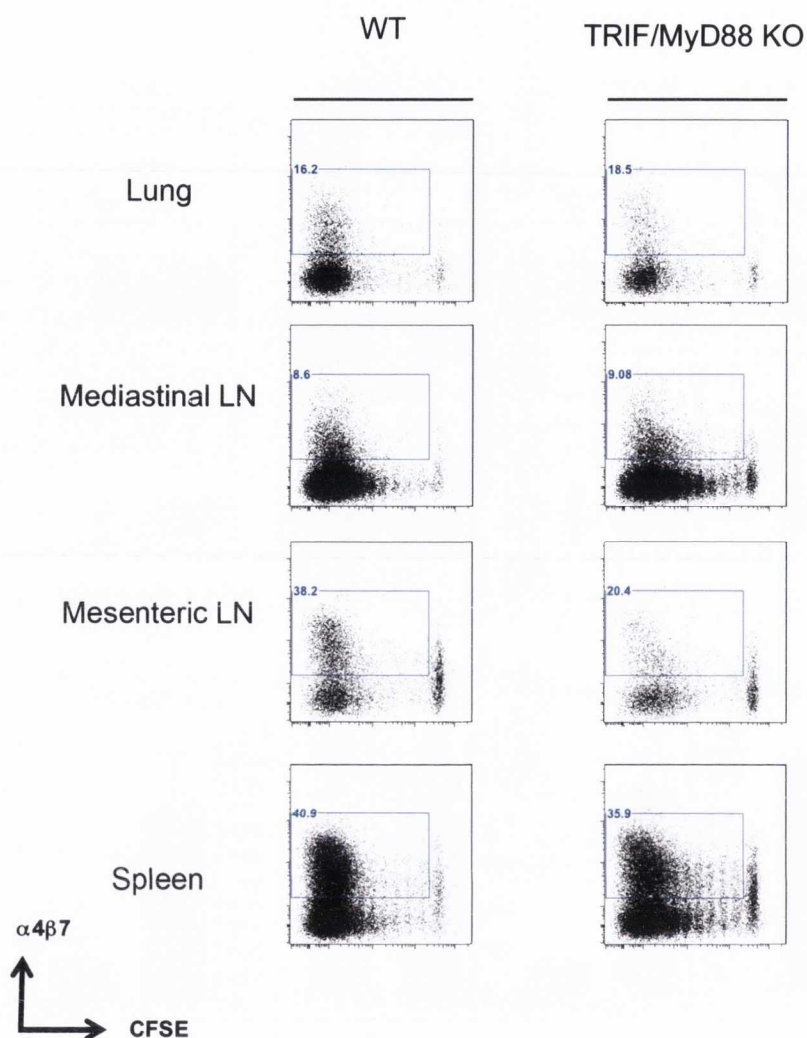


Figure 4.6 Lung DC mediated $\alpha 4\beta 7$ induction is not dependent on TRIF or MyD88 signalling. Representative flow plot revealing the percentage of CFSE^{lo} $\alpha 4\beta 7^{\text{hi}}$ cells (y-axis) compared between mice that were immunized i.n. with ova (100 μ g) plus polyICLC (50 μ g) following the adoptive transfer of CD45.1⁺V α 2⁺CD4⁺OT-II cells into CD45.2⁺ WT or MyD88^{-/-}TRIF^{-/-} mice. Figure 4.6 illustrates a representative of three experiments.

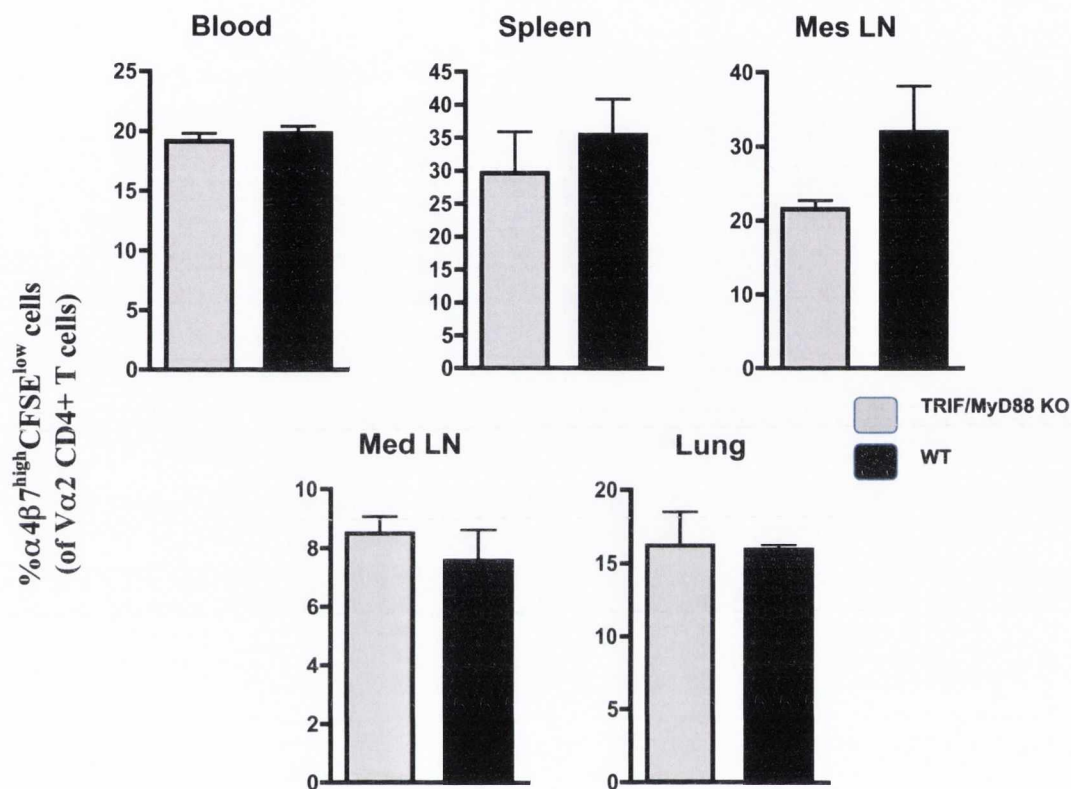


Figure 4.7: Lung DC mediated $\alpha 4\beta 7$ induction is not dependent on TRIF or MyD88 signalling. Cumulative data from three individual experiments revealing the percentage of CFSE^{low} $\alpha 4\beta 7^{\text{high}}$ cells (y-axis) is compared between mice that were immunized i.n. with ova (100 μ g) plus polyICLC (50 μ g) following the adoptive transfer of CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells into CD45.2+ WT or TRIF^{-/-}MyD88^{-/-} mice.

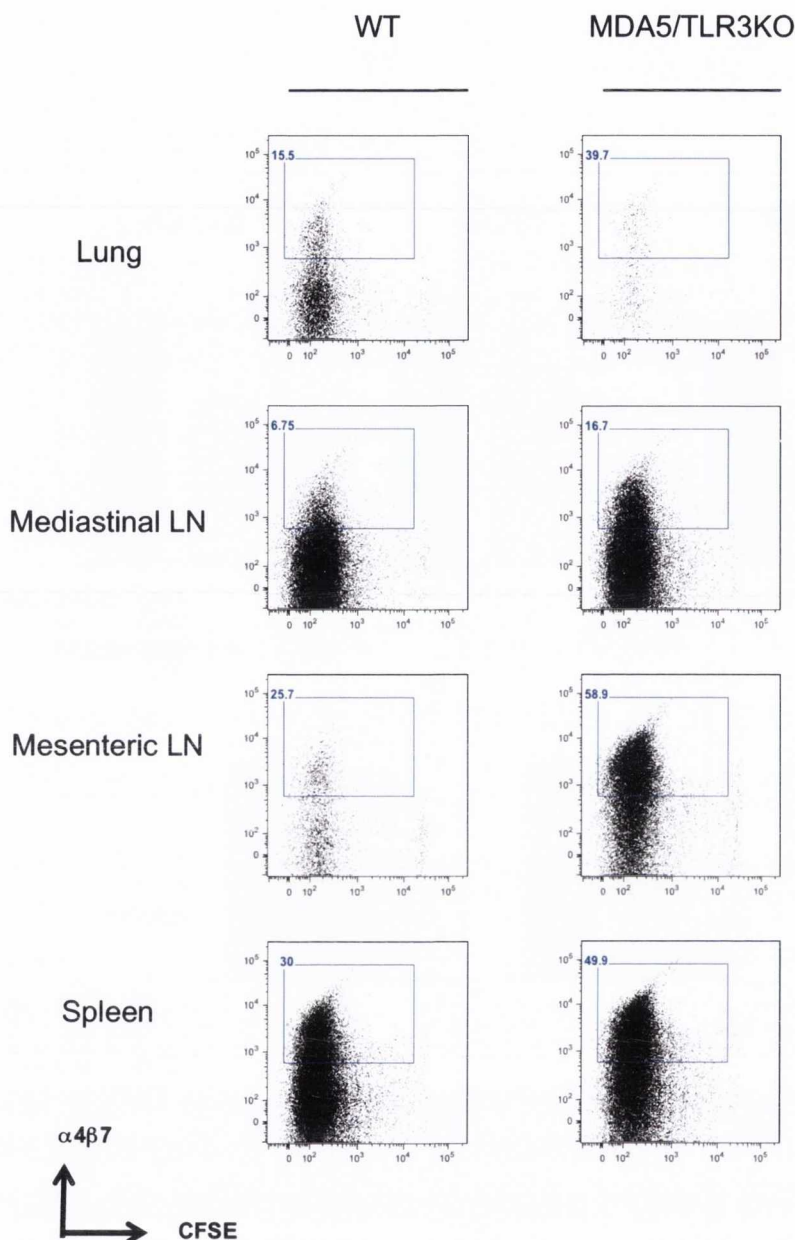


Figure 4.8: Lung DC mediated $\alpha 4 \beta 7$ induction is not dependent MDA5 or TLR 3 signalling. Representative flow plot revealing the percentage of CFSE^{lo} $\alpha 4 \beta 7^{\text{hi}}$ cells (y-axis) compared between mice that were immunized i.n. with ova (100 μ g) plus polyICLC (50 μ g) following the adoptive transfer of CD45.1⁺V α 2⁺CD4⁺OT-II cells into CD45.2⁺ WT or MDA-5^{-/-}TLR3^{-/-} mice. Figure 4.8 illustrates a representative of three experiments.

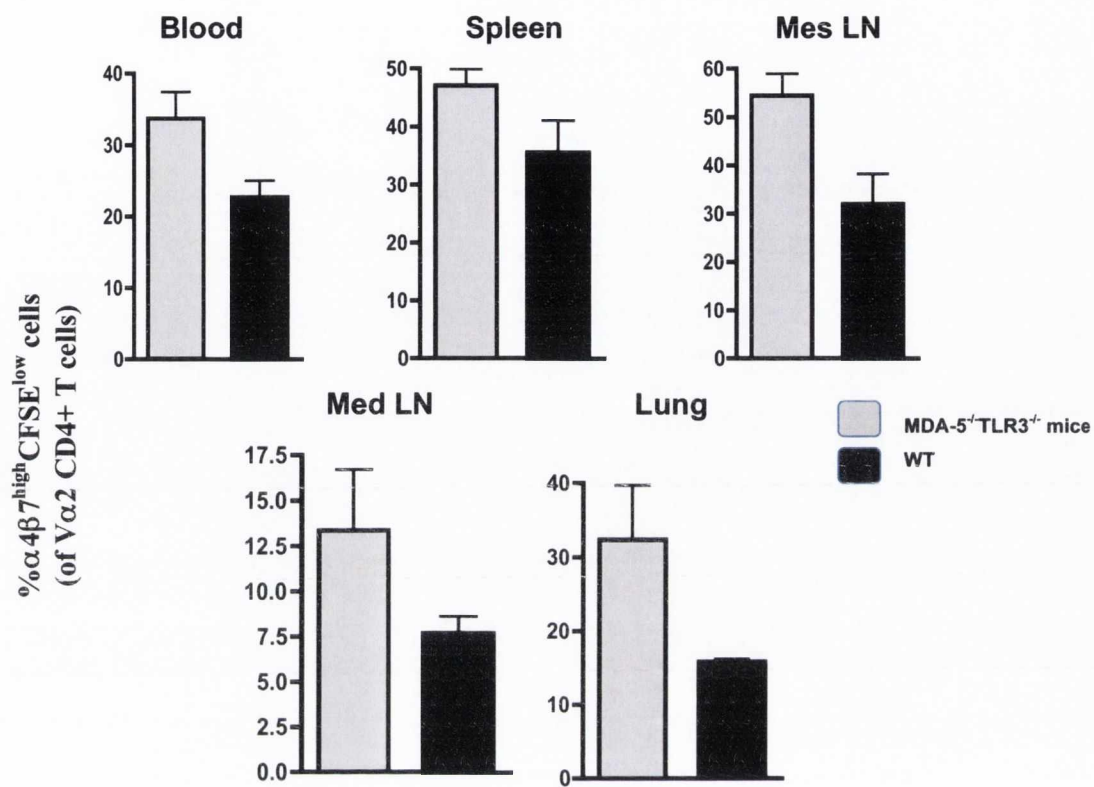


Figure 4.9: Lung DC mediated $\alpha 4 \beta 7$ induction is not dependent on MDA5 or TLR 3 signalling. Cumulative data from three individual experiments revealing the percentage of CFSE^{low} $\alpha 4 \beta 7^{\text{high}}$ cells (y-axis) is compared between mice that were immunized i.n. with ova (100 μ g) plus polyICLC (50 μ g) following the adoptive transfer of CD45.1⁺V α 2⁺CD4⁺OT-II cells into CD45.2⁺ WT or MDA-5^{-/-}TLR3^{-/-} mice.

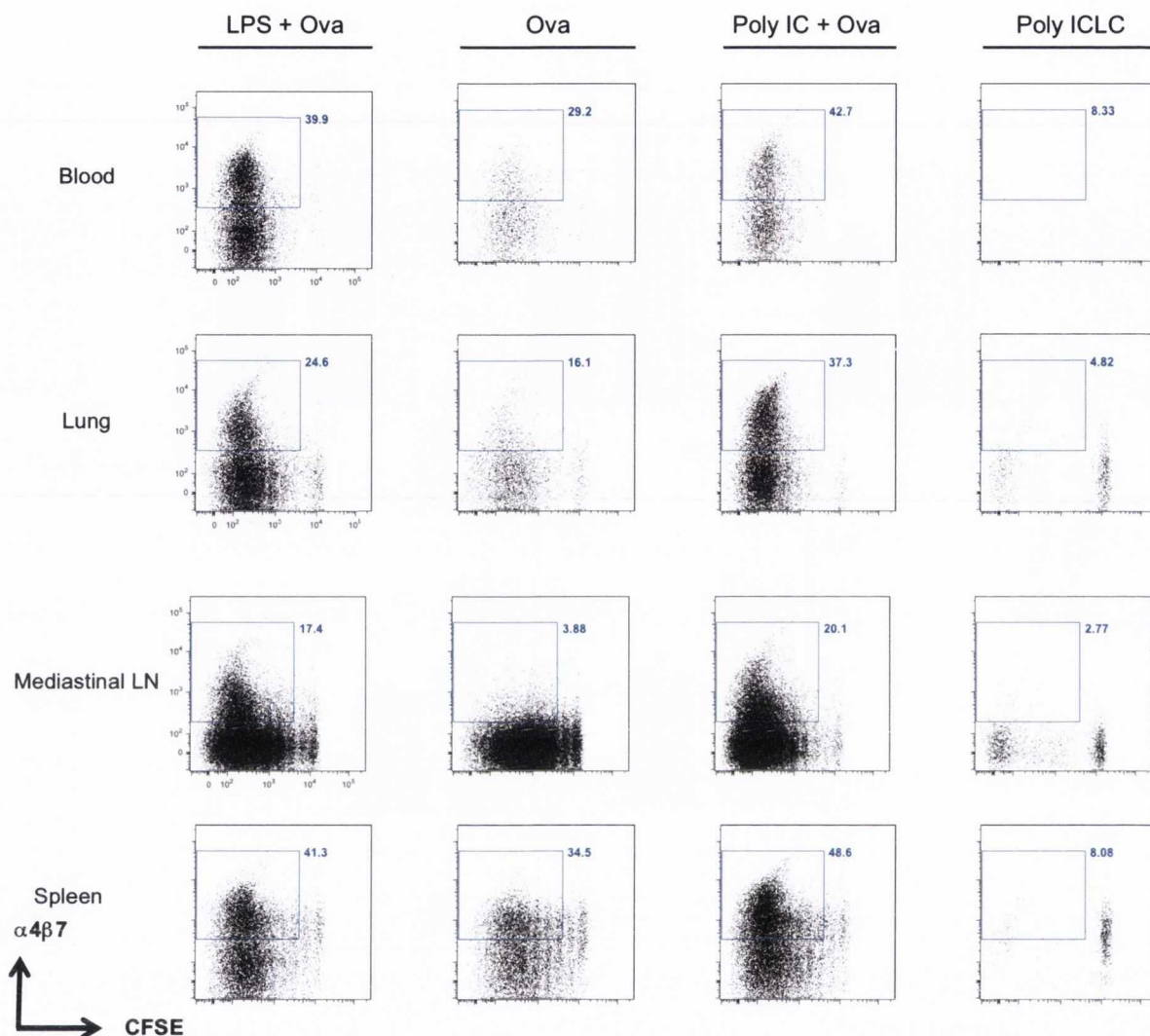


Figure 4.10 Induction of integrin $\alpha 4 \beta 7$ by DCs following i.n. immunization requires antigen but is not adjuvant specific. Representative flow cytometry plots showing the expression of integrin $\alpha 4 \beta 7$ (y-axis) on $CD45.1^+V\alpha 2^+CD4^+OT-II$ cells compared between mice that were immunized i.n. with OVA (100 μ g) alone or, ova (100 μ g) plus LPS (5 μ g) or, ova (100 μ g) plus polyICLC (50 μ g) or, polyICLC (50 μ g) alone following the adoptive transfer of $CD45.1^+V\alpha 2^+CD4^+OT-II$ cells into $CD45.2^+$ recipient mice. 4 days post vaccination the local lymph nodes draining the site of vaccine delivery and systemic compartments were isolated and analysed. Figure 4.10 illustrates a representative of three experiments

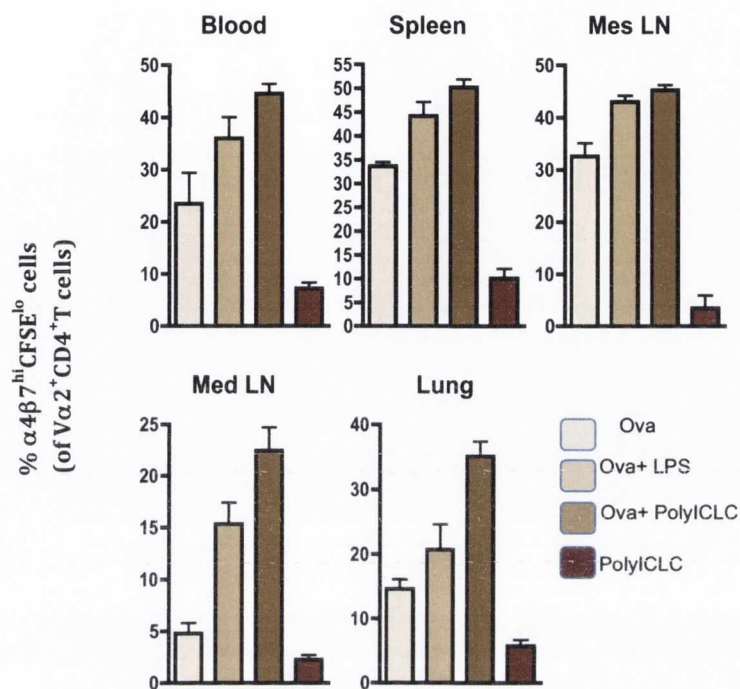


Figure 4.11 Induction of integrin $\alpha 4 \beta 7$ by DCs following i.n. immunization requires antigen but is not adjuvant specific. Cumulative data from three individual experiments revealing the percentage of CFSE^{low} $\alpha 4 \beta 7^{\text{high}}$ cells (y-axis) compared between mice that were immunized i.n. with OVA (100 μ g) alone or, ova (100 μ g) plus LPS (5 μ g) or, ova (100 μ g) plus polyICLC (50 μ g) or, polyICLC (50 μ g) alone following the adoptive transfer of CD45.1⁺V α 2⁺CD4⁺OT-II cells into CD45.2⁺ recipient mice.

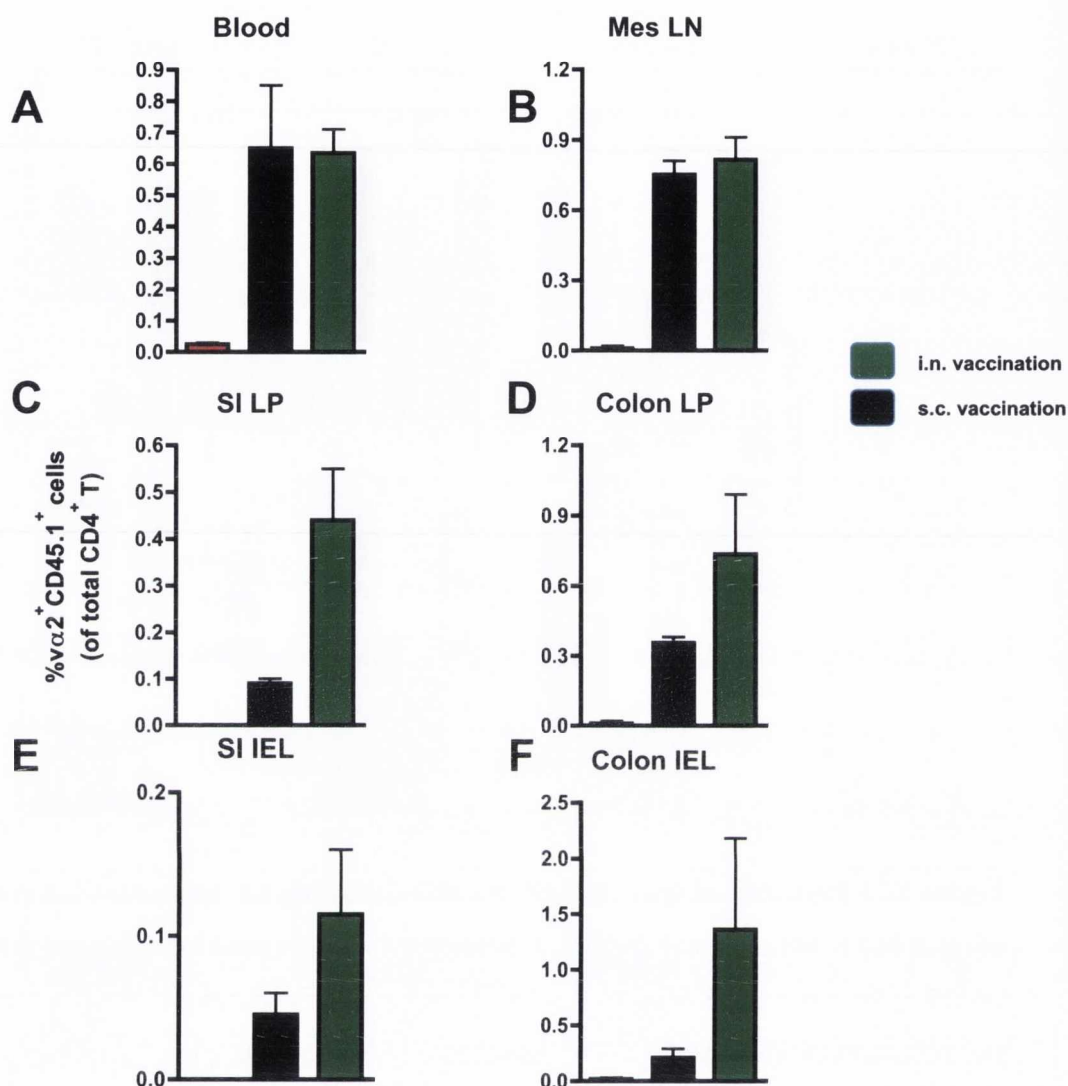


Figure 4.12 Antigen specific, OT-II cells, expand in the gastrointestinal effector sites 4 days post i.n. administration of vaccine. CD45.1⁺Vα2⁺CD4⁺OT-II cells were adoptively transferred to CD45.2⁺ mice followed by immunization with OVA (100μg) plus polyICLC (50μg) after 1 hour. The experimental groups were challenged with 10⁶ PFU *Salmonella*-Ova delivered by oral gavage on day 7 post immunization. Presented above are cumulative data from mice comparing the percentage of the transferred CD45.1⁺Vα2⁺CD4⁺OT-II cells in various tissues including the blood, mesenteric LN, small intestinal (SI) and colonic lamina propria as well as SI and colonic intraepithelial (IEL) compartments

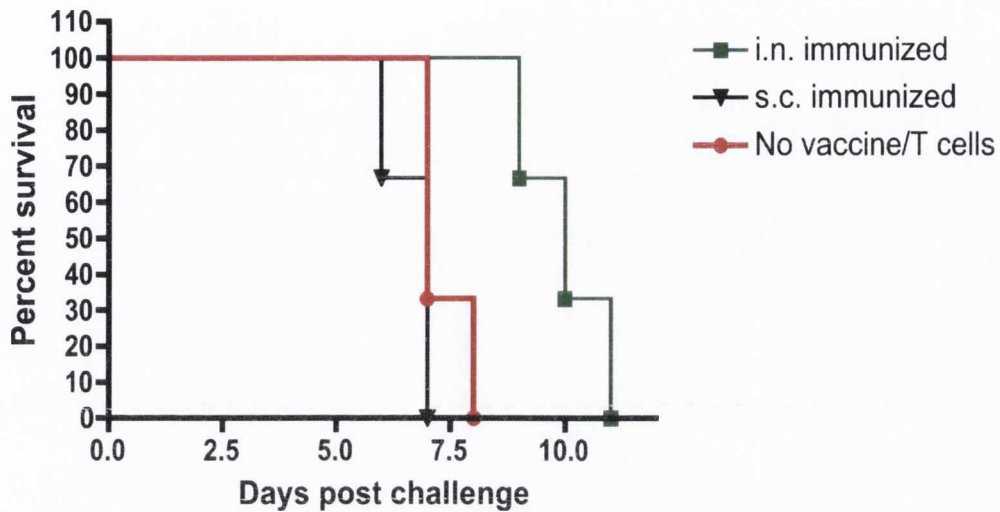


Figure 4.13 I.N. immunization protects mice against enteric challenge with salmonella OVA. CD45.1⁺V α 2⁺CD4⁺OT-II cells were adoptively transferred to CD45.2⁺ mice followed by immunization with ova (100 μ g) plus polyICLC (50 μ g) after 1 hour. The mice were then challenged with 10⁶ PFU *Salmonella*-Ova delivered by oral gavage on day 7 post immunization. Kaplan Meier curves comparing the survival of unimmunized (black) mice or mice immunized i.n. (green) or s.c. (red)

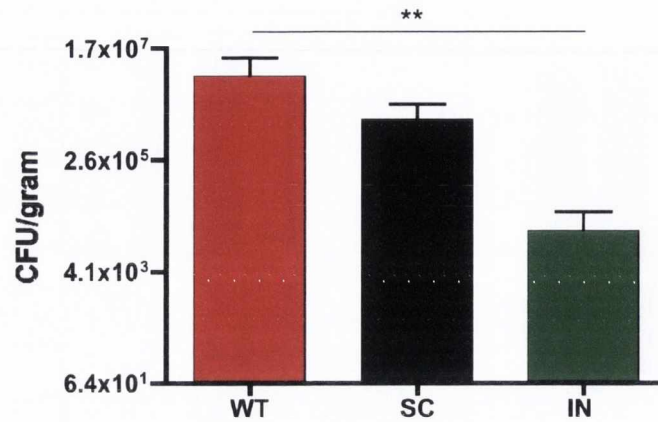


Figure 4.14 I.N. vaccination significantly reduces *Salmonella*-Ova infiltration into the Liver. CD45.1⁺V α 2⁺CD4⁺OT-II cells were adoptively transferred to CD45.2⁺ mice followed by immunization with ova (100 μ g) plus polyICLC (50 μ g) after 1 hour. The mice were then challenged with 10⁶ PFU *Salmonella*-Ova delivered by oral gavage on day 7 post immunization. 6 days post challenge, *Salmonella* colony forming units (CFU)/g stool tissue (y-axis) were determined in the liver of the unimmunized (red), s.c. immunized (black) and i.n. immunized (green) mice, x-axis.

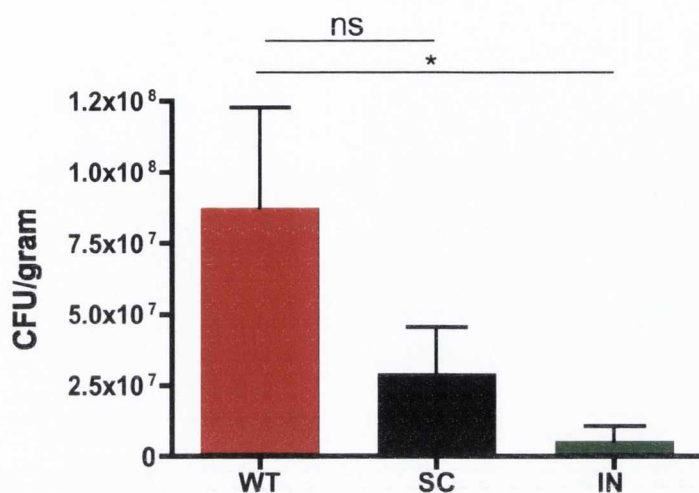


Figure 4.15 I.N vaccination, with heat inactivated *Salmonella* and poly ICLC, results in a significant decrease in bacterial infiltration into the spleen post challenge. Colony forming units (CFU)/g splenic tissue (y-axis) of mice immunized in a prime boost fashion with heat and PFA inactivated *Salmonella* (10^6 PFU) and poly IC ($50\mu\text{g}$) and challenged orally with *Salmonella* on day 7 post boost (10^6 PFU). Red bars depict unimmunized mice, black bars show mice immunized s.c. and green bars represent i.n. immunized mice. Cumulative data from three individual experiments.

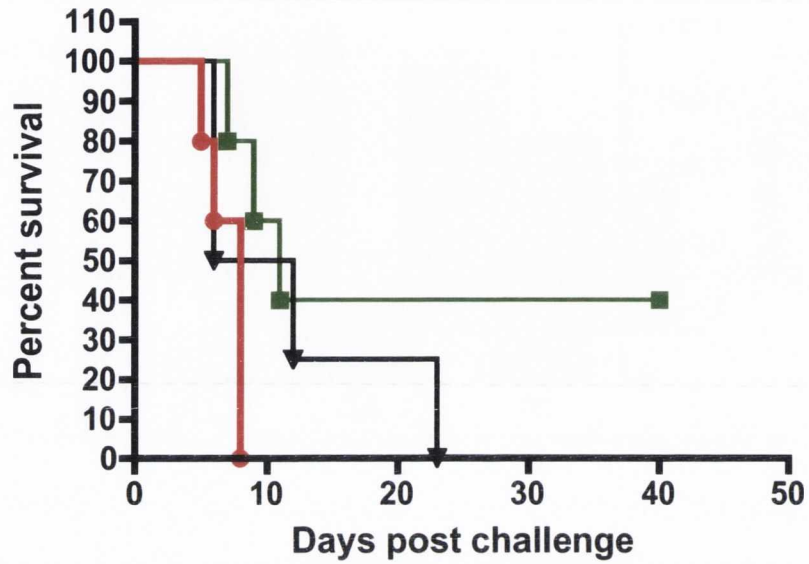


Figure 4.16 I.N. immunization protects against enteric challenge with highly pathogenic *Salmonella*. Kaplan Meier curves comparing the survival of unimmunized (red) mice or mice immunized prime boost i.n. (green) or s.c. (black) with ova (100µg) plus polyICLC (50µg). One week post boost the experimental groups were orally challenged with *Salmonella*-ova (1×10^6 PFU).

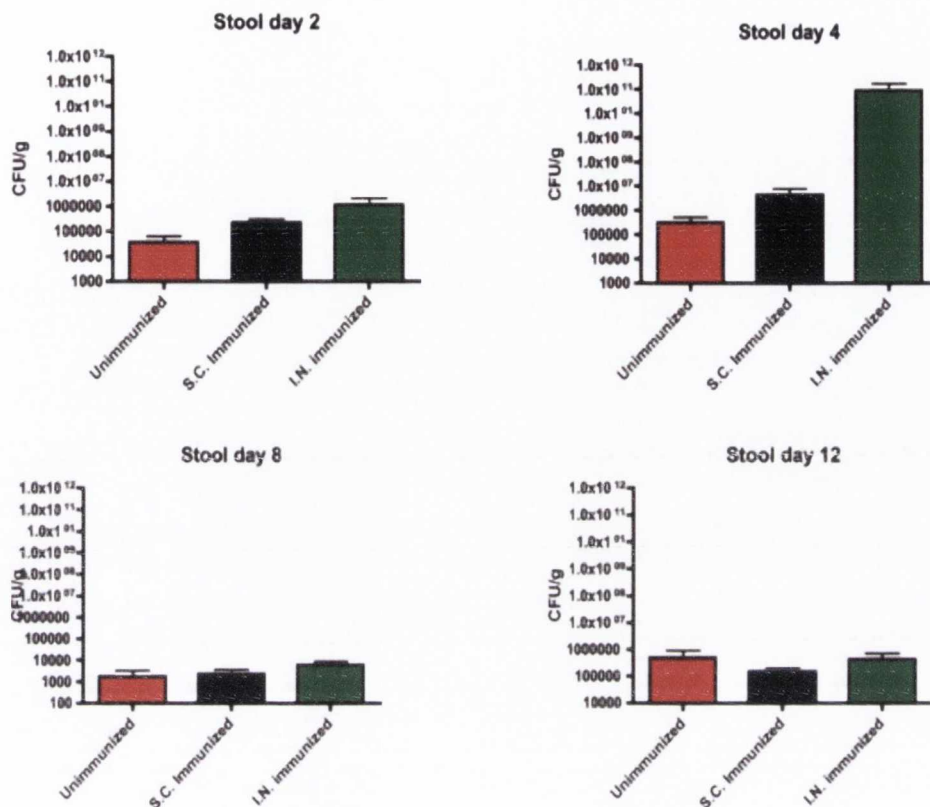
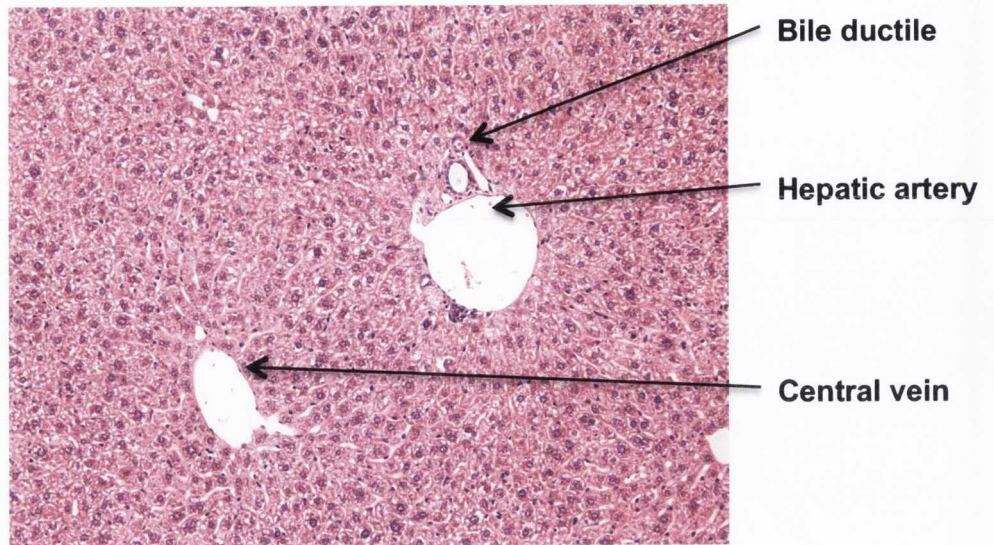


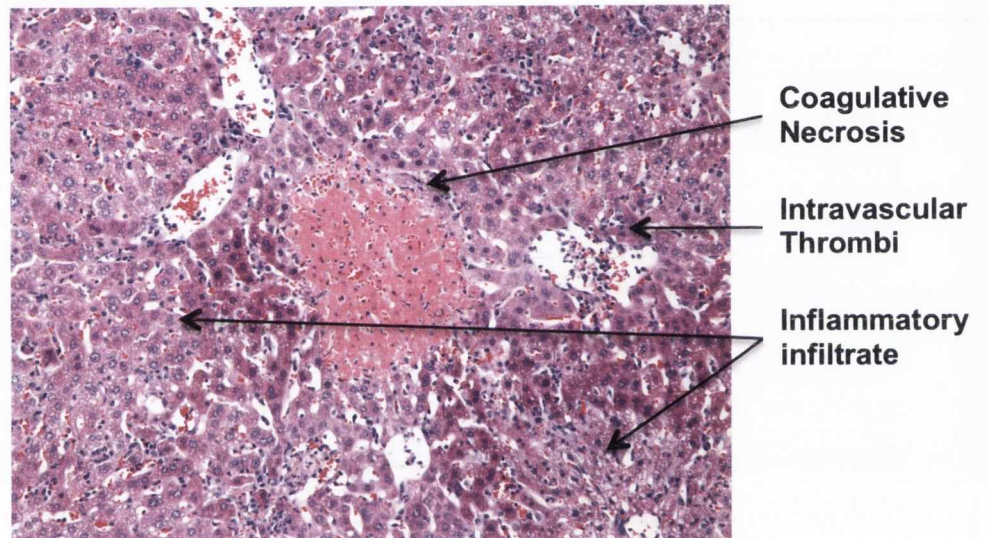
Figure 4.17 I.N. vaccination, with Ova and poly ICLC, protects mice against enteric challenge with *Salmonella*-Ova.

Colony forming units (CFU)/g stool (y-axis) of mice immunized in a prime boost fashion with ova (100µg) plus polyICLC (50µg) against oral challenge with *Salmonella*-Ova (1×10^6 PFU). *Salmonella* colony forming units (CFU)/g stool (y-axis) on days 2, 4, 8 and 12 post challenge in the unimmunized (red), s.c. immunized (black) and i.n. immunized (green) mice, x-axis

A
Wild type



B
Unimmunized



C
Intranasal
Vaccination

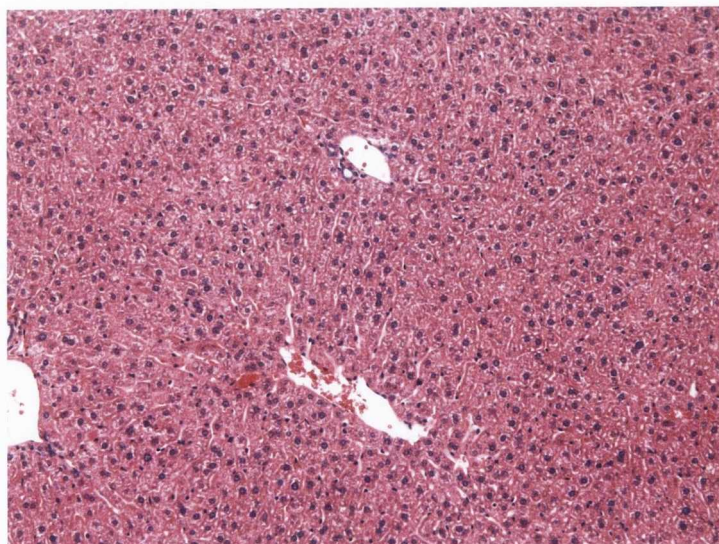
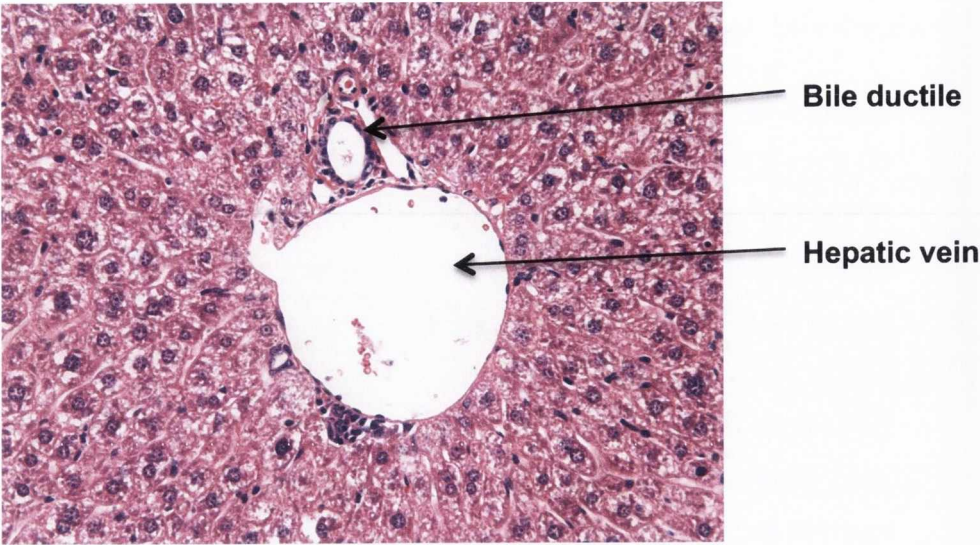


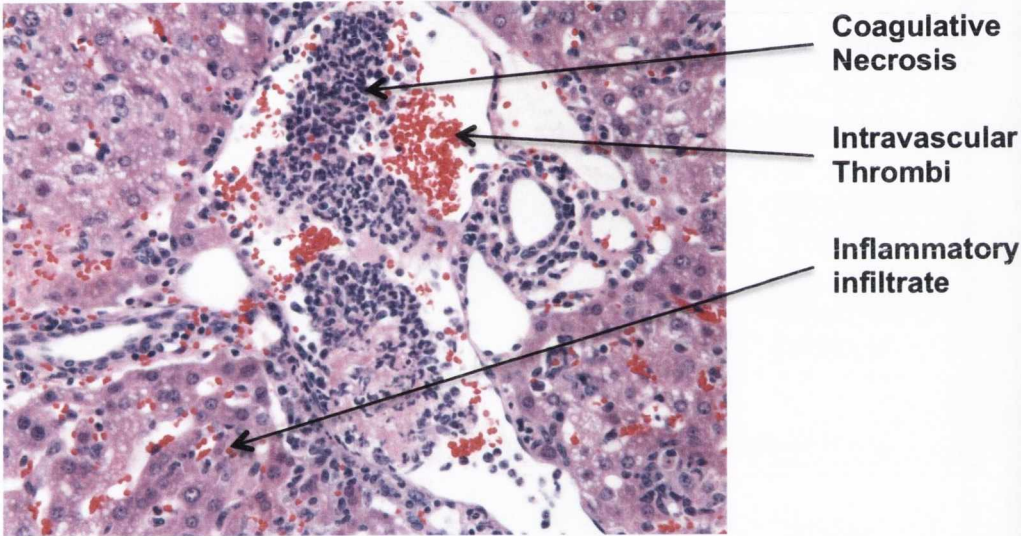
Figure 4.18 Intranasal vaccination, with Ova and poly ICLC, results in protection in the liver following *Salmonella-Ova* challenge.

Hematoxylin & Eosin stained sections examining the Liver (200x mag). C57BL/6 mice were immunized with 100µg of Ova and 50µg of poly ICLC i.n. in a prime boost manner. One week post boost the animals were challenged with 1×10^6 *Salmonella-Ova* by gavage. In addition to the vaccinated mice, wild type C57BL/6 mice were also challenged. The wild type mice were sacrificed on day 6, while the i.n. vaccinated mice were allowed to progress until day 40, the experimental end-point. The liver was removed and preserved in 4% paraformaldehyde. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.18).

A
Wild type



B
Unimmunized



C
Intranasal
Vaccination

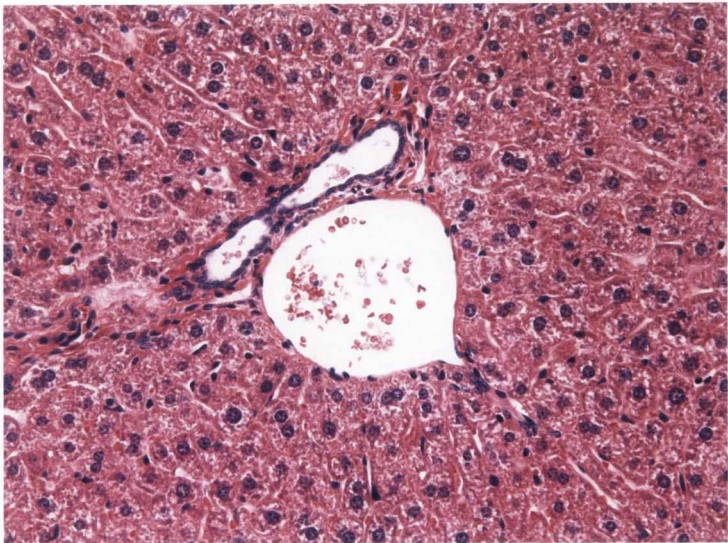
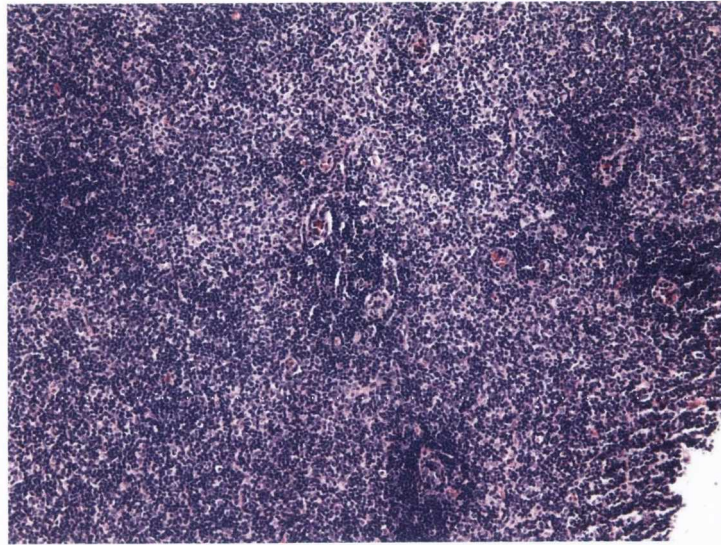


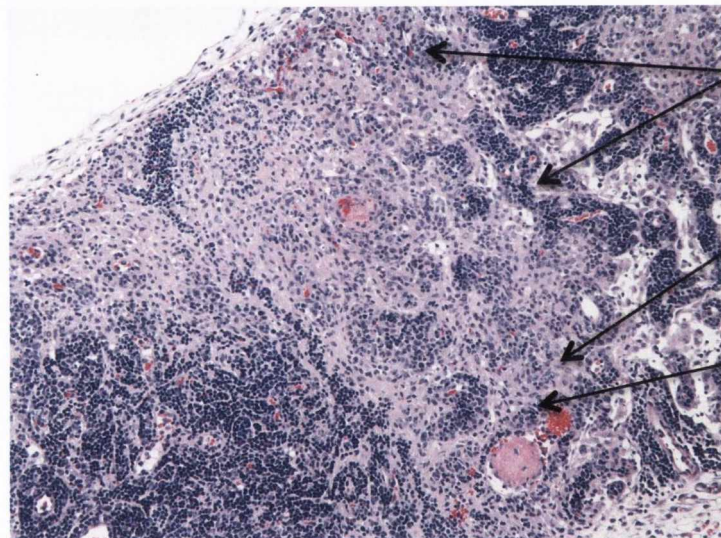
Figure 4.19 Intranasal vaccination, with Ova and poly ICLC, results in protection in the liver following *Salmonella-Ova* challenge.

Hematoxylin & Eosin stained sections examining the Liver (400x mag). C57BL/6 mice were immunized with 100µg of Ova and 50µg of poly ICLC i.n. in a prime boost manner. One week post boost the animals were challenged with 1×10^6 *Salmonella-Ova* by gavage. In addition to the vaccinated mice, wild type C57BL/6 mice were also challenged. The wild type mice were sacrificed on day 6, while the i.n. vaccinated mice were allowed to progress until day 40, the experimental end-point. The liver was removed and preserved in 4% paraformaldehyde. The tissue was embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (H&E) for histopathological analysis (2.2.18).

A
Wild type



B
Unimmunized



Inflammatory
infiltrate

Intravascular
Thrombi

Coagulative
Necrosis

C
Intranasal
Vaccination

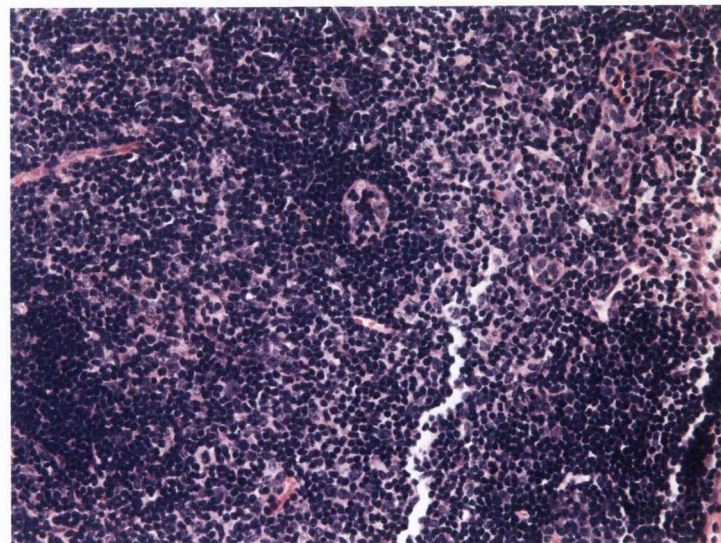
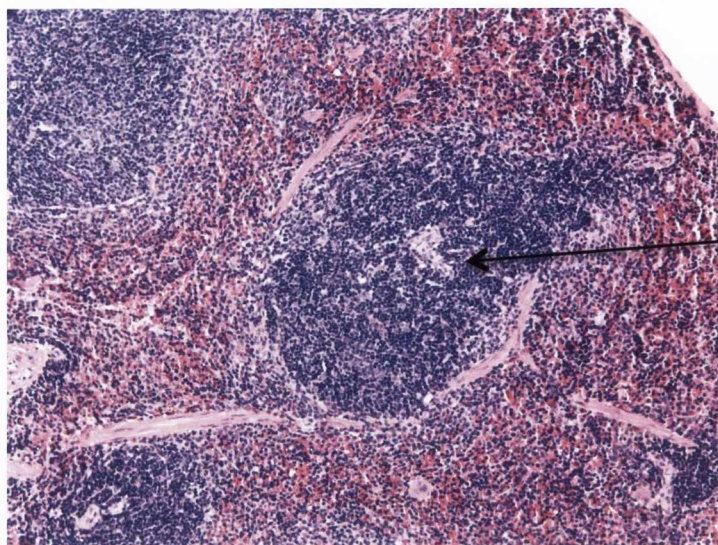


Figure 4.20 Intranasal vaccination, with Ova and poly ICLC, results in protection in the mesenteric lymph node following *Salmonella-Ova* challenge.

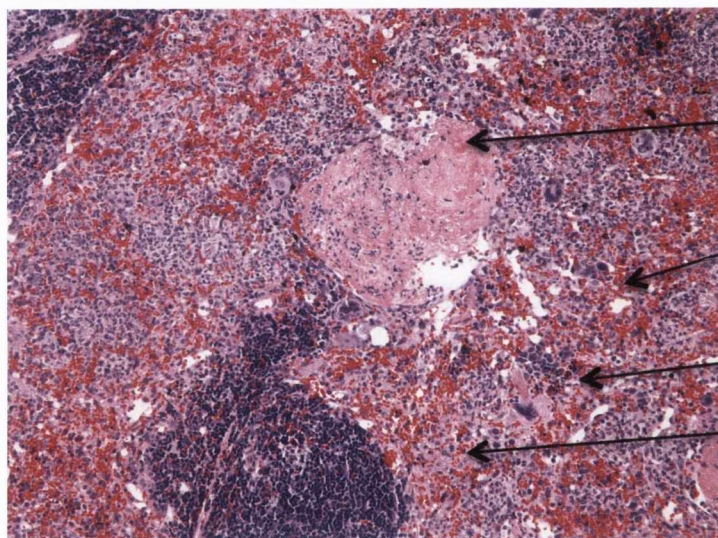
Hematoxylin & Eosin stained sections examining the Mesenteric lymph nodes (200x mag). C57BL/6 mice were immunized with 100µg of Ova and 50µg of poly ICLC i.n. in a prime boost manner. One week post boost the animals were challenged with 1×10^6 *Salmonella-Ova* by gavage. In addition to the vaccinated mice, wild type C57BL/6 mice were also challenged. The wild type mice were sacrificed on day 6, while the i.n. vaccinated mice were allowed to progress until day 40, the experimental end-point. The mesenteric lymph nodes were removed and preserved in 4% paraformaldehyde. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.18).

A
Wild type



White pulp

B
Unimmunized



Inflammatory infiltrate

Coagulative Necrosis

Intravascular Thrombi

Splenic congestion

C
Intranasal Vaccination

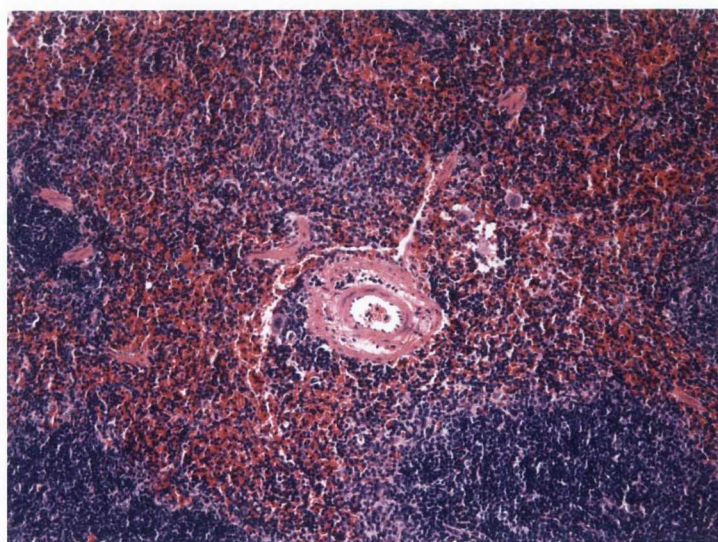


Figure 4.21 Intranasal vaccination, with Ova and poly ICLC, results in protection in the spleen following *Salmonella-Ova* challenge.

Hematoxylin & Eosin stained sections examining the spleen (200x mag). C57BL/6 mice were immunized with 100µg of Ova and 50µg of poly ICLC i.n. in a prime boost manner. One week post boost the animals were challenged with 1×10^6 *Salmonella-Ova* by gavage. In addition to the vaccinated mice, wild type C57BL/6 mice were also challenged. The wild type mice were sacrificed on day 6, while the i.n. vaccinated mice were allowed to progress until day 40, the experimental end-point. The spleen was removed and preserved in 4% paraformaldehyde. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.18)

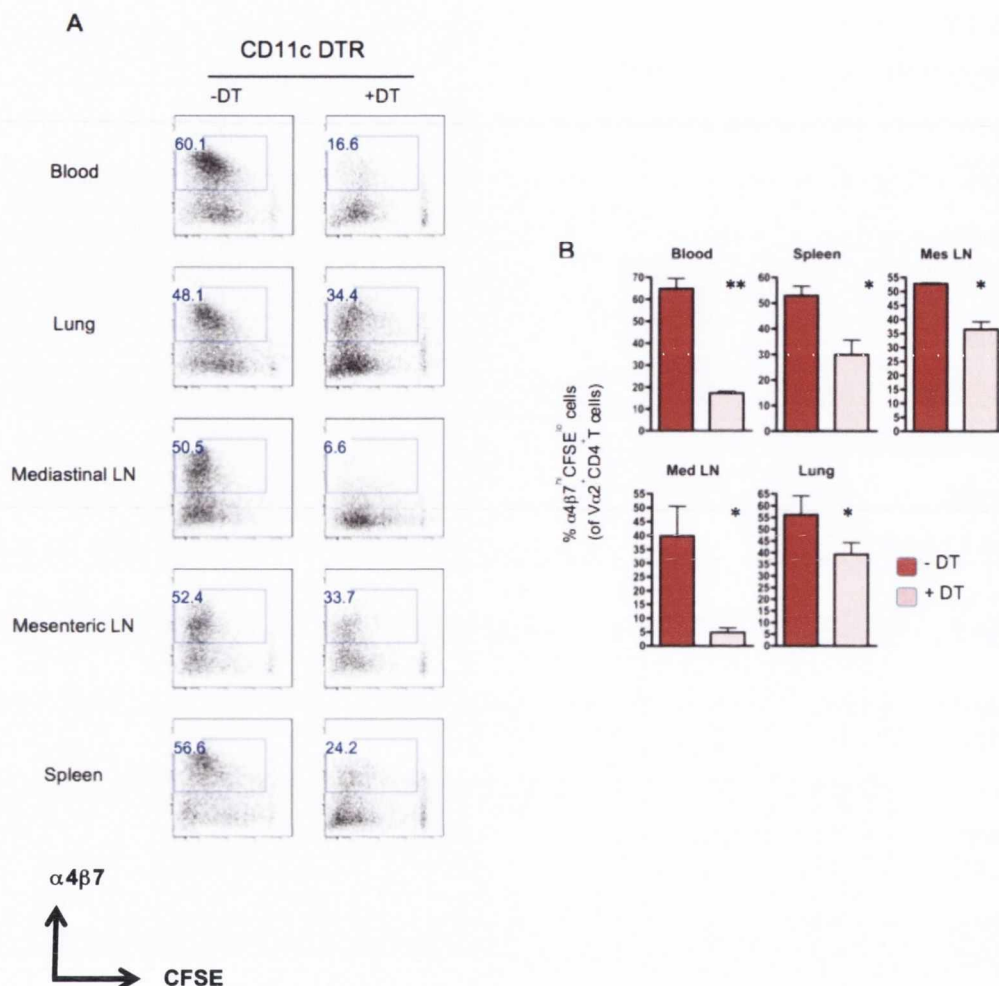


Figure 4.22 Following i.n. immunization, induction of integrin $\alpha 4\beta 7$ is mediated by DCs. (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V $\alpha 2^{+}$ CD4⁺OT-II cells in CD11c- DTR (PBS treated) or CD11c-DTR DT (1 μ g) treated animals, immunized i.n. with ova (100 μ g) and polyICLC (50 μ g). The transferred CD45.1⁺V $\alpha 2^{+}$ CD4⁺OT-II cells were examined after 5 days for the up-regulation of $\alpha 4\beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the blood, lung and mediastinal LN. **(b)** Cumulative data comparing the induction of $\alpha 4\beta 7$ on CD45.1⁺V $\alpha 2^{+}$ CD4⁺OT-II cells (y- axis) between CD11c-DTR or DT treated CD11c-DTR animals followed by i.n. immunization with ova (100 μ g) plus polyICLC (50 μ g).

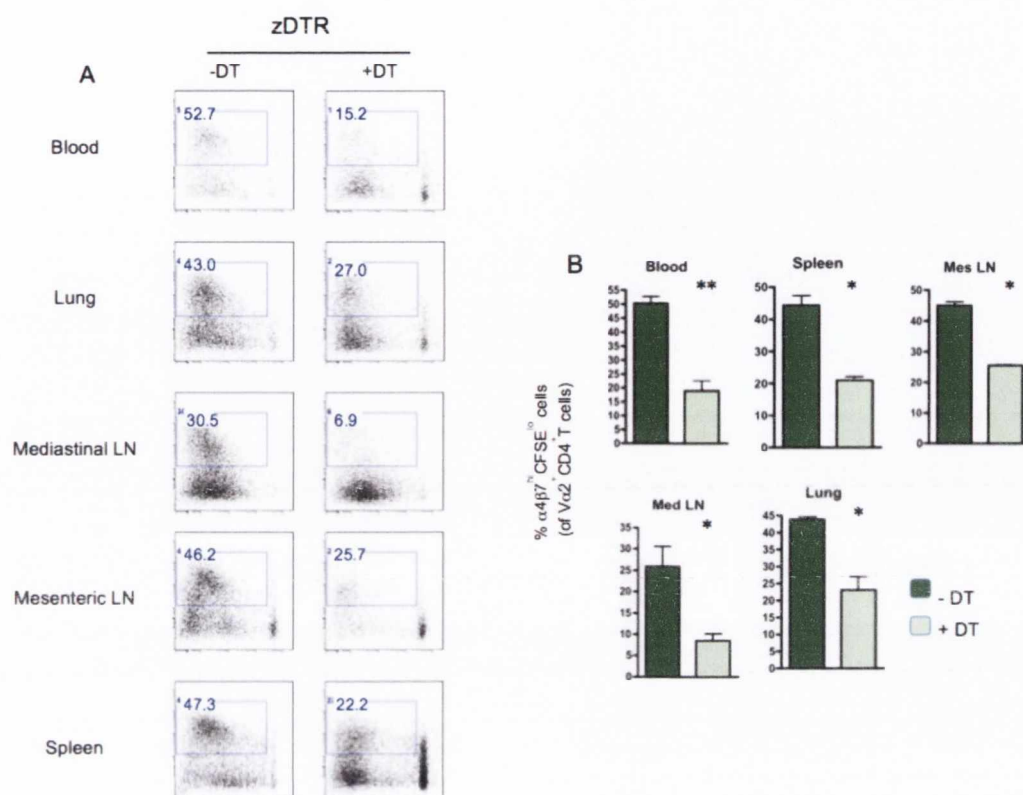


Figure 4.23 Following i.n. immunization, induction of integrin $\alpha 4 \beta 7$ is mediated by DCs. (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4 \beta 7$ on adoptively transferred $CD45.1^+V\alpha 2^+CD4^+OT-II$ cells in zDC-DTR bone marrow chimeric mice administered PBS (left panels) or DT (right panels) one day prior to i.n. immunization with OVA (100 μ g) and polyICLC (50 μ g). The transferred $CD45.1^+V\alpha 2^+CD4^+OT-II$ cells were examined after 5 days for the up-regulation of $\alpha 4 \beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the blood, lung and mediastinal LN. (b) Cumulative data comparing the induction of $\alpha 4 \beta 7$ on $CD45.1^+V\alpha 2^+CD4^+OT-II$ cells (y- axis) in zDC-DTR bone marrow chimeric mice administered PBS or DT followed by i.n. immunization with OVA (100 μ g) plus polyICLC (50 μ g).

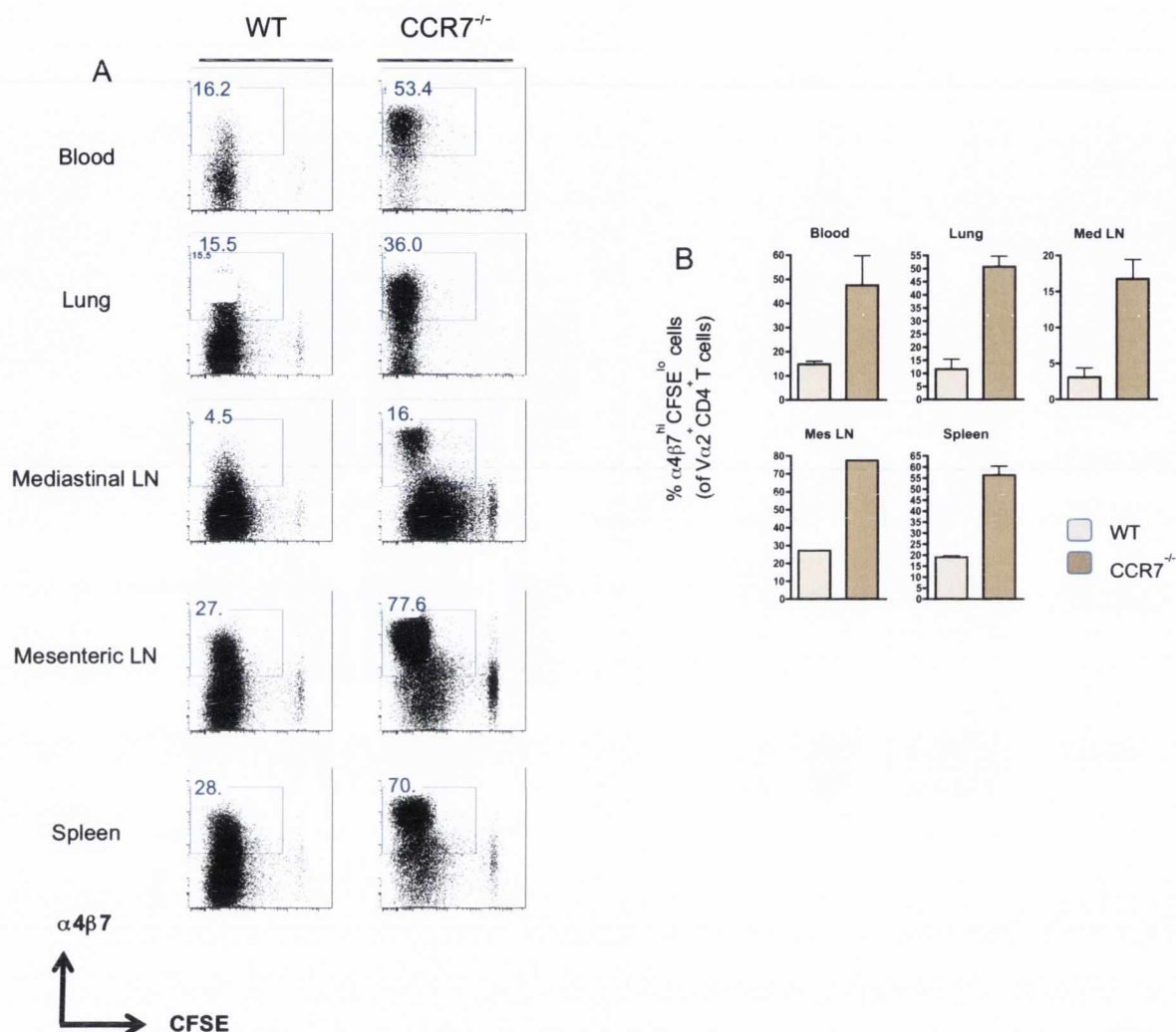


Figure 4.24 Following i.n. immunization, induction of integrin $\alpha 4 \beta 7$ is mediated by lung resident DCs. (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4 \beta 7$ on adoptively transferred CD45.1⁺V $\alpha 2^{+}$ CD4⁺OT-II cells in WT and CCR7^{-/-} animals five days following i.n. immunization with ova (100 μ g) and polyICLC (50 μ g). The transferred CD45.1⁺V $\alpha 2^{+}$ CD4⁺OT-II cells were examined after 5 days for the up-regulation of $\alpha 4 \beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the blood, lung and mediastinal LN. (b) Cumulative data from 3 experiments comparing the induction of $\alpha 4 \beta 7$ on CD45.1⁺V $\alpha 2^{+}$ CD4⁺OT-II cells (y- axis) in WT and CCR7^{-/-} animals followed by i.n. immunization with OVA (100 μ g) plus polyICLC (50 μ g).

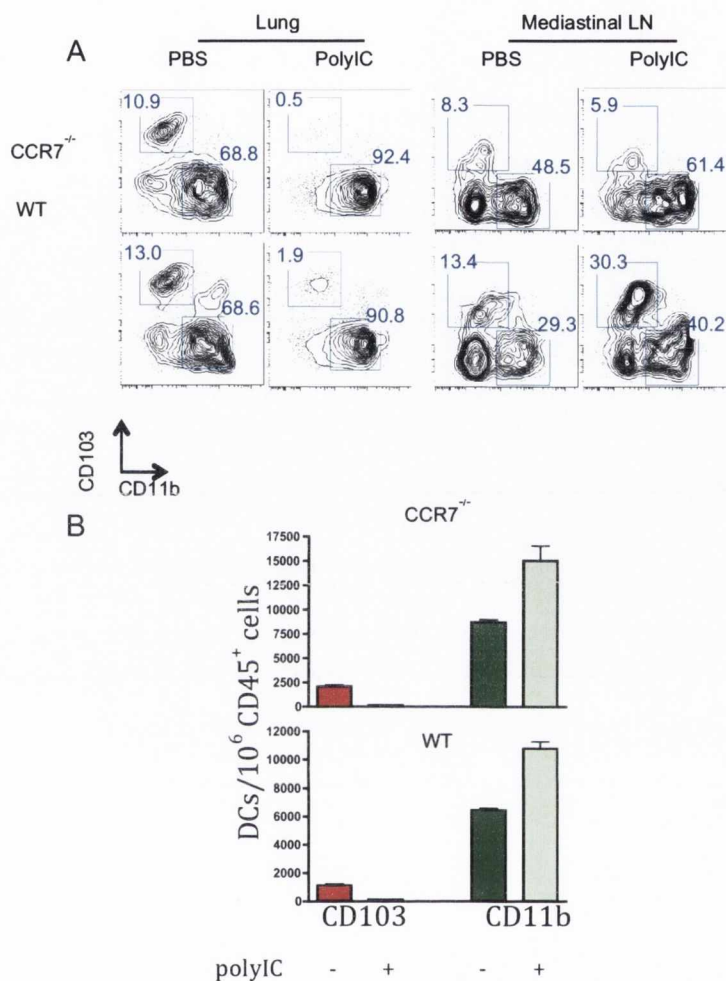


Figure 4.25 Phenotypic analysis of lung resident and migratory DCs. (a) Representative flow cytometry plots illustrating MHCII⁺CD11c^{hi} gated CD103⁺CD11b⁻, CD103⁻CD11b⁺ and CD103⁻CD11b⁻ DCs in the lung and mediastinal lymph node of WT and CCR7^{-/-} animal 24 hours post administration of PBS or poly ICLC (b) Cumulative data.

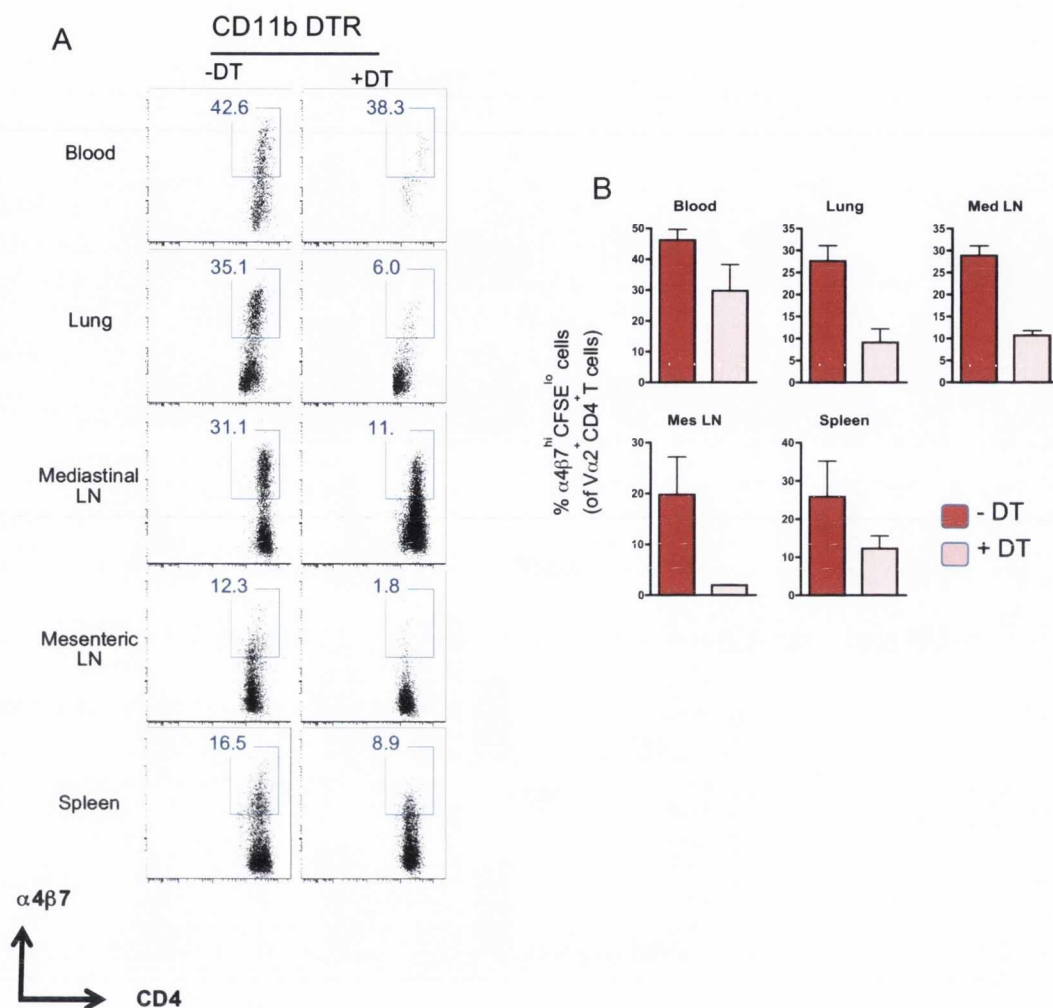


Figure 4.26: Ablation of CD11b⁺ cells attenuates the induction of $\alpha 4\beta 7$ on transferred OT-II cells following i.n. immunization. (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells in CD11b-DTR bone marrow chimeric mice administered PBS (left panels) or DT (right panels) one day prior to i.n. immunization with ova (100 μ g) and polyICLC (50 μ g). The transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells were examined after 5 days for the up-regulation of $\alpha 4\beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the blood, lung and mediastinal LN. (b) Cumulative data from 3 experiments comparing the induction of $\alpha 4\beta 7$ on CD45.1⁺V α 2⁺CD4⁺OT-II cells (y-axis) between CD11b-DTR bone marrow chimeric mice administered PBS or DT followed by i.n. immunization with OVA (100 μ g) plus polyICLC (50 μ g).

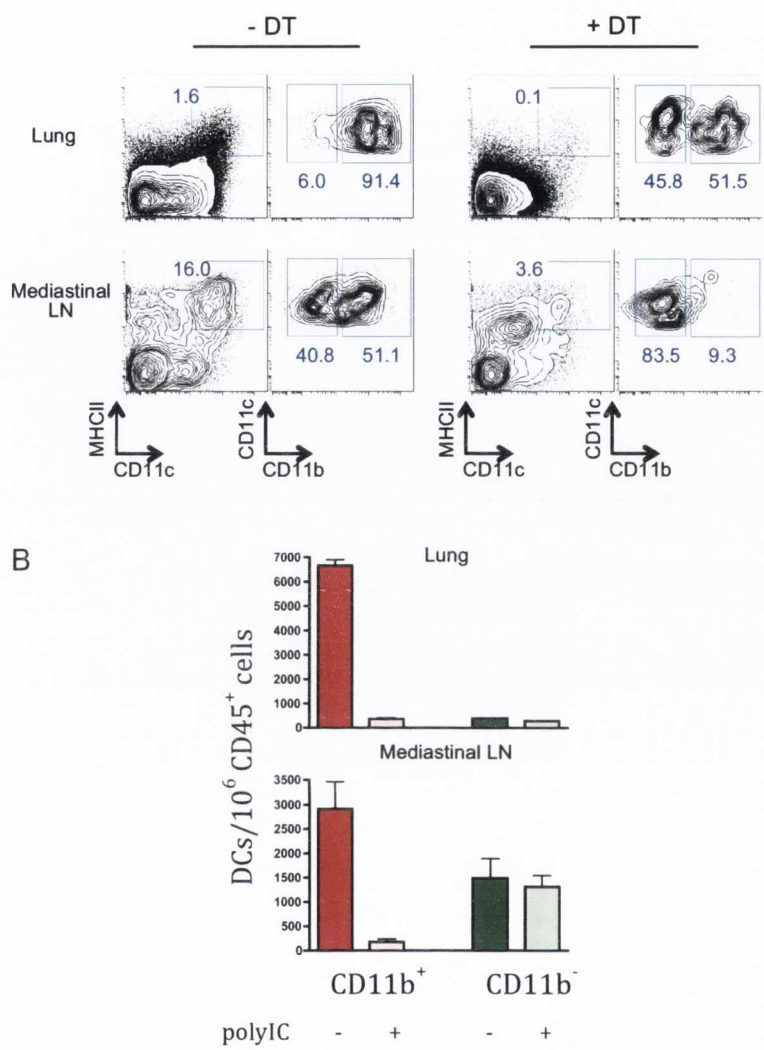


Figure 4.27: Phenotypic analysis of lung CD11b⁺ DC Ablation. (a) Representative flow cytometry plots comparing lung (top panels) and mediastinal LN (bottom panels) of CD11b-DTR bone marrow chimera. MHCII⁺CD11c⁺ gated CD11b⁺ cells are compared between mice administered PBS or DT (b) Cumulative data from 3 experiments showing the absolute number of MHCII⁺CD11c⁺ gated CD11b⁺ and CD11b⁻ cells in the lungs (top panel) and mediastinal LN (bottom panel) following administration of DT.

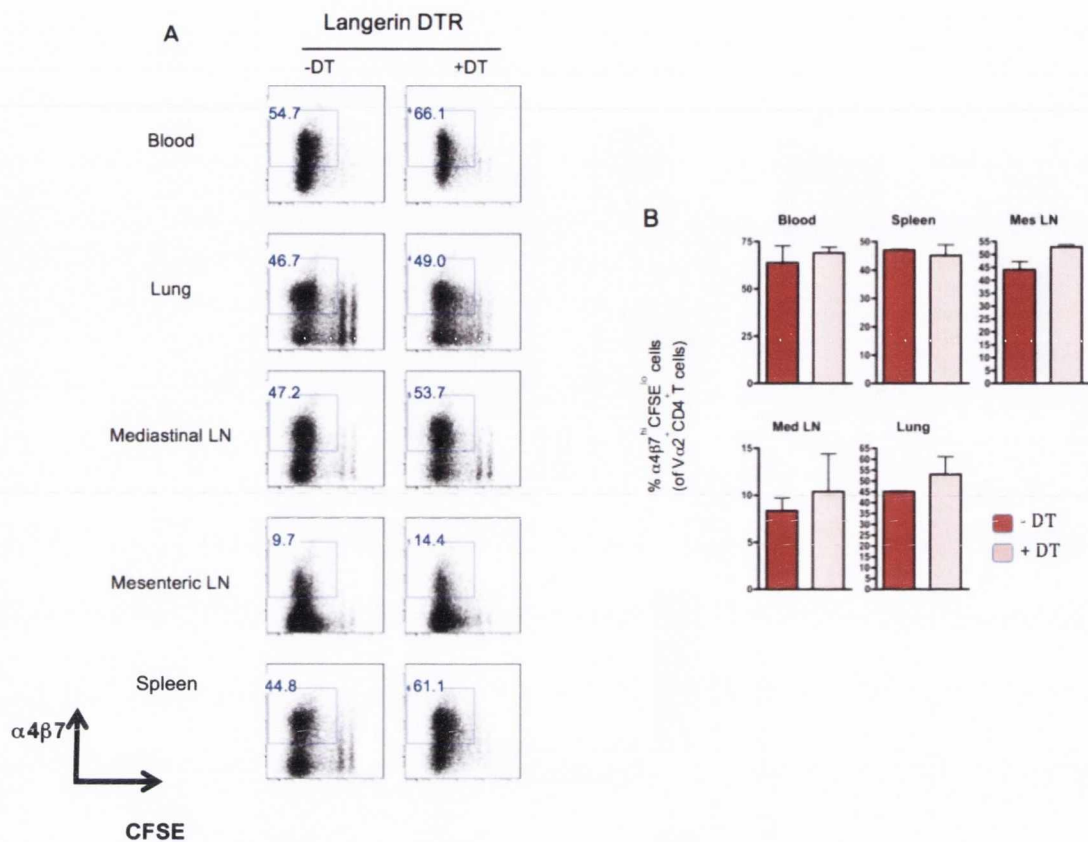


Figure 4.28: Ablation of CD103⁺ DCs does not impact on the induction of $\alpha 4\beta 7$ on transferred OT-II cells following i.n. immunization (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells in langerin-DTR mice administered PBS (left panels) or DT (right panels) one day prior to i.n. immunization with ova (100 μ g) and polyICLC (50 μ g). The transferred CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells were examined after 5 days for the up-regulation of $\alpha 4\beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the blood, lung and mediastinal LN. (b) Cumulative data comparing the induction of $\alpha 4\beta 7$ on CD45.1⁺V $\alpha 2$ ⁺CD4⁺OT-II cells (y-axis) between langerin-DTR bone marrow chimeric mice administered PBS or DT followed by i.n. immunization with OVA (100 μ g) plus polyICLC (50 μ g).

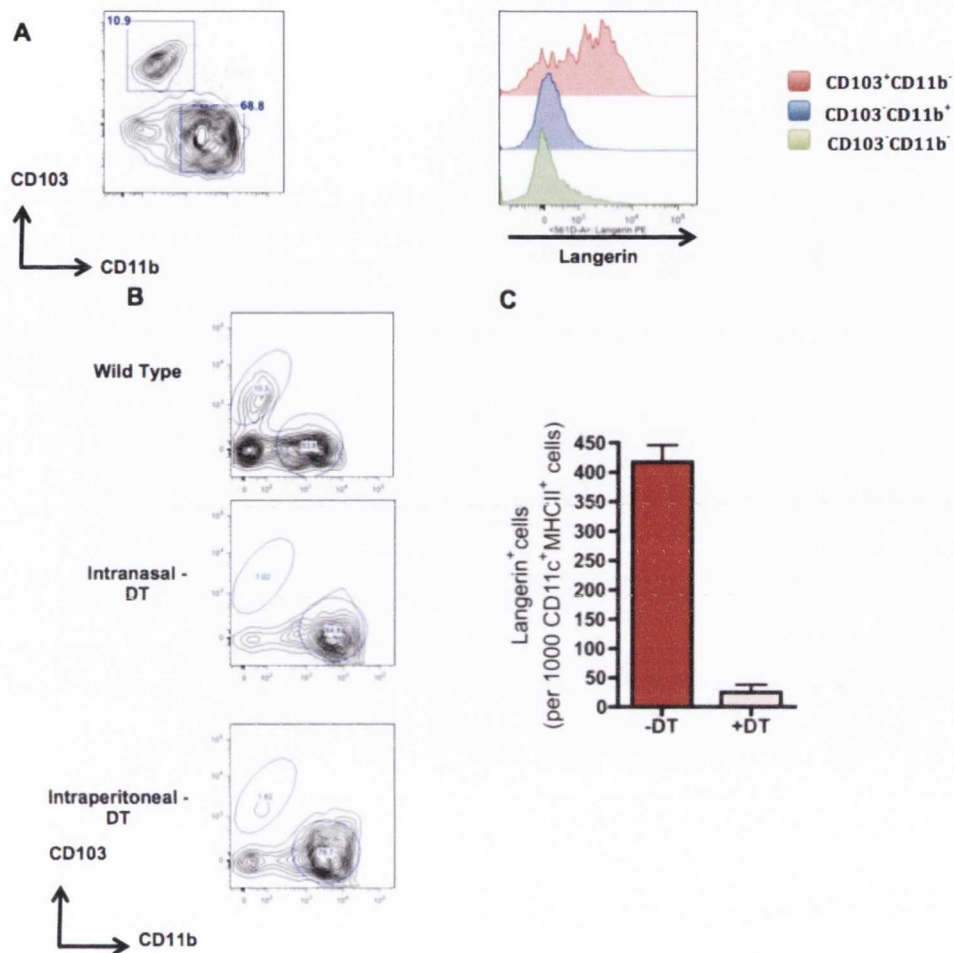


Figure 4.29 Administration of DT i.p. and i.n. results in the ablation of langerin⁺CD103⁺ dendritic cells in langerin DTR mice. 1 μ g DT was administered per mouse (per 25-30 gram). 36 hours either i.p or i.n. to langerin DTR and wild type animals. 36 hours post administration the animals were sacrifice and the lung compartment isolated. (a) Representative flow plots illustrating Langerin expression on lung resident DCs (b) Representative flow plot revealing in vivo ablation of CD103⁺Langerin⁺ DCs (c) Cumulative figure illustrating Langerin⁺ DC depletion.

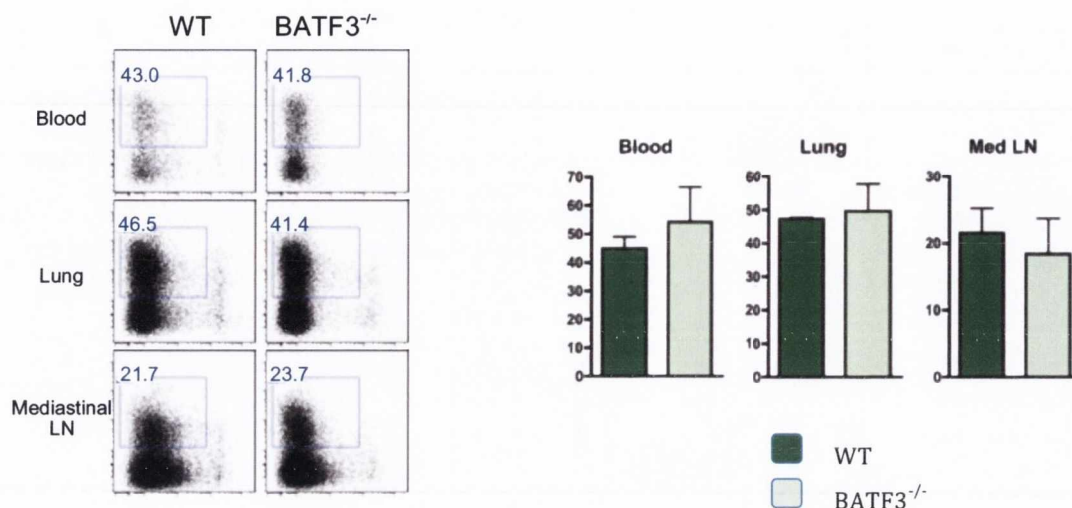


Figure 4.30: Ablation of CD103⁺ DCs does not impact on the induction of $\alpha 4\beta 7$ on transferred OT-II cells following i.n. immunization (a) Representative flow cytometry plots showing the *in vivo* induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells in BATF3^{-/-} mice administered PBS (left panels) or DT (right panels) one day prior to i.n. immunization with OVA (100 μ g) and polyICLC (50 μ g). The transferred CD45.1⁺V α 2⁺CD4⁺OT-II cells were examined after 5 days for the up-regulation of $\alpha 4\beta 7$ (y-axis) on proliferating (CFSE^{low}, x-axis) cells in the blood, lung and mediastinal LN. (b) Cumulative data from 3 experiments comparing the induction of $\alpha 4\beta 7$ on CD45.1⁺V α 2⁺CD4⁺OT-II cells (y-axis) between BATF3^{-/-} bone marrow chimeric mice administered PBS or DT followed by i.n. immunization with OVA (100 μ g) plus polyICLC (50 μ g).

Chapter 5

Lung DC targeting as a strategy to induce GI responses against HIV-1

5.1 Introduction

The GI tract harbours a large complement of immune cells that are preferentially targeted during acute HIV and SIV infections, regardless of the route of virus inoculation (Mehandru et al, 2005). Mucosal GI lymphocytes are in close proximity to the external environment and are phenotypically distinct from peripheral blood lymphocytes; the majority of which (>90%) exhibit a memory phenotype (Schieferdecker et al., 1992). Additionally, up to 70% of these GI lymphocytes have robust expression of the chemokine receptors, CCR5 and CXCR4 (Anton et al., 2000). These receptors provide HIV-1 with an ideal environment to establish infection, acting as essential co-receptors for entry of CCR5-tropic HIV-1 viruses into CD4⁺ T cells. This has been well established in SIV/macaque studies, where during the first 10 days, SIV infected cells in the GI tract were found to be CCR5⁺ memory CD4⁺ T cells (Mehandru et al., 2000).

In other studies of acutely HIV-infected humans and SIV-infected macaques, there was up to a 10-fold higher frequency of infected CD4⁺ T cells in the GI tract compared to peripheral blood. Evidence has revealed that the acute phase is critical for establishing the course of infection, in both humans and macaques. Therefore, having demonstrated that significant viral replication and immune depletion occurs at mucosal sites during acute infection (Mehandru et al., 2005), it has been argued that the goal of an effective HIV vaccine should be to interrupt mucosal transmission at its earliest stages and to prevent viral replication in mucosal tissues.

Given the extraordinary challenge in developing an effective HIV vaccine, most agree that novel approaches need to be identified. Our lab has been developing a strategy to harness the immunizing properties of DCs. The current approach under investigation is to deliver antigens conjugated to monoclonal antibodies (mAbs) that selectively deliver them to DCs in lymphoid tissue. We have been exploring DEC-205 (CD205), a member of the mannose receptor family of C-type lectins that is abundantly expressed on DCs in lymphoid tissues (Pack et al., 2008) and is capable of mediating efficient antigen uptake and presentation (Lahoud et al., 2012). mAbs specific to DEC-205 have been cloned and

engineered to express defined antigenic proteins. These DEC-protein fusion mAbs selectively target DCs *in vivo*, inducing potent immune responses but only when concomitantly administered with a DC maturation signal (Trumpfeller et al., 2006). In a proof of concept study, a single dose of α -DEC-205-Ova fusion mAb co-administered with anti-CD40 (for DC maturation) rapidly targeted DCs, with antigen presentation persisting for >2 weeks.

To extend these concepts to HIV vaccine development, we have made a α -DEC-205-HIV Gag p24 fusion mAb. A single dose of fusion mAb given simultaneously with poly IC and α -CD40 induced significantly more CD4⁺ Th1 type T cells in mice when compared with other HIV vaccine strategies such as high doses of HIV gag protein, HIV gag plasmid DNA or recombinant adenovirus-gag (Nchinda et al., 2010). Poly IC, a synthetic ds-RNA, was chosen as the maturation stimulus since it has the potential to be used in the clinical setting and in mice, ligates to TLR-3 and MDA-5 receptors in DCs and activates them (Caskey et al., 2011). Subsequent studies from our lab have determined that poly IC and poly ICLC alone, in the absence of α -CD40 is an effective adjuvant for achieving systemic CD4⁺ T cell immunity (Caskey et al., 2012). Priming for CD8⁺ T cells utilizing the DEC-205 targeting system is currently weak, however DEC-targeting strikingly boosts CD8⁺ T cells primed with DNA or adenoviral vectors (*unpublished*). Notably, GI mucosal immunity, probably the most relevant to the HIV-1 vaccine development effort, has not been examined using this novel DEC targeted approach thus far.

Mucosal vaccination offers many attractive features including the ease of administration, potential for mass immunization, reduced cost of production, storage and delivery. Given ongoing advances in understanding the mucosal immune system and a critical need for protection at mucosal surfaces for preventative HIV vaccines, interest in mucosal vaccine discovery is accelerating.

Data presented in chapter 4 demonstrated that i.n. vaccination targets lung resident DCs and induces protective immunity to enteric pathogenic challenge. Based on this, the

ability of i.n. vaccination to induce HIV-specific responses in the GI tract, and the lung DC population responsible were investigated.

The studies presented here represent an important step in better understanding GI mucosal immunity, which has direct relevance for HIV vaccine development efforts.

Key objectives of Chapter 5:

- **To determine if HIV specific T cells are induced in the effector sites of the GI tract following i.n. vaccination.**
- **To determine the durability of immune responses in the GI mucosa post i.n. vaccination.**
- **Determine the DC populations responsible for inducing immune responses post i.n. vaccination.**

5.2 Results

5.2.1 Intranasal immunization with α -DEC-205-gag p24 mAb and poly ICLC induces IFN- γ ⁺CD4⁺ T cell responses in the gastrointestinal tract

HIV-1 infection occurs primarily through the vaginal and GI mucosal surfaces, with approximately 80% of new infections being initiated by a single virus species or a few quasispecies (Mehandru et al., 2005). Recent progress in HIV-1 and SIV pathogenesis has revealed that mucosal tissues, primarily the GI tract, are major sites for early viral replication and CD4⁺ T cell destruction, and may be the major viral reservoir, even in patients receiving HAART (Mehandru et al., 2006). This is likely due to the fact that the majority of CD4⁺ T cells co-expressing chemokine receptors required for HIV-1 entry reside in mucosal tissue. As a result the intestinal immune response represents a significant target for HIV-1 infection. Thus, it is increasingly evident that effective therapies and vaccines must be directed towards eliminating HIV-1 in mucosal tissue reservoirs, protecting mucosal CD4⁺ T cells and stimulating effective mucosal immune responses. Given this need to induce HIV-specific responses at mucosal surfaces, we sought to utilize the findings from Chapter 3 and 4 that lung DCs mediate homing of cells to the GI tract in order to generate antigen-specific immunity towards HIV antigens.

In order to assess the capacity of i.n. vaccination as a strategy to induce GI responses, an initial proof of concept study was conducted. C57BL/6 mice were immunized with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g) by the following routes: i.p., i.n., i.m., i.v. and s.c. in a prime-boost regimen (*described in methods*). GI mucosal immune responses were compared to splenic immune responses between different routes of immunization, with control mice immunized with α -DEC-empty vaccine (5 μ g) and poly ICLC (50 μ g) delivered i.p (**Fig. 5.1**). One-week post boost, mononuclear cells were isolated from the small intestinal lamina propria and re-stimulated with a pool of p24 or p17 peptides (1 μ g/ml) and anti-CD28 (1 μ g/ml).

As expected, robust IFN- γ ⁺CD4⁺ T cell responses were observed in the GI tract following i.p. immunization. Interestingly, the i.n. route of immunization also induced significant

levels of antigen specific IFN- γ ⁺CD4⁺ (**Fig. 5.1, 5.2**) T cells comparable to the positive control (i.p.). In contrast C57BL/6 mice immunized with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g) i.m., i.v. or s.c. failed to produce detectable levels of antigen specific T cells in the small intestinal lamina propria (**Fig. 5.2**). This is consistent with data from chapter 3, which revealed that i.n. vaccination enhances the migration of transferred cells to the GI tract but not s.c. vaccination.

5.2.2 Intranasal immunization with α -DEC-205-gag p24 mAb and poly ICLC induces IFN- γ ⁺CD4⁺ T cells in the spleen.

Having established that i.n. vaccination has the capacity to induce robust levels of antigen specific T cells in the GI mucosa, the ability of i.n. vaccination to induce systemic immunity was addressed. It has been shown that i.n. vaccination with BCG can confer protective immunity against *Mycobacterium bovis* challenge (Giri et al., 2006). i.n. vaccination with BCG induces significantly higher immune responses at a local level (specifically the mediastinal LNs, cervical LNs and lung)

C57BL/6 mice were immunized with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g) by the following routes: i.p., i.n., i.m., i.v. and s.c. in a prime-boost regimen (as described previously). One-week post boost splenocytes were isolated and T cells re-stimulated with a pool of p17 or p24 peptides (1 μ g/ml) and anti-CD28 (1 μ g/ml). Splenic responses were best elicited with i.n. immunization; although lower levels of antigen-specific CD4⁺ T cells were also observed with i.p., i.m. and s.c. routes of immunization (**Fig. 5.3, 5.4**)

5.3.3 The lung, nasal associated lymphoid tissues (NALT) and mediastinal lymph nodes are the site of priming following i.n. immunization

Experiments described in chapter 3, demonstrated that following i.n. vaccination, T cell priming and education occurs in the lung and mediastinal LNs (**Fig. 3.3, 3.4**), resulting in enhanced migration of T cells to the GI effector sites. Following the observation that i.n. vaccination induces IFN- γ^+ CD4 $^+$ T cells in the GI lamina propria, the induction of antigen specific CD4 $^+$ T cells in both local and systemic compartments was investigated. Following i.n. vaccination of C57BL/6 animals (prime boost) with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g), mononuclear cells were isolated from the lung, nose and mediastinal LN.

Upon re-stimulation of isolated cells with a pool of p24 or p17 peptides, IFN- γ^+ CD4 $^+$ T cell responses were elicited in the lung and mediastinal LN (**Fig. 5.5, 5.6**). Similarly, IFN- γ^+ CD4 $^+$ T cell responses were also observed at the site of vaccine delivery, the nose (**Fig. 5.6**).

5.2.4 Following i.n. immunization with α -DEC-205-gag p24 mAb and poly ICLC, immunity is predominantly detected in immune effector (and not inductive) sites of the gastrointestinal tract.

Analogous to systemic immune compartments, the GI immune system can be classified into immune inductive (Peyer's patches and MLN) and effector (lamina propria and intra epithelial lymphocytes) sites. As previously stated, the administration of vaccines orally induces the preferential expression of $\alpha 4\beta 7$ on antigen-specific T cells, however, parenteral vaccination does not induce this gut-homing integrin to a significant level (Mora and von Andrian 2006).

The current paradigm of T cell homing to the GI tract involves the induction of $\alpha 4\beta 7$ and CCR9 by MLN and PP DCs; meanwhile those T cells activated in the peripheral lymph nodes acquire the expression of E- and P-ligands, but not gut-homing receptors

(Campbell and Butcher 2002; Mora 2008). These earlier studies have revealed the presence of antigen-specific T cells in the MLN and PPs due to antigen-specific T cell priming in the GI inductive sites.

To determine the site of generation of intestinal immunity, the immune inductive and effector sites were examined separately. C57BL/6 mice were immunized i.n. with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g) using a prime-boost regimen. One week post boost, mice were sacrificed and GI tissues were harvested. Peyer's patch lymphocytes and MLN lymphocytes were examined separately from the small intestinal and colonic lamina propria lymphocytes. Robust levels of IFN- γ ⁺CD4⁺ T cells were detected in the small intestinal and colonic lamina propria but not in the Peyer's patches or MLNSs (**Fig. 5.7, 5.8**). These findings are consistent with data from Chapter 4, revealing that lung DCs have the capacity to induce antigen specific CD4⁺ and CD8⁺ T cells to migrate to the GI tract post i.n. vaccination with priming occurring at local sites.

5.2.5 Anti-DEC p24 immunization enhances gastrointestinal CD4⁺ T cell responses relative to untargeted protein immunization

Current approaches for developing a HIV vaccine have emphasized prime boost strategies, comprising multiple doses of DNA vaccine and recombinant viral vectors. Previous work within the lab of Ralph Steinman, has focused on the development of a protein-based approach that directly harnesses DCs for generating T cell immunity. This vaccination approach delivers antigen to maturing DCs in lymphoid tissues by engineering protein antigens into an antibody to DEC-205, an endocytic receptor expressed on DCs. Work by Trumpfheller *et al* (2006) has revealed the ability of α -DEC-205-gag p24 or p41 to induce more potent, long lived and protective CD4⁺ T cell immunity relative to higher doses of gag protein, HIV gag plasmid DNA, or recombinant adenovirus-gag following i.p and s.c. vaccination. In addition, the response is broad because the primed mice respond to an array of peptides in different MHC haplotypes. However, to date, this approach has not been applied to mucosal vaccination.

In order to assess the quality of CD4⁺ T cells induced by α -DEC-205-gag p24 in the GI tract post i.n. vaccination, C57BL/6 mice were immunized with 0.5, 5 and 15 μ g of p24 protein respectively in combination with poly ICLC (50 μ g) delivered i.n., and compared with 0.5, 5 and 15 μ g of i.n. delivered α -DEC-205-gag p24 and poly ICLC (50 μ g). Consistent with previous studies, the DEC targeting approach was more potent in inducing antigen-specific immunity within the effector compartments of the GI tract compared to untargeted protein vaccination at the doses of 0.5 and 5 μ g (**Fig. 5.9, 5.10**). Enhanced levels of IFN- γ ⁺CD4⁺ T cells were observed in the small intestinal lamina propria and colonic lamina propria utilizing the DEC-205 targeted approach (**Fig. 5.9**). Similarly, the DEC targeted approach was also more potent in inducing immune responses in the lung compartment (**Fig. 5.10**).

5.2.6 Anti-DEC-p24 induces long-term memory CD4⁺ T cells in the gastrointestinal tract

A balance of T cell specificities within the T effector memory and T central memory pools could potentially enable better control of HIV-1 virus during acute infection. Therefore, long-term immunity at mucosal surfaces would be the goal of an ideal HIV vaccine. An effective vaccine should generate sufficient numbers of high avidity effector memory CD4⁺ and CD8⁺ T cells at mucosal sites, to curtail primary infection and prevent dissemination from the mucosal compartment.

To test if long-term intestinal memory could be detected following i.n. vaccination with α -DEC-205-p24, CD4⁺ T responses were measured in the GI tract and systemic compartments 24 weeks after booster vaccination. Robust levels of IFN- γ ⁺CD4⁺ T cells were detected in the lung and spleen (**Fig. 5.11**). Similarly, long term immune responses were detected in the small intestinal lamina propria and colonic lamina propria, both GI effector sites (**Fig. 5.12**). In contrast, i.n. vaccination with untargeted gag-p24 failed to induce IFN- γ ⁺CD4⁺ T cells in the GI effector sites. Furthermore, IFN- γ ⁺CD4⁺ T cells in the systemic sites were also significantly lower compared to the α -DEC-205-p24 targeted vaccine.

Therefore, DEC-205 vaccination, delivered i.n. is capable of inducing long-term memory in the GI tract and other effector sites, critical for the efficacy of a potential HIV vaccine.

5.2.7 After intranasal immunization, antigen-specific T cells are observed in the vaginal mucosa.

Women are increasingly affected by the worldwide HIV epidemic and now account for nearly half the 33 million individuals living with HIV infection, with women in sub-Saharan Africa, accounting for 60% of HIV infections. As the HIV/AIDS epidemic continues to spread, many questions remain unanswered concerning the disease process and many more studies are needed to advance our understanding of HIV pathogenesis in the female reproductive tract. Like the GI tract, tissues of the lower (ectocervix and vagina) and upper (endocervix and uterus) female reproductive tract contain a large abundance of partially activated (Iwasaki 2010), memory CD4⁺ T cells which co-express CCR5 and/or CXCR4.

In order to determine if i.n. vaccination was sufficient to induce antigen-specific T cells in the female reproductive tract, C57BL/6 mice were immunized i.n. with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g) in a prime-boost regimen. One week after the boost, mice were sacrificed and lymphocytes isolated from the female reproductive tract. Robust levels of IFN- γ ⁺CD4⁺ T cells were detected in the female reproductive tract, comparable to the levels observed in the small intestinal lamina propria post i.n. vaccination. As the vaginal tract is one of the primary mucosal surfaces exposed to the virus, the induction of antigen-specific CD8⁺ T cells was also investigated.

In order to determine if i.n. vaccination induced effector CD8⁺ T cells, C57BL/6 mice were primed i.n. with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g). The C57BL/6 mice were subsequently boosted i.n. with NYVAC-gag (1 x 10⁶) and poly ICLC (50 μ g). Two weeks post boost, lymphocytes were isolated from the female reproductive tract and robust levels of IFN- γ ⁺CD8⁺ T cells were observed (**Fig 5.13**). These findings reveal that i.n. vaccination, utilizing various vaccination strategies, is sufficient to induce antigen

specific CD4⁺ and CD8⁺ T cells in the vaginal mucosa.

5.2.7 Langerin⁺ DCs are the predominant immunizing population following i.n. vaccination.

Like the GI tract, the lung is constantly exposed to the external environment, therefore lung DCs play an important role in tuning the optimal immune response (Lambrecht and Hammad 2012). Intraepithelial CD103⁺CD11b⁻ DC are situated in the basolateral layer of the airway epithelium and are capable of extending their dendrites into the airway spaces, endowing them with the capacity to sample antigenic material. This DC subset appears specialized in taking up apoptotic cells and can cross-present soluble inhaled antigens to CD8⁺ T cells (del Rio ML et al., 2007).

Given the important role of Langerin⁺CD103⁺ cDCs in the lung, the effect of Langerin⁺CD103⁺ DC depletion on the induction of immune responses in the local and systemic sites post i.n. vaccination was investigated using Langerin-DTR mice. DT was administered at day -3 and day -1 relative to vaccine delivery on day 0 (**Fig. 5.14**). Upon selective depletion of these cells, as confirmed from phenotypic analysis (**Fig 4.30**), DTR and WT animals were vaccinated i.n. with α -DEC-205-gag p24 (5 μ g) and poly ICLC (50 μ g) using a prime boost protocol. One-week post boost, the animals were sacrificed and mononuclear cells isolated and cultured in the presence of either p17 (control) or p24 (immunizing) peptides and anti-CD28 (1 μ g/ml) to examine cellular recall responses. Both local and systemic sites were examined, to determine the impact of Langerin⁺CD103⁺ depletion on the induction of antigen specific immunity.

Within the local sites of immune induction including the lung, a significant reduction in the frequency of antigen specific IFN- γ ⁺CD4⁺ T cell responses was observed following the ablation of Langerin⁺CD103⁺ DCs (**Fig 5.15 and 5.16a**). Similarly a significant reduction in IFN- γ ⁺CD4⁺ T cell was observed in the spleen (**Fig 5.15 and 5.16b**).

Importantly, IFN- γ ⁺CD4⁺ T cells were significantly reduced in the effector sites of the GI tract (**Fig 5.15**), such as the small intestinal lamina propria and colon (**Fig 5.17a and**

5.17b). Taken together, these data suggest, that Langerin⁺CD103⁺ cDCs are the dominant DC population mediating α -DEC-205-gag p24 priming following i.n. vaccination. Similar to the data generated using the DEC-205 targeting approach, untargeted vaccination also yielded lower levels of antigen specific IFN- γ ⁺CD4⁺ T cells following ablation of lung resident Langerin⁺CD103⁺ DCs. Here, upon Langerin⁺CD103⁺ DCs ablation, DTR and WT animals were vaccinated i.n. with gag p24 (15 μ g) and poly ICLC (50 μ g). One-week post boost, mononuclear cells were isolated from the lung and spleen. Significantly lower levels of IFN- γ ⁺CD4⁺ T cells were observed in these compartments (**Fig 5.18**), reflecting data in Figures 4.14 – 4.16 using DEC targeting, highlighting the unique role of Langerin⁺CD103⁺ DCs in the lung.

To further confirm the specific role of Langerin⁺CD103⁺ cDCs in IFN- γ ⁺CD4⁺ T cell priming in the lung, Langerin-DTR mice were examined following s.c. and i.n. immunization with α -DEC-205-gag p24. IFN- γ ⁺CD4⁺ T cell were observed with equivalent frequency in the lung, spleen and skin draining LNs between the DTR and WT groups following s.c. vaccination (**Fig 5.19**). In contrast a significant reduction was observed in the lung, spleen and mediastinal LN following the ablation of Langerin⁺CD103⁺ DC prior to i.n. vaccination with α -DEC-205-gag p24. Together, these results reveal that Langerin⁺CD103⁺ DCs are the primary lung DC population mediating immune responses following i.n. vaccination.

5.2.8 The administration of Flt3 ligand enhances the T cell response in the gastrointestinal tract and systemic locations post intranasal immunization.

Having established that i.n. vaccination is an effective means to induce gastrointestinal immunity and enhanced systemic immunity (**Fig 5.1 – 5.8**), the ability of Flt3L to enhance this response *in vivo* was investigated. Flt3L is a DC hematopoietin (Lyman 1995) with potential as a therapeutic agent to regulate DC numbers at sites relevant to vaccination, including the skin and skin draining LNs. Flt3L maintains DC numbers at steady-state levels throughout adult life (Merad et al., 2010)

Here, the potential of Flt3L to enhance GI Th1 responses was determined following i.n. vaccination. Age and gender-matched control and experimental mice were injected daily during both the prime and boost phase by IP injection with endotoxin-free (< 0.0064 EU/mg), GMP grade, recombinant human Flt3L (CellDex) at $10\mu\text{g}/\text{mouse}/\text{day}$ diluted in sterile PBS, or by PBS alone (control). At day 8-10 of Flt vs PBS injection, α -DEC205-p24 was delivered i.n. followed by boosting 4 weeks later. Boost vaccination was administered exactly 4 weeks later.

Flt3L administration during vaccination significantly enhanced mucosal immunity at sites relevant to HIV infection, such as the intestinal lamina propria, compared with untreated C57BL/6 mice, which were vaccinated with α -DEC205-gag p24 and adjuvant. A statistically significant increase in CD4^+ $\text{IFN}\gamma$ producing T cells was observed in the lung, lamina propria, spleen and the Peyer's patch (**Fig. 5.20, 5.21**).

5.3 Discussion

The global impact of HIV infection is staggering. Since it was recognized over 27 years ago, the HIV pandemic has resulted in the infection of nearly 60 million people worldwide, nearly half of whom have died from the disease. While the percentage of people newly infected with HIV has stabilized since 2001 the overall number of people living with HIV continues to grow (<http://www.cdc.gov/hiv/>). It is widely accepted that while substantial advances have been made in the field of HIV therapeutics, an effective HIV vaccine would be the optimum solution for the ultimate control of the global AIDS pandemic.

HIV entry occurs through mucosal surfaces with the intestinal mucosa representing a major reservoir for viral replication, amplification, and persistence. CD4⁺ memory T cells expressing CCR5 are the primary targets of HIV infection in the gut mucosa and rapid depletion of this population occurs early during acute infection. As early as 1984, Kotler and colleagues revealed that HIV-1 infected patients had histologic abnormalities of the GI mucosa, lymphocyte depletion and malabsorption (Kotler et al., 1984). Therefore, the induction of HIV specific CD4⁺ and CD8⁺ T cells, in addition to the generation of antibodies capable of blocking the escape of virus from the intestinal mucosa into systemic lymphoid organs, is a critical requirement for an effective HIV vaccine. Moreover the persistence of memory T and B cells at mucosal sites of pathogen entry is critical in developing new vaccination strategies for HIV-1.

Parenterally applied vaccines are highly efficient in promoting systemic immunity but frequently result in weak protection against enteropathogens. This is primarily due to the fact that both effector T cells and antibody producing plasma cells induced by antigen fail to express gut-homing molecules; consequently, “a protective shield in the gut mucosa is not installed properly” (Hammerschmidt et al., 2011). In contrast, it has been well established that oral delivery of effective vaccines allows for the induction of protective mucosal immunity in the intestines. In-fact, several studies have shown that such vaccination strategies can generate more potent mucosal protection than immunization by

injection (Belyakov IM et al., 2007, Belyakov IM et al., 2008, Gallichan et al., 1996, Santosusso M et al., 2005).

Findings presented in chapter 4 (**Fig. 4.17 – 4.22**) demonstrated that i.n vaccination was sufficient to mediate protection against a lethal enteric challenge, mediated by lung DCs which, have the capacity to induce T cells to migrate to the GI effector sites (**Fig 3.1 – 3.7**). The *in vivo* effects of lung DC mediated protection against enteric challenge, promoted further investigations into the ability to lung DCs to induce antigen-specific T cells in the GI mucosa and the lung DC subset responsible. Given the global impact of HIV infection and the discovery that gut mucosa T cell responses have been associated with control of chronic HIV infection (Sankaran S et al., 2005), the potential of i.n. vaccination to generate HIV specific GI tract immunity was addressed.

In order to assess the capacity of i.n. vaccination to induce antigen-specific immune responses at GI effector sites, an initial proof of concept study was conducted. C57BL/6 mice were immunized via a variety of routes, including i.n., i.p., i.v., i.m., and s.c with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g), with control mice immunized with DEC-empty vaccine (5 μ g) and poly I:C (50 μ g) delivered i.p. A booster dose of the vaccine was administered four weeks after priming and mice were sacrificed one week after boost. Interestingly and consistent with preliminary data from chapter 4 (**Fig 4.12**), i.n vaccination elicited robust levels of IFN- γ ⁺CD4⁺ T cells, comparable to that generated by i.p. immunization. While i.p. vaccination has been well established as a route to induce local intestinal immunity (Muri et al., 1995), this is not feasible for large scale human vaccination. In contrast, consistent with the current literature, no antigen specific IFN- γ ⁺CD4⁺ T cells were observed in the GI tract following i.m., i.v., and s.c. vaccine administration. Similarly, splenic T cell responses were best elicited by i.n. immunization, lower levels of antigen specific CD4⁺ T cells were also observed following i.p., i.m. and s.c. immunization. Overall these finding indicate that i.n. vaccination is capable of eliciting robust vaccine specific immune responses in the GI tract.

Having established that i.n. vaccination induces robust GI and systemic responses, it was important to investigate the induction of IFN- γ ⁺CD4⁺ T cells in the lung and other local

sites. Previous studies from Do *et al.*, (2012) investigated cellular immunity at mucosal surfaces following i.n. immunization with α -DEC:LcrV in the presence of poly IC, found IFN- γ^+ CD4 $^+$ T cells in the lung and draining lymph nodes post i.n. immunization, with IFN- γ^+ CD4 $^+$ T cells representing ~6% of CD4 $^+$ T cells in the lung tissue (Do *et al.*, 2012). In the current study, significant numbers of IFN- γ^+ CD4 $^+$ T cells were detected at the site of vaccine administration, the nose and the mediastinal LNs as well as the lung (~20%), following immunization with α -DEC-205-gag p24.

Similar to the systemic immune compartment, the GI immune system can be classified into immune inductive (Peyer's patches and MLN) and effector (lamina propria and intra epithelial lymphocytes) sites. Previous studies from chapter 3 (**Fig. 3.3**) revealed that i.n. vaccine priming and induction of $\alpha 4\beta 7$ occurs in the lung and mediastinal lymph nodes. In order to intestinal immunity, the immune inductive and effector sites were examined separately following i.n. vaccination with α -DEC-205-gag p24. Robust levels of antigen specific IFN- γ^+ CD4 $^+$ T cells were detected in the effector sites (small intestinal and colonic lamina propria) but not inductive (Peyer's patches or MLN) sites.

The current vaccination strategy utilized a novel approach based on targeting the delivery of protein antigens to DCs in the α -DEC-205-gag p24 construct. Gag was chosen, as T cell immunity to gag has been associated with better clinical outcome (Trumpfeller *et al.*, 2012). Following the observation that α -DEC-205-gag p24 delivered i.n. was sufficient to induce antigen specific immunity in the GI tract, the efficacy of i.n. administration of untargeted HIV protein antigens was assessed. Targeting via DEC-205 was significantly more efficient at initiating IFN- γ^+ producing CD4 $^+$ T cell immunity in the small intestinal lamina propria and colonic lamina than untargeted antigen. Similarly, in the lung a significant increase was observed in IFN- γ^+ secreting cells using the DEC-205 targeting strategy. Thus, the use of α -DEC-205 mAb targeting greatly increases the efficiency with which protein antigens elicit T cell immunity, when given i.n. with poly ICLC.

Long-term immunity at mucosal surfaces would be the goal of an ideal HIV vaccine. An effective vaccine should generate sufficient numbers of high avidity effector memory T cells at mucosal sites, ready to be rapidly recalled in a second wave of attack to curtail primary infection and prevent dissemination from the mucosal compartment.

To test if long-term intestinal immune memory could be generated by i.n. vaccination using α -DEC-205-p24, CD4⁺ T responses were measured in the GI tract 24 weeks after booster vaccination. In addition, a direct comparison was performed between DEC-205 targeted and untargeted gag-p24. Significant levels of IFN- γ ⁺CD4⁺ T cells were detected in the small intestinal and colonic lamina propria effector sites following i.n. immunization with α -DEC-205-p24 but not with untargeted gag-p24. Similarly, long term memory was also detected in the lung and spleen, also representing effector tissues in the mouse using the DEC-205 targeting system. As with the GI effector sites, untargeted gag-p24 failed to induce robust levels of IFN- γ ⁺ secreting CD4⁺ T cells in the spleen or lung, however low levels of IFN- γ ⁺CD4⁺ T cells were detected.

Women are increasingly affected by the worldwide HIV epidemic, now accounting for at least half of newly infected individuals. As previously discussed, mucosal sites are the primary sites of HIV transmission, and therefore these tissues are the focus of attention for efforts to prevent HIV infection. Several mechanisms have been proposed to explain how HIV crosses the epithelial barrier of the female reproductive tract; these include the direct passage of the virus/and or infected cells through breaches in the epithelia, uptake by resident mucosal DCs and transcytosis of free virus across polarized epithelial cells (Iwasaki 2010). Following infection, virus amplification occurs rapidly, aided by local inflammation, with systemic dissemination occurring prior to the development of the adaptive immune response, leading to depletion of CD4⁺ T cells, notably those of the gastrointestinal tract.

The female reproductive tract is equipped with a variety of physical and chemical defenses against viral infection. The vagina and ectocervix are lined with a multilayered squamous epithelium; the upper reproductive tract, however, is comprised of a resilient

monolayer of columnar epithelium (Wira, Fahey et al. 2005). The endocervix channel is lined with mucus, which provides protection by entrapping antigenic material. Epithelial cells lining the tract also produce a wide range of soluble factors with anti-HIV properties such as secretory leukocyte protease inhibitor (SLPI), lactoferrin, defensins, cytokines and chemokines (RANTES and MIP-1 β) (Wira, Fahey et al. 2005).

Very few studies have characterized HIV-specific T cell responses in the female reproductive tract of chronically infected individuals, likely due to challenges in obtaining fresh tissue. The use of mouse models has revealed that i.n. infection with Herpes virus results in the development of genital antibodies. A prominent study by Mielcarek *et al* (2000), revealed that i.n. administration with live attenuated *Bordetella pertussis*, from which the pertussis toxin gene was mutated, resulted in the development of anti-FHA IgA and IgG antibodies in the genital tract of mice, both in the vagina and uterus, after a single administration (Mielcarket et al, 2000). In the case of HIV vaccination, while efforts to elicit neutralizing antibodies remain an important but difficult objective, a major focus is to induce strong T cell-mediated immunity, which is associated with control of viral load early after infection with improved clinical outcomes. A successful T-cell based HIV vaccine should elicit broad, strong and polyfunctional CD4⁺ and CD8⁺ T cell responses, and immune memory in the GI mucosa (Keele, Giorgi et al. 2008). This is also true for the female reproductive tract, as detailed studies of acute SIV infection in rhesus macaques have revealed the mechanisms that lead to the rapid expansion of HIV/SIV in tissues, beginning with a small “founder” population of cells near the initial site of entry in the endocervix (Keele, Giorgi et al. 2008). Therefore, the ability of lung DCs to induce HIV specific IFN- γ ⁺ CD4⁺ and CD8⁺ T cells in the vaginal tract post i.n. vaccination was investigated.

Significant numbers of IFN- γ ⁺ secreting CD4⁺ T cells were observed in vaginal tissue. In addition to the induction of CD4⁺ responses, CD8⁺ were subsequently investigated. In order to induce CD8⁺ T cells additional vaccination regimes were investigated. Upon initial priming with α -DEC-205-gag p24 fusion mAb, recipient mice were boosted with NY-VAC-gag and poly ICLC. NY-VAC- gag is a poxvirus-based vector, which has the

capacity to generate antigen specific CD8⁺ T cells. Here IFN- γ ⁺ CD8⁺ T cells were observed in the mucosa. Overall, these studies reveal that i.n. vaccination, targeting lung DCs using DEC-205, is sufficient to induce robust long term immune responses in the vaginal effector and systemic sites.

Having determined that targeting lung DCs induces HIV antigen-specific GI immunity post i.n. vaccination, the lung DC subset responsible was investigated using specific DC ablation systems.

The Langerin⁺CD103⁺ cDC residing in the basolateral layer, have the capacity to extend dendrites into the luminal space to sample/capture antigenic material. These cells fail to develop in mice lacking IRF8, BATF3 and ID2, placing them in the same lineage as CD8 α ⁺ cDCs. This subset also appears specialized in taking up apoptotic cells in the lung and actively migrates to the mediastinal LNs. In some models of inhalation tolerance, the CD103⁺CD11b⁻ cDCs were better at cross-tolerizing CD8 lymphocytes. GeurtsvanKessel *et al.*, have studied antigen presentation by lung DC subsets and found that Langerin⁺CD103⁺ cDCs take up viral antigens and subsequently migrate from their site of residence to the T cell zones of the MedLN (GeurtsvanKessel *et al.*, 2008). The Langerin⁺CD103⁺ DCs was also shown to be more potent at stimulating CD8⁺ T cell responses, in addition to inducing CD4⁺ T cell activity. A number of studies have shown; using the Langerin-diphtheria toxin receptor (DTR), that mice lacking Langerin⁺ DCs had severe deficiencies in antiviral immunity during the initial stages of infection. This may be due to their capacity to selectively uptake apoptotic cells and cross present antigen during viral infections.

Given the important role of Langerin⁺CD103⁺ cDCs in the lung, the effects of Langerin⁺CD103⁺ depletion on the induction of immune responses in the local and systemic sites post i.n. vaccination was investigated. To test a role for Langerin⁺CD103⁺ DC in i.n. vaccine priming Langerin-DTR mice were utilized, here Langerin⁺ DC populations can be selectively depleted by diphtheria toxin treatment (Bennett *et al.*, 2005).

Within the local sites of immune induction such as the lung, a significant reduction in antigen specific IFN- γ ⁺CD4⁺ T cell responses was observed following the ablation of Langerin⁺CD103⁺ DCs. Similarly a significant reduction in IFN- γ ⁺CD4⁺ T cell was observed in the spleen and effector sites of the GI tract, including the small intestinal and colonic lamina propria. Taken together these data suggests that Langerin⁺CD103⁺ cDCs are the dominant DC population mediating α -DEC-205-gag priming following i.n. delivery of vaccine.

In contrast and consistent with a study by Anandasabapathy *et al* (*unpublished*) revealing that s.c. immunization does not depend on Langerin⁺CD103⁺ DC, IFN- γ ⁺CD4⁺ T cell were observed with equivalent frequency between the DTR and WT groups following s.c. vaccination. Together, these results determine that Langerin⁺CD103⁺ DCs in the lung are the dominant immunization DC population following i.n. vaccination. Furthermore, following vaccination of Langerin⁺ DC ablated animals with untargeted gag-p24, a significant decrease in the generation of IFN- γ ⁺CD4⁺ T cell in the lung and spleen was observed, further confirming their role during immunization in the context of untargeted vaccination.

In addition to the Langerin⁺CD103⁺ DCs, another population of lung DCs expressing CD11b are located immediately below the basement membrane in the lung lamina propria. These CD11b⁺ DCs are thought to be closely related to monocytes/macrophages, as they share several markers (F4/80, SIRP- α and CX3CR1) and depend on M-CSF for their development. Evidence has also revealed that at least a proportion of the lung tissue resident CD11b⁺ DCs can be reconstituted by adoptive transfer of pre-cDCs, suggesting a similar developmental pathway to that of cDCs in the central peripheral organs (Pollard AM *et al.*, 1990). It has been speculated that the most likely explanation for these conflicting data is that lung CD11b⁺ cDCs are not a single population, but in-fact are made up of two populations, one derived from a monocytic precursor (~50-60%), and another derived from a pre-cDC precursor (Lambrecht and Hammad. 2012). Studies on the rat have reached a similar conclusion. The role of CD11b⁺ DCs during the context of vaccination has yet to be examined and is currently under investigation.

Following on from the vaccination studies, a strategy to enhance the immune response generated by Langerin⁺ DCs post i.n. immunization was investigated. Flt3L instructs progenitors along a DC developmental pathway (Lyman 2010), regulating the mobilization of pre-DC from the blood, to give rise to IFN- α producing plasmacytoid DCs (pDCs) and CD8 α ⁺ and CD8 α ⁻ conventional DCs (cDCs) in lymphoid organs (Liu K). In lymphoid tissue, Flt3L expands pDC and cDC about 10 fold; mice lacking Flt3L have severely reduced pDC, and cDC (Liu and Nussenzweig 2010). In the spleen, Flt3L biases cDC further to a CD8 α ⁺ and CD205⁺ subset (Bozzacco et al. 2007). This population is specialized to cross-present exogenous antigens on MHC, to ingest dead or dying cells, to produce IL-12 in response to innate and T cell derived cues (Shortman and Heath 2010). Flt3L mobilized spleen DC cross-present microbial antigen to HIV gag specific T cells *ex vivo* (Bozzacco et al. 2007).

In peripheral non-lymphoid tissue including the skin and lungs, Flt3L and its receptor (Flt3) regulate the homeostasis of particular tissue-resident DC (Liu et al 2009) including cross-presenting cDC equivalents. CD103⁺ Langerin⁺ (Edelson 2010) DC are specialized to present viral and tumor antigens to T cells (Health and Carbone 2010). CD103⁺ Langerin⁺ DC share Flt3L dependence (Ginhoux et al., 2011) with CD8 α DC based on analysis of Flt3L and FLK2 receptor knockout mice. Downstream of Flt3L, both CD8 α ⁺ and CD103⁺ subsets are regulated by the kinase mTOR (Sathaliyawala et al. 2010). Following the administration of Flt3L, significantly enhanced levels of IFN- γ ⁺ expressing CD4⁺ T cells were observed in the lung, small intestinal lamina propria and spleen. Interestingly, antigen specific responses were observed in the Peyer's patch and MLNs.

The results from these current studies have revealed that i.n. vaccination, targeting lung DCs results in the induction of robust, long term immune responses in the GI and systemic compartments. Additionally, the Langerin⁺CD103⁺ lung DC is the primary immunizing population following i.n. vaccination. Therefore, targeting of this Langerin⁺CD103⁺ DC population during i.n. vaccination has the capacity to eliciting GI immunity via a novel route of migration to the GI mucosa.

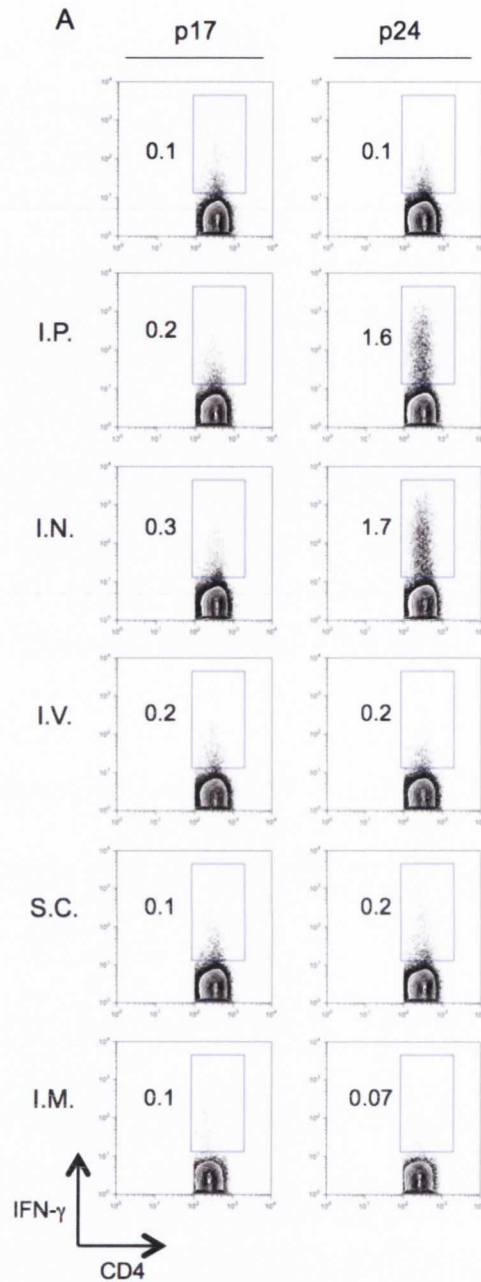


Figure 5.1: Vaccine delivered i.n. and i.p. elicits robust IFN- γ ⁺CD4⁺ T cell responses in the GI lamina propria. C57BL/6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.p., i.n., s.c., i.v. or i.m. α -DEC-205 empty mAb (5 μ g) delivered i.p. served as control. One-week post boost, mononuclear cells were isolated from SILP and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.1 illustrates a representative of three experiments.

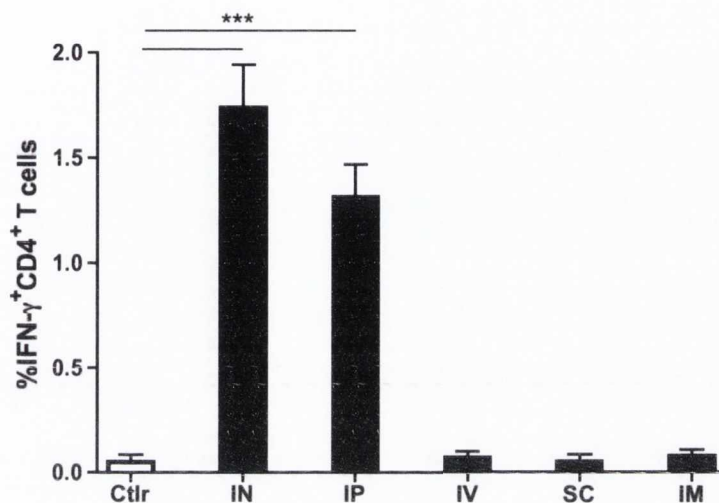


Figure 5.2 Vaccine delivered i.n. and i.p. elicits robust IFN- γ ⁺CD4⁺ T cell responses in the GI lamina propria. C57BL/6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.p., i.n., s.c., i.v., or i.m. DEC-205 empty mAb (5 μ g) delivered i.p. served as control. One-week post boost, mononuclear cells were isolated from SILP and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.2 illustrates a summary of three individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

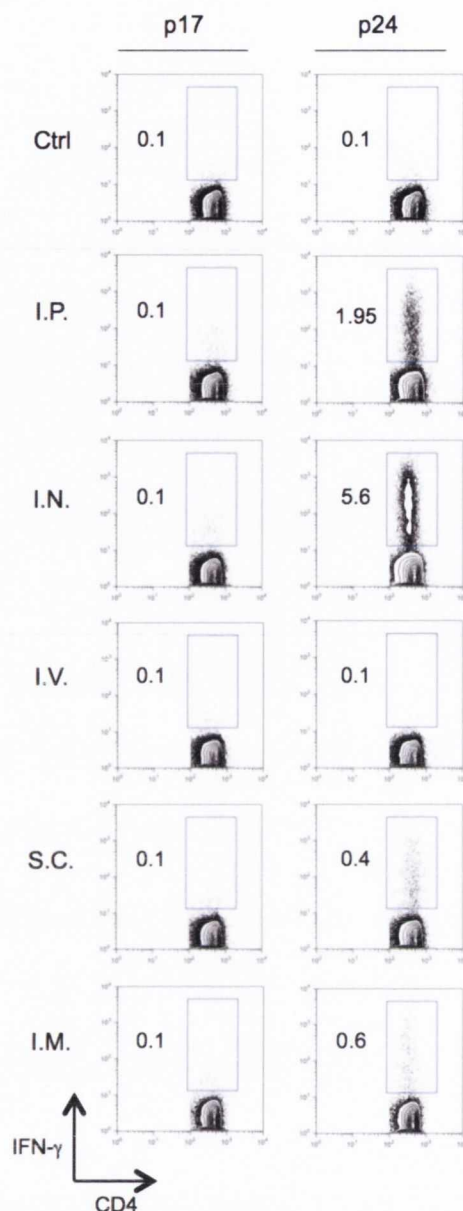


Figure 5.3: Vaccine delivered i.n. and i.p. elicits robust IFN- γ ⁺CD4⁺ T cell responses in the spleen. C57BL/6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.p., i.n., s.c., i.m. or i.v. DEC-205 empty mAb (5 μ g) delivered i.p. served as control. One-week post boost, mononuclear cells were isolated and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.3 illustrates a representative of three experiments, where the animals were primed and boosted by the same route.

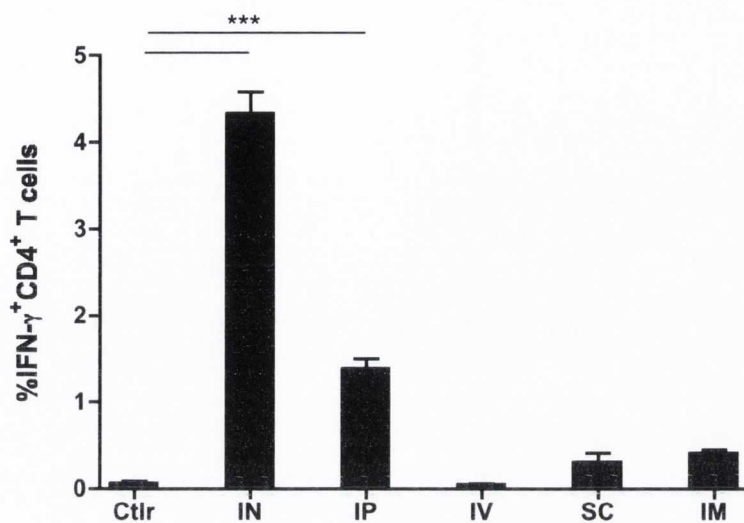


Figure 5.4: Vaccine delivered i.n. and i.p. elicits robust IFN- γ ⁺CD4⁺ T cell responses in the spleen. C57BL/6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.p., i.n., s.c., i.m. or i.v. DEC-205 empty mAb (5 μ g) delivered i.p. served as control. One-week post boost, mononuclear cells were isolated and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Data compiled from three individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

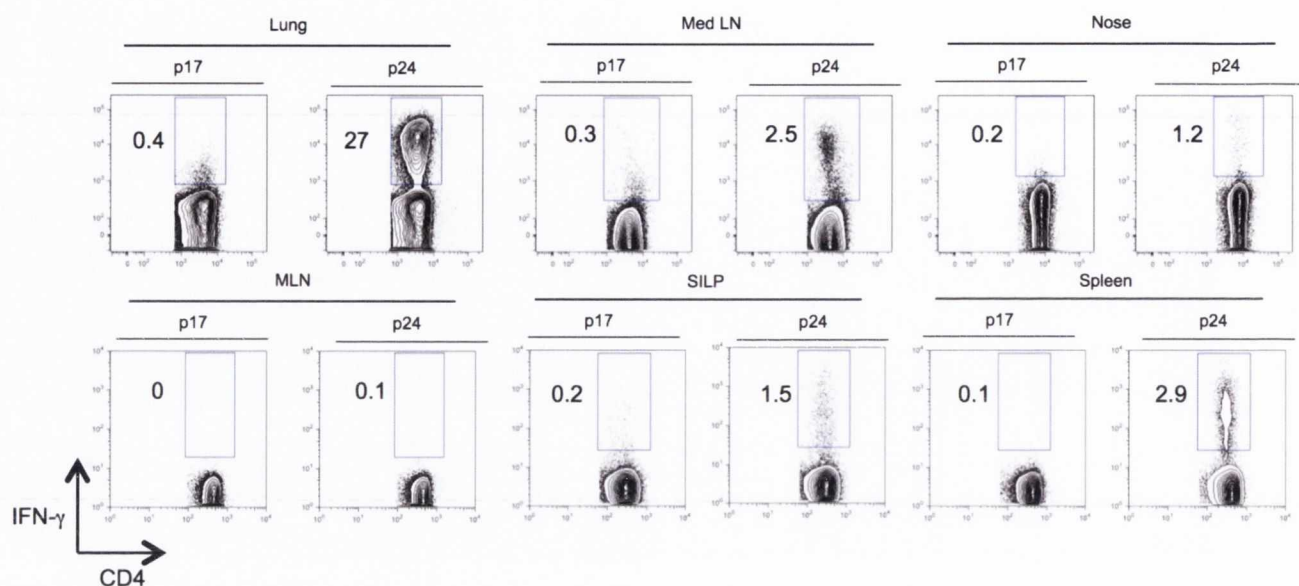


Figure 5.5: The lung, nasal-associated lymphoid tissues (NALT) and mediastinal lymph nodes are the site of priming following i.n. immunization. C57BL/6 mice were immunized i.n. with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g). One-week post boost, nasal-associated lymphoid tissues, mediastinal lymph nodes, spleen, mesenteric lymph nodes, Peyer's patches and intestinal lamina propria cells were isolated and IFN- γ ⁺CD4⁺ T cells were examined by flow cytometry as shown in a representative of three experiments.

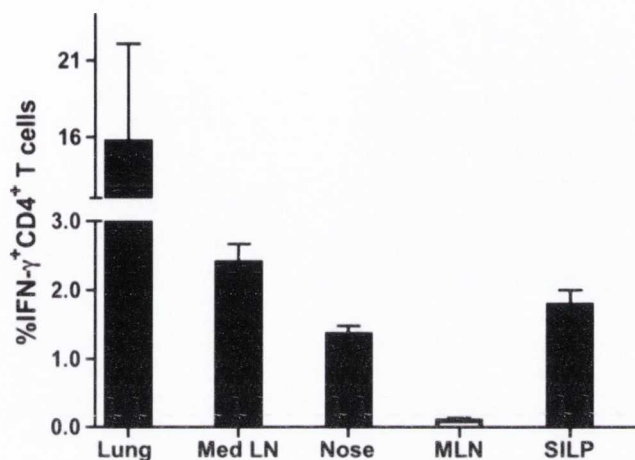


Figure 5.6: The lung, nasal-associated lymphoid tissues (NALT) and mediastinal lymph nodes are the site of priming following i.n. immunization. C57BL/6 mice were immunized i.n. with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g). One-week post boost, nasal-associated lymphoid tissues, mediastinal lymph nodes, spleen, mesenteric lymph nodes, Peyer's patches and intestinal lamina propria cells were isolated and IFN- γ ⁺CD4⁺ T cells were examined by flow cytometry, data compiled from three individual experiments

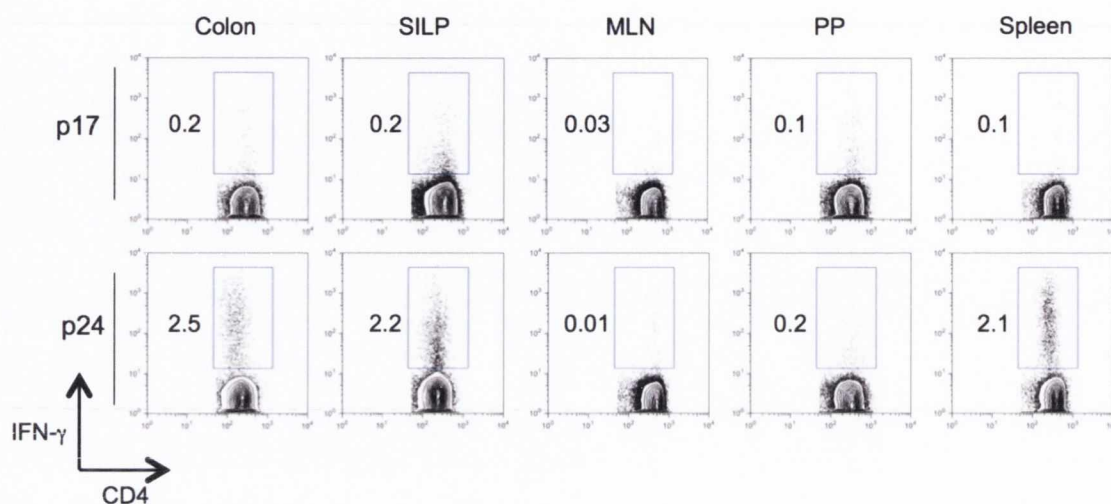


Figure 5.7: Following i.n. immunization, antigen-specific immune responses are elicited in the GI effector but not inductive sites. C57BL/ 6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) in a prime boost fashion. Mononuclear cells were harvested from the colonic and small intestinal lamina propria, mesenteric lymph nodes, Peyer's patches and spleen. One-week post boost, mononuclear cells were isolated and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.7 illustrates a representative of three experiments.

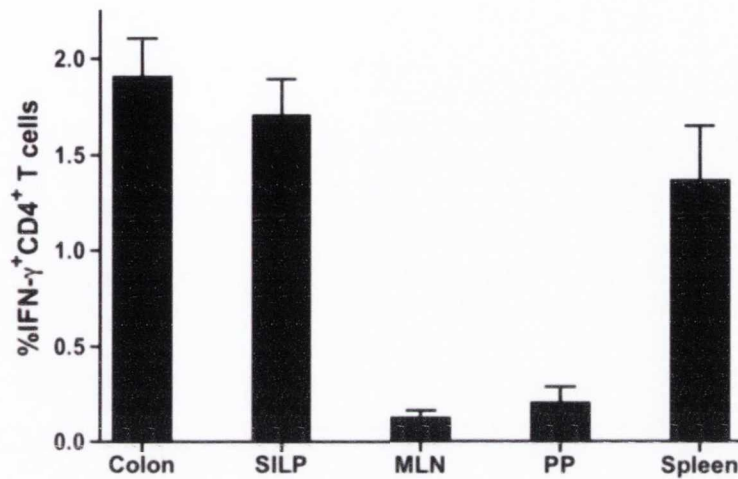


Figure 5.8: Following i.n. immunization, antigen specific immune responses are elicited in the GI effector sites but not inductive sites. C57BL/6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) in a prime boost fashion. Mononuclear cells were harvested from the colonic and small intestinal lamina propria, mesenteric lymph nodes, Peyer's patches and spleen. One-week post boost, mononuclear cells were isolated and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Data compiled from three individual experiments.

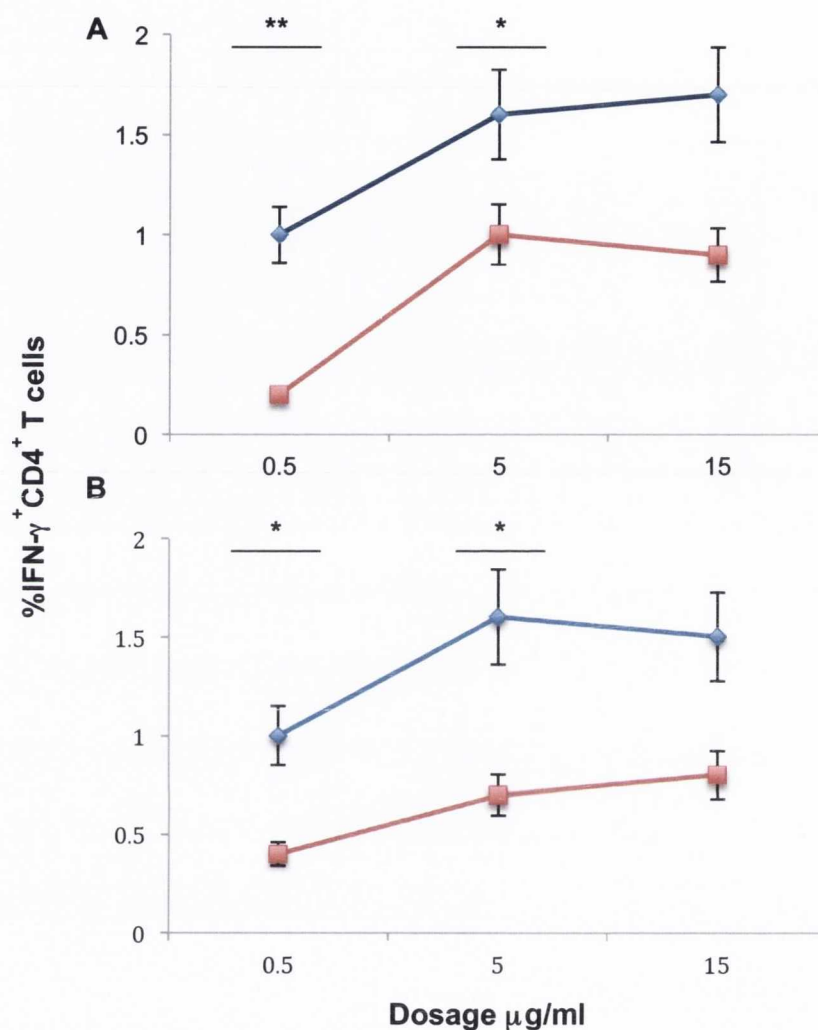


Figure 5.9: DEC targeted HIV p24 vaccination is more potent to elicit GI immune responses than untargeted p24 protein vaccination. C57BL/6 mice were immunized i.n. with escalating doses (0.5μg, 5μg and 15μg) of α-DEC-205-gag p24 (blue) fusion mAb and poly ICLC (50μg) in a prime boost fashion or with untargeted p24 protein (red) vaccination, also administered i.n., at doses of 0.5μg, 5μg and 15μg with poly ICLC (50μg) respectively. (a) SILP, (b) Colon. Data compiled from three individual experiments. *p<0.05, **<p<0.01, ***p<0.001

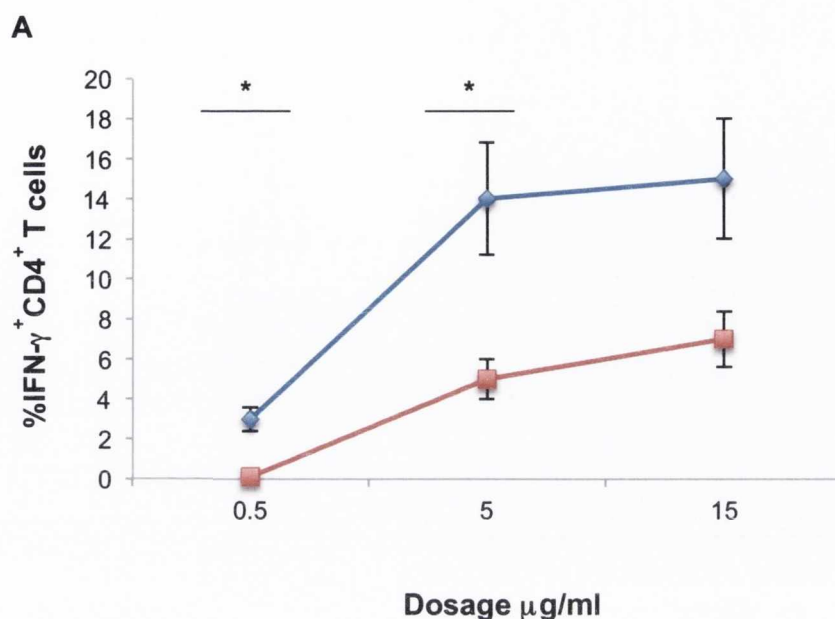


Figure 5.10: DEC targeted HIV p24 vaccination is more potent in inducing lung immune responses than untargeted p24 protein vaccination. C57BL/6 mice were immunized i.n. with escalating doses (0.5μg, 5μg and 15μg) of α-DEC-205-gag p24 (blue) fusion mAb and poly ICLC (50mg) in a prime boost fashion or with untargeted p24 protein (red) vaccination, also administered i.n., at doses of 0.5μg, 5μg and 15μg with poly ICLC (50μg) respectively. Data compiled from three individual experiments. *p<0.05, **<p<0.01, ***p<0.001

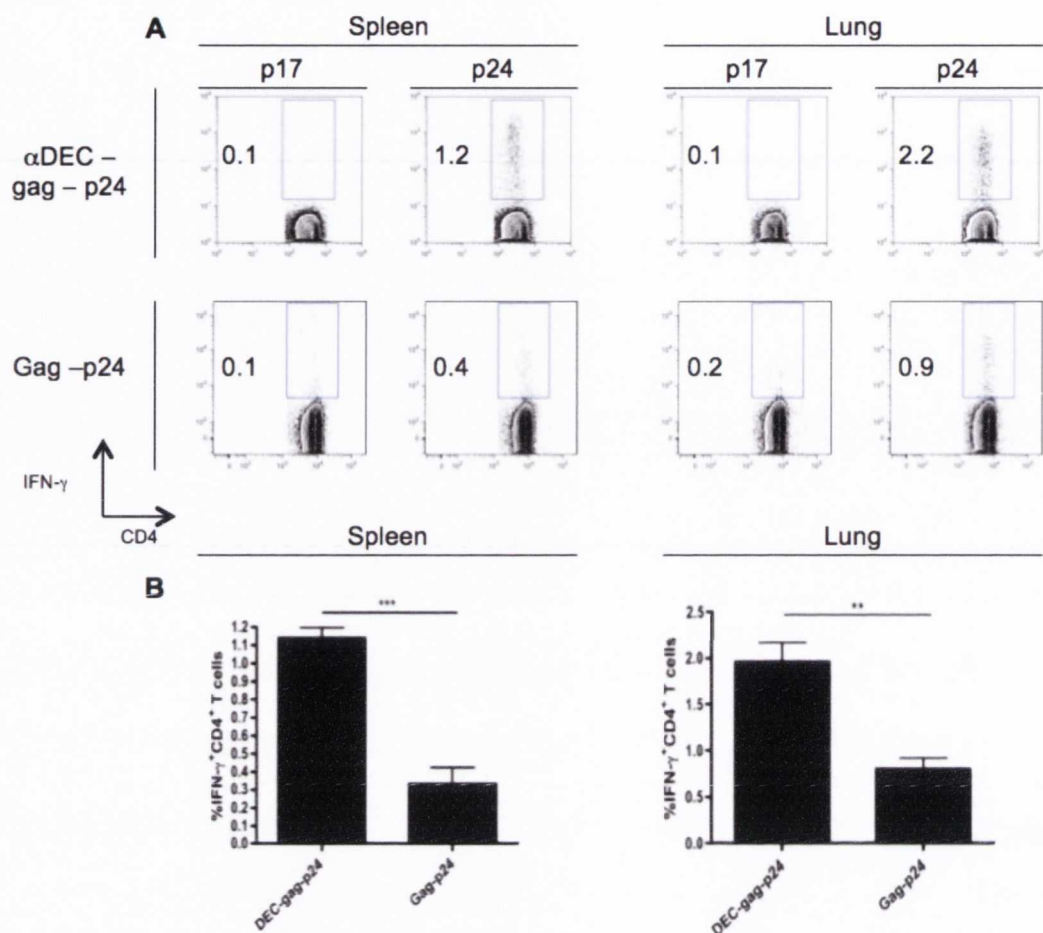


Figure 5.11: DEC targeted HIV p24 vaccination induces long-term memory in systemic sites. C57BL/6 mice were immunized i.n. with α -DEC-205-gag p24 fusion mAb (5 μ g) or gag-p24 (15 μ g) and poly ICLC (50 μ g) in a prime boost fashion. Mice were sacrificed 24 weeks after the boost and lymphocytes were isolated from the spleen and lung. (a) Representative flow plots illustrating IFN- γ ⁺CD4⁺ T cells (b) Cumulative data from three individual experiments. *p<0.05, **<p<0.01, ***p<0.001

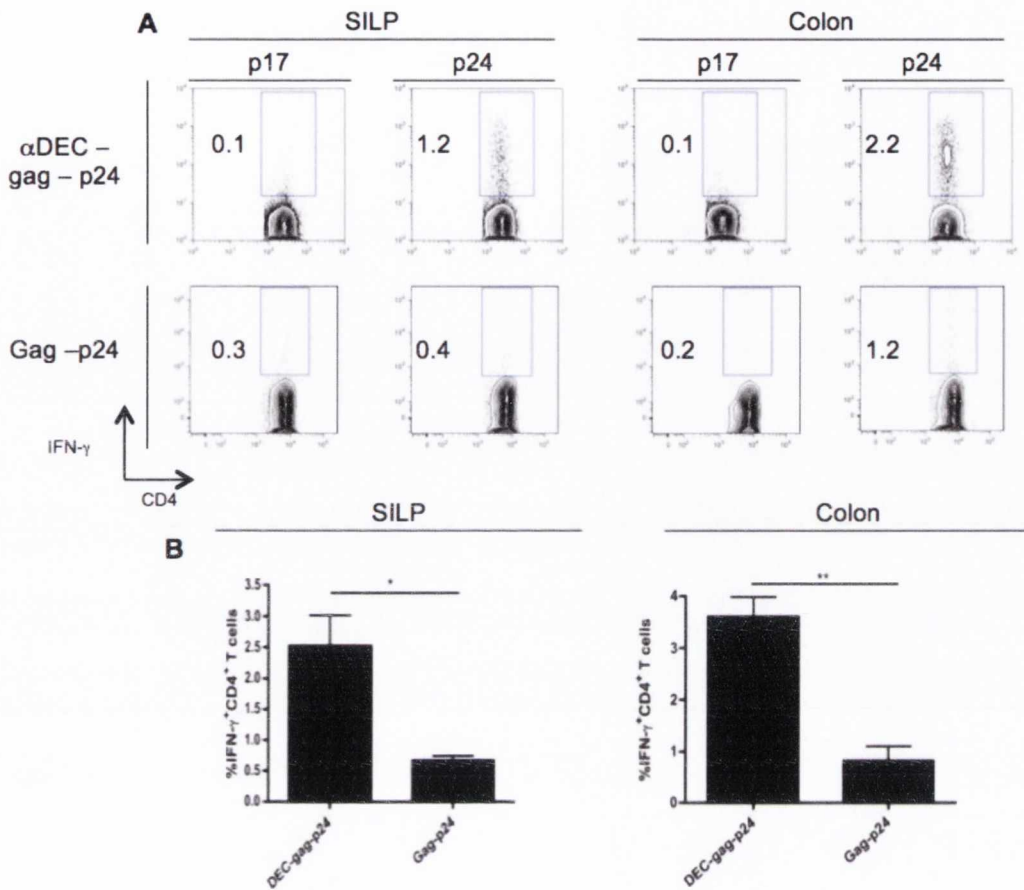


Figure 5.12: DEC targeted HIV p24 vaccination induces long-term memory in GI effector sites. C57BL/6 mice were immunized i.n. with α -DEC-205-gag p24 fusion mAb (5 μ g) or gag-p24 (15 μ g) and poly ICLC (50 μ g) in a prime boost fashion. Mice were sacrificed 24 weeks after the boost and lymphocytes were isolated from the small intestinal lamina propria (SILP) and colonic lamina propria. (a) Representative flow plots illustrating IFN- γ ⁺CD4⁺ T cells (b) Cumulative data from three individual experiments. *p<0.05, **p<0.01, ***p<0.001

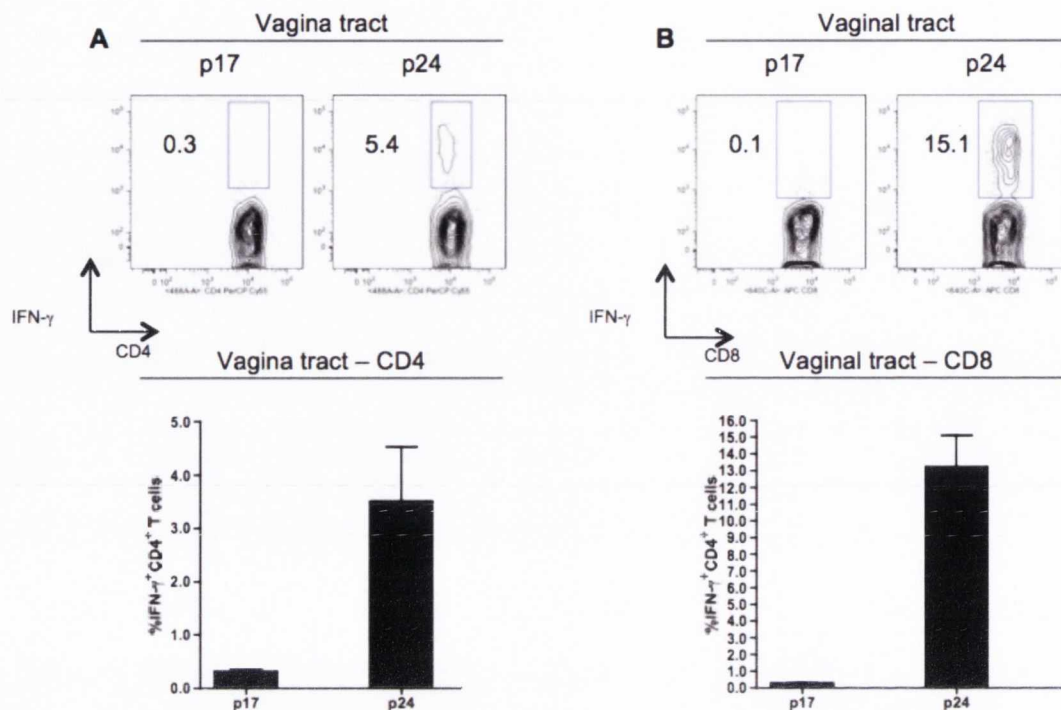


Figure 5.13 DEC targeted HIV-gag and NYVAC-gag vaccination induces antigen-specific CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺ T cells in the female reproductive tract. Female C57BL/ 6 mice were immunized with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) in a prime boost fashion (or in the case of CD8 T cell responses boosted with NYVAC-gag and poly ICLC (50 μ g)). Mononuclear cells were harvested from the reproductive tract and vagina. (a) Representative flow plots illustrating IFN- γ ⁺CD4⁺ T cells (b) Cumulative data from three individual experiments

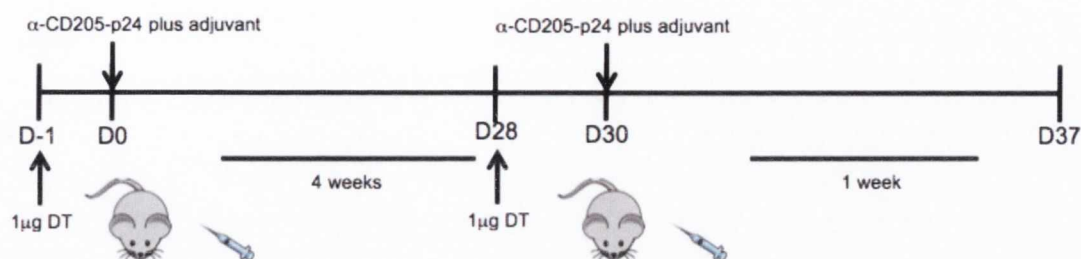


Figure 5.14 Vaccination strategy employed for Langerin⁺CD103⁺ ablation experiment. Langerin DTR and wild type animals received 1μg DT i.p. per average 25-30 gram mouse 36 hours prior to priming. Animals were primed with α-DEC-205-gag p24 fusion mAb (5μg) and poly ICLC (50μg) delivered i.n. 36 hours prior to the boost all groups were administered 1μg of DT and boosted with α-DEC-205-gag p24 fusion mAb (5μg) and poly ICLC (50μg) i.n. WT and DTR groups were sacrificed one-week after boosting

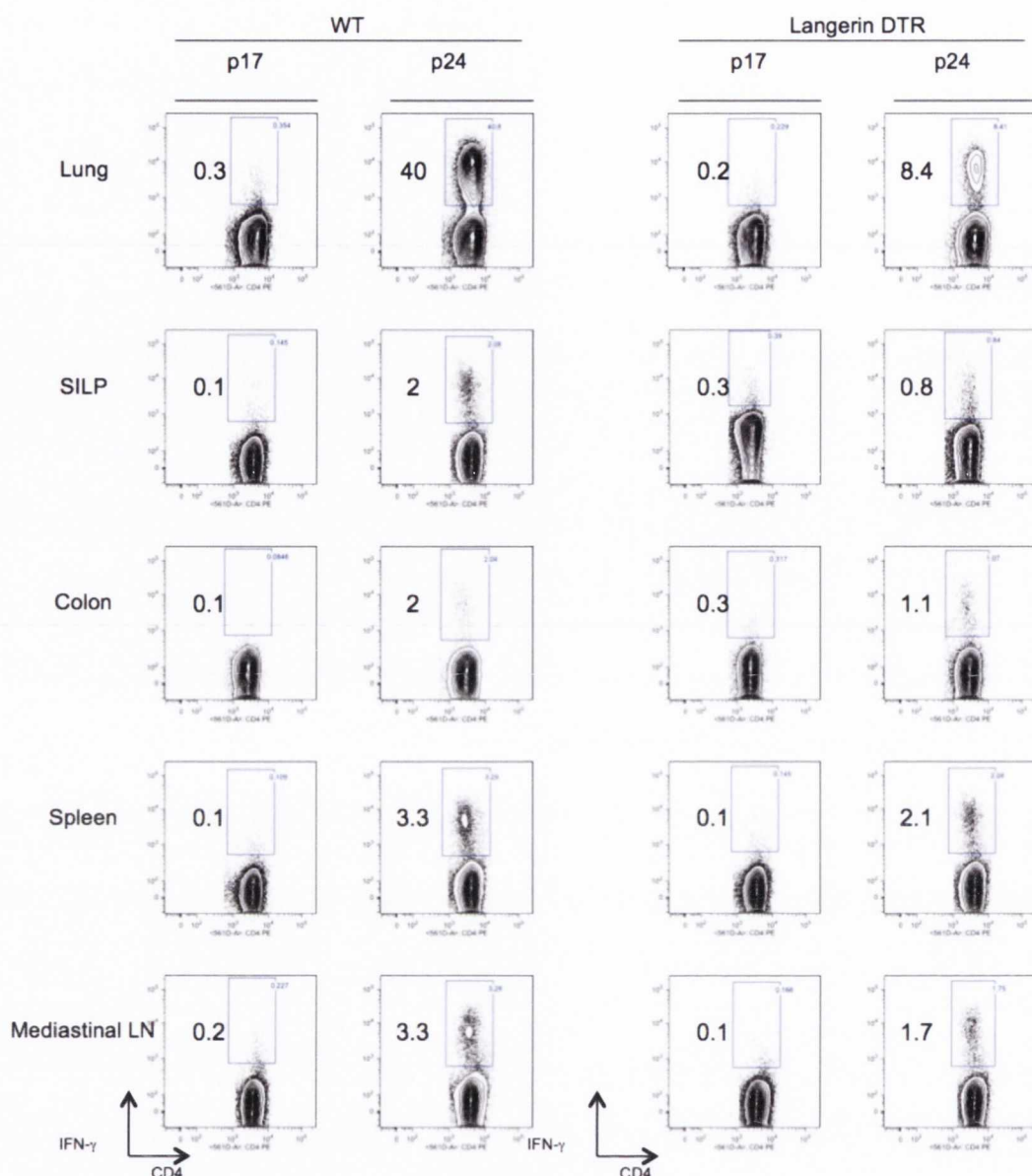


Figure 5.15 Lung resident CD103⁺Langerin⁺ dendritic cells meditate the induction of Th1 immune responses post-intranasal immunization. Langerin DTR and wild type animals received 1 μ g DT i.p. (See methods) Animals were primed with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.n. 36 hours prior to the boost all groups were administered 1 μ g of DT and boosted with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) i.n. One-week post, boost mononuclear cells were isolated from immunological relevant compartments and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.15 illustrates a representative of three experiments.

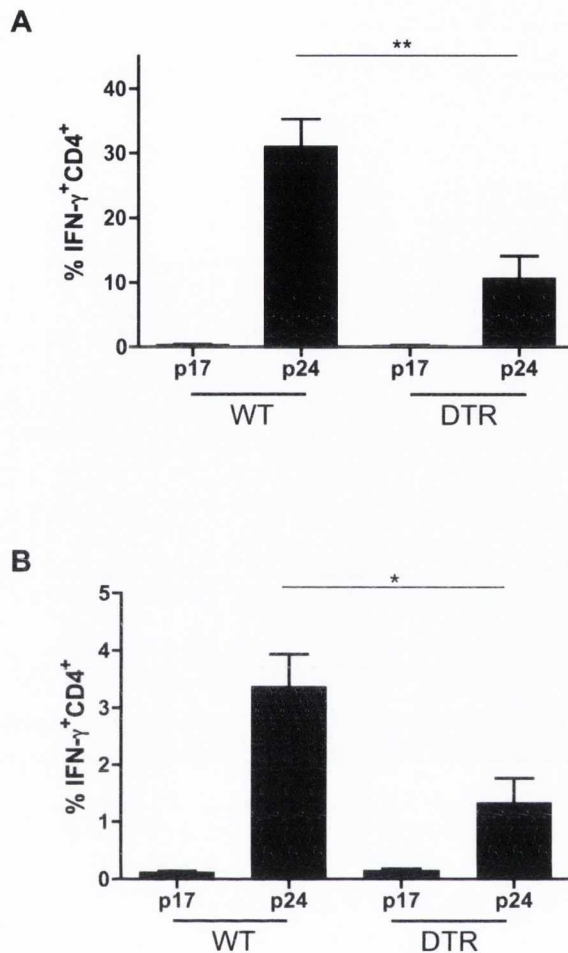


Figure 5.16 Lung resident CD103⁺Langerin⁺ dendritic cells mediate the induction of immune responses in the lung and spleen post-intranasal immunization. Langerin DTR and wild type animals received 1 μ g DT i.p. (see methods). Animals were primed with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.n. 36 hours prior to the boost all groups were administered 1 μ g of DT and boosted with α -DEC-205-gag p24 fusion mAb (5 μ g) with poly ICLC (50 μ g) i.n. One-week post boost, mononuclear cells were isolated from the (a) lung and (b) spleen and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.16 illustrates cumulative data from three individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

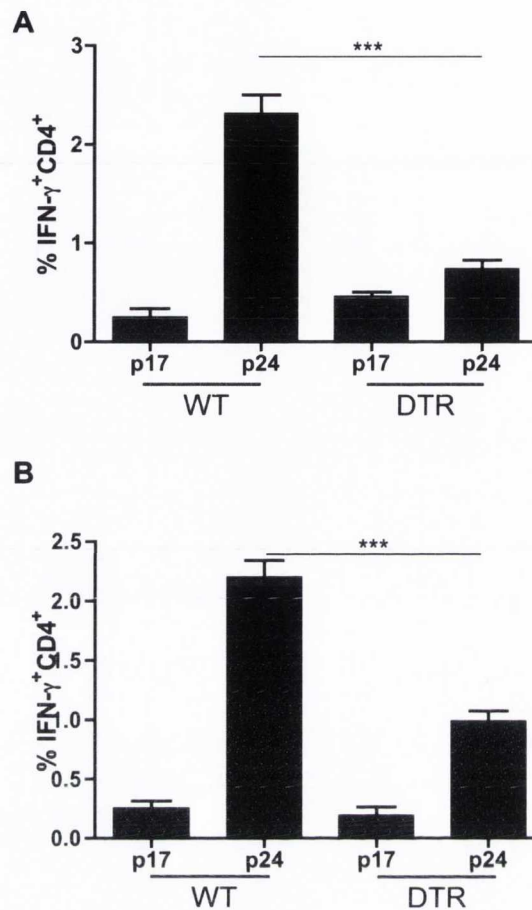


Figure 5.17 Lung resident CD103⁺Langerin⁺ dendritic cells mediate the induction of immune responses in the gastrointestinal tract post-intranasal immunization. Langerin DTR and wild type animals received 1 μ g DT i.p. Animals were primed with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) delivered i.n. 36 hours prior to the boost all groups were administered 1 μ g of DT and boosted with α -DEC-205-gag p24 fusion mAb (5 μ g) and poly ICLC (50 μ g) i.n. One-week post, boost mononuclear cells were isolated from the (a) SILP (B) colon and re-stimulated with anti-CD28 (1 μ g/mL) and p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.17 illustrates cumulative data from three individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

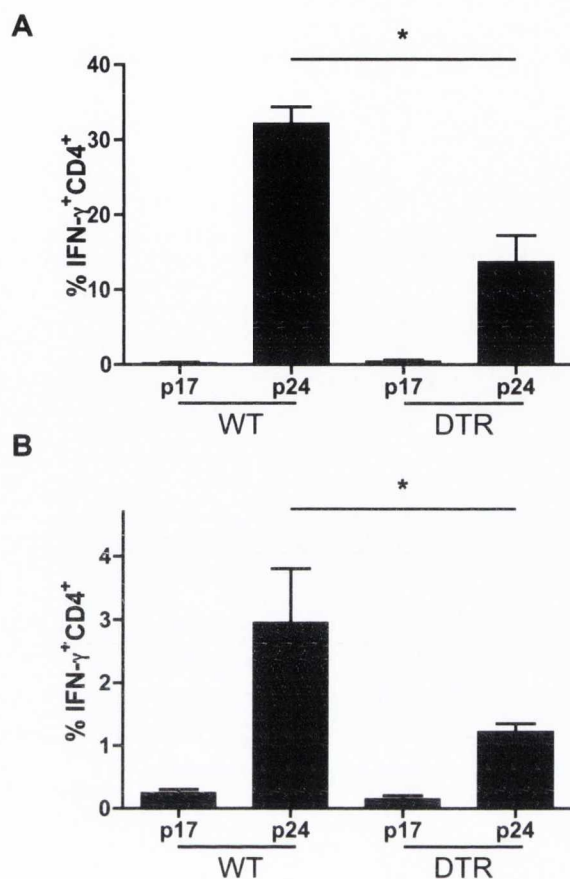


Figure 5.18 Lung resident CD103⁺Langerin⁺ dendritic cells mediate the induction of immune responses in the lung and spleen post-intranasal immunization with untargeted gag-p24. Langerin DTR and wild type animals received 1 μ g DT i.p. Animals were primed with untargeted p24 protein (15 μ g) and poly ICLC (50 μ g) delivered i.n. 36 hours prior to the boost all groups were administered 1 μ g of DT and boosted with gag p24 (5 μ g) with poly ICLC (50 μ g) i.n. One-week post boost, mononuclear cells were isolated from the (a) lung and (b) spleen and re-stimulated with p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Figure 5.18 illustrates cumulative data from two individual experiments. *p<0.05, **p<0.01, ***p<0.001

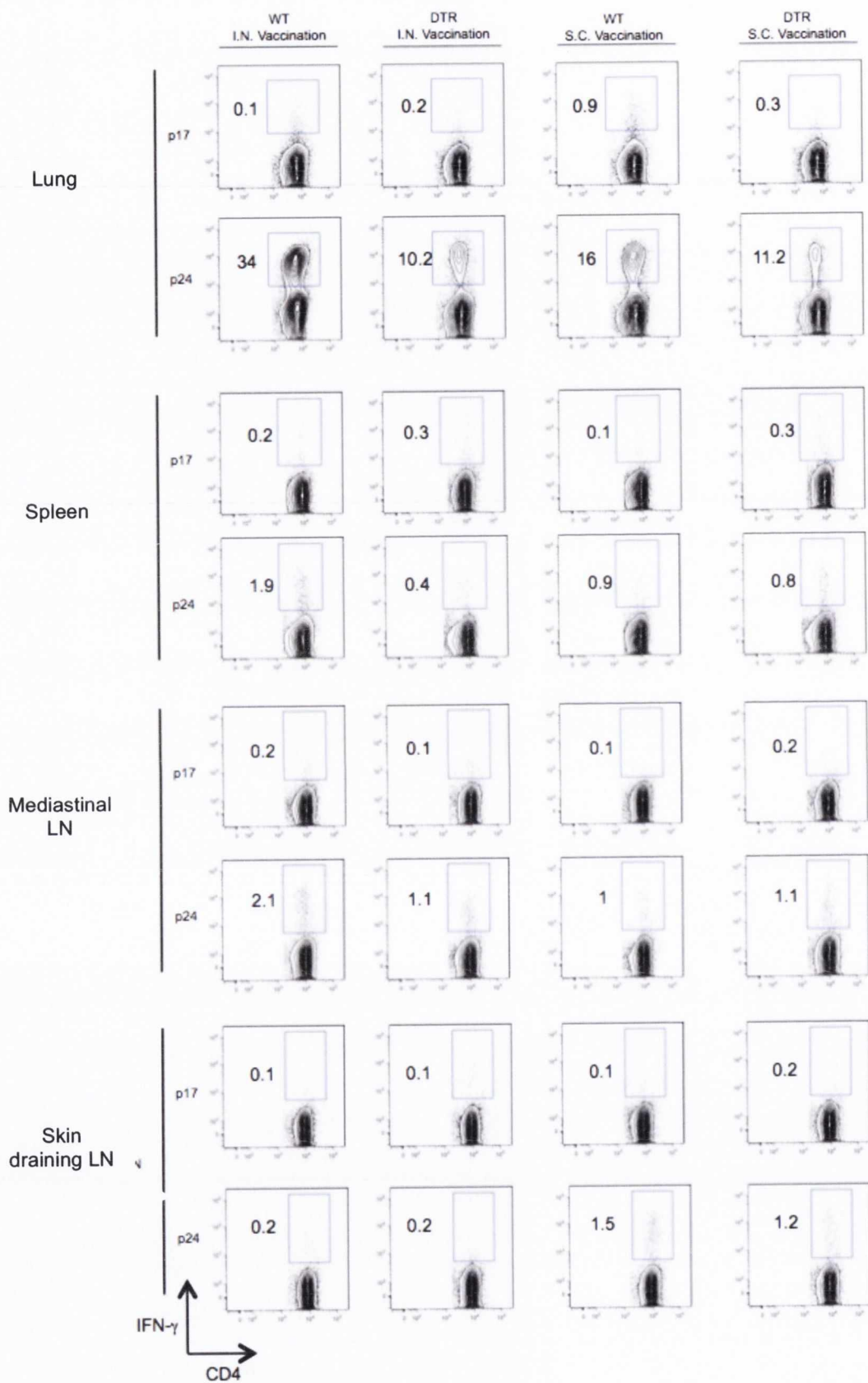


Figure 5.19 Depletion of CD103⁺Langerin⁺ dendritic cells significantly reduces the induction of Th1 immune response following intranasal but not subcutaneous vaccination.

Langerin DTR and wild type animals received 1µg DT i.p. per average 25-30 gram mouse 36 hours prior to priming. Animals were primed with α-DEC-205-gag p24 fusion mAb (5µg) and poly ICLC (50µg) delivered i.n. or s.c. 36 hours prior to the boost all groups were administered 1µg of DT and boosted with α-DEC-205-gag p24 fusion mAb (5µg) with poly ICLC (50µg) i.n. One- week post boost, mononuclear cells were isolated from immunological relevant compartments and re-stimulated with anti-CD28 (1µg/mL) and p24 pools or p17 (control) pools at 1µg/mL for intracellular cytokine staining. Figure 5.19 illustrates a representative of two experiments.

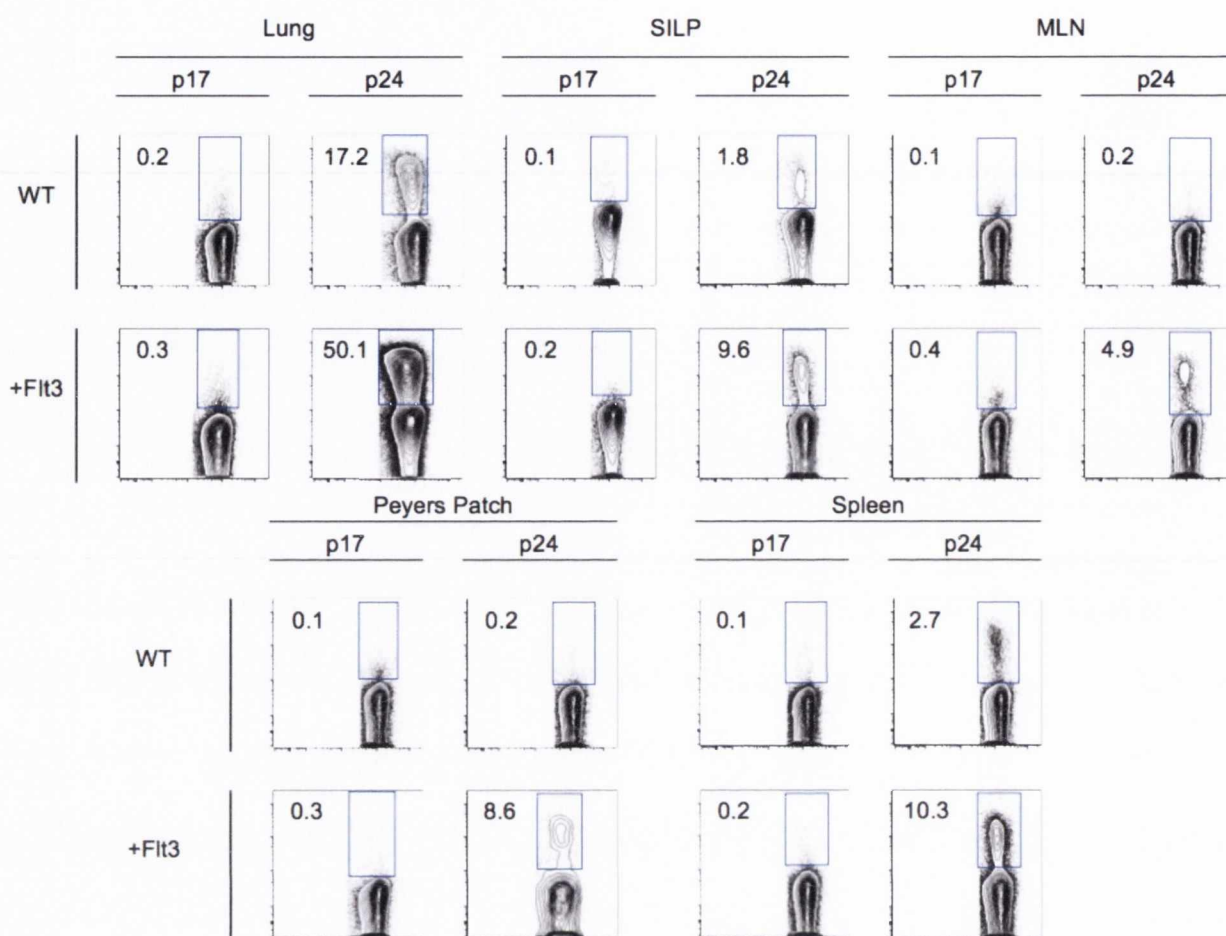


Figure 5.20 Flt3L improves CD4⁺ T-cell IFN γ responses to i.n. HIV vaccine administration. Age and gender matched control and experimental mice were injected i.p. daily during both the prime and boost phase with Flt3L in sterile PBS or PBS alone, for 10 days. At day 8, α -DEC205-gag p24 (5 μ g) and poly ICLC (50 μ g) was administered i.n.. Boost vaccination was administered 4 weeks later. One week post i.n. boost all groups were sacrificed. In mice vaccinated to α CD205-gag p24 peptide challenge with p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Representative of two experiments.

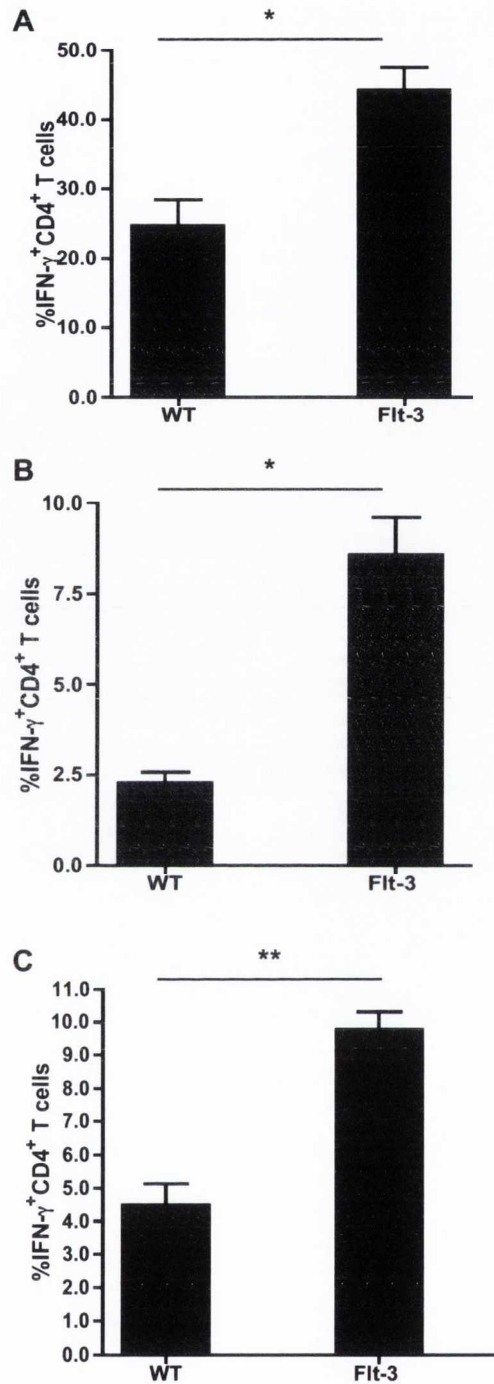


Figure 5.21 Flt3L improves CD4⁺ T-cell IFN γ responses to i.n. HIV vaccine administration. Age and gender matched control and experimental mice were injected i.p. daily during both the prime and boost phase with Flt3L in sterile PBS or PBS alone, for 10 days. At day 8, α -DEC205-gag p24 (5 μ g) and poly ICLC (50 μ g) was

administered i.n.. Boost vaccination was administered exactly 4 weeks later. One week post i.n. boost all groups were sacrificed. In mice vaccinated to α CD205-gag p24 peptide challenge with p24 pools or p17 (control) pools at 1 μ g/mL for intracellular cytokine staining. Reprehensive of two experiments. Figure 5.21 represents cumulative data from two individual experiments, (a) lung, (b) SILP (c) spleen. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Chapter 6

General Discussion

6.1 General Discussion

The term “*common mucosal immunological system*” was first coined by John Bienenstock over 43 years ago. He developed the concept following the discovery that the lymphoid tissues associated with the bronchus were similar to those in the GI tract (Bienenstock and McDermott 1978). However, appreciation of the importance of this concept is only now truly beginning. The mucosal surfaces are immunologically unique, as they act as the primary interface between the host and the physical environment, yet have key barrier functions. The adult human mucosa lines the surfaces of the digestive, respiratory, and genitourinary tracts, covering an immense surface area (400m^2) that is 200 times greater than that of the skin (Holmgren and Czerkinsky 2005; Okayasu, Sutter et al., 2011). Mucosal lymphoid tissues are different from peripheral lymph nodes in their anatomical connections and orientation, cellular composition, and contribution to immune responses. Moreover, different mucosal sites have distinct patterns of organization with respect to immune cells (Coombes and Powrie 2008; Iwasaki 2010).

Despite a recent renaissance of interest in the field of mucosal crosstalk, much of the research undertaken to date has focused on specific individual components of the system. Although much knowledge has been derived from this approach, future research should examine how different components affect each other and how crosstalk is achieved between individual compartments and, more importantly, between various mucosal sites.

The activation of T and B cells generally occurs in the lymph nodes, where lymphocytes also receive their tissue homing capacities. Leukocyte adhesion to the endothelium and subsequent migration into tissues is at the heart of protective and pathological immune responses. Leukocyte adhesion is a multistep process involving the “capture” of lymphocytes by endothelial cells (tethering), following by loose adhesion (rolling), activation and subsequent transmigration of lymphocytes into different tissues. Naïve T and B cells constantly transit between the blood and secondary lymphoid organs, in particular lymph nodes and Peyer's patches, with very high efficiency. This process depends on the expression of L-Selectin (CD62L), LFA-1 and CCR7 (De Calisto et al.,

2011). Naïve B cells are less dependent on CCR7, instead using CXCR4 and CXCR5 to migrate to lymph nodes (Santaolalla and Abreu 2012).

The current paradigm of lymphocyte homing to the GI tract involves the induction of $\alpha 4\beta 7$ and CCR9 by Peyer's Patch and MLN DC in a RA dependent manner. However, the current paradigm is difficult to reconcile with reports of GI T cell responses after i.n. delivery of antigens that do not directly target the GI lymphoid tissue (Esplugues et al., 2011). Collectively, studies suggest that the mucosal immune system is actually one large interconnected network and that individual components are very efficient at sharing information distally.

Here, it was hypothesized that DCs orchestrate crosstalk and homing between distinct mucosal compartments. This study aimed to improve our understanding of this process in order to progress vaccine induced generation of protective immunity. Unexpectedly, it was found that lung DC, like CD103⁺ MLN DCs, up-regulated expression of the gut homing integrin $\alpha 4\beta 7$ *in vitro* and *in vivo* and induced T cell migration to the GI tract. Lung DC mediated $\alpha 4\beta 7$ induction required RA and TGF- β signaling. Furthermore, lung DC targeting by i.n. immunization induced protective immunity against enteric challenge with a highly pathogenic strain of *Salmonella*.

In order to investigate the capacity of non-GI DCs to induce $\alpha 4\beta 7$ on T cells, a screen of DCs isolated from various mucosal and non-mucosal compartments was conducted. CD11c⁺ cells were isolated from murine lung, spleen, MLNs and skin draining lymph nodes and co-cultured with CFSE-labeled ova reactive CD4⁺ T cells from OT-II mice. In agreement with previous studies, MLN DCs significantly up-regulated expression of $\alpha 4\beta 7$ (Mora et al., 2006). Interestingly, lung DCs also had the capacity to induce the expression of $\alpha 4\beta 7$, with up-regulation most prominent on proliferating cells. These findings are in contrast to Jaensson *et al.*, which, revealed that despite the presence of CD103⁺ DCs in a wide range of mucosal murine tissues and secondary lymphoid organs, only GI-associated CD103⁺ DCs had the capacity to efficiently induce the expression of gut homing integrin's on responding T cells *in vitro* (Jaensson et al., 2008).

Following on from these novel observations, *in vivo* analysis was performed. A significantly higher percentage of CFSE^{low} $\alpha 4\beta 7^+$ cells were induced in the lung and mediastinal LN following i.n. immunization than in the skin draining LN following s.c. vaccination. Similar findings were also observed in the case of CD8⁺OT-I cells, revealing the ability of lung DC to induce gut homing on both T cell populations.

Targeting of lung DCs by i.n. delivery was confirmed in a time course experiment where fluorescently labelled antibodies directed against CD11c were administered to C57BL/6 mice i.n. Both CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DC subsets were targeted in the mediastinal LN and lungs with the peak labelling occurring 24 hours post antibody administration. This is consistent with previous reports, revealing that i.t. administration of fluorescently labeled large-molecular-weight antigens results in their uptake by different mediastinal LN DCs. Specifically the CD103⁺ and CD11b⁺ migratory DCs and resident CD8 α ⁺ DCs 12 hours post administration (Vermaelen et al., 2001). In agreement with these studies, following i.t. administration of fluorescent antibodies, CD103⁺ and CD11b⁺ DCs were labeled in the lung tissue and mediastinal LN. Vermaelen *et al.*, (2001) also determined that migration of lung DCs to the mediastinal LNs following antigen capture occurs in a CCR7- and CCR8- dependent manner, this is consistent with data generated from this study revealing CCR7 dependent migration to the mediastinal LNs following fluorescent antibody administration (*data not shown*) (Vermaelen et al., 2001). Despite the appearance of labeled cells in the mediastinal LNs, antibody labelled cells could not be detected in the spleen, MLN or GI induction or effector tissues even after 72 hours.

One possibility for the induction of $\alpha 4\beta 7$ on the transferred cells was the inadvertent swallowing of i.n delivered antigen and thus local priming, proliferation and subsequent dissemination of cells in the MLN. Despite the examination of the transferred CD45.1⁺V α 2⁺CD4⁺ T cells in the MLN during the time course experiment, revealing no proliferation or induction of $\alpha 4\beta 7$, additional strategies to confirm local pulmonary priming were investigated. FTY-720, an inhibitor of lymphocyte egress from lymph

nodes was utilized, whereby CFSE labelled CD45.1⁺ OT-II cells were adoptively transferred to recipient mice and immunized i.n. On days 0 – 3, recipient mice were administered FTY-720. A significant increase in the frequency of transferred cells was noted in the mediastinal LN of mice treated with FTY-720, revealing the expansion and proliferation of these cells in this immune compartment. In contrast, a significant decrease in the frequency of transferred cells was noted in the MLN suggesting that antigen exposure and expansion of the transferred OT-II cells was not occurring in the MLN.

As expected, a significant decrease in the frequency of transferred cells was noted in the small intestine and colon due to their arrest within the mediastinal LN. Thus, based on the time course and FTY experiments, it is possible to confirm that following i.n. delivery of antigen, the induction of $\alpha 4\beta 7$ was localized to the mediastinal LN and lungs and was not due to ingestion of the vaccine. These data extend the work of Ciabattini *et al.*, who have recently demonstrated that following i.n. immunization, antigen specific T cells trafficked to distal skin draining lymph nodes in a CD62L dependent fashion and to the MLNs in an $\alpha 4\beta 7$ dependent fashion (Ciabattini *et al.*, 2011). However, unlike in the present study, mechanisms of induction of $\alpha 4\beta 7$ i.e. the role of lung DCs or the physiological relevance of these pathways was not explored by Ciabattini *et al.* In addition this study revealed that i.t. delivery of vaccine using the OT-II system was sufficient to induce $\alpha 4\beta 7$ on transferred cells, equivalent to i.n. delivery further confirming the role of lung DCs (*data not shown*).

Given the capacity of lung CD11c⁺ DCs to induce $\alpha 4\beta 7$ expression, the ability of the individual lung DC subtypes to induce $\alpha 4\beta 7$ was investigated. DCs within the intestinal compartments have been extensively studied and much is now known regarding their phenotype and function. CD103⁺ DCs in the MLN have the unique ability to induce gut homing phenotypic changes (Annacker *et al.*, 2005; Johansson- Lindbom *et al.*, 2005). Induction of CCR9 and $\alpha 4\beta 7$ expression was initially observed following the co-culture of MLN CD103⁺ DCs from the MLN with T cells after oral antigen administration. In

contrast, these cells failed to induce CCR9 and $\alpha 4\beta 7$ expression on T cells following intraperitoneal (i.p.) antigen injection. Agace and colleagues transferred TCR transgenic OT-I cells into recipient mice and the expression of CCR9 and $\alpha 4\beta 7$ in the MLNs was examined 3 days following i.p. or oral antigen administration. CCR9 and $\alpha 4\beta 7$ endowed the newly activated T cell with the capacity to migrate to the small intestine and exert effector functions (Annacker et al., 2005; Johansson-Lindbom et al., 2005). In contrast the intestinal resident CD11b⁺ DC lack this capacity. Following on from these studies, the ability of the lung DC subsets to induce $\alpha 4\beta 7$ was examined. Lung MHC-II⁺CD11c^{hi} DC were FACS-sorted into CD103⁺CD11b⁻ and CD103⁻CD11b⁺ DC populations and co-cultured with CFSE labelled OT-II cells. Surprisingly, despite the current literature describing distinct functional and anatomical differences, both CD103⁺CD11b⁻ and CD103⁻CD11b⁺ lung DC subsets induced $\alpha 4\beta 7$ integrin with expression of $\alpha 4\beta 7$ being even higher in the CD103⁻CD11b⁺ population.

Having determined the capacity of lung DCs to induce $\alpha 4\beta 7$ expression *in vitro* and *in vivo*. The migratory ability of lung DC primed cells was examined. Anti- $\alpha 4\beta 7$ antibody administration led to a significant reduction in the migration of lung primed CD4⁺ T cells, to the small intestinal and colonic lamina propria as well as the intraepithelial compartments. Conversely, an increase in the percentage of CD45.1⁺V $\alpha 2$ ⁺CD4⁺ T cells was noted in systemic sites such as the blood, spleen and lung where $\alpha 4\beta 7$ is not required for cellular migration. This demonstrated that i.n. immunization-induced OT- II cell migration to the GI tract was mediated by $\alpha 4\beta 7$. It is interesting to note that while migration to GI sites was blocked using an anti- $\alpha 4\beta 7$ antibody, migration to the systemic sites was not. In fact, there was a non-significant increase in the frequency of transferred cells in other sites examined, including the lungs. This lends specificity to the current experimental approach as migration of T cells to the lungs has been shown to be independent of $\alpha 4\beta 7$ /MadCAM but dependent on L-Selectin/PNAd, $\alpha 4\beta 1$ /VCAM-1 and LFA-1 dependent (Xu et al., 2003).

Additionally, the efficiency of lung DC and skin DC primed T cells to migrate to the GI tract was also investigated. Mice were immunized i.n. or s.c. On day 7, post immunization, the frequency of CD45.1⁺V α 2⁺ cells in the small intestines and colon was 3-10 fold higher following i.n. immunization than following s.c. immunization demonstrating that lung DC- primed congenic OT-II cells migrated to the lamina propria with a significantly greater efficiency than skin DC-primed OT-II cells, thereby confirming the previous *in vivo* results.

Vitamin A (retinol) has long been recognized to modulate a broad range of immunological activations (Pino-Lagos, K et al., 2008), a major characteristic of small intestinal CD103⁺ DCs is their enhanced ability to metabolise vitamin A and generate its major active metabolite RA (Blomhoff, R and Blomhoff, HLK. 2006). RA has an unequivocal role in inducing α 4 β 7 (Iwata et al., 2004) and CD103⁺ DCs from PP and MLN are capable of metabolizing vitamin A into RA (Johansson- Lindbom et al., 2005). To investigate the mechanisms mediating α 4 β 7 induction by lung DCs, the presence of retinal to RA oxidizing enzymes- aldehyde dehydrogenases (ALDHs) in lung DCs was investigated, using a fluorescent ALDH substrate- ALDEFLOUR (Yokota et al., 2009). Both CD103⁺CD11b⁻ and CD103⁻CD11b⁺ lung DC subsets demonstrated the presence of ALDH at levels comparable to MLN CD103⁺CD11b⁻ DCs. This is in concurrence with a similar report by Guilliams *et al.*, (Guilliams et al., 2010) although in that study, the ability of lung DCs to induce integrin α 4 β 7 was not examined. As ALDH activated was observed in the lung DCs, the impact of inhibiting retinoic acid receptor signaling on α 4 β 7 induction was determined. Adding 1 μ M RAR- β inhibitor LE540 to DC:OT-II cultures resulted in a significant reduction of α 4 β 7 expression induced by lung DC, therefore the mechanism underlying α 4 β 7 induction appears so be conserved between lung and GI resident DC. Notably, in addition to an *in vitro* culture system, a novel, dominant negative RAR-OT-II Rag^{-/-} mice and TGF- β receptor^{fl/fl}-CD4^{CRE} OT-II Rag^{-/-} mice were used to address the role of RA *in vivo*. Splenic OT-II cells from the dominant negative RAR-OT-II Rag^{-/-} mice and TGF- β receptor^{fl/fl}-CD4^{CRE} OT-II Rag^{-/-} mice were used as donor cells, so local, lung DC subsets in these mice are not relevant here. In agreement with the *in vitro* data, lung induced α 4 β 7 was dependent on RA and TGF-

β signaling *in vivo*, with complete ablation of $\alpha 4\beta 7$ expression of the transferred RAR-OT-II Rag^{-/-} T cells following i.n. vaccination.

Since low levels of integrin $\alpha 4\beta 7$ can be induced by cellular activation, the role of adjuvant in $\alpha 4\beta 7$ induction was investigated. Following CD45.1⁺OT-II cell transfer into naïve CD45.2⁺ hosts, the recipient mice were immunized i.n. with ova alone, ova/LPS, ova/polyICLC and polyICLC alone. Poly ICLC alone did not induce $\alpha 4\beta 7$ whereas ova alone did, demonstrating the need for antigen presentation. Additionally, ova/LPS also induced $\alpha 4\beta 7$ suggesting that $\alpha 4\beta 7$ induction was not adjuvant specific. Additionally, to test for involvement of polyICLC induced, RIG-I and TLR3 mediated DC maturation on $\alpha 4\beta 7$ induction, CD45.1⁺OT-II cells were transferred into wild type, and MDA-5^{-/-}TLR3^{-/-} hosts (McCartney et al., 2009). No significant difference was noted between the MDA-5^{-/-}TLR3^{-/-} and wild type mice. Thus revealing that expression of $\alpha 4\beta 7$ on T cells following i.n. immunization is not induced in a non-specific fashion by polyICLC alone.

In order to determine the role-played by lung DCs in the induction of $\alpha 4\beta 7$ post i.n. immunization, two specific DC ablation strategies were investigated, the CD11c-DTR (Jung et al., 2002) and z-DTR (Meredith et al., 2012a) models. Use of the CD11c DTR leads to the depletion of 85-90% of CD11c⁺ DCs (Bennett and Clausen, 2007). However, this model cannot distinguish between cDCs, activated monocytes and alveolar macrophages, all of which express CD11c and are ablated in this model (Probst et al., 2005; Zammit et al., 2005), the newly described zDC-DTR model was also utilized to selectively deplete the cDC population (Meredith et al., 2012a; Meredith et al., 2012b). This previously uncharacterized zinc finger transcription factor, zDC (Zbtb46, Btbd4), was found to be specifically expressed by cDCs and committed cDC precursors but not by monocytes, pDCs, or other immune cell populations, Meredith *et al.*, (2012a) inserted DTR cDNA into the 3' UTR of the zDC locus to serve as an indicator of zDC expression and as a means to specifically deplete cDCs. Mice bearing this knockin express DTR in cDCs but not other immune cell populations, and DT injection into zDC-DTR bone marrow chimeras results in cDC depletion. In contrast to previously characterized

CD11c-DTR mice, non-cDCs, including pDCs, monocytes, macrophages, and NK cells, were spared after DT injection in zDC-DTR mice (Meredith et al., 2012a; Meredith et al., 2012b).

Significantly attenuated numbers of transferred congenic cells expressing $\alpha 4\beta 7$ were seen in both models, demonstrating that lung DCs induced integrin $\alpha 4\beta 7$ post i.n. vaccination. These results need to be interpreted with the caveat that both DT induced depletion of DCs, and nasal administration of poly ICLC is likely associated with lung inflammation (Tittel et al., 2012). It is speculated that local inflammation is responsible for the variability observed in the levels of $\alpha 4\beta 7$ expressed on transferred cells isolated from various compartments, the expression being high on cells isolated from the lungs compared to the cells isolated from the mediastinal lymph node, blood or from the spleen and MLN where a more profound attenuation of $\alpha 4\beta 7$ was seen.

Thus, based on the data generated by different experimental approaches, it is proposed that following i.n. immunization, lung and mediastinal lymph node resident DCs are targeted which in turn induce the expression of integrin $\alpha 4\beta 7$ on T cells, resulting in their localization to the GI tract. Herein, this provides novel functional evidence of mucosal cross talk mediated by DCs. This also provides a mechanistic insight to the observation of T cell responses in the GI lamina propria following i.n. challenge (Esplugues et al., 2011; Masopust et al., 2010).

One of the most interesting findings from these current studies, with significant relevance to the design of vaccines and targeting strategies, is the role of distinct lung resident DC subsets and their capacity to induce gut homing integrin expression. In contrast to the existing literature showing that CD103⁺ MLN DCs can induce integrin $\alpha 4\beta 7$ whereas the CD11b⁺ MLN DCs cannot, it was determined *in vitro* that $\alpha 4\beta 7$ induction was mediated by both CD103⁺ and CD11b⁺ lung DCs, the expression being higher in the lung CD11b⁺ DC. Since these differences were novel and unexpected further examination of these finding were investigated using *in-vivo* DC depletion models.

In order to deplete the CD103⁺ DC populations, *Langerin DTR* (Bennett et al., 2005) mice were used. Upon ablation of the lung CD103⁺ DC population, no significant differences in the level of $\alpha 4\beta 7$ induction between the langerin depleted and replete mice could be detected. To further confirm the effect of CD103⁺ DC depletion, *Batf3*^{-/-} mice were used, here deletion of the transcription factor *Batf3* results in ablation of the cross presenting, CD103⁺ tissue DCs (Hildner et al., 2008). Again, no significant differences in the induction of $\alpha 4\beta 7$ were observed between wild type and *Batf3*^{-/-} animals. Finally, to deplete the CD11b⁺ lung DCs, CD11b DTR mice were utilized. Both CD11b expressing alveolar macrophages and DCs are depleted in this model; however, the use of zDC-DTR demonstrates the role of classical DCs. Attenuated levels of $\alpha 4\beta 7$ were observed in the mice where CD11b-expressing cells were depleted. Therefore, in contrast of MLN DCs, both CD103⁺ and CD11b⁺ lung DC populations induced integrin $\alpha 4\beta 7$ *in vitro*, although *in vivo*, the CD11b⁺ populations appeared to have a more dominant effect on the induction of $\alpha 4\beta 7$.

Studies have revealed that migratory gut CD103⁺ DCs use dietary and biliary sources of vitamin A to generate RA (Jaensson-Gyllenback et al., 2011), it is therefore speculated that lung DCs either use haematogenous Vitamin A or local stores of vitamin A found with the lung tissue (Dirami et al., 2004; Okabe et al., 1984). Thus, the predominance of CD103⁺ DC in mediating gut homing as in the case of MLN is not observed in case of lung DCs. This further allows investigation into specific DC populations, for example the MLN CD103⁺ DC may not be pre-conditioned to metabolize vitamin A but rather, they may acquire this property based on local environmental factors like the availability of Vitamin A, TLR ligands or other inflammatory stimuli. Recent work by Jaensson-Gyllenback *et al.*, (2011) revealed that bile contained high levels of retinol, induced RA receptor-dependent retinol-metabolizing activity in bone marrow-derived DCs, and imprinted these cells with the ability to generate gut-tropic T cells. Taken together, these results suggest a novel and unexpected role for bile in SILP CD103⁺ DC imprinting (Jaensson-Gyllenback E. et al., 2011). This therefore prompts further investigation into the relative expression of RALDH and the effect of adjuvants on RALDH expression by lung DC subsets. Findings from these studies challenge the dogma that only CD103⁺ gut

resident DCs can recruit T cells to the GI tract. Rather, this data provides compelling evidence that lung DCs, targeted by i.n. immunization, are capable of inducing robust levels of gut homing integrin $\alpha 4\beta 7$, and licensing T cells to migrate to the GI tract.

Having determined this novel route of trafficking to the GI tract, the physiological relevance of such trafficking during the context of pathogenic challenge was investigated. *Salmonella typhimurium*, a pathogen of global significance (Levine, 2006) that is transmitted across the intestinal mucosa and causes a spectrum of diseases ranging from localized intestinal infection to severe systemic illness was chosen (Griffin and McSorley, 2011). Notably, although *Salmonella* is not a lung pathogen, this model was adopted as a tool to examine i.n. vaccine induced protection against *Salmonella* invasion across the GI tract. To confirm protection against enteric challenge with salmonella, three different experimental systems were used: 1) Passive immunization using transferred OT-II cells followed by i.n. immunization where a modest increase in survival was demonstrated. 2) Active prime-boost immunization with ova showing a significantly increased survival and lack of systemic pathology in the i.n. immunized mice, and 3) Active prime-boost immunization using inactivated *Salmonella* showing reduced systemic burden in the i.n. immunized mice. Thus, using multiple experimental systems, lung DCs targeted by i.n. immunization was shown to be able to induce protective immunity within the GI tract. Notably, in mice that received s.c.vaccine, a non- significant increase in survival was also observed. We hypothesize this to be due to the systemic immunity generate by s.c. vaccine and plan to study this further with the use of IgA deficient, CD103^{-/-} and B7^{-/-} mice.

Most current vaccines mediate protection primarily through induction of antibodies (Plotkin SA 2008). However, in many infections such as HIV, malaria and TB, there remains a need for protective T cell immunity. This is particularly true for HIV infection as the GI tract harbours a large complement of immune cells that are preferentially targeted during acute HIV and SIV infections regardless of the route of virus inoculation (Mehandru et al, 2005). As such, induction of mucosal immunity is taking precedence when designing vaccines against HIV-1. Therefore, it has been argued that the goal of an

effective HIV vaccine should be to interrupt mucosal transmission at its earliest stages and to prevent viral production in mucosal tissues.

Vaccination with protein-based vaccines has been underemphasized due to their limited ability to induce strong Th1 immunity. This can be greatly improved by enhancing uptake by DCs, and adjuvants. One approach to improve delivery of protein vaccines to DCs is to introduce the protein into monoclonal antibodies (mAbs) that efficiently target DC receptors, and then co-administer the fusion mAb with an appropriate agonist for DC maturation (Longhi MP et al., 2009). Given the ability of i.n. vaccination to induce protection against an enteric pathogen, mAbs specific to DEC-205 conjugated to relevant HIV proteins were delivered i.n. in an attempt to induce and detect antigen specific immune responses at the intestinal mucosal surfaces.

Because the goal was to compare the efficacy of mucosal and systemic vaccination to induce antigen specific T cells in the effector sites of the small intestine, mice were vaccinated via various routes. Here, it was determined that i.n. vaccination efficiently induced the gag specific IFN- γ ⁺CD4⁺ T cell at the mucosal sites, comparable to vaccine delivered i.p. In contrast, i.v., i.m. and s.c. failed to elicit IFN- γ ⁺CD4⁺ T cells in the GI mucosa, this is in line with the current literature. Additionally, i.n. vaccination induce significant higher levels of gag specific IFN- γ ⁺CD4⁺ T cells in the spleen compared to other routes. These results supporting the need for mucosal immunization to elicit mucosal CD4⁺ T cells are in line with previous reports in the field of infectious disease, showing that mucosal immunization generates a more potent regional humoral and mucosal T cells response compared with systemic immunization (Belyakov IM et al., 1998, Kaufman DR et al., 2008). These mucosal T cells are required to protect against a mucosal infectious challenge, whereas systemic (spleen) T cell response alone failed to control the same infectious challenge (Belyakov IM et al., 1998). Other studies claimed that systemic immunization can overcome immune compartmentalization (Kaufman DR et al., 2008, Hensen SG et al., Liu J et al., 2009). However, in the latter case, the respective efficiency of mucosal and systemic immunization regimen was rarely compared. The requirement of mucosal vaccination to elicit mucosal immunity may be

more restricted with protein-based vaccines than live viral vectors (Belyakov IM et al., 2009). However despite this, data presented here reveal robust induction of GI immune responses post i.n. vaccination. Significant levels of IFN- γ ⁺ expressing CD4⁺ T cells were also found in the lung, local draining LNs and nose. Furthermore, in addition to examining the effector sites of the small intestine, robust IFN- γ ⁺ expressing CD4⁺ T cells were found in the colonic lamina propria but not the GI inductive sites.

To assess the potential of antigen targeting to DCs to improve immunity, various doses of DEC-gag were administered. Similar to previous studies revealing that DEC-205 targeting enhances the generation of antigen specific CD4⁺ T cells in the spleen (Trumpfeller C, Finke JS, et al., 2006). A superior induction of IFN- γ ⁺ expressing CD4⁺ T cells was noted in the DEC-targeted vaccines relative to unconjugated protein in the small intestine, colon, lung and systemic compartments. Findings from these studies reveal that targeting of gag protein to DCs improves CD4⁺ T cell immunization in the GI tract post i.n. immunization relative to other approaches, which are currently undergoing testing in humans.

Long-term immunity at mucosal surfaces would be the goal of an effective vaccine, particularly HIV. Utilizing DEC-205 targeting as a vaccine strategy i.n., long term immunity was observed in the small intestinal lamina propria and colonic lamina propria. Additionally, compared to untargeted protein, DEC-targeting induced robust longterm immunity in the lung and spleen. The current findings indicate that by targeting lung DCs with i.n. vaccination, robust long term IFN- γ ⁺ expressing CD4⁺ T cells can be efficiently induced in both local, systemic and important GI effector sites, highlight a novel route of vaccination to the GI tract.

Lung DCs were originally described in the mouse as a single population of highly-dendritic shaped cells with a high degree of expression of CD11c and MHC-II (Sertl K et al., 1986), it is now clear that at least four different subsets of DCs can be found in the lung. As previously discussed the dominant lung resident DC populations, include the CD103⁺Langerin⁺ and CD11b⁺ DC. Having determined their capacity to induce the

expression of $\alpha 4\beta 7$ *in vitro* and *in vivo*, the distinct roles played by these populations during immunization was investigated. Interestingly, the selective ablation of the CD103⁺Langerin⁺ DC population; significantly reduced the induction of gag specific CD4⁺ T cells in both systemic and mucosal compartments. These findings are in line with other studies revealing that lung resident CD103⁺Langerin⁺ DCs seem specialized in taking up apoptotic cells in the lung, and have enhanced ability to cross-present soluble inhaled antigens (del Rio ML et al., 2007). This functional specialization of DC subsets likely originates from differential expression levels of antigen uptake receptors (Burgdorf et al., 2007) or proteolytic enzymes and chaperon molecules involved in antigen processing (Dudziak D et al., 2007). Despite the fact that CD11b⁺ have the capacity to acquire inhaled antigens and migrate to the draining lymph nodes during steady state and inflammation, the role of this population during immunization remains to be fully determined.

Overall, the results from these studies have contributed significantly to our understanding of the common mucosal immune system. In agreement with several theories stating that the mucosal immune system is an integrated system of tissues, cells and effector molecules, it has been revealed that there is distinct DC mediated mucosal cross talk between a variety of mucosal compartments, particularly the Lung and GI tract. Lung DCs have the capacity to induce the expression of gut homing receptors on T cells *in vitro* and *in vivo*. Lung DCs license T cells to migrate to the intestinal lamina propria via $\alpha 4\beta 7$ post i.n. vaccination. It was determined that lung DC induction of $\alpha 4\beta 7$ was mediated by RA and TGF- β signaling, with lung CD11b⁺ DCs the dominant population mediating $\alpha 4\beta 7$ induction *in vivo*. The physiological relevance of such DC mucosal cross talk was highlighted, revealing that i.n. vaccination protects against enteric challenge with a highly pathogenic strain of *Salmonella*.

From a translation standpoint, a detailed understanding of T cell homing behavior may inspire novel vaccine generation. Although nasal immunization is practiced at the randomized phase II clinical study level for prevention of influenza (Langley JM et al., 2011), this route of immunization is yet to be tested for protection against GI pathogens

in humans. Developing efficacious vaccines against enteric diseases is a global challenge that requires a better understanding of cellular recruitment dynamics at the mucosal surfaces, these studies have revealed that pathways of recruitment of antigen specific immune cells to the gut are much more promiscuous than previously appreciated. There appears to be considerable, albeit hitherto unrecognized, DC orchestrated, mucosal cross talk thereby confirming the classical theory of the *common mucosal immunological system*.

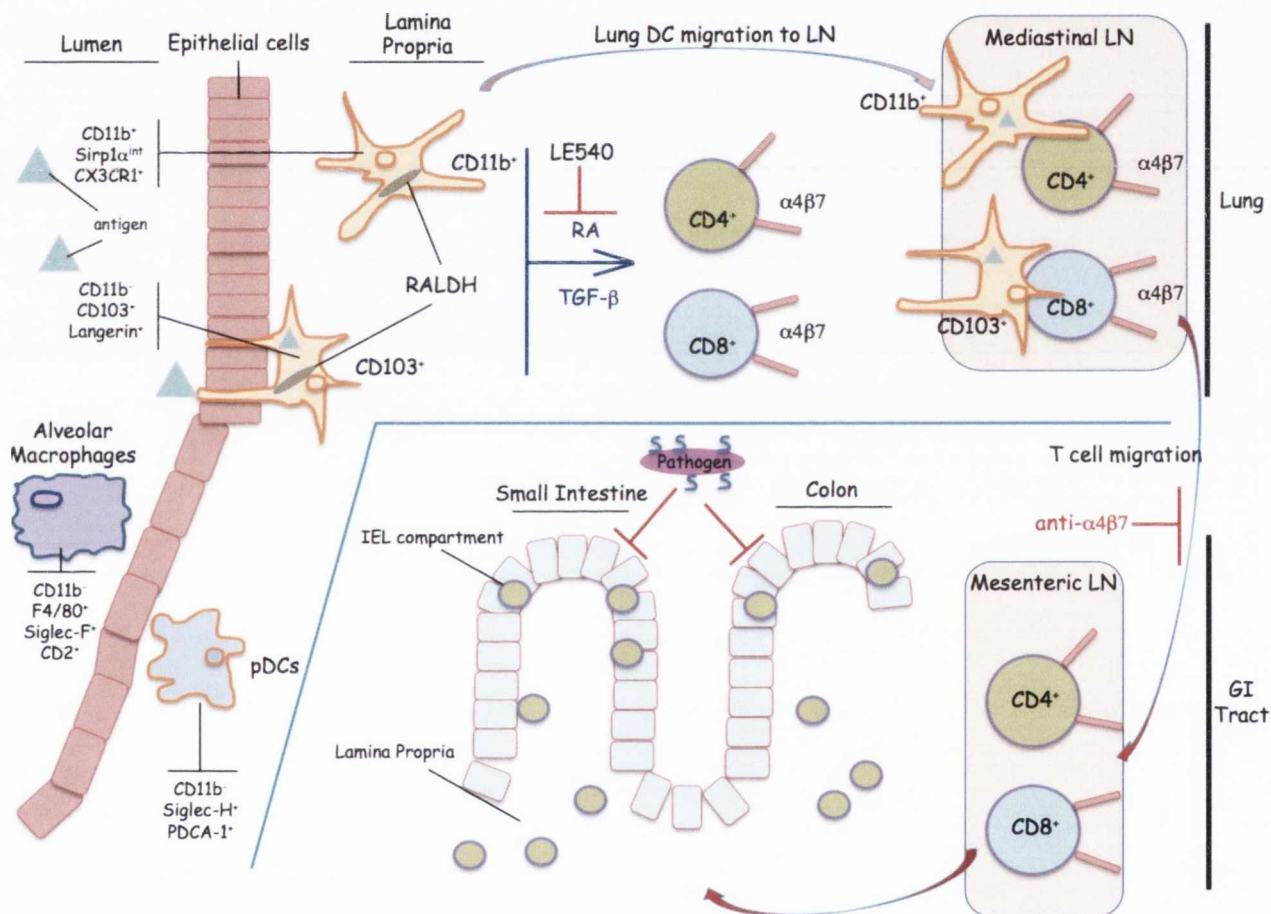


Figure 6.1: Lung DC mediated mucosal crosstalk.

Chapter 7

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Appendix I

Materials

General Materials

Cell Culture Medium – R5:

Rosewell Park Memorial Institute (RPMI) – 1650 (BioSera) was supplemented with 10% heat inactivated (56C for 30 minutes) foetal calf serum (FCS, BioSera), 100mM L-Glutamine (Gibco), 100ug/ml penicillin/streptomycin (Biowest).

Ammonium Chloride Lysis Buffer:

NH₄CL – 0.829 grams (Sigma)

KNCO₃ – 0.109grams (Sigma)

Disodium EDTA – 0.037grams (Sigma)

Distilled water – 100ml

*pH of final ammonium chloride lysis buffer solution is 7.3

20X PBS:

NaCl, 1.4M – 800 grams (Aldrich)

Na₂HPO₄.0.08M – 92 grams (Sigma)

KH₂PO₄, 0.01M – 20 grams (Merck)

KCL, 0.03M – 20 grams (Riedel de Hahn)

*The 20X PBS ingredients were dissolved in 5 litres of distilled water and the final pH was 7.

Materials for ELISA protocol

ELISA wash buffer (1X PBS and 0.05% Tween).

20X PBS – 500-ml

Distilled water – 9.5-L

Tween – 5-ml (Sigma)

Phosphate citrate buffer:

Citric acid** - 10.19 grams (Sigma)

Na₂HPO₄.12H₂O – 36.96 grams (Sigma)

*Phosphate citrate buffer was stored at 4C.

**Anhydrous Citric Acid.

ELISA Developing solution:

OPD Tablet – 1 (Sigma)

Phosphate Citrate Buffer – 25-ml

Hydrogen Peroxide – 15-ul (Sigma).

ELISA Stop Solution:

1M H₂SO₄ – 25-ul (Fluka).

Materials for transfection protocol

2X HBS (500-ml):

HEPES, pH 7.05 (50mM final concentration) – 5.0 grams

KCL (10mM final concentration) – 0.37 grams

Dextrose (12mM final concentration) – 1.0 grams

NaCl (280mM final concentration) – 8.0 grams

Na₂HPO₄-7H₂O (1.5mM final concentration) – 0.2 grams

*pH of final solution is 7.05, bring final volume to 500ml and filter sterilize using a 0.22um filter.

2.5M CaCl₂ (100-ml):

CaCl₂-2H₂O (2.5M final concentration) – 36.75 grams

*Final volume of 100-ml, filter sterilize using a 0.22um filter.

FCS supplemented DMEM Culture medium:

DMEM – 500-ml

FCS (low-IgG) – 50-ml

Glutamine – 5-ml

Antibiotic/mycotic – 5-ml

Cholorquine (100mM):

C₁₈H₂₆ClN₃.2H₃PO₄ (Mw: 515.9) – 5,159 grams

Endotoxin free water – 100ml

Materials for MACS® Buffers:

0.5M EDTA:

EDTA – 20.8 grams
PBS – 100-mls

*The solution is filter sterilised and stored at 4C

MACS® Rinsing/Washing buffer Main Solution:

0.5M EDTA – 2-mls
Sterile PBS – 500-mls

*MACS® Rinsing/Washing bugger had a final pH of 7.3

MACS Running biffer® :

Fecal Calf Serum – 2.5-mls
Sterile PBS – 500mls
0.5M EDTA – 2-mls

*MACS® buffer had a final pH of 7.2

Materials for SDS-PAGE and Western blotting

1.5M Tris-HCL

Tris Base – 18.15 grams
Distilled water – 75-mls

*Solution has a final pH of 8.8 and a final volume of 100-mls

0.5M Tris-HCL pH 6.8

Tris Base – 6 grams
Distilled water – 60-mls

*Solution has a final pH of 6.8 and a final volume of 100-mls

5X Running Buffer

Tris Base – 15 grams
Glycine – 72 grams
SDS – 5 grams

*Solution has a final pH of 8.3 and a final volume of 1000-mls

Transfer Buffer

Tris Base – 0.19 grams
Glycine – 4.32 grams
Distilled water – 240-mls
MeOH – 60-mls
SDS – 0.15 grams

*Solution has a final pH of 8.3

SDS-PAGE Sample Buffer

62.4mM Tris
2% w/v SDS
10% Glycine
0.1% Bromophenol Blue
50mM DTT

Table 2.1: Stacking and Resolving Gels

	Stacking Gel 5%	Resolving Gel 15%
Distilled water	2.3-mls	1.4-mls
30% Bis-acrylamide Mix	5.0-mls	330ul
1M Tris pH 6.8	2.5-mls	
1.5M Tris pH 8.8		250ul
10% Ammonium Persulphate	100ul	20ul
10% SDS	100ul	20ul
TEMED	4ul	2ul

Table 2.2: Antibodies for Flow Cytometry

Antibody	Source	Dilution
CD3 – FITC	Biolegend	1/200
CD45 – FITC	Biolegend	1/200
Mouse IgG1, kappa- FITC	Biolegend	1/200
CD103 – FITC	Biolegend	1/300
CD8 – FITC	Biolegend	1/200
CD8 – PerCP	Biolegend	1/200
CD19 – PerCP	Biolegend	1/200
CD45 – PerCP	Biolegend	1/200
Ly6C – FITC	Biolegend	1/400
CD45 – PB	Biolegend	1/150
CD11b – PB	Biolegend	1/150
CD19 – PB	Biolegend	1/150
CD8 – APC	Biolegend	1/200
B220 – APC	Biolegend	1/300
CD3 – A647	BD – Pharmingen	1/200
CD103 – A647	BD – Pharmingen	1/200
Hamster isotype – A647	Biolegend	1/200
CD11c – APC	BD – Pharmingen	1/200
F4/80 – A647	Biolegend	1/200
CD103 – PE	Biolegend	1/200
Alpha4-Beta7- PE	Biolegend	1/400
MHCII – A700	BD – Pharmingen	1/150
CD11C – PeCy7	BD – Pharmingen	1/700
IFN-gamma – PeCy7	BD – Pharmingen	1/200

Table 2.3: Invitrogen Buffers

Buffer	Composition
Resuspension Buffer (R3)	50mM Tris-HCL, pH 8.0 10mM EDTA
RNase A	20mg/ml in Resuspension Buffer
Lysis Buffer (L7)	0.2mM NaOH 1%(w/v) SDS
Precipitation Buffer (N3)	3.1M Potassium acetate, pH 5.5
Equilibration Buffer (EQ1)	0.1M Sodium acetate, pH5.0 0.6M NaCl 0.15% (w/v) Triton X-100

Wash Buffer (W8)	0.1M Sodium acetate, pH 5.0 825mM NaCl
Elution Buffer (E4)	100mM Tris-HCL, pH 8.0 1.25M NaCl
TE Buffer (TE)	10mM Tris-HCL, pH 8.0 0.1mM EDTA