The Role of Langerin$^+$ CD8α^+ Dendritic Cells in Tumour Immunotherapy

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I. Abstract

The immune system has the potential to selectively target and eliminate tumours cells. However, the induction of an immunosuppressive environment by factors released by tumours cells, or by the tumour stroma, in combination with difficulties in differentiating between healthy and malignant cells, contributes to inefficient or disabled anti-tumour immune responses. A variety of different immunotherapeutic approaches are being developed to tip the balance in favour of anti-tumour immunity. Many of these approaches are designed to stimulate improved activity of T cells with specificity for tumour-associated antigens.

This thesis explores how T cell-mediated responses are initiated and maintained in immunotherapy, with an emphasis on the role of antigen presentation by resident dendritic cells (DCs). An animal model was used in which a DC subset in the spleen that expresses the cell marker langerin could be selectively ablated during the course of therapy. As these DCs have been shown to be uniquely capable of acquiring circulating antigens and cellular debris, and have a heightened capacity for cross-priming $CD8^+$ T cells, it was hypothesised that the function of these cells could play a significant role in determining the outcome of immunotherapies.

A model of adoptive T cell therapy was examined in mice challenged with an intravenously administered lymphoma that formed tumour foci in a variety of locations in the body. Treating established tumours by adoptively transferring *in vitro* activated effector $CD8^+$ T cells significantly increased their symptom-free survival. The protection received by this therapy was dependent on a stimulus being provided by endogenous langerin$^+$ CD8α^+ DCs to the transferred T cells. In the absence of langerin$^+$ CD8α^+ DCs, the proportion and number of transferred anti-tumour $CD8^+$ T cells was lower in the blood and spleen. However, no obvious differences in phenotype and function could be defined. Langerin$^+$ CD8α^+ DCs therefore contribute to the maintenance of an effective $CD8^+$ T cell-based immunotherapy and the role of endogenous

DCs should be taken into consideration during the design of immunotherapies.

To investigate the role of langerin$^+$ CD8α^+ DCs in initiating effector T cell responses, a novel whole-cell vaccine was developed for the treatment of acute myeloid leukaemia (AML). This vaccine exploited the stimulatory functions of invariant natural killer T cells, and was therefore administered intravenously to access the large invariant natural killer T cell compartment of the spleen. The vaccine completely protected mice from developing leukaemia when challenged with AML cells after vaccination, with CD4$^+$ and CD8$^+$ T cells mediating protection. The immune response generated by the vaccine was shown to be completely dependent on langerin$^+$ CD8α^+ DCs. In hosts with established tumours; however, the vaccine was ineffective. This may have been partially due to a reduced function of langerin$^+$ CD8α^+ DCs as their activation phenotype was significantly reduced in the presence of established AML; however, non-specific T cells could still be stimulated via these DCs. Reduced vaccine efficacy was associated with increased number and/or function of suppressor cells, including regulatory T cells and myeloid derived suppressor cells within the host. In addition, in leukemic hosts, the proportion of T cells in the spleen was reduced, and the function of AML-specific CD4$^+$ T cells, but not CD8$^+$ T cells, was impaired. Driving AML-bearing hosts into remission with chemotherapy prior to vaccination enabled the vaccine to protect the host from subsequent AML challenge. Langerin$^+$ CD8α^+ DCs are therefore responsible for initiating the vaccine-induced immune response in this model and their suppression may have contributed to the inefficacy of the vaccine in the presence of established tumours.

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III. List of abbreviations

α-GalCer	α-Galactosylceramide
24G2	anti-CD16/32 FcR blocking antibody
2-ME	2-mercaptoethanol
A20	mouse B cell lymphoma cell line
allo-SCT	allogenic stem cell transplant
AML	acute myeloid leukaemia
Apaf-1	apoptotic protease activating factor 1
APC	antigen presenting cell
APRIL	a proliferation inducing ligand
ATP	adenosine triphosphate
B16	C57BL/6 mouse melanoma cell line
C1498	mouse acute myeloid leukaemia cell line
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
cDC	conventional dendritic cells
CFSE	carboxyflouresceindiacetatesuccinimidyl ester
cIMDM	complete Iscove's modified dulbecco's medium
CTL	cytotoxic T lymphocyte
CTO	cell tracker orange
CXCR	chemokine (C-X-C motif) receptor
DAMP	damage associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	debdritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dox	doxorubicin
DT	diphtheria toxin
DTR	diphtheria toxin receptor
EDTA	ethylenediaminetetraacetic acid
E.G7-OVA	murine thymoma cell line that expresses OVA
ER	endoplasmic reticulum
ERAD	ER-associated destruction pathway
ERAP1	ER-associated aminopeptidase 1
FACS	fluorescence activated cell sorting
FAP	fibroblast activation protein
FBS	foetal bovine serum
FCγR	fragment crystallizable gamma receptor
Foxp3	forkhead box P3
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
HA	hemagglutinin
HLA	human leukocyte antigen
IDO	indoleamine 2, 3-dioxygenase
IFN	interferon
Ii	invariant chain
IL	interleukin
IMDM	Iscove's modified dulbecco's medium
iNKT cells	invariant natural killer T cells

J558	mouse plasmacytoma cell line
KLRG-1	killer cell lectin-like receptor subfamily G member 1
KO	knockout
L	ligand
Lang-EGFPDTR	langerin-diphtheria toxin receptor mice
LCMV	Lymphocytic choriomeningitis virus
LDFB	live dead fixable blue
LPS	lipopolysaccharide
Ly6c	lymphocyte antigen 6 complex, locus C
MCP-1	macrophage chemotactic protein - 1
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MDSC	myeloid derived suppressor cells
NOX2	Nicotinamide adenine dinucleotide phosphate oxidase
NK	natural killer
OT-I	OVA SIINFEKL specific TCR transgenic mice
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PC61	anti-mouse CD25 antibody
PI	propidium iodide
PNAd	peripheral node addressin
PRR	pattern recognition receptor
R	receptor
RBC	red blood cell
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpm	revolutions per minute
rT_{EM}	resident T effector memory
SA	streptavidin
TAP	transporter associated with antigen processing
T_{CM}	T central memory
TCR	T cell receptor
T_{EM}	T effector memory
TGF	transforming growth factor
T_h	T helper
TIL	tumour infiltrating lymphocytes
TLR	toll-like receptors
TNF	Tumour necrosis factor
T_{reg}	regulatory T cell
$V\alpha$	variable α region of the TCR

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Chapter 1: Introduction

1.1 General overview

A tumour arises when a normal cell looses control of its cell cycle, resulting in uncontrolled proliferation. The causative factors are diverse but have been associated with age, weight and contact with carcinogens, such as tobacco smoke. In 2009, cancer was the leading cause of death in New Zealand, accounting for 28.9 % of all deaths (1). It has been estimated that there will be a 45 % increase in deaths attributed to cancer by 2030 worldwide, which indicates an extra 4.3 million new cases of cancer per year (2). Current standard treatments for cancer include surgery, chemotherapy and radiation therapy, however the benefits of these treatments vary significantly depending on the type of cancer and how early it is detected. Surgery can only be performed if the tumour is accessible, and is only beneficial if the tumour has not metastasized to other areas of the body. Chemotherapy and radiotherapy are generally treatments that target proliferating cells, and therefore are not tumour specific. As a result these therapies can cause significant damage to healthy cells, with adverse side effects ranging from nausea to severe morbidity.

The immune system is highly effective at protecting the body from invading pathogens, but it is now recognised that it can prevent the development of tumours within a host. This was made evident by experiments demonstrating that immunodeficient mice develop spontaneous tumours much faster than immunosufficient mice, indicating that the immune system prevents spontaneous and carcinogen-induced tumour development (3). However, the immune system has evolved to avoid attacking healthy tissue, as the risks to the host associated with autoimmunity can be significant. This poses a problem for generating an immune response against tumours because tumour cells derive initially from healthy host cells, and therefore express a largely “normal” repertoire of proteins (or “self-antigens). Immune cells that have the potential to attack tumour cells have largely been removed from the immune system to permit tolerance of self-antigens and prevent autoimmunity. Anti-tumour immunity is therefore reliant on stimulating responses from immune cells that have avoided this depletion process, and are therefore likely to have

only low affinity for self-antigens, or must be targeted to neo-antigens that are unique to the tumour tissue. In addition, immune suppression specifically generated by tumours is capable of preventing the activation and function of an anti-tumour immune response. These themes will be discussed in more detail later in the chapter.

To understand anti-tumour immunity in more detail for this thesis, it is necessary to give a brief overview of the broad features of the immune system, which is currently best understood in the context of defence against pathogens. The immune system can broadly be differentiated into two main components, the innate and adaptive immune response. Both arms have distinct functions that cooperate together to protect the host. The first-line of immune defence consists of the innate immune system, which responds to infectious agents in a generic way and therefore rapidly provides a non-specific, blanket defence in response to exposure to pathogens (4). In contrast, the adaptive immune response takes time to develop, but is highly selective due the process of stimulating only immune cells that have pathogen-specific antigen-receptors. Following activation, these cells undergo clonal expansion and differentiate into effector cells. Importantly, it is now clear that there is cross-talk between the innate and adaptive arms of the immune system, with the early events triggered by innate cells laying the groundwork for an effective adaptive immune response.

The innate immune system consists of a variety of different cells, each of which have distinct functions in eliminating pathogens. These cells include macrophages, neutrophils, DCs, mast cells, eosinophils and natural killer cells (NK cells), each briefly discussed below.

Macrophages, neutrophils and DCs are three main phagocytic innate immune cells. These cells can ingest pathogens and kill them by producing powerful degradative molecules called lysosymes. Macrophages can be found in almost every tissue within the body, the majority of which originate from myeloid precursors in the bone marrow that differentiate into monocytes. Once developed, monocytes circulate in the blood and bone marrow and

migrate into tissues during infection where they can differentiate into macrophages (5). In addition, macrophages can differentiate directly from myeloid precursors in the bone marrow before migrating into tissues. In the context of infection, macrophages can be induced to secrete molecules into the environment that induce inflammation and recruit other cells of the immune system to aid in host-defence (6). Although capable of presenting acquired antigens to antigen-specific cells of the adaptive immune system, their role in coordinating the immune system depends on their activation state. The phenotype of macrophages can range from inflammatory to suppressive, depending on the stimuli they receive (7). A key mechanism of the adaptive immune response is to induce the differentiation of macrophages toward the inflammatory phenotype, thereby aiding the elimination of pathogens. However, in most tumour environments the presence of macrophages is associated with a poor prognosis due to an anti-inflammatory phenotype (7).

Neutrophils traffic in the blood and only migrate into tissues in response to inflammation. They can kill microorganisms by phagocytosis and by the release of reactive oxygen species (ROS) into the phagosome. In addition, neutrophils are able to expel their intracellular components, including their DNA, bound with granules. This structure is able to capture and limit the activity of microorganisms (8). The involvement of neutrophils in the adaptive immune response is limited and not well understood. In the marginal zone of the spleen, neutrophils activate B cells by producing the B cell stimulating cytokine called a proliferation-inducing ligand (APRIL), leading to the production of antibodies by B cells, independently of T cells (9).

DCs are the main focus of this thesis. Like macrophages, they are found in most tissues in the body and can phagocytose cellular material, circulating antigens and whole pathogens. However, unlike macrophages their main role is to coordinate adaptive immune responses. In the steady state (i.e. in the absence of infection), the stimulation provided by DCs to cells of the adaptive immune system can be inhibitory to prevent immune responses against self-tissue. In the presence of signals of infection or evidence of tissue destruction, DCs serve as the most potent stimulators of adaptive immune responses (4).

Phagocytosed antigens are processed and presented in a manner that promotes activation of antigen-specific lymphocytes. These functions will be described later in the thesis, with emphasis on stimulating T cells.

Eosinophils and basophils are cells of the innate immune system that attack pathogens, such as parasites, that are too large to be engulfed by macrophages. When these cells are activated they release toxic molecules that damage pathogens. These cells are not of relevance in this thesis and will not be discussed in any detail.

NK cells are lymphocytes of the innate immune system that are capable of producing and releasing cytotoxic molecules that can cause cell death. Broadly speaking, although fully “armed” with cytotoxic mediators, they remain inactive through interaction with inhibitory molecules expressed by healthy cells. The loss of these molecules, combined with interaction with ‘stress-induced’ stimulatory molecules on infected or perturbed cells, promotes NK activity. NK cells also produce large quantities of cytokines following stimulation, which aid in exacerbating the immune response (10).

The adaptive immune system consists of T and B lymphocytes, each bearing randomly generated antigen-receptors. As noted, adaptive immune responses are slower due to the process of clonal selection of antigen-specific cells. While B cells produce antibodies that play a crucial role in adaptive immunity, including in cancer, this thesis will focus primarily on T cells, which have been shown to have therapeutic activity in many models of cancer, and show promise as effector cells in the clinic.

As a population, T cells are usually divided into two broad categories on the basis of expression of CD4 or CD8. Although not described here, it is important to note that other populations of T cells do exist, such as the innate-like T cells that will be discussed later in the chapter. Broadly speaking, T cells expressing CD4 differentiate into cytokine-secreting T “helper” cells upon activation and function largely by activating or enhancing the functions of other immune cells, including DCs, macrophages and $CD8^+$ T cells. In

contrast, T cells expressing CD8 differentiate into cytotoxic T cells (cytotoxic T lymphocytes; CTL) that are capable of producing cytotoxic molecules and cytokines that can kill target cells. It should be noted that the functions of these two cell types is not exclusive, as $CD4^+$ T cells have been demonstrated to develop cytotoxic functions in some settings (11). Both classes of T cell derive from bone marrow precursors, and mature in the thymus. T cells that recognise self-antigens with high affinity are deleted from the repertoire, and the remaining cells then distribute into the lymphoid organs (12). The process of activating T cells is complex, and is heavily reliant on cues received by antigen presenting cells (APCs), primarily DCs, that provide signals indicating danger to the host. Tissue-resident DCs must acquire antigens and bring them to lymphoid tissues to stimulate T cells specific for those antigens. Alternatively, DCs that reside in lymphoid tissues acquire antigens that drain into the tissue, either from the blood during circulation, or via the lymphatic system. Either way, processed antigens must be presented by mature DCs to induce T cell activation. Once activated, $CD8^+$ T cells are able to migrate to sites of inflammation and kill antigen-expressing or infected cells by releasing cytotoxic molecules (13). They are also capable of targeted release of cytokines, which can improve or exacerbate the immune response. On the other hand, activated $CD4^+$ T cells do not generally have a direct cytotoxic function, but produce cytokines and provide direct stimulation (via receptors) to cells of the innate immune system, such as macrophages, to enhance their host-defence mechanisms (14). Another important role of $CD4^+$ T cells is to aid in progression of the $CD8^+$ T cell response by providing stimulatory signals that enhance the stimulation provided by DCs, a process referred to as “T cell help”. $CD4^+$ T cells perform a similar process to induce the production of antibodies by B cells. $CD8^+$ T cells are the main lymphocyte of the adaptive immune response discussed in this thesis, because of their superior ability to kill tumour cells.

The aim of the research in this thesis was to develop a greater understanding of the interactions between DCs and $CD8^+$ T cells in eliciting anti-tumour immune responses. As will be established over the course of the introduction, there are many subsets of DCs, and it has been hard to elucidate the different

roles of these subsets. The availability of a mouse strain that enables the specific deletion of one DC subset, the langerin$^+$ CD8α^+ DCs in the spleen, enabled an analysis of this subsets function. Importantly, the information generated from this study could aid in the rational design of novel and more effective immunotherapies for the treatment of tumours.

1.2 The structure and immune function of the spleen

This thesis consists of an examination of the function of a specific DC population found within the marginal zone of the spleen. Therefore, a general understanding of the anatomy and function of the spleen is necessary to understand the function of this DC subset and how it has access to antigens from the blood. Immune cells and antigens within the blood enter the spleen through the splenic artery and travel trough the central arterioles, which are surrounded by the periarteriolar lymphoid sheath (a T cell rich area). The central arterioles branch into arterioles that transverse the periarteriolar lymphoid sheath and form capillaries in the marginal sinus and red pulp. The marginal sinus is surrounded by the marginal zone, which contains DCs, macrophages and lymphocytes. Blood within the marginal sinus enters the trabecular vein to exit the spleen, however there are small gaps in the marginal sinus that allows blood to pass into the marginal zone before entering the surrounding red pulp and then re-joining the circulation via venous sinuses (15). Blood passing through the marginal zone enables resident APCs to acquire antigens within the blood.

1.3 Dendritic cells

1.3.1 Antigen processing and presentation

The classical pathways of antigen presentation

To appreciate the importance of DCs in developing an immune response against tumours a deeper understanding of how they process and present antigens is required. There are two classical pathways of antigen presentation and each drives a different response by activating either CD8$^+$ T cells (the

MHC class I presentation pathway) or $CD4^+$ T cells (the MHC class II presentation pathway).

MHC class I presentation pathway

Antigens acquired by DCs, or derived from within the cell as a consequence of infection, are processed into peptide fragments, some of which are ultimately presented on the cell surface in the context of major histocompability complex (MHC) molecules for presentation to T cells. The class of MHC molecule that the peptide is presented on determines whether a $CD4^+$ or $CD8^+$ T cell can recognize it (16, 17); the T cell receptor (TCR) of $CD8^+$ T cells recognise antigens bound to MHC class I and the TCR of $CD4^+$ T cells recognise antigens bound to MHC class II, as demonstrated in figure 1.1A. In the classical model of MHC class I antigen presentation, proteins found within the cell itself ("endogenous" antigens), such as those originating from a virus, are processed and presented on MHC class I. These endogenous proteins enter the core of the proteasome within the cytosol, where they are proteolytically cleaved into short peptides, 2 to 25 amino acids long (18, 19). After degradation, the peptide fragments then enter the lumen of the endoplasmic reticulum (ER) via molecules called transporters associated with antigen processing 1 and 2 (TAP1 and TAP2) (20, 21). The peptides are then cleaved into smaller fragments within the ER by peptidases, such as ER-associated amino peptidase 1 (ERAP1). Within the repertoire of peptides generated will be sequences that can be inserted in the peptide groove of MHC class I molecules (22-24).

The MHC class I complex is composed of both a heavy chain and a beta-2 microglobulin component. Initially following development in the ER, the heavy chain binds to the protein calnexin, which aids in folding of the heavy chain. The folded structure enables binding of ERp57 with calnexin further promoting folding of the heavy chain. Once folded, the heavy chain can form a complex with beta-2 microglobulin, allowing the dissociation of calnexin, and the binding of calreticulin and the TAP complex via tapasin. This creates an unloaded MHC class I molecule receptive to peptides that translocate into the

ER via TAP (25). Binding of a peptide into the peptide binding groove of MHC class I releases the MHC class I/peptide complex from ER proteins, thereby allowing the complex to migrate to the cells plasma membrane via the golgi cisternae for presentation (26). Antigens presented on MHC class I are recognized by TCRs of $CD8^+$ T cells, which when activated provide a cytotoxic response against cells that express that specific antigen (27).

MHC class II antigen pathway

Antigens destined for expression on MHC class II for presentation to $CD4^+$ T cells are exogenous antigens that are captured by APCs and internalized into endosomes (Figure 1.1B). The acquired proteins are then degraded into peptide fragments by proteases within the endosome, creating peptide fragments between 12 and 24 amino acids long that can bind to MHC class II (28-30). The MHC class II molecule is synthesized in the ER. The antigen binding groove ligates to a chaperon protein called the invariant chain (Ii) for stabilization (31). Following stabilization, the MHC class II complex is transported to the endosome where the Ii chain is cleaved by active proteases. A short fragment of the Ii chain remains bound to the MHC class II binding groove and is called the class II associated invariant chain peptide, which can then be displaced by a peptide with higher affinity for MHC class II (32). Once a peptide has bound, the complex migrates to the cell surface to present antigens to $CD4^+$ T cells via a transport vesicle that buds from the endosome (33).

Cross-presentation

The classical pathways do not provide a mechanistic explanation for the observation that $CD8^+$ T cell responses can be stimulated against infectious agents that do not infect DCs. Similarly, tumour antigens would need to access DCs to elicit an anti-tumour $CD8^+$ T cell response. In fact, mice immunized with exogenous antigens have been shown to be capable of developing a cytotoxic $CD8^+$ T cell response, suggesting cross-presentation of these antigens (34). In addition, $CD8^+$ T cell-mediated responses can be generated against injected tumour antigens (35, 36). An explanation for these

observations is that exogenous antigens can be diverted into the MHC class I presentation pathway. The general presentation pathway involved in this process is called cross-presentation, and $CD8^+$ T cell-mediated responses stimulated in this manner are said to have been “cross-primed”. A significant amount of data supports the concept of cross-presentation, and cross-priming, although a variety of different pathways have been proposed. The various theories and their supporting evidence are described below.

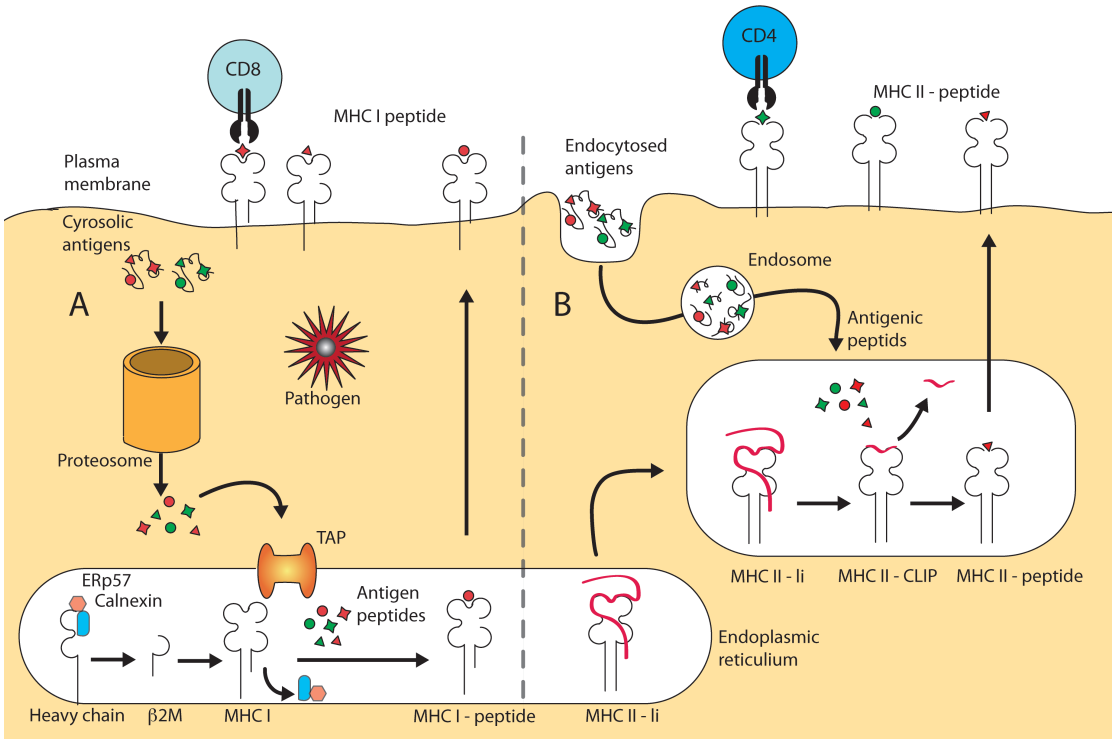


Figure 1.1: Classical models of antigen presentation on MHC class I and MHC class II molecules. (A) Antigens found within the cytosol are processed and presented on MHC class I for the stimulation of $CD8^+$ T cells. (B) Antigens acquired from outside of the cell are acquired into an endosome where they are degraded. The antigens then bind to MHC class II for the stimulation of $CD4^+$ T cells. Adapted from Heath (37).

Endosome to cytosol pathway

One proposed pathway of cross-presentation involves the release of internalized proteins from an endosome into the cytosol where they can be cleaved into fragments by the proteosome. The peptide fragments can then follow the classical MHC class I pathway where they enter the ER via the TAP

complex (Figure 1.2A). Studies have shown that exogenous antigens are present within the cytosol, indicating that the exogenous antigens are able to leave the endosome after acquisition. For example, sub-cellular fractionation, which separates the cytosol from vesicular organelles, has been used to detect exogenous chicken ovalbumin (OVA) protein within the cytosol of DCs following culture with OVA (38). Another study found that the administration of cytochrome C into mice led to a reduction in the amount of $CD8\alpha^+$ DCs. This is because when cytochrome C is present within the cytosol, it will activate the apoptotic protease-activating factor 1 (Apaf-1) molecule, which initiates apoptosis. Therefore, any cell that is able to transport exogenous cytochrome C into the cytosol (which might otherwise enter the MHC class I pathway) will undergo apoptosis. This experiment indicated that $CD8\alpha^+$ DCs are the most efficient cross presenters, which will be discussed in more detail below (39). It has not yet been determined how the antigen is able to leave the endosome but it has been postulated that peptide fragments may simply be able to migrate across the endosome membrane (39) or that the endosome itself ruptures, releasing its contents into the cytosol (27). Regardless of the process, there is strong evidence that DCs can transport exogenous antigens from an endosome into the cytosol for processing on MHC class I.

ER phagosome fusion pathway

When an endosome fuses with part of the ER an ergosome is formed. The development of an ergosome brings together the antigens from the endosome with the machinery from the ER, which enables exogenous antigens to be processed and loaded onto MHC class I within the ergosome. For this to occur the proteins must first exit the ergosome to enter the cytosol and be cleaved by the proteosome, before re-entering the ergosome to bind to MHC class I (Figure 1.2B). Evidence for this pathway has been found by looking for ER proteins within DC phagosomes. The ER proteins TAP, tapasin, ERP57 and Sec61 have been detected within endosomes, indicating that ergosomes had been formed (40). The presence of Sec61 within an ergosome identifies a possible way in which peptides may enter the cytosol. Sec61 is a transporter that shuttles misfolded or mutated proteins from the ER into the cytosol for

destruction as part of the ER-associated destruction pathway (ERAD). Sec61 could therefore also transport peptides from an ergosome into the cytosol for cleavage by the proteosome (41). Sec61 is further implicated in the process of cross-presentation as removing the Sec61 complex with ribonucleic acid (RNA) interference reduces the amount of cross-presentation that occurs (41). After peptides have been degraded in the cytosol they re-enter the endosome via the TAP complex and bind to MHC class I before being exported to the plasma membrane for $CD8^+$ T cell stimulation (40).

Endosome to ER pathway

Another possible method by which cross-presentation occurs is based on studies demonstrating that internalized soluble proteins can exit the phagosome and directly cross the ER membrane to enter the ER lumen (42). Here the peptides can enter the ERAD pathway where they are transported back into the cytosol to be cleaved by the proteosome. The peptide fragments can then re-enter the ER via TAP and be loaded onto MHC class I as part of the normal MHC class I pathway (Figure 1.2C).

Proteosome and TAP independent pathway

It is possible that cross-presentation can occur by a process that does not involve the TAP complex, migration of the exogenous protein into the cytosol or degradation by the proteosome (43). In this pathway, exogenous proteins are degraded into peptides within the endosome, thereby bypassing the requirement to leave the endosome for degradation by the proteosome. It is not known exactly how the peptide encounters MHC class I in his model; however, it is thought to occur after the MHC class I molecule has been exported from the ER and through the Golgi cisternae, as inhibition of protein transport from the ER to the Golgi does not inhibit cross-presentation (44). The antigen; therefore, is likely to be loaded onto MHC class I molecules that have been recycled from the cell surface. In this case, the peptides that were digested in the endosome bind to the recycled MHC class I molecules and are then presented on the plasma membrane for presentation to $CD8^+$ T cells (Figure 1.2D) (45).

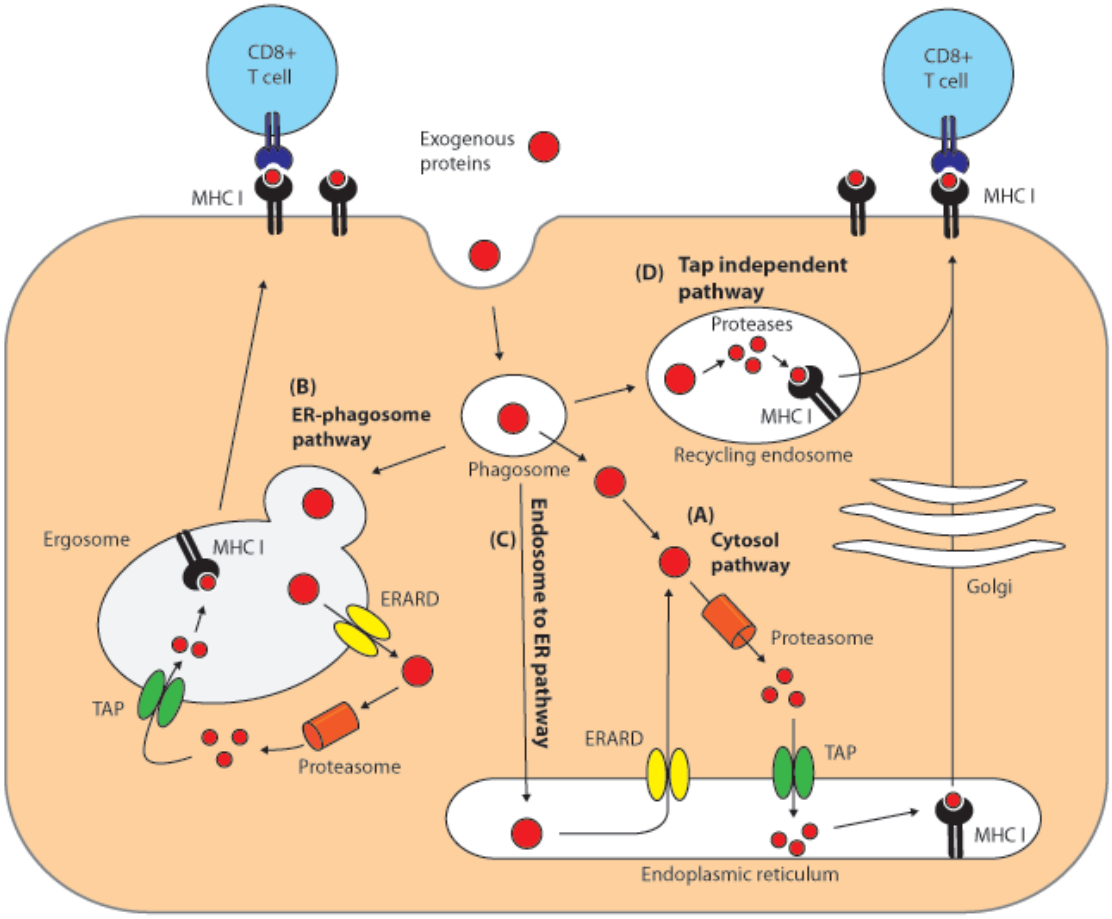


Figure 1.2: Pathways of antigen cross-presentation. (A) The endosome to cytosol pathway involves the exogenous protein leaving the endosome and entering the cytosol, where it is degraded by the proteosome. The antigens then enter the ER via the TAP complex and are loaded onto MHC class I. The MHC I/antigen complex is transported via the Golgi to the cell surface. (B) The ER-phagosome fusion pathway involves the formation of an ergosome by fusion of the endosome and part of the ER. The proteins exit the ergosome and enter the cytosol via the ERARD system to be cleaved by the proteosome. The cleaved antigens then re-enter the ergosome via the TAP complex and are loaded onto MHC I molecules. The MHC I/antigen complex then transits to the cell surface. (C) In the endosome to ER pathway, the acquired exogenous proteins within the endosome enter the cytosol and directly enter the ER. The proteins then enter the ERAD pathway and leave the ER for cleavage by the proteosome. The antigens then re-enter the ER via the TAP complex, are loaded onto MHC I and then the MHC I/antigen complex transits to the cell surface. (D) The proteosome and TAP independent pathway involves the exogenous proteins being cleaved within the endosome and loaded onto MHC I molecules that are recycled from the cell surface. The MHC I/antigen complex then transits to the cell surface. Adapted from Petersen (27).

1.3.2 Dendritic cell activation

The following paragraph is an introduction into the activation and function of DCs, which will be covered in detail below. The presentation of antigenic peptides via MHC molecules are able to stimulate T cells that can recognise the peptide/MHC complex by TCR binding. Depending on the activation status of the DC, the interaction between the TCR and MHC/peptide complex can activate the T cell, cause its deletion, or divert its activity, thereby initiating or preventing a specific immune response (46, 47). Activation is commonly used to describe the phenotypic changes DCs undergo in response to environmental signals that indicate a danger to the host, such as proteins released from dying or damaged cells (48-50). Proteins released from dead or dying cells that activate APCs are called danger associated molecular patterns (DAMPs). Because tumour cells arise from healthy tissue they are not associated with danger signals like bacteria and viruses are. As a result DCs in cancer patients are often not activated effectively and subsequently DCs presenting tumour antigens often inhibit tumour specific T cells instead of activating them (51).

The terms maturation, activation and licensing are used to describe different stages of DC activation. In this report the term ‘maturation’ will be used to describe any process that induces the upregulation of co-stimulatory molecules on the DC plasma membrane, which are essential for T cell activation. ‘Activation’ will be used to describe any process that renders a DC capable of stimulating naïve T cells into proliferating and differentiating into effector T cells. For this to occur the DC must express co-stimulatory molecules and produce cytokines such as interleukin (IL)-12 (49). The term ‘licensing’ will be used to describe the process by which a cell, such as a $CD4^+$ T cell or iNKT cell, activates a DC through receptor binding and cytokine production (52, 53). The maturation of a DC can occur by binding to proinflammatory cytokines, DAMPs or pathogen associated molecular patterns (PAMPs), whereas for a DC to become activated it typically requires licensing in combination with DAMP or PAMP ligation (54, 55).

In the naïve state, DCs express low levels of MHC class I and II and low levels of the co-stimulatory molecules CD40, CD80 and CD86 making them poor T cell activators (56, 57). Naïve DCs are efficient at acquiring antigens in their resident tissues and this is their main function in this state (58). Activated DCs develop a phenotype consisting of a more circular shape, increased cytokine production and increased expression of MHC molecules and co-stimulatory molecules (59, 60). This phenotype provides DCs with the three stimuli required to induce T cell activation, antigen presentation, co-stimulatory molecules and cytokine production. A T cell presented with these stimuli will be activated into inducing an immune response against the presented antigen. Activated DCs also upregulate lymphoid homing molecules such as chemokine (C-C motif) receptor (CCR) 7 and chemokine (C-X-C motif) receptor (CXCR) 4, which allow peripheral DCs to migrate from their resident tissues to T cell areas of lymphoid tissues. Activated DCs are therefore able to interact with and activate T cells in lymphoid tissues (61, 62).

PAMPs and DAMPs

Pattern recognition receptors (PRR) are expressed by innate immune cells and are involved in the detection of pathogens and initiating an immune response. If a DC encounters a PAMP that can be recognised by PRRs then it will develop a more activated phenotype in order to initiate an adaptive immune response (63, 64). Similarly, damage to host cells caused by pathogens can result in DAMPs being released from damaged cells into the extracellular environment, which can also induce DC maturation through recognition by PRRs. Importantly, DAMPs are not released when a healthy cell dies as a consequence of normal cell turnover, because of a controlled form of cell death called apoptosis (65). However, if a cell dies because of immune attack, or as a result of stress associated with rapid growth or a hypoxic environment (perhaps within a tumour), then the dying cell releases DAMPs into the cell environment (66). An example of a DAMP is extracellular adenosine triphosphate (ATP) (67, 68), which in the healthy setting is a source of energy and is found in high intracellular concentrations. However, cell damage can cause ATP to be released into the extracellular environment

where it can induce DC maturation (69). A variety of classes of PRRs are found in DCs, often differentially expressed depending on the DC subtype. The best described are the Toll-like receptors (TLRs), which recognise a broad range of prokaryotic structures, such as glycolipid components of bacterial cell walls, double stranded RNA from some virus species, and unmethylated DNA. They can also recognise some DAMPs, such as HMGB1 (70). Thus, in general, DCs recognising PAMP or DAMP structures via PRRs are induced to mature, and as a result have greater T cell stimulatory capabilities.

Licensing dendritic cells

Although DAMPs and PAMPs have the ability to mature DCs, in many cases this stimulation isn't strong enough to enable them to activate T cells. To activate DCs sufficiently to enable $CD8^+$ T cell activation, licensing is often required. Licensing a DC induces the DC to produce cytokines such as IL-12 and upregulate co-stimulatory molecules that are essential for T cell activation (71). Maturation of a DC by PRR binding results in the upregulation of CD40 on the DC plasma membrane, which can then be ligated by CD40L expressed by the licensing cell (Figure 1.3) (71, 72). Thus, a synergistic effect occurs between PRR binding and licensing (73).

$CD4^+$ T cell licensing

There is ample evidence indicating that $CD4^+$ T cells can regulate the activation status of DCs, aiding in the stimulation of $CD8^+$ T cells. $CD8^+$ T cells primed by DCs in the absence of $CD4^+$ T cell licensing are termed 'helpless T cells' and it was initially believed that helpless T cells were unable to proliferate and differentiate into effector T cells. However, recent studies indicate that helpless $CD8^+$ T cells have a functional effector compartment but a reduced ability to respond to a secondary infection (74). IL-2 is essential during $CD8^+$ T cell priming to develop T cells that can respond to secondary challenge and the absence of IL-2 during priming generates helpless $CD8^+$ T cells (75-77). Interestingly, the IL-2 is not produced by the licensed DC or the helper $CD4^+$ T cell but by the $CD8^+$ T cells themselves (76). The licensing of a

DC by a $CD4^+$ T cell therefore provides DCs with the ability to activate $CD8^+$ T cells, inducing them to produce IL-2 during priming, thereby enabling effective responses to secondary challenges (which will be discussed later).

iNKT cell licensing

As noted earlier, the T cell repertoire includes cells that do not fit into the "conventional" categories of $CD4^+$ or $CD8^+$ T cells. Included are populations that have largely invariant TCR structures, such as iNKT cells, mucosa-associated invariant T (MAIT) cells, and $V\gamma2V\delta2$ T cell in humans. The iNKT cells have been studied in considerable detail, and like $CD4^+$ T cells have been shown to be capable of licensing DCs, which increases their ability to activate $CD8^+$ T cells by inducing co-stimulatory marker expression and cytokine production (53, 73, 78). The invariant TCR alpha chain expressed by iNKT cells ($V\alpha24$-$J\alpha18$ in humans, $V\alpha14$-$J\alpha18$ in mice) recognises glycolipids presented by the MHC class I-like molecule CD1d. The glycolipid agonist α-galactosylceramide (α-GalCer) is able to provide activation of host iNKT cells in all individuals (in humans and many other mammals), thus making it a useful agent to induce DC licensing in immunotherapies. Using α-GalCer to activate iNKT cells has been shown to drive a strong cytotoxic T cell response against the OVA protein in a CD40L-dependent manner, thereby providing mice with increased protection against an OVA-expressing subcutaneous tumour (E.G7-OVA) (53, 79). In addition to the CD40L-dependent stimulation of DCs by iNKT cells, iNKT cells produce IFN-γ and tumour necrosis factor (TNF) α both of which aid in DC maturation (80, 81).

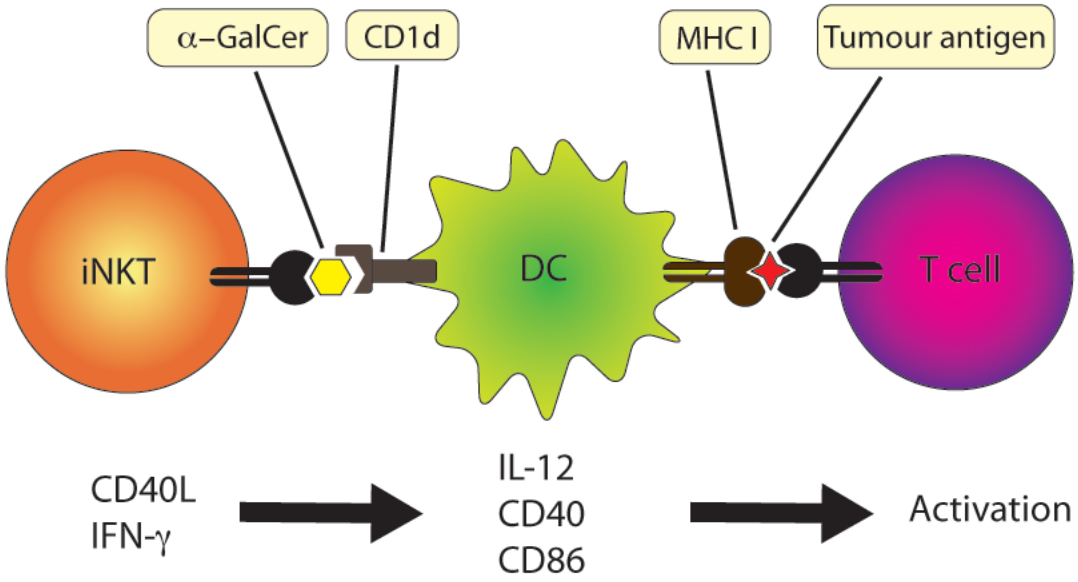


Figure 1.3: Activation of a DC by licensing. The ligation of CD40 on the DC membrane by CD40L on a licensing cell results in the DC upregulating co-stimulatory molecules such as CD40 and CD86 and producing cytokines such as IL-12. This provides the DCs with stronger T cell stimulatory capabilities.

Interestingly there are a few differences in the DC licensing provided by iNKT cells and $CD4^+$ T cells. Following licensing by $CD4^+$ T cells, DCs produce ligands that bind to CCR5 expressed by cytotoxic $CD8^+$ T cells, thereby aiding in the interaction between licensed DCs and $CD8^+$ T cells (82). On the other hand, DCs licensed by iNKT cells express the chemokine CCL17, which recruits cytotoxic T cells expressing the receptor CCR4, which also aids in the interaction between licensed DCs and $CD8^+$ T cells (83). Of note, these two recruitment methods work independently of each other and can therefore be used together to provide a synergistic effect on $CD8^+$ T cell recruitment (73). The combination of $CD4^+$ T cell and iNKT cell licensing could be used in immunotherapeutics to provide enhanced $CD8^+$ T cell stimulation. Because iNKT cells are an important component of this thesis they require their own section and will be discussed in detail at a later point.

1.3.3 Dendritic cell subsets

The DC subsets described in this section refer to those found in mice, as this is the model used in the experiments conducted for this thesis. While there are many similarities with human DCs, and some conclusions can be extrapolated across species, in general it is recognised that there are phenotypic differences, and any functional activities observed in the mouse would ultimately have to be validated in human systems.

It is increasingly becoming apparent that DCs are a heterogeneous cell type with different subsets having distinct locations and functions within the body. The different DC subsets have presumably evolved to have differing antigen acquisition and presentation abilities to respond to different forms of pathogenic insult. They may also have differing roles in inducing and maintaining tolerance to self-antigens. As mentioned above, the important DC subsets involved in developing a $CD8^+$ T cell-mediated immune response against tumours are likely to be those that have the strongest ability to cross-present tumour antigens. It is therefore important to define endogenous DCs with cross-presentation capability, as these may be exploited in the design of immunotherapies. This is a major theme of the thesis.

A major division between DC subsets is the division into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). These cells have differing developmental programs, with pDCs developing in the bone marrow and circulating in the blood as developed pDCs; whereas, cDCs either migrate to tissues in a precursor form and then differentiate into cDCs, or have a long-term precursor in resident tissues that develop cDCs (84). Circulating pDCs are an important combatant against viruses as they are a strong producer of type I IFNs (85, 86). These cells will not be discussed further in this thesis. cDCs can be divided into two groups based on their position within the body, they either reside within secondary lymphoid tissues (the lymphoid resident DCs) or within tissues peripheral to the secondary lymphoid organs (tissue-resident DCs) (Figure 1.4).

Tissue-resident conventional dendritic cells

Tissue resident DCs are also known as migratory DCs as they function as sentinels or scavengers, continuously searching for and acquiring antigens within their resident tissues. Once acquired these DCs can migrate into the lymphoid organs for antigen presentation. The best-characterised tissue resident DC subsets are those found within the skin and these will be used as an example of the different DC populations in peripheral tissues. The skin DC subsets can be differentiated based on their expression of the markers CD11b and langerin (CD207) (87). In the dermis there are two populations of DCs, the $CD11b^+$ $langerin^-$ dermal DCs and the $CD11b^-$ $langerin^+$ dermal DCs (88, 89). The epidermis contains a single subset of DC called Langerhans cells, which expresses both CD11b and langerin (90). The three DC subsets in the skin differ significantly in their functions, for example the $langerin^+$ dermal DCs are the main skin-resident subset involved in antigen cross-presentation (91) and the $CD11b^+$ dermal DCs are more involved in the presentation of antigens on MHC class II to $CD4^+$ T cells. Conversely, the Langerhans cells in the epidermis extend out dendritic processes that allow the acquisition of antigens from surrounding keratinocytes and from the external surface of the skin (92, 93). While they can migrate with antigens to the secondary lymphoid organs, they do not present antigens directly to $CD8^+$ T cells (94-96).

Lymphoid Resident conventional dendritic cells

Lymphoid resident DCs, as the name suggests are found within the secondary lymphoid organs. In lymph nodes they acquire antigens draining through the lymphatic system (97) and in the spleen they acquire antigens circulating within the blood (96, 98). Lymphoid resident DCs can also acquire antigens through a process called antigen transfer, which involves a migratory DC transferring an antigen that was acquired in a peripheral tissue to a lymphoid resident DC (99, 100).

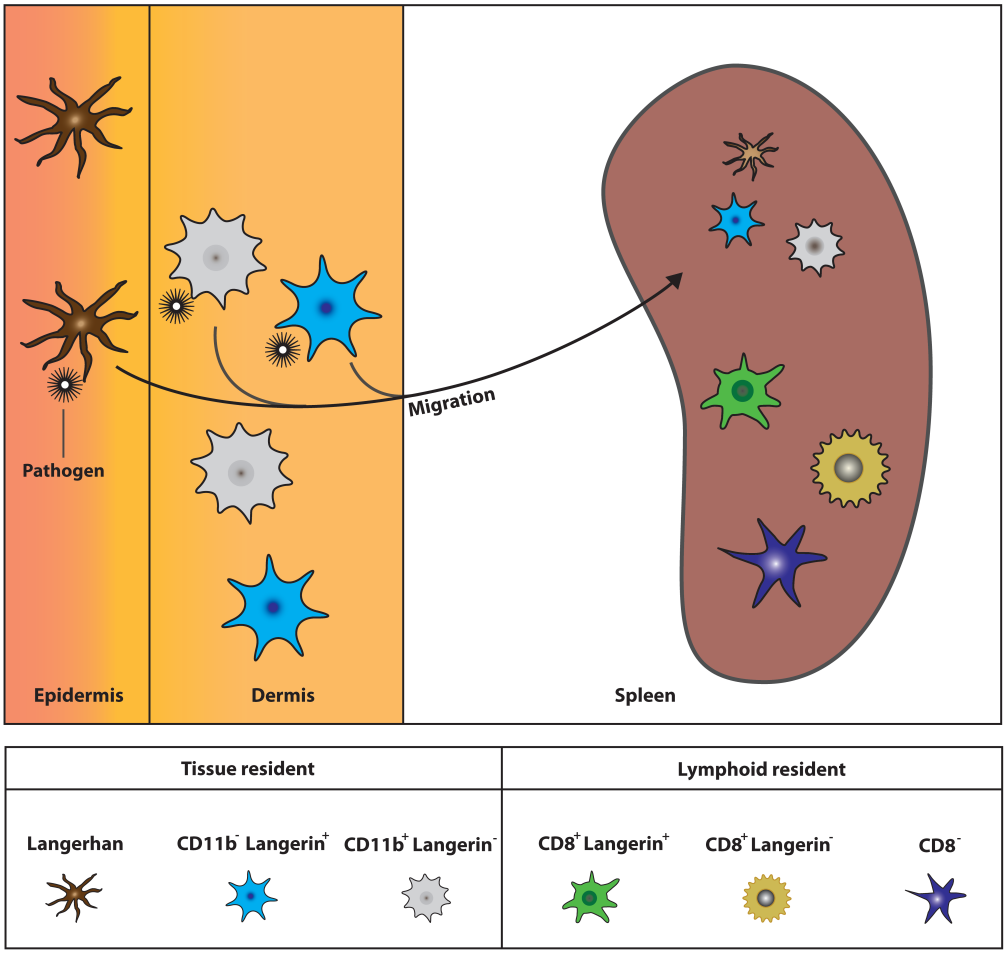


Figure 1.4: Dendritic cell subsets. The lymphoid resident DCs consist of $CD4^-$ $CD8\alpha^-$ DCs, $CD4^+$ $CD8\alpha^-$ DCs, $langerin^-$ $CD8\alpha^+$ DCs and $langerin^+$ $CD8\alpha^+$ DCs. The tissue resident DCs of the skin consist of the Langerhan cells in the epidermis and the $CD11b^+$ $langerin^-$ DCs and $CD11b^+$ $langerin^+$ DCs of the dermis. The tissue resident DCs can acquire antigens in the peripheral tissues and then migrate to the lymphoid tissues for T cell stimulation. Adapted from Heath (87).

The lymphoid resident DCs can be further divided into several groups, based on their function and expression of CD4 and CD8α. Thus, within the spleen there are $CD4^+$ $CD8^-$ DCs, $CD4^-$ $CD8\alpha^+$ DCs (hereafter referred to as $CD8\alpha^+$ DCs) and a $CD4^-$ $CD8^-$ DCs (101). The potent ability of the $CD8\alpha^+$ DCs to cross-present makes them a vital component in the stimulation of $CD8^+$ T cells against exogenous antigens. A major component of this thesis is involved in determining the importance of cross-presenting DCs in generating $CD8^+$ T cell responses against tumours. While $CD8\alpha^+$ DCs have the ability to

present antigens on MHC class II for the stimulation of $CD4^+$ T cells, the $CD8\alpha^-$ DCs (which includes $CD4^+$ $CD8^-$ DCs and $CD4^-$ $CD8\alpha^-$ DCs) are much more proficient at this process (102). Recently it has been shown that separate populations within the $CD8\alpha^+$ subset can be identified. These include the $langerin^+$ $CD8\alpha^+$ and the CX_3CR1^+ DCs (103, 104). The remaining DCs within the $CD8\alpha^+$ population that do not express langerin or CX_3CR1 are referred to as $CD8\alpha^+$ DCs. CX_3CR1^+ DCs lack the hallmark features associated with the other $CD8\alpha^+$ DC subsets, such as IL-12 production and the ability to cross-present. Interestingly, their gene expression profile is more similar to the $CD8\alpha^-$ DCs than the $CD8\alpha^+$. Due to the importance of $langerin^+$ $CD8\alpha^+$ DCs in the thesis they require their own section and will be discussed in detail below.

$Langerin^+$ $CD8\alpha^+$ dendritic cells

$Langerin^+$ $CD8\alpha^+$ DCs can be found within the marginal zone, which surrounds the arteries entering the spleen. This places them in direct contact with blood circulating the body as it drains into the spleen (105). The $langerin^+$ $CD8\alpha^+$ DCs are therefore in an ideal position to scan the blood for antigens and danger signals. These DCs are very competent at phagocytosing apoptotic cells that have been administered intravenously (106). It is therefore likely that dead or dying cells circulating in the blood will be acquired preferentially by the $langerin^+$ $CD8\alpha^+$ DCs. It is also possible that blood-borne tumour cells will similarly be acquired and processed by these DCs, which is a major theme of this thesis.

Within the $CD8\alpha^+$ DC population as a whole, it has been shown that $langerin^+$ DCs are also the most effective subset at cross-priming $CD8^+$ T cells. This was found by injecting mice intravenously with OVA, and then isolating the different DC subsets *in vitro* to determine which subset could most effectively stimulate OVA specific $CD8^+$ T cells (107). If $langerin^+$ $CD8\alpha^+$ DCs do indeed acquire dying tumour cells that are circulating in the blood, it is possible that they will effectively cross-present exogenous tumour antigens to cross-prime

$CD8^+$ T cells. It is also possible that these DCs initiate immunotherapy-induced immune responses that are administered intravenously.

1.3.4 Dendritic cell subsets and cross-presentation

Over decades, research determining which specific APCs are the most efficient at cross-presenting has progressively narrowed the specific cell down from all APCs (17, 55, 108), to $CD11c^+$ DCs (38, 109), to only DCs expressing $CD8\alpha^+$ (45, 110) and finally to the $langerin^+$ $CD8\alpha^+$ DC subset (107). The experiments involved are outlined below.

Although earlier studies indicated that DCs are not the sole cross-presenting APCs it was found that when culturing DCs or macrophages with fluorescent horseradish peroxidase, the fluorescent signal was detected in the cytosol of DCs but not macrophages, a transportation feature that is central to many of the theories of the cross-presentation pathways presented earlier (38). This indicates that macrophages were unable to transport peptides from exogenous proteins into the MHC class I pathway. In addition, this study demonstrated that DCs could present peptides from exogenous OVA protein to stimulate $CD8^+$ T cells, whereas macrophages could not (38). A similar study demonstrated that macrophages infected with the influenza virus were unable to activate $CD8^+$ T cells, however when uninfected DCs were cultured with infected macrophages a significant $CD8^+$ T cell response was induced. This indicates that DCs are more efficient at cross-presentation than macrophages. A further study ruled out the cross-presenting ability of other APCs by depleting all cells expressing CD11c (a commonly used DC marker) in mice. The results from this study revealed that $CD8^+$ T cell proliferation in response to intravenously administered bacteria was ablated in me depleted of $CD11c^+$ cells. This indicates that DCs are required for effective cross-priming of $CD8^+$ T cells (111).

In more recent years, the specific DC subset involved in cross-presentation was identified as $CD8\alpha^+$ lymphoid resident DCs, as only this DC subset activated OVA-specific T cells *in vitro* when isolated from the spleens of mice

that were injected with OVA protein (102, 112). Furthermore, depleting the CD8α^+ DC subset with an anti-CD8α antibody resulted in significantly reduced CD8$^+$ T cell priming in response to cell-associated OVA protein (113). Similarly, the CD8α^+ DCs were implicated as the most efficient cross presenting DCs by administering cytochrome C into mice, which induced apoptosis in cells that could effectively transport the pro-apoptotic protein from the endosome into the cytosol (110). It was found that following cytochrome C administration a majority of DCs expressing CD8α were depleted, although the fact that some remained pointed to some heterogeneity in the CD8α^+ population as a whole (39). This group subsequently found that the CD8α^- DCs did not contain the same ability to present exogenous OVA peptide to CD8$^+$ T cells as the CD8α^+ DCs (39).

Research from my own laboratory has provided strong evidence that the most potent cross-presenting DCs are a subset within the CD8α^+ DCs that express langerin (107). This conclusion was drawn from studies using a transgenic mouse strain in which all langerin-expressing cells can be specifically ablated (114). It was found that cross-presentation of OVA peptides was significantly reduced when the langerin$^+$ DCs were ablated (107). Together these results indicate that a population of DCs expressing both langerin and CD8α are the main cross-presenting cell.

What makes some dendritic cell subsets better at cross-presentation than others?

It remains to be established what specific features enable a DC to be efficient at cross-presentation, however several hypotheses have been developed. It is possible that cross-presenting DCs harbour specialized machinery for cross-presentation or that they have greater access to proteins for cross-presentation (115-117). The former idea is supported by studies that indicate that the CD8α^+ lymphoid resident DCs have enriched levels of proteins involved in MHC class I presentation, such as TAP1, TAP2, Sec61 and ERAP (117), which would enable more acquired antigens to enter the MHC class I pathway. Additionally, it has been proposed that cross-presenting cells have

reduced levels of antigen degradation in the phagosome due to alkalinisation of the phagolysosome lumen by the nicotinamide adenine dinucleotide phosphate NADPH oxidase (NOX2). This prevents proteins within the phagolysosme being rapidly degraded, thereby leaving sufficient antigens for effective presentation. DCs that do not recruit NOX2 have increased acidification of the phagolysosome resulting in increased antigen degradation, thereby reducing the amount of antigens remaining for cross-presentation (118).

It has also been suggested that a factor that determines whether an antigen is cross-presented or not is the method by which the antigen is taken into the cell. In this model, antigens that are acquired by specific receptors are directed towards the cross-presentation pathway and the cross-presenting DCs either express these receptors at higher concentrations than other cells or the receptors are specific to cross-presenting DCs (119). There are several known receptors with a putative antigen acquisition function expressed on the cross-presenting langerin$^+$ CD8α^+ DCs that are not expressed on the CD8α^- DCs, such as DEC205 and DEC207. In particular, CLEC9A which is expressed by CD103$^+$ DCs, including CD8α^+ DCs, recognises an intracellular ligand that is only expressed by a cell upon it's death. It has been demonstrated that necrotic cells acquired by DCs via CLEC9A avoid being rapidly degraded and favours the entry of cell antigens into the MHC class I pathway. In support of this, deficiency in CLEC9A also results in reduced cross-presentation of antigens derived from dead cells (120).

1.3.5 Models of dendritic cell subset depletion

As mentioned above, an effective way to elucidate the roles of specific DC subsets is by determining how an immune response is altered in the absence of that subset. The aim of this thesis was to determine the role of the langerin$^+$ CD8α^+ DCs in activating anti-tumour CD8$^+$ T cells and this was determined by analysing anti-tumour T cell immunotherapies in mice depleted of their langerin$^+$ CD8α^+ DCs. Therefore, to understand the model used in this project an understanding is required of how the langerin$^+$ CD8α^+ DCs can be

depleted in healthy mice. Specifically, a description is provided of a transgenic mouse model called the langerin-diphtheria toxin receptor (DTR) mice (*lang*-EGFPDTR) in which it is possible to specifically deplete the langerin$^+$ cells (114).

Diphtheria toxin receptor

Diphtheria toxin (DT) produced by the bacteria *Corynebacterium diptheriae*, is able to effectively kill human cells by gaining access cell cytoplasm via the human DTR, where it blocks the cells ability to synthesize proteins. The mouse DTR is structurally different from the human counterpart, which significantly reduces the ability of DT to bind to the receptor and thereby enter the cell (121). This renders the murine cell 10^3–10^5 times more resistant to DT than human cells. This three amino acid difference between the murine and human DTRs has been exploited to permit the ablation of specific cell types within mice by genetically engineering the high affinity human DTR to be associated with specific cell markers. This approach has enabled the human DTR to be expressed by the promoters of specific DC markers, thereby associating the human DTR with those markers and permitting the selective ablation of specific cell subsets (122). Cells that internalise DT die through the steady and controlled cell death called apoptosis, thereby preventing the induction of an inflammatory response against debris from the dying cells. The DT depletion model is an inducible and temporary depletion, which gives it advantages over other DC depletion models, such as the Batf3 knock out (KO) mouse (123), which have the Batf3 transcription factor deleted from hosts, resulting in ablated development of the CD8α^+ DCs. The DT model therefore allows the mice to develop and grow as normal, healthy mice, and the specific DC subsets can then be depleted on demand.

CD11c-DTR

The first DTR-based DC depletion model was developed by associating the human DTR to the integrin receptor CD11c, which is expressed on murine DCs (111). By administering 100 ng of the DT intraperitoneally it became possible to deplete all CD11c$^+$ DCs for a period of 24–48 hours, at which point

the cells began to repopulate within the host. This depletion model enabled analysis of the specific functions of DCs as a whole, and as a result the importance of DCs in priming cytotoxic T cells was determined (111). The CD11c-DTR ablation model does have some limitations however, the most significant of which is the inability of these mice to survive for long periods of time following multiple DT treatments. This is likely due to the expression of DTR on non-haematopoietic cells, as the lethality can be overcome by generating bone-marrow chimeras where DTR expression is restricted to haematopoietic cells (124).

Langerin-EGFPDTR

Two separate groups initially developed the *lang*-EGFPDTR mouse model in 2005, by inserting the human DTR into the langerin locus. The aim of these studies was to deplete the epidermal derived Langerhans cells in order to determine their function (125, 126). However, the development of these mice provided a means to deplete all langerin expressing cells including $CD11b^-$ $langerin^+$ DCs found in the dermis, lung, liver, kidneys, small intestine, lymph nodes and also the $langerin^+$ $CD8\alpha^+$ DCs in the spleen. Cells expressing langerin could be depleted in the *lang*-EGFPDTR mice within 24–48 hours by a single intraperitoneal administration of DT, reducing the percent of $CD11c^+$ DCs expression both $CD8\alpha$ and langerin from 9.5% to <0.5% (114). No detectable inflammation was detected following DT treatment and unlike the CD11c-DTR model these mice can withstand multiple DT treatments.

In this thesis the $langerin^+$ $CD8\alpha^+$ DCs in the spleen were analysed using the *lang*-EGFPDTR mouse model. However, due to the depletion of all $langerin^+$ cells following DT treatment, the function of the $langerin^+$ $CD8\alpha^+$ DCs needs to be differentiated from those other subsets. For this reason, the tumour models and immunotherapies were administered intravenously, as it has been demonstrated that the activation of $CD8^+$ T cells with TLR ligands and OVA protein is significantly ablated in mice with surgically removed spleens, relative to mice that had sham surgeries (unpublished data from our laboratory, with permission from T. Osmond). This indicates that DCs in the

spleen were required to activate $CD8^+$ T cells with intravenously administered vaccines. Therefore, immunotherapies administered intravenously in the absence of langerin cells enable the analysis of the functions of langerin expressing cells in the spleen. In addition, the tumour cells were also administered intravenously in this thesis so that the tumour antigens would be preferentially acquired by APCs in the spleen.

1.4 T Cells and the anti-tumour response

The aim of many cancer immunotherapies is to provide tumour-bearing hosts with effector $CD8^+$ T cells capable of targeting and killing tumour cells. T cells are part of the adaptive immune response, which already noted, is slower but much more specific than the innate immune responses. Prior to activation, T cells are said to be in a naïve state, in which they are presented antigens by DCs to determine if the TCR can bind to the peptide/MHC complex. Once an antigen that can be recognised by a T cells TCR is found the T cell is stimulated into immunity or tolerance. Naive T cells remain circulating within the secondary lymphoid organs due to their expression of the lymph node homing receptors CD62L and CCR7, which bind to receptors found at high concentrations in the secondary lymphoid organs (127-129). The importance of sequestering naïve T cells within the lymphoid tissues is to aggregate rare T cells within a specific area so that they are easily accessible to migrating APCs (130).

1.4.1 T cell development

During the development of T cells a rigorous selection process is performed to remove T cells that respond to self-antigens, thereby preventing the development of autoimmune T cells. As noted earlier in the chapter, the implication of removing self-reactive T cells from the immune system in the tumour context is that T cells with TCRs specific for self-antigens that might have served as targets in tumour tissue have also largely been removed during selection. This limits the repertoire of antigens that can be used to generate an anti-tumour immune response. An overriding feature is that tumour antigens must be presented in some way that is unique and

distinguishable from healthy cells. The specific types of tumour antigens will be discussed at a later point.

Following development in the bone marrow, progenitor T cells migrate to the thymus expressing neither CD4 nor CD8 co-receptors (131, 132). The progenitors develop a unique TCR typically composed of an α and β chain. Each chain consists of a variable (V) amino-terminal region, a joining (J) and a constant (C) region. In addition, the β chain has a diversity (D) region. Each region has numerous gene segments and the assembly of these genes through V(D)J recombination creates a unique gene sequence and as a result a unique TCR (133). Following successful recombination, the thymocytes then express both CD4 and CD8. At this stage of development thymocytes undergo a selection process to ensure that the T cells that ultimately enter the circulation can recognise peptides in the context of self-MHC molecules, but do not have high affinity for self-antigens (132, 134). The thymus can be divided into two main regions, the peripheral cortex and the central medulla. Positive selection occurs in the cortex where epithelial cells present MHC class I and II molecules (135, 136). Thymocytes that have TCR receptors that can recognise the MHC molecules are able to survive. The remaining cells are removed from the population due to lack of stimulation ("death by neglect") (137, 138). The surviving cells down-regulate either CD4 or CD8 to become single positive cells, and the marker that remains depends on whether the TCR recognises MHC class I or II. The single positive cells then move into the medulla were negative selection occurs. Self-peptide/MHC molecules are presented to the thymocytes by APCs and those that bind too strongly are killed, thereby removing autoimmune T cells (139). The remaining T cells bind to self-MHC ligand with a mild affinity and these constitute around 1% of the initial T cell population, however this still provides the host with enough T cells to recognize around 10^8 different antigens (140). At this point the T cells leave the thymus and enter secondary lymphoid organs.

A subset of T cells that bind to self-MHC ligand with high affinity survives positive selection and is programmed to have suppressor function. These

regulatory T cells (T_{regs}) suppress anti-self T cells that have escaped negative selection, thereby providing another barrier to avoid an autoimmune response (141, 142). These cells have be defined on the basis of constitutively upregulated expression of the IL-2 receptor α chain (CD25), although a more accurate correlate with suppressor function is expression of the transcription factor forkhead box P3 (Foxp3). This subset of T cell will be described in greater detail at a later point.

1.4.2 T cell activation

In order for a DC to induce the activation of a naïve T cell, it must present an appropriate MHC/peptide complex in combination with co-stimulatory molecules, and also provide the T cell with cytokines, such as IL-12 (130, 143, 144). The CD4 or CD8 co-receptors expressed by the T cell bind to the MHC molecule presented by the DC, thereby stabilizing the TCR/MHC/peptide interaction (145).

An important factor that determines if the T cell is activated or rendered anergic by this interaction is the presence or absence of co-stimulation provided by the APC (146). The best-characterised co-stimulatory molecule interaction is the binding of the T cell molecule CD28 to either CD80 or CD86 on a DC (56). A specialized form of co-stimulation called bidirectional co-stimulation activates both the APC and the T cell. For example, ligation of CD40 on a DC by CD40L presented by a T cell results in co-stimulatory signals provided to the T cell, thereby aiding T cell activation and it also induces the upregulation of CD80 and CD86 on the APC (59, 80).

Naïve T cells undergo a series of changes as they differentiate into effector T cells that allow them to leave the lymph node, enter inflamed tissue, and perform effector functions, which for $CD8^+$ T cells includes a cytotoxic response. The T cell undergoes proliferation to provide a significantly increased population of antigen-specific cells (143). The high affinity IL-2 receptor, CD25, is also upregulated on activated T cells, allowing the cells to respond more effectively to IL-2, which drives the proliferative response (147).

Activated T cells develop a phenotype that allows them to leave the lymphoid tissues by down-regulating the expression of CD62L and CCR7. The markers CD44, CCR2 and CCR5 are upregulated on effector T cells allowing them to migrate into inflamed tissues from the blood where they perform their effector functions (147-149). Effector capabilities are developed in a manner that is specific to the T cell subset, for example $CD8^+$ T cells produce cytotoxic molecules, such as perforin and granzyme B, and upon activation the genes that encode these proteins are expressed (147). These activated $CD8^+$ T cells are now fully differentiated CTLs, and are now equipped to migrate to target tissues, such as tumours, and eliminate antigen-expressing cells.

A T cell response generated against a pathogen must at some point be turned off as an uncontrolled immune response can lead to tissue damage. Following T cell activation, the pool of effector cells enter a programmed response that dictates when the T cell population will reduce in number (150, 151). This reduction is called the contraction phase of the T cell response, with approximately 95% of the effector T cells undergoing apoptosis. The remaining T cells develop into memory cells that initiate a faster adaptive immune response if the antigen is encountered again (152). Memory cells will be discussed below.

1.4.3 T cell functions

Cytotoxic T cells

Perforin and granzyme

$CD8^+$ T cells cause apoptosis upon TCR binding to an antigen/MHC complex on target cells by releasing lytic granules that contain cytotoxic proteins. The cytotoxic proteins are maintained in an inactive form within the lytic granules and only upon their release do they develop cytotoxic potential (13, 153). Cytotoxic proteins called granzymes, particularly granzymes A and B are able to induce apoptosis of a target cell in a caspase dependent or independent pathway (13). Granzyme A induces deoxyribonucleic acid (DNA) damage independent of caspase activity and functions by activating a complex of

molecules containing endonucleases and exonucleases. Once the complex has migrated into the nucleus, the endonuclease cleaves DNA and the endonuclease expands the break by removing bases from the fragment, thereby reducing the chance of DNA repair (154-156). Granzyme B, on the other hand, induces apoptosis via caspase dependent pathways. It can directly activate caspase 3, which also results in DNA fragmentation (157, 158). Granzyme B can also induce mitochondria damage, thereby decreasing mitochondrial function and causing cell death. Mitochondrial damage can also cause the release of cytochrome C, which enters the cytosol and induces activation of the Apaf-1 complex, which in turn induces the activation of caspases 3, 6 and 7, all of which trigger apoptosis (158, 159).

Granzyme A and B are significantly less effective at inducing target cell apoptosis when released alone due to a decreased ability to enter the cell. Granzyme A and B are therefore secreted with another protein called perforin, which forms pores in membranes between 5 to 20 nM in size (160, 161). Following endocytosis of the granzymes and perforin molecules, perforin forms a pore in the endosome, thereby allowing granzyme A and B to enter the cytosol and mediate apoptosis of the target cell (162, 163).

FasL

Cytotoxic $CD8^+$ T cells express FasL (CD178), which can interact with target cells that express Fas (CD95) to induce apoptosis of the target cell in a caspase dependent manner (164, 165). The Fas receptor is expressed on most tissues and also has non-apoptotic functions. The tissue in which Fas is expressed and the presence of other proinflammatory mediators are likely to determine the effect of Fas ligation (166, 167). In the apoptotic inducing setting, the caspase cascade that results from Fas-FasL interaction results in the activation of caspase 8, which activates caspases 3, 6 and 7 directly, thereby resulting in apoptosis (168). Caspase 8 activation also results in damage to the mitochondrial outer membrane, allowing cytochrome C to enter the cytosol and activate the Apaf-1 complex resulting in the induction of apoptosis (169, 170).

Cytokine production

Following activation, $CD8^+$ T cells begin producing cytokines such as IFN-γ, TNF-α and TNF-β (171). There are several functions of IFN-γ that can aid in protection against tumour development. These include promoting apoptosis of tumour cells by upregulating both Fas and FasL on tumour cells, thereby inducing the interaction of these two molecules between tumour cells (172, 173). Furthermore, IFN-γ is able to damage blood vesicles that supply tumours with nutrients, resulting in necrosis of the tumour cells (174). It also aids in the ability of $CD8^+$ T cells to recognize tumour cells by increasing the expression of MHC class I on tumour cells, thus increasing the expression of antigens that $CD8^+$ T cells use to identify their targets (175, 176). The activity of IFN-γ also includes the inhibition of tumour cell proliferation (177, 178) and the activation of macrophages (which will be discussed in the next section). The targets of TNF-α and TNF-β are not tumour cells directly but host cells; however, at high concentrations they can cause haemorrhagic necrosis (179). Both of these cytokines promote tumour destruction by activating DCs, increasing the tumour killing functions of macrophages and NK cells and by promoting the functions of T cells (180, 181). The adhesion molecules E-selectin, intracellular adhesion molecule-2 and vascular cell adhesion molecule-1 are upregulated on endothelial cells in response to TNF interaction, thereby aiding leukocyte recruitment and enhancing the inflammatory response (182). In addition, vascularisation caused by TNFs, increase local blood flow, also aiding immune cell recruitment.

T helper cells

Antigen-specific $CD4^+$ T cells constitute an essential component to immune responses against a variety of pathogens or abnormal cells, including tumour cells. In addition to licensing DCs (as mentioned earlier), $CD4^+$ T cells can mediate tumour protection independent of $CD8^+$ T cells by activating other immune cells. $CD4^+$ T cells have recently been found to also produce cytotoxic molecules such as granzyme B (183-185). The importance of the cytotoxic functions of $CD4^+$ T cells is poorly understood, however it is receiving increased attention.

There are several different subsets of $CD4^+$ helper T cells, each of which is unique in their cytokine repertoire and type of infection that they are specialised to control. The cytokines within the environment during $CD4^+$ T cell priming and the strength by which the TCR binds to the antigen determines the class of helper cell that is generated (186). The helper T cell subsets include T helper (T_h) 1, T_h2, T_h9, T_h17, T_h22, follicular helper T cells and T_{regs}. The functions of the T_h1 cells and T_{regs} are the most relevant subsets in this thesis and the other subsets will not be mentioned further.

T_h1 cells

When stimulated in the presence of IL-12, naïve $CD4^+$ T cells are driven into T_h1 cells (187, 188). In addition, IFN-γ can likewise drive the differentiation of $CD4^+$ T cells into T_h1 cells (189). Activated T_h1 cells produce IL-2, IFN-γ, TNF-α, IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) and contribute to anti-tumour immunity by recruiting phagocytic cells such as macrophages into the tumour bed (190-192). Once within a tumour, the macrophages are activated by two signals produced by T_h1 cells, the ligation of CD40 on the macrophage, and IFN-γ (193). Activated macrophages rapidly degrade material engulfed from the environment, such as tumour material, and begin producing reactive nitrogen species such as nitric oxide and superoxide that can be toxic (191). In addition, activated macrophages can secrete CXCL9 and CXCL10, which can inhibit tumour growth by preventing angiogenesis (192).

In addition to aiding in tumour destruction by recruiting macrophages, T_h1 cells can also kill tumour cells directly by ligating the Fas receptor on the tumour cell, thus inducing apoptosis (194). Licensing DCs is also a critical function of T_h1 cells as it aids in $CD8^+$ T cell priming. In addition, T_h1 cells that enter tumour tissue play a useful role in producing IFN-γ, which has anti-angiogenic properties, and also causes tumour cells to increase the expression of MHC class I, thereby making a better target for the effector $CD8^+$ T cells (176).

Although the research in this thesis is aimed at analysing the role of the langerin$^+$ CD8α^+ DCs in stimulating CD8$^+$ T cells against tumours, the direct anti-tumour activity of T_h1 cells and their ability to aid in the function of CD8$^+$ T cells must be acknowledged. In addition, langerin$^+$ CD8α^+ DCs may play an important role in activating naive CD4$^+$ T cells and aiding their differentiation into T_h1 cells.

Regulatory T cells

As mentioned earlier T_{regs} can develop during the normal process of CD4$^+$ T cell maturation in the thymus. These cells are referred to as natural T_{regs}. However, similar cells with suppressor function can also differentiate from naïve Foxp3$^{-/-}$ CD4$^+$ T cells, when activated in the presence of TGF-β These are called inducible T_{regs} (195). The general function of T_{regs} is to suppress immune responses. This can be to limit responses against self-antigens, or to shut down an immune response against pathogens once they have been cleared. The suppressive nature of T_{regs} has severe implications on tumour immunity and this will be discussed in detail at a later point.

1.4.4 Memory T cells

When the levels of antigen used to generate an effector T cell population has reduced within the host, the T cells with TCRs specific for that antigen will enter the contraction phase (196, 197). A small population of T cells remain following contraction that have developed into long-lived memory T cells to ensure that a strong immune response can be generated if the pathogen to which the antigen derives is detected again.

Memory cells are able to survive within a host through the contraction phase and for a significant period of time thereafter, due to the ability of memory T cells to self-renew in response to IL-7 and IL-15. For this purpose, the receptors IL-7Rα (CD127) and the IL-15Rβ (CD122) are upregulated on memory cells and these markers can be used to identify memory cells (198, 199).

When antigens are encountered a second time, the resulting “secondary” response that is initiated is faster and stronger than a primary response due to the greater number of antigen-specific memory T cells within the host repertoire. In addition, memory cells reside in a primed state that enables a quicker response (200-202). Memory $CD8^+$ T cells also respond to lower levels of antigen than naïve T cells (203, 204) and require less co-stimulation (205).

Memory T cell subsets

The T cells that remain after the contraction phase are divided into distinct subsets of memory cells whose functions cooperate to provide protection upon reinfection. The first two memory subsets discovered are central memory (T_{CM}) and effector memory (T_{EM}) cells (127). The lymphoid homing receptors CD62L and CCR7 are expressed by T_{CM} cells and subsequently they recirculate through the secondary lymphoid organs, like naïve T cells (206). As the most likely sites of secondary infection are in the peripheral tissues, the T_{CM} cells function not as the first line of defence but as a means to repopulate a host with effector T cells when antigen is encountered. This is supported by the high proliferative potential of T_{CM} cells and their ability to produce large quantities of IL-2 upon antigen stimulation (127, 207, 208). On the other hand, T_{EM} cells express low levels of CD62L and CCR7 allowing them to circulate within the blood and enter peripheral sites of inflammation (208). Upon antigen stimulation, T_{EM} cells undergo very little proliferation relative to T_{CM}, but rapidly produce cytokines such as IFN-γ and cytotoxic molecules, such as perforin and granzymes (127, 209).

Recently a subset of memory cell very similar to T_{EM} has been discovered that does not recirculate through the blood like T_{EM} cells but reside solely within tissues. This subset is called resident T_{EM} (rT_{EM}) cells (210). The benefit of the rT_{EM} cells is that they reside within tissues and can therefore respond faster to secondary infection within their resident tissues. They respond to antigen encountered by both attacking the infected or abnormal cells and by recruiting T_{EM} cells circulating in the blood. The overall memory response therefore

consists of three waves, beginning with an attack by the rT_{EM}, followed by recruitment of the migrating T_{EM} cells and ending with the migration of effector T cells that were generated by T_{CM} cells in the lymphoid organs.

Recently, a population of long-lived T cells has been identified that has stem cell-like qualities and these have being termed “memory stem cells” (T_{SCM}) (211). This memory cell subset arises after antigenic stimulation and they have a phenotype similar to that of naïve T cells. In addition, T_{SCM} and naïve T cells are located in the same area of the secondary lymphoid organs. Similar to T_{CM}, T_{SCM} have enhanced proliferative abilities relative to the other T cell populations and are able to generate effector T cells and all memory subsets. It is therefore possible that the generation of T_{SCM} is essential to maintain memory T cell populations.

$CD8^+$ T cells generate in the absence of $CD4^+$ T cell help can develop an effector T cell response, however the memory response generated in the absence of help is significantly impaired (212, 213). Memory cells generated in the absence of help have a reduced ability to produce cytotoxic molecules, such as interferon (IFN)-γ, perforin, granzyme A and B and FasL, and they express more regulatory molecules such as IL-10Rα, TGF-β and TGF-βRII (214). Therefore, although memory cells can be developed in the absence of help, they are unable to respond to secondary challenges (212, 215, 216).

1.5 Invariant Natural Killer T cells

T cells that react with CD1d were termed iNKT cells because of their expression of NK cell and T cell markers. However, it has now been determined that not all iNKT cells express NK markers (217). The discovery of α-GalCer and development of α-GalCer-loaded CD1d tetramers has enabled significant developments in the identification and analysis of iNKT cells (217, 218). These cells are an innate-like cell found in high numbers in the spleen, liver and bone marrow and are able to rapidly produce cytokines in response to stimulation, similar to T_{EM} cells (219). The TCR of iNKT cells is invariant, consisting of an α-chain restricted to the Vα14-Jα18 gene rearrangement in

mice and a limited number of β-chains (218, 220, 221). The TCR recognizes glycolipids, both self and pathogen derived, that are presented on the MHC class I-like molecule CD1d, which is expressed at high levels by DCs (222, 223).

The development of iNKT cells begins the same as for conventional T cells, but differentiate during the double positive stage mentioned earlier (224). Current evidence indicates that the development of the invariant TCR, Vα14-Jα18 is through the random selection of genes during V(D)J recombination as with conventional T cells (225). However, T cells with the invariant TCR bind to CD1d molecules presented with an internally derived antigen by other double positive thymocytes, as opposed to MHC molecules presented by epithelial cells during conventional T cell development (226, 227). The binding of an iNKT cells TCR to CD1d is thought to initiate the iNKT cell developmental program, involving the expansion of the precursor cells and the development of the iNKT cell phenotype. There is evidence indicating that dendritic cells can induce negative selection of iNKT cells in the thymus, as mice that over expressed the CD1d molecule had a significantly decreased population of iNKT cells (228). The increase in CD1d likely caused by stronger binding between the DC and iNKT cell, resulting in more iNKT cells removed during negative selection. Some iNKT cells migrate from the thymus in an immature state and develop further in the periphery. Others remain within the thymus and mature before entering the peripheral tissues (229, 230).

The evolutionary reason why a population of T cells with an invariant TCR exist is unknown, however several theories have been developed. One idea involves the development of iNKT cells as a result of evolutionary selection for a TCR that recognises multiple antigens deriving from pathogens in the ancestral environment (231). Another suggestion involves the fact that iNKT cell activation following infection with LPS-positive bacteria was mediated indirectly via DC activation of T cells following TLR stimulation, whereas LPS-negative bacteria directly activated iNKT cells with cell wall gycolipids. Thus it

is possible that iNKT cells evolved as a defence against bacteria that do not have cell wall ligands that TLRs can recognise (232).

The invariant TCR, in combination with their potent licensing ability, makes iNKT cells an attractive target for immunotherapies, as a single ligand can be used to activate iNKT cells irrespective of an individuals MHC tissue type. An example of a glycolipid that provides a blanket iNKT cell activation stimulus is α-GalCer, which activates iNKT cells in a CD1d-dependent manner. Following administration, α-GalCer rapidly activates iNKT cells enabling them to license DCs in a CD40L-dependent manner (53, 78). Licensed DCs produce IL-12, which stimulates the release of IFN-γ by iNKT cells. The IFN-γ produced by iNKT cells activates NK cells, inducing them to also produce IFN-γ (233). The resulting IFN-γ significantly contributes to an anti-tumour response, including through its anti-angiogenic properties (234, 235). In addition, the activated iNKT cells are able to induce direct killing in a CD1d dependent manner. This was demonstrated by the iNKT cell-mediated killing of $CD1d^+$ AML cells that were pulsed with α-GalCer, largely through the ligation of FasL on the iNKT cell with Fas on the target cell (236, 237). The importance of iNKT cells in mediating an immune response against tumours was made evident by using CD1d and Jα18 KO mice (mice that cannot develop iNKT cells), which were significantly more susceptible to chemically induced tumours, such as prostate and sarcoma tumours (238, 239). Manipulating iNKT cells for the benefit of mediating anti-tumour protection will be discussed in detail later.

1.6 Tumour development and the immune system

Tumours develop when cells escape from various control mechanisms that limit cell-cycle progression. This occurs via genetic and epigenetic mutations that result in the activation of oncogenes and inactivation of tumour suppressor pathways (240, 241). Thus, the cell is able to proliferate without control enabling an exponential increase their population. This results in a significant propagation of these tumour cells, either as a solid node within tissues or as circulating cells within the blood. The difficulty for the immune system to prevent the progression of tumours is largely due to the inability of

the immune system to differentiate between healthy and tumour cells. Ideally an immune response generated against a tumour will not attack healthy cells and for this to occur the tumour antigens used to stimulate the adaptive immune response must be presented in a tumour-specific context. This makes it difficult for the host to develop an endogenous immune response when a malignancy is first initiated, and also makes it difficult to devise immunotherapies directed against established tumours. This difficulty is exacerbated by the immune suppression generated by a tumour, which not only suppresses endogenous responses, but can also be a significant barrier to anti-tumour immunotherapies (242). The interaction between a tumour and the immune system is complex and can range from the immune system being completely ignorant of the tumour to providing complete tumour rejection. A better understanding of this interaction is required to improve immunotherapy.

1.6.1 Tumour immunoediting

Evidence demonstrating that the immune system is capable of identifying tumours as dangerous is indicated by the fact that tumour-specific $CD8^+$ T cells can migrate into tumours. In many cancers, including breast (243), colon (244), ovarian (245) and skin cancers (246), the presence of tumour infiltrating lymphocytes (TILs) is correlated with a better prognosis, thus the immune system is to some degree able to attack tumours. For an effective anti-tumour adaptive immune response, tumour cells and not healthy cells must primarily express the antigen used to activate T cells so as to avoid autoimmunity. Examples of the types of antigens that can be expressed by tumours and recognised by the immune system, are indicated below.

Tumour antigens

Tumour-specific antigens

Tumour specific antigens arise from the genetic instability associated neoplastic transformation, with mutations causing neo-antigens that are expressed by the tumour cell alone (247). The formation of tumour-specific antigens is unpredictable and therefore their detection is rare, making their use as targets for immunotherapy difficult (248). The host's own immune

system is likely to be capable of developing a strong T cell response against this type of antigen, as neo-antigens are effectively ‘foreign’ and therefore T cells recognising these antigens are unlikely to have been deleted through the process of central tolerance. An example of a tumour-specific antigen is the mutated peptide $p53_{210-223}$, with a missense mutation causing an amino acid change from tyrosine to cysteine at position 220 (249). All cells express p53, as it functions as an inhibitor of cell growth by controlling cell cycle progression and apoptosis (250). When mutated, loss of p53 function can contribute to uncontrolled cell growth resulting in tumour development. Importantly, mutated sequences from the p53 protein can be presented on MHC molecules to T cells, providing unique antigens that severe as targets for tumour elimination (251).

Tumour-associated antigens

Tumour-associated antigens are tumour antigens that are typically only expressed in a select number of healthy tissues. For example, expression of cancer/testis antigens (such as NY-ESO-1) is restricted to the testis and ovaries (252, 253). In these healthy tissues the immune system is mildly suppressed, thereby preventing an autoimmune response against these antigens (253). When NY-ESO-1 is expressed on a tumour outside of the testis or ovaries an immune responses that targets the tumour can be generated, while generally avoiding autoimmunity due to the suppressive environment in their original tissues (253).

Other tumour-associated antigens are those that are expressed in a wider variety of healthy cells, but are over expressed by tumour cells (27, 254). For example, the expression of the wild-type p53 protein, which as mentioned, is expressed in all cells, is often over expressed in tumour cells (254). In order to initiate an effective anti-tumour response, the immune system would have to differentiate the tumour cells from healthy cells based not on the presence of the antigen but on the level of expression.

Stromal antigens

Another type of antigen that is an effective target for tumour immunotherapy belongs to the cells that support a tumour and not to the tumour cells themselves (255). These cells are called stromal cells and include fibroblasts, vascular endothelial cells and macrophages. An example is fibroblast activation protein (FAP), which is highly expressed by tumour-associated fibroblasts that often make up a significant portion of the stroma (256). Using a DC-based vaccine to develop an immune response against FAP antigens has proven to be effective in the treatment of colon cancer in mice (257).

1.6.2 Host immune responses to tumours; the elimination phase

The complex interaction between the immune system and a tumour has broadly been differentiated into three different states that are based on the success of the immune system at eradicating a given tumour. These three states consist of elimination, equilibrium and escape. Elimination occurs when the immune system mounts a successful attack on tumour cells. This occurs frequently in healthy individuals by preventing tumour growth and development. This is made evident by experiments involving the treatment of immunodeficient mice with the chemical carcinogen 3-methylcholanthrene to induce sarcomas. Immunodeficient mice developed significantly more tumours following 3-methylcholanthrene treatment compared to wildtype mice, indicating that the immune system is capable of preventing tumour development (258, 259). In addition, the cytotoxic protein perforin has been demonstrated to be essential in protecting mice from developing a spontaneous lymphoma, which further demonstrates that the immune system has the potential to protect a host from tumour development (260).

1.6.3 Host immune responses to tumours; the equilibrium phase

Equilibrium (also known as immune mediated tumour dormancy) is where the immune system and the tumour have reached a state of equilibrium and the rate of tumour growth is equal to the rate that the tumour is being removed by

the immune system. The tumour size within the host is therefore constant due to the immune attack. However, if the host is immunocompromised the tumour will begin to expand (261, 262). An excellent example of the controlled state in which the immune system can hold tumours is seen in cases where a secondary melanoma develops in hosts due to receiving a renal transplant from someone who once had melanoma (263). Some recipients remained tumour-free after transplantation for 16 years before the secondary melanoma developed. This indicates that the tumour cells were present for 16 years, without being able to grow or be eradicated by the immune system (263). Similarly, mice with a dormant B cell lymphoma were symptom free for 60 days but developed tumours rapidly following suppression of the immune system with a CD8 depleting antibody. In addition, inactivating IFN-γ on day 60 of symptom-free survival also induced tumour growth at the original injection site (264). This indicates that $CD8^+$ T cells were actively attacking the tumour cells by producing IFN-γ, which was preventing the tumour from growing but not eradicating the tumour cells completely.

A constant attack on a tumour by the immune system can have the undesired effect of sculpting the tumour to become more immunoresistant. This occurs as the immune system attacks the tumour cells it can recognize, leaving the immune resistant tumour cells behind to grow and repopulate the tumour, thereby developing a more immunoresistant tumour (Figure 1.6) (261).

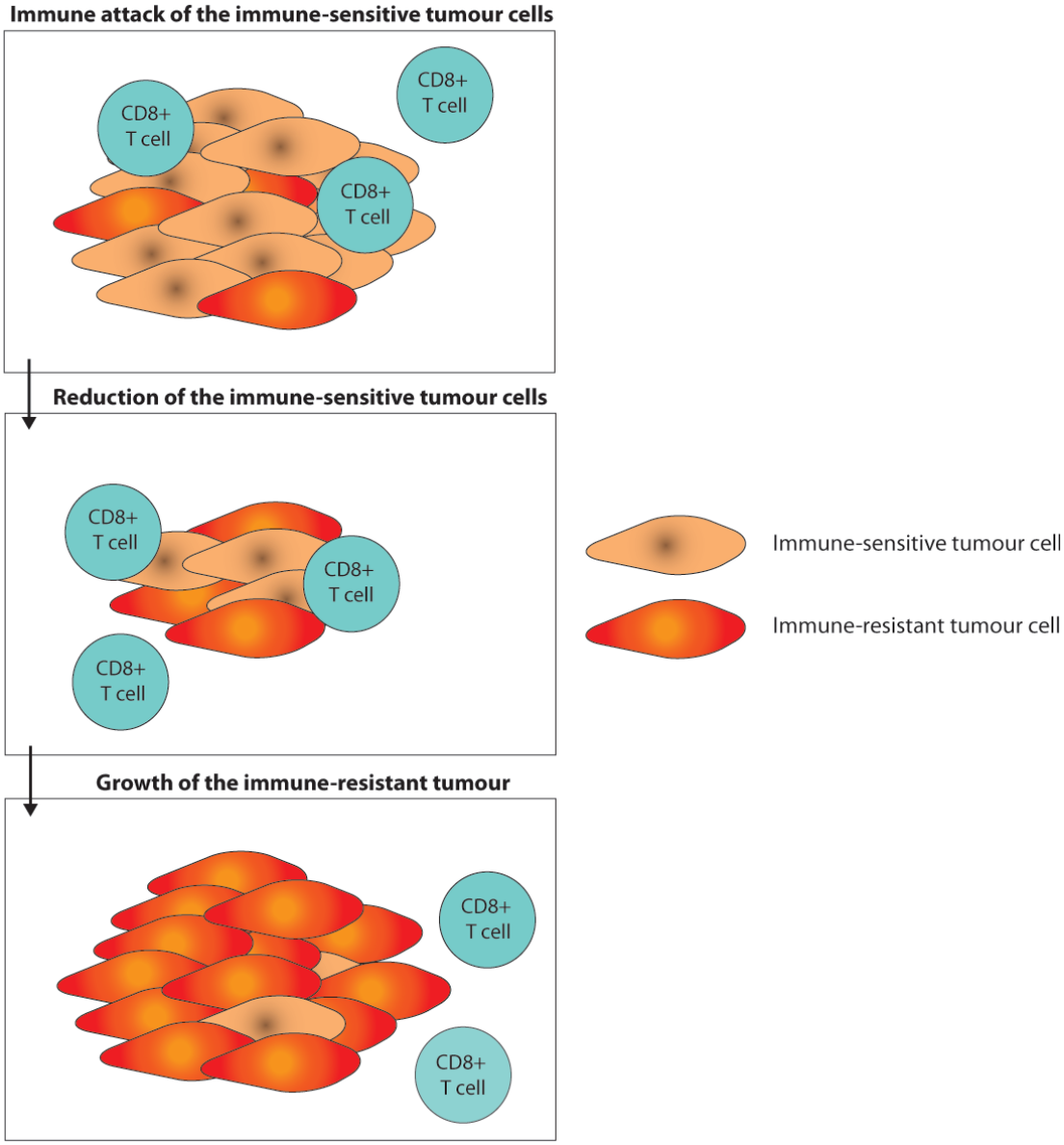


Figure 1.5: Sculpting an immunoresistant tumour by the immune system. The difference between the ability of the immune system to recognize given tumour cells can result in the development a more immunoresistant tumour. This occurs when the immune system attacks and kills the immunosensitive tumour cells while being unable to attack the more immunoresistant tumour cells. The immunoresistant tumour cells are therefore left to grow and repopulate the tumour resulting in a more immunoresistant tumour.

1.6.4 Host immune response to tumours; the escape phase

When the innate and adaptive immune systems cannot eliminate or contain tumour growth, the tumour cells effectively “escape” leading to tumour growth and development. Tumour cells can avoid being attacked by the immune

system by cell modifications that enable the tumour cells to avoid detection or effective attack. In addition, tumours can generate a suppressive environment that is able to shut down or prevent the development of an effective immune response (262).

Tumour cell modification

As mentioned above, cells that survive immune mediated attack are left to grow, thereby developing an immunoevasive tumour. Some tumour cells develop defects in antigen processing or presentation pathways that can result in loss of MHC class I proteins (265, 266), immunoproteosome subunits (267, 268) or downregulation of the TAP complex (269, 270). These mutations impair the ability of the tumour cell to present tumour antigens, thereby making it unrecognizable by the adaptive immune response. In addition, tumour cells can downregulate the IFN-γ receptor, making them resistant to direct killing with IFN-γ (271). Tumour cells can also actively suppress an immune response by upregulating molecules on their surface such as PD-L1, which interacts with receptors on the surface of T cells and dampens their functions or induces apoptosis (272). The secretion of soluble factors can also have a significant immune suppressing function. Sterol metabolites secreted by tumour cells prevent DCs from upregulating CCR7, impairing the ability of DCs to migrate to the lymph nodes for antigen presentation (273). Furthermore, vascular endothelial growth factor (274), and TGF-β (275) produced by tumour cells can suppress DC activation and function. The expression of Indolamine 2, 3-dioxygenase (IDO) by tumour cells also has a variety of suppressive effects, which will be discussed later.

Immunosuppression

The immunosuppressive environment generated by tumours makes it a very difficult disease to treat with immunotherapy. The highly suppressive tumour, AML, provides an appropriate example for tumour-associate immune suppression, due to its clinical implications and due to its use during the research performed for this thesis. Patients with AML, have been found to

have DCs with impaired maturation and functional capabilities in their peripheral blood, and these DCs can drive tolerance in response to leukemic antigens instead of immunity (276). A greater understanding of the immune suppression generated by a tumour would greatly help understand why some current immunotherapies are failing and would aid in the development of new, more effective anti-tumour immunotherapies.

Indolamine 2, 3-dioxygenase

One way that tumour cells can induce immune suppression is by expressing IDO, which the majority of AML cells express (277). IDO is a tryptophan catabolising enzyme that functions by reducing the amount of tryptophan in a host, which is required for T cell proliferation (278). The importance of tryptophan in T cell proliferation is made evident by *in vitro* studies demonstrating that IDO-induced T cell suppression can be reversed by the addition of tryptophan during priming (279). During inflammation, DCs upregulate IDO in response to IFN-γ, as a method to prevent an exaggerated inflammatory response within a host (280).

The metabolites of tryptophan degradation by IDO are called kynurenines and they also generate immunosuppression by inducing apoptosis of thymocytes and functional T_h1 cells (281). The combination of tryptophan deprivation and the presence of kynurenines result in the down-regulation of the $CD8^+$ T cell TCR ζ-chain, causing decreased functionality of cytotoxic T cells (282). Kynurenines also have a distinct effect on $CD4^+$ T cells during priming, driving them to differentiate into immunosuppressive T_{regs} (283). Furthermore, IDO can activate and induce the migration of T_{regs} to a tumour site (283-285). Therefore, IDO suppresses immune responses directly, but can also exacerbate the suppressive environment by activating other suppressive cells.

Regulatory T cells

The immune suppressor cell, T_{regs}, prevent the generation of an immune response in unwarranted situations, thereby decreasing the chance of

developing autoimmunity (286). Some tumours have the ability to inhibit an anti-tumour immune response by activating tumour-antigen specific T_{regs} and thereby evade the immune system. As a result the number of T_{regs} within a tumour-bearing host can be directly correlated to their prognosis (287). Production of the cytokines IL-10 (288) and TGF-β (289) and expression of the inhibitory molecule CTLA-4 (290) are tools by which T_{regs} mediate immune suppression. They also regulate the $CD8^+$ T cell population by absorbing free IL-2, thereby limiting the amount of IL-2 available for $CD8^+$ T cells to utilize for population maintenance and expansion (291). The suppressive capabilities of tumour-induced T_{regs} were demonstrated by showing that mice with AML responded better to the transfer of anti-tumour effector $CD8^+$ T cells when host T_{regs} were depleted prior to transfer (292). The transferred $CD8^+$ T cells in these mice proliferated more and produced more IFN-γ when the T_{regs} were depleted.

As mentioned above T_{regs} can develop directly from the thymus or can be generated by $CD4^+$ T cell differentiation, the T_{regs} generated are called natural and induced T_{regs}, respectively. The type of T_{reg} found in tumours is poorly understood largely due to the difficulty in differentiating them (293). The distinction between the two subsets is important as the method required to deplete tumour-infiltrating T_{regs} may depend on the type infiltrating the tumour. Methods to differentiate between these two subsets are currently being investigated. Initially, expression of the Ikaros family transcription factor 'Helios' was used to identify the natural T_{regs} (294, 295); however, it was later found that Helios could be expressed by both natural and induced T_{regs} (296, 297). It has subsequently been suggested that Helios is upregulated on activated and proliferating T_{regs}. The implication of Helios expression and methods to differentiate between natural and induced T_{regs} are up for debate.

Myeloid derived suppressor cells

Another suppressive cell subset involved in maintaining and controlling an immune response is called the myeloid derived suppressor cells (MDSC). MDSCs constitute a population of progenitor myeloid cells, including immature

DCs, macrophages and granulocytes that are unable to progress into a mature phenotype. There are, broadly speaking, two different subsets of MDSCs in mice that are called the granulocytic and monocytic MDSCs, which express the markers Ly6G and Ly6C, respectively (298). The specific differences between these two subsets are not well understood, however both subsets are able to suppress T cells, with some studies demonstrating different mechanisms of T cell suppression (299). Tumours can increase the number of MDSCs within a host and these can aid the tumour in evading the immune system. Many tumours upregulate both the granulocytic and monocytic population of MDSC but it is the granulocytic population that typically expands the most (298).

The immune suppression generated by MDSCs occurs via several different pathways and most of these are contact dependent. One suppressive process is similar to the tryptophan ablation mediated by IDO, in which MDSCs increase the activity of arginase-1, which catabolises L-arginine, an amino acid also essential for T cell proliferation (300). MDSCs also have upregulated NADPH oxidase and inducible nitric oxide synthase, which results in increased production of ROS and nitric oxide, respectively (301). The production of ROS also suppresses immune responses as indicated by ablation of the suppressive activity of MDSCs that were removed from tumour-bearing mice when ROS production was inhibited (302-304). In addition, MDSCs can exacerbate the immuneosuppressive response of a tumour by increasing the T_{reg} population (305).

1.7 Tumour immunotherapy

The current treatments for tumours largely consist of surgery, radiotherapy and chemotherapy. While these treatments are effective to some degree, in many situations the clinical benefit is short-lived, and is often associated with considerable treatment-related morbidity. The invasiveness of surgery and the non-specific mode of action of radiotherapy and chemotherapy make them significantly harmful, often resulting in severe morbidity or even mortality (306). These therapies have had significant success in treating some

tumours, however many still remain extremely difficult to treat, often only receiving mild benefits from these harmful therapies (307).

Immunotherapy, on the other hand, can be designed to specifically attack each host's individual tumour, thereby limiting the healthy tissue damage that is associated with conventional treatments. In addition, a successful immunotherapy will develop immunological memory to produce long-term protection from tumour relapse. However, there are still many aspects of anti-tumour immunotherapy that need to be fine-tuned to maximise anti-tumour protection. Some of these aspects will be discussed below for the therapies used in the research of this thesis.

1.7.1 Adoptive T cell therapy

The history of adoptive cell therapy

Adoptive T cell therapy is the process of removing tumour-specific T cells from a tumour-bearing host, and then inducing their proliferating *in vitro* to produce large numbers of tumour-specific T cells that can be readministered back into the host (308). The aim of this treatment is to administer a significantly large, and appropriately activated population of antigen-specific effector T cells that can directly attack the tumour. The first model of adoptive T cell therapy involved removing a tumour from mice with pulmonary micrometastasis and culturing the resulting single cell suspension in IL-2. The tumour antigens in combination with IL-2 activated the tumour infiltrating lymphocytes, thereby inducing their proliferation and causing them to attack the tumour cells within the culture (309). By day fifteen the tumour cells had been eradicated leaving behind only the lymphocytes within the culture. Injecting $5x10^6$ of the remaining $CD8^+$ T cells into mice with the same pulmonary micrometastasis resulted in eradication of the established tumour in 96% of the tested mice (309).

There are still many factors that limit the development of an effective adoptive T cell therapy that can readily be adapted to the clinic. These include the difficulty in isolating and expanding tumour infiltrating lymphocytes, the

inability of the T cells to persist for an extended period of time following administration, and the barrier to effective therapy caused by tumour-associated immune suppression. Research to increase the effectiveness of adoptive therapy has included determining the differentiation status and number of T cells that provide the most effective anti-tumour T cell population, in addition to the most suitable environment for the transferred cells (310).

Increasing the number of transferred $CD8^+$ T cells has been shown to have a significant impact on the protective benefit provided by adoptive therapy, with most experiments demonstrating a direct correlation between the number of $CD8^+$ T cells administered and the protection provided to the recipient (310, 311). However, some studies also observed no difference between two significantly different doses of the same transferred $CD8^+$ T cells (312). It was suggested that an immune cascade initiated following T cell transfer was central to tumour killing and therefore increased numbers did not produce a significant difference in survival. The differentiation status of the $CD8^+$ T cells also had significant consequences to the protection provided by adoptive cell therapy. This was made evident by an experiment that involved transferring either $CD62L^{high}$ (naïve T cells) or $CD62L^{low}$ (effector T cells) $CD4^+$ and $CD8^+$ T cells into hosts. While $5x10^6$ $CD62L^{low}$ cells were sufficient to protect mice from a three-day-old fibrosarcoma, $50x10^6$ $CD62L^{high}$ cells provided no protective benefit against the same tumour (313, 314). However, once again the expression of CD62L and the state of differentiation of the transferred T cells have had contradicting results in terms of their ability to provide protection following transfer. Another study demonstrated that $CD8^+$ T cells that have reached an advanced stage of effector cell development had reduced anti-tumour activity, whereas naïve $CD8^+$ T cells, as well as the “early effectors”, which are $CD62L^{high}$ had significant protective capabilities (315). It is thought that the effector $CD8^+$ T cells in this experiment are terminally differentiated and therefore not as effective at providing protection.

Transferring memory cells instead of effector cells may be more effective for adoptive therapy, as they can self-renew and have heightened recall responses (310, 316, 317). Studies examining the most effective memory

subset to transfer have also developed contradicting results. Culturing T cells *in vitro* with IL-15 and/or IL-21 develops a CD62L^{high} T_{CM} phenotype and the transfer of T_{CM} cells provided mice with superior anti-tumour protection compared to T_{EM} cells, possibly due to the stronger ability of T_{CM} cells to proliferate in response to restimulation (318-320). In contrast, the development of T_{EM} cells by culturing T cells with IL-2 has also been found to provide greater anti-tumour protection than T_{CM} cells, possibly due to the expression of adhesion molecules by T_{EM} cells that allow them to migrate to and infiltrate a tumour, thereby providing rapid tumour destruction (313, 314).

Depleting the host immune system with either irradiation or a lymphodepleting chemotherapy prior to adoptive cell therapy has been shown to extend the persistence of T cells following transfer, thereby resulting in more objective response rates in melanoma patients (321). Lymphodepletion aids in T cell persistence both by depleting the regulatory cells generated by a tumour and also by depleting the endogenous T cells that compete with the transferred cells for homeostatic cytokines, such as IL-7 and IL-15 (322, 323).

Determining how to provide the best protection against tumour development with adoptive cell therapy is still under debate and it is likely that the most effective method depends on the type and stage of tumour and the specific immune system of the patient. It is possible that the interaction between the transferred T cells and the endogenous APCs may determine how effective the transferred cells are at providing protection against tumours.

1.7.2 Cancer vaccines that exploit NKT cells

Using a vaccine to generate effector CD8$^+$ T cells against a tumour requires appropriate activation of DCs, particularly DC licensing, which is required to optimally activate CD8$^+$ T cells against a tumour. A single dose of the iNKT cell ligand α-GalCer, administered intravenously, results in a significant activation of splenic DCs within 24 hours due to licensing by the α-GalCer activated iNKT cells (53, 78). In addition, the administration of free α-GalCer provides significant protective benefits to mice with either a melanoma or

thymoma (324, 325). However, the experiments using free α-GalCer in the clinic did not show as much success as the pre-clinical results (326-328).

The limitation of vaccinating with free α-GalCer is the dependency on the effector functions of the innate immune response (325). Studies have now demonstrated the potential of co-administering α-GalCer with a target antigen to induce an effective $CD8^+$ T cell response that is dependent on the stimulation of DCs by iNKT cells (53, 78). To enable this vaccine to generate lymphocytes directed against tumours, whole tumour cells were administered in combination with α-GalCer to provide a source of tumour antigens. This vaccine provided a significant protective benefit to mice with a plasmacytoma (J558) or a thymoma (A20) and the immune response was dependent on the irradiated tumour cells being taken up by DCs in the spleen (329). Subsequent studies have demonstrated that a vaccine consisting of irradiated tumour cells pulsed with α-GalCer provided mice with superior protection against the A20 lymphoma than mice that received irradiated tumour cells with free α-GalCer (330). Interestingly, this immune response was dependent on $CD4^+$ T cells and not $CD8^+$ T cells. Vaccinating mice with irradiated tumour cells pulsed with α-GalCer has subsequently been demonstrated to be an effective treatment for a murine model of glioma (331), melanoma (332), lymphoma (330) and also a model of AML (which differs from the model used in this thesis) (333).

A vaccine consisting of irradiated tumour cells loaded with α-GalCer has the advantage of inducing response to undefined antigens within the autologous whole tumour cells, rather than having to determine if a patient's tumour expresses a given tumour antigen (334). In addition, a vaccine based on whole tumour cells can be used to activate a broad immune response involving $CD4^+$ and $CD8^+$ T cells against a variety of different targets. This greatly decreases the chance of developing a more immunoevasive tumour due to tumour escape. In general, the utility of whole-tumour vaccines will be largely determined by how relatively simple they are to prepare. There is some evidence to suggest that this form of vaccine (regardless of the adjuvant

used) may be more effective in the clinic than therapies targeting defined antigens. An analysis of 1601 patients from 75 published trials in advanced metastatic melanoma showed that 12.6 % of patients that received some form of vaccine with undefined whole tumour antigens had an objective response, compared to 6 % of those that received a vaccine using a defined antigen (335).

When α-GalCer is pulsed onto irradiated tumour cells as an immune adjuvant, *in vivo* DCs will phagocytose the cellular material and the α-GalCer will be presented on the non-classical MHC molecule CD1d for simulation of iNKT cells (336). Activated iNKT cells will then provide a link between the innate and adaptive immune response by licensing DCs (73). The matured DCs will use the tumour antigens acquired from the irradiated tumor cells to induce the activation of naïve T cells into anti-tumour effector T cells (81). The research in this thesis investigates the *in vivo* events that follow administration of such a vaccine designed to treat AML. The availability of *lang*-EGFPDTR made it possible to specifically investigate what role, if any, was played by langerin$^+$ CD8α$^+$ DCs.

1.8 Hypotheses tested in this thesis

It is now evident that DCs are a heterogeneous cell population, with phenotypically distinct subsets that may have different functions in immunity. The role that specific endogenous DC subsets play in different forms of immunotherapy is largely unknown. Due to evidence indicating that langerin$^+$ CD8α$^+$ DCs are efficient at cross-priming CD8$^+$ T cell responses using circulating antigens, it is possible that these cells provide an essential function during *in vivo* immune responses generated by an intravenously administered immunotherapy. With the recent availability of *lang*-EGFPDTR mice, where langerin$^+$ CD8α$^+$ DCs can be depleted from the spleen, it became feasible to study how CD8$^+$ T cell responses are affected in the absence of this subset of DC. The aim of this thesis, therefore, was to anaylse the function of langerin$^+$ CD8α$^+$ DCs in stimulating T cells, in both the naïve and effector settings, following immunotherapy for the treatment of intravenously administered

tumours. For this purpose, two models of immunotherapy were developed, one that utilises effector cells directly (adoptive $CD8^+$ T cell therapy) and another that generates effector cells *in vivo* (tumour/α-GalCer vaccination). With these two models it was possible to determine if $langerin^+$ $CD8\alpha^+$ DCs stimulate transferred effector $CD8^+$ T cells *in vivo* (following transfer) and if they generate effector T cells during a vaccine-induced immune response (Figure 1.6).

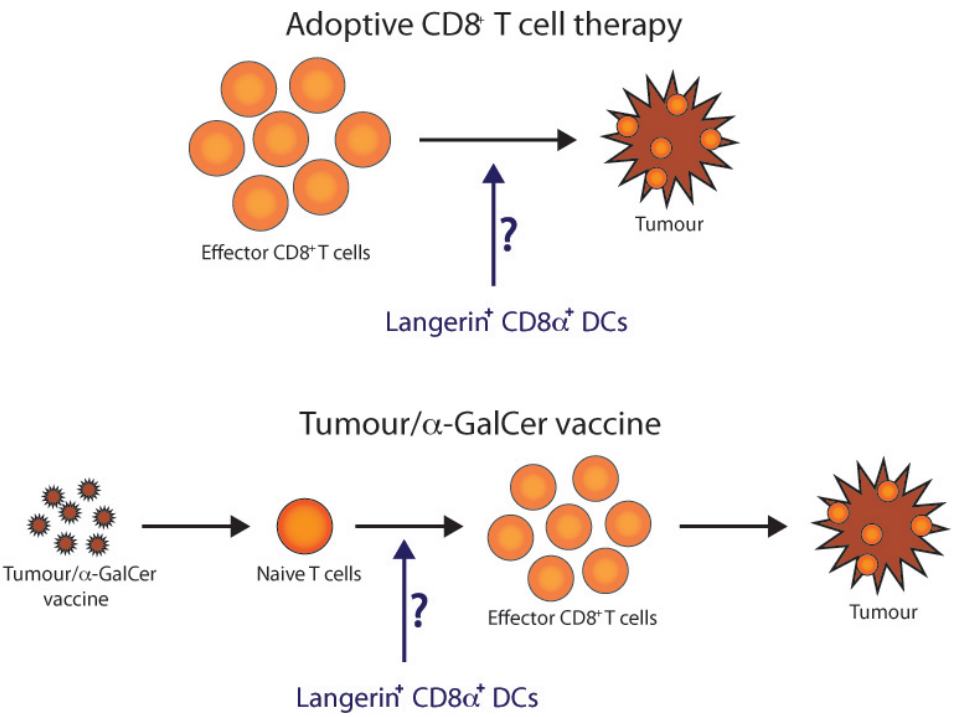


Figure 1.6: Immunotherapy models to determine if $langerin^+$ $CD8\alpha^+$ DCs stimulate and/or generate effector $CD8^+$ T cells. Adoptive therapy is used to determine if $langerin^+$ $CD8\alpha^+$ DCs stimulate effector $CD8^+$ T cells administered for the treatment of intravenous tumours (Top). A vaccine consisting of irradiated tumour cells loaded with α-GalCer is used to determine if $langerin^+$ $CD8\alpha^+$ DCs are involved in generating effector $CD8^+$ T cells during a vaccine-induced immune response for the treatment of intravenously administered tumours.

Adoptive transfer of activated $CD8^+$ T cells has shown promising results in the clinic. However, methods to prolong the survival and function of the transferred T cells are likely to enhance the resulting anti-tumour response. Investigating whether endogenous DCs are involved stimulating adoptively transferred effector $CD8^+$ T cells may provide insight into new methods to improve responses. If endogenous DCs are found to provide stimuli to the transferred T cells it may be possible to enhance the transferred T cell response by either promoting or deterring this interaction. In addition, if the specific stimuli associated with this interaction were determined then it may be possible to enhance tumour elimination by artificially producing or blocking these stimuli. Therefore, in the first part of the thesis, a model of adoptive T cell transfer-based immunotherapy was established, and the following hypothesis was tested "*that resident langerin$^+$ CD8α^+ DCs in the spleen stimulate adoptively transferred effector CD8$^+$ T cells, thereby promoting effective anti-tumour activity of the immunotherapy".*

Vaccination is another immunotherapeutic strategy that relies on the function of T cells, with many models showing that $CD8^+$ T cells are particularly good anti-tumour effector cells. The challenge is to develop simple vaccines with a known mode of activity in order to translate this form of therapy to routine cancer treatment. In this context, resident DCs are likely to play a very significant role. Over the course of this thesis, a novel vaccine consisting of irradiated tumour cells loaded with α-GalCer was developed for the treatment of AML. One of the features of this vaccine is that it required intravenous administration to access splenic iNKT cells, and hence elicit potent activity. With this vaccination model, it was possible to examine the specific role of langerin$^+$ CD8α^+ DCs in the spleen in initiating generating anti-tumour effector T cells. Determining whether this specific DC subset has a role in vaccine-induced responses may significantly contribute to the future design of more potent vaccines. Therefore, in the second part of the thesis, a vaccine-based immunotherapy of AML was used to test the hypothesis "*that resident langerin$^+$ CD8α^+ DCs in the spleen activate naïve T cells following intravenous vaccination, resulting in effective anti-tumour activity*".

1.9 Aims

- Generate two immunotherapy models for the treatment of intravenously administered tumours that use either effector T cells directly or generates effector T cells *in vivo*

- Determine if langerin$^+$ CD8α^+ dendritic cells are required to promote an immunotherapy-based effector CD8$^+$ T cell response

- Determine if langerin$^+$ CD8α^+ dendritic cells are required to generate effector CD8$^+$ T cells following vaccination

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Labware

Product	**Supplier/Distributor**
Acrodisc 13 mm Syringe filters with 0.2 μM membrane	PALL LifeSciences, Cornwall, U.K
Axygen Microtubes 1.7 mL	Axygen Scientific Inc., Union City, CA, U.S.A
BD 25 ml Syringes BD 10 ml Syringes BD 5 ml Syringes	BD Biosciences, Bedford, MA, U.S.A
Falcon Tissue culture plates: 6-well plates, 24-well plates & U-bottom 96-well plates	BD Biosciences, Bedford, MA, U.S.A
Falcon™ Polypropylene conical flasks: 50 ml, & 15 ml	BD Biosciences, Bedford, MA, U.S.A
Falcon Tissue culture flasks: 750 ml & 250 ml	BD Biosciences, Bedford, MA, U.S.A
Falcon Nylon cell strainers 70 μM	BD Biosciences, Bedford, MA, U.S.A
Pre-Seperation filter 30 μm	Miltenyi biotec, Bergisch Gladbach, Germany
PrecisionGlide Needles 25 gauge	BD Biosciences, Bedford, MA, U.S.A
Titertube Microtubes	Biorad, Hercules, CA, U.S.A
Ultra-Fine Insulin syringes 1 ml 29 gauge	BD Biosciences, Bedford, MA, U.S.A

2.1.2 Reagents and Buffers

BD Cytofix/Cytoperm

Purchased from BD Bioscience (CA, U.S.A) and stored at 4 °C.

Bioplex Buffer

- 1 L PBS
- 4 ml EDTA (0.5 M) (Sigma-Aldrich, MO, U.S.A)
- 1 g BSA
- 0.5 ml Tween20 (Sigma-Aldrich, MO, U.S.A)
- 50 μl NaN_3 (Sigma-Aldrich, MO, U.S.A)

Bovine Serum Albumin (BSA)

BSA with low endotoxin levels and no IgG was purchased from ICPbio Ltd (Auckland, New Zealand) in powder form and stored at 4 °C.

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)

Ten 500 μg vials of CFSE were purchased from Molecular Probes, Invitrogen (OR, U.S.A). It was reconstituted in DMSO and stored at -20 °C.

Cell Separation Running Buffer

- 1 L PBS
- 2 mM EDTA (Sigma-Aldrich, MO, U.S.A)
- 10 ml FCS

Cell Tracker Orange (CTO) (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)

1 mg of CTO was purchased from Molecular Probes, Invitrogen (OR, U.S.A). It was reconstituted in DMSO and stored at -20 °C.

Collagenase I

10 mg/ml of collagenase was purchased from Gibco Invitrogen (OR, U.S.A). It was reconstituted in IMDM and stored at -20 °C.

Complete Iscove's Modified Dulbecco's Medium (cIMDM)

- 500 ml iIMDM
- 25 ml FBS
- 5 ml Penstrep (Gibco, Invitrogen)
- 500 μl 2-ME

Cytarabine

Purchased from Pifzer (Perth, Australia) and stored at room temperature.

DNase I

DNase I was purchased from Roche (IN, U.S.A) and stored at -20 °C.

Dulbecco's Phosphate Buffered Saline (dPBS)

Purchased from Gibco Invitrogen (OR, U.S.A).

Diphtheria toxin

1 mg DT in powder form was purchased from Sigma-Aldrich (MO, U.S.A). It was reconstituted in H_20 and stored at -20 °C.

Doxorubicin

10 mg of doxorubicin in a 5 ml vial was purchased from EBEWE Pharma (Unterach, Austria).

Endograde OVA

Low endotoxin chicken OVA was purchased from Profos AG (Regensburg, Germany).

FACS Buffer

- 1 L PBS
- 10 ml FBS
- 5 ml NaN_3
- 4 ml EDTA (0.5 M)

Foetal Bovine Serum (FBS)

Purchased from SAFC Biosciences Sigma-Aldrich (MO, U.S.A) and stored at -20 °C.

α-Galactosyl-ceramide (α-GalCer)

The iNKT cell ligand α-GalCer was manufactured by Industrial Research Ltd (Wellington, New Zealand) as previously described (337).

Incomplete Iscove's Modified Dulbecco's Medium (iIMDM)

Purchased from Gibco Invitrogen (OR, U.S.A).

Liberase

Purchased from Roche (IN, U.S.A) and stored at -20 °C.

Lipopolysaccharides (LPS)

1 mg was purchased from Sigma-Aldrich (MO, U.S.A). It was reconstituted in PBS and sonicated for 30 minutes before being stored at 4 °C.

2 Mercaptoethanol (2 ME)

Purchased from Gibco, Invitrogen (OR, U.S.A) and stored at 4 °C.

Percoll

Purchased from GE Healthcare Biosciences (Uppsala, Sweden).

Phosphate Buffered Saline (PBS)

- 1 sachet of PBS (Gibco, Invitrogen)
- 1 L MilliQ water

Red Blood Cell Lysis Solution

Purchased from Qiagen Sciences (MD, U.S.A) and stored at room temperature.

Running Buffer

- 1 L PBS
- 10 ml FBS
- 4 ml EDTA (0.5 M)

SIINFEKL

The peptide deriving from the OVA protein SIINFEKL (OVA257-264) was purchased from Genscript (NJ, U.S.A). It was reconstituted in H_20 and stored at -20 °C.

2.1.3 Antibodies

Specificity	Fluorophore	Clone	Manufacturer
CD3	FitC	145-2C11	eBioscience (CA, U.S.A)
CD3	PECy7	145-2C11	eBioscience
CD4	A488	GK1.5	eBioscience
CD4	APC	GK1.5	eBioscience
CD4	PE	GK1.5	eBioscience
CD8	Fitc	53-6.7	BioLegend (CA, U.S.A)
CD8	PE	56-6.7	BioLegend
CD8	PerCP	53-6.7	BD Bioscience
CD8	A700	56-6.7	eBioscience
CD8	Pacific Blue	53-6.7	BD Bioscience
CD11b	APC	M1/70	eBioscience
CD11b	Biotin	M1/70	eBioscience
CD11c	PECy7	N418	eBioscience
CD11c	APC	N418	BioLegend
CD25	FitC	7D4	BD Pharmingen
CD25	PE	PC61	eBioscience
CD40	Biotin	3/23	BD Bioscience
CD44	PECy5	IM7	BD Bioscience
CD44	PerCP Cy5.5	IM7	eBioscience
CD45.1	FitC	A20	eBioscience

CD45.1	PE	A20	eBioscience
CD62L	Biotin	MEL-14	BD Bioscience
CD86	PE	GL-1	eBioscience
CD122	FitC	TM-β1	BioLegend
B220	PercP	RA3-6B2	BD Bioscience
FoxP3	PE	FJK-16s	eBioscience
IFN-γ	PE	-	BD Bioscience
IFN-γ	PECy7	XMG1-2	eBioscience
IgG	APC	-	BD Bioscience
KLRG1	PECy7	2F1	eBioscience
Ly6G	PE	1A8	eBioscience
NK1.1	Bio	PK136	eBioscience
Vα2	APC	B20.1	eBioscience
Vα2	V450	B20.1	BD Bioscience

2.1.4 Fluorophores

Fluorophore	**Manufacturer**
DAPI	Invitrogen
Live Dead Fixable Blue (LDFB)	Invitrogen
Propidium Iodide (PI)	Invitrogen
Streptavidin PECy7	BD Bioscience

2.1.5 Cytokines

Granulocyte-macrophage colony stimulating factor (GM-CSF)

Recombinant murine GM-CSF was produced using stationary phase cultures of the murine X63 cell line, modified to secrete the dull length murine GM-CSF protein.

Interleukin 4

Recombinant murine IL-4 was produced using stationary phase cultures of a Chinese Hamster Ovary cell line, modified to secrete full-length murine IL-4 protein.

Concentration of GM-CSF and IL-4

To determine the concentration of GM-CSF and IL-4 isolated from the cultures mentioned above, bone marrow derived DCs (BMDCs) were cultured in different concentrations of the cytokines. The BMDC phenotypes were then compared to BMDCs cultured with known concentrations of GM-CSF and IL-4. The concentrations of GM-CSF and IL-4 that produced BMDCs with similar phenotypes to the control samples were chosen for future experiments.

Interleukin 2

Recombinant human IL-2 was purchased from PeproTech, (NJ, U.S.A).

2.1.6 Tumour Cell Lines

E.G7-OVA

E.G7-OVA is a genetically altered version of the C57BL/6 derived murine lymphoma called EL-4. The genetic manipulation consists of the insertion of DNA coding for the OVA protein into the genetic material of EL-4. E.G7-OVA is therefore EL-4 that expresses OVA (338). E.G7-OVA was obtained from ATCC (VA, U.S.A). EG.7-OVA was grown in cIMDM and cultured in 37 °C with 5 % CO_2.

C1498

C1498 is a murine AML that developed spontaneously in a C57BL mouse at the Jackson Laboratories (ME, U.S.A) in 1941 (339). C1498 was obtained from ATCC (VA, U.S.A) C1498 was grown in cIMDM and cultured in 37 °C with 5 % CO_2.

2.1.7 Mice

Ethics Approval and Housing

All mice were bred and housed at the Biomedical Research Unit of the Malaghan Institute of Medical Research. Groups of age and sex matched mice were used in each experiment. All experiments were carried out within

the provisions of the Animal Welfare Act of New Zealand (1999) and approved by the Victoria University of Wellington Animal Ethics committee.

Mouse Strains

The C57BL/6 inbred mouse strain was obtained from Jackson Labratories (ME, U.S.A). C57BL/6 cells express CD45.2.

Intercrossing C57BL/6 mice with SJL/J mice developed the B6-Sj ptprca congenic model. The cells from these mice therefore express CD45.1 instead of CD45.2. The B6-Sj ptprca mice were imported from the Jackson Labratories (ME, U.S.A)

OT-I mice are from a C57BL/6 origin that have been backcrossed onto B6-Sj ptprc^{a} making the OT-I cells express both CD45.1 and CD45.2. The $CD8^{+}$ T cells from these mice express a transgenic TCR specific for the H-2 Kb–binding peptide of OVA, $OVA_{257–264}$ also known as SIINFEKL (137).

FoxP3-GFP mice are on a C57BL/6 background and have had eGFP knocked into the FoxP3 gene so that the eGFP is produced when FoxP3 is expressed (340). These mice were imported from the University of Washington, U.S.A.

Lang-EGFP mice are on a C57BL/6 background and have eGFP knocked into the langerin gene. eGFP is therefore under the control of the langerin promoter (126).

Lang-EGFPDTR mice are on a C57BL/6 background and have eGFP and the human diphtheria toxin receptor knocked into the langerin gene. The langerin gene is still transcribed without fault and when it is eGFP and DTR is also expressed (126).

2.2 Methods

2.2.1 Immunotherapy development and tumour challenge

Adoptive transfer of effector CD8$^+$ T cells

OT-I lymph node cells were stimulated *in vitro* with DCs loaded with SIINFEKL peptide. To generate DCs, bone marrow from the femur of a C57BL/6 mouse was removed by squirting PBS into one end of the femur and collecting the liquid and bone marrow from the other. The bone marrow was then filtered through a 70 μM cell strainer to remove debris and the cells were centrifuged at 552 x g (Heraeus Multifuge 3 S-R, Kendro Laboratory Products, CT, U.S.A) for 4 minutes. The cells were resuspended in cIMDM and $2x10^6$ cells were added to each well of a six-well plate, in a final volume of 5 ml. Each well received 100 U/ml of IL-4 and GM-CSF and the cells were cultured for seven days. On days three and five of culture another 100 U/ml of IL-4 and GM-CSF were added. After culture the DCs were harvested from the six-well plate and centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes. The cells were resuspended at $1x10^6$ cells/ml and were cultured for four hours with 0.1 μM/ml of SIINFEKL. The DCs were then centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes and resuspended in cIMDM for culture with the T cells.

The naïve OT-I T cells to be activated for adoptive transfer were acquired from the lymph nodes of OT-I mice. The mice were culled by CO_2 asphyxiation and the inguinal, brachial, axillary and mesenteric lymph nodes were removed, placed into PBS and stored on ice. The lymph nodes were then grinded through a 70 μM cell strainer to acquire a single cell suspension. The cells were then centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes to pellet the cells and the supernatant was removed. The cells were resuspended in cIMDM and $5x10^5$ OT-I cells were cultured in a six-well plate with $6.25x10^4$ DCs in a total volume of 5 ml. Four days later the OT-I cells were collected into a single vial and centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes. The cell pellet was resuspended in cIMDM and the cells were placed into a 750 ml flask at $2.5x10^5$ cells/ml with 100 U/ml of IL-2.

The T cells were cultured for 48 hours with new IL-2 administered both days. The following day the cells were collected in 50 ml falcon tubes and centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes. The cells were resuspended in iIMDM and washed two more times. The cells were then resuspended at $25x10^6$ cells/ml and 200 μl ($5x10^6$ cells) were injected into the lateral tail vain of mice.

Irradiated tumour cells loaded with α-GalCer

C1498 cells were cultured in cIMDM at 37 °C with 5 % CO_2. The cells were harvested from the flask, centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for 4 minutes and the cell pellet was resuspended in cIMDM. $10x10^6$ C1498 cells were then added to a 250 ml flask in a total volume of 10 ml and 200 ng of α-GalCer was added. The cells were cultured with the α-GalCer overnight before being harvested from the flask, centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes and washed three times with iIMDM. The cells were resuspended at $3.75x10^6$ cells/ml in iIMDM and irradiated for 32 minutes with gamma radiation (Gammacell 3000 Elan, Best Theratronics Ltd, Ontario, Canada) giving a total of 11,700 grays. The cells were then administered into the lateral tail vein of mice in 200 μl ($7.5x10^5$ cells/mouse).

Cytarabine treatment

Mice treated with cytarabine received three intraperitoneal doses, 24 hours following tumour challenge. Each dose consisted of 3 mg, injected ten hours apart.

Doxorubicin treatment

Mice treated with doxorubicin received a single 240 μg intravenous injection, either 24 hours before or at the same time as adoptive therapy.

E.G7-OVA challenge

E.G7-OVA was cultured in cIMDM at 37 °C with 5 % CO_2 until confluent. The cells were then removed from the flask and placed into a 50 ml falcon tube before being centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes. The cell pellet was resuspended in iIMDM and this was repeated twice to wash the cells, which were then suspended at $5x10^6$ cells/ml. Mice were injected with 200 μl ($1x10^6$ cells) of cells into the lateral tail vain.

C1498 challenge

C1498 was administered similar to E.G7-OVA. It was cultured in cIMDM at 37 °C with 5 % CO_2 until enough cells had grown for the required challenge. The cells were then removed from the flask and placed into a 50 ml falcon tube. The cells were centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes and the cell pellet was resuspended in iIMDM. This was repeated twice to wash the cells, which were then suspended at $5x10^5$ cells/ml and 200 μl ($1x10^5$ cells) was administered into the lateral tail vain of mice.

2.2.2 Tissue preparation

Specific tissues were removed from mice for analysis of the cells within them. The way in which these tissues were prepared is indicated below. The mice were culled by CO_2 asphyxiation and the tissues in question were removed and placed in iIMDM on ice.

Spleen

The spleens were grinded through a 70 μM cell strainer to get a single cell suspension and the cells were centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for 4 minutes. The supernatant was discarded and the cells were resuspended in 1 ml of iIMDM and 1 ml of red blood cell (RBC) lysis buffer for two minutes. The cells were then centrifuged again and resuspended in 5 ml of iIMDM.

Liver

To isolate cells from the liver, excess blood had to first be drained from the liver. To do this, mice were culled and a 25 g needle with a 5 ml syringe was inserted into the hepatic portal vein. The vena cava was then cut with scissors and 5 ml of PBS was then forced into the hepatic vein, thereby forcing blood within the liver to be pushed out the vena cava. This changes the colour of the liver from a dark red to a pale yellow. The liver was removed and placed in iIMDM on ice. The livers were then grinded through a 70 μM cell strainer into a 50 ml falcon tube to get a single cell suspension. The tube was filled with PBS, centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes and the supernatant was aspirated off, leaving behind the cell pellet. The cells were resuspended in a 33% Percoll mix, which was made by diluting Percoll in PBS and 45 ml was added to each liver sample. The cells were then centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for ten minutes without a brake to allow the centrifuge to stop without mixing the cell layers. The supernatant was again aspirated leaving behind the cell pellet, which was resuspended in 2 ml of RBC lysis buffer and incubated for five minutes at 37 °C. The cells were then centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes and resuspended in iIMDM.

Blood

Blood was either removed from live mice by slicing the lateral tail vein 30 minutes after applying the topical aesthetic ELMA (AstraZeneca Limited, Auckland, New Zealand) to the tails. Alternatively blood was drawn from mice that were recently culled by inserting an empty 1 ml tube into the heart and drawing blood into the syringe. For the analysis of cells within the blood, eight-ten drops of blood were collected in a 1.7 ml microtube containing 200 μl of an anticoagulant consisting of PBS with 10 mM EDTA to prevent the blood from clotting. The cells were then incubated with 1 ml of RBC lysis buffer at 37 °C for 30 minutes. The cells were spun at 664 x g (Sigma 1-14 Microfuge, John Morris Scientific Ltd, Auckland, New Zealand) for five minutes to remove the supernatant. For the analysis of blood serum, the blood was paced into an

empty tube to allow clotting. The blood was spun at 664 x g (Sigma 1-14 Microfuge) for five minutes and the serum was removed.

Viable cell counts

To determine the concentration of cells within a single cell suspension, a small aliquot of cells was removed from the sample, which was then diluted with Trypan Blue (Gibco, Invitrogen). A total of 10 μl of the cell mix was then placed on a haemocytometer and the cells were counted under a microscope. The total number of cells could be determined using the following equation:

$$\text{Total cells/ml} = \text{average number of cells within a quadrant} \times \text{dilution factor} \times 10^4$$

2.2.3 Flow Cytometry

Cell surface staining

Molecules expressed by a cell can be used to identify the specific type of cell and its functional capabilities. The molecules can be identified on a cell by using an antibody that has an affinity for specific regions of the desired molecule. A fluorophore attached to the antibody can be detected by flow cytometry, thereby indicating cells that the antibody has bound to and therefore which cells express the marker to which the antibody will bind. To attach the antibody/fluorophore to the cells, a 200 μl aliquot of the sample single cell suspension, containing between one and ten million cells, were transferred into the wells of a 96-well plate. The cells were then spun down at 863 x g (Heraeus Multifuge 3 S-R) for two minutes, the supernatant was tipped off and the cells were washed twice in FACS buffer. Non-specific binding of the antibodies to the FcR was blocked by incubating the cells with anti-CD16/32 clone 24G2 (prepared in house from hybridoma supernatant), for ten minutes at 4 °C. The cells were then centrifuged and the supernatant was removed. The desired antibodies, diluted in PBS, were then added to each cell sample at the required concentrations and the cells were then cultured for 20 minutes at 4 °C. Following this, the cells were washed twice in

FACS buffer, resuspended in 200 μl of FACS buffer and transferred into Titertube microtubes. All flow cytometry was performed on a FACSCalibur or an LSRII (both from BD Bioscience) and analysed using FlowJo software (TreeStar Inc. OR, U.S.A).

Intracellular staining

Prior to intracellular labelling of cytokines the cells were restimulated for 20 hours in 24-well plates ($5x10^6$ cells/well) with anti-CD3 and anti-CD28 antibodies (prepared in house from hybridoma supernatant) at 37 °C. After this incubation 1 μg/ml of monensin was added at to each well and the samples were incubated for a further four hours. Surface markers were then stained as indicated above and the cells were washed with 2 ml of FACS buffer. The samples were then incubated with 1 ml of fixation/permeabilization buffer (BD Bioscience) for 30 minutes at 4 °C before being washed twice with 2 ml of 1 x permeabilization buffer (BD Bioscience). Non-specific binding was again blocked with 24G2 in 1 x permeabilization buffer. Intracellular antibodies were then added in 100 μl of 1 x permeabilization buffer and the cells were incubated for 30 minutes at room temperature. The samples were then washed twice with 2 ml of 1 x permeabilization buffer and suspended in 200 μl of FACS buffer for collection on a FACSCalibur or LSRII (both from BD Bioscience). The data obtained was analysed using FlowJo software (TreeStar Inc.).

Antibody titrations

To determine the concentration at which each antibody should be used, splenocytes were processed and stained as mentioned above, with the antibody diluted in a variety of different volumes including 1:100, 1:200, 1:500 and 1:1000. Each sample was collected on a FACSCalibur or LSRII (both from BD Bioscience). The highest antibody dilution that produced a clear positive stain was used in future experiments. An example of an antibody titration can be found in appendix 1, where the antibody CD8 A700 was titrated. A 1:200 dilution was deemed the appropriate concentration to use.

2.2.4 Proliferation of naïve T cells

To determine the amount of proliferation adoptively transferred naïve T cells undergo *in vivo*, the T cells were stained with CFSE prior to administration. When cells proliferate the cells contents are divided between the daughter cells. If a cells surface is stained with CFSE and the cell divides, the daughter cells receive half of the CFSE stain each. Thus, by using flow cytometry to quantify the fluorescence of CFSE on the transferred T cells it is possible to determine which T cells have proliferated a single time by looking at which cells that have halved their concentration of CFSE. Furthermore it is possible to determine which cells have proliferated up to several times before the stain has been divided to undetectable levels.

Naïve $CD8^+$ OT-I T cells were removed from the inguinal, brachial, axillary and mesenteric lymph nodes of OT-I mice that were culled by CO_2 asphyxiation. The lymph nodes were grinded through a 70 μM cell strainer to acquire a single cell suspension containing the lymphoid cells. The cells were then centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes to form a cell pellet, the supernatant was removed and the lymphoid cells were resuspended at $5x10^6$ in PBS. The CFSE (10 mM) was diluted in PBS to 50 μM, and 25 μl was added to the sample for each ml of cells, which were then immediately vortexed and incubated in a 37 °C water bath for eight minutes. An equal volume of FBS was then added to the cells to stop the CFSE from killing the cells and the cells were then washed once with cIMDM, twice with iIMDM then resuspended in iIMDM at $25x10^6$ cells/ml. Mice were administered 200 μl ($5x10^6$ cells) of cells into the lateral tail vain.

2.2.5 Tumour scoring

In figure 2.7 mice were challenged with $1x10^6$ E.G7-OVA cells and administered different numbers of activated OT-I cells 10-days-later. On day 20 following tumour-challenge, the mice were culled and were analysed for tumour development by necroscopy. The tumours were graded on a scale of 0–2. If no tumour was detected in a particular tissue a zero score was given. In the lymph nodes and spleen, two points was given when the detected

tumour was over 25 mm^2. If the tumour was under this size it was graded a one. In the kidneys and ovaries the tumour was graded two points if the tumour was over 100 mm^2. Under this size and it was graded one point. For hind leg paralysis, a two was appointed if the paralysis involved a complete loss of hind leg movement. If only one leg was paralyzed or if some movement remained a one was appointed. In the liver a two was appointed if more than 10 nodes were detected or if a node was of 25 mm^2 in size or larger. The detection of fewer than 10 nodes under 25 mm^2 resulted in a score of one.

2.2.6 Killing assay

The ability of $CD8^+$ T cells to find and kill cells *in vivo* can be determined by performing a killing assay (341). To do this, target cells expressing the T cells TCR cognate antigen were administered into mice and the amount of target cells that remained in the blood or spleen after 24 hours indicates how well the resident $CD8^+$ T cells could kill those targets. To determine how many target cells were killed, a control cell population was also administered that did not contain the cognate antigen. These cells cannot be recognised by the T cells and therefore can be used as the baseline for 0% killing. Any reduction in the target cell population compared to the non-target control would indicate that killing had occurred. For example, if the non-target cell to target cell ratio is at 1:1 the day after administration then the T cells were unable to kill the target cells and if the ratio is 2:1 then the T cells were able to kill half of the target cell population, etc.

Splenocytes from B6-Sj ptprca mice were used as the target cells as they are $CD45.1^+$ and the experimental mice are $CD45.2^+$. It is therefore possible to differentiate the administered target cells from the host cells based on the expression of CD45.1. Before administration, the target cells were divided into three groups, which were administered the target antigen SIINFEKL at three different concentrations (0.5, 5 and 50 ng). The cells were then incubated at 37 °C for two hours. The non-target control splenocytes did not receive SIINFEKL. The three target populations were then stained with CFSE at a

high, intermediate and low concentration, which was used to differentiate the three target cell populations (not the amount of proliferation, as the splenocytes should not proliferate). The high SIINFEKL population received the strongest staining by being incubated with 1.25 μM of CFSE, the intermediate SIINFEKL population received 0.25 μM of CFSE and the low received 50 nM. The cells were incubated in a 37 °C water bath for eight minutes before an equal volume of FBS was added and the cells washed twice in iIMDM. The non-target control cells were stained with CTO, which is similar but distinguishable from CFSE. This was done by incubating the cells for fifteen minutes at 37 °C with 10 μM of CTO diluted in warm cIMDM. The cells were then centrifuged at 552 x g (Heraeus Multifuge 3 S-R) for four minutes, resuspended in warm cIMDM and incubated for 20 minutes at 37 °C. The four different populations were combined at a 1:1:1:1 ratio and resuspended at $1x10^8$ cell/ml. 200 μl ($20x10^6$ cells) were injected into the lateral tail vein of each mouse. The following day mice were bled and the amount of killed target cells was determined by analysing the remaining transferred cell populations by flow cytometry.

2.2.7 Microscopy

All slides were analysed using an Olympus BX51 microscope (Precision Microscopy Equipment, Wellington, New Zealand) and photos were taken using an Olympus DP70 (Wellington, New Zealand).

Bone marrow

The femur of mice was removed and stored in 4% formalin solution (Sigma-Aldrich, MO, U.S.A). The bones were then decalcified in 10 % formic acid for three days, changing the formic acid daily. Tissue was processed using an automated processor (Tissuetek VIP-5, Sakura Finetek U.S.A Inc, U.S.A). Specimens were fixed in formalin for two hours and dehydrated with increasing concentrations of alcohol for one hour at each concentration (70%, 90% and 100%) before embedding in paraffin wax. Four micron sections were taken using a rotary microtome (model 2235, Leica, Nussloch, Germany),

which were then mounted onto glass slides and manually stained with haematoxylin for ten minutes. After washing with water, the slides were stained with eosin for four minutes (stains made in house). The slides were then cleared with xylene I and II for five minutes. A cover slip was then mounted using Di-N-Butyl Phthalate (DPX, Merck, Darmstadt, Germany).

Blood

Blood was collected in an empty eppendorf tube and one drop was placed on a slide. The drop was then smeared over the length of the slide with a clean cover slip. The blood was then allowed to dry before being stained with a Romanowsky stain variant (Diff-Quick staining) purchased from Siemens Healthcare (Erlangen, Germany). This Consisted of immersing slides in Diff-Quick fixative reagent for five seconds, and blotting away the excess liquid before immersing the slides in Diff-Quick solution I for ten seconds. The slides were again blotted and then immersed in Diff-Quick solution II for seven seconds. The slides were then washed with water and left to dry at room temperature. A cover slip was then mounted using mounting medium (Kirkegaard & Perry Laboratories, Inc, MA, U.S.A).

2.2.8 Cytokine production assay

To determine the type and quantity of cytokines being produced in response to a treatment, a bead-based cytokine assay was performed (Bio-Plex Pro™ Cytokine, Chemokine, and Growth Factor Assay; Biorad Labratories, Inc.) following the distributer's protocol, on either the serum from mice or the supernatant from a splenocyte culture. For the latter, a single cell suspension was made from the spleens of mice as indicated above and the splenocytes were resuspended at $10x10^6$ cells/ml. Each well of a 24-well plate was transferred $1x10^6$ splenocytes of a given sample, in a total volume of 500 μl. One set of the samples remained unstimulated and another was restimulated with $1x10^5$ DCs loaded with C1498 tumour cell lysate (described below). The cells were then cultured at 37 °C for 24 hours, after which the liquid was

collected in a microtube, centrifuged at 616 g (Sigma 1-14 Microfuge) for five minutes and the supernatant was collected for cytokine analysis.

To make the lysate loaded DCs, C1498 cells were freeze thawed six times in liquid nitrogen to lyse the cells. The cells were then filtered through a 40 µm filter to remove clumps. The lysate was then cultured for four hours with bone marrow derived DCs at a ratio of one DC to the lysate from six tumour cells.

2.2.9 Suppression assay

Processed spleens were resuspended in 5 ml of PBS and underlaid with 4 ml of a density separation medium designed to isolate lymphocytes (Lympholyte solution, Cedarlane, Ontario, Canada). The samples were centrifuged at 823 x g (Heraeus Multifuge 3 S-R) for 20 minutes without a brake. The resulting cell pellet was resuspended in 10 ml of PBS and washed twice. The cells were resuspended in running buffer with CD11b-biotin antibody for fifteen minutes at 4 °C before being washed twice in PBS. The cells were resuspended in 200 µl of running buffer per $1x10^7$ cells and this was run through a 30 µm pre-separation filter. For every $1x10^7$ cells, 10 µl of anti-biotin microbeads (Miltenyi biotec, Bergisch Gladbach, Germany) was added and the samples were then incubated at 4 °C for 30 minutes. The samples were run through an automated cell separator (Automacs, Miltenyi biotec, Bergisch Gladbach, Germany) and the positive fraction was washed twice in cIMDM. Lymph nodes were harvested from a C57BL/6 mouse and stained with CFSE as indicated in section 2.2.4. The cells were resuspended at $1x10^6$ cells/ml and anti-CD3 and anti-CD28 were added to the lymphoid cells at 4 µg/ml and 1 µg/ml respectively. Lymphoid cells were cultured in a 96-well plate with $5x10^4$ lymphoid cells per well and the same amount of MDSCs (1:1 ratio), in a total volume of 200 µl. The cells were then cultured for 72 hours before flow cytometry was performed to analyse the dilution of CFSE on both the $CD4^+$ and $CD8^+$ T cells. For the suppression assay with C1498 cells, $5x10^4$ C1498 cells were cultured with the T cells in place of the $CD11b^+$ cells.

2.2.10 Statistical analysis

The statistical significance between groups was determined with a one-way ANOVA followed by the Bonferroni post-test when there were more than two groups with one variable. This analysis was performed, as the sample population is parametric. To determine the statistical difference between only two groups a Mann Whitney test was performed. This test is non-parametric but was chosen as it is a more robust test compared to the unpaired T test, as it includes an analysis of the distribution within groups as well as between them. For the analysis of the statistic difference between two or more groups in a survival assay a Log-rank Mantel Cox test was performed. All statistics performed on a given figure display the standard error of the mean and the tests performed are indicated in the figure legends. Graphpad Prism 5 software (Graphpad Software Inc., San Diego, CA, U.S.A) was used. five animals were chosen because there has been literature precedence for revealing statistically significant differences between groups in similar immunological experiments. Single representative experiments showing statistically significant results are presented throughout. However, to ensure robustness, the experiments were always repeated at least once, and again independently assessed for statistical significance. In some cases, where less than 5 animals were used, again the robustness of the results was confirmed by repeating the experiment with independent statistical analysis.

Chapter 3: The role of langerin^{+} CD8α^{+} dendritic cells in adoptive T cell therapy of established tumours

3.1 Introduction

Endogenous CD8$^+$ T cells activated against tumours are often unsuccessful at preventing tumour progression. This is, in part, due to the development of an immune-suppressive environment by tumours. The *ex vivo* stimulation of T cells in adoptive therapy enables T cell activation in the absence of tumour-associated suppression. Thus, tumour-bearing hosts can be administered a population of appropriately activated tumour-specific T cells. Clinical trials investigating the protective benefits of adoptive CD8$^+$ T cell therapy for the treatment of melanoma found that as many as 70% of recipients developed objective responses, as determined by a decrease in tumour size (321). While the potential of adoptive therapy is evident, it is likely that enhanced protection would be provided if some current limitations could be overcome. One such limitation is the inability of the transferred cells to survive in high numbers for the extended periods of time required for effective anti-tumour activity (342-344). A greater understanding of the interactions and stimuli received by transferred T cells *in vivo* may elucidate new strategies to improve longevity of the transferred cells and hence the level of anti-tumour protection (312, 344).

As DCs are potent APCs, it is possible that transferred CD8$^+$ T cells receive stimuli from endogenous DCs presenting tumour antigens. Evidence indicating the interaction between DCs and endogenous anti-tumour CD8$^+$ T cells is found in studies demonstrating that anti-tumour CD8$^+$ T cell responses within a host are strictly dependent on a match between the MHC haplotype of the host's APCs and that of the host's T cells (17). This suggests that activating anti-tumour CD8$^+$ T cells *in vivo* is dependent on the presentation of tumour antigens by APCs. An Experiment specifically implicating DCs in the activation of anti-tumour CD8$^+$ T cells utilized Batf3 KO mice. Batf3 KO mice are unable to develop CD103$^+$ DCs, which includes the cross-presenting CD8α^+ DCs in lymphoid tissues. These mice were unable to activate anti-tumour effector CD8$^+$ T cells and subsequently could not control the growth of a fibrosarcoma that was effectively controlled in Batf3$^{+/+}$ mice, therefore indicating that CD103$^+$ DCs are essential for anti-tumour CD8$^+$ T cell responses in this model (123).

It remains to be determined if DCs presenting tumour antigens have an effect on transferred effector CD8$^+$ T cells. However, the combination of adoptive CD8$^+$ T cell therapy with a DC-based vaccine can provide a synergistic effect resulting in superior anti-tumour protection provided by the transferred CD8$^+$ T cells (345-347). Endogenous DCs have also been demonstrated to stimulate transferred effector-CD8$^+$ T cells in virally infected hosts, as anti-viral effector CD8$^+$ T cells transferred into influenza-infected hosts proliferated significantly less when depleted of their CD11c$^+$ DCs (348). However, the specific DC subset that stimulated the T cells to proliferate was not determined. It remains to be established if the same stimulatory function applies in a tumour model. Importantly in this context, it is possible that the suppressive environment associated with tumour development may maintain DCs in an immature state, thereby limiting their T cell stimulatory capability (349-351).

The contact dependent interaction between transferred T cells and endogenous DCs was indicated by a study, in which, α-GalCer was loaded onto T cells prior to their transfer. The α-GalCer was transferred directly from the administered T cells to endogenous DCs (352). The anti-tumour activity of the transferred T cells was also enhanced when loaded with α-GalCer, possibly due to licensing of DCs by α-GalCer-activated iNKT cells and then superior stimulation of the transferred T cells by the licensed DCs.

In this chapter, the role of endogenous DCs was investigated in a model of adoptive T cell therapy. Because the stimulation of adoptively transferred CD8$^+$ T cells with tumour antigens requires the exogenous antigens to be cross-primed, the potent cross-priming langerin$^+$ CD8α^+ DCs were analysed (107). In addition to their cross-priming functions, their location in the marginal zone of the spleen places them in an ideal position to acquire antigens from the blood, such as those derived from circulating tumour cells. The langerin$^+$ CD8α^+ DCs were therefore examined in relation to their ability to stimulate adoptively transferred effector CD8$^+$ T cells used for the treatment of an established tumours. For this purpose, the tumour challenge within the following experiments consisted of intravenously administered E.G7-OVA

cells. Intravenously administering the tumour enables the tumour cells to circulate in the blood and drain from circulation into the spleen. This provides a tumour model that enables the acquisition of tumour antigens by splenic DCs, including langerin$^+$ CD8α^+ DCs. In addition, E.G7-OVA expresses the OVA protein, peptides of which can be recognised by the TCRs of OT-I T cells. The tumour cells engraft in a variety of extrasplenic locations and antigens released from these sites are likely to reach the circulation and enter the spleen. Therefore, we have a tumour model that encourages acquisition of tumour antigens by splenic APCs and provides us with access to tumour-specific CD8$^+$ T cells for use in adoptive therapy.

3.1.1 Aims

The aim of this series of experiments was to develop a model of adoptive therapy, consisting of CD8$^+$ T cells used to treat an established tumour, and to determine if the presence or absence of recipient langerin$^+$ CD8α^+ DCs had an effect on the function of the transferred T cells, or the anti-tumour response. The hypothesis addressed is that "*the effective anti-tumour activity of an adoptive transfer-based immunotherapy is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen*."

Specific aims

- To establish whether langerin$^+$ CD8α^+ DCs acquire and cross-present tumour-associated antigens in tumour-bearing animals

- To develop a model of adoptive cell therapy using *in vitro*-activated antigen-specific CD8$^+$ T cells to treat established tumours

- To assess the role of langerin$^+$ CD8α^+ DCs in determining the functional response of effector CD8$^+$ T cells transferred into tumour-bearing animals

- To assess the role of langerin$^+$ CD8α^+ DCs in the anti-tumour response mediated by transferred CD8$^+$ T cells

3.2 Results

3.2.1 Developing a Naïve T cell tumour challenge model

To enable the analysis of langerin$^+$ CD8α^+ DCs in the stimulation of naïve CD8$^+$ T cells, experiments were required to elucidate the number of naïve OT-I T cells and E.G7-OVA cells that would generate an effective tumour-antigen specific CD8$^+$ T cell response *in vivo*. E.G7-OVA is a murine thymoma cell line that has been modified to express OVA protein as a model tumour antigen. Different numbers of E.G7-OVA cells, 1x10^5, 1x10^6 or 6.5x10^6, were administered intravenously into hosts to identify a tumour challenge that develops within a timeframe suitable for experimentation. In all survival experiments, mice were culled at the initial onset of symptoms, as determined by weight loss, or a change in their behaviour as identified by hunching or reduced grooming, and presence of tumours was confirmed by necropsy. Hosts administered 1x10^5 E.G7-OVA cells began to develop symptoms associated with tumour burden at day 40 (Figure 3.1A). Hosts challenged with 1x10^6 or 6.5x10^6 E.G7-OVA cells developed symptoms between days 20 and 32 following tumour challenge. As mice challenged with the lower number of E.G7-OVA cells remained symptom-free for a long period and hosts challenged with the larger two numbers of tumour cells developed symptoms at similar times, the 1x10^6 E.G7-OVA cell tumour challenge was used as the model of tumour challenge throughout this chapter.

Mice intravenously administered 1x10^6 E.G7-OVA cells developed tumours in multiple tissues. In initial experiments, tumour burden was verified by dissection at different times after tumour challenge. Tumours were observable to the naked eye in the liver, kidneys, ovaries, inguinal lymph nodes, spleen and lumbar lymph nodes (Figure 3.2 A–B). Interestingly around 40% of mice developed paralysis of their hind legs, most likely due to a tumour seeding in the spinal cord or brain.

Naïve CD8$^+$ T cells were initially used to ascertain whether tumour antigens could be cross-presented to stimulate tumour-specific CD8$^+$ T cells *in vivo*. For adoptive transfer experiments, CD8$^+$ T cells isolated from OT-I mice were

used, which are genetically altered such that the majority of CD8$^+$ T cells express TCRs specific for the SIINFEKL antigen, which is a H-2K^b-binding peptide epitope from the OVA protein. As the tumour expresses the OVA protein, OT-I CD8$^+$ T cells are essentially model "tumour-specific" CD8$^+$ T cells. The number of naïve OT-I cells that would delay this tumour burden was analysed by intravenously administering different numbers, 500, 5x10^5 or 5x10^6, of naïve OT-I T cells into hosts prior to challenge with 1x10^6 E.G7-OVA cells. Hosts that received either 500 or 5x10^4 naïve OT-I T cells exhibited no delay in the development of symptoms associated with tumour burden when compared to the tumour only control (Figure 3.3). However, hosts that received 5x10^6 naïve OT-I T cells remained symptom-free significantly longer than the other groups and therefore this number of OT-I T cells was used for the remaining naïve T cell experiments.

To determine how many of the 5x10^6 lymphoid cells administered were OT-I CD8$^+$ T cells flow cytometry was performed to analyse the proportion of total lymphoid cells that express CD8 and the OT-I T cell receptor component Vα2. Of the live cells within the lymphoid single cell suspension, 68 % were CD8$^+$ Vα2$^+$ cells and of the total lymphoid cell population, 37.0 % were OT-I T cells (Appendix 2). Therefore, when administering 5x10^6 OT-I lymphoid cells, 1.9x10^6 OT-I T cells are being transferred.

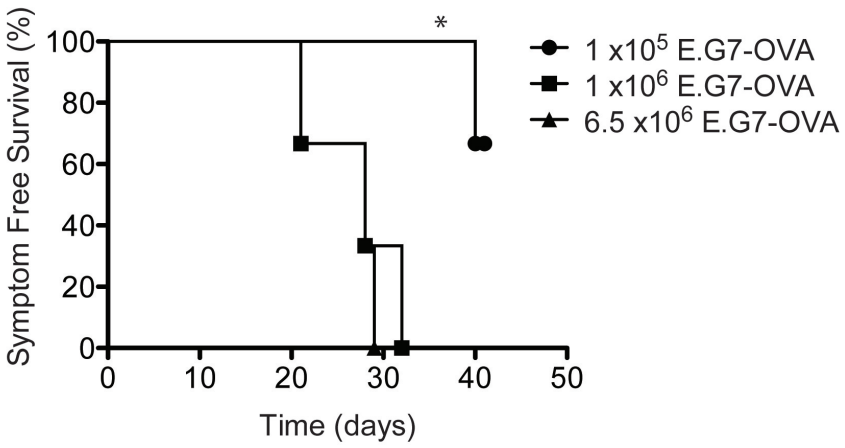


Figure 3.1: Developing the E.G7-OVA tumour challenge model. (A) C57BL/6 mice were intravenously administered 1x10^5, 1x10^6 or 6.5x10^6 E.G7-OVA cells and the symptom-free survival was analysed. This experiment was performed once with three mice per group. *P < 0.05 (Log-rank test for trend).

A

Tissue	Liver	Kidneys	Hind leg paralysis	Ovaries	Inguinal LN	Spleen	Lumbar LN	Total mice
Number of mice	17	15	12	8	3	3	2	28

B

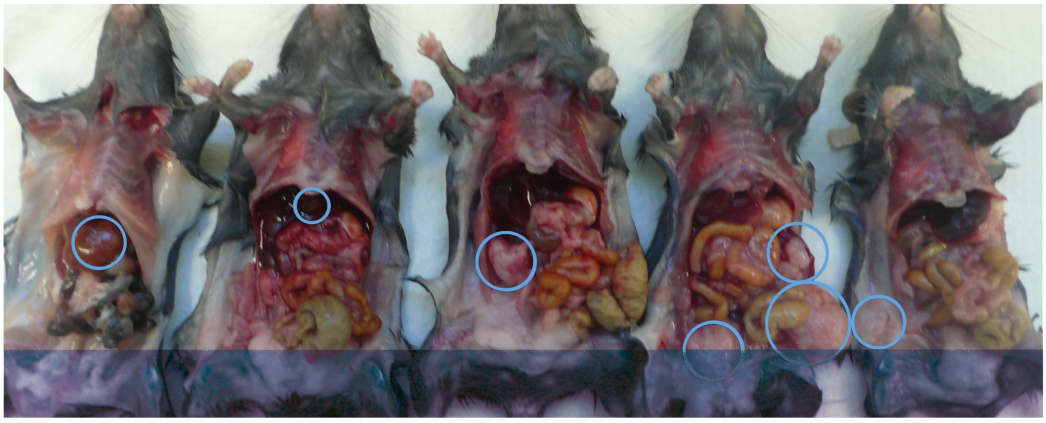


Figure 3.2: E.G7-OVA administered intravenously results in tumour development in a variety of tissues. Mice were administered 1x10^6 tumour cells intravenously and their symptom free survival was followed. Upon the development of symptoms arising from tumour burden the mice were culled. (A) The tissues in which a tumour was found by necropsy are indicated along with the number of mice that had a tumour in that tissue. (B) A photo is used to demonstrate the variety and severity of tumour-burden between mice. The tumours detected are outlined with blue circles.

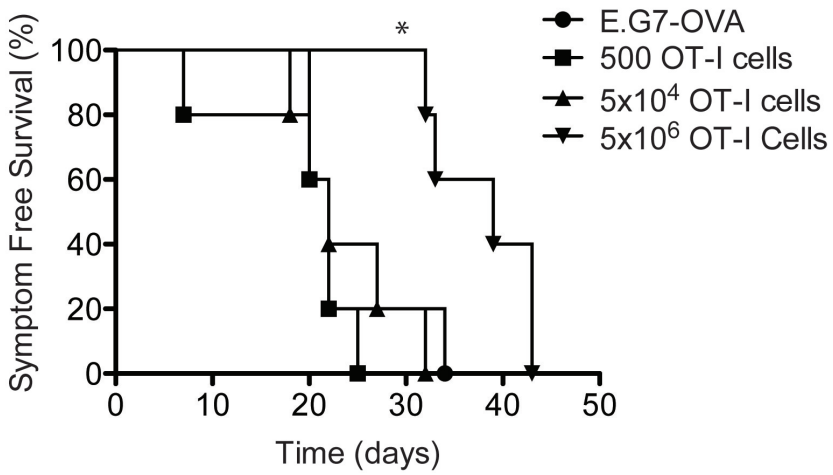


Figure 3.3: Naïve OT-I CD8$^+$ T cells protect mice from tumours in the blood. C57BL/6 mice were intravenously administered 500, 5x10^4 or 5x10^6 naïve OT-I T cells and the following day were challenged with 1x10^6 E.G7-OVA cells. The period that the mice remained symptom-free was determined, and is graphed. This experiment was performed once with five mice per group. However, the survival of tumour-challenged mice with 5x10^6 OT-I T cells has been performed twice. *P < 0.05 (Log-rank test for trend).

3.2.2 Langerin$^+$ CD8α^+ dendritic cells in the spleen acquire and cross-present tumour antigens

In the previous experiments, OT-I T cells were transferred into mice before tumour challenge, thereby maximizing the chance of interaction between the tumour-specific T cells and the tumour, either directly or perhaps with antigenic debris derived from the tumour. It was next investigated whether naïve OT-I T cells could be activated by tumour antigen once the tumour had been established. This may be more indicative that antigens derived from the tumour in its niche environment can be acquired and presented by host APCs. For this purpose, 5x10^6 lymphoid cells, containing naïve CD8$^+$ T cells from CD45.1-expressing OT-I mice were stained with CFSE and administered into CD45.2 expressing mice that had tumours established for one or seven days. Proliferation of the transferred CD8$^+$ T cells was assessed by monitoring CFSE fluorescence by flow cytometry, with a reduction in fluorescence indicative of proliferating cells that have contributed half of their labelled cellular contents to each daughter cell. Flow cytometry was performed on single cell suspensions prepared from the spleens of animals culled three days after T cell transfer, with fluorescent antibodies for CD45.1, CD8 and TCR Vα2 used to identify the transferred CD8$^+$ T cells.

When naïve CD8$^+$ T cells were transferred into mice that had not been challenged with tumour cells, the intensity of the CFSE concentration remained high, indicating that these cells were not proliferating. However, when CD8$^+$ T cells were transferred into mice with tumours established for one or seven days, a significant reduction of CFSE fluorescence was observed, indicating that these CD8$^+$ T cells were receiving a tumour-derived stimulus resulting in their proliferation (Figure 3.4). These data therefore provide evidence that antigens can be presented to CD8$^+$ T cells from established tumours. While it remains possible that the CD8$^+$ T cells were stimulated directly by tumour cells, it was very likely that the antigen had been acquired by resident APCs, and then cross-presented for stimulation of the CD8$^+$ T cells. This tumour model was therefore used in subsequent experiments to explore the possibility that resident APCs such as the splenic

langerin$^+$ CD8α^+ DCs were responsible for the acquisition of tumour antigens, and that these cells could play a role in adoptive T cell therapy with *in vitro* activated CD8$^+$ T cells.

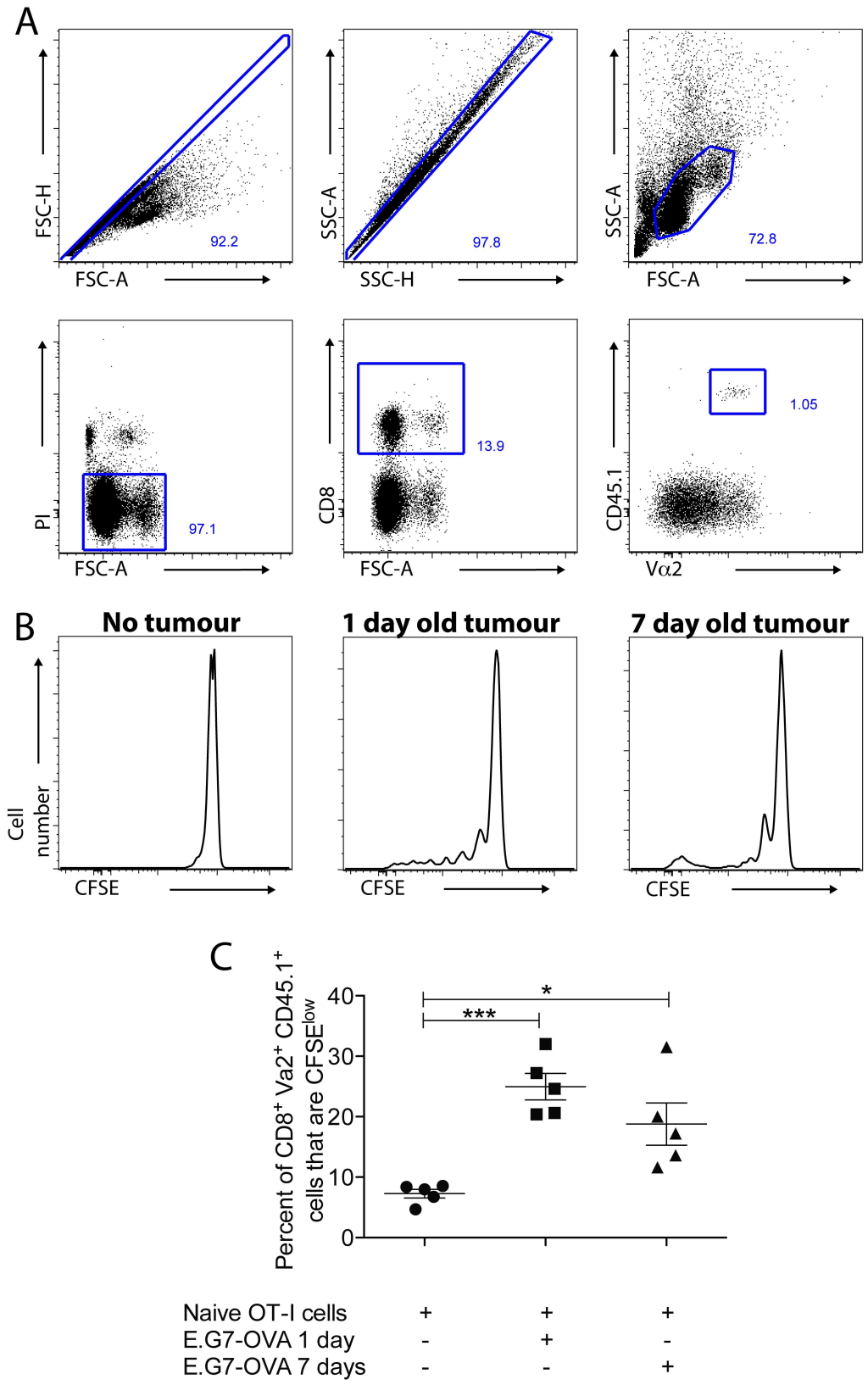


Figure 3.4: Naïve CD8$^+$ T cells proliferate in the spleen of mice with established tumours. CFSE labelled CD8$^+$ T cells were administered into the blood of mice with one or seven day-old tumours. Three days after transfer the spleens were removed and CFSE fluorescence on the transferred cells was analysed by flow cytometry,

using an antibody for CD45.1 to identify the transferred cells. The gating strategy used to identify the transferred CD8$^+$ T cells from a single cell suspension of splenocytes is provided (A). Gating on FSC-A vs. FSC-H and SSC-A vs. SSC-H was performed for every flow cytometric analysis in this thesis but will no longer be displayed. An example of the CFSE dilution observed from each group (B). (C) The percent of transferred CD8$^+$ T cells in the spleen that proliferated is compared between the various groups. This is the result of a single experiment, with five mice per group. *$P < 0.05$, ***$P < 0.001$ (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

To first determine if langerin$^+$ CD8α^+ DCs could acquire and cross-present tumour-derived antigens from established E.G7-OVA tumours, naïve CFSE labelled CD8$^+$ OT-I T cells were transferred into *lang*-EGFPDTR mice that were then tumour challenged; DT was used to deplete the langerin expressing cells in some recipients prior to challenge. Proliferation of the transferred CD8$^+$ T cells was determined by analysing their CFSE fluorescence by flow cytometry three days after transfer. The CD8$^+$ T cells transferred into mice that were not tumour challenged maintained a high CFSE fluorescence indicating that the CD8$^+$ T cells were not proliferating in the absence of antigen. In contrast, the CD8$^+$ T cells transferred into mice that were then tumour challenged displayed a significant decrease in the concentration of CFSE, indicating that the transferred CD8$^+$ T cells proliferated in response to tumour antigens. Mice depleted of langerin$^+$ cells prior to transfer also maintained a high CFSE concentration indicating that the CD8$^+$ T cells were not receiving the proliferation-inducing stimulus even when the tumour antigens were present (Figure 3.5). However, some proliferation was detected, indicating that another DC subset maybe able to induce T cell proliferation to a lesser degree. This experiment suggests that langerin$^+$ CD8α^+ DCs can acquire antigens from the tumour and cross-present them for stimulation of naive antigen-specific CD8$^+$ T cells, thereby negating the idea that the tumour cells are stimulating naive CD8$^+$ T cells directly. Importantly, this readout does not provide information on the quality of the interaction, and whether the outcome is an anti-tumour T cell response, or merely a proliferative response that precedes the induction of tolerance.

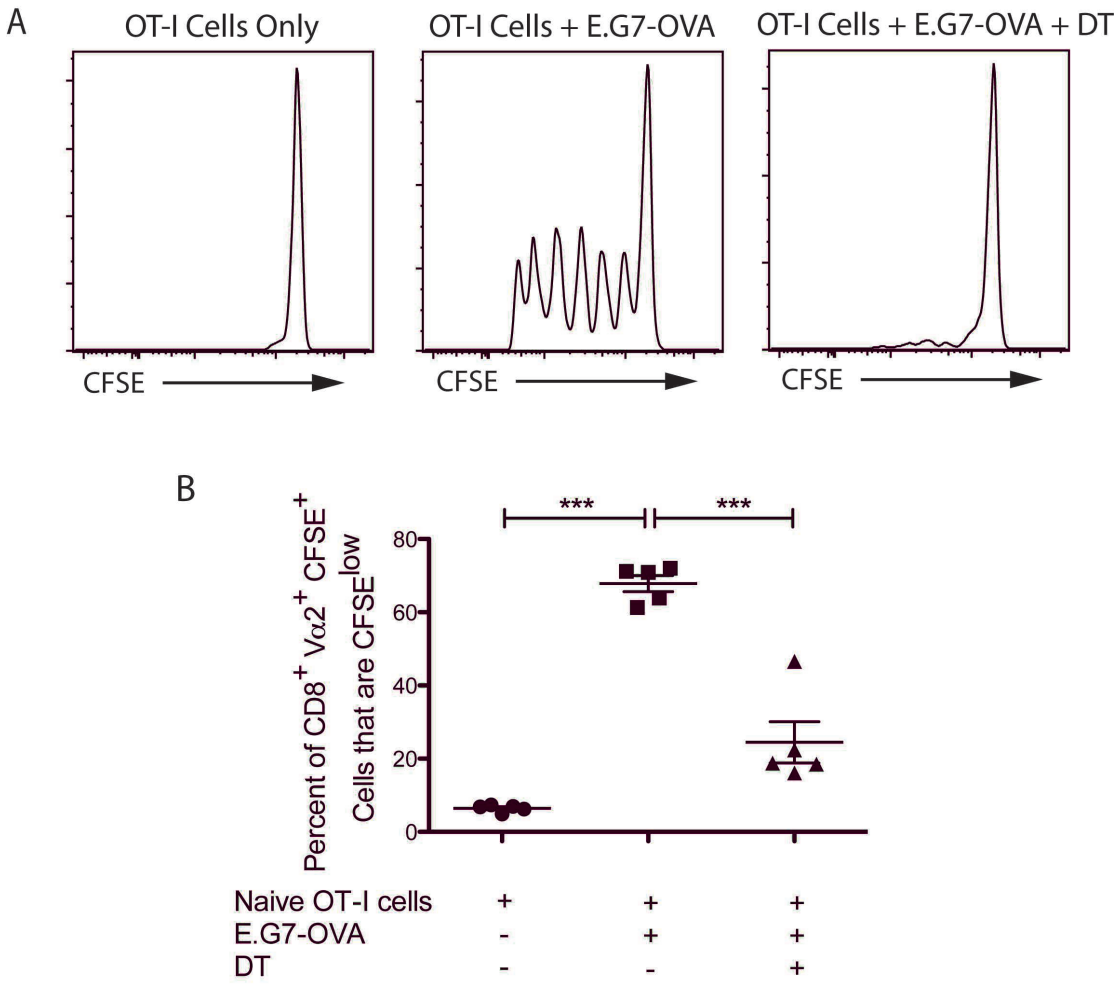


Figure 3.5: Langerin$^+$ CD8α^+ DCs are required for naïve CD8$^+$ T cells to proliferate in response to intravenously administered tumour cells. Naïve OT-I T cells labelled with CFSE were administered into *Lang*-EGFPDTR mice the day prior to challenge with 1x10^6 E.G7-OVA cells. Three days after challenge the spleens were removed and the amount of transferred naïve CD8$^+$ T cells that proliferated was determined by CFSE dilution. One group of mice received two intraperitoneal doses of 350 ng of DT to deplete the langerin$^+$ cells, beginning before OT-I transfer. (A) A histogram representative of CFSE dilution on CD8α^+, Vα2$^+$ and CD45.1$^+$ OT-I T cells from each experimental group. (B) A graph comparing the percent of OT-I T cells found in the spleen that had undergone proliferation as determined by CFSE dilution. This data is a representative of three experiments, with five mice per group. ***P < 0.001 (One-way ANOVA with Bonferroni's Multiple Comparisons Test).

Having established that langerin$^+$ CD8α^+ DCs are required for activation of anti-tumour CD8$^+$ T cells against an intravenous tumour challenge, it was next evaluated whether these DCs are essential for adoptive T cell therapy with activated CD8$^+$ T cells. In order to accomplish this, it was first necessary to demonstrate the ability to activate naïve OVA-specific CD8$^+$ T cells *in vitro* into useful effector T cells for adoptive therapy and then determine the most effective number of CD8$^+$ T cells to transfer, as well as the optimum time to treat mice. The ability to effectively stimulate naïve CD8$^+$ T cells into effector CD8$^+$ T cells *in vitro* was determined by examining the CD8$^+$ T cell phenotype before and after activation. Activation was induced by culturing naïve OT-I T cells for six days with SIINFEKL-pulsed BMDCs, in combination with IL-2 treatment. Flow cytometry, with fluorescent antibodies for CD62L and CD25, was used to determine the activation status before and after the culture period. Before culture, the CD8$^+$ T cells displayed a naïve phenotype with low CD25 and high CD62L expression. During this stimulation around $12x10^6$ naïve T cells could generate over $7.5x10^7$ activated T cells. The T cells cultured with BMDCs, on the other hand, expressed an activated phenotype, with high CD25 and low CD62L expression (Figure 3.6). These studies demonstrate that the T cells were effectively stimulated by the BMDCs and were ready for administration.

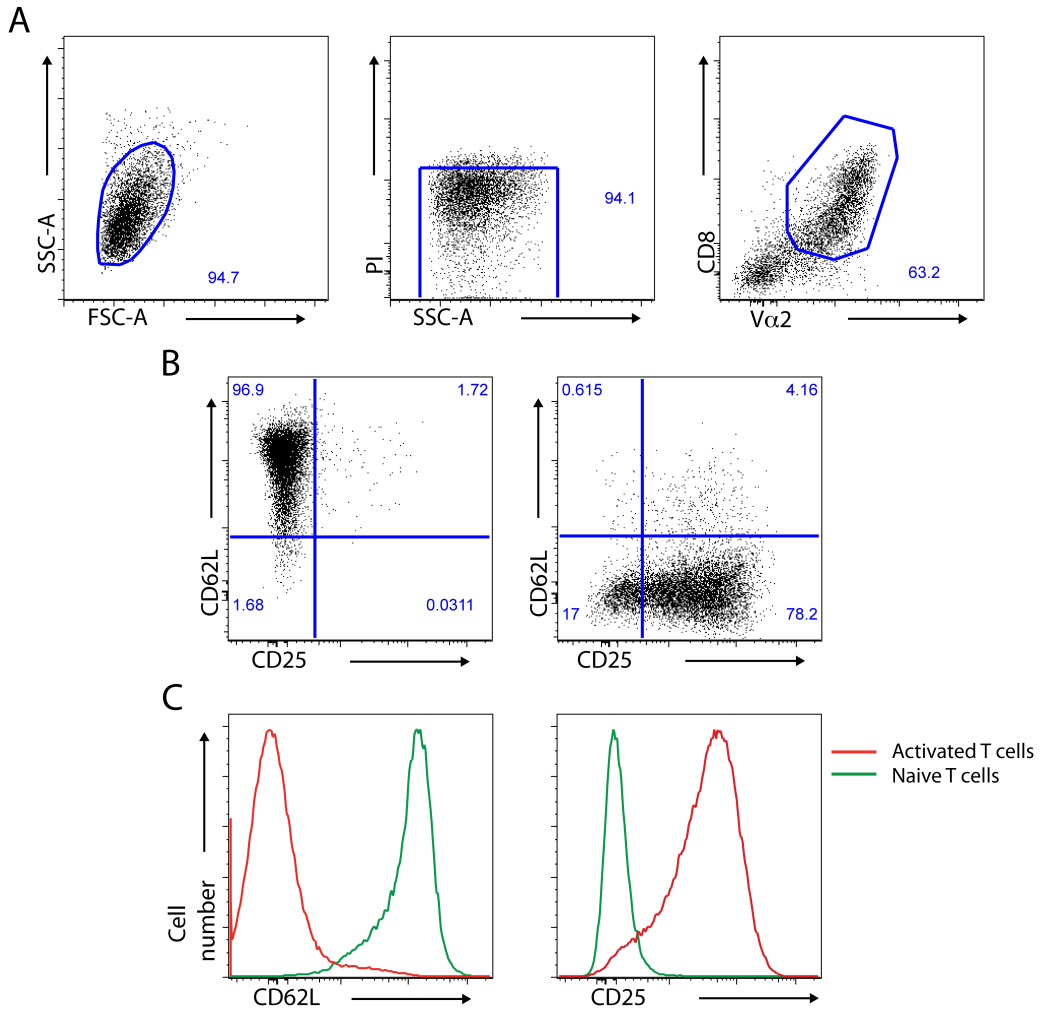


Figure 3.6: CD8$^+$ T cells stimulated *in vitro* express an effector T cell phenotype. Flow cytometry analysis was performed on both naïve and *in vitro* stimulated OT-I T cells to analyse the T cell phenotype after stimulation. The lymph nodes of an OT-I mouse was removed and the naive OT-I T cells were identified by CD8 and Vα2 expression. The same markers were used to identify activated CD8$^+$ T cells removed from culture. (A) An example of the gating strategy used to isolate the OT-I T cells from both the naïve and effector T cell samples. (B) An example of the gating strategy used on both the naïve and stimulated T cells to determine the expression of the lymphoid homing receptor CD62L and the expression of the alpha chain of the IL-2 receptor, CD25. (C) A histogram comparing the expression of CD62L and CD25 on naïve and stimulated T cells. The effector CD8$^+$ T cells are represented in brown in the histograms and naïve T cells are represented in green. This data is representative of three separate experiments.

Next the number of effector CD8$^+$ T cells required to provide mice with effective therapy against the tumour model was determined. Three different doses of T cells were administered into mice ten days after tumour challenge and the development of tumours was assessed by necropsy eleven days later. These time-points were chosen as we needed a model where the mice had an established tumour burden before the administration of OT-I T cells, whilst giving the T cells time to prevent tumour development. In addition, the experiment needed to end before the tumour only control mice required culling. Therefore, day 20 was chosen as the end point and mice were allowed to develop tumours for only 10 days prior to adoptive therapy. After being culled, mice were thoroughly examined and the tissues that developed tumours were scored on a scale from zero to two depending on the severity and size of each tumour and the tumour score for each mouse is combined (Section 2.2.5). The tissues detected with tumours include the ovary, kidney, liver, spleen, inguinal lymph nodes (ILN), auxiliary lymph nodes (ALN), lumbar lymph nodes (LLN) and medistinal lymph nodes (MLN). Some mice developed hind leg paralysis, which was also graded from zero to two depending on the extent of paralysis (Figure 3.7). The results from this experiment indicate that $2x10^4$ and $2x10^5$ effector CD8$^+$ T cells was insufficient to provide any protective benefit to mice, but the administration of $2x10^6$ effector CD8$^+$ T cells resulted in the development of fewer tumours, with only a single, small tumour detected in one mice (Figure 3.7). However, because this was a statistically insignificant result, $5x10^6$ CD8$^+$ T cells were used in future experiments.

Mice	Kidney	Ovary	ILN	ALN	LLN	Liver	MLN	Hind legs	Spleen
EG7 only 1						1		2	
2						1			
3		1							
4		2			2				1
5			2						
2x10^4 OTI 1	1						2	2	
2			2			1			
3		1							
4									
5									
2x10^5 OTI 1								2	
2								2	
3								2	
4	2								
5				2			2		
2x10^6 OTI 1									
2		1							
3									
4									

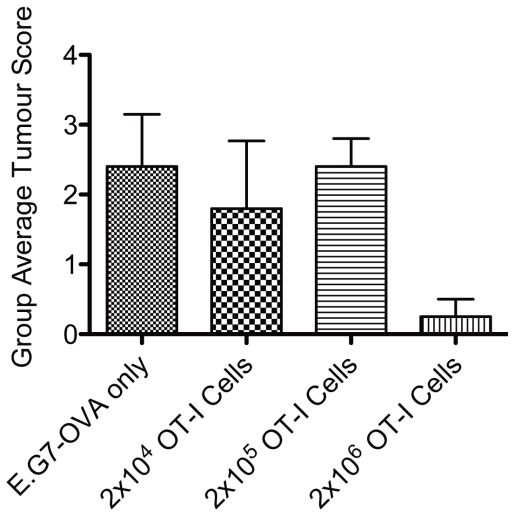


Figure 3.7: Adoptively transferring 2x10^6 effector CD8$^+$ T cells reduces the burden in mice with ten-day-old tumours. *Lang*-EGFPDTR mice were administered E.G7-OVA intravenously and ten days later were administered different doses of effector CD8$^+$ T cells. On day 21 after tumour challenge mice were culled and their tissues were graded for tumour development by necropsy. The tumours in each tissue were graded on a scale of zero to two depending on the size of the tumour, when taking into consideration the size of the tissue itself. Hind leg paralysis was also detected in some mice and the severity was also scaled from zero to two. The score for each mouse is depicted in the table and the average score between groups is graphed. This represents a single experiment with five mice in each group except the group that received 2x10^6 OT-I cells, which had four mice. P = 0.1657

3.2.3 Tumours induce the stimulation of adoptively transferred effector CD8$^+$ T cells

It was next investigated whether established tumours can provide an environment in which adoptively transferred effector CD8$^+$ T cells can be stimulated. Timing of transfer was considered crucial to these experiments as leaving the tumour to grow in the host for too long may allow it to generate a suppressive environment capable of inhibiting the transferred cells, whereas smaller tumours may not provide sufficient antigen to stimulate the CD8$^+$ T cells. Mice were therefore administered 5x10^6 effector OT-I T cells, four, eight or twelve days following tumour challenge. The CD8$^+$ T cell population was analysed in the spleen and blood seven days later by flow cytometry, using fluorescent antibodies for CD45.1, CD8 and Vα2. Control mice were administered effector CD8$^+$ OT-I T cells in the absence of tumour challenge. Interestingly, larger percentages of OT-I T cells were detected in the spleens when tumours were present for twelve days prior to transfer, compared to T cells administered in mice without tumours (Figure 3.8B). In addition, significant increases were observed in the blood of mice that had the tumour for eight or more days' prior to transfer (Figure 3.8C). Furthermore, the ability of the T cells to produce IFN-γ was analysed by intracellular flow cytometry, with a fluorescent antibody for IFN-γ. This showed that a greater percentage of effector OT-I T cells produced IFN-γ when they were administered into mice with twelve-day-old tumours (Figure 3.8D). These results indicate that tumours can provide a stimulus to transferred effector CD8$^+$ T cells, particularly when the tumours had more time to develop a larger mass. This stimulus resulted in accumulation of the transferred T cells in the tissues tested; whether this reflects a proliferative stimulus remains to be ascertained.

Having established a model of adoptive therapy consisting of the administration of 5x10^6 activated CD8$^+$ T cells for the treatment of twelve-day-old tumours, the kinetics of the transferred cells were then assessed. For this purpose, 5x10^6 *in vitro* activated CD8$^+$ T cells were administered into mice with twelve-day-old tumours and blood samples were collected from the mice three, seven and fourteen days after transfer. The percentage of transferred

cells detected by flow cytometry was compared to animals that were transferred effector CD8$^+$ T cells but did not harbour any tumour. Mice that were administered T cells in the absence of tumours consistently maintained a low population of transferred CD8$^+$ T cells in the blood at all time points tested. Mice transferred effector CD8$^+$ T cells in the presence of twelve-day-old tumours had a significantly larger population of transferred T cells in the blood on day seven, however by day fourteen this population had reduced significantly (Figure 3.9). This result indicates that the tumour provided a stimulus to the transferred effector CD8$^+$ T cells that caused more accumulation of the transferred T cells in the blood. It is likely that the transferred T cells underwent proliferation between days three and seven in response to tumour antigens, although tumour induced changes in the recirculation of the transferred T cells between tissues cannot be ruled out.

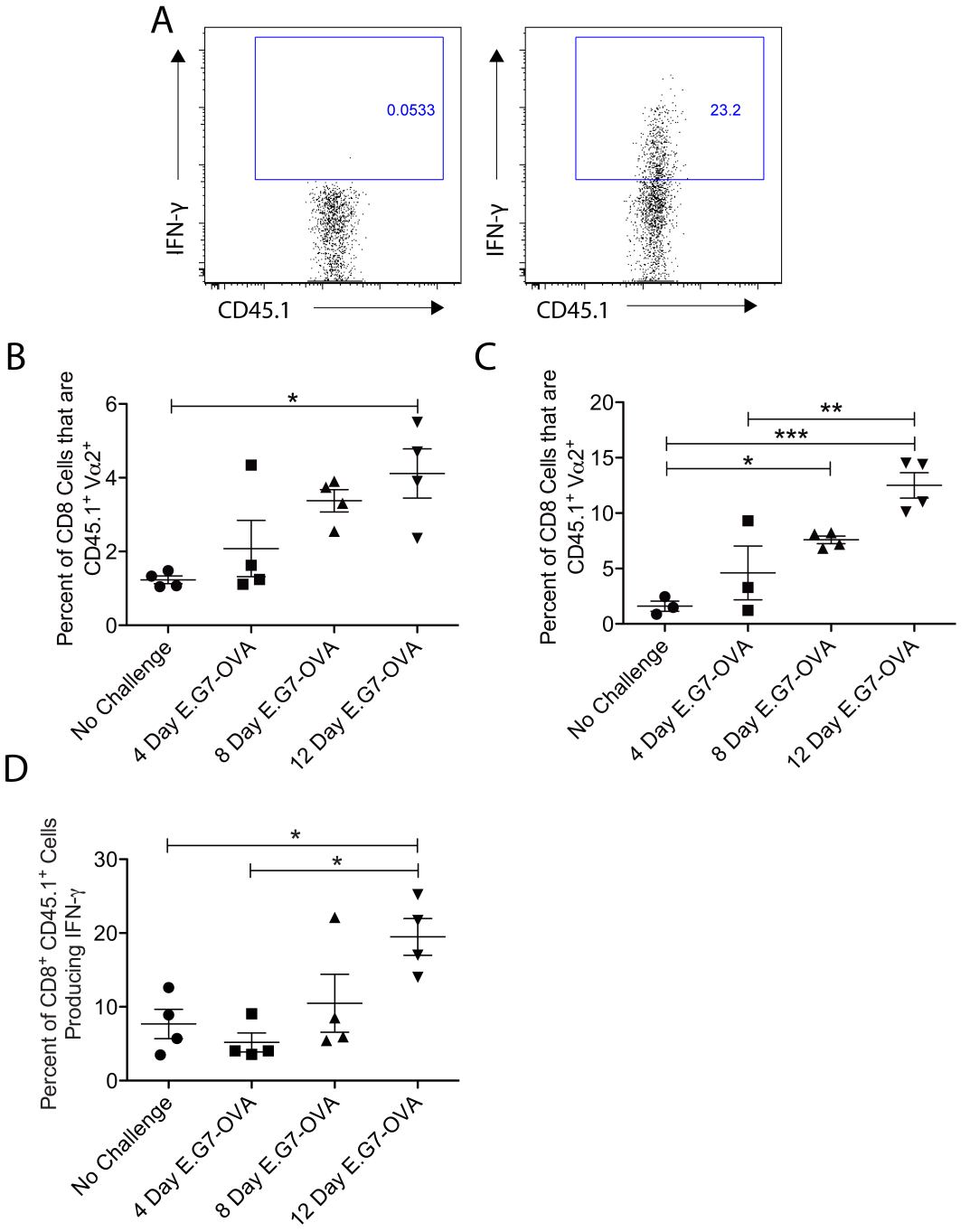


Figure 3.8: Transferred effector CD8$^+$ T cells respond more when the tumours have been allowed to develop for twelve days prior to transfer. *Lang*-EGFPDTR mice were administered 5x10^6 effector OT-I T cells four, eight or twelve days following tumour challenge. Seven days later the T cell populations were compared by flow cytometry by using CD45.1$^+$, CD8$^+$ and Vα2$^+$ expression to identify the transferred effector CD8$^+$ T cells. An example of the gating used to identify the OT-I T cells that are producing IFN-γ is displayed (A), with an isotype negative control antibody used on the left plot, and an IFN-γ antibody on the right. Graphs display the percent of CD8$^+$ cells in (B) the spleen and (C) the blood that are OT-I T cells. The

presence of intracellular IFN-γ within the transferred CD8$^+$ T cells was determined by performing intracellular flow cytometry on the splenocytes using an antibody for IFN-γ and the percent of transferred CD8$^+$ T cells producing IFN-γ is presented (D). This represents a single experiment with four mice per group, however the data are repeated in following experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

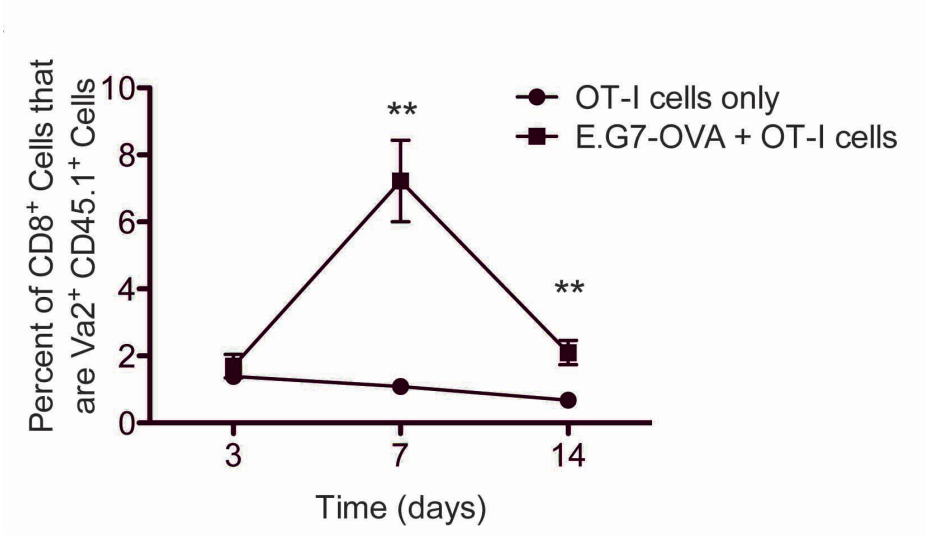


Figure 3.9: Effector CD8$^+$ T cells proliferate after transfer into mice with twelve-day-old tumours. C57BL/6 mice were transferred 5x10^6 *in vitro* activated T cells either in the presence or absence of twelve-day-old tumours. Blood samples were collected three, seven and fourteen days following transfer to analyse the T cell population overtime. The transferred OT-I T cells in the blood were identified by expression of CD8, Vα2 and CD45.1. A line graph showing the percent of CD8$^+$ cells in the blood that are OT-I T cells overtime. This represents a single experiment, with five mice per group, however the data are repeated in following experiments. A T Test with a Mann-Whitney post-test was performed at each time point between the two groups. **P = 0.01.

3.2.4 Langerin$^+$ CD8α^+ dendritic cells are required for the stimulation of adoptively transferred CD8$^+$ T cells in the presence of tumour

Having shown that a tumour derived stimulus induced an increase of *in vitro* activated tumour specific CD8$^+$ T cells in the blood after transfer, it was next investigated whether the potent cross-priming langerin$^+$ CD8α^+ DCs were essential for this stimulation to occur. To do this, *lang*-EGFPDTR mice were administered 5x10^6 effector CD8$^+$ T cells for the treatment of twelve-day-old tumours, with or without DT treatment to deplete langerin$^+$ cells. To ensure that DT treatment was not influencing T cell responses by a mechanism independent of langerin$^+$ cells, DT was also administered into a group of C57BL/6 mice, which do not express the human DTR. Interestingly, in *lang*-EGFPDTR mice depleted of the langerin$^+$ cells prior to adoptive transfer, the accumulation of transferred cells seen in the spleen and blood of tumour-bearing mice was ablated, approaching levels observed in mice that were administered CD8$^+$ T cells in the absence of tumours (Figure 3.10 A–C). The accumulation of the transferred CD8$^+$ T cells in the spleens of tumour-bearing mice was not altered by the administration of DT in C57BL/6 mice, which indicates that DT is not altering the CD8$^+$ T cell response independently of langerin$^+$ depletion (Figure 3.10D). These data demonstrate that the tumour-derived stimulus that induced an increase of CD8$^+$ T cells in the spleen and blood was dependent on langerin$^+$ CD8α^+ DCs.

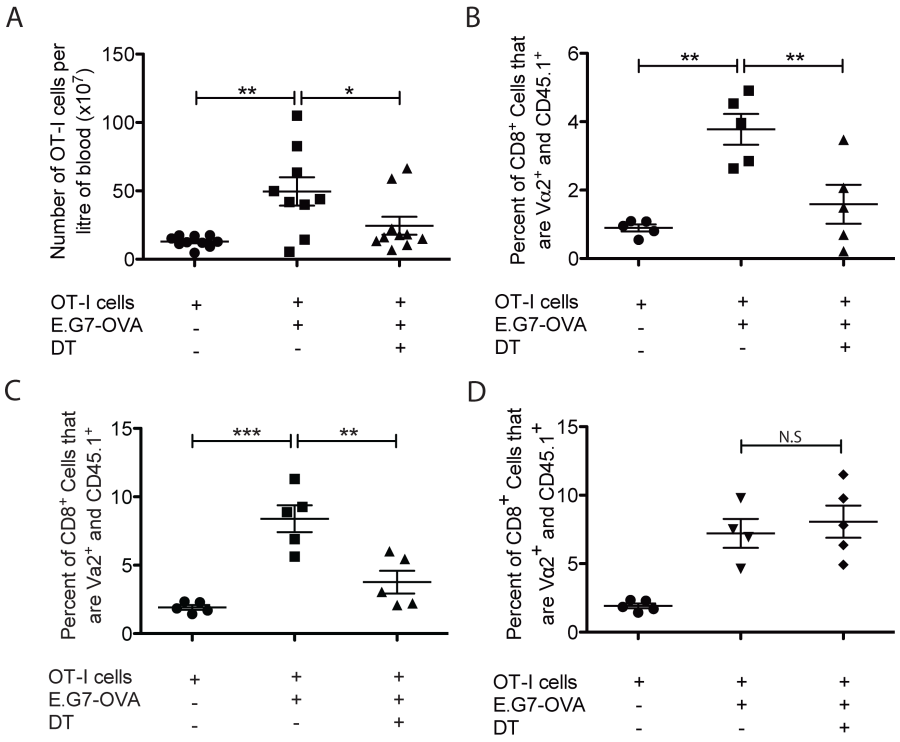


Figure 3.10: The tumour induced increase of transferred effector CD8$^+$ T cells in the spleen and blood is dependent on langerin$^+$ CD8α^+ DCs. *Lang*-EGFPDTR mice were transferred effector OT-I T cells twelve days after the intravenous administration of E.G7-OVA. One group of mice were depleted of the langerin$^+$ cells with 350 ng of DT administered intraperitoneally. Seven days following adoptive transfer the blood and spleens were analysed by flow cytometry using CD8, Vα2 and CD45.1 to identify the transferred CD8$^+$ T cells. The number of OT-I T cells per litre of blood was analysed and is depicted in (A). The proportion of OT-I T cells as a percent of the total CD8$^+$ cell population was also analysed in the blood (B) and spleen (C). The adoptive transfer experiment performed in C57BL/6 mice, analysing the proportion of OT-I T cells as a percent of the total CD8$^+$ cell population in the spleen (D). (A) is the combination of two experiments, (B) is representative of five experiments and (C) and (D) are a representative of four experiments with five mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

3.2.5 The phenotype of adoptively transferred CD8$^+$ T cells is not altered in the absence of langerin$^+$ CD8α^+ dendritic cells

In the absence of the tumour-associated stimulus from the langerin$^+$ CD8α^+ DCs, lower numbers of transferred T cells were found in the blood and spleen. As mentioned in the introduction, effector CD8$^+$ T cells enter the contraction phase of the T cell response when the cognate antigen is reduced, resulting in apoptosis of a significant proportion of the T cell population. It was therefore possible that in the absence of the langerin$^+$ CD8α^+ DCs, the transferred T cells enter the contraction phase due to lack of stimulation. In this case, the CD8$^+$ T cell population in the absence of the langerin$^+$ CD8α^+ DCs stimulation would be lower in number compared to when the langerin$^+$ cells were present (as was observed) and the remaining population would display more of a memory phenotype. To determine if the transferred T cells entered the contraction phase in the absence of langerin$^+$ cells, the phenotype of the transferred effector T cells in the spleen was analysed following transfer into hosts with twelve-day-old tumours, in the presence or absence of langerin$^+$ CD8α^+ DCs. As CD122 is upregulated on memory precursor cells we can use this marker in conjunction with CD62L to identify the different T cell phenotypes (127, 353, 354). T_{CM} cells express CD122 and CD62L, T_{EM} expresses CD122 but not CD62L and effector T cells express neither (354). In addition, KLRG1 can indicate the functional status of the CD8$^+$ T cells, with KLRG1 upregulated on CD8$^+$ T cells that have become terminally differentiated (355). While the effector and T_{EM} and T_{CM} subsets were discernable by this analysis, no significant differences in the proportion of any given phenotype were observed in tumour-bearing animals treated with adoptive therapy in the presence or absence of the langerin$^+$ CD8α^+ DCs and KLRG1 expression also did not differ between these two groups (Figure 3.11). However, CD8$^+$ T cells that were transferred into mice that had not been challenged with tumours displayed more of a T_{CM} phenotype than the CD8$^+$ T cells that were transferred into mice with established tumours (Figure 3.11C). These results therefore indicate that although the absence of the langerin$^+$ CD8α^+ DC stimulus resulted in a reduced accumulation of transferred CD8$^+$ T

cells in the spleen and blood it does not significantly alter their phenotype, which suggests these T cells had not entered the contraction phase.

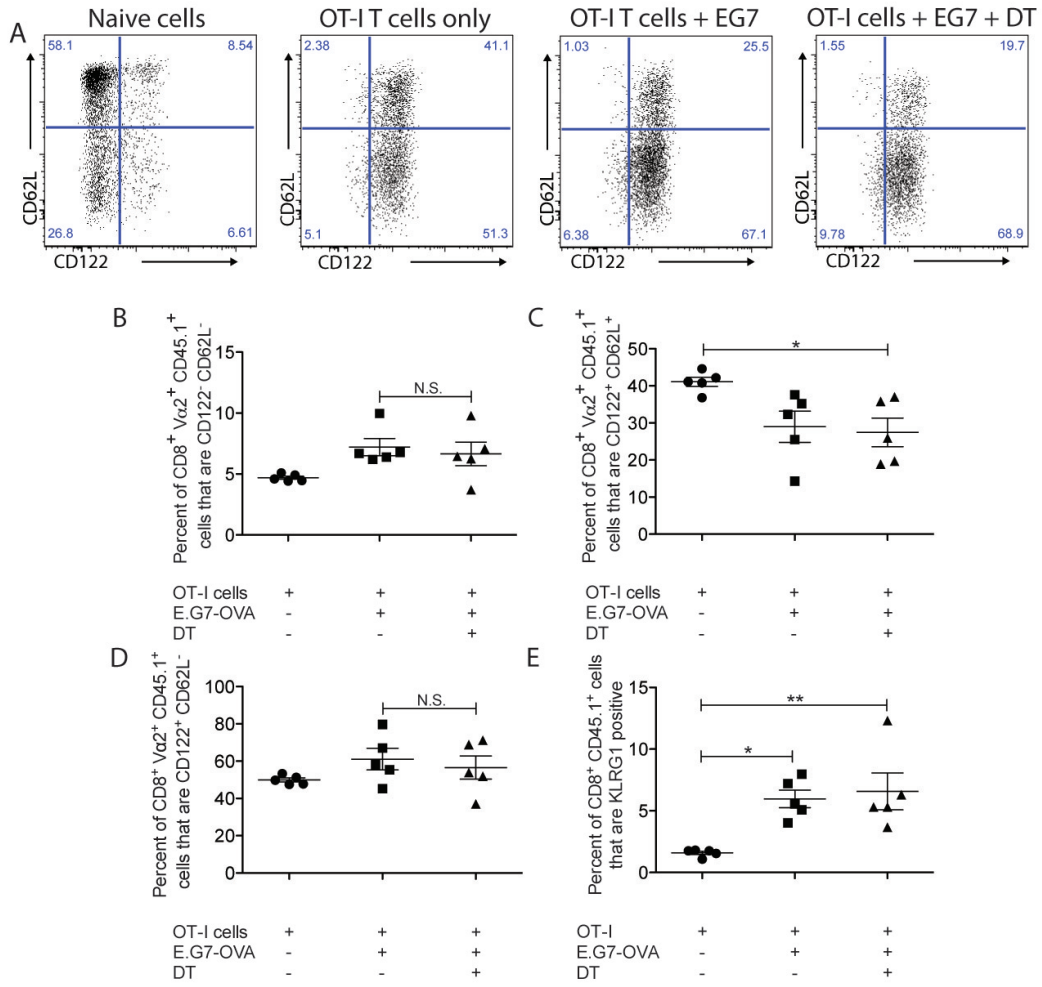


Figure 3.11: The phenotype of transferred CD8$^+$ T cells is unaltered when transferred into mice depleted of their langerin$^+$ CD8α^+ DCs. *Lang*-EGFPDTR mice were transferred effector OT-I T cells twelve days after the intravenous administration of E.G7-OVA. One group of mice were depleted of the langerin$^+$ cells with 350 ng of DT administered intraperitoneally. Seven days following adoptive transfer the phenotype of the OT-I T cells in the spleen was determined by flow cytometry using CD8, Vα2 and CD45.1 to isolate the transferred cells. The gating strategy used to determine the expression of CD62L and CD122 can be found in (A), with naïve cells to demonstrate the negative populations (far left) and an example from each experimental group. (B) The proportion of the OT-I T cell population that is negative for CD122 and CD62L. (C and D) The proportion of the population that is CD122$^+$ and CD62L$^+$ and CD122$^+$ and CD62L$^-$, respectively. (E) The OT-I T cells from the spleen that express KLRG1. *B–D* are a representative of four experiments

and (E) is a representative of two, with five mice per group. *P < 0.05 (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

3.2.6 The functional state of transferred CD8$^+$ T cells is not altered in langerin depleted mice

The studies to this point had shown that a tumour derived stimulus can induce adoptively transferred effector T cells to accumulate in the spleen and blood, however this stimulus did alter their phenotype. It was next investigated whether the langerin$^+$ CD8α^+ DC-dependent stimulation alters the function of the transferred CD8$^+$ T cells in terms of cytokine production and their ability to kill antigen-specific target cells. Analysis of the ability of the CD8$^+$ T cells to produce IFN-γ was determined by intracellular flow cytometry on the splenic OT-I T cell population. This showed that the percentage of transferred cells producing IFN-γ and the MFI of IFN-γ on OT-I T cells was unaffected by transfer into tumour-bearing mice depleted of their langerin$^+$ cells (Figure 3.12 A and B). However, this analysis did not take into account the increased population of transferred CD8$^+$ T cells in the spleen where the differences were quite substantial. When taking into account the population size, there were significantly more transferred CD8$^+$ OT-I T cells producing IFN-γ when the langerin$^+$ cells were present (Figure 3.12C).

The ability of transferred CD8$^+$ T cells to recognize and kill cells loaded with their cognate antigen in tumour-bearing mice was analysed. Target cells consisting of fluorescently labelled splenocytes, loaded with or without peptide, were administered seven days after CD8$^+$ T cell transfer and the following day a blood sample was removed to determine the proportion of target cells that were killed in each group using flow cytometry (Figure 3.13A). Three groups of target cells were used with each group pulsed with a different concentration of the cognate antigen SIINFKEL (0.5, 5 and 50 nM respectively). While peptide specific killing could clearly be observed, no statistically significant difference in the ability of the transferred CD8$^+$ T cells to kill targets was observed when compared in the presence or absence of

langerin$^+$ cells (Figure 3.13B). Of note, mice that received effector CD8$^+$ T cells in the absence of tumours were significantly less effective at killing the 50 nM target cells. This indicates that a tumour-derived stimulus independent of langerin$^+$ cells increases the killing capacity of the transferred CD8$^+$ T cells in this setting (Figure 3.13B). In conclusion, these results indicate that CD8$^+$ T cells administered into tumour bearing mice in the absence of langerin$^+$ cells have similar functional capabilities as those that are transferred in their presence, both in terms of IFN-γ production and their ability to find and kill antigen specific-cells.

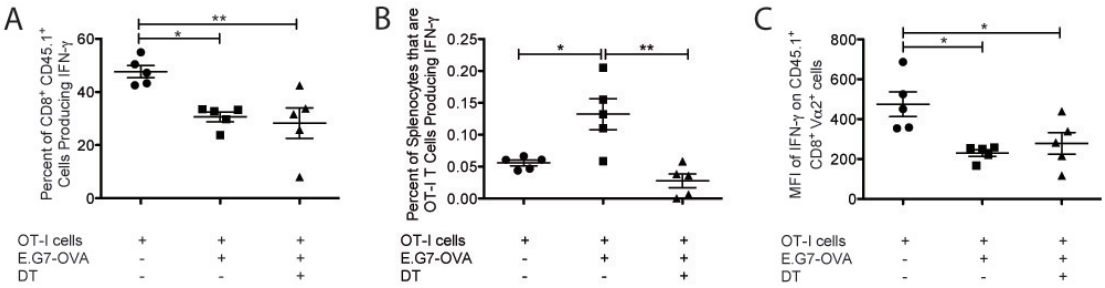


Figure 3.12: The proportion of transferred effector CD8$^+$ T cells producing IFN-γ remains constant in langerin-depleted mice. Effector OT-I T cells were transferred into *lang*-EGFPDTR mice with twelve-day-old intravenously administered tumours. One group of mice were depleted of the langerin$^+$ cells by intraperitoneal administration of 350 ng of DT. Seven days later the spleens were removed from each mouse and intracellular flow Cytometry was performed to determine the proportion of splenic OT-I cells producing IFN-γ, using CD8, Vα2 and CD45.1 to identify the transferred CD8$^+$ T cells. (A) The proportion of OT-I T cells that are positive for intracellular IFN-γ in the spleen. (B) The proportion of total splenocytes that were IFN-γ producing OT-I T cells. (A) and (B) are representatives three experiments, with five mice per group. *P < 0.05, **P < 0.01 (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

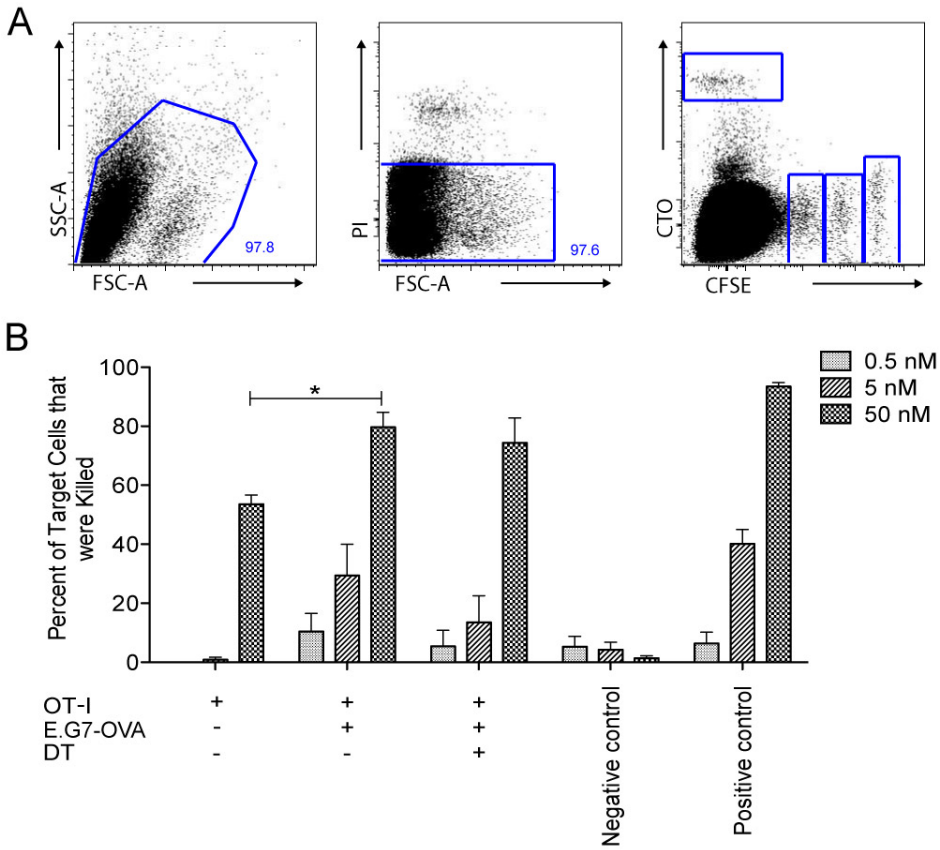


Figure 3.13: Effector CD8$^+$ T cells can effectively kill target cells when administered into tumour-bearing mice depleted of langerin$^+$ cells. *Lang*-EGFPDTR mice were transferred effector OT-I T cells twelve days after the intravenous administration of E.G7-OVA. One group of mice was depleted of langerin$^+$ cells with 350 ng of DT administered intraperitoneally. Seven days following adoptive transfer, target cells were injected intravenously into mice loaded with 0.5, 5 or 50 nM of SIINFKEL and stained with CFSE at a low, medium or high concentration, respectively. A control group of cells containing no target antigen and stained with CTO was also administered. The day after transferring target cells, a sample of blood was removed from each mouse and the three different CFSE$^+$ target cell populations were compared to the non-target CTO$^+$ control to determine how much of the target populations had been killed. The gating strategy used to identify the four different groups of target cells can be found in (A). The positive control group was administered OVA and α-GalCer to activate the transferred CD8$^+$ T cells and the negative control group received no OT-I T cells or treatment. The graph displays the amount of target cells from each group that were killed by the different experimental groups. This figure is representative of two experiments, with five mice per group. *P < 0.05 (One-way ANOVA with a Bonferroni's Multiple comparisons Test on each target cell population).

3.2.7 Anti-tumour activity provided by adoptive T cell transfer is dependent on langerin$^+$ CD8α^+ dendritic cells

Although no significant differences were seen in the CD8$^+$ T cells transferred in the presence or absence of langerin$^+$ DCs with the functional assays assessed, it remained to be established whether the absence of the langerin$^+$ cells would actually have any impact on therapy-induced protection. To determine this mice were administered 5x10^6 effector CD8$^+$ T cells for the treatment of twelve-day-old tumours and the symptom-free-survival was analysed. Mice that were challenged with the tumour alone remained symptom-free for an average of 24 days after challenge (Figure 3.14). In contrast, mice that received adoptive T cell therapy had an extended symptom-free survival by an average of fourteen days, giving them an overall average of 38 days symptom-free after tumour challenge. Interestingly, mice depleted of their langerin$^+$ cells prior to adoptive transfer therapy had an average symptom-free survival of only 28 days. To ensure that DT treatment was not altering the survival of mice independently of adoptive therapy an experiment was conducted comparing the symptom-free survival of mice challenged with E.G7-OVA and treated with or without DT (Appendix 3). In this experiment DT treatment did not alter the symptom-free survival and therefore the reduced protection seen in DT treated mice in figure 3.14 is due to a reduced function of the transferred T cells. This result indicates that the stimulation provided by the langerin$^+$ CD8α^+ DCs is required for the transferred effector CD8$^+$ T cells to mediate effective killing of tumour cells.

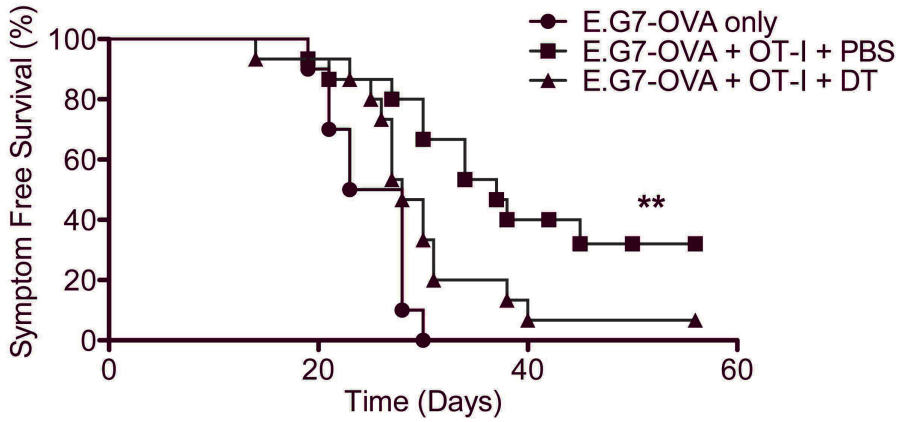


Figure 3.14: Anti-tumour activity provided by adoptive T cell therapy is dependent on langerin$^+$ CD8α^+ dendritic cells. Mice were treated with 5x10^6 effector OT-I T cells twelve days after receiving 1x10^6 E.G7-OVA cells intravenously. One group of mice received 350 ng of DT intraperitoneally two days before transfer and every two to three days to maintain depletion. The health of the mice was closely observed and when a mouse displayed a symptom of disease it was culled. The period of time that the mice remained symptom-free after tumour challenge was recorded and is compared between the groups. This is the combination of three experiments, with 15 mice in the treated groups and 10 in the E.G7-OVA only group. $^{**}P < 0.01$ (Log-rank Mantel Cox Test).

3.2.8 Stimulating endogenous dendritic cells with α-GalCer prior to adoptive T cell therapy

Having found that the treatment of established tumours with adoptive CD8$^+$ T cell therapy was dependent on the stimulus provided by langerin$^+$ CD8α^+ DCs, we aimed to enhance the efficacy of the treatment by stimulating the endogenous DCs prior to transfer. Intravenously administering α-GalCer results in the activation of endogenous DCs through the activation of iNKT cells, which in turn license DCs. Therefore, hosts with twelve-day-old tumours were administered α-GalCer at the same time as the transfer of T cells or 24 hours after. In addition one group received α-GalCer and OT-I T cells in the absence of a tumour challenge. Seven days following transfer the spleens were removed and the OT-I T cell population was analysed. As demonstrated earlier, more transferred T cells were found in the spleens of tumour challenged hosts. Hosts that were administered α-GalCer at the same time as adoptive transfer, with or without tumour challenge did not have the same increase in transferred T cells in the spleen (Figure 3.15). The administration of α-GalCer 24 hours after adoptive therapy had significantly fewer transferred T cells in the spleen compared to hosts that did not receive α-GalCer. This result suggests that this method of DC activation was ineffective at enhancing the stimulus provided by langerin$^+$ CD8α^+ DCs to the transferred T cells.

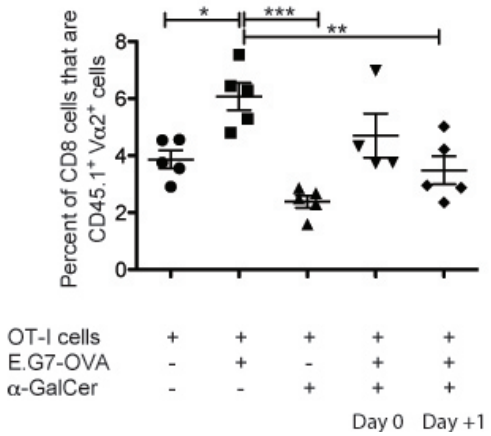


Figure 3.15: Stimulation dendritic cells with α-GalCer prior to adoptive therapy. C57BL/6 mice were challenged with 1x10^6 E.G7-OVA cells and twelve days later were intravenously administered 5x10^6 activated OT-I cells. Two groups of mice received 200 ng of α-GalCer, one of which was not tumour challenged and another group received α-GalCer 24 hours after adoptive transfer. Seven days later the spleens were removed and the number of transferred OT-I T cells was analysed by flow cytometry using CD8, Vα2 and CD45.1 to isolate the transferred cells. The percent of CD8$^+$ cells that expressed Vα2 and CD45.1 was analysed and is displayed. This is the combination of two experiments with five mice per group. *P < 0.05 (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

3.2.9 Increasing tumour debris for cross-presentation with chemotherapy

As the stimulus provided by langerin$^+$ CD8α^+ DCs to the transferred T cells was dependent on the presence of tumours, it is possible that the stimulus will be enhanced by increasing the amount of tumour debris for endogenous DCs to acquire. To increase the amount of tumour debris within the host we treated tumour challenged mice with the chemotherapy doxorubicin. Doxorubicin was used as it is a relatively immunogenic chemotherapy. It has been found to increase the population of CD4$^+$ T cells within hosts, which had also upregulated CD40L (356). In this study, the subsequent interaction of the CD4$^+$ T cells with DCs resulted in enhanced activation and survival of DCs *in vitro*. Therefore, doxorubicin was of interest to enhance the function of the endogenous DCs in this model. Doxorubicin was administered either 24 hours before adoptive T cell transfer or at the same time. Seven days after transfer the spleens were removed and the OT-I T cell population was analysed by flow cytometry. An increase in the transferred T cell population was detected in tumour challenged hosts relative to non-tumour challenged controls. A similar increase was detected in hosts treated with doxorubicin 24 hours prior to adoptive transfer (Figure 3.16). Treating hosts with doxorubicin at the same time as adoptive therapy did not result in the same increase of transferred T cells in the spleen relative to the non-tumour challenged control. The use of doxorubicin in this setting, therefore did not improve the stimulus provided by langerin$^+$ CD8α^+ DCs to the transferred effector CD8$^+$ T cells.

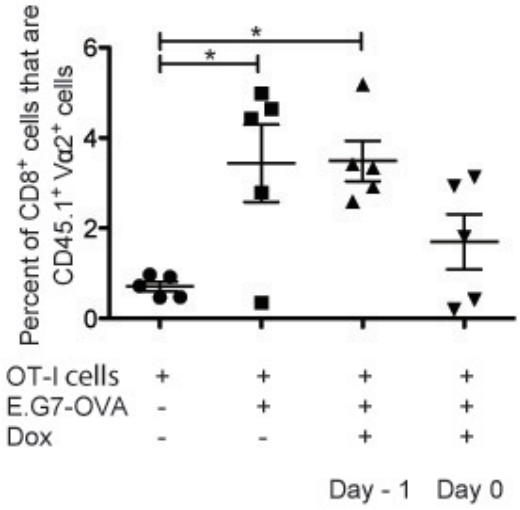


Figure 3.16: Increasing tumour debris for acquisition by endogenous dendritic cells. C57BL/6 mice were challenged with 1x10^6 E.G7-OVA cells and 12 days later received 5x10^6 activated CD8$^+$ T cells. Two groups were treated intravenously with 240 µg of doxorubicin, one group 24 hours before adoptive therapy and another on the same day. Seven days later the spleens were removed and the number of transferred OT-I T cells was analysed by flow cytometry using CD8, Vα2 and CD45.1 to isolate the transferred cells. The percent of CD8$^+$ cells that expressed Vα2 and CD45.1 was analysed and is displayed. This is the combination of two experiments with five mice per group. *P < 0.05 (One-way ANOVA with a Bonferroni's Multiple comparisons Test).

3.3 Discussion

The hypothesis of this study was that the effective anti-tumour activity of an adoptive transfer-based immunotherapy is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen. The data presented here support this hypothesis as the langerin$^+$ CD8α^+ DCs were found to be required for extended maintenance of the transferred T cell population in the spleen and blood, and the presence of the langerin$^+$ CD8α^+ DCs improved their ability to protect against tumour development. The langerin$^+$ CD8α^+ DCs are therefore contributing to maintaining effective immunotherapy, which suggests that the role of endogenous DCs should be taken into consideration during immunotherapy design.

Following adoptive transfer, it is likely that the effector CD8$^+$ T cells contact tumour cells and directly kill them, resulting in an incrfease in tumour debris in the blood. This debris could drain through the marginal zone of the spleen and be acquired by langerin$^+$ CD8α^+ DCs. Due to the potent cross-priming ability of the langerin$^+$ CD8α^+ DCs, antigens from acquired tumour debris could be presented on MHC class I and used to stimulate the transferred CD8$^+$ T cells that enter the spleen. It is possible that the tumour model used provides a pool of circulating cells (although this was assessed and could not be confirmed). It is, however, also possible that within the twelve days prior to adoptive transfer the tumour cells had completely left the blood to seed in distant tissues. In this case, the langerin$^+$ CD8α^+ DCs could come into contact with tumour antigens that have either drained through the lymphatics from tumour tissue, or been transferred to langerin$^+$ CD8α^+ DCs by APCs that migrated from the tumour. It is also possible that the stimulus is independent of antigen presentation by langerin$^+$ CD8α^+ DCs, and that these DCs merely produce cytokines that transferred T cells acquire, such as those that aid in T cell proliferation or survival.

As langerin$^+$ CD8α^+ DCs are efficient at cross-priming CD8$^+$ T cells it is possible that the adoptively transferred CD8$^+$ T cells are not the only CD8$^+$ T cell population affected by the absence of the langerin$^+$ CD8α^+ DCs. It is likely

that endogenous CD8$^+$ T cells are activated following tumour challenge and by depleting the langerin$^+$ CD8α^+ DCs they may not be receiving the same stimulus. However, as DT was administered into hosts 10 days following tumour challenge the depletion of langerin$^+$ CD8α^+ DCs may not be affecting the activation of endogenous naïve CD8$^+$ T cells into effector CD8$^+$ T cells, but preventing the effector cells from receiving a secondary stimulus, similar to the transferred effector CD8$^+$ T cells. In this case, depletion of the langerin$^+$ CD8α^+ DCs is likely reducing the anti-tumour activity of both the transferred and endogenous effector CD8$^+$ T cells.

It was notable that CD8$^+$ T cells that proliferated and developed an effector phenotype following *in vitro* activation appeared to undergo further proliferation following transfer into tumour-bearing mice. A preliminary experiment was conducted, in which CFSE labelled OT-I T cells were administered into mice with 12-day-old tumours. Blood samples were taken from these mice three and seven days following transfer to determine if there was any difference in the CFSE profiles of the transferred T cells in mice depleted of the langerin$^+$ cells (Appendix 4). Because the entire population of transferred cells had significantly downregulated their CFSE, MFI was used to analyses the CFSE data in this experiment. From this preliminary experiment it appears that the T cells proliferate following transfer into tumour-bearing hosts independently of langerin$^+$ cells, as no difference was seen in the CFSE profiles of the transferred T cells in tumour bearing hosts with or without langerin cell depletion. However, this experiment was only completed once and therefore the results have not yet been confirmed. The decrease of transferred T cells in the spleen and blood of mice depleted of langerin$^+$ cells is therefore likely due to reduced survival of the T cells or altered homing. In either instance, the proliferation or accumulation of transferred cells in the spleen and blood indicates an interesting role for the langerin$^+$ CD8α^+ DCs in adoptive therapy.

There is a significant amount of debate surrounding the subset of T cell that provides the most effective protection following transfer into tumour-bearing

hosts. In this study, the transferred CD8$^+$ T cells displayed the phenotype of effector T cells, consisting of low expression of both CD62L and CD122. However, some studies have demonstrated that effector T cells used for adoptive transfer become terminally differentiated resulting in lower T cell survival rates and reduced anti-tumour protection (315, 357). In the current study; however, the effector CD8$^+$ T cells transferred into mice expressed CD122 seven days after transfer (Figure 3.11), suggesting that they have developed into memory precursor cells. Furthermore, nearly 30% of the transferred cells developed CD62L expression, indicating that following transfer into tumour-challenged mice the effector CD8$^+$ T cells can differentiate into both T_{EM} and T_{CM} cells, possibly providing the benefits of both subsets. Differentiation of the transferred cells into memory precursor cells within seven days is a curious finding, as at this point the transferred CD8$^+$ T cell population is still at the peak of the T cell response, as determined by cell number, and the population is yet to enter the contraction phase (Figure 3.8). So the question arises whether the transferred cells are able to form a functional memory population. To test this, effector CD8$^+$ T cells were administered into mice, which were then left for 28 days before being tumour challenged. Interestingly, the transferred cells at this time were able to provide significant protection against tumour development (Appendix 5). This experiment indicates that the transferred effector CD8$^+$ T cells used in this experiment were not becoming terminally differentiated but developed into memory cells, which could be restimulated *in vivo* to provide anti-tumour protection. However, this experiment has only been conducted once and therefore the observations have not been confirmed.

Effector CD8$^+$ T cells transferred into mice that do not have tumours displayed a greater expression of CD62L$^+$ CD122$^+$ cells (Figure 3.11), indicating that the absence of antigen drives the transferred cells into a T_{CM} phenotype. The transferred population of T cells in mice without tumours also displayed a reduced killing ability than the population of cells transferred into mice with established tumours (Figure 3.13). This suggests that there is a tumour-associated stimulus provided to the transferred cells in vivo that is independent of langerin$^+$ CD8α^+ DCs. The reduced killing seen in thenon-

tumour challenged group may be attributed to the slower recall response of resting T_{CM} cells compared to effector cells or T_{EM} cells, which are potentially already attacking tumour cells (354). As the killing function was only analysed for a period of 24 hours this may not have been enough time to see significant activation of T_{CM} cells in mice that were not tumour challenged. The T cells transferred into tumour-bearing mice retained more of a T_{EM} phenotype, possibly due to the continued presence of tumour antigen and these cells may therefore be more primed to kill target cells, enabling a quicker killing response. While the killing function was analysed two and three days after the administration of target cells, the target cells with the highest concentration of SIINFEKL were no longer detectable, likely due to being removed by CD8$^+$ T cell killing. Therefore, this model was not sufficient to analyse delayed killing function.

Another question arising from this study is why the langerin$^+$ CD8α^+ DCs were essential to mediate the protective benefit associated with adoptive transfer when the absence of langerin$^+$ CD8α^+ DCs did not alter the function of the transferred T cells in the assays tested. It would be expected that two CD8$^+$ T cell populations with no significant difference in ability to kill target cells would have a similar anti-tumour protective benefit, however this is not what was observed. It is likely that due to a lack of statistical power the smaller differences between these groups were missed. Another possibility is that the stimulus provided by the langerin$^+$ CD8α^+ DCs is essential to mediate trafficking of the effector CD8$^+$ T cells to tumour sites or to enable infiltration into a tumour. Therefore, although T cell populations administered in the presence or absence of langerin$^+$ CD8α^+ DCs have the same ability to kill target cells, only the group administered in the presence of langerin$^+$ CD8α^+ DCs would be able to migrate to or invade a seeded tumour to mediate tumour killing. In addition, this could account for the increase of transferred cells observed in the spleen and blood of tumour-laden mice, as the langerin$^+$ CD8α^+ DC stimulus may induce the effector CD8$^+$ T cells to accumulate at these sites, assuming this is where the tumour is situated at this point. Unfortunately, due to the nature of the tumour used, in particular the inability

of the tumour to develop consistent solid tumours, it was not possible to assess the ability of the transferred cells to enter a solid tumour.

The implication of this work for immunotherapies is that the protection provided by a given therapy is likely influenced by the host's network of APCs. This needs to be taken into consideration by researchers as the endogenous APCs may be significantly stimulating or inhibiting immunotherapies. By gaining a better understanding of the stimuli provided by host APCs to an immune therapy such as adoptive therapy, it may be possible to enhance or reduce the generated immune response in a manner that favours protection. For example, in this study it would be relevant to determine if the protection provided by the adoptive therapy can be further increased by enhancing the tumour-derived stimulus provided by the langerin$^+$ CD8α^+ DCs. This may be possible by increasing the amount of tumour debris circulating in the blood for the langerin$^+$ CD8α^+ DCs to acquire by treating tumour-bearing mice with chemotherapy (as attempted in figure 3.16) or irradiation or by directly administering irradiated tumour cells prior to transfer (358-361). This idea is supported by research demonstrating that treating tumour-bearing mice with whole-body irradiation activated the host's innate immune system and subsequently adoptively transferred effector CD8$^+$ T cells provided greater anti-tumour protection (362). In addition, mice adoptively transferred naïve CD8$^+$ T cells were provided with greater anti-tumour protection when treated with chemotherapy and a combination of the TLR agonist poly(I:C) and tumour antigens. In these studies, chemotherapy developed a significant increase in the hosts DC population and by activating these cells at the peak of expansion with poly(I:C) and tumour antigens. The transferred anti-tumour CD8$^+$ T cells, in this experiment, were induced to provide superior protection (363). This treatment regime was dependent on the endogenous DCs, as mice depleted of their CD11c$^+$ cells, using the CD11c-DTR mouse model, had significantly reduced numbers of transferred CD8$^+$ T cells in the spleen and lymph nodes. These studies provide evidence to the possibility of enhancing the protection provided by adoptive therapy through the manipulation of endogenous APCs. While figure 3.15 and 3.16 were performed to determine if

DC stimulation would improve the stimulation of the adoptively transferred T cells, these experiments were performed under specific conditions, such as the treatment time, and it is likely that further experiments with different chemotherapies, irradiation or different times of treatment may provide different results. In addition, the activation of the endogenous DCs under certain conditions has been demonstrated to shutdown the cross-presentation pathway, which in this experimental model, would likely lead to reduced stimulation of the transferred T cells (352, 364). The shutting down of the cross-presentation pathway may explain why in figure 3.15 lower proportions of transferred T cells were observed in the spleen when α-GalCer was administered following adoptive therapy. Therefore, to effectively activate DCs in order to enhance adoptive therapy, without shutting-down cross-presentation, different treatments, concentrations and times of treatment would need to be tested.

Tumours typically induce a strong suppressive environment capable of reducing the capacity of DCs to stimulate CD8$^+$ T cells. This can occur by decreasing their co-stimulatory molecules (365), IL-12 production (366), or by inducing abnormalities in a DCs ability to acquire and present antigens (367, 368). The suppressive effect tumours can have on DCs is evident in DCs that have infiltrated a solid melanoma tumour. These DCs display a phenotype consistent with immature DCs and are unable to stimulate CD4$^+$ or CD8$^+$ T cells *in vitro* (349, 369, 370). The langerin$^+$ CD8α^+ DCs have specifically been associated with the induction of CD8$^+$ T cell tolerance in response to intravenously administered apoptotic cells expressing self-antigen (371). In light of this, it is possible that in certain situations the stimulus provided by langerin$^+$ CD8α^+ DCs to transferred effector CD8$^+$ T cells may be reduced or have tolerizing effects. In this situation, the development of effective adoptive CD8$^+$ T cell therapy may require activation of the host's APCs prior to transfer.

It is possible that some subtle differences in experimental results were missed due to small sample size causing type two errors. This may apply to experiments, such as figure 3.7, where a larger sample size may have

provided statistically significant results. Because of the increased cost associated with a larger sample size this was practical.

3.4 Conclusions

Activated CD8$^+$ T cells stimulated *in vitro* for transfer into tumour-bearing hosts, are influenced by the endogenous network of APCs. The results presented in this chapter support this hypothesis as the anti-tumour protection provided by adoptive CD8$^+$ T cell therapy was found to be reduced in the absence of resident langerin$^+$ CD8α^+ DCs. This suggests that langerin$^+$ CD8α^+ DCs have a role in stimulating effector CD8$^+$ T cells. The interaction between the langerin$^+$ CD8α^+ DCs and the transferred T cells resulted in a larger T cell population in the spleen and blood and enhanced protection against tumour development. The endogenous network of APCs therefore needs to be considered when performing adoptive cell therapy in the clinic, and through their manipulation it may be possible to provide enhanced anti-tumour protection.

Chapter 4: The role of langerin^{+} CD8α^{+} dendritic cells in protective immunity generated by an iNKT cell based tumour vaccine

4.1 Introduction

Given the proposed role of langerin$^+$ CD8α^+ DCs in cross-presentation of circulating antigens and the stimulation of CD8$^+$ T cell responses, it was possible that these DCs play a critical role in the generation of effector CD8$^+$ T cells in anti-tumour vaccination strategies, particularly those where vaccines require access to the spleen. For example, “whole cell” vaccines that have been designed to exploit the helper activity of iNKT cells are currently under development, and are often administered intravenously to access the large populations of iNKT cells within the spleen. Colleagues in my laboratory have been engaged in research into this form of vaccine for a number of malignancies, including glioma (331), melanoma (372) and a variety of CNS-associated tumours (Grasso, unpublished). Due to practical matters, such as collection of sufficient tumour tissue, and the ease with which the vaccines can be administered intravenously, haematological malignancies may be an obvious choice to translate this research into the clinic. For such patients, an obvious setting for vaccination will be in morphologic remission after conventional treatment, with the vaccines generated from leukaemic cells collected before treatment.

Acute myeloid leukaemia (AML) is an aggressive haematological malignancy with a dire prognosis without treatment. Most patients achieve morphologic remission after induction chemotherapy, but the majority with poor-risk cytogenetic or molecular features, and most older patients subsequently relapse (373-375). Relapse may be mediated by a small population of chemoresistant leukaemia cells (376, 377) that fall below the threshold used to define remission clinically (374). Long-term disease-free survival can be accomplished in patients with advanced AML, by treating with a chemotherapeutic agent such as cytarabine, followed by allogeneic stem cell transplantation (allo-SCT). However, between 20 and 55% of treated patients relapse (378, 379). Allo-SCT also carries a high morbidity, mortality and cost, and is often precluded by age, co-morbidities or the lack of a suitable donor (380, 381). The low survival rates in combination with the risk associated with

allo-SCT, plainly indicate the necessity for a more effective and tolerable treatment for relapsing AML.

In addition to overexpressing certain self-antigens (382), myeloblasts harbour numerous mutations (383), resulting in expression of tumour-specific antigens capable of eliciting autologous CD4$^+$ and CD8$^+$ T cell responses (384). In fact, expression of mRNA for some antigens has been associated with a favourable prognosis (385, 386), suggesting a level of immune-mediated control that can potentially be exploited in immunotherapy (387). Vaccines based on malignant cells from AML patients may be able to elicit immune responses against multiple leukaemia-specific antigens without needing to first define specific T cell epitopes, which is a drawback to other “targeted” vaccine approaches.

AML may provide a challenge to immunotherapy due to its ability to generate an effective immunosuppressive environment (388-390). Patients suffering from AML have been found to develop DCs with impaired maturation and function that drive tolerance to leukemic cells rather than immunity (276). Similarly, patients with AML have been found to develop NK cells that are deficient in the expression of the activating receptors called the natural cytotoxic receptors. The presence of receptor deficient NK cells correlates with poor survival in AML patients (391). A significant contributor to suppression in AML is IDO, which is expressed on the majority of AML cells (277). This enzyme catabolises the amino acid tryptophan, which is essential for T cell proliferation, while the metabolites produced from this process are able to induce T cell apoptosis (282, 392). Furthermore, the metabolites produced can also cause naïve CD4$^+$ T cells to differentiate into T_{regs}, which have been associated with a worse prognosis in AML (282, 393, 394). As described in the introduction to this thesis, T_{regs} can suppress T cell-mediated immune responses in a variety of different ways, including by producing suppressive cytokines, killing immune cells and reducing free IL-2 from the environment, which is a key cytokine involved in CD8$^+$ T cell proliferation (288, 289, 291).

A simple, whole cell vaccine was developed for the current study that consisted of irradiated murine AML cells loaded with α-GalCer (tumour/α-GalCer). The specific aim of the study was to determine if langerin$^+$ CD8α^+ DCs were involved in the generation of vaccine-induced anti-tumour effector CD8$^+$ T cells in a model of AML. However, as this was a newly developed vaccine that may be used to model a future clinical trial, the study includes an analysis of mechanism of activity, an exploration of the future impact of AML induced immunosuppression, and an attempt to overcome immunosuppression with chemotherapy.

The rationale behind loading the vaccine with α-GalCer is that this specific ligand can be presented on CD1d to stimulate iNKT cells. It has been shown that following the administration of free α-GalCer, activated iNKT cells license DCs in a CD40L-dependent manner, providing them with a superior capacity to stimulate conventional T cells. Potent antigen-specific T cell responses can therefore be generated by administering an antigen in combination with α-GalCer (53, 79-81). Of relevance to this thesis, the DCs involved in stimulation of antigen-specific CD8$^+$ T cell responses to circulating soluble antigens were found to be langerin$^+$ CD8α^+ DCs (107). Once activated, iNKT cells can also stimulate DCs to produce IL-12, which drives the production of IFN-γ by NK cells (233) and may also contribute to anti-tumour activity. The specific DC subset producing IL-12 following iNKT cell stimulation was also found to be langerin$^+$ CD8α^+ DCs (107).

As langerin$^+$ CD8α^+ DCs have been demonstrated to be able to effectively acquire apoptotic cells administered intravenously, it is possible that a vaccine incorporating intravenously administered irradiated tumour cells will be acquired predominantly by langerin$^+$ CD8α^+ DCs (371). In addition, the exogenous nature of the vaccine-derived irradiated tumour cells suggests that the activation of anti-tumour CD8$^+$ T cells will require cross-priming, which is also a prominent function of langerin$^+$ CD8α^+ DCs. However, a colleague conducting research on a similar glioma vaccine showed that anti-tumour activity was independent of these DCs, and was in fact primarily dependent

on CD4$^+$ T cell effectors (331). The outcome of the research outlined in this chapter was therefore of some interest.

4.1.1 Aims

A novel vaccine consisting of irradiated tumour cells loaded with α-GalCer was developed for the treatment of AML. This vaccine-based immunotherapy was used to test the hypothesis "*that effective anti-tumour activity induced by an intravenously administered vaccine is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen*". Determining whether this specific DC subset has a role in vaccine-induced responses may significantly contribute to the future design of more potent vaccines.

Specific aims

- To develop and characterise a tumour/α-GalCer vaccine that provides anti-tumour activity in a mouse model of AML

- To established whether langerin$^+$ CD8α^+ DCs are required for the anti-tumour response elicited by the vaccine

4.2 Results

4.2.1 The Tumour/α-GalCer vaccine completely protects against AML when given prophylactically

An AML-specific vaccine was prepared by incubating the murine AML cell line C1498 with α-GalCer for 18 hours before irradiating them, and then rigorously washing the excess glycolipid off. Fresh vaccine was used for all of the experiments described. To determine if the tumour/α-GalCer vaccine had the capability to generate an immune response that could target and kill AML cells, the vaccine was administered into mice seven days prior to challenge with AML. Unvaccinated animals challenged with AML cells ultimately developed symptoms associated with AML-burden, such as weight loss, hunching or reduced grooming, at which point the mice were immediately culled. Significantly, mice that were prophylactically vaccinated were completely protected from developing symptoms of AML, indicating that the immune response generated was capable of targeting and killing AML cells (Figure 4.1). This experiment was repeated many times, with vaccinated mice remaining symptom-free for the duration of each experiment, with 100 days being the longest period tested.

The detection of leukemic blasts in the peripheral blood is used as a diagnostic tool for AML and their presence causes an elevated white blood cell count. Blood samples where therefore collected from mice that were either vaccinated prior to AML challenge, or were challenged alone, to determine if leukemic blasts could be detected in the blood of AML challenged mice and if these blasts were absent from vaccinated mice. Blood was collected from the AML only control group on the day that they developed symptoms associated with AML burden, prior to being culled. Mice that were prophylactically vaccinated remained symptom-free and therefore blood was taken 40 days after AML challenge. Blood samples were depleted of RBCs and the white blood cells were counted manually with a haemocytometer. Mice that received AML cells alone were found to have significantly higher numbers of white blood cells in their blood compared to naïve mice (Figure 4.2A). Prophylactically vaccinated mice did not have an elevated white blood

cell count and their count corresponded to that of naïve mice. Histology sections from blood and bone marrow samples collected from representative animals showed the expected accumulation of leukemic blasts in unvaccinated mice at the time of symptom onset, but not in those that had received the full α-GalCer-adjuvanted vaccine, suggesting a complete protective response had been induced (Figure 4.2 B–C). Arrows in the blood slides point to leukemic blasts. The tumour cells in the bone marrow slides are so prevalent that healthy cells cannot be identified. This result indicates that AML cells accumulate in the blood and bone marrow following administration, and that the tumour/α-GalCer vaccine prevented their development in these tissues.

Having shown that the vaccine protects mice from AML development, it was then investigated which immune cells were being induced to prevent AML growth. For this purpose, mice were vaccinated prophylactically and either their CD4 or CD8 expressing cells were depleted before AML challenge using specific antibodies. Depletion of the CD4$^+$ and CD8$^+$ cells seven days following treatment is displayed in appendix 6. The vaccine was administered five days before CD4 or CD8 depletion to insure that the depletion removed the effector cells alone and did not interfere with the vaccines ability to generate an effector response. This experiment showed that both CD4$^+$ and CD8$^+$ cells were involved in the vaccine-mediated protection (Figure 4.3). Each depletion setting did not completely ablate the AML protection, likely indicating that the remaining T cell population was able to continue attacking the AML cells. These findings indicate that the tumour/α-GalCer vaccine is capable of preventing AML engraftment and growth in a CD4 and CD8 dependent manner, suggesting CD4$^+$ and CD8$^+$ T cell dependence.

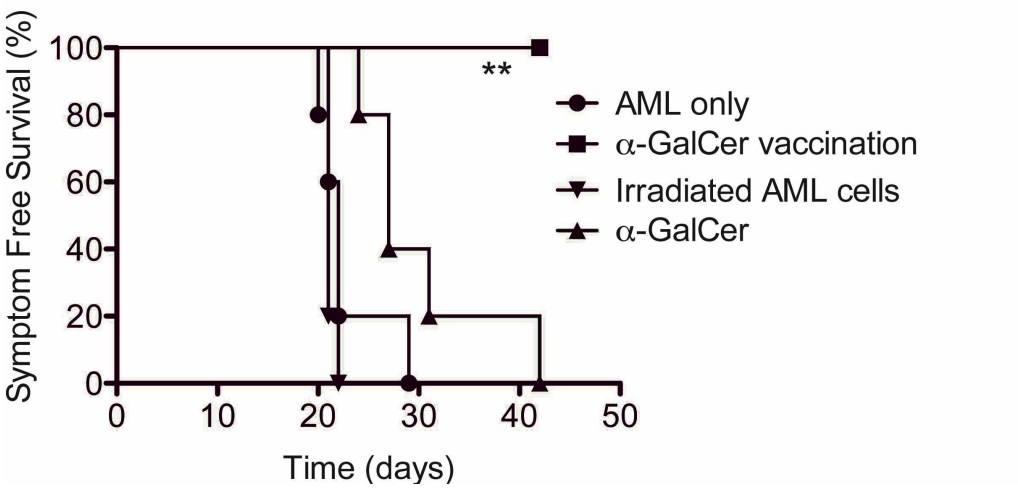


Figure 4.1: Tumour/α-GalCer vaccination protects mice from AML challenge. Mice were vaccinated intravenously with 7.5x10^5 irradiated AML cells loaded with α-GalCer or the individual vaccine contents alone, seven days prior to intravenous challenge with 1x10^5 AML cells. The health of mice was observed closely and the time it took for disease symptoms to develop was recorded and is graphed. This graph represents four experiments with five mice per group. ***P* < 0.01 (log-rank Mantel-Cox test).

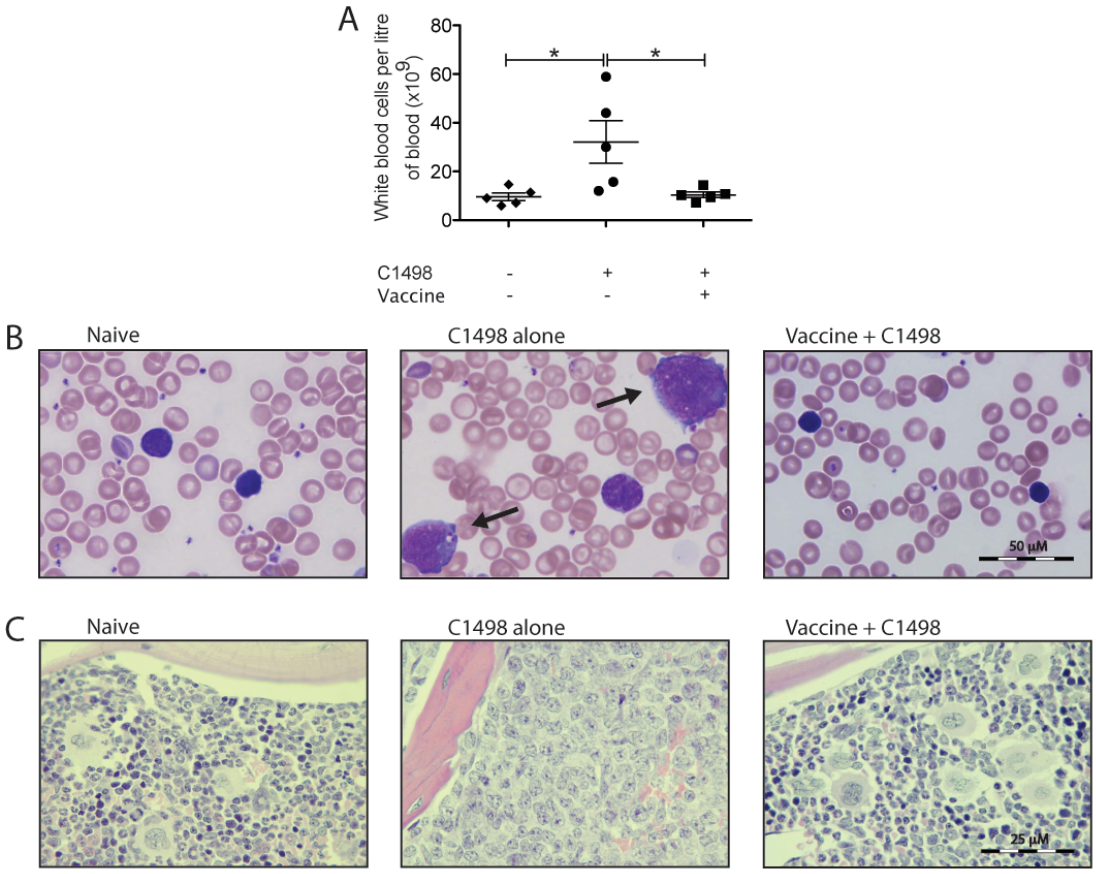


Figure 4.2: The tumour/α-GalCer vaccine prevents the development of leukemic blasts in the blood and bone marrow. One group of mice was administered 7.5x10^5 irradiated AML cells loaded with α-GalCer and seven days later 1x10^5 AML cells were administered into mice. The group that received AML alone was monitored for symptoms associated with AML-burden and upon development a blood sample was taken, the mice were then culled and the femur was removed for histology. The vaccinated group remained disease-free and was culled 40 days following tumour-challenge. (A) The blood samples were depleted of RBCs, the white blood cells were counted with a haemocytometer and the counts graphed. (B) Peripheral blood smear with diff-quick stain and (C) bone marrow histology with hematoxylin and eosin stain taken from a representative naïve control mouse, AML-challenged mouse and vaccinated then AML-challenged mouse. Arrows indicate leukemic blasts. (A) Represents a single experiment with five mice per group. *P < 0.05 (one-way ANOVA with a Bonferroni post test).

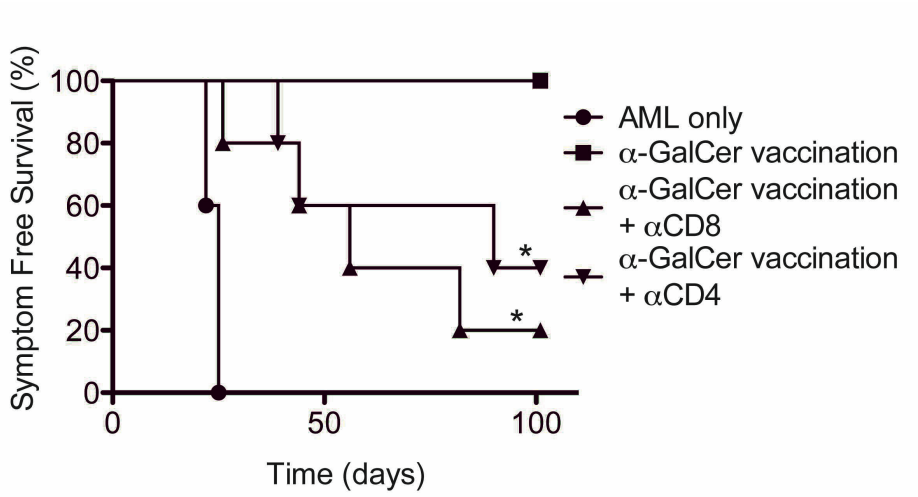


Figure 4.3: The vaccine-induced immune response is dependent on CD4$^+$ and CD8$^+$ cells. Mice were vaccinated intravenously with 7.5x10^5 irradiated AML cells loaded with α-GalCer, seven days prior to intravenous challenge with 1x10^5 AML cells. The health of mice was observed closely and the time it took for disease symptoms to develop was recorded and is graphed. The CD4$^+$ or CD8$^+$ cells were each depleted in one group of mice by intraperitoneal administration of the antibodies anti-CD8 (2.43; 250 µg per mouse) or anti-CD4 (GK1.5; 125 µg per mouse) five, twelve and nineteen days following vaccination and the symptom free survival was determined. This graph represents two experiments with five mice per group. *P < 0.05 (log-rank Mantel-Cox test).

4.2.2 Langerin$^+$ CD8α^+ dendritic cells are essential for the Tumour/α-GalCer vaccine to protect against AML

The requirement of CD8$^+$ cells for full vaccine efficacy suggested that CD8$^+$ T cells were cross-primed with cross-presented AML antigens. It was therefore analysed whether the potent cross-presenting langerin$^+$ CD8α^+ DCs were involved in the generation of the vaccine-induced protection against AML. To test this, *lang*-EGFPDTR mice were depleted of their langerin$^+$ cells by intraperitoneal administration of DT two days prior to vaccination, and the mice were challenged with AML cells seven days after vaccination. Interestingly, depletion of the langerin$^+$ cells prior to vaccination completely abrogated the protection provided by the vaccine and the mice developed AML-associated symptoms at a similar rate to the AML-only control group (Figure 4.4). To ensure that DT treatment was not reducing the symptom-free survival independently of the vaccine, an experiment was performed comparing the symptom-free survival of tumour-challenged mice, with or without DT treatment (Appendix 7). This experiment suggests that DT treatment does not alter the symptom-fee-survival of tumour-challenged mice. It is therefore likely that the function langerin$^+$ CD8α^+ DCs are performing following vaccination is the cross-priming of CD8$^+$ T cells, which were shown in figure 4.3 to be essential for the vaccine to mediate effective anti-AML protection.

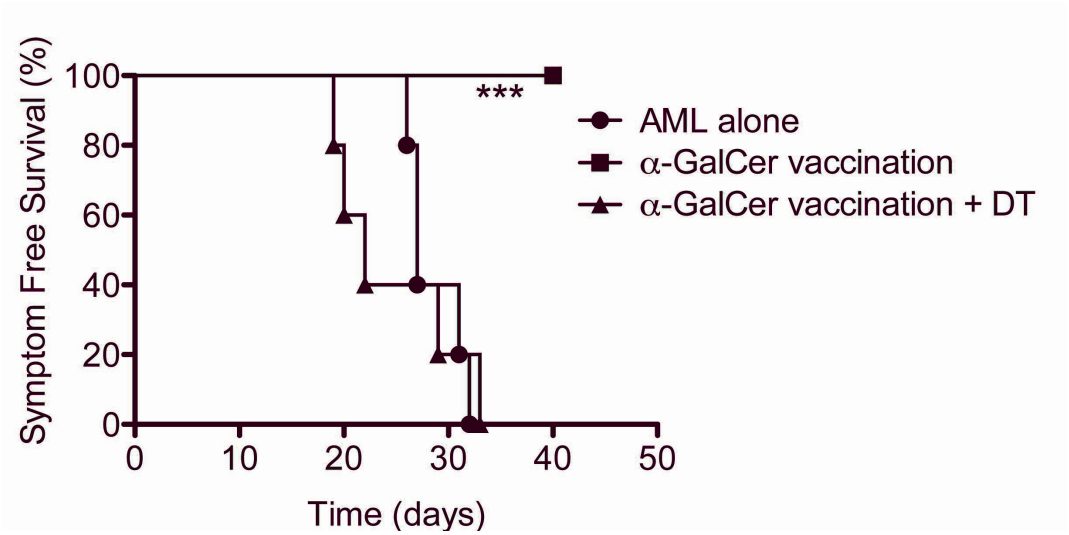


Figure 4.4: Langerin$^+$ CD8α^+ DCs are required for the tumour/α-GalCer vaccine to provide protection against AML. *Lang*-EGFPDTR mice were vaccinated intravenously with 7.5x10^5 irradiated AML cells loaded with α-GalCer seven days prior to challenge with 1x10^5 AML cells. One group of mice received 350 ng of DT administered intraperitoneally two days prior to vaccination and every two to three days after to maintain depletion. The symptom-free survival was analysed and is graphed. This represents two experiments with five mice per group. ***P < 0.001 (log-rank Mantel-Cox test).

4.2.3 Established AML renders the tumour/α-GalCer vaccine ineffective

In the prophylactic setting, the tumour/α-GalCer vaccine was highly effective at protecting mice from AML challenge, however generating an effective anti-tumour immune response is often limited by tumour-mediated immune-suppression. As AML is a particularly known for its ability to generate a strong suppressive environment, the ability of the tumour/α-GalCer vaccine to eradicate established AML was determined. AML cells were administered one week prior to vaccination and the symptom-free survival was analysed. In the therapeutic setting the vaccine was rendered ineffective and mice developed symptoms associated with AML-burden at a similar time as the AML only control mice (Figure 4.5).

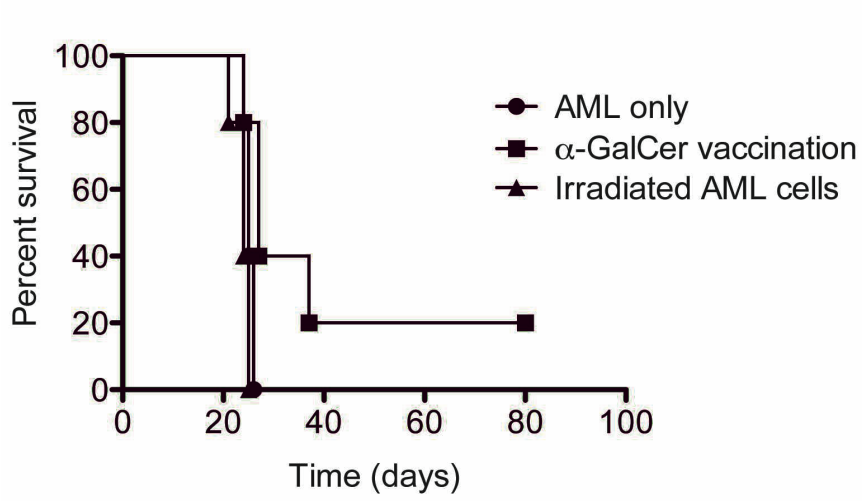


Figure 4.5: Established AML render the tumour/α-GalCer vaccine ineffective. Mice were administered 1x10^5 AML cells intravenously and the cells were allowed to grow and develop for one week before 7.5x10^5 irradiated AML cells loaded with α-GalCer were administered. One group received irradiated AML cells without α-GalCer. The symptom free survival of these mice was recorded. This graph represents three experiments each with five mice per group.

4.2.4 Vaccine-induced iNKT cell activation in hosts with established AML

Having found that the vaccine was ineffective in the therapeutic setting it was necessary to determine whether the established AML had caused suppression of the various vaccine-activated immune cells. Each step of immune activation following vaccination in hosts with established AML was therefore analysed, beginning with iNKT cell activation and expansion, which in turn leads to DCs activation, providing them with enhanced T cell stimulatory capabilities (53, 80, 81, 329). As the langerin$^+$ CD8α^+ DCs are an essential component to the vaccine it was initially investigated whether the vaccine could induce the activation of iNKT cells for DC licensing in the presence of established AML. To test this, AML was allowed to develop in mice for either seven or fourteen days before vaccination and the spleens were removed from mice three days later. Flow cytometry was performed to determine the proportion of iNKT cells within host spleens, using an antibody specific for CD3 and a fluorescent α-GalCer-loaded CD1d tetramer (CD1d tet) to identify the iNKT cells; the gating strategy is indicated in figure 4.6A. Mice vaccinated in the absence of AML had a significant increase in the proportion of CD3$^+$ cells that were iNKT cells three days after vaccination and a similar increase was observed in mice with seven-day-old AML prior to vaccination (Figure 4.6B). The vaccine-induced expansion of the proportion of iNKT cells was significantly lower in mice with fourteen-day-old AML when compared to the vaccine only control group, however the number of iNKT cells had still elevated significantly following vaccination. After being stimulated with α-GalCer, iNKT cells rapidly produce IL-4 and IFN-γ, which can be detected in host serum by performing a multiplex bead-based cytokine assay and used as an indicator of iNKT cell activation and functionality. Mice with seven-day-old AML were found to have as much IL-4 and IFN-γ in their serum following vaccination as mice that received the vaccine alone (Figure 4.6 C and D). These results indicate that although the iNKT cells are being mildly suppressed in hosts with established AML, they are still being activated by the tumour/α-GalCer vaccination and therefore may still be able to effectively license langerin$^+$ CD8α^+ DCs.

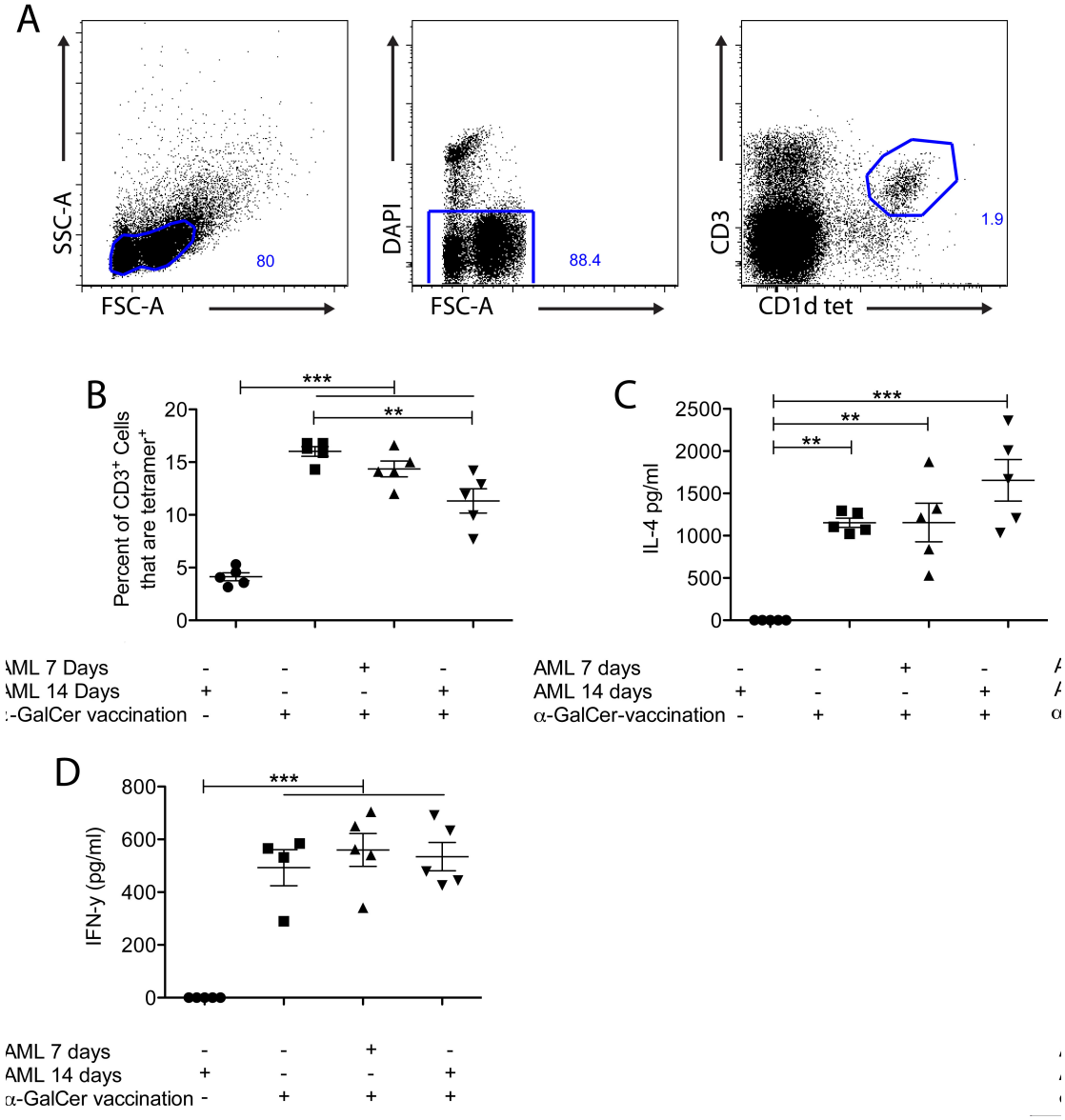


Figure 4.6: iNKT cells can be activated by the tumour/α-GalCer vaccination in mice with established AML. Mice were administered 1x10^5 AML cells intravenously seven or fourteen days before vaccination with 7.5x10^5 irradiated AML cells loaded with α-GalCer. Mice were culled three days following vaccination and flow cytometry was performed on host splenocytes to determine the size of the iNKT cell population. An antibody for CD3 was used in combination with a fluorescent α-GalCer-loaded CD1d tetramer to identify the iNKT cells and the gating strategy used to identify them is displayed in (A). (B) The numbers of iNKT cells from the spleens of mice are graphed. Blood was taken two and five hours following vaccination and the serum was analysed for IL-4 (C) and IFN-γ (D), respectively, with a multiplex bead-based cytokine assay. The results are indicative of three experiments, each with five mice per group. *P < 0.05, **P < 0.01 (one-way ANOVA with a Bonferroni post test).

4.2.5 iNKT cells license dendritic cells following tumour/α-GalCer vaccination in mice with established AML

Having found that the vaccine could significantly activate iNKT cells in hosts with established tumours, the ability of the activated iNKT cells to license DCs was analysed. Mice were vaccinated seven or fourteen days after AML challenge and the spleens were removed 24 hours later for analysis by flow cytometry. An antibody specific for CD11c was used to identify all splenic DCs, with the gating strategy used displayed in figure 4.7A. The activation status of the DCs was determined by expression of CD40 and CD86, which are known to be upregulated in response to a variety of different activation stimuli, including licensing by iNKT cells. No significant difference in the size of the CD11c$^+$ DC population was observed between any of the groups tested (Figure 4.7B). The DCs from vaccinated mice did however express significantly higher levels of the co-stimulatory molecules CD40 and CD86, even in the presence of established AML, which provides some indication that AML-associated suppression did not cause the general inhibition of CD11c$^+$ DCs (Figure 4.7C-D).

As shown in figure 4.4, langerin$^+$ CD8α^+ DCs are essential for the vaccine to generate an effective anti-AML immune response, therefore the suppression of this subset would likely have a significant affect on the vaccine-induced immune response. The activation status of langerin$^+$ CD8α^+ DCs was therefore analysed in therapeutically vaccinated *lang*-EGFP mice so that langerin-expressing cells could be detected. A decrease in the langerin$^+$ CD8α^+ DC population was found in the spleens of vaccinated mice (Figure 4.8B), which corresponds to studies demonstrating that splenic langerin$^+$ CD8α^+ DCs are no longer detected in the spleen following activation with microbial agonists (105, 395). Similar to the total splenic DC population, the langerin$^+$ CD8α^+ DCs upregulated CD40 following vaccination, regardless of the presence of established AML (Figure 4.8C). However, there was less upregulation of CD86 on langerin$^+$ CD8α^+ DCs when the vaccine was administered into mice with well established AML, suggesting that AML-

induced suppression was able to reduce the extent of langerin$^+$ CD8α^+ DC activation following tumour/α-GalCer vaccination (Figure 4.8D).

Langerin$^+$ CD8α^+ DCs produce large quantities of IL-12 (over 500 pg/ml of blood) following activation, which can be detected in the serum of mice five hours following vaccination (107). A multiplex bead-based cytokine assay was performed on host serum to determine if the vaccine induced efficient IL-12 production by langerin$^+$ CD8α^+ DCs in AML-bearing mice. The serum IL-12 levels five hours after vaccination was not significantly different between mice with or without established AML (Figure 4.8E). These data suggest that the vaccine-induced activation of langerin$^+$ CD8α^+ DCs is suppressed in mice with established AML, however their function was not impaired.

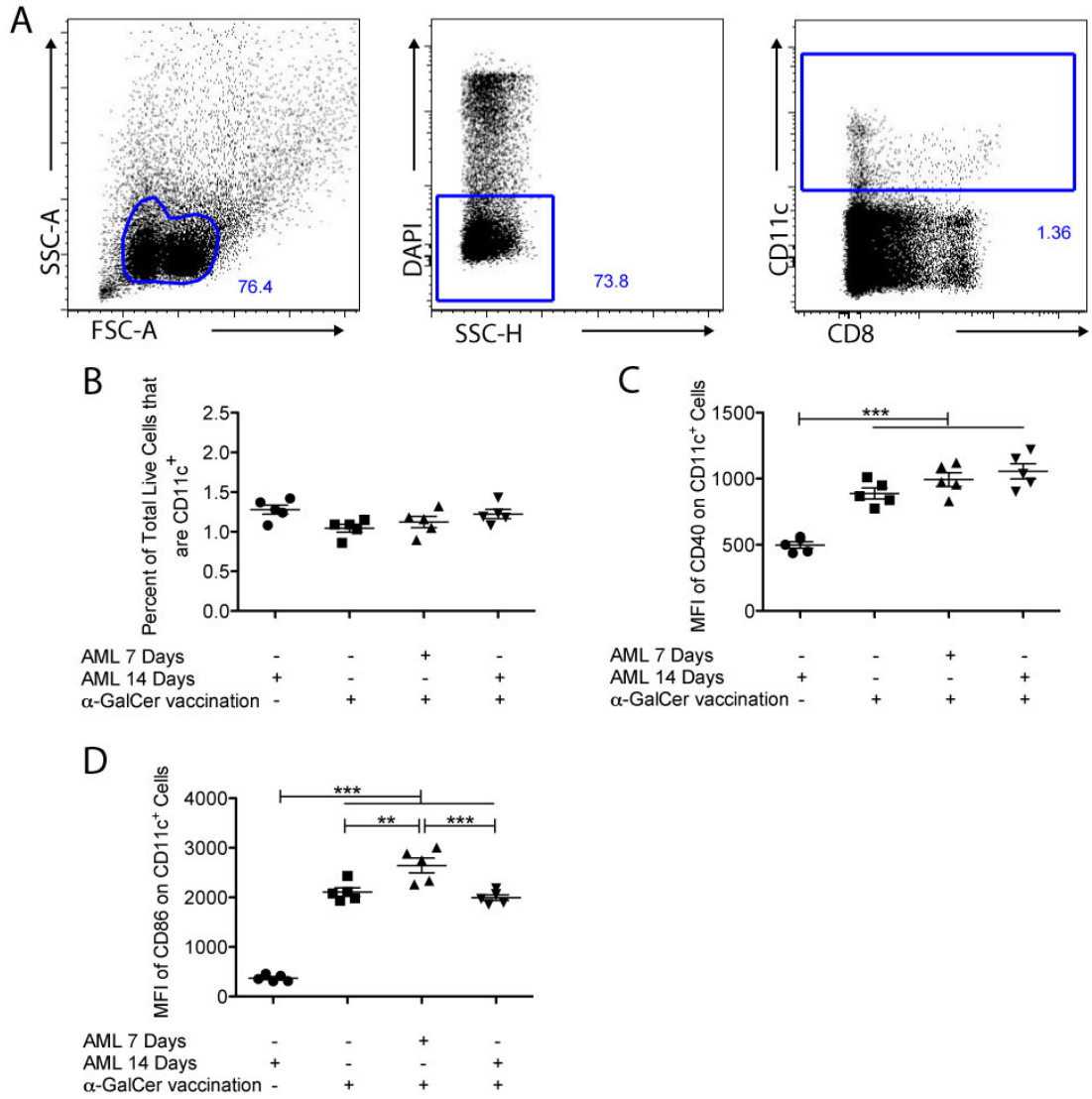


Figure 4.7: Splenic DCs are activated by the tumour/α-GalCer vaccine in hosts with established AML. *Lang*-EGFP mice were administered 1x10^5 AML cells intravenously seven or fourteen days before vaccination with 7.5x10^5 irradiated AML cells loaded with α-GalCer. Mice were culled 24 hours following vaccination and the splenic CD11c$^+$ DC population was analysed by flow cytometry using the gating strategy displayed in (A). The splenic CD11c$^+$ DC populations in each group are compared in (B). CD40 (C) and CD86 (D) were used to determine the activation state of the CD11c$^+$ DCs. The results are indicative of three experiments, with five mice per group. $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA with a Bonferroni post test).

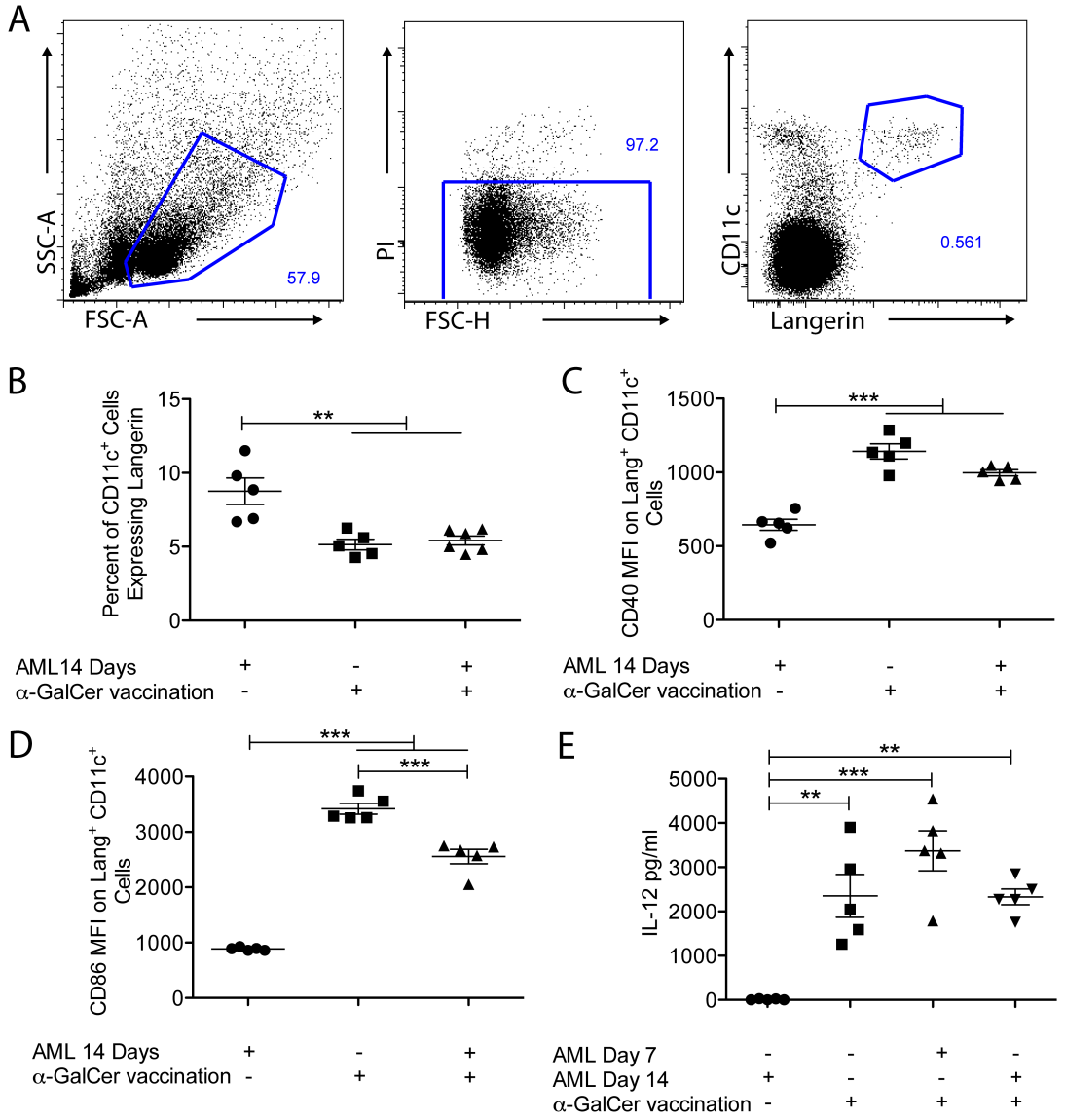


Figure 4.8: The presence of established AML on the vaccine-induced activation of langerin$^+$ CD8α^+ DCs. *Lang*-EGFP mice were administered 1x10^5 AML cells intravenously and were vaccinated with 7.5x10^5 irradiated AML cells loaded with α-GalCer fourteen days later. Mice were culled 24 hours following vaccination and the splenic langerin$^+$ CD8α^+ DC population was analysed by flow cytometry. An anti-CD11c antibody was used to isolate the DCs in the spleen and GFP expression was used to identify langerin$^+$ DCs. The strategy used to isolate the CD11c$^+$ langerin$^+$ DCs is displayed in (A). The number of langerin$^+$ CD8α^+ DCs detected from the mice in each group is graphed in (B) and CD40 (C) and CD86 (D) was used to detect their activation status. Five hours following vaccination mice were bled and the serum was analysed for IL-12 (E). The results are indicative of two independent experiments, each with five mice per group. $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA with a Bonferroni post test).

4.2.6 Established AML does not inhibit non-specific CD8$^+$ T cell activation

It was next examined if CD8$^+$ T cells that are not specific for AML could be activated in hosts with established AML, as this would indicate whether AML induced a generalised suppression of T cell activity. Mice with AML for seven days, or AML-free controls were administered a small population of naive CD8$^+$ T cells from OT-I mice. The OT-I T cells were then stimulated by intravenous administration of OVA protein, which contains the SIINFEKL peptide sequence that serves as the MHC class I binding epitope recognised by OT-I T cells, and α-GalCer to serve as an immune adjuvant. The proportion of CD8$^+$ cells that were OT-I T cells and activation status of the OT-I T cells was determined seven days later. Mice that were administered OVA/α-GalCer in the absence of AML displayed an increased proportion of CD8$^+$ cells that were OT-I T cells in the spleen, compared to mice that were not vaccinated (Figure 4.9A). Treatment with OVA/α-GalCer also differentiated the naïve OT-I T cells into effector T cells as determined by the down-regulation of CD62L and the upregulation of CD44 (Figure 4.9 B–C). Importantly, the OT-I T cell population in mice with established AML also increased in size in response to OVA/α-GalCer vaccination and displayed a CD62L$^-$ CD44$^+$ phenotype characteristic of effector T cells. However, the increase in OT-I T cells in the spleen was significantly lower when stimulated in the presence of AML and they expressed lower levels of CD44, indicating that some non-specific suppression was occurring at the point of T cell activation in the vaccine-induced immune response.

To determine if OT-I T cells stimulated in the presence of established AML had the same ability to mediate antigen-specific killing, the ability of the OT-I T cells to kill target cells was assessed. Mice were vaccinated seven days after AML challenge, and then nine days later syngenic splenocytes were injected as target cells that had been loaded with SIINFEKL peptide and stained with the fluorescent dye CFSE. The following day blood was removed from each mouse to analyse, by flow cytometry, the proportion of target cells that were killed by the OVA/α-GalCer activated OT-I T cells (Figure 4.9D). Mice that

were vaccinated in the absence of AML challenge displayed a significant amount of target cell killing compared to the AML-bearing mice that did not receive OVA/α-GalCer vaccination. Interestingly, mice that were vaccinated seven days after AML challenge displayed a similar level of target cell killing as the AML-free controls. It has previously been demonstrated that the activation of OT-I T cells by OVA/α-GalCer vaccination is dependent on cross-priming by langerin$^+$ CD8α^+ DCs and therefore OT-I T cell activation can be indicative of langerin$^+$ CD8α^+ DC function (107). Overall, these results suggest that the presence of established AML does suppress general T cell activation, however langerin$^+$ CD8α^+ DCs were still able to significantly activate CD8$^+$ T cells that are not specific for AML, into effective cytotoxic lymphocytes.

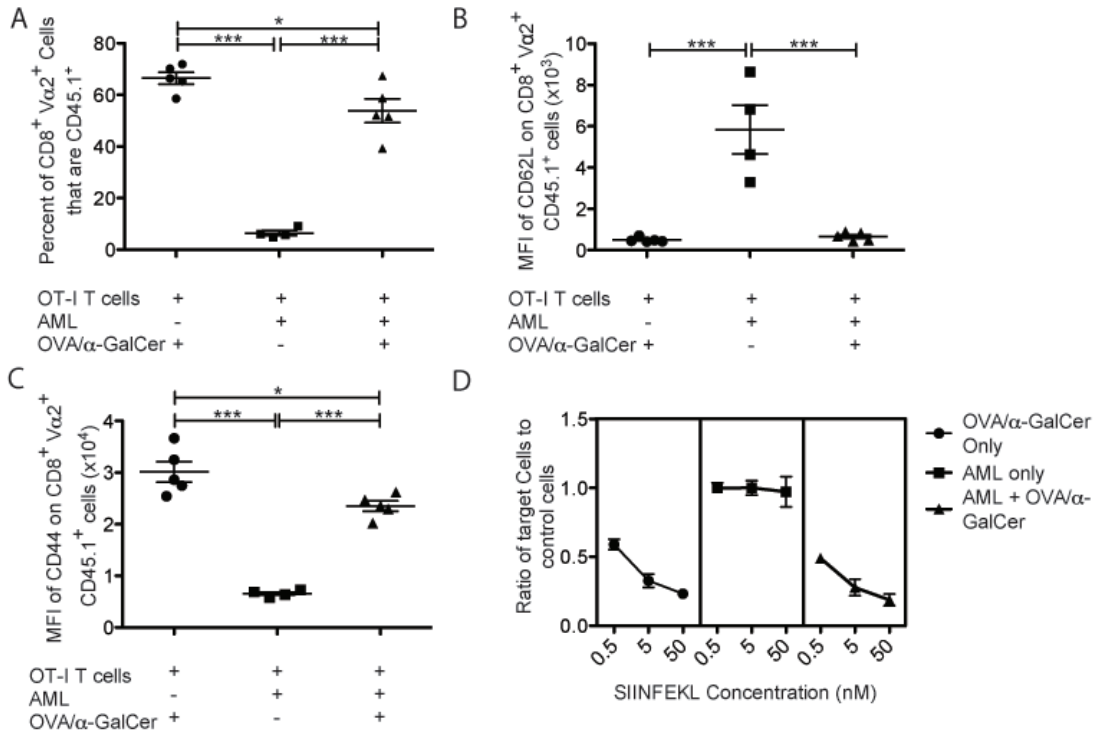


Figure 4.9: Established AML does not prevent T cell activation in an antigen-independent manner. (A, B and C) Mice were administered 1x10^5 AML cells intravenously and six days later mice were injected intravenously with 5x10^6 naïve OT-I T cells. The following day mice were vaccinated with the OVA protein and α-GalCer. Seven days later the spleens were removed and the OT-I T cell population was analysed by flow cytometry for (A) population number, (B) CD62L expression and (C) CD44 expression. OT-I T cells were identified based on cell expression of CD8, Va2 and CD45.1 using the gating strategy displayed in figure 3.7A. (D) Target cells, consisting of splenocytes loaded with one of three concentrations of SIINFKEL (0.5 ng, 5 ng and 50 ng) were administered nine days following vaccination. The groups of target cells could be differentiated based on different CFSE staining concentrations, using the gating strategy seen in figure 3.12. Blood was taken from each mouse the day after target cell administration, and the ratio of each target cell subset was compared to a non-target control to determine the proportion of target cells that were killed. A–C was completed once on spleens and twice on blood, each with five mice per group. (D) represents one experiment with five mice per group. *P < 0.05, ***P < 0.001 (one-way ANOVA with a Bonferroni post test).

4.2.7 Established leukaemia prevents AML-specific CD4$^+$ but not CD8$^+$ T cells from being activated by tumour/α-GalCer vaccination

Having found that non-specific CD8$^+$ T cells could still be activated in the presence of established AML, it was next determined whether AML-specific T cells could also be activated in the same setting. This is relevant as T_{regs} can suppress T cells through antigen-dependent cell-to-cell contact, which enables them to inhibit tumour-specific T cells without hindering non-specific T cells (396). The ability of the vaccine to induce AML antigen-dependent IFN-γ production was therefore assessed in the presence or absence of established AML. The tumour/α-GalCer vaccine was administered seven days following challenge with AML cells and the vaccine-induced immune response was left to develop for one week to ensure that the vaccine had ample time to generate effector T cells. Mice were then culled, the spleens were removed and the splenocytes were cultured for 24 hours with or without DCs loaded with lysate from AML cells for an antigen-dependent restimulation. After 24-hours of culture the supernatant was removed and a multiplex bead-based cytokine assay was performed to determine how much IFN-γ was produced by each group. Antigen-dependent restimulation of the splenocytes resulted in a significant increase in the production of IFN-γ from each group. The splenocytes from vaccinated mice produced more IFN-γ during the 24-hour culture than the groups that were not vaccinated and this was not reduced when the splenocytes were from mice with established AML (Figure 4.10). This result indicates that although the presence of established AML abrogates the protective benefit provided by the tumour/α-GalCer vaccine, it did not prevent the production of IFN-γ by AML-specific T cells.

To determine if the presence of established AML altered the phenotype or IFN-γ production of either CD4$^+$ or CD8$^+$ T cells separately following vaccination, flow cytometry was performed on splenocytes harvested from therapeutically vaccinated mice seven days after vaccination. The gating strategy used to identify CD4$^+$ and CD8$^+$ T cells is depicted in figure 4.11A. Mice that were vaccinated in the absence of established AML had CD8$^+$ T

cells that displayed a significant upregulation of the activation marker CD44 and more of them produced IFN-γ when compared to CD8$^+$ T cells from non-vaccinated mice (Figure 4.11 B–C). The CD8$^+$ T cells from mice with AML were similarly activated in response to vaccination. CD4$^+$ T cells from mice vaccinated in the absence of AML challenge also upregulated CD44 and more of them produced IFN-γ when compared to the non-vaccinated control group (Figure 4.11 D–E). However, when vaccinated in the presence of established AML the CD4$^+$ T cells did not display the same upregulation of CD44 as the vaccinated control group and less of them were found to be IFN-γ^+. These data suggest that CD4$^+$ T cell activation is inhibited in hosts with established AML but CD8$^+$ T cell activation is unimpeded. It is possible that CD4$^+$ T cell inhibition may be why the vaccine is ineffective in mice with established AML.

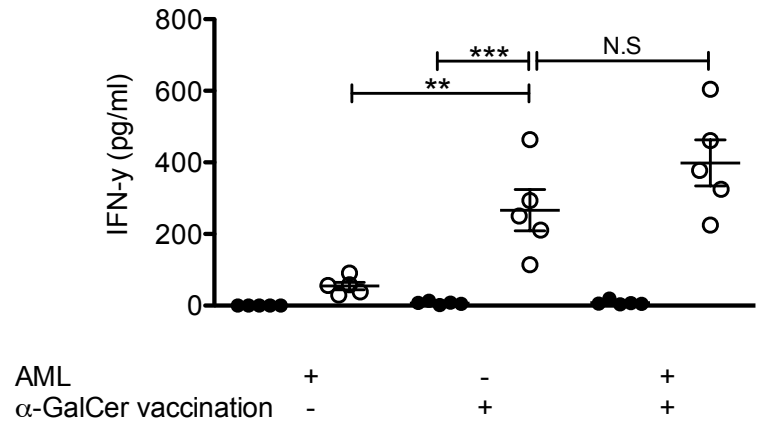


Figure 4.10: Antigen-specific T cells can be activated in the presence of established AML. C57BL/6 mice were administered 1x10^5 AML cells intravenously and were vaccinated with 7.5x10^5 irradiated AML cells loaded with α-GalCer seven days later. The vaccine-induced immune response was then allowed to develop for one week before the mice were culled, the spleens were removed and 1x10^6 splenocytes were cultured for 24 hours. A multiplex bead-based cytokine assay was performed on the resulting supernatant and the level of IFN-γ produced was determined. One set of samples was not restimulated (closed circles) and another set was restimulated by culture with 1x10^5 DCs loaded with the lysate from the AML cell line C1498, at a ratio of one DC to the lysate of six AML cells (open circles). This graph represents three experiments, each of which had five mice per group. *P < 0.05, **P < 0.01 (one-way ANOVA with a Bonferroni post test).

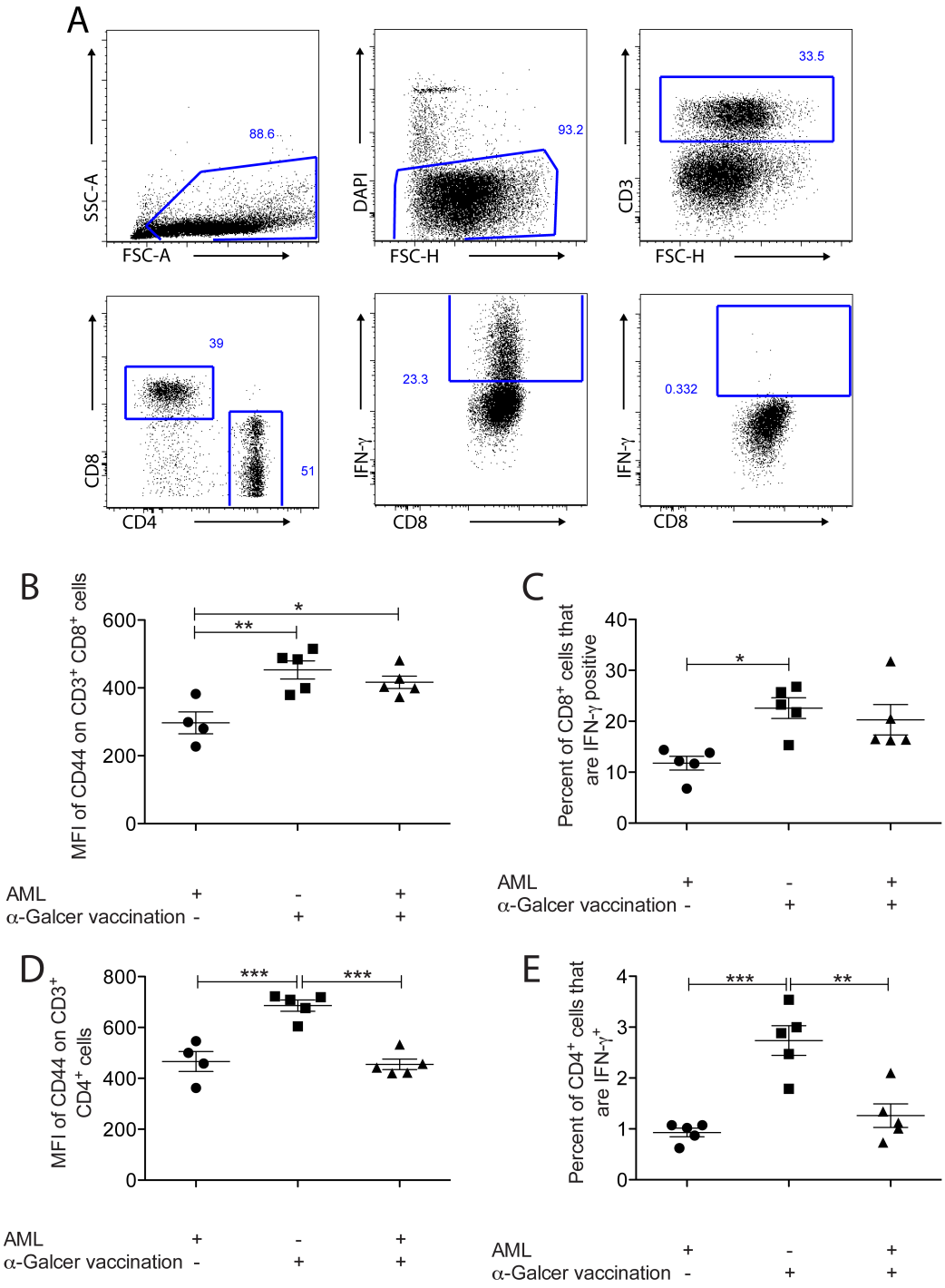


Figure 4.11: Vaccine-induced CD4$^+$ T cells but not CD8$^+$ T cells are suppressed by the presence of established AML. C57BL/6 mice were administered 1x10^5 AML cells intravenously and were vaccinated with 7.5x10^5 irradiated AML cells loaded with α-GalCer seven days later. The vaccine-induced immune response was then allowed to develop for one week before the mice were culled and the spleens removed. The activation status of the splenic T cells was then analysed by flow cytometry using an antibody for CD3 to identify T cells and antibodies for CD4 and CD8 to differentiate

the T cell populations. Anti-CD44 was used to determine the activation status of the T cells and an IFN-γ antibody to determine their functionality. The gating strategies are displayed in A. (B) The MFI of CD44 on CD8$^+$ T cells and (D) CD4$^+$ T cells. The percent of CD8$^+$ or CD4$^+$ T cells that were IFN-γ positive is displayed in (C) and (E), respectively. This figure represents two experiments with five mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with a Bonferroni post test).

4.2.8 AML-induced T_{regs} suppress the vaccine-mediated protection

The previous result suggested that AML-specific CD4$^+$ T cells are inhibited by the presence of established AML, however the mode by which this occurs had not been analysed. It was therefore determined if the presence of established AML was associated with elevated levels of suppressor cells. The immune suppressor cell T_{regs}, are often found in higher numbers in AML-hosts and therefore the number of T_{regs} in mice with established AML was analysed (282, 397). Mice were administered 1x10^5 AML cells intravenously and the tumour was left to develop for 20 days to exacerbate the potential development of AML-associated suppressor cells. Mice were then culled and the number of T_{regs} in the livers and spleens was determined by flow cytometry. The liver was analysed as this tissue most consistently developed tumours in hosts and as T_{regs} have previously been found to increase in the liver in C1498 treated hosts (292). The identification of T_{regs} was based on expression of CD4 and Foxp3 (340); the gating used to detect these cells is displayed in figure 4.12A. Foxp3 expression was determined by intracellular antibody staining against Foxp3 or by conducting experiments in Foxp3-EGFP mice that express enhanced green fluorescent protein under the Foxp3 promoter (Figure 4.12 B–C). Mice with established AML were found to have significantly increased numbers of CD4$^+$ FoxP3$^+$ cells in the liver compared to non-AML challenged control mice (from 4.1% of CD4$^+$ cells in the naïve mice to 29.7% in AML-bearing mice; Figure 4.12 D–E), however no significant difference was found in the spleens (Appendix 8).

To determine if the AML-associated increase in T_{regs} was responsible for the lack of protection provided by the vaccine in the therapeutic setting, T_{regs} were depleted from AML-bearing mice prior to vaccination using the monoclonal antibody PC61. This antibody specifically binds to the receptor CD25, which is expressed at high levels on T_{regs}, resulting in their depletion (398). After intraperitoneal administration, PC61 depleted 97% of T_{regs} in the spleen and blood for a minimum of one week following treatment (Appendix 9). In order to

avoid depleting vaccine-induced effector CD4$^+$ and CD8$^+$ T cells, which upregulate CD25 upon activation, PC61 was administered six days prior to vaccination. Treating mice with PC61 at this time enabled the depletion of T_{regs}, while the excess circulating PC61 antibody will largely be cleared before the development of effector CD4$^+$ or CD8$^+$ T cells. Mice were challenged with AML cells and the following day one group of mice received a single injection of PC61. One week after AML challenge the mice were vaccinated and their symptom-free survival was analysed. Mice depleted of T_{regs} prior to therapeutic vaccination had an increase in median symptom-free survival by 10 days (Figure 4.13A). To ensure that PC61 was not providing mice with any protective benefit that is independent from the vaccine, the symptom-free survival of mice treated with PC61 alone or PC61 plus the tumour/α-GalCer vaccine was compared (Figure 4.13B). Administration of PC61 alone provided no protection against onset of AML-associated symptoms and therefore the protection seen in figure 4.13A was due to the efficacy of the tumour/α-GalCer vaccine in the absence of T_{regs}. Therefore, tumour-induced T_{regs} are able to suppress to vaccine-induced immune response.

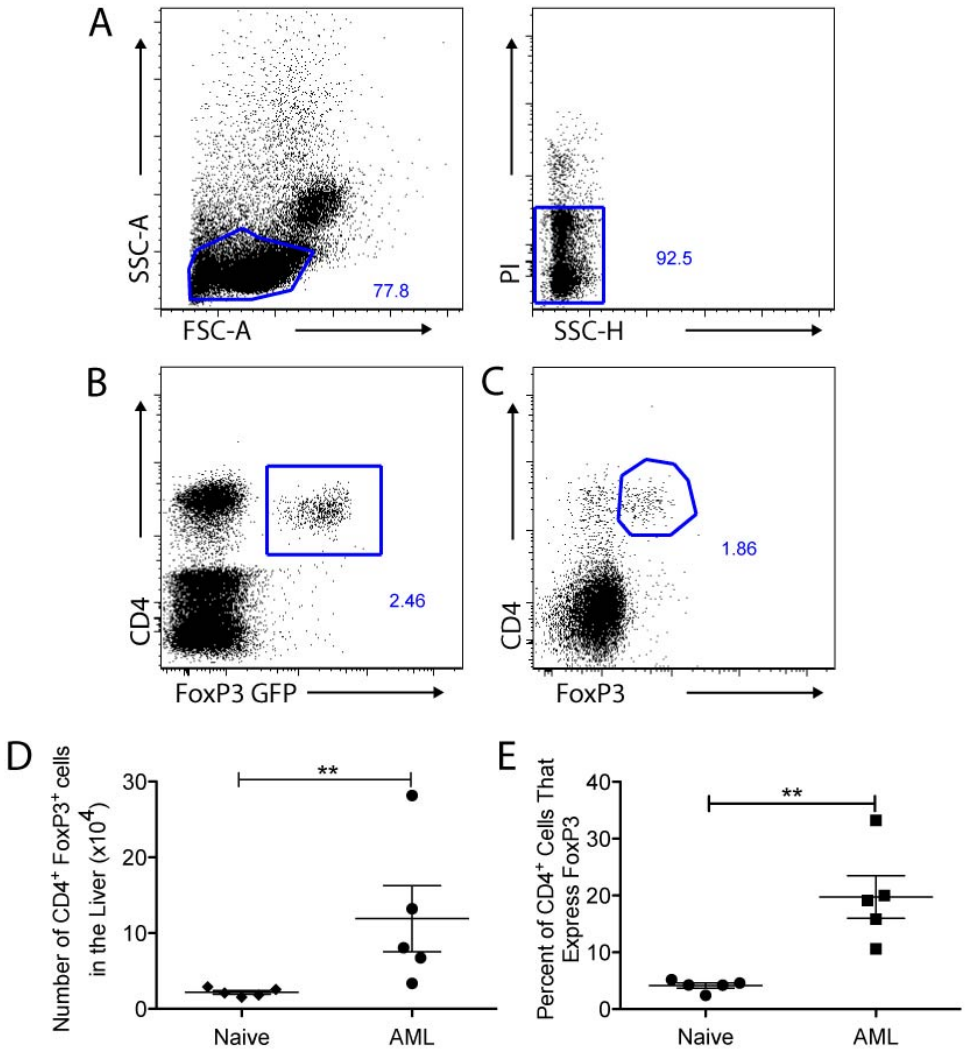


Figure 4.12: T_{regs} are significantly elevated in AML-bearing mice. Mice were administered 1x10^5 AML cells intravenously and were culled 20 days later. The population of T_{regs} in the liver was analysed by flow cytometry using the gating strategy displayed in (A). Foxp3 expression was determined by using a CD4 antibody in combination with GFP expression from FoxP3-GFP mice (B) or a Foxp3 antibody (C). The levels of T_{regs} between the two groups are graphed in (D) and (E), using a Foxp3 antibody and Foxp3-GFP mice respectively. This represents three experiments, with five mice per group $^{**}P < 0.01$ (t-test with Mann Whitney).

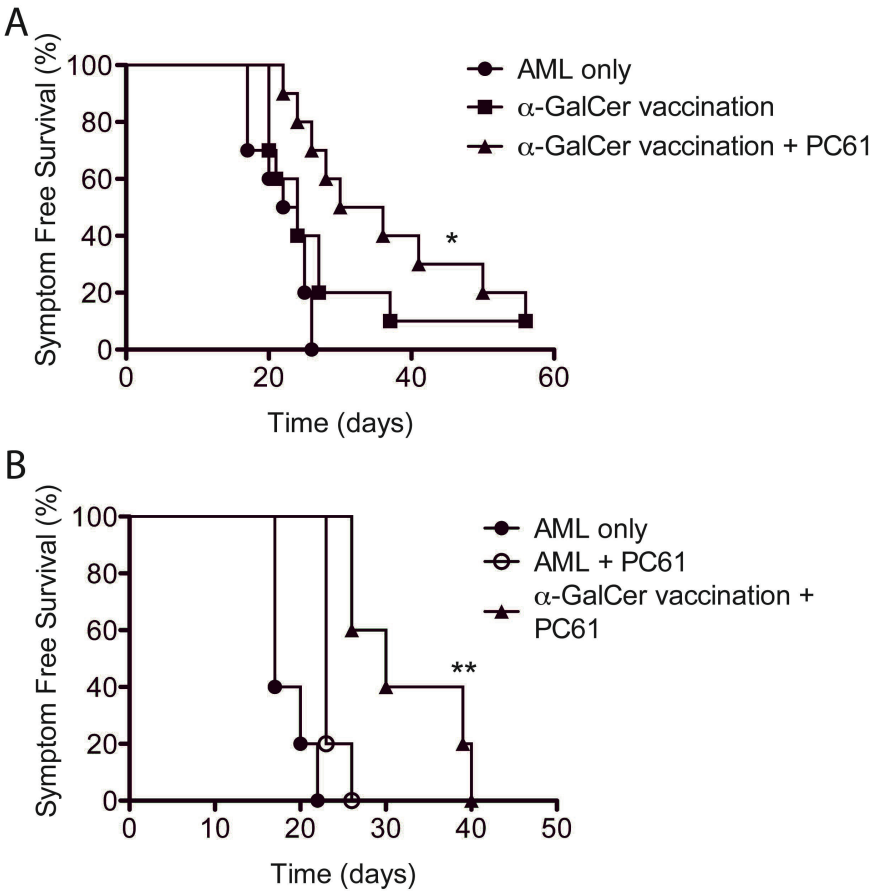


Figure 4.13: AML-associated T_{regs} inhibit the vaccine-induced protection against AML. (A) Depicts the symptom-free survival of mice that were administered $1x10^5$ AML cells and were vaccinated with $7.5x10^6$ irradiated AML cells loaded with α-GalCer seven days later. One group of vaccinated mice were depleted of CD25$^+$ cells (T_{regs}) prior to vaccination by intraperitoneal injection of 100 mg of PC61 the day after AML challenge. (B) The survival of AML challenged mice treated either with PC61 alone or PC61 in addition to tumour/α-GalCer vaccination is compared. A represents two experiments with five mice per group *P < 0.05 (log-rank Mantel-Cox test). (B) represents one experiment with five mice per group. **P < 0.01 (log-rank Mantel-Cox test).

4.2.9 AML establishment increases the number of myeloid derived suppressor cells

Another immunosuppressive cell type associated with tumour development is the MDSC. A substantially higher number of MDSCs are found in the blood of high-risk leukaemia patients compared to low-risk leukemic patients or healthy controls, and these have been associated with T cell tolerance (399). The MDSC population in mice with established AML was therefore analysed to determine if they could be involved in inhibiting the vaccine-induced AML protection in the therapeutic setting. Mice were challenged with AML cells, which were allowed to grow and develop for 20 days in order to exacerbate the AML-associated suppression. At day 20, the mice were culled and the number of MDSCs in the spleen was determined by flow cytometry, using antibodies to identify the CD11b$^+$ Ly6G$^+$ and the CD11b$^+$ Ly6C$^+$ MDSCs; the gating strategy is displayed in figure 4.14A (298). Interestingly, a significant increase in the Ly6G$^+$ MDSC population was observed in the spleens of AML-bearing mice relative to naïve mice, however no difference was observed in the CD11b$^+$ Ly6C$^+$ MDSCs (Figure 4.14 B and C). These data therefore indicate the possibility that MDSCs, like T_{regs}, inhibit the vaccine-induced immune response.

The suppressive quality of the MDSCs from these mice was also analysed by determining their ability to suppress T cell proliferation. Naive T cells stained with CFSE were cultured with MDSCs isolated from the spleens of AML-bearing or naïve mice. The naïve T cells were then stimulated with anti-CD3 and anti-CD28 antibodies and T cell proliferation was determined by CFSE dilution. The MDSCs from mice with established AML significantly suppressed the proliferation of CD4$^+$ T cells but not CD8$^+$ T cells, when compared to the culture of MDSCs from naïve mice (Figure 4.14E). Thus, the MDSCs from AML hosts were more immunosuppressive.

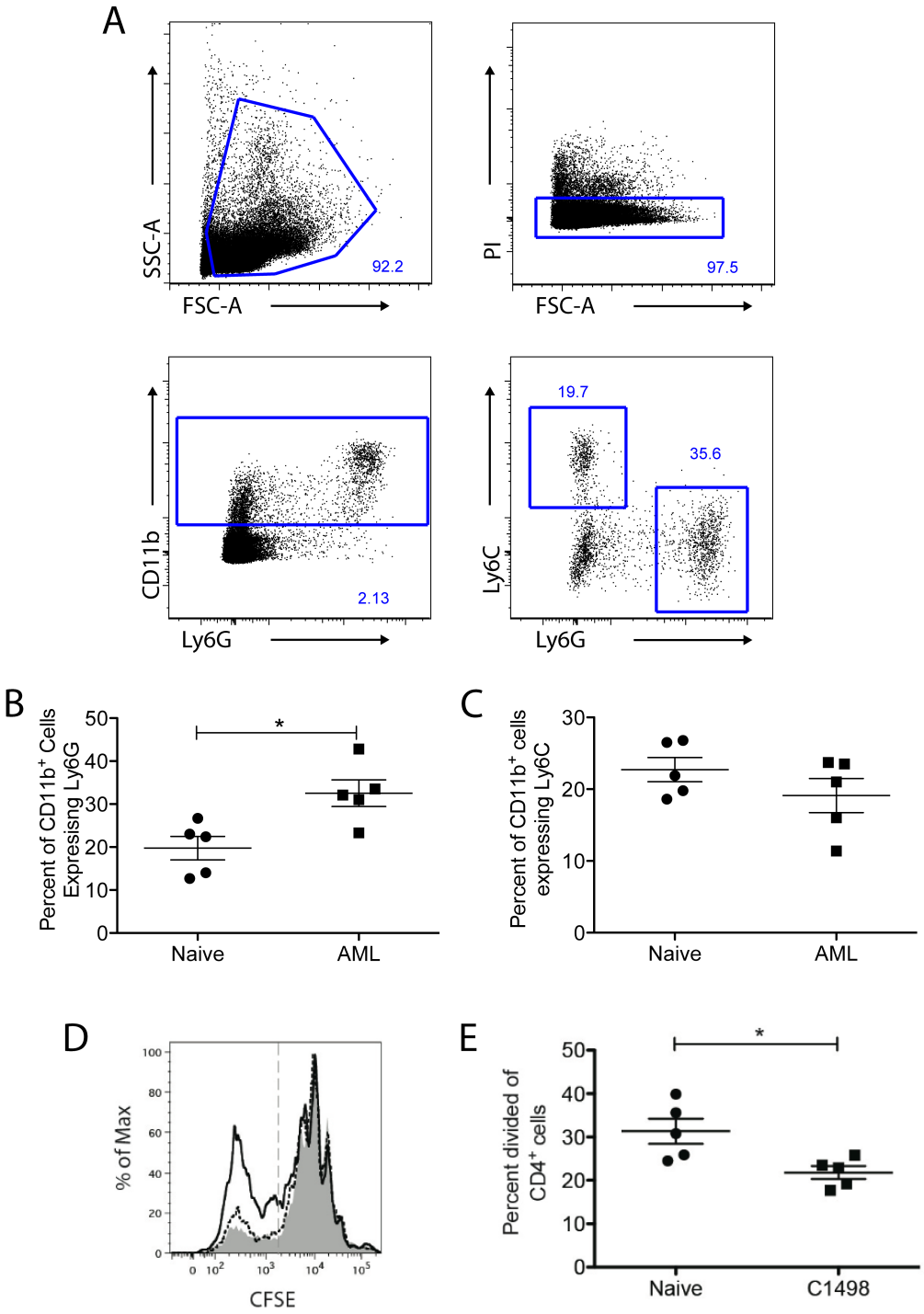


Figure 4.14: AML-bearing mice have a significantly elevated number of MDSCs in the spleen. C57BL/6 mice were administered 1x10^5 AML cells intravenously and were culled 20 days later. The MDSC population in the spleen was analysed by flow cytometry using antibodies to identify the CD11b$^+$ and Ly6G$^+$ granulocytic MDSCs, using the gating strategy displayed in (A). The percent of CD11b$^+$ cells that were Ly6G$^+$ (B) or Ly6C$^+$ (C) between the two groups is graphed. CD11b$^+$ cells were isolated from the spleens and cultured with CFSE labelled T cells (at a 1:1 ratio) that were stimulated with anti-CD3 and anti-CD28 for 72 hours. The ability of the MDSCs

to suppress T cell proliferation was then analysed by CFSE dilution using an anti-CD3 antibody to isolate the T cells. (D) A representative histogram of CFSE dilution is displayed for CD4$^+$ cells cultured with CD11b$^+$ splenocytes from naïve mice (black line) or tumour challenged mice (dotted line). Unstimulated T cells are represented in the plots as the shaded section. (E) The percent of CD4$^+$ cells that had diluted CFSE is graphed. This represents three experiments with five mice per group *P < 0.05 (t-test with Mann Whitney).

4.2.10 C1498 AML cells suppress T cell proliferation

Having found that there are a variety of suppressive factors involved in hosts with established tumours it is possible that the tumour cells themselves are able to suppress the function of the vaccine induced immune response. To determine if C1498 cells have T cell suppressive capabilities, C1498 cells or CD11b$^+$ splenocytes from naive mice were cultured with CFSE stained naïve T cells. The T cells were then stimulated with anti-CD3 and anti-CD28 antibodies and T cell proliferation was determined by CFSE dilution. Both CD4$^+$ and CD8$^+$ T cells were significantly suppressed when cultured with C1498 cells. Interestingly, CD4$^+$ T cell proliferation was significantly more suppressed when cultured with C1498 cells than CD8$^+$ T cells. This may explain why the vaccine-induced CD4$^+$ T cells were suppressed in the presence of established tumours.

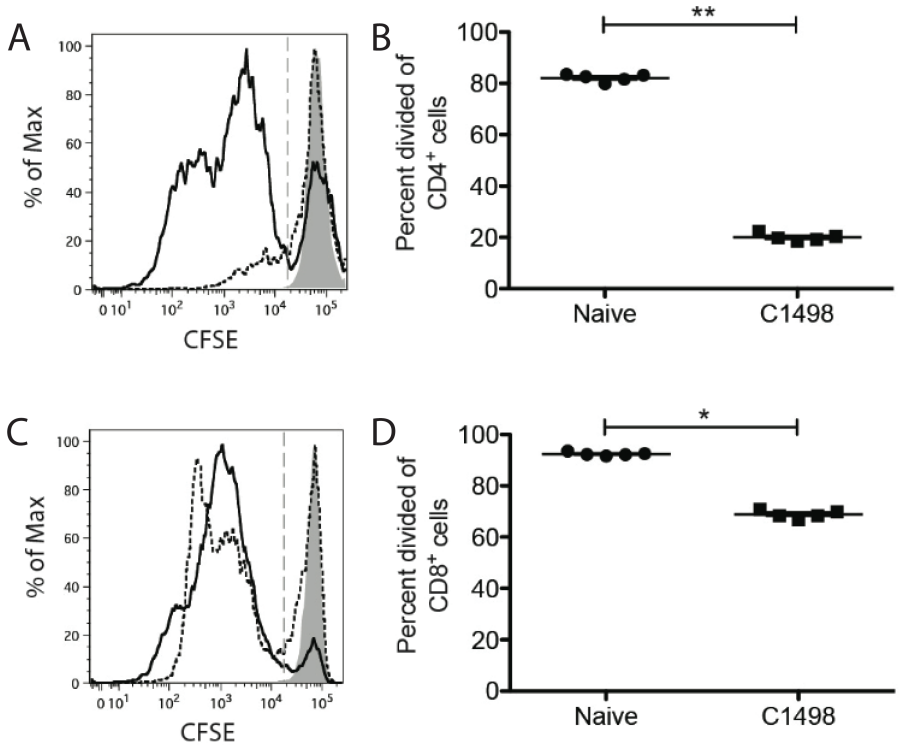


Figure 4.15: C1498 cells suppress T cell proliferation. C1498 cells or CD11b$^+$ cells isolated from the spleens of naive mice were cultured with CFSE labelled naïve T cells that were stimulated with anti-CD3 and anti-CD28 for 72 hours. The ability of the C1498 cells to suppress T cell proliferation was then analysed by CFSE dilution using an anti-CD4 and anti-CD8 antibodies to identify the T cells. A representative histogram of CFSE dilution is displayed for CD4$^+$ (A) and CD8$^+$ (C) cells cultured with naïve splenocytes (black line) or C1498 cells (dotted line). Unstimulated T cells are represented in the plots as the shaded section. The percent of CD4$^+$ (B) or CD8$^+$ (D) cells that had diluted CFSE is graphed. This figure represents three experiments, each with five mice per group. *P < 0.05, **P < 0.01 (t-test with Mann Whitney).

4.2.11 The effect of chemotherapy on AML-associated immune suppression

As the first line of treatment for AML is chemotherapy, which often drives patients into remission, it was of interest to determine if chemotherapy would alter the immune environment in a manner that favours immunotherapy. Chemotherapies, which can significantly reduce tumour burdens, are often associated with the generation of an immunosuppressive environment (400). As this may impact the ability of a vaccine to be effective when administered during remission, the immune system had to be analysed following chemotherapy. For this purpose, we examined the effect of the chemotherapy cytarabine. Cytarabine was chosen because it is used clinically for the treatment of AML and therefore is of relevance to this model (401). In addition, after cytarabine treatment the neutrophil and lymphocyte compartment has been demonstrated to return to normal within 6 days and this treatment provided an environment for effective immunotherapy (402). Therefore, the effect of cytarabine treatment on T cells was analysed in mice with AML. While the T cell population was reduced in the spleens of mice with untreated AML, mice treated with cytarabine after AML administration had similar numbers of CD8$^+$ and CD4$^+$ T cells as naïve controls (Figure 4.16 A–B). Moreover, while expression of CD44 on CD8$^+$ and CD4$^+$ T cells was reduced in animals with AML, whereas in cytarabine-treated animals the expression was similar to that seen in naïve healthy controls (Figure 4.16 C–D).

The population of suppressor cells in AML bearing mice was analysed in hosts receiving either no treatment or the chemotherapeutic agent cytarabine to determine if cytarabine reduced immune suppression. Cytarabine was administered 24 hours following AML challenge and nineteen days later the immune environment of the host was analysed. The period of nineteen days following cytarabine treatment was chosen, as this would allow repopulation of the host's immune compartments that had been depleted by the chemotherapeutic treatment. The T_{reg} population in the liver was analysed and as was seen previously was significantly larger in the AML only control

group relative to naïve mice (Figure 4.17). Mice treated with cytarabine following AML challenge displayed no reduction in the T_{regs} population in the liver relative to the tumour only control.

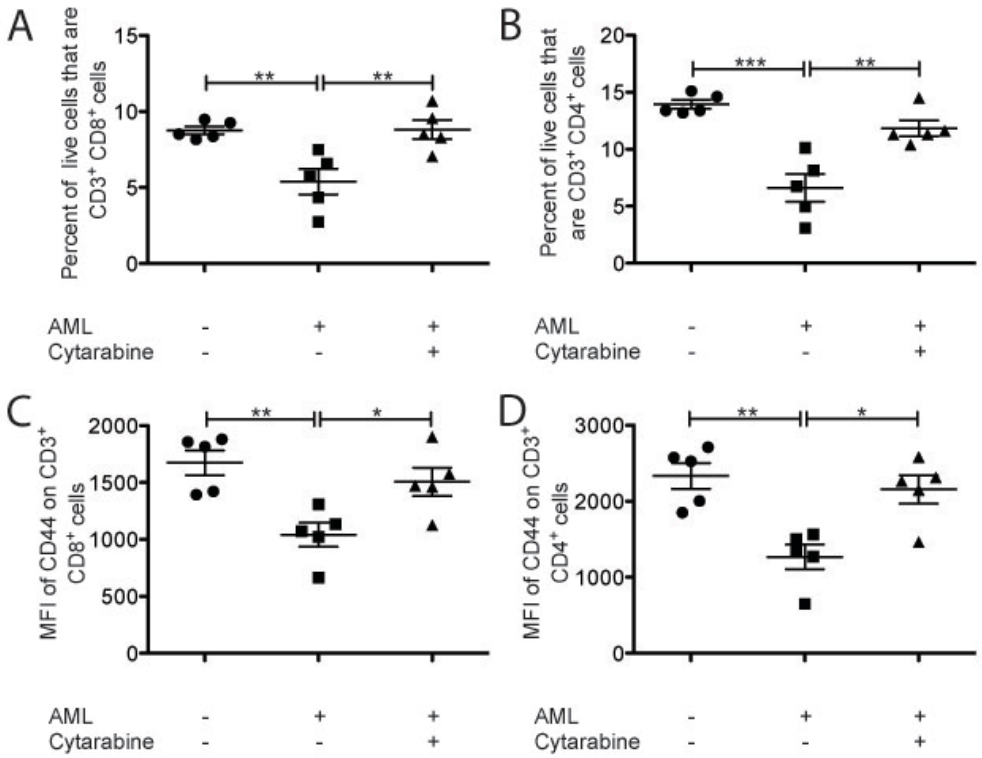


Figure 4.16: Cytarabine reduces AML-associated T cell suppression. Mice were challenged with 1x10^5 AML cells and 24 hours later a course of chemotherapy was initiated. Three doses of cytarabine were administered ten hours apart, each consisting of 3 mg administered intraperitoneally. (A–D) The T cell populations in the spleen were analysed. T cells were identified as CD3$^+$ cells expressing either CD8 or CD4. The proportion of live cells expressing CD3 and CD8 (A) or CD3 and CD4 (B) is graphed. The MFI of CD44 on the CD3$^+$ CD8$^+$ cells (C) and CD3$^+$ CD4$^+$ cells (D) is graphed. This figure represents three experiments, each with five mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with a Bonferroni post test).

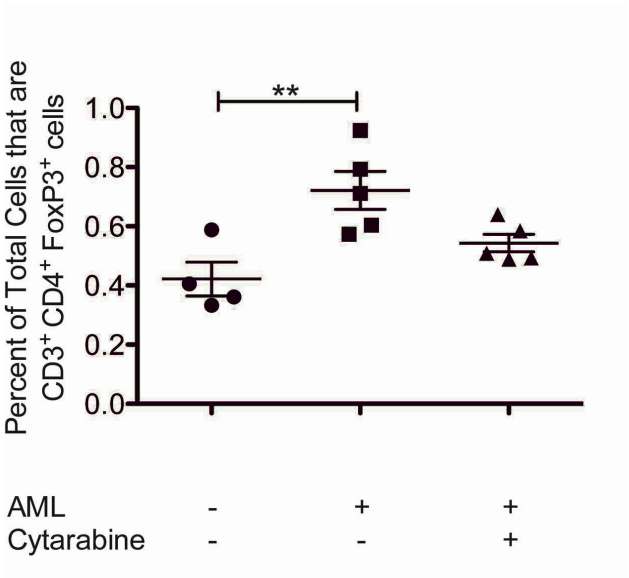


Figure 4.17: The effect of cytarabine on AML-associated T_{regs}. Mice were administered 1x10^5 AML cells intravenously and the following day one group of mice was intraperitoneally administered three 3 mg doses of cytarabine, administered ten hours apart. The mice were culled 20 days after AML challenge and the population of T_{regs} in the liver was analysed by flow cytometry using the gating strategy displayed in 4.12A. An antibody was used to identify the Foxp3 positive cells. The graph compares the percent of all live cells from the liver that are CD3$^+$ CD4$^+$ and FoxP3$^+$ cells. This represents a single experiment with five mice per group. ***P* < 0.01 (one-way ANOVA with a Bonferroni post test).

4.2.12 The tumour/α-GalCer vaccine is effective following chemotherapy

As most AML patients achieve remission with chemotherapy, the most likely use for a vaccine-based therapy will be an attempt to prevent relapse post-chemotherapy. To examine whether the tumour/α-GalCer vaccine would have any efficacy in this setting, AML-bearing mice were administered cytarabine and then were vaccinated while still symptom-free, or received no further treatment. Cytarabine treatment alone was sufficient to significantly delay the onset of symptoms of tumour-burden when compared to untreated animals, with no increase in protection associated with vaccination (Figure 4.18A). However, it was when the surviving animals were rechallenged with an elevated dose of AML intravenously that the benefit of the combined treatment became most apparent. All animals that received the vaccine post-chemotherapy were completely protected from rechallenge, whereas all animals that received cytarabine chemotherapy alone developed symptoms associated with AML-burden within 20 days (Figure 4.18B). Thus, chemotherapy-induced remission provided an environment for effective vaccination with tumour/α-GalCer to protect against rechallenge.

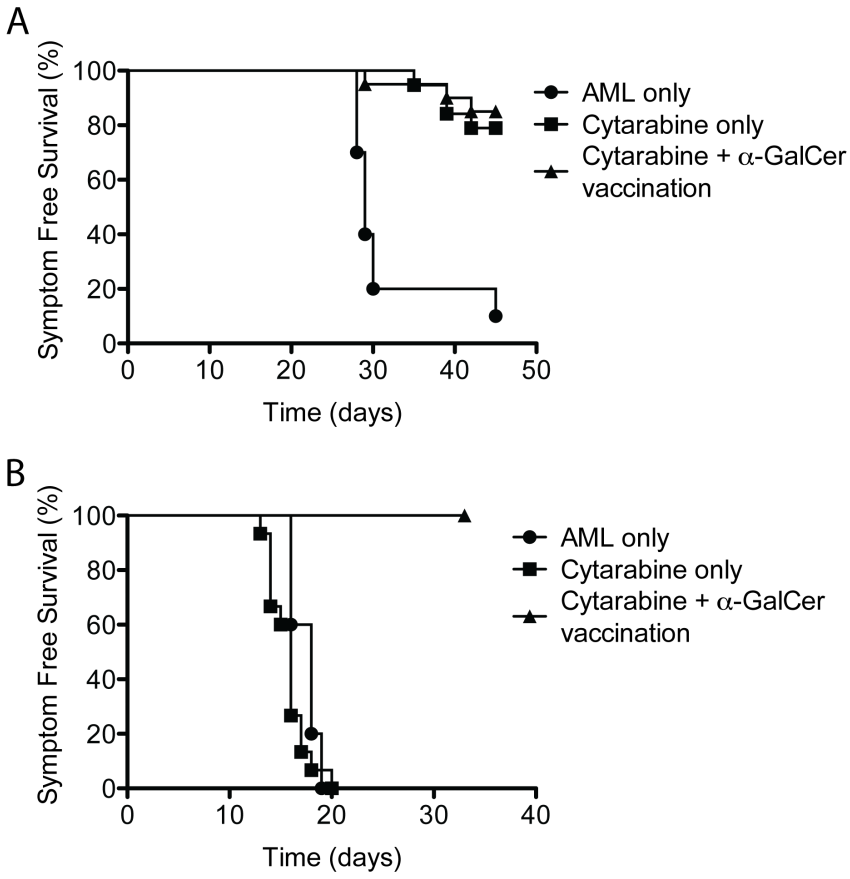


Figure 4.18: The tumour/α-GalCer vaccine is effective following chemotherapy. Mice were challenged with 1x10^5 AML cells and 24 hours later a course of chemotherapy was initiated. Three doses of cytarabine were administered ten hours apart, each consisting of 3 mg administered intraperitoneally. (A) On day 23 one group of the chemotherapy treated mice were vaccinated with 7.5x10^5 irradiated AML cells loaded with α-GalCer and their symptom-free survival was analysed. (B) The remaining mice were then rechallenged on day 45 with 5x10^5 AML cells and the symptom-free survival was again followed. This graph represents two experiments, with five mice in the AML only groups and ten mice in the cytarabine treated groups. (A-B) $P < 0.0001$ (Log-rank Mantel-Cox Test).

4.3 Discussion

The experiments presented in this chapter show that vaccinating mice with the tumour/α-GalCer vaccine prior to AML challenge prevented the accumulation of leukemic blasts in the blood and bone marrow and provided hosts with complete protection against AML development. The hypothesis tested here, that an intravenously administered vaccine is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen, was supported as depletion of the langerin$^+$ CD8α^+ DCs made the anti-AML vaccine ineffective. If the AML had time to establish prior to vaccination then the anti-AML protection was ablated, which was to some degree associated with suppression of langerin$^+$ CD8α^+ DCs and CD4$^+$ T cells. Hosts with established AML had elevated numbers of the suppressor cells T_{regs}, which contributed significantly to the inhibition of the vaccine in the therapeutic setting, and had increased numbers of MDSCs with heightened suppressor function *in vitro*. In addition, the C1498 cells themselves were able to suppress T cell proliferation *in vitro*, which suggests they may have been able to suppress the vaccine-induce immune response. Driving AML-bearing hosts into remission with chemotherapy enabled the vaccine to develop an immune response capable of protecting mice from AML rechallenge. The results from this chapter may significantly contribute to the future design of more potent vaccines.

Following administration, the tumour/α-GalCer vaccine must stimulate iNKT cells by presenting α-GalCer on CD1d. Either the irradiated AML cells do this directly, as C1498 cells express CD1d, or the tumour/α-GalCer complex is acquired by a host DCs, which then present α-GalCer on CD1d to iNKT cells. Liu et al. showed support for the latter with an α-GalCer-adjuvanted plasmacytoma vaccine, by demonstrating that DCs that acquired the administered irradiated tumour cells had higher expression levels of CD1d than other DCs (329). Furthermore, using an α-GalCer-adjuvanted vaccine to treat a murine glioma provided mice with significant anti-tumour protection even though the glioma cell line used, GL261, did not express CD1d (331).

While it is likely that α-GalCer is presented by resident cells with the vaccine used in this study, more work is required to formally establish this. However, what is clear is that resident cells are required for the resultant anti-AML activity, with langerin$^+$ CD8α^+ DCs critically involved, which suggests that cellular material is transferred to host APCs.

The administration of free α-GalCer in combination with tumour antigens has been demonstrated to be effective at activating anti-tumour CD8$^+$ T cells in a manner that was dependent on DC licensing by iNKT cells (53, 78). In this thesis, α-GalCer was loaded into AML cells that were then irradiated, as this has proved to provide a more efficient anti-tumour response than administering irradiated tumour cells in combination with α-GalCer in other published models (330), and in work conducted by colleagues in my laboratory (Hunn, unpublished). Using whole tumour cells in the vaccine enables the generation of anti-tumour CD4$^+$ and CD8$^+$ T cells against multiple antigens, in addition to avoiding the complications of needing to identify specific tumour antigens. Importantly, loading the α-GalCer onto whole tumour cells reduces the effective dose, which has been shown to limit the "exhaustion" phenotype that is typically seen in iNKT cells when high doses of free α-GalCer are used (331). When iNKT cells become exhausted, they are no longer able to provide the helper function required for vaccine activity (Dickgreber et al, unpublished observation), which limits the ability to re-vaccinate until the exhaustion period is over, which can take several weeks in animal models.

This α-GalCer-adjuvanted vaccine strategy is an effective treatment for a variety of different murine tumours (330-332), inducing another model of AML (AML-ETO9a) where vaccine-induced activity was seen in both the prophylactic and therapeutic settings (333). The difference in activity in the therapeutic setting compared to the work published here may reflect the different tumour cells used, as the evidence provided here shows that this malignant clone promotes a highly suppressive environment. Intrinsic qualities

of the tumour may be relevant with AML-ETO9a, making it potentially more sensitive to T cell mediated killing, by expressing a more immunogenic range of tumour antigens. In support of this, the AML1/ETO oncogene mutation, which is expressed by these tumour cells is associated with a favourable outcome in the clinic (403).

Although the α-GalCer-adjuvanted vaccine strategy has been studied in a variety of different tumour models, the type of effector cell generated by the vaccine that protects against tumour development varies in each setting. For the treatment of a murine lymphoma model (EL-4), the vaccine was dependent on iNKT cells and NK cells but independent of CD4$^+$ and CD8$^+$ T cells (404). In contrast, protection mediated by a vaccine for the treatment of the murine lymphoma (A20) was dependent on activation of CD4$^+$ T cells but not CD8$^+$ T cells (330), as was a vaccine for the treatment of an orthotopic model of glioma (GL261) (331). The effector functions of both the CD4$^+$ and CD8$^+$ T cells were required for the treatment of a model of melanoma (B16) (332), as was seen for the AML model described here (Figure 4.3). Interestingly, this result was different to the study of AML-ETO9a, where anti-AML activity was dependent on the CD8$^+$ T cells, iNKT cells and NK cells, but was independent of CD4$^+$ T cells (333). The differences between the effector cells generated in these studies is likely due to the different tumour cell lines used, such as the different tumour antigens presented and perhaps differences in sites and modes of uptake of the vaccine-derived material. For example, Shimizu et al., found that their live B16 melanoma vaccine was killed in the spleen by iNKT cells (332) and the vaccine was dependent of CD4$^+$ and CD8$^+$ T cells. In contrast, tumour cells that were lethally irradiated for the vaccine may have died in different tissues, such as the mediastinal lymph nodes as was found by Hunn et al., and in this case the vaccine was dependent on CD4$^+$ T cells but not CD8$^+$ T cells (331). Different sites and modes of cell death would alter the location, subset and activation status of the DCs that acquired the tumour antigen. Different sites and modes of cell death may result in different danger signals and phagocytosis signals being

released that could alter the location, subset and activation status of the DCs that acquired the tumour antigen as well.

The hypothesis of this study was that effective anti-AML activity induced by an intravenously administered vaccine is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen. The data presented here support this hypothesis, as the protection mediated by the tumour/α-GalCer vaccine was dependent on langerin$^+$ CD8α^+ DCs. However, langerin is expressed by other APCs in different locations, including the lung (405), where the vaccine is likely to circulate. Due to the intravenous administration of the vaccine and the likelihood of the irradiated AML cells circulating to the spleen, it is probable that the splenic langerin$^+$ CD8α^+ DCs are the langerin$^+$ cells that have an essential role in generating the vaccine-induced effector CD8$^+$ T cells. In addition, splenectomised mice fail to elicit potent CD8$^+$ T cell responses to soluble antigens co-injected with α-GalCer (Osmond, unpublished observation), providing strong evidence that it is the splenic cells that are required. In addition, langerin$^+$ CD8α^+ DCs have been shown to be proficient at acquiring irradiated cells in the blood, such as those within the vaccine (371). In contrast, an α-GalCer-adjuvanted glioma cell based vaccine could still provoke anti-tumour responses in splenectomised mice, with responses generated in the lung-draining lymph nodes (331). However, this vaccine was shown to function independent of CD8$^+$ T cells and langerin-expressing cells.

After acquiring the irradiated AML cells, the langerin$^+$ CD8α^+ DCs could present the α-GalCer on CD1d for the stimulation of iNKT cells and cross-present antigens from the AML cell for presentation on MHC class I. It should be noted however that α-GalCer can be presented by other DC subsets (107). Once iNKT cells have been activated by α-GalCer presented on CD1d they are able to license DCs (78, 81). The combination of the iNKT cell licensing with the irradiated AML cells provides DCs with the three signals required to activate T cells; antigens from the irradiated tumour cell presented via MHC, the co-stimulatory molecules required for T cell activation and cytokine

production resulting from licensing. Of note, the presence of established tumours suppressed the vaccine-induced activation of langerin$^+$ CD8α^+ DCs relative to the vaccine only control group, however they were still significantly activated as they upregulated CD40 and CD86 after vaccination and produced significant quantities of IL-12. While langerin$^+$ CD8α^+ DCs are efficient at cross-priming CD8$^+$ T cells, it is possible that langerin$^+$ CD8α^+ DCs stimulate both CD4$^+$ and CD8$^+$ T cells, as they are likely to be able to more efficiently acquire the vaccine-derived irradiated AML cells from the blood than the other DC subsets (371). However, it cannot be ruled out that the other DC subsets are acquiring antigens and directly presenting them on MHC II for the stimulation of CD4$^+$ T cells.

It was demonstrated that iNKT cells are activated following vaccination, however it was not determined if the iNKT cells or NK cells perform any tumour cell killing as a result of vaccination. Figure 4.3 demonstrates that both CD4$^+$ and CD8$^+$ effector cells are essential for the vaccine-induced immune response to mediate AML cell killing, however CD4 is also expressed by a subset of iNKT cells and therefore by depleting all CD4$^+$ cells with the anti-CD4 antibody the CD4$^+$ iNKT cells may have also been removed from the host. It is therefore possible that the depletion of the CD4$^+$ iNKT cells may have contributed toward the decreased protection provided by the vaccine in this experiment. Further evidence for the role of CD4$^+$ T cells was from studies in vaccinated MHC II$^{-/-}$ mice, which were not protected from AML development due to the inability to present antigens to CD4$^+$ T cells (Appendix 10). Furthermore, in these depletion experiments, the ablation of CD8$^+$ cells with an anti-CD8 antibody could have also have depleted the CD8α^+ DCs, which includes the langerin$^+$ CD8α^+ DCs, however the depleting antibody was not administered until five days after vaccination, at which point the langerin$^+$ CD8α^+ DCs are likely to have performed their vaccine induced stimulation of T cells. However, this does not rule out the potential of iNKT cells or NK cells to provide some anti-tumour protection as a result of vaccination. By performing an experiment, similar to figure 4.3, where both CD4$^+$ and CD8$^+$ cells were

depleted, it may have been possible to determine if cells other than the CD4$^+$ and CD8$^+$ T cells function as effectors. Because the vaccine-induced protection was not completely ablated with the single depletion experiments, it is possible that the double depletion will do this. If this did occur then the effector cells likely consist solely of CD4$^+$ and CD8$^+$ T cells and the residual survival seen with the single depletions is due to the remaining T cell population. If the double depletion does not reduce the vaccine-induced symptom-free survival further than the single depletion then it is likely that the CD4$^+$ T cells function by enhancing CD8$^+$ T cell killing, which would explain the similar ablation of symptom-free survival with both single depletions. In addition, if this is the case, other effector cells, such as NK or NKT cells, are performing some effector function to maintain the protection seen in the single depletion experiments.

The development of AML in mice generated an increase in the number of T_{regs} within the host, although it was not determined whether they were developed within the thymus as natural T_{regs} and proliferated in the presence of tumours or if they differentiated from naïve CD4$^+$ T cells as induced T_{regs}. The activity of IDO has been demonstrated to differentiate CD4$^+$ T cells into CD4$^+$ Foxp3$^+$ T_{regs} due to the metabolites of tryptophan catabolism, specifically 3-hydroxyanthranillic acid, which induce DCs to produce TGF-β (282, 393, 406). The increase in T_{regs} observed in this study may be the result of stimulation by metabolites of tryptophan catabolism, as IDO can be expressed by AML cells (393, 407). In addition, C1498 cells have been demonstrated to express the T cell inhibitory molecule PDL-1 (408) and the expression of PDL-1 on tumour cells has been correlated with an increase in the number and function of T_{regs} (409). It would have been possible to determine if these pathways were methods by which T_{regs} were generated in this model by initially determining if the C1498 cells used in these experiments express IDO or PDL-1 and then analysing if the T_{reg} population still increased in tumour-bearing hosts when these pathways were blocked. For example, treating hosts with the IDO inhibitor, 1-methyl tryptophan, may have prevented the increase in tumour-

associated T_{regs}, which would implicate IDO as an inducer of T_{regs} (393). A similar experiment could be conducted using anti-PD-1 to block the binding of PDL-1 expressed by tumour cells to PD-1 expressed by T cells (410).

The tumour/α-GalCer vaccine was ineffective in the presence of established tumour, but efficacy was partially restored by depletion of T_{regs} before vaccination. While the vaccine was able to activate iNKT cells, DCs and CD8$^+$ T cells in the presence of established AML, CD4$^+$ T cells were significantly inhibited in terms of activation status and IFN-γ production. However, figure 4.10 shows that the overall levels of vaccine-induced antigen-specific IFN-γ production were not altered by tumour-associated suppression. When comparing figure 4.11 C and E, it is obvious that a much greater proportion of CD3$^+$ cells producing IFN-γ are CD8$^+$ T cells and as these are not suppressed, the amount of IFN-γ produced is largely unaffected by the presence of established AML. This would perhaps imply that CD4$^+$ T cells play a critical role other than as a large provider of IFN-γ. This could possibly include functions such as DC licensing, as the helper function of CD4$^+$ T cells and iNKT cells is synergistic (411), or the activation of other immune cells such as macrophages, that can then attack AML cells.

It is possible that the vaccine-induced T cells become suppressed after leaving secondary lymphoid organs, as the AML-associated suppression may prevent the T cells from migrating to or entering the sites of AML cell accumulation, such as the bone marrow. In addition, it is possible that the T_{regs} that induce the immune suppression reside within these sites and therefore only suppresses T cells following migration (412). Tumours that recruit T_{regs} have been found to have increased levels of the suppressive cytokines IL-10 and TGF-β (413) and effector T cells within tumours display reduced proliferation and perforin and granzyme B production (414-416). A study demonstrating the therapeutic benefit of an α-GalCer-adjuvanted vaccine for the treatment of melanoma found that more CD8$^+$ T cells infiltrated the tumour when the T_{regs} were depleted (417). Therefore, by looking at the T

cell population in the spleen it is possible that we are analysing the immune response in sites of relatively little immunosuppression. It would have been interesting to examine the suppressive environment in the bone marrow as AML cells accumulate at this sight and it may have been possible to detect significantly more T_{regs} in this compartment. By examining the vaccine in hosts depleted of T_{regs} prior to therapeutic vaccination it may have been possible to determine if T_{regs} were preventing the infiltration of T cells into the bone marrow. In addition, analysis of the activation status of T cells within the bone marrow may have provided interesting results.

There are other factors that may cause the therapeutic vaccine to be ineffective besides the suppression generated by AML. As AML is typically a fast growing tumour cell, in the therapeutic setting the disease may have progressed to a point where the AML cells were able to grow faster than they could be removed by the immune system. In addition, the AML cells may be edited by the endogenous immune system prior to vaccination, thereby making it a more immunoresistant cell that cannot be attacked by the vaccine-induced immune response (418). This is unlikely to be the case in the animal model; however, as the AML is only administered seven days prior to vaccination. It would have been possible to test this by challenging mice with C1498 for seven days and then removing tumour cells from the bone marrow or blood. The tumour cells, which may have been immunoedited *in vivo*, could then be expanded by culture. It would then be possible to determine if these cells had become resistant to the vaccine-induced immune response by challenging mice with these tumour cells 7 days following vaccination with the original tumour cells. If the mice were protected from tumour development by the vaccine, as seen in figure 4.1, then the tumour cells would not have been made resistant by immunoediting.

As the majority of AML patients achieve complete morphologic remission following chemotherapy it seems likely that this setting may provide a suitable environment for using an α-GalCer-adjuvanted vaccine strategy to prevent

relapse (375, 419). However, this had to be examined as chemotherapies are also able to generate immunosuppressive environments, including by developing T_{regs} (400). Here, we used cytarabine, which is in routine clinical use for both intensive and palliative treatment of AML, to induce remission and invoke an environment suitable for subsequent vaccination. Hosts with established AML had a reduced percent of T cells in the spleen, however this was restored to levels comparable to naïve mice by treatment with cytarabine. These data imply that the suppressive environment generated by AML is partially ablated with cytarabine treatment, and by vaccinating at this point hosts were protected from AML rechallenge. The rechallenge at this point was a model of AML relapse, which occurs in the majority of AML patients treated with chemotherapy. However, due to time constraints, relapse of the C1498 AML model following cytarabine treatment was not a feasible experimental model. Previous studies have demonstrated that mice relapse following treatment of AML with cytarabine (402, 420), and this has been demonstrated to occur in the model used in this chapter (Appendix 11). It would; however, have been more appropriate to examine the ability of the tumour/α-GalCer to prevent natural relapse following cytarabine treatment.

It should be noted once again that a lack of statistical power might have caused some type two errors within the experiments of this chapter, such as for the IL-4 production in the presence of 14-day-old tumours in figure 4.6C. Therefore, some differences between the groups may not have been detectable. It is possible that with a larger sample size these differences would have been distinguishable.

4.4 Conclusion

The experiments presented in this chapter show that a vaccine consisting of irradiated AML cells loaded with α-GalCer can prevent the development of AML following challenge. The vaccine-induced immune response was dependent on the langerin$^+$ CD8α^+ DCs, and involved CD4$^+$ and CD8$^+$ T cells for anti-tumour effector function. In contrast to the efficacy of the vaccine in

the prophylactic setting, the vaccine was ineffective in mice with established AML, which was attributed in part to T_{regs}, and may involve suppressive activity of MDSCs and the tumour cells themselves. Although the langerin$^+$ CD8α^+ DCs were suppressed in the presence established tumours they were still significantly activated by the vaccine and therefore langerin$^+$ CD8α^+ DC suppression is not likely to be the method by which the vaccine efficacy was suppressed. By driving hosts into remission with chemotherapy prior to vaccination, it was possible to illicit responses that protected from AML rechallenge. A potential clinical application for a similar vaccine is therefore in a setting of chemotherapy-induced morphologic remission to prevent relapse. Knowledge of the importance of langerin$^+$ CD8α^+ DCs for vaccine efficacy may aid in the future design of more potent anti-tumour vaccines.

Chapter 5: General discussion

DCs are critical for eliciting T cell mediated immunity, however their functions in specific T cell-mediated immunotherapies is not well defined. There is considerable heterogeneity within the DC population that may be related to functional differences, including differing T cell stimulatory capabilities. This thesis addresses the possibility that specific DC subsets are involved in promoting effector $CD8^+$ T cells for effective cancer immunotherapy. Experiments were conducted in a murine model system, which has the advantage of having advanced DC phenotyping techniques, including ablation models, such as the langerin-targeted conditional ablation model described here. While the human counterparts to murine DCs have not been well defined, the overall concepts addressed in this thesis, namely that specific DC subsets in the host are more effective than others in mediating $CD8^+$ T cell immunity, will need to be considered when developing effective immunotherapies.

Recent studies have indicated some key features and functions of murine splenic langerin$^+$ CD8α^+ DCs, most relevant of which is their potent ability, relative to other splenic DCs, to cross-prime $CD8^+$ T cells and to produce IL-12 (107). In addition, the position of these DCs in the marginal zone of the spleen suggests a function in scanning the blood for debris. These features suggest that they may be an effective DC subset at activating or maintaining immunotherapy associated $CD8^+$ T cells. The aim of this study was therefore to determine if langerin$^+$ CD8α^+ DCs were required to stimulate effector $CD8^+$ T cells in a model of adoptive therapy and generate effector $CD8^+$ T cells following vaccination.

In chapter three, the langerin$^+$ CD8α^+ DCs were found to play a role in the anti-tumour response in a murine model of adoptive T cell therapy. The tumour model used was an intravenously administered lymphoma, which circulated and seeded at multiple locations in the host, including the liver, kidneys, ovaries, inguinal lymph nodes, spleen and lumbar lymph nodes. Initial analysis indicated that the model tumour antigen, OVA, was presented to the immune system in a manner that permitted recognition by naive OVA-

specific $CD8^+$ T cells. This likely reflected release of the antigen from the tumour and uptake of antigen by resident DCs, as the T cell proliferation was significantly reduced in absence of langerin$^+$ CD8α^+ DCs. Furthermore, sufficient naive OVA-specific $CD8^+$ T cells became activated to induce some anti-tumour activity. In the setting of adoptive T cell therapy, the absence of langerin$^+$ CD8α^+ DCs resulted in reduced anti-tumour efficacy of activated OVA-specific $CD8^+$ T cells, which was associated with less transferred $CD8^+$ T cells in the blood and spleen. The langerin$^+$ CD8α^+ DCs were therefore capable of stimulating and enhancing the anti-tumour activity of effector $CD8^+$ T cells during immunotherapy. The hypothesis tested, *that the effective anti-tumour activity of an adoptive transfer-based immunotherapy is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen*, has therefore been supported by this study. The functional status of specific resident DC populations is therefore an aspect to be considered when developing adoptive T cell therapies, to ensure appropriate endogenous stimulation.

In chapter four it was demonstrated that langerin$^+$ CD8α^+ DCs were required for the efficacy of a whole tumour cell-based vaccine that was administered intravenously to harness the stimulatory potential of iNKT cells. This chapter involved the development and characterisation of a tumour/α-GalCer vaccine that provides mice with protection against AML. The results show that anti-leukaemia activity generated by the vaccine was ablated in animals depleted of langerin$^+$ CD8α^+ DCs. It is therefore likely that following vaccination and iNKT cell licensing, langerin$^+$ CD8α^+ DCs use vaccine-derived tumour cells as a source of antigen for the stimulation of endogenous naïve T cells to generate anti-tumour effector $CD8^+$ T cells. Activated $CD4^+$ T cells were also required for anti-AML activity; however, without a defined tumour antigen it was not possible to establish whether langerin$^+$ CD8α^+ DCs stimulated both sets of T cell effectors. The hypothesis tested, *that effective anti-tumour activity induced by an intravenously administered vaccine is dependent on the function of resident langerin$^+$ CD8α^+ DCs in the spleen*, is supported by this research. The functional status of specific resident DC populations is therefore an aspect that should also be considered during the development of

effective vaccine-mediated therapy, particularly in settings such as haematological malignancies, where whole-tumour vaccines may be practical.

5.1 The strengths and weaknesses of the *lang*-EGFPDTR mouse model

The *lang*-EGFPDTR mouse model is an effective way to deplete langerin$^+$ DCs, as they can be selectively depleted by DT administration. Mouse models that deplete DCs by knocking out genes essential to specific DC subsets may result in the mice having developmental issues as a result of the absence of these cells during growth. However, the selective depletion with the *lang*-EGFPDTR model bypasses the implications of langerin DC depletion during mouse development. However, this model does have one significant flaw for this study, which is that the splenic langerin$^+$ CD8α^+ DC subset is not the only DC subset that expresses langerin. Therefore, other langerin$^+$ DC subsets are also depleted by DT administration, which includes langerhan cells in the epidermis and a CD103$^+$ langerin$^+$ DC subset found in the dermis and lung. This was taken into consideration during study design and intravenous tumours were used in these experiments to encourage the interaction between the tumour cells and the splenic DCs as the tumour circulates through the blood. Likewise, the intravenous administration of the two immunotherapies in these studies also promoted interaction with the langerin$^+$ CD8α^+ DCs. However, this does not rule out the possibility that the other langerin$^+$ DC subsets have an essential role in stimulating CD8$^+$ T cells against tumours administered intravenously.

It would be possible to specifically determine if the splenic langerin$^+$ CD8α^+ DCs were the langerin$^+$ DC subset required during adoptive CD8$^+$ T cell therapy or for the tumour/α-GalCer vaccine by analysing the symptom-free survival of mice following treatment with either therapy in splenectomised mice. If splenectomised mice did not have a delay in their symptom-free survival, as DT treated *lang*-EGFPDTR mice did, then the vaccine functioned independently of splenic DCs and therefore the splenic langerin$^+$ CD8α^+ DCs,

and vice versa. A similar study performed by researchers within my lab group found that splenectomised mice fail to elicit potent $CD8^+$ T cell responses to soluble antigens co-injected with α-GalCer (Osmond, unpublished observation), which suggests that splenic DCs are required.

5.2 Experimental limitations

There are a variety of different ways to analyse CFSE dilution plots, some of which are more appropriate than others. For example, analysis of CFSE dilution using replication index analyses the fold expansion of the proliferating cells rather than of the total cell population. However, these forms of analyses require differentiation between the division generations. Because of an inability to distinguish between he generations in some of the experimental CFSE plots, such as is seen in figure 4.14D and figure 4.15 A and C, it was not possible to use proliferation algorithms to analyse the individual generations. For this reason, the percent of cells that had divided was analysed in all experiments for consistency, excluding the experiment conducted in appendix 5, where all cells had downregulated CFSE and therefore percent divided was also not an appropriate analysis. In this experiment the MFI of CFSE was used for analysis.

Throughout this thesis the function of the langerin$^+$ CD8α^+ DCs has been examined with particular attention given to their role in stimulating $CD8^+$ T cells. While we did not determine specifically what the stimuli langerin$^+$ CD8α^+ DCs provide to $CD8^+$ T cells we have suggested that it is likely the result, in part, of the cross-presentation of tumour antigens and the cross-priming of $CD8^+$ T cells. For the experiments in chapter 3, It would have been relevant to specifically determine if this was occurring by isolating langerin$^+$ CD8α^+ DCs from the spleens of mice, pulsing them with or without OVA and/or an activating stimulus and culturing them with effector OT-I T cells. It is possible that this would have enabled us to determine whether the stimuli provided by langerin$^+$ CD8α^+ DCs was dependent on their activation status and/or antigen presentation by analysing the subsequent T cell response. However, due to a

difficulty in isolating langerin$^+$ CD8α^+ DCs from hosts and maintaining them in culture for extended periods of time, this was not possible.

It is possible that some subtle differences in experimental results were missed due to small sample size. This may apply to experiments looking at effector phenotype of activated cells transferred into tumour challenged hosts, where no obvious difference were observed in the presence or absence of langerin$^+$ CD8α^+ DCs. Increasing the sample size may have provided more informative results; however, this would have increased the expense and practicality of performing of these experiments.

5.3 Langerin$^+$ CD8α^+ dendritic cells and their human counterpart

The information found in this thesis regarding the langerin$^+$ CD8α^+ DCs will be most beneficial to human studies if a human counterpart to the langerin$^+$ CD8α^+ DC is found with similar function. A population of CD11c$^+$ CD123$^-$ CD141$^+$ DCs is claimed to be similar to the CD8α^+ DCs and this subset has been demonstrated to be efficient at cross-presenting tumour antigens and to produce high levels of IL-12, which are both traits characteristic of langerin$^+$ CD8α^+ DCs (447). This DC subset is found in a variety of non-lymphoid tissues, and is therefore perhaps more similar to the broader mouse population of CD103$^+$ DCs, which does include the splenic CD8α^+ DC population (448). It may therefore be possible to specifically identify multiple DC subsets with the human CD141$^+$ DCs, one of which may include a heightened propensity for cross-priming, as is the case for murine langerin$^+$ CD8α^+ DCs.

5.4 The role of langerin$^+$ CD8α^+ dendritic cells in adoptive T cell therapy of established tumours

This study has made it evident that resident langerin$^+$ CD8α^+ DCs provide a stimulus in the context of adoptive T cell therapy with activated CD8$^+$ T cells;

however, it was not determined what the specific stimulus consists of. Some key characteristics of the langerin$^+$ CD8α^+ DCs may be relevant. One such characteristic is the strong ability of the langerin$^+$ CD8α^+ DCs to produce IL-12 in response to different activation stimuli, such as TLR stimulation and "feedback" from activated iNKT cells (107). IL-12 has a direct effect on a variety of cells but its most relevant functions include inducing IFN-γ production by NK cells, T_h1 cells and CD8$^+$ T cells and inducing CD8$^+$ T cell proliferation (421-423). The IFN-γ produced by NK cells in response to IL-12 also inhibits TGF-β signalling and thereby blocks its suppressive effects on CD8$^+$ T cell proliferation (424).

Other cytokines that may be the stimulus provided by the langerin$^+$ CD8α^+ DCs to the transferred effector CD8$^+$ T cells include IL-2 and IL-15, which induce T cell proliferation and increase T cell survival *in vivo.* It is likely that the cytokines IL-2 or IL-15 would be presented in *trans* to T cells. Specifically, DCs would present IL-2 bound on CD25 (IL-2Rα) to T cells expressing IL-2Rβ and IL-2Rγ, thereby bringing together the three IL-2 receptor subunits on the T cell in combination with IL-2 (425). IL-15 would be presented on IL-15Rα on the DC, which would bind to IL-2 receptor beta and gamma subunits expressed by T cells, as with IL-2. The *trans* presentation of these cytokines would enable the antigen-specific T cells to provide a stronger response against the antigen due to increased survival and proliferation (426). Treating patients with IL-2 following adoptive cell therapy has been studied extensively and has been demonstrated to improve the persistence of transferred cells, thereby enhancing the anti-tumour protection provided by the therapy (427-430).

IL-15 is produced at varying levels by different DC subsets, with the splenic CD8α^+ DC population being the superior producers (431, 432). In addition to inducing T cell proliferation, IL-15 has the potential to aid adoptive therapy by preventing activation-induced apoptosis, and thereby enhancing T cell survival (433). The *trans* presentation of IL-15 by DCs induces T cell homeostatic proliferation and the development of memory cells (432). In

addition, IL-15 can convert tolerant $CD8^+$ T cells into immunogenic $CD8^+$ T cells for use in adoptive therapy (433, 434). This cytokine has also been used in combination with adoptive therapy, similarly to IL-2, to enhance the persistence of transferred T cells (318). Interestingly, not only was it found to be as successful as IL-2 at improving the anti-tumour protection provided by adoptive therapy but the benefit received when combining adoptive therapy with IL-2 treatment was dependent on the production of IL-15 by endogenous immune cells (318). This indicates that IL-2 may drive the *in vivo* production of IL-15, which then induces T cell proliferation and increases the survival of transferred cells. It is therefore plausible that upon transfer of effector $CD8^+$ T cells into tumour-bearing mice, the T cells bind to tumour antigens presented on MHC molecules by langerin$^+$ CD8α^+ DCs, which results in the production of IL-2 and/or IL-15, thereby inducing proliferation or increasing the survival of the transferred cells. In order to specifically determine if the stimulus is IL-2 or IL-15, an experiment involving the administration of these cytokines following adoptive therapy in mice depleted of langerin cells may determine if the effect of langerin depletion on T cell proliferation is ablated. In addition, a similar response to langerin depletion may be generated by the transferring T cells into mice that have restricted production of IL-2 or IL-15 by DCs, either by blocking the cytokine receptors or by substituting IL-2 KO or IL-15 KO DCs into mice.

The signals required for the stimulation of naïve $CD8^+$ T cells has been studied extensively; however, the stimuli required to stimulate effector $CD8^+$ T cells is not well understood. The potent ability of the langerin$^+$ CD8α^+ DCs to cross-prime $CD8^+$ T cells (107) makes it possible that the level of tumour antigen presentation by these DCs is an important feature of the stimulus presented to the transferred T cells. However, a preliminary experiment that involved increasing the presence of endogenous antigen by intravenously administering OVA protein prior to adoptive therapy in mice without tumours did not induce proliferation of the transferred cells (Appendix 12). This preliminary study suggests that the presentation of tumour antigen alone is insufficient to effectively stimulate transferred effector $CD8^+$ T cells. However,

this experiment needs to be repeated before this can be confirmed. The inability of the langerin$^+$ CD8α^+ DCs to stimulate the transferred cells in this setting is presumably because the endogenous DCs were in an immature state and therefore the antigen was presented in the presence of signal one (antigen presentation) but the absence of signals two and three (co-stimulation and cytokines respectively). Therefore, it is likely that the stimulus presented by langerin$^+$ CD8α^+ DCs includes signal two and/or three. To gain further insight into the stimuli presented to effector CD8$^+$ T cells by langerin$^+$ CD8α^+ DCs it may be beneficial to analyse the stimuli required for restimulation of memory cells, although the specific signals required are still debated. Earlier studies demonstrated that only signal one was required for memory CD8$^+$ T cell reactivation in mice (435, 436), however the experiments performed in these studies were all *in vitro.* More recent studies now indicate that CD28 co-stimulation is essential for effective memory recall (437-439). This was determined by intraperitoneally challenging C57BL/6 mice with PR8 influenza A virus and then intranasially rechallenging them 60 days later. Blocking CD28 co-stimulation with an anti-CD28 antibody significantly reduced the number of antigen specific CD8$^+$ T cells that accumulated in the lung following rechallenge, compared to isotype control mice (438). The number of IFN-γ^+ CD8$^+$ T cells and the cytotoxic function of the CD8$^+$ T cells was also significantly reduced in the absence of co-stimulation and the ability of mice to clear the secondary infection was impaired (438). Another recent study found that recall of memory CD8$^+$ T cells upon secondary challenge was dependent on both CD28 and CD27 stimulation (440). Here, memory CD8$^+$ T cells were generated by intraperitoneally administering OVA with an anti-CD40 antibody and 23 days later mice were rechallenged intravenously with the MHC class I binding OVA antigen, SIINFEKL, and anti-CD40. Blocking CD80/86 or CD70, the CD28 and CD27 co-receptors, respectively, with antibodies prior to rechallenge significantly reduced the anti-CD40 mediated expansion of OVA-specific memory CD8$^+$ T cells (440). The requirements for CD28 and CD27 stimulation was also seen for memory recall in mice rechallenged with peptide and the TLR agonist LPS (440). These studies suggest that the stimuli provided by langerin$^+$ CD8α^+ DCs to effector

$CD8^+$ T cells may include antigen/MHC, CD80/86 and CD70 binding to the TCR, CD28 and CD27 expressed by the T cell, respectively. $Langerin^+$ $CD8\alpha^+$ DCs may be able to provide these signals, which are associated with activated DCs, as a result of being stimulated by DAMPS that have been released from tumour cells, either killed by the endogenous immune system or by the transferred effector $CD8^+$ T cells.

Signal three typically consists of IL-12 or IFN-α and these function to extend the proliferation of $CD8^+$ T cells by promoting IL-2 signalling (441), which is essential for both the generation of memory $CD8^+$ T cells and for their effective recall during secondary challenge. The IL-2 required to generate memory $CD8^+$ T cells that could respond to secondary challenge, derived from the $CD8^+$ T cells themselves (76). Furthermore, memory $CD8^+$ T cells generated in $CD28^{-/-}$ mice following intranasal inoculation with the vaccinia virus produced significantly less IL-2 (437), which suggests that memory precursor $CD8^+$ T cells produce IL-2 as a result of CD28 co-stimulation during priming. It was therefore hypothesised that memory $CD8^+$ T cells also require CD28 co-stimulation during secondary challenge to induce their production of IL-2. This was confirmed by restoring the recall response of memory $CD8^+$ T cells generated in $CD28^{-/-}$ mice by administering IL-2 intraperitoneally following rechallenge. These observations perhaps suggest that in the experiments conducted in this thesis, the transferred effector $CD8^+$ T cells receive antigen and co-stimulation from $langerin^+$ $CD8\alpha^+$ DCs, which ultimately stimulates IL-2 production by the $CD8^+$ T cells, thus inducing their proliferation.

The evidence that resident DCs can play a significant role in determining the function of transferred cells may be extremely beneficial to the area of adoptive cell therapy. It is possible that deliberately activating resident DCs *in vivo* following transfer, or providing the transferred cells an equivalent stimulus *in vitro* prior to transfer, could serve to enhance the anti-tumour activity of the T cells in the tumour-laden host. For example, it may be possible to enhance the protection provided by adoptive therapy by ensuring

that langerin$^+$ CD8α^+ DCs are activated prior to transfer by vaccinating mice with a tumour/α-GalCer vaccine, using the relevant tumour cell, prior to adoptive therapy. The results in chapter four demonstrate that the tumour/α-GalCer vaccine activates langerin$^+$ CD8α^+ DCs. In addition, the administration of irradiated tumour cells may increase the amount of antigen acquired by the langerin$^+$ CD8α^+ DCs for presentation to the transferred cells and thereby promote their stimulation. The combination of these two therapies may also provide enhanced anti-tumour protection as the hosts will have been transferred a population of effector CD8$^+$ T cells targeted against a specific antigen and the vaccine will activate naïve T cells against multiple antigens from the tumour. The importance of the langerin$^+$ cells could be determined in this experiment by depleting them prior to vaccination.

The main implication of this study is that the endogenous immune system has a significant effect on an immunotherapy and the ability of an immunotherapy to provide anti-tumour protection may largely depend on the status of the endogenous immune system prior to administration.

5.5 The role of langerin$^+$ CD8α^+ dendritic cells in initiating an immune response generated by an iNKT cell-based vaccine

A vaccine consisting of irradiated tumour cells pulsed with α-GalCer protected mice against AML challenge in a CD4$^+$ and CD8$^+$ T cell dependent manner, with initiation of the anti-tumour response dependent on langerin$^+$ CD8α^+ DCs in the spleen. Given the key role of CD8$^+$ T cells, the involvement of langerin$^+$ CD8α^+ DCs is likely due to their ability to effectively cross-prime CD8$^+$ T cells. It is not clear what role CD4$^+$ T cells have in this therapy but it is possible that their main role was in performing a helper function by licensing DCs and enabling them to more effectively stimulate CD8$^+$ T cells. However, it is also possible that the CD4$^+$ T cells mediated tumour-killing either directly, similar to CD8$^+$ T cells, or indirectly by inducing other immune cells such as macrophages to attack tumour cells.

When administered therapeutically, the vaccine was unable to delay the development of symptoms associated with AML burden. This may have been partially due to tumour-associated suppression of langerin$^+$ CD8α^+ DCs as their activation, following vaccination, was significantly reduced in tumour-bearing hosts. However, the expression of the co-stimulatory molecules CD40 and CD86 were still significantly upregulated in tumour-bearing hosts following vaccination, relative to naïve hosts. Langerin$^+$ CD8α^+ DCs produce large quantities of IL-12 following activation and serum IL-12 correlates with langerin$^+$ CD8α^+ DC activation. Serum IL-12 levels were similar following vaccination in mice with or without established AML, indicating effective DC activation. Furthermore, the vaccine was able to activate CD8$^+$ T cells in mice with established AML. These results likely indicate that the langerin$^+$ CD8α^+ DCs were functionally activated by vaccination in the presence of established tumours.

CD4$^+$ T cells were significantly inhibited in mice with established AML as indicated by reduced expression of the activation marker CD44 and a reduced proportion of CD4$^+$ T cells producing IFN-γ. If the vaccine activated CD4$^+$ T cells functioned by licensing DCs then it is probable that their inhibition would result in DCs with reduced expression of the activation markers CD40 and CD86 and less serum IL-12, due to the lack of CD4$^+$ T cell help. However, this is not what was observed as CD11c$^+$ DCs were similarly activated following vaccination in mice with or without established AML. In addition, as some residual anti-AML activity was seen in the absence of CD8$^+$ T cells, other CD4$^+$ T cell functions are likely involved independently of CD8$^+$ T cells. Therefore, it is possible that vaccine-activated CD4$^+$ T cells are involved in the killing of AML cells, either directly by producing cytotoxic molecules or indirectly by activating macrophages to mediate killing. The direct killing of AML cells by CD4$^+$ T cells is unlikely as the AML cell line used, C1498, does not express MHC class II and therefore the CD4$^+$ T cells would be unable to recognise the AML cells for a direct attack (442). As the proportion of CD4$^+$ T cells capable of producing IFN-γ was significantly reduced in tumour-bearing mice, it is possible that the decreased CD4$^+$ T cell-derived IFN-γ is why the

vaccine was ineffective therapeutically. It should be noted here that significantly fewer $CD4^+$ T cells were capable of producing IFN-γ relative to $CD8^+$ T cells (Figure 4.11 C and E). However, the amount of IFN-γ required for a $CD4^+$ T cell to activate an MHC class II expressing cell, such as a macrophage, may be relatively low due to the formation of a synapse between the two cells upon TCR engagement. Therefore, an essential function of $CD4^+$ T cells in the vaccine-mediated anti-AML immune response may be the activation of innate cells, such as macrophages, which then attack the AML cells. In fact, $CD4^+$ T cell-mediated activation of macrophages has been demonstrated to provide anti-tumour responses in a variety of tumour models including, intraocular tumours (443), melanoma (191), myeloma (444) and interestingly a B cell lymphoma (192).

Mice with established AML had elevated numbers of MDSCs in their spleens, which were demonstrated *in vitro* to have a significant suppressive effect on T cell activation. It would therefore be of interest to determine how the vaccine would perform therapeutically in mice depleted of their MDSCs. A recent study determined that β-glucan, a cell wall component of various pathogens, including yeast, fungi and bacteria, aids in driving immature MDSCs into a more differentiated cell type. This reduced their suppressive function and as a result aided in the activation of anti-tumour $CD4^+$ and $CD8^+$ T cells (445). Treating mice with β-glucan, prior to therapeutic vaccination may therefore provide an enhanced anti-tumour immune response. It would also be interesting to determine how long the vaccine would delay the onset of symptoms associated with AML development in leukemic mice treated with β-glucan and depleted of T_{regs}.

The C1498 AML cells also had significant suppressive functions on T cells. The method by which AML cells suppress T cell proliferation is unknown, however as leukaemia cells typical express IDO it is likely that the depletion of tryptophan by IDO is responsible for the reduced T cell proliferation (407). It is possible that the leukaemic cells in AML-bearing mice were responsible for the inhibition of the vaccine in the therapeutic setting. It would therefore be

beneficial to reduce the amount of AML cells within a host prior to vaccination to reduce the amount of suppressor cells, as would be done with a chemotherapeutic agent such as cytarabine. This may explain why the vaccine was able to generate an immune response in tumour-bearing hosts following cytarabine treatment, as the suppressive C1498 cells may have largely been removed, leading to a more appropriate environment for vaccination. The suppressive functions of both the tumour-associated MDSCs and the C1498 cells had a larger impact on $CD4^+$ T cells, relative to $CD8^+$ T cells. This is interesting, as the vaccine appeared to be ineffective in tumour-bearing hosts due to the suppression of $CD4^+$ T cells. Therefore, it seems likely that the role these suppressive cells played a significant role in suppressing the vaccine-induced $CD4^+$ T cells in tumour-bearing hosts, resulting in ineffective vaccination. However, it is possible that the suppressive effect of MDSCs observed in figure 4.14 was not actually a suppressive quality of MDSCs, but of AML cells that had upregulated CD11b *in vivo*, although we have no evidence of this occurring.

The tumour/α-GalCer vaccine is potentially a feasible treatment to prevent AML relapse in the clinic, as a characteristic of AML is the accumulation of leukemic blasts in bone marrow, which can be aspirated from bone marrow fluid for vaccine manufacture; this procedure is routinely performed as a diagnostic test for AML. Vaccination has the obvious benefit over allo-SCT as the difficulties involved in finding a suitable donor are not required. In addition, the fact that the tumour derives from the host may indicate that the vaccine will also be much more tolerable than allo-SCT, although this is not validated. Clearly, evidence of the clinical efficacy of this vaccine would be required while comparing its function to allo-SCT. Current studies show that allo-SCT reduces the relapse rate from 52 % in the chemotherapy only arm to 36 % with the combined treatment (446). Clinical grade α-GalCer has already been developed and administered in humans. Patients with solid tumours received an intravenous dose of α-GalCer ranging from 50-4800 $\mu g/m^2$ on days one, four and fifteen of a four week cycle. No adverse effects were seen in any of the patients treated, however no clinical responses were detected either

(326). This is likely due to the use of α-GalCer as a sole agent, which would largely be dependent on cells of the innate immune system such as NK and NKT cells. In mouse models there is compelling evidence that α-GalCer is more efficient as an adjuvant, however this remains to be formally tested in humans (53, 78).

5.6 Future directions

Having found a function of langerin$^+$ CD8α$^+$ DCs in promoting tumour immunotherapies it would be beneficial to the improvement of immunotherapies in the clinic if the stimulus provided by langerin$^+$ CD8α$^+$ DCs to transferred effector CD8$^+$ T cells was determined. This information may provide unique ways to enhance the efficacy of adoptive cell therapy by stimulating endogenous APCs. This research would likely require isolating langerin$^+$ CD8α$^+$ DCs from tumour challenged mice and analysing their ability to stimulate CD8$^+$ T cells *in vitro*. The stimulation of T cells under these conditions could be compared to stimulation by langerin$^+$ CD8α$^+$ DCs pulsed with antigen and/or an activating stimulus, or antigen pulsed langerin$^+$ CD8α$^+$ DCs with the addition of cytokines to the culture, such as IL-2 or IL-15. These experiments may help elucidate what the specific stimuli provided to the transferred CD8$^+$ T cells is, which would then enable *in vivo* experiments to be conduced in an attempt to replicate these stimuli in langerin$^+$ depleted mice. For example, if the langerin$^+$ CD8α$^+$ DCs are found to be stimulating the transferred T cells by producing IL-15, it may be possible to provide this stimuli by administering IL-15 into hosts, before or after adoptive transfer, thereby eliminating the therapies dependency on langerin$^+$ CD8α$^+$ DCs. Once the stimuli are determined, it would be possible to perform research with the aim of improving the function of adoptive therapies by either enhancing the function of the endogenous DCs or by producing these stimuli artificially. Therefore, research conducted into combining another therapy with adoptive CD8$^+$ T cell therapy, with the aim of improving the stimuli provided by langerin$^+$ CD8α$^+$ DCs to the transferred T cells, may provide results translatable to the clinic. This was examined in figures 3.15 and 3.16; however, this was examined under limited conditions and significantly more

work could be done in this area, including the use of a variety of different treatments, with different treatment times and doses. It would then be relevant to apply these experiments to another tumour model, such as a melanoma, as this may be more applicable to the clinic. However, in this tumour model the role of the endogenous DCs would first need to be confirmed. The hypothesis of this research would be "*that stimulating endogenous DCs enhances the efficacy of adoptively transferred CD8$^+$ T cells for the treatment of tumours*".

Further experiments into the tumour/α-GalCer vaccine would also be beneficial, as this could prove to be a promising replacement for the current post-remission therapy for AML. Future experiments into this vaccine should include the development of a model, which incorporates remission of AML following cytarabine treatment, in place of the secondary challenge used in this thesis. The tumour/α-GalCer vaccine could then be compared to allo-SCT in its ability to prevent AML relapse. It would also be interesting to compare allo-SCT and the tumour/α-GalCer vaccine for the treatment of different models of AML, particularly humanized models of leukaemia and spontaneous leukaemia's. In the latter of which, the vaccine would need to be generated from the bone marrow of a mouse that developed a spontaneous tumour and then be administered into another host, which would more closely resemble what would occur in the clinic with this vaccine. The hypothesis of this research would be "*that a tumour/α-GalCer vaccine for the treatment relapsing acute myeloid leukaemia is more effective that allo-SCT*".

5.7 Novel findings

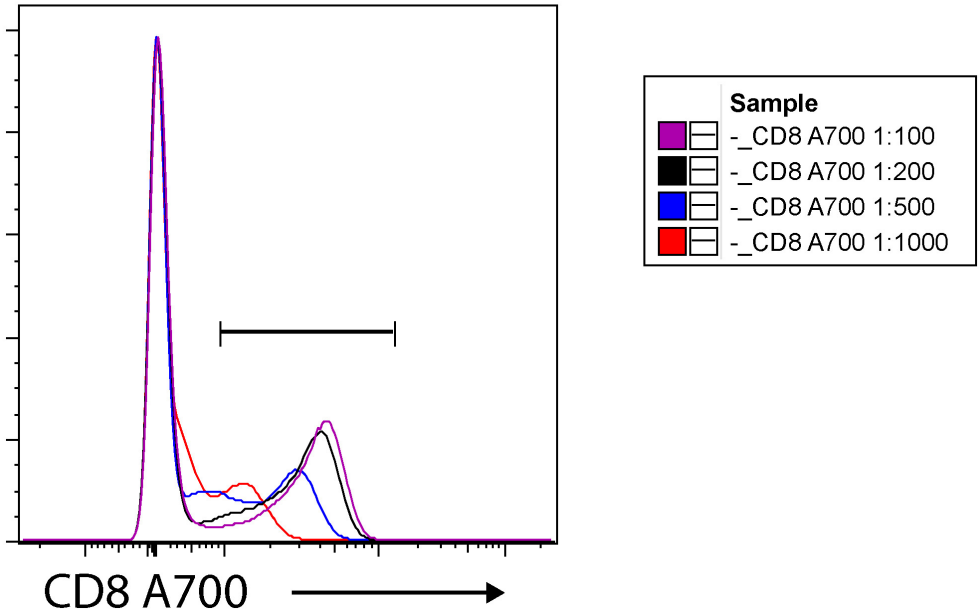
The aim of this thesis was to gain an understanding of the function of langerin$^+$ CD8α^+ DCs in stimulating effector CD8$^+$ T cells and generating effector CD8$^+$ T cells from naive T cells following immunotherapy for intravenously administered tumours. We demonstrate for the first time that effector CD8$^+$ T cells transferred for the treatment of an intravenous lymphoma are stimulated by langerin$^+$ CD8α^+ DCs *in vivo*. This stimulation resulted in the accumulation of transferred effector CD8$^+$ T cells in both the blood and spleen and provided the hosts with greater protection mediated by

the immunotherapy. Furthermore, a vaccine-induced immune response, which generated $CD4^+$ and $CD8^+$ effector T cells, was dependent on the function of langerin$^+$ CD8α^+ DCs for the vaccine to protect against AML development. Therefore, this research provides a novel function for the endogenous splenic langerin$^+$ CD8α^+ DCs in stimulating and generating effector $CD8^+$ T cells following T cell-based immunotherapies for the treatment of intravenous tumours. The implication of this information is that immunotherapies administered for the treatment of intravenously administered tumours is likely to be dependent on the function of endogenous splenic DCs. Therefore, the functional status of the splenic DCs may determine the protective benefit provided by some immunotherapies. The endogenous network of APCs should therefore be taken into consideration during the design of novel immunotherapies.

A novel vaccine for the treatment of AML was also characterised within this thesis. It was found to generate an immune response capable of targeting and killing AML cells. However, the established tumour generated a suppressive environment capable of inhibiting the protective function of the vaccine, despite stimulating iNKT cells and leading to DC activation. We have demonstrated a successful combination of immunotherapy following chemotherapy. Given the suppressive environment of the C1498 AML cells, it is likely that the reduction of these cells via cytarabine treatment aided in reducing some tumour-associated immunosuppression and thereby developing an immune environment suitable for immunotherapy. This finding is relevant clinically as there is an unmet need for effective post-remission therapies for AML that have reduced toxicity, cost and dependency on suitable donors, compared to the current post-remission therapy, allo-SCT.

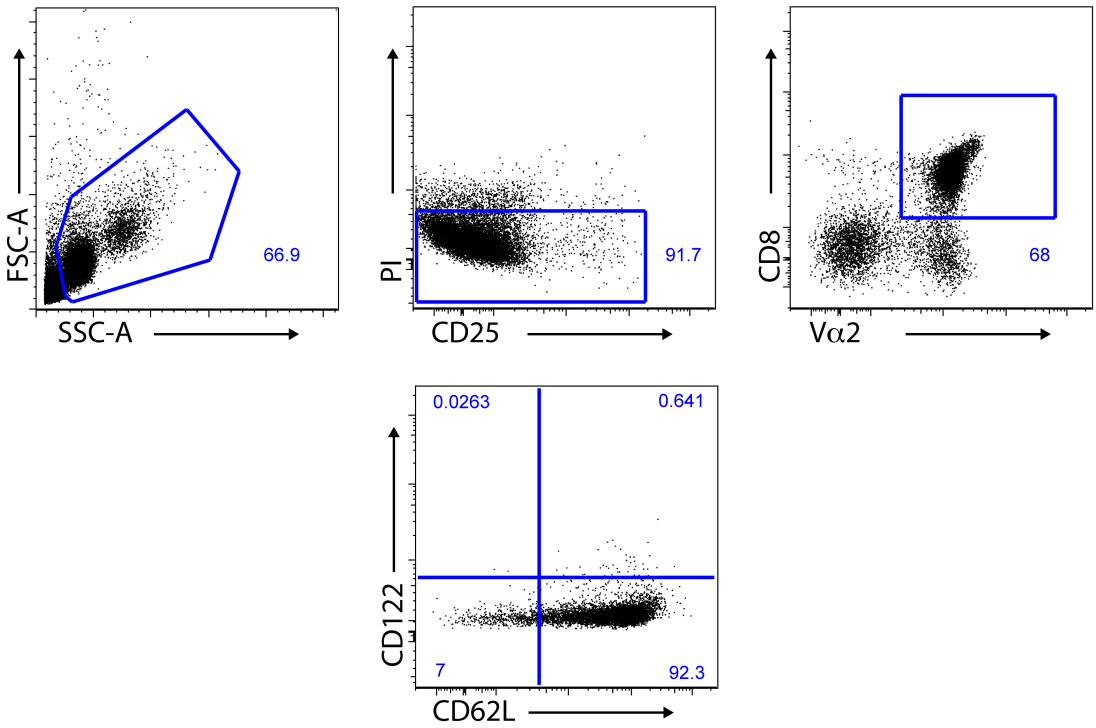
VI. Appendices

I. Appendix 1



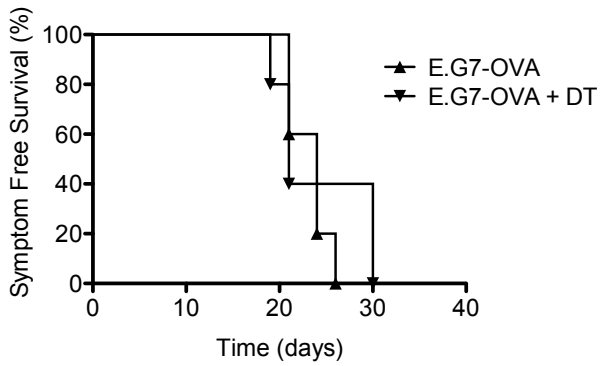
Appendix figure 1. Antibody titration. Splenocytes were removed from a CF7BL/6 mouse. The spleenocytes were separated into different groups for staining. Samples were stained with the antibody CD8 A700, at a dilution of 1:100, 1:200, 1:500 or 1:1000. Flow cytometry was performed on the samples to determine the lowest concentration of antibody that enabled differentiation between the positive and negative populations. The dilution 1:200 was chosen as an appropriate concentraion.

II. Appendix 2



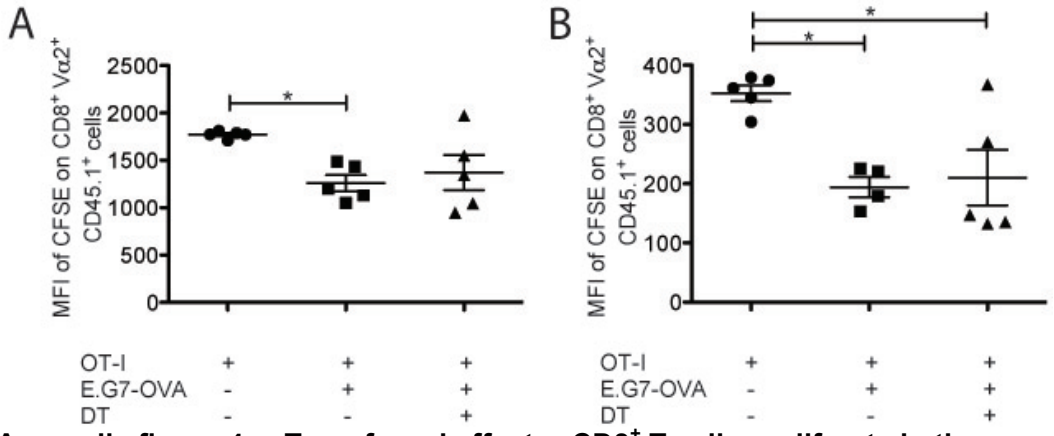
Appendix figure 2. The proportion of OT-I lymphoid cells that are OT-I CD8$^+$ T cells. Lymph nodes were removed from a naïve OT-I mouse. Flow cytometry was performed on the single cell suspension using antibodies for CD8 and Vα2 to identify the cells that were CD8$^+$ OT-I T cells. The gating strategy used to identify these cells is depicted on the top row, with the proportions of cells that express CD8 and Vα2. In addition, the proportion of these cells that express CD62L and CD122 is also displayed (bottom row). This experiment was performed twice.

III. Appendix 3



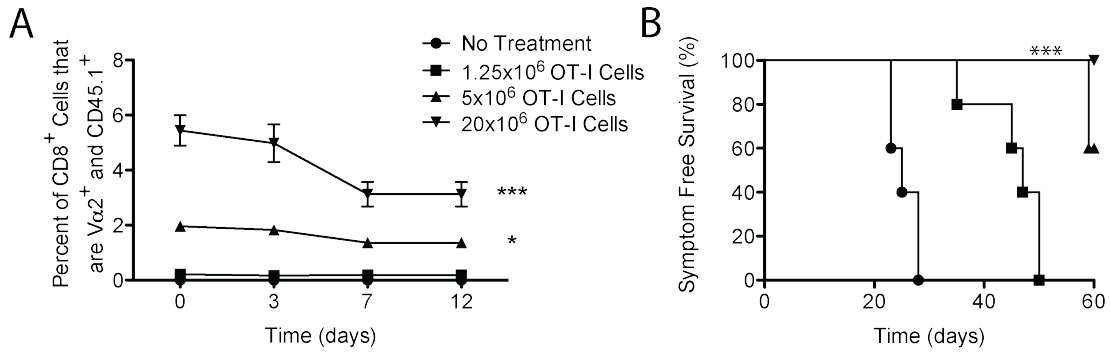
Appendix figure 3. Treatment of E.G7-OVA-bearing hosts with DT does not alter the symptom-free survival. *Lang*-EGFPDTR mice were intravenously administered $1x10^6$ E.G7-OVA cells. One group of tumour challenged mice were treated with 350 ng of DT intraperitoneally 2 days before tumour challenge and every 2–3 days later and the symptom-free survival was analysed. This represents a signal experiment with five mice per group. $*P < 0.05$ (Log-rank Mantel-Cox Test).

IV. Appendix 4



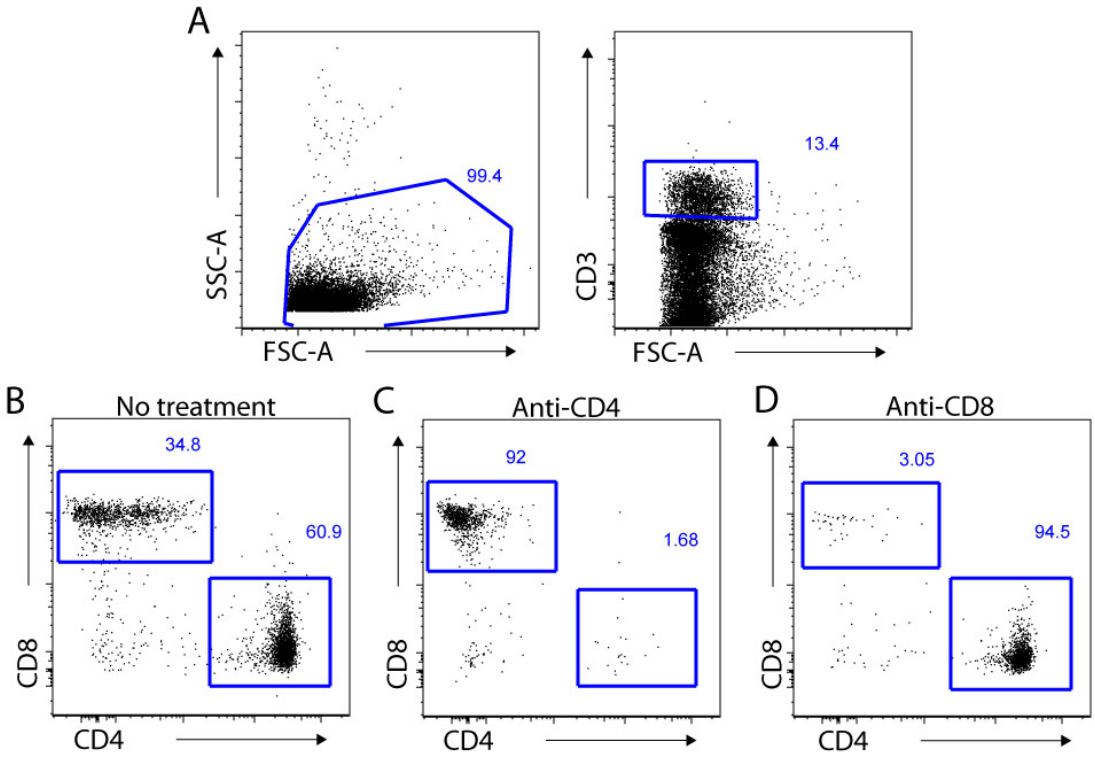
Appendix figure 4. Transferred effector CD8$^+$ T cells proliferate in the presence of established tumours. *Lang*-EGFPDTR mice were intravenously administered 1x10^6 E.G7-OVA cells and 12 days later were administered 5x10^6 activated OT-I T cells labelled with CFSE. One group of tumour challenged mice were treated with 350 ng of DT intraperitoneally 2 days prior to transfer and every 2–3 days following. Blood samples were taken 3 days (A) and 7 days (B) after transfer and flow cytometry was performed to analyse the CFSE expression on the transferred T cells using CD8, Vα2 and CD45.1 to identify them. The MFI of CFSE on the transferred cells is displayed. This represents a single experiment with five mice per group. *P < 0.05 (one-way ANOVA with a Bonferroni post test).

V. Appendix 5



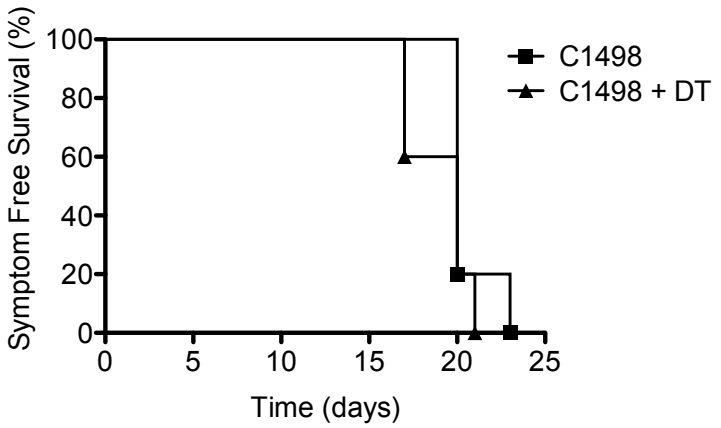
Appendix figure 5. Transferred effector CD8$^+$ T cells develop memory. C57BL/6 mice received 1.25x10^6, 5x10^6, 2x10^7 or no activated OT-I T cells intravenously. (A) On days 3, 7 and 12 following transfer a blood sample was taken from each host and flow cytometry was performed to identify the transferred T cell population using CD8, Vα2 and CD45.1 expression. The percent of CD8+ cells that are Vα2$^+$ and CD45.1$^+$ over time is displayed. (B) 28 days following transfer, 1x10^6 E.G7-OVA cells were administered into each group and the symptom free survival was analysed. This represents a single experiment with five mice per group. (A) *P < 0.05, ***P < 0.001 (one-way ANOVA with a Bonferroni post test). (B) ***P < 0.001 (Log-rank Mantel-Cox Test).

VI. Appendix 6



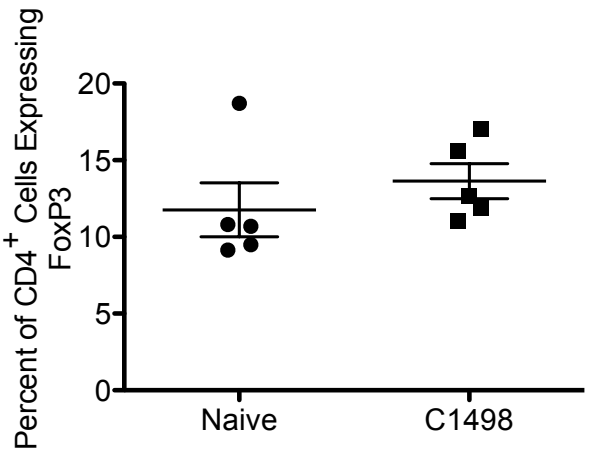
Appendix figure 6. CD4 and CD8 cell depletion. C57BL/6 mice were treated intraperitoneally with either anti-CD8 (2.43; 250 µg per mouse) or anti-CD4 (GK1.5; 125 µg per mouse). Seven days later the spleens were removed and flow cytometry was performed to analyse the remaining T cell populations. T cells were identified by CD3 expression and CD4 and CD8 were used to identify the $CD4^+$ and $CD8^+$ T cells. This experiment was performed once with two mice per group.

VII. Appendix 7



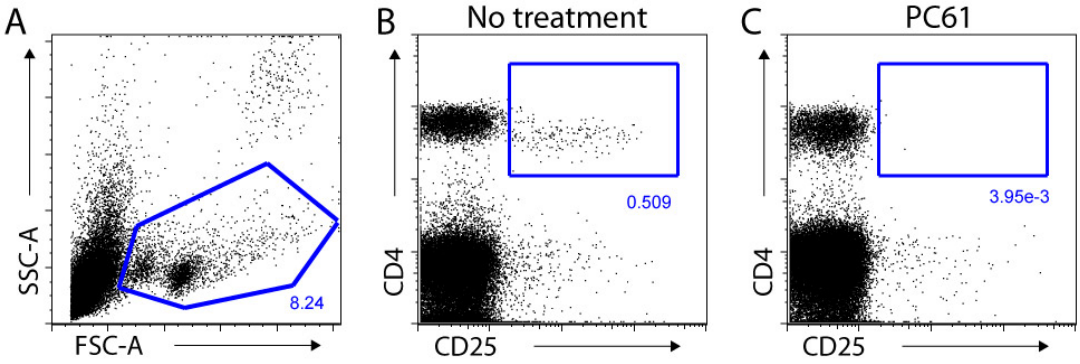
Appendix figure 7. Treatment of E.G7-OVA-bearing hosts with DT does not alter the symptom-free survival. *Lang*-EGFPDTR mice were intravenously administered $1x10^5$ C1498 cells. One group of tumour challenged mice were treated with 350 ng of DT intraperitoneally 2 days before tumour challenge and every 2–3 days later and the symptom-free survival was analysed. This represents a signal experiment with five mice per group. $*P < 0.05$ (Log-rank Mantel-Cox Test).

VIII. Appendix 8



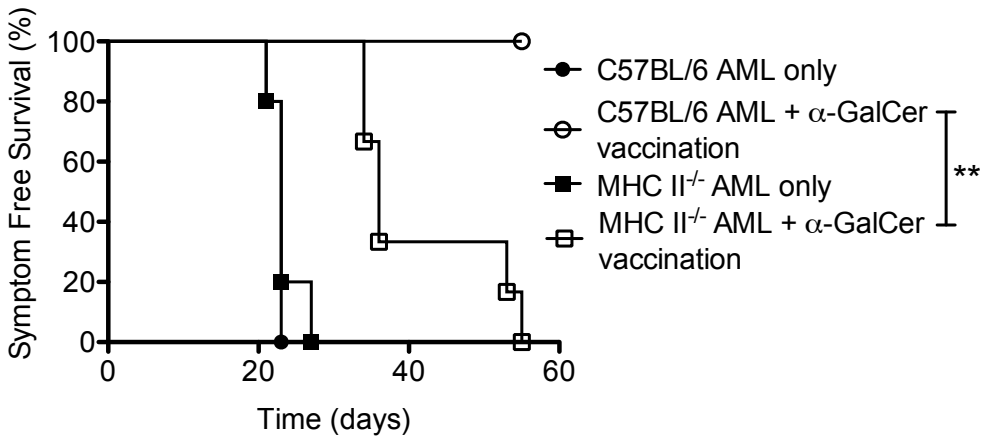
Appendix figure 8. C1498 does not increase the Treg population in the spleen. Mice were administered $1x10^5$ AML cells intravenously and were culled 20 days later. The population of T_{regs} in the spleen was analysed by flow cytometry using the gating strategy displayed in figure 4.14A. Foxp3 expression was determined by using a CD4 antibody in combination with GFP expression from FoxP3-GFP mice. The proportion of $CD4^+$ cells expressing FoxP3 is graphed. This represents two experiments, with five mice per group $^{**}P < 0.01$ (t-test with Mann Whitney).

IX. Appendix 9



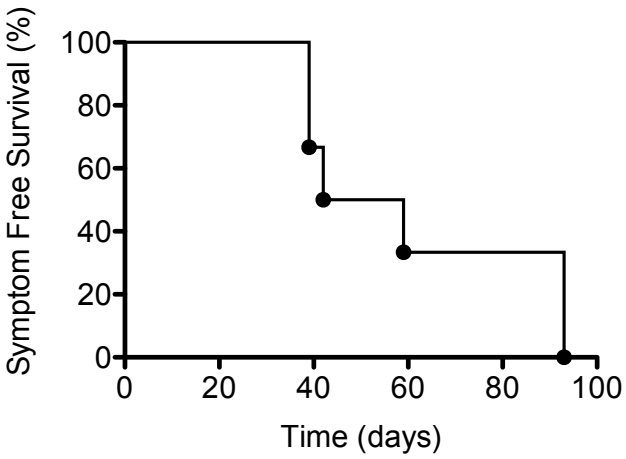
Appendix figure 9. Regulatory T cell depletion with PC61. C57BL/6 mice were treated intraperitoneally with 100 µg of PC61 or left untreated. Seven days later the spleens were removed and the T_{reg} population in was analysed by flow cytometry using CD4 and CD25 for T_{reg} identification. Flow plots demonstrate the effect of PC61 on the $CD4^+$ $CD35^+$ cells. This experiment was performed once with two mice per group.

X. Appendix 10



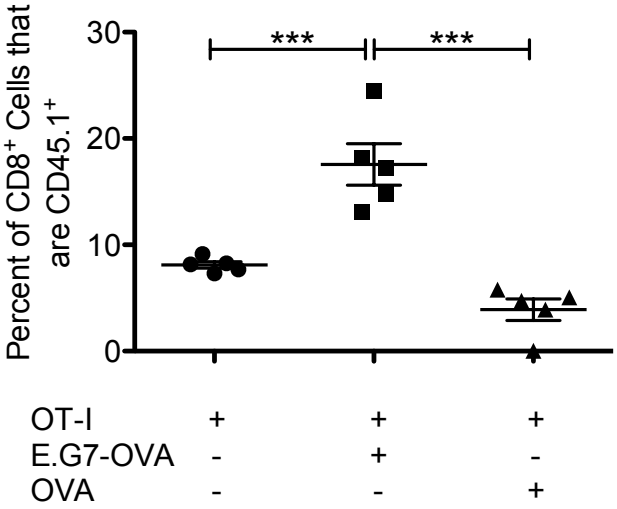
Appendix figure 10. The tumour/α-GalCer vaccine is ineffective in MHCII$^{-/-}$ mice. One group of C57BL/6 and one group of MHC II$^{-/-}$ mice were treated with 7.5x10^5 irradiated AML cells loaded with α-GalCer. Seven days later the mice were challenged with 1x10^5 C1498 cells along with another group of naïve mice from each mouse strain. The symptom-free survival was assessed. This represents a single experiment with five mice per group. ***P* < 0.01 (Log-rank Mantel-Cox Test).

XI. Appendix 11



Appendix figure 11. Hosts administered cytarbine for the treatment of AML relapse. C57BL/6 mice were challenged with $1x10^5$ AML cells and 24 hours later a course of chemotherapy was initiated. Three doses of cytarabine were administered ten hours apart, each consisting of 3 mg administered intraperitoneally. The symptom-free survival was followed. This represents the combination of two experiments with three mice per experiment.

XII. Appendix 12



Appendix figure 12. Antigen presentation alone does not stimulate transferred T cells. C57BL/6 mice were intravenously administered $5x10^6$ activated OT-I T cells. One group of mice was challenged with $1x10^6$ E.G7-OVA cells 12 prior to adoptive transfer and another received 200 µg of OVA protein 24 hours prior. Seven days later a sample of blood was extracted from each host and flow cytometry was performed to identify the transferred T cell population using CD8, Vα2 and CD45.1. The proportion of $CD8^+$ cells that express Vα2 and CD45.1 is displayed. This represents a single experiment with five mice per group. ***$P < 0.001$ (one-way ANOVA with a Bonferroni post test).

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