**MECHANISMS OF INDUCTION OF CD8⁺ T CELL
RESPONSES BY DENDRITIC CELLS**

By

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Abstract

Splenic CD8 α ⁺ dendritic cells (DCs) have been described as key antigen presenting cells for the induction of CD8⁺ T cell responses to circulating antigen. This is through a heightened capacity to acquire and present the antigens via the process of cross-presentation, expression of high levels of the co-stimulatory and adhesion molecules required to stimulate CD8⁺ T cells, and the capacity to release high levels of the cytokines required to drive differentiation of CD8⁺ T cells into cytotoxic T lymphocytes (CTLs). However, recent research has indicated that the splenic CD8 α ⁺ DC population is more heterogeneous than originally thought. A previous study from my own laboratory suggested that a population of CD8 α ⁺ DCs that express the c-type lectin langerin primarily possess the heightened functions previously attributed to the total CD8 α ⁺ population. Therefore, the aim of this thesis research was to explore this subset of DCs in more detail, with specific emphasis on gaining mechanistic insight into their ability to elicit CD8⁺ T cell responses to circulating proteins.

In the first section of this thesis, the hypothesis that the splenic langerin⁺ CD8 α ⁺ DCs were the critical subset involved in the induction of strong systemic CD8⁺ T cell responses to circulating antigen was tested in detail. This was examined using a genetically modified mouse model in which langerin-expressing cells could be easily identified and/or specifically depleted. It was first shown that the induction of CD8⁺ T cell responses to the model antigen ovalbumin was dependent on entry into the spleen in the presence of appropriate stimulation, which in these studies was provided by agonists for the toll-like receptors (TLRs) and/or signals from innate-like lymphocytes called natural killer T (NKT) cells. The primary targets for these signals were shown to be splenic langerin⁺ CD8 α ⁺ DCs, as CD8⁺ T cell responses were significantly reduced in hosts depleted of these cells within the spleen. Furthermore, agonists for TLRs that were not expressed by langerin⁺ CD8 α ⁺ DCs failed to enhance T cell responses. The langerin⁺ CD8 α ⁺ DCs were shown to be located in the marginal zone of the spleen, where they could readily screen the blood for antigens, and their function was critical to the induction of CD8⁺ T cell responses within six hours of antigen delivery.

Interestingly, other local langerin-negative antigen presenting cells (APCs) were shown to be capable of cross-presentation, but with significantly reduced capacity to prime CD8⁺ T cell responses. Therefore, in the second section of this thesis the hypothesis that the langerin-negative APCs were capable of contributing to CD8⁺ T cell responses with appropriately timed stimuli was investigated. One of the downstream effects of inducing NKT cell activation at the time of priming was shown to be the “pre-conditioning” of langerin-negative DCs, allowing them to respond strongly to subsequent TLR ligation. Using *SiglecH*-DTR mice, it was shown that plasmacytoid DCs (which are langerin-negative) were pre-conditioned by NKT cell activation, allowing them to respond more actively to the delayed TLR stimulation by producing significantly enhanced levels of IFN- α . This factor was also potentially responsible for “feeding back” to the CD8 α ⁺ DCs (including langerin-expressing CD8 α ⁺ DCs), to enhance their function, as indicated by increases in cytokine production. Significantly, the major langerin-negative DC populations, defined as CD8 α ⁻ DCs, were pre-conditioned to have an enhanced cytokine release response to subsequent stimulation through TLR7, a receptor not expressed by langerin-positive DCs. This enhanced ability to respond to TLR7 ligation permitted these langerin-negative APCs to contribute to increased CD8⁺ T cell accumulation, with enhanced functional activity. Importantly, the CD8⁺ T cell response induced remained significantly dependent on initial cross-priming by langerin⁺ CD8 α ⁺ DCs, and it was only through pre-conditioning that langerin-negative APCs could contribute to enhancing the T cell response.

In the third section of this thesis, the hypothesis that the CD8⁺ T cell responses generated in the presence of langerin⁺ CD8 α ⁺ DCs were phenotypically and functionally distinct from those responses generated in their absence was tested. No obvious differences were seen in CD8⁺ T cell homing, memory phenotype, re-stimulatory capacity, and expression of key molecules involved in metabolic function, survival and cytolytic function. However, *in vivo* cytotoxic function several weeks after priming was comparable, suggesting that this function was not related to initial burst size, providing some evidence of difference in function between CD8⁺ T cells primed in the presence or absence of langerin⁺ CD8 α ⁺ DCs.

In summary, the splenic langerin⁺ CD8α⁺ DCs are the major subset responsible for cross-priming CD8⁺ T cell responses to circulating antigen, and for interpreting multiple stimulatory signals for enhancing the response. However, effective CD8⁺ T cell responses can be generated in their absence, particularly when antigens are provided in the context of appropriately temporally phased stimuli.

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List of Abbreviations

2-ME	2-mercaptoethanol
α GalCer	α -Galactosylceramide
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC	(fluorophore) Allophycocyanin
AutoMACS	Auto magnetic-activated cell sorting
β 2	β 2-microglobulin
BCR	B cell receptor
BDCA3	Blood dendritic cell antigen 3
BSA	Bovine serum albumin
cDC	Conventional dendritic cell
cDNA	Complementary deoxyribonucleic acid
CDP	Common dendritic precursor
CFSE	Carboxyfluorescein succinimidyl ester
Chi3l3	Chitinase 3-like 3
cIMDM	Complete Iscove's Modified Dulbecco's Medium
CLIP	Class II-associated invariant-chain peptide
CTL	Cytotoxic T lymphocyte
CTO	Cell tracker orange
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DCIR2	Dendritic cell inhibitory receptor 2
dH ₂ O	Distilled water
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum

FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage-colony stimulating factor
Grwd1	Glutamate-rich WD repeat containing 1
GSL	Glycosphingolipid
GTPase	Guanosine-5'-triphosphatase
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1
IFN- α	Interferon-alpha
IFN- γ	Interferon-gamma
iGb3	Isoglobotrihexosylceramide
Ii	Invariant chain
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-7R	Interleukin-7 receptor
IL-12	Interleukin-12
IL-12R	Interleukin-12 receptor
IMDM	Iscove's Modified Dulbecco's Medium
i.p.	Intraperitoneal
i.v.	Intravenous
KLRG1	Killer cell lectin-like receptor G1
LN	Lymph node
LPS	Lipopolysaccharide
Lyz1	Lysozyme 1
MDP	Monocyte and dendritic precursor
MHC	Major histocompatibility complex
MIIC	MHC class II compartments
MPL	Monophospholipid A
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NaN ₃	Sodium azide

NaOH	Sodium hydroxide
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NOX2	NADPH oxidase complex 2
ns	Not significant
OVA	Ovalbumin
Pam3Cys	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
pH	Power of hydrogen
PI	Propidium iodide
Poly I:C	Polyinosinic:polycytidylic acid
Pre-DC	Precursor dendritic cell
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SA	Streptavidin
TAE buffer	Tris-acetate EDTA buffer
TAP	Transporter of antigen processing
TCR	T cell receptor
T _{CM}	Central memory T cell
T _{eff}	Effector T cell
T _{EM}	Effector memory T cell
Tgfb β	Transforming growth factor, beta-induced
Th cell	T helper cell
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor-alpha
T _M	Memory T cell
T _{pcm}	Precursor memory T cell
Treg	Regulatory T cell
T _{SCM}	Stem cell memory T cell

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Chapter 1: General Introduction

The immune system is a complex network of immune cells located in many tissues throughout the host, with the purpose of defending the host from attack by invading pathogens and for regulating the host's own cells to prevent dysregulation. Understanding the interplay between the networks of immune cells is important for manipulating immune responses for treatment of diseases.

The immune cells develop in the bone marrow and thymus of mammals and reside within peripheral tissues and the secondary lymphoid tissues of the lymph nodes and spleen^{1, 2}. Innate immune cells have broad antigen-specificity by using pattern-recognition receptors that recognise pathogen-associated molecular patterns from foreign pathogenic material, such as bacterial cell wall components, and respond immediately to the threat by phagocytosing the pathogen or by releasing toxic factors to induce inflammatory responses and kill the pathogen^{3, 4}. In contrast, adaptive immune cells, such as B lymphocytes and CD4⁺ and CD8⁺ T lymphocytes, have randomly generated receptors, and undergo a process of clonal selection after antigen exposure to amplify clones that specifically recognise determinants (or “epitopes”) within specific antigen structures. Thus, upon direct recognition of antigen through the B cell receptor (BCR) on B cells or recognition of peptide antigens presented by antigen presenting cells (APCs) via the major histocompatibility complex (MHC) molecules to the T cell receptor (TCR) on T cells, the adaptive immune cells are activated against that particular antigen, proliferate extensively, and elicit effector functions to fight the disease. After the disease has cleared, memory cells remain that respond with an increased rate of effector function upon re-encounter of antigen⁵. The adaptive immune response is an important process for providing a large, highly specific response that can directly target and eliminate the pathogenic threat, and provide long-term capacity to limit re-infection.

B cells are activated to produce antibodies that can recognise and bind to the pathogen resulting in blocking of the pathogen from infecting host cells⁶. The antibodies can also bind to pathogens to cause opsonisation, which can trigger elimination by phagocytic cells that recognise the constant region of the antibody structure (Fc)⁷. Antibodies that bind cellular structures can trigger direct

cytotoxicity by natural killer (NK) cells, again mediated via recognition of the Fc region. As the TCR structures are randomly generated, it is necessary for T cells to undergo a process of positive selection in the thymus to retain only those cells with capacity to recognise self MHC structures, while cells that recognise peptide epitopes from self-antigens with strong avidity have to be negatively selected⁵. The result is release from the thymus of a large pool of T cells with high specificity for a wide range of antigens, but with limited self-reactivity that could potentially cause autoimmunity.

Activation of T cells requires two signals. The first is presentation of antigen in the form of peptide to the TCR via MHC molecules. The second is the provision of co-stimulatory molecules, CD86/CD80 that bind to CD28 on the T cells, which drives production of the T cell proliferation factor IL-2⁸. With only antigen presentation and no co-stimulation, the T cells undergo limited proliferation, and may become refractory to further stimulation, or even depleted. The adaptive immune system will therefore “tolerate” the antigenic targets of these cells, and any surviving T cells are often described as “tolerised”; no immune response is mounted against the pathogenic source of the antigen⁹. The third signal for the full activation of CD8⁺ T cells is provision of pro-inflammatory cytokines, such as IL-12 and type I IFN, that aid in the activation and differentiation of the T cells^{10; 11}. Upon stimulation with this third signal, the T cells activate and differentiate into effector subsets that possess different functional immune responses depending on the signals received. Of particular interest to this thesis are the CD8⁺ T cells that can be induced to become cytotoxic T lymphocytes through expression of the lytic molecules perforin and granzyme for killing antigen-specific targets¹².

The immune rich spleen is an important site for APCs, which will be discussed in greater detail later in this chapter, to present peptide antigens on MHC molecules to T cells for the induction of strong antigen-specific responses to circulating antigens, such as bacterial pathogens. The spleen is a lymphoid organ that filters blood and is abundant in macrophages within the red pulp. Blood enters the spleen through the splenic artery, which branches into the central arterioles before either releasing blood into the red pulp or the marginal zone of the white pulp nodules.

Some arterioles even connect straight into the venous blood vessels rather than being filtered through the cellular compartment of the spleen. Red pulp tissue macrophages are situated in the red pulp of the spleen to remove deficient red blood cells from the circulation. The release of blood into the marginal zone delivers antigens to marginal zone macrophages, marginal metallophilic macrophages, dendritic cells (DCs) and marginal zone B cells. Within the follicle of the white pulp nodules are the follicular and B-1 B cells, and the T cells are primarily within the periarteriolar lymphoid sheath (often referred to as the T cell zone) that surrounds the central arteriole. Some DCs can also be found within this area. Upon administration of antigen, the marginal zone APCs acquire the antigens and the DCs migrate to the T cell zone to present antigen, either causing tolerance or activation of the T cells depending on the co-stimulatory ability of the DCs^{13; 14; 15; 16; 17}.

The involvement of the spleen in the induction of strong antigen-specific CD8⁺ T cell responses to circulating antigens has been investigated extensively in murine models. A summary of the current knowledge is reviewed in detail in this chapter to identify where further research is required to understand the immune interactions and important cellular networks that drive or enhance CD8⁺ T cell responses.

1.1 The role of antigen presenting cells in adaptive immune responses

1.1.1 Antigen presenting cells

APCs, including macrophages, dendritic cells (DCs) and B cells^{18; 19}, are very important mediators for the adaptive immune system, although there are still many functions and cellular processes that have not been clearly defined.

APCs can be characterised as cells that are able to acquire exogenous antigens from the extracellular environment, and process and present the antigen on major histocompatibility complex class II (MHC class II) molecules to CD4⁺ T cells. This is in comparison to all nucleated cells being capable of presenting endogenously generated antigens on major histocompatibility complex class I molecules to CD8⁺ T cells²⁰. The MHC class II molecule is restricted to APCs,

providing a useful surface marker for identifying APCs during analysis. APCs can also be characterised as cells that, upon activation from interactions with pathogenic molecules on the pattern-recognition receptors, are capable of providing surface-bound and soluble co-stimulatory molecules to prime antigen-specific T cells²¹. As already noted, in the absence of co-stimulation T cells are tolerised against the antigen presented on MHC molecules⁹. In addition to the B7 molecules CD80 and CD86, which bind to CD28 on T cells, to induce IL-2 production^{22; 23; 24}, another molecule involved in co-stimulation is CD40 on APCs, which binds to the CD40L (CD154) on T cells. Binding of the CD40L to CD40 enhances the activatory function of the APCs, including increased CD86/80 expression and increased IL-12 production, for further enhancing T cell responses^{25; 26}. The potent stimulatory signal IL-12p70, consisting of IL-12p40 and IL-12p35 subunits²⁷, is important for activating immune cells and inducing the production of IFN- γ from multiple cell types, including CD8⁺ T cells, natural killer (NK)²⁸ and natural killer T (NKT)²⁹ cells. IL-12p70 also induces the differentiation of naive CD4⁺ T cells into T helper 1 (Th1) cells³⁰, which produce the cytokines IFN- γ and TNF- α for an enhanced inflammatory responses, including increased activation of APCs engaged in pathogen killing; meanwhile IL-12 production inhibits the differentiation of CD4⁺ T cells into IL-4 cytokine expressing T helper 2 cells (Th2) which are thought to limit helminth infections, but can be the cause of allergic responses^{31; 32}.

The ability of APCs to acquire exogenous antigen is an adaptive approach that allows the APCs to “police” the entire host by scanning for foreign proteins and peptides and to induce specific immune responses against the source of the foreign material. The degree to which APCs can induce T cell responses relies in part on the functional capabilities of the APC. B cells are classified as an APC; however, B cells have limited general capacity for endocytosis (apart from uptake of specific antigen via the BCR)³³ and therefore provide lower presentation compared to DCs. Also, while macrophages are highly proficient at endocytosis³⁴, they have minimal MHC class II expression and co-stimulation compared to DCs¹⁹. Therefore DCs can be classified as the most efficient APC subset, with high antigen presentation³⁵ and co-stimulatory capacity^{19; 36}.

1.1.2 Antigen presentation pathways

Antigen presentation is a key characteristic of APCs. However, as well as the classical pathways of presentation, to be described in detail below, there are also the more recently characterised pathways of antigen cross-presentation.

1.1.2.1 Classical antigen presentation pathways

1.1.2.1.1 Presentation of acquired antigens via MHC class II to CD4⁺ T cells

Multiple cellular machinery processes are required for the classical MHC class II presentation of antigens that have been acquired by APCs to stimulate CD4⁺ T cells, as illustrated in Figure 1.1. These processes involve the phagocytosis, pinocytosis or receptor-mediated endocytosis of proteins into the APC, with the endocytic vesicles then fusing with lysosomes. The proteins are then degraded by proteases in lysosomes, creating peptides of ~ 15 to 24 amino acids long³⁷, that can bind to MHC class II molecules. Before peptide-loading occurs, the MHC class II molecules are contained within the endoplasmic reticulum (ER) where they are bound by the invariant chain (Ii) that prevents endogenous peptides binding to the antigen-binding site. Exocytic vesicles containing the MHCII-Ii complexes bud off from the ER, transfer through the golgi and then fuse with vesicles containing the degraded exogenous peptides to form endosome/lysosome-like compartments termed the MHC class II compartments (MIIC). Through a lowered pH of ~5-6, a complement of many cysteine proteases including Cathepsin S^{38; 39; 40}, the invariant chain is degraded leaving low-affinity CLIP (class II-associated invariant-chain peptide) in the peptide binding groove. The H-2M (or HLA-DM in humans) molecule then dissociates CLIP from the class II molecules⁴¹ and CLIP is replaced with the specific peptides that have a high affinity for the binding groove, resulting in peptide-MHC class II complexes. These complexes are then transported to the cell surface where antigen is presented to antigen-specific CD4⁺ T cells⁴².

Figure 1.1. Classical antigen presentation pathways.

Endogenous proteins in the cytosol are degraded by the proteasome into short peptides. The short peptides are transported into the endoplasmic reticulum (ER) by transporter of antigen-processing (TAP) molecules where they are loaded onto major histocompatibility complex class I (MHCI) molecules (each MHCI comprised of three α chains and a β 2-microglobulin molecule). The MHCI-peptide is transported through the golgi body to the cell surface where the peptide is presented to the T cell receptor (TCR) of CD8⁺ T cells. Exogenous protein is endocytosed and then degraded into long peptides in the lysosome. Meanwhile MHCII molecules bound by the inactivating Invariant chain (Ii) molecule are transported from the ER through the golgi body into the MHC class II compartment (MIIC) where they merge with the lysosomes containing the long exogenous peptides. Proteases degrade the Ii to low affinity class II-associated invariant-chain peptide (CLIP), H2-M molecules dissociate CLIP from the peptide-binding groove to be replaced with long peptides. The MHCII-peptide complexes are transported to the cell surface where the peptide is presented to CD4⁺ T cells.

1.1.2.1.2 Presentation of endogenous antigens via MHC class I to CD8⁺ T cells

There is also a classical presentation pathway where peptide fragments from proteins manufactured within the cell (endogenous antigens) are presented on MHC class I molecules, as illustrated in Figure 1.1. Endogenous proteins within cells include proteins from viruses and bacteria that may have infected the cells^{43; 44}; therefore it is crucial that all cells possess the ability to process and present endogenous proteins for presentation on MHC class I molecules, to allow effective immune recognition by CD8⁺ T cells. This antigen presentation pathway involves endogenous proteins in the cytosol undergoing degradation to short peptides by the proteasome. These peptides are then transported into the lumen of the ER by transporter of antigen-processing (TAP) molecules where the short peptides, ~8-10 amino acids³⁷ long, can then bind to peptide-specific MHC class I molecules within the ER. Exocytic vesicles containing the peptide-MHC class I complexes bud off from the ER and travel through the golgi to the cell surface, where they can present the peptide antigen to CD8⁺ T cells with the antigen-specific T cell receptor (TCR)^{42; 45}.

1.1.2.2 *Antigen cross-presentation pathways*

In 1976, Bevan had noted that a cytotoxic CD8⁺ T response could be induced against antigens introduced exogenously from adoptively transferred allogeneic cells^{46; 47}, a process that could not be explained by the classical pathway above where only endogenous antigens could be presented on MHC class I molecules to CD8⁺ T cells. It was hypothesised that antigens could be “cross-presented”, which would involve the uptake of exogenous antigens into APCs followed by cross-over into the MHC class I presentation pathway. In this way, the exogenous antigens could be presented on MHC class I molecules to CD8⁺ T cells. Antigen cross-presentation is a desired process for responding to intracellular infections that do not specifically infect APCs. It is also required in vaccination strategies using exogenous antigens to induce cytotoxic CD8⁺ T cell responses to intracellular bacteria or viral infections, or diseases such as cancer³⁷.

There are several theories about how antigen cross-presentation occurs. The first major theory is the phagosome-to-cytosol pathway, as illustrated in Figure 1.2. This involves escape of exogenous antigens from phagosomes into the cytosol where they are degraded further by the proteasome and, therefore, processed similarly to endogenous antigens that are presented on MHC class I molecules. This was shown experimentally by inhibition of the proteasome and/or mutations in the TAP molecules, which prevented the presentation of peptides from exogenous protein to CD8⁺ T cells⁴⁸. The failure to detect cross-presentation in TAP^{-/-} mouse models clearly indicates a dependence on transfer from the cytosol to the ER for cross-priming of CD8⁺ T cells to administered exogenous antigen⁴⁹. This pathway of cross-presentation allows the identification of cross-presenting cells by administration of cytochrome c. The presence of cytochrome c in the cytosol would usually indicate mitochondrial damage and induce apoptosis of the cell⁵⁰. Therefore, when cross-presenting cells that cross-present via the phagosome-to-cytosol pathway take up exogenously administered cytochrome c, they undergo apoptosis when the cytochrome c enters the cytosol^{51; 52}.

However, while some forms of exogenous antigen are highly dependent on TAP and the proteasome, there are also exogenous antigens that do not rely on TAP. For example, iron oxide beads containing chicken ovalbumin protein (OVA) are heavily dependent on TAP, whereas poly-lactide poly-glycolide microspheres containing OVA can still be cross-presented in a TAP-deficient model⁵³. This led to the other main cross-presentation theory, which is the vacuolar pathway, as illustrated in Figure 1.2. Here, exogenous proteins are degraded in the phagolysosome where they encounter MHC class I molecules from vesicles, either being recycled from the cell surface or being transported from the ER⁴⁵. Shen et al⁵³ demonstrated that TAP-independent cross-presentation relies heavily on cysteine proteases to process the exogenous proteins. They further identified Cathepsin S, a cysteine protease found in vacuoles, to have a crucial role in TAP- and proteasome-independent cross-presentation for processing exogenous proteins in vacuoles.

Figure 1.2. Cross-presentation pathways

The phagosome-to-cytosol pathway involves endocytosed exogenous proteins escaping the phagosome into the cytosol. They are then processed as in Figure 1.1, where the proteasome degrades the molecules into short peptides which are transported by transporter of antigen-processing (TAP) into the endoplasmic reticulum (ER). The peptides are loaded onto major histocompatibility complex class I (MHC I) molecules and transported through the golgi body to the cell surface for presentation to the T cell receptor (TCR) of CD8⁺ T cells. The vacuolar pathway involves the phagosome (containing endocytosed exogenous protein) merging with the lysosome and vacuoles containing MHC I molecules (either from the ER or recycled from the cell surface). The proteins are degraded into short peptides by proteases and loaded onto MHC I for transport to the cell surface and presentation to CD8⁺ T cells.

The pathway of antigen presentation that occurs after acquisition of exogenous antigen depends on the characteristics of the APC subset that obtains and processes the antigen. For example, the inhibition of endosome acidification improves cross-presentation of soluble antigens including Hepatitis C virus protein and OVA^{54; 55}. This is also seen with the cross-presentation of different forms of OVA administration including alum-OVA and cell-associated OVA⁵⁵. This is due to restricted degradation of antigens by acidic proteases in phagolysosomes. As different DC populations possess varying levels of acidity in their phagosomes⁵⁶, this provides an excellent example of why certain DC subsets would have superior antigen cross-presentation ability.

1.1.3 Dendritic cells

As mentioned above, DCs are superior APCs due to their high antigen presentation and co-stimulatory capacities. However, even within the DC population there is high heterogeneity (Figure 1.3), with different subsets possessing higher capacity for different functions. Within the mouse, the DC populations have been widely studied and characterised.

DCs originate from a monocyte and dendritic precursor in the bone marrow, which then diverges into either the common DC precursor or monocytes^{57; 58}. The monocytes can then be differentiated into monocyte-derived inflammatory DCs by stimulation with IL-4 and GM-CSF⁵⁹. The generation of these cells from bone marrow *in vitro* has been used for many dendritic cell studies^{60; 61; 62}. Meanwhile, the common dendritic precursor can be differentiated into either the plasmacytoid DC (pDC) or conventional DC (cDC)^{57; 58}. The pDCs are identified as SiglecH⁺ B220⁺ CD11c^{low}⁶³. This subset has some antigen cross-presentation ability in humans⁶⁴ and in mice⁶³, although it remains controversial. However, while pDCs are somewhat capable of these other functions, such as cross-presentation, the most identifiable function of this subset is their high responsiveness to nucleic acid molecules, resulting in large type I IFN production by these cells^{65; 66}.

Figure 1.3. Dendritic cell populations and differentiation.

An original monocyte and dendritic precursor (MDP) differentiates into either the common dendritic precursor (CDP) or monocytes, which can further be differentiated into the monocyte-derived inflammatory DC. The CDP can then differentiate into the plasmacytoid DC (pDC) population or the pre-DC. The pre-DC differentiates into the migratory non-lymphoid tissue DC populations, including the epidermal Langerhans cells and the dermal CD103⁺ and CD11b⁺ populations. The pre-DC also differentiates into the lymphoid tissue DC populations, which include the CD4⁺, CD4⁺CD8 α ⁻, CD8 α ⁺ subsets. Within the CD8 α ⁺ population there are langerin⁺, CX₃CR1⁺ and langerin⁻ CX₃CR1⁻ subsets.

The cDCs can further be differentiated into the migratory and lymphoid tissue resident DCs based on their location and movement. Migratory DCs are located in the periphery and transport antigens from the periphery to lymphoid tissues⁶⁷. A well-studied migratory DC is the Langerhans cell in the epidermis. This subset is also involved in carrying antigen to the draining lymph nodes⁶⁸, albeit at a slower migration rate than the dermal DCs⁶⁸, where it is able to induce CD8⁺ T cell responses⁶⁹. There are several migratory DCs in the dermis, including the CD103⁻ langerin⁻ CD11b⁺ DCs, CD103⁻ langerin⁻ CD11b⁻ DCs, CD103⁺ langerin⁺ CD11b^{low} DCs and CD103⁻ langerin⁺ CD11b^{low} DCs⁷⁰. However, most researchers separate the murine dermal DCs into CD103⁺ vs CD11b⁺ DCs for functional characterisation. For example, the CD103⁺ dermal DCs in the skin have been

identified as the primary subset for uptake and transport of herpes simplex virus antigens to cutaneous lymph nodes for cross-presentation to CD8⁺ T cells, while both the CD103⁺ and CD11b⁺ DCs are capable of CD4⁺ T cell induction⁷¹. This has also been shown in lungs, where the CD103⁺ DCs in the lung are the superior subset for uptake of flu antigen from virally infected cells, transport of flu antigen to the mediastinal lymph node (lung draining lymph node), and induction of virus-specific CD8⁺ T cells⁷². However, there is some controversy with this theory, as other research has indicated the superiority of the CD11b⁺ DCs in taking up flu antigens in the lung and migrating to the mediastinal lymph node to cross-present antigens to CD8⁺ T cells⁷³.

In comparison to the migratory DCs, lymphoid tissue resident DCs are already situated within lymphoid tissues¹ to filter blood and lymph fluids for circulating antigens. There has been more research into these cells, with distinct functions of the different subpopulations a lot more defined, particularly in murine spleen. The resident splenic DC populations can be separated into the CD8 α ⁺ DCs, CD4⁺ DCs, or CD8 α ⁻CD4⁻ DCs^{74; 75; 76}. While most conventional DCs are dependent on the Irf8 transcription factor, the CD8 α ⁺ DCs are also developmentally dependent on the Batf3 transcription factor. In contrast, the CD4⁺ DCs are dependent on the Irf4 and Irf2 transcription factors^{77; 78; 79; 80}. The populations show markedly different gene expression profiles via microarray analysis, including the genes for the c-type lectins CD205 and CD207 (langerin) and Clec9a, receptors involved in antigen uptake and presentation such as Trem14, and intracellular molecules involved in cross-presentation machinery such as Sec61B and Tap molecules, showing upregulation in CD8 α ⁺ DCs in spleen and other lymphoid tissues^{81; 82}. In comparison, the CD4⁺ DCs show preferential gene expression of molecules such as the c-type lectins Clec4a4 (DCIR2) and Clec4a2 (DCIR1), and H2-DMb2 and Lamp1 involved in antigen presentation via MHC class II^{82; 83; 84}. There is accumulating evidence to suggest that CD4⁺ and CD4⁻CD8 α ⁻ DCs preferentially present exogenous antigens via MHC class II molecules to CD4⁺ T cells, while CD8 α ⁺ DCs have a superior capacity to cross-present exogenous antigens via MHC class I molecules to cross-prime CD8⁺ T cells^{49; 52; 85}.

This superior cross-priming is in part due to the high capacity of the CD8 α^+ DCs to endocytose dead/dying cells, allowing them to acquire more antigens for cross-priming^{15; 86}. However, this is not the only mechanism, as even when all of the DC subsets were shown to successfully take up similar amounts of soluble OVA, the CD8 α^+ DCs would preferentially cross-present to CD8 $^+$ T cells while the CD8 α^- DCs would preferentially present to CD4 $^+$ T cells⁸⁵. This could also be due to the CD8 α^+ DCs possessing increased machinery for MHC class I presentation, as mentioned above⁸². CD8 α^+ DCs also have a higher pH in the phagosome and endosome compartments, which, as indicated in 1.1.2.2 above, results in higher levels of cross-presentation due to the differential processing of exogenous proteins in phagolysosomes to peptides that can be further processed for presentation via MHC class I molecules. The alkalisation of the phagosomes and endosomes in CD8 α^+ DCs is due to the expression of the small GTPase Rac2 causing the development of NADPH oxidase complex 2 (NOX2) in phagosomes, resulting in production of reactive oxygen species in the phagosomes, which increases the pH. In contrast, the CD8 α^- DCs express Rac1, which causes the development of NOX2 at the plasma membrane, so the phagosomes are maintained at a lower pH level⁵⁶. CD8 α^+ DCs have also been observed to contain a high amount of the protease inhibitor cystatin c. Cystatin c is an inhibitor of cathepsin S, the protease involved in processing of the invariant chain for peptide loading onto MHC class II molecules. Theoretically, the increased inhibitor of increased MHC class II peptide loading would provide another explanation for why the CD8 α^+ DCs preferentially present antigen via MHC class I than class II, however this has not been observed by experimental data so far⁸⁷.

The CD8 α^+ DCs have also been characterised as superior co-stimulators for T cell priming, with increased IL-12⁸⁸, CD86 and CD40 expression⁷⁵, contributing to their proficient role as “cross-primers” of CD8 $^+$ T cell responses to exogenous antigen. In this context, CD8 α^+ DCs have been shown to produce chemokines, such as CCL17⁸⁹, to attract CD8 $^+$ T cells to increase MHC class I-peptide interactions with the TCR. To add to this, they are also exclusively XCR1 $^+$, which binds with XCL1 secreted from CD8 $^+$ T cells to stabilise the interaction for increased T cell activation^{90; 91}. The location of the CD8 α^+ DCs within the spleen is critical, in that

they have been shown to both localise within the marginal zone with the marginal metallophilic and marginal zone macrophages, as well as in the T cell area^{16; 92}. This indicates that they are important mediators between the different populations of immune cells within the spleen and may be critical for supporting immunological network interactions, which is a major topic investigated within this thesis.

It is now clear that the CD8 α^+ DCs in murine spleen can be subdivided further, with a large proportion expressing langerin^{93; 94}, a marker initially associated with Langerhans cells, but also seen on other DCs, including the dermal DCs described earlier. The c-type lectin langerin (also known as CD207) was first identified as being involved in internalisation of cell membrane components for the formation of birbeck granules in epidermal Langerhans cells^{95; 96; 97; 98}. A convenient model for identifying langerin⁺ CD8 α^+ DCs in the spleen is the *lang-EGFP* mice, developed by Kissenpfennig et al⁶⁸ for easier analysis of the dermal and epidermal langerin⁺ DCs, which provides the bright expression of enhanced green fluorescent protein (EGFP) in all langerin-expressing cells. While splenic langerin⁺ CD8 α^+ DCs have an immune tolerising maintenance function in steady-state conditions¹⁶, upon activation they have been shown as critical for cross-priming of antigen-specific CD8⁺ T cell responses due to high capacity for cross-presentation and expressing high levels of co-stimulatory molecules⁵². In the spleen these DCs are situated within the marginal zone, allowing them to have prime access to antigens being filtered through the spleen. They have been shown to express higher levels of CD40 and CD86 than their langerin-negative counterparts, and also produce more IL-12 and have a superior ability to endocytose dead and dying cells^{16; 52; 92}. In fact, many of the attributes described above in relation to the CD8 α^+ DC population as a whole can be attributed to the langerin⁺ subpopulation; this is a major theme investigated in this thesis. How these cells can induce such strong CD8⁺ T cell responses to systemic circulating antigens is of great interest, as they are likely to be key mediators of CD8⁺ T cell responses to systemic pathogen-derived antigens, and can perhaps be exploited in intravenous vaccine applications⁵²;

Adding to the heterogeneity within the CD8 α^+ DC population, there are also CD8 α^+ DCs that express CX₃CR1, which have very different phenotypical and functional attributes to the typical CD8 α^+ DC characterisation¹⁰⁰. In comparison to the traditional CD8 α^+ DCs, these CX₃CR1⁺ DCs do not produce high IL-12, do not cross-present antigen and are developmentally dependent on the transcription factor E2-2 instead of the transcription factor Batf3 that the other CD8 α^+ DCs are dependent on for their development¹⁰⁰. While researchers have analysed CX₃CR1-expressing CD8 α^+ DCs versus their CX₃CR1-negative counterparts¹⁰⁰, and langerin-expressing CD8 α^+ DCs versus their langerin-negative counterparts⁵², the relationships between these different cells has not been clearly defined. For example, whether the langerin-negative population overlaps entirely with the CX₃CR1 population has not been described. Some of these questions will be addressed in this thesis.

1.2 The role of dendritic cells in CD8⁺ T cell responses

1.2.1 Induction of CD8⁺ T cell responses

The induction of CD8⁺ T cell responses is desirable in responses to intracellular infections, and may be exploited in vaccines for disease therapy, including tumour immunotherapy. The main effector function desired in these responses (although not the only one) is cytotoxic killing function of activated CD8⁺ T cells, which can serve to eliminate infected cells, or neoplastic tissue³⁷. The CD8⁺ T cells are activated by APCs through presentation of MHC class I-peptide complexes to the TCR (described in detail in section 1.1.2), through co-stimulatory molecule interactions such as CD80/86-CD28^{23; 101}, and a so-called “third signal” mediated by IL-12 binding to the IL-12R¹². This results in activated CD8⁺ T cells that are capable of increased IFN- γ production¹⁰². These cells can in turn activate other immune cells, for example macrophage activation for protection against bacterial infection¹⁰³. The CD8⁺ T cells become cytotoxic T lymphocytes (CTLs) with increased expression of perforin and granzymes¹² that serve to degrade multiple cellular components within targeted tissue. For example, they target the lamins that maintain the integrity of the nuclear membrane¹⁰⁴, they cause DNA fragmentation¹⁰⁵ and they directly induce the caspase pathways for apoptosis^{106; 107; 108}. The CTLs are

also capable of inducing apoptosis in target cells via the Fas/FasL pathway of caspase induction^{109; 110}. Further to this, IFN- γ has been shown to have a role in up-regulating Fas on target cells, which helps to enhance Fas-mediated killing by CTLs¹¹¹. Research to understand the immune cell involvement and immunological processes required to induce potent cytotoxic T cell responses is very relevant for the study of bacterial infections⁴⁴ and viral infections¹¹², and may also contribute to therapies for diseases such as cancer^{37; 113}. Because of the high professional APC functions of DCs, different methods are being investigated for specifically targeting DCs by vaccination in order to induce strong antigen-specific CTL responses, which are briefly reviewed below²⁴.

1.2.2 *Vaccination strategies*

Using the host's own immune system to induce responses against disease is appealing both in a prophylactic sense to prevent infection, and also as a therapy to treat existing conditions such as cancer. It is particularly appealing in the cancer setting due to the high specificity of the adaptive immune system, together with long-term memory, and is not generally associated with the toxic side effects observed with other cancer treatments, such as chemotherapy and radiotherapy¹¹⁴. There are several methods for using the host immune system for therapeutic treatment, including activating antigen-specific T cells *ex vivo* for adoptive T cell therapy¹¹⁵, injecting antigens with immunostimulatory adjuvants to activate local APCs¹¹⁶, directly injecting stimuli into tumour sites to activate DCs and T cells within the tumour¹¹⁷ and activating and loading antigens onto DCs *ex vivo* for reintroduction into hosts to activate T cells *in vivo*¹¹⁸. Because DCs are highly professional APCs that can be manipulated to provide high co-stimulation as well as present antigens efficiently to induce specific strong antigen-specific T cell responses, the use of DC vaccines has been researched intensely for immunotherapy^{118; 119}. Successful DC immunotherapy was observed in a very small study of human patients with B cell lymphoma, where immunotherapy treatments resulted in tumour regression^{120; 121}. Further work has described the T cell responses elicited *in vivo* in humans with the use of DC vaccine models^{122; 123}. Most clinical studies have involved collection of a patient's peripheral blood mononuclear cells (PBMCs) from the blood to generate monocyte-derived DCs, which are then pulsed

with known antigens prior to injecting them back into the patient. Although there has been promising early phase data, with evidence of increased anti-tumour responses and improved clinical outcome, this has yet to be reproducibly seen at phase III level, with the exception of Provenge, a therapy for hormone-refractory prostate cancer that was approved by the FDA in 2010^{124; 125; 126}.

The process for maturing and activating DCs in culture to provide optimal DCs for vaccination has been thoroughly studied to improve DC yield viability, maturation/activation status and function^{127; 128; 129}; and those methods applied with varying degrees of success in various cancers, such as melanoma¹³⁰, prostate cancer^{131; 132} and multiple brain tumours¹³³. However, further study in mice using this vaccination method demonstrated that, instead of the injected DCs being required for the direct activation of CD8⁺ T cells, a significant component of the response involves host APCs that acquire antigens^{99; 134}. For example, Petersen *et al*⁹⁹ demonstrated that the administration of antigen-loaded activated allogeneic DCs, that would be unable to directly stimulate T cells inside of the host due to incompatible MHC expression, was capable of inducing strong CD8⁺ T cell responses. This meant that host DCs were acquiring the antigen and the injected DCs were acting as couriers of the antigen. Further to this, when they used a mouse depletion model to deplete the langerin⁺ DCs within the mice, they saw a significant decrease in the CD8⁺ T cell response. This indicated that it was the host langerin⁺ DCs that were important for acquiring the antigen and inducing the antigen-specific CD8⁺ T cell responses to the systemic circulating antigen. This coincided with research showing the superior uptake of dead/dying cells by langerin⁺ CD8α⁺ DCs within the marginal zone of the spleen¹⁶.

Due to the superior cross-priming function of the CD8α⁺ DCs, there has been a lot of recent research into ways of specifically targeting antigens to these cells. For example, by conjugating antigens to DEC205-, Clec9A- or langerin-targeting antibodies, it has been possible to target antigens directly into cross-presenting cells, with evident increases in T cell responses^{135; 136}. Although, whether specific targeting is the best method for activating effective immune responses and whether the delivery of the antigen is specific and safe enough is still being

debated¹³⁷. There remains a need to fully define an appropriate molecular target to provide optimal antigen-specific CD8⁺ T cell responses, and the best method of antigen delivery (for example antibody-mediated or packaged such as in exosomes) has yet to be determined. In this context, it is possible that negative side effects result from off-target delivery.

In the absence of specific targeting of antigens to DCs, the evidence provided above suggests that inducing CD8⁺ T cell responses to systemically administered antigens (either soluble or cell-associated) will be critically dependent on langerin⁺ CD8 α ⁺ DCs^{52; 99}. Vaccination strategies that exploit this route of administration would therefore potentially benefit from targeting these cells. However, how the different DC populations co-ordinate with one another to mediate these responses, if indeed they do, is a topic of this thesis.

1.2.3 Long-term memory CD8⁺ T cell responses

The induction of large, highly functional effector CD8⁺ T cell responses can limit infection and be greatly beneficial for treatment against active disease as described above. However, memory responses can also be beneficial for providing further protection from recurring disease^{138; 139; 140}. While effector T cells are characterised as short-lived T cells that have large effector responses (described in 1.2.1) to primary immune responses, memory T cells are characterised as slowly replicating T cells with long-term survival that are able to proliferate rapidly upon re-encountering antigen^{141; 142}. Memory T cells can be separated into two main categories, which are effector memory T cells (T_{EM}) and central memory T cells (T_{CM})¹⁴³. T_{EM} cells possess homing receptors for inflamed tissues and reside within the periphery where they have immediate effector function upon re-encountering antigen. In comparison, T_{CM} cells reside within lymphoid organs and are slower to be re-stimulated by re-encountered antigen, but they have more proliferative potential^{143; 144}.

There have been different theories about how memory CD8⁺ T cells develop¹⁴⁵. Memory T cells were originally thought to develop from effector T cells that survived the contraction phase of the primary CD8⁺ T cell response¹⁴⁴.

However, more recent research has shown that upon T cell activation, there is an early differentiation of the replicating cells that generates the short-lived effector cells (KLRG1⁺CD127⁻) and a separate population of precursor memory cells (KLRG1⁻CD127⁺)¹⁴⁶. It is the precursor memory cells that then survive after the terminally differentiated effector cells have undergone apoptosis, and the precursor memory cells then become either T_{CM} or T_{EM} cells that remain within the host, prepared to become activated once more upon re-stimulation^{147; 148}. There is also more recent research to indicate that upon initial interactions with antigen, there is also another population of memory T cells produced, called stem cell memory T cells (T_{SCM}). These cells are very long-lived, self-renewing and they are theorised to be multipotent, being the precursors of both T_{EM} and T_{CM}^{149; 150}. Ongoing research is being conducted to identify more markers related to phenotype. For example, enhanced expression of the T-bet transcription factor has been shown to correlate with increased short-lived effector cell differentiation¹⁵¹.

Research by both Stemberger et al¹⁵² and Gerlach et al¹⁵³ demonstrated that an individual T cell has the potential to develop into an effector or memory cell, indicating that it is not because of variations between T cells, such as TCR avidity, that causes different populations; rather, the naïve T cell itself has the potential to acquire either phenotype depending on the signals provided at the time of activation. Attempts are now being made to identify mechanisms by which a naïve T cell differentiates into an effector cell or a precursor memory cell through the use of different modes of T cell stimulation. For example, while both high and low antigen doses result in effector T cell development, higher antigen doses provide longer and more stable DC-T cell interactions that induce an early change in the transcriptional profile of the cells, which results in increased memory T cells¹⁵⁴. Research to improve the memory T cell response has also found that fatty acid metabolism is very important in the generation of memory T cells, as dysfunctional fatty acid metabolism in CD8⁺ T cells results in induction of effector T cells, but not memory¹⁵⁵. This is because effector T cells rely on glycolytic metabolism, whereas memory T cells (and naïve T cells) rely on fatty acid oxidation¹⁵⁶. Inhibiting the intracellular kinase mammalian target of rapamycin (mTOR) with the immunosuppressive drug rapamycin has also been shown to increase precursor

memory T cell generation¹⁵⁷. This is because mTOR is involved in the glycolytic pathway and, therefore, blocking the enhancement of glycolysis by mTOR results in improved memory T cell generation as the T cells are forced to use fatty acid metabolism¹⁵⁸. Further to this, T cell expression of the chemokine receptor CXCR3 has been shown to have a role in the induction of short-lived effector T cells, due to the accumulation of T cells at the site of antigen and inflammation via CXCR3-mediated attraction. This causes a stronger stimulation of CD8⁺ T cells, resulting in terminal differentiation into the short-lived effector cells¹⁵⁹. Inflammatory cytokines, such as IL-12 and type I IFN, have also been shown to increase the effector T cell differentiation^{151; 160}. Therefore, the high inflammatory profile generated by the langerin⁺ CD8 α ⁺ DCs warrants further investigation in the context of memory/effector profile of CD8⁺ T cell responses they stimulate; this is another theme of this thesis.

1.3 Enhancing dendritic cell-induced CD8⁺ T cell responses

1.3.1 *Enhancing APC activation with TLR agonists*

Without appropriate activation signals, APCs are unable to efficiently activate CD8⁺ T cell responses, and may actually cause tolerisation of antigen-specific T cells¹⁶. In addition, these DCs may also induce immune-suppressing CD4⁺ T cells called T regulatory cells (Tregs) that inhibit CD8⁺ T cell priming by a number of mechanisms, including the sequestering of CD80/86 molecules via their binding to CTLA-4 on the Tregs¹⁶¹. The higher the activation status of the APCs, the more co-stimulatory molecules are expressed for increased priming of CD8⁺ T cells⁵². In viral or bacterial infections, the infectious agents include pathogen associated molecular patterns that are recognised via pattern-recognition receptors, such as the toll-like receptors (TLRs), resulting in activation of APCs¹⁶². Toll receptors were originally found in drosophila fruitfly with an involvement in development. The TLRs, conserved across multiple species, were identified to be similar to these toll receptors with a functional importance in host defence by recognition of pathogenic molecular patterns^{163; 164}.

Known functional TLRs include TLRs 1-9 in mice, which can be detected on different immune cells at varying levels¹⁶⁵. These TLRs can be stimulated using agonists that have molecular features common to infectious agents. For example, double-stranded RNA, which is a feature of many viruses during their replication¹⁶⁶, is recognised by TLR3. This interaction can be modelled using the synthetic compound polyinosinic:polycytidylic acid (Poly I:C)¹⁶⁷. Single-stranded RNA, which is also a feature of viruses, is recognised by TLR7 and 8. TLR7/8 activation can be modelled by using the synthetic agonist resiquimod¹⁶⁸. The unmethylated bacterial DNA typical of prokaryotes is recognised by TLR9 and can be modelled using synthetic unmethylated CpG¹⁶⁹. The flagellin on motile bacteria is recognised by TLR5¹⁷⁰. Peptidoglycans and lipopeptides, components from gram-positive bacteria, are recognised by TLR2 dimerised with either TLR6 or TLR1, respectively¹⁷¹. An effective and commonly used synthetic lipopeptide for stimulating TLR2 is N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (Pam3Cys)¹⁷¹. Another bacterial wall component, which is recognised by TLR4 is bacterial lipopolysaccharide (LPS) from gram-negative bacteria¹⁷². The TLRs have been identified to respond to endogenous ligands derived within the host as well. Examples of these endogenous ligands are “danger” signals released by stressed cells, including high mobility group box 1 (HMGB1) protein and heat shock proteins recognised by TLR2 and TLR4, and messenger RNA that is recognised by TLR3, as reviewed by Tsan et al¹⁷³.

Except for the intracellular endosomally located TLR7/8, TLR9 and TLR3, the majority of the TLRs are located on the cellular surface¹⁷⁴. The TLRs signal via common intracellular signalling molecules, including the most commonly shared adaptor proteins MyD88 and TRIF, and the signalling molecules of the IRAK family and IRF family^{175; 176}. The resultant signals from TLR stimulation activate transcription factors, such as NF- κ B, for the up-regulation of cytokines and surface co-stimulatory molecules¹⁶⁷. For example, DCs are activated by TLR3 stimulation to produce cytokines such as IL-12 and type I IFNs, and TLR stimulation up-regulates the expression of co-stimulatory molecules such as CD80, CD86 and CD40^{177; 178}.

Because of the strong activation signals provided by TLR stimulation, TLR agonists are being used in multiple research methods as adjuvants for enhancing T cell priming. For example, injection of TLR agonists directly into tumours in mice has shown an improvement of anti-tumour responses for disease regression¹¹⁷. The stimulation of DC with multiple TLR agonists for improved activation has also been investigated for improving DC vaccine strategies⁶⁰. They have been used in clinical trials for a variety of diseases ranging from warts to cancer as a single agent as well as in combination with therapies such as DC-based immunotherapy^{179; 180}. TLRs are expressed with different degrees on various cell types^{165; 181; 182}. This variation is also observed between CD8 α^+ and CD8 α^- splenic DCs, where CD8 α^+ DCs are TLR3⁺ TLR7⁻ and CD8 α^- DCs are TLR7⁺ TLR3⁻¹⁸³. Understanding which DCs receive the most benefit from TLR-induced activation, and how best to activate those DCs, would significantly improve advances in treatments for inducing strong CD8⁺ T cell responses for immune therapy.

1.3.2 CD4⁺ T cell help

The term “T cell help” was initially used to describe the molecular interactions provided by antigen-specific CD4⁺ T cells to B cells to help antibody production, with CD40 signalling a key component¹⁸⁴. A similar role has been shown to be important in the initiation of CD8⁺ T cell responses, with CD4⁺ T cells “licensing” DCs to stimulate more effective CD8⁺ T cell responses, again with CD40 signalling playing a key role^{185; 186}.

The presentation of MHC class II-peptide complexes to CD4⁺ T cells combined with co-stimulation, for example CD40-CD40L and CD80/86-CD28 binding, induces CD4⁺ T cell activation to produce T helper (Th) cells. There are several different types of Th responses that can be induced, including the allergy and parasite infection-related Th2 cells^{187; 188}, and autoimmune-related Th17 cells¹⁸⁹. However, another T helper response, the pro-inflammatory Th1 cell response, is significantly involved in aiding inflammatory responses for increased CD8⁺ T cell priming by DCs^{190; 191}.

These Th1 cells differentiate in response to IL-12 production¹⁹², therefore indicating an important relationship between activated IL-12-producing CD8 α^+ DCs and the Th1 development. The Th1 cells aid in CD8 $^+$ T cell responses by activating APCs through the CD40-CD40L interaction to further enhance their CD8 $^+$ T cell priming ability by increasing cytokine and surface co-stimulatory molecule expression^{185; 193}. They also produce IL-2 to directly activate CD8 $^+$ T cells¹⁹⁴. Binding of TCR to MHC class II-peptide antigen complexes induces CCL3 and CCL4 chemokine production by CD4 $^+$ T cells and DCs, resulting in enhanced recruitment of CD8 $^+$ T cells expressing the corresponding chemokine receptor CCR5¹⁹⁵. This therefore causes CD8 $^+$ T cells to be attracted to the site of an activated APC. As well as aiding in the initial priming of CD8 $^+$ T cells¹⁹⁰, CD4 $^+$ T cell help also contributes to secondary CD8 $^+$ T cell responses mediated by memory cells. An example of this was shown by the high expression of CD27 in CD8 $^+$ T cells that were primed in the presence of CD4 $^+$ T cell. CD27 ligation during re-exposure to antigen results in high autocrine IL-2 production by CD8 $^+$ T cells and high proliferation. In comparison, CD8 $^+$ T cells primed in the absence of CD4 $^+$ T cell help have reduced CD27 and produce very little IL-2 and overall have a reduced potential for secondary memory response¹⁹⁶. Research utilising CD4 $^+$ T cell help to enhance CD8 $^+$ T cell responses is being undertaken in diseases such as HIV¹⁹⁷ and cancer, for example using NY-ESO-1 peptides for both CD4 $^+$ and CD8 $^+$ T cells for treatment of NY-ESO-1-expressing ovarian cancer¹⁹⁸.

1.3.3 *NKT cell help*

As well as the CD4 $^+$ T cell help described in 1.3.2 above, help can also be provided by innate cells, such as natural killer (NK) cells, and innate-like T cells, such as $\gamma\delta$ T cells and natural killer T (NKT) cells. Innate-like T cells are CD3 $^+$ T cells that often have limited T cell receptor specificity and exhibit many features of NK cells, including innate cytotoxic function. These innate and innate-like cells have been shown to augment CD8 $^+$ T cell responses against a variety of infections. For example, NK cells increase CD8 $^+$ T cell responses to herpes simplex virus¹⁹⁹ and $\gamma\delta$ T cells aid in the CD8 $^+$ T cell responses to west nile virus²⁰⁰. The role of NKT cells in providing help to CD8 $^+$ T cells²⁰¹ is a topic of this thesis, and will therefore be described below in some detail.

Semi-invariant natural killer T (NKT) cells are innate T cells with a semi-invariant $\alpha\beta$ TCR. These invariant NKT cells are called type I NKT cells and the TCRs are V α 14 paired with limited V β chain diversity (typically V β 8, V β 7 and V β 2) in mice²⁰² and V α 24 paired with V β 11 in humans²⁰³. These murine V α 14 NKT cells develop within the thymus²⁰² and in extra-thymic sites²⁰⁴, and develop into either CD4⁺ or CD4⁻CD8⁻ cells^{205; 206}. They are found in a variety of locations including the spleen, liver, lymph nodes and thymus²⁰⁷. NKT cells depend on APC presentation of glycolipids on the MHC class I-like molecule CD1d to the TCR²⁰⁸ of NKT cells, along with stimuli from the APC, to result in strong cytokine production for enhanced immune responses²⁹. Activated NKT cells strongly produce a multitude of cytokines including IFN- γ and IL-4, which are important for activating different T cells in immune responses^{209; 210}, and activation induces cytotoxic activity²⁰⁸ including cytotoxic effects against tumour cells²¹¹ indicating possible uses of NKT cell activation for cancer immunotherapy. The V α 14-expressing type I NKT cells constitute a major population of the NKT cells in mice, however there is also a population of type II NKT cells that constitute a minor population of NKT cells in mice²¹² and have a more varied TCR repertoire (including V α 3, V α 8, V β 3 and V β 8 as some of the more dominantly used TCRs)²¹³. The type II NKT cells preferentially recognise the self-glycolipid 3-sulfated galactosyl ceramide (sulfatide) from myelin²¹² and have been shown to oppose the effects of type I NKT cells; for example, type I NKT cell-induced asthma can be negated by activation of type II NKT cells by treatment with sulfatide²¹⁴. The different NKT cell groups have also been shown to have opposing effects in other disease treatment such as tumours, with type I NKT cells having an antitumour effect with suppression of tumour growth while type II NKT cells suppress this antitumour effect causing recurrence of the tumour²¹⁵. NKT cells also provide protection against viral and bacterial infections^{216; 217}; for example, IFN- γ production by NKT cells significantly increases survival against a pulmonary *Streptococcus pneumoniae* infection through the downstream increase in neutrophil accumulation and increase in cytokines such as TNF- α ²¹⁸.

A known synthetic NKT cell ligand with potent NKT activation is α -galactosylceramide (α GalCer)²¹⁹. This compound was originally derived from naturally occurring agelasphins from the marine sponge *Agelas mauritanus*. Derivatives of these agelasphins have been explored with varying combinations of sugars (for example, galactosyl and glucosyl), α -linkages or β -linkages, and varying attached lipid chains, and some of these derivatives have anti-tumour activity²²⁰. In particular, the Agelasphin9b had especially good anti-tumour activity *in vivo* against B16 murine melanoma and also showed a high activity in mixed lymphocyte reactions, indicating a role in inducing lymphocyte proliferation²²¹. This Agelasphin9b led to the development of α GalCer, which has further been described as an excellent inducer of anti-tumour activity against B16 melanoma, with high lymphocyte proliferation induction^{222; 223}. As well as the α -linkages showing improved effects for NKT activation, the fatty acyl chain and sphingosine base lengths are also important, as shortening of these components reduced the proliferative effects of the ligand²⁰⁸. The use of α GalCer has also been shown to be protective against other tumours, for example, the Colon26 adenocarcinoma²²⁴. Nakagawa et al²²⁴ showed that a large dose of 100 μ g/kg of α GalCer injected intravenously directly into mice, after they had been challenged with an intrasplenic dose of Colon26 adenocarcinoma, provided protection against liver metastases and increased survival of the animals.

NKT cells also depend on cytokine production by APC, such as IL-12, for activation. This was identified by the observation that the tumour protection observed with NKT activation was similar to the therapeutic outcome observed in animals treated with intraperitoneal IL-12²²⁴. The similar activities of α GalCer and IL-12 administration led to investigation into the mechanisms of how α GalCer was enhancing the immune response, and how it was related to IL-12. Through the CD1d- α GalCer complexes on DCs binding to the V α 14 TCR on NKT cells, the DCs and NKT cells then interact via CD40-CD40L interactions, as well as through CD80/86 and CD28, to aid in NKT activation²⁰⁸. The presentation of α GalCer specifically activates NKT cells for IFN- γ and IL-4 production in a CD40-CD40L interaction dependent manner between DCs and NKT cells, which is also critically dependent on IL-12 production²⁹. Further to this, α GalCer stimulation also

enhances the expression of the IL-12 receptor on NKT cells via CD1d-TCR interactions; therefore, the increased IFN- γ production by NKT cells and resultant IL-12 stimulation by DCs increases the NKT cell activation further for an activation feedback loop²⁹. Because of the importance of IL-12 for NKT stimulation, and the high production of IL-12 by langerin⁺ CD8 α ⁺ DCs stimulated by NKT activation, this coincides with the reduction in CD8⁺ T cell cross-priming when langerin⁺ CD8 α ⁺ DCs are depleted before administration of antigen with α GalCer⁵². Therefore, activation of NKT cells provides “help” to enhance CD8⁺ T cell responses.

Research into immunisation strategies using α GalCer as an adjuvant indicates that it can enhance immune responses to circulating antigens. The use of α GalCer as an adjuvant in vaccine strategies has shown success for malaria treatment by increasing CD8⁺ T cell responses to malaria parasites in liver stages of the infection²²⁵. Co-administration of α GalCer and soluble protein intravenously can induce strong CD8⁺ T cell responses with antigen-specific anti-tumour protection^{226; 227}. Importantly, TLR agonists can be used in combination with α GalCer and antigen to significantly enhance DC induction of antigen-specific CD8⁺ T cell responses²²⁸. Use of α GalCer as an adjuvant has also been shown to increase both effector and memory antigen-specific CD8⁺ T cell populations, indicating that there is also an improved quality in the CD8⁺ T cell response generated with NKT activation^{229; 230; 231}. Therefore, this makes α GalCer an ideal adjuvant to use for providing NKT cell help to induce stronger CD8⁺ T cell responses *in vivo*.

Some investigation has been carried out to analyse the importance of CD8 α ⁺ DC and NKT cell interactions for NKT cell help in CD8⁺ T cell cross-priming. For example, the work by Semmling et al⁸⁹ showed the importance of the CD8 α ⁺ DC population for integrating multiple signals from NKT cells and CD8⁺ T cells for increased CD8⁺ T cell cross-priming. Further work has identified the localisation of NKT cells to the marginal zone of the spleen upon administration of a glycolipid such as α GalCer, where interactions occur between NKT cells and cells in the marginal zone where APCs such as langerin⁺ CD8⁺ DCs reside^{232; 233}. Interactions

between NKT cells and pDCs through OX40 and OX40L binding, when pDCs localise to the marginal zone after antigen and α GalCer administration, have been shown to increase cross-talk between pDCs and conventional DCs for increased CD8⁺ T cell responses²³⁴. However, the direct involvement of langerin⁺ CD8 α ⁺ DC-NKT interactions has not been defined.

1.4 Analysis of T cell responses using transgenic mouse models

The general aim of the study described in this thesis was to examine the role of CD8 α ⁺ DC responses to circulating antigens, with a particular emphasis on defining the roles of distinct subpopulations of CD8 α ⁺ DCs within the spleen. This was made possible by the availability of transgenic mouse strains in which langerin-expressing CD8 α ⁺ DCs can be identified, and specifically depleted. The following section provides a background to the experimental tools that were used to assess T cell mediated immune responses, including a description of the transgenic mice used.

1.4.1 Antigen-specific T cells

For the analysis of antigen-specific T cell responses, T cells from transgenic mice with a TCR specific for a known antigen can be utilised for measuring antigen-specific T cell responses²³⁵. A common antigen-specific model that is well studied is the OTI transgenic mouse that has CD8⁺ T cells with V α 2⁺ V β 5⁺ TCR specific for the OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) from chicken egg ovalbumin (OVA) protein²³⁶ bound to the MHC class I molecule H-2K^{b237; 238}. Treatment with the soluble OVA protein, OVA protein-loaded DCs *in vitro*, or SIINFEKL-loaded DCs, have all shown the ability to induce measurable CD8⁺ T cell responses^{55; 239}. Also T cell function can be analysed by the use of disease-associated cells, such as tumour cells and viruses, modified to express the OVA protein^{240; 241}. These modified OVA-expressing disease cells are particularly useful for analysing the antigen-specific CD8⁺ T cell response induced by APCs that are more adept at phagocytosing whole cells for antigen processing and presentation²⁴². For example, the CD8 α ⁺ DCs, and more specifically the langerin⁺ subset, are more adept at engulfing apoptotic cells for acquiring antigens¹⁶. Therefore, cross-presentation of OVA peptide to the OTI

CD8⁺ T cells would occur after langerin⁺ CD8α⁺ DCs had engulfed apoptotic OVA-expressing diseased cells.

While flow cytometry with fluorescent tetrameric H-2K^b/SIINFEKL complexes can be used to detect endogenous OVA₂₅₇₋₂₆₄ peptide-specific CD8⁺ T cells²²⁷, the OTI T cells provide a convenient fully controllable and measurable model system. Care has to be used with this system, however, as low numbers of CD8⁺ T cells isolated from OTI mice transferred into recipients for experimentation generally mimics responses by natural endogenous numbers, but transfer of large numbers causes competition for antigen resulting in suboptimal activation^{243; 244}. Despite this, transfer of large numbers can be used to assess for the presence of SIINFEKL antigen *in vivo*²⁴⁵. The use of OTI mice that are congenic for CD45.1 provides a further marker for accurate analysis by flow cytometry when adoptively transferred into CD45.2⁺ hosts^{52; 99}. Using this transgenic mouse model has already been useful for indicating the importance of langerin⁺ CD8α⁺ DCs in inducing strong antigen-specific CD8⁺ T cell responses to soluble protein⁵².

1.4.2 Fluorescent protein expression for cellular analysis

The analysis of langerin⁺ CD8α⁺ DCs has previously been difficult in C57BL/6 mice due to the low expression of langerin compared to BALB/c mice²⁴⁶. Antibodies against the langerin c-type lectin can be used in BALB/c mice, but little to no langerin can be detected on lymphoid resident CD8α⁺ DCs using these antibodies in C57BL/6 mice, possibly due to lower expression²⁴⁷. However, the transgenic OVA₂₅₇₋₂₆₄-specific T cells model described above is dependent on recognition of SIINFEKL peptide presented on H2-K^b molecules²⁴⁸, so can only be used on an H-2^b background, such as C57BL/6 mice²⁴⁹. Therefore an accurate way to identify langerin⁺ CD8α⁺ DCs in C57BL/6 mice was required, so they could be analysed in the context of OVA-specific CD8⁺ T cell responses.

To improve the analysis tools of langerin⁺ cells in mouse epidermis, Kissenpfennig et al⁶⁸ developed *lang*-EGFP mice, where DNA encoding enhanced green fluorescent protein (EGFP) was inserted after the langerin promoter for EGFP expression by langerin⁺ cells in mice. While this transgenic model was initially

developed to explore the role of langerin-expressing skin APCs, it is also possible to identify langerin⁺ CD8 α ⁺ DCs in the spleen. The *lang*-EGFP mice have very bright EGFP expression by all langerin⁺ cells, making flow cytometric and microscopic analysis of the cells more accurate and efficient for visualisation^{68; 250}. The *lang*-EGFP mice were generated using 129/Sv background embryonic stem cells that have higher langerin expression⁶⁸, so it is possible that some extra langerin expression still exists in the C57BL/6 back-crossed *lang*-EGFP mice. Through this higher expression of langerin compared to C57BL/6 mice and due to the bright EGFP production of all langerin⁺ cells, including the very low langerin-expressing CD8 α ⁺ DCs, the lymphoid resident langerin⁺ CD8 α ⁺ DCs could be extensively studied in these C57BL/6 background *lang*-EGFP mice⁵².

1.4.3 Functional and cellular knockout models

An obvious method to identify the importance of various cell types in the immune system is to develop transgenic knockout mice that lack certain genes for cellular subset development. The CD8 α ⁺ DCs described earlier in this chapter are developmentally dependent on the transcription factor Batf3 and therefore Batf3^{-/-} mice are effectively CD8 α ⁺ DC knockout mice. Depletion of the CD8 α ⁺ DCs by Batf3 deficiency has been shown to result in reduced CD8⁺ T cell responses²⁵¹.

As well as depleting particular cell subsets, knockout mice can be used to deplete specific cellular functions. One example is the CD1^{-/-} mice²⁵² that are incapable of presenting glycolipids on CD1d molecules to NKT cells for positive selection, and are therefore NKT cell-deficient²⁹. Cells from these mice have previously been used to assess the importance of NKT cell help in CD8⁺ T cell priming^{227; 253}. Other knockout models used in this thesis are TLR-deficient strains, which cannot respond to specific TLR-ligands. For example, TLR4^{-/-} mice have a severely reduced response to the LPS derivative monophosphoryl lipid A (MPL)^{172; 254}, and TLR2^{-/-} mice do not seem to respond to Pam3Cys¹⁸².

While knockout mouse models can be useful for removing specific cellular subsets or for removing particular cellular functions, they have the drawback of compensatory mechanisms that are invoked when a particular function has been

continuously depleted, which makes analysis difficult. For example, while *Irf8*^{-/-} mice are deficient in *Irf8*-dependent DCs, due to the loss of regulation of haematopoiesis they also develop spontaneous leukaemia²⁵⁵. The limitations of these knockout models indicated the need for inducible knockout models, which are reviewed below.

1.4.4 Diphtheria toxin receptor inducible depletion models

Because of the limited uses of knockout mouse models, inducible depletion models are more appropriate for specific kinetic analysis with only short-term changes in the homeostasis of the host immune cells. An inducible mouse model method that is becoming increasingly popular is the use of targeted expression of the human or simian diphtheria toxin receptor (DTR; a heparin-binding epidermal growth factor-like growth factor precursor)^{68; 256}. The primate DTR has a higher sensitivity to diphtheria toxin (DT) than murine DTR^{257; 258}. This provides a useful research technique, where expression of primate DTR on a specific murine cellular subset would cause the depletion of that specific subset when DT is administered, with little to no effect on murine DTR⁺ cells. DT is a protein derived from *Corynebacterium diphtheriae*. DT was identified as a soluble factor that, at distant sites from the *C. diphtheriae* bacterial infection, would cause massive tissue necrosis and organ failure, often resulting in cardiac arrest²⁵⁹. DT is composed of an A and B subunit. The B subunit binds to the DTR, causing the DT and DTR to be endocytosed. This allows the access of the A subunit to the intracellular components of the DTR⁺ cell. Protein synthesis within the cell is then inhibited by ADP-ribosylation of elongation factor 2, which is catalysed by the A subunit. This results in rapid apoptosis, as the cells are unable to synthesise any proteins^{256; 259}. By targeted transgenic expression of the DTR gene, mouse models have been used for the depletion of several different cell subsets, including CD11c⁺ cells²⁵⁶, CD11b⁺ cells²⁶⁰, langerin⁺ cells⁶⁸ and SiglecH⁺ cells²⁶¹. This provides a powerful tool for analysing the involvement of different cellular subsets at varying stages of immune responses.

In particular, the *lang*-DTREGFP model, developed by Kissenpfennig et al⁶⁸ to express both the DTR and EGFP under the control of the langerin promoter, has

been used extensively to analyse the langerin⁺ dermal DCs and the epidermal langerhans cells^{68; 250; 262; 263}. These cells can be efficiently depleted with 400 ng to 1 µg of DT injected intraperitoneally, and they remain depleted of langerin-expressing cells in the skin for 7-14 days before they begin to repopulate^{264; 265}. However, the lymphoid tissue resident langerin⁺ CD8α⁺ DCs have been less characterised and required further analysis for accurate use of the model in this thesis. While previous research⁵² had shown that depletion of all langerin⁺ DCs using this *lang*-DTREGFP model can reduce systemic CD8⁺ T cell responses, this thesis provides a more detailed analysis of this observation with a specific emphasis on the involvement of the splenic langerin⁺ CD8α⁺ subset.

Overall, the transgenic tools described provided the basis of the methodology used in the studies described in this thesis.

1.5 Aims

Splenic CD8 α^+ DCs have been described as a prominent APC subset for cross-priming antigen-specific CD8 $^+$ T cell responses to circulating antigens. However, within this population, it is the langerin-expressing CD8 α^+ DCs that may account for this enhanced cross-priming ability. The aim of this thesis was to better understand the role of langerin-expressing CD8 α^+ DCs in cross-priming by exploiting the capacity to specifically deplete these cells in transgenic models to assess how the stimulation and differentiation of CD8 $^+$ T cell responses is affected.

- The first part of the thesis addresses the hypothesis that the splenic langerin $^+$ CD8 α^+ DCs are critical in inducing CD8 $^+$ T cell responses to systemically administered antigen.

Specific aims were to:

- Establish a method for inducing antigen-specific CD8 $^+$ T cell responses to systemically administered antigens
- Define the location and phenotype of the critical DCs involved in this CD8 $^+$ T cell response
- Conduct a preliminary investigation into the role of different DC subsets in this response by exploiting the use of ligands for differentially expressed TLRs

- The second part of the thesis addresses the hypothesis that langerin-positive and langerin-negative DCs can co-ordinate their activity to enhance CD8 $^+$ T cell responses to systemically administered antigen

Specific aims were to:

- Identify any possible langerin $^-$ DCs contributing to the induction of CD8 $^+$ T cell responses to circulating antigen
- Investigate how to enhance the coordinated activity of other DCs potentially involved in the CD8 $^+$ T cell response
- Determine if a langerin $^+$ CD8 α^+ DC independent CD8 $^+$ T cell response could be induced by optimising langerin $^-$ DC involvement in cross-priming

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- The last part of the thesis addresses the hypothesis that CD8⁺ T cell responses generated in the presence of langerin⁺ CD8α⁺ DCs are phenotypically and functionally distinct from those responses generated in their absence.

Specific aims were to:

- Investigate whether CD8⁺ T cell responses induced by intravenous administration of antigen and adjuvant acquire a different pattern of distribution in mice depleted of langerin⁺ CD8α⁺ DCs
- Determine if the langerin⁺ CD8α⁺ DCs affect the phenotype and killing function of CD8⁺ T cells
- Assess the impact of anti-tumour function in langerin⁺ DC-deficient mice

Chapter 2: Materials and Methods

2.1 Reagents and Buffers

α -Galactosylceramide (α GalCer)

- 500 μ g α GalCer (CN046; Industrial Research Limited, New Zealand)
- 1 ml dH₂O

The α GalCer was dispersed in sterile distilled water (dH₂O), incubated at 37°C for 10 min and sonicated for 10 min. The dispersed α GalCer was stored at 4°C.

Agarose gel

- 2 g UltraPure Agarose (Invitrogen, Life Technologies, New Zealand)
- 100 ml 1X TAE buffer (Invitrogen, Life Technologies)

The agarose was added to the water, stirred and heated using a microwave until the agarose had dissolved forming a 2% agarose solution. The solution was stored at 65°C until required to keep it from solidifying. To make the gel, a volume of 30 ml of agarose solution was aliquoted and 3 μ l of 10,000X SYBR Safe DNA Gel Stain (Invitrogen, Life Technologies) mixed into the solution before being poured into the mould. Once set, the well-forming comb was removed from the gel and the gel was submerged in 1X TAE buffer ready to use.

Alserver's solution

- 20.5 g dextrose (D-glucose) (AnalaR, BDH, Middle East)
- 7.9 g sodium citrate.2H₂O (Sigma-Aldrich, New Zealand)
- 4.2 g NaCl (Sigma-Aldrich)
- dH₂O
- 1 M citric acid (AnalaR, BDH)

The dextrose, sodium citrate and NaCl were dissolved in dH₂O and made up to 1 L total volume for final concentrations of 114 mM, 27 mM and 71 mM, respectively. The buffer was then adjusted to pH 6.1 using 1 M citric acid. The buffer was stored at room temperature.

AutoMACS (Auto magnetic-activated cell sorting) buffer

- 1 L PBS (Gibco, Invitrogen)
- 10 mL foetal calf serum (FCS; Sigma-Aldrich)
- 4 ml 0.5 M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich)

FCS was thawed and added (1% final concentration), along with EDTA (2 mM final concentration), to the autoclaved, sterilised PBS and stored at 4°C.

Brefeldin A

- 5 mg brefeldin A (Sigma-Aldrich)
- 250 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich)

Brefeldin A was dissolved in DMSO with 10 min sonification. The solution was then used immediately, with any remaining volume stored at -20°C.

Buprenorphine

- 500 µl 0.3 mg/ml Buprenorphine (Renckitt Benckiser Pharmaceuticals, New Zealand)
- 4.5 ml DPBS (Gibco, Invitrogen)

Stock 0.3 mg/ml Buprenorphine was diluted 10X to 30 µg/ml in DPBS and stored at 4°C.

Carprofen

- 300 µl 50 mg/ml Carprofen (Norbrook Laboratories, NZ)
- 15 ml DPBS

Stock 50 mg/ml Carprofen was diluted 50X to 1 mg/ml in DPBS and stored at 4°C.

Cell Tracker Orange chloromethyl-benzoyl-aminotetra-methyl-rodamine (CMTMR)

- 1 mg Cell Tracker Orange CMTMR (CTO; Invitrogen, Life Technologies)

- 180 µl DMSO

CTO was dissolved in DMSO for a stock concentration of 10 mM. Aliquots of 10 µl were stored at -20°C.

CFSE (carboxyfluorescein succinimidyl ester)

- 2 mg Vybrant carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) Cell Tracer Kit (Invitrogen, Life Technologies)
- 4.5 ml DMSO

CFSE was dissolved in DMSO to a concentration of 10 mM. The stock CFSE was aliquoted into 10 µl aliquots for storage at -20°C.

Collagenase type I/DNase I digestion media

- 216 mg Collagenase type I (Gibco, Invitrogen)
- 1.08 ml 10 mg/ml DNase I (Roche, New Zealand)
- 90 ml IMDM

The collagenase type I was dissolved in the media and DNase I was added.

Complete IMDM (cIMDM)

- 500 ml IMDM (Gibco, Life Technologies)
- 500 µl 1,000X 2-mercaptoethanol (2-ME; Gibco, Life Technologies)
- 5 ml Penicillin-Streptomycin (Gibco, Life Technologies)
- 25 ml FCS

Foetal calf serum, Penicillin-Streptomycin and 2-mercaptoethanol were added to IMDM for a final concentration of 5%, 1% and 1X, respectively. The cIMDM was stored at 4°C.

Cytochrome c

- 1 g cytochrome c (Sigma-Aldrich)
- 4 ml DPBS (Gibco, Invitrogen)

Cytochrome c was dissolved in DPBS and aliquoted into 120 µl aliquots for storage at -20°C.

DAPI (4',6-diamidino-2-phenylindole)

- 10 mg DAPI (Invitrogen, Life Technologies)
- 50 ml dH₂O

The DAPI was dissolved in sterile dH₂O to a stock concentration of 200 µg/ml. The aliquot in current use was stored at 4°C to avoid repeated freeze-thawing while the reserve aliquots were stored at -20°C.

Diphtheria toxin (DT)

- 1 mg DT (Sigma-Aldrich)
- 1 ml dH₂O

The DT was dissolved in sterile dH₂O, creating a 1 mg/ml stock solution, and aliquots were stored at -20°C until required.

DNase I

- 100 mg DNase I (Roche)
- 5 ml IMDM (Gibco, Life Technologies)

DNase I was diluted in IMDM, creating a 20 mg/ml stock, aliquoted out and stored at -20°C until required.

Dulbecco's Phosphate Buffered Saline (DPBS)

Pre-sterilised bottles of 500 ml DPBS (Gibco, Life Technologies) was stored at 4°C and used under sterile conditions.

Fluorescence-activated cell sorting (FACS) buffer

- 1 L PBS
- 10 mL FCS

-
- 2 ml 5% NaN₃ (Sigma-Aldrich)
 - 4 ml 0.5 M EDTA

FCS was thawed and added, along with EDTA and NaN₃, to the PBS for final concentrations of 1%, 0.01% and 2 mM, respectively. The buffer was then stored at 4°C.

Foetal calf serum (FCS)

Frozen aliquots of FCS (Sigma-Aldrich) were stored at -20°C and thawed immediately prior to use.

Goat serum

Frozen aliquots of goat serum (Sigma-Aldrich) were stored at -20°C and thawed immediately prior to use.

Iscove's Modified Dulbecco's Medium (IMDM)

Pre-sterilised bottles of IMDM (Gibco, Lif Technologies) were stored at 4°C and used under sterile conditions.

Ketamine/Xylazine

- 1 ml 100 mg/ml Ketamine (Phoenix Pharm Distributors Ltd, New Zealand)
- 0.5 ml 20 mg/ml Xylazine (Phoenix Pharm Distributors Ltd)
- 8.5 ml DPBS

Stock Ketamine and Xylazine were diluted in DPBS to final concentrations of 10 mg/ml and 1 mg/ml, respectively, and stored at 4°C. For intradermal and intranasal vaccinations, a lower concentration of 0.5 mg/ml xylazine was used.

Liberase

- 5 mg Liberase TL (Roche)
- 2 ml dH₂O

Liberase TL was dissolved in dH₂O, creating a stock concentration of 2.5 mg/ml, before being aliquoted and stored at -20°C until required.

Liberase/DNase digestion buffer

- 0.5 ml IMDM
- 62.5 µl of 2.5 mg/ml Liberase TL (Roche)
- 5 µl of 20 mg/ml DNase I (Roche)

Liberase TL and DNase I were thawed and added to IMDM immediately prior to use.

Monensin

- 13.857 mg monensin sodium salt (Sigma-Aldrich)
- 1 ml methanol (Scharlau, Germany)

Methanol was added to monensin for a final stock concentration of 20 mM and stored at -20°C.

Monophospholipid A (MPL)

- 1 mg MPL (Sigma-Aldrich)
- 1 ml DPBS

MPL was added to DPBS for a final stock concentration of 1 mg/ml, sonicated for 2 h and stored at 4°C.

NaOH hydrolysing buffer

- 10 ml 5 M NaOH (Sigma-Aldrich)
- 1 L dH₂O

A volume of 10 ml 5 M NaOH was added to 1 L of dH₂O to make a final concentration of 50 mM NaOH. The solution was stored at room temperature.

Ovalbumin (OVA)***Endograde:***

- 250 mg OVA (Hyglos GmbH, Germany)
- 10 ml dH₂O

OVA was dissolved in sterile dH₂O, creating a stock concentration of 25 mg/ml, aliquoted out and stored at -20°C.

Non-endograde:

- 1 g OVA (Sigma-Aldrich)
- 20 ml dH₂O

OVA was dissolved in sterile dH₂O, creating a stock concentration of 50 mg/ml, aliquoted out and stored at -80°C.

OVA₂₅₇₋₂₆₄ peptide (SIINFELK)

- 1 mg OVA₂₅₇₋₂₆₄ peptide (GenScript, NJ, USA)
- 20 µl DMSO

The peptide was dissolved in DMSO for a stock concentration of 50 mM, which was stored at -20°C.

Pam3Cys (N-palmitoyl-S-dipalmitoylglycerol Cys-Ser-(Lys)4)

- 2 mg Pam3Cys (EMC microcollections GmbH, Germany)
- 2 ml dH₂O

Pam3Cys was dissolved in sterile distilled water and sonicated for 10 min before being stored at 4°C.

Percoll gradient solution

- 15 ml percoll (GE Healthcare Bio-Sciences AB, Sweden)
- 30 ml DPBS
- 1 ml Alservers solution

The components were added together immediately prior to use.

Phosphate Buffered Saline (PBS)

- 95.5 g Dulbecco's Phosphate Buffered Saline (PBS; Gibco, Life Sciences)
- 10 L dH₂O

The 95.5 g PBS was dissolved in 1 L of dH₂O, sterilised by autoclaving, and the solution was diluted 10X further in sterile dH₂O.

PBS (+ 10 mM EDTA)

- 500 ml PBS
- 10 ml 0.5 M EDTA

A volume of 10 ml of 0.5 M EDTA was added to 500 ml PBS, creating a stock concentration of 10mM EDTA, and was stored at room temperature.

Poly I:C (polyinosinic:polycytidylic acid)

- 25 mg Poly I:C (InvivoGen, CA, USA)
- 10 ml dH₂O

Poly I:C was dissolved in dH₂O to a final stock concentration of 2.5 mg/ml and stored in aliquots at -20°C.

Resiquimod

- 5 mg resiquimod (Tocris Bioscience, UK)
- 5 ml dH₂O

Resiquimod was dissolved in dH₂O to a final stock concentration of 1 mg/ml, sonicated for 10 min and stored in aliquots at -80°C.

Saponin buffer

- 1 g saponin (Sigma-Aldrich)

-
- 1 g bovine serum albumin (BSA; Sigma-Aldrich)
 - 1 L PBS

Saponin and BSA were dissolved in PBS, then aliquoted and stored long-term at -20°C or short-term at 4°C.

Sort buffer

- 100 ml Roswell Park Memorial Institute (RPMI) 1640 Medium without phenol red (Gibco, Life Technologies)
- 1 ml 0.5 M EDTA
- 2 ml FCS

The EDTA and FCS were added to the RPMI for final concentrations of 5 mM and 20%, respectively. The buffer was stored at 4°C.

Trypsin-EDTA

Frozen aliquots of Trypsin-EDTA (TE; Gibco, Life Technologies) were stored at -20°C and thawed immediately prior to use.

Tris-HCl neutralising buffer

- 121.14 g UltraPure Tris (Invitrogen, Life Technologies)
- Hydrochloric acid (HCl; Techgrade; Biolab-chemicals, Israel)
- 1 L dH₂O

A weight of 121.14 g Tris was dissolved in 1 L dH₂O to make a final concentration of 1 M and HCl was added to adjust the buffer to a pH of 7.5. The buffer was stored at room temperature.

***Würzburger buffer*⁹³**

- 500 ml DPBS
- 5 ml FCS
- 5 ml 0.5 M EDTA

- 0.5 ml 20 mg/ml DNase I

The FCS and DNase aliquots were thawed and added, along with EDTA, to the DPBS at final concentrations of 1%, 5 mM and 0.02 mg/ml, respectively. The buffer was stored at 4°C and used under sterile conditions.

2.2 Antibodies

All antibodies were titrated (example in Appendix 1) before first use.

Table 2.1. Table of antibodies.

Specificity	Fluorophore	Clone	Manufacturer
Affinipure F(ab') ₂ fragment goat anti-human IgG	APC	N/A	Jackson ImmunoResearch Laboratories, Inc. (PA, USA)
Anti-rat IgG (goat)	Dylight 488	Poly4054	Biolegend
B220	Biotin	RA3-6B2	BD Pharmingen (Becton Dickinson, CA, USA)
	PerCP	RA3-6B2	BD Pharmingen
CD3	Biotin	145-2c11	eBioscience (CA, USA)
CD4	APC	GK1.5	eBioscience
	Biotin	GK1.5	eBioscience
	eFluor 450	RM4-5	eBioscience
CD8 α	AlexaFluor 700	53-6.7	eBioscience
	FITC	SK1	eBioscience
	Pacific Blue	53-6.7	BD Pharmingen
	PE	53-6.7	Biolegend
CD11b	APC-Cy7	M1/70	BD Pharmingen
	Biotin	M1/70	Biolegend

CD11c	APC PE-Cy7	HL3 N418	BD Pharmingen Biolegend
CD19	eFluor 450	1D3	eBioscience
CD24	PE	M1/69	eBioscience
CD40	PE	3/23	BD Pharmingen
CD44	PE-Cy5	IM7	BD Pharmingen
CD45.1	Biotin FITC PE	A20 A20 A20	BD Pharmingen Biolegend Biolegend
CD45.2	PE	104	BD Pharmingen
CD103	APC	2E7	eBioscience
CD122	FITC	TM-beta1	eBioscience
CD127	Biotin	SB/199	Biolegend
CD169	Purified rat	3D6.112	Abcam
CD326	APC-Cy7	G8.8	Biolegend
CX3CL1- humanIgG Fc fusion protein (CX3CR1)	Supernatant		Provided by Steffen Jung, Werzmann Institute of Science, IL, USA)
DEC-205	Biotin	NLDC-145	Biolegend
GR-1	APC	RB6-8C5	BD Pharmingen
H-2K ^b	eFluor 450	AF6-88.5.5.3	eBioscience
H-2K ^b -SIINFEKL	Biotin	25.D1.16	eBioscience
IgG1κ isotype	Biotin FITC Pacific Blue	P36.2.8.1 R3-34 RTK2071	eBioscience BD Pharmingen Biolegend
IgG2ακ isotype	eFluor 450 FITC PE	eBM2a eBM2a R35-95	eBioscience eBioscience BD Pharmingen
IgG2βκ isotype	PE	eB149/10H5	eBioscience
IL-2	PE	JES6-5H4	BD Pharmingen
IL-12p40	APC	C15.6	BD Pharmingen

	PE	C15.6	BD Pharmingen
IFN- γ	Pacific Blue	XMG1.2	Biolegend
KLRG1	PE-Cy7	2F1	eBioscience
Langerin	Biotin	L31	eBioscience
SiglecH	PE	eBio44C	eBioscience
SiglecF	PE	E50-2440	BD Pharmingen
Streptavidin	AlexaFluor 555	N/A	Invitrogen
	FITC	N/A	BD Pharmingen
	PE	N/A	BD Pharmingen
	PECy7	N/A	BD Pharmingen
	PerCP	N/A	BD Pharmingen
	APC-AlexaFluor 750	N/A	Invitrogen
TNF- α	FITC	MP6-XT22	eBioscience
	PE	MP6-XT22	BD Pharmingen
V α 2	APC	B20.1	BD Pharmingen
	PE	B20.1	BD Pharmingen

2.3 Mice

2.3.1 Ethics approval and housing

All mice used for this thesis research were bred and housed in the Biomedical Research Unit of the Malaghan Institute of Medical Research, Wellington, New Zealand. Experimental protocols were approved by the Victoria University Animal Ethics Committee and performed according to their guidelines. Mice were used between 6-12 weeks of age and were age and sex-matched where possible.

2.3.2 Mouse strains

C57BL/6J and Balbc/ByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and B6.SJL-*Ptprc^aPep3^b*/BoyJArc mice were from the Animal Resource Centre (Canning Vale, Western Australia). OTI mice, expressing the transgenic V α 2 TCR specific for an H-2 K^b-binding peptide of OVA (OVA₂₅₇-

²⁶⁴)²³⁸, were originally provided by Frank Carbone (Melbourne University, Australia). *SiglecH*-DTR mice were kindly provided by Christiane Ruedl (Nanyang Technological University, Singapore). OTIxB6.SJL-*Ptprc^aPep3^b*/BoyJArc mice were crossed in-house to allow for CD45.1 identification of adoptively transferred OTI cells in CD45.2 mice, as were the *siglecH*-DTRxC57BL/6 mice. *Lang*-EGFP and *lang*-DTREGFP mice were kindly provided by Bernard Malissen (Centre d'Immunologie de Marseille-Luminy, France). TLR2^{-/-} mice were obtained from the Research Institute for Microbial Diseases (Osaka University, Japan). Batf3^{-/-} mice were kindly provided by Kenneth M. Murphy (Washington University School of Medicine, MO, USA).

2.4 *In vivo* cell depletion

2.4.1 *Langerin-DTREGFP and SiglecH-DTR depletion*

An aliquot of 1 mg/ml stock DT was thawed and diluted in DPBS to 1.75 µg/ml. A volume of 200 µl, 350 ng DT, was injected intraperitoneally into mice at days as indicated per experiment. As a control, 200 µl DPBS was intraperitoneally injected into control mice.

2.4.2 *Cytochrome c*

Mice were warmed under a lamp and placed into restrainers. A volume of 200 µl containing 5 mg cytochrome c was injected into the lateral tail vein.

2.5 Splenectomy

Mice were anaesthetised with 100 mg/kg Ketamine and 10 mg/kg Xylazine i.p., followed by s.c. administration of 0.1 mg/kg Buprenorphine for perioperative analgesia. Lacrilube (Allergan New Zealand Ltd, NZ) was applied to corneas to prevent dessication. The surgical area was sterilised with ethanol, followed by an incision on the left flank to access the spleen. The primary artery and vein were tied to sever blood flow to the spleen, which was followed quickly by cutting of the blood vessels to release the spleen. The incision was sutured and mice recovered on

a heat pad. Subcutaneous administration of 5 mg/kg Carprofen was used for the two days following surgery for post-operative analgesia.

2.6 Tissue preparation

2.6.1 Blood

2.6.1.1 White blood cells

Mice were placed in a restrainer, warmed under a heat lamp to increase blood volume, and an incision was made in the lateral tail vein. Blood was collected (4-6 drops) into 1.5 ml eppendorf tubes containing 200 μ l PBS (+ 10 mM EDTA). One ml of red blood cell lysis solution (Qiagen, CA, USA) was added to each tube and incubated at 37°C for 30 min. The white blood cells were pelleted by centrifugation at $706 \times g$ for 5 min in a Biofuge Fresco (Heraeus, ThermoFisher Scientific, New Zealand), the supernatant removed, and the cells resuspended in 200 μ l FACS buffer for flow cytometric analysis.

2.6.1.2 Serum

Mice were placed in a restrainer, warmed under a heat lamp to increase blood volume, and an incision was made in the lateral tail vein. Blood was collected (8-10 drops) into 1.5 ml eppendorf tubes, stored at 4°C overnight, and centrifuged at $13,250 \times g$ for 10 min. The top serum layer within the tube after centrifugation was transferred into new tubes and stored at -20°C until analysis.

2.6.2 Spleen

Mice were culled by asphyxiation, followed by cervical dislocation. Spleens were collected and placed into IMDM resting on ice. Spleens were injected with 0.5 ml Liberase/DNase digestion buffer and incubated at 37°C for 30 min. Spleens were then dissociated by mulching through 70 μ m filters (Becton Dickinson), washed with IMDM, then the cells were pelleted by centrifugation at $645 \times g$ for 4 min in a Megafuge 4 KR (Heraeus, ThermoFisher Scientific). Supernatant was discarded and red blood cells were lysed by brief vortexing with 3 ml red blood cell

lysis solution. Cells were pelleted again by centrifugation, the supernatant was discarded, and cells were washed with DPBS. For extraction of T or B cells from spleens, the tissue was dissociated immediately after extraction, without incubating with Liberase/DNase digestion buffer.

2.6.3 Lymph nodes

Mice were culled by asphyxiation, followed by cervical dislocation. The outer skin layer was cut and peeled away for collection of the Inguinal, Auxiliary and Brachial lymph nodes with tweezers. The muscle wall was cut and spread for access to the mesenteric lymph node. All lymph nodes were collected in IMDM on ice, mulched through a 70 μm filter and then washed with IMDM. Cells were pelleted by centrifugation at $645 \times g$ for 4 min, supernatant discarded and the cells were washed with DPBS.

2.6.4 Liver

Mice were culled by asphyxiation, followed by cervical dislocation. The outer skin and muscle wall were cut and peeled away exposing the liver. The liver was perfused by injecting 1 ml of DPBS into the hepatic portal vein while cutting the posterior vena cava, to allow drainage of the flow-through. The liver was then excised and placed in cIMDM on ice. The liver was mulched through a 70 μm filter, washing through with DPBS. The cells were collected into a 50 ml Falcon tube and the volume was made up to 50 ml with DPBS. Cells were pelleted by centrifugation at $363 \times g$ for 10 min and the supernatant was removed by aspiration. To extract the white blood cells, the liver cells were subjected to a percoll gradient. Liver cells were mixed with Percoll Gradient Solution by vortexing, and centrifuged at $1,008 \times g$ for 10 min with the brake off. The supernatant, along with the hepatic cells, was aspirated and the red blood cells lysed by incubation for 5 min at 37°C with red blood cell lysis solution. The white blood cells were washed twice with DPBS and aliquoted into 96 well plates to be stained for flow cytometry.

2.6.5 *Bone marrow*

Mice were culled by asphyxiation, followed by cervical dislocation. Legs were removed from the body, the skin and muscle was removed from the bones and the bones were placed in IMDM on ice. Cutting the sinew at the knee joint disconnected the femur and tibia. The ends of each bone were cut, and the marrow was collected into tubes by using a syringe and needle to flush IMDM through the bone. The marrow was then mulched through a 70 μm filter, centrifuged at $645 \times g$ for 4 min, the supernatant discarded, and the red blood cells lysed with brief vortexing with red blood cell lysis solution. The cells were then washed with DPBS and aliquoted into 96 well plates to be stained for flow cytometry.

2.6.6 *Lung*

Mice were culled by asphyxiation, followed by cervical dislocation. The skin was cut and peeled away, followed by opening of the chest cavity. The lungs were harvested from the chest cavity and placed into IMDM on ice. The lungs were then dissected into small pieces and placed into collagenase type I/DNase I digestion media for 1 h at 37°C. The lungs were mulched through a 70 μm strainer, pelleted by centrifugation at $645 \times g$ for 8 min and the supernatant was aspirated. The red blood cells were lysed by brief vortexing with red blood cell lysis solution and the cells were washed with DPBS. The white blood cells were enriched via CD45 microbead enrichment (detailed in section 1.7.1) and the enriched cells were aliquoted into 96 well plates to be stained for flow cytometry.

2.6.7 *Viable cell counts*

An aliquot of 10 μl of cells was added to 190 μl 0.4% trypan blue (Gibco, Life Technologies) at a known dilution. A small volume of the mix was placed onto a haemocytometer. Under the microscope, the viable cells were identified as white cells, whereas the dead cells were blue. The cells were counted and the total cell count calculated using the following formula:

$$\text{Total cell number} = (\text{Average cell count per ninth area}) \times (\text{dilution factor}) \times (10^4) \times (\text{total volume})$$

2.7 Cell enrichment

2.7.1 *Microbead enrichment*

Cells were isolated from splenic tissue, as described in 2.6.2, and washed in Würzburger buffer, with centrifugation and discarding of supernatant. For enrichment of DCs, cells were resuspended in a total of 100 µl/spleen consisting of 80 µl Würzburger buffer and 20 µl of CD11c Microbeads (Miltenyi Biotec GmbH, Germany), followed by 15 min incubation at 4°C. For enrichment of white blood cells from the lung, a total of 150 µl/lung was used consisting of 135 µl Würzburger buffer and 15 µl of CD45 Microbeads (Miltenyi Biotec GmbH) and incubated for 15 min at 4°C. For OTI cell enrichment, cells were either incubated with B220-biotin and CD4-biotin or with CD45.1-biotin antibody (see Table 2.1) in FACS buffer, followed by 15 min incubation at 4°C with 80 µl Würzburger buffer and 20 µl anti-biotin Microbeads (Miltenyi Biotec) per spleen. After bead incubation, cells were washed with Würzburger buffer, resuspended in 3 ml per spleen, and filtered through a 30 µm pre-separation filter column (Miltenyi Biotec). An AutoMACS machine (Miltenyi biotec) was then used to magnetically sort the bead-positive (CD45, CD45.1 and CD11c) or bead-negative (B220 and CD4) cells, which were collected in fresh tubes. The enriched cells were then used for further analysis, while the unwanted cells were discarded.

2.7.2 *Dynabead enrichment*

Cells were isolated from lymph node and splenic tissue as described in sections 2.6.2 and 2.6.3. Cells were then washed in Würzburger buffer, counted and resuspended at 1×10^8 cells/ml. A CD8 flowcomp dynabead kit (Invitrogen, Life Technologies) was then used to isolate the CD8⁺ cells following the manufacturer's instructions. This involved incubating the cells for 10 min at 4°C with 50 µl of CD8 antibody (provided in the kit) per 1×10^8 cells. The cells were washed once with Würzburger buffer, resuspended in 1 ml of buffer, and 150 µl of dynabeads (provided in the kit) per 1×10^8 cells was added. The sample was incubated for a further 15 min at 4°C, with intermittent mixing of beads and cells to prevent the

beads from settling at the bottom of the tube and therefore not binding to cells. The sample tube was placed in a magnetic column for 1 min and the negative unbound cells were transferred to a waste tube. The bead-bound cells were washed with 1 ml of Würzburger buffer before being placed in the magnetic column for 1 min for any remaining negative cells to be separated from the positive cells. The bead-bound cells were then resuspended in 1 ml of Releasing Buffer (provided in the kit), to elute the beads from the cells, and incubated at room temperature for 10 min. The sample was placed in the magnetic column for 1 min to separate the unbound beads from the cells. The positive cells were removed from the tube and placed into a fresh tube, which was also treated in the same manner again to remove any remaining contaminating beads. The positive cells were washed a final time with Würzburger buffer.

2.8 Antigen-specific T cell transfer

The OTIxB6.SJL-*Ptprc^aPep3^b*/BoyJArc mice contain CD8⁺ T cells that are specific for the SIINFEKL peptide found within chicken ovalbumin protein (OVA)²³⁸. The lymph nodes and spleen were collected and processed as described in sections 2.6.2 and 2.6.3. The samples were enriched for the CD8⁺ T cells via CD8 dynabead enrichment as described in 2.7.2. The final CD8⁺ population was washed in DPBS and resuspended at 5×10⁴ cells/ml. Mice were warmed under a heat lamp and placed in a restrainer. A volume of 200 µl, therefore 1×10⁴ OTI T cells, was injected into mice intravenously via the lateral tail vein. The mice were then left overnight to allow the T cells to circulate. Remaining unused cells were checked for purity by flow cytometry staining with CD8-FITC and CD4-APC for 10 min at 4°C. An example of the purity results is shown in Appendix 2.

2.9 Vaccination

2.9.1 Intravenous vaccination

Antigen and stimuli, as indicated per experiment, were combined for a total volume of 200 µl per mouse. OVA, αGalCer and Pam3Cys were used at 200 µg/mouse, 200 ng/mouse, and 20 µg/mouse, respectively. M1-OVA (kindly

provided by Prof John Fraser, University of Auckland, New Zealand) was used at 2 µg/mouse, with 1 µg/mouse of OVA (the molar equivalent) used as a control in the same experiments. For T cell stimulation, Poly I:C was used at 33.3 µg/mouse and resiquimod was used at 6.67 µg/mouse. For stimulation for DC intracellular cytokine staining, Poly I:C was used at 50 µg/mouse and resiquimod was used at 10 µg/mouse. Mice were warmed under a heat lamp and placed into restrainers. The 200 µl vaccine volume was injected into the lateral tail vein.

2.9.2 *Intranasal vaccination*

Antigen and stimuli, as indicated per experiment, were combined for a total volume of 50 µl per mouse. OVA, αGalCer and Pam3Cys were used at 200 µg/mouse, 200 ng/mouse, and 20 µg/mouse, respectively. Mice were administered 100 mg/kg Ketamine and 3 mg/kg Xylazine intraperitoneally to anaesthetise them for the procedure. Mice were laid on their backs and the 50 µl volume of vaccine was placed on the nostrils using a pipette. The vaccine was taken into the lungs upon inhalation. During recovery, mice were kept warm and monitored.

2.9.3 *Intradermal vaccination*

Antigen and stimuli, as indicated in the experimental data, were combined for a total volume of 30 µl per mouse. OVA, αGalCer and Pam3Cys were used at 200 µg/mouse, 200 ng/mouse, and 20 µg/mouse, respectively. Mice were administered 100 mg/kg Ketamine and 3 mg/kg Xylazine intraperitoneally to anaesthetise them for the procedure. Mice were laid on their sides, the ear was laid flat along the rounded surface of a 1.5 ml tube to make the ear more accessible and the 30 µl vaccine volume was injected gently into the skin of the ear. During recovery, mice were kept warm and monitored.

2.9.4 *Intramuscular vaccination*

Antigen and stimuli, as indicated in the experimental data, were combined for a total volume of 50 µl per mouse. OVA, αGalCer and Pam3Cys were used at 200 µg/mouse, 200 ng/mouse, and 20 µg/mouse, respectively. Mice were restrained by

hand, the fur on the thigh flattened and sterilised by application of 70% ethanol. The 50 μ l vaccine volume was injected into the caudal thigh muscle. Aspiration with the syringe was used during injection to assess the presence of blood to ensure the injection was not entering the intravenous route.

2.10 Tumour cell culture and challenge

2.10.1 In vitro cell culture

Adherent B16.OVA and suspension EG7.OVA tumour cells were cultured in tissue flasks with complete IMDM. They were also supplemented with 1% Geneticin (G418; Gibco, Life Technologies) to promote production of OVA protein by the tumour cells. The cells were harvested when 70-80% confluent, the adherent cells first being incubated with Trypsin-EDTA for 5 min at 37°C to remove them from the flask surface.

2.10.2 Subcutaneous tumour challenge

Tumour cells were cultured and harvested as described in section 2.10.1 and washed twice with IMDM to remove the FCS. The cells were counted, resuspended at 2.5×10^6 cells/ml in IMDM, and filtered through a 70 μ m filter to separate any cell clumps into a single cells suspension. A volume of 100 μ l (5×10^5 cells) was then injected subcutaneously into each mouse in the left flank. The tumours were monitored over time as they grew and mice were culled once the tumour size had grown to the ethical limits of 200 mm², or culled earlier if mice showed signs of illness.

2.11 Flow cytometry

2.11.1 Cell surface staining

Cells were obtained from various tissues by the protocols outlined in section 2.6. Cells were washed and then resuspended with FACS buffer before an aliquot of each sample was pipetted into wells of a 96 well u-bottom plate. Cells were pelleted in the plates by centrifugation at $1,008 \times g$ for 2 min. Supernatant was removed by

turning the plate upside-down. Non-specific Fc-mediated binding of antibodies was then prevented in most experiments by a 10 min 4°C incubation of each sample with 50 µl of FACS buffer containing 2.4G2 Fc-blocking reagent (collected in-house from supernatant from 2.4G2 hybridoma cells). Cells were washed with FACS buffer, centrifuged and the supernatant was discarded. Antibody mixes for cell surface markers were diluted into FACS buffer at concentrations determined by titration. Each cell pellet was mixed with 50 µl of the antibody mixture, followed by an incubation of 10 min at 4°C. Cells were again washed with FACS buffer, centrifuged and the supernatant discarded. Cells were resuspended in FACS buffer containing the viability dyes DAPI or propidium iodide (PI; BD Pharmingen) to ensure that only live cells with an intact cell membrane to prevent the DAPI or PI dyes from entering the cell were included in the final analysis. Flow cytometry analysis was then performed using a BD FACSort, BD FACSCalibur or BD LSR II SORP (Becton Dickinson) instrument and analysed using FlowJo (version 9) software (TreeStar, Inc., OR, USA). Fluorescence minus one (FMO) controls were also used where required (example in Appendix 3). Briefly, this included the full staining panel of antibodies minus one antibody, therefore allowing for comparison to background fluorescence from the other fluorophores.

2.11.2 Intracellular staining

2.11.2.1 Dendritic cells

After cells had been stained in 96 well plates with antibodies targeting cell surface markers (as described in section 2.11.1), the cells were then also stained with live/dead fixable blue viability dye (Invitrogen, Life Technologies) to provide identification of live versus dead cells during flow cytometry analysis. This viability dye was used instead of DAPI or PI to allow discrimination of live versus dead cells post fixation when the cellular membrane has lost integrity due to the intracellular staining process. The cells were then stained intracellularly using a Fix and Perm Cell Permeabilisation Kit (Invitrogen, Life Technologies) following the manufacturers instructions. In brief, cells were washed of excess cell surface antibodies using FACS buffer, pelleted by centrifugation at $1,008 \times g$ for 2 min and fixed by applying 100 µl of Solution A reagent (provided in the kit) to each sample

for 15 min at room temperature in the dark. Cells were then washed and pelleted before addition of 50 μ l of Solution B reagent (supplied in the kit) containing antibodies for intracellular staining. Solution B provided permeabilisation of the cells for the antibodies to access the intracellular target ligands. Cells were incubated for 20 min at room temperature in the dark before again being washed and pelleted by centrifugation. Cells were resuspended in FACS buffer for analysis on a BD LSRII SORP.

2.11.2.2 *T cells*

To detect measurable levels of cytokine production intracellularly in CD8⁺ T cells at seven days after administration of antigen, the T cells were re-stimulated immediately before analysis. This was performed by resuspending splenocytes (obtained and processed as described in section 2.6.2) at a concentration of 10×10^6 cells/ml in cIMDM. A volume of 200 μ l (consisting of 2×10^6 cells) was aliquoted into wells of a 96 wells plate, and a final concentration of 5 μ g/ml of OVA₍₂₅₇₋₂₆₄₎ SIINFEKL peptide was added to each well. The plates were incubated at 37°C for 5.5 h. To prevent cytokine release during the re-stimulation, a final concentration of 3 μ g/ml brefeldin A was added to each well at the beginning of the re-stimulation. A final concentration of 20 μ M monensin was added after the second hour of the re-stimulation. After the incubation, the cells were washed with cIMDM to remove excess peptide and stained for cell surface markers (as described in section 2.11.1), including the viability dye live/dead fixable blue to allow discrimination of live cells during later flow cytometry analysis. The cells were then washed with FACS buffer to remove excess antibodies and fixed with 100 μ l of formalin containing 4% paraformaldehyde (Invitrogen, Life Technologies) per well for 20 min on ice. The cells were then pelleted by centrifugation at $1,008 \times g$ for 2 min, the formalin discarded, and the cells were permeabilised with 200 μ l of saponin buffer per well for 30 min at room temperature. The cells were pelleted and the saponin buffer discarded, before addition of 50 μ l saponin buffer per well containing antibodies against cytokines. The Fc-blocking reagent 2.4G2 was included to prevent non-specific binding. The cells were incubated with the antibodies for 30 min at room temperature before excess unbound antibodies were washed away with two washes

of saponin buffer. The cells were resuspended in FACS buffer for analysis on a BD LSRIISORP.

2.11.3 *In vivo proliferation*

To be able to analyse the proliferation of antigen-specific CD8⁺ T cells *in vivo*, CFSE was used to bind green fluorescent dye to all of the proteins within the T cells. As the cells proliferated, the CFSE would then be diluted evenly between the daughter cells, providing an incremental dilution of the CFSE dye²⁴⁰. Lymph nodes from OTIxB6.SJL-*Ptprc^aPep3^b*/BoyJArc mice were first collected and processed as described in section 2.6.3. The cells were resuspended at 2×10^7 cells/ml in DPBS and filtered through a 70 μ m filter. Stock CFSE of 10 mM concentration was diluted to 2 μ M in DPBS. An equal volume of diluted CFSE was then added to the cells for a final concentration of 1 μ M. The cells were incubated for 7 min at room temperature in the dark before an equal volume of FCS was added to stop the staining. Cells were pelleted by centrifugation at $645 \times g$ for 4 min. The cells were washed twice with cIMDM before being resuspended in IMDM at a concentration of 2.5×10^7 cells/ml. A volume of 200 μ l (5×10^7 cells) was injected intravenously into each mouse. White blood cells were collected from blood two days later, as described in section 2.6.1.1. T cell proliferation was detected by flow cytometry, measuring CFSE dilution as indicated by the decrease in fluorescent intensity in the green channel.

2.11.4 *VITAL assay*

For the analysis of the killing ability of antigen-specific cells, a VITAL assay²⁶⁶ was used to provide known target numbers that could be measured and calculated to compare the functionality between different treatment groups. The procedure first involved the acquisition of spleens from naïve mice, as described in section 2.6.2 (without the liberase/DNase digestion). The splenocytes were resuspended in 8 ml cIMDM and separated into four tubes. SIINFEKL (OVA₂₅₇₋₂₆₄) peptide from OVA protein was incubated for 2 h at 37°C at a concentration of 50 nM, 5 nM and 0.5 nM in the first three tubes, while the fourth tube received no peptide. The tube that received no peptide was then washed three times with

cIMDM and resuspended in 5 ml of warm cIMDM. Cytotracker orange (CTO) was diluted from a stock concentration of 10 mM to an intermediate dilution of 20 μ M in 5 ml of warm cIMDM before being added to the 5 ml of cells for a final concentration of 10 μ M CTO. The cells were incubated for 15 min at 37°C, pelleted by centrifugation, resuspended in 10 ml of warm cIMDM and incubated for a further 20 min at 37°C. The cells were washed twice with cIMDM and a third time with IMDM. During the CTO incubations, the three peptide-loaded samples were washed twice with IMDM, once with DPBS and resuspended in 5 ml DPBS. Dilutions of 5 μ M, 1 μ M and 0.2 μ M CFSE were made in DPBS and 5 ml was added to the 5 ml of high, medium and low peptide-loaded cells, respectively. The cells were incubated for 7 min before the staining was quenched by addition of 10 ml of FCS. The cells were washed twice with cIMDM, once with IMDM and the three CFSE samples were combined with the CTO sample. The combined cells were counted, pelleted and resuspended in IMDM for intravenous administration of approximately 3×10^6 cells per mouse.

White blood cells were collected from blood 15 h later, as described in section 2.6.1.1, and the populations of CTO and CFSE cells were analysed by flow cytometry. To calculate the percentage of antigen-specific killing that had occurred per sample, cell counts were first normalised to the negative control (CTO-positive) cells by dividing each CFSE cell count by the corresponding CTO cell count (termed the CFSE:CTO ratio). The following formula was then used:

$$\% = 100 - ((\text{"CFSE:CTO ratio"} / \text{"Average Naïve CFSE:CTO ratio"}) \times 100)$$

2.11.5 Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was used to purify adoptively transferred CD45.1⁺ OTI T cells from spleens of CD45.2⁺ hosts for either RNA extraction or adoptive transfer into naïve recipients. Spleens were collected and processed as described in section 2.6.2 (without the liberase/DNase digestion). The OTI cells were then enriched as described in section 2.7.1 using AutoMACS Microbead enrichment. Enrichment of CD45.1-positive cells was used for cells destined for RNA extraction while the CD4 and B220 depletion was used for cells

destined for adoptive transfer (see section 2.7.1 for details). Cells were counted, washed and incubated for 10 min at 4°C with antibodies for CD8, CD45.1 and V α 2 in sort buffer. Cells were washed and resuspended in sort buffer at 1.5×10^7 cells/ml for separation on a BD FACSVantage DiVa (Becton Dickinson). The purified population of CD8⁺ CD45.1⁺ V α 2⁺ cells either underwent RNA extraction, as detailed in section 2.12.1, or were adoptively transferred into naïve recipients intravenously at 1×10^6 cells/mouse.

2.12 *In vitro* killing assay

To assess the cytotoxic capabilities of the cross-primed CD8⁺ T cells, the cells were purified and titrated into an *in vitro* killing assay to directly compare the amount of killing occurring with equal numbers of T cells. On day six after antigen was administered to the animals, 1.5 ml of T cell lymphoma (EL4) cells were aliquoted into 24 well culture plates at 3×10^5 cells/ml in cIMDM. A final concentration of 1.1 μ Ci/ml tritiated ([3H])-thymidine (PerkinElmer, MA, USA) was added to each well and left overnight to allow the incorporation of [3H]-thymidine into the DNA of the proliferating cells. On day seven, at the peak of the CD8⁺ T cell response, spleens were obtained from antigen-administered mice and processed as described in section 2.6.2. The OTI CD8⁺ T cells were enriched via microbead enrichment, as described in section 2.7.1, with enrichment for CD45.1⁺ cells. The OTI T cells were further purified via FACS, as described in section 2.11.5, with positive sorting for CD8, CD45.1 and V α 2. The purified cells were resuspended at 5×10^6 cells/ml in cIMDM and titrated 3 fold for a series of 6-8 dilutions. The [3H]-thymidine incorporated EL4 cells were washed, resuspended at 1×10^5 cells/ml and 50 μ l (5×10^4 cells) were aliquoted into 96 well plates. A final concentration of 5 nM SIINFEKL (OVA₂₅₇₋₂₆₄) peptide from OVA protein was added to half of the EL4 cells and incubated for 45 min at 37°C. The EL4 cells were centrifuged at 1,008 $\times$ g for 2 min to pellet the cells and the peptide was washed from the EL4 cells with cIMDM. Each well was resuspended in 5 μ l cIMDM and 100 μ l of each of the purified T cell dilutions was added in triplicate to the EL4 cell wells with and without peptide. Media only, T cell only and EL4 only controls were included as positive and negative controls for measuring [3H]-thymidine. The T

cells were left to kill the target EL4-peptide cells for 3.5 h at 37°C before being harvested onto filter paper using a cell harvester (TOMTEC, Germany). Betaplate scintillation fluid (PerkinElmer) was added to the dried filter paper and the beta radioactivity was measured using a 1450 MICROBETA PLUS Liquid Scintillation Counter (WALLAC, PerkinElmer Inc).

To calculate the percentage of antigen-specific killing that had occurred per sample, counts per minute (CPM) were first normalised to the average negative control (EL4 cells only) by dividing each effector to target (E:T) ratio concentration by the average EL4 cells only value from the same harvested plate (termed the sample:neg ratio). The following formula was then used:

$$\% = 100 - ((\text{"sample:neg ratio"} / \text{"Average Naïve sample:neg ratio"}) \times 100)$$

2.13 RNA analysis

2.13.1 RNA extraction

Cells for RNA extraction were washed in DPBS, pelleted by centrifugation at $1,254 \times g$ for 5 min and the supernatant was removed. RNA extraction was then performed using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. The cell pellet was vigorously resuspended in Buffer RLT Plus (provided in the kit; containing 2-Mercaptoethanol) to lyse the cells and the lysate was filtered through a QIAshredder column (provided within the kit) at $13,250 \times g$ for 2 min to completely disrupt cellular particles. The lysate was then added to a genomic DNA column and centrifuged at $8,000 \times g$ for 30 sec to remove genomic DNA from the sample. A total volume of 70% ethanol was added to the flow-through and the lysate plus ethanol were added to an RNeasy spin column (provided in the kit), for RNA extraction, and centrifuged at $8,000 \times g$ for 15 sec to remove all components from the column except for the RNA that was bound to the column. The flow-through was discarded and the RNA sample within the column was washed once with Buffer RW1 and centrifuged at $8,000 \times g$ for 15 sec. The flow-through was discarded and the column was washed twice with Buffer RPE, with centrifugation of $8,000 \times g$ for 15 sec for the first wash and $8,000 \times g$ for 2 min for

the second wash. The column was centrifuged for a further 1 min at $13,250 \times g$ to remove any excess liquid from the column. Warm water was then added directly to the membrane of the RNA column, incubated at room temperature for 1 min to allow the water to bind to the RNA and then centrifuged at $8,000 \times g$ for 1 min to elute the RNA. The eluted water/RNA was transferred to a clean tube, the concentration was calculated using a NanoDrop-1000 Spectrophotometer with NanoDrop-1000 version 3.8.1 software (ThermoFisher Scientific), and the RNA samples were stored at -80°C until required for polymerase chain reaction (PCR) analysis.

2.13.2 Reverse transcription PCR

2.13.2.1 Reverse transcription PCR for real-time quantitative PCR

The I-Script cDNA Synthesis Protocol (Bio-Rad, CA, USA) was used for reverse transcription, following the manufacturer's instructions. Added together in an RNase-free tube was 4 μl of 5X iScript Reaction Mix, 1 μl of iScript Reverse Transcriptase, 250 ng of RNA, and DNase/RNase free water to make a total of 20 μl . The samples were placed in a BioRad iCycle PCR machine with parameters of 5 min at 25°C , 30 min at 42°C , and 5 min at 85°C for the primers to bind, the reverse transcriptase to transcribe the RNA to cDNA, and the inactivation of the reverse transcriptase enzyme. The samples were then held at 4°C .

2.13.2.2 Reverse transcription PCR for PCR array

The RT² First Strand Kit (Qiagen) was used for cDNA synthesis using the manufacturer's instructions. Any contaminating genomic DNA was removed from the samples by first cleaning the RNA samples with a genomic DNA elimination mix. A total volume of 10 μl was compiled using 500 ng RNA, 2 μl of Buffer GE and RNase-free water. The mix was incubated for 5 min at 42°C before being placed on ice for 1 min. For each 10 μl of genomic DNA elimination mix, a corresponding reverse-transcription mix of total volume 10 μl was compiled. The reverse transcription mix consisted of 4 μl of 5X Buffer BC3, Control P2, RE3 Reverse Transcriptase Mix and RNase-free water. The reverse-transcription mix and

genomic DNA elimination mix were added, mixed by pipetting, and incubated for 15 min at 42°C. The sample was then incubated at 95°C for 15 min to inactivate the reverse transcriptase enzyme. Finally, 91 µl of RNase-free water was added to each reaction and the samples placed on ice in preparation for the real-time quantitative PCR array procedure.

2.13.3 Real-time quantitative PCR

2.13.3.1 Real-time quantitative PCR procedure

Real-time quantitative PCR was carried out in 20 µl reaction volumes with 10 µl SYBR Green Master Mix, 3 µl primer, 2 µl sterile distilled water and 12.5 ng cDNA. Primers to 18s ribosomal RNA were used to normalise the amount of nuclear material. The 7500 Real Time PCR System machine with 7500 Software version 2.0.6 (Applied Biosystems, Life Technologies) was used for real-time PCR quantification. The parameters used were 1 cycle of 94°C for 15 min to activate the DNA polymerase and denature the DNA; then 40 cycles of 94°C for 15 s to separate the DNA strands, 55°C for 30 s to allow the primers to anneal, and 72°C for 35 s to allow extension of the DNA strands. The amount of DNA was measured at the end of each cycle of the 72°C step using SYBR green as the fluorescent detector.

Table 2.2. Table of real-time quantitative PCR primers. All primers were from Qiagen.

Gene	Name	Assay name	Reference Sequence accession number
<i>Bcl2</i>	B cell leukaemia/lymphoma 2	Mm_Bcl2_1_SG	NM_009741.4
<i>Casp3</i>	Caspase 3	Mm_Casp3_1_SG	NM_001284409.1
<i>Casp8</i>	Caspase 8	Mm_Casp8_1_SG	NM_001080126.1
<i>Chi3l3</i>	Chitinase 3-like 3	Mm_Chi3l3_1_SG	NM_009892.2
<i>Grwd1</i>	Glutamate-rich WD repeat containing 1	Mm_Grwd1_1_SG	NM_153419.2
<i>Il7r</i>	Interleukin 7 receptor	Mm_Il7r_1_SG	NM_008372.4
<i>Klrg1</i>	Killer cell lectin-like receptor subfamily G, member 1	Mm_Klrg1_1_SG	NM_016970.1
<i>Lyz1</i>	Lysozyme 1	Mm_Lyz1_1_SG	NM_013590.4
<i>Rn18s</i>	18S ribosomal RNA	Mm_Rn18s_2SG	NR_003278.3
<i>Serpine2</i>	Serine (or cysteine) peptidase inhibitor, clade E, member 2	Mm_Serpine2_1_SG	NM_009255.4
<i>Tbx21</i>	T-box 21 (aka Tbet)	Mm_Tbx21_1_SG	NM_019507.2
<i>Tgfbi</i>	Transforming growth factor, beta-induced	Mm_Tgfbi_1_SG	NM_009369.4

2.13.3.2 Real-time quantitative PCR analysis

Excel software was used to analyse the real-time quantitative PCR data. Ct (cycle threshold) values were normalised by calculating the difference between the sample Ct value and the corresponding 18s Ct value, termed the Δ Ct value. The difference in normalised Δ Ct between the untreated and treated cell RNA was calculated, termed the $\Delta\Delta$ Ct, and the fold change of transcript determined by using the equation:

$$\text{Fold change} = 2^{-\Delta\Delta\text{Ct}}$$

2.14 DNA analysis

2.14.1 DNA extraction

For genotyping analysis of the *Batf3*^{-/-} mice, tail tips were collected for DNA extraction. Tail tips were numbed with Emla anaesthetic topical cream (AstraZeneca, UK) 30 min before tail tips were cut off with dissection scissors into 1.5 ml tubes. Adding 200 µl of 50 mM NaOH to each tube and incubating for 1 h and 95°C hydrolysed the tail tips. The samples were left to cool to room temperature before the NaOH was inactivated by 1M Tris-HCl (pH 7.5) neutralising buffer. The concentration of DNA was then measured using the NanoDrop-1000 Spectrophotometer with NanoDrop-1000 version 3.8.1 software.

2.14.2 PCR

The PCR reactions were carried out in 20 µl reaction volumes with 10 µl 2X MyTaq Red Mix (Bioline, UK), 1 µl forward primer, 1 µl reverse primer, 6 µl sterile distilled water and 2 µl of DNA template. Two mixtures were made per sample, one for *Batf3* and the other for the Neomycin insert cassette. The primers (Sigma-Aldrich) used for the PCR reactions are listed in table 2.3.

Table 2.3. Table of PCR primers used for genotyping *Batf3*^{-/-} mice.

Gene target	Primer sequence
Basic leucine zipper transcription factor, ATF-like 3 (<i>Batf3</i>)	Exon 1: Forward: 5-GAACCAGCCGCAGGTATTGG-3 Reverse: 5-ACAAATTGACCGGTCCGACA-3
Neomycin cassette	Forward: 5-CGGTTCTTTTGTCAAGAC-3 Reverse: 5-ATCCTCGCCGTCGGGCATGC-3

The samples were placed in a BioRad iCycle PCR machine. The parameters used for the *Batf3* reaction were 1 cycle of 94°C for 5 min to activate the DNA polymerase and denature the DNA; then 35 cycles of 94°C for 30 s to separate the DNA strands, 64°C for 30 s to allow the primers to anneal, and 72°C for 60 s to

allow extension of the DNA strands; followed by 1 cycle of 72°C for 5 min, then held at 4°C. The neomycin reaction was performed using the same parameters except for using an annealing temperature of 58°C for 30 s instead of 64°C for 30 s.

The final products were then analysed by applying them to wells of a 2% agarose gel and running for 1 h at 100 volts. The DNA products were visualised in the gel by UV excitation of the SYBR Safe DNA Gel Stain reagent using a GelDoc XR and Quantity One software (Bio-Rad).

2.15 Immunofluorescence

Spleens were collected from euthanised mice and frozen in moulds with optimal cutting temperature (OCT) freezing medium (Tissue-Tek Sakura, The Netherlands) using liquid nitrogen. The frozen tissues were stored at -80°C until required. The tissues were cut into 8 µm sections using a cryostat and adhered onto Superfrost Plus microscope slides (ThermoFisher Scientific). The sections were fixed for 5 min using formalin containing 4% paraformaldehyde. The sections were washed with PBS and non-specific antibody binding blocked by incubating for 30 min at room temperature in the dark with 5% goat serum in PBS. To inhibit non-specific binding of streptavidin and biotin, the sections were treated with Streptavidin/Biotin blocking Kit (Vector, In Vitro, New Zealand). This involved washing the sections with PBS, incubating at room temperature in the dark with avidin reagent for 10 min, washing with PBS and incubating at room temperature in the dark with biotin reagent for 10 min. The sections were washed with PBS and incubated with Langerin-biotin antibody in 1% goat serum in PBS at room temperature in the dark. The sections were washed twice with PBS before staining with Streptavidin-AlexaFluor 555 antibody in the dark at room temperature in 1% goat serum. The sections were washed with PBS, excess PBS was removed and then Dapi fluoromount (SouthernBiotech, AL, USA) was added before coverslipping the slides. The fluorescent AlexaFluor 555 and DAPI were visualised using an Olympus BX51 microscope and Cell^F software (Olympus, New Zealand).

2.16 Serum analysis

Analysis of IFN- α in serum was performed using a Flowcytomix kit (eBioscience) following the manufacturer's instructions. Using a 96-well filter plate on a vacuum manifold, the wells were pre-wet with 1X Assay Buffer. The plate was removed from the vacuum manifold and 25 μ l of the serially diluted standards was aliquoted in duplicate into the allocated wells. Assay Buffer was added to the designated "blank" control wells, while 25 μ l of each serum sample was aliquoted into the designated sample wells. A volume of 25 μ l of 1:40 Bead Mixture and 50 μ l of 1:40 Biotin-Conjugate Mixture was added to each well. The plate was protected from light and incubated for 2 h at room temperature on a plate shaker at 500rpm. The wells were then washed twice with Assay Buffer using the vacuum manifold. Removed from the vacuum, 100 μ l of Assay Buffer was added to each well along with 50 μ l of 1:31.25 Streptavidin-PE Solution. The plate was again protected from light and incubated for 1 h at room temperature on a plate shaker at 500rpm. The wells were washed twice with Assay Buffer using the vacuum manifold and the stained beads were resuspended in 500 μ l final volume of Assay Buffer for flow cytometry analysis. The data was analysed using the FlowCytomixPro software (eBioscience) to calculate the concentration of IFN- α compared to the known standard controls.

2.17 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 software (CA, USA). Student's unpaired, two-tailed t-test was used to calculate significance between 2 groups. Between 3 or more samples, one-way analysis of variance (ANOVA) with Tukey's post test was used. When using two parameters with multiple groups, two-way ANOVA with Bonferroni's post test was used. P values of $P < 0.05$ were considered to be significant.

Chapter 3: The importance of splenic langerin⁺ CD8α⁺ dendritic cells for cross-priming CD8⁺ T cell responses to circulating antigens

3.1 Introduction

The induction of potent CD8⁺ T cell responses is important for the elimination of infected or neoplastic tissue, because activated CD8⁺ T cells can differentiate into cytotoxic lymphocytes (CTLs) with antigen-specific targeted cytotoxic function^{267; 268}. The cytotoxic function of the CD8⁺ T cells includes targeted release of perforin and granzymes¹² for killing cells^{104; 105}, and induction of apoptosis via the Fas/FasL pathway^{109; 110}. They can also produce IFN-γ¹⁰², which activates other useful effector cells such as phagocytic macrophages¹⁰³. Antigen presenting cells (APCs), especially dendritic cells (DCs), are responsible for inducing T cell responses through their capacity for acquiring, processing and presenting antigens to specific T cells with all of the co-stimulatory and accessory stimuli required to drive T cell proliferation and differentiation^{99; 118; 119; 134; 269}.

It is now recognised that there is considerable heterogeneity among APCs, including DCs, with significant differences in functional activity that can potentially contribute to the initiation of different types of immune responses, as described in detail in Chapter 1. The induction of CD8⁺ T cell responses to circulating exogenous antigens relies on the ability of APCs to cross-present antigen^{46; 47}. In this process, exogenous antigen is acquired by an APC, and then due to several different possible pathways (reviewed in Chapter 1), enters the major histocompatibility complex (MHC) class I pathway of antigen presentation. In mice, CD8α⁺ DCs in murine spleen have been shown to be superior to other splenic DCs at the process of cross-presentation^{49; 85}, and therefore likely to play a key role in inducing CD8⁺ T cell responses to pathogen-derived antigens, or tumour-derived antigens, that reach circulation. They may also be ideal targets for vaccination strategies.

Another important function of APCs is the production of cytokines, such as IL-12. A heightened capacity to release IL-12 is a feature common to murine CD8α⁺ DCs and has been shown to be important for activating multiple immune cell types including natural killer (NK) cells and invariant natural killer T (iNKT) cells^{28; 29}, and inducing T helper 1 (Th1) CD4⁺ T cell responses³⁰. Importantly, enhanced

activation of T cell help through Th1 and iNKT activation by DCs enhances priming of CD8⁺ T cells^{190; 201}. This makes the CD8α⁺ DCs a critical APC, not only for cross-presentation, but also for CD8⁺ T cell priming.

The intention of the work described in this thesis was to investigate the role of different APCs in eliciting CD8⁺ T cell responses. This knowledge will be useful information for understanding how responses are generated to pathogen-derived antigens, but importantly, may also inform the design of vaccination strategies where CD8⁺ T cell responses are desired, such as in cancer. It was hypothesised that potent CD8⁺ T cell responses would involve CD8α⁺ DCs, but may also involve co-ordinated activity of many different APCs providing the right signals are provided. By concentrating on intravenously administered antigens, the focus was intended to be on the involvement of APCs in the spleen. To this end, it was necessary to develop and explore a model system to induce specific CD8⁺ T cell responses, and to then use new tools, such as specific DC ablation models, to investigate the APCs involved.

Previous work from our laboratory using the *lang*-DTREGFP ablation model has narrowed down the superior functions of cross-presentation and IL-12 production to a subpopulation of langerin-expressing cells within the CD8α⁺ population¹¹⁶. The fact that potent CD8⁺ T cell responses were raised to antigens in the circulation (provided appropriate stimulatory signals were present) suggested the langerin-expressing cells were likely to be in the spleen, as this is a key point of entry of blood borne antigens to the lymphoid tissue. The fact that the langerin⁺ CD8α⁺ DCs could be isolated from spleen and used to stimulate CD8⁺ T cells provided evidence to support this theory. However, there are other langerin-expressing cells in the host^{68; 270; 271}, including langerin-expressing APC in the lung that could participate in responses to circulating antigens, so it remained possible that some of these cells played a role in cross-priming. The work in this chapter addresses this possibility.

Another way to examine the role of different APCs in eliciting CD8⁺ T cell responses was to exploit the fact that toll-like receptor (TLR) expression is varied

across different cell subsets (as reviewed in Chapter 1). Therefore, different TLR agonists can be used to activate a desired APC type, albeit a rather blunt approach given the rather broad expression of some receptors on different immune cells. Previous work from our group had shown that the capacity of DCs to respond to TLR ligation could be significantly improved by initiating interactions with NKT cells using the NKT cell ligand α-galactosylceramide (αGalCer)^{116; 228}. While all DCs in the spleen respond to the NKT cell activation triggered by αGalCer, langerin⁺ CD8α⁺ DCs were the primary subset involved in the enhanced IL-12 production observed, and also the enhanced CD8⁺ T cell responses to co-administered antigens. The work presented in this thesis exploits the different expression profiles of TLR receptors to investigate whether other DCs are, or can be, involved in this response by analysing responses induced in the presence of specific TLR ligands and/or αGalCer.

To accomplish this, it was first necessary to develop and analyse an appropriate model of a CD8⁺ T cell response to study. The initial work in this chapter defines such a model. Previous studies conducted in our laboratory used a model consisting of intravenous administration of chicken egg ovalbumin protein (OVA) with αGalCer^{99; 227}. Here the possibility of using additional TLR ligation was investigated focussing initially on the ligand Pam3Cys, which targets TLR2 and is known to be expressed on all of the major DC populations in the spleen¹⁸³, and then using the TLR ligands resiquimod (TLR7)¹⁶⁸ and Poly I:C (TLR3)¹⁶⁷, which activate CD8α⁻ and CD8α⁺ DCs, respectively¹⁸³, allowing for selective activation of particular subsets.

3.2 Aims

The purpose of this research was to develop and analyse an appropriate model to study the role of different DCs in CD8⁺ T cell responses to intravenous administration of antigens. While prior studies had provided some strong evidence that splenic langerin⁺ CD8α⁺ DCs are the key cell type important for cross-priming T cells, there remained some experimental approaches that could be used to test this further. Therefore, in this chapter the hypothesis that “*the splenic langerin⁺ CD8α⁺ DCs are critical in inducing CD8⁺ T cell responses to systemically administered antigen*”, was subject to further investigation.

Specific aims were:

- To establish a method for inducing antigen-specific CD8⁺ T cell responses to systemically administered antigens
- To define the location and phenotype of the critical DCs involved in this CD8⁺ T cell response
- To conduct an investigation into the role of different DC subsets in this response by exploiting the use of ligands for differentially expressed TLRs

3.3 Results

3.3.1 Intravenous administration of antigen and activatory stimuli induces systemic CD8⁺ T cell responses

Intravenous administration of protein antigens and appropriate activatory compounds can induce systemic CD8⁺ T cell responses. For example, co-administration of antigens with TLR ligands or αGalCer, alone or in combination, can provide the signals needed to stimulate strong CD8⁺ T cell responses¹¹⁶. To establish a sensitive model to explore such responses to the model antigen OVA, naive C57BL/6 mice were donated a small cohort of CD8⁺ T cells from OTI mice, which express a transgenic TCR specific for OVA₂₅₇₋₂₆₄ peptide complexed with the H-2K^b MHC class I molecule^{237; 238}. In this way, it was possible to use flow cytometry with antibodies for the transgenic TCR to enumerate antigen-specific CD8⁺ T cells in blood over time after administration of OVA. The combination of OVA with the TLR2 ligand Pam3Cys and the NKT cell agonist αGalCer has previously been shown to reproducibly produce a potent response that could reliably be measured in the blood, and was therefore used in initial studies. As shown in Figure 3.1A and B, this response peaks at around day 7, with the accumulation of antigen-specific CD8⁺ T cells remaining significantly higher than in controls that had not been provided antigen for more than 20 days. The additive effect of Pam3Cys with OVA and αGalCer, which had previously been reported¹¹⁶, was reconfirmed in this experiment, as shown by the significant increase in the expansion and maintenance of the CD8⁺ T cell response compared to responses induced in the presence of Pam3Cys or αGalCer alone (Figure 3.1B). To rule out the possibility that this response was due to the TLR2 agonist acting directly on the T cells, OTI CD8⁺ T cells were transferred into TLR2^{-/-} mice before stimulating them *in vivo* with OVA, αGalCer and Pam3Cys. No difference was seen between the OVA+αGalCer and the OVA+αGalCer+Pam3Cys groups (Figure 3.1C), suggesting that the enhancing effect of Pam3Cys was not direct stimulation of CD8⁺ T cells, but rather involves other TLR2-positive cells, presumably APCs. The experiments presented in Figure 3.1 are typical of the many experiments conducted throughout this thesis, with statistically significant responses observed to antigens co-administered with different activatory stimuli. However, it should be pointed out

that the overall size of the response varied considerably between individual experiments (note the differences in peak population size Figure 3.1B versus 3.1C), which likely reflects subtle changes in the number and quality of the transferred transgenic T cells.

The intravenous route of administration could permit access to many APC subsets, as well as specific lymphoid-rich tissues, as the antigen and stimuli circulate throughout the blood. Studies conducted earlier within the laboratory suggested that the bulk of activity was mediated by APCs in the spleen^{99; 116}. In this context, it was notable that responses to OVA and Pam3Cys and/or αGalCer administered via the intradermal (Figure 3.2A) and intranasal (Figure 3.2B) routes were negligible. The intramuscular route was able to induce a significant systemic CD8⁺ T cell expansion (Figure 3.2C), however not as strongly as the intravenous route (Figure 3.2D). Also, while the addition of Pam3Cys or αGalCer could increase the CD8⁺ T cell response with the intramuscular administration, the TLR2 agonist and NKT cell agonist could not act synergistically to produce an even greater response. Thus, the intravenous route of free OVA, αGalCer and Pam3Cys combined together is the best method, from the administration routes utilised, for inducing strong systemic antigen-specific CD8⁺ T cell responses.

Figure 3.1 Combining antigen and αGalCer with Pam3Cys enhanced the CD8⁺ T cell response.

(A) Gating strategy for flow cytometric analysis of OVA-specific CD8⁺ T cells. (B) C57BL/6 mice had OTI cells adoptively transferred one day before treatment with the indicated combinations of OVA, αGalCer and Pam3Cys intravenously. Blood was analysed at days 7, 14 and 21 by flow cytometry. The p values indicated on the graph show significant difference compared to the T cell only group. Data shown are n = 5 per group and are from 3 independent experiments that all gave similar results. (C) TLR2^{-/-} mice were adoptively transferred OTI cells 1 day before treatment with the indicated combinations of OVA, αGalCer and Pam3Cys intravenously. Blood was analysed at day 7 by flow cytometry. Data shown are n = 2-5 per group and is from 1 independent experiment. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

Figure 3.2 Intravenous antigen administration is superior to intranasal, intradermal and intramuscular routes to induce systemic CD8⁺ T cell responses.

Mice were adoptively transferred OTI cells 1 day before treatment with the indicated combinations of OVA, αGalCer and Pam3Cys either intradermally (A), intranasally (B), intramuscularly (C), or intravenously (D). Blood was collected at day 7 after antigen administration to analyse the CD8⁺ T cell response by flow cytometry. (A-C) Data shown are $n = 5$ per group and is from 1 independent experiment each. (D) Data shown are $n = 5$ per group and is a representative of 3 independent experiments showing similar results. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, ** $p < 0.01$, *** $p < 0.001$. Statistical significance indicated was in comparison to the OVA only group.

3.3.2 Splenic CD8α⁺ DCs are important for the CD8⁺ T cell response

It remained possible that the intravenous administration route would allow access of the antigen and stimuli to immune-rich sites other than the spleen, such as the lungs and liver. The liver and spleen are both sites that contain high numbers of NKT cells^{224; 272}, therefore making them ideal targets for intravenous αGalCer. The use of splenectomised mice has been previously utilised to assess the dependence on splenic immune cells with intravenous antigens⁸⁹. Therefore, intravenous OVA and αGalCer administration was used on splenectomised mice with the inclusion of the TLR2 agonist Pam3Cys to determine if the splenic cells were required for cross-presenting antigen and responding to the multiple stimuli to cross-prime CD8⁺ T cells. C57BL/6 mice were splenectomised before OTI T cells were transferred and then stimulated *in vivo*. The effect of spleen removal was significant, with a decrease in the CD8⁺ T cell response to almost naïve levels (Figure 3.3). This data therefore suggests that the cells in the spleen are required for generation of a CD8⁺ T cell response to intravenously administered antigen and stimuli.

As mentioned earlier, within the spleen are a population of CD8α⁺ DCs that have been shown to have a strong capacity for cross-presentation of acquired antigens, and therefore play an important role in stimulating CD8⁺ T cells^{49; 85}. The development of CD8α⁺ DCs relies on the expression of the transcription factor Batf3⁷⁷, and in Batf3^{-/-} mice (Figure 3.4A) the CD8α⁺ DC population is largely absent from birth²⁵¹. Intravenous administration of OVA with Pam3Cys and/or αGalCer in these mice failed to induce proliferation of a cohort of transferred OTI T cells, indicating that there was an inability of the remaining APCs to cross-prime the CD8⁺ T cell response (Figure 3.4B). Taken with the results from splenectomised animals, these data suggest that splenic CD8α⁺ DCs are indeed critical cells for inducing the bulk of the CD8⁺ T cell response to systemic antigens.

Figure 3.3 The spleen is required for the systemic CD8⁺ T cell response.

Mice were either splenectomised (SplX) or surgically manipulated without removal of spleen (Sham) and left to recover. OTI cells were adoptively transferred 1 day before intravenous administration of OVA with αGalCer and Pam3Cys. Blood was collected at day 7 after antigen administration to analyse the CD8⁺ T cell response by flow cytometry. (A) Representative dot plots. (B) Graphed data. Data shown are n = 5 per group and is from 1 independent experiment. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, ***p<0.001.

Figure 3.4 The expression of Batf3 is required for the induction of CD8⁺ T cell responses to intravenous antigens.

(A) DNA was extracted from tail tips of mice, and amplified by PCR for analysis of the presence of either Batf3 or the neomycin insertion cassette. Batf3^{+/+} C57BL/6 controls (C1 and C2) show presence of Batf3 compared with Batf3^{-/-} mice (B1-10), and negative water control (Neg). DNA ladders (L) were used to indicate DNA sizes. (B) Mice were adoptively transferred OTI cells 1 day before intravenous administration with the indicated antigen and stimuli. Blood was collected at day 7 after antigen administration to analyse the CD8⁺ T cell response by flow cytometry. Data shown are n = 5 per group and is from 1 independent experiment. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p<0.05, ***p<0.001.

3.3.3 *Langerin-expressing CD8α⁺ DCs are located in the marginal zone of the spleen*

It has been previously published that a subset of splenic CD8α⁺ DCs that express langerin are more efficient at cross-priming CD8⁺ T cells than their langerin-negative CD8α⁺ DC counterparts because they can express high levels of co-stimulatory molecules and cytokines in response to stimulation by TLR ligands, or stimulation through interactions with NKT cells¹¹⁶. Another possible reason is that they are preferentially located in the marginal zone of the spleen, where they can rapidly screen the blood for antigens, and can interact directly with accessory cells such as NKT cells. This was therefore investigated using fluorescence microscopy. The spleen structure contains red and white pulp. The red pulp contains red pulp tissue macrophages for filtering the blood, while the white pulp contains concentrated nodules of B and T cells. Surrounding the white pulp regions are the marginal zone macrophages (SIGN-R1⁺) and marginal metallophilic macrophages (CD169⁺; Figure 3.5A), as well as DCs that can also be found throughout the white pulp^{14; 15; 16}. Using DAPI to stain the nuclei of all cells, and therefore identify the densely populated white pulp area, further staining with a langerin antibody showed a population of langerin⁺ cells as residing in the marginal zone of the spleen (Figure 3.5B and C). This correlated with other published data^{15; 16}, however the antibody could only identify langerin expression in BALB/cByJ background mice and not the C57BL/6 mice (Figure 3.5D). This inability to detect langerin expression in C57BL/6 mice is thought to be due to lower levels of langerin protein²⁴⁶, as discussed in chapter 1.

3.3.4 *Functionally distinct populations of CD8α⁺ DCs based on expression of langerin and CX₃CR1*

The earlier report of Farrand *et al* (2009)¹¹⁶ on the important role of splenic langerin⁺ CD8α⁺ DCs in cross-presentation and IL-12 production had been conducted in *lang*-EGFP and *lang*-DTREGFP mice⁶⁸. The expression of enhanced green fluorescent protein (EGFP) from the langerin promoter in these mice enabled phenotyping of the langerin⁺ and langerin⁻ DC subsets by flow cytometry, while co-expression of the DTR from the langerin promoter in *lang*-DTREGFP mice enabled

specific ablation of the langerin⁺ subset. However, this earlier research was conducted before a third CD8α⁺ population of DCs was identified based on expression of CX₃CR1 (Figure 3.6A) which, unlike most CD8α⁺ DCs, does not rely on Batf3 for development and does not produce IL-12 or cross-present antigen¹⁰⁰. This new observation raised the possibility that the data of Farrand *et al* (2009)¹¹⁶ describing langerin⁺ CD8α⁺ DCs as the most potent cross-priming subset simply represented a difference between the more “conventional” Batf3-dependent CD8α⁺ population and this functionally distinct CX₃CR1⁺ population. To examine whether this was the case, we assessed the phenotype of the different CD8α⁺ subsets in *lang*-EGFP mice, where flow cytometry using a CX₃CL1-human IgG fusion protein in combination with antibodies to CD8α and CD11c, and eGFP expression, could be used to discriminate the cells. In fact, three populations were discerned, suggesting that the langerin⁺ cells in the study of Farrand *et al* (2009)¹¹⁶ comprised two populations; CX₃CR1⁺ cells, and CX₃CR1⁻ cells. The *lang*-EGFP mice were treated with αGalCer and Pam3Cys, or left naïve, and the different DC subsets were analysed for surface expression of CD40 and intracellular IL-12p40 expression. The langerin⁺ CD8α⁺ subset expressed the highest levels of both IL-12p40 and CD40 in response to the stimuli (Figure 3.6B and C). The langerin⁻ CX₃CR1⁻ cells did not exhibit this same response, suggesting that langerin expression does correlate with differences in functional response within the Batf3-dependent CD8α⁺ cells, as the Farrand *et al* (2009) study had implied¹¹⁶.

Previous research in BALB/c mice had indicated that all of the CD8α⁺ DCs in mouse spleen expressed langerin⁹², therefore suggesting that the presence of three CD8α⁺ DC populations observed in *lang*-EGFP was potentially a feature of the C57BL/6 background. The same analysis was therefore conducted in BALB/c mice, this time using an antibody to detect langerin-expressing cells. Flow cytometric analysis revealed that not only were there three populations of CD8α⁺ DCs (langerin⁺, CX₃CR1⁺ and langerin⁻ CX₃CR1⁻), but again it was the population with langerin-expression that had the highest IL-12p40 production upon αGalCer stimulation (Figure 3.6 D and E).

Figure 3.5 Langerin⁺ CD8 α ⁺ DCs are found in the marginal zone of murine spleen.

Sections from frozen BALB/c (A, B and C) and C57BL/6 (D) spleens were stained with target antibody and counter-stained with DAPI for nuclei identification, before the cells were visualised by fluorescence microscopy. Dashed white lines indicate marginal zones around white pulp areas. (A) Section showing CD169-Dy488 (green), along with DAPI (blue). Image is at 10x magnification. (B) Control for langerin antibody staining; stained with SA-A555 (red) without langerin-biotin antibody, along with DAPI (blue). Image is at 40x magnification. (C) Spleen section showing DAPI only (blue), langerin-A555 only (red), and the merged image at 40x. Data presented is a representative of 3 independent experiments. (D) DAPI (blue) and langerin-A555 (red) in merged stain in C57BL/6 at 40x. Data is from 1 independent experiment.

The increase in the OVA peptide-specific CD8⁺ T cell response after OVA administration requires processing and presentation of antigen by cross-presentation. To assess which cells were involved in cross-presentation, mice were treated *in vivo* with cytochrome c, which induces apoptosis when it enters the cytosol. As cells that cross-present antigens are generally efficient at transferring acquired antigens into the cytosol, these cells are ablated preferentially when cytochrome c is administered^{50; 51}. An analysis of the three different splenic DC populations based on expression of langerin and CX₃CR1 24 hours after the administration of cytochrome c showed that the langerin⁺ CD8α⁺ DCs were the only subset significantly depleted (Figure 3.7), suggesting that they have a heightened capacity to transfer exogenous antigens into the cytosol.

The capacity to process and present peptides derived from acquired antigens on MHC class I molecules was then investigated. To examine this, an antibody against the complex of MHC class I H-2K^b with OVA₂₅₇₋₂₆₄ (SIINFEKL) peptide²⁷³ was used to detect the presentation of processed OVA by splenic DCs 15 hours after OVA protein was administered intravenously (Figure 3.8A). Analysis by flow cytometry showed the langerin⁺ CD8α⁺ DCs presented the highest levels of processed peptide, as defined by increased fluorescence expression after anti-H-2K^b/SIINFEKL staining. Small but non-significant increases in fluorescence were seen on the langerin⁻ CD8α⁺ DCs and CD8α⁻ DCs (Figure 3.8B and C). Because of the large amount of OVA protein needed for these experiments, inexpensive commercial OVA was used that may contain low levels of endotoxin. A potential concern was that any endotoxins present may have caused activation in the DCs, perhaps making these cells more prone to non-specific binding of the antibody by the langerin⁺ CD8α⁺ DCs²⁷⁴. To rule this out, another group of mice were treated with TLR4 agonist monophosphoryl lipid-A (MPL) to induce activation without any OVA protein present. No increased binding of the anti-H-2K^b/SIINFEKL was observed on DCs from these animals (Figure 3.8B and C). These data suggest that the langerin⁺ CD8α⁺ DCs have a superior capacity for cross-presentation of acquired antigens.

In summary, the existence of a third CX₃CR1⁺ CD8α⁺ DC subset has not confounded the interpretation that it is the langerin⁺ CD8α⁺ DCs found within the marginal zone of the spleen that responds most effectively to *in vivo* stimulation, and have a heightened capacity for cross-presentation.

Figure 3.6 Langerin⁺ CD8α⁺ DCs express more CD40 and IL-12 in response to stimulation than the other CD8α⁺ DC subsets.

(A and D) Flow cytometry gating strategy for analysing the different CD11c^{hi} DCs in *lang*-EGFP (A) or BALB/cByJ (D) mouse spleen. (B and C) *Lang*-EGFP mice were left naïve or treated *i.v.* with αGalCer and Pam3Cys, 2 h later injected *i.v.* with 250 µg brefeldin A and 4 h later spleens were extracted, CD11c-enriched and analysed by flow cytometry. (B) Percentage of IL-12p40⁺ cells within each DC subset. Data shown are n = 6 per group with 3 experimental repeats compiled. (C) Mean fluorescence intensity of CD40 in each DC subset. Data shown are n = 4 per group with 2 experimental repeats compiled. (E) BALB/cByJ mice were left naïve or treated *i.v.* with αGalCer, 2 h later injected *i.v.* with 250 µg brefeldin A, 4 h later spleens extracted and analysed for percentage of IL-12p40⁺ cells within each DC subset by flow cytometry. Data shown is n = 5 per group and is a representative of 2 independent experiments showing similar results. Statistical analysis was performed using Student's t test, showing mean ± SEM, ***p<0.001, ns = not significant. Unless indicated, statistics in (E) are in comparison to Naïve controls.

Figure 3.7 Langerin⁺ CD8 α ⁺ DCs are depleted by cytochrome c treatment.

Mice were treated *i.v* with 5 mg equine cytochrome c. After 24 h, spleens were harvested for analysis of DC subset numbers by flow cytometry. Data shown are n = 3 per group and is from 1 independent experiment. Statistical analysis performed using Two-Way ANOVA with Bonferroni's post test, showing mean $\pm$ SEM, *p<0.05, ns = not significant.

Figure 3.8 The langerin⁺ CD8α⁺ DC subset has higher cross-presentation ability than other DCs.

Lang-EGFP mice were injected intravenously with either 5 mg OVA, 5 µg MPL, or were left Naïve. After 15 h, spleens were collected, processed and analysed by flow cytometry for SIINFEKL peptide presentation by DCs. (A) Gating strategy of DCs for analysis. (B) Example histograms of the SIINFEKL peptide presentation on H-2K^b molecules of the DC subsets indicated. Histograms indicate Naïve (shaded grey), OVA (Blue) and MPL (red). (C) Graphed data indicating expression of H-2K^b/SIINFEKL on the DC subsets with the indicated treatments. Data shown are n = 2 per group and is a representative of 2 independent experiments showing similar results. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p<0.05.

3.3.5 *Diphtheria Toxin administration into lang-DTREGFP mice depletes langerin⁺ CD8α⁺ DCs within 4 hours, lasting for 3-4 days*

The use of *lang*-DTREGFP mice has been extensively established for analysing the Langerhans cells and langerin⁺ dermal DCs⁶⁸. The use of this depletion model for splenic langerin⁺ CD8α⁺ DCs has also provided data suggesting that these cells are primarily responsible for inducing CD8⁺ T cell responses to circulating antigens¹¹⁶; a key result that underpins this thesis. These earlier studies used two sequential doses of 350ng DT to successfully deplete the majority of the splenic CD8α⁺ DCs¹¹⁶. To be able to fully understand the timeframe of the involvement of splenic langerin⁺ CD8α⁺ DC in immune responses, the depletion kinetics of the *lang*-DTREGFP mouse model was studied in more detail. The DT was administered at different times before the spleens were harvested and the proportions of different DC populations were analysed by flow cytometry. The langerin⁺ CD8α⁺ DCs were depleted by four hours after one dose of 350ng *i.p* (Figure 3.9A). The number of langerin⁺ CD8α⁺ DCs was severely reduced 24 hours after DT administration (data not shown), correlating with the proportion data. Analysing the presence of langerin⁺ CD8α⁺ DCs after longer time points showed that the langerin⁺ CD8α⁺ DCs were still depleted after two days, but repopulation was seen by four days after DT administration (Figure 3.9B). One dose of 350 ng of DT can therefore successfully deplete the langerin⁺ CD8α⁺ DCs in a very short time of less than four hours, and the proportions remain low for over two days.

Figure 3.9 After DT administration langerin⁺ CD8 α ⁺ DCs are depleted by 4 hours and repopulate by 4 days.

Lang-DTREGFP mice were administered DT and spleens were harvested at the indicated timepoints to assess the presence of langerin⁺ CD8 α ⁺ DCs by flow cytometry. (A) Early timepoints to assess length of time until langerin⁺ CD8 α ⁺ DCs are depleted by DT. (B) Late timepoints to assess the length of time until langerin⁺ CD8 α ⁺ DCs return. Data shown are n = 3-5 per group and are from 1 independent experiment each. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, ***p<0.001. Significant differences indicated are in comparison to Naïve controls.

3.3.6 *Langerin⁺ CD8α⁺ DCs are required early in the immune response*

With the kinetics of DT-mediated depletion of langerin⁺ CD8α⁺ DCs defined, it was possible to investigate the kinetics of involvement of these DCs in response to circulating antigens. The antigen-specific CD8⁺ T cell response peaks at day seven after intravenous administration of OVA, αGalCer and Pam3Cys (Figure 3.1; ¹¹⁶), and it had previously been shown that the depletion of langerin⁺ CD8α⁺ DCs by administration of DT for the two days preceding stimulation results in a significant, but not complete reduction in the CD8⁺ T cell response¹¹⁶. The results from Figure 3.9 suggest that the langerin⁺ CD8α⁺ DCs would only have been depleted for 3-4 days in this experiment. It was therefore investigated whether sustained depletion for the full seven days would impact the reduction further. Figure 3.10A shows that the effect of one dose of DT one day prior to stimulation was sufficient to significantly reduce the CD8⁺ T cell response at day seven and that continued administration of DT had no further impact, suggesting the langerin⁺ CD8α⁺ DCs are important very early on in the response. Some DT models, such as the CD11c-DTR model, have shown off-target effects with the use of DT^{256; 275}, so C57BL/6 controls were used to prove that the results seen in Figure 3.10A were from the effect of langerin⁺ DC depletion, as the use of DT on C57BL/6 mice had no effect on the CD8⁺ T cell response (Figure 3.10B). To further define the kinetics of langerin⁺ CD8α⁺ DC involvement in generating the CD8⁺ T cell response to circulating antigens, mice were depleted of langerin⁺ DCs at various timepoints within the first 24 hours of the immune response. These studies showed that langerin⁺ CD8α⁺ DCs are important during the first 6-8 hours after antigen administration to generate the large CD8⁺ T cell response observed at day seven (Figure 3.10C).

Interestingly, previous research has shown that the treatment of mice with αGalCer ultimately results in a reduction of the number of CD8α⁺ DCs, including a reduction in the langerin⁺ subset, by 24 hours²⁷⁶, which was attributed to activation-induced cell death involving TNF-α. Given the early function required by langerin⁺ CD8α⁺ DCs to elicit CD8⁺ T cell responses, the timing of this activation-induced cell death was investigated further. Analysis of the spleen by flow cytometry at

different times after intravenous administration of αGalCer showed significant reduction in langerin⁺ CD8α⁺ DCs as early as six hours (Figure 3.11A). This same effect could also be seen in BALB/cByJ mice (Figure 3.11B), using the langerin antibody to identify the langerin⁺ DCs. Although this is a quick response, there would still be time for the langerin⁺ CD8α⁺ DCs to prime CD8⁺ T cell responses, as indicated by the earlier experiment in Figure 3.9. It should be noted that some langerin⁺ CD8α⁺ DCs do survive for longer periods; the possibility that these cells have been “conditioned” by the stimulatory environment, and can still participate in inducing responses if provided more stimulation is investigated elsewhere in this thesis.

Figure 3.10 Langerin⁺ CD8α⁺ DCs are required early during the immune response.

Lang-DTREGFP (A and C) and C57BL/6 (B) mice were adoptively transferred OTI cells on day -1, before *i.v* administration of OVA with αGalCer and Pam3Cys on day 0. OTI T cell expansion was measured in blood by flow cytometry on day 7. (A and C) Mice were administered PBS (white bars) as a vehicle control, or DT (grey bars) at the indicated day (A) or hour (C) time points relative to vaccination. The hour time points in C were adjusted for the 4 h depletion time determined in Figure 3.9A. Data shown are n = 4-5 per group and are representatives of 2 independent experiments each showing similar results. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. Significant differences indicated are in comparison to PBS controls. (B) C57BL/6 mice were administered DT or the PBS vehicle control 1 day before vaccination, to control for possible off-target effects of DT treatment. Data shown are n = 5 per group and is a representative of 3 independent experiments showing similar results. Statistical analysis was performed using Student's *t* test, showing mean ± SEM, ns = not significant.

Figure 3.11 Treatment with αGalCer depleted langerin⁺ CD8α⁺ DCs.

(A) *Lang*-DTREGFP mice were administered OVA, αGalCer and Pam3Cys intravenously and spleens were harvested from mice at indicated timepoints after vaccination, along with a control that received DT at day -1 (white bar). Presence of langerin⁺ CD8α⁺ DCs was determined by flow cytometry. (B) BALB/cByJ mice were treated with αGalCer and spleens were harvested at the indicated time points to determine the presence of langerin⁺ CD8α⁺ DCs. Data shown are n = 5 per group and are from 1 independent experiment each. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, ***p<0.001. Significant differences indicated are in comparison to 0 h groups.

3.3.7 *Enhancing CD8⁺ T cell responses to circulating antigens with TLR ligation requires the receptor to be expressed by CD8α⁺ DCs*

The potent responses examined in the previous experiments were a result of synergy between signals from activated NKT cells and ligation of TLR2²²⁸. To determine whether integration of these signals was via langerin⁺ CD8α⁺ DCs, or could perhaps involve other APCs, we exploited the differential expression TLRs by subsets of DCs. In particular, we exploited the known differences in expression of TLRs between CD8α⁺ DC and CD8α⁻ DC, thereby subdividing the stimulatory capacities of the major resident DC subtypes in the spleen. While CD8α⁺ DC express both TLR2 and TLR3, they do not TLR7. On the other hand, CD8α⁻ DC express TLR2 and TLR7, but not TLR3⁷⁷. By combining injection of OVA and αGalCer with ligands for these different receptors (Figure 3.12), we showed that OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell responses were enhanced when TLR2 or TLR3 ligands were used (Pam3Cys or PolyI:C, respectively), but not when a TLR7 ligand was used (resiquimod). Therefore, even when stimulated directly, CD8α⁻ DC could not significantly contribute to the response. These experiments were conducted in *lang*-DTREGFP mice, so it was possible to examine the effect of depleting langerin⁺ cells before the antigens were administered. The OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell responses were considerably reduced in the absence of langerin⁺ cells when TLR2 or TLR3 ligands were used, although not completely ablated, implying a limited role for some TLR2⁺ or TLR3⁺ APCs that did not express langerin. Notably, administration of the TLR7 ligand did not enhance CD8⁺ T cell responses over those seen with OVA and αGalCer alone, and absence of langerin⁺ cells almost completely inhibited the T cell response, suggesting it was not possible to invoke the activity of CD8α⁻ DCs to contribute to the CD8⁺ T cell response with this TLR ligand.

Overall, these results suggest that langerin⁺ CD8α⁺ DCs can integrate TLR-mediated signals to improve induction of CD8⁺ T cell responses. In the context of the contemporaneous administration of antigens and ligands assessed here, the introduction of a ligand for a TLR expressed by other CD8α⁻ APCs was unable to contribute to the response.

Figure 3.12 CD8α⁺ DC activation enhances cross-priming, dependent on the langerin⁺ subset.

Lang-DTREGFP mice were administered DT (white bars) or PBS (grey bars), and OTI cells were adoptively transferred, 24 h before they were treated with OVA along with either αGalCer, Pam3Cys, poly I:C, or resiquimod intravenously, as indicated. Blood was collected 7 days after treatment for flow cytometric analysis to assess OTI expansion. Data shown are $n = 5$ per group are representatives of 2-3 independent experiments each showing similar results. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$.

3.3.8 *While other APCs are capable of cross-presentation, langerin⁺ CD8α⁺ DCs are required for cross-priming*

It is possible that the critical role of langerin⁺ CD8α⁺ DCs in supporting cross-priming of CD8⁺ T cells is indirect; that is, they may respond to stimulation in such a manner that the local environment is altered, and other APCs can then contribute to the immune response. While the experiment in figure 3.8 points to a superior ability of langerin⁺ CD8α⁺ DCs to cross-present antigen, it is possible that this assay was not sensitive to detect low level cross-presentation by langerin-negative cells. Indeed, if such langerin-negative cross-presenting cells exist, as has been previously reported^{63; 71}, they may be able to contribute to cross-priming if appropriately conditioned, which may be a downstream role of activated langerin⁺ CD8α⁺ DCs. To assess this, an experimental design was used to examine cross-presentation *in vivo*. To do this, CFSE-labeled OVA_{257–264}-specific CD8⁺ T cells were transferred into animals that had received OVA (and activation stimuli) in hosts that were either langerin⁺ DC-depleted, or left langerin⁺ DC-sufficient, enabling analysis of T cell proliferation in response to cross-presentation in the absence of langerin⁺ CD8α⁺ DCs. Another experimental group was left with langerin⁺ DCs present for the treatment with OVA and stimuli, but then depleted at 12 hours later, before the addition of CD8⁺ T cells (Experimental design is depicted in Figure 3.13A). By assessing T cell proliferation in animals depleted of langerin-expressing cells after administration of antigen and stimuli, it was possible to determine the role of langerin⁺ CD8α⁺ DCs in modifying the immune environment without these cells being able to directly stimulate CD8⁺ T cells.

Assessing proliferation of CFSE-labeled OVA_{257–264}-specific CD8⁺ T cells early (within 3 days) after transfer into antigen-bearing mice is known to be a very sensitive measure of antigen-presentation *in vivo*, reflecting presence of the H-2K^b-binding epitope in the host through cross-presentation²⁴⁰. However, proliferation and accumulation of OVA_{257–264}-specific CD8⁺ T cells is only sustained over longer periods if the antigen has been cross-presented in a stimulatory fashion; that is, the T cells have been cross-primed. Interestingly, in the groups that were depleted of langerin⁺ CD8α⁺ DCs cross-presentation was still observed (Figure 3.13B and C;

measured by CFSE dilution due to cellular division), indicating that APCs other than langerin⁺ CD8α⁺ DCs were able to cross-present. However, by assessing CD8⁺ T cell expansion and accumulation in the blood at day 5 after CD8⁺ T cell transfer, the group that had langerin⁺ CD8α⁺ DCs depleted were unable to efficiently cross-prime the immune response (figure 3.13D). This was in comparison to the large CD8⁺ T cell peak in the group that had langerin⁺ CD8α⁺ DCs present during the entire immune response. It had been hypothesised that the langerin⁺ CD8α⁺ DCs were required to initiate the correct immune environment, and therefore the mice depleted of langerin⁺ CD8α⁺ DCs after 12 hours of stimulation would be able to efficiently prime CD8⁺ T cells. However, this was not the case, as when the langerin⁺ CD8α⁺ DCs were not present at the same time as the CD8⁺ T cells, there was no increase in cross-priming of CD8⁺ T cells detected. This data also indicates that there must be some langerin⁺ CD8α⁺ DCs that survive the early activation-induced cell death observed with administration of αGalCer (Figure 3.11), as the langerin⁺ DCs were still required for directly interacting with the CD8⁺ T cells 24 hours after αGalCer administration. This is explored later in this thesis.

Figure 3.13 Langerin⁺ CD8α⁺ DCs are not the only APC capable of cross-presenting to initiate CD8⁺ T cell division, but are required for optimal cross-priming to induce T cell expansion and accumulation.

C57BL/6 and *lang*-DTREGFP mice were administered OVA, αGalCer and Pam3Cys *i.v* on day -1 and 5x10⁶ CFSE-stained OTI cells were transferred on day 0. Some groups were given DT *i.p* either before manipulation, or 12 h between OVA treatment and OTI transfer. (A) Diagram of the experimental groups. (B and C) Proliferation of OTI cells at day 2 measured by CFSE dilution of the cells present in blood. B, Representative histograms. C, Graphed data. (D) OTI accumulation in blood on days 2, 5 and 7. Data shown are n = 5 per group and is a representative of 2 independent experiments showing similar results. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM ***p>0.001.

3.4 Discussion

The purpose of this research was to study the role of different DCs in inducing CD8⁺ T cell responses to intravenous administration of antigens, with the hypothesis that the splenic langerin⁺ CD8α⁺ DCs are critical in this response. Intravenous administration of antigen with αGalCer to activate NKT cells (which in turn activate APCs), or agonists for TLR activation, induced strong systemic CD8⁺ T cell responses that were shown to be dependent on cross-presenting splenic langerin-expressing CD8α⁺ DCs primarily located in the marginal zone of the spleen, during the first six hours of the immune response. While other APCs were indicated as being capable of some cross-presentation of antigen, it was the langerin⁺ CD8α⁺ DCs that were critically required for efficient cross-priming to increase CD8⁺ T cell accumulation.

The intravenous route of administration results in antigen and stimuli being filtered through several immune-rich organs, including the spleen. Splenectomy of mice prior to T cell stimulation with intravenous OVA and activation stimuli indicated that the splenic cells were critically required for the induction of the systemic CD8⁺ T cell response (Figure 3.3), and use of the CD8α⁺ DC-deficient Batf3^{-/-} mice and the langerin-ablation model indicated a reliance on langerin⁺ CD8α⁺ DCs (Figure 3.4). Therefore, the antigen and stimuli initially passes through the marginal zone of the spleen where the langerin⁺ CD8α⁺ DCs encounter the antigen for cross-presentation. The administration of αGalCer has recently been shown to induce localisation of the NKT cells to the marginal zone of the spleen where the αGalCer is presented by marginal zone APCs²³². Therefore, the langerin⁺ CD8α⁺ DCs are located in an optimal position in the spleen not only for early antigen uptake for cross-presentation, but also to activate NKT cells. The TLR2 agonist is in itself capable of activating APCs to produce increased cytokines and co-stimulatory molecules^{116; 277}. However, this agonist works in cooperation with NKT cell activation to induce superior cytokine release by APCs, specifically the langerin⁺ CD8α⁺ DCs, and enhances co-stimulatory molecule expression and chemokine production^{29; 89; 226}. The NKT cells also produce a multitude of cytokines, including large amounts of IFN-γ and IL-4²⁷⁸. Importantly, NKT cells

have been shown to provide CD40 signalling to enhance APC function in a manner similar to classical T helper cells. The interactions between NKT cells and APCs are likely to induce chemokine production by DCs⁸⁹, facilitating increased recruitment of CD8⁺ T cells. The splenic CD8α⁺ DCs have been identified as an important APC target for this NKT cell interaction⁸⁹, supporting the concept that the langerin-expressing population may be an important APC for interacting with NKT cells to enhance CD8⁺ T cell responses. Further research in our lab has identified an increased expression of the NKT cell-induced chemokine CCL17⁸⁹ and of CXCL10, the ligand for CXCR3⁺ CD8⁺ T cells²⁷⁹, by the langerin-expressing population after αGalCer administration *in vivo* (Petersen *et al*, *unpublished data*), therefore suggesting the langerin⁺ CD8α⁺ DCs may be important for attracting CD8⁺ T cells to the site of antigen presentation for enhanced CD8⁺ T cell responses. The langerin⁺ CD8α⁺ DCs have also been shown to migrate to the T cell zone after antigen encounter¹⁶, where they would likely have increased interactions with CD8⁺ T cells to enhance CD8⁺ T cell responses. Overall, activated langerin⁺ CD8α⁺ DCs may serve to increase CD8⁺ T cell accumulation at the antigen presentation site, followed by increased delivery of stimuli for priming.

The administration of this same antigen and stimuli combination via routes other than intravenous delivery was not so effective at stimulating T cell responses (Figure 3.2). The alternative routes would require increased doses of stimuli to compensate for the reduced infiltration of the components through the tissues. For example, Courtney *et al*²⁸⁰ used 2 μg αGalCer per mouse via the intranasal route to activate NKT cells without causing anergy that results from systemic administration; this is in comparison to the 200 ng dose used in this thesis research. The cells that would gain access to the antigen and stimuli may also not require the same stimuli, as different cells show preferences for different stimuli^{165; 181; 182}. While the intradermal and intranasal routes were not effective at inducing a systemic response, the intramuscular route was able to induce a small but significant response (Figure 3.2C). This route would allow filtration of the antigen and stimuli slowly into the bloodstream, therefore acting as a slow release intravenous administration²⁸¹. The effect of the intramuscular route gives translatable significance to this research, as the intramuscular route is a common route of administration for multiple disease

treatments in humans^{282; 283}. However, for the purposes of this research, where a strong systemic response is required for the study of the APCs involved in cross-priming, the intravenous route was preferable. The intravenous administration of OVA antigen with αGalCer and Pam3Cys induced very strong measurable CD8⁺ T cell responses, therefore allowing more in-depth study into the T cell response and mechanisms inducing this response.

The splenic CD8α⁺ DC population has previously been attributed the role of the optimal cross-priming DC subset, however the research shown in this thesis, as well as further research undertaken by Petersen *et al* (*manuscript in preparation*), has indicated that there are three phenotypically and functionally distinct populations of CD8α⁺ DCs. The CX₃CR1⁺ CD8α⁺ DCs do not cross-present antigen and do not provide co-stimulation or IL-12 production. Meanwhile, the langerin⁺ CD8α⁺ DCs are major IL-12 producers and antigen cross-presenters in comparison to the langerin⁻ CX₃CR1⁻ CD8α⁺ DCs. These langerin⁻ CX₃CR1⁻ CD8α⁺ DCs may be precursors to the langerin-expressing population, as they have the ability to develop langerin expression *in vivo*, and the functions associated with langerin⁺ CD8α⁺ DCs, such as increased phagocytosis of dying cells, upregulation of CD86 and CD40, and increased IL-12 production (Petersen *et al*, *manuscript in preparation*). This indicates that the langerin⁻ CD8α⁺ DCs have the potential to be optimal cross-priming APCs, but it is only once they have acquired this langerin⁺ differentiated state that they are capable of high stimulation and cross-presentation for cross-priming. Analysis of the three different populations was also confirmed in BALBc/ByJ mice with similar population proportions to the C57BL/6 mouse background populations, contrasting previous research that showed all CD8α⁺ DCs to be langerin-positive⁹². It is possible that the included analysis of CX₃CR1 expression refined the analysis of the different CD8α⁺ DC populations. Therefore, this research is applicable to other mouse strains and not limited to only the *lang-DTREGFP* and *lang-EGFP* mouse models. This research could also indicate that the human equivalent of the CD8α⁺ mouse DC, the CD141⁺ DC^{284; 285}, is more functionally equivalent to specifically the langerin⁺ CD8α⁺ DC subset in mice, and therefore research focussing on the langerin-expressing population may be more

indicative of the immune functions of human CD141⁺ DCs. Mechanistic studies of cross-presentation may benefit from focussing on langerin-expressing DCs.

For studying langerin⁺ cells, researchers have previously used the *lang*-DTREGFP mouse model⁶⁸. However, due to the extensive research focused on Langerhans cells when using this depletion model, there has not been as widely studied kinetics of the lymphoid tissue resident langerin⁺ CD8α⁺ DCs. Therefore, the depletion model needed to be defined to be able to critically assess the exact timed involvement of these DCs. It was determined that a dose of 350 ng DT *i.p.*, a third of the 1 µg dose used for depleting Langerhans cells and dermal langerin⁺ DCs⁶⁸, was enough to deplete the splenic langerin⁺ DCs by four hours after administration, with the depletion being maintained for a period of 2-4 days (Figure 3.9). Also of note was that the DT could be given for multiple doses over the period of a week without any ill effects observed in the mice²⁸⁶. This was unlike the *CD11c*-DTR mice that have shown toxic side effects of multiple DT doses^{256; 275}. By applying these known depletion times to a time-course depletion experiment using OVA antigen and stimuli to stimulate the CD8⁺ T cell response, it was possible to determine that the langerin⁺ CD8α⁺ DCs were only required within the first 6-8 hours of the immune response (Figure 3.10). This also established that for further research in this thesis, the maintained depletion of the langerin⁺ CD8α⁺ DCs was not required, as they only needed to be depleted during the initial antigen administration.

Previous study using αGalCer has indicated that the αGalCer stimulation of NKT cells results in a reduction of the CD8α⁺ DCs, and the langerin⁺ CD8α⁺ DCs, within 24 hours of administration²⁷⁶. In this thesis research, the reduction of langerin⁺ CD8α⁺ DCs could be detected as early as 6-12 hours (Figure 3.11). Combined with the data from Figure 3.10, showing that the langerin⁺ CD8α⁺ DCs were only required during the first 6-8 hours of the immune response after administration of αGalCer, this suggested that the langerin⁺ CD8α⁺ DCs carry out their important function early in the immune response before their activity is inhibited by a reduction in the population. This is most likely due to αGalCer stimulation of NKT cells causing a release of cytokines (such as TNF-α)²⁷⁶ that are

toxic to the heavily activated and matured langerin⁺ CD8α⁺ DCs and the activity of the langerin⁺ CD8α⁺ DCs is therefore negatively regulated by the activated NKT cells to prevent further activation. While there is a major reduction in the proportion of langerin⁺ CD8α⁺ DCs, it is possible that some cells remain and are able to influence the immune response. This is investigated later in this thesis.

The preferential cross-priming of the langerin⁺ CD8α⁺ DC population is not a splenic DC-specific phenomenon, as it is a common occurrence for CD103⁺ DC populations in many tissues, not just the langerin⁺ CD8α⁺ CD103⁺ DCs in the spleen, to show preferential acquisition of antigen and presentation to T cells. For example, in the skin and lung the CD103⁺ and CD11b⁺ DCs have preferences for acquiring, transporting, and presenting antigens to induce T cell responses in the draining lymph node^{71; 72; 73}. This has also been shown in the gut and islet DC populations, with the CD103⁺ DCs showing preference for antigen acquisition and migration to lymphoid tissue for T cell stimulation compared to the CX3CR1⁺ CD11b⁺ DCs^{287; 288}. This is most likely due to a similar developmental relationship of the various cross-presenting CD103⁺ populations throughout the different tissues, as they are all developmentally dependent on the transcription factors Batf3 and Irf8, and have similar gene expression profiles⁷⁷. However, there is also evidence of other DCs, such as pDCs, possessing a capacity for antigen cross-presentation⁶³. The involvement of other APCs versus langerin⁺ CD8α⁺ DCs in the antigen cross-presentation and priming functions was examined (Figure 3.13) and it was observed that, while other APCs were capable of antigen cross-presentation to initiate CD8⁺ T cell proliferation, it was the langerin⁺ CD8α⁺ DCs that were critical for interpreting the multiple stimulatory signals to efficiently cross-prime the CD8⁺ T cells.

The majority of data described in this chapter was using the TLR2 agonist Pam3Cys, which is expressed on multiple cell types including CD8α⁺ and CD8α⁻ DCs in the spleen¹⁸³. Using the TLR3 agonist Poly I:C also showed a dependence on langerin⁺ CD8α⁺ DCs for cross-priming (Figure 3.12). However, when the mice were administered the TLR7 agonist resiquimod to specifically activate the TLR7-expressing CD8α⁻ DCs, the DCs were not able to increase priming. In fact, CD8⁺ T cell responses observed in the animals treated with OVA, αGalCer and resiquimod

resulted only from the response to OVA and αGalCer by langerin⁺ CD8α⁺ DCs, as expected from previous experiments. These results indicated a strong dependence on the langerin⁺ CD8α⁺ DCs to mediate the immune interactions occurring in the spleen after contemporaneous administration of multiple stimuli, as the CD8α⁻ DCs were not able to contribute to the immune response without the langerin⁺ CD8α⁺ DCs present. However, it may be possible to administer the stimuli and antigen at different times to firstly determine if the langerin⁺ CD8α⁺ DCs can be involved in the immune response at later timepoints during the immune response, and secondly to determine if the activation status of other DC populations can be altered to increase CD8⁺ T cell priming. This is explored later in this thesis.

In summary, in this chapter it was established that systemic CD8⁺ T cell responses to circulating antigens were severely reduced in the absence of splenic langerin⁺ CD8α⁺ DCs for the first 6-8 hours after antigen and stimuli were administered. These data therefore provide supportive evidence for the hypothesis that the splenic langerin⁺ CD8α⁺ DCs are important for cross-priming CD8⁺ T cell responses.

Chapter 4: Conditioned langerin⁻ DCs can contribute to CD8⁺ T cell priming after initial cross-priming by langerin⁺ CD8 α ⁺ DCs

4.1 Introduction

Splenic CD8 α^+ DCs are an important antigen presenting cell (APC) type for inducing strong systemic CD8⁺ T cell responses. Evidence provided in this thesis, together with an earlier published study, suggest that this function is largely due to the langerin-expressing cells within the CD8 α^+ dendritic cell (DC) population¹¹⁶. Analysis of langerin⁺ CD8 α^+ DCs has shown higher levels of co-stimulatory molecules both in the steady state and in response to activation. They are also sensitive to cytochrome c exposure *in vivo*, suggesting acquired antigens are readily transferred into the cytosol, which is a key feature required for cross-presentation. However, it was shown in Chapter 3 that langerin⁻ APCs that are also capable of cross-presenting antigen, but were unable to prime the antigen-specific CD8⁺ T cell response.

The ability of the langerin⁻ APCs to cross-present, but not prime CD8⁺ T cell responses was possibly due to lack of appropriate, and timely, activation stimuli such as the NKT cell-mediated signals and various toll-like receptor (TLR) agonists^{183; 289} used in the previous chapter. It has been shown that timing can be a major factor in eliciting immune responses. For example, the introduction of TLR stimulation before antigen exposure has been shown to inhibit antigen uptake by APCs *in vitro*²⁹⁰. Also, the introduction of type I IFN early to immature DCs before they have experienced CD40 to CD40-ligand (CD40L) interactions with CD8⁺ T cells or NKT cells can inhibit the ability of these DCs to produce IL-12. In comparison, exposure of type I IFN to mature DCs that have already experienced CD40 to CD40L interactions causes these DCs to become strong producers of IL-12 for T cell priming²⁹¹. Recent research has indicated that through α GalCer administration to activate natural killer T (NKT) cells, plasmacytoid DCs (pDCs) can become activated to aid in the cross-priming of conventional DCs (cDCs)²³⁴. In this respect, it may be possible that activated NKT cells provide signals to “condition” other DC populations within the langerin-negative fraction, and therefore these cells may respond more effectively to delayed administration of activatory stimuli. This may be the case in a natural biological system, where a host may be exposed to pathogen-derived glycolipids that trigger the rapid function that

is typical of NKT cells, and subsequently receive other stimulatory signals associated with the pathogen, such as TLR agonists.

Therefore, this chapter investigates the possibility that through changes in timing of stimuli, it may be possible to invoke contributions of langerin⁻ APCs to the CD8⁺ T cell response, and that the response induced may ultimately reflect coordinated involvement of both langerin⁺ and langerin⁻ APCs.

4.2 Aims

The purpose of this research was to identify if other DC subsets were capable of contributing to CD8⁺ T cell responses, or if the langerin⁺ CD8 α ⁺ DCs were irreplaceable. The involvement of various DCs was investigated to determine the coordinated relationships between different cells for enhanced CD8⁺ T cell responses. It was hypothesised “*that langerin⁻ DCs were capable of contributing to CD8⁺ T cell responses to circulating antigen under the appropriate stimulatory conditions*”. In particular, the timing and influence of activated NKT cells as a powerful and rapid source of activatory signals, together with TLR stimulation, was investigated in this context.

Specific aims were to:

- Determine if activation of NKT cells can create a “conditioned” immune environment that permits langerin⁻ DCs to be further activated with TLR stimulation to contribute to the induction of CD8⁺ T cell responses to circulating antigen
- Investigate the roles of langerin⁺ DCs and langerin⁻ DCs in this context

4.3 Results

4.3.1 *Langerin⁺ APCs can enhance the CD8⁺ T cell response if appropriately conditioned and activated*

The results in Chapter 3 indicated that while langerin⁺ CD8α⁺ DCs are the optimal cross-priming cell type, other APCs are also able to cross-present. This suggests they have potential to aid in cross-priming of CD8⁺ T cells, but perhaps only if they could be appropriately activated. Previous work in our laboratory, using varied administration times of stimuli, has indicated that the TLR2 agonist Pam3Cys can be administered 24 hours after antigen and αGalCer to induce either the same, or even higher, CD8⁺ T cell responses than if the stimuli had been administered simultaneously, suggesting that signals from activated NKT cells had created a conditioned environment in which APCs remained sensitive to TLR ligation. This conditioning period was lost between 24 and 48 hours after αGalCer had been administered (Farrand *et al*, unpublished observation). This experiment was repeated here, with “pre-conditioning” with αGalCer at the time of administration of OVA, creating an environment in which administration of Pam3Cys 24 hours later actually induced a stronger CD8⁺ T cell response than if the stimuli were administered together; importantly the response was always stronger with the combination, regardless of timing, than with αGalCer alone (figure 4.1A). Interestingly, if the langerin⁺ CD8α⁺ DCs were depleted at 12 hours after OVA and αGalCer were administered, the absence of langerin⁺ CD8α⁺ DCs impeded the ability of the Pam3Cys to increase priming of CD8⁺ T cells. This suggested that the langerin⁺ CD8α⁺ DCs were important for directly responding to the TLR agonist in this case. It was therefore investigated whether different TLR agonists could be used in a similar model to invoke the activity of other conditioned DCs, including langerin⁺ DCs.

Similarly to the stimuli used in Chapter 3, the TLR3 agonist Poly I:C was used to stimulate TLR3⁺ CD8α⁺ DC, while the TLR7 agonist resiquimod was used to stimulate TLR7⁺ CD8α⁺ DCs. The experimental setup was similar to that described above, with OVA and αGalCer administered 24 hours before administration of the TLR agonists. Pre-treatment with αGalCer before stimulation

of CD8 α^+ DCs with Poly I:C resulted in an increased response over administering the stimuli simultaneously, although this did not reach statistical significance. However, as expected the T cell response was dependent on langerin⁺ CD8 α^+ DCs (Figure 4.1B). In contrast, using resiquimod after α GalCer caused a significant increase in the CD8⁺ T cell response (Figure 4.1C). It was observed that this treatment had a severe toxic effect in some of the animals, suggesting the occurrence of a cytokine storm from the enhanced CD8 α^- DC activation. Importantly, when the stimuli were administered simultaneously, there was no such benefit to the CD8⁺ T cell response compared to α GalCer with antigen alone. This suggests that signals from activated NKT cells in response to α GalCer had created a conditioned environment that enabled TLR7⁺ CD8 α^- DCs to contribute to the cross-primed response at this later timepoint. Indeed, the increase observed with the addition of resiquimod was shown to be independent of langerin⁺ CD8 α^+ DCs, and hence likely to be through conditioned langerin⁻ cells, as langerin⁺ DC depletion before resiquimod administration did not reduce the CD8⁺ T cell response.

Further to the increased accumulation of antigen-specific CD8⁺ T cells observed in blood, the functional capacity of the T cells was also examined. Spleens were extracted at the peak of the CD8⁺ T cell response at day seven and the antigen-specific T cells were re-stimulated with SIINFEKL peptide to increase cytokine production by the activated cells. The administration of α GalCer with OVA antigen resulted in a functional CD8⁺ T cell response, as indicated by intracellular production of IFN- γ and TNF- α (Figure 4.2). However, pre-conditioning of langerin⁻ DCs via α GalCer administration 24 hours prior to resiquimod administration resulted in an enhanced cytokine production. While this experiment was only performed once and therefore can only give an indication of the functional activity of the CD8⁺ T cells, it indicated a trend towards an increased functional antigen-specific CD8⁺ T cell response. Therefore, pre-conditioned langerin⁻ DCs can contribute towards both increased antigen-specific CD8⁺ T cell accumulation and increased functional capacity of the accumulated CD8⁺ T cells.

Figure 4.1 Langerin⁺ APCs are capable of aiding in cross-priming if pre-conditioned with αGalCer.

OTI cells were transferred into *lang*-DTREGFP mice 24 h before co-administration of OVA and αGalCer, along with either Pam3Cys (A), Poly I:C (B), or resiquimod (C) i.v, as indicated. Twelve h after OVA and αGalCer, DT (white bars) or PBS (grey bars) was given i.p and 12 h later again the indicated groups received TLR agonist (Delayed TLR ligand). Blood was collected 7 days after OVA administration for flow cytometric analysis to assess OTI expansion. Data shown are $n = 5$ per group and is representative of 2-3 independent experiments showing similar results. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$.

Figure 4.2 Pre-conditioned APCs enhance the functional CD8⁺ T cell response.

OTI cells were transferred into C57BL/6 mice 24 h before administration of OVA and αGalCer, with resiquimod administered 24 h after. Spleens were collected 7 days after OVA administration, OTI cells re-stimulated with 5 μg/ml SIINFEKL peptide incubation for 5.5 h in the presence of brefeldin A. Intracellular cytokine expression was analysed by flow cytometry. (A) Example flow cytometry dot plots. (B and C) Numbers of IFN-γ (B) and TNF-α (C) positive OTI cells. Data shown are n = 3-4 per group and is from 1 independent experiment. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p<0.05.

4.3.2 *Pre-conditioning of langerin⁻ APCs results in langerin-independent activation of all splenic DC subsets*

The pre-conditioning of CD8 α ⁻ DCs mediated by NKT cells in response to α GalCer before stimulation with the TLR7 agonist resiquimod enhances CD8⁺ T cell priming (as shown in figure 4.1 above). To assess what this temporal arrangement of stimuli had on DC phenotype, mice were administered α GalCer followed by stimulation with the TLR7 agonist resiquimod 24 hours later. Six hours after resiquimod administration, flow cytometry was then used to analyse intracellular IL-12p40 as an indicator of activation of the splenic DC subsets; this included DCs defined on the basis of both langerin and CX₃CR1 expression. In the absence of resiquimod administration, langerin⁺ CX₃CR1⁻ CD8 α ⁺ DCs produced the most IL-12p40 in response to α GalCer alone. Interestingly, administration of resiquimod together with α GalCer failed to induce significant IL-12p40 in any DCs. However, pre-conditioning by activating NKT cells altered the response to resiquimod, with all of the DC subsets becoming capable of producing IL-12p40, including subsets that are TLR7⁺ (CD8 α ⁻ DCs) and TLR7⁻ (langerin⁻ CD8 α ⁺ DCs) (Figure 4.3). These data suggest that direct TLR ligation was not required for the IL-12 response, implying that conditioning helped influence release of a soluble mediator that triggered IL-12 production.

It was possible that the langerin⁺ CD8 α ⁺ DCs were directly responsible for the pre-conditioning factor(s) that enabled langerin⁻ DCs to be further activated. To investigate this, splenic DCs in *lang*-DTREGFP mice were pre-conditioned by administration of α GalCer 24 hours before addition of resiquimod. By treating with DT either before addition of α GalCer or before addition of resiquimod, it was possible to determine the importance of langerin⁺ CD8 α ⁺ DCs at the different stages in activation of the splenic DC subsets. Analysis of intracellular IL-12p40 within the CD8 α ⁺ and CD8 α ⁻ DC subsets after stimulation showed that, as expected, CD8 α ⁺ DCs were the main manufacturers of IL-12p40 in response to α GalCer. As in the previous experiment (Figure 4.3), significant proportions of CD8 α ⁻ DCs produced IL-12p40 in response to stimulation when the animals had been pre-conditioned by NKT cell activation. However, the depletion of langerin⁺ CD8 α ⁺

DCs both before and after α GalCer administration had no impact on this response, suggesting that langerin⁺ CD8 α ⁺ DCs were not directly responsible for creating the conditioned environment that enhanced responses to the delayed administration of TLR7 agonist (Figure 4.4).

Figure 4.3 Pre-conditioning DCs with αGalCer results in increased activation in all DC subsets with delayed administration of TLR7 agonist.

Lang-EGFP mice were left naïve or treated *i.v.* with αGalCer and/or 10 μg resiquimod, as indicated, or with the resiquimod delayed by 24 h from αGalCer administration. A) Two h later mice were injected *i.v.* with 250 μg brefeldin A. Four h later spleens were extracted, CD11c-enriched by AutoMACS enrichment, and analysed by flow cytometry for percentage of IL-12p40⁺ cells within each DC subset (as in chapter 3). Data shown are n = 4-6 per group and represents 2 experiments compiled. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p>0.05, **p>0.01, ***p>0.001. Unless indicated, statistical significance is in comparison to Naïve populations.

Figure 4.4 Pre-conditioning allows activation of all DCs in a langerin⁺ DC-independent manner.

Lang-DTREGFP mice were left naïve or treated *i.v.* with αGalCer and 10 μg resiquimod delayed by 24 h from αGalCer administration. Indicated groups received DT *i.p.* in conjunction with stimuli, or 12 h between αGalCer and delayed resiquimod. Two h later mice were injected *i.v.* with 250 μg brefeldin A. Four h later spleens were extracted, CD11c-enriched and analysed by flow cytometry for percentage of IL-12p40⁺ cells within each DC subset. Data shown are n = 4 per group and represents 2 independent experiments compiled. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p>0.05. Statistical significance is in comparison to Naïve controls.

4.3.3 *Plasmacytoid DCs are partially responsible for the increased activation of the DC subsets after pre-conditioning*

Recent studies^{234; 292} have identified an important interaction between pDCs and NKT cells, which through CD1d- α GalCer-TCR binding and O_x40-O_x40L interactions, leads to increased activation of pDCs. This pDC activation has been shown to enhance the cross-priming ability of conventional DCs for increased CD8⁺ T cell responses, through the release of type I IFN²³⁴. Therefore, the pre-conditioning induced by administration of α GalCer in the previous experiments may have involved NKT cell-mediated activation of pDCs. As pDCs express TLR7, it is possible that these cells were also critical for triggering the heightened responses observed to delayed resiquimod, potentially through the production of IFN- α .

To identify the involvement of pDCs, *siglecH*-DTR mice were used so that SiglecH⁺ pDCs could be depleted by administration of DT. Initial flow cytometric analyses were conducted to assess intracellular IL-12p40 as an activation marker. While injection of α GalCer alone unexpectedly did not induce significant IL-12p40 production by langerin⁺ CD8 α ⁺ DCs in these animals, pre-conditioning with α GalCer followed by treatment with resiquimod resulted in significantly enhanced proportions of IL-12p40 producing CD8 α ⁺ and CD8 α ⁻ DCs. Interestingly, the depletion of pDCs resulted in a reduction of IL-12p40 producing CD8 α ⁺ DCs (Figure 4.5). These results suggest two things. First, TLR7⁺ CD8 α ⁻ DCs needed to be pre-conditioned before they could be activated to release IL-12 by direct stimulation with resiquimod. Second, enhanced IL-12p40 production by TLR7⁺ CD8 α ⁺ DCs in response to resiquimod was reliant on the activation of pDCs.

Previous studies²⁹¹ have identified the importance of appropriate timing of type I IFN exposure on the activation of DCs. The exposure of IFN- α to immature DCs inhibits the CD40-CD40L interaction, resulting in reduced IL-12 production. Conversely, exposure of IFN- α to mature DCs that have already experienced the CD40-CD40L interaction induces a strong IL-12 response, resulting in improved CD8⁺ T cell cross-priming. Because of the high production of type I IFN by pDCs^{65; 66}, this indicated a possible mechanism by which the pDCs are involved in

aiding the cross-priming effect of the CD8 α^+ DCs. Therefore, the amount of IFN- α in mouse serum six hours after administration of resiquimod was analysed to determine if the pre-conditioning by activation of NKT cells improves type I IFN production. It was highly evident that, while resiquimod alone or α GalCer with resiquimod was only able to induce a small amount of IFN- α , the pre-conditioning effect of α GalCer before stimulation with resiquimod greatly enhanced IFN- α production (Figure 4.6A). Using *lang*-DTREGFP mice, it was shown that langerin⁺ CD8 α^+ DC depletion did not impact the production of IFN- α (Fig 4.6B). Instead, as hypothesised, the pDCs were the cell subset responsible for the increased type I IFN, as shown by depletion of the SiglecH⁺ pDCs in *siglecH*-DTR mice causing a significant decrease in the serum IFN- α levels (Fig 4.6C).

Figure 4.5 Langerin⁺ CD8α⁺ DC-independent pre-conditioning of DCs is mildly dependent on pDCs.

The pDCs were depleted by treatment of *siglecH*-DTR mice with DT *i.p* on days -1 and -2, or were treated with PBS as a control (as indicated on graph). Mice were then left naïve or on day 0 were treated *i.v* with αGalCer and/or 10 μg resiquimod, as indicated, or with the resiquimod delayed by 24 h from αGalCer administration. Two h later mice were injected *i.v* with 250 μg brefeldin A. Four h later spleens were extracted, CD11c-enriched by AutoMACS enrichment, and analysed by flow cytometry for percentage of IL-12p40⁺ DCs. Data shown is n = 4 and is representative of 3 independent experiments showing similar results. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean ± SEM, *p>0.05, **p>0.01.

Figure 4.6 Delaying TLR7 agonist activates all DCs in a pDC-dependent manner via IFN- α .

C57BL/6 (A), *lang*-DTREGFP (B) or *siglecH*-DTR (C) mice were left naïve or were treated *i.v* with α GalCer and/or 10 μ g resiquimod, as indicated, or with the resiquimod delayed by 24 h from α GalCer administration. Indicated groups received DT *i.p.* in conjunction with stimuli, or 12 h between α GalCer and delayed resiquimod. Mice were bled 6 h later to collect serum. IFN- α was measured by flow cytometry using a flowcytomix fluorescent bead assay kit. Data shown are $n = 3$ -5 per group and is representative of either 2 independent experiments showing similar results (A) or 2 experiments compiled (B and C). Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, ** $p < 0.01$, *** $p < 0.001$.

4.3.4 *Pre-conditioning of langerin⁻ APCs does not result in a fully langerin-independent CD8⁺ T cell response*

The data from earlier in this chapter had indicated that langerin⁻ APCs preconditioned in the presence of NKT cell activation can be activated with delayed TLR7 agonists to increase T cell priming, and that this TLR-driven activity was independent of langerin⁺ CD8 α ⁺ DCs (figure 4.4 and 4.1C). However, it was unknown if this phasing of stimuli made the whole response independent of langerin⁺ CD8 α ⁺ DCs. To address this, *lang*-DTREGFP mice were depleted of langerin-positive cells 24 hours before pre-conditioning and therefore the langerin⁺ CD8 α ⁺ DCs were not present for any involvement in the CD8⁺ T cell response. Interestingly, it was found that without the initial interactions with langerin⁺ CD8 α ⁺ DCs, the CD8⁺ T cell response was severely reduced (Figure 4.7). This indicated that the langerin⁺ CD8 α ⁺ DCs were still critically required for initiating the CD8⁺ T cell cross-priming, and that it was only after this event that conditioned langerin⁻ APCs could be triggered to aid in development of the T cell response.

Figure 4.7 Pre-conditioning of DCs does not abolish langerin⁺ CD8 α ⁺ DC-dependent cross-priming of CD8⁺ T cells.

OTI cells were transferred into *lang*-DTREGFP mice and they were administered either 350 ng DT or 200 μ l PBS *i.p.* The next day, mice were administered OVA and α GalCer *i.v.* One day later, mice received 6.67 μ g resiquimod. Blood was collected 7 days after OVA administration for flow cytometric analysis to assess OTI accumulation. Data shown are $n = 3-5$ per group and is representative of 2 independent experiments showing similar results. Statistical analysis performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, *** $p > 0.001$.

4.4 Discussion

Splenic langerin⁺ CD8 α ⁺ DCs are critically involved in inducing CD8⁺ T cell responses to circulating antigen¹¹⁶, however the potential involvement of other DCs, particularly in the context of multiple stimuli, is relatively undefined. Given the rapid function of innate-like T cells such as NKT cells in response to activation with specific ligands, it was assessed here whether langerin⁺ DCs could be conditioned by the stimulatory environment caused by NKT cell activation to become capable of contributing to driving CD8⁺ T cell responses. This was confirmed in figure 4.1 and 4.2, where administration of resiquimod 24 hours after antigen and α GalCer resulted in an increase in both CD8⁺ T cell accumulation and function, suggesting TLR7-expressing cells can become involved in the response. In fact, the stimulatory environment caused by NKT cell activation effectively conditioned all of the DCs examined to exhibit an increased activation response to the TLR7 agonist, which was characterised by increased percentages of IL-12 producing cells including the TLR7-negative CD8 α ⁺ DC populations (Figure 4.3). The “conditioning factor” was not dependent on langerin-expressing cells being present at the time the TLR agonist was given (Figure 4.4). Using *siglecH*-DTR mice, it was shown that pDCs that were conditioned by NKT cell activation were involved in the generally increased activation response, specifically the enhanced IL-12 response of CD8 α ⁺ DCs (Figure 4.5). The pDCs themselves responded more actively to the delayed TLR7 stimulation by producing significantly enhanced levels of IFN- α , which was possibly the factor that drove the enhanced cytokine production by CD8 α ⁺ DCs (Figure 4.5 and 4.6). The CD8 α ⁺ DCs were also conditioned to have an enhanced response to subsequent TLR7 stimulation, which could be mediated directly through the TLR (Figure 4.5). However, while the conditioning caused by NKT cell activation permitted langerin⁺ DCs to contribute towards stimulation of CD8⁺ T cell responses, this could only be achieved in an environment in which cross-priming was initially stimulated by langerin⁺ CD8 α ⁺ DCs (Figure 4.7).

Earlier studies have indicated a dependence on CD8 α ⁺ DCs for the induction of CD8⁺ T cell responses via NKT cell stimulation with α GalCer⁸⁹. This was shown to be through the induction of CCL17 chemokine to attract CCR4⁺ CD8⁺ T cells⁸⁹,

which was described as an alternative “helper” function to that of classical CD4⁺ T helper cells which produce CCL5 to attract the CCR5⁺ CD8⁺ T cells¹⁹⁵. Microarray data from our lab (Petersen, *unpublished data*) showed that upon α GalCer administration the langerin⁺ CD8 α ⁺ DCs demonstrated a 300-fold increase in mRNA production of CCL17 compared with langerin⁻ DCs. Therefore, it is possible that the dependence on langerin⁺ CD8 α ⁺ DCs for the initial cross-priming of CD8⁺ T cells is in part due to the initial interactions with NKT cells to attract CD8⁺ T cells to the antigen presentation site. This theory is further supported by previous research indicating that the APCs in the marginal zone of the spleen, where the langerin⁺ CD8 α ⁺ DCs reside to optimally receive antigens from the blood¹⁶, are crucially important for a DC-dependent localisation of NKT cells to the marginal zone after α GalCer administration²³². Therefore, there is potential for langerin⁺ CD8 α ⁺ DCs to be contributing to activation and coordination of both NKT cells and CD8⁺ T cells. Bone marrow chimera experiments using CD1d^{-/-} mice and experiments involving inhibition of CCL17 *in vivo* to investigate the relationship between langerin⁺ CD8 α ⁺ DCs and NKT cells did not provide conclusive results (*data not shown*), however, so this theory could not be proven.

The potential cross-presenting capacity of other DC subsets has been indicated in previous research⁶³, and the data presented in this thesis further confirms that other DC populations can contribute to CD8⁺ T cell responses. For example, it was demonstrated that early IFN- α inhibited CD40-CD40L induced IL-12 production, whereas late IFN- α exacerbated IL-12 production causing an increase in CD8⁺ T cells. This coincides with previously published data highlighting the effect of type I IFN on STAT signalling pathways in immature versus mature DCs for the control of IL-12 production²⁹¹. It was also observed that NKT cell pre-conditioned pDCs were responsible for enhancing CD8 α ⁺ DC activation to further stimuli, which coincides with previously published data that showed the activation of pDCs via interactions with NKT cells through Ox40-Ox40L resulted in increased conventional DC activation for increased cross-priming of CD8⁺ T cells²³⁴.

This research showed that the potent stimulatory environment created by NKT cell activation was able to condition langerin⁻ APCs to be capable of enhancing CD8⁺ T cell responses, given an appropriate secondary stimulus, but did not show evidence of CD8⁺ T cell responses that were completely independent of langerin⁺ CD8 α ⁺ DCs. These results therefore validate the concept of targeting CD8 α ⁺ DCs^{137; 293; 294}, and more specifically the langerin⁺ CD8 α ⁺ DCs¹³⁵, for vaccination strategies. However, this data also indicates that vaccination strategies that utilise the activity of multiple DC subsets may in fact be able to enhance CD8⁺ T cell responses further for improved immunotherapy.

The effect of administration of α GalCer to activate NKT cells resulted in a lowered activation threshold in DC populations, as demonstrated in this chapter. This is in comparison to a reduced function, such as reduced antigen uptake, after exposure to TLR agonists²⁹⁰. This therefore indicates a variable that would need to be considered when applying stimuli as adjuvants in a vaccine in hosts that may have recently been exposed to pathogen molecules. Pre-exposure to various pathogenic components provides resident immune cells with the potential to not respond to vaccines or to exhibit hyper-activation, causing cytotoxicity. In this chapter, experiments involving the delayed administration of TLR7 agonist after pre-conditioning with α GalCer resulted in mild to severe cytotoxicity, with a very large increase in a milieu of cytokines (Farrand *et al*, *unpublished observation*). Conversely, when a TLR ligand was administered before α GalCer, the immune response was severely reduced with an absence of an antigen-specific CD8⁺ T cell response (Farrand *et al*, *unpublished observation*).

Administration of α GalCer to activate NKT cells had a significant effect on the immune environment of the spleen. While it was shown here that DCs become conditioned to respond more effectively to a secondary stimulus, this does raise the question of the role of these APCs in the absence of such a second round of stimulation. It remains to be established, for example, whether conditioned langerin-negative APCs that have cross-presented antigen can meaningfully interact with antigen-specific CD8⁺ T cells.

In summary, this chapter highlights the central role of langerin⁺ CD8α⁺ DCs in initiating the induction of CD8⁺ T cell responses, while also describing the potential involvement of other DCs. Evidence was found in support, rather than rejection, of the hypothesis “that langerin⁺ DCs were capable of contributing to CD8⁺ T cell responses”. This chapter highlights the fact that pathogenic infection involving multiple activatory stimuli can have a profound effect on the APCs of the spleen, and the timing of stimuli can influence which APCs become involved in raising adaptive immune responses. This knowledge may be important for optimally designing vaccines for the activation of superior cell types for efficient coordination of cellular functions to enhance immune responses, such as antigen-specific CD8⁺ T cell responses.

Chapter 5: Splenic langerin⁺ CD8 α ⁺ DCs and langerin⁻ DCs induce similar phenotypic and functional changes in responding CD8⁺ T cells

5.1 Introduction

In Chapter 3, it was established that the splenic langerin⁺ CD8 α ⁺ dendritic cells (DCs) were required for the induction of strong antigen-specific CD8⁺ T cell accumulation in the blood of mice administered systemic antigen and stimuli. However, the qualitative aspects of T cell responses are determined by the microenvironment at the time of priming, with the phenotype of the antigen presenting cells (APCs) present within the lymphoid tissue having an impact on T cell development. Therefore, the phenotype and function of the CD8⁺ T cell responses generated in the presence or absence of langerin⁺ CD8 α ⁺ DCs was investigated in more detail, with the hypothesis that the CD8⁺ T cell responses would be phenotypically and functionally distinct.

The induction of CD8⁺ T cell responses to systemic proteins involves cross-priming by DCs, with these cells also playing a role in programming the differentiation state of the primed cells. In this context, it is known that the capacity for chemotaxis by T cells is programmed in the priming process, with studies showing that T cells acquire a migratory preference depending on the lymphoid tissue they were primed in, suggesting the differential APCs in the lymphoid tissue influence the chemokine receptor expression in T cells^{295; 296}. For example, priming with peyer's patches DCs in comparison to lymph node and splenic DCs induces increased expression of the α 4 β 7 integrin for homing to the small intestine²⁹⁷. It was therefore possible that the depletion of langerin⁺ CD8 α ⁺ DCs at the time of priming may have resulted in the activated T cells acquiring a bias to home to tissues other than the blood and spleen, which is explored in this chapter.

It is possible that CD8⁺ T cell responses induced under different conditions can acquire differences in the effector (T_{eff}) versus precursor memory (T_{pem}) phenotypes (as defined in Chapter 1), which may have an impact on the kinetics of a response and also on effector functions, such as cytotoxicity and the ability to be re-stimulated. In this context, Joshi *et al*¹⁵¹ demonstrated that increased exposure to bacteria-induced inflammation, including IL-12, induces larger short-lived CD8⁺ T_{eff} cell responses, whereas using antibiotics to abrogate inflammation early in the

immune response reduces the T_{eff} cell development. Given the involvement of langerin⁺ CD8α⁺ DCs in producing large amounts of IL-12⁵² it is possible that large short-lived CD8⁺ T_{eff} cell responses predominate when these DCs are present. These cells may rapidly decrease in number as they undergo apoptosis²⁹⁸, leaving a predominantly memory cell population within the host. It therefore remained to be established whether the large CD8⁺ T cell responses seen in the presence of langerin⁺ CD8α⁺ DCs are predominantly short-lived terminally differentiated T cells and whether responses induced in the absence of these DCs differ with respect to memory phenotype.

In addition to size, kinetics and distribution of the CD8⁺ T cell response, questions still remain about the role of langerin⁺ CD8α⁺ DCs in determining the functional quality of induced responses. For example, what is the effect of langerin⁺ CD8α⁺ DC depletion on the induction of CD8⁺ T cells with antitumour activity? While enhanced CD8⁺ T cell responses might be expected to correlate with improved tumour protection³⁷, the reduced CD8⁺ T cell responses in langerin⁺ DC-deficient mice have yet to be correlated with reduced tumour protection. What is also unknown is the effect of langerin⁺ CD8α⁺ DC depletion on the functional quality of the CD8⁺ T cells at a later time when a disease relapse may occur.

In summary, a number of questions remain about the role of langerin⁺ CD8α⁺ DC in determining the phenotype and function of induced CD8⁺ T cell responses to soluble antigens. Addressing these questions will be of particular relevance to understanding how responses are generated to circulating antigens that enter the spleen. This information may also be of use in the design of immunotherapy that can exploit the potent cross-priming ability of langerin⁺ CD8α⁺ DCs.

5.2 Aims

The purpose of this research was to determine what impact langerin⁺ CD8 α ⁺ DCs have on the phenotype and function of CD8⁺ T cell responses induced to circulating antigens. It was hypothesised that CD8⁺ T cell responses generated in the presence of langerin⁺ CD8 α ⁺ DCs are phenotypically and functionally distinct from those responses generated in their absence.

Specific aims were to:

- Investigate whether CD8⁺ T cell responses induced by intravenous administration of antigen and adjuvant acquire a different pattern of distribution in mice depleted of langerin⁺ CD8 α ⁺ DCs
- Determine if the langerin⁺ CD8 α ⁺ DCs affect the phenotype and killing function of CD8⁺ T cells
- Assess the impact of anti-tumour function in langerin⁺ DC-deficient mice

5.3 Results

5.3.1 *Langerin⁺ CD8 α ⁺ DC depletion reduces CD8⁺ T cell accumulation throughout the lymphoid tissues*

As described in Chapter 3, and in prior studies⁵², splenic langerin⁺ CD8 α ⁺ DCs play an important role in inducing CD8⁺ T cell responses to systemic antigens. However, this was measured by accumulation of the antigen-specific CD8⁺ T cells in blood and spleen only, without assessing other anatomical locations, including other lymphoid tissues. Therefore, to determine if the langerin⁺ CD8 α ⁺ DCs influenced accumulation of CD8⁺ T cells at these other sites, antigen was administered together with stimulation provided by the NKT cell agonist, α -galactosylceramide (α GalCer), and the TLR2 agonist Pam3Cys, to animals with or without prior depletion of langerin⁺ CD8 α ⁺ DCs, and then flow cytometry was used to assess accumulation of antigen-specific T cells in bone marrow, liver, and various lymph nodes. To enable detection of the induced T cell population at the different locations, it was necessary to transfer a cohort of OVA₂₅₇₋₂₆₄-(SIINFEKL)-specific T cells from OTI mice before the antigens were administered. Also, the combination of α GalCer and Pam3Cys was selected to drive the response, as these two compounds work synergistically to provide particularly large responses (as observed in Chapter 3). As expected, the accumulation of CD8⁺ T cells in the blood was significantly reduced in the absence of langerin⁺ CD8 α ⁺ DCs (Figure 5.1A). The analyses of different organs showed that most of the transgenic CD8⁺ T cells accumulated in the spleen and liver, regardless of whether langerin⁺ CD8 α ⁺ DCs were present at the time of priming. However, both the proportion of and number of CD8⁺ T cells in these locations was significantly reduced when langerin⁺ CD8 α ⁺ DCs had been depleted. In fact, this same trend was seen in all of the lymphoid tissues analysed (Figure 5.1B, C and D). The similar pattern of distribution of the cells between the different organs was observed (Figure 5.1E), suggesting that the changes in DC function at the time of priming did not significantly affect T cell homing.

Figure 5.1 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming reduces the accumulation of CD8⁺ T cells throughout the lymphoid tissues.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated intravenously with OVA, α GalCer and Pam3Cys. Mice were culled at day 7 after vaccination and the various tissues were collected and processed to analyse the CD8⁺ T cell response by flow cytometry. (A) Percentage of OVA-specific cells within CD8⁺ cells in blood. (B) Flow cytometry dot plot examples of the various tissue CD8⁺ T cell responses. Data was graphed as either percentage (C) or number (D) of OVA-specific CD8⁺ T cells within total live cells in the indicated tissues. (E) Proportion of total OTI cells within each tissue. Data shown are n = 5 per group and is from 1 independent experiment. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, *p>0.05, **p>0.01, ***p>0.001.

5.3.2 Reduced CD8⁺ T cell responses by langerin⁺ CD8 α ⁺ DC depletion correlates with reduced in vivo killing function

The reduced accumulation of CD8⁺ T cells observed in langerin⁺ CD8 α ⁺ DC-depleted mice is dramatic at the peak of the response. However, data from our lab has previously indicated that the kinetics of the CD8⁺ T cell response may be altered in the langerin⁺ CD8 α ⁺ DC depleted mice, with the DT treated group demonstrating a delayed increase in the CD8⁺ T cell accumulation⁵². To determine if the difference observed at the peak of the CD8⁺ T cell response was due to an altered kinetics of the CD8⁺ T cells, data from multiple experiments involving mice treated with OVA, α GalCer and Pam3Cys was combined. The large numbers of mice combined showed that the langerin⁺ CD8 α ⁺ DC-depleted mice did not have a delayed CD8⁺ T cell response but, in fact, showed the same pattern of accumulation with an overall reduced proportion than the langerin⁺ DC-sufficient mice (Figure 5.2). This indicated that the depletion of langerin⁺ CD8 α ⁺ DCs did not alter the kinetics of the CD8⁺ T cell response, other than to reduce the T cell accumulation.

To determine if there was an altered cytotoxic functional activity in the CD8⁺ T cells at the peak of the effector response or when memory cells had developed, langerin⁺ DC-deficient and -sufficient mice were treated with OVA, α GalCer and Pam3Cys and a VITAL assay²⁶⁶ was undertaken. Briefly, this involved intravenous administration of naïve CFSE-stained SIINFEKL peptide-loaded splenocytes, as well as control CTO-stained unloaded splenocytes, into the host mice. By assessing the presence of the CFSE and CTO positive cells in the blood of mice 18 hours later, the percentage of antigen-specific killing by the SIINFEKL peptide-specific CD8⁺ T cells could be measured. It was determined that langerin⁺ DC-depleted mice had significantly reduced antigen-specific killing occurring at the peak of the response, in comparison to DT-treated C57BL/6 controls where no change occurred (Figure 5.3). Comparatively, at 32 days, when the CD8⁺ T cell proportions would have returned to similar levels between the groups, there was no significant difference in the direct killing response between the treatment groups. This was most likely due to the development of memory cells in the DT group that were capable of responding more readily and effectively to antigen exposure. Therefore, the

increased level of T cell accumulation at the peak of the response or similar T cell accumulation at the later timepoint correlated with the level of killing function. This suggests that the langerin⁺ DC-sufficient mice have increased functional immune responses due to the increased numbers of CD8⁺ T cells induced by langerin⁺ CD8α⁺ DCs.

Figure 5.2 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming results in reduced CD8⁺ T cell accumulation in blood, but with similar kinetics to non-depleted mice.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated intravenously with OVA, α GalCer and Pam3Cys. Mice were bled at the indicated days after vaccination and analysed for CD8⁺ T cell accumulation by flow cytometry. Data from 5 independent experiments were compiled in the depicted graph with $n=3-21$ per time-point. Statistical analysis was performed using Student's *t* test, showing mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5.3 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming results in reduced cytotoxic activity at the peak of the response, but does not affect responses assayed after one month.

Lang-DTREGFP (A) or C57BL/6 (B) mice were administered DT or PBS 24 h before they were treated with OVA, α GalCer and Pam3Cys intravenously. At day 7 (full bars) or day 32 (striped bars) they were each given CFSE-labelled, SIINFEKL-peptide loaded splenocytes intravenously, as well as CTO-labelled naïve splenocytes as a negative control. Blood was collected 18 h later for flow cytometric analysis of the percentage of CFSE-labelled cells killed. Data shown are $n = 5$ per group and is representative of 2 independent experiments showing similar results. Statistical analysis was performed using One-Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, *** $p < 0.001$, ns = not significant.

5.3.3 CD8⁺ T cells primed in the absence of langerin⁺ CD8 α ⁺ DCs do not have an impaired cytotoxic function or cytokine production

In section 1.3.2, the langerin⁺ DC-sufficient mice were shown to have a higher *in vivo* killing response to peptide-loaded target cells. However, this was not able to demonstrate whether it was due to the increased CD8⁺ T cell accumulation in these mice, or because the CD8⁺ T cells had gained an enhanced killing function on a per cell basis. To investigate if the CD8⁺ T cells were individually more effective at killing, the OVA-peptide specific CD8⁺ T cells were sorted at the peak of the response at day seven by fluorescence-activated cell sorting (FACS). Because of the negligible numbers of antigen-specific CD8⁺ T cells in naïve mice at day seven, CD8⁺ T cells were purified from a littermate of the original CD8⁺ T cell donor. The sorted T cells were titrated and incubated with tritiated thymidine ([³H]-thymidine)-incorporated EL4 lymphoma cells that had been pre-loaded with SIINFEKL (OVA₂₅₇₋₂₆₄) peptide. The incorporated [³H]-thymidine remaining in each assay well was analysed using liquid scintillation counting, with higher counts per minute (CPM) indicating the presence of intact target cells and lower CPM indicating killing of target cells had occurred. The CD8⁺ T cells primed in langerin⁺ DC-sufficient mice were shown to have the same killing ability as the langerin⁺ DC-deficient mice (Figure 5.4). Therefore, the increased *in vivo* killing observed in figure 5.3 was due to the increased accumulation of CD8⁺ T cells rather than an increased cytotoxic capacity per cell. Re-stimulation of the OVA-specific CD8⁺ T cells with SIINFEKL peptide *in vitro* at day seven allowed the analysis of intracellular cytokines being produced by the activated T cells. The production of IFN- γ , TNF- α and IL-2 was not impaired in CD8⁺ T cells primed in langerin⁺ CD8 α ⁺ DC deficient mice (Figure 5.5), correlating with the *in vitro* cytotoxicity data.

Figure 5.4 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming does not impact the cytotoxic killing capacity of the CD8⁺ T cells.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated intravenously with OVA, α GalCer and Pam3Cys. Spleens were collected 7 days after vaccination, enriched for OTI cells by CD45.1 sorting using AutoMACS, and the OTI cells were purified by FACS, along with naïve OTI cells pre-enriched using CD8 dynabead enrichment from an OTI mouse. The sorted cells were titrated and incubated with SIINFEKL-loaded EL4 target cells containing [3H]-thymidine for 3.5 h, followed by analysis of killing by liquid scintillation counting. Counts were adjusted to EL4 only controls and antigen specific killing was calculated relative to naïve controls. Antigen-specific killing is graphed with a titrated effector to target (E:T) ratio. Data shown are from $n = 5-18$ spleens combined and is a representative of 2 independent experiments showing similar results. Statistical analysis was performed using Two-Way ANOVA with Bonferroni's post test, showing mean $\pm$ SEM, *** $p < 0.01$, ns = not significant.

Figure 5.5 Langerin⁺ CD8 α ⁺ DC depletion does not impact on amount of cytokine production by CD8⁺ T cells, but increases the total number of CD8⁺ T cells for increased total number of cytokine-producing CD8⁺ T cells.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated intravenously with OVA, α GalCer and Pam3Cys. Spleens were collected 7 days after vaccination, processed and re-stimulated for 5.5 h with 5 μ g/ml SIINFEKL peptide in the presence of brefeldin A to inhibit cytokine release. The cells were stained for surface markers, fixed, permeabilised, stained for intracellular cytokines and analysed by flow cytometry. Data shown are $n = 4-5$ per group and is a representative of 2 independent experiments showing similar results. Statistical analysis was performed using One-

Way ANOVA with Tukey's post test, showing mean $\pm$ SEM, with no significance detected.

5.3.4 *Langerin⁺ CD8 α ⁺ DC-dependent cross-priming has no impact on early qualitative CD8⁺ T cell responses*

The similar cytotoxicity and cytokine production in langerin⁺ and langerin⁻ DC cross-primed CD8⁺ T cells indicated strong functional similarities. The next step was to investigate the difference in memory and effector development. The T_{pcm} versus T_{eff} phenotype of the CD8⁺ T cell responses was therefore investigated by real-time quantitative PCR and flow cytometry to measure the CD127⁺ KLRG1⁻ (T_{pcm}) and KLRG1⁺ CD127⁻ (T_{eff}) cells¹⁴⁶. Seven days after antigen and stimuli were administered, at the peak of the CD8⁺ T cell response, RNA was extracted from purified OTI CD8⁺ T cells for real-time quantitative PCR analysis. There was a similar reduction in *Il7r* (CD127) and increase in *Klrg1* (KLRG1) compared to naïve controls in CD8⁺ T cells cross-primed by either langerin⁺ or langerin⁻ DCs (Figure 5.6A). The flow cytometric analysis of the CD8⁺ T cell surface marker phenotype at day 7 (gating strategy in figure 5.6B) correlated with the PCR data, where there was little to no detectable CD127, and an increase in KLRG1⁺ cells in both CD8⁺ T cell populations. This shows a similar proportion of terminally differentiated effector cells (Figure 5.6C) and precursor memory cells (Figure 5.6D). Therefore, this data suggested that the langerin⁺ CD8 α ⁺ DCs have no impact on effector or memory cell development.

To further analyse any phenotypical or functional differences between the CD8⁺ T cells induced in the absence or presence of langerin⁺ CD8 α ⁺ DCs, the RNA expression of a selection of genes was analysed by quantitative real-time PCR. This selection of genes was chosen from previously published work describing the differences in mRNA expression between the T_{pcm} and T_{eff} populations of CD8⁺ T cells²⁹⁹, as well as common apoptosis markers to establish apoptosis versus survival potential in the cells. The expression of *Tbx21* (the transcription factor T-bet) indicates development of a T_{eff} phenotype¹⁵¹ and RNA expression analysis of this gene confirmed there was no bias towards a T_{eff} phenotype in the CD8⁺ T cell populations (Figure 5.7). For apoptotic and survival status of the CD8⁺ T cells, the RNA expression of *Caspase 3*, *Caspase 8*, *Bcl2* and *Serpine2* were analysed, with

results indicating that langerin⁺ CD8 α ⁺ DCs do not induce a different survival/apoptosis bias. For functional gene expression analysis, RNA expression of genes for ribosome biogenesis (glutamate-rich WD repeat containing 1; *Grwd1*)³⁰⁰, antimicrobial protein (lysozyme 1; *Lyz1*)³⁰¹, metabolism (chitinase 3-like 3; *Chi3l3*)^{302; 303} and migration (transforming growth factor, beta-induced; *Tgfb1*)^{299; 304} were measured. The increased or reduced mRNA expression in most of the markers suggested an increased proportion of T_{eff} cells in both CD8⁺ T cell populations (Figure 5.7), especially the correlation of the increased *Chi3l3* and decreased *Grwd1* seen in previous research²⁹⁹. However, there is a trend towards an increase in possible T_{eff} proportions from langerin⁻ DC stimulated CD8⁺ T cells as seen by an increase in *Tbx21* and *Chi3l3*, but this does not correlate with the flow cytometry data from figure 5.6 demonstrating no significant change in the expression of KLRG1.

In summary, this data suggests that the langerin⁺ CD8 α ⁺ DCs do not induce different proportions of phenotypically and functionally different CD8⁺ T cells. Instead they induce an increased number of activated CD8⁺ T cells with a similar phenotype and functional gene expression as CD8⁺ T cells induced in langerin⁺ DC-depleted mice.

Figure 5.6 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming does not alter the proportion of effector and precursor memory cells.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated with OVA, α GalCer and Pam3Cys intravenously. (A) Spleens were collected on day 7, enriched for CD45.1⁺ cells, and OTI cells were purified by FACS and RNA was extracted. Real-time quantitative PCR was used to assess mRNA production of KLRG1 and CD127. Data shown are $n = 10$ -20 spleens per group pooled together and are representative of 2 independent experiments showing similar results. Values below the red dotted line at 0.5 are considered significant while the red dotted line at 1 = no change. (B, C and D) Spleens were collected on day 7 for flow cytometric analysis of CD8⁺ T cell expansion and phenotype. (B) Gating strategy for OTI (CD45.1⁺V α 2⁺) activated (CD44⁺), effector (KLRG1⁺CD127⁻), and precursor memory (KLRG1⁻CD127⁺) cells. (C and D) Percentage of CD44⁺ cells that are terminally differentiated effector cells (C) or precursor memory cells (D). Data shown are $n = 3$ per group and are representative of 2 independent experiments showing similar results. Statistical analysis was performed using Student's *t* test, showing mean $\pm$ SEM, ns = not significant.

Figure 5.7 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming has no impact on the expression pattern of genes involved in survival, differentiation, metabolism and function by activated CD8⁺ T cells.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated with OVA, α GalCer and Pam3Cys intravenously. At day 7 OTI T cells were fluorescence-activated cell sorted (FACS) from spleens, RNA was extracted, and real-time PCR was used to analyse the indicated genes. Red dashed lines indicate no change (1.0), significantly decreased level (<0.5), or significantly increased level (>2.0) of expression. Data shown is from $n = 10$ -20 pooled spleens and is representative of 2 independent experiments showing similar results.

5.3.5 *Langerin⁺ CD8 α ⁺ DC-induced CD8⁺ T show similar responses to re-stimulation*

The re-stimulatory capacity of T cells initially activated in the presence or absence of langerin⁺ CD8 α ⁺ DCs was investigated. At the peak of the CD8⁺ T cell response (Figure 5.8A), after OVA, α GalCer and Pam3Cys treatment, the spleens of treated mice were harvested and the CD8⁺ T cells purified by fluorescence-activated cell sorting (FACS; Figure 5.8B). Equal numbers of the cells were then transferred into naïve mice that were boosted with OVA and α GalCer. The cells were observed to have equal re-stimulatory capability, as they had the same level of increased accumulation of CD8⁺ T cells over the period of one week (Figure 5.8C).

The re-stimulated CD8⁺ T cells also had a similar phenotype, as there were the same proportions of activated CD44⁺ CD122⁺ cells observed (Figure 5.9A). Within the activated CD8⁺ T cells, there was an earlier increased proportion of effector cells in the langerin⁺ DC-sufficient mice (Figure 5.9B). This suggested that the CD8⁺ T cells generated in langerin⁺ DC-sufficient mice have an increased potential for generating more terminally differentiated T cells, in comparison to the higher proportion of T_{eff} cells already generated in the langerin⁺ DC-deficient mice in figure 5.6. Like the proportion of activated T cells, the proportion of T_{pcm} cells was the same in both the presence and absence of langerin⁺ CD8 α ⁺ DCs, suggesting the DCs do not influence memory development (Figure 5.9C).

Altogether, this data suggests that potential for re-stimulation of CD8⁺ T cells is not greatly altered by cross-priming responses in langerin⁺ DC-deficient mice from langerin⁺ DC-sufficient mice. However, there are differences in the kinetics of T_{eff} cell development between the treatment groups.

Figure 5.8 Langerin⁺ CD8 α ⁺ DC depletion at the time of priming has no impact on the re-stimulatory ability of the induced CD8⁺ T cells.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated with OVA, α GalCer and Pam3Cys intravenously. Spleens were collected on day 7, the OTI cells were CD45.1-enriched using AutoMACS and purified using FACS. The sorted cells (1×10^6 /mouse) were transferred into naïve C57BL/6 mice and re-stimulated with OVA and α GalCer intravenously. (A) Flow cytometric analysis of CD8⁺ T cell expansion in *lang*-DTREGFP spleens at day 7 (pre-sorted). (B) Purification of OTI cells for adoptive transfer (post-sorted). (C) C57BL/6 mice were bled at days 5, 7 and 9 after re-stimulation for flow cytometric analysis of CD8⁺ T cell expansion. Data shown are $n = 10$ -20 per group of pooled spleens and $n = 5$ per treatment group. Data are representative of 2 independent experiments showing similar results. Statistical analysis was performed using Student's *t* test, showing mean $\pm$ SEM, *** $p < 0.001$.

Figure 5.9 The phenotype of CD8⁺ T cells after re-stimulation is largely unaffected by depletion of langerin⁺ CD8 α ⁺ DCs at the time of initial priming.

Lang-DTREGFP mice were administered DT or PBS, and OTI cells were adoptively transferred, 24 h before they were treated with OVA, α GalCer and Pam3Cys intravenously. Spleens were collected on day 7, the OTI cells were CD45.1-enriched using AutoMACS and purified using FACS. The sorted cells (1×10^6 /mouse) were transferred into naïve C57BL/6 mice and re-stimulated with OVA and α GalCer intravenously. C57BL/6 mice were bled at days 5, 7 and 9 after re-stimulation for flow cytometric analysis of CD8⁺ T cell phenotype. (A) Percentage of activated cells within the OTI population determined by CD122 and CD44 expression. (B and C) Percentage of CD122⁺CD44⁺ cells that are terminally differentiated effector cells (B) or precursor memory cells (C). Data shown are $n = 5$ per group and are representative of 2 independent experiments showing similar results. Statistical analysis was performed using Student's *t* test, showing mean $\pm$ SEM, *** $p < 0.001$.

5.3.6 Langerin⁺ CD8α⁺ DCs provide minimal therapeutic anti-tumour protection

Achieving effective CD8⁺ T cell-mediated therapy in animals with established tumours is a difficult task, particularly as immune suppression may be occurring in tumour-burdened mice. Low anti-tumour activity can be attributed to down-regulation of tumour antigens by tumour cells and production of suppressive cytokines by tumours, as well as the increase in immunosuppressive cells. Immunosuppressive cells include regulatory T cells (Tregs), which can inhibit the activation and proliferation of T_{eff} cells³⁰⁵, and myeloid-derived suppressor cells, which suppress cytotoxic T and NK cell functions and further increase Treg populations in the tumour³⁰⁶. However, even with immunosuppression occurring in the tumour setting, the quality of the CD8⁺ T cell response is still likely to be a contributing factor in determining therapeutic outcome. Two therapeutic models were therefore investigated to assess the functional activity of T cells induced in the presence or absence of langerin⁺ CD8α⁺ DCs; mice engrafted with the melanoma, B16.OVA, or the T cell lymphoma, EG7.OVA, subcutaneously. Four days later, the mice were depleted of langerin⁺ DCs or left untreated. The next day they were administered OVA, αGalCer and Pam3Cys to induce a tumour-specific CD8⁺ T cell response. In both tumour models, the administration of antigen and stimuli induced tumour protection compared to naïve controls (Figure 5.10). However, while there was a trend for improved tumour protection in langerin⁺ DC-sufficient mice, there was very little to no significant difference in the tumour protection between the groups in both tumour models. This suggests that the increased accumulation of CD8⁺ T cells induced by langerin⁺ CD8α⁺ DCs does not result in increased anti-tumour function.

Figure 5.10 Langerin⁺ CD8 α ⁺ DCs induce minimal increased tumour protection therapeutically.

Lang-DTREGFP (A and C) and C57BL/6 (B and D) mice were challenged with 1×10^6 EG7.OVA (A and B) or 1×10^5 B16.OVA (C and D) subcutaneously. At day 4 after tumour challenge they were administered either DT or PBS and at day 5 given OVA, α GalCer and Pam3Cys intravenously. Tumour growth was measured over time. Data shown are $n = 5$ per group and are from 1 independent experiment each. Statistical analysis was performed using Two-Way ANOVA with Bonferroni's post test, showing mean $\pm$ SEM, * $p < 0.05$, ns = not significant.

5.4 Discussion

Previous work has indicated an ability to induce strong CD8⁺ T cell responses upon systemic administration of appropriate antigen and stimuli^{226; 227}. The induction of these responses has been observed to rely on the cross-priming effect of the langerin⁺ CD8 α ⁺ DCs within mouse spleen⁵², as also seen in chapter 3 in this thesis. However, the effect on the functional and phenotypical quality of these strongly induced CD8⁺ T cell responses has not been extensively studied. In fact, the majority of the analysis of the CD8⁺ T cell response, and therefore the interpretation of the data, relied on accumulation of the T cells in either blood or spleen of mice. If the effect of langerin⁺ CD8 α ⁺ DC depletion resulted in the CD8⁺ T cells having a different homing capacity then the CD8⁺ T cells may have accumulated elsewhere, explaining the lack of T cells in the blood and spleen. Therefore, a wider range of organs were analysed in figure 5.1 for the presence of the CD8⁺ T cells, to confirm that the langerin⁺ CD8 α ⁺ DC depletion purely resulted in reduced CD8⁺ T cells throughout many tissues in the host, rather than the cells acquiring a different homing capacity. It was indeed determined that the CD8⁺ T cells were reduced overall, as the numbers were reduced in all tissues analysed. This result first reconfirmed that the langerin⁺ CD8 α ⁺ DCs are required for induction of strong systemic CD8⁺ T cell responses, as well as disproved the hypothesis that, from the tissues analysed, the CD8⁺ T cells may have accumulated elsewhere in the host due to different homing capacity. However there are also other tissues such as the gut, lung and skin where the T cells may also reside and further research should investigate this possibility before confidently disproving the hypothesis.

Previously published data⁵², as well as the T cell accumulation difference of the langerin-induced and not langerin-induced CD8⁺ T cell responses (Figure 5.2), led to the hypothesis that there may be more short-lived T_{eff} cells at the peak of the response in the langerin⁺ DC-sufficient mice resulting in improved killing function. An *in vivo* killing assay (VITAL assay) demonstrated an increased antigen-specific killing of target cells at the peak of the response in langerin⁺ DC-sufficient mice (Figure 5.3, suggesting there might in fact be a higher proportion of short-lived T_{eff}

cells at the peak of the response in the langerin⁺ DC-sufficient mice that provide the increased killing function. However, an *in vitro* killing assay with equal numbers of antigen-specific CD8⁺ T cells and intracellular cytokine analysis demonstrated an equal cytotoxic ability and cytokine production (Figures 5.4 and 5.5). Analysis of the activated phenotype of the langerin⁺ DC cross-primed CD8⁺ T cells showed no difference in the proportion of T_{eff} and T_{pcm} cells compared to langerin⁺ DC-deficient mice (Figure 5.6). PCR analysis of the T cells at the peak of the response correlated with the flow cytometry data to again suggest there is no difference in the proportion of T_{eff} versus T_{pcm}, as well as there was no obvious difference in effector functional responses or in apoptosis and survival (Figures 5.6 and 5.7). When equal numbers of *in vivo* activated CD8⁺ T cells were administered to naïve hosts and re-stimulated, the cells could be equally re-stimulated; however the langerin⁺ DC-primed CD8⁺ T cells had the potential for further T_{eff} differentiation (Figure 5.8 and 5.9). This data in total suggests that proportionally there is very little difference between the treatment groups, but the langerin⁺ CD8 α ⁺ DCs induce CD8⁺ T cells that can further expand upon increased antigen and stimuli administration. This may be due to limited antigen exposure preventing maximum expansion of the CD8⁺ T cells when the antigen-specific CD8⁺ T cell numbers increase to such high levels that they are competing for antigen³⁰⁷.

In comparison to the large difference in CD8⁺ T cell accumulation between the langerin⁺ DC-sufficient and -deficient mice at the peak of the response, at day 32 there were fairly similar proportions of antigen-specific CD8⁺ T cells within the CD8⁺ population (Figure 5.2). Also in comparison to the peak of the response, at the later time of day 32 there was no significant difference in the killing function of the cells (Figure 5.3). Previous research has found that the initial size of the T cell population at the peak of the response determines the eventual size of the memory pool³⁰⁸. Therefore, the potential for memory generation would have had to be higher in the langerin⁺ DC-depleted mice to be able to generate similar long-term survival numbers with similar functional memory when the initial CD8⁺ T cell populations were so significantly different in size. This indicates that while there were no differences detected in the T_{eff} versus T_{pcm} development at the peak of the

response, there must be some undetected differences in the CD8⁺ T cells generated in the absence of langerin⁺ CD8α⁺ DCs.

These results seen could also have been due to several experimental design problems. One possible issue is that in some research, it is not until later in the response that a precursor memory/ effector differentiation can be identified in the mice^{146; 309}. Later time points were not chosen in this research, however, because the T cell numbers remaining at the later time points were at very low levels where the data might have been skewed due to small numbers. Another possible issue is that the CD127 antibody to detect T_{pcm} cells did not appear particularly effective. Both clones used successfully in the literature, the A7R34¹⁴⁶ and SB/199³¹⁰ clones, were tested in this research and neither were able to show clear positive results. However, the flow cytometry data was supported by the corresponding reduction of CD127 mRNA expression measured by real-time quantitative PCR. Most likely, the peak of the response at day 7 in my model was during the initial period where a significant reduction in CD127 is observed, as has been seen with LCMV-specific CD8⁺ T cells where CD127 expression is drastically reduced by day 5 after stimulation²⁹⁹. A time course assessing CD127 expression over time in differentiating CD8⁺ T cells would confirm this theory. This was not done in this thesis research, as my focus was to investigate the T cells at the peak of the response.

Other research by Petersen *et al* (*unpublished data*) has shown an importance of langerin⁺ CD8α⁺ DCs in anti-tumour immune responses. The functional relevance of the langerin⁺ CD8α⁺ DC-induced CD8⁺ T cells is important for identifying the relevance of taking advantage of the cross-priming role of langerin⁺ CD8α⁺ DCs in various immunotherapies, including tumour immunotherapy. There are vaccine strategies that target antigen to specific cell subsets^{136; 293}, including targeting to langerin⁺ CD8α⁺ DCs¹³⁵. This has become especially more relevant with the identification of the human BDCA3⁺ DCs that show functional and phenotypical similarities to the CD8⁺ DCs in mice^{285; 311; 312}. These cells are able to also induce antigen-specific CD8⁺ T cell responses by cross-priming and, therefore, a high anti-tumoural function of these T cells would need to be achieved for viable

tumour immunotherapy. Because the importance of CD4⁺ T cells has more recently been shown as important for anti-tumoural responses, for example the newly defined Th9 responses have been shown to aid in mouse melanoma research^{313; 314}, the functional relevance of the cytotoxic CD8⁺ T cell response is even more questionable as it is heavily dependent on CD4⁺ T cells in circumstances such as these. Therefore, the anti-tumour function of the early peak of the CD8⁺ T cell response induced by langerin⁺ CD8α⁺ DCs was compared with the significantly reduced CD8⁺ T cell response in the langerin⁺ CD8α⁺ DC-depleted mice. A trend towards a slight improvement of anti-tumour function with the langerin⁺ CD8α⁺ DC-induced CD8⁺ T cell response could be seen with a therapeutic administration of antigen and stimuli after a tumour was already established (Figure 5.10), although this was not highly significant. This trend provided a correlation with the increased early killing function in figure 5.3 and indicated that the increased CD8⁺ T cell response induced by langerin⁺ CD8α⁺ DCs was able to contribute slightly to anti-tumour activity. However, the small CD8⁺ T cell response generated in the absence of langerin⁺ CD8α⁺ DCs was also capable of providing strong anti-tumour protection. This suggests two possible theories, the first being that the small number of CD8⁺ T cells generated in the absence of langerin⁺ CD8α⁺ DCs is capable of providing enough anti-tumour function. The *in vitro* cytotoxicity assay supports the capability of the CD8⁺ T cells to kill, however the *in vivo* cytotoxicity data suggests that there are not enough cytotoxic T cell numbers to efficiently kill target cells. Another theory is that while the CD8⁺ T cells can contribute to anti-tumour activity, other cells that have not been studied in this thesis may be more significantly involved in the cytotoxic activity to protect against tumours. An example, as mentioned above, is the Th9 cells that are important for anti-B16 melanoma tumour activity via production of IL-9 and granzyme B³¹⁴.

While there was a difference in antigen-specific killing function between the CD8⁺ T cell populations at the peak of the response, which also translated to a slightly improved early tumour protection, there was no difference in function seen between the memory T cell populations at the later timepoint of day 32. While an induction of CD8⁺ T cells could be used to treat existing tumours detected early, or other diseases where a strong cytotoxic CD8⁺ T cell response could be used for

treatment, for example, in viral infections³¹⁵, the ability to develop efficient memory CD8⁺ T cells would be useful against further challenge at a later time. In this instance, there is no benefit to the long-term immune response by using langerin⁺ CD8α⁺ DCs for the induction of CD8⁺ T cells, as the same memory function was developed in the absence of langerin⁺ CD8α⁺ DCs.

One limiting factor with the data in this thesis is that it uses OVA-specific CD8⁺ T cells and only OVA protein and peptide as the model antigen. Further work to expand on the relevancy of the langerin⁺ CD8α⁺ DCs would include using a broader range of antigens to confirm that the data is applicable across multiple models.

In summary, this chapter demonstrates that langerin⁺ CD8α⁺ DCs are able to cross-prime CD8⁺ T cell responses and cause significant expansion resulting in an increased *in vivo* function, without any major effect on the quality at the peak of the response. However, *in vivo* cytotoxic function several weeks after priming was comparable, suggesting that this function was not related to initial burst size, providing some evidence of difference in function between CD8⁺ T cells primed in the presence or absence of langerin⁺ CD8α⁺ DCs. Therefore, there is limited data to support the hypothesis that the langerin⁺ CD8α⁺ DCs induce phenotypically and functionally distinct T cell responses.

Chapter 6: General Discussion

6.1 The *lang*-DTREGFP model is a useful model for analysing the importance of langerin⁺ CD8α⁺ dendritic cells

This thesis sought to determine the role of the murine splenic langerin⁺ CD8α⁺ dendritic cell (DC) subset in inducing CD8⁺ T cell responses to circulating antigen. Through intravenous antigen administration, the adoptive transfer of antigen-specific CD8⁺ T cells and surgical removal of the spleen, the splenic langerin⁺ CD8α⁺ DCs were confirmed as containing a crucial cross-priming population with high co-stimulatory⁵² and endocytic¹⁶ capacities. The *lang*-DTREGFP mouse model was a major tool used to analyse these DCs by specifically depleting langerin-expressing cells prior to, or during, antigen administration. Although the *lang*-DTREGFP model has been widely used for the analysis of dermal and epidermal migratory langerin⁺ DCs^{68; 250; 262; 263}, the model has not previously been described in detail for the analysis of splenic langerin⁺ CD8α⁺ DCs. Here it was shown that the treatment of these mice with diphtheria toxin (DT) via an intraperitoneal route induced depletion within four hours of the splenic langerin-expressing diphtheria toxin receptor (DTR)-positive cells, with the depletion maintained for two days and the cells fully replaced by four days.

The depletion kinetics of the splenic langerin⁺ CD8α⁺ DCs was markedly different to langerin⁺ epidermal and dermal DCs^{264; 265} where depletion was maintained 7-14 days. The lymphoid resident DCs are known to reconstitute lymphoid tissues within a short timeframe after depletion of populations^{316; 317; 318}, possibly due to development from different precursors than the epidermal DCs³¹⁹. For example, it was reported that clodronate liposome treatment depleted all of the phagocytosing populations in lymphoid tissues, but while the marginal zone and marginal metallophilic macrophages took 7-14 days to reconstitute the spleen, the DCs were reconstituted in the shorter period of seven days³²⁰. Understanding the kinetics of depletion in the *lang*-DTREGFP mouse model allowed the specific timed depletion of langerin⁺ CD8α⁺ DCs to detail the critical role for cross-priming CD8⁺ T cell responses within the first six hours of antigen administration.

With the early reconstitution of the langerin⁺ CD8 α ⁺ DCs in mouse spleen, long-term studies would require the repeated administration of DT to maintain the depletion. However, other models, such as the *CD11c*-DTR model, have severe side effects upon a second dose of DT²⁵⁶. *CD11c*-DTR mice can endure one dose of DT, however a second dose can be fatal. This is thought to be due to effects of the DT on CD11c⁺ non-hematopoietic radio-resistant cells, as lethally irradiated CD11c-DTR mice that are supplemented with wild-type bone marrow experience severe weight loss and die after multiple DT treatments²⁷⁵. In contrast, in this thesis the *lang*-DTREGFP mice tolerated multiple DT doses every two days over the period of a week, with no ill effects observed and the depletion could be maintained with no resistance to DT. In a study conducted with a colleague in my laboratory, we found that one dose every two days could be administered for a period of up to three weeks with no weight loss and no serum toxicity markers²⁸⁶. *CD11c*-DTR mice have also shown the side effect of increased neutrophilia after treatment with DT only³²¹. This has been hypothesised to be because of an inflammatory response to the CD11c⁺ cell death upon DT administration³²², rather than being a direct off-target effect of the DT as DT-treated C57BL/6 control mice are not affected by DT³²¹, and possibly due to the absence of chemokine and growth factor repression by DC that would control neutrophil populations in homeostatic conditions³²². Nevertheless, we observed no neutrophilia in response to administration of DT in the *lang*-DTREGFP mouse model (Appendix 4, *published data*²⁸⁶). Overall, these studies indicated that the *lang*-DTREGFP model is a safe and reproducible model for the analysis of langerin⁺ CD8 α ⁺ DCs.

At the outset of this study, the *lang*-DTREGFP model held many advantages over other models to explore the function of DCs. Previously, knockout models such as the *Irf8* and *Batf3* transcription factor knockout mice were used to investigate diseases and immune responses in the absence of CD8 α ⁺ DCs and other lymphoid cells^{251; 255}. However, these models have a major flaw for the study of specific CD8 α ⁺ DC immune responses, which is that these mice have a continuous deficiency in CD8 α ⁺ DCs. They cannot be used to specifically ablate the CD8 α ⁺ DC populations at specific time points in the immune response to identify important functional timing. The development of mouse models with human or simian DT

receptor expression in specific immune cells allowed the transient depletion of those immune cells for more detailed research.

The *CD11c*-DTR model has been used in many studies of DC function, and represented a significant breakthrough in analysis of DC function. However, this model is flawed, in that other populations of cells also express significant levels of CD11c. Therefore, it is not a DC-specific depletion model and, as detailed in Table 6.1, these other cells have been shown to be depleted by DT treatment in these mouse models^{256; 275; 323}. The *siglecH*-DTR mouse model was developed during this study to deplete plasmacytoid DCs (pDCs); however, it poses the same problem as the *CD11c*-DTR model where there is Siglec-H expression on microglia³²⁴ and some macrophage populations in lymphoid tissues. While DT does not affect lymphoid macrophages³²⁵, the depletion of microglia has not been confirmed and could severely impact immunology studies involving the brain. The *lang*-DTREGFP model does not have this problem, as only DCs express langerin and therefore it is less likely to cause any off-target affects. However, there are multiple DC populations that are langerin⁺, such as a population of migratory dermal DCs, the epidermal Langerhan's cells and the lymphoid resident CD8 α ⁺ DCs⁶⁸. These langerin-expressing cells can be observed in the spleen, lung, many lymph nodes and the skin, and they are also depleted significantly by the 350 ng dose of DT used throughout this thesis to deplete the splenic langerin⁺ CD8 α ⁺ DCs (Appendix 5, *published data*²⁸⁶). While removal of the spleen of live mice has indicated that the splenic population of cells is important for cross-priming CD8⁺ T cell responses, it remains possible that other langerin⁺ DC populations may at least in part be capable of enhancing those CD8⁺ T cell responses. To assess this, future experiments could detail the phenotypical and functional responses of other non-splenic langerin⁺ DCs over time after initial antigen administration. For example, the presentation of antigen and expression of co-stimulatory molecules could be analysed by *in vitro* studies on cells sorted from these tissues after antigen administration to determine the cross-priming potential of these cells. Also, the presence of antigen-specific CD8⁺ T cells in the various tissues and the early expression level of activation markers on these T cells would indicate if the cells are obtaining a heightened

activation state due to the antigen presenting cells (APCs) within the different lymphoid tissues.

During the undertaking of this thesis research the *Clec9a*-DTR mouse model was developed and used to investigate the involvement of DCs in cross-priming³²⁶. The c-type lectin receptor CLEC9A (DNGR-1) is an endocytic receptor³²⁷ expressed by CD103⁺ DCs in non-lymphoid tissues and CD103⁺ CD8 α ⁺ DCs in lymphoid tissues²⁸⁴, including both langerin-positive and langerin-negative populations. Depletion of the CLEC9A⁺ cells results in a reduction of CD4⁺ and CD8⁺ T cell activation in an experimental cerebral malaria model³²⁶, indicating that the depletion of CLEC9A⁺ DCs ablates the ability of the CD8 α ⁺ DCs to cross-prime CD8⁺ T cell responses. The success of this model poses a question as to the relevance of the *lang*-DTREGFP mouse model, as the *Clec9a*-DTR model would also be depleting langerin⁺ CD8 α ⁺ DCs. However, data from my laboratory has specifically investigated the functional differences between langerin⁺ CD8 α ⁺ DCs and langerin⁻ CD8 α ⁺ DCs, to demonstrate key differences between the subsets. The langerin⁺ subset possesses higher apoptosis of dying cells, superior cross-presentation, IL-12 production in response to various agonists, and the langerin⁻ subset has the potential to differentiate into terminally differentiated langerin-expressing cells (Petersen *et al*, *manuscript in preparation*). Therefore, this data indicates that the langerin-expressing subset of CD8 α ⁺ DCs in the spleen is highly relevant for studying immune responses and provides support as to the relevance of using the *lang*-DTREGFP mouse model in the studies executed in this thesis.

Overall, this research indicates that the *lang*-DTREGFP mouse model is a useful model with no toxicity side effects for specific analysis of splenic langerin⁺ CD8 α ⁺ DC involvement in cross-priming CD8⁺ T cells. However, other langerin-expressing populations than the splenic DCs are also depleted by administration of DT and therefore should be taken into consideration for all experiments using this model.

Table 6.1. Summary of the known details of the discussed DTR mouse models.

Model	Cells depleted with DT	Multiple DT dose	Side effects	Reference
<i>CD11c</i> -DTR	DCs (except Langerhans cells), intraepithelial lymphocytes, CD8 ⁺ LN T cells, marginal zone macrophages, marginal metallophilic macrophages	No	Severe weight loss followed by lethality with multiple doses, neutrophilia	256; 275; 321; 323
<i>SiglecH</i> -DTR	Plasmacytoid DCs, possibly microglia (not tested)	Yes	None detected	Chapter 4; 325
<i>Lang</i> -DTREGFP	Langerhans cells, dermal langerin ⁺ DCs, langerin ⁺ CD8 α ⁺ DCs	Yes	None detected	Chapter 3; 68; 286
<i>Clec9a</i> -DTR	CD103 ⁺ DCs, minor pDC depletion	Yes	Unknown	326

6.2 The characteristics of classical CD8 α ⁺ DCs are attributed to the langerin-expressing subset

This thesis sought to determine the roles of the langerin-expressing subset of CD8 α ⁺ DCs for CD8⁺ T cell induction by investigating the known functions of CD8 α ⁺ DCs in cross-priming CD8⁺ T cells. It was shown that the splenic langerin⁺ CD8 α ⁺ DCs were in the optimal position in the marginal zone of the spleen for antigen uptake and were shown by flow cytometry to possess high antigen presentation of OVA peptide from OVA protein on MHC class I. They were also observed to possess a large capacity for IL-12 production and high CD40 expression. They also contributed to chemokine production (measured by mRNA expression; *data not shown*) involved with increasing CD8⁺ T cell recruitment such as CCL17⁸⁹, rather than innate cells such as monocytes, neutrophils and eosinophils such as CCL12³²⁸, CXCL5³²⁹ and CCL11³³⁰.

The high levels of presentation of OVA peptide from injected soluble OVA protein corresponds with previous research demonstrating the superior ability of the CD8 α ⁺ DCs population as a whole to cross-present antigen to CD8⁺ T cells, while CD4⁺ DCs mainly present peptides to CD4⁺ T cells⁸⁵. However, the results from this thesis indicate that it is specifically the langerin-expressing subset that serves this function. Previously the CD8 α ⁺ DC population as a whole was attributed the role of releasing high levels of IL-12⁸⁸, and providing the most significant CD40⁷⁵ and chemokine⁸⁹ expression for enhancing CD8⁺ T cell responses, but this thesis research identifies that it is the langerin⁺ population of the CD8 α ⁺ DCs that possess these superior functions.

Research undertaken in my laboratory has purified langerin⁺ CD8 α ⁺ DCs, langerin⁻ CD8 α ⁺ DCs and CD8 α ⁻ CD24⁺ DCs from mouse spleen. Upon transferring these different subsets into naïve hosts, the CD8 α ⁻ CD24⁺ DCs differentiate into CD8 α ⁺ DCs and the langerin⁻ CD8 α ⁺ DCs differentiate into langerin-expressing cells. Meanwhile, the langerin⁺ CD8 α ⁺ DCs do not lose langerin-expression and instead are unable to survive long-term (Petersen *et al*, *manuscript in preparation*) and, therefore, the langerin⁺ phenotype may be a

terminally differentiated phenotype. This would mean that the early precursor $CD24^+$ DCs develop into $CD8\alpha^+$ DCs³³¹ that have minor antigen presentation and co-stimulatory ability and those characteristics then increase as the DCs differentiate into langerin-expressing cells. The exact phenotyping of the progression of DC differentiation could be studied by sorting and comparing $CD24^+ CD8\alpha^-$, langerin $^- CD8\alpha^+$ and langerin $^+ CD8\alpha^+$ DCs. This would allow for the functional analysis of these separate populations by *in vitro* assays, such as incubations with $CD8^+$ T cells and $CD4^+$ T cells to assess T cell stimulation capabilities. Results from this thesis suggest that in these experiments there would be a progression of increased cross-priming ability as the cells became more differentiated. However, these experiments would prove hard to accomplish with the langerin $^+$ population as they are a small population within the entire spleen and they do not show great survival with manipulative techniques (Deborah Knight and Ian Hermans, *personal communications*).

The langerin $^+ CD8\alpha^+$ DCs are also severely reduced due to the strong stimulus of α GalCer-activated NKT cells, as shown in this thesis; therefore, sorting langerin $^+ CD8\alpha^+$ DCs from mice that had α GalCer-stimulated NKT cells to activate the DCs would provide limited cell numbers. Perhaps a stimulus that provided a measurable activation response without being too strong for the sensitive langerin $^+ CD8\alpha^+$ DCs would be more effective at providing activation for analysis without depleting the cell numbers limiting analysis. For example, instead of α GalCer the NKT cell ligands iGb3 or GSL1 could be used to activate the DCs, as they have shown the ability to aid in NKT stimulation for enhanced $CD8^+$ T cell cross-priming^{332; 333}. However, while other research has used various NKT cell ligands to activate NKT cells and aid in cross-priming responses to $CD8^+$ T cells, the survival of splenic langerin $^+ CD8\alpha^+$ DCs after stimulation with those ligands requires further investigation.

Overall, this research indicates that the attributes previously associated with $CD8\alpha^+$ DC characteristics, for resulting in large $CD8^+$ T cell responses, are more attributable to the langerin $^+ CD8\alpha^+$ DC population.

6.3 Langerin⁺ CD8 α ⁺ DCs are responsible for increasing antigen-specific CD8⁺ T cell responses with no impact on the quality of the response

The role of splenic langerin⁺ CD8 α ⁺ DCs in defining the quality of CD8⁺ T cell responses to systemic antigen was investigated. While an obvious proportional difference in antigen-specific CD8⁺ T cells was observed in the blood and spleens of mice either depleted or not depleted of langerin⁺ DCs, it was possible that the CD8⁺ T cells had developed different chemotactic ability and had sequestered in other organs as differential chemokine receptor expression can be induced from the same original population of CD8⁺ T cells³³⁴. Analysing multiple lymphoid tissues proved that the CD8⁺ T cells were reduced throughout the langerin⁺ DC-depleted mice, and analysis of effector versus precursor memory markers by both flow cytometry and PCR analysis did not reveal any obvious difference in the proportion of memory and effector cells. This was interesting as the high IL-12 levels produced by langerin⁺ CD8 α ⁺ DCs were hypothesised to aid in the increase of effector T (T_{eff}) cell responses¹⁵¹. Functional analysis *in vivo* indicated that at the peak of the response at day seven, when there was the largest difference in CD8⁺ T cell accumulation, the higher T cell accumulation correlated with an increased antigen-specific killing function *in vivo*. By adding equal numbers of CD8⁺ T cells from the langerin⁺ DC-deficient and -sufficient group into an *in vitro* assay, they were found to have similar cytotoxic capacity. They were also shown to have similar cytokine production, as measured by intracellular cytokine staining. Therefore, this indicated that the increased CD8⁺ T cell accumulation would be the cause of the enhanced *in vivo* killing, rather than the individual cells possessing an enhanced function.

It is thought that approximately 90-95% of the CD8⁺ T cell population dies after the peak of the antigen-specific response and the remaining 5-10% become long-lived memory cells that can respond to re-stimulation³⁰⁸. This would therefore suggest that in my research there should be a larger number of surviving CD8⁺ T cells from the large langerin⁺ DC-induced CD8⁺ T cell population compared to the smaller langerin⁻ DC-induced population. However, this was not observed by assessing the accumulation of CD8⁺ T cells in the blood of the antigen-administered

mice over time. In fact, a much larger proportion of the CD8⁺ T cells induced in langerin⁺ DC-deficient mice survived long-term while the langerin⁺ CD8α⁺ DC-induced CD8⁺ T cells reduced severely in number. The similar long-term accumulation levels in the different CD8⁺ T cell populations were most likely memory cells, as they showed a similar ability to respond to re-challenge and kill target cells. This suggests that even though no early differences in the qualitative response could be determined, the langerin⁻ DC-induced CD8⁺ T cells did in fact possess a higher potential for memory development. The assays and analysis carried out in this thesis research was not able to identify any early indications of a different qualitative potential in the CD8⁺ T cells. However, it is possible that analyses performed at later time points, when memory development would be occurring, would give some indication as to the differentiation processes that are driving a larger long-term survival population in the langerin⁻ DC-induced CD8⁺ T cells.

The increased accumulation of antigen-specific CD8⁺ T cells in langerin⁺ CD8α⁺ DC-sufficient mice at the peak of the response at day seven was able to target and kill splenocytes presenting peptide, however both the large langerin⁺ DC-induced CD8⁺ T cell response and the small langerin⁻ DC-induced CD8⁺ T cell response were shown to be effective in a tumour setting. This indicates that there is possibly a difference in the quality of the response, with the langerin⁻ DC-induced CD8⁺ T cells possessing a better anti-tumour function on a per cell basis. However, the *in vitro* cytotoxicity assay and intracellular cytokine data do not support this theory of a different anti-tumour potential. Previous research has suggested that less differentiated CD8⁺ T cells provide better anti-tumour function than highly differentiated effector T cells, as they are able to persist longer to eliminate the tumour cells for a longer period³³⁵; but again, the analysis of CD8⁺ T cell phenotypical differences between the populations has not shown any major differences to indicate a potential for better survival and long-term memory persistence that would correlate with the literature.

Another possibility as to why no difference was observed in the anti-tumour function between the langerin⁺ and langerin⁻ DC-induced CD8⁺ T cells is because of the involvement of CD4⁺ T cells. It has recently been found that a subset of CD4⁺ T

helper cells, named T helper 9 (Th9) due to the preference for IL-9 production, are critically involved in tumour models such as B16 melanoma^{313; 314; 336}. The production of IL-9 by these cells is important for inhibiting tumour growth and the cells were also capable of direct tumour lysis through the production of granzyme B³¹⁴. This indicates that the tumour models may not be particularly good models for analysing the disease antigen-specific functional responses of CD8⁺ T cells, as CD4⁺ T cells have significant effects that were not measured in this thesis research. The ability of the langerin⁺ versus the langerin⁻ DCs to induce specialised CD4⁺ T cell responses, such as the Th9 cells, could be investigated in future anti-tumour studies to amass a wider understanding of all the effector cells involved in anti-tumour protection. It is possible that while the langerin⁺ CD8 α ⁺ DCs are efficient at cross-priming large CD8⁺ T cell responses, the langerin⁻ DCs may have a superior function in inducing these specialised T helper responses. Further work could also investigate the ability of the langerin⁺ CD8 α ⁺ DCs to induce antigen-specific CD8⁺ T cell responses to systemic bacterial infections and viral infections, as other researchers use these methods for inducing and investigating cytotoxic T lymphocyte (CTL) responses^{86; 142}. In this situation, it may also be useful to determine the relationship between the langerin⁺ CD8 α ⁺ DCs and NKT cells using a bacterially-derived NKT cell ligand, such as GSLs^{333; 337}, as it would be more disease relevant than the artificial marine sponge-derived α GalCer due to the constant presence and challenge of bacteria in hosts.

The conclusion from this research (illustrated in Figure 6.1) was that despite functional differences in langerin⁺ and langerin⁻ CD8 α ⁺ DCs in the spleen (with the langerin⁺ CD8 α ⁺ DCs having enhanced IL-12 production and CD40 expression), no obvious downstream impact could be detected in the quality of T cell responses generated in the absence of langerin⁺ cells, other than a sharp decrease in numbers of activated CD8⁺ T cells detected.

Figure 6.1. Theory of CD8⁺ T cell effector vs memory development in langerin⁺ DC-sufficient vs -deficient mice.

(A) In the presence of langerin⁺ CD8α⁺ DCs there is high antigen presentation along with co-stimulation and IL-12 production, resulting in large CD8⁺ T cell responses of effector T cells (T_{eff}) and pre-cursor memory T cells (T_{pcm}). The large numbers of CD8⁺ T cells correlates with high functional activity. (B) In comparison, in the absence of langerin⁺ CD8α⁺ DCs there is a reduced antigen presentation and stimulation, therefore reduced numbers of CD8⁺ T cells and reduced functional activity. (A and B) Over time the memory populations (T_m) develop into relatively equal numbers with similar memory function, suggesting that the langerin⁺ DCs induce CD8⁺ T cells with a higher potential for memory development.

6.4 The coordinated activity of multiple cellular subsets for improved CD8⁺ T cell responses

While splenic langerin⁺ CD8 α ⁺ DCs are important for inducing large CD8⁺ T cell responses to systemic antigen through their superior ability to cross-prime, a goal of this thesis was to assess the potential role of other DCs in contributing to CD8⁺ T cell responses. It was observed that langerin⁻ APCs were capable of cross-presenting antigen to induce CD8⁺ T cells, but lacked an ability to efficiently prime the T cells. By initially pre-conditioning pDCs and CD8 α ⁻ DCs with α GalCer-mediated NKT cell activation, it was possible to stimulate those DCs with a TLR7 agonist to contribute to the priming of CD8⁺ T cell responses. The pDCs were pre-conditioned by NKT cell stimulation to possess an enhanced capacity for type I IFN production in response to further TLR7 stimulation, which served to activate the CD8 α ⁺ DCs that did not express TLR7. The CD8 α ⁻ DCs were also pre-conditioned by NKT cell stimulation to possess an enhanced activatory response upon further TLR7 stimulation. However, while the delayed TLR7-mediated enhancement of CD8⁺ T cell priming was independent of the langerin⁺ CD8 α ⁺ DCs, the original cross-priming of CD8⁺ T cells was still significantly dependent on the langerin⁺ CD8 α ⁺ DCs.

The observation of multiple APC subsets contributing to cross-presentation correlates with other research, which has shown some limited role of other APCs to contribute to antigen cross-presentation; for example, OVA antigen targeted to the Siglec-H endocytic receptor expressed on pDCs results in cross-presentation of that antigen to CD8⁺ T cells⁶³. The ability to activate these other APCs to contribute to CD8⁺ T cell responses could potentially benefit vaccination strategies by including multiple cellular subsets for an enhanced immune response. The activation of pDCs by NKT cells has been shown to contribute to the cross-priming ability of cDCs²³⁴, and research in this thesis demonstrates a method by which conditioning these cells with α GalCer-mediated NKT activation followed by TLR7 stimulation can make these cells be activated and used in conjunction with other APCs, such as langerin⁺ CD8 α ⁺ DCs, at different stages in the immune response to improve CD8⁺ T cell responses. The activation of pDCs in the research by Shimizu *et al*²³⁴ demonstrated

that the pDCs required signalling induced by interaction with HMGB1, a damage-associated molecular pattern molecule, as well as OX40-OX40L interactions from activated NKT cells. The HMGB1 is released from dying cells within 8-32 hours after stimulation, as observed with apoptosing RAW 264.7 (mouse macrophage-like cells), Jurkat cells (human T cell lymphoma) and HeLa cells (human cervical cancer cells)^{338; 339}. In the case of studies in this thesis, it is possible that the sensitive langerin⁺ CD8 α ⁺ DCs that die 6-8 hours after exposure to α GalCer-induced NKT cell stimulation release such damage signals during the pre-conditioning of pDCs with α GalCer-induced NKT activation. The release of damage signals remains to be proven, however, and one way to prove this would be to measure the release of various damage-associated molecules *in vitro* and *in vivo* after α GalCer administration in the presence and absence of langerin⁺ CD8 α ⁺ DCs.

The studies in the thesis and previous research can be summarised into a theory about the coordinated activity of multiple cell types for enhanced CD8⁺ T cell responses, as illustrated in Figures 6.2 and 6.3. The splenic marginal zone langerin⁺ CD8 α ⁺ DCs are the first DCs to acquire antigen and α GalCer from blood filtered through the spleen; they present α GalCer to NKT cells that localise to the marginal zone of the spleen²³², which in turn further activates the langerin⁺ CD8 α ⁺ DCs and causes the attraction of CD8⁺ T cells by chemokine production⁸⁹. The langerin⁺ CD8 α ⁺ DCs then initiate antigen presentation and co-stimulation (Figure 6.2A). The NKT cells meanwhile are able to interact with the pDCs, presumably via OX40-OX40L interactions as CD1d expression by pDCs has previously been shown to not be required for activation by NKT cells²⁹², and also with CD8 α ⁻ DCs, causing them to become more sensitised to later TLR stimulation such as with the TLR7 agonist resiquimod (Figure 6.2B). TLR stimulation of the pre-conditioned pDCs results in the production of a large amount of type I IFNs that can further activate remaining CD8 α ⁺ DCs that have not died due to α GalCer-induced NKT cell activation sensitivity. The CD8 α ⁻ DCs and pDCs are able to provide stimulation and antigen presentation, along with the cross-priming from CD8 α ⁺ DCs that are still remaining, to further enhance CD8⁺ T cell responses.

Without langerin⁺ CD8 α ⁺ DCs present at the time of antigen and α GalCer administration, there is a severely reduced initiation of the CD8⁺ T cell cross-priming, and therefore very minor initial CD8⁺ T cell response for the NKT cell pre-conditioned pDCs and CD8 α ⁻ DCs to enhance (Figure 6.3A). Without the NKT cell activation, there is only the limited increase in stimulatory ability of the langerin⁺ CD8 α ⁺ DCs in response to TLR stimulation (such as TLR2 ligation by Pam3Cys) for CD8⁺ T cell priming to occur; there is also no pre-conditioning of the CD8 α ⁻ DCs and pDCs for an enhanced response to TLR stimulation to manipulate these DCs to contribute to CD8⁺ T cell priming (Figure 6.3B).

The exact role of the different DCs could be confirmed using mixed bone marrow chimeras, for example, using *SiglecH*-DTR bone marrow mixed with CD1^{-/-} would allow the confirmation of the important interaction between pDCs and NKT cells. Further mixed bone marrow chimeras could be used for the analysis of the IL-12 and CD40 expression by langerin⁺ CD8 α ⁺ DCs and also the use of Bm1 mice, which have a defect in the H-2K^b molecule and therefore cannot present SIINFEKL OVA-peptide to CD8⁺ T cells²³⁸, would allow the determination of the importance of antigen presentation by the various DC subsets. Imaging studies to follow the interactions of the various cellular subsets involved at different points in the immune response, for example, like previous research demonstrating the migration of splenic langerin⁺ CD8 α ⁺ DCs to T cell areas after stimulation^{16; 92}, would also confirm the direct interactions between the cell subsets, such as specifically the langerin⁺ CD8 α ⁺ DCs interacting with NKT cells in the marginal zone.

Overall this research indicates a central role of the langerin⁺ CD8 α ⁺ DCs in initiating CD8⁺ T cell responses, using NKT cell activation to enhance CD8⁺ T cell priming. By beginning the cross-priming of the CD8⁺ T cells, the langerin⁺ CD8 α ⁺ DCs then provide the opportunity for NKT cell pre-conditioned pDCs and CD8 α ⁻ DCs to participate in priming CD8⁺ T cells. This research highlights the possibility of differentially timing adjuvants in vaccines to enhance multiple DC activation for increased CD8⁺ T cell responses. This is in contrast to research that focuses on targeting one particular DC subset for the induction of CD8⁺ T cell responses^{135; 136;}

These data point to a broader coordinated role of DCs in adaptive immune responses to antigens entering the spleen, and suggest that the threshold for invoking different DC populations can be determined by the milieu of stimuli that accompany the antigen into the lymphoid tissue. For example, bacteria expressing glycolipids that stimulate NKT cells may condition many different DCs to become exquisitely sensitive to TLR ligation, meaning that several DC subtypes can now participate in driving a suitable immune response. The ability of the host to receive and integrate multiple stimuli to become more sensitive to pathogenic challenge may control many others aspect of adaptive immunity, not just the CD8⁺ T cell priming investigated here.

A)
Without prior pre-conditioning of pDCs
and CD8 α ⁻ DCs with NKT cell stimulation

B)
With prior pre-conditioning of pDCs and
CD8 α ⁻ DCs with NKT cell stimulation

Figure 6.2. Schematic of induction of CD8⁺ T cell responses through the network of interactions between various cellular subsets.

A) Moderate CD8⁺ T cell response with langerin⁺ CD8 α ⁺ DCs as the major contributor to cross-priming. B) Large induction of CD8⁺ T cell responses by pre-conditioning CD8 α ⁻ DCs and pDCs with NKT cell activation 24 h prior to the addition of the TLR7 agonist resiquimod for increased activation.

Figure 6.3. Schematic of importance of langerin⁺ CD8 α ⁺ DCs and NKT cells for induction of CD8⁺ T cell responses.

A) Reduced CD8⁺ T cell response in the absence of langerin⁺ CD8 α ⁺ DCs for initial cross-priming, even when pDCs and CD8 α ⁻ DCs have been pre-conditioned with NKT cell stimulation. B) Reduced CD8⁺ T cell response without help from NKT cell activation.

6.5 Future directions

This thesis outlines progressive research into the functional roles of the splenic langerin⁺ CD8 α ⁺ DCs in mice for inducing CD8⁺ T cell responses. The next step in this research would be to compare this murine langerin⁺ CD8 α ⁺ population with the equivalent human population. It has previously been theorised that the BDCA3⁺ (CD141⁺) population of human DCs found in the bone marrow, tonsils, lymph nodes, blood and spleen are the equivalent of the murine CD8 α ⁺ DCs due to the comparative expression of DNNGR-1 (CLEC9A), XCR1, Batf3, TLR3, CD40, CD86, and the high IL-12 production and cross-presentation ability^{285; 311; 340}. However, this thesis has indicated that the attributes previously associated with the total CX3CR1⁻ CD8 α ⁺ DC population may in fact be more applicable to the langerin⁺ population. Therefore, research should be undertaken to compare the langerin⁺ CD8 α ⁺ and langerin⁻ CD8 α ⁺ populations with the BDCA3⁺ human DCs to confirm if the BDCA3⁺ DCs are in fact the equivalent of the murine langerin⁺ CD8 α ⁺ DCs.

The exact functions of the various DCs involved in interactions with NKT cells after antigen and α GalCer administration for inducing CD8⁺ T cell responses still requires further investigation, as the mechanisms of activation by the CD8 α ⁻ and pDCs have not been described in this research. Methods to accomplish this analysis could include depletion models with depletion of DCIR2 (dendritic cell inhibitory receptor-2) or Dectin-1 (DC-associated C-type lectin 1), molecules expressed on CD8 α ⁻ DCs^{341; 342}, and further investigation using the *SiglecH*-DTR mice, to determine what factors have changed when the CD8 α ⁻ DCs or the pDCs are absent during the immune response. To specifically deplete certain cellular functions, the different cellular depletion models could be used in mixed bone marrow chimeras with functional knockout models, such as IL-12p40^{-/-} and CD40^{-/-} mice. This would allow the functional depletion in only specific cell types, allowing the importance of functional attributes of particular DC subsets to be elucidated. An example of where this technique would be useful is to understand the antigen-presentation requirements by the NKT cell pre-conditioned DCs. It is not clear from this thesis research whether antigen presentation is required by the pre-conditioned

DCs or if they are only adding to the priming of CD8⁺ T cells. Therefore, the bone marrow chimeras would be useful for specifically depleting the antigen presentation capability of the CD8 α ⁻ and pDCs by using bone marrow from Tap1^{-/-} or Bm1 mutant mice. Tap1^{-/-} and Bm1 mice are both incapable of presenting SIINFEKL peptide from the OVA protein to the antigen-specific CD8⁺ T cells and, therefore, the use of these mice would indicate the importance of antigen presentation by the different DC subsets.

Understanding the specific contributions of different APC subsets and knowing how to manipulate those populations for enhanced T cell activation is very beneficial for future vaccine strategy research. Many researchers are currently attempting to target antigens to specific DC subsets, such as Clec9a, DEC-205 and langerin, for utilising the superior cross-presenting and co-stimulatory functions of those cells^{135; 136; 343}. However, this thesis research indicates that manipulating multiple subsets to create an activatory cell network may be more beneficial for improving CD8⁺ T cell responses. Side effects seen in the past using un-targeted antigens and stimuli have included off-target effects from the antigen being presented by the incorrect functional cell type and the antigen not being able to get to the desired functional cell type¹³⁷ and, as seen in this thesis, induction of too much inflammation resulting in toxicity (*unpublished observation*). Therefore, careful consideration would have to be taken with the method for activating multiple cellular subsets. Studies could attempt to use targeting strategies in combination with pre-conditioning treatments, for enhanced activation of particular cellular subsets while ensuring the antigen is acquired primarily by the major cross-priming subset for optimal antigen uptake and presentation.

Further investigation is also required as to the functional benefit of the pre-conditioned DC-induced CD8⁺ T cells. Recent research has indicated that while early exposure of CD8⁺ T cells to IL-12 results in large highly functional effector cells that are able to infiltrate tumour tissue, exposure to type I IFN causes the T cells to be programmed into less functional cells with a reduced capacity for tumour tissue infiltration³⁴⁴. Because of the high type I IFN levels observed in serum with pre-conditioned pDCs, this may result in less functional CD8⁺ T cells. Preliminary

data in this thesis has indicated that the CD8⁺ T cells induced in a high type I IFN environment (due to the NKT cell pre-conditioned pDCs) have an increased function in regards to IFN- γ and TNF- α production. Further experiments that sort the activated T cells for *in vitro* killing assay analysis would identify the antigen-specific killing function of the CD8⁺ T cells.

The application of this murine DC knowledge of pre-conditioning DCs with the CD1d-restricted NKT cells is problematic when applied to the human situation, as humans possess multiple CD1-restricted cells and only a small percentage are the CD1d-restricted NKT cells. In previous research mice have been modified to express the various CD1 molecules³⁴⁵ and, therefore, future research should assess the importance of langerin⁺ CD8 α ⁺ DCs for presenting these other CD1-restricted ligands. That knowledge could then be applied to the human DC subsets to allow a wider repertoire of possible pre-conditioning agonists to be explored. Another aspect is to consider the effect that MHC class II presentation to CD4⁺ T cells has on the CD8⁺ T cell response. Aarntzen *et al*³⁴⁶ recently showed in a human trial that inclusion of MHC class II presentation by a DC vaccine increased the clinical outcome of melanoma due to improved help from CD4⁺ T cells to CD8⁺ T cells. Whether the NKT cell interactions with langerin⁺ CD8 α ⁺ DCs and the pre-conditioning of CD8 α ⁻ and pDCs is enough help for the induced CD8⁺ T cell responses, or whether ensuring the activation of CD4⁺ T cells would also be beneficial, was not investigated in this thesis. This was due to previous research showing a superior presentation to CD8⁺ T cells by the langerin⁺ CD8 α ⁺ DCs with no superior presentation to CD4⁺ T cells⁵². However, further research should investigate the role of CD4⁺ T cells in the pre-conditioned CD8 α ⁻ CD4⁺ DC population, as they preferentially present antigen to CD4⁺ T cells⁸⁵. Therefore, they may be improving CD8⁺ T cell responses by improving CD4⁺ T cell help, and CD4⁺ T cells have also been shown to improve CD8⁺ T cell memory¹⁹⁶.

6.6 Conclusions

This thesis has clearly shown useful techniques for the identification of a DC subpopulation with superior cross-priming attributes in murine models. These langerin⁺ CD8 α ⁺ DCs are important for presenting antigen, providing stimulation, activating NKT cells and influencing CD8⁺ T cell responses early in the immune response, which enhances CD8⁺ T cell accumulation throughout the host resulting in enhanced antigen-specific function. After the initial cross-priming of CD8⁺ T cells by the langerin⁺ CD8 α ⁺ DC subset, temporal phasing of stimuli allows the pre-conditioning of other DCs that are then capable of further enhancing CD8⁺ T cell responses. This knowledge could be applied to the improvement of vaccine strategies for inducing large antigen-specific CD8⁺ T cell responses for the treatment of various diseases.

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Appendices

Appendix 1

Figure A1 Antibody titration.

Naïve splenocytes from CD45.1⁺ and CD45.2⁺ mice were mixed and stained with different dilutions of CD45.1-FITC antibody, as indicated in the legend. The fluorescence was measured by flow cytometry. The most dilute concentration of 1:1000 was able to provide identification of the positive cells, the same level of detection as the concentrated 1:100 dilution, without non-specific binding, so was chosen as the concentration of antibody to use.

Appendix 2

Figure A2 CD8 dynabead purification analysis.

Spleens and lymph nodes were collected, processed, and the cells were enriched using a FlowComp dynabead kit. A sample of the pre-sorted (Pre-sort) cells and the post-sorted cells (CD8-enriched (Positive) and CD8-depleted (Negative)) were stained with fluorescently tagged antibodies against CD4 and CD8 for analysis on a BD FACSCalibur. The resulting percentages of CD4⁺, CD8⁺ and CD4⁻ CD8⁻ cells are indicated on the flow cytometry dot plots.

Appendix 3

Figure A3 Fluorescence minus one (FMO) control.

Spleens were collected, processed and the splenocytes were stained with either the complete antibody panel, or were incubated with all antibodies minus the CD44-PE antibody. The CD44⁺ cells could be correctly identified, as shown on the dot plots, in comparison to the background fluorescence from the other fluorophores in the FMO control.

Appendix 4

Figure A4 DT treatment in *lang*-DTREGFP mice does not induce neutrophilia.

Lang-DTREGFP mice were either administered PBS or 350 ng DT i.p. After 24 h, spleens were harvested and analysed by flow cytometry for the presence of neutrophils (CD11b^{hi}Gr-1^{hi}). Data shown are $n = 3$ per group and is representative of 2 independent experiments showing similar results. Statistical analysis was performed using Student's t test, showing mean $\pm$ SEM, ns = not significant.

Appendix 5

Figure A5 Two doses of 350 ng DT depletes multiple subsets of langerin-expressing DCs in *lang*-DTREGFP mice.

Lang-DTREGFP mice were administered either PBS or 350 ng DT i.p two days consecutively. After 24 h from the second administration, the indicated tissues were collected, processed and analysed for the presence of langerin-expressing DCs by flow cytometry. Langerin-expressing cells were separated into lymphoid DCs (CD11c⁺langerin⁺CD8 α ⁺), dermal DCs (CD11c⁺langerin⁺CD8 α ⁻CD326^{low/-}), and Langerhans Cells (CD11c⁺langerin⁺CD8 α ⁻CD326^{hi}). Data shown is n = 5 per group and is representative of 2 independent experiments showing similar results. Statistical analysis was performed using Student's t test between PBS and DT groups, showing mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001, ns = not significant.

Publications

Publications arising from work presented in this thesis:

Prendergast KA and **Osmond TL**, Ochiai S, Hermans IF, Kirman JR. Sustained *in vivo* depletion of splenic langerin⁺ CD8 α ⁺ dendritic cells is well-tolerated by *lang*-DTREGFP mice, *Journal of Immunological Methods* (2014) 406: p104-109

Tang C, Knight DA, **Osmond TL**, Hermans IF, Petersen TR. Batf3-independent langerin⁺ CX₃CR1⁺ CD8 α ⁺ splenic DC represent a precursor for classical cross-presenting CD8 α ⁺ DC, *Journal of Leukocyte Biology* (In Press)

Manuscript submitted:

Osmond TL, Farrand KJ, Painter GF, Ruedl C, Petersen TR, Hermans IF. The optimal cross-priming function of murine langerin⁺ CD8 α ⁺ DCs can be aided by pre-conditioning CD8 α ⁺ DCs.