The fates of dendritic cells and antigen regulate CD4$^+$ T cell responses

Joel Zhi-Iong <u>Ma</u>

A thesis

submitted to the Victoria University of Wellington

in fulfilment of the requirements for the degree of

Doctor of Philosophy

in Biomedical Science

Victoria University of Wellington

2010

Abstract

The rapid activation of effector T cells by antigen-presenting dendritic cells (DCs) is necessary to contain and eradicate pathogens. Upon eradication of the pathogens by effector T cells, the immune response eventually resolves, and the clearance of residual antigen is necessary to prevent immune cell exhaustion or immunopathology.

It has been proposed that the elimination of antigen-presenting DCs by $CD8^+$ cytotoxic T cells (CTLs) limits the duration of antigen presentation, hence resolving ongoing immune responses. However, inter-DC antigen transfer spreads antigens for further antigen presentation and may reduce the effect of CTL-mediated DC killing. The aim of my thesis was to examine the impact of CTL-mediated DC killing and inter-DC antigen transfer on the induction and the quality of resulting T cell responses.

Initial experiments established that CTLs eliminated antigen-bearing DCs mainly through the cytolytic molecule perforin, whereas FasL played a minor role. CTL-mediated DC killing prevented antigen-bearing DCs from stimulating naïve $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes. Thus, CTLs regulated the clonal expansion of naïve T cells by controlling the survival of antigen-presenting DCs.

The efficiency of CTL-mediated DC killing depended on the method of antigen loading onto DCs, and to a lesser extent, the method of generating CTLs. Surprisingly, efficient CTL-mediated DC killing that completely prevented the accumulation of injected DCs in the lymph nodes did not abolish T cell proliferation, indicating that other antigen presenting cells (APCs) were inducing the residual T cell proliferation when the antigen-bearing DCs were eliminated by CTLs.

Further investigations revealed that the antigen from the injected DCs was transferred to host DCs. In the absence of direct antigen presentation by injected DCs, host DCs stimulated local T cell proliferation but did not induce a systemic effector T cell response. In contrast, in the presence of efficient CTL-mediated DC killing, inter-DC antigen transfer enabled the host DCs to stimulate T cell proliferation. These T cells then developed into

functional effector T cells. In conclusion, in the absence of inter-DC antigen transfer, CTL-mediated DC killing reduces the size of T cell responses. However, in the presence of inter-DC antigen transfer, the impact of CTL-mediated DC killing is reduced, hence influencing the size and quality of T cell responses. My findings shed light on how CTL-mediated DC killing and inter-DC antigen transfer regulate immune responses and how DC vaccine regimens for immunotherapy can be improved.

Acknowledgements

I am most grateful to my supervisor, Professor Franca Ronchese, for giving me this opportunity to pursue my Ph.D. Her insight, patience and encyclopaedic knowledge of immunology have and will continue to spur me on in my quest for scientific knowledge. I would not have completed my Ph.D. without her guidance and support. I would also like to thank my co-supervisor, Professor Graham Le Gros, for his advice and constructive criticism. His foresight and experiences have opened my mind to think outside the box. Under his directorship, the Malaghan Institute of Medical Research has been a conducive and intellectually challenging environment for my Ph.D. study. I would also like to thank my administrative supervisor, Dr. Anne La Flamme, for facilitating my Ph.D. study.

I am very grateful for the support that all my colleagues in the Malaghan Institute of Medical Research have given me. I would like to specially thank Dr. Mark Yang Jianping, who showed me the ropes of immunology when I first started off. Mark has been a great and indispensable mentor to me. I am indebted to Evelyn Hyde and Dr. Noriyuki Enomoto for their willingness to help me complete some of my 'technically impossible' experiments and to impart their knowledge to me. I am grateful for the assistance that Antonia Richter has provided me during her internship. I would like to thank my past and present colleagues in our group, in particular, Sonai Lim, Sabine Kuhn and Jim Qin, for their support and encouragement. I would also like to thank Dr. Clare Slaney, Assoc. Professor David Slaney, Dianne Sika-Paotanu and Stephanie Steiger. They have been invaluable friends and colleagues. I owe my study to the valiant mice, which sacrificed their lives for the betterment of mankind.

I want to express my heartfelt thanks to my parents and my aunt Alicia for their sacrifice, constant prayers and support. They have been my pillar of strength and motivation. I am very blessed to have my wonderful wife, Mei Yin, with me in New Zealand. Her being here has motivated me to complete my Ph.D. She has been my most important source of encouragement, company and advice. No words can describe how important my wife and my family are to me. Finally, hallelujah! He has brought me thus far, and will bring me even further!

Table of contents

List of figures

List of tables

Abbreviations

ACT	Ammonium chloride Tris
AICD	Activation-induced cell death
ALPS	Autoimmune Lymphoproliferative Syndrome
AP-1	Activated protein-1
APC	Antigen presenting cell
APC (fluorochrome)	Allophycocyanin
BM-DC	Bone marrow-derived dendritic cell
BrdU	Bromodeoxyuridine
CCR	CC chemokine receptor
CD	Cluster of differentiation, cluster of designation
CFSE	Carboxy-fluorescein diacetate succimimidyl ester
cIMDM	Complete IMDM
CLIP	Class II-associated invariant chain peptide
CMTMR	Chloromethyl-benzoyl-aminotetramethyl-rhodamine
CO_2	Carbon dioxide
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dH_2O	Distilled water
DISC	Death inducing signalling complex
DN DCs	Double negative DCs
DTR	Diphtheria Toxin Receptor
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FADD	Fas-associated protein via death domain
FCS	Fetal calf serum
FHL	Familial Hemophagocytic Lymphohistiocytosis
FITC	Fluorescein isothiocyanate
flt-3	Fms-like tyrosine kinase-3
FSC	Forward scatter
Gld	General lymphoproliferative disorder
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp33	Lymphocytic choriomeningitis virus glycoprotein 33 (KAVYNFATM)
GVHD	Graft versus host disease
HEV	High endothelial venules
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
i.v.	Intravenous
IFNγ	Interferon gamma
IL	Interleukin
IMDM	Iscoves' modified Dulbecco's medium
LC	Langerhans cells

LCMV	Lymphocytic choriomeningitis virus
lpr	Lymphoproliferation
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MACS	Magnetic cell separation
MAP kinase	Mitogen-activated protein kinases
MFI	Median fluorescence intensities
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MOG	Myelin oligodendrocyte glycoprotein
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	Natural Killer T cells
Nup98	Nucleoporin 98
OT-I	Ovalbumin transgenic-I
OT-II	Ovalbumin transgenic-II
OVA	Ovalbumin
OVA-TR	Ovalbumin conjugated to Texas Red
OVA$_{323-339}$	Ovalbumin MHC class II peptide (ISQAVHAAHAEINEAGR)
OVAtr	Ovalbumin transgenic
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll A protein
Pfp	Pore forming protein, perforin
PI	Propidium iodide
PKO	Perforin-deficient
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxygen species
Rpm	Revolutions per minute
s.c.	Subcutaneous
SEM	Standard error of mean
SIINFEKL	Ovalbumin MHC class I peptide (SIINFEKL)
SPI-6	Serine protease inhibitor-6
SSC	Side scatter
STAT	Signal Transducers and Activator of Transcription
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Tumour growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated protein via death domain
TRAIL	TNF-related apoptosis-inducing ligand
Treg	CD4$^+$ T regulatory cells
TSLP	Thymic stromal lymphopoietin

UPS	Ubiquitin-proteasome system
wt	Wild type
ZAP	Zeta-chain-associated protein kinase

Chapter 1

General Introduction

The immune system can be broadly characterised into innate and adaptive immunity. The innate immune system acts as the frontline defence against pathogens. The innate immune cells mobilise rapidly in the face of invading pathogens and respond in a non-antigen specific manner. Innate immune cells do not 'remember' the pathogens they have encountered before and cannot provide immunological memory for the host. In contrast, the adaptive immunity requires a longer time to mobilise than innate immunity. Adaptive immune cells respond and target pathogens that the immune cells recognise. Adaptive immune cells also remember the past pathogens they have encountered and if the same pathogen is seen again, these memory adaptive immune cells respond quickly to eliminate the pathogen. At the onset of pathogen invasion, the communication between the innate and adaptive immunity is essential for a well co-ordinated immune response. Dendritic cells (DC) play an important role in bridging the innate and adaptive immunity. DCs are potent activators of the adaptive immunity and are important regulators of the immune responses. Aberrant DC accumulation, prolonged DC survival, or ablation of DCs lead to autoimmune pathology (Chen et al., 2006; Ohnmacht et al., 2009), or delayed adaptive immune responses (GeurtsvanKessel et al., 2008).

In this chapter, I will provide the background information on the characteristics and functions of DCs, the origins of DCs, the different DC subsets and the types of T cell responses induced by DCs. I will discuss about the transfer of antigens between DCs. Lastly, I will elaborate on why it is important that DC survival and the duration of DC antigen presentation are regulated.

1.1 Dendritic cells and the 'Langerhans cell' paradigm

In 1973, Steinman R. M. and Cohn Z. A. identified DCs in murine spleen preparations and in other lymphoid organs based on their unique morphology (Steinman and Cohn, 1973). They characterised the phenotype, functions and population percentage in lymphoid tissues of these newly identified cells (Steinman et al., 1975; Steinman and Cohn, 1974; Steinman et al., 1974). Two subsequent seminal papers by Steinman and colleagues demonstrated the capacity of DCs in stimulating T lymphocytes. In the first study, Steinman purified DCs

from spleens and tested their ability to stimulate a mixed lymphocyte reaction (MLR) compared against whole spleen suspensions, macrophages, and preparations depleted of T and B cells (Steinman and Witmer, 1978). This study showed that DCs alone could stimulate MLR more potently than other cell types, and conversely, cell suspensions lacking DCs only stimulated MLR weakly. In the second study, with the aid of a then-newly developed specific antibody against DCs (Nussenzweig et al., 1982), Steinman depleted the DCs from spleen preparations and examined both proliferative and cytotoxic responses in MLR (Steinman et al., 1983). Depleting DCs in spleen preparations using the DC-specific antibody abrogated T cell proliferation and reduced cytotoxic responses. The study also showed that the re-introduction of purified DCs could restore MLR stimulatory capacity. These early studies in mice provided evidence that DCs were potent activators of T cells.

Paul Langerhans first observed DCs in the skin although at that time, these cells were thought to be of neural origin. Accordingly these cells were named Langerhans cells. Early characterisation of Langerhans cells and the realisation that Langerhans cells were related to the cells reported by Ralph Steinman in the 1980s threw light on the functions of DCs (Romani et al., 1989; Schuler and Steinman, 1985). When freshly isolated, murine Langerhans cells were proficient in taking up and processing intact antigens but did not activate T cells. However, during the course of *in vitro* culture, these Langerhans cells lost their antigen uptake efficiency and became potent T cells stimulator. Other studies during that period showed that mature Langerhans cells migrated out of skin transplants (Larsen et al., 1990) and lost their ability to phagocytose particular antigens (Reis e Sousa et al., 1993) and that DCs migrated from the blood into the spleen (Austyn et al., 1988).

From these studies in mice, the 'Langerhans cell paradigm' was formulated (Fig. 1.1). Immature DCs are proficient in antigen capture but inefficient in stimulating T cells. During the process of maturation, the DCs lose their phagocytic abilities, become potent T cell stimulators and migrate out of the peripheral tissues into the draining lymph nodes. In the draining lymph nodes, these mature DCs present antigens to naïve T cells and induce T cell responses.

The 'Langerhans cell paradigm' derived from these studies was based on the observations on the conventional DCs in the non-lymphoid tissues. The conventional DC is one of the two members belonging to the DC family. The other member under the DC family tree is called plasmacytoid DC. Conventional and plasmacytoid DCs differ in their development, phenotype, morphology, migratory behaviour and functions. For example, plasmacytoid DCs resemble lymphoblasts or plasma cells, are phenotypically recognised as $B220^+CD11c^{lo}$ in mice and secrete large amounts of their signature cytokine type 1 interferon (IFN), whereas conventional DCs have a dendrite-like morphology, are $B220^-$ $CD11c^{hi}$ in mice, and secrete lower amounts of type I IFN in response to RNA viruses compared to plasmacytoid DCs (Colonna et al., 2004). The 'Langerhans cell paradigm' addresses the functional development and phenotypic changes in conventional DCs, and hereafter I shall focus my discussion on conventional DCs and designate conventional DCs as DCs for simplicity.

Immature Langerhans cell	Mature Langerhans cell
- Reside in the epidermis - Proficient at antigen capture - Poor T cell stimulator - Express low levels of MHC class II and co-stimulatory molecules	- Migrate to draining lymph nodes - Poor at antigen capture - Potent T cell stimulator - Express high levels of MHC class II and co-stimulatory molecules
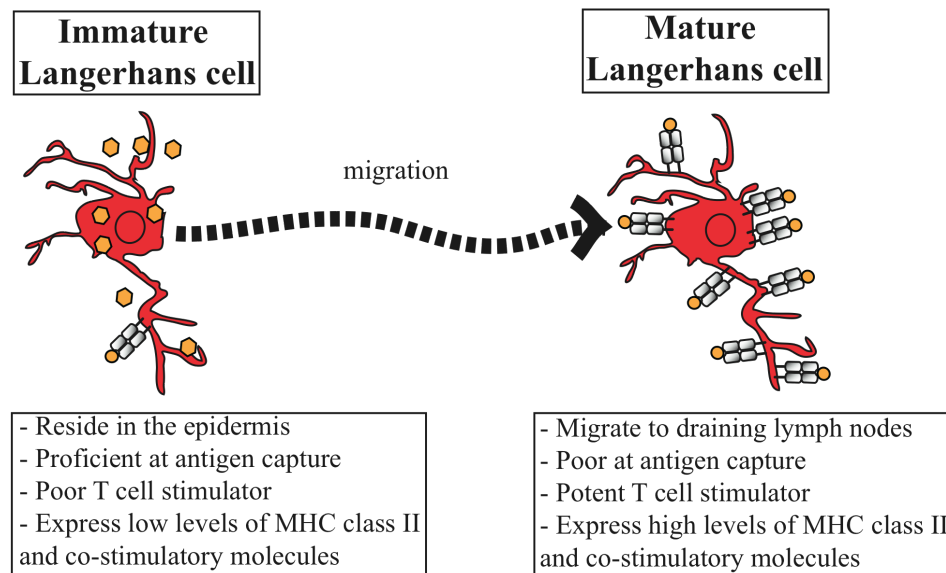
Fig. 1.1. **The Langerhans cell paradigm delineates DCs into immature and mature stages.**

1.2. DCs are professional APCs

Nearly all cell types can be considered as antigen-presenting cells (APC) because they are capable of presenting antigens to $CD8^+$ T cells through the expression of MHC class I molecules. However, to qualify as a professional APC the cell must fulfil two criteria. The first criterion is that the cell must be able to present antigens to naive $CD4^+$ T cells,

therefore the cell must express MHC class II and be able to internalise and process antigens via the MHC class II pathway. The second criterion is that the cell must express co-stimulatory molecules that can lead to the activation of T cells. Although some authors have defined DCs, B cells and macrophages as professional APCs, others propose that B cells and macrophages are not as 'professional' as DCs in terms of antigen presentation (Trombetta and Mellman, 2005). B cells are poor at antigen uptake but specialise in antibody production. B cells cannot stimulate naïve CD4$^+$ T cells although they can stimulate memory CD4$^+$ T cells (Ronchese and Hausmann, 1993). Macrophages are highly proficient in antigen uptake and processing; but they express low levels of MHC class II and co-stimulatory molecules and are poor at activating CD4$^+$ T cells. While macrophages and B cells can present endogenous proteins on MHC class I to CD8$^+$ T cells, neither of them can present exogenous proteins on MHC class I to CD8$^+$ T cells as efficiently as DCs. Moreover, DCs are functionally plastic, located in strategic areas of the body and made up of a heterogeneous population (Trombetta and Mellman, 2005). These characteristics enable DCs to function as highly proficient professional APCs.

1.3. Different functional stages of DCs

In the 'Langerhans cell paradigm', the term 'maturation' has been used to delineate the acquisition of the ability to stimulate naïve T cells and induce effector T cell responses (Villadangos and Heath, 2005). However, accumulating evidence has shown that immature DCs also stimulated naïve T cells but induced T cell tolerance instead (Finkelman et al., 1996; Steinman et al., 2003), emphasising the need to redefine the functional stages of DCs. Several investigators have used different terminology to define DC functional stages and to the best of my knowledge, there is no standard guideline for defining the different stages of DCs (Lutz and Schuler, 2002; Reis e Sousa, 2006; Villadangos and Heath, 2005). In my study, I have categorised different DC functional stages into immature, steady-state, activated and licensed (Fig. 1.2). At these different stages, DCs perform and specialise in different functions. The different functional stages of DCs will be detailed in the following sections.

Immature DCs

naïve T cell

Activation stimulus

spontaneous migration

migration

migration

proliferates & develops

Steady-state DCs

Activated DCs

Licensed DCs

CD4⁺ T helper cell

naïve T cell

naïve T cell

naïve CD8⁺ T cell

proliferates & develops

proliferates & differentiates into

proliferates & differentiates into

T cell tolerance

T cell tolerance

Effector T cells

Cytotoxic T lymphocyte

develops into

Memory CD8⁺ T cell

Fig. 1.2. **DCs have different functional stages that induce different types of T cell responses.**

1.3.1. Immature DCs are efficient in antigen capture and induce T cell tolerance

Most DCs in the body exist in an immature status. Immature DCs are constantly sampling their environment (Lindquist et al., 2004; Ng et al., 2008). They are proficient in antigen capture because they have a wide variety of mechanisms such as receptor-mediated endocytosis, phagocytosis and macropinocytosis to take up different forms of antigen. In receptor-mediated endocytosis, immature DCs use a range of receptors such as CD205 (Jiang et al., 1995), mannose receptor (Sallusto et al., 1995), Fc receptors such as FcγRI, FcγRII (Regnault et al., 1999) and FcεRI (Jurgens et al., 1995), and scavenger receptors such as CD36 (Platt et al., 1998). In phagocytosis, immature DCs take up particulate antigens through receptor-mediated actin polymerisation. The types of particulate antigens taken up by immature DCs include bacteria, fungi, parasites, apoptotic cells and necrotic debris. In macropinocytosis, immature DCs internalise soluble antigens in an actin cytoskeleton-dependent manner. Immature DCs also utilise macropinocytosis constitutively (Sallusto et al., 1995).

Although immature DCs are efficient in antigen uptake, the antigens taken up are not rapidly degraded to be loaded onto MHC class II molecules but remain in the lysosomal compartments for several days (Inaba et al., 2000) due to low proteolytic efficiency (Fiebiger et al., 2001). Immature DCs also express low levels of MHC class II molecules, partly because they rapidly recycle MHC class II molecules from the cell surface (Askew et al., 2000; Wilson et al., 2004). This forms a situation whereby few MHC class II molecules are available for the loading of degraded antigens thus leading to fewer antigens presented on the cell surface (Colledge et al., 2002; Inaba et al., 2000; Turley et al., 2000; Veeraswamy et al., 2003).

Immature DCs also express low levels of co-stimulatory molecules such as CD80, CD86 and CD40 (Inaba et al., 1994; Larsen et al., 1992; Vremec and Shortman, 1997; Wilson et al., 2003). Co-stimulatory molecules CD80 and CD86 on DCs engage CD28 on T cells. Sufficient co-stimulatory engagement is necessary for full activation of T cells. While these immature DCs induce T cell proliferation, the T cells are removed from circulation or become unresponsive to secondary antigenic stimulation and do not develop into effector T

cells (Bonifaz et al., 2002; Dhodapkar et al., 2001; Hawiger et al., 2001; Scheinecker et al., 2002). Taken together, immature DCs are specialists in antigen capture because of the accessibility to various antigen uptake mechanisms; and are poor in activating effector T cells because they provide minimum antigenic and insufficient co-stimulatory signals to the T cells (Hernandez et al., 2001; Kearney et al., 1994).

1.3.2. Steady-state DCs induce T cell tolerance

Immature DCs in the peripheral tissues spontaneously migrate to the draining lymph nodes in the absence of inflammatory molecules or pathogenic stimuli (Hemmi et al., 2001; Mishima, 1966; Ruedl et al., 2000; Vermaelen et al., 2001). They carry along with them antigens captured from their environment. These DCs are referred to as steady-state DCs. In the skin, the spontaneous migration of steady-state DCs has been proposed to be induced by the disruption of E-cadherin adhesion between the DCs and neighbouring cells (Jiang et al., 2007). Steady-state DCs upregulate MHC class II expression, express moderate levels of co-stimulatory molecules but do not express inflammatory cytokines (Jiang et al., 2007; Ruedl et al., 2000). Similar to lymph node resident immature DCs, antigen-presenting steady-state DCs induce T cell proliferation but these T cells do not develop into functional effector T cells (Jiang et al., 2007; Kurts et al., 1999; Waithman et al., 2007). Constitutive removal of DCs under steady-state conditions leads to spontaneous autoimmunity, indicating the importance of immature and steady-state DCs in maintaining T cell tolerance (Ohnmacht et al., 2009).

1.3.3. Activated DCs prime functional effector T cells

Immature DCs become activated DCs when they encounter inflammatory cytokines or pathogen associated molecules patterns (PAMPs). Different types of PAMPs bind to receptors such as Toll-like receptors (TLR) in the DCs and activate intracellular signalling molecules such as MyD88 (Janeway and Medzhitov, 2002). Inflammatory cytokines such as TNF-α and IL1β are recognised by corresponding cytokine receptors on the immature DCs. Other signals that induce DC activation include necrotic cells (Sauter et al., 2000) and immunoglobulins (Regnault et al., 1999). Activated DCs downregulate macropinocytosis

and non-specific phagocytosis (Garrett et al., 2000; Platt et al., 2010), although receptor-mediated endocytosis and receptor-mediated phagocytosis in activated DCs remain intact (Platt et al., 2010). Activated DCs increase MHC class II synthesis transiently, and express high levels of MHC class II, CD80 and CD86. Antigen processing through MHC class II pathway is upregulated by the acidification of lysosomal compartments. The translocation of MHC class II molecules to the cell surface is increased and the recycling of surface MHC class II molecules is reduced (Cella et al., 1997; Chow et al., 2002; Pierre et al., 1997). This results in an increase in surface MHC class II-peptide complexes (Cella et al., 1997; Villadangos et al., 2001). The expression and half-life of MHC class I complexes in DCs can also be increased by activation signals (Delamarre et al., 2003; Rescigno et al., 1998). The changes in activated DCs allow them to retain and present the antigens captured in an immature state to antigen-specific naïve T cells. In the activated state, DCs express high levels of co-stimulation molecules such as CD80, CD86 and CD40, which fully activate naïve T cells into functional effector T cells (Fujii et al., 2004)

In peripheral DC populations such as Langerhans cells, DC activation is associated with increased migratory behaviour and migration from the peripheral tissues to the draining lymph nodes. These activated peripheral DCs upregulate matrix metalloproteases to navigate through the extracellular matrix to the lymph vessels (Ratzinger et al., 2002). Activated peripheral DCs also upregulate CCR7 expression in order to home into the paracortex of draining lymph nodes through the recognition of CCL19 and CCL21 chemokine gradients (Martin-Fontecha et al., 2003). Naïve T cells also reside in the paracortex of draining lymph nodes; hence the chances of activated peripheral DCs encountering antigen-specific naïve T cells are increased.

1.3.4. Licensed DCs prime functional effector and memory CD8^{+} T cells

The interaction between CD40 on immature DCs and CD40L on CD4^{+} T cells also induces DC activation (Caux et al., 1994; Schuurhuis et al., 2000). CD4^{+} T cell-helped DC activation has been termed 'DC licensing' (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). The functional difference between the activated DCs described in 1.2 and licensed DCs is observed in the type of CD8^{+} T cells induced by the respective

types of DCs. Activated DCs can stimulate CD8^{+} T cells into CTLs but these CTLs have poor cytotoxic effector functions, short life span and die from TRAIL-mediated death (Janssen et al., 2005). In contrast, with the help provided by CD4^{+} T cells, licensed DCs induce functional effector and memory CTLs (Janssen et al., 2003; Smith et al., 2004). Licensed DCs primed CTLs even after the signals from CD4^{+} T cells were removed, indicating that the DCs retained the information provided by CD4^{+} T cells (Smith et al., 2004; van Mierlo et al., 2004). The signals sent from the licensed DCs to the CTLs include CD27-CD70 interaction (Ballesteros-Tato et al., 2010), decreased interaction between programmed cell death (PD)-1 and PD ligands (Keir et al., 2007), or increased CTL autocrine production of IL-2 through CD27-CD70 interaction (Peperzak et al., 2010).

Other immune cells can also license DCs. NK cells have been shown to license DCs to induce memory CD8^{+} T cell responses (Mocikat et al., 2003). A recent study on NKT cells has shown that NKT cells licensed DCs and induced DCs to produce CCL17 to attract naïve CD8^{+} T cells (Semmling et al., 2010). The expression of CD40L on DCs also provide optimal signals for the priming of CD8^{+} T cell responses without CD4^{+} T cell help (Johnson et al., 2009).

1.4. Different subsets of DCs are found in different locations

DCs arise from precursor cells that originate from the bone marrow (BM) haematopoietic stem cells. These DC precursors migrate from the bone marrow to different tissues. The constant influx of DC precursors into the tissues continually replaces DCs that migrate and eventually undergo apoptosis in the lymph nodes or spleens, resulting in a constant DC turnover. The differentiation of precursors to DCs is largely dependent on fms-like tyrosine kinase-3 (flt-3) ligand (McKenna et al., 2000). Certain types of DCs, like the Langerhans cells, can self-renew *in situ* and are not replaced by circulating precursors under steady state conditions (Merad et al., 2002). The differentiation of precursors to Langerhans cells is also dependent on TGF-β1 (Borkowski et al., 1996) and M-CSF (Ginhoux et al., 2006) but is flt-3 ligand independent (Ginhoux et al., 2009). The development of some DCs, like the skin-derived dermal DCs (King et al., 2010) or *lamina propria* DCs (Bogunovic et al.,

2009; Varol et al., 2009), are less dependent on flt-3 ligand and depend on GM-CSF instead.

DCs are found in different organs and tissues, such as the skin, lymph nodes, spleen, lung, intestines, liver, kidneys, pancreas and eyes (Forrester et al., 2010; Helft et al., 2010). These DCs comprise of different DC subtypes and can be distinguished by their phenotypic markers. Some DCs express similar phenotypic markers but are found in different tissues (del Rio et al., 2010). However, all these DC subsets share a dendritic morphology, express high levels of CD11c and MHC class II, and are potent T cell stimulators. In this study, I will focus on DCs found in the skin and skin-draining lymph nodes.

1.4.1. Different DC subsets are found in the skin

In the skin, Langerhans cells (LC) occupy the interstitial spaces between keratinocytes in the epidermis (Valladeau and Saeland, 2005). The LC precursors populate and proliferate rapidly in the epidermis after birth (Elbe et al., 1989). As mentioned earlier, the development of precursors into LCs under steady-state conditions is dependent on TGF-β1 and M-CSF as mice devoid of these cytokines lack epidermal LCs (Borkowski et al., 1996; Ginhoux et al., 2006). Steady-state LCs reside in the epidermis for their entire lifespan and turn over slowly as observed from bromodeoxyuridine (BrdU) labelling of dividing cells (Kamath et al., 2002; Merad et al., 2002). LCs are usually immobile but move their dendrites occasionally to survey the environment (Ng et al., 2008; Nishibu et al., 2006). Under steady-state conditions, some LCs spontaneously migrate to the skin-draining lymph nodes, possibly due to the disruption of E-cadherin anchorage onto neighbouring keratinocytes (Jiang et al., 2007). The activation of LCs leads to increased LC migration into the skin-draining lymph nodes. Among the various phenotypic markers, LCs express high levels of CD207/langerin (Valladeau et al., 2000), CD205 (Jiang et al., 1995) and E-Cadherin.

Dermal DCs, like LCs, are CD205$^+$CD8^{lo}. Dermal DCs can be broadly characterised as CD103$^+$ and CD11b$^+$ DCs. CD103$^+$ DCs also express CD207 (Ginhoux et al., 2007; Henri et al., 2010). The development of dermal DCs is largely dependent on flt-3 ligand. Mice

lacking flt-3 or flt-3 ligand lack CD103$^+$ dermal DCs and have reduced numbers of CD11b$^+$ dermal DCs (Ginhoux et al., 2009). GM-CSF also plays a role in the development of dermal DCs as mice lacking GM-CSF also have reduced numbers of CD103$^+$ and CD11b$^+$ dermal DCs (King et al., 2010; Kingston et al., 2009). Under steady-state conditions, dermal DCs exhibit different behavioural patterns from LCs as they actively crawl through the interstitial space of the dermis. Some dermal DCs also migrate to the skin-draining lymph nodes at steady-state conditions. Upon encounter with pathogenic molecules or parasites, dermal DCs decreased their migratory speed and changed their morphology, possibly to facilitate antigen capture (Ng et al., 2008). These activated dermal DCs then migrate to the draining lymph nodes.

In the skin-draining lymph nodes, three lymph node resident DC populations that can be distinguished by their expressions of CD205, CD8 and CD4 have been characterised (Vremec and Shortman, 1997). CD8$^+$CD205$^+$CD4$^-$ DCs are found in the paracortex of the draining lymph nodes. The anatomical location of CD8$^-$CD205$^-$CD4$^-$ DCs in the draining lymph nodes is still unclear although an early study indicated that these DCs were not found in the T or B cell areas (Witmer and Steinman, 1984). The frequency of CD8$^-$CD205$^-$CD4$^+$ DCs is very low in the draining lymph nodes (Shortman and Liu, 2002) and their anatomical location in the draining lymph nodes is also unknown. Besides these lymph node-resident DCs, Langerhans cells and dermal DCs are also found in distinct areas of the paracortex under steady-state conditions (Kissenpfennig et al., 2005) and accumulate in the draining lymph nodes upon skin inflammation or infection.

Monocytes circulating in the blood can be recruited to the inflammatory or infected sites. These recruited monocytes subsequently differentiate into DCs. For example, during skin inflammation, circulating monocytes are recruited to the inflamed epidermis. The recruited monocytes down-regulate the monocyte marker Gr-1, upregulate MHC class II expression, and differentiate into LCs (Ginhoux et al., 2006). Monocytes have also been shown to differentiate into dermal DCs and lymph node resident DCs during an infection with *Leishmania major* (Leon et al., 2007). These monocyte-derived skin DCs are also found in the skin-draining lymph nodes. They exhibit an activated DC phenotype and are able to induce effector T cells.

1.5. Different DC subsets have different functions

Studies have indicated that the different DC subsets have different functions. There is some evidence indicating that the different functions are inherent in the DC subsets and DC subsets of a certain lineage have similar functions. Here, I summarise the evidence from different DC subsets located in different tissues according to their different functions.

The ability to present antigens to $CD4^+$ and $CD8^+$ T cells vary among the DC subsets. All DCs can present antigens to $CD8^+$ T cells when they are infected with pathogens or presenting endogenous antigens. However, not all DCs can capture exogenous antigens and present them to $CD8^+$ T cells; this process is referred to as cross-presentation and will be discussed in 1.7.3. DCs that are proficient in cross-presenting to $CD8^+$ T cells include lymphoid resident $CD8^+CD205^+$, skin derived $CD103^+$ dermal DCs, $CD103^+$ intraepithelial lung DCs and LCs (Bedoui et al., 2009; del Rio et al., 2007; den Haan et al., 2000; Stoitzner et al., 2006). On the contrary, $CD8^-CD205^-$ DCs are better at stimulating $CD4^+$ T cells than $CD8^+CD205^+$ DCs (Dudziak et al., 2007). Similarly, skin-derived $CD11b^+$ dermal DCs and $CD11b^+$ lung DCs are also better at priming $CD4^+$ T cells than $CD103^+$ dermal DCs (Bedoui et al., 2009; del Rio et al., 2007).

Different DC subsets can capture antigens through different mechanisms. $CD8^+CD205^+$ DCs express a unique set of proteins and receptors distinct from lymphoid resident $CD8^-CD205^-$ DCs (Burgdorf et al., 2007; Dudziak et al., 2007; Schnorrer et al., 2006). Unlike $CD8^-CD205^-$ DCs, $CD8^+CD205^+$ DCs are also capable of phagocytosing apoptotic cells and exosomes (Iyoda et al., 2002; Segura et al., 2007). Recent evidence has shown that within the splenic $CD8^+CD205^+$ DCs, the DCs expressing CD103 and CD207 are efficient in phagocytosing apoptotic cells (Qiu et al., 2009). This is interesting because the expression of CD103 in dermal DCs has also been proposed to enable $CD103^+$ dermal DCs to reach across the basement membrane into the epidermis to take up antigens (Bursch et al., 2007). CD103 binds to E-Cadherin, which is expressed on all the epidermal cells including LCs. In support of this proposal, $CD103^+$ intraepithelial lung DCs extend their dendrites into the airway lumen (Sung et al., 2006). This phenomenon of extending dendrites across the epithelia layers has also been observed in intestinal DCs where they

sample for bacteria across the epithelium (Rescigno et al., 2001). The evidence indicates that the expression of certain molecules enables the DC subset to perform specific functions.

Different DC subsets induce different types of T cell responses. $CD8^+CD205^+$ splenic DCs produced IL-12 after microbial stimulation *in vivo* (Reis e Sousa et al., 1997) and skewed $CD4^+$ T cells to Th1 responses. Alternatively, there is also evidence that $CD8^+CD205^+$ splenic DCs are able to induce $CD4^+$ T cells into regulatory T cells in the absence of exogenous TGFβ. In contrast, $CD8^-CD205^-$ DCs were only able to do so when TGFβ was added (Yamazaki et al., 2008). $CD11b^+$ DCs from the Peyer's patches produced IL-10 and skewed $CD4^+$ T cells to Th2 responses better than $CD8^+$ and $CD8^-CD11b^-$ DCs from the Peyer's patches (Iwasaki and Kelsall, 2001). Intestinal $CD103^+$ DCs, but not $CD103^-$ DCs, were also able to induce $CD4^+$ regulatory T cells (Coombes et al., 2007).

Different DC subsets of a certain lineage have similar functions. As mentioned previously, lymphoid resident $CD8^+$ DCs and $CD103^+$ DCs in the peripheral tissues are proficient cross-presenters to $CD8^+$ T cells. They also have unique antigen capture mechanisms that facilitate their cross-presenting functions and are distinct from lymphoid resident or peripheral DCs that express CD11b but not CD8 nor CD103. In mice lacking the transcription factor Batf3 (basic leucine zipper transcription factor, ATF-like 3), $CD103^+$ dermal DCs (Edelson et al., 2010) and lymphoid resident $CD8^+$ DCs (Hildner et al., 2008) are absent whereas other DC subsets are intact. This supports the notion that different DC subsets are inherently distinct and fulfil different functions in the body.

1.6. DCs can be cultured from precursors *ex vivo*

Although DCs are found in many tissues and organs in the body, one major difficulty when studying DCs is the low frequency of DCs found in the body. When an early study showed that adding GM-CSF in murine BM haematopoietic stem cells could generate large numbers of DCs (Inaba et al., 1992), the use of *in vitro* cultured DCs greatly opened up the field of DCs. Subsequent studies on generating DCs from human PBMCs added GM-CSF and IL-4 to yield immature DCs (Romani et al., 1994; Sallusto and Lanzavecchia, 1994).

GM-CSF differentiates and mobilises myeloid cells (Metcalf et al., 1986), whereas IL-4 inhibits the development of macrophages (Jansen et al., 1989). The culture of murine BM cells with GM-CSF/IL4 generates immature DCs, which have CD11c^{hi}CD11b^{+} phenotype. Upon treatment with activation stimuli such as LPS, these immature DCs acquire an activated phenotype observed from the upregulation of MHC class II and co-stimulatory molecules. When injected subcutaneously, activated GM-CSF/IL-4 murine DCs migrate into the paracortex in the draining lymph nodes via a CCR7-dependent manner (Martin-Fontecha et al., 2003). These DCs produce cytokines such as TNF-α and CCL-2 and are similar to inflammatory DCs induced by *Listeria monocytogenes* infection *in vivo* (Serbina et al., 2003; Xu et al., 2007).

The culture of murine BM cells with flt-3 ligand also generated large numbers of DCs that consisted of three DC populations – CD11b^{+}CD11c^{hi} and CD24^{+}CD11c^{hi} DCs and B220^{+}CD11c^{lo} DCs (Brasel et al., 2000; Brawand et al., 2002). CD11b^{+}CD11c^{hi} and CD24^{+}CD11c^{hi} DCs closely resemble CD8^{-} and CD8^{+} DCs residing in lymphoid organs (Brasel et al., 2000; Naik et al., 2005). This is in accordance with the finding that flt-3 ligand gives rise to multiple types of DCs *in vivo* (McKenna et al., 2000).

1.7. DCs present antigens to CD4^{+} and CD8^{+} T cells via MHC molecules

DCs present antigens on MHC class II to CD4^{+} T cells, MHC class I to CD8^{+} T cells and CD1 to NKT cells (Bendelac et al., 2007). It was previously thought that antigens loaded on MHC class II or MHC class I were derived from exogenous and endogenous cellular proteins respectively. This delineation has been blurred after it was demonstrated that in some cases, DCs could present antigens from exogenous proteins to CD8^{+} T cells via MHC class I (Brossart and Bevan, 1997). This process has been referred to as 'cross-presentation'.

1.7.1 DCs present exogenous antigens on MHC class II molecules to CD4^{+} T cells

DCs take up proteins from the extracellular environment via macropinocytosis, phagocytosis and receptor-mediated endocytosis. The endocytosed proteins are carried in

endocytic vesicles and are delivered to endosomes. The protein-carrying endosomes then fuse with lysosomes. The acidification in the lysosomes then activates endosomal and lysosomal proteases, which degrade the endocytosed proteins into peptide fragments (Trombetta et al., 2003). MHC class II molecules are assembled in the endoplasmic reticulum (ER). An invariant chain, Ii, binds in the groove of the MHC class II molecule to prevent binding of peptides and partly-folded proteins (Riberdy et al., 1992). It also directs the MHC class II molecules to the peptide-carrying endosomes where peptide loading occurs. Subsequent cleavage of the invariant chain leaves a short peptide fragment CLIP (class II-associated invariant chain peptide) in the groove of the MHC class II molecule (Avva and Cresswell, 1994). The CLIP-MHC class II complex is then stabilised by HLA-DM, which also catalyses the release of CLIP fragment and the loading of the peptide fragments onto the empty MHC class II molecules (Denzin and Cresswell, 1995). The peptide-loaded MHC class II complex is then transported to the cell surface of the DC.

1.7.2 DCs present endogenous antigens on MHC class I molecules to CD8^{+} T cells

Like all other nucleated cells, DCs also degrade their cytosolic proteins by the ubiquitin-proteasome system (UPS) into peptides (Pamer and Cresswell, 1998). These peptides are then transported by TAP1 and TAP2 molecules into the ER. In the ER, the partially folded MHC class I chains bind to calnexin (Suh et al., 1996). The binding of β_2-microglobulin to the MHC class I chains releases the MHC class I from calnexin. This MHC class I-β_2-microglobulin complex then binds to other chaperone proteins including calreticulin and tapasin (Sadasivan et al., 1996). The binding of the MHC class I-chaperone complex to TAP via tapasin allows the loading of peptides onto the MHC class I molecule. The loading of peptides then releases the MHC class I molecule from the chaperone proteins (Lehner et al., 1998). The peptide-loaded MHC class I complex is subsequently transported to the cell surface of the DC. Following this, the DC then presents the peptide loaded onto MHC class I molecules to the antigen-specific CD8^{+} T cells. This is also known as the classical MHC class I pathway.

1.7.3. DCs cross-present exogenous antigens on MHC class I molecules to CD8$^+$ T cells

While all nucleated cells can degrade cytosolic proteins and present them via the classical MHC class I pathway, DCs are also able to process exogenous antigens and present them on MHC class I molecules. This pathway is referred to as 'cross-presentation'. Cross-presentation was first proposed to explain how the presentation of exogenous antigens could prime CD8$^+$ T cells (Bevan, 1976, 1989). Although some *in vitro* evidence has shown that macrophages and B cells can cross-present (Ke and Kapp, 1996; Kovacsovics-Bankowski et al., 1993), CD8$^+$ DCs, and more recently identified, CD24$^+$ and CD103$^+$ DCs, are the DC subsets that can cross-present to naïve CD8$^+$ T cells effectively (Bedoui et al., 2009; Carbone et al., 1998; del Rio et al., 2007; den Haan et al., 2000; Pooley et al., 2001). Recent evidence has shown that under certain conditions, other DC subsets can also cross-present to CD8$^+$ T cells (Ballesteros-Tato et al., 2010; den Haan and Bevan, 2002; McDonnell et al., 2010).

It is not clearly demonstrated why certain DC subsets are cross-presenting antigens to CD8$^+$ T cells while others don't. The consensus is that cross-presenting DCs are inherently different from non cross-presenting DCs. In support of this, cross-presenting DCs express higher levels of distinct proteins involved in MHC class I antigen processing (Dudziak et al., 2007) and have lower phagosomal acidity (Savina et al., 2009) compared to non cross-presenting DC subsets. While the uptake of antigens for cross-presentation is distinct from other antigen presentation pathways (Burgdorf et al., 2008), certain receptors used to take up antigens are selectively expressed on some DC subsets that cross-present (Belz et al., 2002; Burgdorf et al., 2007; Caminschi et al., 2008). The DC subsets that can cross-present share the same DC lineage because Batf3-deficient mice lack the cross-presenting DC subsets characterised by the phenotypic markers CD8 and CD103 (Hildner et al., 2008). Taken together, the evidence reported has been consistent with the notion that the ability to cross-present to CD8$^+$ T cells is inherent only in the cross-presenting DC subset.

Although the mechanisms of cross-presentation are not fully elucidated, there are at least three mechanisms that have been put forward (Burgdorf and Kurts, 2008; Heath and Carbone, 2001a; Yewdell et al., 1999). In the first mechanism, captured antigens are

degraded and directly loaded onto MHC class I molecules in a TAP-independent manner in the endosomes (Kurotaki et al., 2007). The other two mechanisms are mediated in a TAP-dependent manner and the antigens are degraded by the cytoplasmic proteasome. These two TAP-dependent mechanisms differ in the location of antigen-loading onto MHC class I molecules. The first TAP-dependent mechanism proposes that antigen-loading onto MHC class I molecules occurs in the ER (Ackerman et al., 2006); whereas in the second TAP-dependent mechanism, antigens are loaded in the endosomes (Ackerman et al., 2003).

1.8. The peptide-MHC complexes on DCs engage TCRs on T cells

The peptide-MHC complexes on the cellular surface of DCs engage the TCR complexes on the T cells. TCRs are made up of α and β chains. The α chains contain V and J gene segments, whereas the β chains consist of V, D and J gene segments. The rearrangements of the TCR genes occur during T cell development in the thymus. The developing T cells in the thymus are called thymocytes. After rearranging their TCR chains, thymocytes have to interact with self peptide-self MHC molecules complexes on the thymic epithelial cells to continue their development; otherwise they undergo apoptosis due to neglect. This is known as positive selection (Starr et al., 2003). Thymocytes that successfully interact with self peptide-self MHC molecules complexes cease their TCR gene rearrangements to prevent the occurrence of T cells with dual TCR specificity, which may give rise to undesirable immune responses. During positive selection, thymocytes can develop into either $CD4^+$ or $CD8^+$ T cells, depending on the binding of co-receptor CD4 or CD8 to the respective MHC molecules.

The positively selected thymocytes then encounter the stromal or bone-marrow derived thymic cells that present a broad range of tissue-specific antigens. When the TCRs on the positively selected thymocytes bind too strongly to the self peptide-MHC complexes on the thymic stromal cells or thymic DCs, the thymocytes are removed by clonal deletion. This process is referred to as negative selection, which removes autoreactive thymocytes in the thymus (Starr et al., 2003). This process of positive and negative selection is also known as central tolerance.

Thymocytes that are not negatively selected develop into either CD4$^+$ or CD8$^+$ T cells. Newly developed CD4$^+$ and CD8$^+$ T cells that have not encountered their cognate antigens are referred to as naïve T cells. These naïve T cells leave the thymus and circulate into the T cell zone of secondary lymphoid organs such as the lymph nodes and spleens, where they reside while awaiting antigenic stimuli.

1.9. The activation status of DCs determines their ability to induce T cell responses

DCs are the most potent APCs at inducing *de novo* T cell responses from naïve T cells. When DCs present antigens to naïve T cells, the engagement of the TCR initiates intracellular signalling into the T cells. The earliest intracellular events occurring after TCR engagement is the activation of protein tyrosine kinase (Gauen et al., 1994; Samelson and Klausner, 1992). Activation of protein tyrosine kinase then elicits downstream intracellular signalling events, including the phosphorylation of the CD3ε, ζ-associated protein (ZAP)-70 and MAP kinases; increase in intracellular calcium; activation of transcription factors such as NFκB, nuclear factor of activated T cells (NFAT) and activator protein (AP)-1 (Cantrell, 1996; Fooksman et al., 2010). These transcription factors then translocate into the nucleus and induce gene transcription.

At the inter-cellular level, the naïve CD8$^+$ T cells undergo multiple transient encounters with the antigen-presenting DCs during the first few hours of DC-T cell interaction (Mempel et al., 2004). The surface expression of activation markers CD69 and CD44 on these T cells are also upregulated during the transient DC-T cell interactions. This is followed by the formation of stable interactions between the DC and T cell. Unlike CD8$^+$ T cells, naïve CD4$^+$ T cells are able to form stable interactions with antigen-presenting DCs upon their first encounter (Celli et al., 2007). Other activation markers such as CD25 are upregulated and the T cells begin to make cytokines such as IL-2 and IFNγ. After the prolonged DC-T cell interaction, the T cells undergo clonal expansion through several rounds of cellular division. The T cells dissociate from the antigen-presenting DCs, migrate rapidly and exit the draining lymph nodes (Mempel et al., 2004; Stoll et al., 2002).

The engagement of cognate peptide-MHC complexes on DCs and antigen-specific TCR on T cells initiates T cell clonal expansion. However, the fate of these dividing T cells depends on the co-stimulatory signals provided by the DCs. Sufficient co-stimulatory signals are necessary for the full activation of naïve T cells. During inflammation or infection, DCs upregulate the expression of co-stimulatory molecules and become activated DCs. The quintessential co-stimulatory ligands are CD80 and CD86, which are highly expressed on activated DCs. CD80 and CD86 bind to CD28 on the T cells. There are other co-stimulatory molecules and some of them belong to the TNF super family, including OX40/OX40L, 4-1BB/4-1BBL and CD27-CD70 (Watts, 2005). The engagement of CD28 on the T cell by CD80 or CD86 drives the cell cycle progression of T cells (Boonen et al., 1999), enhances the expression of anti-apoptotic protein Bcl-X_L (Boise et al., 1995), and lowers the antigen stimulation threshold required to induce cellular divisions of the T cells (Iezzi et al., 1998). The expression levels of co-stimulatory molecules on the antigen-presenting DCs are important because T cells do not become fully activated when receiving co-stimulatory signals provided by other DCs that are not presenting the antigen directly (Sporri and Reis e Sousa, 2005). When naïve T cells receive an antigenic stimulus and sufficient co-stimulation from activated or licensed DCs, the T cells undergo clonal expansion, produce effector cytokines such as IFNγ and/or produce cytolytic molecules, and are referred to as effector T cells. These effector T cells carry out cell-mediated adaptive immunity against various pathogens and tumours. They also develop into functional long-lived memory T cells that are capable of responding to secondary antigenic stimuli (Badovinac et al., 2005; Wang et al., 2006).

When DCs, such as immature or steady-state DCs, do not express sufficient co-stimulatory molecules to fully activate the naïve T cells, naïve T cells undergo clonal expansion and produce effector cytokines transiently. These T cells eventually disappear from circulation or become unresponsive to secondary antigenic stimulation (Bonifaz et al., 2002; Hawiger et al., 2001; Hernandez et al., 2001; Huang et al., 2003; Kearney et al., 1994; Scheinecker et al., 2002). These processes are referred to as T cell deletional tolerance or T cell anergy respectively. T cell tolerance and anergy are important because DCs also present self-antigens and may stimulate autoreactive T cells that have escaped negative selection. T cell tolerance and anergy are forms of peripheral tolerance to restrain unwanted immune

responses in the absence of inflammation or infection and prevent the occurrence of autoimmune diseases.

1.10. Antigens are transferred from DC to DC

Besides being able to present antigens directly to T cells, DCs bearing the antigen can pass antigen to other DCs (Allan et al., 2006; Belz et al., 2004a; Inaba et al., 1998; Kleindienst and Brocker, 2003; Qu et al., 2009). Antigen transfer is not limited to inter-DCs because macrophages have also been shown to capture antigens and transfer them to DCs for antigen presentation to T cells (Backer et al., 2010). Furthermore, intercellular protein transfer has been demonstrated on numerous occasions in different immune cells, for example, T cells can acquire MHC molecules from DCs and B cells can acquire B cell receptors from other B cells (Davis, 2007; Rechavi et al., 2009).

Inter-DC antigen transfer may occur through DC secretion of exosomes (Luketic et al., 2007; Segura et al., 2005), engulfing apoptotic DCs (Inaba et al., 1998; Kleindienst and Brocker, 2003), transfer of plasma membrane from live DCs (Harshyne et al., 2001), through gap junctions (Neijssen et al., 2005) or membrane nanotubes (Chinnery et al., 2008). The antigen transferred may come in the form of proteins (Norbury et al., 2004; Shen and Rock, 2004), peptides (Neijssen et al., 2005) or MHC-peptide complexes (Qu et al., 2009).

The major caveat of some of these findings is that inter-immune cell protein transfer has only been shown in an *in vitro* system. For example, inter-immune cell protein transfer via the transfer of plasma membrane, also known as trogocytosis, has been shown *in vitro* (Chaudhri et al., 2009; Harshyne et al., 2001) but has not been visualised *in vivo*. Formation of membrane nanotubes between immune cells has also been observed mostly in *in vitro* models (Davis and Sowinski, 2008) and to date, only the membrane nanotubes between DCs was observed *in vivo* (Chinnery et al., 2008).

The physiological purpose of inter-DC antigen transfer is still unclear. There are speculations that inter-DC antigen transfer serves to increase the number of antigen-

presenting DCs, hence amplifying the immune responses. In support of this, inter-DC antigen transfer is very efficient and occurs as early as eight hours after viral infection (Allan et al., 2006). On the other end of the spectrum, inter-DC antigen transfer may function to tolerise T cells and prevent auto-reactivity. This is supported by reports showing that DCs taking up apoptotic bodies induce T cell tolerance (Inaba et al., 1998; Liu et al., 2002; Sauter et al., 2000; Steinman et al., 2000). Furthermore, in the absence of activation stimuli, most DCs exist in an immature state and induce T cell tolerance or anergy (Bonifaz et al., 2002; Hawiger et al., 2001; Scheinecker et al., 2002). Spreading the antigen across a large pool of immature DCs can potentially induce T cell tolerance or anergy. Thus, inter-DC antigen transfer may serve to spread the antigen for immune amplification during inflammation or infection, or to restrain self-reactive immune responses from developing in the absence of inflammation or infection.

1.11. DCs induce CD4$^+$ T cell responses

DCs can influence the development of the effector T cells through the production of cytokines. This is best demonstrated by the differentiation of activated CD4$^+$ T cells into distinct different types of CD4$^+$ T helper (Th) cells. When immature or steady-state DCs encounter PAMPs, PAMPs signal through TLRs and activate the DCs. Depending on the type of PAMPs involved, DCs can produce pro-inflammatory cytokines IL-6, IL-12, TNFα and IL-23 (Goriely et al., 2008; Langenkamp et al., 2000; Trinchieri and Sher, 2007). DCs can also produce immunoregulatory cytokines such as TGFβ (Yamazaki et al., 2008). These cytokines can skew the CD4$^+$ T cells towards different pathways.

Th1 cells produce their signature cytokine IFNγ and mediate immune protection and responses against intracellular bacteria and tumours (Mosmann and Coffman, 1989; Paul and Seder, 1994). When activated DCs produce IL-12, IL-12 activates innate cells such as NK cells to produce IFNγ, which activates intracellular signal molecule Stat1 in the CD4$^+$ T cells, resulting in the up-regulation of the transcription factor T-bet. T-bet then induces gene transcription and epigenetic changes in CD4$^+$ T cells, leading to IFNγ production and the up-regulation of IL-12 receptor. IL-12 also binds to IL-12 receptor on the CD4$^+$ T cells and signals through the intracellular signalling molecule Stat4. This induces IFNγ

production, which then reinforces the expression of IL-12 receptor. Thus, a positive feedback loop is formed between IL-12 and IFNγ, which induces effector CD4$^+$ T cells to differentiate into CD4$^+$ Th1 cells (Hsieh et al., 1993; Seder et al., 1993).

Th17 cells mediate immune responses against certain extracellular bacteria and fungi. IL-6 produced by DCs acts in combination with TGFβ produced by other cells or by DCs themselves to activate the intracellular protein STAT3 in the CD4$^+$ T cells. STAT3 induces the nuclear receptor RORγt and RORα (Yang et al., 2008). The activation of RORγt and RORα induces the differentiation of CD4$^+$ T cell into Th17 cells. Besides producing other cytokines, Th17 cells produce their signature cytokine IL-17 (Korn et al., 2009). The production of IL-23 by DCs also helps expand and maintain the population of Th17 cells and promotes the production of IL-17 (Aggarwal et al., 2003; Cua et al., 2003).

The Foxp3$^+$CD25$^+$CD4$^+$ regulatory T (Treg) cells are immunosuppressive and mediate protection against autoreactive or excessive immune responses that are deleterious to the host. They play an important role in peripheral tolerance. DCs can induce Treg cells from naïve CD4$^+$ T cells through the production of TGFβ (Yamazaki et al., 2008). TGFβ induces the expression of transcription factor Foxp3 in CD4$^+$ T cells. Intestinal DCs also metabolise retinoic acid, which facilitates the induction of Treg cells (Coombes et al., 2007; Sun et al., 2007). Furthermore, DCs also induce the production of IL-2 by effector T cells(Yamazaki et al., 2007). IL-2 activates the intracellular protein STAT5 in CD4$^+$ T cells. IL-2 and STAT5 are important in the development of Treg cells (Antony et al., 2006; Burchill et al., 2007), the maintenance of Treg cell population and the expression of Foxp3 in Treg (Fontenot et al., 2005).

Th2 cells mediate immune responses against extracellular parasites and helminths. DCs also induce CD4$^+$ T cells to differentiate into Th2 cells. However, unlike the clear-cut cases of Th1, Th17 and Treg cells, how DCs induce Th2 cell development is still unresolved. This is because while DCs produce Th1-, Th17- and Treg-inducing cytokines, DCs do not produce Th2-inducing cytokines, such as IL-4 (Le Gros et al., 1990; Swain et al., 1990) and thymic stromal lymphoietin (TSLP) (Ziegler and Liu, 2006). Some studies have proposed that DCs use other intercellular signalling molecules such as CD40 (MacDonald et al.,

2002) and OX40L (Ito et al., 2005; Wang et al., 2006) to induce Th2 cell differentiation. Others have proposed that DCs induce Th2 cell differentiation through a TLR-4 dependent and MyD88-independent pathway (Kaisho et al., 2002). More recently, a study suggested that DCs cooperate with basophils in a reactive oxygen species (ROS)-mediated manner to induce Th2 cell differentiation (Tang et al., 2010). Although the exact mechanism of how DCs initiate Th2 cell differentiation remains unclear, there is accumulating evidence supporting the role of DCs in Th2 cell development. For example, the soluble extracts from the eggs of *Schistosoma mansori* activated DCs and these DCs induced Th2 responses (de Jong et al., 2002; MacDonald et al., 2001). From these pieces of evidence, DCs induce the development of Th2 cells through mechanisms that have not been fully clarified.

1.12. DCs induce CTL development

DCs are the most potent APCs at cross-presentation (Heath and Carbone, 2001b) and also play a central role in inducing the development of naïve CD8$^+$ T cell into cytolytic effector T cells (Jung et al., 2002; Probst and van den Broek, 2005; Zammit et al., 2005). When naïve CD8$^+$ T cells encounter DCs presenting cognate antigens in the draining lymph nodes, antigenic stimulation via TCR-peptide-MHC class I interactions induces the antigen-specific naïve CD8$^+$ T cells to undergo several rounds of cell divisions. Cellular division leads to a striking increase in the numbers of antigen-specific CD8$^+$ T cells (Blattman et al., 2002; Butz and Bevan, 1998; Murali-Krishna et al., 1998). As mentioned previously, the interaction of co-stimulatory molecules such as CD80 or CD86 expressed on the antigen-presenting DCs with CD28 expressed on the antigen-specific naïve CD8$^+$ T cells is necessary to drive these dividing CD8$^+$ T cells into fully activated cytolytic effector T cells (Andreasen et al., 2000; Liu et al., 1997; Shedlock et al., 2003). Other co-stimulatory molecules that activate naïve CD8$^+$ T cells into cytolytic effector T cells include the 4-1BB/4-1BB ligand pathway (Tan et al., 1999). These cytolytic effector T cells are referred to as cytotoxic T lymphocytes (CTL).

The presence of cytokines produced by the innate immune cells or activated DCs during infection also helps to maximise the development of CTLs. IL-12 and Type I IFN have been shown to promote the proliferation and survival of CTLs (Curtsinger et al., 1999;

Marrack et al., 1999). IL-12 also stimulates the CTLs to produce IFNγ (Curtsinger et al., 2003). Another important signal during the activation of naïve CD8$^+$ T cells is the licensing of antigen-presenting DCs by CD4$^+$ T cells. The presence of CD4$^+$ T cells is necessary for the development of long-lived memory CD8$^+$ T cells. This is because in the absence of CD4$^+$ T cell help, antigen-presenting DCs induce CD8$^+$ T cells that undergo activation-induced cell death (AICD). AICD of CD8$^+$ T cells is mediated through the TRAIL-TRAIL receptor pathway (Janssen et al., 2005). In the absence of CD4$^+$ T cell help, CD8$^+$ T cells also fail to respond to secondary challenge (Janssen et al., 2003).

After undergoing clonal division and acquiring effector functions, CTLs migrate to the sites of infection (Dudda et al., 2004; Mora et al., 2003), eliminate pathogen-infected cells directly through cytolytic mechanisms or enhance anti-viral responses through the secretion of effector cytokines. The expression of the appropriate peptide-MHC class I complexes on the pathogen-infected cells enables the antigen-specific CTLs to recognise and target the infected cells. Once the antigen-specific TCRs of the CTLs engage the peptide-MHC class I complexes on the infected cells, the CTLs can either deploy their cytolytic molecules in the direction of the infected cell or engage the receptors that induce apoptosis on the infected cell.

Following the pre-determined CTL program, the population size of antigen-specific CTLs contracts (Badovinac et al., 2002), whereby most of the CTLs undergo apoptosis, leaving some CTLs behind as long-lived memory CD8$^+$ T cells (Butz and Bevan, 1998; Murali-Krishna et al., 1998). These long-lived memory CD8$^+$ T cells are maintained at a steady level throughout the lifespan of the host (Hammarlund et al., 2003). Upon the exposure to the same pathogen, these memory CD8$^+$ T cells mount a quick response. They are localised in non-lymphoid tissues or in draining lymph nodes (Sallusto et al., 1999) and are maintained at a higher frequency than the naïve CD8$^+$ T cells. This provides the host with enhanced immunological protection against re-exposure to the pathogens.

1.13. CTLs use different cytolytic mechanisms to induce target cell apoptosis

Upon TCR-peptide-MHC class I engagement, CTLs produce various cytolytic proteins that can induce the apoptosis of target cells through different mechanisms. These mechanisms can be grouped into receptor-mediated and granule-mediated cytolytic pathways (Russell and Ley, 2002). The receptor-mediated cytolytic pathway initiates apoptosis when the death ligands on the CTLs bind to the corresponding receptors on the target cell. CTLs synthesize most of the death ligands *de novo* although they store a small portion of these death ligands in vesicles. CTLs translocate the pre-stored or newly synthesized death ligands to their cell surface. Because CTLs produce most of the death ligands upon target engagement, the time taken to induce target cell apoptosis depends on the time taken to synthesize the death ligands. It is also essential that the target cell expresses the corresponding death receptors.

In granule-mediated cytolytic pathway, CTLs store cytolytic proteins in granules. Upon interaction with the target cell, the CTL re-orients the granules in the direction of the target cell. The CTL then releases the cytolytic proteins into the immunological synapse between the CTL and the target cell. The cytolytic proteins enter the target cell and induce apoptosis. Because the cytolytic proteins are pre-stored, the CTLs can release these cytolytic proteins quickly and induce rapid target cell apoptosis.

1.13.1 FasL on CTLs binds to Fas on target cells to induce apoptosis in target cells

The ligands and receptors that induce cytolysis in receptor-mediated cytolytic pathway generally belong to the TNFα super family. The members in the TNFα family that induce cytolysis include Fas ligand (FasL), TNFα and TRAIL (Fig. 1.3). The receptor for FasL is Fas; the receptors for TNFα are the TNFα receptor 1 and 2 (TNFαR1/2) and death receptor (DR)-3; and the receptors for TRAIL are DR-5. The FasL-Fas pathway is the quintessential receptor-mediated cytolytic mechanism (Lowin et al., 1994b).

The FasL-Fas pathway is initiated by the binding of FasL on the CTL to Fas on the target cell. The ligation of FasL to Fas recruits the intra-cellular protein Fas-associated via death domain (FADD) (Ju et al., 1994; Strasser et al., 2009). Other death receptors such as TNFα

receptors and TRAIL receptors also converge on intracellular signalling proteins such as FADD or TNF-receptor 1-associated via death domain (TRADD) (Bodmer et al., 2000; Sprick et al., 2000). FADD binds to the intracellular portions of Fas through the interactions of death domains and recruits caspase 8, forming the death-inducing signalling complex (DISC). Recruited caspase 8 then undergoes auto-proteolytic cleavage and becomes fully activated caspase 8. The activated form of caspase 8 activates executioner caspases 3 and 7, which cleave other cellular proteins, leading to target cell apoptosis. Caspase 10 is also recruited into the DISC complex to induce the downstream apoptosis cascade.

1.13.2 CTLs secrete perforin and granzymes to induce apoptosis in target cells

Perforin and granzymes are predominantly expressed in CTLs and NK cells, although $CD4^+$ Treg cells express perforin and granzyme B (Boissonnas et al., 2010). When the CTL engages the target cell via the TCR-peptide-MHC class I complex, the Golgi apparatus, microtubule-organising centre and cytotoxic granules in the CTL are reorientated and directed towards the immunological synapse formed between the CTL and the target cell. Perforin and preformed granzymes are stored in cytotoxic granules. Perforin and preformed granzymes are released into the tight inter-cellular junction between the CTL and target cell (Fig. 1.3). The perforin and granzymes then diffuse across the immunological synapse into the target cell.

The general consensus is that perforin requires calcium to bind to target cell membrane; is necessary for the delivery of granzymes into the target cell; and forms channels through cell membranes. However, exactly how perforin delivers granzymes into the target cell and how perforin acts as a cytolytic molecule are still debated. Initial evidence has shown that perforin inserts itself into the plasma membrane of target cells and forms membrane pores (Tschopp et al., 1986). Because perforin forms a channel in the cell membrane, the cytolytic mechanism of action was initially thought to allow granzymes to diffuse into the target cell and induce cell apoptosis (Shi et al., 1992; Tschopp and Nabholz, 1990). However, some evidence has indicated that the pore size of perforin was too small for granzymes to pass through and that perforin delivers granzymes without obvious plasma

membrane pore formation (Metkar et al., 2002). There are also suggestions that when perforin disrupt the integrity of the plasma membrane, the plasma membrane repair mechanism takes place and somehow facilitate the uptake of granzymes (Keefe et al., 2005). Other studies have also proposed that granzymes can enter target cells independent of perforin and that perforin form pores in endosome-like vesicles in the target cells, hence mediating the release of granzymes into the cytosol of target cells (Browne et al., 1999; Froelich et al., 1996; Pinkoski et al., 1998). Though the precise mechanism of perforin-mediated killing is unknown, studies using perforin-knockout (PKO) mice showed that these mice were more susceptible to viral infections than wt mice (Kagi et al., 1994a). PKO CTLs were less cytolytic and showed defects in inducing membrane damage and apoptosis compared to perforin functional CTLs (Lowin et al., 1994a). These pieces of evidence indicate the critical role of perforin in CTL-mediated cytolytic functions.

Granzyme A and B are the most well characterised of a family of 11 serine proteases (Chowdhury and Lieberman, 2008; Trapani et al., 2000). Human granzyme B has been shown to activate caspase-dependent pathway to initiate apoptosis through cleaving after aspartic residues in pro-caspase 3 and 8 (Atkinson et al., 1998; Medema et al., 1997; Yang et al., 1998) and caspase-independent pathways such as cleaving the proapoptotic Bid protein (Barry et al., 2000; Sutton et al., 2000). Human granzyme A induces target cell death similar to apoptosis in a caspase-independent manner (Beresford et al., 1999). In mice, knocking out granzyme A led to increased susceptibility to poxvirus (Mullbacher et al., 1996), whereas knocking out granzyme B in CTLs allowed the recipient mice to survive better in acute graft versus host disease (Graubert et al., 1996). Mice deficient of both granzyme A and B also could not control poxvirus infection (Mullbacher et al., 1999), indicating that CTLs employ granzyme A and B to mediate cytolytic functions. However, other studies have shown that granzyme A and B were not required for the clearance of tumours (Davis et al., 2001; Smyth et al., 2003). Granzyme A- and B-deficient CTLs were also able to lyse target cells but did not mediate target cell DNA fragmentation (Davis et al., 2001; Regner et al., 2009; Simon et al., 1997). Infection of PKO or granzyme A and B knockout mice with murine cytomegalovirus revealed that PKO mice suffered more immune pathological damage and did not survive the infection, whereas granzyme A and B knockout mice could control the infection and exhibited minimal immune pathology (van

Dommelen et al., 2006). Clearly, the cytolytic mechanism of action of granzyme A, granzyme B and perforin are distinct from one another.
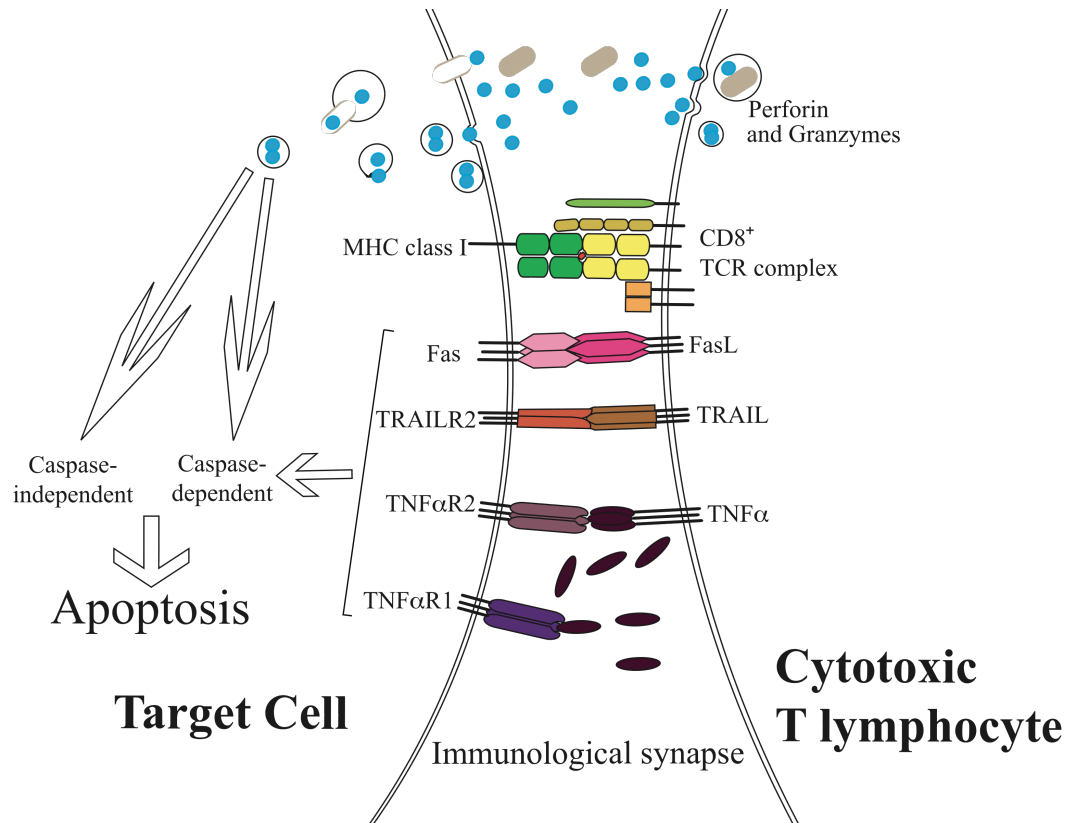
Fig. 1.3. **The CTL uses a variety of cytolytic molecules to induce apoptosis in target cell.**

1.14. CD8$^+$ T cell cytotoxicity is important in eliminating antigen-loaded DCs

The development of CTLs is crucial in controlling and resolving bacterial and viral infections. In order to terminate the pathogen-induced immune responses and return the immune responses back to homeostasis, a few checkpoints are necessary. Firstly, the eradication of the pathogens and pathogen-infected cells are needed to remove the antigen and inflammation sources. Secondly, the numerical contraction of pathogen-specific CTLs that have expanded and accumulated during the immune response is necessary to release space and growth factors for the generation of other immune responses. The removal or the suppression of other participating immune cells is also necessary to terminate the existing immune responses. Lastly, the clearance of APCs that have taken up pathogens or are still presenting pathogenic antigens is also necessary to prevent induction of new immune

responses. There is an increasing number of evidence indicating that the APCs, in particular DCs, are targeted and eliminated by CTLs and other immune cells through cytolytic mechanisms. These studies have provided some evidence to show that regulating the duration of DC antigen presentation is important in controlling immune pathology and initiating beneficial immune responses.

Earlier studies have proposed that the inability of T cells to eliminate antigen-presenting cells through perforin or FasL enhance lymphocyte proliferation and cytokine production and could lead to immune pathology (Sad et al., 1996; Spielman et al., 1998). In the study by Sad et al., *in vitro* experiments showed that PKO CTLs were less cytolytic, proliferated more rigorously and produced more IL-2 and IFNγ than wt CTLs when stimulated by APCs. Using cell lines expressing low or high levels of Fas, Sad et al. also showed that lesser Fas expression on the APCs enhanced cytokine production and proliferation of PKO CTLs and concluded that CTLs limited their activation and cytokine production through the elimination of APCs. Spielman et al. examined this issue in mice deficient of perforin and FasL and observed that the immune pathology in the pancreas of these mice was due to increased monocyte/macrophages infiltration. By testing the cytolytic ability of the CTLs from mice deficient of perforin and FasL on macrophages, Spielman et al. concluded that the immune pathology observed in these mice was a consequence of defective cytolysis of APCs by CTLs.

Because DCs are proficient APCs to CD8$^+$ T cells, it is likely that these DCs become targets of CTLs. Infection by immunosuppressive clone 13 LCMV resulted in a CD8-mediated loss of splenic DCs, and a transient anti-LCMV CTL response, which led the authors to hypothesize that the disappearance of the virally-infected DCs were due to the anti-viral CTLs (Borrow et al., 1995). This is not surprising because CTLs eliminate viral-infected cells. However, LCMV gp33 peptide-specific CTLs induced by DC immunisation in wt mice and the adoptive transfer of TCR transgenic mice specific for gp33 peptide were later shown to be capable of eliminating gp33-bearing DCs (Hermans et al., 2000; Ludewig et al., 2001). Furthermore, the elimination of antigen-bearing DCs by CTLs prevented the generation of anti-tumour CTLs (Hermans et al., 2000). These studies provide evidence for

the notion that the duration of antigen presentation by DCs is regulated by CTL-mediated elimination.

Studies using PKO CTLs or PKO mice also provided evidence for CTL-mediated DC elimination. Peptide-loaded DCs could be recovered from mice that received PKO CTLs but not wt CTLs, indicating that antigen-bearing DCs were eliminated by CTLs in a perforin-dependent manner (Yang et al., 2006). The duration of DC antigen-presentation in a secondary influenza infection was also enhanced in PKO mice (Belz et al., 2007). Other evidence implicating perforin in the regulation of DC induction of T cell responses are shown in PKO mice. Enhanced accumulation of antigen-specific CD8$^+$ T cells in PKO mice was observed after multiple immunisations with peptide-loaded DCs (Yang et al., 2006). Infection of PKO mice with LCMV showed that CTLs in these mice accumulated excessively, produced more IFNγ than wt CTLs, resulting in increased immunopathology and increased mortality (Matloubian et al., 1999). Moreover, DCs were involved in the aberrant accumulation and activation of CTLs in LCMV-infected PKO mice (Borrow et al., 1995). *Listeria monocytogenes* infection of PKO mice also led to an increased expansion of CTLs (Badovinac et al., 2000). These observations in PKO mice are reminiscent of familial hemophagocytic lymphohistiocytosis (FHL) patients. FHL patients suffer from perforin gene defects and are characterised by uncontrolled activation and accumulation of CD8$^+$ T cells (Stepp et al., 1999). Taken together, the evidence supports the notion that DC antigen presentation is regulated by CTLs through perforin-mediated cytolytic pathway.

The regulation of subsequent immune responses through CTL-mediated DC killing is further shown in studies on human and mice lacking the death receptor-mediated cytolytic pathway. Patients suffering from autoimmune lymphoproliferative syndrome (ALPS) have defects in apoptosis-related genes such as the Fas, FasL and TRAIL genes, which then affect the homeostasis of lymphocytes and result in autoimmune manifestations including aberrant accumulation of lymphocytes, elevated amounts of double negative T cells and autoantibodies. In a patient suffering from ALPS type II, DCs from this patient were resistant to TRAIL-mediated apoptosis and aberrant DC accumulation was observed (Wang et al., 1999). ALPS type II patients are defective in caspase 10 gene, which also plays a downstream role in Fas-mediated apoptosis (Wang et al., 2001). Moreover, ALPS type IB

(Straus et al., 1999) and IA (Wu et al., 1996) are also observed in mice suffering from lymphoproliferative (*lpr*) and general lymphoproliferative (*gld*) diseases respectively. These *lpr* and *gld* mice do not express Fas and FasL respectively due to their respective mutations in Fas and FasL genes (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). The evidence in support of regulating DCs by Fas-FasL pathway was reported in *lpr* mice whereby increased DC frequency was observed (Fields et al., 2001). In mice where Fas was selectively knocked out in DCs, there was an accumulation of DCs and manifestations of systemic autoimmunity were observed (Stranges et al., 2007). This indicates that Fas-FasL-mediated deletion of DCs is necessary to prevent autoimmune manifestations. In addition, CTLs were also not able to eliminate Fas-deficient DCs (Stranges et al., 2007), providing support for the notion that CTLs regulate DC antigen presentation through Fas-FasL cytolytic pathway. The importance in controlling the DC antigen presentation was demonstrated when prolonged DC survival led to an aberrant accumulation of DCs, and in turn, resulted in enhanced T cell proliferation and autoimmune manifestations (Chen et al., 2006).

There is other evidence indicating that DCs are protected from CTL killing in certain conditions. Some *in vitro* studies have shown that DCs are resistant to apoptosis induced by cytolytic molecules. Using DCs from various sources, some studies have shown that DCs were resistant to apoptosis induced by Fas-FasL pathway because they expressed FLIP, which inhibited the Fas signalling pathway (Ashany et al., 1999; Leverkus et al., 2000; Rescigno et al., 2000; Willems et al., 2000). One study showed that serum-free treated DCs cultured from PBMCs expressed Bcl-X_L, thus protecting them from Fas-mediated DC killing (Lundqvist et al., 2002). Another study showed that activation of murine and human DCs by LPS or CD40L increased serine protease inhibitor protein expression and become resistant to CTL cytolysis *in vitro* (Medema et al., 2001). This was further supported by observations that activated DCs were resistant to CTL-mediated killing *in vivo* (Mueller et al., 2006). Both Mueller et al. and Medema et al. also showed that antigen-specific CD4^{+} T cells protected DCs from CTL elimination *in vivo* and *in vitro* respectively (Medema et al., 2001; Mueller et al., 2006). A study by Watchmaker et al. later showed that human memory CD8^{+} T cells produced TNFα to induce the expression of serine protease inhibitor protein in DCs such that these DCs were resistant to cytolysis *in vitro* (Watchmaker et al., 2008).

Thus far, with the exception of the study conducted by Mueller et al., studies conducted under *in vivo* conditions have shown that DCs are sensitive to CTL cytolysis. *In vivo* live imaging of draining lymph nodes in mice has shown that effector and memory CTLs established interactions with cognate antigen-loaded DCs and induced DC apoptosis (Guarda et al., 2007a). While LPS-activated DCs expressed serine protease inhibitor protein, the expression of this inhibitor did not protect them from CTL-mediated killing *in vivo* (Andrew et al., 2008). Memory CTLs also did not protect DCs from cytolysis *in vivo* because memory CTLs induced by DC immunisation (Hermans et al., 2000) or by influenza infection (Belz et al., 2007) eliminated antigen-presenting DCs. Although some types of CD4$^+$ T cells such as CD4$^+$ Th2 cells have been shown to protect DCs from CTL killing, CD4$^+$ Th1 cells did not protect antigen-bearing DCs from cytolysis (Medema et al., 2001). Other types of CD4$^+$ T cells also induced DC apoptosis directly. For example, *in vivo* imaging of draining lymph nodes showed that Foxp3$^+$ CD4$^+$ Treg cells induced DCs apoptosis and this was mediated via a perforin-dependent mechanism *in vivo* (Boissonnas et al., 2010).

The evidence from these *in vivo* and *in vitro* studies may appear conflicting. However, because DCs take active measures to protect themselves from CTL-mediated killing at least *in vitro*, this is indicative that CTL-mediated elimination of DCs is important in regulating immune responses. In line with this, CTL-mediated DC killing has been shown to prevent the induction of anti-tumour (Hermans et al., 2000) and alloreactive T cell responses (Laffont et al., 2006). Moreover, when DCs cannot undergo apoptosis due to the expression of caspase inhibitor protein (Chen et al., 2006) or deletion of apoptosis inducing proteins (Stranges et al., 2007), the immune responses become dys-regulated resulting in immune pathology. Taken together, these studies support the notion that cytolytic elimination of DCs by CTLs or other immune cells plays a crucial role in resolving immune responses, restraining unwanted immune responses, and may have an impact on the initiation of beneficial immune responses.

1.15. Aims of this study

While it is clear that CTLs remove pathogens and undergo numerical contraction after the resolution of primary immune responses, there is a lack of clear evidence showing whether CTLs also mediate resolution through the cytolytic elimination of antigen-presenting DCs. How CTLs mediate DC killing is also not fully defined. When CTLs eliminate target cells, apoptosis ensues and the cellular materials from the apoptotic cells can be taken up by other DCs and presented to other T cells (Parish et al., 2009). The CTL-mediated elimination of antigen-presenting DCs may result in antigen transfer from the dying DCs to other DCs. Moreover, antigens can be transferred among DCs without the need for CTLs to release the cellular contents of the target cell (Allan et al., 2006; Kleindienst and Brocker, 2003; Luketic et al., 2007). This leads to a dilemma because if CTLs eliminate DCs to resolve immune responses, but antigen transfer among DCs spreads the antigens for further antigen presentation, no decrease in the immune responses would be expected.

In this study, I hypothesize that CTLs eliminate antigen-presenting DCs through cytolytic molecules, thereby preventing the induction of T cell responses. My second hypothesis is that inter-DC antigen transfer does not induce T cell responses in the presence of CTL-mediated DC killing.

To address these hypotheses, I examined the CTL-mediated regulation of DC survival, the cytolytic mechanisms involved, the impact of CTL-mediated DC killing and inter-DC antigen transfer on the induction of subsequent T cell responses. The following aims are addressed in the three results chapters of this thesis. They are:

1. To characterise the mechanisms of CTL-mediated DC elimination, and examine the impact of DC elimination on T cell proliferation
2. To evaluate DC elimination and its impact on T cell proliferation using different methods of antigen loading on DCs and different methods of generating CTLs
3. To examine the impact of CTL-mediated DC killing and inter-DC antigen transfer on the induction of T cell proliferation and the quality of the resulting T cell responses

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Labwares

Items	Source
Acrodisc® 32 mm syringe filter with 0.2 μm Supor® membrane	PALL Life Sciences, Cornwall, U.K.
1 ml Tuberculin syringes & 10 ml syringes	Becton Dickinson (BD) Biosciences, CA, USA
1.0 μm pore size, PET track-etched membrane 12 well format cell insert	
Falcon® polystyrene sterile conical tubes: Blue Max 50 ml and Blue Max Jr. 15 ml	
Falcon® polystyrene sterile multiwall tissue culture plates: 6 well, 24 well and Microtest™ U-bottom 96 well plates	
Falcon® polystyrene sterile tissue culture flasks: 200 ml & 600 ml	
Falcon® polystyrene sterile serological pipettes	
Nylon cell strainers: 40 & 70 μm	
PrecisionGlide™ needles: 18, 20, 25 & 27.5 gauge (G)	
Ultra-Fine™ needle insulin syringes (29 G): 0.3, 0.5 & 1 ml	
Nylon gauze: 70 μm	NZ Filter Specialists Ltd., Auckland NZ
30 μm MACS pre-separation filters	Miltenyi Biotech, GmbH, Germany
Large cell MACS columns	Miltenyi Biotech, GmbH, Germany
Petri dish, 90 mm diameter	Labserv, Auckland, New Zealand

2.1.2. Reagents

Reagents and materials	Source
2-mercapto-ethanol (2-ME) 55 mM solution	Sigma-Aldrich, MO, USA
Anti-CD4 MACS microbeads	Miltenyi Biotech, GmbH, Germany
Anti-CD8α MACS microbeads	Miltenyi Biotech, GmbH, Germany
Ammonium chloride powder	Sigma-Aldrich, MO, USA
BD Cytofix/Cytoperm kit	BD Bioscience, CA, USA
Betaplate scintillator	PerkinElmer Life Sciences and Analytical Sciences, MA, USA
Carboxy-fluorescein diacetate succinimidyl ester (CFSE)	Molecular Probes, Invitrogen, CA, USA
DNase I powder	Roche, IN, USA
Ethlenediaminetetraacetic Acid (EDTA) powder	Sigma-Aldrich, MO, USA
Fetal calf serum (FCS)	Invitrogen, CA, USA
Dextran fluorescein 40,000 MW anionic (FITC dextran)	Molecular Probes, Invitrogen, CA, USA
LCMV glycoprotein gp_{33-41} (KAVYNFATM) peptide	Mimotopes, Victoria, Australia
Granulocyte macrophage colony-stimulating factor (GM-CSF)	GM-CSF producing murine X63 cell line, gift from Dr. Antonius Rolink (Basel Institute of Immunology, Basel, Switzerland)
GolgiStop	BD Pharmingen, CA, USA
Iscove's modified Dulbecco medium (IMDM) supplemented with GlutaMAX^{TM}, 25 mM HEPES buffer and 3.024 mg/l NaHCO$_3$	Invitrogen, CA, USA
Liberase CI powder	Roche, IN, USA

Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0111:B4	Sigma-Aldrich, MO, USA
Interleukin (IL)-2	IL-2 producing IL2L6 cell line, generated by the modification of murine J558 parental line (Traunecker et al., 1991)
IL-4	IL-4 producing Chinese hamster ovary cell line, gift from Dr. Antonius Rolink (Basel Institute of Immunology, Basel, Switzerland)
IL-6	Gift from A/P Christiane Ruedl (Nanyang Technological University, Singapore)
Orange fluorescent dye chloromethyl-benzoyl-aminotetramethyl-rhodamine (CMTMR, also known as 'Cell Tracker Orange')	Molecular Probes, Invitrogen, CA, USA
Ovalbumin (OVA) protein powder	Sigma-Aldrich, MO, USA
OVA$_{257-264}$ (SIINFEKL) peptide	Mimotopes, Victoria, Australia
OVA$_{323-339}$ (ISQAVHAAHAEINEAGR) peptide	Mimotopes, Victoria, Australia
Penicillin-streptomycin	Invitrogen, CA, USA
Phosphate buffer saline (PBS), CaCl$_2$ and MgCl$_2$ free	Invitrogen, CA, USA
Propidium iodide (PI)	BD Biosciences, CA, USA
Sodium azide powder	Sigma-Aldrich, MO, USA
Stem cell factor	Gift from A/P Christiane Ruedl (Nanyang Technological University, Singapore)
Streptavadin MACS microbeads	Miltenyi Biotech, GmbH, Germany
Tris powder	Invitrogen, CA, USA
Tritiated thymidine (6-Methyl-^{3}H thymidine)	Amersham Biosciences, Little Chalfont, UK

Type II Collagenase powder	Invitrogen, CA, USA

2.1.3. Antibodies

Antibodies used to minimise non-specific antibody binding

Monoclonal Antibodies	Clone	Source
Anti-FcγRII/III receptor	2.4G2	Purified in-house

Antibodies used to label DCs and other APCs

Monoclonal Antibodies	Clone	Source
Anti-MHC-II (I-A)[b]	3JP	Purified in-house
Anti-CD11c	N418	Purified in-house
Anti-CD11b	M1/70	BD Pharmingen, CA, USA
Anti-CD80	16-10A1	BD Pharmingen, CA, USA
Anti-CD86	GL1	BD Pharmingen, CA, USA
Anti-CD40	3/23	BD Pharmingen, CA, USA
Anti-F4/80	CI:A3-1	Purified in-house
Anti-CD95	Jo2	BD Pharmingen, CA, USA

Antibodies used to label T cells

Monoclonal Antibodies	Clone	Source
Anti-CD3ε	2C11	BD Pharmingen, CA, USA
Anti-CD8	2.43	Purified in-house
Anti-CD4	GK1.5	Purified in-house
Anti-CD4	GK1.5	BD Pharmingen, CA, USA
Anti-Vα2	B20.1	BD Pharmingen, CA, USA
Anti-Vβ5.1/5.2	MR9-4	BD Pharmingen, CA, USA
Anti-CD62L	MEL-14	BD Pharmingen, CA, USA

Anti-CD44	IM7	BD Pharmingen, CA, USA
Anti-CD178	MFL4	BD Pharmingen, CA, USA

Antibodies used to label C57BL/6 and CD45-congenic B6.SJL-*Ptprc*[a] cells

Monoclonal Antibodies	Clone	Source
Anti-CD45.2	104	eBiosciences, CA, USA
Anti-CD45.1	A20	BD Pharmingen, CA, USA

Antibodies used to deplete lineage-positive cells and congenic B6.SJL-*Ptprc*[a] cells

Monoclonal Antibodies	Clone	Source
Anti-CD3ε	145-2C11	eBiosciences, CA, USA
Anti-B220	6B2	Purified in-house
Anti-CD19	1D3	BD Pharmingen, CA, USA
Anti-NK1.1	PK136	eBiosciences, CA, USA
Anti-DX5	DX5	eBiosciences, CA, USA
Anti-Ter119	Ter119	eBiosciences, CA, USA
Anti-Gr-1	RB6-8C5	BD Pharmingen, CA, USA
Anti-Thy1.2	53-2.1	eBiosciences, CA, USA
Anti-CD45.1	A20	eBiosciences, CA, USA

Antibodies used for flow cytometry sorting

Monoclonal Antibodies	Clone	Source
Anti-CD11c	HL3	BD Pharmingen, CA, USA
Anti-CD8	53-6.7	BD Pharmingen, CA, USA
Anti-CD205	205yekta	eBiosciences, CA, USA

Antibodies used for intracellular labelling

Monoclonal Antibodies	Clone	Source
Anti-IFNγ (Rat IgG1 κ isotype)	XMG1.2	BD Pharmingen, CA, USA
Rat IgG1 κ isotype	R3-34	BD Pharmingen, CA, USA

Antibodies used for secondary labelling of cells

Monoclonal Antibodies	Clone	Source
Streptavadin-APC	Streptavadin	BD Pharmingen, CA, USA
Streptavadin-FITC	Streptavadin	BD Pharmingen, CA, USA
Streptavadin-PE	Streptavadin	BD Pharmingen, CA, USA
Streptavadin-PerCP	Streptavadin	BD Pharmingen, CA, USA

2.1.4. Buffer compositions

Buffer	Buffer composition
Ammonium Chloride Tris (ACT)	0.144 M Ammonium chloride ph 7.4 17 mM Tris ph 7.4
Complete medium (cIMDM)	500 ml IMDM 1% Penicillin-streptomycin 5×10^{-5} M 2-ME 5% FCS
Fluorescent activated cell sorter (FACS) buffer	10 mM EDTA pH 8.0 0.01% NaN_3 2% FCS
Wuerzburger buffer	1% FCS 10 μg/ml DNAse I 5 mM EDTA pH 8.0 500 ml PBS

2.2. Cell lines

The Nup98 HoxB4 OVA transgenic haematopoietic stem cell (HSC) line were a gift from A/P Christiane Ruedl. Briefly, the HSCs were derived from act-mOVA mice (Ehst et al., 2003) and transfected with nucleus protein nucleoporin 98 (Nup98) and transcription factor HoxB4 (Ruedl et al., 2008). The transfection with Nup98 and HoxB4 allows the self-renewal of HSCs for several weeks *in vitro* (Ruedl et al., 2008). This stem cell line was maintained in culture with cIMDM containing IL-6 and stem cell factor in 90 mm petri dishes.

2.3. Mice

2.3.1. Maintenance and ethical approval

All mice were bred and maintained on standard laboratory food and water *ad libitum* in the Biomedical Research Unit of the Malaghan Institute of Medical Research. The mouse strains were maintained by brother × sister mating. Sex matched mice between 6 – 14 weeks of age were used for all experiments. All experimental protocols were approved by Victoria University Animal Ethics Committee and performed in accordance with institutional guidelines.

2.3.2. Mouse strains

C57BL/6 breeding pairs were originally obtained from the Jackson Laboratory (Bar Harbour, ME, USA) and bred at the Malaghan Institute of Medical Research, Wellington, New Zealand by brother × sister mating.

C57BL/6-background OT-I and OT-II T cell receptor (TCR)-transgenic mice were gifted by Prof. Francis Carbone (University of Melbourne, Melbourne, Australia). T cells from OT-I and OT-II mice express $V\alpha2^+V\beta5.1/5.2^+$ TCRs, which are specific for ovalbumin $(OVA)_{257-264}$ presented on $H2-K^b$, or $OVA_{323-339}$ on $I-A^b$, respectively. The OT-II TCR

transgenic mice were tested for the expression of Vα2 and Vβ5.1/5.2 in peripheral blood by flow cytometry.

Perforin knockout (PKO) OT-I mice were generated by crossing the OT-I and C57BL/6 PKO mice twice at the Malaghan Institute of Medical Research, Wellington, New Zealand(Kagi et al., 1994a). The presence of the inactivated perforin allele in PKO OT-I mice was determined by PCR as in Kagi et al (Kagi et al., 1994a).

CD45-congenic B6.SJL-*Ptprc*[a] mice were from the Animal Resources Centre, Perth, Australia.

OT-I × B6.SJL-*Ptprc*[a] mice were bred at the Malaghan Institute of Medical Research, Wellington, New Zealand.

OT-II × B6.SJL-*Ptprc*[a] mice were bred at the Malaghan Institute of Medical Research, Wellington, New Zealand.

C57BL/6-background MRL-FAS^{lpr} mice were purchased from the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, and bred at the Malaghan Institute of Medical Research, Wellington, New Zealand.

MHC II$^{-/-}$ B6Aa^0/Aa^0 mice were gifted by Dr. Horst Blüthmann (Hoffmann-La Roche, Basel, Switzerland). MHC II$^{-/-}$ B6Aa^0/Aa^0 mice were routinely tested for the expression of MHC class II to confirm that they were indeed MHC class II knockouts (Appendix 1).

2D2 mice expressing transgenic TCRs specific for the MOG$_{35-55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) presented by Ia^b were obtained from Harvard Medical School (Boston, MA, USA) (Petersen et al., 2004). The 2D2 mice were maintained by breeding C57BL/6 males with 2D2 TCR transgenic females.

2.3.3. Mouse manipulation

Mice were sacrificed by CO_2 asphyxiation, followed by cervical dislocation.

2.4. Methods – Cell culture

2.4.1. General cell culture

All cells were cultured in cIMDM at 37 °C with 5% CO_2 and 95% humidity.

2.4.2. Culture from bone marrow precursors

Muscles and connective tissues were removed from the femurs and tibias of the euthanised mice. The ends of the bones were cut and the bones were flushed with IMDM using a 25 G needle and a 10 ml syringe. The BM cells flushed out from the tibias and femurs were collected in 50 ml Falcon tubes. Clumps of cells were disrupted through vigorous pipetting and vortexing. The cell suspension was then passed through a 70 μm cell strainer. The BM cells were subjected to centrifugation at 300 × g for 10 min and washed with IMDM. Live BM cells were identified using Trypan blue dye exclusion and counted using a haemocytometer. After counting the BM cells, they were resuspended at 2×10^6 cells per 5 ml in cIMDM supplemented with 10 ng/ml GM-CSF and 20 ng/ml IL-4 (Garrigan et al., 1996). 5 ml of the BM cell suspension were pipetted into each well of the 6-well plates. The 6-well plates containing BM cells were then incubated for 7 days. On the 3rd and 5th days of GM-CSF/IL-4 treatment, approximately 2 ml from each well in 6-well plates was aspirated gently and fresh cIMDM supplemented with 10 ng/ml GM-CSF and 20 ng/ml IL-4 was added to replace the aspirated media. The percentage of DCs in the BM cell culture was determined using anti-CD11c and anti-CD11b antibodies. Greater than 70% of cells recovered from GM-CSF/IL-4 cultures were CD11b$^+$CD11c$^+$ (Fig. 3.6.2a and Fig. 4.1a).

2.4.3. Generating DCs from Nup98-HoxB4 murine HSCs

Nup98-HoxB4 OVA-transgenic (OVAtr) murine HSCs were cultured in 90 mm petri dishes, containing cIMDM supplemented with 0.1% IL-6 and 10% stem cell factor. IL-6 and stem cell factor were kindly provided by A/P Christiane Ruedl (Nanyang Technological University, Singapore). HSCs were sub-cultured every 3rd day. OVAtr HSCs were harvested from 90 mm petri dishes by gently agitating with the pipette. HSCs were then washed with IMDM to remove the stem cell factor and IL-6. After washing, HSCs were cultured in GM-CSF and IL-4 in the same manner as BM cells in 2.4.2, except that HSCs were plated at 1×10^6 cells/well in 6-well plates. Greater than 90% of cells recovered from the GM-CSF/IL-4 cultures were CD11b$^+$CD11c$^+$ and will be referred to as OVAtr DCs in this thesis (Fig. 4.3.1a).

2.4.4. Activation of cultured DCs

On the 6th day of culture in GM-CSF/IL-4, LPS was added at a final concentration of 100 ng/ml to the cells cultured from the OVAtr HSCs and BM cells and incubated for 18-24 h. Non-adherent cells were harvested on day 7 by gentle agitation with the pipette. Greater than 70% and 90% of cells recovered from the GM-CSF/IL-4 cultures of BM cells and OVAtr HSCs, respectively, were CD11b$^+$CD11c$^+$ cells (Fig. 3.6.2a and Fig. 4.3.1a). LPS-treated DCs cultured from the BM cells and OVAtr HSCs were MHC class II^{hi}CD80^{hi}CD86^{hi}CD40^{hi} (Fig. 3.6.2b and Fig. 4.3.1b).

2.4.5. Loading BM-DCs with peptides

On the 7th day of culture, LPS-treated DCs were subjected to centrifugation at $300 \times$ g for 10 min. The cells were then counted and resuspended in cIMDM at 1×10^6 cells/ml. SIINFEKL, OVA$_{323-339}$, or gp$_{33-41}$ peptides were added into the DC suspensions at the appropriate concentrations and incubated at 37 °C for 4 h. After incubation, DCs were washed twice in IMDM, counted and centrifuged.

2.4.6. Labelling DCs with CFSE

DCs were cultured and activated as described in 2.4.2 and 2.4.4. DCs were washed with PBS and counted. After counting, the cells were resuspended to a concentration of 5×10^6 cells/ml in PBS. A final concentration of 1 µM CFSE was added to the cells. Upon the addition of CFSE, the cells were vortexed and incubated for 10 min at 37 °C. Equal volumes of FCS and IMDM were added to the cell suspensions to stop the CFSE-labelling reactions. The cells were then washed twice thoroughly with IMDM and resuspended in the appropriate volume of IMDM or cIMDM.

2.4.7. Labelling DCs with CMTMR

DCs were cultured and activated as described in 2.4.2 and 2.4.4. DCs were counted and resuspended in pre-warmed cIMDM at a concentration of 5×10^6 cells/ml. 10 µM CMTMR was added to the cell suspension, which was then incubated for 15 min at 37 °C. The cells were then centrifuged and resuspended in pre-warmed cIMDM before being incubated for another 20 min at 37 °C. After the 20 min incubation, the labelled cells were subsequently washed twice thoroughly with IMDM.

2.4.8. Loading BM-DCs with OVA protein

On the 5th day of GM-CSF/IL-4 treatment of BM cells, non-adherent cells were harvested by gentle pipetting, followed by centrifugation at $300 \times g$ for 10 min. The cells were counted and resuspended in fresh cIMDM supplemented with 10 ng/ml GM-CSF and 20 ng/ml IL-4. In the meantime, OVA protein powder was weighed and allowed to thoroughly dissolve in PBS to make a stock concentration of 40 mg/ml. The OVA protein solution was then filtered through a 32 mm syringe filter with 0.2 µm membrane pore size under sterile conditions. The filtered OVA protein solution was added to the DC suspension at a final concentration of 2 mg/ml. In some experiments, the OVA protein solution was serially diluted and the diluted OVA protein solution was added to the DC suspension at a final concentration of 0.02 mg/ml or 0.004 mg/ml.

After adding the appropriate OVA protein concentration into the DC suspension, the DCs were resuspended at 2×10^6 cells per 5 ml in cIMDM and left in the incubator at 37 °C overnight. The next day, the DC culture was treated with LPS. The percentage of DC+OVA was determined using anti-CD11c and anti-CD11b antibodies. Greater than 70% of cells recovered after the loading of OVA protein were CD11b$^+$CD11c$^+$ (Fig. 4.1a).

2.5. Methods – T cell purification

2.5.1. Preparation of lymph node and spleen cell suspensions by tissue disruption

Lymph nodes were made into cell suspensions by pressing with a 1 ml syringe plunger through a 70 μm cell strainer. Lymphocytes were collected in 50 ml tubes. The lymphocytes were then centrifuged, washed with Wuerzburger buffer and stored on ice before T cell purification.

Spleens were cut into small pieces using a pair of scissors. The pieces of spleens were then disrupted by pressing with a 1 ml syringe plunger through a 70 μm cell strainer. Splenocytes were treated with ACT buffer for 5 min at 37 °C to lyse the red blood cells. Splenocytes were then washed with Wuerzburger buffer and stored on ice before T cell purification.

2.5.2. T cell purification using magnetic cell separation (MACS)

CD4$^+$ T cells were enriched from the lymph nodes and spleens of naïve OT-II or OT-II B6.SJL-*Ptprc*^{a} mice, while CD8$^+$ T cells were enriched from the lymph nodes and spleens of naïve OT-I or OT-I B6.SJL-*Ptprc*^{a} mice. Lymph nodes and spleens of the abovementioned mice were harvested and prepared as described in 2.5.1. After preparation, both lymphocytes and ACT-treated splenocytes were centrifuged and pooled together in Wuerzburger buffer. The mixture of lymphocytes and splenocytes was filtered through a 70 μm cell strainer again. The cell mixture was counted and resuspended to 1×10^6 cells per 9 μl in Wuerzburger buffer. To enrich for CD4$^+$ T cells, 1 μl of anti-CD4 MACS microbeads

for every 1×10^6 cells was added. To enrich for CD8$^+$ T cells, 1 µl of anti-CD8α MACS microbeads for every 1×10^6 cells was added. The cell mixture was incubated with the MACS microbeads for 15 min on ice. The cells were mixed well periodically during the incubation. After the incubation, 0.1 µl of Wuerzburger buffer for every 1×10^6 cells was added and the cells were centrifuged to remove the unbound microbeads. The cells were then resuspended to 200×10^6 cells/ml in Wuerzburger buffer and passed through a 30 µm MACS pre-separation filter for positive magnetic selection (POSSEL program) on the AutoMACS machine (Miltenyi Biotec GmbH, Germany). The purity of enriched CD4$^+$ T cells was greater than 90% (Appendix 2).

2.6. Methods – *In vitro* T cell activation with peptide-pulsed BM-DCs

BM-DCs were cultured, activated and loaded with 0.1 µM SIINFEKL as described in 2.4.2, 2.4.4 and 2.4.5. In the meantime, lymph nodes were harvested from naïve OT-I or OT-I PKO mice and made into cell suspensions as described in 2.5.1, except that the lymphocytes were resuspended in cIMDM. The lymphocytes were counted and adjusted to 0.5×10^6 cells/ml with cIMDM. After the 4 h incubation with the peptide, the SIINFEKL-loaded DCs were washed with IMDM once and resuspended to 0.0625×10^6 cells/ml in cIMDM. 1 ml of SIINFEKL-loaded DC mix, 1 ml of lymphocyte mix, and 3 ml of cIMDM were added into each well of the 6-well plates. Each well contained 0.0625×10^6 SIINFEKL-loaded DCs and 0.5×10^6 OT-I lymphocytes in a total cIMDM volume of 5 ml. The plates were left to incubate at 37 °C for 4 days. On the 4th day, activated OT-I CD8$^+$ T cells were collected and washed with IMDM once. The activated CD8$^+$ T cells were counted and resuspended to 0.25×10^6 cells/ml in cIMDM supplemented with 100 U/ml of IL-2. The cells were then left in the incubator overnight. The next day, additional 100 U/ml of IL-2 was added to the activated CD8$^+$ T cells. After 2 days in IL-2, the activated OT-I CD8$^+$ T cells were then harvested and washed in IMDM twice. Greater than 95% of T cells recovered from the *in vitro* activation culture of OT-I or OT-I PKO lymphocytes were Vα2$^+$Vβ5.1/5.2$^+$ (Fig. 3.1.1 and Fig. 3.5.2). The recovered OT-I or OT-I PKO CD8$^+$ T cells bore the effector phenotype CD62L^{lo}CD44^{hi} (Fig. 3.1.1 and Fig. 3.5.2).

2.7. Methods – Labelling of CD178 (FasL) on CTLs

In vitro activated OT-I CTLs prepared as described in 2.6 were washed with FACS buffer and incubated with anti-FcγRII/III for 10 min at 4 °C in 96-well plates. 10 μM SIINFEKL or media only was added together with anti-CD178 antibody into each well containing OT-I CTLs. The CTLs were incubated for 30 min or 2 h at 37 °C. After incubation, *in vitro* activated OT-I CTLs were washed with FACS buffer twice and resuspended in 100 – 150 μl FACS buffer. The appropriate concentration of PI was added 5 – 10 min before the cells were monitored by flow cytometry.

2.8. Methods – *In vitro* T cell proliferation assays

2.8.1. *In vitro* T cell proliferation assays using thymidine uptake

Lymphocytes were harvested as described in 2.5.1 except that they were resuspended in cIMDM. Lymphocytes were resuspended in the appropriate volume. The lymphocytes were then plated in 96-well plates together with either peptide-loaded DCs prepared as in 2.4.5 or protein-loaded DCs prepared as in 2.4.8 and incubated for 2 days at 37 °C. After the incubation, 1 μCi tritiated thymidine was added into the wells and the cells were incubated for another 6 h. Following the incubation with thymidine, the cells were harvested using a Harvester 96® (Tomtec, CT, USA). Thymidine incorporation was detected using Wallac 1450 MicrobetaPlus Liquid Scintillation Counter (PerkinElmer Life Sciences and Analytical Sciences, formerly Wallac Oy) and acquired using Wallac 1450 MicroBeta Windows Workstation ver. 2.70.004.

2.8.2. *In vitro* T cell proliferation assays using CFSE dilution

CD4$^+$ and CD8$^+$ T cells were enriched and labelled with CFSE as described in 2.5.2 and 2.4.6. In some experiments, CFSE-labelled CD4$^+$ T cells were plated in 24-well plates, together with either protein-loaded DCs prepared as in 2.4.8 or DCs prepared as in 2.4.4 for

4 or 5 days at 37 °C. After the incubation, cells were harvested and labelled with antibodies against cell surface markers as described in 2.14.1 and monitored by flow cytometry.

2.9. Methods – *Ex vivo* DC manipulation

2.9.1. Preparation of lymph node cell suspensions by enzymatic digestion

Lymph nodes were collected in IMDM and were broken into pieces using 18 G needles. The lymph nodes were then incubated in IMDM containing 100 μg/ml DNase I and 0.1 mg/ml Liberase CI for 25 min at 37 °C. After 25 min of incubation, EDTA was added at a final concentration of 10 mM and the digested lymph nodes were incubated for another 5 min at 37 °C. The digested lymph nodes were then pressed through a 70 μm cell strainer, and washed once with Wuerzburger buffer for subsequent experiments.

2.9.2. Enriching DCs for *in vitro* T cell proliferation assays

$CD11c^+$ DCs were enriched from the brachial and axillary draining lymph nodes of mice. Brachial and axillary lymph nodes were harvested and prepared as described in 2.9.1. After preparation, the lymphocytes were incubated with anti-FcγRII/III antibodies for 15 – 20 min at 4 °C. The lymphocytes were then incubated with anti-CD3ε, anti-CD19, anti-B220, anti-NK1.1, anti-DX5, anti-Ter119, anti-Gr-1 and anti-Thy1.2 biotinylated antibodies for 30 min at 4 °C. In some experiments, anti-CD45.1 biotinylated antibody was also added. The cells were mixed well periodically during incubation. After antibody incubation, the lymphocytes were washed twice with Wuerzburger buffer. The lymphocyte mixture was counted and resuspended to 1×10^6 cells per 9 μl in Wuerzburger buffer. 1 μl of streptavidin MACS microbeads for every 1×10^6 cells was added and the lymphocytes were incubated for 15 min at 4 °C. After incubation, 0.1 μl of Wuerzburger buffer for every 1×10^6 cells was added and the lymphocytes were centrifuged to remove the unbound microbeads. The lymphocytes were resuspended to 200×10^6 cells/ml in Wuerzburger buffer and passed through a 30 μm MACS pre-separation filter for negative magnetic

selection (DEPLETE program) on the AutoMACS machine (Miltenyi Biotec GmbH, Germany). The cell compositions of the different fractions are shown in Appendix 12. The purity of the enriched DCs for each experiment is shown together with the results. The enriched CD11c$^+$ DCs were then incubated with CFSE-labelled CD8$^+$ T cells or CD4$^+$ T cells in 96-well plates and incubated for 3 or 5 days, respectively.

2.9.3. Fluorescence activated cell sorting (FACS) of DCs for *in vitro* T cell proliferation assays

Purified DCs from 2.9.2 were resuspended at 2×10^6 cells/ml in Wuerzburger buffer and labelled with anti-CD205, anti-CD8 and anti-CD11c antibodies as described in 2.14.1 under sterile conditions. Labelled DCs were resuspended at 4×10^6 cells/ml and sorted using a FACSVantage SE DiVa (Becton Dickinson, CA, USA). Unlabelled and single labelled samples for each fluorochrome were used to set the voltage and compensation parameters. The sorted DC subpopulations were then incubated with CFSE-labelled CD8$^+$ T cells or CD4$^+$ T cells in 96-well plates and incubated for 3 or 5 days, respectively.

2.10. Methods – *In vitro* T cell restimulation assays

2.10.1. Adoptive transfer of T cells for *in vitro* T cell restimulation assays

CD4$^+$ T cells were enriched as described in 2.5.2. CD4$^+$ T cells were resuspended in appropriate volumes of IMDM. 2×10^6 CD4$^+$ T cells, or a mixture of 5×10^6 CTLs and 2×10^6 CD4$^+$ T cells in 300 μl IMDM were injected i.v. into recipient mice through the lateral tail vein.

2.10.2. Preparation of spleen cell suspensions by enzymatic digestion

Spleens were collected in IMDM containing 100 μg/ml DNase I and 0.1 mg/ml Liberase CI. The IMDM containing DNase I and Liberase CI was then injected into various locations on each spleen for better digestion. The spleens were then incubated in the same

IMDM mixture for 25 min at 37 °C. After 25 min of incubation, EDTA was added at a final concentration of 10 mM and the digested spleens were incubated for another 5 min at 37 °C. The digested spleens were then pressed through a 70 μm cell strainer, and washed once with IMDM. After the wash, the splenocytes were resuspended in cIMDM.

2.10.3. Restimulating T cells with peptide *in vitro*

Splenocytes were prepared as described in 2.10.2. Splenocytes were counted and resuspended to 6×10^6 cells/ml. 1 ml of splenocytes was dispensed into each well of 6-well plates. OVA$_{323-339}$ was added to some of the wells containing splenocytes at a final concentration of 10 μM. Some wells contained 6×10^6 splenocytes and 10 μM OVA$_{323-339}$. In some wells, no OVA$_{323-339}$ was added to the splenocytes. The splenocytes were then left in the incubator for 15 h at 37 °C. After incubation, GolgiStop was added to each well to prevent the export of proteins from the Golgi bodies. The splenocytes were incubated in GolgiStop for 5 h at 37 °C. Following the 5 h incubation, the splenocytes were harvested and washed in IMDM. The splenocytes were then labelled with antibodies as described in 2.14.1 and 2.14.2.

2.11. Methods – *In vivo* DC killing assays

2.11.1. Generation of endogenous CTL responses using BM-DC immunisation

BM-DCs were cultured, activated and loaded with 0.1 μM SIINFEKL as described in 2.4.2, 2.4.4 and 2.4.5. SIINFEKL-loaded BM-DCs were then resuspended at 1×10^6 cells/ml in IMDM. 100 μl of SIINFEKL-loaded DCs were injected s.c. into the flank of each recipient mouse.

2.11.2. Adoptive transfer of CTLs for DC killing assays

In vitro activated CTLs prepared as in 2.6 were adoptively transferred into recipient mice. In most experiments, 5×10^6 CTLs in 300 μl IMDM were injected i.v. into the lateral tail vein of each recipient mouse. In some experiments, 1×10^6 or 10×10^6 CTLs were transferred.

2.11.3. Preparing DCs for DC killing assays

BM-DCs were cultured and activated as described in 2.4.2 and 2.4.4. The DCs were harvested and split into two tubes. The first tube of DCs was loaded with SIINFEKL peptide at a final concentration of 0.1 μM (DC+SIINFEKL) as described in 2.4.5. The second tube of DCs was not loaded with SIINFEKL (DC only). After incubation, both groups of DCs were washed twice in IMDM and counted. The DC+SIINFEKL group was labelled with CFSE as described in 2.4.6. The DC only group was labelled with CMTMR as described in 2.4.7. After labelling, both groups of DCs were counted and resuspended at 1×10^6 cells/ml. CFSE-labelled DC+SIINFEKL and CMTMR-labelled DC only were mixed in equal numbers. In some DC killing experiments, OVAtr DCs or OVA-loaded DCs prepared as described in 2.4.3 or 2.4.8 respectively, were labelled with CFSE and mixed with equal numbers of CMTMR-labelled DC only. The mixture of CFSE-labelled DCs and CMTMR-labelled DCs was then centrifuged and resuspended to 20×10^6 cells/ml. The DC mixture was injected s.c. into the forelimbs of recipient mice (Appendix 14), hence one forelimb of each mouse received 0.5×10^6 cells/ml of DC+SIINFEKL and 0.5×10^6 cells/ml of DC only.

2.11.4. Preparation of lymph node cell suspensions by enzymatic digestion

Brachial and axillary lymph nodes were prepared as described in 2.9.1, except that they were digested in 100 μg/ml DNase I and 2.4 mg/ml Type II Collagenase for 1 h at 37 °C. The digested lymphocytes were then washed and prepared for analysis by flow cytometry.

2.12. Methods – *In vivo* T cell proliferation assays

2.12.1. Adoptive transfer of T cells for T cell proliferation assays

CD4$^+$ and CD8$^+$ T cells were enriched and labelled with CFSE as described in 2.5.2 and 2.4.6. CFSE-labelled CD4$^+$ or CD8$^+$ T cells were adoptively transferred into recipient mice. In most experiments, 1×10^6 CFSE-labelled CD4$^+$ or CD8$^+$ T cells in 300 µl IMDM were injected i.v. into the lateral tail vein of each recipient mouse. In some experiments, 2×10^6 CFSE-labelled CD4$^+$ T cells were transferred.

In experiments involving DC killing and T cell proliferation, CTLs were mixed with CFSE-labelled CD4$^+$ or CD8$^+$ T cells. The T cell mixture contained either CTLs and CFSE-labelled CD4$^+$ T cells, or CTLs and CFSE-labelled CD8$^+$ T cells. In most experiments, a mixture of 5×10^6 CTLs and 1×10^6 CFSE-labelled T cells in 300 µl IMDM were transferred. In some experiments, recipient mice received 1×10^6 CTLs and 1×10^6 CFSE-labelled T cells, or 10×10^6 CTLs and 1×10^6 CFSE-labelled T cells instead. The T cell mixture was injected i.v. into the lateral tail vein of each recipient mouse.

2.12.2. Preparing DCs for T cell proliferation assays

BM-DCs were cultured and activated as described in 2.4.2 and 2.4.4. The DCs were loaded with 0.1 µM SIINFEKL for *in vivo* CD8$^+$ T cell proliferation assays; or loaded with 1 µM OVA$_{323-339}$, 10 µM OVA$_{323-339}$, 1 µM OVA$_{323-339}$ and 10 µM SIINFEKL, or 1 µM OVA$_{323-339}$ and 10 µM gp$_{33-41}$, for *in vivo* CD4$^+$ T cell proliferation assays at 37 °C for 4 h. After incubation, the peptide-loaded DCs were washed twice in IMDM and counted. The peptide-loaded DCs were resuspended to 2×10^6 cells/ml in IMDM. Each recipient mouse was injected s.c. into one of their forelimbs with 0.1×10^6 DCs in 50 µl IMDM (Appendix 14).

In some experiments, OVA-loaded DCs prepared as described in 2.4.8 were washed twice and counted before being resuspended to 4×10^6 cells/ml in IMDM. Each recipient mouse

was injected s.c. into one of their forelimbs with 0.2×10^6 DCs or 0.5×10^6 DCs in 50 µl IMDM (Appendix 14).

In other experiments, OVAtr DCs prepared as described in 2.4.3 and 2.4.4 washed twice and counted before being resuspended to 4×10^6 cells/ml in IMDM. Each recipient mouse was injected s.c. into one of their forelimbs with 0.2×10^6 DCs in 50 µl IMDM.

2.12.3. Preparation of lymph node and spleen cell suspensions by tissue disruption

The axillary and brachial lymph nodes were sandwiched between 2 pieces of 70 µm gauze and perforated with 18 G needles. The draining lymph nodes were then pressed with a 1 ml syringe plunger. Cells were collected from the disrupted draining lymph nodes by agitation with IMDM using a pipette. The cells were filtered through pieces of 70 µm gauze twice and washed with FACS buffer thrice.

The spleen cell suspensions were prepared and treated with ACT buffer as described in 2.5.1. The ACT reaction was stopped by the addition of IMDM and the cells were spun down. After centrifugation, the cells were resuspended in FACS buffer and filtered through pieces of 70 µm gauze into 15 ml Falcon tubes. This process was performed twice. After filtering and washing, the cells were resuspended to 5 ml.

200 µl of lymphocytes or splenocytes prepared above were aliquoted into the corresponding wells of a 96-well plate. The plate was stored on ice for fluorescent antibody labelling as described in 2.14.1.

2.13. Methods – Tracking transfer of FITC-dextran *in vivo*

On the 6th day of culture, FITC-dextran was added to a final concentration of 100 µg/ml into wells that contained BM-DCs or OVAtr DCs prepared as described in 2.4.2 or 2.4.3, respectively. The DCs were incubated in FITC-dextran for 1 h at 37 °C. After the 1 h incubation, the DCs were treated with LPS for 18-24 h. Following the LPS incubation, the

DCs were washed twice with IMDM and counted. FITC-dextran-loaded DCs were labelled with CMTMR as described in 2.4.7. After CMTMR labelling, the DCs were resuspended to 10×10^6 cells/ml. Each forelimb of recipient mouse were injected s.c. with 0.5×10^6 DCs in 50 μl IMDM (Appendix 14).

2.14. Methods – Flow cytometry

2.14.1. Labelling the cell surface molecules with fluorescence-conjugated antibodies

Cells were washed in FACS buffer and resuspended at $1 - 2 \times 10^7$ cells/ml. The cell suspension was dispensed into the wells of a 96-well plate. The plate was centrifuged at $370 \times g$ for 2 min and the plate was flicked to remove the supernatants from the wells. The plate was then tapped at the sides to resuspend the cell pellet. The cells were first incubated in anti-FcγRII/III antibodies for 10 min on ice. After the 10 min incubation, fluorescence-conjugated antibodies against cell surface markers were added at the appropriate dilutions and the cells were incubated for another 10 min on ice. Following the completion of this incubation, the labelled cells were washed twice by adding 200 μl of FACS buffer and spun down at $370 \times g$ for 2 min. If a secondary antibody was required, the cells labelled with the biotinylated primary antibodies were further incubated with the appropriate dilutions of a streptavidin-conjugated fluorochrome for 10 min on ice. After the incubation, the cells were washed twice with FACS buffer and resuspended in $100 - 150$ μl FACS buffer. The appropriate concentration of PI was added $5 - 10$ min before the cells were analysed by flow cytometry.

2.14.2. Labelling intracellular molecules with fluorescence-conjugated antibodies

Cells were labelled with antibodies against cell surface makers as described in 2.14.1. After cell surface labelling and washing in FACS buffer, the cells were incubated with 200 μl of BD Cytofix/Cytoperm solution for 20 min at 4 °C. The cells were washed twice in $1 \times$ BD Perm/Wash buffer. The cells were then incubated with antibodies against cytokines, or the

respective isotype control antibodies for 30 min on ice. After the 30 min incubation, the cells were washed thrice in BD Perm/Wash buffer. During each wash, the cells were allowed to sit in the buffer for 10 min before centrifugation to minimise background staining. After the three washes, the cells were resuspended in 200 – 300 μl FACS buffer and monitored by flow cytometry.

2.14.3. Acquisition of cells on flow cytometry

Antibody-labelled cells were analysed on a FACSort, FACScalibur, or LSRII SORP flow cytometer (Becton-Dickson, CA, USA). The live cells were identified as PI$^-$ cells. Cells prepared as described in 2.14.2 were identified based on their forward scatter (FSC) and side scatter (SSC) properties. Unlabelled cells, PI-treated unlabelled cells and single-labelled cells for each fluorochrome-conjugated antibody were used to adjust the channel voltages and compensate for the spectral overlap between fluorochromes used. In some experiments, single-labelled cells for CFSE and single-labelled cells for CMTMR were used to calibrate the voltages and compensation overlap. FlowJo version 9.0.2 (Treestar Inc, CA, USA) was used to analyse data captured on the flow cytometers.

2.15. Methods – Analysis of data collected

2.15.1. *In vivo* DC killing assays

The analysis of flow cytometry data for *in vivo* DC killing assays was performed using FLOWJO version 9.0.2 software (TreeStar, Oregan, USA) and was as follows: First, dead cells were excluded with PI staining. CFSE$^+$ DCs and CMTMR$^+$ DCs were then gated and counted. The gates for CFSE$^+$ DCs and CMTMR$^+$ DCs were determined using control mice that were injected with singly-labelled DCs. The ratio of CFSE$^+$ DCs to CMTMR$^+$ DCs in each sample was normalised to the control group in each experiment to account for discrepancies in counting. To obtain absolute numbers, total lymphocyte numbers were counted using a *Z2* Coulter Particle Count and Size Analyzer (Beckman Coulter,

California, USA) that was set up to detect cell size of $4 - 10$ µM. Absolute numbers of DCs were calculated as (DC/live cells) × total cell numbers.

2.15.2. *In vivo* and *in vitro* T cell proliferation assays

The analysis of flow cytometry data for *in vivo* T cell proliferation assays was performed using FLOWJO version 9.0.2 software (TreeStar, Oregan, USA) and was as follows: First, dead cells were excluded with PI staining. Adoptively transferred T cells were gated using one of the following gates- $CD4^+CD45.2^+$, $CD4^+CD45.1^+$, $CD8^+CD45.2^+$, or $CD8^+CD45.1^+$ live cells. The gates for the transferred T cells were determined using single-labelled and double-labelled cells. $CFSE^{hi/int}$ cells were then gated on the transferred T cells. T cells that were $CFSE^{hi}$ and had not undergone any CFSE dilution were referred to as undivided cells. The gating on undivided T cells was determined using control mice that were injected with DCs not loaded with peptides or proteins. These control mice were included in all the *in vivo* T cell proliferation experiments conducted (data not shown). T cells that had undergone at least one CFSE dilution were referred to as divided cells. Highly divided T cells were cells that had undergone more than four CFSE dilutions. Total lymphocyte or splenocyte numbers were counted using a Z2 Coulter Particle Count and Size Analyzer that was set up to detect cell size of $4 - 10$ µM. Absolute numbers of divided T cells are calculated as (divided T cells/live cells) × total cell numbers.

In some *in vitro* T cell proliferation data, $V\alpha2^+CD4^+$ or $V\alpha2^+CD8^+$ cells were gated from PI^- T cells. $CFSE^{hi/int}$ cells were then gated on the $V\alpha2^+CD4^+$ and $V\alpha2^+CD8^+PI^-$ T cells. T cells that had undergone at least one CFSE dilution were referred to as divided cells.

2.15.3. *In vitro* T cell restimulation assays

The analysis of flow cytometry data for *in vivo* T cell restimulation assays was performed using FLOWJO version 9.0.2 software (TreeStar, Oregan, USA) and was as follows: Fixed cells were first gated on the FSC and SSC. $CD4^+CD45.1^+$ T cells were then gated from the FSC/SSC gate. $CD4^+CD45.1^+$ T cells expressing IFNγ were gated. The same gates were

applied to the corresponding $CD4^+CD45.1^+$ T cells labelled with isotype-matching antibody.

2.15.4. Software used for data analysis

Microsoft Excel 2008 for Mac OS (Microsoft Corporation, Washington, USA) and GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego California USA) were used to create tables and graphs and for statistical analysis.

2.15.5. Statistical analysis

Data was tested for normality using D'Agostino and Pearson omnibus test. The results showed that representative data from CTL-mediated DC killing, DC accumulation in the draining lymph nodes, and $CD4^+$ T cell proliferation were consistent with a Gaussian distribution (Appendix 3). Statistical analysis comparing two groups was conducted using Student's *t*-test. F-test was used to determine the equality of variances. Exact probability values for Student's *t*-test and F-test were calculated using Microsoft Excel 2008. Statistical analysis comparing three groups or more was conducted using one-way ANOVA with Bonferroni's post-test. When analysing two factors in three groups or more, two-way ANOVA with Bonferroni's post-test was used to determine the statistical significance. Bonferroni's correction was used to address the errors in inference when comparing multiple groups. Mean and standard errors of mean are shown in the graphs.

Chapter 3

Cytolytic mechanisms involved in CTL-mediated DC elimination

Our group and others have shown that antigen-presenting DCs are eliminated by CTLs through the cytolytic molecule perforin (Belz et al., 2007; Yang et al., 2006). Others have provided evidence that CTLs eliminate DCs through the cytolytic molecule FasL (Stranges et al., 2007). These studies provide evidence that CTLs actively remove antigen-presenting DCs.

The physiological relevance of why CTLs eliminate DCs has not been formally established. However, there are some indications that CTL-mediated DC elimination functions as a form of negative feedback to downregulate immune responses that may otherwise lead to immunopathology. When the survival of DCs was prolonged, excessive T cell proliferation and autoreactive immune responses were observed (Chen et al., 2006). Furthermore, CTLs suppressed the induction of alloreactive CD4$^+$ T cells and CD4$^+$ T cell-mediated allograft rejection through the perforin-mediated elimination of allogeneic DCs (Laffont et al., 2006).

There is other circumstantial evidence indicating that cytolytic molecules regulate T cell responses by controlling DC survival. These were reported by studies in patients or mice carrying defective cytolytic molecules or other downstream molecules in the cytolytic pathways. In perforin-deficient patients, aberrant accumulation of T cells was observed (Stepp et al., 1999). Mice deficient in perforin (PKO mice) also showed increased numbers of effector T cells when infected with bacteria (Badovinac et al., 2000) or virus (Matloubian et al., 1999), or immunised with peptide-loaded DCs (Yang et al., 2006). The increase in effector T cells observed in PKO mice was due to the failure of CTLs in removing antigen-presenting DCs (Yang et al., 2006). Patients deficient in caspase-10 showed increased numbers of T cells and DCs (Wang et al., 1999). DCs in these caspase-10 deficient patients were resistant to TRAIL-mediated killing. Mice deficient in Fas (*lpr* mice) or FasL (*gld* mice) also showed an aberrant accumulation of abnormal T cells (Watanabe-Fukunaga et al., 1992). This could be due to the increased DC frequency observed in *lpr* mice (Fields et al., 2001). Furthermore, when Fas was selectively knocked out in DCs, some of the manifestations seen in *lpr* mice were also observed (Stranges et al., 2007).

The evidence described suggests that if CTLs do not regulate the survival of antigen-presenting DCs tightly, immunopathology will develop. Thus, closer examination of the cytolytic killing mechanisms regulating the survival of antigen-presenting DCs and the effects of DC elimination on the induction of subsequent immune responses are necessary.

The aims of this chapter are:

1) To characterise and evaluate the contributions of the cytotoxic pathway(s) used by CTLs to eliminate DCs

2) To examine the effects and contributions of the cytotoxic pathway(s) mediating DC elimination on $CD4^+$ and $CD8^+$ T cell proliferation

3.1. *In vitro* activated $V\alpha2^+V\beta5.1/5.2^+$ OT-I CTLs are $CD62L^{lo}CD44^{hi}$

To generate a pure population of OT-I CTLs, OT-I TCR transgenic lymphocytes were activated *in vitro* with SIINFEKL-loaded DCs as described in Chapter 2. I started off by testing different concentrations of SIINFEKL loaded on DCs and different numbers of SIINFEKL-loaded DCs to OT-I lymphocyes (Table 3.1.1). The goal of this optimisation was to obtain $V\alpha2^+V\beta5.1/5.2^+$ OT-I CTLs expressing low CD62L and high CD44. When 0.5×10^6 OT-I lymphocytes were incubated with 62500 DCs loaded at $0.1\mu M$ SIINFEKL, this condition yielded 1.41×10^6 OT-I CTLs (Table 3.1.2). This culture condition generated populations consisting of greater than 95% of $CD62L^{lo}CD44^{hi}V\alpha2^+V\beta5.1/5.2^+$ OT-I CTLs (Fig. 3.1.1) and the recovery of CTLs was sufficient for adoptive transfer (Table 3.1.2).

Next, I used the optimised *in vitro* condition and tested different *in vitro* resting conditions for the OT-I CTLs (Table 3.1.3). OT-I CTLs were incubated with IL-2 at either 125000 or 250000 cells/ml. After 2 days of incubation, the number of OT-I CTLs increased (Table 3.1.4) and greater than 95% of cells were $CD62L^{lo}CD44^{hi}V\alpha2^+V\beta5.1/5.2^+$ (Fig. 3.1.2). 250000 cells/ml was chosen as the resting condition for the OT-I CTLs because the number of OT-I CTLs recovered was sufficient for adoptive transfer. Under the optimised *in vitro* activation and resting conditions, OT-I CTLs acquired cytolytic functions as they were able to eliminate SIINFEKL-loaded DCs *in vivo* (Fig. 3.5.3) and antigen-loaded EL-4 cell line *in vitro* (Robinson et al., 2010).

Table 3.1.1. Optimisation of culture conditions for *in vitro* activation of OT-I lymphocytes

SIINFEKL (µM) loaded on DCs	Ratio of DC to OT-I lymphocytes				
	6:1	1:1	1:2	1:4	1:8
10	1.2: 0.2 *	N.D.	N.D.	N.D.	N.D.
	N.D.**	1: 1	N.D.	N.D.	N.D.
	N.D.	0.5: 0.5	N.D.	N.D.	N.D.
	N.D.	0.25:0.25	N.D.	N.D.	N.D.
1	1.2: 0.2	N.D.	N.D.	N.D.	N.D.
	N.D.	1: 1	0.5: 1	N.D.	N.D.
	N.D.	0.5: 0.5	0.25: 0.5	N.D.	N.D.
0.1	1.2: 0.2	N.D.	N.D.	N.D.	N.D.
	N.D.	1: 1	0.5: 1	0.25: 1	0.125: 1
	N.D.	0.5: 0.5	0.25: 0.5	0.125: 0.5	**0.0625: 0.5**

* DC no. to OT-I lymphocytes no. ($\times 10^6$)
** N.D. not done

Table 3.1.2. Recovery of OT-I CTLs after *in vitro* culture incubation with DCs in Table 3.1.1

SIINFEKL (µM) loaded on DCs	Ratio of DC to OT-I lymphocytes				
	6:1	1:1	1:2	1:4	1:8
10	0.44 *	N.D.	N.D.	N.D.	N.D.
	N.D. **	1.23	N.D.	N.D.	N.D.
	N.D.	0.89	N.D.	N.D.	N.D.
	N.D.	0.45	N.D.	N.D.	N.D.
1	0.17	N.D.	N.D.	N.D.	N.D.
	N.D.	1.31	1.21	N.D.	N.D.
	N.D.	0.77	0.76	N.D.	N.D.
0.1	0.2	N.D.	N.D.	N.D.	N.D.
	N.D.	2.68	1.30	2.53	3.5
	N.D.	0.74	1.83	1.77	1.41

* No. of OT-I CTLs recovered on day 4 ($\times 10^6$)
** N.D. not done

Conditions tested
10 µM SIINFEKL

1.2×10^6 DCs

0.2×10^6 OT-I

1 µM SIINFEKL

0.5×10^6 DCs

0.5×10^6 OT-I

0.1 µM SIINFEKL

0.0625×10^6 DCs

0.5×10^6 OT-I
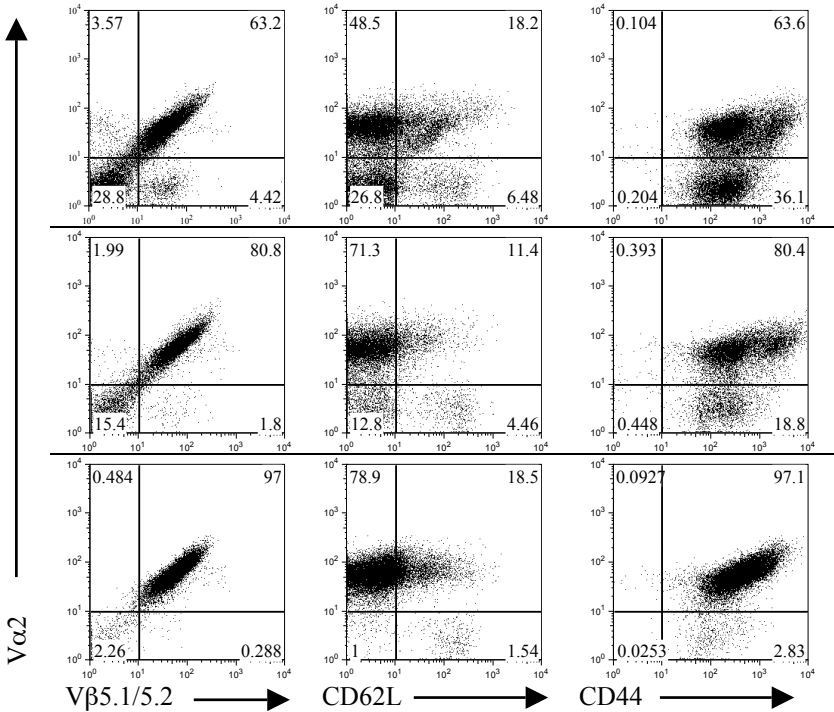
Fig. 3.1.1. **Different *in vitro* activation culture conditions for naïve OT-I lymphocytes yield CTLs of different phenotypes.** Total lymphocytes prepared from OT-I transgenic mice were cultured in the indicated culture conditions with SIINFEKL-loaded LPS-activated DCs for 4 days. Activated T cells were then rested in IL-2 for 2 days. These *in vitro* expanded T cells were examined for the expression of Vα2 and Vβ5.1/5.2 by flow cytometry. Vα2⁺Vβ5.1/5.2⁺, Vα2⁺CD62L⁺ and Vα2⁺CD44⁺ cells are shown as percentages of live cells. One representative experiment of three is shown. The condition selected for further experiments shown in bold.

Table 3.1.3. Optimisation of *in vitro* resting conditions for activated OT-I T cells

In vitro priming conditions		*In vitro* resting conditions	
SIINFEKL (μM) loaded on DCs	Ratio of DCs to OT-I lymphocytes	DC no. : OT-I lymphocyte no. ($\times 10^6$)	
		0.125 *	0.25
0.1	1:8	0.25: 2 **	0.25: 2
		0.125: 1	0.125: 1
		0.0625: 0.5	**0.0625: 0.5**

* T cell concentration in resting culture ($\times 10^6$/ml)
** DC no. to OT-I lymphocyte no. ($\times 10^6$)

Table 3.1.4. Recovery of OT-I CTLs after *in vitro* activation and 2 days in IL-2 in Table 3.1.3

In vitro priming conditions		*In vitro* resting conditions	
SIINFEKL (μM) loaded on DCs	Ratio of DCs to OT-I lymphocytes	No of CTLs recovered ($\times 10^6$)	
		0.125 *	0.25
0.1	1:8	11.73 **	22.78
		15.13	24.48
		12.58	24.82

* T cell concentration in resting culture ($\times 10^6$/ml)
** No. of CTLs recovered ($\times 10^6$)
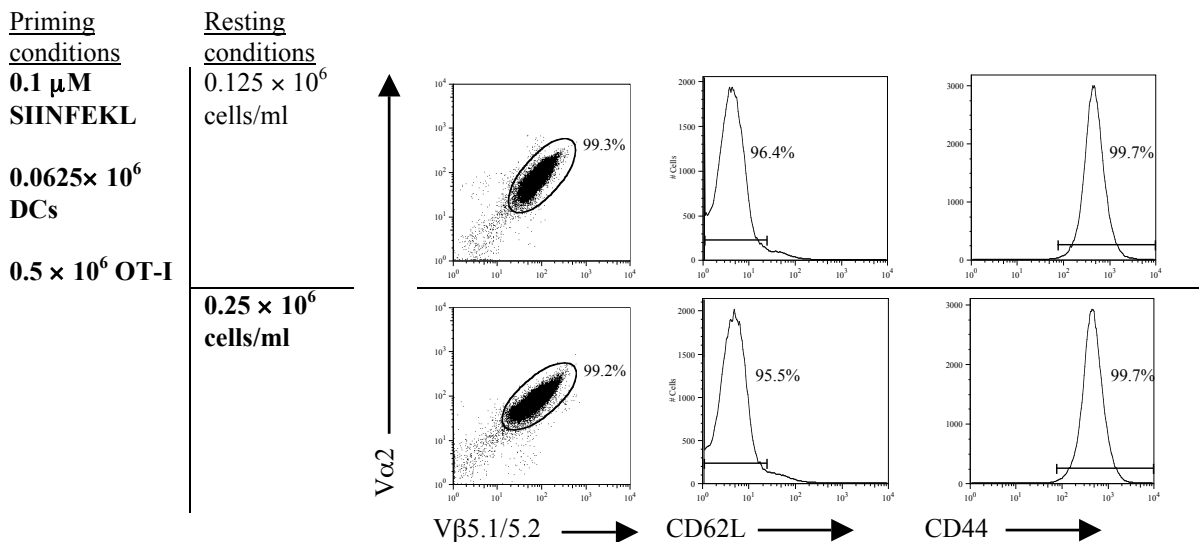
Fig. 3.1.2. **Optimised *in vitro* activation culture condition for OT-I lymphocytes yields a homogenous population of CD62L^{lo}CD44^{hi}Vα2$^+$Vβ5.1/5.2$^+$ T cells after resting in IL-2.** Total lymphocytes prepared from OT-I transgenic mice were cultured in the indicated culture conditions with SIINFEKL-pulsed LPS-activated DCs for 4 days. Activated T cells were then rested in IL-2 at different cell numbers per ml for 2 days. These *in vitro* activated T cells were examined for the expression of Vα2 and Vβ5.1/5.2 by flow cytometry. Vα2$^+$Vβ5.1/5.2$^+$, Vα2$^+$CD62L$^+$ and Vα2$^+$CD44$^+$ cells are shown as percentages of live cells. One representative experiment out of three is shown. The condition selected for further experiments shown in bold.

3.2. CTLs prevent DCs from accumulating in the draining lymph nodes

It has been proposed that CTLs target antigen-bearing DCs in the peripheral tissues (Yang et al., 2006), or in the draining lymph nodes (Guarda et al., 2007a). To investigate whether DC killing by CTLs prevent DC entry into the draining lymph nodes, CFSE-labelled DC loaded with SIINFEKL (DC+SIINFEKL) were mixed with CMTMR-labelled control DCs (DC only) in equal numbers and injected s.c. into the forelimbs of mice (Hermans et al., 2004) (Appendix 14). The experimental setup is shown in Fig. 3.2.1a. Six hours after DC injection, half of these mice received *in vitro* activated CTLs i.v. while the rest did not. These mice were then sacrificed at the indicated time points and CFSE$^+$ DCs and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. To determine the number of DCs that had reached the lymph nodes at the time of CTL transfer, one group of mice was sacrificed at the time of CTL injection. This is indicated as time point 0 h.

At 0 h, similar low numbers of injected CFSE$^+$ and CMTMR$^+$ DCs were recovered from the draining lymph nodes of host mice (Fig. 3.2.1b). In mice that did not receive CTLs, similar numbers of both DC populations were also recovered at 8, 24, 48 and 72 h. Over time, increasing numbers of CMTMR$^+$ DCs were found in the draining lymph nodes of mice that received CTLs and in control mice (Fig. 3.2.1c). However, in mice that received CTLs, the number of CFSE$^+$ DCs plateaued at 24 h and remained significantly lower than the control group at 48 h and 72 h. This indicates that CTLs prevent the entry of DCs into the draining lymph nodes through DC killing.
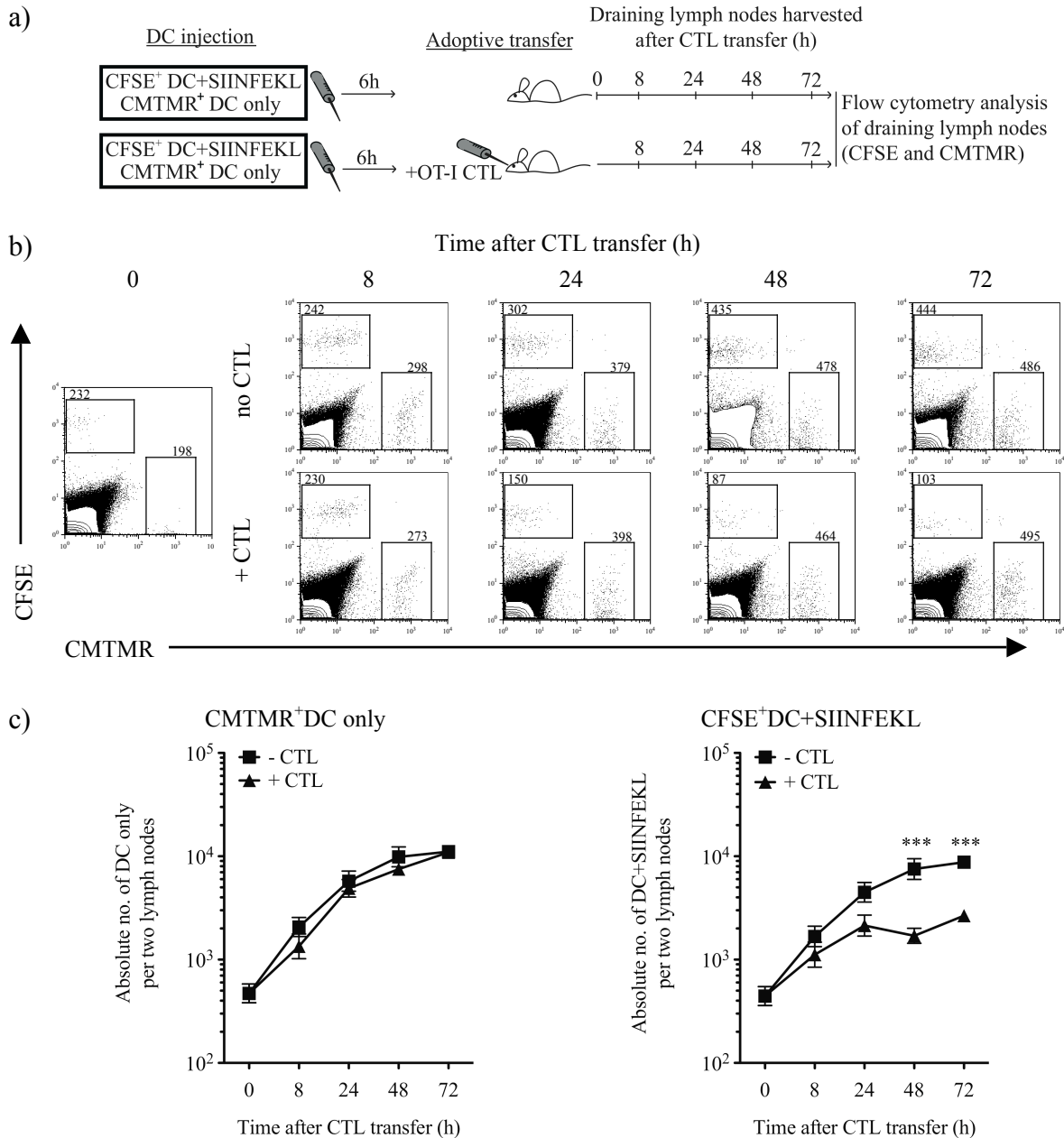
Fig. 3.2.1. **DCs do not accumulate in the draining lymph nodes over time in the presence of CTL-mediated DC killing. (a)** CFSE-labelled DCs loaded with SIINFEKL (DC+SIINFEKL) were mixed in equal numbers with CMTMR-labelled control DCs (DC only) and injected s.c. into the forelimbs of C57BL/6J mice. 6 h later, half of these mice received *in vitro* activated CTLs i.v. while the rest did not. These mice were then sacrificed and draining lymph nodes were harvested at the indicated time points. Time point 0 h corresponds to the time of CTL injection. CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. **(b)** CFSE$^+$ and CMTMR$^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. **(c)** Absolute numbers of CMTMR$^+$ DC only and CFSE$^+$ DC+SIINFEKL in the draining lymph nodes are shown. One representative experiment of two with 3 – 6 mice per group is shown. Statistical significance was determined using the two-way ANOVA with Bonferroni's correction. *** $p < 0.001$

Next, I asked how CTLs would influence DC accumulation into the draining lymph nodes if DCs were given more time to migrate. The experiment setup is shown in Fig. 3.2.2a. $CFSE^+$ DC+SIINFEKL and $CMTMR^+$ DC only were mixed in equal numbers and injected into the forelimbs of mice. These injected DCs were then given 6 h or 24 h to migrate to the draining lymph nodes before *in vitro* activated CTLs were transferred. As a control, one group did not receive CTLs. 72 h after DC injection, cell suspensions were prepared from draining lymph nodes and $CFSE^+$ and $CMTMR^+$ cells were monitored by flow cytometry.

When CTLs were given 6 h after DC injection, low numbers of $CFSE^+$ cells were detected compared to the control group, whereas the number of $CMTMR^+$ cells was similar (Fig. 3.2.2b). The number of $CFSE^+$ cells did not increase even though the antigen-bearing DCs had more time to migrate into the draining lymph nodes (18 h + 48 h). When CTLs were given 24 h after DC injection, the number of $CFSE^+$ cells recovered was similar to the control group (Fig. 3.2.2c), although some CTL-mediated DC killing was observed (Fig. 3.2.2d). Taken together, these results suggest that the presence of CTLs prevents the entry of antigen-loaded DCs into the draining lymph nodes and this cannot be overcome even if these DCs were given more time to reach the draining lymph nodes.

a)

DC injection

Adoptive transfer

CFSE+ DC+SIINFEKL
CMTMR+ DC only

6h
→
+OT-I CTL

18h   48h

CFSE+ DC+SIINFEKL
CMTMR+ DC only

24h
→
+OT-I CTL

48h

Flow cytometry analysis
of draining lymph nodes
(CFSE and CMTMR)

CFSE+ DC+SIINFEKL
CMTMR+ DC only

72h

b)   Time after DC injection (h)   -   6   24

CTL transfer   -   +   +

CFSE

CMTMR

c)

Absolute no. of
DC per two lymph nodes

Time after
DC challenge (h)   -   6   24

CTL transfer   -   +   +

■ CFSE+ DC+SIINFEKL
□ CMTMR+ DC only

d)

Normalised ratio of
DC+SIINFEKL to DC only

Time after
DC challenge (h)   -   6   24

CTL transfer   -   +   +

Fig. 3.2.2. **CTLs prevent the entry of DCs into the draining lymph nodes. (a)** CFSE-labelled DCs loaded with SIINFEKL (DC+SIINFEKL) and CMTMR-labelled control DCs (DC only) were mixed in equal numbers and injected s.c. into the forelimbs of C57BL/6J mice. 6 h or 24 h later, some of these recipient mice received *in vitro* activated CTLs i.v., while the control group did not receive CTLs. 72 h after DC injection, CFSE+ and CMTMR+ DCs in the draining lymph nodes were monitored by flow cytometry. (**b**) CFSE+ and CMTMR+ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. (**c**) Absolute numbers of CMTMR+ DC only and CFSE+ DC+SIINFEKL in the draining lymph nodes are shown. (**d**) The ratios of CFSE+ DC+SIINFEKL to CMTMR+ DC only were normalised to the ratios derived from untreated mice. The experiment was performed once with 3 mice per group.

3.3. CTLs inhibit CD4$^+$ T cell proliferation by eliminating DCs

To determine if CTLs affect the induction of CD4$^+$ T cell proliferation, CTLs and CFSE-labelled OT-II CD4$^+$ T cells harvested from naïve OT-II mice were mixed and transferred into one group of mice (Fig. 3.3a). The control group received CFSE-labelled OT-II CD4$^+$ T cells only. 24 h later, DCs loaded with SIINFEKL and OVA$_{323-339}$ were injected to the forelimbs of these recipient mice. 3 days later, CD4$^+$ T cell proliferation in the draining lymph nodes was monitored by flow cytometry.

In mice that received CTLs, most of the CD4$^+$ T cells remain undivided (Fig. 3.3b). The percentage and number of divided CD4$^+$ T cells were significantly lower in the mice that received CTLs than in control mice (Fig. 3.3c. and Fig. 3.3d.). This shows that CTLs inhibit the induction of CD4$^+$ T cell proliferation. This is similar to the results reported by Guarda et al (Guarda et al., 2007a).

a)

b)

c) d)
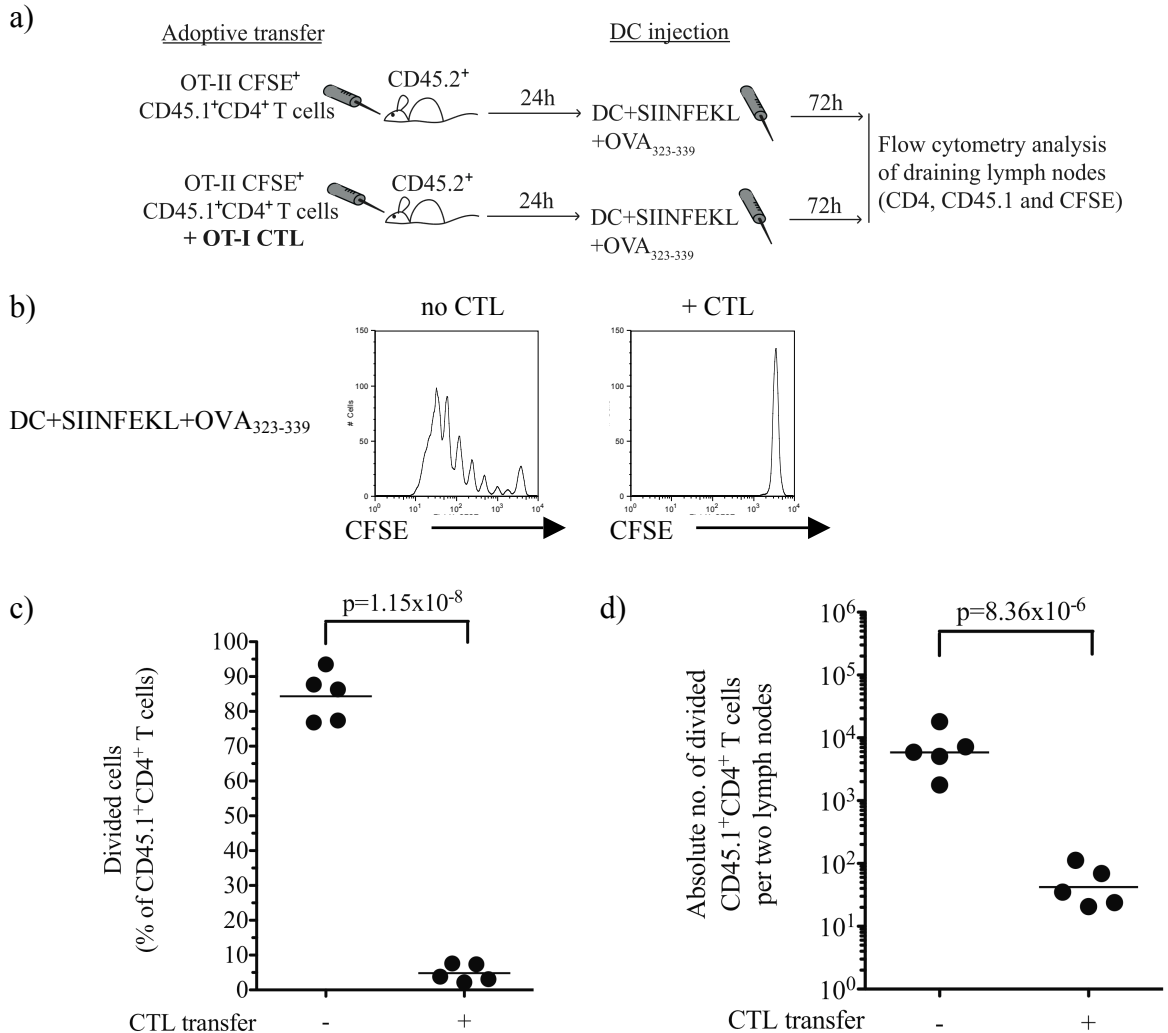
Fig. 3.3. **CTLs inhibit CD4$^+$ T cell proliferation through DC elimination.** (**a**) C57BL/6J mice received *in vitro* activated OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. As a control, one group of mice received only CD4$^+$ T cells. After 24 h, these mice were injected s.c. into their forelimbs with LPS-activated DCs loaded with SIINFEKL and OVA$_{323-339}$. 3 days later, CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiement was performed once with 5 mice per group. Statistical significance was determined with two tailed Student's *t*-test.

3.4. CTLs inhibit CD4$^+$ T cell proliferation in an antigen-specific manner

To determine if CTLs inhibit the CD4$^+$ T cell proliferation in an antigen-specific manner, DCs were loaded with either a combination of SIINFEKL and OVA$_{323-339}$ or the irrelevant peptide gp33 and OVA$_{323-339}$. DCs loaded with SIINFEKL and OVA$_{323-339}$ or DCs loaded with gp33 and OVA$_{323-339}$ were injected into mice that had previously received either a mixture of CFSE-labelled OT-II CD4$^+$ T cells and OT-I CTLs, or CD4$^+$ T cells only (Fig. 3.4a). 3 days later, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

CD4$^+$ T cell proliferation was observed in all mice that received DCs loaded with gp33 and OVA$_{323-339}$ regardless of the presence of CTLs (Fig. 3.4b). In mice that received CTLs and DCs loaded with SIINFEKL and OVA$_{323-339}$, the majority of CD4$^+$ T cells remained undivided whereas most of the CD4$^+$ T cells divided in the corresponding control group that did not receive CTL (Fig. 3.4b). This result is similar to that shown in Fig. 3.3. The percentage and number of divided CD4$^+$ T cells were significantly higher in mice that received CTLs and DC+gp33+OVA$_{323-339}$ than those that received CTLs and DC+SIINFEKL+OVA$_{323-339}$ (Fig. 3.4c and 3.4d). Similar results were obtained when comparing the percentage and number of highly divided CD4$^+$ T cells (>4 divisions) between mice that received CTLs and DC+gp33+OVA$_{323-339}$ and mice that received CTLs and DC+SIINFEKL+OVA$_{323-339}$ (data not shown). This shows that CTLs inhibit CD4$^+$ T cell proliferation in an antigen-specific manner.
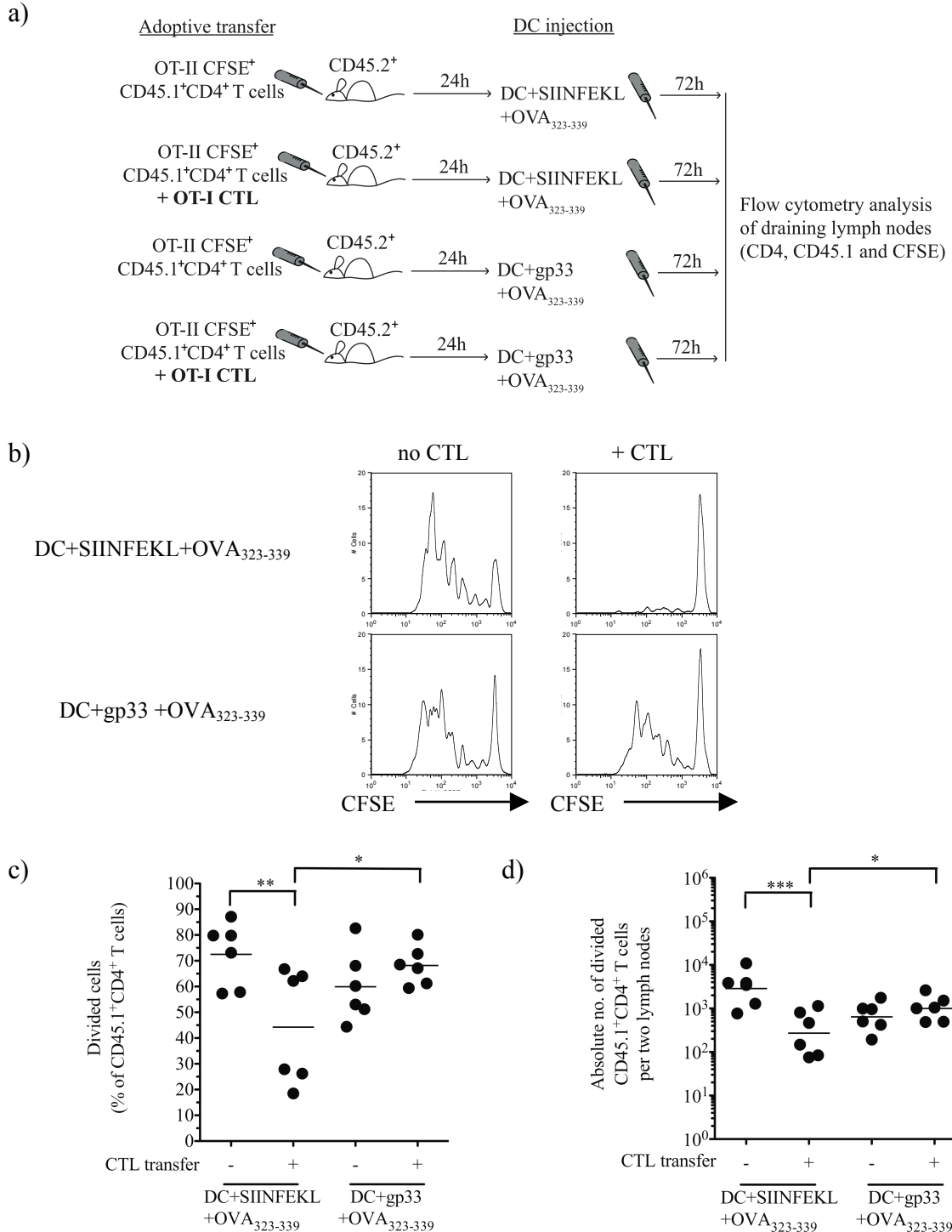
Fig. 3.4. **CTLs inhibit CD4$^+$ T cell proliferation in an antigen-specific manner. (a)** C57BL/6J mice received *in vitro* activated OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. As a control, some mice received CD4$^+$ T cells only. After 24 h, these mice were injected s.c. into their forelimbs with either LPS-activated DCs loaded with SIINFEKL and OVA$_{323-339}$ or DCs loaded with gp33 and OVA$_{323-339}$. 3 days later, CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. **(b)** CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. **(c)** The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. **(d)** Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 6 mice per group. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. * p<0.05, ** p<0.01, *** p<0.001.

3.5. Perforin-mediated elimination of DCs regulates T cell proliferation

3.5.1. PKO CD8^{+} T cells respond to antigens better than wt CD8^{+} T cells

The granules-mediated cytolytic pathway used by CTLs requires perforin (Kagi et al., 1994a; Lowin et al., 1994a). To determine whether knocking out perforin in T cells affects their ability to respond to antigen, total lymphocyte suspensions were prepared from wt and PKO OT-I mice and incubated with DCs loaded with serially diluted SIINFEKL concentrations for 48 h. Thymidine uptake was subsequently examined. PKO OT-I CD8^{+} T cells were capable of responding to SIINFEKL-loaded DC stimulation and did so better than wt T cells (Fig. 3.5.1).
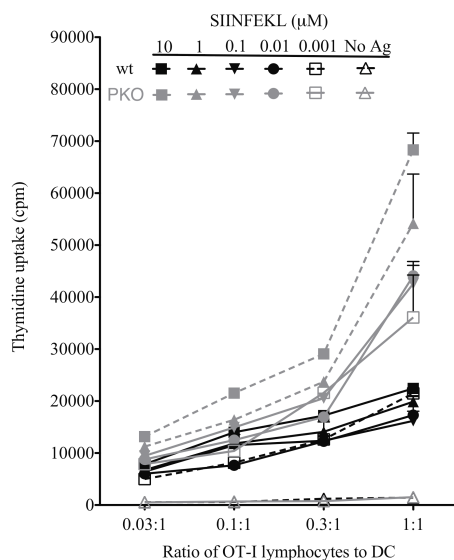
Fig. 3.5.1. **PKO OT-I lymphocytes can respond to SIINFEKL-loaded DC stimulation better than wt OT-I lymphocytes.** Total lymphocyte suspensions from wt and PKO OT-I mice were prepared and serially diluted. The lymphocytes were then incubated with DCs loaded with the indicated serially diluted SIINFEKL concentrations. 48 h later, the cells were incubated with thymidine for 6 h. Thymidine uptake in wt and PKO OT-I lymphocytes was then measured. The experiment was performed once with triplicate wells.

3.5.2. *In vitro* activated wt and PKO CTLs express similar phenotypic markers

To examine if perforin is important in preventing DC entry to the lymph nodes, perforin knockout (PKO) OT-I T cells were used. Wt and PKO OT-I lymphocytes were cultured as previously determined in Fig. 3.1.2. 98.6% of the live PKO CTLs were Vα2[+]Vβ5.1/5.2[+], as were the wt CTLs (Fig. 3.5.2a). In contrast to naïve T cells, greater than 98% of wt and PKO CTLs had low CD62L and high CD44 expression (Fig. 3.5.2b. and 3.5.2c). Thus, *in vitro* activation of PKO OT-I lymphocytes yielded CTLs that were phenotypically similar to wt OT-I CTLs.
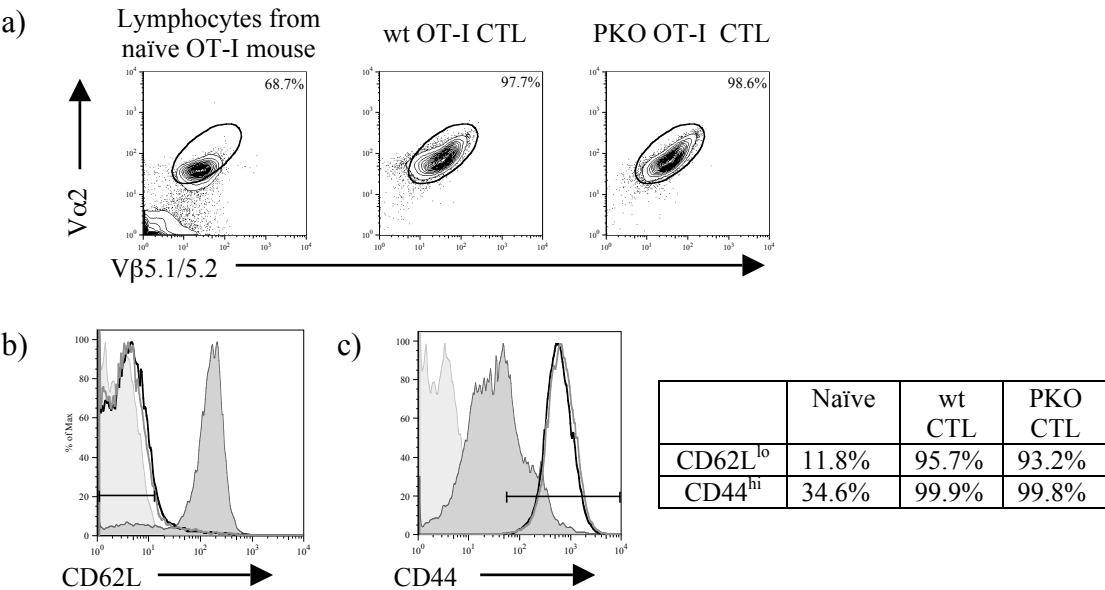
Fig 3.5.2. **The phenotypes of *in vitro* activated wt and PKO OT-I CTLs are similar.** OT-I lymphocytes harvested from wt or perforin knockout (PKO) OT-I mice were activated as described in Chapter 2. *In vitro* activated CTLs were examined for the expression of Vα2 and Vβ5.1/5.2 by flow cytometry. Lymphocytes from naïve OT-I mouse were used as a control. (**a**) Vα2$^+$Vβ5.1/5.2$^+$ cells are shown as a percentage of live cells. Expressions of (**b**) CD62L and (**c**) CD44 were examined to determine their effector phenotypes. Black and dark grey solid lines depict wt and PKO CTLs respectively. Naïve OT-I lymphocytes are shown as dark grey filled areas. Light grey filled areas represent the unstained control. The percentages of CD62L^{lo}Vα2$^+$Vβ5.1/5.2$^+$ cells and CD44^{hi}Vα2$^+$Vβ5.1/5.2$^+$ cells are shown. One representative experiment of five is shown.

3.5.3. Perforin is essential to CTL-mediated DC elimination

Next, I asked whether perforin in CTLs was necessary in preventing DC accumulation in the draining lymph nodes. Groups of mice received either *in vitro* activated wt or PKO OT-I CTLs. As a control, some mice did not receive CTLs. 24 h later, three different DC mixtures were then prepared. The first DC mixture contained equal numbers of CFSE-labelled DCs loaded with 100×10^{-9} µM SIINFEKL and CMTMR-labelled DCs not loaded with SIINFEKL (DC only). The second DC mixture contained equal numbers of CFSE-labelled DCs loaded with 10µM SIINFEKL and CMTMR-labelled DCs only. The last DC mixture contained equal numbers of CFSE-labelled DCs not loaded with SIINFEKL and CMTMR-labelled DCs not loaded with SIINFEKL. Recipient mice were injected s.c. into their forelimbs with either one of the three aforementioned DC mixtures. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ cells in the draining lymph nodes were monitored by flow cytometry.

In mice that did not receive CTLs, similar numbers of CFSE$^+$ and CMTMR$^+$ cells were collected from the draining lymph nodes (Fig. 3.5.3a). Wt CTLs did not affect CFSE$^+$ or CMTMR$^+$ DCs when both populations were not loaded with SIINFEKL (Fig. 3.5.3b). The number of CFSE$^+$ DCs loaded with 10 μM SIINFEKL was significantly lower in mice that received wt compared to mice that did not receive CTLs (Fig. 3.5.3c). When perforin was knocked out in CTLs, more CFSE$^+$ DCs loaded with 10 μM SIINFEKL were recovered compared to mice that received wt CTLs. When DCs were loaded with 100×10^{-9} μM SIINFEKL, DC killing was very low as similar numbers of CFSE$^+$ and CMTMR$^+$ cells were recovered in mice that received wt or PKO CTLs (Fig. 3.5.3b).

A separate experiment was carried out to determine if increasing the number of PKO CTLs would prevent the accumulation of DCs in the draining lymph nodes. Mice received different numbers of wt or PKO CTLs. As before, control mice did not receive CTLs. These mice were then given a mixture of CFSE$^+$ DC loaded with SIINFEKL and CMTMR$^+$ DC only. After 48 h, CFSE$^+$ and CMTMR$^+$ cells in the draining lymph nodes were monitored by flow cytometry.

In both groups of mice that received wt or PKO CTLs, the ratio of DC+SIINFEKL to DC only showed a decreasing trend when the number of CTLs was increasing (Fig. 3.5.3d). Increasing the number of PKO CTLs did not eliminate DC+SIINFEKL totally as the ratio of DC+SIINFEKL to DC only in mice that received 10×10^6 PKO CTLs was 0.2. In contrast, nearly all the DC+SIINFEKL were absent when 5×10^6 and 10×10^6 wt CTLs were present.

Taken together, the results show that in order to eliminate antigen-loaded DCs and prevent their entry into the draining lymph nodes, CTLs require sufficient antigen presented on the target DCs. The results also show that increasing the ratio of CTLs to antigen-bearing DCs also increases DC killing. While CTLs eliminate DCs through perforin, other killing pathway(s) are involved since knocking out perforin in CTLs does not totally abolish DC elimination.

a)

no CTL   wt CTL   PKO CTL

No Ag

Concentration of SIINFEKL on DCs (µM) 100×10^{-9}

10

Not performed

CFSE

CMTMR

DC only

b)

Normalised ratio of DC±SIINFEKL to DC only

CTL transfer   -   wt   -   wt   PKO   -   wt   PKO
DC+SIINFEKL (µM)   No Ag 100×10^{-9} 10

c)

■ CFSE⁺ DC+SIINFEKL
□ CMTMR⁺ DC only

Absolute no. of DCs per two lymph nodes

CTL transfer   -   wt   -   wt   PKO
DC+SIINFEKL (µM)   No Ag   10

d)
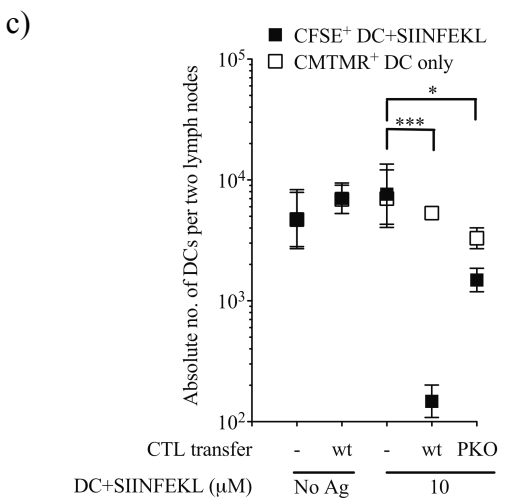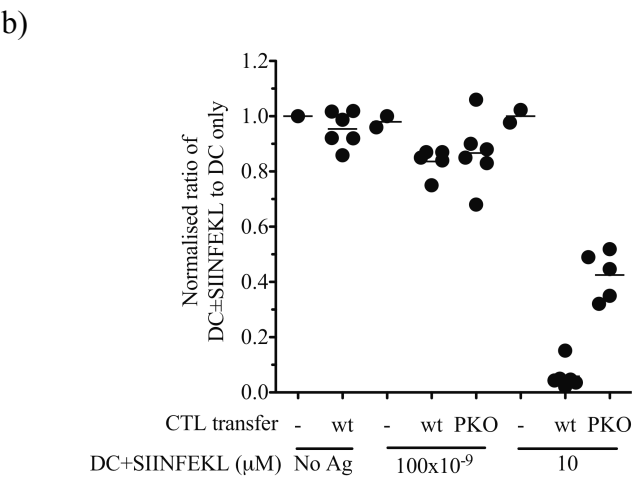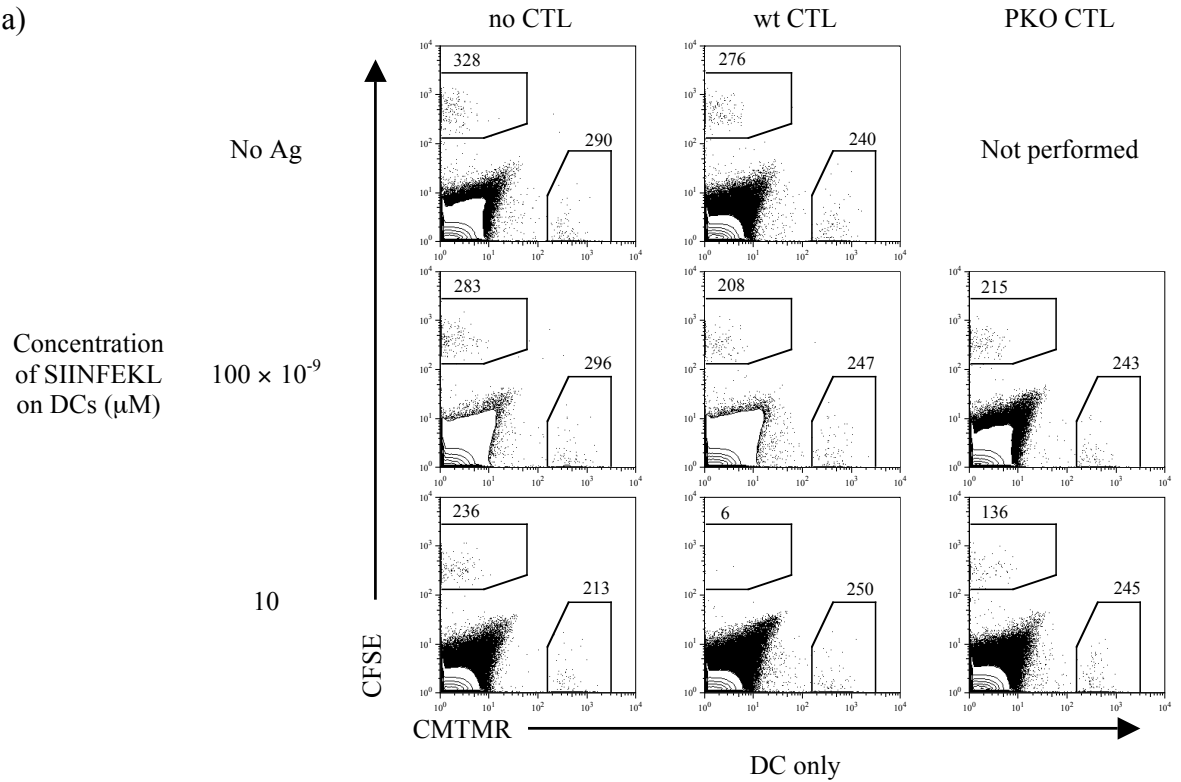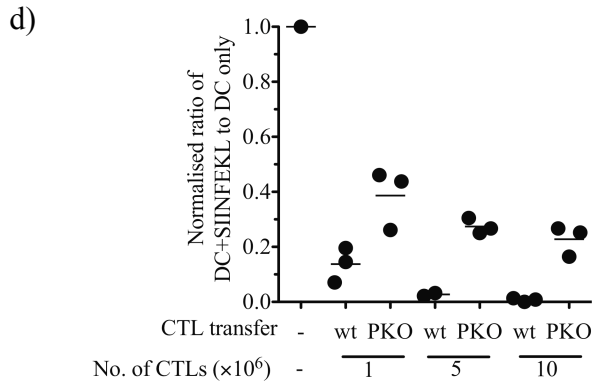
Fig 3.5.3. **CTL elimination of antigen-loaded DCs *in vivo* is perforin- and antigen concentration-dependent.** Groups of B6.SJ *ptprca* mice received *in vitro* activated wt or PKO OT-I CTLs. As a control, some mice did not receive CTLs. 24 h later, some recipient mice were injected s.c. with a DC mixture containing equal numbers of $CFSE^+$ DCs loaded with 100×10^{-9} μM SIINFEKL (DC+SIINFEKL) and $CMTMR^+$ DCs not loaded with antigen (DC only). Some recipient mice were injected s.c. with a different DC mixture containing equal numbers of $CFSE^+$ DCs loaded with 10 μM SIINFEKL and $CMTMR^+$ DCs only. Other recipient mice were injected s.c. with a DC mixture containing equal numbers of $CFSE^+$ DCs not loaded with SIINFEKL and $CMTMR^+$ DCs only. 48 h after DC injection, $CFSE^+$ and $CMTMR^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**a**) $CFSE^+$ and $CMTMR^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. (**b**) The ratios of $CFSE^+$ DC±SIINFEKL to $CMTMR^+$ DC only were normalised to the ratios derived from untreated mice (-) that received the same DC mixture. Results are from one experiment. (**c**) Absolute numbers of $CFSE^+$ and $CMTMR^+$ DCs in the draining lymph nodes are shown. One representative experiment of two with $1 - 3$ mice per group is shown in 3.5.3a and b. These two separate experiments are pooled together and shown in 3.5.3c. $CFSE^+$ DCs among various groups were analysed for statistical significance using the one-way ANOVA with Bonferroni's method. * p<0.05, *** p<0.001. (**d**) Groups of C57BL/6J received 1, 5, 10×10^6 wt or PKO OT-I CTLs or no CTLs. These mice were injected s.c. with the mixture of $CFSE^+$ DC+SIINFEKL and $CMTMR^+$ DC only as in 3.5.3a, except that DCs were pulsed with 0.1 μM SIINFEKL before labelling with CFSE. The ratios of $CFSE^+$ DC+SIINFEKL to $CMTMR^+$ DC only were normalised to the ratios derived from untreated mice. The experiment was performed once with $1 - 3$ mice per group.

3.5.4. CTLs inhibit $CD4^+$ T cell proliferation through perforin-mediated DC elimination

Having identified perforin as an important mediator of DC elimination (Fig. 3.5.3), I asked if CTLs inhibited $CD4^+$ T cell proliferation through perforin-mediated DC killing. The experimental design is shown in Fig. 3.5.4a. Mice received either CFSE-labelled OT-II $CD4^+$ T cells and wt OT-I CTLs, $CD4^+$ T cells and PKO OT-I CTLs, or $CD4^+$ T cells without CTLs. 24 h later, DCs loaded with SIINFEKL and $OVA_{323-339}$ were injected s.c. to these mice. Some mice were injected with DC loaded with $OVA_{323-339}$ only. 3 days later, $CD4^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

Wt DCs loaded with SIINFEKL and $OVA_{323-339}$ induced strong $CD4^+$ T cell division in mice that did not receive CTLs, whereas $CD4^+$ T cell division was significantly reduced in mice that received wt CTLs (Fig. 3.5.4b). This result is similar to Fig. 3.3. When perforin

was knocked out in CTLs, $CD4^+$ T cell division was restored (Fig. 3.5.4c and 3.5.4d). CTLs did not reduce $CD4^+$ T cell division when DCs were not loaded with SIINFEKL, indicating that CTLs did not affect $CD4^+$ T cell proliferation through non-antigen-specific mechanisms (Fig. 3.4). This shows that perforin is important in the CTL-mediated inhibition of $CD4^+$ T cell proliferation.

a)

b)

c)

d)
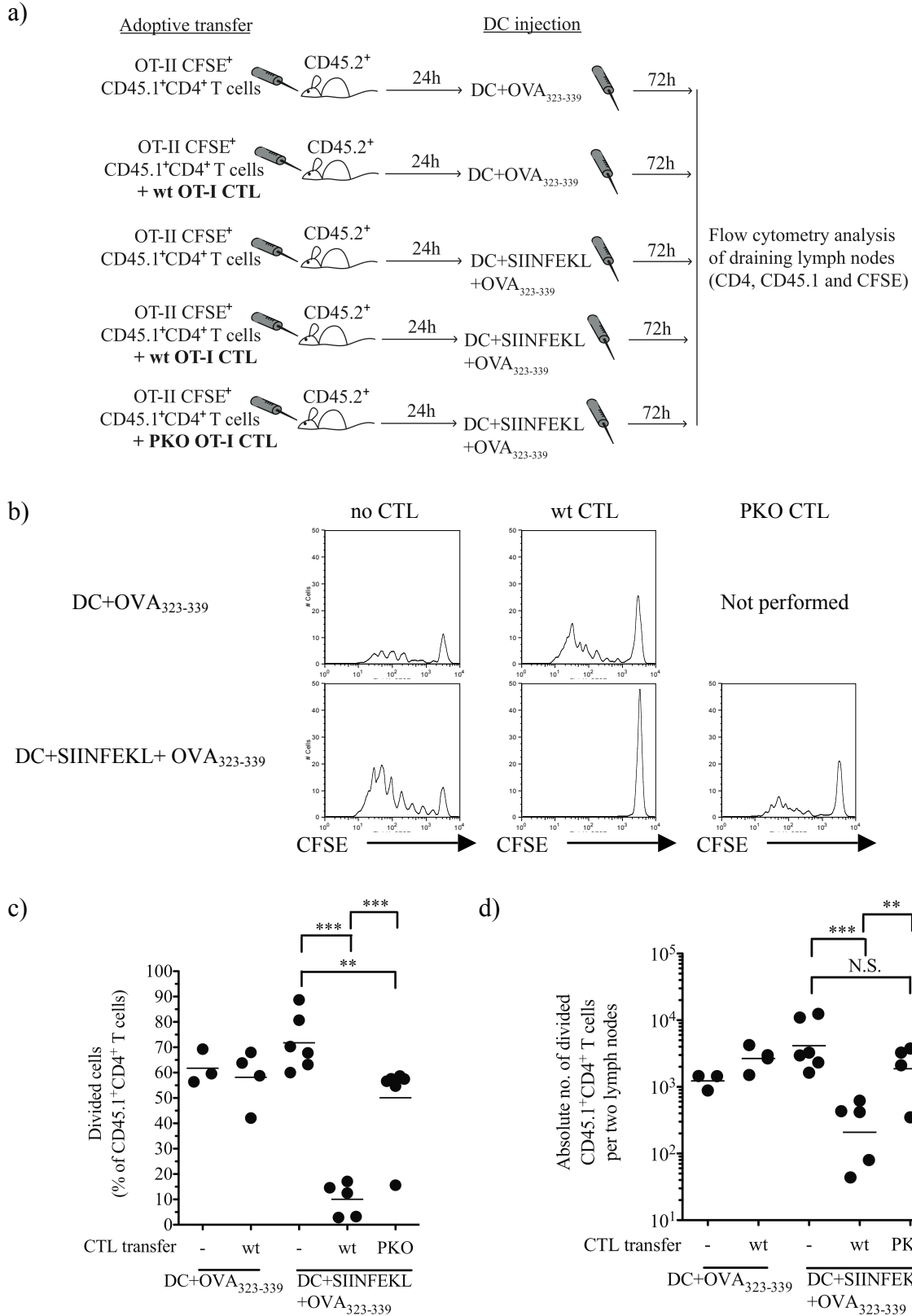
Fig 3.5.4. **CTLs inhibit CD4$^+$ T cell proliferation through perforin-mediated DC killing. (a)** C57BL/6J mice received *in vitro* activated wt or PKO OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. As a control, some mice received CD4$^+$ T cells only. After 24 h, some recipient mice were injected s.c. into their forelimbs with LPS-activated DCs loaded with SIINFEKL and

$OVA_{323-339}$. Other recipient mice received DCs loaded with $OVA_{323-339}$ instead. 3 days later, $CD45.1^+CD4^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in $CD45.1^+CD4^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of $CD45.1^+CD4^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided $CD45.1^+CD4^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with $3 - 6$ mice per group. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. ** $p<0.01$, *** $p<0.001$, N.S.= $p>0.05$.

3.5.5. CTLs inhibit $CD8^+$ T cell proliferation through perforin-mediated DC elimination

A similar experiment was carried out to address if CTLs inhibited $CD8^+$ T cell proliferation through perforin-mediated DC elimination. The experimental setup is shown in Fig. 3.5.5a. Mice were given CFSE-labelled $CD8^+$ T cells from naïve wt OT-I mice and wt OT-I CTLs, or $CD8^+$ T cells and PKO CTLs, or $CD8^+$ T cells only. 24 h later, DCs loaded with SIINFEKL were injected s.c. into these mice. 3 days later, $CD8^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

In mice that received wt CTLs, $CD8^+$ T cell division was significantly reduced compared to the control group (Fig. 3.5.5b). The reduction in $CD8^+$ T cell division was significantly alleviated when perforin was knocked out (Fig. 3.5.5c and 3.5.5d). Similar results were obtained when the percentage and number of highly divided $CD8^+$ T cells (>4 divisions) were compared among the three groups (data not shown).

Taken together, CTLs inhibit the induction of $CD8^+$ and $CD4^+$ T cell proliferation through perforin-mediated DC killing. However, it should be noted that $CD8^+$ T cells divided even though antigen-loaded DCs were killed. This proliferation could be induced by the few surviving antigen-loaded DCs, or by host APCs that had taken up antigens from the injected DCs. This will be examined in chapter 5.

a)

b)

c)   d)
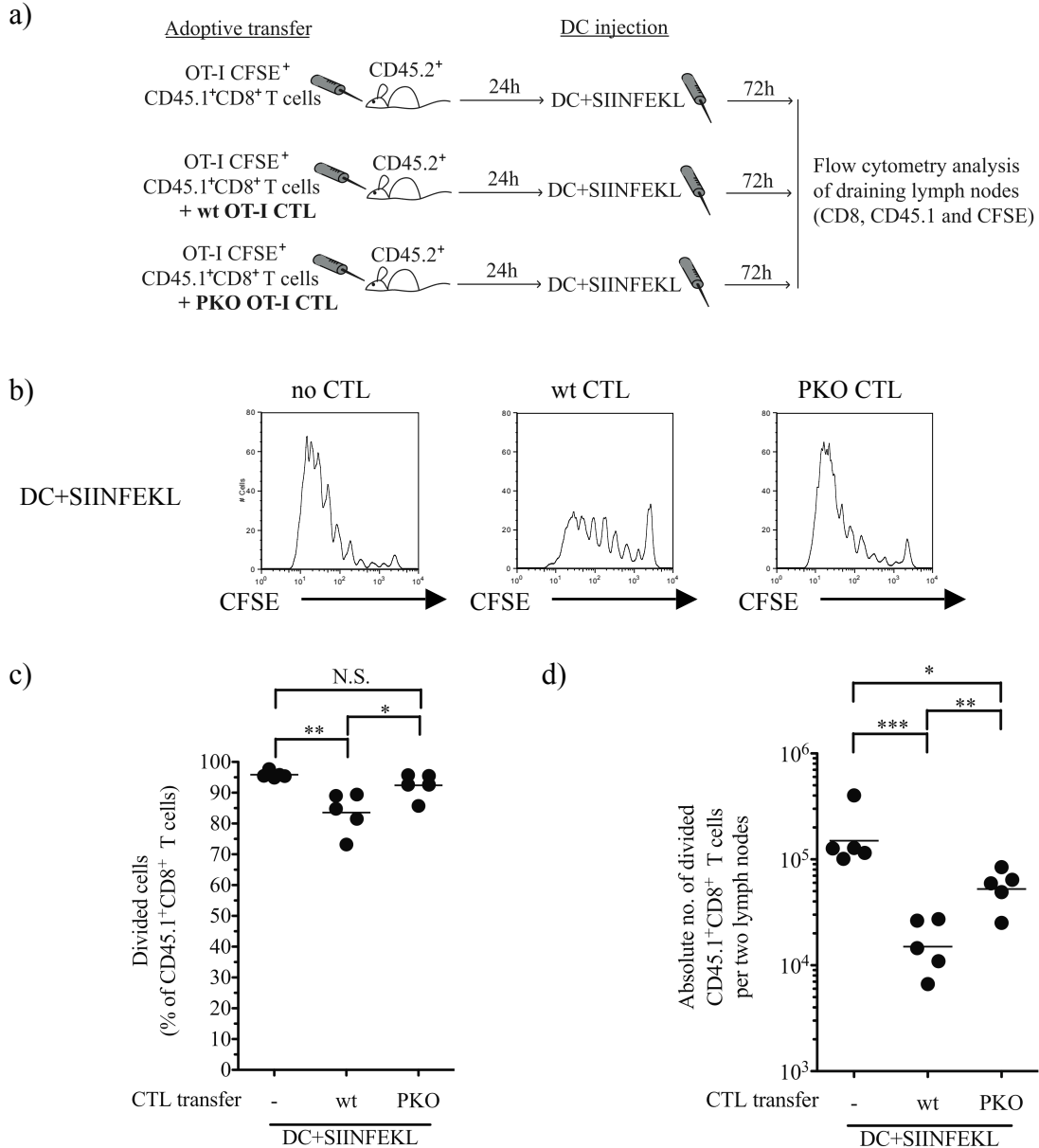
Fig. 3.5.5. **CTLs inhibit CD8$^+$ T cell proliferation in a perforin-dependent manner.** (**a**) C57BL/6J mice received *in vitro* activated wt or PKO OT-I CTLs and CFSE-labelled OT-I CD45.1$^+$CD8$^+$ T cells. As a control, some mice received only CD8$^+$ T cells. After 24 h, these mice were injected s.c. into their forelimbs with LPS-activated DCs loaded with SIINFEKL. 3 days later, CD45.1$^+$CD8$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD8$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD8$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD8$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 5 mice per group. Statistical significance was determined using the one-way ANOVA with Bonferroni's method. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, N.S.= $p>0.05$.

3.6. FasL-mediated elimination of DCs plays a minor role in regulating T cell proliferation

3.6.1. *In vitro* activated OT-I CTLs express FasL upon antigen stimulation

Previous observations in Fig. 3.5.3 showed that the inactivation of perforin pathway was not sufficient to abolish DC elimination. This suggests that other cytolytic killing mechanisms are involved in CTL-mediated DC elimination. Besides perforin, another cytolytic pathway is mediated by the interaction of FasL on the CTLs with Fas on the target cell (Lowin et al., 1994b). I asked if *in vitro* activated OT-I CTLs expressed FasL. OT-I lymphocytes were activated and rested as described in Chapter 2. OT-I CTLs were then incubated with SIINFEKL for 30 min or 2 h. FasL expression on OT-I CTLs was monitored by flow cytometry.

As previously shown in Fig. 3.1.1, Fig. 3.1.2 and 3.5.2, greater than 95% of the *in vitro* activated OT-I CTLs were CD44^{hi}Vα2$^+$ (Fig 3.7.1a). FasL expression in CD44^{hi}Vα2$^+$ CTLs was only detected when CTLs were stimulated with SIINFEKL. It has been reported that CTLs store some FasL in granules and express pre-stored FasL during the early stages of CTL-target cell interaction, whereas most of the FasL are synthesized *de novo* and expressed later than pre-stored FasL (He and Ostergaard, 2007). In line with this, a small percentage of CTLs expressed low levels of FasL after 30 min stimulation with SIINFEKL, whereas the expression of FasL was detected on the majority of the CTLs after 2 h stimulation with SIINFEKL (Fig. 3.6.1b and c). This result shows that the OT-I CTLs used in this study express FasL on their cell surface upon antigenic stimulation.

a)
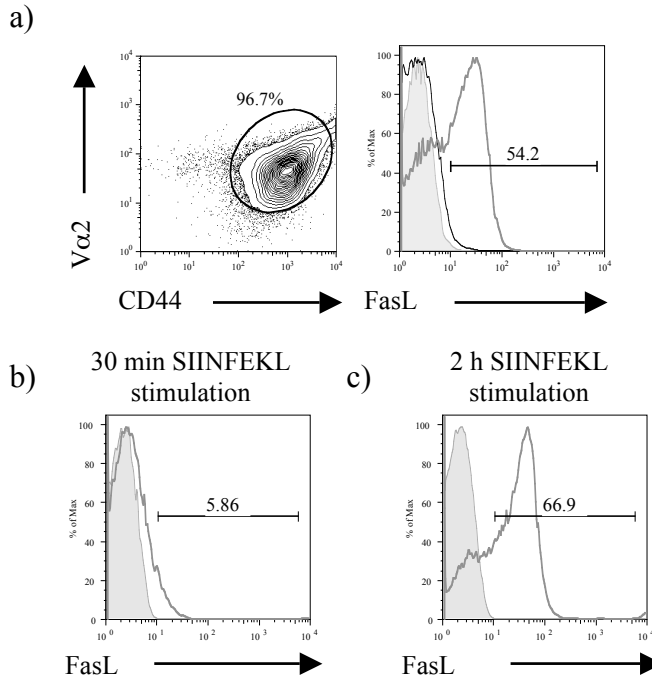
Fig. 3.6.1. **In vitro activated OT-I CTLs express FasL after in vitro antigen stimulation.** OT-I CTLs were generated as described in Chapter 2. After culture, OT-I CTLs were incubated with anti-FasL antibody and SIINFEKL. In some cultures, only anti-FasL antibody was added. After 30 min or 2 h incubation, the expression of FasL in OT-I CTLs was monitored by flow cytometry. (**a**) $V\alpha2^+CD44^+$ OT-I CTLs are shown in the contour plot. The percentage of $V\alpha2^+CD44^+$ OT-I CTLs expressing FasL is shown in the histogram. Dark grey lines represent the expression of FasL in OT-I CTLs with the addition of SIINFEKL. Black lines represent no SIINFEKL added. Filled light grey areas represent the unstained control. The percentages of OT-I CTLs expressing FasL after (**b**) 30 min, and (**c**) 2 h SIINFEKL incubations are shown in the histograms. The experiment was performed once.

3.6.2. *lpr* DCs do not express Fas but are phenotypically similar to wt DCs

To investigate if CTLs used FasL to eliminate DCs, DCs were cultured from B6.MRL-FAS^{lpr} (*lpr*) mice. These mice carry a Fas receptor mutation, hence the CTLs cannot induce apoptosis of target cells through FasL-Fas pathway (Watanabe-Fukunaga et al., 1992).

I started off by characterizing bone marrow (BM)-derived DCs from these *lpr* mice. Bone marrow cells were prepared from wt and *lpr* mice and cultured as described in Chapter 2. Some DCs were treated with LPS, while some were left untreated. After treatment, all DCs were incubated with a panel of antibodies to characterise the phenotype and activation status.

Culturing *lpr* bone marrow cells *in vitro* yielded similar percentages of CD11b$^+$CD11c$^+$ live cells as wt cultures (Fig. 3.6.2a). The percentages of CD11c$^+$ cells expressing high MHC class II, CD40, CD86 and CD80 before and after LPS treatment were also similar in wt and *lpr* cultures (Fig. 3.6.2b and Table 3.6.1). Unlike wt DCs, DCs cultured from *lpr* mice did not express Fas (Fig. 3.6.2c). Fas expression on wt DCs was also upregulated after LPS treatment.

a)

b)
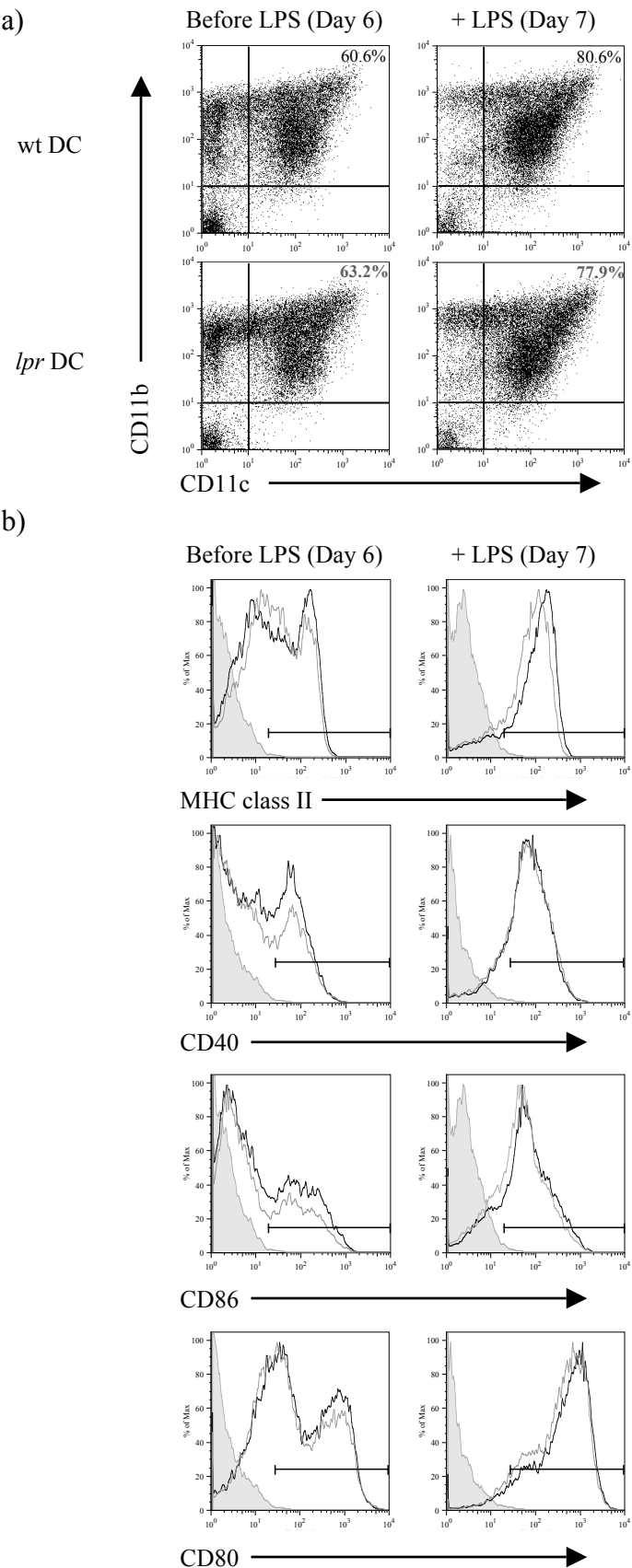
Fig. 3.6.2. *In vitro* **cultures of wt and *lpr* BM cells yield similar percentages of CD11c⁺CD11b⁺ cells.** Bone marrow cells were prepared from C57BL/6J (wt) and *lpr* mice and cultured with GM-CSF and IL-4 for 6 days *in vitro*. On day 6, GM-CSF/IL-4 cell cultures were treated with LPS for 24 h. Cells were harvested on day 6 (before LPS) and day 7 (24 h after LPS) and analysed for expressions of CD11c, CD11b, MHC class II, CD40, CD86 and CD80. (**a**) The expressions of CD11b and CD11c in cultured cells are shown in dot plots. The percentages of CD11c⁺CD11b⁺ cells are shown. (**b**) The expressions of activation markers in CD11c⁺ cells are shown in histograms. Wt DCs are depicted in black while *lpr* DCs are depicted in dark grey. The unstained controls are shown as filled light grey areas. The percentages of live CD11c⁺ cells expressing the activation markers are shown in Table 5. The experiment was performed once. (**c**) The expressions of Fas and CD11c in cultured cells treated with or without LPS are shown as percentages in dot plots. The experiment was performed once.

c)
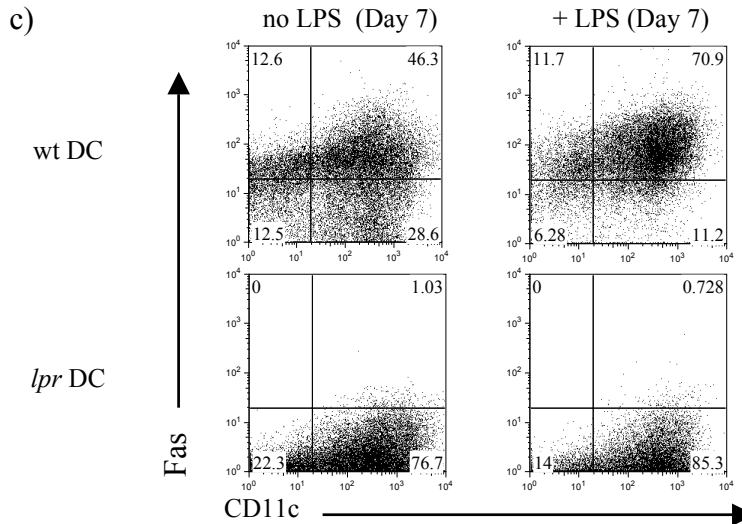
Table 3.6.1. Percentage of wt and *lpr* CD11c$^+$ cells expressing activation markers*

Markers	Before LPS (Day 6)		+ LPS (Day 7)	
	wt DC	*lpr* DC	wt DC	*lpr* DC
MHC class II	54.5%	56.2%	84.7%	81.7%
CD40	32.5%	28.3%	80.1%	78.7%
CD86	37.9%	32.3%	78.5%	75.0%
CD80	63.9%	67.3%	93.1%	92.6%

* Percentages of CD11c$^+$ cells expressing high levels of activation markers as gated in Fig. 3.6.2b.

3.6.3. *lpr* DCs are similar to wt DCs in terms of migratory capacity

Having shown that *lpr* DCs were phenotypically similar to wt DCs (Fig. 3.6.2), I asked if defective Fas affected the ability of DCs to migrate to draining lymph nodes. To address this, wt and *lpr* DCs were labelled with CMTMR and CFSE respectively. Equal numbers of CFSE$^+$ *lpr* DCs and CMTMR$^+$ wt DCs were mixed and injected s.c. into the forelimbs of mice. 24 h and 48 h later, CFSE$^+$ and CMTMR$^+$ cells in the draining lymph nodes were monitored by flow cytometry. The number of *lpr* DCs that accumulated in the draining lymph nodes 24 h and 48 h after DC injection was similar to that of wt DCs (Fig. 3.6.3a).

To determine if defective Fas affected the survival of DCs *in vivo*, I examined the numbers of wt and *lpr* DCs *in vivo* over a two-week period. Because host cells might take up the fluorescent dyes over time, CD45.2$^+$ DCs were injected into congenic CD45.1$^+$ hosts in this experiment. As before, CMTMR$^+$ wt and CFSE$^+$ *lpr* DCs were mixed in equal numbers

before injecting into mice. CFSE$^+$ and CMTMR$^+$ cells in draining lymph nodes were monitored by flow cytometry at the indicated time points.

The number of wt and *lpr* DCs accumulating in the draining lymph nodes increased between 24 to 48 h after DC injection (Fig. 3.6.3b and c). Thereafter, the number of wt and *lpr* DCs in the draining lymph nodes decreased sharply after four days. Although more *lpr* than wt DCs were observed seven days after DC injection, both DC populations dropped to similar numbers 10 days after DC injection. The numbers of wt and *lpr* DCs observed two weeks after DC injection were similar to those of wt and *lpr* DCs observed at 24 h after DC injection. Therefore, both wt and *lpr* DCs migrate and accumulate in the draining lymph nodes similarly.

a)

Type of DCs   wt   *lpr*   wt   *lpr*

Time after DC injection (h)   24   48

b)

Time after DC injection (Days)

lpr DC

CFSE

CMTMR

wt DC

c)

Time after DC challenge (Days)   1   2   4   7   10   14
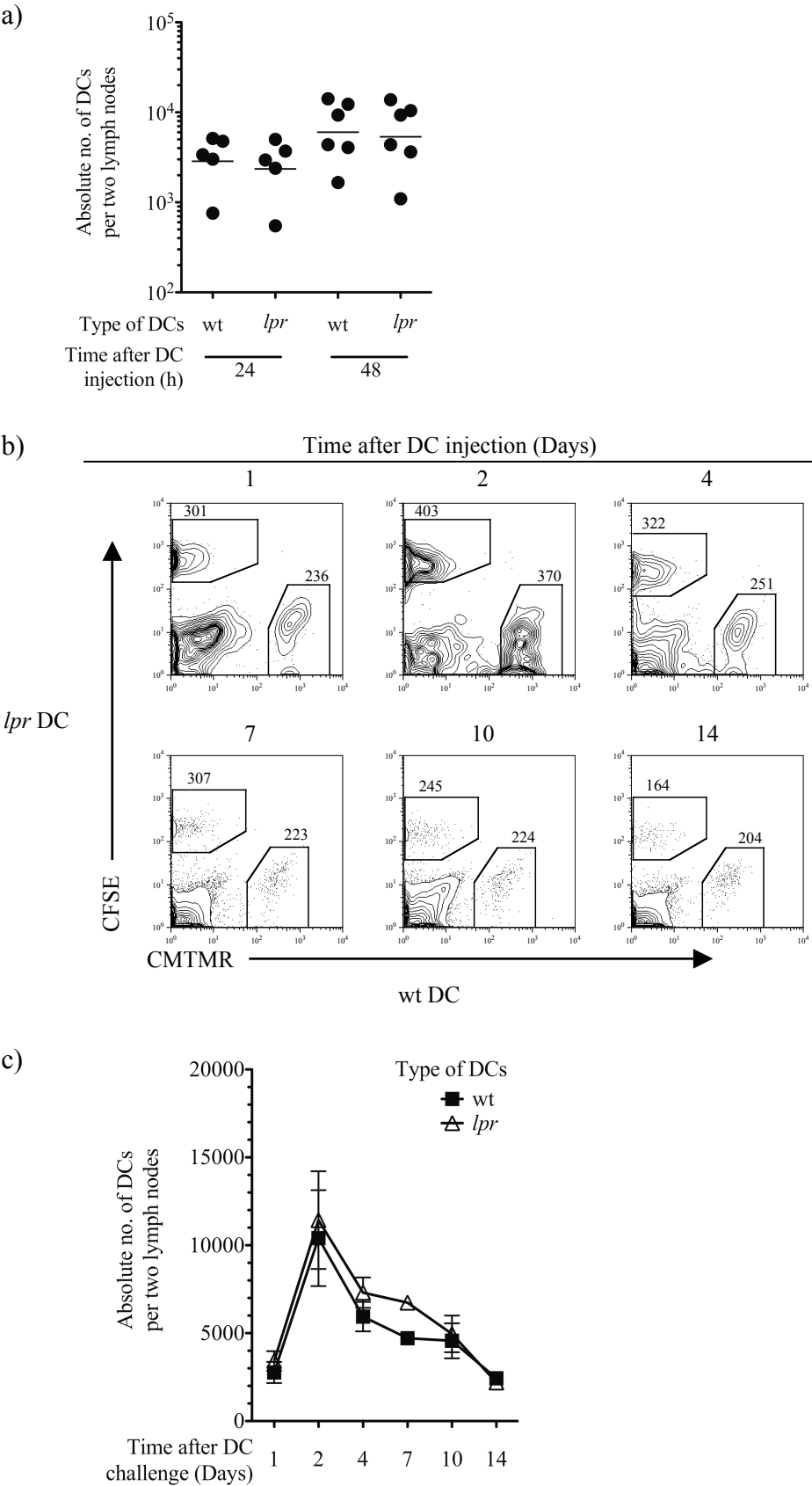
Fig. 3.6.3. **The numbers of wt and *lpr* DCs in the draining lymph nodes increase and then decline at the same rate.** (**a**) LPS-activated wt and *lpr* DCs were labelled with CMTMR and CFSE respectively. CFSE-labelled *lpr* DCs were mixed in equal numbers with CMTMR-labelled wt DCs injected s.c. into the forelimbs of recipient mice. 24 h and 48 h later, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**a**) Absolute numbers of CFSE$^+$ or CMTMR$^+$ DCs in the draining lymph nodes are shown. The experiment was performed once with 3 mice per group. (**b**) The experiment was carried out as in 3.6.3a, except that CMTMR$^+$CD45.2$^+$ wt and CFSE$^+$CD45.2$^+$ *lpr* DCs into the forelimbs of congenic B6.SJ *ptprca* mice. CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry at the indicated time points over 2 weeks. CD45.2$^+$CFSE$^+$ and CD45.2$^+$CMTMR$^+$ DCs were gated on live cells and shown in representative dot plots from individual mice. The number of events in each gate is shown. (**c**) Absolute numbers of CD45.2$^+$CFSE$^+$ CD45.2$^+$CMTMR$^+$ DCs in the draining lymph nodes from 3.6.3b are shown. The experiment was performed once with 2 – 3 per group.

3.6.4. *lpr* DCs induce *in vitro* T cell proliferation as effectively as wt DCs

I went on to ask if defective Fas expression affected the ability of DCs to present antigens to T cells. To investigate this, wt and *lpr* DCs were loaded with either SIINFEKL or OVA$_{323-339}$ at the indicated concentrations for 4 h. SIINFEKL-loaded or OVA$_{323-339}$-loaded DCs were then serially diluted and incubated with OT-I or OT-II lymphocytes respectively. 48 h later, the cells were incubated with thymidine for 6 h before thymidine uptake was measured.

Both wt and *lpr* DCs stimulated comparable levels of thymidine uptake by OT-I and OT-II lymphocytes (Fig. 3.6.4a and 3.6.4b). This shows that *lpr* DCs are able to present both MHC class I and II peptides to CD8$^+$ and CD4$^+$ T cells, respectively, and stimulate T cell division.
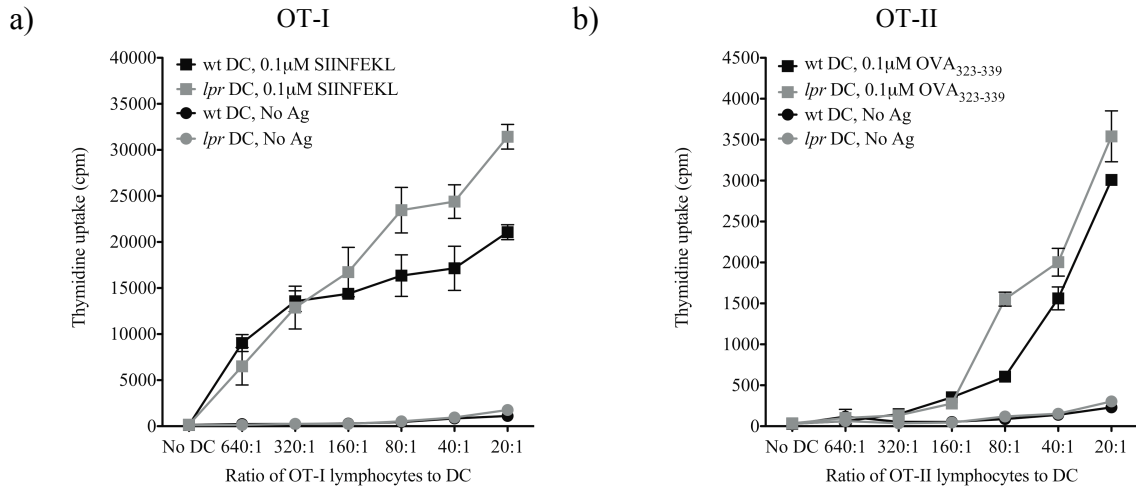
Fig. 3.6.4. *lpr* **DCs present antigens and stimulate CD8$^+$ and CD4$^+$ T cell proliferation as efficiently as wt DCs.** Wt and *lpr* DCs were loaded with either SIINFEKL or OVA$_{323\text{-}339}$ at the indicated concentrations for 4 h. SIINFEKL-loaded and OVA$_{323\text{-}339}$-loaded DCs were serially diluted and incubated with 2 × 10^6 lymphocytes/ml prepared from either OT-I or OT-II total lymphocyte suspensions. 48 h later, the cultures were incubated with thymidine for 6 h before thymidine uptake was measured. Thymidine uptake in (**a**) OT-I and (**b**) OT-II lymphocytes was then measured. The experiment was performed once with triplicate wells.

3.6.5. Perforin contributes more significantly to DC killing than FasL-mediated mechanisms

I sought to dissect the relative contributions of perforin and FasL in DC elimination. The experimental setup is shown in Fig. 3.6.5a. Mice received *in vitro* activated wt OT-I CTLs, PKO OT-I CTLs, or no CTLs. 24 h later, two different DC mixtures were prepared. The first DC mixture contained equal numbers of CFSE-labelled wt DCs loaded with SIINFEKL and CMTMR-labelled *lpr* DCs not loaded with SIINFEKL (DC only). The second DC mixture contained equal numbers of CFSE-labelled *lpr* DCs loaded with SIINFEKL and CMTMR-labelled *lpr* DCs only. Some recipient mice were injected with the first DC mixture, while others were injected with the second DC mixture. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ cells in the draining lymph nodes were monitored by flow cytometry.

In mice that did not receive CTLs, similar numbers of SIINFEKL-loaded DCs and DCs only were recovered (Fig. 3.6.5b). DC killing was evident in mice that received wt CTLs because most SIINFEKL-loaded wt DCs could not be recovered (Fig. 3.6.5c and d). Knocking out perforin in CTLs significantly lowered DC killing more than knocking out

Fas in DCs. When both perforin and Fas were knocked out, DC killing was very low but was not totally abolished. This indicates that perforin and FasL pathways mediate most of DC killing although some other cytolytic molecule(s) may play a very minor role.
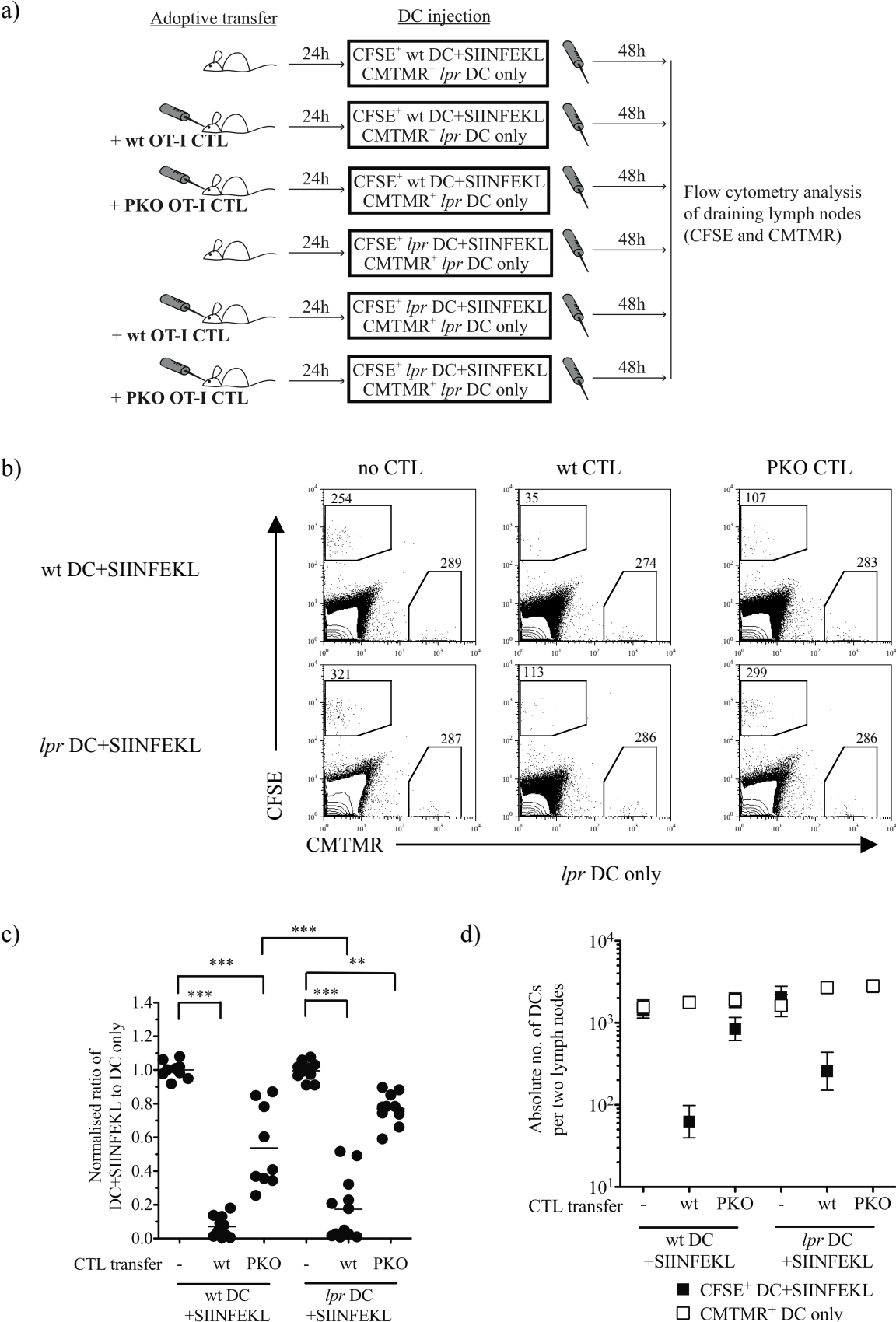
Fig. 3.6.5. **Perforin contributes more significantly to DC killing than FasL-mediated mechanisms**

in vivo. (**a**) Groups of B6.SJ *ptprca* mice received *in vitro* activated wt or PKO OT-I CTLs, or no CTLs. 24 h later, some recipient mice were injected s.c. into their forelimbs with a DC mixture containing equal numbers of CFSE-labelled wt DCs loaded with SIINFEKL (DC+SIINFEKL) and CMTMR-labelled *lpr* DCs not loaded with SIINFEKL (DC only). Other recipient mice were injected with another DC mixture containing equal numbers of CFSE-labelled *lpr* DCs loaded with SIINFEKL (DC+SIINFEKL) and CMTMR-labelled *lpr* DCs not loaded with SIINFEKL (DC only). 48 h after DC injection, $CFSE^+$ and $CMTMR^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**b**) $CFSE^+$ and $CMTMR^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. (**c**) The ratio of $CFSE^+$ DC+SIINFEKL to $CMTMR^+$ DC only was normalised to the ratio derived from untreated mice that received the same DC mixture. (**d**) Absolute numbers of $CFSE^+$ and $CMTMR^+$ DCs in the draining lymph nodes are shown. Two separate experiments with 3 mice per group are pooled together and shown. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. ** $p<0.01$, *** $p<0.001$.

3.6.6. CTLs regulate $CD4^+$ T cell proliferation through perforin- but not FasL-mediated DC killing

To investigate the effects of perforin- and FasL-mediated DC elimination on $CD4^+$ T cell proliferation, mice received *in vitro* activated wt or PKO OT-I CTLs, or no CTLs (Fig. 3.6.6a). 24 h later, some recipient mice were injected s.c. with wt DCs loaded with SIINFEKL and $OVA_{323-339}$, while others were injected s.c. with *lpr* DCs loaded with SIINFEKL and $OVA_{323-339}$. 3 days after DC injection, $CD4^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

In mice that did not receive CTLs, both wt and *lpr* DCs elicited strong $CD4^+$ T cell proliferation (Fig. 3.6.6b). $CD4^+$ T cell division was strongly inhibited by wt CTLs when mice received wt DCs. This result is similar to Fig. 3.3. When Fas was knocked out in DCs, a significant reduction in $CD4^+$ proliferation was observed in the percentage, but not in the absolute number. This suggested that FasL-mediated DC killing did not play a significant role in inhibiting $CD4^+$ T cell proliferation (Fig. 3.6.6c and 3.6.6d). When perforin was knocked out in CTLs, $CD4^+$ T cell division was restored to comparable levels to the control group that did not receive CTLs. This result is similar to Fig. 3.5.4. When both perforin and Fas were knocked out, $CD4^+$ T cell division was also restored to similar levels as the control group that did not receive CTLs. This result shows that $CD4^+$ T cell proliferation is regulated by CTLs through perforin-mediated, but not FasL-mediated, killing of antigen-bearing DCs.

a)

Adoptive transfer   DC injection

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells   CD45.2$^+$ 24h   wt DC+SIINFEKL
+OVA$_{323-339}$ 72h

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells
+ wt OT-I CTL   CD45.2$^+$ 24h   wt DC+SIINFEKL
+OVA$_{323-339}$ 72h

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells
+ PKO OT-I CTL   CD45.2$^+$ 24h   wt DC+SIINFEKL
+OVA$_{323-339}$ 72h

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells   CD45.2$^+$ 24h   *lpr* DC+SIINFEKL
+OVA$_{323-339}$ 72h

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells
+ wt OT-I CTL   CD45.2$^+$ 24h   *lpr* DC+SIINFEKL
+OVA$_{323-339}$ 72h

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells
+ PKO OT-I CTL   CD45.2$^+$ 24h   *lpr* DC+SIINFEKL
+OVA$_{323-339}$ 72h

Flow cytometry analysis
of draining lymph nodes
(CD4, CD45.1 and CFSE)

b)

Fig. 3.6.6. **CTLs regulate CD4$^+$ T cell proliferation through perforin-mediated, but not FasL-mediated, DC killing.** (**a**) C57BL/6J mice received *in vitro* activated wt or PKO OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. As a control, some mice received CD4$^+$ T cells only. After 24 h, these mice were injected s.c. into their forelimbs with LPS-activated wt or *lpr* DCs loaded with SIINFEKL and OVA$_{323-339}$. 3 days later, CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 4 – 5 mice per group is shown. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. *p<0.05, ** p<0.01, N.S.= p>0.05.

3.6.7. CTLs regulate CD8$^+$ T cell proliferation through perforin- but not FasL-mediated DC killing

To characterise the effects of perforin- and FasL-mediated DC elimination on CD8$^+$ T cell proliferation, mice received *in vitro* activated wt or PKO OT-I CTLs, or no CTLs (Fig. 3.6.7a). 24 h later, some recipient mice were injected s.c. with wt DCs loaded with SIINFEKL while others were injected s.c. with *lpr* DCs loaded with SIINFEKL. After 48 h, CD8$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

In mice that did not receive CTLs, both wt and *lpr* DCs elicited strong CD8$^+$ T cell division (Fig. 3.6.7b). CD8$^+$ T cell division was inhibited, but not abolished, by wt CTLs when mice received wt DCs. When Fas was knocked out in DCs, CD8$^+$ T cell division was significantly reduced, suggesting that FasL-mediated DC killing did not play a significant

role in inhibiting CD8$^+$ T cell proliferation (Fig. 3.6.7c and 3.6.7d). When perforin was knocked out in CTLs, CD8$^+$ T cell division in mice that received PKO CTLs and wt DCs was restored to comparable levels to the control group. This result is similar to Fig. 3.5.5. When both perforin and Fas were knocked out, CD8$^+$ T cell division was also restored to similar levels as the control group. Similar results were obtained when the percentage and number of highly divided CD8$^+$ T cells (>4 divisions) were compared among the various groups (data not shown).

This result shows that the CTLs regulate CD8$^+$ T cell proliferation through perforin-mediated, but not FasL-mediated killing of antigen-bearing DCs. However, it should be noted that robust CD8$^+$ T cell division was observed even when few DCs were found in the draining lymph nodes (Fig. 3.5.3, Fig. 3.5.5, Fig 3.6.5 and Fig 3.6.7). The robust CD8$^+$ T cell division in the presence of DC killing could be induced by the few surviving SIINFEKL-loaded DCs that had reached the draining lymph nodes or by host APCs that had taken up antigens from the injected DCs. These possibilities will be examined in chapter 5.

a)

b)
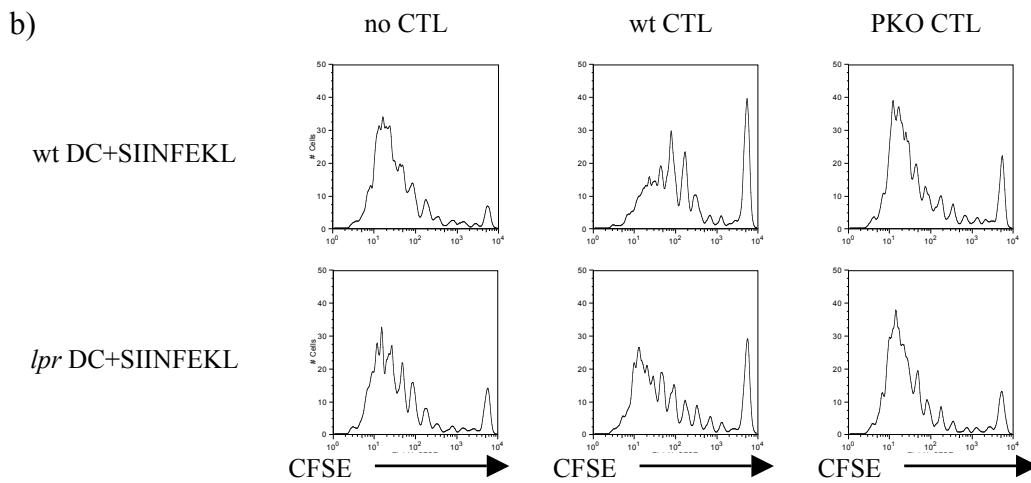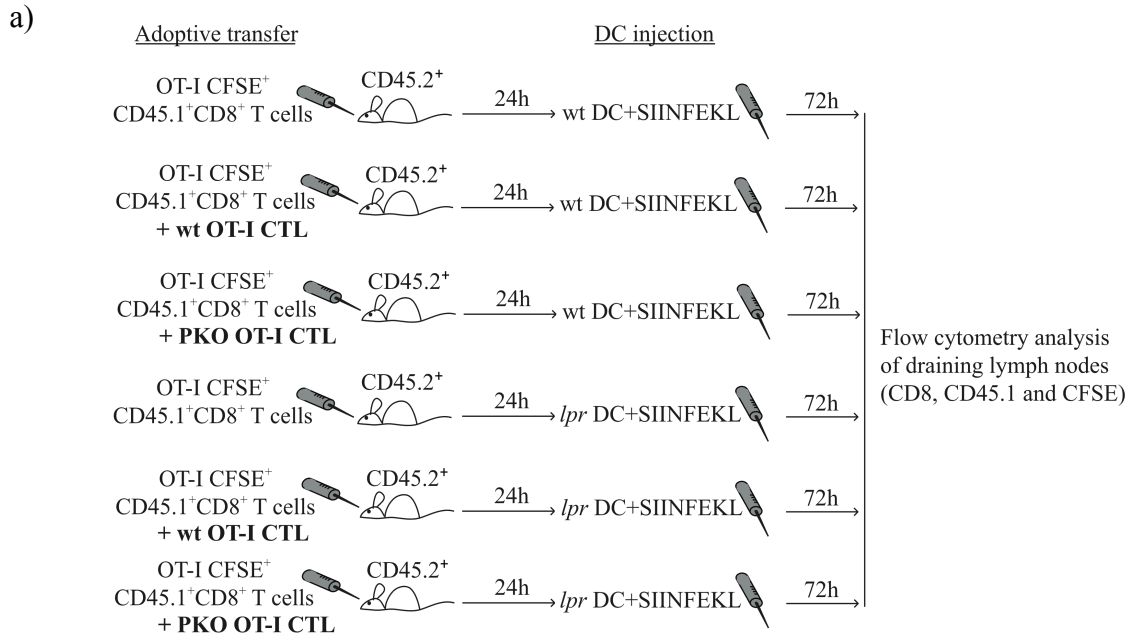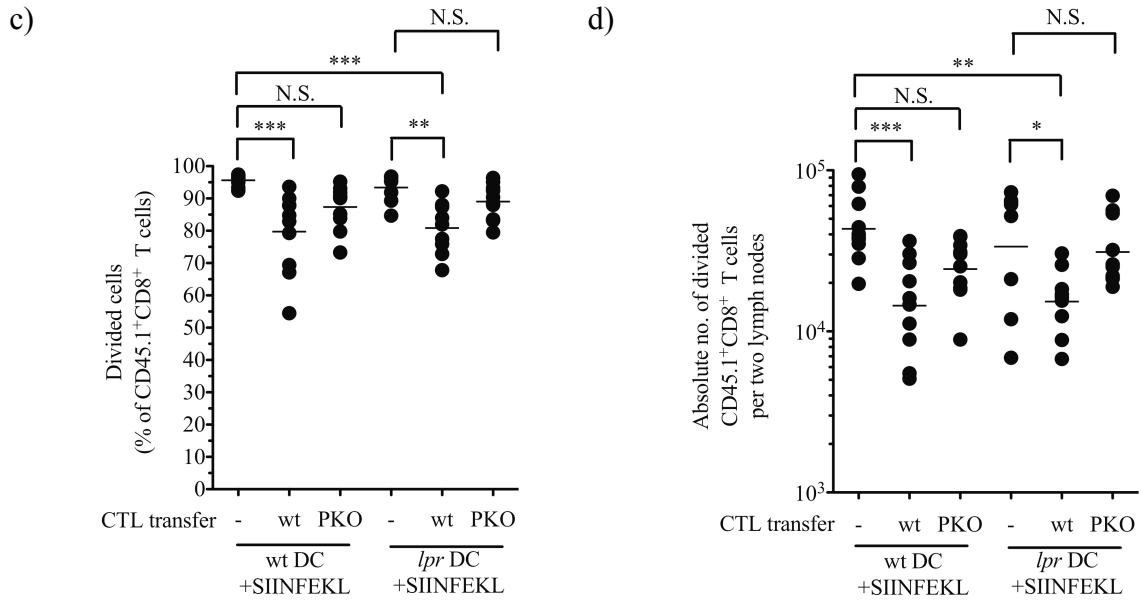
Fig. 3.6.7. **CTLs regulate CD8$^+$ T cell proliferation through perforin-, but not FasL-mediated DC killing.** (**a**) C57BL/6J mice received *in vitro* activated wt or PKO OT-I CTLs and CFSE-labelled OT-I CD45.1$^+$CD8$^+$ T cells. As a control, some mice received CD8$^+$ T cells only. After 24 h, these mice were injected s.c. into their forelimbs with LPS-activated wt or *lpr* DCs loaded with SIINFEKL. 3 days later, CD45.1$^+$CD8$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD8$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD8$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD8$^+$ T cells in the draining lymph nodes are shown. Two separate experiments with 3 – 5 mice per group are pooled together and shown. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. * p<0.05, N.S.= p>0.05.

3.7. Discussion

In this chapter, I have shown that CTLs regulate naïve T cell proliferation through the cytolytic elimination of antigen-loaded DCs. Elimination of antigen-presenting DCs is mediated significantly through perforin, with non-significant contributions from the Fas-FasL pathway. The cytolytic elimination of DCs prevents them from accumulating in the draining lymph nodes. Because the accumulation of DCs in the draining lymph nodes is prevented, DCs can no longer interact with naïve T cells and the induction of naïve T cell proliferation is limited. Thus, CTLs serve as a gatekeeper in regulating the size of downstream T cell responses (Fig. 3.7).
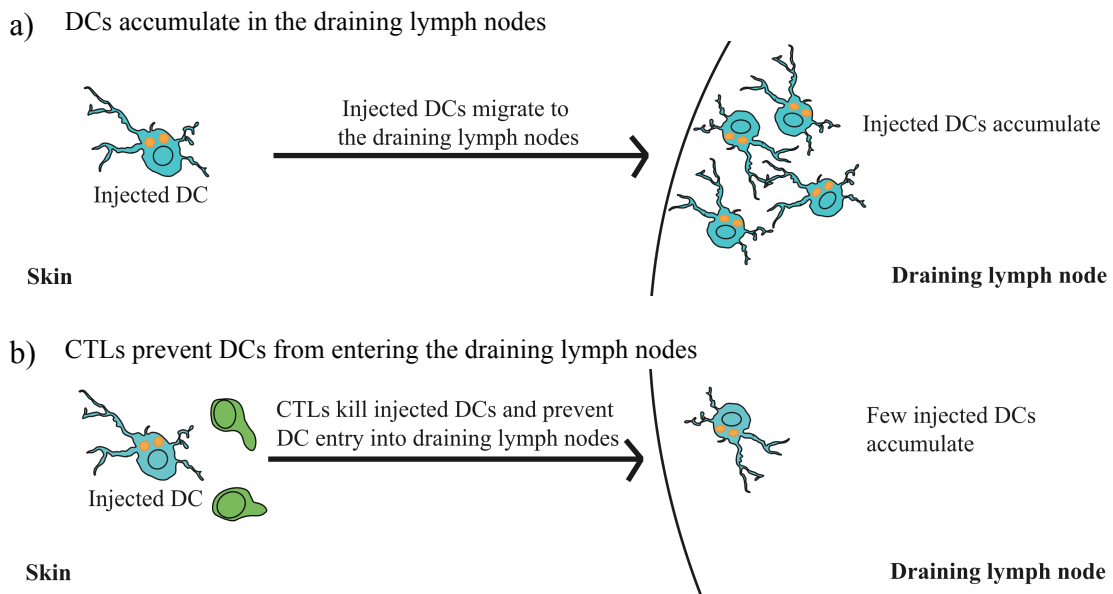
Fig. 3.7. **CTLs prevent DCs from entering the draining lymph nodes**.

3.7.1. Location of CTL-mediated DC elimination

The location of CTL-mediated DC elimination is contentious. In one study using the LCMV model, when antigen-loaded DCs were allowed to migrate into the draining lymph nodes before the transfer of CTLs, DCs that had reached the draining lymph nodes were protected from CTL-mediated killing (Yang et al., 2006). The number of DCs injected into the ear was also reduced in the presence of CTLs, indicating that DCs were eliminated outside the draining lymph nodes. In another study using the OVA model, when DCs were allowed to migrate into the draining lymph nodes before CTL transfer, these DCs were eliminated by CTLs in the draining lymph nodes (Guarda et al., 2007a). This was demonstrated using real time 2 photon intra-vital imaging.

The discrepancy between the studies of Yang et al. and Guarda et al. may be due to a few reasons. Firstly, although CTL-mediated DC killing was visualised in the draining lymph nodes, the DC killing in the draining lymph nodes was not quantified. It is possible that some DCs were killed in the draining lymph nodes but this DC killing would be expected to have little impact on the induction of immune responses. This was not adequately addressed by Guarda. In their experiments visualising DC killing in the draining lymph nodes, DCs were injected into the recipient mice before the adoptive transfer of CTLs (Guarda et al., 2007b). In contrast, in their experiments showing that DC killing reduced T cell proliferation, CTLs and naïve T cells were transferred into the receipient mice before DCs were injected. The authors showed that when CTLs were transferred before the injection of DCs, DCs did not accumulate in the draining lymph nodes, hence did not induce T cell proliferation. This is similar to the results reported by Yang et al (Yang et al., 2006) and Hermans et al (Hermans et al., 2000). In order to demonstrate that DC killing in the draining lymph nodes can reduce the generation of immune responses, T cell proliferation needs be examined when DCs are injected into mice before CTL transfer. However, this was not shown by Guarda et al (Guarda et al., 2007a).

Secondly, different antigen models- LCMV versus OVA- and the different T cell transgenic mice were also used in the two studies (Guarda et al., 2007a; Yang et al., 2006). In my study using the OVA model, when DCs were given before the transfer of CTLs, some DC

killing was observed. This DC killing could be occuring in the draining lymph nodes as reported by Guarda et al and could be mediated by the adoptively transferred CTLs that were found in the draining lymph nodes (Guarda et al., 2007a) (Appendix 4). In contrast, no DC killing was reported in the LCMV model when DCs were given before CTL transfer (Yang et al., 2006). It should be noted that although some DC killing occurred in the draining lymph nodes in my study, the number of antigen-loaded DCs recovered in mice that received CTLs was similar to mice that did not receive CTLs. This suggests that DC killing in the draining lymph nodes does not reduce the number of antigen-loaded DCs greatly. Unlike the CTLs used in my study and the studies of Yang and Guarda, other studies have indicated that memory CTLs eliminate DCs in the draining lymph nodes (Belz et al., 2007; Hermans et al., 2000). This is because memory CTLs express cytolytic molecules rapidly, allowing them to eliminate DCs in the draining lymph nodes (Belz et al., 2007). Thus, the type of CTLs influences the location of CTL-mediated DC killing.

My results also showed that when CTLs were transferred after DC injection, the number of antigen-bearing DCs that had reached the draining lymph nodes did not increase and remain constant. When DCs were given more time to accumulate in the draining lymph nodes before the transfer of CTLs, the number of antigen-bearing DCs found in the draining lymph nodes increased. This suggests that the presence of existing CTLs prevent the entry of DCs into the draining lymph nodes (Fig. 3.7). Taken together, this evidence supports two scenarios. In the first scenario, effector CTLs eliminate antigen-bearing DCs in the non-lymphoid tissues, thereby preventing the DCs from entering the draining lymph nodes to induce *de novo* T cell responses. The few DCs that survive non-lymphoid CTL-mediated killing are then eliminated in the draining lymph nodes.

The second scenario occurs only when memory CD8$^+$ T cells are present. Antigen-bearing DCs enter the draining lymph nodes and activate memory CD8$^+$ T cells into effector CTLs. These CTLs eliminate the antigen-bearing DCs in the draining lymph nodes to terminate DC antigen presentation, then circulate out into the non-lymphoid tissues to eliminate the antigen-bearing DCs, thus preventing more antigen-bearing DCs from entering the draining lymph nodes.

3.7.2. Different cytolytic killing mechanisms used by CTLs for DC killing

The cytotolytic functions of CTLs are mediated through various molecules. Of these cytolytic molecules, two of the most well-documented are perforin and FasL (Kagi et al., 1994b; Lowin et al., 1994b). The different functions thought to be mediated by perforin and FasL were predicted from the phenotypes observed in PKO (Kagi et al., 1994a), *lpr* (Watanabe-Fukunaga et al., 1992), *gld* (Takahashi et al., 1994) and Fas-null (Adachi et al., 1996) mice. Observations made from humans who suffer from FHL (Stepp et al., 1999) and various types of ALPS (Fisher et al., 1995; Rieux-Laucat et al., 1995; Straus et al., 2001; Wang et al., 1999; Wu et al., 1996) further emphasised the delineation of the physiological purposes of perforin and FasL. Notwithstanding the distinct niches occupied by perforin and FasL in the body, other studies have shown that their different cytolytic pathways complement and compensate to some degree when either one of them is inactivated (Ando et al., 1997; Braun et al., 1996; Janssen et al., 2010; Maeda et al., 2005; Price et al., 2005). Other factors such as the different TCR-antigen affinities (Cao et al., 1995; Kessler et al., 1998) and different CTL activation conditions (Aung and Graham, 2000) have also been reported to determine whether CTLs use perforin or FasL to kill target cells.

The sensitivity of target cells to perforin and FasL has to be considered as well. Some cells are resistant to FasL because they express caspase-inhibitors. For example, macrophages (Perlman et al., 1999), DCs (Ashany et al., 1999) and certain tumour cell lines (Medema et al., 1999) have been shown to be resistant to FasL cytolysis because these cells express cFLIP (cellular Fas-associated death domain-like IL-1β-converting enzyme). Some cells are resistant to FasL because they express low levels of Fas (Yang et al., 1995). Some tumour cell lines have been shown to be resistant to perforin-mediated killing because perforin bind poorly to the surfaces of these cells (Lehmann et al., 2000). Although the reason why perforin binds poorly to the tumour cells is not elucidated, there are suggestions that the expression of cathepsin B protects the tumour cells from perforin-mediated killing (Balaji et al., 2002). Thus, the distinct functional niches occupied by perforin and FasL are also defined by the susceptibility of the target cells to the different CTL cytolytic mechanisms.

Some studies have shown that CTLs eliminate DCs through perforin (Belz et al., 2007; Laffont et al., 2006; Yang et al., 2006), while others have shown that DCs are eliminated by CTLs through FasL (Stranges et al., 2007). However, the cytolytic contributions of perforin and FasL to CTL-mediated DC killing have not been examined in the same experiment using similar conditions. In my study, I have shown that perforin is a significant and critical component of DC elimination. On the other hand, knocking out Fas in DCs only reduces the killing of antigen-bearing DCs slightly.

Why CTLs eliminate DCs more through perforin than FasL is not clear. In my study, the OT-I CTLs expressed some FasL after 30 min of incubation with SIINFEKL *in vitro*. More OT-I CTLs acquired increased FasL expression when these CTLs were incubated for 2 h with SIINFEKL. This is in line with previous studies which have shown that the early and late FasL expressions are from a pre-existing pool of FasL, or from *de novo* protein synthesis, respectively (He et al., 2010; He and Ostergaard, 2007). FasL expression on CTLs alone is insufficient to induce target cell death because unlike perforin, FasL-mediated killing requires the expression of Fas on target cells. In my study, LPS-activated DCs increase Fas expression and this has also been shown in previous studies (Stranges et al., 2007). Thus, FasL-Fas mechanism contributes little to DC killing but this is not because CTLs or DCs do not express FasL or Fas respectively.

It is possible that the DCs express anti-apoptotic proteins that protect them from FasL-mediated killing *in vitro* (Ashany et al., 1999). However, other studies have indicated that the expression of anti-apoptotic proteins in DCs does not necessarily confer protection from CTL killing. For example, DCs used in my study have been shown to express serine protease inhibitor (SPI)-6 (Andrew et al., 2008), which reportedly protects DCs from CTL-mediated killing *in vitro* (Medema et al., 2001). However, the DCs expressing SPI-6 remained sensitive to CTL-mediated killing *in vivo* (Andrew et al., 2008). Taken together, the current evidence indicates that CTLs predominantly use perforin to mediate DC killing, although FasL also makes a small contribution to DC killing as well.

CTLs are reported to eliminate DCs through other cytolytic molecules independent of perforin and FasL (Ludewig et al., 2001). Other cytolytic molecules include members of the

TNFα super family such as TRAIL (Mirandola et al., 2004) and TNFα (Balkwill, 2006), and members of the serine protease family such as granzymes (Chowdhury and Lieberman, 2008). CTLs have been shown to utilise TRAIL and TNFα to eliminate viral infected cells (Brincks et al., 2008; White and Harty, 1998) and tumour cells (Pitti et al., 1996; Poehlein et al., 2003; Prevost-Blondel et al., 2000). Studies of ALPS Type II patients indicated that DCs obtained from these patients were resistant to TRAIL-mediated killing, thus contributing to the accumulation of abnormal T cells and DCs (Wang et al., 1999). CTLs also express granzymes and have been shown to utilise grazymes to mediate apoptosis in target cell (Revell et al., 2005; Shresta et al., 1999), although recent evidence indicates that granzyme B and A are not required for CTL cytolytic functions (Regner et al., 2009). In my study, when perforin and Fas were stimultaneously knocked out, CTL-mediated DC killing was greatly reduced and similar numbers of antigen-loaded DCs and control DCs could be recovered. This indicates that perforin and FasL mediate most of the CTL cytolytic functions (Kojima et al., 1994; Lowin et al., 1994b). Thus, while CTLs express and utilise cytolytic molecules other than perforin and FasL, the role of other cytolytic molecules in DC killing may not be easily demonstrated due to the significant contributions from perforin and FasL to DC killing.

3.7.3. Protection of DCs from CTL-mediated DC killing

There have been conflicting evidence regarding the susceptibility of antigen-bearing DCs to CTL-mediated killing. Several *in vitro* studies have reported that DCs are protected from FasL-mediated killing through the expression of anti-apoptotic molecules (Ashany et al., 1999; Leverkus et al., 2000; Lundqvist et al., 2002; Rescigno et al., 2000; Willems et al., 2000). The expression of SPI-6 is also reported to protect DCs from perforin/granzyme-mediated CTL-mediated killing *in vitro* (Medema et al., 2001). LPS- and anti-CD40 treatments induce DC activation and have also been shown to protect activated DCs from CTL-mediated killing (Medema et al., 2001; Mueller et al., 2006). DC interaction with other T cells such as CD4^{+} T cells *in vivo* (Mueller et al., 2006) and memory CD8^{+} T cells *in vitro* (Watchmaker et al., 2008) has also been reported to protect DCs from CTL-mediated killing. Furthermore, DC apoptosis is inhibited when DCs form immunological

synapses with naïve CD4$^+$ T cells, indicating that the survival of DCs is critical for the initiation of immune responses (Riol-Blanco et al., 2009).

In vivo studies have shown that DCs are susceptible to perforin- and FasL-mediated killing. *In vivo* DC killing was reduced by 20-30% when Fas was knocked out in DCs, indicating that CTLs eliminated DCs in a FasL-dependent manner (Stranges et al., 2007). While the expression of SPI-6 has been shown to protect DCs from perforin/granzyme-mediated killing *in vitro* (Medema et al., 2001), DCs expressing SPI-6 remained sensitive to killing by CTLs *in vivo* (Andrew et al., 2008). Unlike the LPS-treated splenic DCs (Mueller et al., 2006), LPS-activated BM-DCs remained susceptible to CTL-mediated killing *in vivo* even though LPS-treatment increased the expression of SPI-6 in BMDCs (Andrew et al., 2008). In my study, LPS-treated BMDCs were used throughout and they too remained sensitive to CTL-mediated killing. DCs were also susceptible to memory CD8$^+$ T cell cytolytic killing in an *in vivo* influenza model (Belz et al., 2007), and in a DC immunisation model (Hermans et al., 2000). Moreover, *in vivo* live imaging has shown that DCs are eliminated by CTLs (Guarda et al., 2007a). Although CD4$^+$ T cells have been shown to protect DCs from CTL-mediated killing, DCs were eliminated through FasL-Fas pathway after interacting with antigen-specific CD4$^+$ T cells *in vitro* (Matsue et al., 1999) and disappeared from the draining lymph nodes after interaction with CD4$^+$ T cells *in vivo* (Ingulli et al., 1997). Other types of CD4$^+$ T cells such as the Foxp3$^+$ CD4$^+$ regulatory T cells also induced DC apoptosis in a perforin-dependent manner *in vivo* (Boissonnas et al., 2010).

It is unclear why DCs are sensitive to CTL-mediated killing in some models but not in others. However, because DCs actively protect themselves from killing by CTLs at least *in vitro*, this indicates that DC survival is actively regulated through the expression of anti-apoptotic proteins in DCs and CTL-mediated DC killing. The regulation of DC survival is important because prolonged DC survival and aberrant DC accumulation lead to pathological immune responses (Chen et al., 2006). Taken together, the survival of DCs and the duration of DC antigen presentation are subjected to CTL-mediated regulation.

3.7.4. CTL-mediated elimination of DCs and T cell proliferation

Sufficient DC numbers and antigen concentration are required for the activation of naïve T cells (Henrickson et al., 2008; Martin-Fontecha et al., 2003) (Appendices 5 and 6). When CTLs eliminate antigen-bearing DCs, CTLs lower the number of available antigen-presenting DCs in the draining lymph nodes to induce T cell proliferation (Guarda et al., 2007a). In my study, $CD4^+$ and $CD8^+$ T cell proliferation were inhibited when antigen-presenting DCs were eliminated by CTLs. This is in line with previous studies (Guarda et al., 2007a; Hermans et al., 2000). My study also has shown that CTLs regulate naïve T cell proliferation mainly through perforin-mediated DC killing, whereas FasL-mediated DC killing was not observed to play any regulatory role. Thus, by limiting the availability of antigen-presenting DCs through perforin-mediated elimination, CTLs function as a regulator of naïve T cell clonal expansion.

It has been proposed that the physiological function of CTL-mediated DC killing is to downregulate immune responses that may otherwise lead to immunopathology (Guarda et al., 2007a; Hermans et al., 2000; Laffont et al., 2006; Ronchese and Hermans, 2001; Yang et al., 2006). While there is no formal evidence demonstrating the proposed physiological function, one study has shown that the presence of effector $CD8^+$ T cells prevent the induction of alloreactive $CD4^+$ T cells and $CD4^+$ T cell-mediated allograft rejection through perforin-mediated DC killing (Laffont et al., 2006). There is also other circumstantial evidence. For example, when CTLs failed to eliminate DCs, the accumulation in DCs led to the increased expansion of effector T cells (Yang et al., 2006). My study shows that CTLs prevent the initation of T cell responses through cytolytic elimination of DCs, thus supporting the notion that CTLs eliminate DCs to terminate unnecessary immune responses or prevent the induction of *de novo* immune responses.

Chapter 4

The impact of different antigen loading methods on DC killing and CD4$^+$ T cell proliferation

In Chapter 3, I have shown that DCs loaded with peptides were recognised by antigen-specific CTLs and were eliminated through perforin-dependent killing, leading to a decrease in subsequent antigen presentation to naïve T cells.

Because most physiological antigens presented by DCs via the cross-presentation and classical MHC class I pathways are derived from exogenous or endogenous proteins respectively (Rock and Goldberg, 1999; Rock and Shen, 2005), it is vital to characterise how the killing of DCs by CTLs is influenced by the way antigens are loaded onto DCs, and whether this will affect the induction of subsequent T cell responses.

Another aspect that requires investigation is the type of CTLs used. Adoptive transfer of *in vitro* activated OT-I CD8$^+$ T cells was previously used to examine the DC killing mechanisms in Chapter 3. Unlike the OT-I CTLs, host effector CD8$^+$ T cells are polyclonal and have different avidities to a particular epitope as the different TCRs on the polyclonal T cells interact to varying degrees with the peptide-MHC molecule complexes on the APCs. Avidity is important in controlling the type of T cell responses. For example, high avidity T cells proliferated when low antigen doses were presented by APCs (Alexander-Miller et al., 1996a), whereas high avidity T cells underwent activation induced T cell death (AICD) when high antigen doses were presented by APCs (Alexander-Miller et al., 1996b). In contrast, low avidity T cells proliferated when high doses of antigen were presented by APCs. Very low antigen doses could also induce T cell anergy (Korb et al., 1999). Thus, in contrast to OT-I CTLs, host effector CD8$^+$ T cells generate different types of immune responses due to their polyclonal nature. The inherent differences between OT-I CTLs and the host effector CD8$^+$ T cells may have an impact on CTL-mediated DC killing and the subsequent induction of T cell responses. Host effector CD8$^+$ T cells can be generated through peptide-loaded DC immunisation (Porgador and Gilboa, 1995), hence the effects of physiologically relevant host effector CD8$^+$ T cells (host CTLs) on DC killing and on subsequent T cell responses can be examined.

In this chapter, I will address the following aims:

1) To establish whether CTLs raised *in vivo* via DC immunisation, and transgenic CTLs activated *in vitro*, eliminate DCs loaded with soluble protein *in vivo*

2) To establish whether CTLs raised *in vivo* via DC immunisation, and transgenic CTLs activated *in vitro*, eliminate DCs expressing endogenous protein *in vivo*

3) To evaluate how CTLs affect the induction of CD4$^+$ T cell proliferation by eliminating DCs loaded with soluble protein or expressing endogenous protein *in vivo*

4.1. DCs incubated with soluble protein have a highly activated phenotype

The process of uptake and presentation of exogenous proteins on MHC class I molecules is known as cross-presentation (Carbone and Bevan, 1990). To characterise the phenotype of DCs cross-presenting protein antigens, DCs were loaded with soluble OVA protein and their activation phenotypes were determined.

As described in Chapter 2, BM-DCs were cultured for 5 days and then loaded with 2 mg/ml of soluble OVA protein for 2 days. On the 6th day of culture, some of these DCs were treated with LPS, whereas others were not. On the 7th day of culture, all of these DCs were examined for the expression of various co-stimulatory molecules and phenotypic markers.

The percentage of $CD11c^{+}CD11b^{+}$ DCs was similar in all cultures regardless of OVA incubation or LPS treatment (Fig. 4.1a). The percentages of MHC class II^{hi}, $CD40^{hi}$, $CD80^{hi}$ and $CD86^{hi}$ $CD11c^{+}$ DCs were highest in DCs loaded with OVA protein (DC+OVA) and treated with LPS (Fig. 4.1b and Table 4.1.1). DC+OVA that were not treated with LPS also showed high activation phenotype. The high percentage and MFI observed in non-LPS treated DC+OVA were likely due to the presence of endotoxins in the OVA protein. LPS treatment increased the activation phenotype of DCs not loaded with OVA protein. The MFI of the various co-stimulatory molecules also reflected a similar trend (Fig. 4.1b and Table 4.1.2).

a)

b)
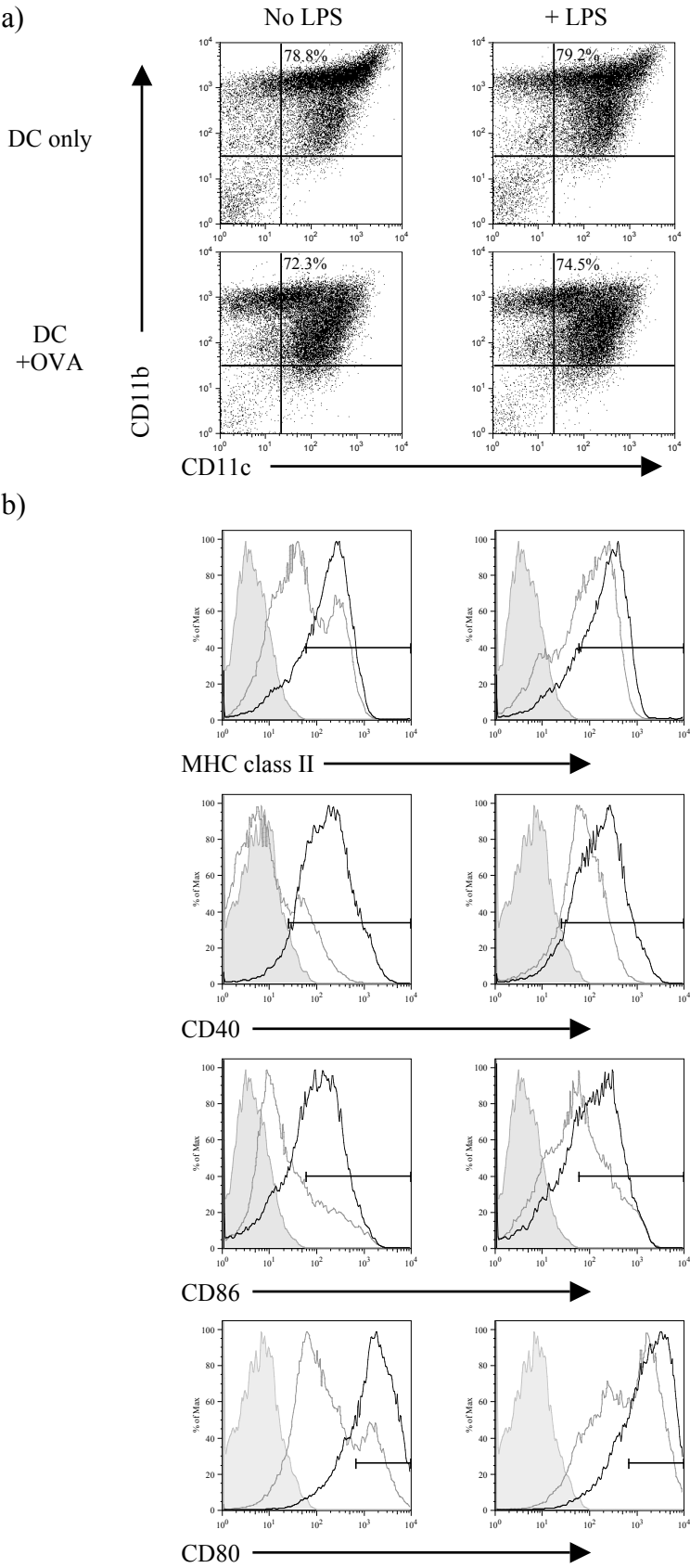
Fig. 4.1. **Culture of BM-DCs loaded with soluble OVA protein yields CD11c$^+$CD11b$^+$ cells with an activated phenotype.** Bone marrow cells were prepared from C57BL/6J mice and cultured with GM-CSF and IL-4 for 7 days *in vitro*. On day 5 of culture, some cells were incubated with OVA protein (DC+OVA) while others were not. On day 6 of culture, LPS was added to some cell cultures. 24 h after LPS treatment, LPS-treated and non-LPS treated cells were harvested and analysed for expressions of various phenotypic and activation markers. (**a**) The expressions of CD11b and CD11c in cultured cells are shown in the dot plots. The percentages of CD11c$^+$CD11b$^+$ cells are shown. (**b**) The expressions of activation markers in CD11c$^+$ cells are shown in histograms. Untreated DCs (DC only) are depicted in grey while DC+OVA are depicted in black. The unstained controls are shown as filled light grey areas. The percentages and MFIs of live CD11c$^+$ cells expressing the activation markers are shown in Tables 4.1 and 4.2. The experiment was performed once.

Table 4.1.1. Expression of activation markers on BM-DC cultured with OVA protein

Activation marker	No LPS		+ LPS	
	DC only	DC+OVA	DC only	DC+OVA
MHC class II	43.9%*	77.2%	61.8%	78.8%
CD40	25.3%	93.2%	84.6%	92.2%
CD86	25.5%	63.1%	47.5%	65.9%
CD80	23.4%	75.5%	49.3%	76%

* Percentages of activation marker highly expressed in CD11c$^+$ cells as gated in Fig. 4.1b.

Table 4.1.2. MFIs of activation markers on BM-DC cultured with OVA protein

Activation marker	No LPS		+ LPS	
	DC only	DC+OVA	DC only	DC+OVA
MHC class II	50.6	134	70.2	154
CD40	9.87	156	70.2	156
CD86	24.1	75.1	51.8	90
CD80	178	1336	535	1455

* MFIs of activation markers expressed in CD11c$^+$ cells in Fig. 4.1b.

4.2. Host and *in vitro* activated CTLs eliminate DCs loaded with OVA protein but do not affect the subsequent induction of CD4$^+$ T cell proliferation

4.2.1. Host CTLs eliminate DCs loaded with OVA protein

My first experiment asked if host CTLs eliminated DCs loaded with OVA protein. Fig. 4.2.1a. illustrates the experimental setup. To induce host CTL responses, mice were immunised with DCs loaded with SIINFEKL (DC+SIINFEKL). Some mice were immunised with DCs not loaded with SIINFEKL (DC only), or did not receive immunisation (not immunised). 7 days later, CFSE$^+$ DCs loaded with OVA protein (DC+OVA) and CMTMR$^+$ DCs without OVA were mixed in equal numbers and injected s.c. into the forelimbs of immunised and non-immunised mice. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ cells in draining lymph nodes were monitored by flow cytometry.

In the group immunised with DC+SIINFEKL, the number of CFSE$^+$ DCs was greatly reduced compared to mice immunised with DC alone or non-immunised mice (Fig. 4.2.1b and c). This shows that host CTLs eliminate DCs loaded with OVA protein.
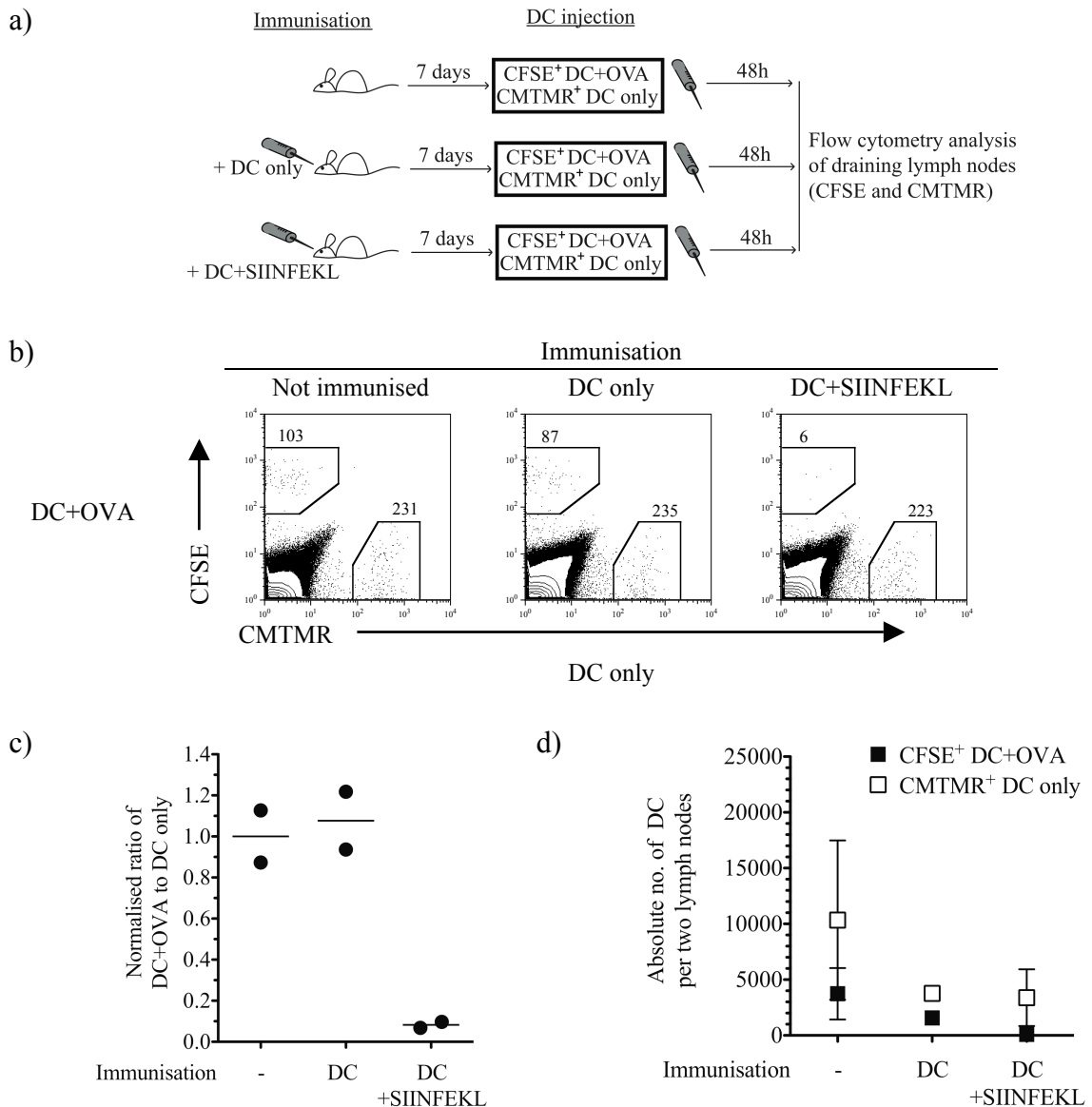
Fig. 4.2.1. **Host CTLs eliminate DCs loaded with OVA protein. (a)** Groups of C57BL/6J mice were immunised with DC loaded with SIINFEKL (DC+SIINFEKL), or DC not loaded with SIINFEKL (DC only) or did not receive immunisation (not immunised). 7 days later, CFSE-labelled DCs incubated with OVA protein (DC+OVA) were mixed with CMTMR-labelled control DC (DC only) in equal numbers and were injected s.c. into their forelimbs. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. **(b)** CFSE$^+$ and CMTMR$^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. **(c)** The ratios of CFSE$^+$ DC+OVA to CMTMR$^+$ DC only were normalised to the ratios derived from non-immunised mice. **(d)** Absolute numbers of CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes are shown. One representative experiment of two with 2 – 3 mice group is shown.

4.2.2. Host CTLs do not inhibit CD4$^+$ T cell proliferation induced by DCs loaded with OVA protein

Having determined that antigen-loaded DC immunisation generated host CTLs capable of eliminating DCs loaded with OVA protein (Fig. 4.2.1), I went on to ask if the host CTLs would inhibit CD4$^+$ T cell proliferation induced by DCs loaded with OVA protein.

The experimental setup is shown in Fig. 4.2.2a. Groups of mice were either immunised with DCs loaded with SIINFEKL (DC+SIINFEKL), or DCs not loaded with SIINFEKL (DC only), or did not receive immunisation. 6 days later, CFSE-labelled OT-II CD4$^+$ T cells were injected i.v. into these mice. 24 h after the adoptive transfer of CD4$^+$ T cells, 0.5 × 10^6 OVA protein-loaded DCs were injected s.c. into the forelimbs of the recipient mice. 3 days later, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

Compared to mice that were not immunised with DCs, the percentage of divided CD4$^+$ T cells was significantly reduced in mice immunised with DC only and DC+SIINFEKL (Fig. 4.2.2c). However, the number of divided CD4$^+$ T cells in mice immunised with DC+SIINFEKL was significantly higher than in mice immunised with DC only (Fig. 4.2.2e). This result was unexpected because OVA-loaded DCs were eliminated by host CTLs (Fig. 4.2.1) and fewer OVA-loaded DCs in the draining lymph nodes should induce lesser CD4$^+$ T cell proliferation.

It was possible that some OVA-loaded DCs escaped CTL-mediated killing and a sufficient number of OVA-loaded DCs ended up in the draining lymph nodes. A separate experiment was then conducted whereby a lower number of DCs loaded with OVA protein (0.2 × 10^6 DCs) was injected into mice that were previously immunised with DC alone, or DC+SIINFEKL, or into mice that did not receive immunisation. 3 days after DC injection, CD4$^+$ T cells in draining lymph nodes were examined for CFSE dilution by flow cytometry.

Mice immunised with DC+SIINFEKL showed a significantly reduced CD4$^+$ T cell division compared to mice that did not receive immunisation, although this reduction was not significant when compared to mice immunised with DC only (Fig. 4.2.2d). Under this condition, the number of divided CD4$^+$ T cells was similar in all groups (Fig. 4.2.2f). Taken together, these results show that although host CTLs eliminate OVA-loaded DCs, the CTL-mediated DC killing does not lower CD4$^+$ T cell proliferation significantly.

a)

Immunisation   Adoptive transfer   DC injection

CD45.1$^+$ 6 days   OT-II CFSE$^+$ 24h   DC+OVA   72h
                          CD45.2$^+$CD4$^+$
                          T cells

+ DC only   CD45.1$^+$ 6 days   OT-II CFSE$^+$ 24h   DC+OVA   72h   Flow cytometry analysis
                                     CD45.2$^+$CD4$^+$ of draining lymph nodes
                                     T cells   (CD4, CD45.2 and CFSE)

+ DC+SIINFEKL   CD45.1$^+$ 6 days   OT-II CFSE$^+$ 24h   DC+OVA   72h
                                         CD45.2$^+$CD4$^+$
                                         T cells

b)

Immunisation

0.2×10^6 DC+OVA

Not immunised   DC only   DC+SIINFEKL

CFSE   CFSE   CFSE

0.5×10^6 DC+OVA injected

c)

Divided cells (% of CD45.2$^+$CD4$^+$ T cells)

Immunisation   -   DC   DC+SIINFEKL

e)

Absolute no. of divided CD45.2$^+$CD4$^+$ T cells per two lymph nodes

Immunisation   -   DC   DC+SIINFEKL

0.2×10^6 DC+OVA injected

d)

Divided cells (% of CD45.1$^+$CD4$^+$ T cells)

Immunisation   -   DC   DC+SIINFEKL

f)

Absolute no. of divided CD45.1$^+$CD4$^+$ T cells per two lymph nodes

Immunisation   -   DC   DC+SIINFEKL

Fig. 4.2.2. **The elimination of DCs loaded with OVA protein by host CTLs does not reduce CD4$^+$ T cell proliferation.** (**a**) B6.SJ *ptprca* mice were immunised with DCs loaded with SIINFEKL (DC+SIINFEKL), or DC not loaded with SIINFEKL (DC only), or did not receive immunisation. 6 days later, CFSE-labelled OT-II CD45.2$^+$CD4$^+$ T cells were injected i.v. into these recipient mice. 24 h later, 0.5×10^6 or 0.2×10^6 LPS-activated DCs loaded with OVA protein (DC+OVA) were then injected s.c. into the forelimbs of these mice. 3 days later, CD45.2$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c, d**) The percentages of CD45.2$^+$CD4$^+$ or CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**e, f**) Absolute numbers of divided CD45.2$^+$CD4$^+$ or CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 5 mice per group for each condition. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. * p<0.05, ** p<0.01, N.S.= p>0.05.

4.2.3. Host CTL-mediated DC killing has little impact on the accumulation of divided CD4$^+$ T cells in the spleens

The division of CD4$^+$ T cells was also examined in the spleens of mice that were immunised with DC only, DC+SIINFEKL, or did not receive immunisation 7 or 8 days after DC+OVA injection. The experimental setup is shown in Fig. 4.2.3a.

Few divided CD4$^+$ T cells were recovered from the spleens of all mice. Compared to mice immunised with DC only, the percentage of divided CD4$^+$ T cells in the spleens of mice immunised with DC+SIINFEKL was not significantly different at either day 7 or 8 after DC+OVA injection (Fig. 4.2.3b and c). However, the number of divided CD4$^+$ T cells in the spleens of mice immunised with DC+SIINFEKL was significantly lower than in mice immunised with DC only 7 days, but not 8 days, after DC+OVA injection (Fig. 4.2.3d). The differences between the percentage and number of divided CD4$^+$ T cells could be due to experimental variations. This might lead to the variable results observed in Fig. 4.2.3d.

The results show that host CTLs eliminate DCs loaded with OVA protein but this has little impact on the number of divided CD4$^+$ T cells circulating in the spleens of recipient mice.

a)

b)

c)

d)
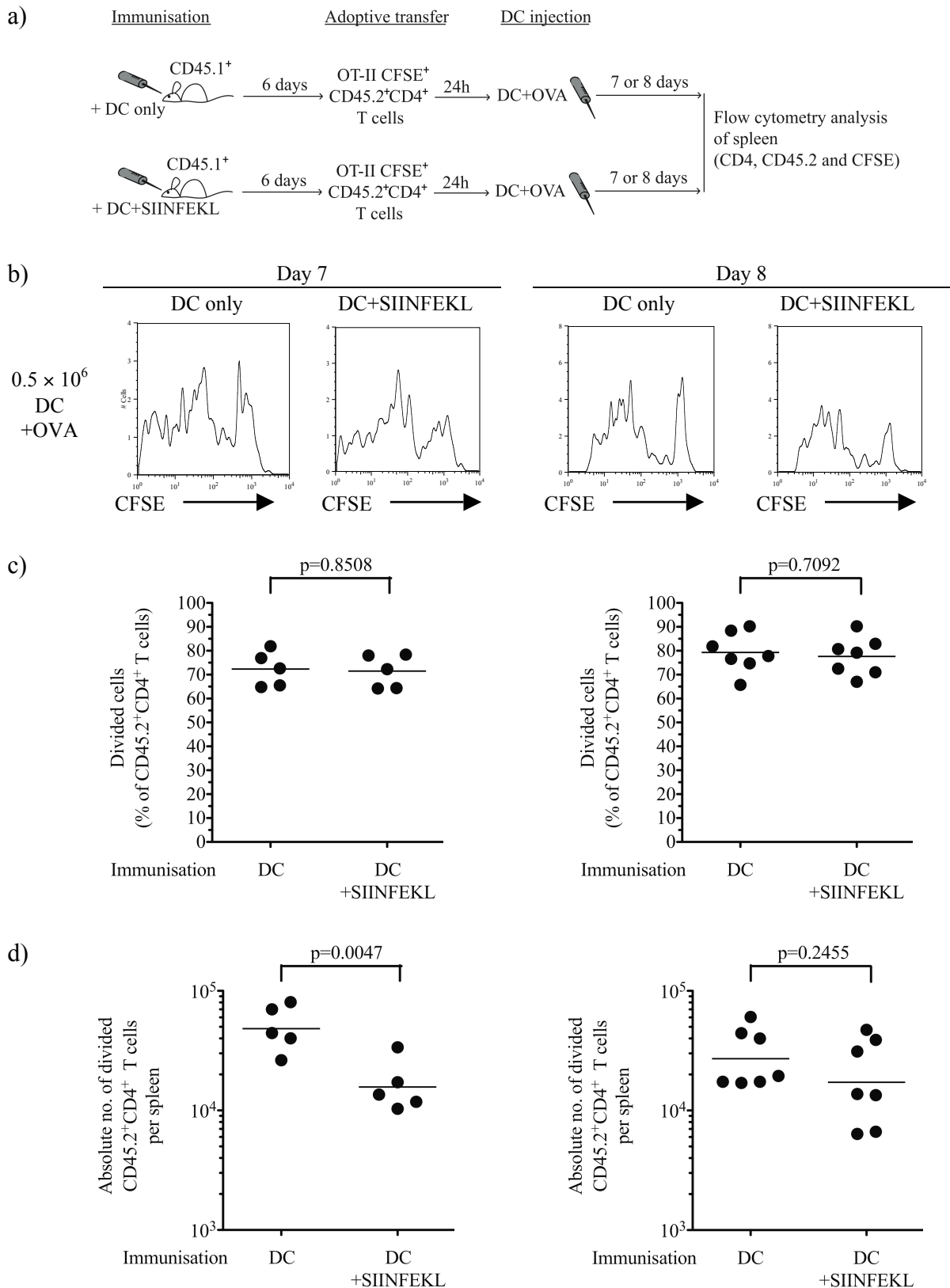
Fig. 4.2.3. **Host CTL-mediated DC killing has little impact on the accumulation of divided CD4$^+$ T cells in the spleens.** (**a**) B6.SJ *ptprca* mice were immunised with DCs loaded with SIINFEKL

(DC+SIINFEKL), or DCs not loaded with SIINFEKL (DC only). 6 days later, CFSE-labelled OT-II CD45.2$^+$CD4$^+$ T cells were injected i.v. into these recipient mice. 24 h later, LPS-activated DCs loaded with OVA protein (DC+OVA) were injected s.c. into the forelimbs of these mice. 7 or 8 days later, CD45.2$^+$CD4$^+$ T cells in the spleens were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.2$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.2$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.2$^+$CD4$^+$ T cells in the draining lymph nodes are shown. For Day 7, the experiment was performed once with 5 mice per group for Day 7. For Day 8, two separate experiments with 4 – 5 mice per group were pooled together and shown. Statistical significance was determined with two tailed Student's *t*-test.

4.2.4. *In vitro* activated CTLs do not inhibit CD4$^+$ T cell proliferation induced by DCs loaded with OVA protein

Because the adoptive transfer of *in vitro* activated transgenic OT-I CTLs inhibited CD4$^+$ T cell proliferation induced by peptide-loaded DCs (Fig 3.3), I asked if the adoptive transfer of CTLs would inhibit CD4$^+$ T cell proliferation induced by DCs that were loaded with OVA protein.

The experimental setup is shown in Fig. 4.2.4a. Mice received CFSE-labelled OT-II CD4$^+$ T cells and 5×10^6 (low) or 10×10^6 (high) numbers of *in vitro* activated OT-I CTLs. Some mice received only CD4$^+$ T cells without CTLs. 24 h later, DCs loaded with OVA protein were injected s.c. into these recipient mice. 3 days after DC injection, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

The majority of CD4$^+$ T cells divided in all groups irrespective of the number of CTLs transferred (Fig. 4.2.4b). The percentages and numbers of divided CD4$^+$ T cells in mice that received low or high numbers of CTLs were not significantly different from the control group (Fig. 4.2.4c and d). This result shows that transferring low or high number of *in vitro* activated CTLs in mice does not reduce the CD4$^+$ T cell proliferation.

It was possible that some OVA-loaded DCs escaped CTL-mediated killing even though a high number of *in vitro* activated CTLs was transferred. Because transferring high numbers of CTLs into mice did not reduce CD4$^+$ T cell division, the number of CTL was kept at 10×10^6 and the number of DC+OVA injected were reduced from 0.5×10^6 cells to 0.2×10^6 cells. I asked if the CD4$^+$ T cell division would be inhibited under these conditions.

However, even when 0.2×10^6 DC+OVA was injected, the percentage and number of divided CD4$^+$ T cells were not significantly different from the control group (Fig. 4.2.4e and f). The results in Fig. 4.2.4. showed that *in vitro* activated CTLs did not reduce CD4$^+$ T cell proliferation that was stimulated by DCs loaded with OVA protein. This result was different from the results showing that *in vitro* activated CTLs reduced the CD4$^+$ T cell proliferation induced by SIINFEKL- and OVA$_{323-339}$-loaded DCs (Fig. 3.3). One possible reason for this discrepancy is that unlike peptide-loaded DCs, OVA protein-loaded DCs were not completely eliminated by CTLs, thereby allowing sufficient OVA protein-loaded DCs to accumulate in the draining lymph nodes. This is addressed in the next section.

a)

Adoptive transfer   DC injection

OT-II CFSE$^+$ CD45.1$^+$ 24h   72h
CD45.2$^+$CD4$^+$ T cells   →   DC+OVA   →

OT-II CFSE$^+$ CD45.1$^+$ 24h   72h
CD45.2$^+$CD4$^+$ T cells   →   DC+OVA   →   Flow cytometry analysis
+ Low number   of draining lymph nodes
of OT-I CTL   (CD4, CD45.2 and CFSE)

OT-II CFSE$^+$ CD45.1$^+$ 24h   72h
CD45.2$^+$CD4$^+$ T cells   →   DC+OVA   →
+ High number
of OT-I CTL

b)   No. of CTLs transferred ($\times 10^6$)

         no CTL   5   10

0.5 × 10^6
DC+OVA

    CFSE →   CFSE →   CFSE →

                        0.5 × 10^6 DC+OVA injected

c)   N.S.   d)   N.S.

               N.S.   N.S.

No. of CTL   No. of CTL
transferred (x10^6)   -   5   10   transferred (x10^6)   -   5   10

Fig. 4.2.4. *In vitro* **activated CTLs do not inhibit CD4$^+$ T cell proliferation induced by DCs loaded with OVA protein.** (**a**) B6.SJ *ptprca* mice were injected i.v. with CFSE-labelled OT-II CD45.2$^+$CD4$^+$ T cells and 5×10^6 or 10×10^6 *in vitro* activated OT-I CTLs. As a control, some mice received CD4$^+$ T cells only. After 24 h, these mice were injected s.c. into their forelimbs with 0.5×10^6 or 0.2×10^6 LPS-activated DCs loaded with OVA protein. 3 days later, CD45.2$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.2$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.2$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.2$^+$CD4$^+$ T cells in the draining lymph nodes are shown. Two separate experiments with 3 − 4 mice per group are pooled together and shown. In a separate experiment, C57BL/6J mice were injected i.v. with CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells and 10×10^6 *in vitro* activated OT-I CTLs, or CD4$^+$ T cells only. After 24 h, these mice were injected s.c. into the forelimbs with 0.2×10^6 LPS-activated DCs loaded with OVA protein. 3 days later, CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**e**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**f**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 5 mice per group. Statistical significance was determined with one-way ANOVA with Bonferroni's correction or two tailed Student's *t*-test. N.S.= p>0.05.

4.2.5. *In vitro* activated CTLs do not eliminate DCs loaded with OVA protein completely

To investigate if *in vitro* activated CTLs eliminated DCs loaded with OVA, mice received 10×10^6 *in vitro* activated OT-I CTLs while the control group did not. Fig 4.2.5a shows the experimental setup. DCs loaded with OVA protein were labelled with CFSE and mixed in equal numbers with CMTMR-labelled DCs not loaded with OVA. The DC mixture was then injected s.c. into the forelimbs of recipient mice 24 h after CTL transfer. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ cells in the draining lymph nodes were monitored by flow cytometry.

In mice that received CTLs, much fewer CFSE$^+$ cells were collected compared to CMTMR$^+$ cells (Fig. 4.2.5b). However, some CFSE$^+$ DC+OVA survived CTL-mediated killing and accumulated in the draining lymph nodes of mice that received CTLs (Fig. 4.2.5c and d). These DC+OVA were probably sufficient to induce the OT-II CD4$^+$ T cell proliferation observed in Fig. 4.2.4.
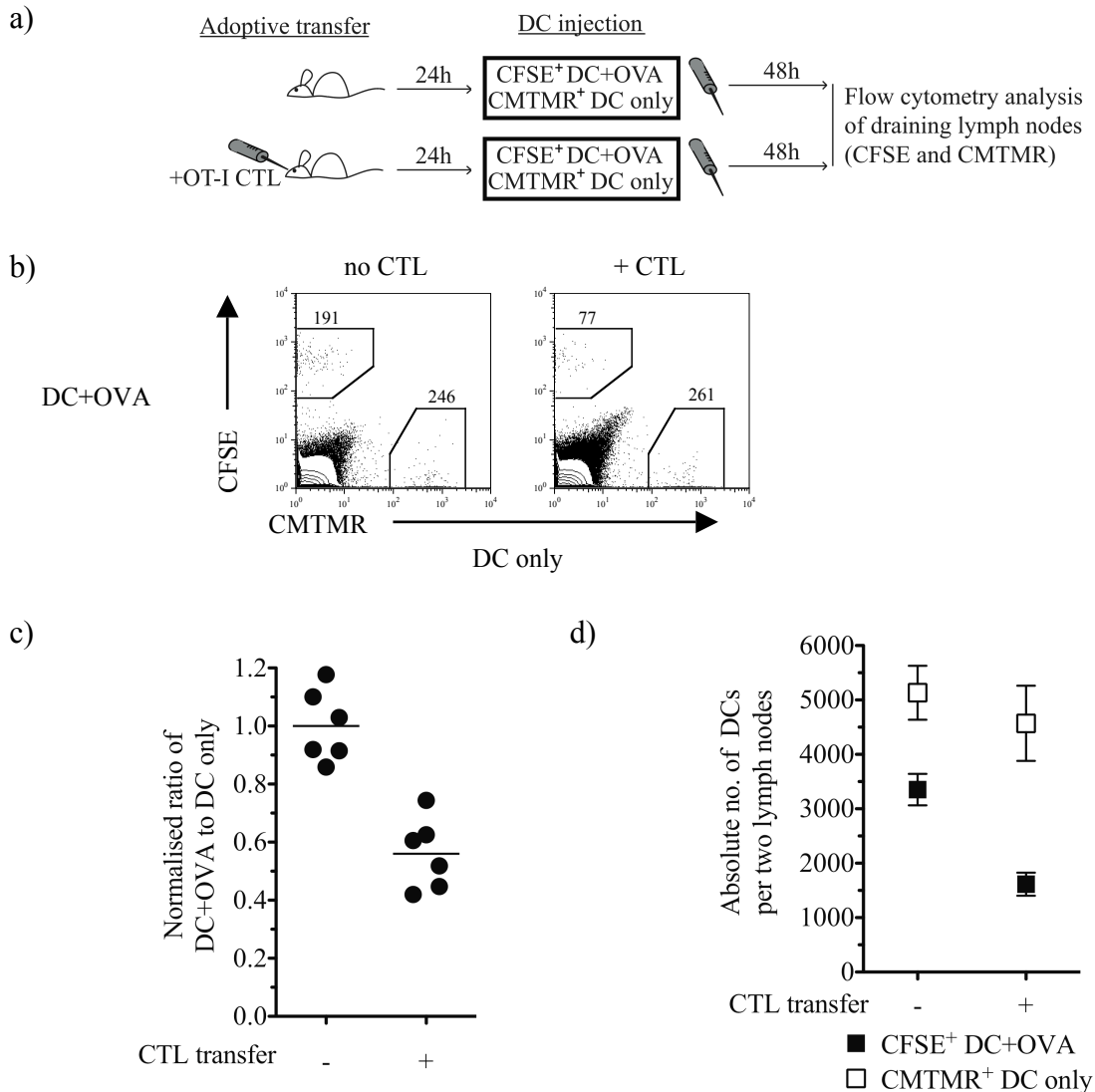
Fig. 4.2.5. **DCs loaded with OVA protein are not completely eliminated by *in vitro* activated CTLs.** (**a**) C57BL/6J mice received 10×10^6 *in vitro* activated OT-I CTLs (+ CTL) or no CTLs. 24 h later, CFSE-labelled DCs loaded with OVA protein (DC+OVA) were mixed with CMTMR-labelled control DCs (DC only) in equal numbers and were injected s.c. into the forelimbs of recipient mice. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**b**) CFSE$^+$ and CMTMR$^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. (**c**) The ratios of CFSE$^+$ DC+OVA to CMTMR$^+$ DC only were normalised to the ratios derived from mice that did not receive CTLs. (**d**) Absolute numbers of CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes are shown. The experiment was performed once with 3 mice per group.

4.3. Elimination of OVAtr DCs by *in vitro* activated CTLs, but not host CTLs, inhibits CD4$^+$ T cell proliferation

4.3.1. DCs cultured from immortalised OVAtr murine HSCs are phenotypically similar to wt BM-DCs

Using DCs loaded with OVA protein, I have shown that DC elimination by host or *in vitro* activated CTLs did not reduce CD4$^+$ T cell proliferation (Fig. 4.2.1 to 4.2.5). One possible reason for this finding is that not all the DCs loaded with OVA protein were eliminated by CTLs. Moreover, OVA-loaded DCs presented peptide-MHC class I complexes to varying degrees (Burgdorf et al., 2008), indicating that some OVA-loaded DCs might not be targeted for killing by CTLs. I asked if DCs presenting OVA protein via the classical MHC class I pathway would be a better target for CTL elimination and if the elimination of these DCs would then influence the induction of CD4$^+$ T cell proliferation. To address this, DCs were cultured from OVA-transgenic (OVAtr) murine haematopoietic stem cells (HSC). These OVAtr HSCs were generated through the immortalisation of HSCs derived from act-mOVA mice (Ruedl et al., 2008). Act-mOVA mice expressed OVA – H-2K^b fusion proteins that localised to the cell surface (Ehst et al., 2003).

In vitro culture of OVAtr HSCs yielded a higher percentage of CD11c$^+$CD11b$^+$ DCs than cultures from C57BL/6J mice (Fig. 4.3.1a). Higher percentages and MFIs of OVAtr DCs expressed MHC class II and CD80 compared to the wt DCs (Fig. 4.3.1b and Table 4.3.1). The MFI and percentage of OVAtr DCs expressing CD40 and CD86 were similar to the wt DCs (Table 4.3.2). Thus, GM-CSF/IL-4 and LPS treatment of these OVA-expressing HSCs yielded a high population of activated OVAtr DCs.

a)

b)
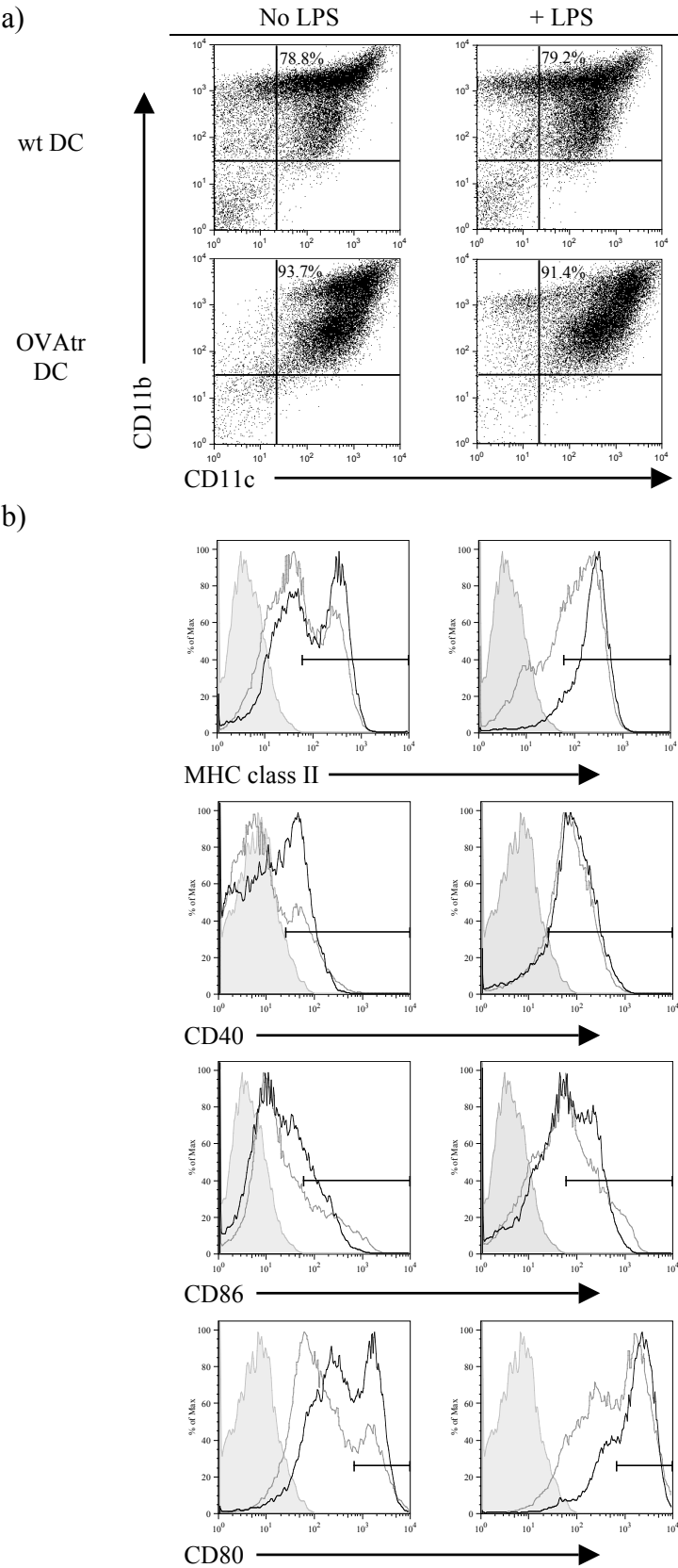
Fig. 4.3.1. *In vitro* **culture of OVAtr HSCs yields a high percentage of CD11c⁺CD11b⁺ OVAtr DCs.** Bone marrow cells from C57BL/6J (wt) mice and OVAtr HSCs were cultured with GM-CSF and IL-4 for 6 days *in vitro*. On day 6, some GM-CSF/IL-4 cell cultures were then treated with LPS for 24 h as described in Chapter 2. Some cell cultures were left untreated. Cells were harvested after 24 h and analysed for expressions of various phenotypic and activation markers. (**a**) The expressions of CD11b and CD11c in cultured cells are shown in dot plots. The percentages of CD11b⁺CD11c⁺ cells are shown. (**b**) The expressions of activation markers in CD11c⁺ cells are shown in histograms. Wt DCs are depicted in grey while OVAtr DCs are depicted in black. The unstained controls are shown as filled light grey areas. The percentages and MFIs of live CD11c⁺ cells expressing the activation markers are shown in Tables 8 and 9. The experiment was performed once together with that in Fig. 4.1 at the same time.

Table 4.3.1. Expression of activation markers on CD11c^{+} wt and OVAtr cells

	No LPS		+ LPS	
Activation marker	DC only	OVAtr DC	DC only	OVAtr DC
MHC class II	43.9%*	55.8%	61.8%	88%
CD40	25.3%	36.6%	84.6%	85.4%
CD86	25.5%	23.1%	47.5%	50.9%
CD80	23.4%	39.5%	49.3%	71.9%

* Percentages of activation markers highly expressed on CD11c^{+} cells as gated in Fig. 4.3.1b.

Table 4.3.2. MFIs of activation markers on CD11c^{+} wt and OVAtr cells

	No LPS		+ LPS	
Markers	DC only*	OVAtr DC	DC only	OVAtr DC
MHC class II	50.6	77.4	70.2	190
CD40	9.87	11.2	70.2	72.5
CD86	24.1	19.1	51.8	52.9
CD80	178	383	535	1163

* MFIs of activation markers expressed on CD11c^{+} cells in Fig. 4.3.1b.

4.3.2. Host CTLs eliminate OVAtr DCs

Host CTLs induced by SIINFEKL-loaded DC immunisation eliminated DCs loaded with exogenous OVA protein effectively (Fig. 4.2.1). I asked if the loading of endogenous OVA protein onto MHC class I molecules in DCs would also lead to efficient DC killing by host CTLs.

The experimental setup is shown in Fig. 4.3.2a. To induce host CTL responses, mice were immunised with DCs loaded with SIINFEKL (DC+SIINFEKL). Some mice were immunised with DCs loaded without SIINFEKL (DC only), or did not receive immunisation (not immunised). 7 days later, CFSE-labelled OVAtr DCs were mixed in equal numbers with CMTMR-labelled wt control DCs and were injected s.c. into the forelimbs of recipient mice. 48 h after DC injection, CFSE[+] and CMTMR^{+} cells in the draining lymph nodes were monitored by flow cytometry.

More CFSE[+] OVAtr DCs were collected compared to CMTMR[+] control DCs in mice that were immunised with DC only or did not receive immunisation, suggesting that DCs from immortalised HSC cultures had a better migration or survival advantage compared to DCs

from normal BM cultures. In contrast, CFSE$^+$ OVAtr DCs were nearly absent in mice immunised with DC+SIINFEKL (Fig. 4.3.2b and c). The number of OVAtr DC accumulating in the draining lymph nodes was less than 50 in mice immunised with DC+SIINFEKL (Fig. 4.3.2d). This result indicates that host CTLs eliminate OVAtr DCs effectively.
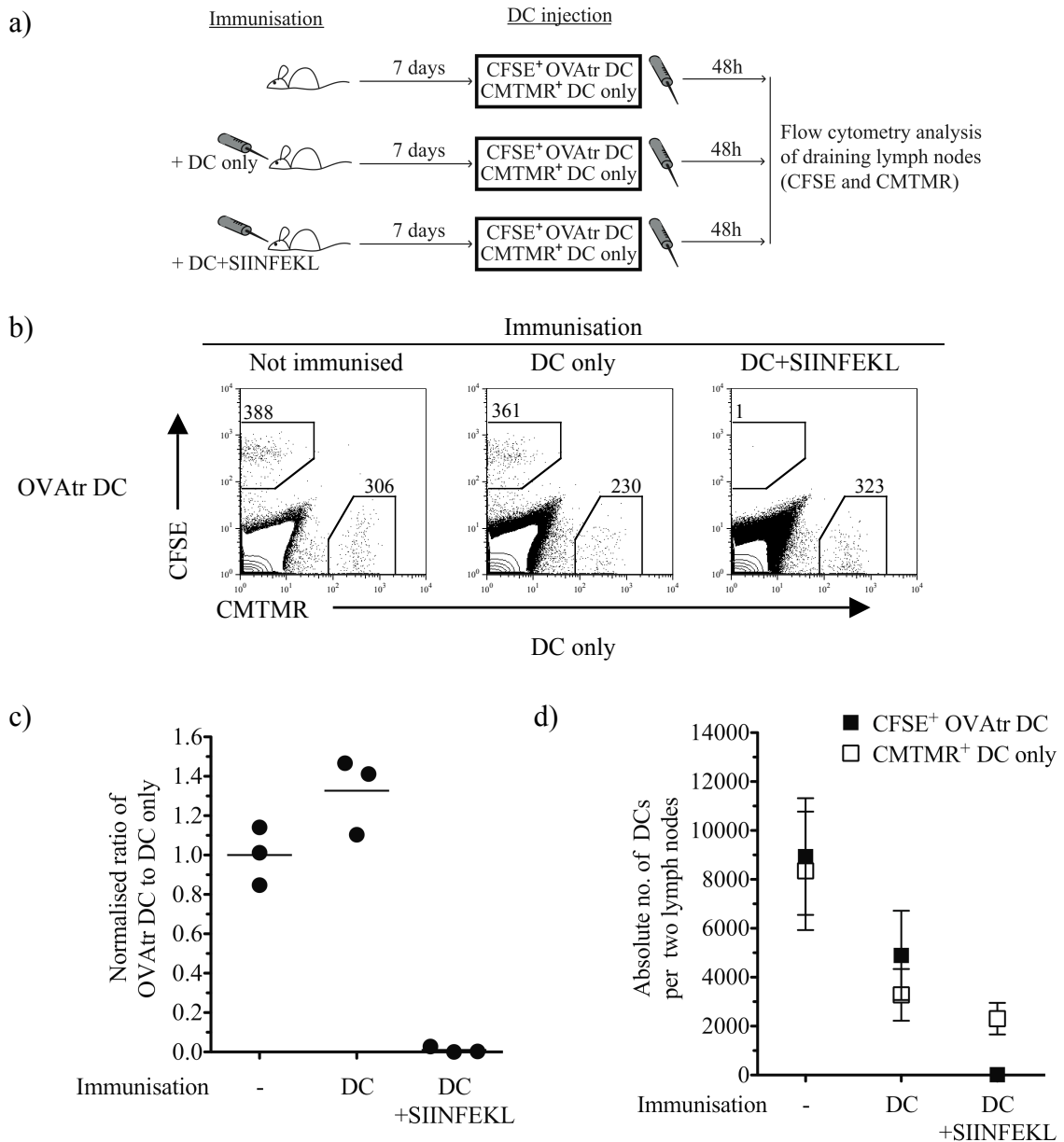
Fig. 4.3.2. **Host CTLs eliminate OVAtr DCs.** (**a**) C57BL/6J mice were immunised with DCs loaded with SIINFEKL (DC+SIINFEKL), or DCs not loaded with SIINFEKL (DC only), or did not receive immunisation (not immunised). 7 days later, CFSE-labelled OVAtr DC and CMTMR-labelled wt DC (DC only) were mixed in equal numbers and injected s.c. into the forelimbs of the recipient mice. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**b**) CFSE$^+$ and CMTMR$^+$ DCs are shown in representative dot plots from individual mice.

The number of events in each gate is shown. (**c**) The ratios of CFSE$^+$ OVAtr DC to CMTMR$^+$ DC only were normalised to the ratios derived from mice that did not receive immunisation. (**d**) Absolute numbers of CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes are shown. The experiment was performed once with 3 mice per group.

4.3.3. Host CTLs do not affect CD4$^+$ T cell proliferation induced by OVAtr DCs

Because most of the OVAtr DCs were eliminated by host CTLs (Fig. 4.3.2), I tested if host CTL-mediated elimination of OVAtr DCs would inhibit CD4$^+$ T cell proliferation.

The experimental setup is shown in Fig. 4.3.3a. Mice were immunised with DCs loaded without (DC only), or DCs loaded with SIINFEKL (DC+SIINFEKL), or did not receive immunisation (not immunised). 6 days later, CFSE-labelled OT-II CD4$^+$ T cells were injected i.v. into the recipient mice. 24 h following the adoptive transfer of CD4$^+$ T cells, OVAtr DCs were injected s.c. into the forelimbs of these mice. 3 days after DC injection, CD4$^+$ T cells in draining lymph nodes were examined for CFSE dilution by flow cytometry.

Although the percentages of divided CD4$^+$ T cells were lower in mice that were immunised with DC only or DC+SIINFEKL compared to control mice, the difference was not significant (Fig. 4.3.3b and c). The number of divided CD4$^+$ T cells was similar in all groups (Fig. 4.3.3d). When comparing the percentage and number of highly divided CD4$^+$ T cells (>4 divisions), no significant difference was observed between mice that were immunised with DC only and DC+SIINFEKL (data not shown). This result shows that although host CTLs eliminate OVAtr DCs effectively, this does not affect the induction of CD4$^+$ T cell proliferation.

a)

b)

Immunisation

OVAtr DC

c)

d)
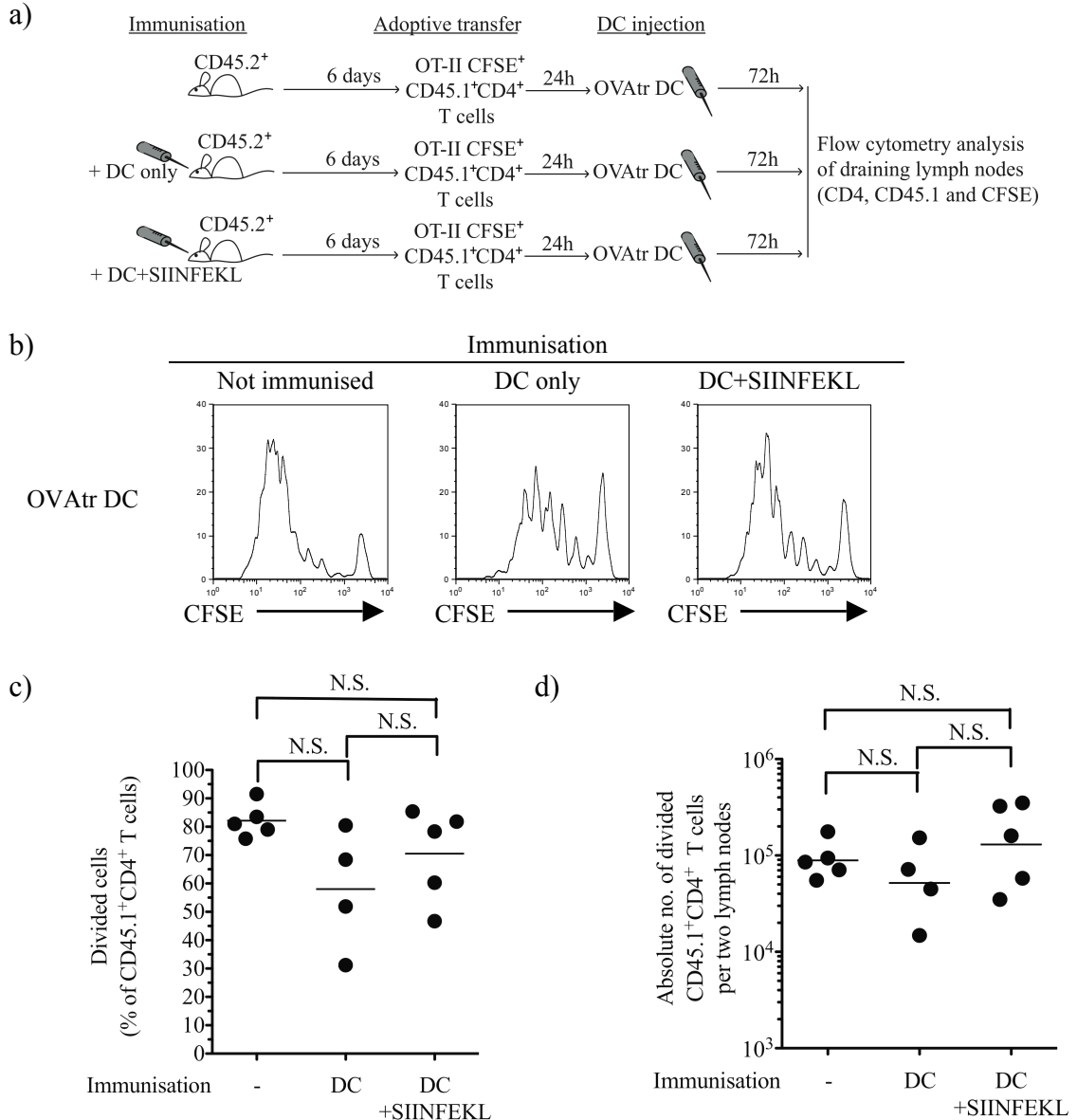
Fig. 4.3.3. **Host CTL-mediated elimination of OVAtr DCs does not inhibit CD4$^+$ T cell proliferation.** (**a**) C57BL/6J mice were immunised with DCs loaded with SIINFEKL (DC+SIINFEKL), or DCs not loaded with SIINFEKL (DC only), or did not receive immunisation (not immunised). 6 days later, CFSE$^+$ OT-II CD45.1$^+$CD4$^+$ T cells were injected i.v. into these mice. 24 h later, OVAtr DCs were injected s.c. in the forelimbs of the recipient mice. 3 days later, CD45.1$^+$CD4$^+$ T cells were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 5 mice per group. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. N.S.= p>0.05.

4.3.4. *In vitro* activated CTLs eliminate OVAtr DCs

In vitro activated CTLs were capable of eliminating SIINFEKL-loaded DCs effectively (Fig. 3.5.3). *In vitro* activated CTLs also eliminated OVA-loaded DCs but the elimination of OVA-loaded DCs was less efficient than that of SIINFEKL-loaded DCs (Fig. 4.2.5). The differences observed between CTL-mediated killing of SIINFEKL-loaded DCs and OVA-loaded DCs could be due to the different mechanisms used to load SIINFEKL peptide or exogenous OVA protein onto MHC class I molecules, or different amount of peptides loaded on MHC class I molecules. I asked if the loading of endogenous OVA protein onto MHC class I molecules on DCs would lead to efficient DC killing by *in vitro* activated CTLs.

To determine if *in vitro* activated CTLs eliminated OVAtr DCs, one group of mice were given *in vitro* activated CTLs while the control group did not receive CTLs. The experimental setup is shown in Fig. 4.3.4a. 24 h later, CFSE$^+$ OVAtr DCs and CMTMR$^+$ wt control DCs (DC only) were mixed in equal numbers and injected s.c. into these recipient mice. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ cells were monitored by flow cytometry.

Although in the control group the number of CFSE$^+$ OVAtr DCs was higher than the number of CMTMR$^+$ DC only, CFSE$^+$ OVAtr DCs were nearly absent in mice that received CTLs (Fig 4.3.4b and c). This result indicates that *in vitro* activated CTLs eliminated OVAtr DCs effectively.

a)

b)

c)   d)
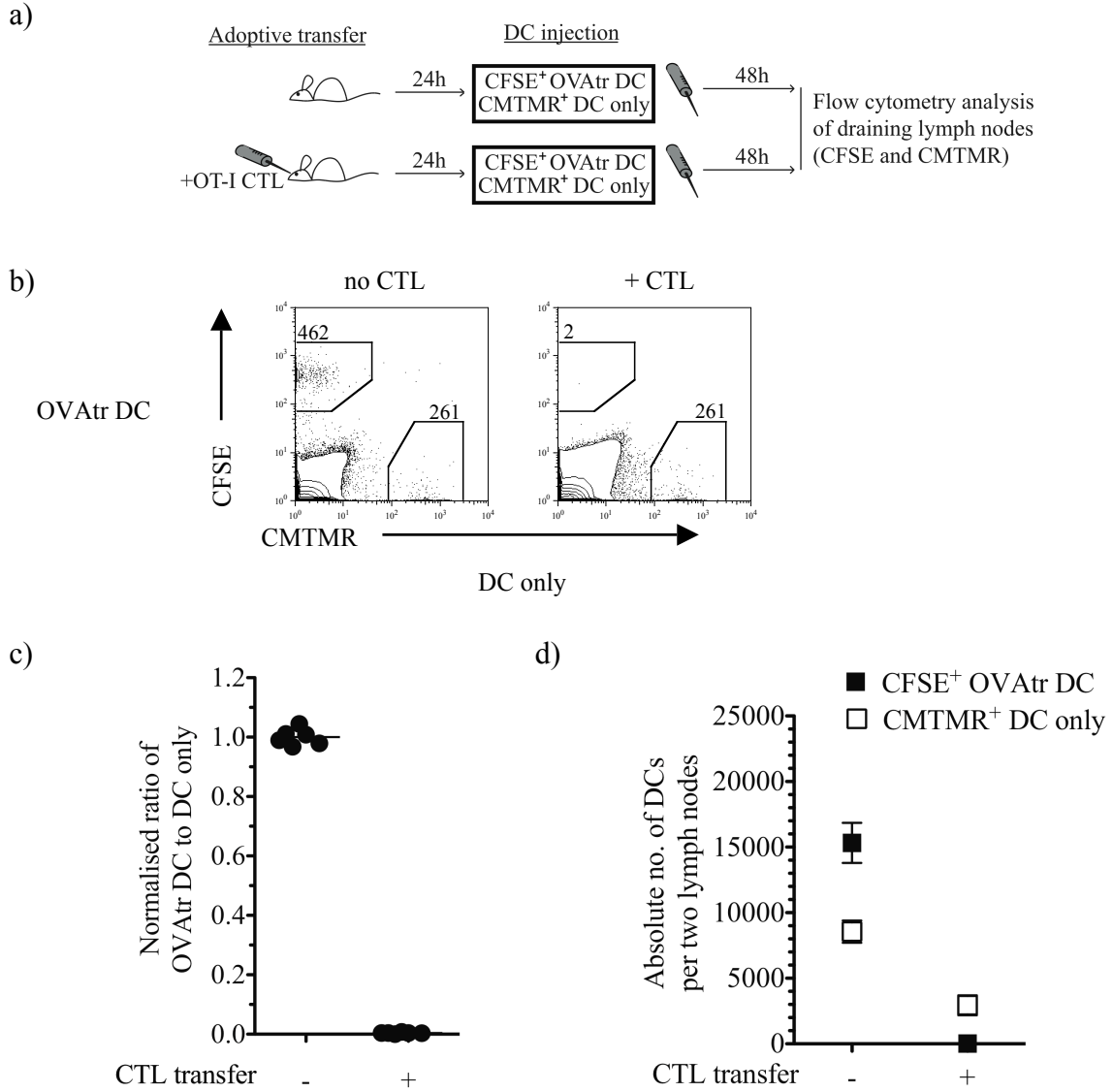
Fig. 4.3.4. *In vitro* **activated CTLs eliminate OVAtr DCs.** (**a**) C57BL/6J mice were injected i.v. with 10×10^6 *in vitro* activated OT-I CTLs (+ CTL) or no CTLs. 24 h later, CFSE$^+$ OVAtr DCs and CMTMR$^+$ wt DCs (DC only) were mixed in equal numbers and injected s.c. into the forelimbs of recipient mice. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**b**) CFSE$^+$ and CMTMR$^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. (**c**) The ratios of CFSE$^+$ OVAtr DCs to CMTMR$^+$ DC only were normalised to the ratios derived from mice that did not receive CTLs. (**d**) Absolute numbers of CFSE$^+$ and CMTMR$^+$ cells in the draining lymph nodes are shown. One representative experiment of two with 3 mice per group is shown.

4.3.5. *In vitro* activated CTL-mediated elimination of OVAtr DCs inhibits CD4$^+$ T cell proliferation

One possible reason why *in vitro* activated CTLs could not inhibit CD4$^+$ T cell proliferation induced by OVA-loaded DCs is because the CTLs did not completely eliminate the OVA-loaded DCs (Fig. 4.2.5). Because *in vitro* activated CTLs eliminated OVAtr DCs (Fig. 4.3.4) as effectively as SIINFEKL-loaded DCs (Fig. 3.5.3), I asked whether the effective CTL-mediated elimination of OVAtr DCs would inhibit CD4$^+$ T cell proliferation.

To investigate if *in vitro* activated CTL-mediated elimination of OVAtr DCs inhibited CD4$^+$ T cell proliferation, mice received CFSE-labelled OT-II CD4$^+$ T cells and *in vitro* activated CTLs (Fig. 4.3.5a). Some mice received only CD4$^+$ T cells. 24 h later, OVAtr DCs were injected s.c. into the forelimbs of these mice. 3 days after DC injection, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

Although the majority of CD4$^+$ T cells divided in both groups (Fig. 4.3.5b), the percentage and number of divided CD4$^+$ T cells were significantly lower in mice that received CTLs than in the control mice (Fig. 4.3.5c and d). This result shows that *in vitro* activated CTLs inhibited the induction of CD4$^+$ T cell proliferation through the elimination of OVAtr DCs. However, it should be noted that although antigen-presenting DCs did not accumulate in the draining lymph nodes, the induction of CD4$^+$ T cell proliferation was not abolished.

a)

Adoptive transfer   DC injection

OT-II CFSE$^+$ CD45.2$^+$
CD45.1$^+$CD4$^+$ T cells $\xrightarrow{24h}$ OVAtr DC $\xrightarrow{72h}$

OT-II CFSE$^+$ CD45.2$^+$
CD45.1$^+$CD4$^+$ T cells $\xrightarrow{24h}$ OVAtr DC $\xrightarrow{72h}$
+ **OT-I CTL**

Flow cytometry analysis
of draining lymph nodes
(CD4, CD45.1 and CFSE)

b)

no CTL   + CTL

OVAtr DC

CFSE $\longrightarrow$ CFSE $\longrightarrow$

c)

p=0.0014

Divided cells
(% of CD45.1$^+$CD4$^+$ T cells)

CTL transfer   -   +

d)

p=4.17x10^{-6}

Absolute no. of divided
CD45.1$^+$CD4$^+$ T cells
per two lymph nodes

CTL transfer   -   +
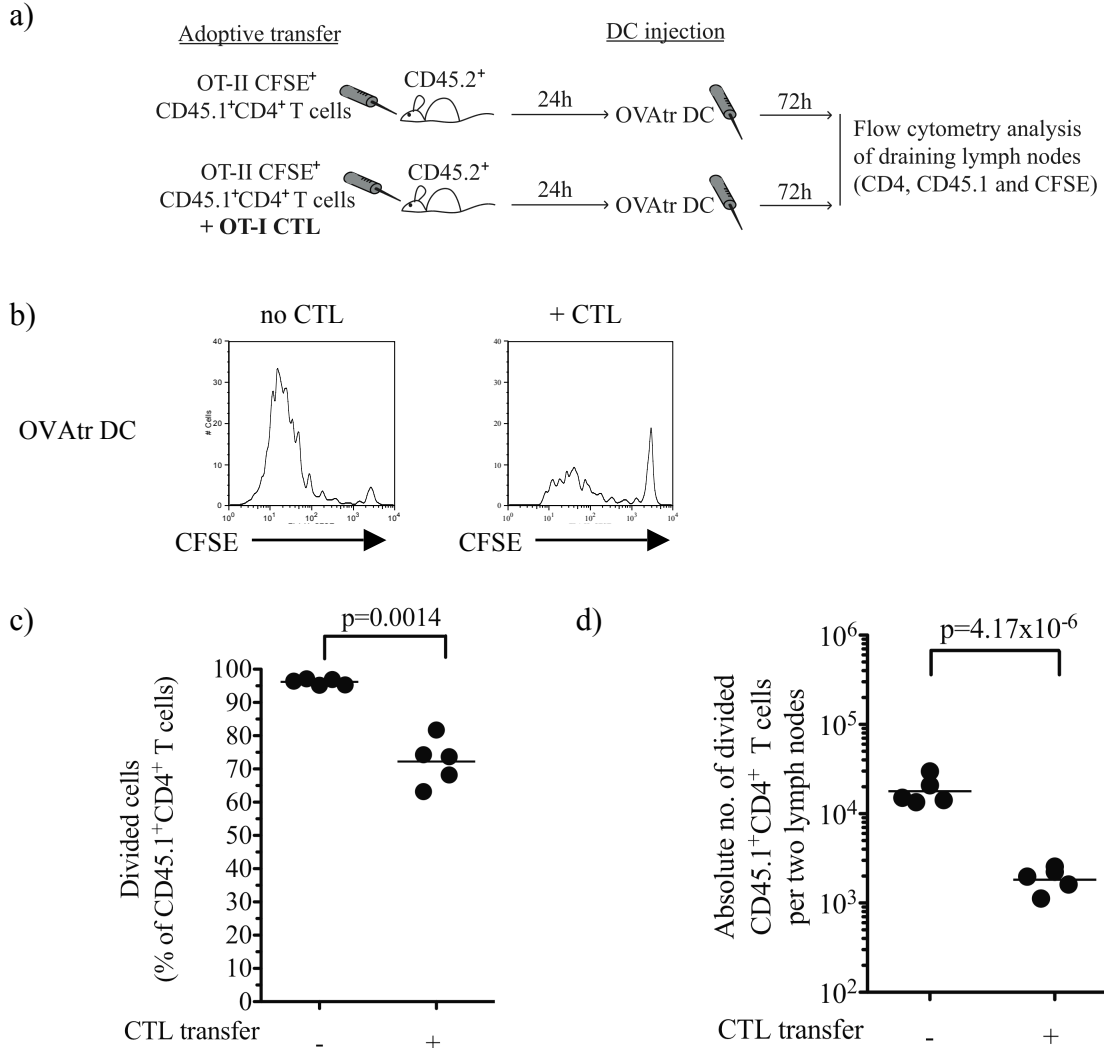
Fig. 4.3.5. *In vitro* **activated CTL-mediated elimination of OVAtr DCs inhibits CD4$^+$ T cell proliferation.** (**a**) C57BL/6J mice were injected i.v. with *in vitro* activated OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. Some mice received only CD4$^+$ T cells. 24 h later, OVAtr DCs were then injected s.c. into the forelimbs of the recipient mice. 3 days later, CD45.1$^+$CD4$^+$ T cells were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. One representative experiment of two with 5 mice per group is shown. Statistical significance was determined with two tailed Student's *t*-test.

4.4. Discussion

In this chapter, I have shown that host CTLs induced by peptide-loaded DC immunisation and or by the adoptive transfer of *in vitro* activated OT-I CTLs targeted OVA-loaded DCs and OVAtr DCs for elimination. In particular, DC elimination was highly efficient when OVAtr DCs were used as targets. This was not the case when DCs loaded with soluble OVA protein were used, as half of these DCs escaped CTL killing and accumulated in detectable numbers in the draining lymph nodes. The elimination of OVA-loaded DCs by host CTLs, or *in vitro* activated CTLs, was not sufficient to inhibit CD4$^+$ T cell proliferation. Neither did the efficient elimination of OVAtr DCs by host CTLs reduce CD4$^+$ T cell proliferation. Only *in vitro* activated OT-I CTLs inhibited the proliferation of CD4$^+$ T cells induced by OVAtr DCs significantly, although large numbers of these divided CD4$^+$ T cells were still observed. Overall, the results indicate that the different MHC class I loading mechanisms used by DCs have different implications in terms of CTL-mediated DC killing and induction of subsequent T cell responses. In addition, the method of CTLs generation may also influence the induction of T cell responses.

4.4.1. Types of CTLs used and their effect on CD4$^+$ T cell responses

It has long been established that immunisation with DCs loaded with MHC class I peptide induced antigen-specific CTLs that can confer protection against tumours expressing that particular antigen (Porgador and Gilboa, 1995; Porgador et al., 1996). An advantage of peptide-loaded DC immunisation over adoptive transfer of transgenic CTLs is that DC immunisation induces polyclonal T cell responses. In my study, the quality of the polyclonal CTLs was comparable to, if not better than, the OT-I TCR transgenic CTLs that recognised a single immunodominant epitope as both types of CTLs were able to eliminate OVA-loaded DCs and OVAtr DCs efficiently. That said, although the killing function of CTLs induced by peptide-loaded DC immunisation was intact, the elimination of OVAtr DCs by host CTLs did not reduce CD4$^+$ T cell proliferation. In contrast, a significant reduction of CD4$^+$ T cell proliferation was observed when OT-I CTLs were used. One possible reason for the differences in reducing CD4$^+$ T cell proliferation observed could be due to the numbers of CTLs present and the different avidities. In my study, OT-I CTLs

were transferred at 5×10^6 or 10×10^6 cells into the recipient mice. All the transferred OT-I CTLs could potentially target the OVAtr DCs for killing. In contrast, the immunisation of mice with SIINFEKL-loaded DCs induced polyclonal host CTLs. The number of polyclonal host CTLs could not be controlled for and might vary from mouse to mouse. Moreover, because of their polyclonal nature, not all the host CTLs generated by DC immunisation targeted OVAtr DCs with the same avidity. Although the differences between OT-I and host CTLs were not obvious in eliminating OVAtr DCs, it remained possible that these differences accumulated and affected the induction of CD4$^+$ T cell proliferation instead. It is also possible that OT-I and host CTLs produce different levels of cytokines although it is unclear how the different levels of cytokines can account for the abovementioned discrepancy.

It is also striking that while both types of CTLs were very effective in DC killing, neither of them could abolish CD4$^+$ T cell proliferation. This cannot be due to the different types of CTLs used as a large number of CD4$^+$ T cells divided in the presence of OT-I CTLs or host CTLs. I will discuss this in 4.4.3.

4.4.2. Different MHC class I presentation pathways and the induction of CD4$^+$ T cell responses

Although *in vivo* and *in vitro* activated CTLs were functionally comparable in terms of DC killing, the susceptibility of DCs to CTL-mediated elimination varied according to how antigens were loaded onto the DCs. Distinct antigen loading pathways are employed when DCs cross-present exogenous proteins or present endogenous proteins. Not all the BM-DCs took up exogenous OVA protein, even when loaded with protein *in vitro* for two days (Delamarre et al., 2003) (Appendix 7). Although the process of cross-presenting soluble OVA protein was enhanced by the treatment of LPS and disruption of DC clusters by repeated pipetting *in vitro* (Delamarre et al., 2003), a combination of LPS and cluster disruption did not ensure that all DCs took up OVA protein *in vitro* (Appendix 7). LPS-activated OVA-loaded DCs also presented OVA peptide-MHC class I complexes in varying amounts (Burgdorf et al., 2008), indicating that CTLs might not eliminate all the OVA-loaded DCs because not all of the DCs presented antigens to CTLs. This is unlike

OVAtr DCs, which produce endogenous OVA protein ubiquitously under the actin promoter and present high amounts of OVA peptide-MHC class I complexes(Ehst et al., 2003). Thus, the difference in CTL-mediated killing of OVA-loaded DCs and OVAtr DCs is most likely due to the different amounts of peptide-MHC class I complexes on the cell surface of DCs.

The different sensitivities of DCs to CTL-mediated killing could be the reason why different CD4$^+$ T cell proliferative responses were observed after immunisation with OVA-loaded DCs and OVAtr DCs. More OVA$^+$ DCs than OVA-loaded DCs that survived CTL-mediated elimination (Burgdorf et al., 2008) (Appendix 7 and Fig. 4.2.5), therefore it was more likely that some of these DCs were presenting OVA protein on MHC class II but not on MHC class I. In contrast, all the OVAtr DCs should present SIINFEKL-MHC class I complexes for CTL-mediated killing because the peptides derived from these degraded cellular proteins were loaded onto MHC class I molecules due to continuous protein turnover (Rock and Goldberg, 1999), leaving no OVAtr DCs to present to CD4$^+$ T cells.

4.4.3. Residual CD4$^+$ T cell proliferation when DCs fail to accumulate in the draining lymph nodes

Interestingly, when OVAtr DCs did not accumulate in the draining lymph nodes due to DC killing by host or *in vitro* activated CTLs, substantial CD4$^+$ T cell division was observed. There are a few possible reasons for these observations.

Some studies have shown that DCs injected s.c. could be found in the spleens of mice and the DCs that migrated to the spleen could induce T cell responses (Mullins et al., 2003). However, we were not able to find BM-DCs injected s.c. into the spleens of mice (Huck et al., 2008). When BM-DCs were injected in the ears of mice, most of the DCs accumulated at the site of injection while some DCs migrated to the draining lymph nodes (Yang et al., 2006). Thus, the residual CD4$^+$ T cell proliferation is unlikely to be induced by DCs that have migrated to the spleen from the forelimbs of the recipient mice.

Another possible reason is that OVAtr DCs might be inherently different from the BM-DCs because OVAtr DCs were cultured from immortalised HSCs. In my study, I have shown that OVAtr DCs were phenotypically similar to BM-DCs. Ruedl et al have also previously shown that DCs cultured from immortalised HSCs were similar to the DCs cultured from BM cells (Ruedl et al., 2008). Furthermore, in my study, the residual $CD4^+$ T cell proliferation was not observed only after OVAtr DCs injection. I have shown that OT-I CTLs also eliminated both SIINFEKL-loaded BM-DCs and OVAtr DCs efficiently (Fig. 3.5.3). However, residual $CD8^+$ T cell proliferation was observed even though a few SIINFEKL-loaded BM-DCs accumulated in the draining lymph nodes (Fig. 3.5.5). Thus, the residual $CD4^+$ T cell proliferation is not an artefact of using OVAtr DCs cultured from immortalised HSCs.

It is possible that although CTLs prevented OVAtr DCs from entering the draining lymph nodes, OVAtr DCs could induce $CD4^+$ T cell expansion in the non-lymphoid tissues. However, this has only been shown to occur during HSV infection and for re-activation of HSV memory T cells (Wakim et al., 2008). Naïve T cells do not have access to non-lymphoid tissues (Mackay et al., 1990). Thus, the residual $CD4^+$ T cell proliferation is not induced by OVAtr DCs in the non-lymphoid tissues.

In my study, a few OVAtr DCs that survived CTL-mediated killing were found in the draining lymph nodes and these DCs could have induced $CD4^+$ T cell proliferation. Sufficient number of antigen-presenting DCs is necessary for inducing T cell proliferation. For example, when 0.18×10^6 antigen-bearing DCs were injected, approximately 180 injected DCs reached the draining lymph nodes and induced very little $CD4^+$ T cell proliferation (Martin-Fontecha et al., 2003). In my study, when CTLs eliminated OVAtr DCs, an average of 24 OVAtr DCs was recovered from the draining lymph nodes and induced sizable $CD4^+$ T cell proliferation (~70% $CD4^+$ T cells proliferated). Thus, the residual $CD4^+$ T cell proliferation is unlikely to be induced only by the few OVAtr DCs that escaped CTL-mediated killing.

One plausible explanation for the residual $CD4^+$ T cell proliferation is the transfer of antigens from OVAtr DCs to host DCs. Host DCs could have captured the antigens carried

by the injected DCs and presented them to the CD4$^+$ T cells in the draining lymph nodes. Indeed, inter-DC antigen transfer has been shown in a viral infection model (Allan et al., 2006), and DC immunisation models (Kleindienst and Brocker, 2003; Luketic et al., 2007). This possibility will be addressed in chapter 5.

Chapter 5

Host DCs present antigens carried by injected DCs to T cells

In Chapter 4, I have shown that while host effector CD8$^+$ cells or *in vitro* activated CTLs eliminated a large proportion of DCs cross-presenting OVA protein, the induction of CD4$^+$ T cell proliferation was not affected. I have also shown that although the presentation of endogenous proteins led to complete DC elimination and significant inhibition of CD4$^+$ T cell proliferation, this CD4$^+$ T cell proliferation was not abolished. Furthermore, the CD8$^+$ T cell proliferation was still detectable when the SIINFEKL-presenting DCs were completely eliminated as shown in Chapter 3. Because the extent of T cell proliferation depends on the number of antigen-presenting DCs that reach the draining lymph nodes (Martin-Fontecha et al., 2003), the presence of T cell division when most antigen-presenting DCs are eliminated is a conundrum.

One likely explanation for this conundrum is that when no injected DCs appears to reach the draining lymph nodes, the host DCs take up antigens from the injected DCs and present these antigens to CD4$^+$ and CD8$^+$ T cells. Recent evidence suggests that antigen-bearing DCs may transfer antigens to other DCs. Skin- and lung-derived migratory DCs have been shown to ferry viral antigens from the skin to the resident DCs in the draining lymph nodes (Allan et al., 2006; Belz et al., 2004b). Antigens were subsequently transferred from migratory DCs to the lymph node resident DCs. This enables the resident DCs to stimulate T cell responses. Such interactions between the migratory and non-migratory DCs highlight the existence of interplay amongst the heterogeneous population of DCs; and in my study, suggest that the migrating injected DCs may also transfer antigens to host DCs.

In this chapter, I will address the following aims:

1) To examine if the low number of antigen-presenting DCs that survive CTL elimination is sufficient to induce T cell proliferation
2) To investigate if host DCs are involved in presenting antigens loaded onto injected DCs to T cells
3) To examine the quality of effector T cells when host DCs present antigens carried by injected DCs that cannot present antigens directly to T cells

5.1. Host cells take up cellular materials of injected DCs *in vivo*

Previous observations showed that OVA transgenic (OVAtr) DCs were completely eliminated by host and transferred CTLs (Fig. 4.3.2 and Fig. 4.3.4). However, when DCs were completely killed, the $CD4^+$ T cell proliferation was not abolished (Fig. 4.3.3 and Fig. 4.3.5). I hypothesized that materials from the injected DCs were being taken up by host DCs, allowing the host DCs to induce $CD4^+$ T cell proliferation.

Fig. 5.1a illustrates how the experiment was carried out. To show whether host cells were taking up materials from injected DCs *in vivo*, $CD45.2^+$ OVAtr DCs were incubated with FITC-conjugated dextran and subsequently labelled with CMTMR ($FITC^+CMTMR^+$) so as to clearly distinguish them from the $CD45.1^+$ host DCs. As controls, some DCs were incubated with FITC-conjugated dextran only ($FITC^+$) but not labelled with CMTMR, whereas some DCs were not incubated with FITC-conjugated dextran but labelled with CMTMR ($CMTMR^+$) (Appendix 8c). These DCs were injected into $CD45.1^+$ hosts that had previously received *in vitro* activated CTLs or no CTLs. 2 days after DC administration, DC populations in the draining lymph nodes were monitored by flow cytometry. Three DC populations were recovered. Injected OVAtr DCs were $CD45.1^-CMTMR^+$ (Population #1 in Fig. 5.1b) and the respective single stain controls are shown in Appendix 8a. Host DCs that took up materials from injected DCs were $CD45.1^+CMTMR^-FITC^+$ (Population #3 in Fig. 5.1b) and the respective single stain controls are shown in Appendix 8b. A small population of $CD45.1^+CMTMR^+$ cells (Population #2 in Fig. 5.1b) was observed and could be host cells that have taken up CMTMR or injected DCs that have taken up CD45.1.

As observed in previous experiments (Fig. 4.3.4), the number of injected DCs ($CD45.1^-CMTMR^+$, #1) was reduced in mice that received CTLs compared to control mice (Fig. 5.1c). In accordance with my hypothesis, some $CD45.1^+$ host cells were $FITC^+$ (Fig. 5.1c). The number of $CD45.1^+CMTMR^-FITC^+$ host cells (#3) was also lower in mice that have received CTLs compared to control mice, suggesting that host cells that captured antigens from the injected DCs might have become CTL targets.

In the same experiment, some groups of mice received wt DCs that were not loaded with added antigens. No CTL-mediated elimination of CD45.1$^-$CMTMR$^+$ DCs (#1) was observed. The number of CD45.1$^+$CMTMR$^-$FITC$^+$ host cells (#3) was similar between mice that have received CTLs and control mice (Fig. 5.1d). Taken together, the results suggest that host cells take up the materials from injected DCs. It is also interesting to note that although CTLs mediated apoptosis of the OVAtr DCs, the demise of OVAtr DCs did not increase the number of host cells that had taken up materials from injected DCs. Instead, a slight decrease of CD45.1$^+$CMTMR$^-$FITC$^+$ host cells was observed. This might indicate that host DCs were eliminated by CTLs (Mueller et al., 2006).

a)

b) Gating strategy

c) Mice receiving OVAtr DC

d) Mice receiving wt DC only
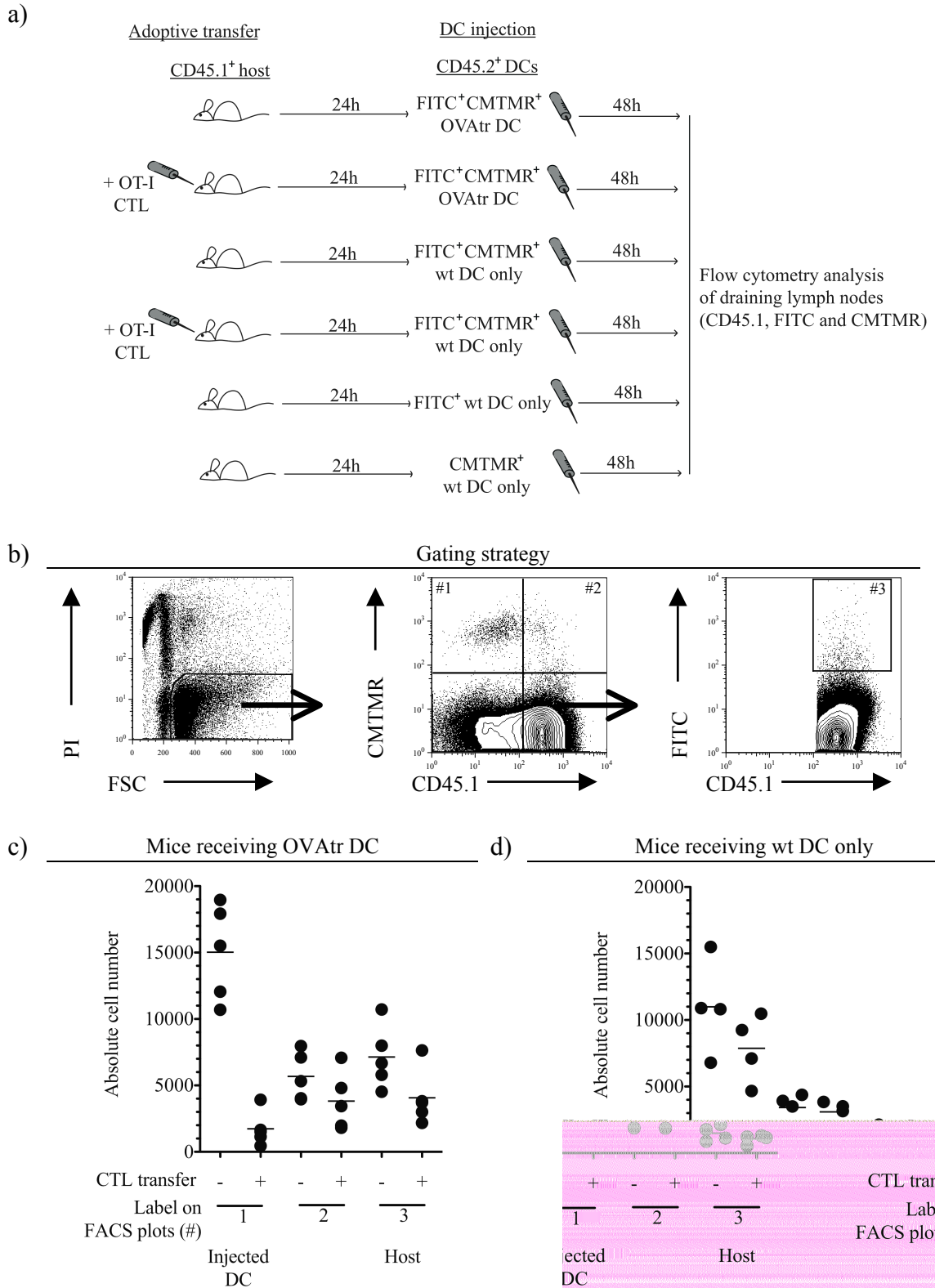
Fig. 5.1. **Host cells acquire materials from injected DCs *in vivo*.** (**a**) OVAtr DCs or wt DCs were incubated with FITC-dextran and subsequently activated with LPS. 24 h after LPS treatment, these FITC-dextran DCs were labelled with CMTMR. These CMTMR$^+$ FITC-dextran OVAtr DCs were then injected s.c. into the forelimbs of B6.SJ *ptprca* mice that were previously injected with *in vitro* activated OT-I CTLs 24 h prior to DC injection. As a control, some mice did not receive CTLs. 48 h after DC

injection, FITC$^+$, CMTMR$^+$ and CD45.1$^+$ cells in the draining lymph nodes were monitored by flow cytometry. (**b**) The gating strategy used to analyse the results is illustrated. Population #1 represents the injected CD45.1$^-$CMTMR$^+$ DCs. Populations #2 and #3 represent CD45.1$^+$CMTMR$^+$ cells and host CD45.1$^+$CMTMR$^-$FITC$^+$ cells respectively. (**c** and **d**) Absolute numbers of different DC populations in the draining lymph nodes are shown. One representative experiment of two with 1 – 5 mice per group is shown.

5.2. OVA produced by OVAtr DCs is taken up by other DCs and presented to CD4$^+$ T cells *in vitro*

Although I have shown that host cells took up materials from OVAtr DCs in Fig 5.1, the transfer of OVA protein from the OVAtr DCs to host cells was not measured directly.

To determine whether the OVA protein from OVAtr DCs was transferred to other cells for presentation to CD4$^+$ T cells, an *in vitro* CD4$^+$ T cell proliferation assay was performed using transwell chambers with 1.0 μm pore size (Fig. 5.2). 0.1×10^6 LPS-activated wt DCs and 0.3×10^6 CFSE$^+$ OT-II CD4$^+$ T cells were plated in the bottom insert of the trans-well. In some wells, 2D2 CD4$^+$ T cells, which are specific for an irrelevant myelin oligodendrocyte glycoprotein (MOG) peptide, were used as a specificity control. In the top insert, OVAtr DCs were plated from 0.1×10^6 to 1.2×10^6. As positive controls, OVA protein or OVA$_{323-339}$ peptide, instead of OVAtr DCs, was added in the top insert of some wells. As a negative control, some wells did not receive any antigens or OVAtr DCs. 5 days later, CD4$^+$ T cells were examined for CFSE dilution by flow cytometry.

In the absence of antigen or OVAtr DCs, no CD4$^+$ T cell proliferation was observed. Wt DCs in the bottom insert were able to induce strong CD4$^+$ T cell proliferation as observed in wells where OVA$_{323-339}$ peptide was added. Similarly, strong CD4$^+$ T cell proliferation was also observed in wells where soluble OVA protein was added, showing that these wt DCs in the bottom insert were capable of capturing, processing and presenting exogenous soluble protein. CD4$^+$ T cell proliferation was observed when OVAtr DCs were added. As the number of OVAtr DCs increased, more CD4$^+$ T cells underwent cell division. This proliferation was antigen-specific as 2D2 CD4$^+$ T cells remained undivided even at the highest number of OVAtr DCs. The proliferation of OT-II, but not 2D2 CD4$^+$ T cells, indicated that OVA protein released by OVAtr DCs was taken up by other DCs and

presented to antigen-specific CD4$^+$ T cells. However, I cannot rule out the possibility that the OVAtr DCs can reach across the transwells via membrane nanotubes to present antigens to CD4$^+$ T cells in the bottom insert (Chinnery et al., 2008).
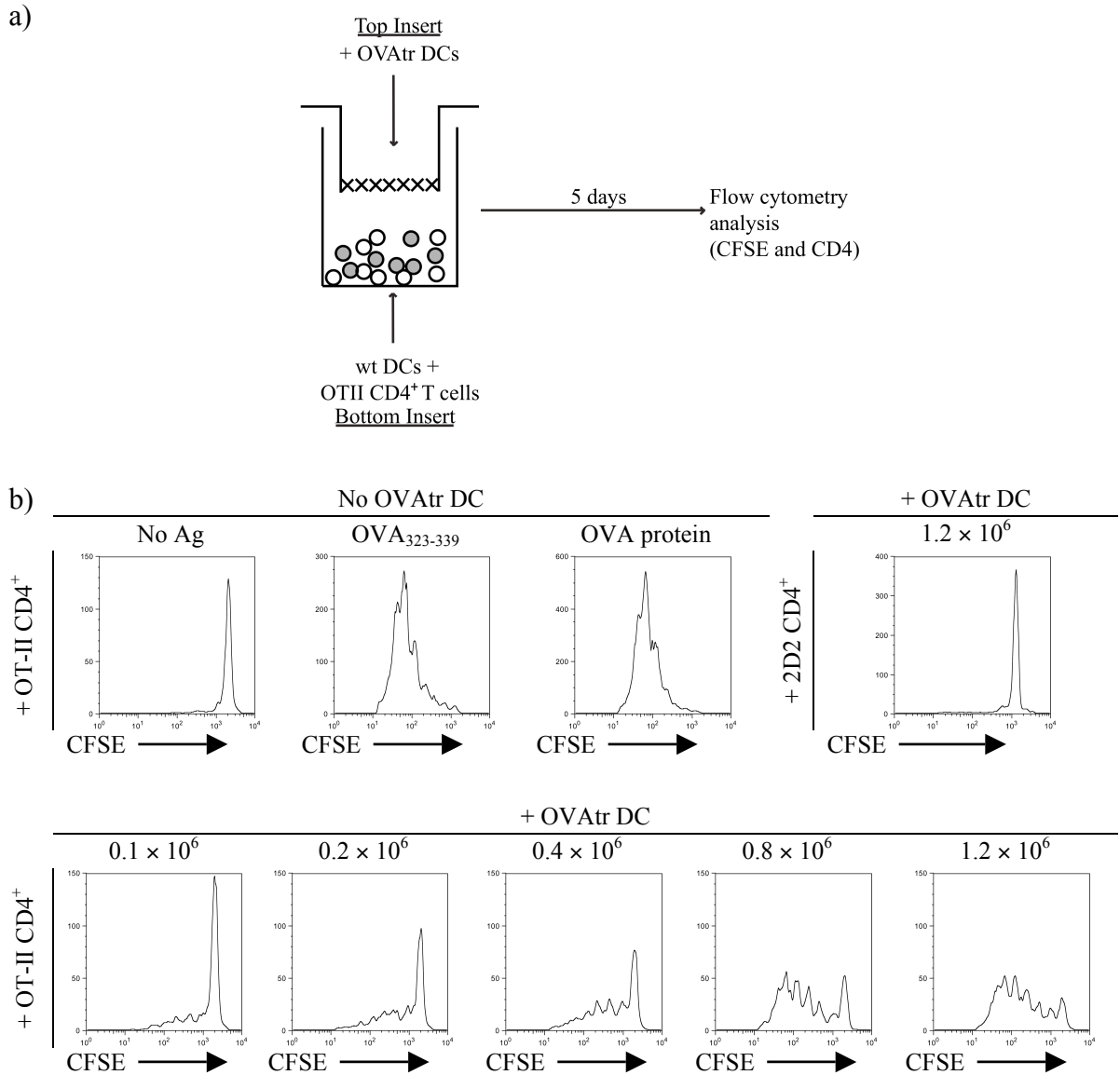
Fig. 5.2. **OVAtr DCs release functional OVA that is captured and presented by other DCs to T cells.** LPS-activated DCs were plated with CFSE-labelled OT-II CD4$^+$ T cells at 0.1×10^6 DC to 0.3×10^6 CD4$^+$ T cells in the bottom insert. As a control, 2D2 CD4$^+$ T cells were also used. In the top insert, OVAtr DCs were plated from 0.1×10^6 to 1.2×10^6 cells. The control wells received OVA$_{323-339}$, OVA protein or no antigen. The pore size of the transwell is 1.0 μm. 5 days later, CD4$^+$ proliferation was examined for CFSE dilution by flow cytometry. (**a**) The schematic diagram of the transwell setup is shown. (**b**) CFSE dilution in CD4$^+$ T cells is shown as representative histograms from individual wells. The experiment was performed once with duplicate wells.

5.3. MHCII$^{-/-}$ DCs loaded with OVA protein are eliminated by *in vitro* activated CTLs *in vivo*

Because host cells can capture and present antigens derived from OVAtr DCs, it is also plausible that a similar situation may occur with the DCs loaded with soluble OVA protein. I have previously shown in Fig. 4.2.5 that not all DCs loaded with soluble OVA protein (DC+OVA) were killed by OVA-specific CTLs *in vivo* and these surviving DCs accumulated in the draining lymph nodes. One possibility is that these surviving DCs were the ones inducing the strong CD4$^+$ T cell proliferation observed in my previous results (Fig. 4.2.4).

To prevent the surviving DCs from presenting antigens directly to CD4$^+$ T cells, I used MHC class II$^{-/-}$ DCs (MHCII$^{-/-}$) that were cultured from the bone marrow cells of B6Aa^0/Aa^0 mice. As shown in Appendix 9, MHCII$^{-/-}$ DCs were unable to induce the proliferation of CFSE-labelled OT-II cells *in vitro*, whereas wt DCs induced strong OT-II proliferation. This result shows that MHCII$^{-/-}$ DCs do not present antigens to CD4$^+$ T cells.

Having confirmed that MHCII$^{-/-}$ DCs did not present antigens to CD4$^+$ T cells, I asked if these DCs were eliminated by CTLs in a similar fashion to their wild type counterpart.

The experimental setup is illustrated in Fig. 5.3a. Mice were injected i.v. with *in vitro* activated CTLs while the control group were not. Wt or MHCII$^{-/-}$ DCs loaded with soluble OVA protein (wt DC+OVA or MHCII$^{-/-}$ DC+OVA) were labelled with CFSE. As an endogenous control, some MHCII$^{-/-}$ DCs were not loaded with OVA and labelled with CMTMR instead (DC only). The CFSE$^+$ DC+OVA were mixed with CMTMR$^+$ DCs in equal numbers and injected s.c. into the forelimbs 24 h after the transfer of CTLs. After 48 h, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry.

Although the numbers of CFSE$^+$ wt DCs and CMTMR$^+$ DCs differed slightly in the control mice, much fewer CFSE$^+$ wt DCs were collected compared to CMTMR$^+$ cells in mice that received CTLs (Fig. 5.3b). Similarly, fewer CFSE$^+$ MHCII$^{-/-}$ DCs were collected in mice

that received CTLs compared to the control group. Approximately 40-50% of these CFSE$^+$ MHCII$^{-/-}$ DC+OVA survived CTL elimination (Fig. 5.3c.). These results indicate that CTLs eliminate antigen-presenting MHCII$^{-/-}$ DCs.
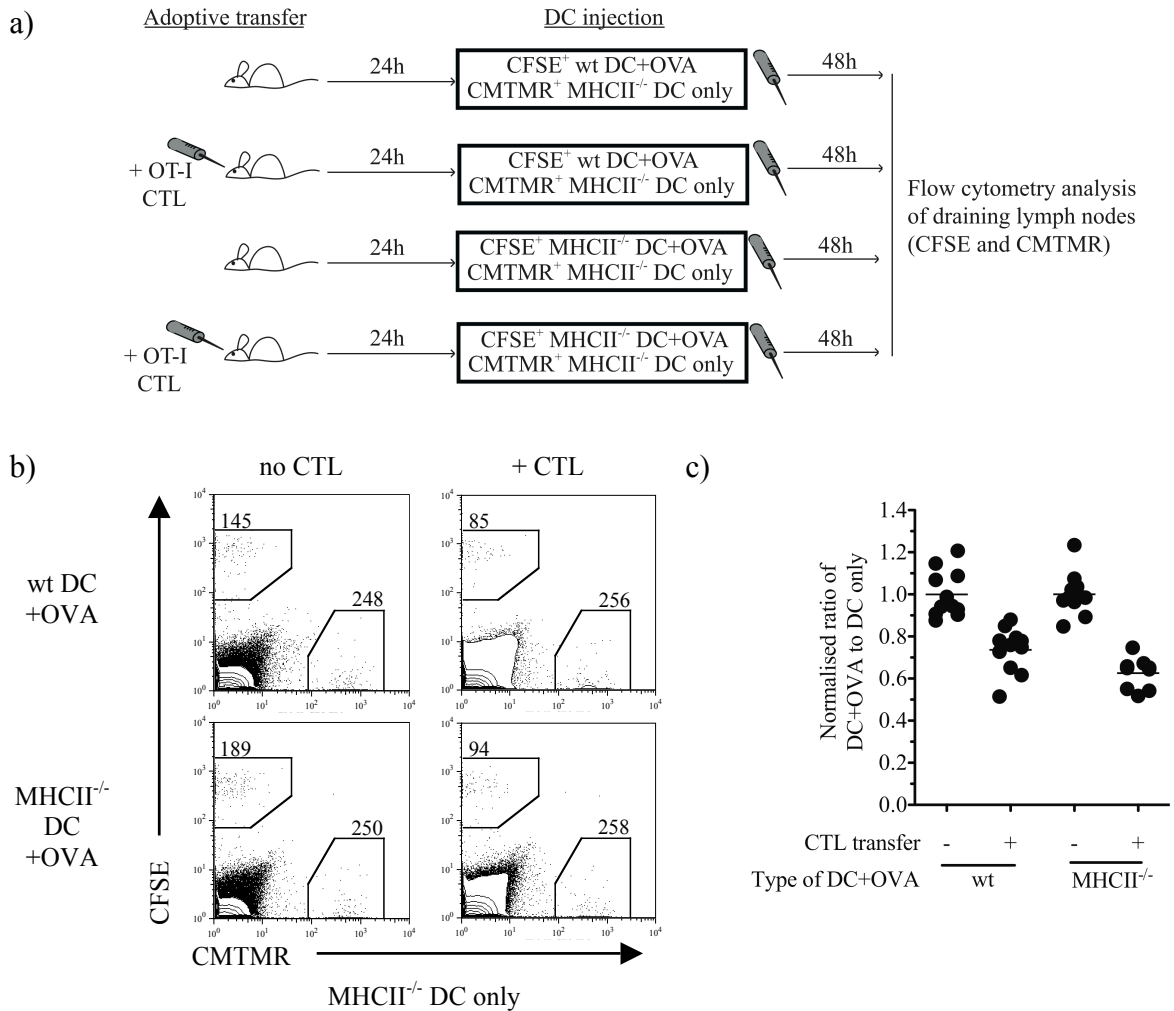
Fig. 5.3. **MHCII$^{-/-}$ DCs loaded with soluble OVA are eliminated by OT-I CTLs *in vivo*.** (**a**) Groups of C57BL/6J mice were injected i.v. with *in vitro* activated OT-I CTLs (+ CTL) or no CTL. 24 h later, CFSE-labelled wt or MHCII$^{-/-}$ DC loaded with OVA protein (DC+OVA) were mixed with CMTMR-labelled control MHCII$^{-/-}$ DC (DC only) in equal numbers and were injected s.c. into the forelimbs of these mice. 48 h after DC injection, CFSE$^+$ and CMTMR$^+$ DCs in the draining lymph nodes were monitored by flow cytometry. (**b**) CFSE$^+$ and CMTMR$^+$ DCs are shown in representative dot plots from individual mice. The number of events in each gate is shown. (**c**) The ratios of CFSE$^+$ wt or MHCII$^{-/-}$ DC+OVA to CMTMR$^+$ DC only were normalised to the ratios derived from their respective untreated mice. Two separate experiments with 2 – 3 mice per group are pooled together and shown.

5.4. Host APCs induce CD4$^+$ T cell proliferation when the OVA-loaded DCs cannot present antigens directly to CD4$^+$ T cells

In chapter 5.3, I have shown that MHCII$^{-/-}$ DCs could not present antigens to CD4$^+$ T cells and were eliminated by CTLs. Because these DCs could not directly present antigens to CD4$^+$ T cells, I used them to address the contributions of host APCs to the induction of CD4$^+$ T cell proliferation.

The experimental setup is shown in Fig. 5.4a. Mice were injected i.v. with CD4$^+$ T cells and *in vitro* activated CTLs. As a control, some mice were injected with CD4$^+$ T cells only. 24 h later, wt or MHCII$^{-/-}$ DCs loaded with OVA protein were injected s.c. to these mice. 3 days after DC injection, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

In the groups that received wt DCs, the majority of CD4$^+$ T cells underwent cell divisions regardless of the presence of CTLs (Fig 5.4b). Fewer cell divisions were observed in both the control and CTL groups that received MHCII$^{-/-}$ DCs, suggesting that CD4$^+$ T cell proliferation was reduced. This difference was reflected in the percentage and number of divided CD4$^+$ T cells and was statistically significant (Fig. 5.4c and 5.4d). While some differences in CD4$^+$ T cell proliferation were observed between the groups that received CTLs and the groups that did not, the difference was not significant. CD4$^+$ T cell division observed in mice injected with MHCII$^{-/-}$ DCs was not due to the carryover of OVA protein in the injection medium (Appendix 10).

As CD4$^+$ T cell proliferation was observed after MHCII$^{-/-}$ DCs were injected, this result showed that the host APCs made substantial contributions to MHC class II restricted antigen presentation.

a)

Adoptive transfer   DC injection

OT-II CFSE$^+$ CD45.2$^+$ host
CD45.1$^+$CD4$^+$ T cells → 24h → wt DC+OVA → 72h →

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells → 24h → wt DC+OVA → 72h →
+ OT-I CTL

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells → 24h → MHCII$^{-/-}$ DC+OVA → 72h →

OT-II CFSE$^+$
CD45.1$^+$CD4$^+$ T cells → 24h → MHCII$^{-/-}$ DC+OVA → 72h →
+ OT-I CTL

Flow cytometry analysis
of draining lymph nodes
(CD4, CD45.1 and CFSE)

b)

c)

d)
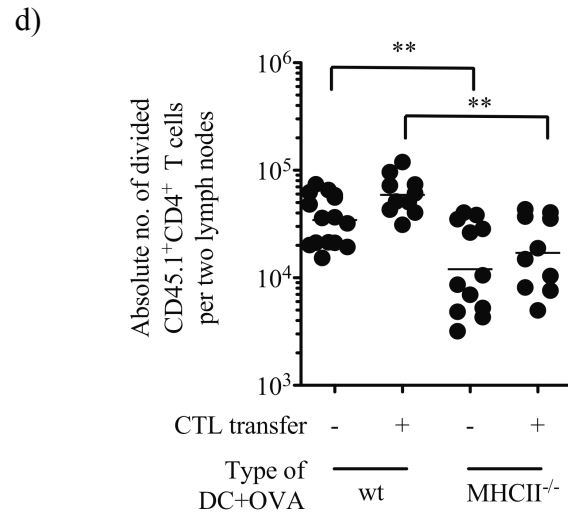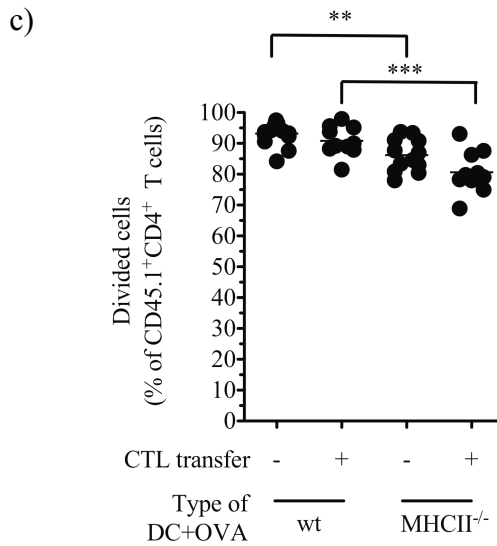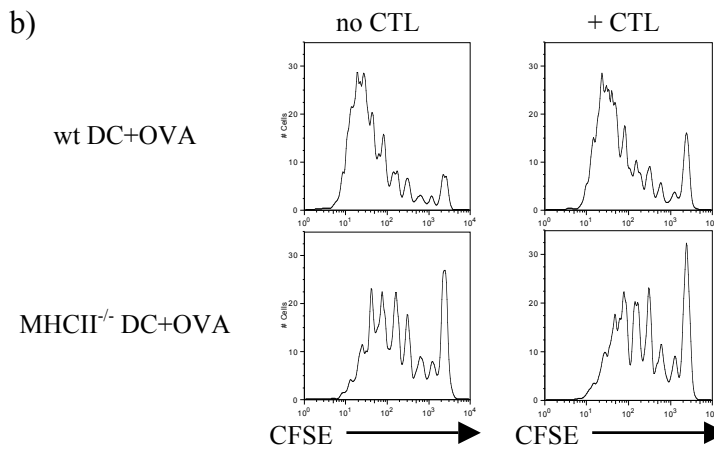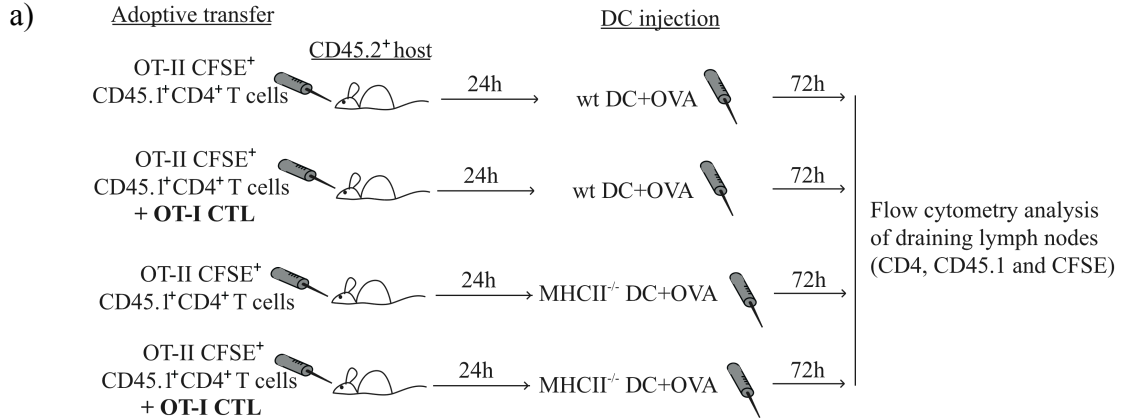
Fig. 5.4. **CD4$^+$ T cell proliferation is reduced, but not abolished, when injected DCs cannot present antigens directly to CD4$^+$ T cells.** (**a**) C57BL/6J mice were injected i.v. with *in vitro* activated OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. As a control, some mice received only CD4$^+$ T cells. After 24 h, these recipient mice were injected s.c. into their forelimbs with LPS-activated wt or MHCII$^{-/-}$ DCs previously loaded with 2 mg/ml of OVA protein. 3 days later, CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. Two separate experiments with 5 mice per group are pooled together and shown. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. ** p<0.01, *** p<0.001.

5.5. Loading of high antigen dose on MHCII$^{-/-}$ DCs induces CD4$^+$ T cell proliferation *in vivo*

To examine whether the antigen dose loaded onto the injected DCs was important to the host APCs for inducing CD4$^+$ T cell responses, MHCII$^{-/-}$ DCs loaded with a high (2 mg/ml) or low (0.02 mg/ml) OVA dose were used (Fig. 5.5a). Some MHCII$^{-/-}$ DCs pulsed with OVA$_{323-339}$ acted as a control, whereas wt DCs loaded with a low OVA dose served as a positive control. The DCs were injected s.c. into mice that had received CFSE-labelled CD4$^+$ T cells 24 h before. 3 days after DC injection, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

The majority of CD4$^+$ T cells underwent cell divisions in mice that received MHCII$^{-/-}$ DC+high OVA or wt DC+low OVA (Fig. 5.5b). However, CD4$^+$ T cell proliferation was abolished in mice that received MHCII$^{-/-}$ DC+low OVA and those that received MHCII$^{-/-}$ DC+OVA$_{323-339}$ (Fig. 5.5c and d). This suggests that host APCs were involved in inducing CD4$^+$ T cell proliferation only when a high antigen dose was loaded onto the injected DCs, but not when a low antigen dose was used.

a)

b)

c)

d)
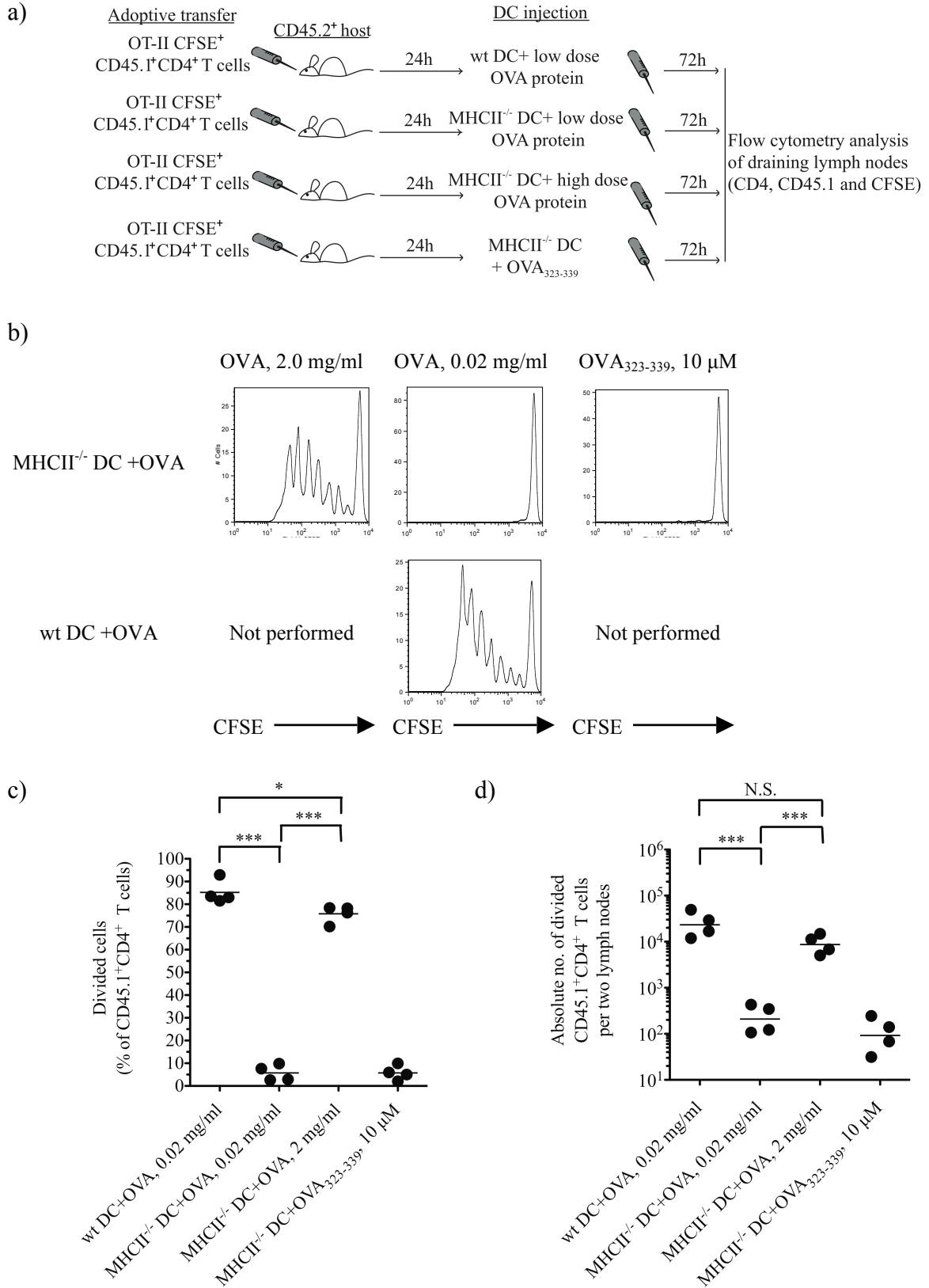
Fig. 5.5. **At a low antigen dose, CD4$^+$ T cell proliferation is lost when injected DCs cannot present antigens directly to CD4$^+$ T cells.** (**a**) C57BL/6J mice were injected i.v. with CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. After 24 h, these recipient mice were injected s.c. into their forelimbs with LPS-activated MHCII$^{-/-}$ DCs previously loaded with either 0.02 mg/ml or 2 mg/ml of OVA protein. As a

control, some mice received MHCII$^{-/-}$ DCs loaded with 10 μM OVA$_{323-339}$, or wt DCs loaded with 0.02 mg/ml of OVA protein. 3 days later, CD45.1^{+}CD4^{+} T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1^{+}CD4^{+} T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1^{+}CD4^{+} T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1^{+}CD4^{+} T cells in the draining lymph nodes are shown. The experiment was performed once with 4 mice per group. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. * $p<0.05$, *** $p<0.001$, N.S.= not significant

5.6. CTL-mediated elimination of injected DCs reduces CD4^{+} T cell proliferation in the absence of host APC participation

To investigate if the CTL-mediated elimination of injected DCs affected CD4^{+} T cell proliferation when host APCs did not participate in antigen presentation, DCs were incubated with low concentration of OVA protein (0.004 mg/ml) before injection into mice (Fig 5.6a). At low OVA dose, there was no uptake of antigens by host DCs (Fig. 5.5). To target the injected DCs for CTL elimination, these DCs were also pulsed with 10 μM SIINFEKL. These DCs were then injected s.c. into mice that had received either CD4^{+} T cells and CTLs or CD4^{+} T cells only 24 h before. 3 days after DC injection, CD4^{+} T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry.

In mice that received no CTL and wt DC+OVA+SIINFEKL, vigorous CD4^{+} T cell division was observed, indicating that at this extremely low OVA concentration, the proliferation of CD4^{+} T cells was still detectable (Fig. 5.6b). In the group that received CTL and wt DC+OVA+SIINFEKL, some CD4^{+} T cell division was observed but was significantly lower than that in the control group (Fig. 5.6c and d). The reduction of CD4^{+} T cell division in the presence of CTLs was similar to the decrease observed when using DCs pulsed with SIINFEKL and OVA$_{323-339}$ (Fig. 3.3), indicating that when the injected DCs were loaded with a low antigen dose, DC elimination by CTLs nearly abolished CD4^{+} T cell proliferation. The residual CD4^{+} T cell proliferation was likely induced by the injected DCs that survived CTL-mediated elimination. In the groups that received MHCII$^{-/-}$ DC+OVA+SIINFEKL or MHCII$^{-/-}$ DC+OVA, no CD4^{+} T cell proliferation was observed regardless of the presence of CTLs (Fig. 5.6b to Fig. 5.6d). These results extend my previous findings, showing that at low antigen doses, only the injected DCs induced CD4^{+}

T cell division (Fig. 5.5) and that CTL-mediated elimination of injected DCs significantly reduced subsequent CD4$^+$ T cell proliferation (Fig. 3.3).

a)

b)
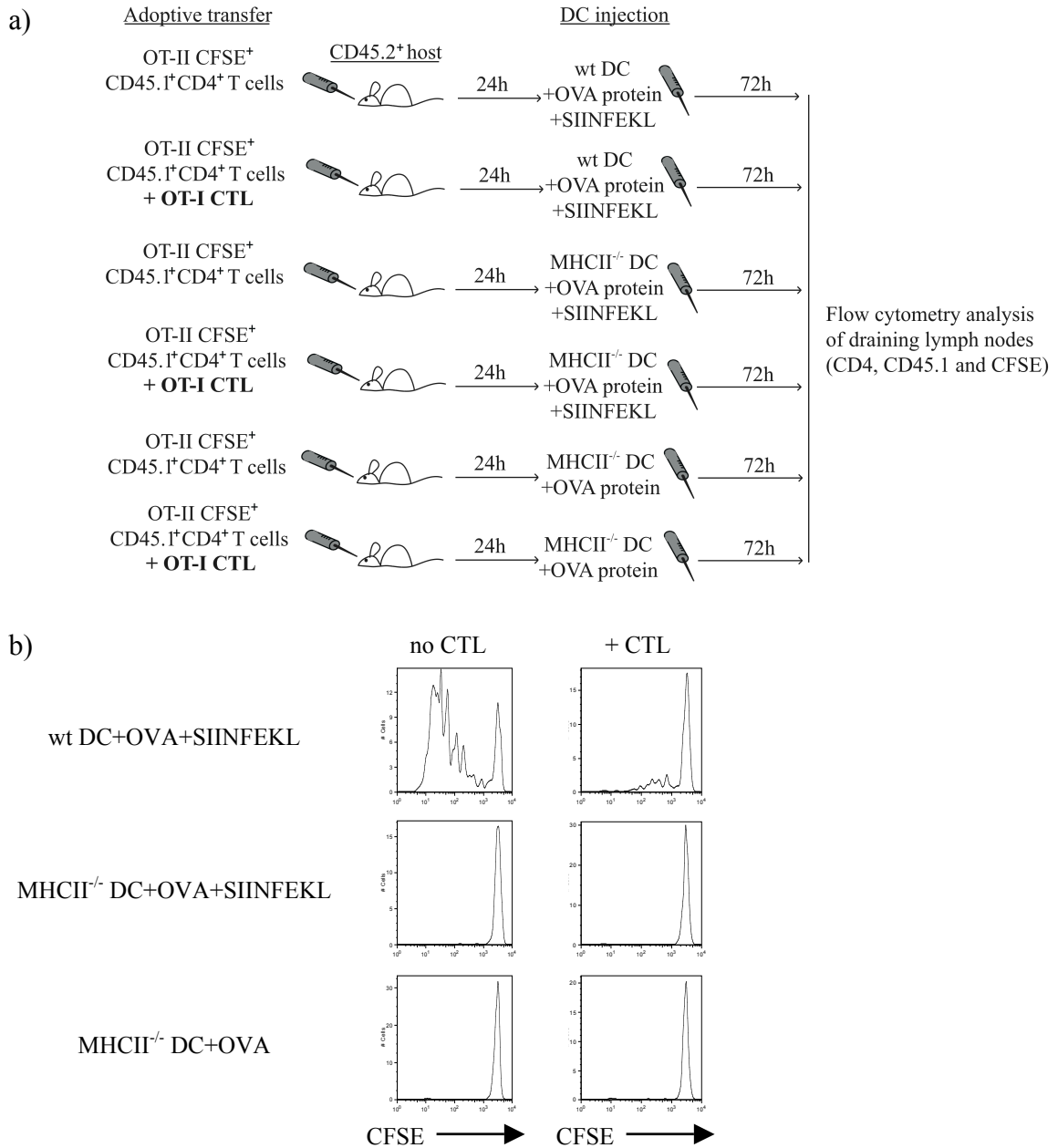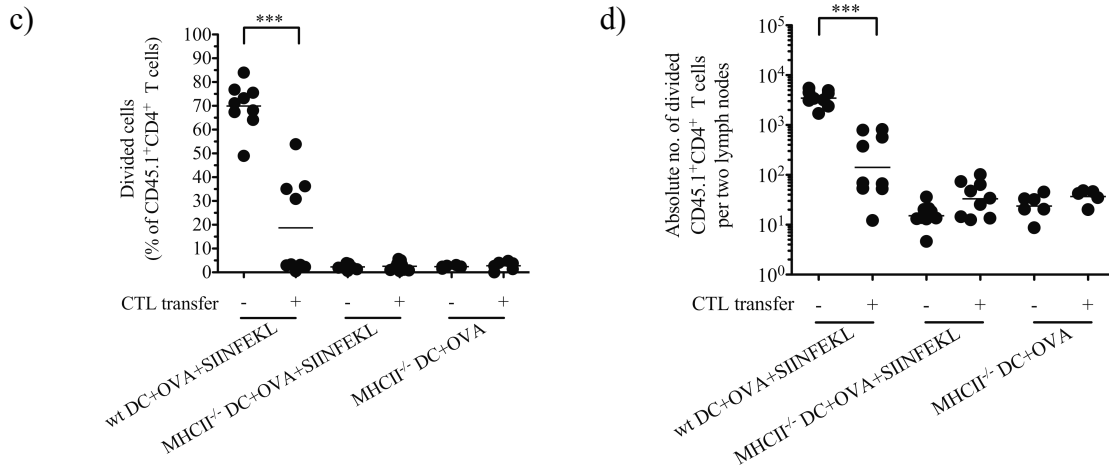
Fig. 5.6. **CTLs reduce CD4$^+$ T cell proliferation induced by injected DCs in the absence of host APC participation.** (**a**) C57BL/6J mice were injected i.v. with *in vitro* activated OT-I CTLs and CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. As a control, some mice received only CD4$^+$ T cells. After 24 h, these recipient mice were injected s.c. into their forelimbs with LPS-activated wt or MHCII$^{-/-}$ DCs previously loaded with 0.004 mg/ml of OVA protein and 10 μM SIINFEKL. Some mice received MHCII$^{-/-}$ DCs loaded with OVA only. 3 days later, CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. Two separate experiments with 4 – 5 per group are pooled together and shown. Statistical significance was determined with one-way ANOVA with Bonferroni's correction. *** p<0.001.

5.7. Host DCs present antigens carried by injected DCs to T cells

5.7.1. Host DCs present antigens carried by injected DCs to CD4$^+$ T cells

To assess the involvement of host DCs in presenting antigens carried by injected DCs to T cells, I adopted a published DC enrichment protocol using negative selection with magnetic beads (Bedoui et al., 2009; Lee et al., 2009). The DC enrichment protocol is described in Chapter 2. I modified the protocol to include the depletion of injected DCs using the CD45.1 congenic marker to enrich for host CD11c$^+$ DCs (Appendices 11 and 12). I used this protocol to characterise the involvement of host DCs in presenting antigens carried by injected DCs to CD4$^+$ and CD8$^+$ T cells.

The experimental design is illustrated in Fig. 5.7.1a. CD45.1$^+$ DCs previously loaded with OVA protein (DC+OVA) were injected s.c. into the forelimbs of C57BL/6J mice. As a control, one group of mice received CD45.1$^+$ DCs that were not loaded with OVA (DC

only). 24 h after DC injection, brachial and axillary draining lymph nodes were harvested and digested into cell suspensions. The cell suspensions made from mice that received DC+OVA were aliquoted equally into two tubes before the addition of antibodies. One of the two aliquots was incubated with a mixture of antibodies against T cell, B cell, NK cell and monocyte lineage markers and anti-CD45.1 antibody to enrich for CD45.2$^+$CD11c$^+$ cells (host only). The other aliquot was incubated with a mixture of antibodies against lineage markers to enrich for CD45.2$^+$ and CD45.1$^+$CD11c$^+$ cells (total). After the incubation with antibodies, antibody-coated cells were labelled with magnetic beads and were removed using magnetic separation. Following magnetic depletion of antibody-coated cells, the enriched cells were serially titrated and co-incubated with CFSE-labelled OT-II CD4$^+$ T cells for 4 or 5 days to detect for antigen presentation on MHC class II. OT-II CD4$^+$ T cells were then examined for CFSE dilution by flow cytometry.

The percentages of host CD11c$^+$CD45.2$^+$ cells after depletion were comparable between the groups containing host DCs and total DCs in mice that received DC+OVA (Fig 5.7.1b). No injected DCs (CD11c$^+$CD45.1$^+$) was detected in either groups, possibly because of the low number (0.2×10^6) of CD45.1$^+$ DCs injected. OT-II CD4$^+$ T cells proliferated slightly when co-incubated with a mixture of injected and host CD11c$^+$ cells for 4 days, whereas no proliferation was observed with host CD11c$^+$ cells only (Fig. 5.7.1c). However, after 5 days of co-incubation, some proliferation of OT-II T cells was observed in both groups containing host DCs and total DCs. Host CD11c$^+$ cells induced slightly lower OT-II T cell proliferation than total DCs (Fig. 5.7.1c). Consistent with my previous observations (Fig. 5.4 to Fig. 5.6), the results show that the host DCs present antigens acquired from injected DCs to CD4$^+$ T cells.

a)

DC injection   Enrich for CD11c$^+$ DCs   Co-incubate with:
                      through magnetic depletion
                                                       OT-II CFSE$^+$
CD45.1$^+$ CD45.2$^+$ host   CD4$^+$ T cells
                                          Total
                                   (CD45.1$^+$ & CD45.2$^+$)
DC+OVA   [mouse]   24h   Flow cytometry analysis
                                                       OT-II CFSE$^+$ after 4 and 5 days
                                          Host only   CD4$^+$ T cells   (CD4, CFSE and Vα2)
                                        (CD45.2$^+$)

CD45.1$^+$ CD45.2$^+$ host

DC only   [mouse]   24h   Host only   OT-II CFSE$^+$
                                      (CD45.2$^+$)   CD4$^+$ T cells

b)

Before separation   After separation
                              Total   Host only

CD45.2

CD11c

CD45.1

c)

Day 4 proliferation   Day 5 proliferation

Divided cells/ well (% of Vα2$^+$CD4$^+$)
DC/ well (x10^3)

□ DC only
○ DC+OVA (Total)
● DC+OVA (Host only)

Fig. 5.7.1. **Host DCs present antigens carried by injected DCs to CD4$^+$ T cells.** (**a**) C57BL/6J mice were injected s.c. into their forelimbs with LPS-activated OVA-loaded CD45.1$^+$ wt DCs (DC+OVA) or wt DCs not loaded with OVA protein (DC only). 24 h later, brachial and axillary lymph nodes were harvested and digested into cell suspensions. Cell suspensions from the DC+OVA group were split into halves. One aliquot was incubated with a mixture of antibodies against lineage markers and anti-CD45.1 antibody to enrich for CD45.2$^+$CD11c$^+$ cells (host only) and the other aliquot was incubated with a mixture of antibodies against lineage markers to enrich for total CD11c$^+$ cells (total) through magnetic separation. The enriched CD11c$^+$ cells were then serially diluted and cultured with 10×10^4 CFSE-labelled OT-II CD4$^+$ T cells per well for either 4 or 5 days. After incubation, OT-II CD4$^+$ T cells were examined for CFSE dilution by flow cytometry. (**b**) Aliquots of samples before and after magnetic depletion were stained with anti-CD11c, -CD45.2 and -CD45.1 antibodies to determine the percentages of host and injected DCs present. (**c**) The percentages of Vα2$^+$CD4$^+$ T cells that had divided at least once are shown. One representative experiment of two with triplicate wells is shown.

5.7.2. Host DCs present antigens carried by injected DCs to CD8$^+$ T cells

To characterise the role of host DCs presenting antigens carried by injected DCs to CD8$^+$ T cells, a similar experiment using CFSE-labelled OT-I CD8$^+$ T cells co-incubated with the enriched DCs for 3 days was carried out. The experiment is illustrated in Fig. 5.7.2a.

The percentages of host DCs (CD11c$^+$CD45.2$^+$) after depletion were comparable between the groups containing host DCs and total DCs in mice that received DC+OV (Fig. 5.7.2b). The majority of OT-I CD8$^+$ T cells underwent cell divisions when OT-I T cells were incubated with total or host CD11c$^+$ cells (Fig. 5.7.2c). In conclusion, host DCs are able to present antigens carried by injected DCs to both CD8$^+$ and CD4$^+$ T cells.

a)

b)

c)

Fig. 5.7.2. **Host DCs present antigens carried by injected DCs to CD8^{+} T cells.** (**a**) C57BL/6J mice were injected s.c. into their forelimbs with LPS-activated OVA-loaded CD45.1^{+} wt DCs or CD45.1^{+} wt DCs not loaded with OVA protein (DC only). 24 h later, brachial and axillary lymph nodes were harvested and digested into cell suspensions. Cell suspensions from the DC+OVA group were split into halves. One aliquot was incubated with a mixture of antibodies against lineage markers and anti-CD45.1 antibody to enrich for CD45.2^{+}CD11c^{+} cells (host only) and the other aliquot was incubated with a mixture of antibodies against lineage markers to enrich for total CD11c^{+} cells (total) through magnetic separation. Enriched CD11c^{+} cells were then serially diluted and cultured with 10 × 10^{4} CFSE-labelled OT-I CD8^{+} T cells per well for 3 days. After incubation, OT-I CD8^{+} T cells were examined for CFSE dilution by flow cytometry. (**b**) Aliquots of samples before and after magnetic depletion were stained with anti-CD11c and -CD45.2 antibodies to determine the percentages of host DCs present. (**c**) The percentages of Vα2^{+}CD8^{+} T cells that had divided at least once are shown. One representative experiment of two with triplicate wells is shown.

5.7.3. Both peptide and protein antigens carried by injected DCs can be transferred to and presented by host DCs to T cells

Having shown in Fig. 5.7.1 and Fig. 5.7.2 that host DCs could take up OVA protein loaded onto injected DCs and present them to $CD4^+$ and $CD8^+$ T cells, I asked if the host DCs could also present with other forms of antigens carried by the injected DCs.

Fig. 5.7.3a. illustrates the experimental setup. I compared the proliferative responses of $CD8^+$ and $CD4^+$ T cells when the injected DCs had been loaded with either OVA protein or OVA peptides. Similar to previous experiments (Fig. 5.7.1 and Fig. 5.7.2), $CD45.2^+$ mice were injected with $CD45.1^+$ DCs on the forelimbs. These DCs were previously loaded with either OVA protein or SIINFEKL and $OVA_{323-339}$. 24 h after DC injection, total or host DCs were enriched through magnetic depletion and subsequently co-incubated with $CD8^+$ OT-I or $CD4^+$ OT-II T cells for 3 or 5 days, respectively. OT-I $CD8^+$ and OT-II $CD4^+$ T cells were then examined for CFSE dilution by flow cytometry.

The percentages of host DCs ($CD11c^+CD45.2^+$) recovered from draining lymph nodes were comparable among all the groups (Fig. 5.7.3b). The majority of $CD8^+$ OT-I T cells divided when incubated with total $CD11c^+$ cells or host $CD11c^+$ cells from mice that received DC+SIINFEKL+$OVA_{323-339}$ (Fig. 5.7.3c). Similar to previous observations (Fig. 5.7.2), both total $CD11c^+$ cells and host $CD11c^+$ cells prepared from mice that received DC+OVA induced OT-I T cell division.

The majority of $CD4^+$ OT-II T cells divided in all groups regardless of whether the DCs were total or host only (Fig. 5.7.3d). These results show that both peptide and protein antigens carried by the injected DCs are transferred to and presented by the host DCs to $CD8^+$ and $CD4^+$ T cells. In this study, the transfer of peptides from injected to host DCs occurred when the injected DCs were loaded with 10 μM SIINFEKL and 10 μM $OVA_{323-339}$. Other studies have indicated that peptide transfer between DCs occur at peptide dose lower than that used in this study (Luketic et al., 2007).

a)

Co-incubate with:

Enrich for CD11c⁺ DCs
through magnetic separation

OT-I CFSE⁺
CD8⁺ T cells
Flow cytometry analysis
after 3 days
(CD8, CFSE and Vα2)

Total
(CD45.1⁺ & CD45.2⁺)

DC injection

OT-II CFSE⁺
CD4⁺ T cells
Flow cytometry analysis
after 5 days
(CD4, CFSE and Vα2)

CD45.1⁺   CD45.2⁺ host

DC+OVA   24h

OT-I CFSE⁺
CD8⁺ T cells
Flow cytometry analysis
after 3 days
(CD8, CFSE and Vα2)

Host only (CD45.2⁺)

OT-II CFSE⁺
CD4⁺ T cells
Flow cytometry analysis
after 5 days
(CD4, CFSE and Vα2)

OT-I CFSE⁺
CD8⁺ T cells
Flow cytometry analysis
after 3 days
(CD8, CFSE and Vα2)

Total
(CD45.1⁺ & CD45.2⁺)

OT-II CFSE⁺
CD4⁺ T cells
Flow cytometry analysis
after 5 days
(CD4, CFSE and Vα2)

CD45.1⁺   CD45.2⁺ host

DC
+SIINFEKL
+OVA₃₂₃₋₃₃₉   24h

OT-I CFSE⁺
CD8⁺ T cells
Flow cytometry analysis
after 3 days
(CD8, CFSE and Vα2)

Host only (CD45.2⁺)

OT-II CFSE⁺
CD4⁺ T cells
Flow cytometry analysis
after 5 days
(CD4, CFSE and Vα2)

OT-I CFSE⁺
CD8⁺ T cells
Flow cytometry analysis
after 3 days
(CD8, CFSE and Vα2)

CD45.1⁺   CD45.2⁺ host

DC only   24h   Host only (CD45.2⁺)

OT-II CFSE⁺
CD4⁺ T cells
Flow cytometry analysis
after 5 days
(CD4, CFSE and Vα2)

b)

Before separation   After separation

Total   Host only

DC+OVA

DC
+SIINFEKL+OVA₃₂₃₋₃₃₉
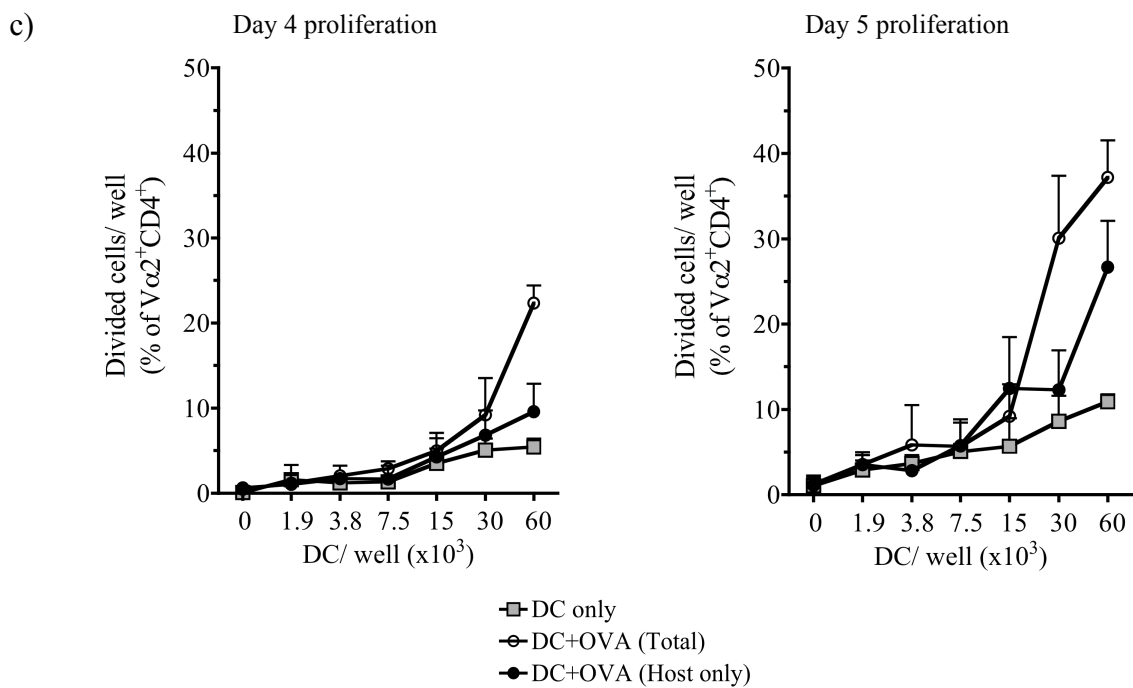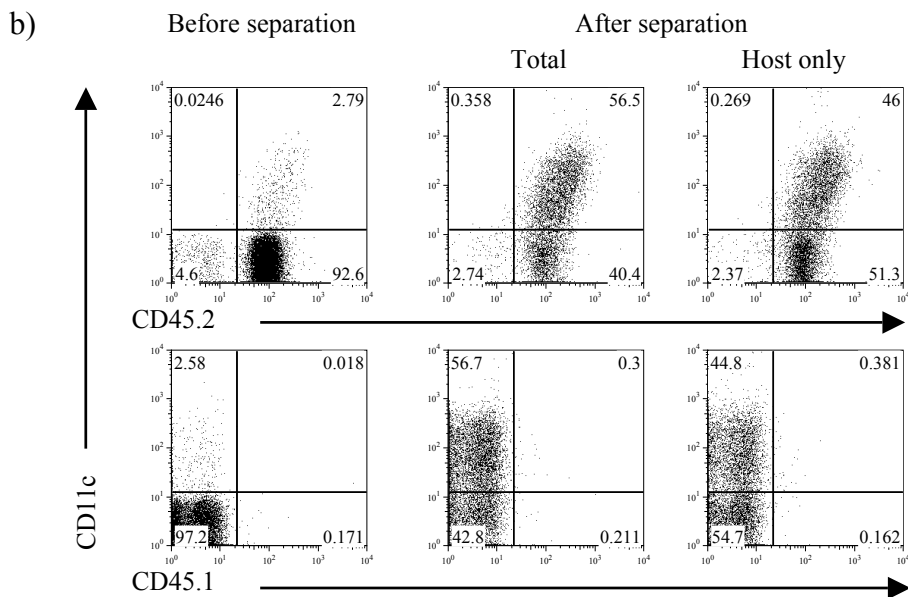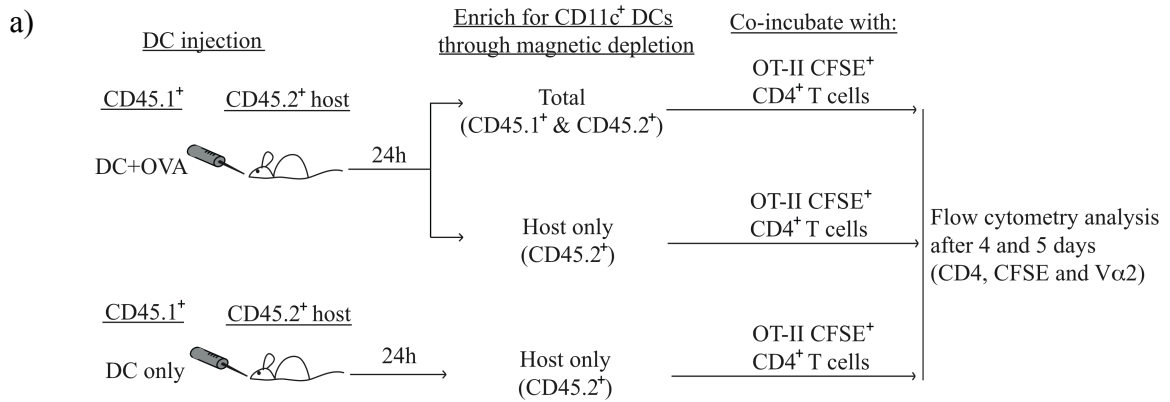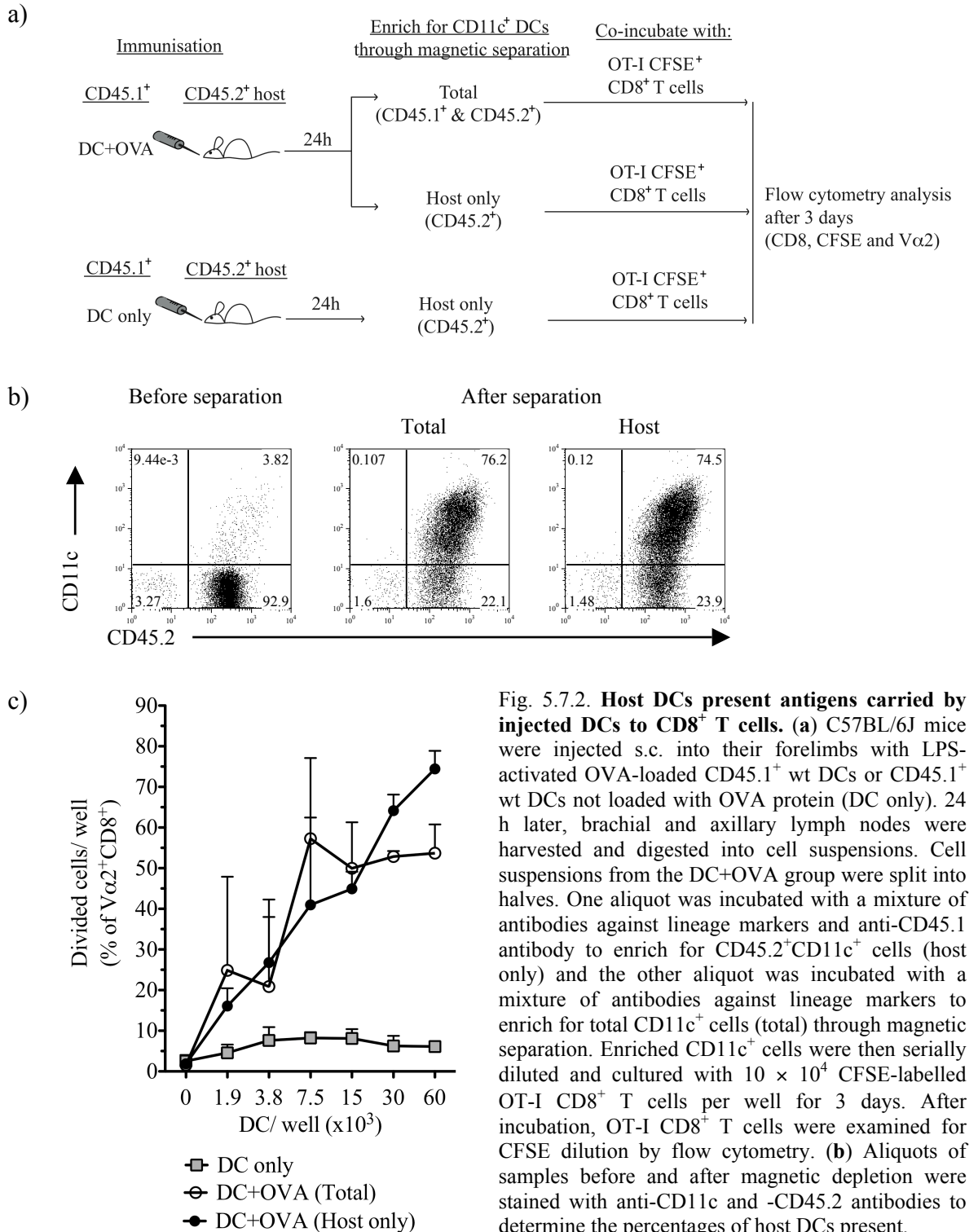
CD11c

CD45.2

Fig. 5.7.3. **Host DCs present protein and peptide antigens carried by injected DCs to CD8+ and CD4+ T cells.** (**a**) C57BL/6J mice were injected s.c. into their forelimbs with LPS-activated CD45.1+ wt DCs previously loaded with OVA protein (DC+OVA), or 10 μM SIINFEKL and 10 μM OVA$_{323-339}$ (DC+SIINFEKL+OVA$_{323-339}$), or not loaded with any added antigens (DC only). 24 h later, brachial and axillary lymph nodes were harvested and digested into cell suspensions. Cell suspensions from the DC+OVA and DC+SIINFEKL+OVA$_{323-339}$ groups were split into halves. One aliquot from each group was incubated with a mixture of antibodies against lineage markers and anti-CD45.1 antibody to enrich for CD45.2+CD11c+ cells (host only) through magnetic separation. The other aliquot from each group was incubated with a mixture of antibodies against lineage markers to enrich for total CD11c+ cells (total) through magnetic separation. Enriched CD11c+ cells were then serially diluted and cultured with 10×10^4 CFSE-labelled OT-I CD8+ T cells per well for 3 days or 10×10^4 CFSE-labelled OT-II CD4+ T cells per well for 4 days. After incubation, OT-I CD8+ and OT-II CD4+ T cells were examined for CFSE dilution by flow cytometry. (**b**) Aliquots of samples before and after magnetic depletion were stained with anti-CD11c and -CD45.2 antibodies to determine the percentages of host DCs present. The percentages of (**c**) Vα2+CD8+ or (**d**) Vα2+CD4+ T cells that had divided at least once are shown. The experiment was performed once with triplicate wells for DC+OVA and DC only; and with duplicate wells for DC+SIINFEKL+OVA$_{323-339}$.

5.7.4. Host DCs present antigens carried by injected DCs transiently

Because *in vivo* CD4$^+$ T cell proliferation was already observed within 2 to 3 days after DC injection (Fig. 4.2.2, Fig. 4.2.4 and Fig. 5.4 to 5.6), the time when the host DCs interact with naïve T cells must have already occurred within the first 2 days (24 h and 48 h) after DC injection.

To examine how long host DCs presented captured antigens from injected DCs for, host DCs were recovered from draining lymph nodes 24 h and 48 h after DC injection and their ability to present to CD8$^+$ T cells was determined. To examine if the presence of DC killing would affect antigen presentation of host DCs, *in vitro* activated CTLs were transferred into some groups of mice to induce DC elimination. Fig. 5.7.4a illustrates the experimental setup.

The percentages of host DCs (CD11c$^+$CD45.2$^+$) recovered from draining lymph nodes after depletion were comparable between groups at the 24 h and 48 h, respectively (Fig. 5.7.4b). OT-I CD8$^+$ T cell division was observed when OT-I T cells were incubated with host DCs harvested 24 h after DC injection. In the presence of CTLs, OT-I CD8$^+$ T cell division was reduced compared to the control mice that did not receive CTLs (Fig. 5.7.4c). Host CD11c$^+$ cells that were prepared 48 h after DC injection only induced unsubstantial OT-I T cell division, regardless of the presence of CTLs. The results suggest that host DCs present antigens carried by injected DCs to CD8$^+$ T cells transiently. Furthermore, the presence of CTLs reduce, but does not abolish, the antigen presentation by the host DCs, suggesting that some host DCs presenting captured antigens are not eliminated by CTLs.

a)

Enrich for CD11c⁺ DCs
through magnetic separation

Adoptive transfer   DC injection   Co-incubate with:

CD45.2⁺ host   CD45.1⁺

24h   DC+OVA   24h   Host only   OT-I CFSE⁺
(CD45.2⁺)   CD8⁺ T cells

24h   DC+OVA   24h   Host only   OT-I CFSE⁺   Flow cytometry analysis
(CD45.2⁺)   CD8⁺ T cells   after 3 days
+OT-I CTL   (CD8, CFSE and Vα2)

24h   DC only   24h   Host only   OT-I CFSE⁺
(CD45.2⁺)   CD8⁺ T cells

OT-I CFSE⁺
24h   DC+OVA   48h   Host only   CD8⁺ T cells
(CD45.2⁺)

24h   DC+OVA   48h   Host only   OT-I CFSE⁺   Flow cytometry analysis
(CD45.2⁺)   CD8⁺ T cells   after 3 days
+OT-I CTL   (CD8, CFSE and Vα2)

24h   DC only   48h   Host only   OT-I CFSE⁺
(CD45.2⁺)   CD8⁺ T cells

b)

24 h after DC injection   48 h after DC injection

Before separation   After separation   Before separation   After separation

no CTL

| 0.0148 | 4.01 | 0.0602 | 69.9 | 0.0139 | 2.74 | 0.27 | 75.4 |
| 1.85 | 94.1 | 1.58 | 28.4 | 4.09 | 93.2 | 3.19 | 21.2 |

+ CTL

| 4.74e-3 | 4.18 | 0.132 | 65.6 | 0.0194 | 3.7 | 0.289 | 79.2 |
| 2.39 | 93.4 | 1.94 | 32.3 | 4.99 | 91.3 | 3.54 | 16.9 |

CD11c

CD45.2

c)

Fig. 5.7.4. **Presentation of captured antigens by host DCs is transient and is reduced by CTLs.** (**a**) C57BL/6J mice were injected i.v. with *in vitro* activated OT-I CTLs, whereas the control groups did not receive any CTLs (no CTL). 24 h later, mice were injected s.c. into their forelimbs with LPS-activated CD45.1$^+$ wt DCs previously loaded with OVA (DC+OVA) or wt DCs not loaded with OVA protein (DC only). Brachial and axillary lymph nodes were harvested and digested into cell suspensions either 24 h or 48 h after DC injection. Cell suspensions were incubated with a mixture of antibodies against lineage markers and anti-CD45.1 antibody to enrich for host CD11c$^+$ cells through magnetic separation. Enriched CD11c$^+$ cells were then serially diluted and cultured with 10 × 10^4 CFSE-labelled OT-I CD8$^+$ T cells per well for 3 days. After incubation, OT-I CD8$^+$ T cells were examined for CFSE dilution by flow cytometry. (**b**) Aliquots of samples before and after magnetic depletion were stained with anti-CD11c and -CD45.2 antibodies to determine the percentages of host DCs present. (**c**) The percentages of Vα2$^+$CD8$^+$ T cells that had divided at least once are shown. The experiment was performed once with triplicate wells.

5.8. Host skin-derived DCs contribute most to presenting antigens carried by injected DCs to CD8$^+$ and CD4$^+$ T cells

Host DCs in the skin draining lymph nodes can be categorised into lymph node resident and migratory skin-derived DCs. To identify which host DC subpopulation was presenting the antigens carried by the injected DCs to CD8$^+$ and CD4$^+$ T cells, host CD11c$^+$ cells from draining lymph nodes were sorted according to their expressions of CD11c, CD205 and CD8 (Fig. 5.8a). Sorting CD11c$^+$ DCs yielded populations of CD205$^+$CD8^{lo} (skin-derived DCs), CD205$^+$CD8^{hi} (CD8$^+$ DCs) and CD205$^-$CD8$^-$ (double negative DCs) cells that were each greater than 95% pure (Fig. 5.8b). These DC subpopulations were then co-incubated with CFSE-labelled OT-I CD8$^+$ or OT-II CD4$^+$ T cells. OT-I and OT-II T cells were examined for CFSE dilution by flow cytometry 3 or 5 days later, respectively.

The strongest CD8$^+$ and CD4$^+$ T cell divisions were induced by host skin-derived DCs (Fig 5.8c and d). Host lymph node resident CD8$^+$ DCs also presented antigens to CD8$^+$ T cells, but not to CD4$^+$ T cells. Host lymph node resident double negative DCs did not present antigens to either CD8$^+$ or CD4$^+$ T cells. Although the injected DCs were CD205$^+$CD8$^-$ (Appendix 13), it is unlikely that the injected DCs were sorted together with host skin-derived DCs because injected DCs were depleted and could not be detected by flow cytometry (Fig. 5.7.1 to 5.7.4).

These results show that migratory skin-derived DCs capture and present antigens from injected DCs to CD8$^+$ and CD4$^+$ T cells. Lymph node resident CD8$^+$ DCs also participate in presenting transferred antigens to CD8$^+$ T cells.

a)

Co-incubate with:

Sort based on CD11c,
CD205 and CD8

Skin-derived
DCs

OT-I CFSE⁺
CD8⁺ T cells → Flow cytometry analysis after 3 days (CD8, CFSE and Vα2)

OT-II CFSE⁺
CD4⁺ T cells → Flow cytometry analysis after 5 days (CD4, CFSE and Vα2)

DC injection

CD45.1⁺   CD45.2⁺ host
DC+OVA →[24h]→ Enriched for CD11c⁺ host DCs (CD45.2⁺) →

CD8⁺ DCs

OT-I CFSE⁺
CD8⁺ T cells → Flow cytometry analysis after 3 days (CD8, CFSE and Vα2)

OT-II CFSE⁺
CD4⁺ T cells → Flow cytometry analysis after 5 days (CD4, CFSE and Vα2)

Double
negative
DCs

OT-I CFSE⁺
CD8⁺ T cells → Flow cytometry analysis after 3 days (CD8, CFSE and Vα2)

OT-II CFSE⁺
CD4⁺ T cells → Flow cytometry analysis after 5 days (CD4, CFSE and Vα2)

Skin-derived
DCs

OT-I CFSE⁺
CD8⁺ T cells → Flow cytometry analysis after 3 days (CD8, CFSE and Vα2)

OT-II CFSE⁺
CD4⁺ T cells → Flow cytometry analysis after 5 days (CD4, CFSE and Vα2)

CD45.1⁺   CD45.2⁺ host
DC only →[24h]→ Enriched for CD11c⁺ host DCs (CD45.2⁺) →

CD8⁺ DCs

OT-I CFSE⁺
CD8⁺ T cells → Flow cytometry analysis after 3 days (CD8, CFSE and Vα2)

OT-II CFSE⁺
CD4⁺ T cells → Flow cytometry analysis after 5 days (CD4, CFSE and Vα2)

Double
negative
DCs

OT-I CFSE⁺
CD8⁺ T cells → Flow cytometry analysis after 3 days (CD8, CFSE and Vα2)

OT-II CFSE⁺
CD4⁺ T cells → Flow cytometry analysis after 5 days (CD4, CFSE and Vα2)

Fig. 5.8. **Transferred antigens from injected DCs are presented by host skin-derived DCs to CD8+ and CD4+ T cells.** (**a**) C57BL/6J mice were injected into their forelimbs with LPS-activated OVA-loaded CD45.1+ wt DCs (DC+OVA) or wt DCs not loaded with OVA protein (DC only). Brachial and axillary lymph nodes were harvested and digested into cell suspensions 24 h after DC injection. Cell suspensions were enriched for host CD11c+ cells through magnetic separation. Enriched CD11c+ cells were sorted based on their expressions of CD205 and CD8, which yielded CD205+CD8lo (skin-derived DCs), CD205+CD8hi (CD8+ DCs) and CD205-CD8- (double negative DCs) populations. These three sorted DC populations were then serially diluted and cultured with 10 × 10^4 CFSE-labelled OT-I CD8+ or OT-II CD4+ T cells per well for 3 or 5 days, respectively. After incubation, OT-I CD8+ and OT-II CD4+ T cells were examined for CFSE dilution by flow cytometry. (**b**) The percentages of CD11c+ cells and different DC populations in the samples before and after flow cytometry sorting are shown. The percentages of (**c**) Vα2+CD8+ or (**d**) Vα2+CD4+ T cells that had divided at least once are shown. Two separate experiments with duplicate wells for DC+OVA, and single wells for DC only are pooled together and shown.

5.9. Systemic effector CD4$^+$ T cells responses need the participation of injected DCs

In the experiments presented thus far, I have shown that inter-DC antigen transfer enabled host DCs to present antigens to CD8$^+$ and CD4$^+$ T cells (Fig. 5.7.1 to Fig. 5.8). To determine if CD4$^+$ T cells stimulated by host and injected DCs, or by host DCs only, acquire similar effector functions, IFNγ production by these CD4$^+$ T cells was examined. The ability of these CD4$^+$ T cells to produce IFNγ was also examined in the presence of CTL-mediated DC elimination.

The experimental design is depicted in Fig. 5.9.1. Mice received CTLs and OT-II CD45.1$^+$CD4$^+$ T cells, or OT-II CD45.1$^+$CD4$^+$ T cells only. 24 h later, these mice were injected s.c. with OVAtr DC, wt DC+OVA or MHCII$^{-/-}$ DC+OVA. As controls, some mice were injected with MHCII$^{-/-}$ DC+OVA$_{323-339}$ or MHCII$^{-/-}$ DC only. 19 days after DC injection, the spleens of the mice were harvested, digested and made into single cell suspensions. The cell suspensions were then restimulated with OVA$_{323-339}$ in cIMDM. Some cell suspensions were left in culture without the addition of OVA$_{323-339}$ in cIMDM. After peptide restimulation for 20 h, the production of IFNγ by CD4$^+$ T cells was monitored by flow cytometry.

The OT-II CD45.1$^+$CD4$^+$ T cells were present and produced IFNγ upon OVA$_{323-339}$ restimulation in groups that were injected with OVAtr DCs or wt DC+OVA (Fig. 5.9.2a and c). In groups that received MHCII$^{-/-}$ DC+OVA, MHCII$^{-/-}$ DC+OVA$_{323-339}$ or MHCII$^{-/-}$ DC only, OT-II CD4$^+$ T cells were nearly absent and IFNγ-producing CD4$^+$ T cells could not be detected. In all groups, the presence of CTLs did not affect the production of IFNγ by OT-II CD4$^+$ T cells (Fig. 5.9.2b and c).

Similar percentages of host CD4$^+$ T cells were detectable in all groups. However, host CD4$^+$ T cells produced IFNγ only in groups that were injected with OVAtr DCs or wt DC+OVA, but not in groups that received MHCII$^{-/-}$ DC+OVA, MHCII$^{-/-}$ DC+OVA$_{323-339}$ or MHCII$^{-/-}$ DC only (Fig. 5.9.2d). The IFNγ production by host CD4$^+$ T cells was not antigen-specific as the host CD4$^+$ T cells, but not OT-II CD4$^+$ T cells, produced IFNγ when no OVA$_{323-339}$ was added to the culture (Fig. 5.9.2e).

These results showed that although host DCs presented antigens carried by injected MHCII$^-$$^{/-}$ DCs to induce CD4$^+$ T cell proliferation (Fig. 5.4 and Fig. 5.7.1), the stimulated CD4$^+$ T cells did not accumulate in the spleens of mice. This suggests that the priming of effector CD4$^+$ T cells need the participation of the injected DCs. This result also showed that although CTLs eliminated OVAtr DCs and prevented the DCs from accumulating in the draining lymph nodes (Fig. 4.3.4), the quality of effector CD4$^+$ T cells induced by host DCs through inter-DC antigen transfer was not compromised.
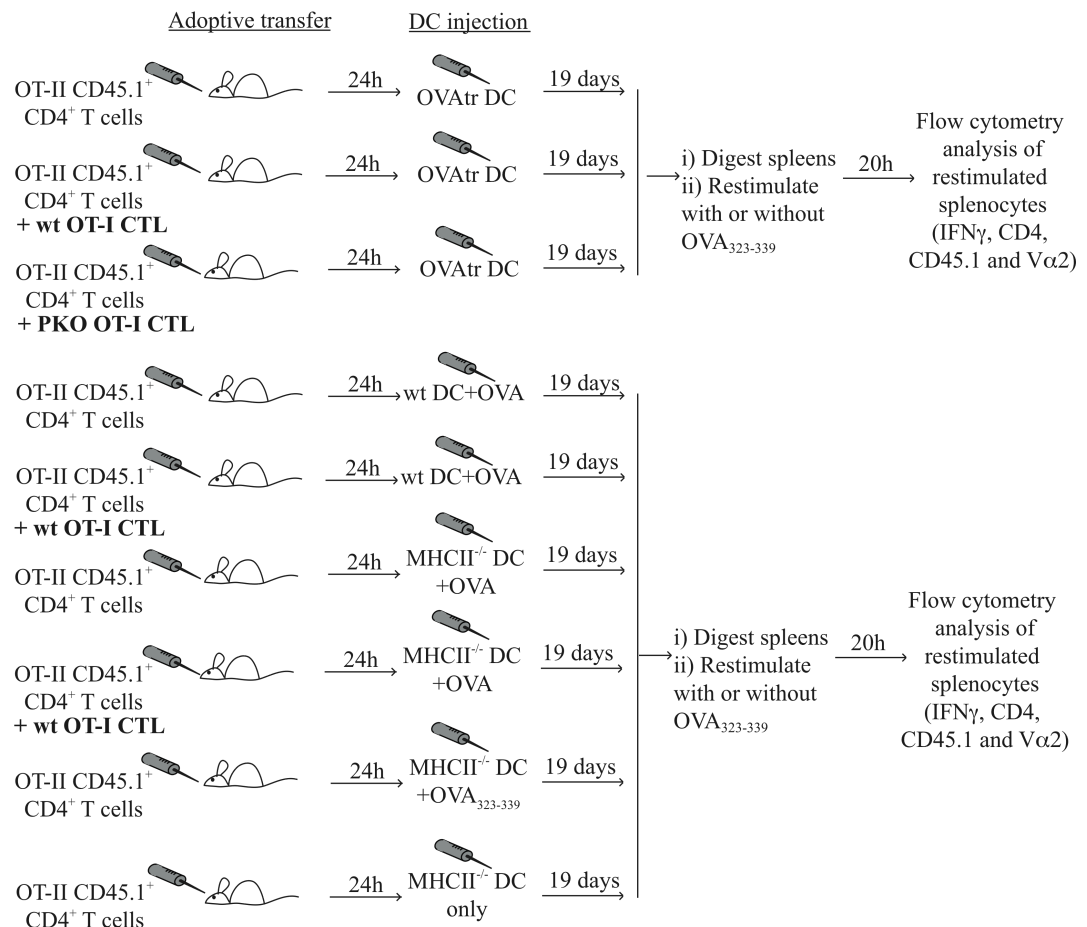
Fig. 5.9.1. **The experimental setup for Fig. 5.9.2. is illustrated.**

e)

■ IFNγ$^+$CD45.1$^+$CD4$^+$ T cells
□ Isotype$^+$CD45.1$^+$CD4$^+$ T cells
▲ IFNγ$^+$ host CD4$^+$ T cells
△ Isotype$^+$ host CD4$^+$ T cells
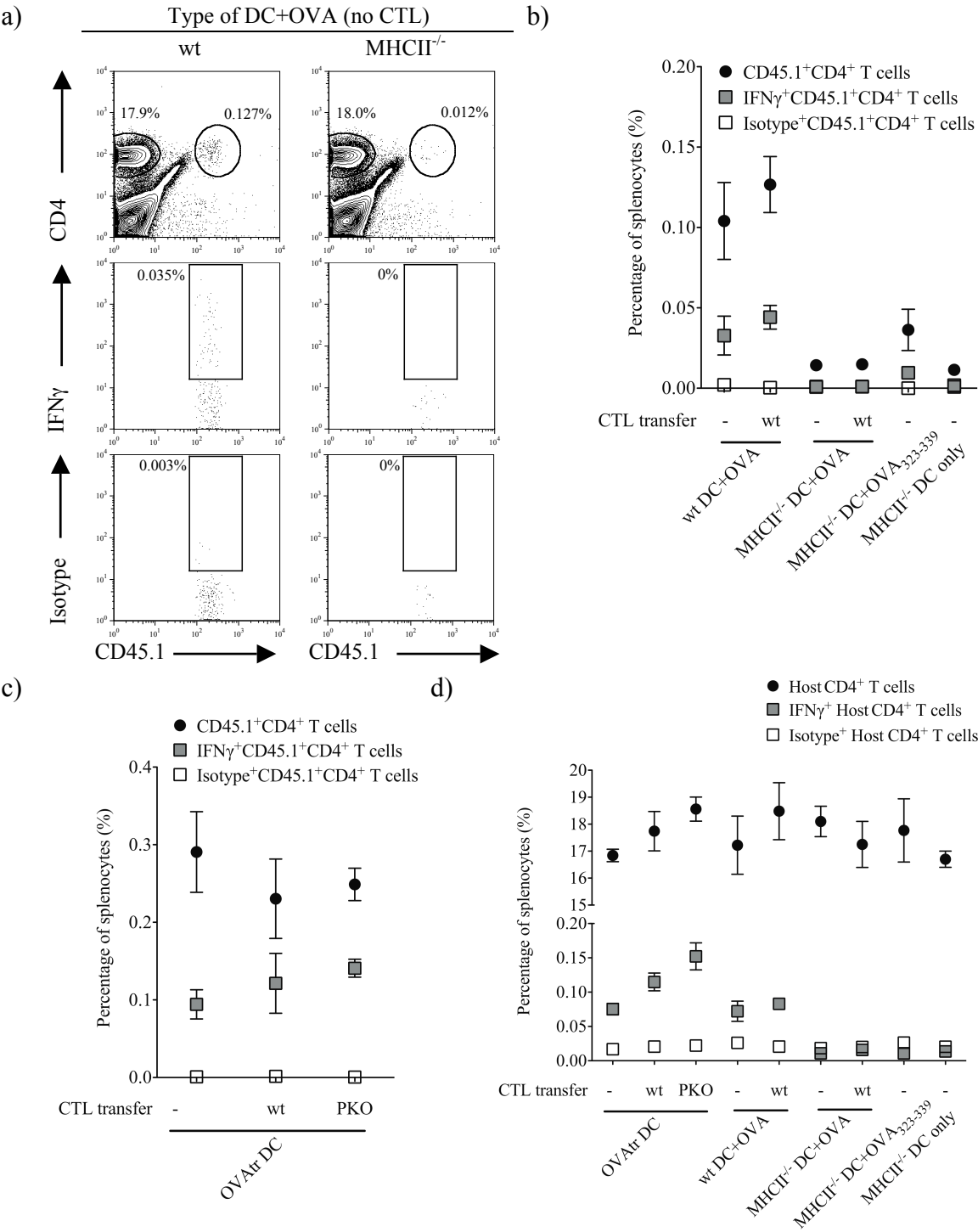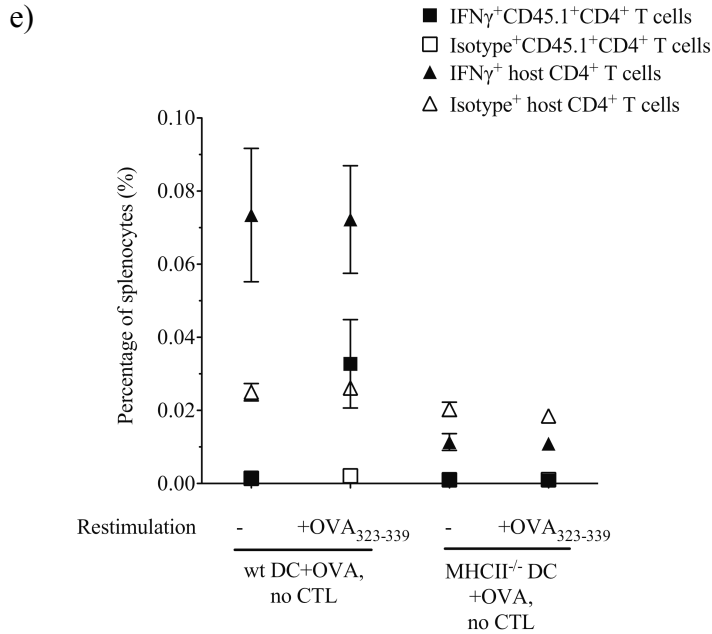
Fig. 5.9.2 **Effector CD4$^+$ T cells do not accumulate in the spleens when injected DCs cannot present antigens directly to CD4$^+$ T cells.** The experimental setup is illustrated in Fig. 5.9.1. C57BL/6J mice were injected i.v. with either OT-II CD45.1$^+$CD4$^+$ T cells and *in vitro* activated OT-I CTLs, or OT-II CD4$^+$ T cells only. After 24 h, these mice were injected s.c. into their forelimbs with LPS-activated OVAtr DCs, wt DC+OVA or MHCII$^{-/-}$ DC+OVA. As controls, some mice received MHCII$^{-/-}$ DCs loaded with OVA$_{323-339}$ or MHCII$^{-/-}$ DCs only. 19 days later, spleens were harvested, digested and made into cell suspensions. The cell suspensions were then restimulated with OVA$_{323-339}$ in cIMDM. Some cell suspensions were left in culture without the addition of OVA$_{323-339}$ in cIMDM. After 20 h of restimulation, IFNγ production by CD4$^+$ T cells was monitored by flow cytometry. (**a**) Host CD4$^+$ and OT-II CD45.1$^+$CD4$^+$ T cells from individual mice that received OT-II CD4$^+$ T cells only and wt DC+OVA or MHCII$^{-/-}$ DC+OVA are shown. IFNγ-producing OT-II CD45.1$^+$CD4$^+$ T cells and the corresponding isotype controls are shown. (**b** and **c**) The percentages of total CD45.1$^+$CD4$^+$ T cells, IFNγ-producing CD45.1$^+$CD4$^+$ T cells and the corresponding isotype controls are shown. (**d**) The percentages of host CD4$^+$ T cells, IFNγ-producing host CD4$^+$ T cells and the corresponding isotype controls are shown. (**e**) The percentages of IFNγ-producing host CD4$^+$ T cells, IFNγ-producing OT-II CD45.1$^+$CD4$^+$ T cells and the corresponding isotype controls are shown. One representative experiment out of two with 3 – 5 mice per group is shown.

5.10. Discussion

In this chapter, I showed that the cellular materials of injected DCs and the antigens loaded on them could be transferred to host cells. The transferred antigens were presented by host antigen-presenting cells to CD4$^+$ T cells and induced CD4$^+$ T cell proliferation. I also showed that host DCs presented the antigen carried by injected DCs to CD4$^+$ and CD8$^+$ T cells. The presentation of transferred antigens by host DCs was transient. Among the host DC subpopulations, the skin-derived DCs contributed the most to presenting antigens carried by injected DCs to CD4$^+$ and CD8$^+$ T cells. Host lymph node resident CD8$^+$ DCs also presented the transferred antigens to CD8$^+$ T cells. Although host DCs present antigens captured from injected DCs and induced CD4$^+$ T cell proliferation, the antigen presentation by injected DCs was necessary to prime effector CD4$^+$ T cells. When CTLs prevented the entry of injected DCs into draining lymph nodes, inter-DC antigen transfer enabled the host DCs to induce CD4$^+$ T cell proliferation. The stimulated CD4$^+$ T cells subsequently developed into effector T cells in the presence of CTL-mediated DC killing. Overall, this chapter highlights two key aspects, the type of immune responses generated from inter-DC cooperation, and the interplay between CTL-mediated DC killing and inter-DC antigen transfer (Fig. 5.10.1 and Fig. 5.10.2).

5.10.1. Contributions of host and injected DCs to T cell proliferation

Antigen transfer among DC populations has been suggested by a number of reports (Allan et al., 2006; Belz et al., 2004a; Inaba et al., 1998; Kleindienst and Brocker, 2003; Luketic et al., 2007; Qu et al., 2009). One example of antigen transfer among DCs was described by Inaba et al (Inaba et al., 1998). In that study, the transfer of MHC class II-restricted peptide between injected DCs and host DCs in the T cell area of draining lymph nodes was demonstrated by using antibodies against the MHC class II-peptide complex. In a different study, the peptides transferred from injected DCs to host DCs induce antigen specific CD4$^+$ T cell clonal expansion *in vivo* (Kleindienst and Brocker, 2003). In that study, the T cell clonal expansion was significantly lacking in the absence of antigen presentation by host DCs, even though the injected DCs alone could induce CD4$^+$ T cell expansion. In my study, compared to the CD4$^+$ T cell expansion induced by host and injected DCs, host DCs

presenting antigens captured from injected DCs induced significantly lower CD4$^+$ T cell expansion (Fig. 5.10.1b). The decrease in T cell proliferation could be due to the alteration in antigen-presenting DC numbers in the draining lymph nodes. This is because the generation of effector T cells in the draining lymph nodes has been shown to be inversely related to antigen dose, the stability of peptide-MHC complexes on the DCs, and the antigen-presenting cell density (Henrickson et al., 2008). In considering the notion of altering DC numbers, my study showed that CTL-mediated killing of DCs loaded with OVA protein did not reduce CD4$^+$ T cell expansion, although approximately 50% of the OVA-loaded DCs injected were eliminated. This indicated that host DCs presenting transferred antigens were sufficient to induce CD4$^+$ T cell proliferation. This is probably because host DCs constitute the biggest proportion of DC presenting antigens to T cells, since only a few percent of the injected DCs reach the draining lymph nodes (Martin-Fontecha et al., 2003).

5.10.2. Induction of effector T cells by host and injected DCs

Besides having superior numerical advantage over the injected DCs in the draining lymph nodes, host DCs have been shown to prime CD62L^{lo} effector CD4$^+$ T cells that produce IL-2, suggesting that host DCs presenting antigens carried by injected DCs were sufficient to generate effector T cell responses (Kleindienst and Brocker, 2003). In my study, when host DCs were presenting antigens captured from injected MHCII$^{-/-}$ DCs, only a few effector CD4$^+$ T cells were found in the spleens of mice. The few effector CD4$^+$ T cells did not make IFN-γ upon re-stimulation 19 days after DC injection. Because these CD4$^+$ T cells proliferated in the draining lymph nodes but did not appear in the spleens, this would indicate that the presentation of transferred antigens by host DCs alone failed to generate systemic effector CD4$^+$ T cell responses (Fig. 5.10.2b). In my study, splenocytes were re-stimulated with OVA$_{323-339}$ 19 days after DC injection and examined for IFN-γ production, whereas in the study by Kleindienst and Brocker, lymphocytes were re-stimulated with PMA and ionomycin 5 days after DC injection and examined for IL-2 production (Kleindienst and Brocker, 2003). This could account for the differences in the type of CD4$^+$ T cells observed between this study and the study by Kleindienst and Brocker. Nonetheless, in support of the notion that effector T cells were not generated when host DCs were

presenting antigens captured from injected DCs, tolerised CD4$^+$ T cells have been shown to produce effector cytokines such as IL-2 and IFN-γ early after antigen encounter but this effector cytokine production was lost afterwards (Huang et al., 2003). Moreover, targeting antigens to DCs *in vivo* through CD205 antibody induced CD4$^+$ T cell proliferation and IL-2, but not IFN-γ, production, and these CD4$^+$ T cells eventually disappeared from the draining lymph nodes and spleen (Hawiger et al., 2001).

There are a few possibilities as to why host DCs do not prime effector CD4$^+$ T cells when the injected DCs cannot present antigen directly to CD4$^+$ T cells. Firstly, as mentioned before, the density of these antigen-presenting DCs in the draining lymph nodes is likely to be altered in the absence of presentation by the injected DCs. This can potentially translate to insufficient antigen stimuli to cross the threshold necessary for the development of effector functions (Iezzi et al., 1998).

Secondly, the injected DCs are phenotypically highly activated, hence they are able to provide T cells with the optimal co-stimulatory signals. In contrast, host DCs are phenotypically immature (De Smedt et al., 2001; Wilson et al., 2003). Targeting the antigen directly to host DCs without activation signals led to T cell tolerance, whereas simultaneous addition of antigen and DC activation stimuli induced effector T cells (Bonifaz et al., 2002; Hawiger et al., 2001). It is likely that in the absence of direct antigen presentation by activated injected DCs and inflammation, host DCs do not provide sufficient co-stimulatory signals to the CD4$^+$ T cells. It can be argued that in a tripartite interaction, the host DCs supply the antigen signal whereas injected DCs provide the co-stimulatory signals to prime effector T cells. Against this, it has been shown that when the CD4$^+$ T cells did not receive antigenic and co-stimulatory signals from the same DC, naïve CD4$^+$ T cells underwent proliferation but did not develop into effector CD4$^+$ T cells (Sporri and Reis e Sousa, 2005). This is in line with my results showing that through the presentation of antigens captured from the injected DCs, host DCs can induce the proliferation of CD4$^+$ T cells but did not induce systemic effector CD4$^+$ T cell responses.

Overall, this indicates the importance of injected DCs in producing effector T cell responses and directing the type of T cell immune response generated.

5.10.3. Effects of CTL-mediated DC killing and antigen transfer on effector T cell development

I have shown that while CTL-mediated DC killing prevented DC accumulation in the draining lymph nodes (Fig. 5.10.1a), the antigens were transferred to the host DCs which then initiated CD4$^+$ T cell proliferation (Fig. 5.10.1c). These CD4$^+$ T cells developed into functional effector T cells that could produce IFN-γ upon antigenic re-stimulation (Fig. 5.10.2a). This is surprising because by preventing the accumulation of antigen-loaded activated DC in the draining lymph nodes, CTLs essentially deprive the CD4$^+$ T cells of sufficient co-stimulatory activation. However, in the presence of CTL-mediated DC killing, effector CD4$^+$ T cells were present in the spleens and responded to antigen restimulation. In contrast, I have shown that even though OVA-loaded activated MHCII$^{-/-}$ DCs accumulated in the draining lymph nodes and could provide sufficient co-stimulatory signals, CD4$^+$ T cells proliferated but did not accumulate in the spleens.

This begets a question of how the CD4$^+$ T cells develop into effector T cells when activated injected DCs are prevented from entering the draining lymph nodes by CTLs. I propose the following: after CD4$^+$ T cells have received incomplete signals from host DCs that have presented antigens captured from injected DCs, the CD4$^+$ T cells migrate from the draining lymph nodes to the site of injection (Campbell and Butcher, 2002), where most of the injected DCs reside (Yang et al., 2006). CD4$^+$ T cells subsequently receive antigen and co-stimulation signals from the injected DCs in the non-lymphoid tissues. The fully activated CD4$^+$ T cells then develop into functional effector T cells. In support of this, CD4$^+$ T cells required re-exposure to antigen-presenting cells and polarising cytokines to differentiate fully into effector T helper cells (Bajenoff et al., 2002). The development of effector CD4$^+$ T helper cells also required priming by DCs that had been directly activated by LPS (Sporri and Reis e Sousa, 2005). Moreover, DCs in the non-lymphoid tissues expanded the number of tissue-resident memory CD8$^+$ T cells during herpes simplex virus (HSV) infection (Wakim et al., 2008).

In order to demonstrate that the injected DCs in the non-lymphoid tissues are priming the CD4$^+$ T cells, I propose the following experiment: Mice will be injected i.v. with OT-I

CTLs and OT-II CD4$^+$ T cells, or OT-II CD4$^+$ T cells. 24 h later, CCR7$^{-/-}$ DCs loaded with OVA protein or CCR7$^{-/-}$ MHC class II$^{-/-}$ DCs loaded with OVA protein will be injected s.c. into the forelimbs of the recipient mice. 7 and 19 days later, splenocytes will be harvested, digested and restimulated by OVA$_{323-349}$ in cIMDM. The production of IFNγ by CD4$^+$ T cells will then be examined. Because CCR7$^{-/-}$ DCs do not migrate into the draining lymph nodes (Martin-Fontecha et al., 2003), the stimulated CD4$^+$ T cells need to migrate to the non-lymphoid tissues to encounter the injected CCR7$^{-/-}$ DCs. By comparing the result obtained from CCR7$^{-/-}$ DCs to that from CCR7$^{-/-}$ MHC class II$^{-/-}$ DCs, the experiment should provide evidence that will elucidate if the activated DCs at the site of injection are important for the development of effector CD4$^+$ T cells.

5.10.4. Efficiency of antigen transfer between injected and host DCs

Visualisation and direct quantification of the antigen transfer from injected DCs to host DCs showed that inter-DC antigen transfer was remarkably efficient (Inaba et al., 1998). In a HSV infection, the detection of antigen transfer through antigen specific CD8$^+$ T cell expansion revealed that the transfer of antigens from migratory to lymph node resident DCs occurred at least eight hours after infection (Allan et al., 2006). My results showed that 24 h after DC injection, host DCs presented antigens carried by injected DCs on MHC class-I and class-II molecules. Because antigen transfer among DCs occurs early during T cell priming, the physiological purpose of inter-DC antigen transfer has been proposed to amplify the immune responses by increasing the availability of DCs presenting antigens to T cells (Allan et al., 2006; Mueller et al., 2002). In other circumstances, it has been suggested that inter-DC antigen transfer serves to induce T cell tolerance to prevent self-reactivity (Inaba et al., 1998). This is because host DCs are immature and they induce T cell tolerance in the absence of activation stimuli (Bonifaz et al., 2002; Hawiger et al., 2001). The physiological purpose of inter-DC antigen transfer probably depends on the situation encountered by the DCs.

5.10.5. Antigen transfer between injected and host DCs in the draining lymph node

It has been proposed that migratory DCs from the skin (Allan et al., 2006) or the lungs (Belz et al., 2004b) ferry antigen from the periphery to the lymph node resident CD8^{+} DCs. In support of this, the kinetics of inter-DC antigen transfer coincides with the kinetics of skin-derived migratory DCs reaching the draining lymph nodes (Allan et al., 2006). Furthermore, because the injection of apoptotic or necrotic peptide-loaded DCs did not induce T cell proliferation, it was suggested that viable antigen-loaded DCs transferred their antigen to the host DCs in the draining lymph nodes and not at the site of injection (Kleindienst and Brocker, 2003). My results showed that host DCs presented transferred antigens 24h after DC injection during which the number of DCs was accumulating in the draining nodes (Fig. 3.6.3). Moreover, my DC sorting experiments showed that host lymph node resident CD8^{+} DCs presented the MHC class I-restricted antigen carried by injected DCs. Taken together, these results support the notion that some MHC class I-restricted antigen transfer between CD8^{+} DCs and migratory DCs takes place in the draining lymph nodes.

5.10.6. Antigen transfer between injected and host DCs in the skin

Besides occurring in the draining lymph nodes, a case could be argued for inter-DC antigen transfer taking place in the non-lymphoid tissues or at the site of injection, i.e. the skin. It has been proposed that in a HSV-1 model, CD103^{+} dermal DCs acquire the viral antigens in the skin rather than in the draining lymph nodes due to the different kinetics of antigen presentation by the CD103^{+} dermal DCs compared to the lymph node resident CD8^{+} DCs (Bedoui et al., 2009). It is also interesting to note that CD103^{+} dermal DCs cross-present both HSV and antigens from the keratinocytes, although HSV antigens and antigens from the keratinocytes are restricted to the epidermal layer, hence should only be accessible to Langerhans cells (Bedoui et al., 2009; Henri et al., 2010). Although the proposition of antigen transfer from Langerhans cells to dermal DCs (Ginhoux et al., 2007) has been re-addressed and argued against by Henri et al (Henri et al., 2010), the model used in their studies addresses a steady-state scenario. However, this situation could be different in viral infections, for example, HSV-1 and HSV-2 infections lead to massive cell death in DCs

(Jones et al., 2003). The large quantity of apoptotic bodies from dying DCs could be taken up by other DCs and presented to CTLs (Albert et al., 1998). Although this has never been formally shown, this could be the case for HSV-infected Langerhans cells and CD103^{+} dermal DCs (Bosnjak et al., 2005).

My results showed that host skin-derived DCs presented most of the transferred antigens acquired from the injected DCs. This could indicate that inter-DC antigen transfer could have occurred in the skin where the antigen-loaded DCs were introduced, or that the antigen transfer to the host skin-derived DCs could have occurred in the draining lymph nodes since DCs migrate from the skin to the draining lymph nodes at steady state (Henri et al., 2010; Merad et al., 2002) and during inflammation (De Smedt et al., 1996; Merad et al., 2000). However, I showed that even when the entry of antigen-loaded DCs into the draining lymph nodes was prevented by CTLs, robust T cell proliferation was observed. Besides, antigen-loaded DCs at the site of injection remained viable, and in abundance, although their numbers were markedly reduced by specific CTLs (Yang et al., 2006). Notably, I showed that the low numbers of host skin-derived DCs induced robust CD8^{+} T cell proliferation, whereas host lymph node resident CD8^{+} DCs induced weaker T cell responses. This suggests that the antigen density is higher on the host skin-derived DCs compared to host lymph node resident CD8^{+} DCs and is probably due to greater exposure to the antigens loaded on the injected DCs. In line with this, my study showed that the antigen transfer from injected DCs to host DCs was dependent on the antigen dose loaded onto the injected DCs, indicating that the antigen concentration at the injection site determines whether inter-DC antigen transfer occurred. In contrast to the low frequency of antigen-loaded DCs that escaped CTL killing in the draining lymph nodes, the availabilities of antigen-loaded DCs and antigen in the non-lymphoid tissues are likely to serve as the main sources of antigen for inter-DC antigen transfer. In conclusion, my study showed that inter-DC antigen transfer occurred mostly in the peripheral tissues, while some antigen transfer took place in the draining lymph nodes.

5.10.7. Antigen transfer via apoptotic DCs

Antigen transfer from DC to DC could occur via apoptotic DCs or apoptotic bodies, as the uptake of apoptotic cells by DCs has been described *in vitro* (Inaba et al., 1998) and *in vivo* (Fleeton et al., 2004; Huang et al., 2000). In addition, lymph node resident CD8^{+} DCs specialised in the uptake of apoptotic bodies (Iyoda et al., 2002) and these CD8^{+} DCs have been shown to be recipients of antigen transfer (Allan et al., 2006). Furthermore, the elimination of target cells by CTLs released antigens for antigen presentation by DCs (Kurts et al., 1998b; Parish et al., 2009). It is possible that the CTL-mediated DC killing releases the materials of the deceased DCs, and this may lead to greater uptake by host APCs. However, this was not observed in my study. Instead, the number of host cells that took up materials of the injected DCs was reduced in the presence of CTLs. This could be because the host cells became susceptible to killing by CTLs after antigen transfer. Taken together, there is evidence supporting the notion that inter-DC antigen transfer occurs through apoptotic DCs.

5.10.8. Antigen transfer via exosomes

Exosomes have been proposed to facilitate antigen transfer among DCs (Thery et al., 2002b). In support of this, DCs cultured *in vitro* have been shown to secrete exosomes (Segura et al., 2005). The exosomes secreted by DCs bore functional MHC-peptide complexes, could activate T cell responses (Thery et al., 2002a) and induced anti-tumour responses (Zitvogel et al., 1998). My *in vitro* transwell experiments indicated that direct cell-cell contact was not necessary for antigen transfer between two DCs. Although my study could not distinguish between exosomes and apoptotic bodies, other studies have shown evidence that inter-DC antigen transfer occurred through exosomes. For example, the exosomes containing MHC-peptide complexes were recovered from the draining lymph nodes of mice immunised with SIINFEKL-loaded DCs (Luketic et al., 2007). These exosomes also induced T cell proliferation and conferred tumour protection. It should be noted that antigen transfer occurred only when viable antigen-loaded DCs, but not apoptotic ones, were injected (Kleindienst and Brocker, 2003). Thus, it is likely that the

pool of viable DCs at the site of injection are transferring antigens to the host skin-derived DCs via the secretion of exosomes.

5.10.9. Host DC subpopulations involved in antigen transfer

Host skin-derived DCs consist of five populations residing in the epidermis and dermis (Henri et al., 2010). Among them, $CD207^+CD103^+$ dermal (Bedoui et al., 2009; Henri et al., 2010) and Langerhans cells (Stoitzner et al., 2006) have been shown to cross-present antigens to $CD8^+$ T cells. All the dermal DCs and Langerhans cells can present antigens to $CD4^+$ T cells to varying degrees (Bedoui et al., 2009; Stoitzner et al., 2006). Accordingly, because my DC sorting experiment did not segregate the host skin-derived DCs into these subpopulations, these DCs collectively were able to present MHC class-I and -II antigens carried by the injected DCs remarkably well.

In the draining lymph node and spleen, the resident DCs can be categorised into three populations $CD8^+CD205^+CD4^-$, $CD4^+CD8^-CD205^-$ and $CD8^-CD4^-CD205^-$ DCs (Henri et al., 2001; Vremec et al., 2000). $CD8^+CD205^+CD4^-$ DCs, also commonly denoted as $CD8^+$ DCs, have been shown to cross prime $CD8^+$ T cells but have limited MHC class-II presenting ability (den Haan et al., 2000; Dudziak et al., 2007). Their ability to cross present antigens lies in their intrinsic expression of proteins involved in MHC class-I processing and receptor-mediated antigen uptake (Burgdorf et al., 2007; Dudziak et al., 2007; Schnorrer et al., 2006). In accordance with this, my results showed that host lymph node resident $CD8^+$ DCs presented MHC class-I, but not MHC class-II, restricted antigens carried by the injected DCs.

Because $CD4^+CD8^-CD205^-$ and $CD8^-CD4^-CD205^-$ DCs do not express CD205 and $CD4^+CD8^-CD205^-$ DCs constitute a minor proportion of lymph node resident DCs (Shortman and Liu, 2002), these two DC populations were grouped together as $CD8^-CD205^-$ DCs, which were denoted as double negative DCs, in my DC sorting experiment. While it was not surprising that host lymph node resident double negative (DN) DCs did not present MHC class-I restricted antigens (den Haan et al., 2000; Dudziak et al., 2007; Qiu et al., 2009), it was interesting that DN DCs did not present MHC class-II restricted

antigens either. This was contrary to reports that these DN DCs could activate CD4$^+$ T cells (Dudziak et al., 2007; Pooley et al., 2001). Although lymph node resident DCs existed in an immature phenotype and were functionally immature under steady state conditions *in vivo* (De Smedt et al., 2001; Wilson et al., 2003), it as unlikely that DN DCs were unable to activate T cells due to this reason because their lymph node resident counterparts, CD8$^+$ DCs, could induce CD8$^+$ T cell proliferation. Furthermore, *ex vivo* manipulation of DCs could induce DC activation (Wilson et al., 2003). I cannot rule out the possibility that DN DCs presented very low amounts of antigens, which could not be detected by OT-II CD4$^+$ T cells. Other possibilities as to why DN DCs did not capture antigens from other DCs and present these antigens to CD4$^+$ T cells are discussed in subsequent sections.

5.10.10. Location of host DC subpopulations and accessibility to antigen-loaded injected DCs

One possible explanation for why DN DCs do not present transferred antigens to CD4$^+$ T cells may lie in their spatial location in the draining lymph nodes. Early studies by Witmer and Steinman showed that MHC class-II expressing cells found in the paracortex where T cells reside, or the B cell area in the lymph nodes did not stain for anti-33D1 antibody, suggesting that DN DCs did not reside in these locations (Witmer and Steinman, 1984). The MHC class-II expressing cells in the T cell zone are likely to be CD8$^+$ DCs as they express low levels of CD207 (Kissenpfennig et al., 2005) while the MHC class-II expressing cells in the B cell area are likely to be follicular DCs (Cyster et al., 2000). In the spleen, DN DCs are located in the marginal zone, whereas CD8$^+$ DCs are found in the T cell area (Dudziak et al., 2007). This is similar in the Peyer's patch where DN DCs and CD8$^+$ DCs are anatomically segregated (Iwasaki and Kelsall, 2000). The distinct localisations of DC subpopulations in various lymphoid organs suggest that CD8$^+$ DCs and DN DCs have different access to the antigen-loaded injected DCs.

In considering whether DN DCs have access to transferred antigens, the location of the antigen-bearing DCs in the lymph nodes has to be examined. DCs that carry the antigens into the draining lymph nodes are the injected DCs and the host skin-derived DCs. Among the host skin-derived DCs, classical dermal DCs and Langerhans cells have been shown to

localise in the outer paracortex but occupy distinct paracortical locations (Kissenpfennig et al., 2005). It is likely that CD207$^+$CD103$^+$ dermal DCs occupy similar paracortical regions as the Langerhans cells since it was not known at that time that a population of dermal DCs also expressed CD207 (Bursch et al., 2007; Poulin et al., 2007). Antigen-bearing host skin-derived DCs have been shown to preferentially localise in the paracortex near the high endothelial venules (HEV) (Bajenoff et al., 2003). Injected DCs enter the draining lymph nodes through the afferent lymphatic vessels, pass through the subcapsular sinus and reside in the outer paracortex (Bajenoff et al., 2003). These injected antigen-loaded DCs interact with antigen-specific T cells in the outer paracortex in the proximity of HEVs. Because CD8$^+$ DCs also reside in the paracortex, it stands to reason that the antigen-bearing incoming DCs and CD8$^+$ DCs are in close proximity for antigen transfer to occur (Kleindienst and Brocker, 2003). On the contrary, it remains a possibility that DN DCs in the draining lymph nodes may not have encountered the antigen-bearing DCs.

5.10.11. Intrinsic properties of DN DCs and antigen transfer

Besides the unresolved location of the DN DCs in the draining lymph nodes, the intrinsic properties of DN and CD8$^+$ DCs have to be considered. I have previously discussed that antigen transfer has been proposed to occur via apoptotic cells or exosomes. It has been shown that CD8$^+$, but not DN, DCs selectively take up apoptotic cells (Iyoda et al., 2002) and exosomes (Segura et al., 2007). This is not because DN DCs are impaired in antigen capture since they can capture soluble proteins and peptides. The functional differences in antigen capture between DN and CD8$^+$ DCs are likely to be due to the differential expression of various messenger transcripts and proteins (Dudziak et al., 2007), supporting the idea that different DC subpopulations have specialised functions (Villadangos and Schnorrer, 2007). Thus, it is possible that the intrinsic properties of DN DCs do not allow them to participate in inter-DC antigen transfer.

a)   No antigen transfer occurs when a low antigen dose is loaded onto injected DCs

Injected DCs migrate to
the draining lymph nodes

Injected DC

Injected DCs accumulate and
induce T cell proliferation

CTLs kill injected DCs and prevent
DC entry into draining lymph nodes

Injected DC

Few surviving injected DCs
induce weak T cell proliferation

Skin

Draining lymph node

b)   Inter-DC antigen transfer occurs when a high antigen dose is loaded onto injected DCs

Injected DC

Injected DCs and host
skin-derived DCs migrate
to the draining lymph nodes

Injected DCs accumulate and
induce T cell proliferation

Host lymph node resident
CD8$^+$ DCs also capture
antigens and induce CD8$^+$
T cell proliferation

Host skin-derived DC
capture antigens from
injected DCs

Host skin-derived DCs
present the captured antigens
and induce T cell proliferation

Skin

Draining lymph node

c)   Inter-DC antigen transfer occurs even when injected DCs are killed by CTLs

Injected DC

CTLs kill injected DCs
but host skin-derived DCs
carry antigens

Few surviving injected DCs
induce weak T cell proliferation

Host lymph node resident
CD8$^+$ DCs also capture
antigens and induce CD8$^+$
T cell proliferation

Host skin-derived DC
capture antigens from
injected DCs

Host skin-derived DCs
present the captured antigens
and induce T cell proliferation
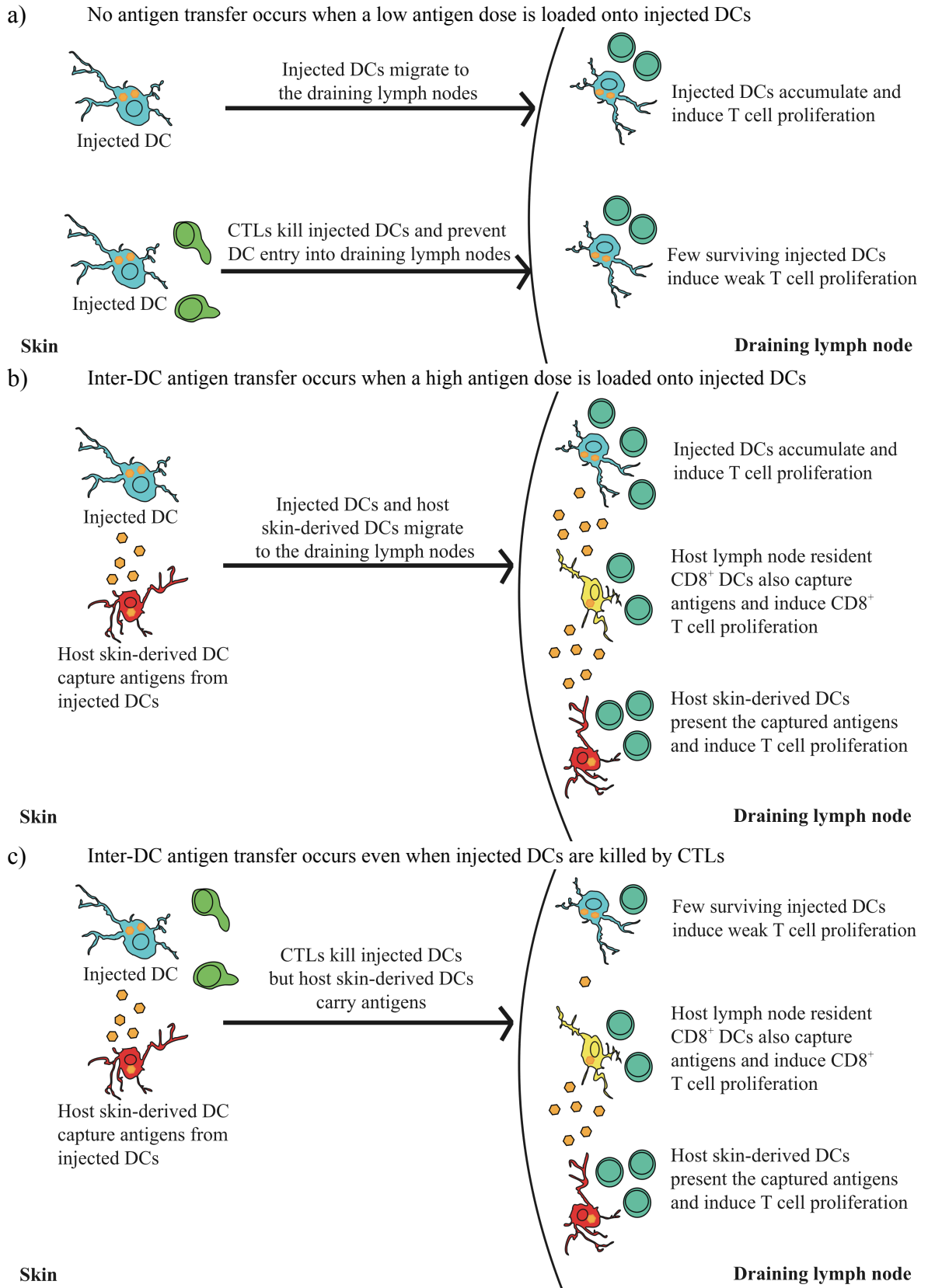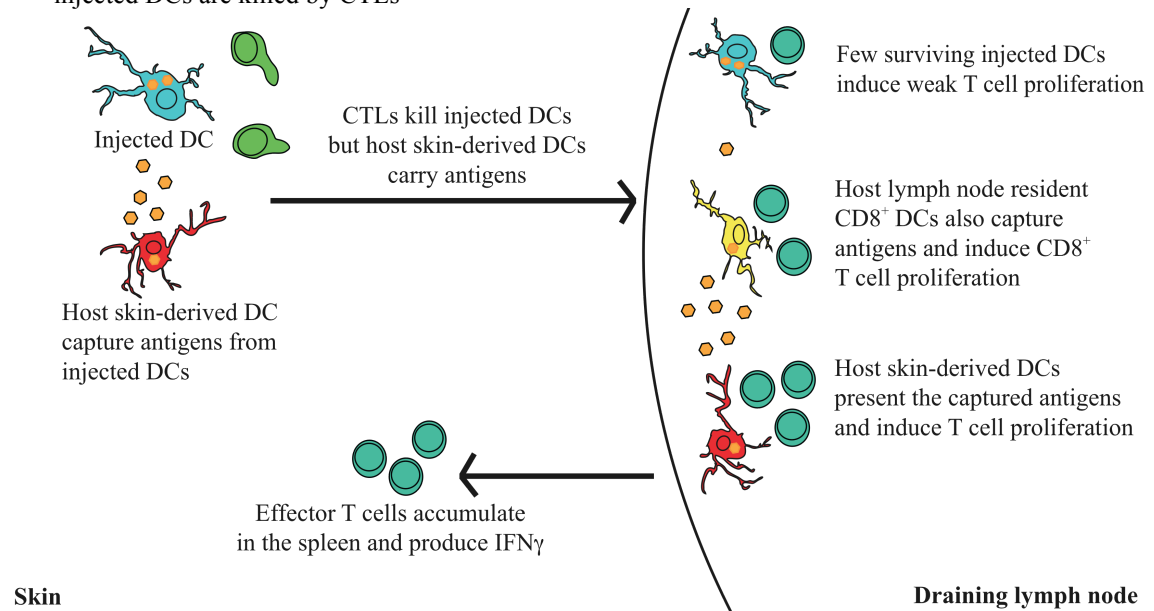
Skin

Draining lymph node

Fig. 5.10.1. **The effects of CTL-mediated DC killing and inter-DC antigen transfer on T cell proliferation.**

a) IFNγ producing effector T cells are induced by host DCs presenting captured antigens even when injected DCs are killed by CTLs

Injected DC

CTLs kill injected DCs but host skin-derived DCs carry antigens

Host skin-derived DC capture antigens from injected DCs

Few surviving injected DCs induce weak T cell proliferation

Host lymph node resident CD8$^+$ DCs also capture antigens and induce CD8$^+$ T cell proliferation

Host skin-derived DCs present the captured antigens and induce T cell proliferation

Effector T cells accumulate in the spleen and produce IFNγ

Skin

Draining lymph node

b) Host DCs presenting captured antigens fail to induce systemic effector T cell responses when injected MHCII$^{-/-}$ DCs cannot present antigens directly to T cells
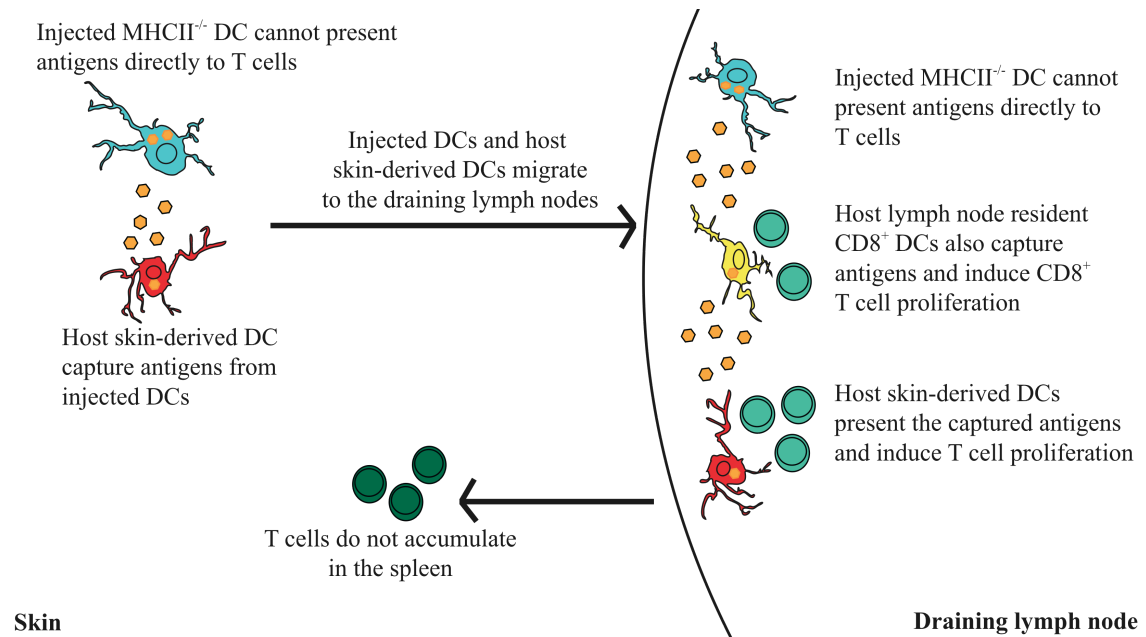

Injected MHCII$^{-/-}$ DC cannot present antigens directly to T cells

Injected DCs and host skin-derived DCs migrate to the draining lymph nodes

Host skin-derived DC capture antigens from injected DCs

Injected MHCII$^{-/-}$ DC cannot present antigens directly to T cells

Host lymph node resident CD8$^+$ DCs also capture antigens and induce CD8$^+$ T cell proliferation

Host skin-derived DCs present the captured antigens and induce T cell proliferation

T cells do not accumulate in the spleen

Skin

Draining lymph node

Fig. 5.10.2. **The type of T cell responses induced by CTL-mediated DC killing and inter-DC antigen transfer.**

Chapter 6

General Discussion

My first hypothesis states that CTLs eliminate antigen-presenting DCs through cytolytic molecules, thereby preventing the induction of T cell responses. In order to address the first hypothesis, I characterised the mechanisms of CTL-mediated DC elimination and examined the impact of DC elimination on T cell proliferation. Results from my study showed that CTLs regulated T cell proliferation by eliminating DCs. CTL-mediated DC killing occurred mostly through perforin, whereas FasL played a minor role.

I also set out to evaluate how different methods of antigen loading onto DCs, types of CTLs and methods of CTL generation might affect DC elimination and the resulting T cell responses. My study showed that CTL-mediated DC killing was affected by the method of antigen loading onto DCs, and to a lesser extent, the method of generating CTLs. Surprisingly, when CTLs killed DCs efficiently and prevented DC accumulation in the draining lymph nodes, this did not abolish T cell proliferation. This suggested that other APCs were inducing the residual T cell proliferation when the antigen-loaded DCs were eliminated by CTLs.

In order to investigate the residual T cell proliferation, I examined the impact of CTL-mediated DC killing and inter-DC antigen transfer on the induction of T cell proliferation and the quality of T cells induced. Antigens were transferred from the antigen-bearing DCs to host DCs, allowing the host DCs to induce T cell proliferation. Different host DC subsets participated in this inter-DC antigen transfer. These observations contradicted my second hypothesis, which states that inter-DC antigen transfer does not induce T cell responses in the presence of CTL-mediated DC killing. My study also showed that although CTL-mediated DC killing reduced the T cell proliferation, inter-DC antigen transfer allowed the host DCs to participate in amplifying T cell expansion. The stimulated T cells could subsequently develop into effector T cells. In conclusion, the interplay between CTL-mediated DC killing and inter-DC antigen transfer reduces the impact of DC killing on T cell proliferation, and influences the size and quality of T cell responses. The findings of my study are important in understanding how CTL-mediated DC killing and inter-DC antigen transfer interact and regulate subsequent immune responses; and in designing better DC vaccine regimens for immunotherapy.

6.1. Limitations of my study and other possibilities

Although the use of *in vitro* activated TCR transgenic CTLs and CD4$^+$ T cells from naïve TCR transgenic mice enabled us to dissect the immune responses, these TCR transgenic T cells may be more sensitive to the model antigen OVA than host T cells induced by DC immunisation are to physiological antigens. TCR transgenic T cells are transferred by the hundreds of thousands into C57BL/6J mice, whereas in a physiological setting, host naïve T cell numbers range from tens to a few thousands in one mouse (La Gruta et al., 2010; Moon et al., 2007). Adoptive transfer of large numbers of identical TCR transgenic T cells may also lead to intraclonal competition (Hataye et al., 2006). TCR transgenic T cells are monoclonal and recognise a specific epitope of an antigen, whereas host T cells consist of different T cell clones that recognise a specific epitope of an antigen; or may recognise different epitopes of an antigen (Felix et al., 2007). The limitations of using OT-I T cells were partly circumvented in my study through the use of peptide-loaded DC immunisation to induce polyclonal host CTLs. I have shown that these polyclonal host CTLs eliminated antigen-loaded DCs. Similar observations were reported by Belz et al. in a viral infection model (Belz et al., 2007), indicating that DC killing occurred under physiological conditions.

In my study, CTL-mediated elimination of antigen-bearing DCs reduced CD4$^+$ T cell proliferation. However, under some physiological conditions, the same DC may not be able to present antigens to both CTLs and CD4$^+$ T cells. This is because different DC subtypes exist in the body. Lymphoid resident CD8$^-$CD205$^-$ DCs and CD11b$^+$ dermal DCs do not cross-present to CD8$^+$ T cells efficiently, but can present to CD4$^+$ T cells. The lymphoid resident CD8$^+$ DCs and the CD103$^+$ dermal DCs are potent cross-presenters to CD8$^+$ T cells, but present to CD4$^+$ T cells inefficiently. Herein lie two questions. The first question asks if CTLs selectively eliminate the cross-presenting DC subsets compared to the non-cross-presenting DC subsets. This question can be partly addressed by using a T cell hybridoma cell line to detect the presentation of SIINFEKL on the different DC subsets in the presence of CTLs as reported by Belz et al (Belz et al., 2007). The second question asks how CTL-mediated elimination of the cross-presenting DC subsets will regulate CD4$^+$ T cell responses when the non-cross-presenting DC subsets are poorly eliminated and

continue to induce CD4$^+$ T cell responses. This question can be addressed using a mouse model lacking the non-cross-presenting DC subsets. However, to the best of my knowledge, a mouse model lacking the non-cross-presenting DC subsets has not been identified. The closest approximation to this hypothetical mouse model would be the CD11b-DTR mice (Duffield et al., 2005) because CD11b is expressed on non-cross-presenting DC subsets. After removing CD11b$^+$ cells from the CD11b-DTR mice through diphteria toxin treatment, the impact of CTL-mediated DC killing on CD4$^+$ T cell proliferation can be determined. The results from this experiment will provide some indications on whether CTLs regulate CD4$^+$ T cell responses through selective killing of the cross-presenting DC subset.

While this and other studies have shown that the OVA antigen and other forms of antigens are transferred among DC populations (Kleindienst and Brocker, 2003; Luketic et al., 2007; Qu et al., 2009), it is unclear whether tumour cell lysates, apoptotic or necrotic cells taken up by one DC population are passed onto another DC population. Furthermore, my study has shown that inter-DC antigen transfer was dependent on the antigen dose loaded onto the DCs. It can be argued that the phenomenon of inter-DC antigen transfer is an artefact of loading excess antigens onto DCs. Against this, skin-derived DCs have been shown to pass viral antigens to lymph node resident CD8$^+$ DCs during HSV infection (Allan et al., 2006), indicating that inter-DC antigen transfer did occur under physiological conditions.

Ex vivo manipulation of DCs through magnetic enrichment and flow cytometry sorting is likely to activate the otherwise immature DCs (Wilson et al., 2003). This may skew the results such that DCs that carry the antigen but do not express sufficient co-stimulatory molecules may induce T cell responses because the *ex vivo* manipulation of DCs activates them. In my study, this limitation was partly addressed through the use of MHCII$^{-/-}$ DCs to show that host APCs were involved in capturing antigens from injected MHCII$^{-/-}$ DCs and presenting the antigens to CD4$^+$ T cells. Although CD11c-DTR mice are not available in our laboratory, these mice would be useful to formally demonstrate that host DCs are involved in inter-DC antigen transfer *in vivo* (Jung et al., 2002). This is because when CD11c-DTR mice are treated with diphtheria toxin, DCs will be removed *in vivo*. By injecting these mice with OVA protein-loaded MHCII$^{-/-}$ DCs, the results from this

experiment will provide supporting evidence to show whether DCs are truly participating in the antigen transfer *in vivo*.

6.2. DC killing and inter-DC antigen transfer for the maintenance of memory T cells

The targeting of antigen-loaded DCs by CTLs for apoptosis has been proposed to be a form of negative feedback to restrain unwarranted immune responses (Belz et al., 2007; Ronchese and Hermans, 2001; Yang et al., 2006). Cells undergoing apoptosis package their cellular contents such as proteins and DNA into apoptotic bodies and these are rapidly phagocytosed by macrophages and DCs (Cohen et al., 1992; Rubartelli et al., 1997). Horizontal spreading of cellular contents from deceased cells to other cells can thus be achieved through apoptosis. Indeed, CTL-mediated killing of target cells released the otherwise inaccessible tissue antigens, which were then taken up by DCs and presented to other self-reactive T cells (Kurts et al., 1998a; Parish et al., 2009). One question arising from this is the physiological purpose of releasing antigens from DCs by CTL-mediated elimination since DC killing is to act as a negative feedback for subsequent immune responses. Moreover, when DCs are loaded with high amounts of antigens, antigen transfer may occur without the need of releasing antigens through DC killing. As mentioned in Chapter 1, inter-DC antigen transfer thus appears to undermine the proposed physiological function of CTL-mediated DC killing.

It is conceivable that following the resolution of primary immune responses through DC elimination and clearance of antigen source, the purpose of transferring antigen released from DCs before, or after CTL-mediated killing to other non-targeted DCs, is to serve as an antigen reservoir for the antigen-specific maintenance of memory T cells (Woodland and Kohlmeier, 2009). In support of this, residual influenza viral antigen presentation lasted for up to two months after influenza infection, and influenza-specific memory CD8$^+$ T cells retained in the lungs had an activated phenotype (Zammit et al., 2006). Influenza-specific memory CD4$^+$ T cells could also be generated from naïve CD4$^+$ T cells through persisting antigen presentation (Jelley-Gibbs et al., 2005). Further examination on the source of antigen reservoir revealed that low and high amounts of antigen persisted in tissue-specific DCs and macrophages respectively (Matthews et al., 2007; Wikstrom et al., 2010). Because

these macrophages did not induce T cell proliferation (Matthews et al., 2007), it was likely that the residual antigen in the DCs was the source for the maintenance of the memory T cells (Zammit et al., 2006). Thus, while CTLs eliminate antigen presenting DCs to downregulate the immune responses during the process of antigen clearance, the transfer of antigen from DC to DC may allow for the persistence of a pool of low antigen load, thereby maintaining or promoting memory T cell population.

6.3. Implications of DC killing and inter-DC antigen transfer for DC immunotherapy

By eliminating antigen-loaded DC vaccines, CTLs can limit the efficiency of DC vaccines in generating T cell responses (Hermans et al., 2000; Yang et al., 2006) (Chapter 3). Because multiple DC vaccines are often given to patients so as to induce sufficient protection against diseases, subsequent DC vaccine injections could be rendered ineffective due to existing CTLs or memory CD8$^+$ T cells generated from the previous DC vaccine injection. Indeed, multiple DC immunisations led to a gradual decline in antigen-specific CD8$^+$ T cells when these DCs were eliminated by CTLs, whereas an accumulation of these T cells was observed in the absence of DC killing (Yang et al., 2006). To the best of my knowledge, the phenomenon of DC killing in DC immunotherapy has never been documented in clinical trials because it may require invasive procedures to procure tissues for assessing the killing of the DC vaccines. I have shown that if the antigen dose loaded on the DC vaccines was sufficient for inter-DC antigen transfer, the quality of effector T cells was not affected even though DC killing reduced the size of the T cell responses. Exploiting the occurrence of antigen transfer from DC vaccines to host DCs may be potentially beneficial in overcoming CTL-mediated DC killing. Morever, the transfer of antigen from DC vaccines to host DCs occurred regardless of the method of antigen-loading on DCs. This allows room to choose the form of antigen when designing DC vaccines. Further experiments using DC vaccines loaded with cell lysates will reveal if inter-DC antigen transfer can be exploited using cell lysates.

In considering the proposal to harness inter-DC antigen transfer for DC immunotherapy, one obvious issue is that host DCs, at least in the case of tumour infiltrating DCs, may be dysfunctional, thus affecting the generation of anti-tumour immune responses (Gerner and

Mescher, 2009; Stoitzner et al., 2008). I have also shown that if host DCs were the only APCs presenting transferred antigens, the quality of effector T cells was less favourable than when both injected and host DCs presented antigens. This can be avoided by injecting adjuvants together with DC vaccines, which have been shown to improve T cell and anti-tumour responses (Petersen et al., 2010; Tough et al., 1997). Thus, coupled with a well-designed prime and boost vaccination strategy, the combination of different therapies with DC vaccines is a prime candidate for immunotherapy against various diseases.

6.4. Future studies

I have hypothesized in 6.2 that the physiological relevance of the interaction between inter-DC antigen transfer and CTL-mediated DC killing is to maintain memory T cells. In order to test this hypothesis, I would first examine how long the antigens persist in the DCs after the antigen are transferred. This result will indicate if inter-DC antigen transfer serves to maintain memory T cells. Secondly, I would examine if CTL-mediated DC killing releases antigens loaded onto the DCs and whether the released antigens are taken up by other DCs. This result will show whether CTL-mediated DC killing facilitates inter-DC antigen transfer. When taken together, these results will provide evidence that will partly address whether the interaction between inter-DC antigen transfer and CTL-mediated DC killing is to maintain memory T cells.

The interaction between inter-DC antigen transfer and CTL-mediated DC killing has never been characterised under physiological conditions. I propose the following experiment to address this question under steady-state conditions. This experiment requires a mouse model whereby one DC subset is made to produce high or low levels of OVA protein constitutively, whereas the other DC subsets do not. This can be achieved by expressing OVA under the control of langerin promoter so that only langerin$^+$ DCs express OVA. These mice will be transferred with OT-I CTLs and DC killing will be determined by evaluating the numbers and percentages of the OVA-producing DC subset. Antigen transfer from the OVA-expressing DC subset to other DC subsets can be determined through sorting the different DC subsets and incubating the sorted DC subsets with CFSE-labelled T cells as described in chapter 5.8. These results will provide evidence that will address the

interaction of inter-DC antigen transfer and CTL-mediated DC killing under steady-state conditions.

I have shown that inter-DC antigen transfer allowed the host DCs to prime effector $CD4^+$ T cells in the presence of CTL-mediated DC killing (Fig. 5.9). I have hypothesized that this was because the injected DCs in the non-lymphoid tissues were providing optimal signals to the $CD4^+$ T cells (Chapter 5.10.3). To address this hypothesis, I have proposed the use of $CCR7^{-/-}$ and $CCR7^{-/-}MHCII^{-/-}$ DCs (Chapter 5.10.3). By comparing the $CD4^+$ T cell responses in mice that received $CCR7^{-/-}$ or $CCR7^{-/-}MHCII^{-/-}$ DCs loaded with OVA protein, the result will indicate if the $CD4^+$ T cells are receiving activation signals from DCs in the non-lymphoid tissues.

While CTL-mediated DC killing and inter-DC antigen transfer have been examined separately in previous studies (Allan et al., 2006; Guarda et al., 2007a; Hermans et al., 2000; Kleindienst and Brocker, 2003; Luketic et al., 2007; Stranges et al., 2007; Yang et al., 2006), my study is the first to examine the interplay between CTL-mediated DC killing and inter-DC antigen transfer and their impact on the resulting T cell responses. My findings also emphasize the importance of tracking the fates of DCs and the antigen they carry. The future work described above continues to investigate how these two inter-cellular phenomena interact and influence the generation of immune responses. Results from the proposed future work will better our understanding of how the interactions among immune cells affect the generation and quality of immune responses.

References

Ackerman, A.L., Giodini, A., and Cresswell, P. (2006). A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. Immunity *25*, 607-617.

Ackerman, A.L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proc Natl Acad Sci U S A *100*, 12889-12894.

Adachi, M., Suematsu, S., Suda, T., Watanabe, D., Fukuyama, H., Ogasawara, J., Tanaka, T., Yoshida, N., and Nagata, S. (1996). Enhanced and accelerated lymphoproliferation in Fas-null mice. Proc Natl Acad Sci U S A *93*, 2131-2136.

Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., and Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem *278*, 1910-1914.

Albert, M.L., Sauter, B., and Bhardwaj, N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature *392*, 86-89.

Alexander-Miller, M.A., Leggatt, G.R., and Berzofsky, J.A. (1996a). Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc Natl Acad Sci U S A *93*, 4102-4107.

Alexander-Miller, M.A., Leggatt, G.R., Sarin, A., and Berzofsky, J.A. (1996b). Role of antigen, CD8, and cytotoxic T lymphocyte (CTL) avidity in high dose antigen induction of apoptosis of effector CTL. J Exp Med *184*, 485-492.

Allan, R.S., Waithman, J., Bedoui, S., Jones, C.M., Villadangos, J.A., Zhan, Y., Lew, A.M., Shortman, K., Heath, W.R., and Carbone, F.R. (2006). Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity *25*, 153-162.

Ando, K., Hiroishi, K., Kaneko, T., Moriyama, T., Muto, Y., Kayagaki, N., Yagita, H., Okumura, K., and Imawari, M. (1997). Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. J Immunol *158*, 5283-5291.

Andreasen, S.O., Christensen, J.E., Marker, O., and Thomsen, A.R. (2000). Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8+ effector T cell responses. J Immunol *164*, 3689-3697.

Andrew, K.A., Simkins, H.M., Witzel, S., Perret, R., Hudson, J., Hermans, I.F., Ritchie, D.S., Yang, J., and Ronchese, F. (2008). Dendritic cells treated with lipopolysaccharide up-regulate serine protease inhibitor 6 and remain sensitive to killing by cytotoxic T lymphocytes in vivo. J Immunol *181*, 8356-8362.

Antony, P.A., Paulos, C.M., Ahmadzadeh, M., Akpinarli, A., Palmer, D.C., Sato, N., Kaiser, A., Hinrichs, C.S., Klebanoff, C.A., Tagaya, Y.*, et al.* (2006). Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. J Immunol *176*, 5255-5266.

Ashany, D., Savir, A., Bhardwaj, N., and Elkon, K.B. (1999). Dendritic cells are resistant to apoptosis through the Fas (CD95/APO-1) pathway. J Immunol *163*, 5303-5311.

Askew, D., Chu, R.S., Krieg, A.M., and Harding, C.V. (2000). CpG DNA induces maturation of dendritic cells with distinct effects on nascent and recycling MHC-II antigen-processing mechanisms. J Immunol *165*, 6889-6895.

Atkinson, E.A., Barry, M., Darmon, A.J., Shostak, I., Turner, P.C., Moyer, R.W., and Bleackley, R.C. (1998). Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. J Biol Chem *273*, 21261-21266.

Aung, S., and Graham, B.S. (2000). IL-4 diminishes perforin-mediated and increases Fas ligand-mediated cytotoxicity In vivo. J Immunol *164*, 3487-3493.

Austyn, J.M., Kupiec-Weglinski, J.W., Hankins, D.F., and Morris, P.J. (1988). Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone. J Exp Med *167*, 646-651.

Avva, R.R., and Cresswell, P. (1994). In vivo and in vitro formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. Immunity *1*, 763-774.

Backer, R., Schwandt, T., Greuter, M., Oosting, M., Jungerkes, F., Tuting, T., Boon, L., O'Toole, T., Kraal, G., Limmer, A.*, et al.* (2010). Effective collaboration between marginal metallophilic macrophages and CD8+ dendritic cells in the generation of cytotoxic T cells. Proc Natl Acad Sci U S A *107*, 216-221.

Badovinac, V.P., Messingham, K.A., Jabbari, A., Haring, J.S., and Harty, J.T. (2005). Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nat Med *11*, 748-756.

Badovinac, V.P., Porter, B.B., and Harty, J.T. (2002). Programmed contraction of CD8(+) T cells after infection. Nat Immunol *3*, 619-626.

Badovinac, V.P., Tvinnereim, A.R., and Harty, J.T. (2000). Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. Science *290*, 1354-1358.

Bajenoff, M., Granjeaud, S., and Guerder, S. (2003). The strategy of T cell antigen-presenting cell encounter in antigen-draining lymph nodes revealed by imaging of initial T cell activation. J Exp Med *198*, 715-724.

Bajenoff, M., Wurtz, O., and Guerder, S. (2002). Repeated antigen exposure is necessary for the differentiation, but not the initial proliferation, of naive CD4(+) T cells. J Immunol *168*, 1723-1729.

Balaji, K.N., Schaschke, N., Machleidt, W., Catalfamo, M., and Henkart, P.A. (2002). Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. J Exp Med *196*, 493-503.

Balkwill, F. (2006). TNF-alpha in promotion and progression of cancer. Cancer Metastasis Rev *25*, 409-416.

Ballesteros-Tato, A., Leon, B., Lund, F.E., and Randall, T.D. (2010). Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. Nat Immunol *11*, 216-224.

Barry, M., Heibein, J.A., Pinkoski, M.J., Lee, S.F., Moyer, R.W., Green, D.R., and Bleackley, R.C. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol *20*, 3781-3794.

Bedoui, S., Whitney, P.G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R.S., Wojtasiak, M., Shortman, K., Carbone, F.R.*, et al.* (2009). Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat Immunol *10*, 488-495.

Belz, G.T., Smith, C.M., Eichner, D., Shortman, K., Karupiah, G., Carbone, F.R., and Heath, W.R. (2004a). Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. J Immunol *172*, 1996-2000.

Belz, G.T., Smith, C.M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F.R., and Heath, W.R. (2004b). Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. Proc Natl Acad Sci U S A *101*, 8670-8675.

Belz, G.T., Vremec, D., Febbraio, M., Corcoran, L., Shortman, K., Carbone, F.R., and Heath, W.R. (2002). CD36 is differentially expressed by CD8+ splenic dendritic cells but is not required for cross-presentation in vivo. J Immunol *168*, 6066-6070.

Belz, G.T., Zhang, L., Lay, M.D., Kupresanin, F., and Davenport, M.P. (2007). Killer T cells regulate antigen presentation for early expansion of memory, but not naive, CD8+ T cell. Proc Natl Acad Sci U S A *104*, 6341-6346.

Bendelac, A., Savage, P.B., and Teyton, L. (2007). The biology of NKT cells. Annu Rev Immunol *25*, 297-336.

Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F., and Heath, W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478-480.

Beresford, P.J., Xia, Z., Greenberg, A.H., and Lieberman, J. (1999). Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity *10*, 585-594.

Bevan, M.J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med *143*, 1283-1288.

Bevan, M.J. (1989). Immunology. Stimulating killer cells. Nature *342*, 478-479.

Blattman, J.N., Antia, R., Sourdive, D.J., Wang, X., Kaech, S.M., Murali-Krishna, K., Altman, J.D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med *195*, 657-664.

Bodmer, J.L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J., and Tschopp, J. (2000). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol *2*, 241-243.

Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., Liu, K., Jakubzick, C., Ingersoll, M.A., Leboeuf, M.*, et al.* (2009). Origin of the lamina propria dendritic cell network. Immunity *31*, 513-525.

Boise, L.H., Minn, A.J., Noel, P.J., June, C.H., Accavitti, M.A., Lindsten, T., and Thompson, C.B. (1995). CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity *3*, 87-98.

Boissonnas, A., Scholer-Dahirel, A., Simon-Blancal, V., Pace, L., Valet, F., Kissenpfennig, A., Sparwasser, T., Malissen, B., Fetler, L., and Amigorena, S. (2010). Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. Immunity *32*, 266-278.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C., and Steinman, R.M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med *196*, 1627-1638.

Boonen, G.J., van Dijk, A.M., Verdonck, L.F., van Lier, R.A., Rijksen, G., and Medema, R.H. (1999). CD28 induces cell cycle progression by IL-2-independent down-regulation of p27kip1 expression in human peripheral T lymphocytes. Eur J Immunol *29*, 789-798.

Borkowski, T.A., Letterio, J.J., Farr, A.G., and Udey, M.C. (1996). A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells. J Exp Med *184*, 2417-2422.

Borrow, P., Evans, C.F., and Oldstone, M.B. (1995). Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression. J Virol *69*, 1059-1070.

Bosnjak, L., Miranda-Saksena, M., Koelle, D.M., Boadle, R.A., Jones, C.A., and Cunningham, A.L. (2005). Herpes simplex virus infection of human dendritic cells induces apoptosis and allows cross-presentation via uninfected dendritic cells. J Immunol *174*, 2220-2227.

Brasel, K., De Smedt, T., Smith, J.L., and Maliszewski, C.R. (2000). Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood *96*, 3029-3039.

Braun, M.Y., Lowin, B., French, L., Acha-Orbea, H., and Tschopp, J. (1996). Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. J Exp Med *183*, 657-661.

Brawand, P., Fitzpatrick, D.R., Greenfield, B.W., Brasel, K., Maliszewski, C.R., and De Smedt, T. (2002). Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. J Immunol *169*, 6711-6719.

Brincks, E.L., Katewa, A., Kucaba, T.A., Griffith, T.S., and Legge, K.L. (2008). CD8 T cells utilize TRAIL to control influenza virus infection. J Immunol *181*, 4918-4925.

Brossart, P., and Bevan, M.J. (1997). Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. Blood *90*, 1594-1599.

Browne, K.A., Blink, E., Sutton, V.R., Froelich, C.J., Jans, D.A., and Trapani, J.A. (1999). Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol *19*, 8604-8615.

Burchill, M.A., Yang, J., Vogtenhuber, C., Blazar, B.R., and Farrar, M.A. (2007). IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. J Immunol *178*, 280-290.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P.A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. Science *316*, 612-616.

Burgdorf, S., and Kurts, C. (2008). Endocytosis mechanisms and the cell biology of antigen presentation. Curr Opin Immunol *20*, 89-95.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat Immunol *9*, 558-566.

Bursch, L.S., Wang, L., Igyarto, B., Kissenpfennig, A., Malissen, B., Kaplan, D.H., and Hogquist, K.A. (2007). Identification of a novel population of Langerin+ dendritic cells. J Exp Med *204*, 3147-3156.

Butz, E.A., and Bevan, M.J. (1998). Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity *8*, 167-175.

Caminschi, I., Proietto, A.I., Ahmet, F., Kitsoulis, S., Shin Teh, J., Lo, J.C., Rizzitelli, A., Wu, L., Vremec, D., van Dommelen, S.L.*, et al.* (2008). The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. Blood *112*, 3264-3273.

Campbell, D.J., and Butcher, E.C. (2002). Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. J Exp Med *195*, 135-141.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annu Rev Immunol *14*, 259-274.

Cao, W., Tykodi, S.S., Esser, M.T., Braciale, V.L., and Braciale, T.J. (1995). Partial activation of CD8+ T cells by a self-derived peptide. Nature *378*, 295-298.

Carbone, F.R., and Bevan, M.J. (1990). Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. J Exp Med *171*, 377-387.

Carbone, F.R., Kurts, C., Bennett, S.R., Miller, J.F., and Heath, W.R. (1998). Cross-presentation: a general mechanism for CTL immunity and tolerance. Immunol Today *19*, 368-373.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., and Banchereau, J. (1994). Activation of human dendritic cells through CD40 cross-linking. J Exp Med *180*, 1263-1272.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature *388*, 782-787.

Celli, S., Lemaitre, F., and Bousso, P. (2007). Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4+ T cell activation. Immunity *27*, 625-634.

Chaudhri, G., Quah, B.J., Wang, Y., Tan, A.H., Zhou, J., Karupiah, G., and Parish, C.R. (2009). T cell receptor sharing by cytotoxic T lymphocytes facilitates efficient virus control. Proc Natl Acad Sci U S A *106*, 14984-14989.

Chen, M., Wang, Y.H., Wang, Y., Huang, L., Sandoval, H., Liu, Y.J., and Wang, J. (2006). Dendritic cell apoptosis in the maintenance of immune tolerance. Science *311*, 1160-1164.

Chinnery, H.R., Pearlman, E., and McMenamin, P.G. (2008). Cutting edge: Membrane nanotubes in vivo: a feature of MHC class II+ cells in the mouse cornea. J Immunol *180*, 5779-5783.

Chow, A., Toomre, D., Garrett, W., and Mellman, I. (2002). Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. Nature *418*, 988-994.

Chowdhury, D., and Lieberman, J. (2008). Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol *26*, 389-420.

Cohen, J.J., Duke, R.C., Fadok, V.A., and Sellins, K.S. (1992). Apoptosis and programmed cell death in immunity. Annu Rev Immunol *10*, 267-293.

Colledge, L., Bennett, C.L., Reay, P.A., and Blackburn, C.C. (2002). Rapid constitutive generation of a specific peptide-MHC class II complex from intact exogenous protein in immature murine dendritic cells. Eur J Immunol *32*, 3246-3255.

Colonna, M., Trinchieri, G., and Liu, Y.J. (2004). Plasmacytoid dendritic cells in immunity. Nat Immunol *5*, 1219-1226.

Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med *204*, 1757-1764.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T.*, et al.* (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature *421*, 744-748.

Curtsinger, J.M., Lins, D.C., and Mescher, M.F. (2003). Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J Exp Med *197*, 1141-1151.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K., and Mescher, M.F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol *162*, 3256-3262.

Cyster, J.G., Ansel, K.M., Reif, K., Ekland, E.H., Hyman, P.L., Tang, H.L., Luther, S.A., and Ngo, V.N. (2000). Follicular stromal cells and lymphocyte homing to follicles. Immunol Rev *176*, 181-193.

Davis, D.M. (2007). Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. Nat Rev Immunol *7*, 238-243.

Davis, D.M., and Sowinski, S. (2008). Membrane nanotubes: dynamic long-distance connections between animal cells. Nat Rev Mol Cell Biol *9*, 431-436.

Davis, J.E., Smyth, M.J., and Trapani, J.A. (2001). Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. Eur J Immunol *31*, 39-47.

de Jong, E.C., Vieira, P.L., Kalinski, P., Schuitemaker, J.H., Tanaka, Y., Wierenga, E.A., Yazdanbakhsh, M., and Kapsenberg, M.L. (2002). Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J Immunol *168*, 1704-1709.

De Smedt, T., Butz, E., Smith, J., Maldonado-Lopez, R., Pajak, B., Moser, M., and Maliszewski, C. (2001). CD8alpha(-) and CD8alpha(+) subclasses of dendritic cells undergo phenotypic and functional maturation in vitro and in vivo. J Leukoc Biol *69*, 951-958.

De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., Urbain, J., Leo, O., and Moser, M. (1996). Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. J Exp Med *184*, 1413-1424.

del Rio, M.L., Bernhardt, G., Rodriguez-Barbosa, J.I., and Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. Immunol Rev *234*, 268-281.

del Rio, M.L., Rodriguez-Barbosa, J.I., Kremmer, E., and Forster, R. (2007). CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. J Immunol *178*, 6861-6866.

Delamarre, L., Holcombe, H., and Mellman, I. (2003). Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. J Exp Med *198*, 111-122.

den Haan, J.M., and Bevan, M.J. (2002). Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo. J Exp Med *196*, 817-827.

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med *192*, 1685-1696.

Denzin, L.K., and Cresswell, P. (1995). HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. Cell *82*, 155-165.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C., and Bhardwaj, N. (2001). Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med *193*, 233-238.

Dudda, J.C., Simon, J.C., and Martin, S. (2004). Dendritic cell immunization route determines CD8+ T cell trafficking to inflamed skin: role for tissue microenvironment and dendritic cells in establishment of T cell-homing subsets. J Immunol *172*, 857-863.

Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.W., Park, C.G*., et al.* (2007). Differential antigen processing by dendritic cell subsets in vivo. Science *315*, 107-111.

Duffield, J.S., Forbes, S.J., Constandinou, C.M., Clay, S., Partolina, M., Vuthoori, S., Wu, S., Lang, R., and Iredale, J.P. (2005). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest *115*, 56-65.

Edelson, B.T., Kc, W., Juang, R., Kohyama, M., Benoit, L.A., Klekotka, P.A., Moon, C., Albring, J.C., Ise, W., Michael, D.G*., et al.* (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. J Exp Med *207*, 823-836.

Ehst, B.D., Ingulli, E., and Jenkins, M.K. (2003). Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. Am J Transplant *3*, 1355-1362.

Elbe, A., Tschachler, E., Steiner, G., Binder, A., Wolff, K., and Stingl, G. (1989). Maturational steps of bone marrow-derived dendritic murine epidermal cells. Phenotypic and functional studies on Langerhans cells and Thy-1+ dendritic epidermal cells in the perinatal period. J Immunol *143*, 2431-2438.

Felix, N.J., Donermeyer, D.L., Horvath, S., Walters, J.J., Gross, M.L., Suri, A., and Allen, P.M. (2007). Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. Nat Immunol *8*, 388-397.

Fiebiger, E., Meraner, P., Weber, E., Fang, I.F., Stingl, G., Ploegh, H., and Maurer, D. (2001). Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. J Exp Med *193*, 881-892.

Fields, M.L., Sokol, C.L., Eaton-Bassiri, A., Seo, S., Madaio, M.P., and Erikson, J. (2001). Fas/Fas ligand deficiency results in altered localization of anti-double-stranded DNA B cells and dendritic cells. J Immunol *167*, 2370-2378.

Finkelman, F.D., Lees, A., Birnbaum, R., Gause, W.C., and Morris, S.C. (1996). Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion. J Immunol *157*, 1406-1414.

Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middleton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell *81*, 935-946.

Fleeton, M.N., Contractor, N., Leon, F., Wetzel, J.D., Dermody, T.S., and Kelsall, B.L. (2004). Peyer's patch dendritic cells process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice. J Exp Med *200*, 235-245.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol *6*, 1142-1151.

Fooksman, D.R., Vardhana, S., Vasiliver-Shamis, G., Liese, J., Blair, D.A., Waite, J., Sacristan, C., Victora, G.D., Zanin-Zhorov, A., and Dustin, M.L. (2010). Functional anatomy of T cell activation and synapse formation. Annu Rev Immunol *28*, 79-105.

Forrester, J.V., Xu, H., Kuffova, L., Dick, A.D., and McMenamin, P.G. (2010). Dendritic cell physiology and function in the eye. Immunol Rev *234*, 282-304.

Froelich, C.J., Orth, K., Turbov, J., Seth, P., Gottlieb, R., Babior, B., Shah, G.M., Bleackley, R.C., Dixit, V.M., and Hanna, W. (1996). New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem *271*, 29073-29079.

Fujii, S., Liu, K., Smith, C., Bonito, A.J., and Steinman, R.M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med *199*, 1607-1618.

Garrett, W.S., Chen, L.M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J.E., and Mellman, I. (2000). Developmental control of endocytosis in dendritic cells by Cdc42. Cell *102*, 325-334.

Garrigan, K., Moroni-Rawson, P., McMurray, C., Hermans, I., Abernethy, N., Watson, J., and Ronchese, F. (1996). Functional comparison of spleen dendritic cells and dendritic cells cultured in vitro from bone marrow precursors. Blood *88*, 3508-3512.

Gauen, L.K., Zhu, Y., Letourneur, F., Hu, Q., Bolen, J.B., Matis, L.A., Klausner, R.D., and Shaw, A.S. (1994). Interactions of p59fyn and ZAP-70 with T-cell receptor activation motifs: defining the nature of a signalling motif. Mol Cell Biol *14*, 3729-3741.

Gerner, M.Y., and Mescher, M.F. (2009). Antigen processing and MHC-II presentation by dermal and tumor-infiltrating dendritic cells. J Immunol *182*, 2726-2737.

GeurtsvanKessel, C.H., Willart, M.A., van Rijt, L.S., Muskens, F., Kool, M., Baas, C., Thielemans, K., Bennett, C., Clausen, B.E., Hoogsteden, H.C.*, et al.* (2008). Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. J Exp Med *205*, 1621-1634.

Ginhoux, F., Collin, M.P., Bogunovic, M., Abel, M., Leboeuf, M., Helft, J., Ochando, J., Kissenpfennig, A., Malissen, B., Grisotto, M.*, et al.* (2007). Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. J Exp Med *204*, 3133-3146.

Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., Price, J., Yin, N., Bromberg, J., Lira, S.A.*, et al.* (2009). The origin and development of nonlymphoid tissue CD103+ DCs. J Exp Med *206*, 3115-3130.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.M., Stanley, E.R., Randolph, G.J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. Nat Immunol *7*, 265-273.

Goriely, S., Neurath, M.F., and Goldman, M. (2008). How microorganisms tip the balance between interleukin-12 family members. Nat Rev Immunol *8*, 81-86.

Graubert, T.A., Russell, J.H., and Ley, T.J. (1996). The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. Blood *87*, 1232-1237.

Guarda, G., Hons, M., Soriano, S.F., Huang, A.Y., Polley, R., Martin-Fontecha, A., Stein, J.V., Germain, R.N., Lanzavecchia, A., and Sallusto, F. (2007a). L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. Nat Immunol *8*, 743-752.

Guarda, G., Hons, M., Soriano, S.F., Huang, A.Y., Polley, R., Martin-Fontecha, A., Stein, J.V., Germain, R.N., Lanzavecchia, A., and Sallusto, F. (2007b). L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. In Nat Immunol, pp. 743-752.

Hammarlund, E., Lewis, M.W., Hansen, S.G., Strelow, L.I., Nelson, J.A., Sexton, G.J., Hanifin, J.M., and Slifka, M.K. (2003). Duration of antiviral immunity after smallpox vaccination. Nat Med *9*, 1131-1137.

Harshyne, L.A., Watkins, S.C., Gambotto, A., and Barratt-Boyes, S.M. (2001). Dendritic cells acquire antigens from live cells for cross-presentation to CTL. J Immunol *166*, 3717-3723.

Hataye, J., Moon, J.J., Khoruts, A., Reilly, C., and Jenkins, M.K. (2006). Naive and memory CD4+ T cell survival controlled by clonal abundance. Science *312*, 114-116.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., and Nussenzweig, M.C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med *194*, 769-779.

He, J.S., Gong, D.E., and Ostergaard, H.L. (2010). Stored Fas ligand, a mediator of rapid CTL-mediated killing, has a lower threshold for response than degranulation or newly synthesized Fas ligand. J Immunol *184*, 555-563.

He, J.S., and Ostergaard, H.L. (2007). CTLs contain and use intracellular stores of FasL distinct from cytolytic granules. J Immunol *179*, 2339-2348.

Heath, W.R., and Carbone, F.R. (2001a). Cross-presentation in viral immunity and self-tolerance. Nat Rev Immunol *1*, 126-134.

Heath, W.R., and Carbone, F.R. (2001b). Cross-presentation, dendritic cells, tolerance and immunity. Annu Rev Immunol *19*, 47-64.

Helft, J., Ginhoux, F., Bogunovic, M., and Merad, M. (2010). Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. Immunol Rev *234*, 55-75.

Hemmi, H., Yoshino, M., Yamazaki, H., Naito, M., Iyoda, T., Omatsu, Y., Shimoyama, S., Letterio, J.J., Nakabayashi, T., Tagaya, H.*, et al.* (2001). Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-beta1-dependent cells. Int Immunol *13*, 695-704.

Henri, S., Poulin, L.F., Tamoutounour, S., Ardouin, L., Guilliams, M., de Bovis, B., Devilard, E., Viret, C., Azukizawa, H., Kissenpfennig, A.*, et al.* (2010). CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. J Exp Med *207*, 189-206.

Henri, S., Vremec, D., Kamath, A., Waithman, J., Williams, S., Benoist, C., Burnham, K., Saeland, S., Handman, E., and Shortman, K. (2001). The dendritic cell populations of mouse lymph nodes. J Immunol *167*, 741-748.

Henrickson, S.E., Mempel, T.R., Mazo, I.B., Liu, B., Artyomov, M.N., Zheng, H., Peixoto, A., Flynn, M.P., Senman, B., Junt, T*., et al.* (2008). T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. Nat Immunol *9*, 282-291.

Hermans, I.F., Ritchie, D.S., Yang, J., Roberts, J.M., and Ronchese, F. (2000). CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. J Immunol *164*, 3095-3101.

Hermans, I.F., Silk, J.D., Yang, J., Palmowski, M.J., Gileadi, U., McCarthy, C., Salio, M., Ronchese, F., and Cerundolo, V. (2004). The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. J Immunol Methods *285*, 25-40.

Hernandez, J., Aung, S., Redmond, W.L., and Sherman, L.A. (2001). Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. J Exp Med *194*, 707-717.

Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S*., et al.* (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science *322*, 1097-1100.

Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., and Murphy, K.M. (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science *260*, 547-549.

Huang, C.T., Huso, D.L., Lu, Z., Wang, T., Zhou, G., Kennedy, E.P., Drake, C.G., Morgan, D.J., Sherman, L.A., Higgins, A.D*., et al.* (2003). CD4+ T cells pass through an effector phase during the process of in vivo tolerance induction. J Immunol *170*, 3945-3953.

Huang, F.P., Platt, N., Wykes, M., Major, J.R., Powell, T.J., Jenkins, C.D., and MacPherson, G.G. (2000). A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. J Exp Med *191*, 435-444.

Huck, S.P., Tang, S.C., Andrew, K.A., Yang, J., Harper, J.L., and Ronchese, F. (2008). Activation and route of administration both determine the ability of bone marrow-derived dendritic cells to accumulate in secondary lymphoid organs and prime CD8+ T cells against tumors. Cancer Immunol Immunother *57*, 63-71.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89-95.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med *176*, 1693-1702.

Inaba, K., Turley, S., Iyoda, T., Yamaide, F., Shimoyama, S., Reis e Sousa, C., Germain, R.N., Mellman, I., and Steinman, R.M. (2000). The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. J Exp Med *191*, 927-936.

Inaba, K., Turley, S., Yamaide, F., Iyoda, T., Mahnke, K., Inaba, M., Pack, M., Subklewe, M., Sauter, B., Sheff, D.*, et al.* (1998). Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. J Exp Med *188*, 2163-2173.

Inaba, K., Witmer-Pack, M., Inaba, M., Hathcock, K.S., Sakuta, H., Azuma, M., Yagita, H., Okumura, K., Linsley, P.S., Ikehara, S.*, et al.* (1994). The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. J Exp Med *180*, 1849-1860.

Ingulli, E., Mondino, A., Khoruts, A., and Jenkins, M.K. (1997). In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. J Exp Med *185*, 2133-2141.

Ito, T., Wang, Y.H., Duramad, O., Hori, T., Delespesse, G.J., Watanabe, N., Qin, F.X., Yao, Z., Cao, W., and Liu, Y.J. (2005). TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J Exp Med *202*, 1213-1223.

Iwasaki, A., and Kelsall, B.L. (2000). Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. J Exp Med *191*, 1381-1394.

Iwasaki, A., and Kelsall, B.L. (2001). Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. J Immunol *166*, 4884-4890.

Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R.M., and Inaba, K. (2002). The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J Exp Med *195*, 1289-1302.

Janeway, C.A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197-216.

Jansen, J.H., Wientjens, G.J., Fibbe, W.E., Willemze, R., and Kluin-Nelemans, H.C. (1989). Inhibition of human macrophage colony formation by interleukin 4. J Exp Med *170*, 577-582.

Janssen, E.M., Droin, N.M., Lemmens, E.E., Pinkoski, M.J., Bensinger, S.J., Ehst, B.D., Griffith, T.S., Green, D.R., and Schoenberger, S.P. (2005). CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature *434*, 88-93.

Janssen, E.M., Lemmens, E.E., Gour, N., Reboulet, R.A., Green, D.R., Schoenberger, S.P., and Pinkoski, M.J. (2010). Distinct roles of cytolytic effector molecules for antigen-restricted killing by CTL in vivo. Immunol Cell Biol.

Janssen, E.M., Lemmens, E.E., Wolfe, T., Christen, U., von Herrath, M.G., and Schoenberger, S.P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature *421*, 852-856.

Jelley-Gibbs, D.M., Brown, D.M., Dibble, J.P., Haynes, L., Eaton, S.M., and Swain, S.L. (2005). Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. J Exp Med *202*, 697-706.

Jiang, A., Bloom, O., Ono, S., Cui, W., Unternaehrer, J., Jiang, S., Whitney, J.A., Connolly, J., Banchereau, J., and Mellman, I. (2007). Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. Immunity *27*, 610-624.

Jiang, W., Swiggard, W.J., Heufler, C., Peng, M., Mirza, A., Steinman, R.M., and Nussenzweig, M.C. (1995). The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature *375*, 151-155.

Johnson, S., Zhan, Y., Sutherland, R.M., Mount, A.M., Bedoui, S., Brady, J.L., Carrington, E.M., Brown, L.E., Belz, G.T., Heath, W.R.*, et al.* (2009). Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells. Immunity *30*, 218-227.

Jones, C.A., Fernandez, M., Herc, K., Bosnjak, L., Miranda-Saksena, M., Boadle, R.A., and Cunningham, A. (2003). Herpes simplex virus type 2 induces rapid cell death and functional impairment of murine dendritic cells in vitro. J Virol *77*, 11139-11149.

Ju, S.T., Cui, H., Panka, D.J., Ettinger, R., and Marshak-Rothstein, A. (1994). Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. Proc Natl Acad Sci U S A *91*, 4185-4189.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Jurgens, M., Wollenberg, A., Hanau, D., de la Salle, H., and Bieber, T. (1995). Activation of human epidermal Langerhans cells by engagement of the high affinity receptor for IgE, Fc epsilon RI. J Immunol *155*, 5184-5189.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994a). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31-37.

Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994b). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science *265*, 528-530.

Kaisho, T., Hoshino, K., Iwabe, T., Takeuchi, O., Yasui, T., and Akira, S. (2002). Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. Int Immunol *14*, 695-700.

Kamath, A.T., Henri, S., Battye, F., Tough, D.F., and Shortman, K. (2002). Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. Blood *100*, 1734-1741.

Ke, Y., and Kapp, J.A. (1996). Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. J Exp Med *184*, 1179-1184.

Kearney, E.R., Pape, K.A., Loh, D.Y., and Jenkins, M.K. (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity *1*, 327-339.

Keefe, D., Shi, L., Feske, S., Massol, R., Navarro, F., Kirchhausen, T., and Lieberman, J. (2005). Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. Immunity *23*, 249-262.

Keir, M.E., Freeman, G.J., and Sharpe, A.H. (2007). PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. J Immunol *179*, 5064-5070.

Kessler, B., Hudrisier, D., Schroeter, M., Tschopp, J., Cerottini, J.C., and Luescher, I.F. (1998). Peptide modification or blocking of CD8, resulting in weak TCR signaling, can activate CTL for Fas- but not perforin-dependent cytotoxicity or cytokine production. J Immunol *161*, 6939-6946.

King, I.L., Kroenke, M.A., and Segal, B.M. (2010). GM-CSF-dependent, CD103+ dermal dendritic cells play a critical role in Th effector cell differentiation after subcutaneous immunization. J Exp Med *207*, 953-961.

Kingston, D., Schmid, M.A., Onai, N., Obata-Onai, A., Baumjohann, D., and Manz, M.G. (2009). The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. Blood *114*, 835-843.

Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhe, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D.*, et al.* (2005). Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity *22*, 643-654.

Kleindienst, P., and Brocker, T. (2003). Endogenous dendritic cells are required for amplification of T cell responses induced by dendritic cell vaccines in vivo. J Immunol *170*, 2817-2823.

Kojima, H., Shinohara, N., Hanaoka, S., Someya-Shirota, Y., Takagaki, Y., Ohno, H., Saito, T., Katayama, T., Yagita, H., Okumura, K.*, et al.* (1994). Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. Immunity *1*, 357-364.

Korb, L.C., Mirshahidi, S., Ramyar, K., Sadighi Akha, A.A., and Sadegh-Nasseri, S. (1999). Induction of T cell anergy by low numbers of agonist ligands. J Immunol *162*, 6401-6409.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. (2009). IL-17 and Th17 Cells. Annu Rev Immunol *27*, 485-517.

Kovacsovics-Bankowski, M., Clark, K., Benacerraf, B., and Rock, K.L. (1993). Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. Proc Natl Acad Sci U S A *90*, 4942-4946.

Kurotaki, T., Tamura, Y., Ueda, G., Oura, J., Kutomi, G., Hirohashi, Y., Sahara, H., Torigoe, T., Hiratsuka, H., Sunakawa, H.*, et al.* (2007). Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells via an endosomal pathway. J Immunol *179*, 1803-1813.

Kurts, C., Heath, W.R., Kosaka, H., Miller, J.F., and Carbone, F.R. (1998a). The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). J Exp Med *188*, 415-420.

Kurts, C., Miller, J.F., Subramaniam, R.M., Carbone, F.R., and Heath, W.R. (1998b). Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. J Exp Med *188*, 409-414.

Kurts, C., Sutherland, R.M., Davey, G., Li, M., Lew, A.M., Blanas, E., Carbone, F.R., Miller, J.F., and Heath, W.R. (1999). CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose. Proc Natl Acad Sci U S A *96*, 12703-12707.

La Gruta, N.L., Rothwell, W.T., Cukalac, T., Swan, N.G., Valkenburg, S.A., Kedzierska, K., Thomas, P.G., Doherty, P.C., and Turner, S.J. (2010). Primary CTL response magnitude in mice is determined by the extent of naive T cell recruitment and subsequent clonal expansion. J Clin Invest *120*, 1885-1894.

Laffont, S., Coudert, J.D., Garidou, L., Delpy, L., Wiedemann, A., Demur, C., Coureau, C., and Guery, J.C. (2006). CD8+ T-cell-mediated killing of donor dendritic cells prevents alloreactive T helper type-2 responses in vivo. Blood *108*, 2257-2264.

Langenkamp, A., Messi, M., Lanzavecchia, A., and Sallusto, F. (2000). Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol *1*, 311-316.

Larsen, C.P., Ritchie, S.C., Pearson, T.C., Linsley, P.S., and Lowry, R.P. (1992). Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. J Exp Med *176*, 1215-1220.

Larsen, C.P., Steinman, R.M., Witmer-Pack, M., Hankins, D.F., Morris, P.J., and Austyn, J.M. (1990). Migration and maturation of Langerhans cells in skin transplants and explants. J Exp Med *172*, 1483-1493.

Le Gros, G., Ben-Sasson, S.Z., Seder, R., Finkelman, F.D., and Paul, W.E. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. J Exp Med *172*, 921-929.

Lee, H.K., Zamora, M., Linehan, M.M., Iijima, N., Gonzalez, D., Haberman, A., and Iwasaki, A. (2009). Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection. J Exp Med *206*, 359-370.

Lehmann, C., Zeis, M., Schmitz, N., and Uharek, L. (2000). Impaired binding of perforin on the surface of tumor cells is a cause of target cell resistance against cytotoxic effector cells. Blood *96*, 594-600.

Lehner, P.J., Surman, M.J., and Cresswell, P. (1998). Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line .220. Immunity *8*, 221-231.

Leon, B., Lopez-Bravo, M., and Ardavin, C. (2007). Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. Immunity *26*, 519-531.

Leverkus, M., Walczak, H., McLellan, A., Fries, H.W., Terbeck, G., Brocker, E.B., and Kampgen, E. (2000). Maturation of dendritic cells leads to up-regulation of cellular FLICE-inhibitory protein and concomitant down-regulation of death ligand-mediated apoptosis. Blood *96*, 2628-2631.

Lindquist, R.L., Shakhar, G., Dudziak, D., Wardemann, H., Eisenreich, T., Dustin, M.L., and Nussenzweig, M.C. (2004). Visualizing dendritic cell networks in vivo. Nat Immunol *5*, 1243-1250.

Liu, K., Iyoda, T., Saternus, M., Kimura, Y., Inaba, K., and Steinman, R.M. (2002). Immune tolerance after delivery of dying cells to dendritic cells in situ. J Exp Med *196*, 1091-1097.

Liu, Y., Wenger, R.H., Zhao, M., and Nielsen, P.J. (1997). Distinct costimulatory molecules are required for the induction of effector and memory cytotoxic T lymphocytes. J Exp Med *185*, 251-262.

Lowin, B., Beermann, F., Schmidt, A., and Tschopp, J. (1994a). A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. Proc Natl Acad Sci U S A *91*, 11571-11575.

Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994b). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature *370*, 650-652.

Ludewig, B., Bonilla, W.V., Dumrese, T., Odermatt, B., Zinkernagel, R.M., and Hengartner, H. (2001). Perforin-independent regulation of dendritic cell homeostasis by CD8(+) T cells in vivo: implications for adaptive immunotherapy. Eur J Immunol *31*, 1772-1779.

Luketic, L., Delanghe, J., Sobol, P.T., Yang, P., Frotten, E., Mossman, K.L., Gauldie, J., Bramson, J., and Wan, Y. (2007). Antigen presentation by exosomes released from peptide-pulsed dendritic cells is not suppressed by the presence of active CTL. J Immunol *179*, 5024-5032.

Lundqvist, A., Nagata, T., Kiessling, R., and Pisa, P. (2002). Mature dendritic cells are protected from Fas/CD95-mediated apoptosis by upregulation of Bcl-X(L). Cancer Immunol Immunother *51*, 139-144.

Lutz, M.B., and Schuler, G. (2002). Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol *23*, 445-449.

MacDonald, A.S., Straw, A.D., Bauman, B., and Pearce, E.J. (2001). CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. J Immunol *167*, 1982-1988.

MacDonald, A.S., Straw, A.D., Dalton, N.M., and Pearce, E.J. (2002). Cutting edge: Th2 response induction by dendritic cells: a role for CD40. J Immunol *168*, 537-540.

Mackay, C.R., Marston, W.L., and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med *171*, 801-817.

Maeda, Y., Levy, R.B., Reddy, P., Liu, C., Clouthier, S.G., Teshima, T., and Ferrara, J.L. (2005). Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. Blood *105*, 2023-2027.

Marrack, P., Kappler, J., and Mitchell, T. (1999). Type I interferons keep activated T cells alive. J Exp Med *189*, 521-530.

Martin-Fontecha, A., Sebastiani, S., Hopken, U.E., Uguccioni, M., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2003). Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med *198*, 615-621.

Matloubian, M., Suresh, M., Glass, A., Galvan, M., Chow, K., Whitmire, J.K., Walsh, C.M., Clark, W.R., and Ahmed, R. (1999). A role for perforin in downregulating T-cell responses during chronic viral infection. J Virol *73*, 2527-2536.

Matsue, H., Edelbaum, D., Hartmann, A.C., Morita, A., Bergstresser, P.R., Yagita, H., Okumura, K., and Takashima, A. (1999). Dendritic cells undergo rapid apoptosis in vitro during antigen-specific interaction with CD4+ T cells. J Immunol *162*, 5287-5298.

Matthews, K.E., Karabeg, A., Roberts, J.M., Saeland, S., Dekan, G., Epstein, M.M., and Ronchese, F. (2007). Long-term deposition of inhaled antigen in lung resident CD11b-CD11c+ cells. Am J Respir Cell Mol Biol *36*, 435-441.

McDonnell, A.M., Prosser, A.C., van Bruggen, I., Robinson, B.W., and Currie, A.J. (2010). CD8alpha+ DC are not the sole subset cross-presenting cell-associated tumor antigens from a solid tumor. Eur J Immunol *40*, 1617-1627.

McKenna, H.J., Stocking, K.L., Miller, R.E., Brasel, K., De Smedt, T., Maraskovsky, E., Maliszewski, C.R., Lynch, D.H., Smith, J., Pulendran, B.*, et al.* (2000). Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood *95*, 3489-3497.

Medema, J.P., de Jong, J., van Hall, T., Melief, C.J., and Offringa, R. (1999). Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. J Exp Med *190*, 1033-1038.

Medema, J.P., Schuurhuis, D.H., Rea, D., van Tongeren, J., de Jong, J., Bres, S.A., Laban, S., Toes, R.E., Toebes, M., Schumacher, T.N.*, et al.* (2001). Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells. J Exp Med *194*, 657-667.

Medema, J.P., Toes, R.E., Scaffidi, C., Zheng, T.S., Flavell, R.A., Melief, C.J., Peter, M.E., Offringa, R., and Krammer, P.H. (1997). Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. Eur J Immunol *27*, 3492-3498.

Mempel, T.R., Henrickson, S.E., and Von Andrian, U.H. (2004). T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature *427*, 154-159.

Merad, M., Fong, L., Bogenberger, J., and Engleman, E.G. (2000). Differentiation of myeloid dendritic cells into CD8alpha-positive dendritic cells in vivo. Blood *96*, 1865-1872.

Merad, M., Manz, M.G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I.L., Cyster, J.G., and Engleman, E.G. (2002). Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol *3*, 1135-1141.

Metcalf, D., Burgess, A.W., Johnson, G.R., Nicola, N.A., Nice, E.C., DeLamarter, J., Thatcher, D.R., and Mermod, J.J. (1986). In vitro actions on hemopoietic cells of recombinant murine GM-CSF purified after production in Escherichia coli: comparison with purified native GM-CSF. J Cell Physiol *128*, 421-431.

Metkar, S.S., Wang, B., Aguilar-Santelises, M., Raja, S.M., Uhlin-Hansen, L., Podack, E., Trapani, J.A., and Froelich, C.J. (2002). Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. Immunity *16*, 417-428.

Mirandola, P., Ponti, C., Gobbi, G., Sponzilli, I., Vaccarezza, M., Cocco, L., Zauli, G., Secchiero, P., Manzoli, F.A., and Vitale, M. (2004). Activated human NK and CD8+ T cells express both TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. Blood *104*, 2418-2424.

Mishima, Y. (1966). Melanosomes in phagocytic vacuoles in Langerhans cells. Electron microscopy of keratin-stripped human epidermis. J Cell Biol *30*, 417-423.

Mocikat, R., Braumuller, H., Gumy, A., Egeter, O., Ziegler, H., Reusch, U., Bubeck, A., Louis, J., Mailhammer, R., Riethmuller, G*., et al.* (2003). Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity *19*, 561-569.

Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., and Jenkins, M.K. (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity *27*, 203-213.

Mora, J.R., Bono, M.R., Manjunath, N., Weninger, W., Cavanagh, L.L., Rosemblatt, M., and Von Andrian, U.H. (2003). Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature *424*, 88-93.

Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol *7*, 145-173.

Mueller, S.N., Jones, C.M., Smith, C.M., Heath, W.R., and Carbone, F.R. (2002). Rapid cytotoxic T lymphocyte activation occurs in the draining lymph nodes after cutaneous herpes simplex virus infection as a result of early antigen presentation and not the presence of virus. J Exp Med *195*, 651-656.

Mueller, S.N., Jones, C.M., Stock, A.T., Suter, M., Heath, W.R., and Carbone, F.R. (2006). CD4+ T cells can protect APC from CTL-mediated elimination. J Immunol *176*, 7379-7384.

Mullbacher, A., Ebnet, K., Blanden, R.V., Hla, R.T., Stehle, T., Museteanu, C., and Simon, M.M. (1996). Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. Proc Natl Acad Sci U S A *93*, 5783-5787.

Mullbacher, A., Waring, P., Tha Hla, R., Tran, T., Chin, S., Stehle, T., Museteanu, C., and Simon, M.M. (1999). Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. Proc Natl Acad Sci U S A *96*, 13950-13955.

Mullins, D.W., Sheasley, S.L., Ream, R.M., Bullock, T.N., Fu, Y.X., and Engelhard, V.H. (2003). Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. J Exp Med *198*, 1023-1034.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177-187.

Naik, S.H., Proietto, A.I., Wilson, N.S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M.H., O'Keeffe, M., Shao, Q.X., Chen, W.F.*, et al.* (2005). Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol *174*, 6592-6597.

Neijssen, J., Herberts, C., Drijfhout, J.W., Reits, E., Janssen, L., and Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. Nature *434*, 83-88.

Ng, L.G., Hsu, A., Mandell, M.A., Roediger, B., Hoeller, C., Mrass, P., Iparraguirre, A., Cavanagh, L.L., Triccas, J.A., Beverley, S.M.*, et al.* (2008). Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. PLoS Pathog *4*, e1000222.

Nishibu, A., Ward, B.R., Jester, J.V., Ploegh, H.L., Boes, M., and Takashima, A. (2006). Behavioral responses of epidermal Langerhans cells in situ to local pathological stimuli. J Invest Dermatol *126*, 787-796.

Norbury, C.C., Basta, S., Donohue, K.B., Tscharke, D.C., Princiotta, M.F., Berglund, P., Gibbs, J., Bennink, J.R., and Yewdell, J.W. (2004). CD8+ T cell cross-priming via transfer of proteasome substrates. Science *304*, 1318-1321.

Nussenzweig, M.C., Steinman, R.M., Witmer, M.D., and Gutchinov, B. (1982). A monoclonal antibody specific for mouse dendritic cells. Proc Natl Acad Sci U S A *79*, 161-165.

Ohnmacht, C., Pullner, A., King, S.B., Drexler, I., Meier, S., Brocker, T., and Voehringer, D. (2009). Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J Exp Med *206*, 549-559.

Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I--restricted antigen processing. Annu Rev Immunol *16*, 323-358.

Parish, I.A., Waithman, J., Davey, G.M., Belz, G.T., Mintern, J.D., Kurts, C., Sutherland, R.M., Carbone, F.R., and Heath, W.R. (2009). Tissue destruction caused by cytotoxic T lymphocytes induces deletional tolerance. Proc Natl Acad Sci U S A *106*, 3901-3906.

Paul, W.E., and Seder, R.A. (1994). Lymphocyte responses and cytokines. Cell *76*, 241-251.

Peperzak, V., Xiao, Y., Veraar, E.A., and Borst, J. (2010). CD27 sustains survival of CTLs in virus-infected nonlymphoid tissue in mice by inducing autocrine IL-2 production. J Clin Invest *120*, 168-178.

Perlman, H., Pagliari, L.J., Georganas, C., Mano, T., Walsh, K., and Pope, R.M. (1999). FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. J Exp Med *190*, 1679-1688.

Petersen, T.R., Gulland, S., Bettelli, E., Kuchroo, V., Palmer, E., and Backstrom, B.T. (2004). A chimeric T cell receptor with super-signaling properties. Int Immunol *16*, 889-894.

Petersen, T.R., Sika-Paotonu, D., Knight, D.A., Dickgreber, N., Farrand, K.J., Ronchese, F., and Hermans, I.F. (2010). Potent anti-tumor responses to immunization with dendritic cells loaded with tumor tissue and an NKT cell ligand. Immunol Cell Biol *88*, 596-604.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787-792.

Pinkoski, M.J., Hobman, M., Heibein, J.A., Tomaselli, K., Li, F., Seth, P., Froelich, C.J., and Bleackley, R.C. (1998). Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. Blood *92*, 1044-1054.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A., and Ashkenazi, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem *271*, 12687-12690.

Platt, C.D., Ma, J.K., Chalouni, C., Ebersold, M., Bou-Reslan, H., Carano, R.A., Mellman, I., and Delamarre, L. (2010). Mature dendritic cells use endocytic receptors to capture and present antigens. Proc Natl Acad Sci U S A *107*, 4287-4292.

Platt, N., da Silva, R.P., and Gordon, S. (1998). Recognizing death: the phagocytosis of apoptotic cells. Trends Cell Biol *8*, 365-372.

Poehlein, C.H., Hu, H.M., Yamada, J., Assmann, I., Alvord, W.G., Urba, W.J., and Fox, B.A. (2003). TNF plays an essential role in tumor regression after adoptive transfer of perforin/IFN-gamma double knockout effector T cells. J Immunol *170*, 2004-2013.

Pooley, J.L., Heath, W.R., and Shortman, K. (2001). Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol *166*, 5327-5330.

Porgador, A., and Gilboa, E. (1995). Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. J Exp Med *182*, 255-260.

Porgador, A., Snyder, D., and Gilboa, E. (1996). Induction of antitumor immunity using bone marrow-generated dendritic cells. J Immunol *156*, 2918-2926.

Poulin, L.F., Henri, S., de Bovis, B., Devilard, E., Kissenpfennig, A., and Malissen, B. (2007). The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. J Exp Med *204*, 3119-3131.

Prevost-Blondel, A., Roth, E., Rosenthal, F.M., and Pircher, H. (2000). Crucial role of TNF-alpha in CD8 T cell-mediated elimination of 3LL-A9 Lewis lung carcinoma cells in vivo. J Immunol *164*, 3645-3651.

Price, G.E., Huang, L., Ou, R., Zhang, M., and Moskophidis, D. (2005). Perforin and Fas cytolytic pathways coordinately shape the selection and diversity of CD8+-T-cell escape variants of influenza virus. J Virol *79*, 8545-8559.

Probst, H.C., and van den Broek, M. (2005). Priming of CTLs by lymphocytic choriomeningitis virus depends on dendritic cells. J Immunol *174*, 3920-3924.

Qiu, C.H., Miyake, Y., Kaise, H., Kitamura, H., Ohara, O., and Tanaka, M. (2009). Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. J Immunol *182*, 4127-4136.

Qu, C., Nguyen, V.A., Merad, M., and Randolph, G.J. (2009). MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells. J Immunol *182*, 3650-3659.

Ratzinger, G., Stoitzner, P., Ebner, S., Lutz, M.B., Layton, G.T., Rainer, C., Senior, R.M., Shipley, J.M., Fritsch, P., Schuler, G.*, et al.* (2002). Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. J Immunol *168*, 4361-4371.

Rechavi, O., Goldstein, I., and Kloog, Y. (2009). Intercellular exchange of proteins: the immune cell habit of sharing. FEBS Lett *583*, 1792-1799.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P.*, et al.* (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med *189*, 371-380.

Regner, M., Pavlinovic, L., Koskinen, A., Young, N., Trapani, J.A., and Mullbacher, A. (2009). Cutting edge: rapid and efficient in vivo cytotoxicity by cytotoxic T cells is independent of granzymes A and B. J Immunol *183*, 37-40.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nat Rev Immunol *6*, 476-483.

Reis e Sousa, C., Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R.N., and Sher, A. (1997). In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J Exp Med *186*, 1819-1829.

Reis e Sousa, C., Stahl, P.D., and Austyn, J.M. (1993). Phagocytosis of antigens by Langerhans cells in vitro. J Exp Med *178*, 509-519.

Rescigno, M., Citterio, S., Thery, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S., and Ricciardi-Castagnoli, P. (1998). Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. Proc Natl Acad Sci U S A *95*, 5229-5234.

Rescigno, M., Piguet, V., Valzasina, B., Lens, S., Zubler, R., French, L., Kindler, V., Tschopp, J., and Ricciardi-Castagnoli, P. (2000). Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1beta, and the production of interferon gamma in the absence of IL-12 during DC-T cell cognate interaction: a new role for Fas ligand in inflammatory responses. J Exp Med *192*, 1661-1668.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol *2*, 361-367.

Revell, P.A., Grossman, W.J., Thomas, D.A., Cao, X., Behl, R., Ratner, J.A., Lu, Z.H., and Ley, T.J. (2005). Granzyme B and the downstream granzymes C and/or F are important for cytotoxic lymphocyte functions. J Immunol *174*, 2124-2131.

Riberdy, J.M., Newcomb, J.R., Surman, M.J., Barbosa, J.A., and Cresswell, P. (1992). HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. Nature *360*, 474-477.

Ridge, J.P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature *393*, 474-478.

Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I.A., Debatin, K.M., Fischer, A., and de Villartay, J.P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science *268*, 1347-1349.

Riol-Blanco, L., Delgado-Martin, C., Sanchez-Sanchez, N., Alonso, C.L., Gutierrez-Lopez, M.D., Del Hoyo, G.M., Navarro, J., Sanchez-Madrid, F., Cabanas, C., Sanchez-Mateos, P.*, et al.* (2009). Immunological synapse formation inhibits, via NF-kappaB and FOXO1, the apoptosis of dendritic cells. Nat Immunol *10*, 753-760.

Robinson, M.J., Ronchese, F., Miller, J.H., and La Flamme, A.C. (2010). Paclitaxel inhibits killing by murine cytotoxic T lymphocytes in vivo but not in vitro. Immunol Cell Biol *88*, 291-296.

Rock, K.L., and Goldberg, A.L. (1999). Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu Rev Immunol *17*, 739-779.

Rock, K.L., and Shen, L. (2005). Cross-presentation: underlying mechanisms and role in immune surveillance. Immunol Rev *207*, 166-183.

Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M., and Schuler, G. (1994). Proliferating dendritic cell progenitors in human blood. J Exp Med *180,* 83-93.

Romani, N., Koide, S., Crowley, M., Witmer-Pack, M., Livingstone, A.M., Fathman, C.G., Inaba, K., and Steinman, R.M. (1989). Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. J Exp Med *169*, 1169-1178.

Ronchese, F., and Hausmann, B. (1993). B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. J Exp Med *177*, 679-690.

Ronchese, F., and Hermans, I.F. (2001). Killing of dendritic cells: a life cut short or a purposeful death? J Exp Med *194*, F23-26.

Rubartelli, A., Poggi, A., and Zocchi, M.R. (1997). The selective engulfment of apoptotic bodies by dendritic cells is mediated by the alpha(v)beta3 integrin and requires intracellular and extracellular calcium. Eur J Immunol *27*, 1893-1900.

Ruedl, C., Khameneh, H.J., and Karjalainen, K. (2008). Manipulation of immune system via immortal bone marrow stem cells. Int Immunol *20*, 1211-1218.

Ruedl, C., Koebel, P., Bachmann, M., Hess, M., and Karjalainen, K. (2000). Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes. J Immunol *165*, 4910-4916.

Russell, J.H., and Ley, T.J. (2002). Lymphocyte-mediated cytotoxicity. Annu Rev Immunol *20*, 323-370.

Sad, S., Kagi, D., and Mosmann, T.R. (1996). Perforin and Fas killing by CD8+ T cells limits their cytokine synthesis and proliferation. J Exp Med *184*, 1543-1547.

Sadasivan, B., Lehner, P.J., Ortmann, B., Spies, T., and Cresswell, P. (1996). Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity *5*, 103-114.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med *182*, 389-400.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-

stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med *179*, 1109-1118.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708-712.

Samelson, L.E., and Klausner, R.D. (1992). Tyrosine kinases and tyrosine-based activation motifs. Current research on activation via the T cell antigen receptor. J Biol Chem *267*, 24913-24916.

Sauter, B., Albert, M.L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med *191*, 423-434.

Savina, A., Peres, A., Cebrian, I., Carmo, N., Moita, C., Hacohen, N., Moita, L.F., and Amigorena, S. (2009). The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. Immunity *30*, 544-555.

Scheinecker, C., McHugh, R., Shevach, E.M., and Germain, R.N. (2002). Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J Exp Med *196*, 1079-1090.

Schnorrer, P., Behrens, G.M., Wilson, N.S., Pooley, J.L., Smith, C.M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E.*, et al.* (2006). The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. Proc Natl Acad Sci U S A *103*, 10729-10734.

Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R., and Melief, C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schuler, G., and Steinman, R.M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. J Exp Med *161*, 526-546.

Schuurhuis, D.H., Laban, S., Toes, R.E., Ricciardi-Castagnoli, P., Kleijmeer, M.J., van der Voort, E.I., Rea, D., Offringa, R., Geuze, H.J., Melief, C.J.*, et al.* (2000). Immature dendritic cells acquire CD8(+) cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli. J Exp Med *192*, 145-150.

Seder, R.A., Gazzinelli, R., Sher, A., and Paul, W.E. (1993). Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc Natl Acad Sci U S A *90*, 10188-10192.

Segura, E., Amigorena, S., and Thery, C. (2005). Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. Blood Cells Mol Dis *35*, 89-93.

Segura, E., Guerin, C., Hogg, N., Amigorena, S., and Thery, C. (2007). CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo. J Immunol *179*, 1489-1496.

Semmling, V., Lukacs-Kornek, V., Thaiss, C.A., Quast, T., Hochheiser, K., Panzer, U., Rossjohn, J., Perlmutter, P., Cao, J., Godfrey, D.I.*, et al.* (2010). Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. Nat Immunol *11*, 313-320.

Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., Kuziel, W.A., and Pamer, E.G. (2003). TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity *19*, 59-70.

Shedlock, D.J., Whitmire, J.K., Tan, J., MacDonald, A.S., Ahmed, R., and Shen, H. (2003). Role of CD4 T cell help and costimulation in CD8 T cell responses during Listeria monocytogenes infection. J Immunol *170*, 2053-2063.

Shen, L., and Rock, K.L. (2004). Cellular protein is the source of cross-priming antigen in vivo. Proc Natl Acad Sci U S A *101*, 3035-3040.

Shi, L., Kraut, R.P., Aebersold, R., and Greenberg, A.H. (1992). A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J Exp Med *175*, 553-566.

Shortman, K., and Liu, Y.J. (2002). Mouse and human dendritic cell subtypes. Nat Rev Immunol *2*, 151-161.

Shresta, S., Graubert, T.A., Thomas, D.A., Raptis, S.Z., and Ley, T.J. (1999). Granzyme A initiates an alternative pathway for granule-mediated apoptosis. Immunity *10*, 595-605.

Simon, M.M., Hausmann, M., Tran, T., Ebnet, K., Tschopp, J., ThaHla, R., and Mullbacher, A. (1997). In vitro- and ex vivo-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. J Exp Med *186*, 1781-1786.

Smith, C.M., Wilson, N.S., Waithman, J., Villadangos, J.A., Carbone, F.R., Heath, W.R., and Belz, G.T. (2004). Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. Nat Immunol *5*, 1143-1148.

Smyth, M.J., Street, S.E., and Trapani, J.A. (2003). Cutting edge: granzymes A and B are not essential for perforin-mediated tumor rejection. J Immunol *171*, 515-518.

Spielman, J., Lee, R.K., and Podack, E.R. (1998). Perforin/Fas-ligand double deficiency is associated with macrophage expansion and severe pancreatitis. J Immunol *161*, 7063-7070.

Sporri, R., and Reis e Sousa, C. (2005). Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. Nat Immunol *6*, 163-170.

Sprick, M.R., Weigand, M.A., Rieser, E., Rauch, C.T., Juo, P., Blenis, J., Krammer, P.H., and Walczak, H. (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity *12*, 599-609.

Starr, T.K., Jameson, S.C., and Hogquist, K.A. (2003). Positive and negative selection of T cells. Annu Rev Immunol *21*, 139-176.

Steinman, R.M., Adams, J.C., and Cohn, Z.A. (1975). Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. J Exp Med *141*, 804-820.

Steinman, R.M., and Cohn, Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med *137*, 1142-1162.

Steinman, R.M., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med *139*, 380-397.

Steinman, R.M., Gutchinov, B., Witmer, M.D., and Nussenzweig, M.C. (1983). Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. J Exp Med *157*, 613-627.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C. (2003). Tolerogenic dendritic cells. Annu Rev Immunol *21*, 685-711.

Steinman, R.M., Lustig, D.S., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. J Exp Med *139*, 1431-1445.

Steinman, R.M., Turley, S., Mellman, I., and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med *191*, 411-416.

Steinman, R.M., and Witmer, M.D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc Natl Acad Sci U S A *75*, 5132-5136.

Stepp, S.E., Dufourcq-Lagelouse, R., Le Deist, F., Bhawan, S., Certain, S., Mathew, P.A., Henter, J.I., Bennett, M., Fischer, A., de Saint Basile, G.*, et al.* (1999). Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science *286*, 1957-1959.

Stoitzner, P., Green, L.K., Jung, J.Y., Price, K.M., Atarea, H., Kivell, B., and Ronchese, F. (2008). Inefficient presentation of tumor-derived antigen by tumor-infiltrating dendritic cells. Cancer Immunol Immunother *57*, 1665-1673.

Stoitzner, P., Tripp, C.H., Eberhart, A., Price, K.M., Jung, J.Y., Bursch, L., Ronchese, F., and Romani, N. (2006). Langerhans cells cross-present antigen derived from skin. Proc Natl Acad Sci U S A *103*, 7783-7788.

Stoll, S., Delon, J., Brotz, T.M., and Germain, R.N. (2002). Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science *296*, 1873-1876.

Stranges, P.B., Watson, J., Cooper, C.J., Choisy-Rossi, C.M., Stonebraker, A.C., Beighton, R.A., Hartig, H., Sundberg, J.P., Servick, S., Kaufmann, G.*, et al.* (2007). Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity *26*, 629-641.

Strasser, A., Jost, P.J., and Nagata, S. (2009). The many roles of FAS receptor signaling in the immune system. Immunity *30*, 180-192.

Straus, S.E., Jaffe, E.S., Puck, J.M., Dale, J.K., Elkon, K.B., Rosen-Wolff, A., Peters, A.M., Sneller, M.C., Hallahan, C.W., Wang, J.*, et al.* (2001). The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. Blood *98*, 194-200.

Straus, S.E., Sneller, M., Lenardo, M.J., Puck, J.M., and Strober, W. (1999). An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. Ann Intern Med *130*, 591-601.

Suh, W.K., Mitchell, E.K., Yang, Y., Peterson, P.A., Waneck, G.L., and Williams, D.B. (1996). MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. J Exp Med *184*, 337-348.

Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R., and Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med *204*, 1775-1785.

Sung, S.S., Fu, S.M., Rose, C.E., Jr., Gaskin, F., Ju, S.T., and Beaty, S.R. (2006). A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J Immunol *176*, 2161-2172.

Sutton, V.R., Davis, J.E., Cancilla, M., Johnstone, R.W., Ruefli, A.A., Sedelies, K., Browne, K.A., and Trapani, J.A. (2000). Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. J Exp Med *192*, 1403-1414.

Swain, S.L., Weinberg, A.D., English, M., and Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. J Immunol *145*, 3796-3806.

Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell *76*, 969-976.

Tan, J.T., Whitmire, J.K., Ahmed, R., Pearson, T.C., and Larsen, C.P. (1999). 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. J Immunol *163*, 4859-4868.

Tang, H., Cao, W., Kasturi, S.P., Ravindran, R., Nakaya, H.I., Kundu, K., Murthy, N., Kepler, T.B., Malissen, B., and Pulendran, B. (2010). The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. Nat Immunol *11*, 608-617.

Thery, C., Duban, L., Segura, E., Veron, P., Lantz, O., and Amigorena, S. (2002a). Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. Nat Immunol *3*, 1156-1162.

Thery, C., Zitvogel, L., and Amigorena, S. (2002b). Exosomes: composition, biogenesis and function. Nat Rev Immunol *2*, 569-579.

Tough, D.F., Sun, S., and Sprent, J. (1997). T cell stimulation in vivo by lipopolysaccharide (LPS). J Exp Med *185*, 2089-2094.

Trapani, J.A., Davis, J., Sutton, V.R., and Smyth, M.J. (2000). Proapoptotic functions of cytotoxic lymphocyte granule constituents in vitro and in vivo. Curr Opin Immunol *12*, 323-329.

Traunecker, A., Oliveri, F., and Karjalainen, K. (1991). Myeloma based expression system for production of large mammalian proteins. Trends Biotechnol *9*, 109-113.

Trinchieri, G., and Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol *7*, 179-190.

Trombetta, E.S., Ebersold, M., Garrett, W., Pypaert, M., and Mellman, I. (2003). Activation of lysosomal function during dendritic cell maturation. Science *299*, 1400-1403.

Trombetta, E.S., and Mellman, I. (2005). Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol *23*, 975-1028.

Tschopp, J., Masson, D., and Stanley, K.K. (1986). Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. Nature *322*, 831-834.

Tschopp, J., and Nabholz, M. (1990). Perforin-mediated target cell lysis by cytolytic T lymphocytes. Annu Rev Immunol *8*, 279-302.

Turley, S.J., Inaba, K., Garrett, W.S., Ebersold, M., Unternaehrer, J., Steinman, R.M., and Mellman, I. (2000). Transport of peptide-MHC class II complexes in developing dendritic cells. Science *288*, 522-527.

Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., Moore, K., Kleijmeer, M., Liu, Y., Duvert-Frances, V., Vincent, C., Schmitt, D., Davoust, J.*, et al.* (2000). Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity *12*, 71-81.

Valladeau, J., and Saeland, S. (2005). Cutaneous dendritic cells. Semin Immunol *17*, 273-283.

van Dommelen, S.L., Sumaria, N., Schreiber, R.D., Scalzo, A.A., Smyth, M.J., and Degli-Esposti, M.A. (2006). Perforin and granzymes have distinct roles in defensive immunity and immunopathology. Immunity *25*, 835-848.

van Mierlo, G.J., Boonman, Z.F., Dumortier, H.M., den Boer, A.T., Fransen, M.F., Nouta, J., van der Voort, E.I., Offringa, R., Toes, R.E., and Melief, C.J. (2004). Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol *173*, 6753-6759.

Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., Fehling, H.J., Hardt, W.D., Shakhar, G., and Jung, S. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity *31*, 502-512.

Veeraswamy, R.K., Cella, M., Colonna, M., and Unanue, E.R. (2003). Dendritic cells process and present antigens across a range of maturation states. J Immunol *170*, 5367-5372.

Vermaelen, K.Y., Carro-Muino, I., Lambrecht, B.N., and Pauwels, R.A. (2001). Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. J Exp Med *193*, 51-60.

Villadangos, J.A., Cardoso, M., Steptoe, R.J., van Berkel, D., Pooley, J., Carbone, F.R., and Shortman, K. (2001). MHC class II expression is regulated in dendritic cells independently of invariant chain degradation. Immunity *14*, 739-749.

Villadangos, J.A., and Heath, W.R. (2005). Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm. Semin Immunol *17*, 262-272.

Villadangos, J.A., and Schnorrer, P. (2007). Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol *7*, 543-555.

Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000). CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J Immunol *164*, 2978-2986.

Vremec, D., and Shortman, K. (1997). Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. J Immunol *159*, 565-573.

Waithman, J., Allan, R.S., Kosaka, H., Azukizawa, H., Shortman, K., Lutz, M.B., Heath, W.R., Carbone, F.R., and Belz, G.T. (2007). Skin-derived dendritic cells can mediate deletional tolerance of class I-restricted self-reactive T cells. J Immunol *179*, 4535-4541.

Wakim, L.M., Waithman, J., van Rooijen, N., Heath, W.R., and Carbone, F.R. (2008). Dendritic cell-induced memory T cell activation in nonlymphoid tissues. Science *319*, 198-202.

Wang, J., Chun, H.J., Wong, W., Spencer, D.M., and Lenardo, M.J. (2001). Caspase-10 is an initiator caspase in death receptor signaling. Proc Natl Acad Sci U S A *98*, 13884-13888.

Wang, J., Zheng, L., Lobito, A., Chan, F.K., Dale, J., Sneller, M., Yao, X., Puck, J.M., Straus, S.E., and Lenardo, M.J. (1999). Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell *98*, 47-58.

Wang, Y.H., Ito, T., Homey, B., Watanabe, N., Martin, R., Barnes, C.J., McIntyre, B.W., Gilliet, M., Kumar, R., Yao, Z., *et al.* (2006). Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells. Immunity *24*, 827-838.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314-317.

Watchmaker, P.B., Urban, J.A., Berk, E., Nakamura, Y., Mailliard, R.B., Watkins, S.C., van Ham, S.M., and Kalinski, P. (2008). Memory CD8+ T cells protect dendritic cells from CTL killing. J Immunol *180*, 3857-3865.

Watts, T.H. (2005). TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol *23*, 23-68.

White, D.W., and Harty, J.T. (1998). Perforin-deficient CD8+ T cells provide immunity to Listeria monocytogenes by a mechanism that is independent of CD95 and IFN-gamma but requires TNF-alpha. J Immunol *160*, 898-905.

Wikstrom, M.E., Batanero, E., Judd, S.R., Wiqvist, K., Holt, P.G., and Stumbles, P.A. (2010). Lung homing T-cell generation is dependent on strength and timing of antigen delivery to lymph nodes. Immunol Cell Biol.

Willems, F., Amraoui, Z., Vanderheyde, N., Verhasselt, V., Aksoy, E., Scaffidi, C., Peter, M.E., Krammer, P.H., and Goldman, M. (2000). Expression of c-FLIP(L) and resistance to CD95-mediated apoptosis of monocyte-derived dendritic cells: inhibition by bisindolylmaleimide. Blood *95*, 3478-3482.

Wilson, N.S., El-Sukkari, D., Belz, G.T., Smith, C.M., Steptoe, R.J., Heath, W.R., Shortman, K., and Villadangos, J.A. (2003). Most lymphoid organ dendritic cell types are phenotypically and functionally immature. Blood *102*, 2187-2194.

Wilson, N.S., El-Sukkari, D., and Villadangos, J.A. (2004). Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. Blood *103*, 2187-2195.

Witmer, M.D., and Steinman, R.M. (1984). The anatomy of peripheral lymphoid organs with emphasis on accessory cells: light-microscopic immunocytochemical studies of mouse spleen, lymph node, and Peyer's patch. Am J Anat *170*, 465-481.

Woodland, D.L., and Kohlmeier, J.E. (2009). Migration, maintenance and recall of memory T cells in peripheral tissues. Nat Rev Immunol *9*, 153-161.

Wu, J., Wilson, J., He, J., Xiang, L., Schur, P.H., and Mountz, J.D. (1996). Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. J Clin Invest *98*, 1107-1113.

Xu, Y., Zhan, Y., Lew, A.M., Naik, S.H., and Kershaw, M.H. (2007). Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol *179*, 7577-7584.

Yamazaki, S., Bonito, A.J., Spisek, R., Dhodapkar, M., Inaba, K., and Steinman, R.M. (2007). Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors. Blood *110*, 4293-4302.

Yamazaki, S., Dudziak, D., Heidkamp, G.F., Fiorese, C., Bonito, A.J., Inaba, K., Nussenzweig, M.C., and Steinman, R.M. (2008). CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. J Immunol *181*, 6923-6933.

Yang, J., Huck, S.P., McHugh, R.S., Hermans, I.F., and Ronchese, F. (2006). Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo. Proc Natl Acad Sci U S A *103*, 147-152.

Yang, X., Stennicke, H.R., Wang, B., Green, D.R., Janicke, R.U., Srinivasan, A., Seth, P., Salvesen, G.S., and Froelich, C.J. (1998). Granzyme B mimics apical caspases. Description of a unified pathway for trans-activation of executioner caspase-3 and -7. J Biol Chem *273*, 34278-34283.

Yang, X.O., Pappu, B.P., Nurieva, R., Akimzhanov, A., Kang, H.S., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Schluns, K.S.*, et al.* (2008). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity *28*, 29-39.

Yang, Y., Mercep, M., Ware, C.F., and Ashwell, J.D. (1995). Fas and activation-induced Fas ligand mediate apoptosis of T cell hybridomas: inhibition of Fas ligand expression by retinoic acid and glucocorticoids. J Exp Med *181*, 1673-1682.

Yewdell, J.W., Norbury, C.C., and Bennink, J.R. (1999). Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. Adv Immunol *73*, 1-77.

Zammit, D.J., Cauley, L.S., Pham, Q.M., and Lefrancois, L. (2005). Dendritic cells maximize the memory CD8 T cell response to infection. Immunity *22*, 561-570.

Zammit, D.J., Turner, D.L., Klonowski, K.D., Lefrancois, L., and Cauley, L.S. (2006). Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. Immunity *24*, 439-449.

Ziegler, S.F., and Liu, Y.J. (2006). Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. Nat Immunol *7*, 709-714.

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. (1998). Eradication of established murine

tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med *4*, 594-600.

Appendices

Appendix 1
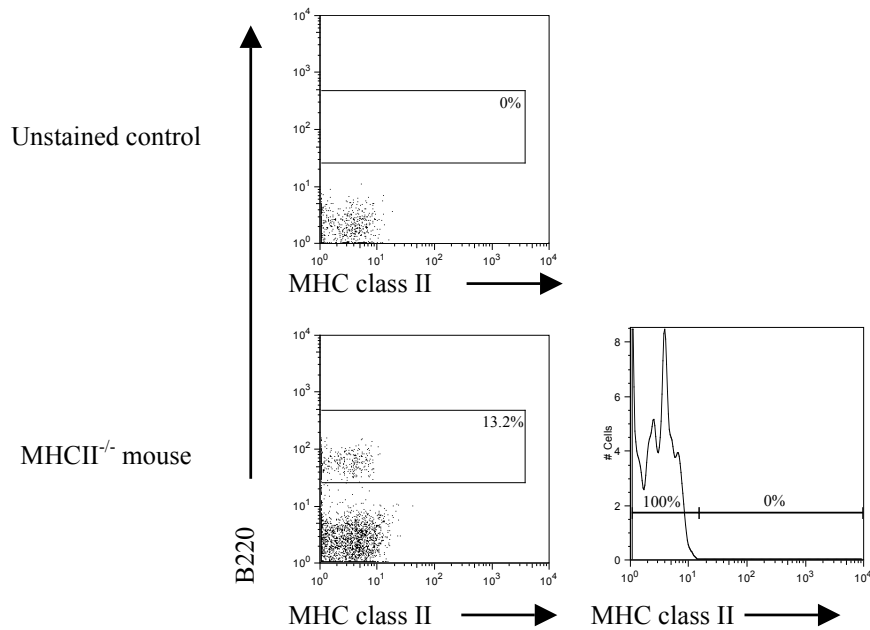
Appendix 1. **B6Aa^0/Aa^0 mice do not express MHC class II.** B6Aa^0/Aa^0 mice were tail-bled and the blood collected was processed and analysed for the expression of B220 and MHC class II by flow cytometry. The percentage of cells expressing B220 and MHC class II is shown in a representative dot plot from an individual B6Aa^0/Aa^0 mouse and the unstained control. The percentage of B220$^+$ cells expressing MHC class II is shown in a representative histogram.

Appendix 2
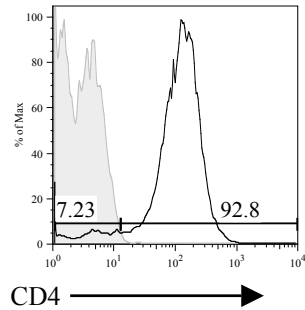
Appendix 2. **CD4$^+$ T cells are enriched from OT-II lymphocytes after positive selection.** Lymphocytes and splenocytes were enriched for CD4$^+$ T cells as described in Chapter 2. An aliquot of the enriched sample was examined for CD4 expression by flow cytometry. The expression of CD4 in the lymphocytes is shown as percentages in the representative histogram. This result is representative of several experiments.
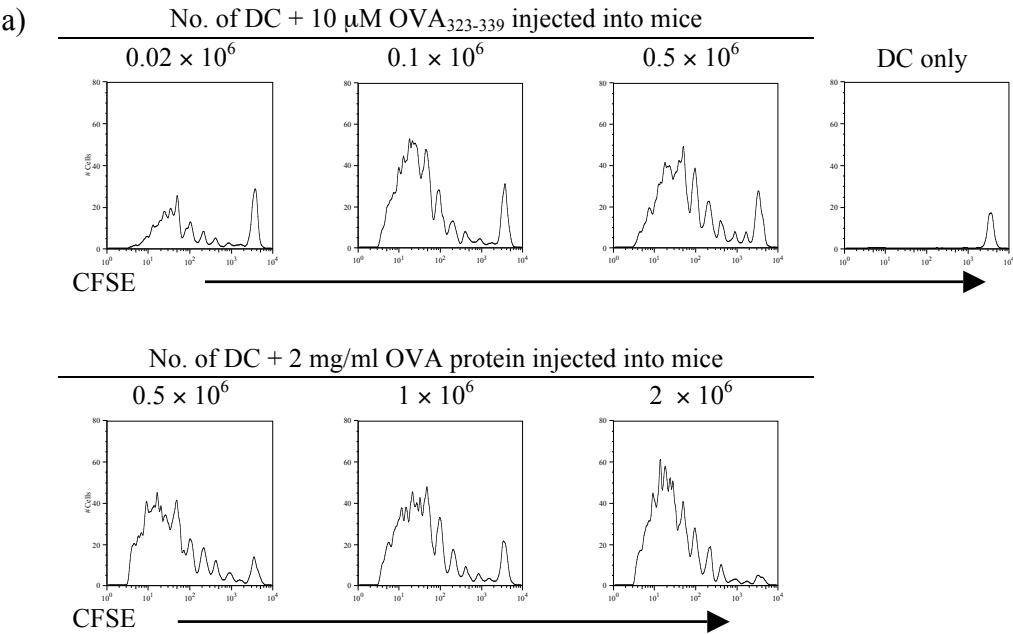
Appendix 3

a)

b)

c)

d)
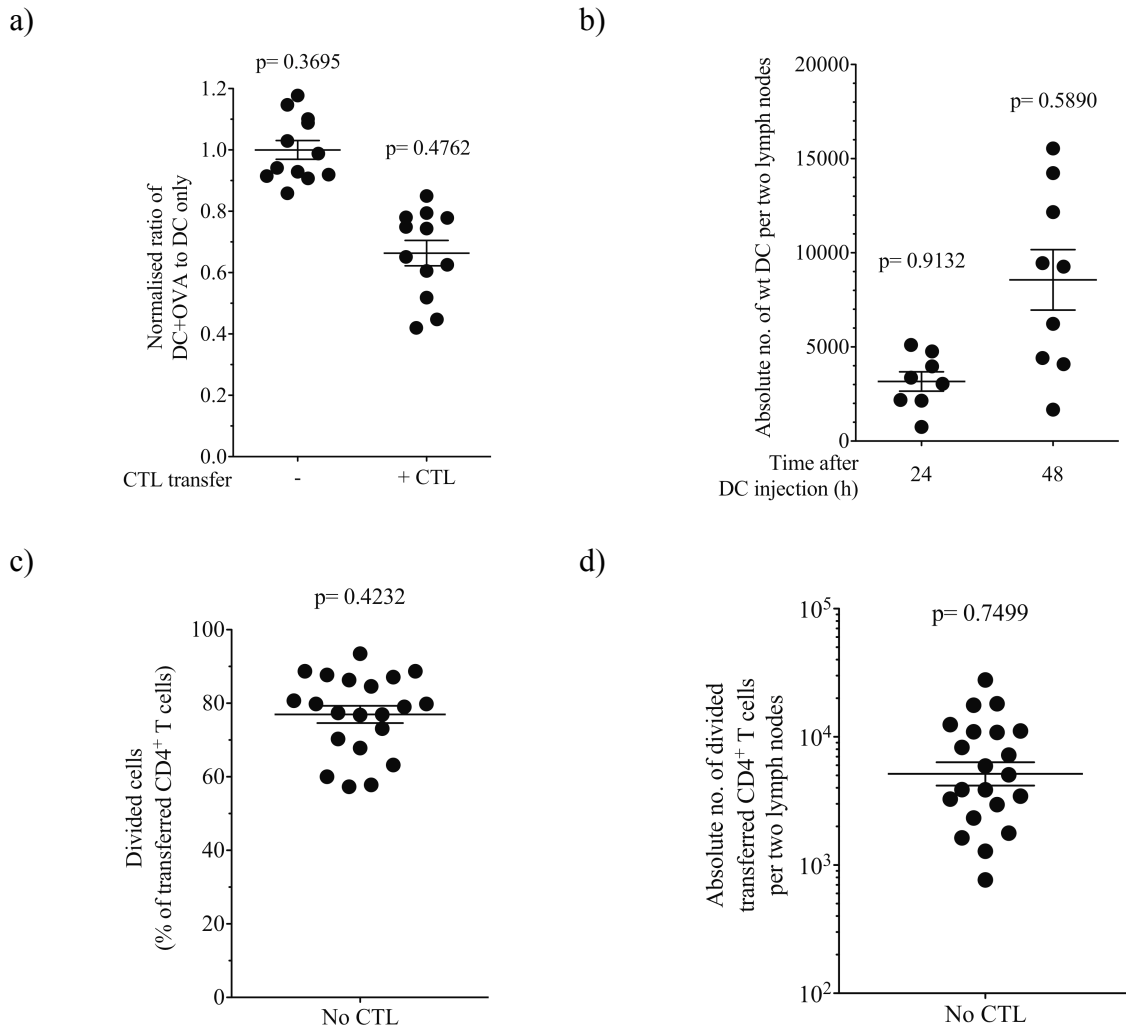
Appendix 3. **CTL-mediated DC killing, DC accumulation in the draining lymph nodes and CD4^{+} T cell division are consistent with a Gaussian distribution.** (**a**) The experiment was carried out as described in Fig. 4.2.5. Results are pooled from two separate experiments carried out under similar conditions. (**b**) The experiment was carried out as described in Fig. 3.6.3. Results are pooled from two separate experiments carried out under similar conditions. (**c, d**) The experiment was carried out as described in Fig. 3.3. Results are pooled from 4 separate experiments carried out under similar conditions. Statistical significance was determined with D'Agostino and Pearson omnibus normality test at a 95% confidence interval. $p > 0.05$ is considered to be consistent with a Gaussian distribution.
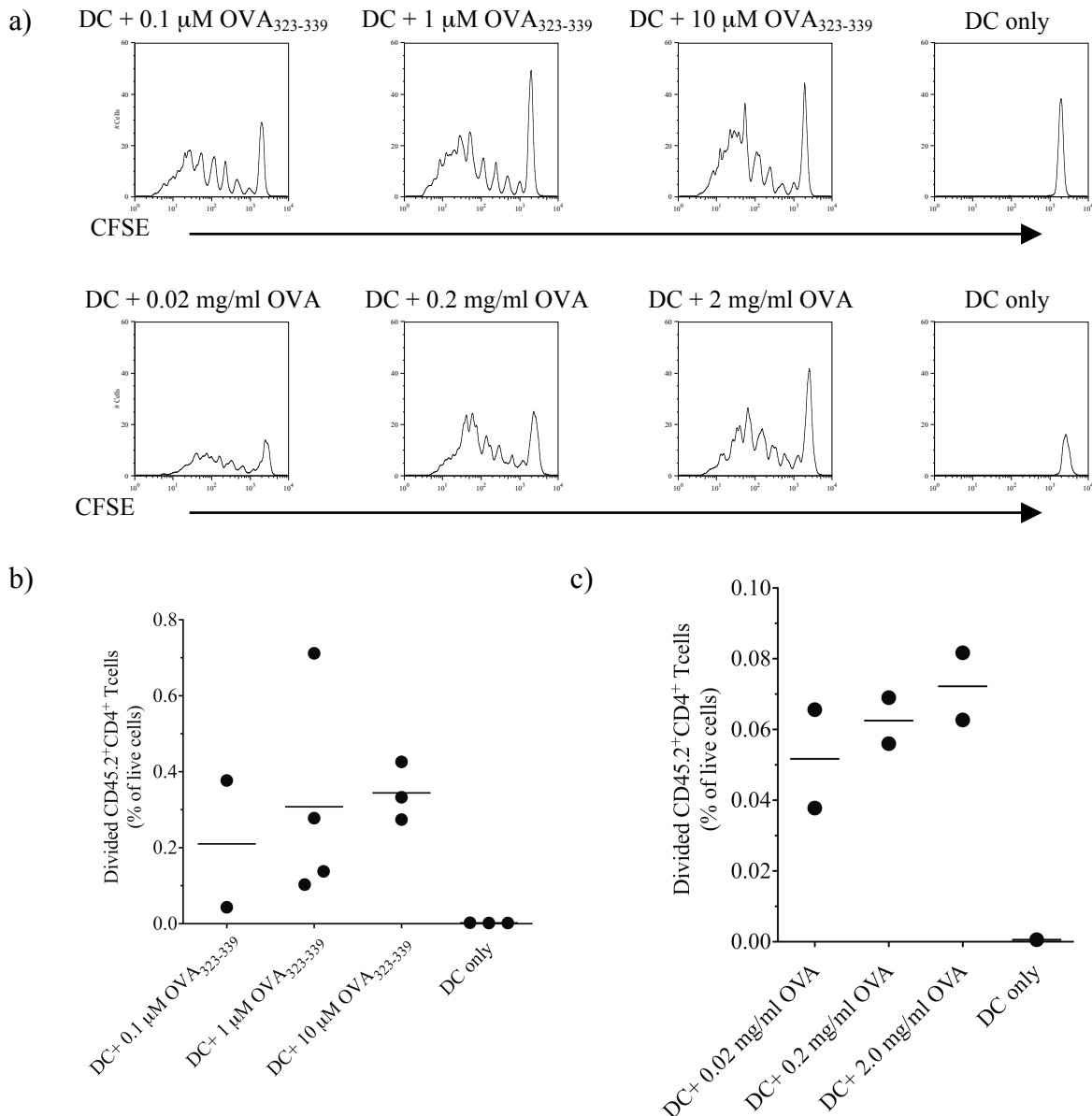
Appendix 4

a)

b)
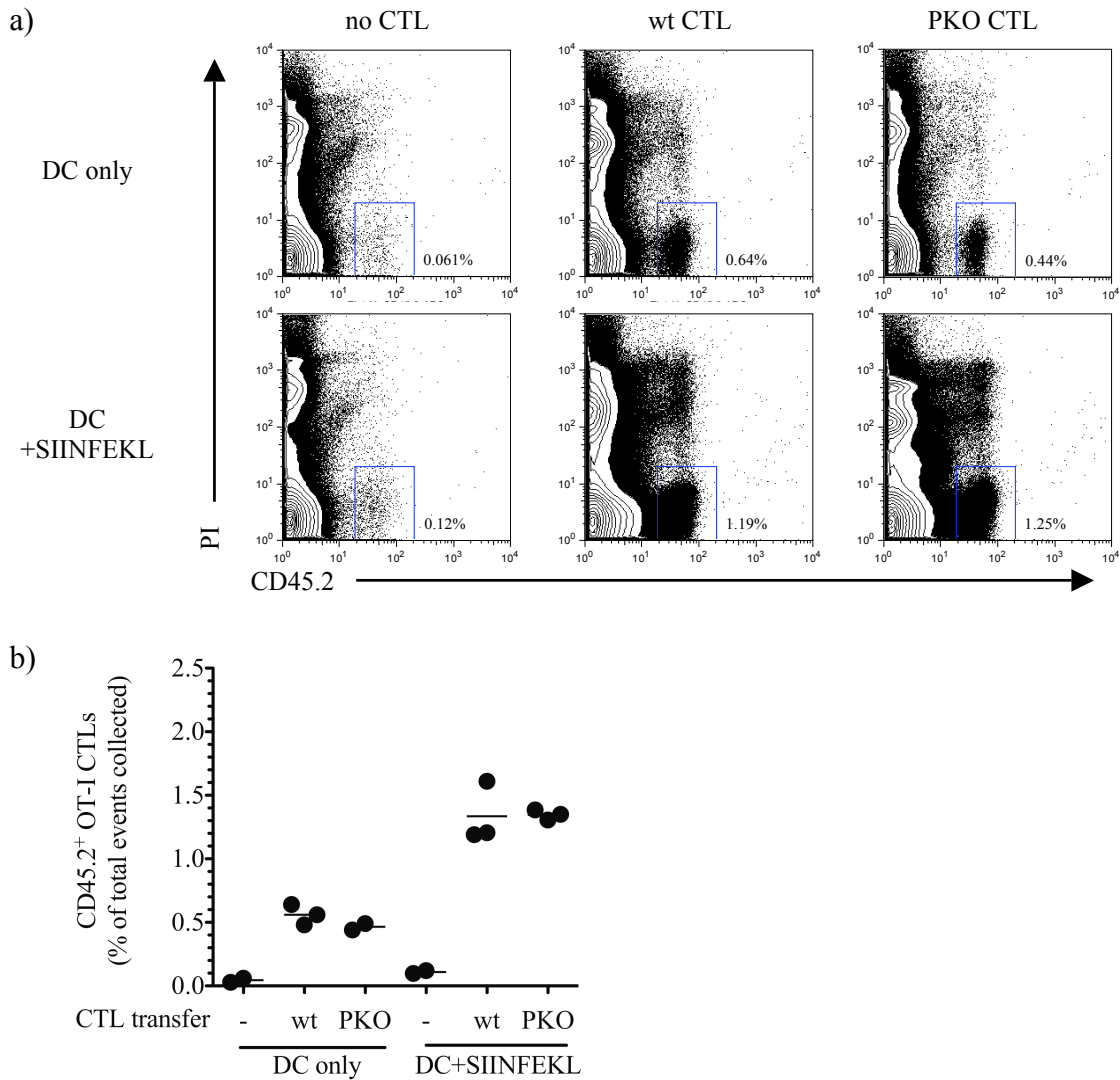
Appendix 4. **CTLs circulate in the draining lymph nodes.** Groups of B6.SJ *ptprca* mice received *in vitro* activated wt or PKO CD45.2$^+$ OT-I CTLs. As a control, some mice did not receive CTLs. 24 h later, *lpr* DCs loaded with SIINFEKL were mixed in equal numbers with *lpr* DC only and injected s.c. into the forelimbs of recipient mice. After 48 h, CD45.2$^+$ OT-I CTLs in the draining lymph nodes were monitored by flow cytometry. (**a**) CD45.2$^+$ OT-I CTLs are shown in representative contour plots from individual mice. (**b**) The percentages of CD45.2$^+$ OT-I CTLs in the draining lymph nodes are shown. The experiment was performed once with 2 – 3 mice per group.
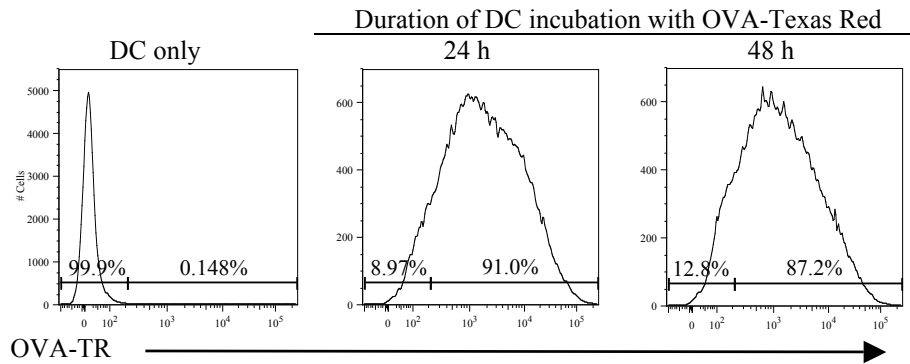
Appendix 5

a)

No. of DC + 10 μM OVA$_{323-339}$ injected into mice

CFSE

No. of DC + 2 mg/ml OVA protein injected into mice

CFSE

b)

No. of DCs injected (x10^6)

DC+OVA$_{323-339}$ DC+OVA
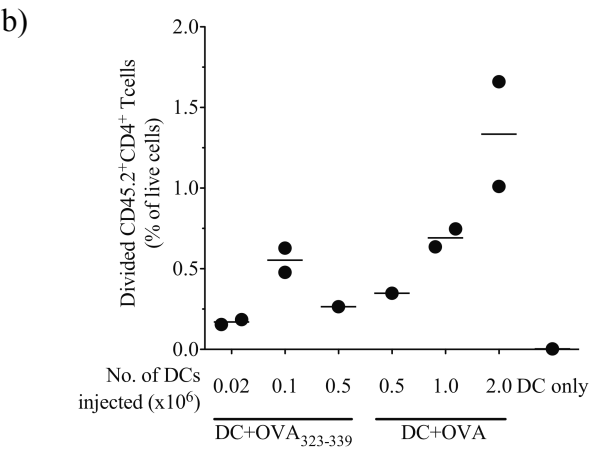
Appendix 5. **CD4$^+$ T cell division is dependent on the number of antigen-loaded DCs injected.** B6.SJ *ptprca* mice received CFSE-labelled OT-II CD45.2$^+$CD4$^+$ T cells. 24 h later, different numbers of DCs loaded with OVA$_{323-339}$ or OVA protein were injected into the forelimbs of recipient mice. 3 days later, CD4$^+$ T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**a**) CFSE dilution in CD45.2$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**b**) The percentages of CD45.2$^+$CD4$^+$ T cells that had divided at least once are shown. The experiment was performed once with 1 – 3 mice per group.

Appendix 6

a)

b)   c)



Appendix 6. **CD4^{+} T cell division is dependent on the antigen dose loaded on the DCs.** B6.SJ *ptprca* mice received CFSE-labelled OT-II CD45.2^{+}CD4^{+} T cells. 24 h later, DCs loaded with different concentrations of OVA$_{323-339}$ or DCs loaded with different concentrations of OVA protein were injected into the forelimbs of recipient mice. 3 days later, CD4^{+} T cells in the draining lymph nodes were examined for CFSE dilution by flow cytometry. (**a**) CFSE dilution in CD45.2^{+}CD4^{+} T cells is shown as representative histograms from individual mice. (**b** and **c**) The percentages of CD45.2^{+}CD4^{+} T cells that had divided at least once are shown. The results of OVA$_{323-339}$ and OVA protein are from two separate experiments with 1 – 4 mice per group.
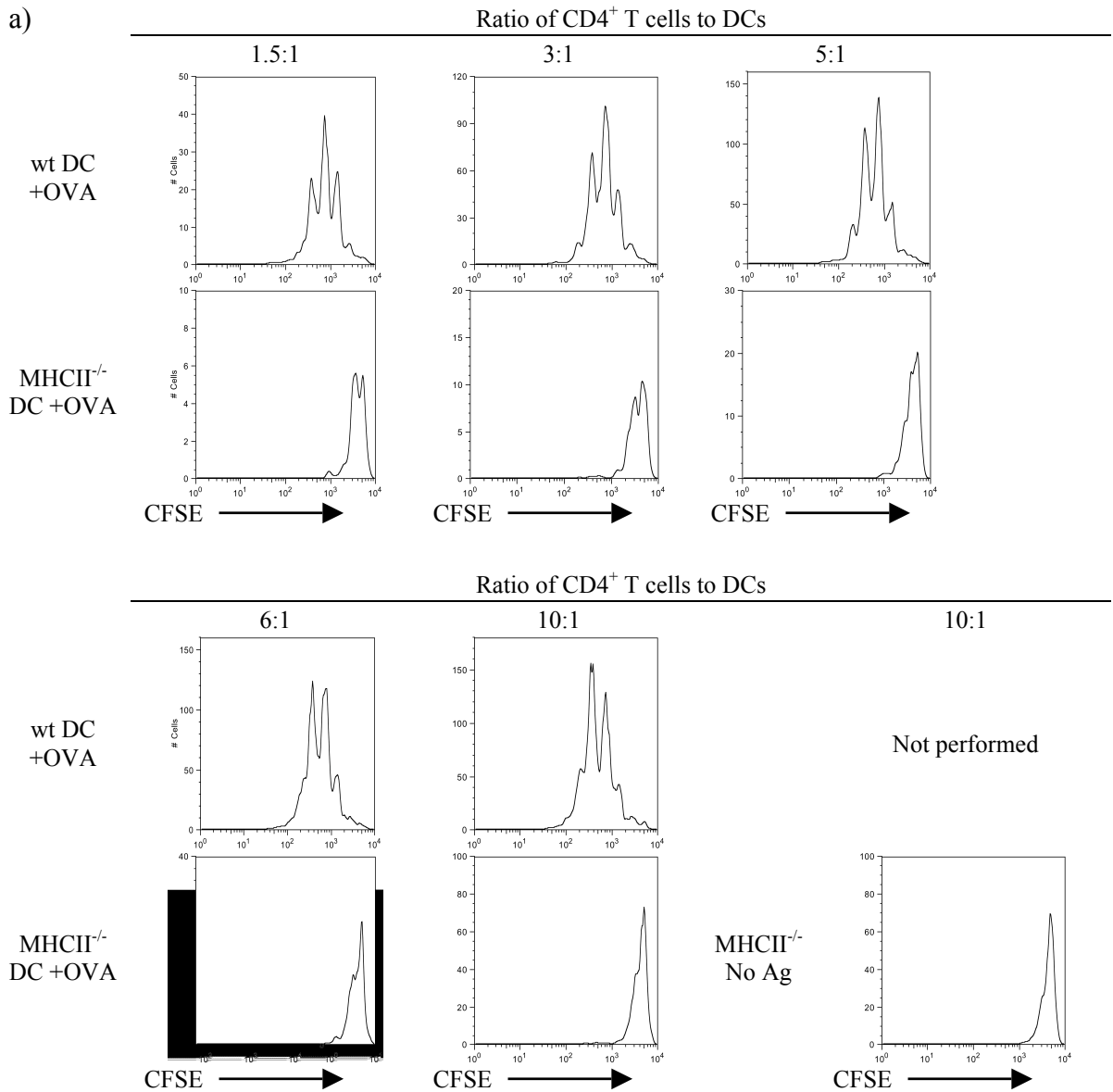
Appendix 7

Appendix 7. **Not all DCs take up soluble OVA protein even after prolonged incubation.** Bone marrow DCs were prepared as described in Chapter 2. Some of these DCs were incubated with OVA protein conjugated to Texas Red (OVA-TR) on day 5 of culture. In some wells, OVA-TR was added to DCs 4 h before adding LPS on day 6 of culture. As a control, some DCs did not receive any OVA-TR. LPS was added to the DC culture on day 6. The DCs were harvested on day 7 and analysed for CD11c^{+} and OVA-TR^{+} cells by flow cytometry. The percentages of CD11c^{+} cells that are OVA^{+} or OVA^{-} are shown in each histogram. The experiment was performed once.

Appendix 8

a)

c)
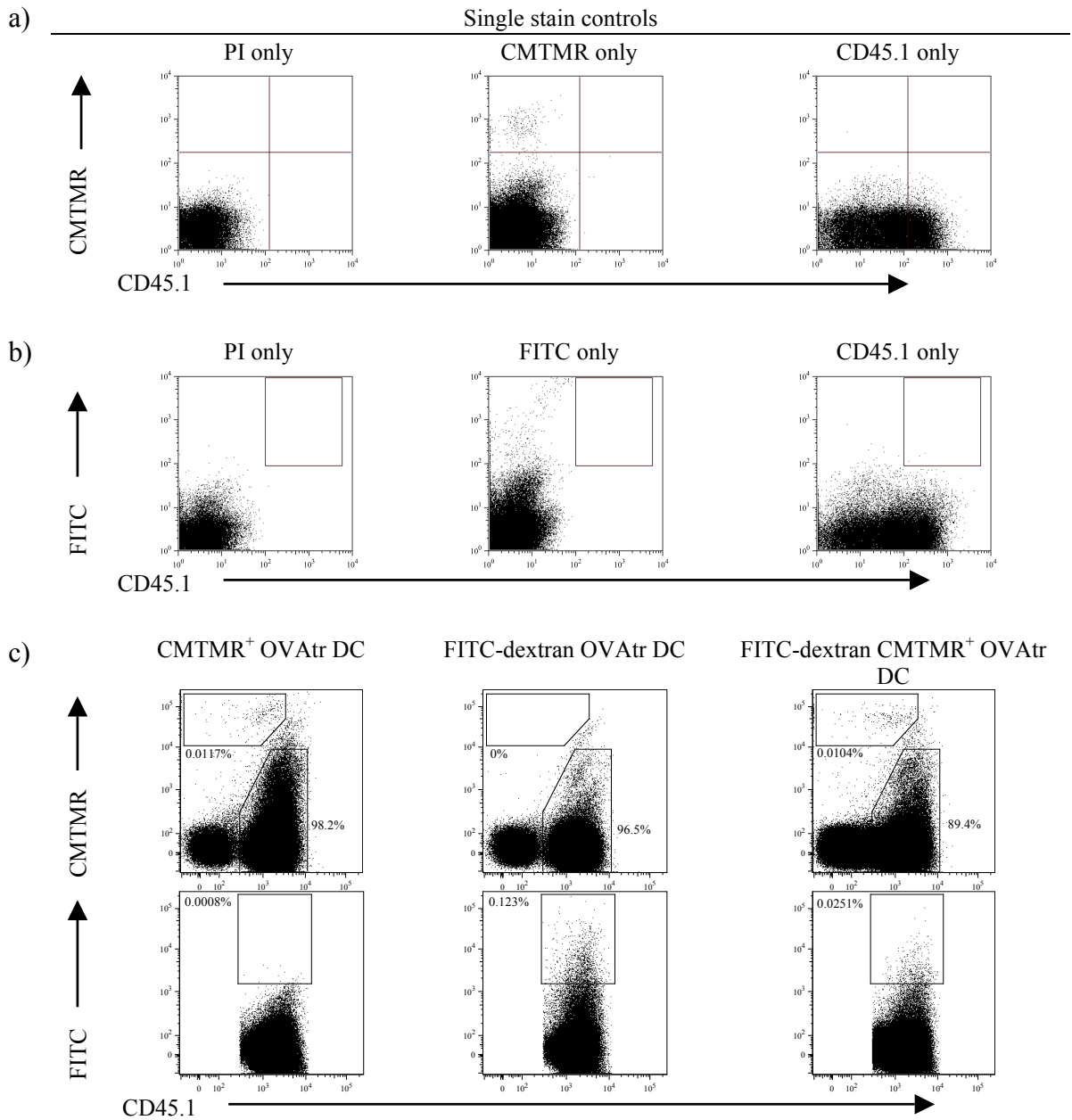
Appendix 8. **Host cells acquire materials from injected DCs.** This experiment was carried out together with Fig. 5.1 at the same time. The methodology is described in Fig. 5.1. (**a**) CMTMR-labelled CD45.1⁻ OVAtr DCs are shown. (**b**) CD45.1⁻ OVAtr DCs incubated with FITC-dextran are shown. (**c**) A separate experiment with 1 – 5 mice per group was carried out as described in Fig. 5.1.
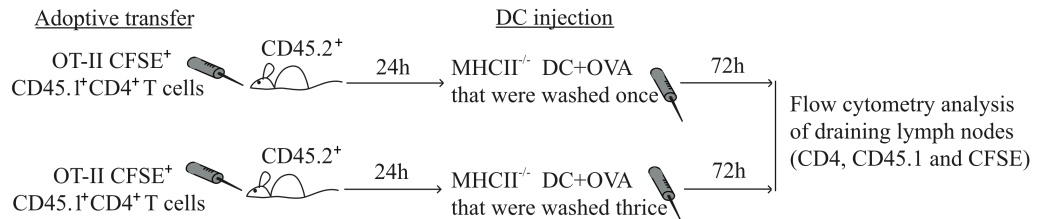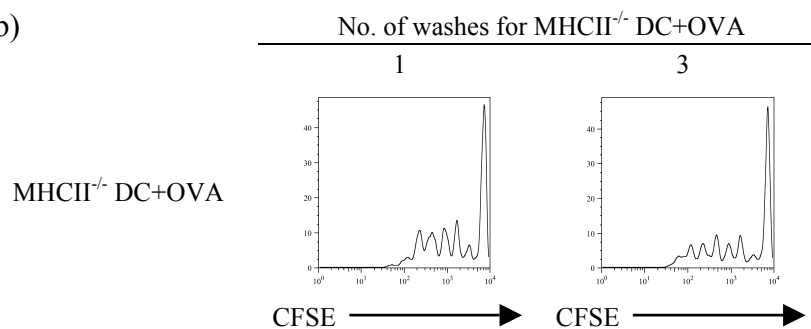
Appendix 9

a)

Appendix 9. MHCII^{-/-} DCs cannot induce CD4⁺ T cell proliferation. DCs were cultured from the bone marrows of wt and B6Aa^0/Aa^0 (MHCII$^{-/-}$) mice as described in Chapter 2. These DCs were loaded with OVA protein and subsequently activated with LPS (DC+OVA). LPS-activated DC+OVA were then plated at 0.01×10^6 cells per well and co-incubated with serially diluted numbers of CFSE-labelled OTII CD4$^+$ T cells at the indicated ratios. As a negative control, MHCII$^{-/-}$ DCs that were not loaded with OVA protein (No Ag) were used. 4 days after co-incubation, CD4$^+$ T cells were examined for CFSE dilution by flow cytometry. CFSE dilution of CD4$^+$ T cells is shown as representative histograms from individual wells. The experiment was performed once with duplicate wells for each condition.
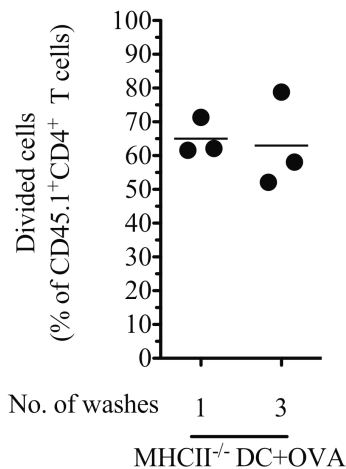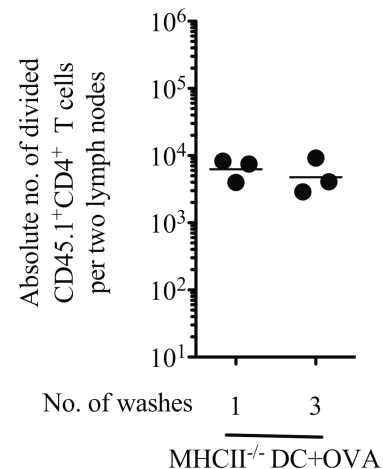
Appendix 10

a)

b)

No. of washes for MHCII$^{-/-}$ DC+OVA

MHCII$^{-/-}$ DC+OVA

c)

d)

Appendix 10. **CD4$^+$ T cell proliferation is not due to the carryover of OVA protein in the injection medium.** (**a**) C57BL/6J mice were injected i.v. with CFSE-labelled OT-II CD45.1$^+$CD4$^+$ T cells. OVA protein-loaded MHCII$^{-/-}$ DCs were washed once or thrice with IMDM before injection into the forelimbs 24 h after CD4$^+$ T cell transfer. 3 days later, CD45.1$^+$CD4$^+$ T cells were examined for CFSE dilution by flow cytometry. (**b**) CFSE dilution in CD45.1$^+$CD4$^+$ T cells is shown as representative histograms from individual mice. (**c**) The percentages of CD45.1$^+$CD4$^+$ T cells that had divided at least once are shown. (**d**) Absolute numbers of divided CD45.1$^+$CD4$^+$ T cells in the draining lymph nodes are shown. The experiment was performed once with 3 mice per group.
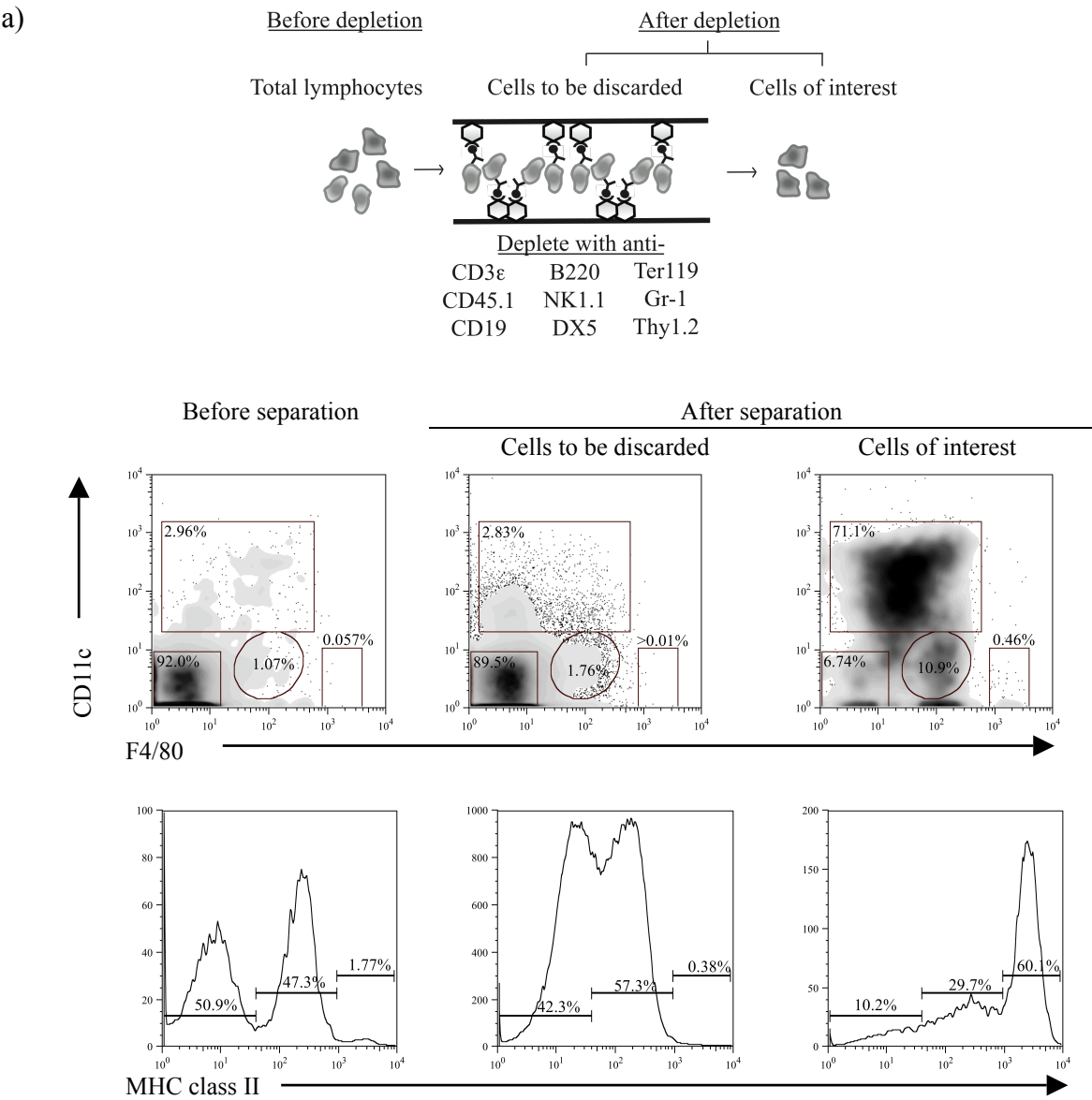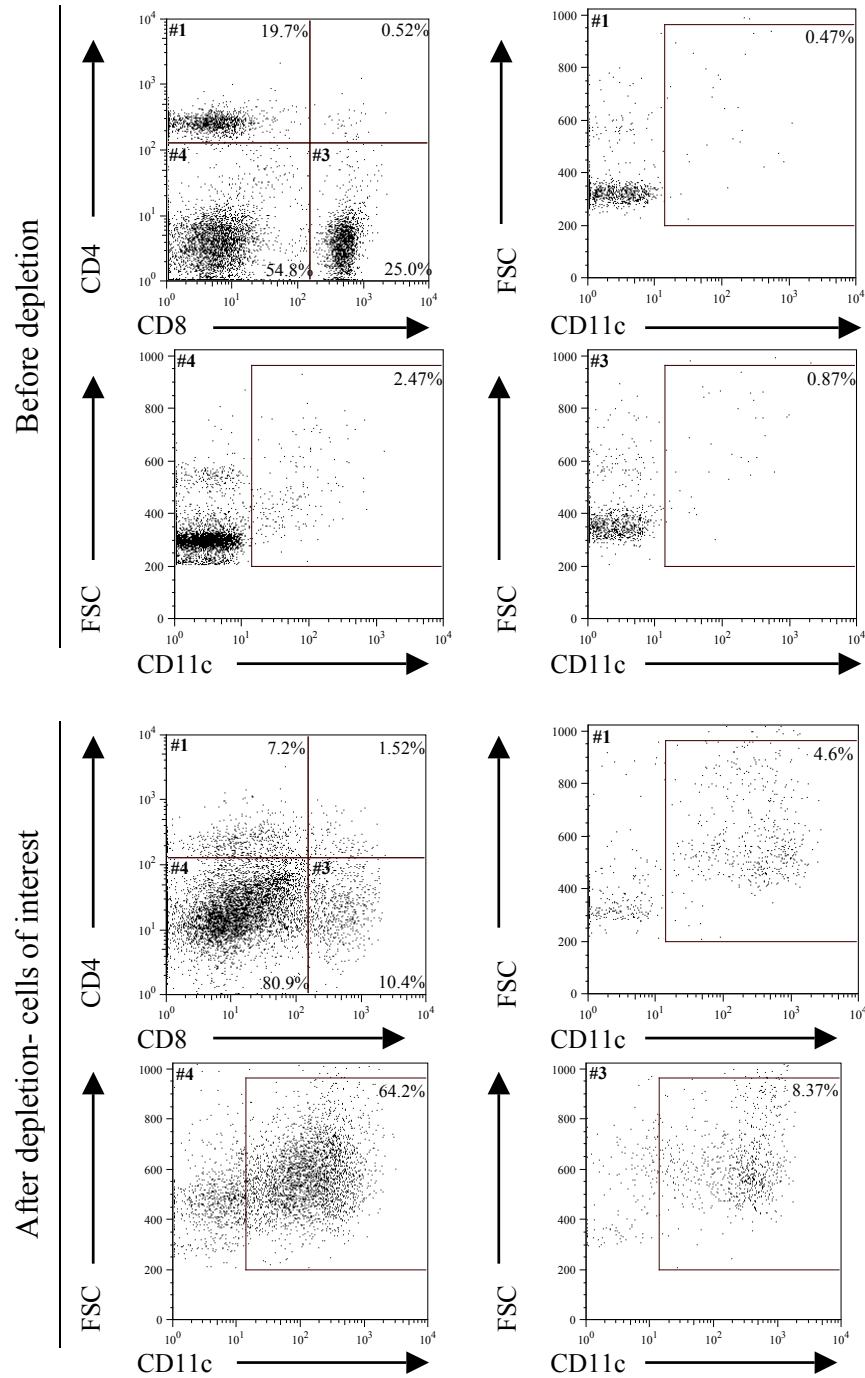
Appendix 11

a)
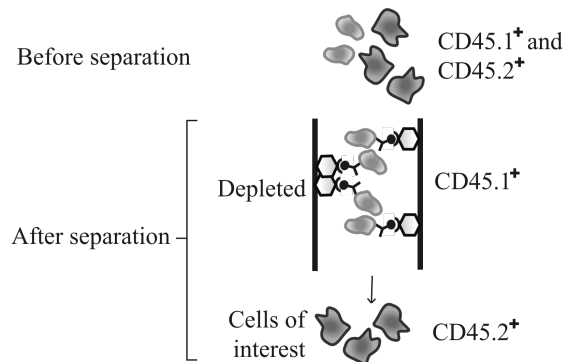
b)
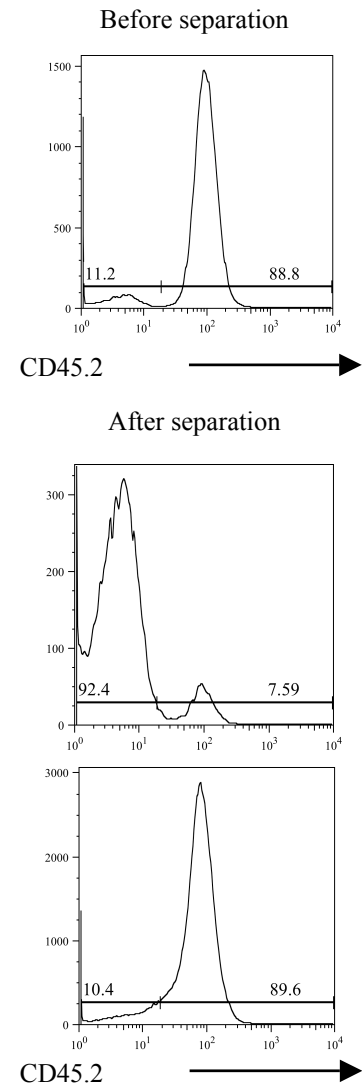
Appendix 11. **CD45.2$^+$ cells are enriched using magnetic depletion.** (**a**) Brachial and axillary lymph nodes from four C57BL/6J (CD45.2$^+$) and one B6.SJ *ptprca* (CD45.1$^+$) mice were pooled together, digested and made into cell suspensions. Cells were labelled with biotinylated anti-CD45.1 and were subsequently depleted of CD45.1$^+$ cells (cells of interest) with magnetic beads. After magnetic separation, the depleted cells were then analysed for the expression of CD45.2 using flow cytometry analysis. (**b**) Expression of CD45.2 in lymphocytes is shown as histograms. CD45.2$^+$ or CD45.2$^-$ cells are shown as percentages of live cells. The experiment was performed once.

Appendix 12

a)

Before depletion

After depletion

Total lymphocytes   Cells to be discarded   Cells of interest

Deplete with anti-

CD3ε	B220	Ter119
CD45.1	NK1.1	Gr-1
CD19	DX5	Thy1.2

Before separation   After separation

Cells to be discarded   Cells of interest

CD11c

F4/80

MHC class II

Appendix 12- continued

d)

Appendix 12. **Depletion of other cell types using magnetic beads enriches for DCs.** C57BL/6J mice were injected s.c. into their forelimbs with LPS-activated CD45.1[+] wt DCs previously loaded with OVA protein (DC+OVA) or wt DCs not loaded with OVA protein (DC only). 24 h later, brachial and axillary lymph nodes were harvested, digested and made into cell suspensions. Cell suspensions were enriched for CD11c^{+} cells through depletion using a cocktail of antibodies and magnetic beads described in Chapter 2. Aliquots of these cell suspensions before and after depletion

were then analysed for the expressions of CD11c and F4/80, MHC class II, CD4 and CD8. (**a**) The schematic diagram of negative selection is shown. The percentages of live cells expressing (**b**) CD11c and F4/80, (**c**) MHC class II, (**d**) CD4 and CD8 are shown. The labels #1, #3 and #4 represent the top left, bottom right and bottom left quadrants of the dot plots. The corresponding histograms are labelled #1, #3 and #4, respectively. The experiment was performed once.

Appendix 13

a) <u>GM-CSF/IL-4 treated BM cells</u>

b) <u>GM-CSF/IL-4 treated BM cells</u>

CD11c$^+$ cells   CD11c$^-$ cells
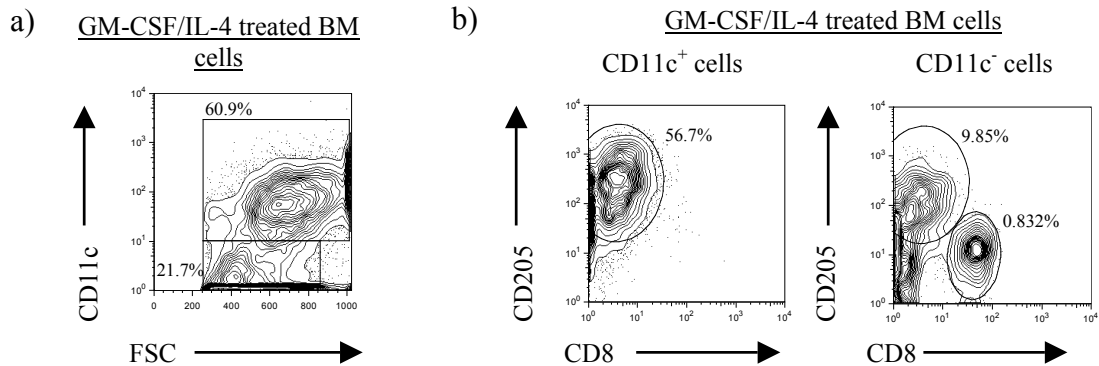
Appendix 13. **BM cells cultured with GM-CSF/IL-4 give rise to CD11c$^+$CD205$^+$CD8$^-$ cells.** BM cells were prepared as described in Chapter 2. LPS was added to the BM cells on day 6 of culture. 24 h after adding LPS, GM-CSF/IL-4 treated cells were harvested, labelled with anti-CD11c, anti-CD205 and anti-CD8 antibodies and analysed by flow cytometry. (**a**) The expression of CD11c in GM-CSF/IL-4 treated BM cells is shown in the contour plot. The percentages of CD11c$^+$ and CD11c$^-$ cells are shown. (**b**) The expressions of CD205 and CD8 in CD11c$^+$ and CD11c$^-$ GM-CSF/IL-4 treated BM cells are shown in the contour plots. The percentages of CD11c$^+$CD205$^+$CD8$^-$ cells, CD11c$^-$CD205$^+$CD8$^-$ cells and CD11c$^-$CD205$^+$CD8$^+$ cells are shown. The experiment was performed once.

Appendix 14

Site of
injection

Point of
entry

Legend

.... Needle travels
beneath the skin

Appendix 14. **Subcutaneous injection into the forelimbs of mice.**