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# Spi6 in Dendritic Cell priming of CD8 T cell responses to virus

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This is a statement to declare that all the work presented here was performed by the author unless otherwise stated

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# ABSTRACT

Spi6 is a serine protease inhibitor. Its main function is inhibition of granzyme B (GrB): it is therefore an anti-apoptotic protein in cytotoxic T cells (CTL), where leakage of GrB from cytotoxic granules into the cytosol can trigger apoptosis. CTLs use GrB release to kill infected cells but they can also induce apoptosis in antigen-loaded mature dendritic cells (DC) during priming. Mature DCs upregulate Spi6 and *in vitro* have higher resistance to CTL-induced apoptosis compared to immature ones: when Spi6 is absent, this resistance is lost. However, whether Spi6 protects DCs from CTL-mediated apoptosis *in vivo* is still under debate. Using mice deficient in Spi6, the project focuses on the role of Spi6 in DC survival during the priming of naïve and memory anti-Lymphocytic Choriomeningitis virus (LCMV) CD8 T cell responses. $CD8\alpha^+$ DCs are professional antigen presenting cells responsible for cross-presentation of viral antigens in secondary lymphoid organs. Upon maturation, Spi6 is expressed by $CD8\alpha^+$ DC *in vivo*. In our model, Spi6 KO DC antigen-presentation ability was comparable to wild-type (WT) but their survival was impaired. This resulted in defective expansion of WT LCMV-specific CD8 T cells. A similar requirement for Spi6 was found for DC priming of memory CD8 T cell expansion. GrB KO CD8 T cells rescued the priming defect in Spi6 KO mice during both primary and secondary responses, demonstrating GrB is the physiological target of Spi6 in DCs. Thus, GrB is a major immunosuppressive agent controlling the DC priming of anti-viral T cell mediated immunity.

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# ABBREVIATIONS

APC: Antigen-presenting cell

BCR: B-cell receptor

BMDDC: Bone marrow-derived dendritic cell

cDC: Conventional dendritic cell

CL13: Clone 13

CTL: Cytotoxic T cell

DC: Dendritic cell

DN: Double-negative

DNA: Deoxyribonucleic acid

DP: Double positive

EEC: Early effector cell

FRC: Follicular reticular cell

GP: Glyco protein

GrA: Granzyme A

GrB: Granzyme B

HEC: High endothelial cell

HEV: High endothelial venule

ICAM intercellular adhesion molecule-1

IFN: Interferon

IL: Interleukin

IPC: Interferon-producing cell

IS: Immunological synapse

LCMV: Lymphocytic Choriomeningitis virus

LFA-1: lymphocyte function-associated antigen-1

LN: Lymph node

LPS: Lipopolysaccharide

MHC: Major histocompatibility complex

MPEC: Memory-precursor effector cell

NK: Natural killer

NP: Nucleo protein

PAMP: pathogen-associated molecular pattern

PCD: Programmed cell death

PI-9: Proteinase inhibitor 9

pDC: Plasmacytoid dendritic cell

Pfn: Perforin

p.i.: Post-infection

PRR: Pattern recognition receptor

RNA: Ribonucleic acid

RT-PCR: Real-time poly chain reaction

SLEC: Short-lived effector cell

SN: Single-negative

SP: Single-positive

Spi6: Serine protease inhibitor 6

TCR: T-cell receptor

TEC: Thymic epithelial cell

TLR: Toll-like receptor

TNF: Tumor necrosis factor

VCAM: vascular cell adhesion molecule

# 1. INTRODUCTION

## 1.1 Motivation, assumptions and research challenges

Infectious diseases are still today a very high priority in the agenda of the WHO, due to the high mortality rates they have among children and young adults, especially in developing countries (WHO). Six major diseases (tuberculosis, pneumonia, diarrhoea, HIV/AIDS, malaria and measles) cause the majority (90%) of deaths, and piling evidence suggests that some diseases once thought to be unrelated to pathogenic agents, like cancers, are or the greatest part the result of chronic infections. Viral infections in particular are at the origin of at least 4 of these 6 threatening diseases and cause chronic conditions which account for major health burdens in developed countries, like hepatitis and AIDS. New strategies are needed in order to develop vaccines to successfully prevent especially lethal infections and innovative therapeutic approaches are desirable to tackle chronic diseases.

Dendritic cells (DCs) are the main switch responsible for the activation of immune responses against viral pathogens, mediated by CD8 T cells. CD8 T lymphocytes are the cell type mainly responsible for anti-viral immunity as they are able to release cytotoxic agents. In order for antiviral responses to be properly and efficiently developed, dendritic cells need to be preserved and functional. Enhancement of DC survival is desirable to achieve improved activation of CD8 T cell responses. Dendritic cells are thus an ideal target of new therapeutic methodologies aimed at manipulating and ameliorating immune responses to viruses. CD8 T cells response manipulation includes efficient activation of a *de novo* response, which is needed for a vaccine to be effective, and re-stimulation of anergic or unresponsive CD8 T cells in treatment of chronic infections. The same CD8 T cell response is also highly involved in eradicating cancer cells, thus further widening the possible applications of therapeutic strategies improving DCs survival. Vaccination strategies that involve targeting of DCs with specific antigens to be used for activation of immune responses have already been tested, and proved to be promising (Belz et al. 2004).

## 1.2 Key contributions and achievements

This project's results have significance in the context of designing therapeutic strategies involving the preservation of dendritic cells. In the project are presented the results from the study of the role of Serine protease inhibitor (Spi)-6 in protecting dendritic cells involved in priming CD8 T cell responses against virus. A controversy recently was brought up when *in vitro* results supporting a protective role for Spi6 were disproved by *in vivo* studies. The controversy was addressed by this project.

The novelty of this study is in the analysis of DC survival in absence of Spi6 expression an *in vivo* mouse model. The model, a KO mouse for the protein of interest, Spi6, allowed the study of physiological expression and function of Spi6 in two mouse DC subsets. The use of a KO mouse model for the protein of interest represents a novel approach to study Spi6 function in DCs.

Key contributions include the identification of specific upregulation of Spi6 in CD8α DCs but not in pDCs in the spleen of infected mice, the demonstration that Spi6 absence in DCs induces defective CTL responses and the corroboration that granzyme B (GrB) is the cause behind DC and CTL deficient levels in absence of Spi6.

This project is therefore important in sight of a potential use of DCs to manipulate CD8 T cell responses. Applications include but are not limited to vaccination design, treatment of chronic diseases, tumours and prevention or treatment of transplantation complications due to rejections.

## 1.3 Road map

The background information available in the literature to better understand the two main cell types involved, DCs and CD8 T cells, together with a description of Spi6 and its function, are laid down in chapter 2 (2.2). In the same chapter are described the viral model (2.7), the animal model of choice and the hypothesis on which the project is based (2.8.4). The materials and methods used to investigate Spi6 role in DCs during the priming of CD8 T cell responses are described in Chapter 3, while the results obtained from experimental investigations are reported in Chapter 4. Lastly, the discussion of the findings supported by these results and future work are illustrated in Chapter 5.

# 2. BACKGROUND

The human body is furnished with an extremely clever and sophisticated defence apparatus, called the immune system. The immune system is a complex network of cells and molecular mediators which extend throughout our body and provides protection against pathogens, be they viruses, bacteria, parasites, or fungi. It can be divided into two arms, which interact closely with each other: innate immunity and adaptive immunity. CD8 T cells are the cellular mediators responsible for the killing of virally infected cells and are part of the adaptive arm of the immune response. These cells are nonetheless unable to recognise an infected cell unless previously instructed by a member of the innate immunity: dendritic cells. DCs can recognise invading pathogens and interact with CD8 T cells to instruct them on the type and levels of invader. They therefore play a key role in activation and shaping of the CD8 T cell responses. Spi6 is a serine protease inhibitor expressed by DCs, but its function in these cells has, to date, not been clearly defined. This protein is expressed by CD8 T cells as well where it protects them against self-inflicted death due to a cytotoxic molecule called GrB. The same function is predicted in DCs. To study Spi6 function in DCs, a viral infection model has been used, the Lymphocytic Choriomeningitis virus (LCMV).

## 2.1 Innate and adaptive immunity: Two sides of the same coin

Innate immunity is present in all life forms from plants to animal and it represents the first line of defence against infections. It includes anatomical barriers like mucosae and intestinal flora and it involves non-specific reactions to pathogen invasion triggered by its cellular components which are Mast cells, Macrophages, Neutrophils, Dendritic cells, Eosinophils, Natural killer (NK) cells, and γδ T cells. Considered part of the innate immune system are also the complement system, which allows opsonisation of pathogens when bound to antibodies, and cytokines and

chemokines. Examples of innate responses are septic shock (due to the systemic release of high levels of IL-1 and TNF-α) and the initiation of allergic reactions (due to local release of histamine by mast cells). The innate system is mobilised immediately upon pathogen invasion due to the recognition of PAMPs (pathogen-associated molecular patterns), specific molecular patterns maintained in pathogens, like lipopolysaccharide (LPS) and double strand RNA (dsRNA). PAMPS are recognized by receptors called PPRs (pattern recognition receptors) expressed by various innate cells. The responses triggered when the components of the innate system are activated are non-specific and often systemic (Akira 2011). Moreover, this system does not provide long-lasting protection but rather quick and immediate defensive reactions.

Adaptive immunity, on the contrary, is able to respond to pathogen invasion with a specific and local set of measures and cells to almost any pathogen and it can evolve (in the best of options) together with the pathogen during infection. Each response is based on the recognition of specific antigens derived from pathogen protein degradation via dedicated receptors like the T cell receptor (TCR) and the B cell receptor (BCR). These receptors, on the membrane of the main player of adaptive immunity, B and T cells, are able to recognize a very high number of antigens due to two main mechanisms: the V(D)J recombination of their loci in both T and B cells and successive somatic hypermutations in B cells. The adaptive system is dependent on the innate system in that it needs to be activated by it, and cannot mount a response in absence of the latter.

What distinguish the adaptive immune arm from the innate one are not just the pathogen specificity of its responses and the need for activation, but also the duration and the "memory" of them. In fact, each adaptive response creates a set of cells able to reactivate in case of repeated pathogen invasion, called memory cells, which will be faster and more efficient in clearing the pathogen (Ahmed and Gray 1996). The adaptive immunity consists of two main arms: a humoral one, made by B cells and the antibodies they produce, and a cellular one, made by CD4 T helper, CD4 T regulatory and CD8 T cells. B cells can recognise a pathogen through their BCR. The activation of this receptor will trigger the production of antibodies and, once the pathogen is defeated, the B cell is set to become a long-lasting, quiescent antibody

reserve called plasma cell. T cells, instead, are broadly divided into CD4 and CD8 T cells. CD4 T cells are responsible for helping B cells and CD8 T cells reach the proper activation status and also have immune regulatory functions. CD8 T cells are the ones responsible for targeted and direct killing of infected or tumour cells. They have cytotoxic properties and their activation is sought-after in the design of vaccines and therapeutic strategies. For these characteristics, this project focussed on CD8 T cells. The specificity of the adaptive response also depends on the expression of major histocompatibility complex (MHC) molecules which are surface proteins loaded with peptides derived from the processing of intracellular molecules expressed in all the cells, with a few exceptions, like red blood cells.

The two arms of the immune system are thus clearly interconnected, as the adaptive system needs to be activated by the innate system to be able to carry on its defensive functions. The cellular component of the innate immunity which is responsible for the cross-talk between the adaptive and immune arms is the Dendritic Cells (DCs). Discovered by the Nobel Prize Laureate Ralph Steinman in 1973 (Steinman and Cohn 1973), these cells instruct T and B cells to develop either immunity or tolerance by presenting antigens acquired from the environment and by delivering costimulatory signals. The signals depend on the type of pathogen and, modality of the infection, the level of inflammation and other parameters and are of main importance to regulate the specificity of the adaptive immune response in terms of activation and extent.

## 2.2 CD8 T cells

CD8 T cells are the main cell component of the adaptive immune responses against intracellular pathogens and tumours. They are identified by the expression of a transmembrane glycoprotein called CD8 whose role is that of co-receptor to the TCR. CD8 T cells are mainly responsible for the killing of cancer cells and cells infected by intracellular pathogens like viruses and intracellular bacteria. Activation through contact with an antigen-loaded mature DC is necessary for activation and proliferation to be triggered. Once activated, CD8 T cells can recognise an infected or

tumour cell by means of interaction mediated by their TCR with peptides mounted on a MHC-I molecule and with the MHC itself. Once recognised, the infected cell can be killed by released cytotoxic molecules or via the engagement of death receptors. Once the infection is cleared, CD8 memory cells, progeny of the activated killer cells, develop to form a long-lasting quiescent population, ready to be activated by successive pathogen invasions.

### 2.2.1 The T cell receptor

The TCR is a highly variable surface protein composed by two chains which is always complexed with CD3 molecules, forming the TCR complex (see Figure 1). By recognising specific antigens loaded onto MHC, the TCR complex mediates, in concert with other receptors, activation and effector functions of the T cells (Janeway 1992). Also, the TCR is solely responsible for giving T cells their specificity. The TCR molecule is a heterodimer composed by one α- and one β-chain. Each chain can be further divided into a constant (C) segment, the portion anchored to the membrane, a J (joining) segment and a variable (V) segment, which is the portion sticking out towards the intracellular space. The β-chain has a similar structure but also contains a D (diversity) segment between the V and the J. Each variable region has 3 different complementary determining regions (CDR) which are responsible for the recognition of MHC-loaded antigens. In thymocytes (the precursors of CD8 naïve T cells), during differentiation to naïve cells, a clever process of rearrangement of the exons of the gene segments V, D and J takes place in each of the chain loci. The result of this rearrangement is a unique combination translated in each T cell, leading to as many T cells with different specificity as binding and recognising sites can be formed by V (D) J rearrangements (Janeway 1992). In the beta-chain, Dβ-Jβ rearrange first, while Vβ to DJ rearranges when the thymocytes are in the double negative (DN) stage of differentiation in the thymus. The TCR β chain then binds to a pre-TCR α chain, and the thymocytes proceed in their proliferation and progress to the double positive (DP) developmental stage. The TCR α-chain will undergo rearrangement only after the proliferation. This is further described below (Chapter 2.2.2)

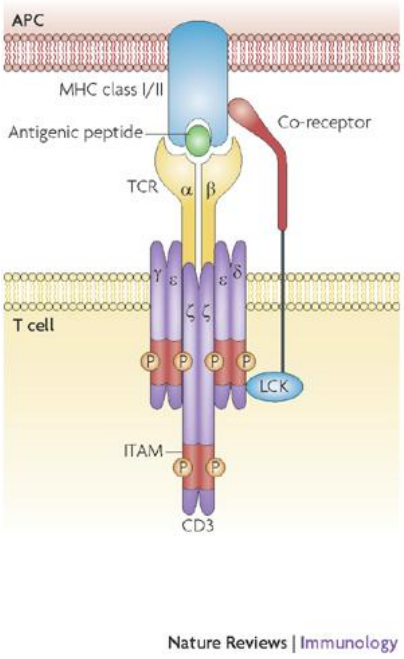


**Figure 1: TCR complex: in yellow the two chains of the TCR (α and β), in purple the CD3 complex, in red the co-receptor (CD8 or CD4) and in light blue the MHC-I/II on the target or dendritic cell (Gascoigne 2008).**

It has been calculated that the TCR rearrangement can produce $10^{15}$–$10^{20}$ unique TCR structures. In humans in particular there are $10^{12}$ naïve αβ T cells in the circulation of an adult, while in mice there are about $10^{8}$ T cells. The number of possible combinations of 11 amino acids, which is the average length of antigens loaded onto a MHC molecule, can be as high as $10^{18}$, although not all of them would be able to successfully associate with the MHC binding pocket (Miles et al. 2011).

The CD3 molecule associated with the TCR is responsible for the signal transduction of the TCR: the TCR has in fact very few cytosolic amino acids, while the CD3 has long cytoplasmic tails. Studies in which it was tried to express the TCR without CD3 showed how co-expression of these two molecules is required (Weiss and Stobo 1984). The exact way TCR and CD3 interact is not fully understood, but recent studies showed how a conformational change happens in the TCR upon binding of the same to a pMHC (peptide-loaded MCH). The signals triggered by ligation of the TCR complex support CD8 T cell maturation to the effector status and stimulate proliferation, as reviewed below (Chapter 2.2.4).

### 2.2.2 CD8 T cell development

CD8 T cells develop from hematopoietic stem cells (HSC) which, once mobilised from the bone marrow, are released in the circulation and periodically enter the thymus. It is not fully clarified at which step of differentiation these cells reach the thymus, but it is known that after leaving the BM, the HSC can proliferate and differentiate into common lymphoid progenitors (CLPs). Some of these will enter the thymus and settle there, thus becoming thymus-settling progenitors (TSP) and subsequently earliest T-lineage progenitors (ETP). ETPs, T cell precursors, enter the thymus via the post-capillary venules which penetrate the thymus in between the cortical and the medullary regions.

The entry rate is regulated and one of the molecules implicated in this process is P-selectin. P-selectin is in fact expressed on thymic epithelium and its levels have been shown to correspond to thymic niche availability (Rossi et al. 2005). Sphingosine-1-phosphate (S1P) is an intracellular intermediate in the membrane sphingolipid metabolism and it is produced intracellularly by most cell types. Its extracellular levels seem to be correlated to the exit of mature lymphocytes from the thymus (in the same way in which, as we will see later, seems to work regulating egression from LN and spleen of T cells). Studies from Cyster and colleagues have indicated the egress regulation might be in place through the maintenance of a gradient between the blood and the (higher) thymic concentration of S1P (Cyster 2005; Cyster and Schwab 2012).

The ETPs can proliferate in the thymus giving origin to T cells which do not express either CD8 or CD4 and are therefore called double negative (DN, i.e. $CD8^-$ $CD4^-$) lymphoid precursors (Schwarz and Bhandoola 2006).

The ETPs, defined as $CD8^-$ $CD4^-$ $CD44^+$ $CD25^-$ or DN-1, undergo a maturation process which involves a migration in the thymus and during which the cells acquire specific surface markers and rearrange their TCR. DN-1 cells moving deeper into the cortex of the thymus express $CD25^+$ first, and then $CD117^-$ (DN2). During the third stage (DN3), they become $CD44^-$ $CD25^+$ and localise in the external area of the cortex (see Figure 2). In the next step the precursors undergo their first selection, called β selection, which requires the β-chain to be properly bound to the pre-TCR-α-chain and mounted on the T cell surface to allow the cell to proceed in its differentiation

process and interact with stromal cells. The cells that pass this checkpoint become $CD8^+$ $CD4^+$ (DP), which is the most abundant sub-population in the thymus cortex. DPs then move to the medullar region to functionally differentiate and become $CD8^+$ or $CD4^+$ single positive (SP) (Anderson and Jenkinson 2001; Singer et al. 2008). Passage from DP to SP happens through further selection, positive and negative, and requires the cells to pass a number of checkpoints. The positive selection happens in the cortex, where weak recognition of self-peptides mounted on MHC of cortical thymic epithelial cells (cTEC) by the newly formed TCR, triggers survival and maturation. The machinery that produces antigens to be presented by cTEC is different from the one in peripheral DCs (reviewed extensively in (Klein et al. 2009). Only TCRs with strong and intermediate affinity allow for positive selection of T cells, while failure to recognise MHC molecules induces death by neglect. The negative selection happens in the medullary region: here the same peptides are expressed by medullary TECs and DCs which, when the avidity of the T cell for pMHC is too strong, transmit an apoptotic signal, thus mediating the elimination of auto-reactive T cells (Ashton-Rickardt and Tonegawa 1994).

In the thymus there is a strict correlation between localisation and maturation stage, suggesting that the orchestration of migration plays a fundamental role in the correct development of CD8 and CD4 T cells. Studies with 2-photon microscopy revealed that precursors move slowly and randomly before positive selection, then create temporary interactions with TECs for the positive selection itself, and only move more quickly once this phase is concluded (Witt et al. 2005). The peculiarity of thymic selection is the expression, by TECs, of genes previously thought to be exclusively expressed by peripheral specific organs (Kyewski and Derbinski 2004). These antigens can be cross-presented by medullary DCs or by the TECs and are not homogenously distributed in the medulla. These self-peptides also have a degree of cross-reactivity, thus allowing for the selection of a wide repertoire of T cells (Hu et al. 1997). The total time a precursor resides in the thymus for selection has been estimated in about a week (Shortman et al. 1990).

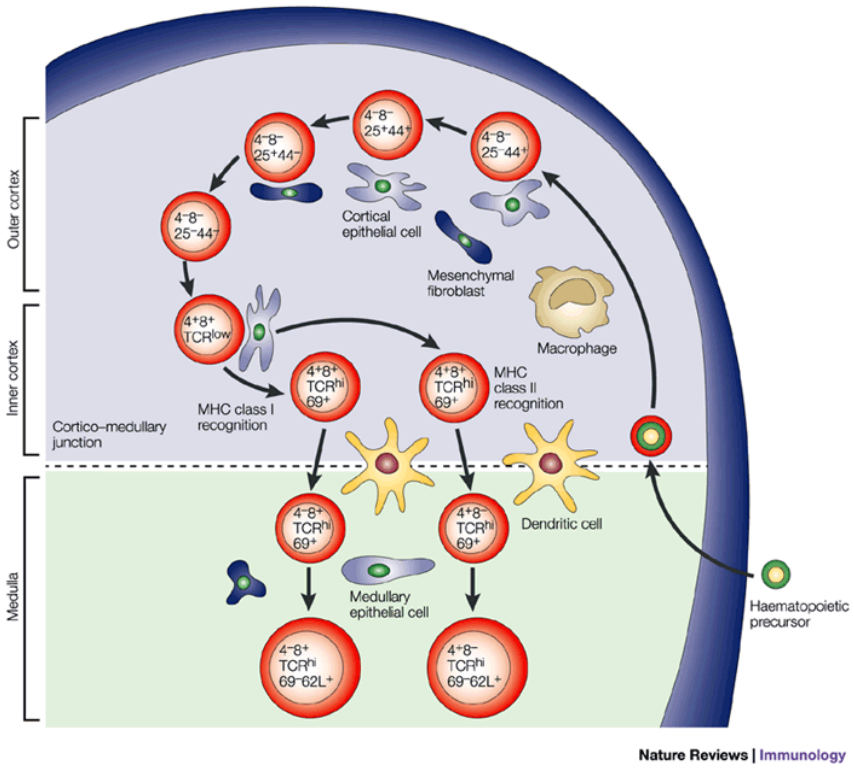


**Figure 2: Thymocyte development: migration through the thymus and maturation to single positive T cells (Anderson and Jenkinson 2001).**

The selection process ensures that CD8 T cells do not trigger any auto-immune reaction and at the same time instructs these cells to tolerance mechanisms. When the selection process is terminated, CD8 T cells are released in the blood stream in the so-called naïve form. This happens approximately 4-5 days after the positive selection has terminated (McCaughtry et al. 2007). Each cell leaving the thymus will have a different TCR and therefore different specificity of reaction, and will circulate in the blood stream in a naïve status, ready to receive activation signals.

### 2.2.3 CD8 T cell migration to lymph nodes

Naïve CD8 T cells circulate in the blood stream and home to secondary lymphoid organs to sample pathogen-derived antigens. The journey of a naïve CD8 T cell to a lymphoid organ (the lymph node in this case for simplicity), starts with the

extravasation from the capillary bed through the endothelial layer which happens via a process called tethering, mediated by selectins. The following step is the "rolling" of the T cell on the endothelial layer. During the rolling some receptors on endothelial cells are activated and integrin conformation is changed, increasing the affinity and avidity and allowing the T cell to adhere to the endothelial cells (HECs) and start the transendothelial migration. At this stage, chemokines produced by the HECs and bound by the basal lamina, especially CCL21, are crucial for efficient T cell entry into the lymph node (LN). In order to enter secondary lymphoid organs from the blood, CD8 T cells must express homing receptors, such as the selectin CD62L, the chemokine receptor CCR7 and the integrins $\alpha_4\beta_1$ (VLA-4) and $\alpha_L\beta_2$ (LFA-1). Expression of CCR7, which binds CCL12, together with L-selectin and LFA-1 (lymphocyte function-associated antigen-1) allows selective entry into the LN. One exception to this is central memory T cells, which can enter the LN in absence of CCR7 following CXCR4 signalling (Scimone et al. 2004).

Thus, in naïve CD8 T cells the fundamental receptors are CCR7 and L-selectin. CCR7 ligands, CCL19 and CCL21, are expressed by HECs in the LN and bound by the basal lamina and it is currently believed that these drive extravasation by creating a gradient which guides the T cell across the endothelium. Once inside the LN, the signals that guide T cells to the T areas and direct them around are not known: CCR7 deletion affects only marginally T cell migration inside the LN (Okada and Cyster 2007). Some studies with pertussis toxin, which can block the function of the G-proteins used by chemoattractant receptors to trigger the intracellular pathway, resulted in almost total inhibition of T cell migration in the LN (Huang et al. 2007).

### 2.2.4 Naïve CD8 T cell priming

Naïve CD8 T cells circulate in the blood and lymphatic system without specific direction in the steady state; in inflammatory conditions they can mature and acquire effector function. Priming of CD8 T cells happens mainly in the lymphoid organs, where mature DCs migrate to following chemotactic signals. Maturation from naive to cytotoxic T cell (CTL) is triggered by a number of signals, the first of which is the

recognition by the CD8 TCR of a peptide in a MHC-I context (pMHC). When recognition happens in a secondary lymphoid organ (mainly spleen and lymph nodes), and the MHC-I is expressed by a DC, activation of the CD8 T cell can be triggered (Figure 3). As explained by Polly Matzinger in 1994 (Matzinger 1994), the activation program is triggered in the CD8 T cell when the TCR-MCH-I interaction is associated with the delivery of inflammatory and co-stimulation signals from the DC. Activation leads to expression of *de novo* proteins, like GrB and perforin, to a high rate of proliferation, and to reorganisation of both cytosolic and membrane structures. CD8 T cells are therefore unable to kill unless activated by an antigen-presenting cell, and more specifically by a DC.

#### 2.2.4.1 Costimulation requirement

Costimulation represents the second signal needed for CD8 T cell activation. Upon binding of the pMHC (complex of peptide and MHC-I) on the DC, the distribution of surface molecules in the CD8 T cell is reorganized to bring specific coreceptors (like CD40L and CD28) close to the TCR, ready to receive additional co-stimulatory signals. Several receptors have co-stimulation properties: among these, the most widely studied and the first to be identified is CD28. Not only CD28 ligation triggers a signalling cascade that activates cytokine production and promotes cell survival, but it also endorses proliferation through strengthening of the cell metabolism. CD28 is constitutively expressed by CD8 T cells, and it is the first coreceptor to be engaged after MHC recognition. It binds CD80 and CD86 on DCs, and its engagement activates the PI-3K-PDK1-Akt pathway which induces NF-kB translocation to the nucleus (Macintyre et al. 2011). CD28 signalling enhances TCR signalling in a quantitative more than qualitative way (Acuto and Michel 2003): its binding can promote the expression of co-stimulatory receptors OX40 and CD137, which reinforce the costimulatory signal when engaged by OX40L and 4-1BBL stimulation. OX40 and 4-1BB signals act along the same pathways as CD28 but more downstream (Watts 2005). The signal from these “secondary” costimulatory receptors is therefore important to sustain the immune response and fully activate CTLs.

Among costimulatory receptors also are some with a negative regulatory function, in that they can downregulate activation signals. Among these are CTLA-4, which has strong homology to CD28 but binds CD80 and CD86 with a higher affinity than

CD28 (Walunas et al. 1994), ICOS, upregulated with T cell activation (Hutloff et al. 1999) and PD-1, expressed on CD8 T cells upon activation. PD-1 is also upregulated in chronic infections where it is correlated to the exhaustion state of CD8 T cells (Barber et al. 2006).

Other costimulatory signals are delivered by cytokines released by the DCs. IL-2 for example is involved in the terminal differentiation of CD8 T cells, the expression of cytotoxic molecules and sustains proliferation. IL-2 has a quantitative effect, as the *in vitro* culture of naïve CD8 T cells with higher concentration of IL-2 results in more efficient cytotoxic T cells compared with cells differentiated in lower IL-2 conditions (Pipkin et al. 2010).

Once fully activated, CTLs undergo a rapid expansion phase during which the duplicating time is 6 hours (Badovinac et al. 2007).

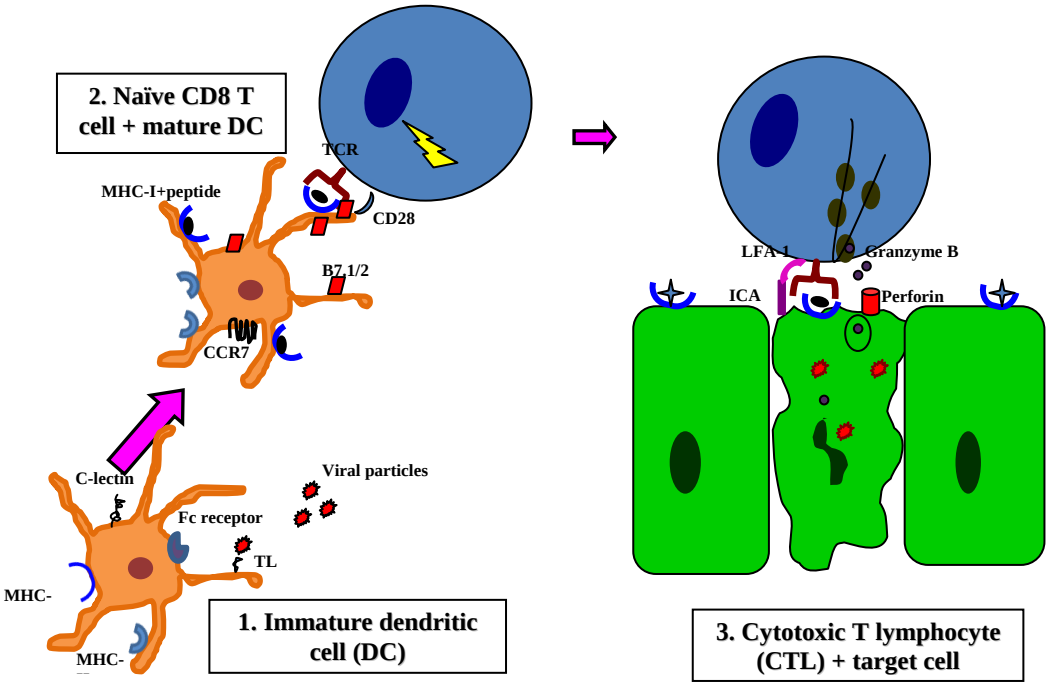


**Figure 3: Schematic representation of DC maturation (1-2), priming of a CD8 T cell (2), maturation of CD8 T cell to CTL (3) and CTL cytotoxic function on target cells (3).**

### 2.2.5 CD8 T cell activation

Upon activation, CD8 T cells undergo a series of changes among which increased uptake of glucose, iron and amino acids, and a metabolic switch from oxidative phosphorylation to aerobic glycolysis (Michalek and Rathmell 2010). The cell metabolism needs to adapt to the increased fuel request to sustain high-level proliferation and gene expression (Jacobs et al. 2008). The glucose metabolism in particular is highly increased as glucose availability impacts CTL proliferative and effector capacity (Maciver et al. 2008). In the resting state, CD8 T cells are mainly fuelled by an oxidative metabolism, which depends on growth factors to be maintained. During activation, aerobic glycolysis is activated by TCR triggering and costimulation (Frauwirth et al. 2002) and glucose transporter Glu-1 is upregulated to provide for the enhanced fuel needs (Rathmell et al. 2000).

The clustering, or aggregation, of TCR molecules and the conformational changes in the TCR/CD3 complex trigger intracellular signalling from the cytoplasmic tail of CD3. Studies by Chan and colleagues showed how CD3 signalling is dependent on phosphorylation of the ITAM tyrosines in the cytoplasmic tail, which is a recruitment site for intracellular signalling mediators like ZAP-70 and PTK (Chan et al. 1992). Signalling through the TCR therefore activates an intracellular pathway that goes via PLC$\gamma$, an increase in intracellular $Ca^{2+}$ (Imboden and Stobo 1985) and activation of DAG, which in turns activates NF-kB. Other effects of TCR signalling are cytoskeleton remodelling and membrane fluidity increase. Actin reorganization is in fact key for the cell to be polarised when activated. The CD8 T cell needs to round up and accumulate F-actin (filamentous) at the immunological synapse (IS), the point of contact between the T cell and the APC (Burkhardt et al. 2008) where the MTOC (microtubular organisation centre) moves towards (Kupfer et al. 1987). Also, cytoskeleton reorganisation is needed for the formation of the distal pole, a negative regulator of this process, which sequesters away the negative regulators (Burkhardt et al. 2008).

TCR stimulation activates downstream pathways that can stimulate upregulation of the translation machinery, thus inducing death receptors and cytotoxic molecule expression (Salmond et al. 2009). TCR stimulation also induces MAPK and mTOR (mammalian target of rapamycin) and immunosuppressive drug used for CCR5

downregulation in HIV kidney transplant patients (Heredia et al. 2003). MTOR, also activated by IL-2 signalling (Rao et al. 2010), controls cell growth proliferation and metabolism and it also seems to be implicated in CD8 memory differentiation (see below).

TCR stimulation, through involvement of the small GTPase Ras proximity-1 (Rap-1), also increases affinity of integrins for their ligand (Menasche et al. 2007). The most important integrins affected by activation are LFA-1 which binds ICAM (intercellular adhesion molecule-1) and VLA-4 (very late antigen-4) which binds VCAM (vascular cell adhesion molecule) and fibronectin. Activation of these integrins is critical for the migration of CTLs outside the LN to the peripheral tissues (as described below).

Once fully activated, CTLs undergo a rapid expansion phase during which the duplicating time is 6 hours (Badovinac et al. 2007). Of note is that proliferating cells are much more responsive to cytokine stimuli than non-proliferating ones.

### 2.2.6 Cytotoxic T cell granule content and killing function

The main differences between naïve CD8 T cells and CTL are in the surface receptor composition and in the presence and content of the so-called cytotoxic granules in the cytoplasm. The granules are lysosomal structures containing cytotoxic enzymes whose intactness is highly regulated. Granules are maintained at a very acid pH and granzymes, which are basic, are bound to the acidic serglycin proteoglycan to keep them stable and inactive. The content of these granules, together with the expression of Fas ligand (FasL), is what allows CTLs to kill upon recognition of the pathogenic antigens in MHC-I context in periphery.

#### 2.2.6.1 Granzymes

Granzymes (granule enzymes) are serine proteases homologous to trypsin, whose transcriptional regulation seems to be dependent on two stimuli: activation of the TCR and cytokine (of the $\gamma_c$ family) signalling, mainly IL-21 and IL-15 (Chowdhury

and Lieberman 2008). IL-15 in fact induces expression of GrA, GrB, IFN-γ, and FasL in lymphocytes (Ye et al. 1996) and IL-21 specifically enhances GrB transcription. There are 7 different granzymes in humans and 10 in mice, independently activated and transcribed. These are coded in three main clusters both in humans and mice: GzmA, GzmK on chromosome 5 (humans) and 13 (mice), GzmB and GzmH (humans) or GzmC (mice) on chromosome 14 of each species, GzmM on chromosome 19 (humans) and 10 (mice). The GzmB gene cluster also includes cathepsin G and mast cell chymase. Moreover, this last cluster is encoding for GrD, E, F, G, L and N (Russell and Ley 2002).

Analysis at the mRNA and protein level showed that the expression machinery for granzymes is regulated in a two-step process with upregulation of the production of mRNA first, equipping the cell with a quick response to further stimuli. This is verifiable for example in NKs which, once activated, show high levels of GrB mRNA but low protein levels (Fehniger et al. 2007). The same has been shown for memory cells (Kaech et al. 2002) and human pDCs. Granzymes are translated with a leading peptide: this directs them to the ER first, where this signalling peptide is cleaved off and a mannose-6-phospate (M6P) sequence is added with the scope of successively directing them to lysosomes in the form of inactive zymogens. Granules are, in fact, specialised secretory lysosomes. Once in the granules they are activated by removal of the N-terminal dipeptide by cathepsin C and stored bound to highly acidic molecules, the serglycin proteoglycans, until degranulation is triggered (Smyth and Trapani 1995). The serglycins form the electron dense core visible with electron microscopy and make sure the granules are densely packed (Grujic et al. 2005). Granzyme B and granzyme A are the most abundant in CTL granules both in humans and mice.

**Granzyme B** is the most studied of these granzymes and is present in both humans and mice. In humans, but not in mice, GrB is expressed by pDCs upon activation (Rissoan et al. 2002) and also by mast cells (Hirst et al. 2001). GrB can induce rapid apoptosis of target cells *in vitro* in a direct or indirect way (Martin et al. 1996). The specificity of this enzyme is different in the two species. GrB has a specific target in cleaving after aspartic acid residues and in particular after the tetrapeptide IEFD in mouse and IEPD in humans. Therefore, the human GrB, but not the murine one, can

directly cleave the targets of key caspases, such as Bcl-2-interacting domain (Bid) a pro-apoptotic molecule, and the inhibitor of caspase-3-activated DNase (ICAD) (Cullen et al. 2007). The activation of these two molecules causes caspase 3-dependent DNA fragmentation (Shresta et al. 1995) and the induction of permeability of the outer mitochondrial membrane (Talanian et al. 1997; Sutton et al. 2000). The specific cleaving site of GrB makes it particularly specific for pro-caspase-3, but other caspases are nonetheless targets of GrB, among which are caspases 2, 7, 8, 9, 10. Other intracellular targets of both human and mouse GrB include apoptotic nucleases and PARP (poly-ADP-ribose polymerase) and DNA-PK (protein kinase). In antigen-specific CTLs, GrB upregulation is extremely quick, and protein levels can be detected as early as within 2 days of an immune response, according to data tracking GrB expression in CD8 T cells during an influenza infection model (Bannard et al. 2009; Bannard et al. 2009). Growing evidence suggests that GrB can be expressed in other cell types in presence or absence of perforin and can have an extracellular role. GrB expressed by T regulatory cells has a perforin-dependent role in regulating the immune response (Gondek et al. 2005). Also, GrB has been shown to cleave vitronectin, a protein that allows extracellular matrix organisation (Buzza et al. 2005) and cartilage proteoglycans during inflammation and infection. This function could facilitate lymphocyte migration and cause tissue destruction and anchorage-dependent death. In humans, GrB levels are detectable and considerably higher than normal in serum during inflammation, for example in patients with HIV infection or rheumatoid arthritis, and bacteremia (Lauw et al. 2000). In the secreted form, GrB is inhibited by trypsin inhibitors antithrombin III and α-2-microglobulin (Spaeny-Dekking et al. 2000), while the cytoplasmic form is inhibited by serine protease inhibitor 6 (Spi6) in mice (Zhang et al. 2004). In some cases, the increase of soluble GrB is accompanied by the increase in soluble (released) serpin inhibitors although this is believed to be connected to release from damaged cells rather than secretion, as serpins do not have leading sequences for exocytosis (Rowshani et al. 2005). GrB KO mice can control and clear viral infections but have a delay in the induction of apoptosis, specifically DNA fragmentation (Zajac et al. 2003).

The second more abundant granzyme in human and mouse is **granzyme A**. This granzyme is a triptase with a serine protease activity and it is regulated, in the same way as GrB, at a post-translational and activation level (Kummer et al. 1996). It has a different specificity from GrB therefore activating different downstream pathways. Its

structure is also quite different as it dimerizes to exert its cleaving function. GrA also has a well known extracellular proinflammatory activity as it cleaves pro-IL-1β (Irmler et al. 1995; Young et al. 2000). Among other substrates is SET, a reticulum-associated oxidative stress response complex. SET is part of a DNA repair complex and when cleaved it allows for nucleases to be released and cut DNA into fragments (Fan et al. 2003). The dynamics of apoptosis induced by GrA are slower than for GrB.

Studies with mice lacking both GrA and GrB have shown how other granzymes can compensate for lack of these two enzymes. Also, the phenotype and development of these mice are normal. Viral infections are cleared normally, although with a slightly different dynamic of clearance and apoptosis induced by GrA/GrB KO CTL is time-wise and morphologically different from a WT (Waterhouse et al. 2006; Hoves et al. 2011; Trapani 2011).

#### 2.2.6.2 Perforin

Also released from the granules is **perforin**, a protein thought to be responsible for the delivery of granzymes to the target cell. Perforin (pfn) is a highly conserved pore-forming protein sharing the structure of complement. It is stored in the granules complexed with reticulin, a calcium-storage protein which contains a KDEL sequence for ER retention in its C-terminal. Reticulin is also released upon degranulation and the rise in $Ca^{2+}$ concentration permits a conformational change that unlocks perforin free (Andrin et al. 1998). Initial studies predicted a model of pore-forming function for perforin, hence the name, that would allow GrB to enter the target cell, but these were then challenged by the discovery that suboptimal concentration of perforin do not create pores in the membrane but still allow GrB entry (Metkar et al. 2002).

The role of this protein and the exact mechanism through which it works has been widely discussed and a consensus has still not been reached. Some models propose that perforin polymerizes in presence of $Ca^{2+}$ creating temporary permeable holes in the membrane to allow the passage of granzyme and ions (Liu et al. 1995). Other groups have reported the ability of GrB to enter the cells in absence of perforin through a mannose-6-phospate receptor (Motyka et al. 2000) or through $Ca2^{+}$ induced-cell repair mechanisms (Keefe et al. 2005). Despite controversial data, perforin is considered to be necessary for the cytolytic activity. Also unclear is the

mechanism that activates granzymes once the protein has reached the cytosolic compartment of the target cell: some evidence suggests a role for perforin in keeping the granzyme inactive until it reaches its target (Voskoboinik and Trapani 2006). Perforin-KO mice are severely immunodeficient and, during infection, a high accumulation of antigen-specific CD8 T cells is seen. CTLs in pfn KO mice are unable to perform cytotoxic activities and to deliver granzymes to the target cell (Kagi et al. 1996; Matloubian et al. 1999). Ashton-Rickardt's lab demonstrated in 2001 that pharmacological inhibition of pfn could improve survival of CTLs (Opferman et al. 2001).

#### 2.2.6.3 Granulysin

Granulysin is a cationic protein that belongs to the saposin-like family of lipid-binding proteins. It is expressed in two forms: a 15KDa that is constitutively secreted, and a 9KDa form that is stored and released from the granules together with granzymes and perforin. It has lytic properties and has been reported to lyse Gram positive bacteria and fungi (Stenger et al. 1998). For this reason, portions of this protein are being studied as drugs with antibiotic application (Krensky and Clayberger 2009). It seems to enter the target cell through cationic interaction with the membrane lipids. It triggers the intrinsic apoptotic pathway through release of the CytC from the mitochondria (Kaspar et al. 2001). It has also been shown to act as a chemoattractant for mature DCs, human monocytes, NK cells and T cells (Deng et al. 2005). However, granulysin is not expressed in mice.

### 2.2.7 Egress from lymphoid organs and migration of effector cytotoxic T cells to the site of infection

Once undergone proliferation antigen-specific effector cells need to leave the LN (or spleen) to reach the tissue invaded by the pathogen and kill the infected cells.

Egress from the LN is controlled by downregulation of certain receptors and upregulation of others.

During viral infections, local cytokines and chemokines are produced both by the innate and the incoming adaptive arms of the immune system: they are needed to elicit, direct and sustain the T cell response *in loco*. Local inflammation in particular will create two waves of chemokines at the site of viral infection (Figure 5). A first wave is represented by chemokines expressed by cells of the innate system upon TLR stimulation, like Type I interferons (IFN-α/β) attracting NK and CD4 T cells. A second wave is started by these NKs and CD4 T cells, which induce release of MCP-1 from macrophages and MIP-1α and IFN-γ from T cells. Release of IFN-γ from local DCs induces a strong influx of antigen-specific CTLs to the infection site, resulting in a stronger cytolytic response (Nansen et al. 2000). CD8 T cell production of chemokines like MIP-1α stimulates macrophage production of other chemokines like MCP-1. The cytokines RANTES, MIP-1α and β, IP-10 and MCP-1 in particular appear to dominate during viral infections independent of virus type, organ site or phase of infection (Figure 4). As a consequence to the locally released chemokines, T cells that migrate to the site of infection express CCR2, CCR5 and CXCR3, binding the above ligands in a redundant but regulated way (Figure 5).

Upregulated CXCR3, a chemokine receptor, leads the cell journey to the peripheral tissues (Groom and Luster 2011). Ligands for this receptor (Figure 5) are the chemokines CXCL9 (also known as MIG: monokine induced by γ interferon), CXCL10 (or IP-10: interferon-induced protein of 10 kDa), CXCL11 (or I-TAC: interferon-inducible T cell α chemoattractant) which induce migration of activated T cells *in vitro* and *in vivo*. Regulation of CXCR3 expression by t-bet has linked this transcription factor to CTL effector function activation.

The role of CXCR3 in driving migration and differentiation of effector CD8 T cells is reviewed by Groom and Luster (Groom and Luster 2011), who explain that t-bet-deficient mice experience a defect in CD8 T cell migration due to absence of CXCR3. The ligands for CXCR3, CXCL9 and CXCL10, are induced in a TNF-dependent way in the lumen of high endothelial venules (HEV) in LN and also in a type II interferon-dependent way, locally at the site of inflammation (Groom and Luster 2011). The source of these chemokines is mainly peripheral DCs (Figure 5). These ligands can

also bind GAG (glucosamminoglycan)-containing molecules on the extracellular matrix thus prolonging their half life and activity *in loco* and maximizing the chemoattractant function for activated CD8 T cells.

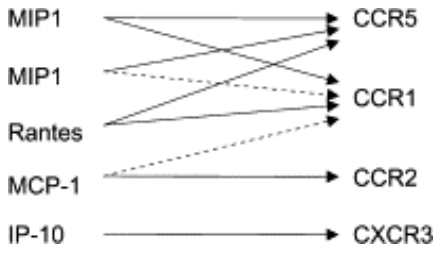


**Figure 4: Chemokines redundancy in viral infection: chemokines and their receptors (Thomsen et al. 2003).**

Also upregulated in activated CD8 T cells are VLA-4 and LFA-1 (Issekutz and Issekutz 2002), while CCR7 and L-selectin, not required for migration to the inflamed tissues, are downregulated (Thomsen et al. 2003). During an infection about 90% of the CD8 T cells will upregulate LFA-1 and VLA-1, but only a small fraction of these will be antigen-specific. Thomsen and colleagues demonstrated that I-CAM and V-CAM, receptors for LFA-1 and VLA-4 respectively, are required for migration of effector CD8 T cells to the site of inflammation as when these are knocked-down the migration is impaired. They also studied the specific role of selectins and integrins in a viral model of infection and verified that, while selectins are important, their absence, especially of E- and P-selectins expressed by endothelial cells, does not interfere with the immune response to a big extent. Also, L-selectin, which is not expressed by early effector cells, is later gradually re-upregulated in memory cells. This is correlated, among other things, to the capacity of these cells to re-access the LN, so that the number of memory cells that can access LN slowly increases with time (Thomsen et al. 2003).

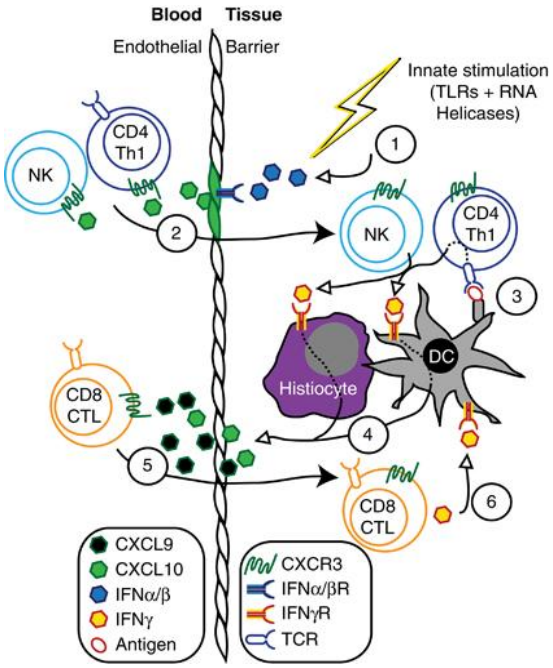


**Figure 5: Local and systemic induction of chemokines at the site of infection (Groom and Luster 2011).**

Chemokines released at the infection site are not the only players in the organisation of lymphocyte migration from the secondary lymphoid organs (SLOs): locally, lymphocyte retention or egress is regulated by S1P levels and by the lipid G protein-coupled S1P receptor 1 (S1P1) expressed on CD8 T cells. At the very beginning of an infection, naïve T cells need to be kept in the LN or spleen for longer to increase their chances to encounter mature DCs. S1P1 binds S1P, which is released in the blood and lymph by lymphatic endothelial cells but is present at low concentrations in the lymphatic tissues (Pappu et al. 2007). S1P1 is downregulated during CD8 T cell migration in the blood in the naïve status and it is upregulated shortly after entrance in the SLOs, possibly by the local concentration of its ligand S1P (Lo et al. 2005). Upregulation of CD69, driven by interferon signaling, is also thought to be downregulating S1P1 in T cells during the early stages of the immune response, so to release CTL from the SLOs (Shiow et al. 2006; Bankovich et al. 2010). This process is also associated with the upregulation of IL-6 which induces increased expression of ICAM-1 and increased presentation of CCL21 on the HEV. These molecules induce a temporary decrease of the egress of T cells from LN and a transient increase in the homing. The process is called LN shutdown and it is aimed at facilitating T cell priming by prolonging naïve cells persistence in the LN stroma. A downregulation of

the homeostatic chemokine CCL21 and its receptor follows LN shutdown around day 3 p.i., according to Mueller and colleague (Mueller et al. 2007). CCL21 and IL-6 thus work in concert with S1P1 regulation on the T effector cells and the local concentration of S1P to allow egress of CTLs from the LN.

### 2.2.8 Effector function and killing pathways

Once mature, the CTL leaves the lymphoid tissue through the efferent lymphatic vessel and enters the blood stream in order to reach the infected tissue. The cell will then extravasate from the blood vessels to the infected tissue following chemotactic and inflammatory signals. Once in the damaged tissue, identification and binding of the target cell is rapid and followed by a polarization of the T cell and triggering of degranulation.

The recognition of the target cell happens via TCR-pMHC binding: the TCR of the CD8 T cell recognises a peptide in a MHC-I context on the target cell and the binding leads to the formation of an IS. Studies showed that the localisation of the IS between the effector and the target cell does not always coincide with that of the TCR-MHC complex, thus suggesting that mobility of the surface molecules is increased upon ligation of the TCR on a CD8 T cell (Favier et al. 2001). A single CTL can interact and kill several target cells, while surviving its own deadly weapons thanks to expression of Spi6 at cytoplasmic level. Once activated, in fact, the production of cytotoxic molecules is sustained and continuous, to the extent that granzymes, other than re-fill new granules, can be directly secreted in a constitutive, granule-independent way. This is proven by the absence of the granule-leading sequence in these granzymes (Isaaz et al. 1995). As mentioned before, the highly targeted killing mechanism exerted through apoptosis can at this point be induced in two ways: release of granule-contained cytotoxic molecules and binding of Fas-FasL.

#### 2.2.8.1 Granule-dependent secretory pathway

Upon recognition of the cognate antigen in MHC-I context, microtubule structures form to allow the movement of the granules towards the IS, where the cytotoxic enzymes will be released (Kupfer et al. 1983). The granules then align along the microtubules and move towards the IS (Bossi and Griffiths 2005). The granule membrane fuses with the CTL plasma membrane, specifically releasing its content in the IS: this process is called degranulation.

During degranulation, which is targeted and localised, the key players are granzymes and perforin. Once a granule fuses with the external membrane of the CTL, its content is released into the extracellular space between the two cells and perforin allows the granzymes to enter the target cell. As described above, the exact mechanism through which perforin mediates the entry of GrB into the target cells is still not clear. Granzymes are thought to dissociate from serglycin once in the intracellular space (Raja et al. 2005), but several ways have been proposed for entry into the cells, among which are association with lipids of the target cell plasma membrane due to cationic interactions (Bird et al. 2005), use of receptors and endocytosis due to perforin-mediated change in $Ca^{2+}$ concentration endocytosis (Keefe et al. 2005).

The first hypothesis about the way pfn mediates GrB entry described membrane damage in the target cells, but this was contradicted by Tschopp's group which described formation of pores creating physical access for GrB in presence of pfn (Duke et al. 1989). A later work by Metkar showed that GrB, when released from the granules, is associated to serglycin to stabilise it (Metkar et al. 2002). These results opened the way to studies describing how serglycin associates through its negative charge to glucosamminoglycans on the membrane of the target cell thus having a fundamental role in GrB entry (Raja et al. 2005). Other groups showed how the uptake of GrB could be mediated by a M6P protein, CI-MSP (Motyka et al. 2000): the role of this protein as a receptor for GrB was confuted when Trapani's group showed that CI-MPR KO mice were still susceptible to CTL-mediated killing (Trapani et al. 2003).

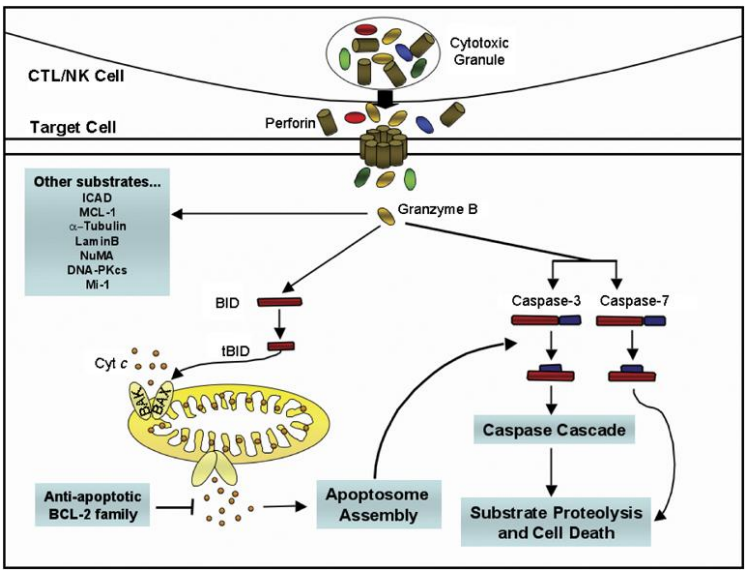


**Figure 6: GrB-mediated apoptosis activation. The main targets of human GrB are shown (Cullen and Martin 2008).**

Once GrB reaches the cytosol of the target cell, it induces apoptosis mainly through the activation of the caspase cascade which causes hydrolysis of cytoplasmic and nuclear proteins leading ultimately to a contained disassembly of the cell, apoptosis (Figure 6). Capases are proteases present in the cytoplasm of a cell in an inactive form (Alnemri et al. 1996) and able to cleave targets which induce cytokine release, stress signals induction and apoptosis effector molecules. GrB can activate caspases directly through proteolysis of the inactive form, or through induction of permeability of the outer mitochondrial membrane. This in turn induces the release of cytochrome C into the cytosol and suppression of transmembrane potential and targeting of the pro-apoptotic molecule Bid (Talanian et al. 1997; Sutton et al. 2000). Activation of pro-caspase 3 in particular induces caspase 3-dependent DNA fragmentation (Shresta et al. 1995) and cleavage of Bid, a member of the pro-apoptotic family Bcl-2 (Sutton et al. 2000). In humans, as seen in chapter 2.2.6.1, GrB activates the caspase pathway through cleavage and activation of caspase-3, but it can also indirectly activate it through cleavage of caspase targets. Another target of the human but not the mouse GrB is ICAD, an inhibitor of CAD DNase (caspase-activated DNase). Proteolytic cleavage of ICAD induces CAD release, which mediates the internucleosomal degradation of DNA which is one of the hallmarks of CTL-induced apoptosis. CTLs protect themselves against the action of GrB thanks to an intracellular serine protease inhibitor called PI-9 in humans and Spi6 in mice. This is upregulated with CTL

maturation and it inhibits GrB in the cytosol by binding it irreversibly in an SDS-stable complex in Western blot (Phillips et al. 2004). This protein and its role will be further described below (see Chapter 2.6).

Granzyme A can enter the mitochondria, where it cleaves a component of the electron transport chain complex-I, thus stimulating a caspase-independent apoptosis program (Martinvalet et al. 2005). Once the mitochondrial redox function is damaged, the oxidative stress response complex (SET), itself a target of GrA, is activated. These mediators induce DNA fragmentation, and the fragments, much larger than those created by GrB, are released into cytoplasm (Beresford et al. 2001). The process is delayed compared to GrB-induced DNA fragmentation, working as a sort of back-up.

Among the other granzymes is granzyme C in mouse, homologous to granzyme H in human. Both these granzymes have chymotryptic activities that induce caspase-dependent and CAD-independent death when released by NK cells (Hou et al. 2008). Two viral substrates have been identified, but no physiological ones: adenoviral proteins DBP and the previously described GrB inhibitor (Andrade et al. 2007).

Granzyme Z is also a tryptase, it is encoded downstream of GrA and it is expressed in low amounts. GrK causes a caspase-independent death which is perforin-dependent and connected to loss of mitochondrial function, but this is inhibited in cells overexpressing bcl-2 (MacDonald et al. 1999)

Granzyme M is different from the other granzymes and it is, in fact, more closely homologous to a neutrophil protease. It is mostly expressed in NK cells and γδ T cells. Its role in apoptosis triggering is still not clear. More studies are needed to clarify how it engages apoptotic pathways and to further prove its role in inhibiting PI-9, which has been shown *in vitro* but not confirmed *in vivo* (Mahrus et al. 2004).

#### 2.2.8.2 The engagement of the death receptors

CTL can mediate apoptosis, also called programmed cell death (PCD), of infected and cancer cells in two ways. The first is through release of cytotoxic activity via degranulation, while the second is exerted through the engagement of the death receptors Fas (or CD95) on the CTL, and Fas ligand (FasL) on the target cell,

belonging to the tumour necrosis factor (TNF) receptor superfamily. Upon binding the interaction of the two receptors triggers trimerization of the Fas receptor which recruits caspase-8 and activates the caspase cascade (Berke 1995) and ultimately apoptosis via DNA fragmentation.

When pMHC-TCR binding occurs in the context of an infection, degranulation is the major death pathway activated. Released granzymes enter the target cell, the action of GrB and GrA intracellularly is predominant and it induces different apoptosis pathways which include caspase activation and mitochondrial damage, with different dynamics. Fas-FasL triggering is involved in regulation of lymphocyte homeostasis. Apoptotic cells are then cleared by macrophages of other phagocytes without adding to the inflammatory status of the organism, which is why this pathway is so important. The granule secretory pathway is the one on which this project focuses as it is the main mechanism used by CTL to eliminate LCMV, the viral model of choice.

### 2.2.9 Clonal contraction and memory formation

In acute infection, following the elimination of a pathogen, antigen-specific CTLs represent a big fraction of the total T-cell repertoire. When a CD8 T cell is properly primed for activation, a reprogramming of its metabolism, transcriptome and protein expression is triggered. The reprogramming results in maturation to a fully effective CTL and proliferation occurs at a very high rate making these cells the predominant population in the T-cell repertoire. Studies from Badovinac show that a single CD8 T cell can undergo up to 19 cell divisions, which can bring the number of daughter cells close to 500,000: the mean division time is estimated to be 4-6hrs (Badovinac and Harty 2007). Once the pathogen is cleared, the removal of CD8 T effector cells starts: this phase is called contraction phase and it is characterised by high levels of apoptosis. About 90% of the effector CD8 T cells have to be eliminated to restore T-cell homeostasis, while a little fraction remains to form the memory pool (Figure 7).

The mechanism through which the apoptosis program is triggered in these cells has not been uncovered, but hypotheses suggest that pathogen levels and DC feedback on activation status are responsible for activating apoptotic programs and the end of the expansion phase.

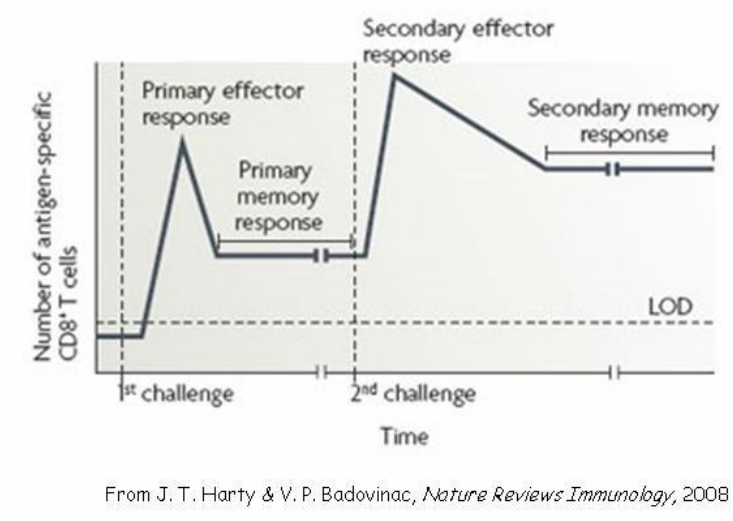


**Figure 7: CD8 primary and secondary responses dynamics. After the peak of the response, a contraction phase occurs, resulting in the formation of memory The same is repeated at each subsequent re-invasion by the same pathogen (Harty and Badovinac 2008).**

Among the first factors involved in the triggering of contraction are the decrease in antigen levels and the lack of inflammatory signals and growth factors. As seen earlier, the environment influences the maintenance of the response, both by furnishing growth factors and regulating the intake of glucose. When these are lacking, or decline, survival of the CTLs is at risk. Recent studies proved that addition of cytokines like IL-15 (Yajima et al. 2006) and IL-2 (Blattman et al. 2003) during the contraction phase could rescue effector cells from apoptosis.

In this context it is interesting to determine whether contraction is actively regulated during the expansion phase, or the fate of each cell is pre-determined. Prlic and Bevan showed that effector T cell fate is not regulated by competition for survival and growth factors: they demonstrated that CTL function is not affected by this competition and by higher numbers of effector cells (Prlic and Bevan 2008). Another

interesting and controversial result came from Harty's lab, who showed that the amount of antigen *per se* does not seem to influence the initiation of the contraction phase or its extension (Badovinac et al. 2002).

Mounting evidence suggests that anti-apoptotic molecules are particularly involved in shaping the fate of the effector cells during the contraction phase. Members of the Bcl family seem to be particularly important in this process. Bim, IL-7R, and IFN-γ all promote contraction after infection by non-cytololytic viruses like LCMV (Haring et al. 2005). Studies on cell death during contraction demonstrated that caspase-independent apoptosis is triggered (Nussbaum and Whitton 2004), which cannot be rescued by overexpression of antiapoptotic molecules like bcl-2 or bcl-xl (Petschner et al. 1998). Bcl-2 is downregulated at the peak of response, initiating apoptotic events but it acts in concert with Bim: a deficit in contraction and higher levels of memory are observed when Bim is removed (Pellegrini et al. 2003). The lack of Bim might be responsible for rescuing the terminally differentiated effector cells (Prlic and Bevan 2008), but cells formed in these conditions are not good memory cells in that they lack the ability to undergo basal proliferation and can therefore survive only for a limited time after antigen clearance (Wojciechowski et al. 2006).

Among cytokines contributing to the contraction phase and to the shaping of the memory pool, IL-7 seems to favour the skewing of the pool towards memory. Absence of IL-7Rα, one of the two subunit of IL-7 receptor, results in lack of memory, but IL-7 absence only results in impaired memory if the IL-7Rα is stimulated (Klonowski et al. 2006). Forced IL-7Rα expression on a CD8 T cell subset normally expressing low levels of this did not skew the cell population towards becoming memory (Hand et al. 2007). Also, IL-12 seems to influence memory formation as its absence allows the creation of a larger memory pool (Pearce and Shen 2007). The lack of type-I IFN receptors also produces a deficient memory compartment and leads to defective clearance. Lack of type-I IFN can also induce chronic infection in LCMV-infected mice according to the results published by Hand and colleagues (Hand et al. 2007).

Fundamental in the transition between memory precursor and memory cell is also the level of IL-12. In absence of IL-12, more memory IL7Rα$^{hi}$ cells and fewer T effector cells are developed. Also, IL-12 inhibits IL-2 production and IL-7Rα expression. IL-12 acts on CD8 T cells directly through the IL-12Ra and signals

intracellularly towards the effector destiny (Kieper et al. 2001; Pearce and Shen 2007).

Transcription factors are also involved in shaping the contraction phase and memory formation. As briefly mentioned above, involvement of mTOR in the commitment of cells towards a memory phenotype has been described (Araki et al. 2009). Studies in LCMV-infected mice showed that administration of rapamycin, inhibitor of the mTORC1 (the complex which signals downstream from mTOR), induces a higher number and better "quality" of memory cells. The study showed that low doses of rapamycin administered at day 0-8 p.i. resulted in increased numbers of memory cells due to decreased contraction, while administration during the contraction phase (day 8-30 p.i.) resulted in accelerated transition from effector to memory and in a better quality in terms of survival and effector function of the memory pool. The same paper showed that mTOR action is cell intrinsic, despite rapamycin having an effect on other cell types, like DC (Araki et al. 2009). One explanation as to why there is such difference depending on the timing of rapamycin administration might come from the work of Thomson and colleagues, who showed that mTOR seems to form complexes with different proteins/regulators, thus having different function depending on the cell phase (Thomson et al. 2009). T-bet involvement in memory development was also observed. Expression of t-bet is induced by IL-12 in CD8 T cells and in turn induces the expression of CD25 and KLRG1, driving the cell to a terminally differentiated status. In a study by Chang and colleagues the correlation between higher t-bet levels and a more terminally differentiated memory phenotype, effector-like, was demonstrated, while a lower amount of t-bet correlated with a less differentiated memory phenotype. The observation that antigen-specific stimulated CD8 T cells from IL-2ra$^{-/-}$ mice (IL-2RA is also called CD25) can differentiate into memory precursors to a higher frequency than control cells supports the results, as CD25 is linked to terminal differentiation (Chang et al. 2011).

Chemokines and their receptors are also involved in memory formation and clonal contraction. A recent study conducted by Woodland's lab has shown how CXCR3 and CCR5 have a role in determining the beginning of the contraction phase and memory generation (Kohlmeier et al. 2011). They showed that CCR5 KO and

CXCR3 KO mice have a lower contraction and higher levels of memory cells after LCMV infection.

Notably, several molecules, including cytokines, chemokines, transcription factors and surface receptors have been associated with contraction of the primary response and memory development. The mechanism underlying this high apoptotic rate is still not clear, but it is likely to require a combination of signals and the interaction of regulatory molecules. This need for multiple signals, including cytokines like IL-12, IFN-γ and activation of BIM, mirrors the three signals required for CD8 T cell activation, pMHC, costimulation and inflammation. This suggests that three or more signals might also be required for contraction and memory formation.

## 2.3 CD8 T memory cells

Once the infection is cleared, 90-95% of the antigen-specific CTLs die through PCD: this phase is called contraction (Sprent and Surh 2002). The 5-10% that remains will form a pool of long-lasting memory cells: resting mature specialized CD8 T cells that survive in absence of antigenic stimulation (Lau et al. 1994), have a basal proliferation status and can easily and quickly re-activate effector functions (Barber et al. 2003) upon re-encounter with the antigen (Whitmire et al. 2008). These memory cells also have less stringent requirement for costimulatory signals compared to naïve CD8 T cells (Masson et al. 2008). Antigen-specific memory cells are in much higher frequencies than the initial antigen-specific pool.

### 2.3.1 How is the CD8 T cell memory pool formed?

The third phase of a CD8 T cell response, after proliferation and contraction, involves the appearance of memory cells. No agreement has been reached to date on a CD8 memory development model. How a heterogeneous population of effector and memory cells can descend from a single naïve CD8 T cell, whether the fate of an effector cell is decided during the effector phase or during the contraction phase, whether a memory cell has to go through the effector phase at all: all these questions have several answers based on the results obtained with various models. A study where reporter genes were inserted after the promoter of GrB or IFNγ showed how memory cells can arise from cells whose transcription path for GrB and IFNγ has been activated (Bannard et al. 2009). Other studies showed how memory cells can develop from cells which have not gone through an effector phase (Manjunath et al. 2001). A definite answer has still not been found.

A number of factors influence memory development. One of the main factors is the interplay of the 3 signals received by a CD8 naïve T cell during priming: MHC-peptide signalling, costimulation and inflammatory signals. Among other factors

influencing memory development are the number of antigen-specific precursor cells, the antigen load, the inflammation severity and duration, the extent of CD4 help, the nature of the pathogen and the localisation of the infection.

Some studies support a linear model, where memory cells are the next developmental phase of effector cells. Some others support an asymmetrical model, where the origin of memory cells is dictated during DC priming and during the first, asymmetrical, division generating to two different pools of cells which are the effector and the memory cells. Lately, much attention has been given to the role of inflammation during the development of effector responses, with data supporting the theory that CTLs follow a default pathway regulated by the levels of inflammation. Three main models have been proposed:

A) The first model, called of "Linear differentiation" was proposed in 1999 by Ashton-Rickardt. He demonstrated that a population of fully differentiated CD8 effector T cells that had undergone 5 divisions *in vitro* could differentiate into memory cells (Opferman et al. 1999). This model was broadened in 2003 by Ahmed who suggested that when a naive CD8 T cell is primed it can differentiate into one of two subsets of memory cells with different characteristics and expressing different markers, like CCR7 and CD62L. The ones that have low replicative capacity and are $CD62L^{lo}$ $CCR7^{-}$ are called effector memory ($T_{EM}$) cells: they will survive at the end of the contraction phase and, later on, will develop into $CD62^{hi}$ central memory, $T_{CM}$ (Wherry et al. 2003). The main difference between these two subsets is the anatomic location: $CD62L^{hi}$ cells reside in the secondary lymphoid organs while $CD62L^{lo}$ reside in peripheral tissues and spleen due to the lack of expression of the homing receptor to the lymphoid tissue. These two populations also differ in terms of function; the first being able to quickly proliferate and the second being able to quickly exert effector functions instead. A variant of this model is the self-renewal model (Ahmed et al. 2009) that describes the possibility that effector cells can still revert to the $T_{EM}$ state after having developed into $T_{CM}$. One of the key assumptions of this model is that, since effector CD8 T cells are very susceptible to apoptosis, some anti-apoptotic molecule must be switched on in the cells that progress to being memory cells (Kaech et al. 2002).

B) A second model, termed "Fixed lineage model" (Figure 8) accounts for the concomitant presence at early stages of the immune response of memory-destined and terminally-differentiated effector cells. This model maintains early commitment of the activated CD8 T cell into an effector or memory cell, like an early fate decision (Sallusto and Lanzavecchia 2001). This theory accounts for memory cells developing directly from naïve CD8 T cells, without going through the effector phase. The existence of a precursor subset, nonetheless, does not imply that the determination of the memory fate is cell-autonomous; this might depend on APC costimulatory signals or antigen load or inflammatory environment. It has in fact been shown that a weakly inflammatory environment can lead to the formation of a bigger-than-average memory cell pool in relation to the levels of effector cells at the peak (Badovinac and Harty 2007). A further widening of this model is represented by the asymmetrical model. This states that a primed naive CD8 T cell is directed by DCs to divide asymmetrically into two daughter cells with different properties: one will retain the effector machinery, which means expression of GrB, IFN-γ and CD25 and will be $CD62L^{lo}$, and the other will be $CD62L^{hi}$ and have no effector functions (Beuneu et al. 2010). In this model the contribution of DCs to the formation of memory is fundamental.

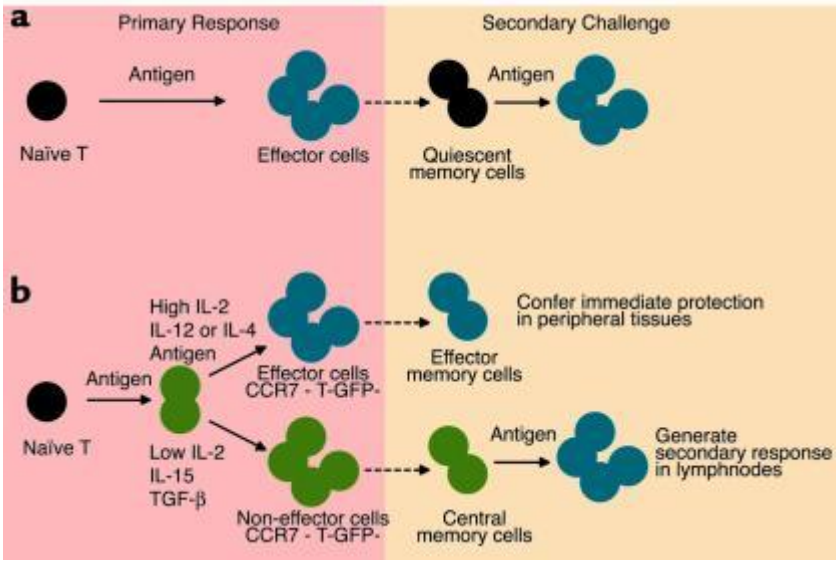


**Figure 8: Memory development models: a) linear and b) fixed lineage model (Sallusto and Lanzavecchia 2001).**

C) Having seen a high level of heterogeneity in *in vitro* experiments showing memory cell development from both effector and naïve cells (Manjunath et al. 2001), Ahmed and Gray formulated the "Decreasing potential model" (Figure 9) in an attempt to explain this discrepancy. The theory describes TCR stimulation as the determinant for T cell fate: when TCR stimulation is strong, the CD8 T cell will develop into an effector cell with short survival and poor proliferative capacity; reduced TCR stimulation would allow instead for the development of a cell with memory characteristics of long survival and proliferation potential (Ahmed and Gray 1996). This model is challenged by the finding that even when very high antigen stimulation occurs, a memory pool is nonetheless created (Prlic et al. 2006). Badovinac's study demonstrated that long-term exposure to antigen shapes the creation of a memory pool in the sense of generating an early subset of $CD127^+$ (IL-7Rα) and $CD62L^+$ (L-selectin) cells but with no effect on its size, compared to short-term exposure (Badovinac and Harty 2007). The role of antigen in influencing memory development was also studied by Sakar and colleagues who showed an inverse correlation between antigen and memory (Sarkar et al. 2008). Another version of this theory is the Progressive differentiation (Figure 9), proposed by Lanzavecchia and Sallusto, which considers the total of the stimulation signal strength received by a CD8 T cell during its life as determining for the fate of the cell. According to this model, CD8 T cells progress into differentiation stages due to the accumulation of activation signals (Lanzavecchia and Sallusto 2002). Both models predicts that an intermediate activation signal will drive the CD8 T cell to memory differentiation, while a too strong signal will induce terminally differentiated effector cells destined to die.

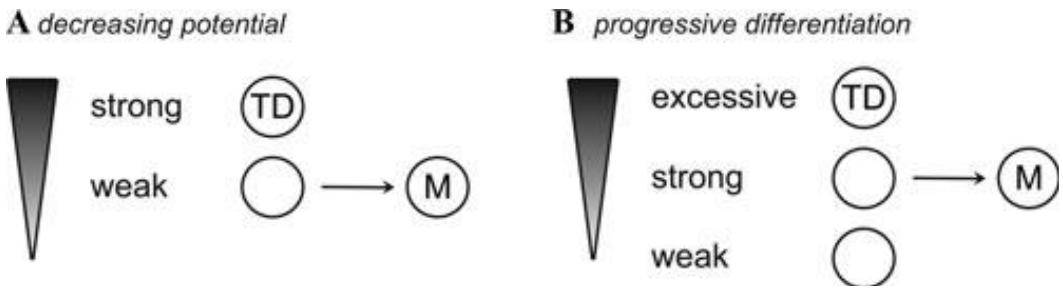


**Figure 9: Decreasing potential (A) and progressive differentiation models (B). TD: terminally differentiated cell; M: memory cell (Gerlach et al. 2011).**

Of increasing importance is the role of inflammation in controlling the balance between effector and memory cells. This emerged from experiments in which the ratio between memory cells and effector cells was measured under controlled conditions. Lower inflammatory conditions were seen to induce shorter effector peaks and earlier memory cell development. Studies conducted by Badovinac in 2005 also demonstrated that reducing the inflammation during an infection accelerates the transition of effector into memory for CD8 responses (Badovinac et al. 2005). A follow-up paper showed that inflammatory signals at different stages of the CD8 response can shape memory formation and influence its acceleration or slowing down. Inflammatory cytokines like IL-12 and IFN-γ, when present at day 2 p.i. but not before, can favour the generation of terminally differentiated effector cells, thus reducing the formation of the memory pool. In absence of high levels of inflammation, homeostatic cytokines like IL-15 and IL-7 drive the formation of memory (Sandau et al. 2010). The hypothesis states that CD8 T cells follow a default pathway of memory differentiation unless they receive inflammatory stimuli: when inflammatory signals are conveyed, the effector phase is sustained and maintained for longer. This is consistent with previous work showing that effector CTLs primed in a highly inflammatory milieu acquire a terminally differentiated effector phenotype and are short-lived, while in absence of inflammatory signals these cells become anergic or die (Joshi et al. 2007). In particular, exposure to inflammatory cytokines like IL-12 and IL-4 induce the transcription factors T-bet and blimp-1 which in turn enhance expression of KLRG-1 and CD25, molecules highly expressed on terminally differentiated effector cells, see Chapter 2.3.2 (Kalia et al. 2010). T-bet is therefore involved in differentiation of CD8 T cells to the effector state.

When thinking about how memory is formed, from these main models emerges an important issue regarding **when** this memory fate is written into the antigen-specific CD8 cell baggage. Three models, describing fate determination before the first division (or at division 0, D0), during the first division (D1), or after the first division (D1+) are illustrated in Figure 10.

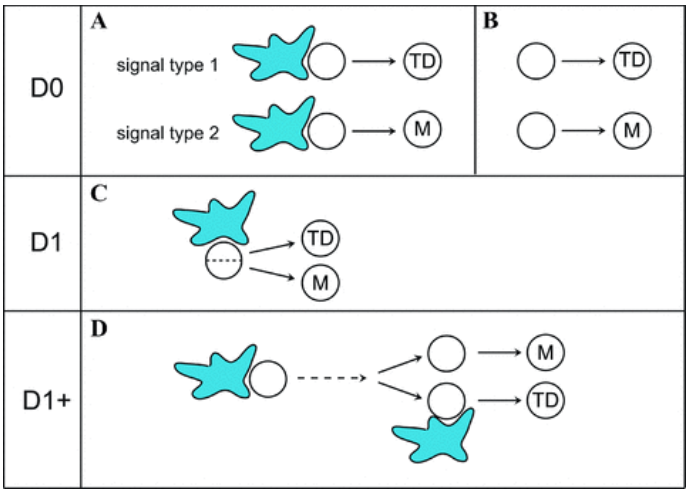


**Figure 10: Memory fate decision models. Three models currently account for memory fate determination before the first division (D0), during the first division (D1) or after it (D1+). TD is terminal differentiation and M is memory cell (Gerlach et al. 2011).**

The D0 model predicts that different signals can give rise to one specific subset (Figure 10, A), and this can be due to the fact that naïve CD8 T cells are intrinsically different and thus harbour the possibility of developing in only one subset (Figure 10, B). The hypothesis is that CD8 T cell destiny could be shaped by the combination of several signals received before the first cell division. Indirect evidence supporting this model,suggested by Farber (Farber 2000), comes from studies where antigen availability was controlled both *in vitro* and *in vivo* showing a correlation between the levels of antigen and the size of the memory pool (Prlic et al. 2006). Direct evidence can be found instead in Gerlach's work which, with a very elegant technique of gene barcoding, allowed the identification of both effector and memory cells deriving from the same naïve CD8 T cell after LM infection (Gerlach et al. 2010). This evidence is quite compelling and it is reinforced by further gene profiling studies of memory cells (Turner et al. 2003). In another very elegant experiment the YFP reporter signal was fused to the IFNγ gene and its expression measured. Different intensity in the YFP was seen in antigen-specific T cells before the first division but after contact with a DC. The same study reported stability of YFP through successive divisions of each single T cell, and dependence on the signal intensity from the antigen-loaded MHC-I on the DC.

For the model predicting fate commitment at D1, during the first division, evidence comes from Reiner's lab where activated but not dividing cells were isolated after LM infection *in vivo* and analysed with confocal microscopy revealing polarised expression of different molecules including CD8, LFA-2, IFN-γR (Chang et al. 2007). This is in line with the common knowledge that during mitosis the intracellular pericentriolar material separates unevenly. Another study stated that the first division is critical and decisive to the fate of the daughter cells as it results in an uneven distribution of cellular machinery and especially of the transcription factor t-bet as described above (Chang et al. 2011). In more recent works, CD8 T cells were activated *in vitro* and differences in CD8 expression were seen among CD8 T cells, so that some were $CD8^{lo}$ and others $CD8^{hi}$. To test whether the difference was significant, $CD8^{lo}$ and $CD8^{hi}$ T cells were adoptively transferred into WT mice and analysed for their ability to clear a bacterial infection. $CD8^{hi}$ cells were associated with a greater reduction of bacterial load, and these cells were described as DC-proximal (Oliaro et al. 2010).

Studies involving CFSE-labelled T cells which had already undergone divisions, like the experiment described for the linear differentiation model, showed that fate commitment can happen at a later stage than D1, thanks to the integration of different signals (Opferman et al. 2001; Sarkar et al. 2008).

At present, data support a model in which memory cells can derive and be derived directly from both naïve and effector cells and whose fate is decided after the first division. Whether this happens through an asymmetrical division or depending on TCR/antigen stimulation is not yet clear but these two models might well coexist.

### 2.3.2 Central CD8 T cell memory and CD8 T effector memory

All the tree models have in common that a memory-committed precursor population can be clearly identified early during effector responses. The molecules used to identify and distinguish memory from effector cells are KLRG-1 (killer cell lectin-like receptor G-1), CD127 and CD62L. The memory committed precursor population

can be identified and characterised by IL-7Rα$^{hi}$ (CD127$^{hi}$) and KLRG-1$^{lo}$ and especially IL-7Rα is functionally required for the transition to memory cells (Kaech et al. 2003). Interestingly, IL-7Rα expression has been linked to memory precursors and has been shown to be fundamental for memory development (Schluns et al. 2000), but IL-7 itself has not. In fact, there seem to be another ligand to the IL-7Rα receptor which activates it in absence of IL-7.

Two subsets of memory cells had been initially described, as seen above: effector memory and central memory. CD8 T central memory ($T_{CM}$) cells express CD62L and CCR7 for homing to SLOs, they can proliferate and revert to $T_{EM}$ when primed (Sallusto et al. 1999). CD8 T effector memory ($T_{EM}$) cells are CD62L$^-$ and CCR7$^-$: they constitutively express GrB and pfn, migrate to the site of infection because of expression of CCR4, CXCR3 and CCR5, and quickly reactivate effector functions.

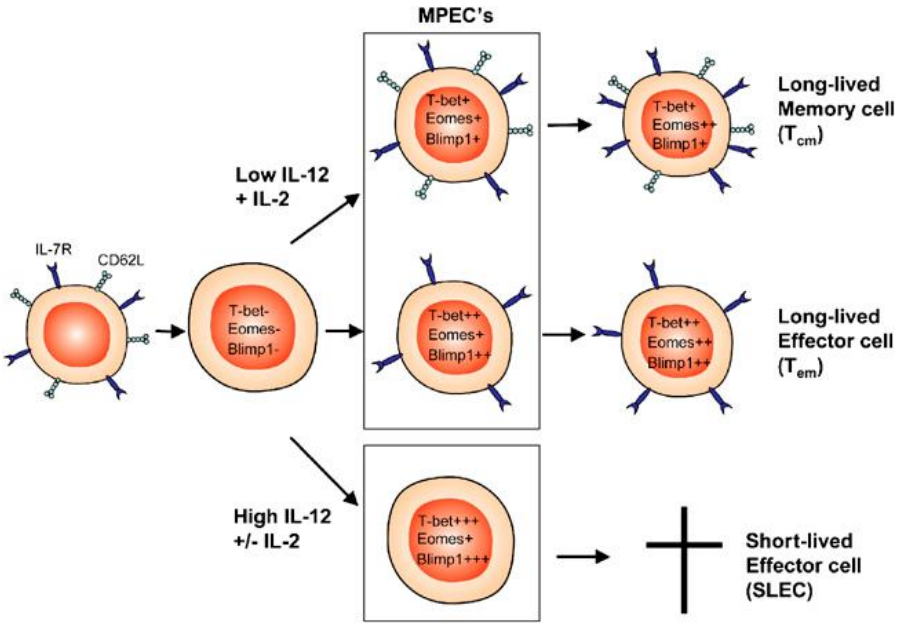


**Figure 11: CD8 memory cell populations. Development of memory cell subsets memory precursor effector cells (MPEC), short-lived effector cells (SLEC) and is dependent on cytokines like IL-2 and upregulation of transcription factors t-bet and Blimp-1 (Kallies 2008).**

Recent findings have shown that the memory population is non-uniform in terms of gene expression, localisation, and proliferating and differentiating capabilities. The $T_{EM}$ subset has therefore been subdivided into two subsets based on the observed

capacity of some $T_{EM}$ to de-differentiate into $T_{CM}$. The $T_{CM}$ population is considered a less differentiated population due to its proliferation and self-renewal capacity: a transitional subset, able to revert to $T_{CM}$ and a terminally differentiated, end-stage subset of $T_{EM}$. These finding were obtained by Lefrancois's work which showed the existence of memory precursor effector cells (MPEC) which are IL-7Rhigh and KLRG-1low, short-lived effector cells (SLEC) which are IL-7Rα- KLRG-1+, and early effector cells (EEC) which are IL-7Rα- KLRG-1- (see Figure 11) Interestingly, the last of these three subpopulations (EECs), can develop into the other two when adoptively transferred and properly stimulated (Lefrancois and Obar 2010). The developmental paths of these cells are shown in Figure 11. Very elegant experiments, where a single CD8 naïve cell was adoptively transferred, showed how that single naïve CD8 T cell can generate both effector and memory cells (Stemberger et al. 2007; Gerlach et al. 2010).

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## 2.4 Homeostasis of CD8 T cell numbers and attrition

In the steady state, a homeostatic system exists to maintain a balance between long-lived memory cells, mature naïve CD8 T cells and newly formed ones so that the total number of CD8 T lymphocytes is constant. The homeostasis is believed to be maintained by competition of the T cells for the limited amount of IL-7 and CCL19 produced by the follicular reticular cells (FRC). As IL-7 and CCL19 availability is limited, the cells which do not have access to them will not proliferate and eventually die. In 2007, Luther's lab demonstrated that the homeostasis of these cells is maintained in the periphery by the release of IL-7 by FRCs in the spleen and LN (Link et al. 2007). Additionally, CD8 T cells undergo homeostatic proliferation, which has been shown to be TCR-dependent, as it is abrogated in $MHC^{-/-}$ mice (Ernst et al. 1999). According to mouse studies, naïve T cells can re-circulate in the body for up to 8 weeks before being eliminated (Tough and Sprent 1994). As a result, the total number of the T cell repertoire is maintained at a stable level in steady state conditions. Memory cells represent a small percentage of the T cell repertoire in an individual during young age, but they become a larger part of the total T cell population during adult age. The homeostasis of CD8 memory cells, which does not depend on antigen and MHC molecules, has been shown to be regulated by IL-7 as well (Schluns et al. 2000).

During infections, the homeostasis mechanism is overcome by other signals. When a new CD8 T cell response is established, a phenomenon called "attrition" causes the existent memory pool and some of the unrelated naive CD8 T cells to die through apoptosis induced by anergic contacts with mature DCs during the first days of the expansion phase.

Jiang and colleagues demonstrated that high levels of type-I IFNs are associated with early stages of inflammation between day 2 and 4 of an immune response. The levels of type-I IFNs also drive general upregulation of CD69. CD69 is a C-type lectin considered the earliest activation marker on lymphocytes, as explained in Figure 12: its function is to act as a costimulatory molecule. During infections, due to the systemic diffusion of inflammatory cytokines, non-specific T lymphocytes can

undergo cytokine-driven phenotypic changes — so-called bystander activation (Figure 12). Moreover, as reported above, an upregulation of CCL21 which induces CD8 T cell migration to the LN favouring bystander cell apoptosis, is associated with the early stage of infections, day 1-2 (Mueller et al. 2007). Type-I IFNs have also specifically been correlated to the apoptosis of these non-antigen-specific bystander T cells (both naïve and memory). The death of bystander T cells does not interfere with antigen-specific responses.

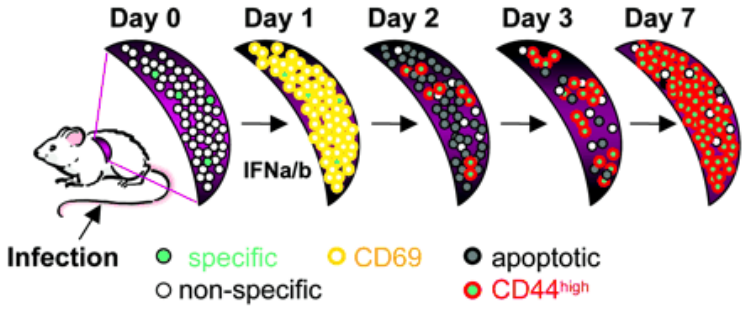



**Figure 12: CD69 and CD44 expression upregulation during the initial phases of the CD8 T response and attrition mechanism in bystander activated T cells (Jiang et al. 2003).**

The phenomenon of attrition facilitates the expansion of antigen-specific effector T cells as it physically increases the space available in the lymphoid organ. Attrition also allows for new immunogenic antigen-specific responses not to be overwhelmed by existing immune responses in the case of a re-infection with the same pathogen or a variant, as it is the case when different virus strains co-infect a host.

## 2.5 Antigen presenting cells

B and T cells which belong to the adaptive arm of the immune system are, unlike the ones belonging to the innate arm, not fully activated when stimulated by pathogen-derived signals. Pathogen-derived antigens need to be presented in the context of MHC molecules, together with costimulatory and danger signals delivered by a professional antigen presenting cell (APC). Many cells can function as APCs, namely B cells, macrophages, and epithelial cells when activated by inflammatory signals. Among these cells, nonetheless, non-hematopoietic cells like epithelial cells lack costimulatory signalling molecules and are not able to initiate a MHC-I-restricted CD8 T cell response (Sigal et al. 1999). Bone-marrow derived B cells, macrophages and DCs can deliver co-stimulatory signals and initiate a MHC-II-restricted response. Nonetheless, only DCs are necessary to activate CD8 T cell responses. Since CD8 T cells are mainly responsible for direct elimination of viral responses and DCs are the cells in charge of activating them, DCs are a key player for the development and regulation of viral responses.

Dendritic cells were first described by Ralph Steinman in the 1970s. He discovered a new cell type while performing an investigation on macrophage features in killing microbes. The new cell type had a branched morphology, loose adherence to plastic and high content in vacuoles and mitochondria. Because of the shape, Steinman called them dendritic cells, but their function remained not known for several years after. Steinman himself conducted more studies on these newly discovered cells, and characterized them as antigen presenting cells (Steinman and Cohn 1973). Steinman received the Nobel Prize in 2011 for this discovery and the successive work he carried on to study this cell type.

In this chapter the characteristics of dendritic cells, their development and classification in humans and mice will be firstly described. Afterwards, the processes typical of the immature and the mature status will be presented (antigen uptake, pathogen sensing, maturation, presentation and costimulation of T cells). Finally, the

two main secondary lymphoid organs will be described, followed by a more detailed description of the two mouse subsets of interest for this project, CD8α$^+$ DC and plasmacytoid DCs.

### 2.5.1 Antigen-presenting cell specific functions

Naive CD8 T cells circulate between lymphoid tissues and the blood stream in a naïve, inert status. This activation is carried out by APCs. Among APCs, DCs are the predominant cell type and because of their ability to fully activate naïve CD8 and CD4 T cells and to deliver costimulatory signals and inflammatory signals, they are called "professional" APCs. They have specific functions which include the ability to present uptaken antigens on their MHC class I and class II molecules and to convey activation, survival or apoptotic signals to T cells. DCs are the connectors between innate and adaptive immunity as they are able to stimulate T cells and drive T cell responses based on the signals received from the innate system.

As mentioned, non-hematopoietic cells are unable to present antigens to CD8 T cells, while bone-marrow derived B cells, DCs and macrophages have been shown to cross-present *in vitro* (Bellone et al. 1997). A following experiment studying B cells and DC cross-presentation functions, showed that *in vivo* cross-presentation is not impaired when B cells are ablated (Schoenberger et al. 1998), but it is abrogated when phagocytes are ablated (Debrick et al. 1991). In 2001 Kurts demonstrated that in phagocyte-ablated mice, expression of MHC-I-restricted DCs can restore CD8 priming/response, thus establishing that these cells have a fundamental role in cross-presentation to CD8 T cells (Kurts et al. 2001). Further proof of their key function in cross-presentation was provided when conditional ablation of DC was performed by Lang's group in 2001. A mouse was engineered to express the diphtheria toxin receptor (DTR) under the control of the murine CD11c gene promoter (Brocker et al. 1997). This gene encodes the CD11c subunit of the CD11c/CD18 β integrin, which is expressed in all DC type in both immature and mature state except for the Langerhans cells which only upregulate it when activated. When the CD11c-DTR mice were injected with DT all and only the cells expressing the DT receptor (i.e. the DCs) were

target of its killer action, as mice are otherwise insensitive to DT (Jung et al. 2002). This system allowed the induction of temporary ablation of DCs. The study reported that elimination of DCs negatively affected the proliferation of CTL precursors after pathogenic challenge. It also showed that DCs are efficiently replaced in 3 days.

DCs are a rare cell type widely distributed across all tissues: soon after their discovery, it was noticed that several different cell types, sharing a common hematopoietic origin and the same functions, but with different lineage precursors, fell under the definition of dendritic cell. When talking about dendritic cells, we refer to a heterogeneous population, consisting of cells with different specific tasks, tissue distribution, and surface molecules.

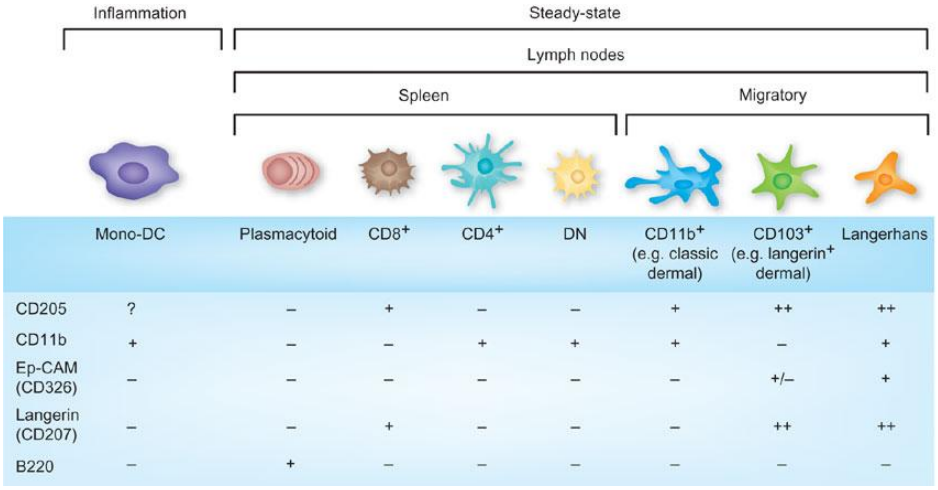
### 2.5.2 Human and mouse denritic cell subpopulations

DCs are a very heterogenic cellular population. Several subtypes can be identified based on surface markers and tissue localization. In humans, DCs were until not long ago mainly divided into lymphoid and myeloid cells based on the lineage, but recent studies have demonstrated that lineage origin is not a clear distinctive method and a marker-based approach has now been adopted. In mice, some subsets inhabit secondary lymphoid organs and others patrol the peripheral tissues. All the subsets are defined by the levels of their surface markers (like CD8, CD4, CD11c, CD205 and CD11b, CD24) and when possible by a specific function, although more and more studies report a certain plasticity in the function of these cells (see Figure 13).

**Human DCs**, the study of which mainly comes from *in vitro* work, as reviewed by Shortman and Liu (Shortman and Liu 2002), are now being categorized based on surface markers and the technique used to derive them from a $CD34^+$ precursor. The classification of human blood-derived DCs, the most easily accessible, is based on expression of BDCA-1, -2 and -3 (blood dendritic cell antigen).

In particular, according to a recent nomenclature definition there are 3 types of DC in the human blood, two of which are considered both immature and precursors (Ziegler-Heitbrock et al. 2010). The first two sets are myeloid DCs: one subset is

BDCA-1/CD1c$^{+}$, which identifies a myeloid DC population, and the other one is BDCA-3/CD141$^{+}$ which seems to represent the human counterpart of murine CD8α$^{+}$ DC as they are also Clec9A$^{+}$ (Dzionek et al. 2000). Both these subtypes are CD123$^{dim}$ CD11c$^{bright}$, rather monocytoid in appearance, express CD45RO and spontaneously develop into typical mature DCs even when cultured without any exogenous cytokines. DCs in the third subset, considered immature, are called plasmacytoid DCs and are BDCA-2$^{+}$ (CD303), a C-type lectin transmembrane glycoprotein that can internalize antigen for presentation to T cells, and also CD123$^{bright}$ CD11c$^{-}$.




| | Mono-DC | Plasmacytoid | CD8$^{+}$ | CD4$^{+}$ | DN | CD11b$^{+}$ (e.g. classic dermal) | CD103$^{+}$ (e.g. langerin$^{+}$ dermal) | Langerhans |
|---|---|---|---|---|---|---|---|---|
| CD205 | ? | – | + | – | – | + | ++ | ++ |
| CD11b | + | – | – | + | + | + | – | + |
| Ep-CAM (CD326) | – | – | – | – | – | – | +/– | + |
| Langerin (CD207) | – | – | + | – | – | – | ++ | ++ |
| B220 | – | + | – | – | – | – | – | – |

**Figure 13: DC subpopulations in mice and principal markers for identification (Heath and Carbone 2009).**

**Mouse DCs** are mainly classified as mucosal DC and non-mucosal conventional DC (cDC). The latter including migratory cells (Langerhans cells and dermal DCs), resident CD8α$^{+}$ and CD8α$^{-}$ DC which are found in LN and spleen, and plasmacytoid DCs (pDCs). Furthermore, in inflammatory conditions, monocytes can differentiate into DCs (Lopez-Bravo and Ardavin 2008). Due to the nature of my research and the model used, the focus is going to be on mouse subsets at this stage.

Migratory DCs can be subdivided into immature dermal or interstitial DCs and immature epidermal or Langerhans cells (LCs), which represent respectively approximately 35% and 25% of total cDCs (Kamath et al. 2002) and can be found in

steady state each patrolling the respective layer of the skin. Migratory DCs constantly migrate from the peripheral tissues to the SLOs via the lymphatic system, but in absence of inflammation they are not retained: they have a role in antigen transport and transfer to residential DCs in spleen and LNs, which will be described later (Belz et al. 2004; Allan et al. 2006). Langerhans cells ($CD9^+$ $CD68^+$) are thymus-derived and found in the epidermis and in the oral, respiratory and genital mucosa. They have unique membrane-bound organelles, whose role is unknown, called Birbeck granules, and express the C-type lectin marker Langerin (Ebner et al. 2004).

Interstitial, dermal, or submucosal cells have a more peripheral distribution: in humans they display specific surface markers as CD11c and CD1c, and express high levels of HLA-DR both in the immature and in the mature stage. Nevertheless, both of these subgroups behave in the same way: they are mainly phagocytic cells for most of their lifespan, but undergo phenotypical and functional changes upon stimulation, becoming efficient antigen-presenting cells. In mouse these cells are $Langerin^+$ and $CD103^+$ and can, under appropriate stimulatory conditions, cross-present antigens (Kaplan 2010). Studies with influenza virus have shed a light on the role of this subpopulation as an early presentation tool. Depletion of lung DCs in fact could impaire T cell expansion. The specific location where this happens in the lung is not clear still, but studies are ongoing (McGill et al. 2008).

Resident DCs in the lymphoid organs are distinguished by expression of the CD8α homodimer, different from the CD8 heterodimer (CD8αβ) expressed by CD8 T cells, and divided into $CD8\alpha^+$ and $CD8\alpha^-$. $CD8\alpha^-$ DC can be further subdivided into $CD8\alpha^-$ $CD4^-$ and $CD8\alpha^-CD4^+$ DC subsets (Villadangos and Schnorrer 2007), but because no exclusive functions in immune responses have been specifically ascribed to either of these subsets, the two are rarely described individually. In general, $CD8\alpha^-$ DCs are better at presenting antigens on MHC-II context and thus induce CD4 T cell responses (Dudziak et al. 2007), with production of IFN-γ and IL-4 (Soares et al. 2007), while $CD8\alpha^+$ DCs are better at cross-presenting to CD8 T cells and uptaking apoptotic cells (Belz et al. 2004). The distribution of these DC subtypes varies in the different lymphoid organs (see Figure 14): $CD8\alpha^+$ and $CD8\alpha^-$ represent respectively the 25% and 75% of DCs in the spleen for example (Kamath et al. 2000). The

composition of the DC population in spleen and LNs will be further developed in the appropriate chapters below (see 2.5.9.1 and 2.5.9.2).

Plasmacytoid DCs will also be the subject of a chapter below. They have lower levels of CD11c and high levels of the B220 surface marker (CD45R), are less involved in presentation of antigens but have been shown to be efficient producers of type I interferon (Villadangos and Young 2008).

Monocyte-derived DC are present in humans and the correspondent subset is called inflammation-derived DC n mice as they are induced in inflammatory status and seem to be involved in antigen presentation to CD4 T cells when resident and migratory DC are not enough (Shortman and Naik 2007).

| | Blood-derived | | | Tissue-derived | |
|---|---|---|---|---|---|
| | CD4 DC | CD8 DC | DN DC | Langerhans cells | Interstitial DC |
| Location | | | | | |
| Spleen | Yes | Yes | Yes | | |
| Cutaneous lymph nodes | Yes | Yes | Yes | Yes | Yes |
| Visceral lymph nodes | Yes | Yes | Yes | | Yes |
| Surface markers | | | | | |
| CD4 | +++ | | | | |
| CD8 | | +++ | | | |
| CD205 | | ++ | | +++ | + |
| CD11b | +++ | | +++ | +++ | +++ |
| Maturity | Immature | Immature | Immature | Mature | Mature |
| CD40, CD80, CD86 | + | + | + | ++ | ++ |
| MHC II | ++ | ++ | ++ | +++ | +++ |
| Ag. processing[b] | +++ | +++ | +++ | +/– | +/– |
| T cell proliferation[c] | – | – | – | +++ | +++ |

**Figure 14: Conventional DC population in mouse spleen and LNs. Modified from (Villadangos and Heath 2005).**

## 2.5.3 Dendritic cell origin and development

DCs are cells of hematopoietic origin, their progenitors originate in the bone marrow (BM) and are then released in the circulation from which they colonise all tissue, except for the brain, eyes and testes (Hart 1997). Notably, DC progenitors can be found in the bone marrow (BM), blood, peripheral lymphoid tissues and various

organs at various differentiation stages and, depending on the subset, they can develop and mature into differentiated DCs in the BM or peripherally.

The search for a common DC progenitor has just recently been fruitful. Studies conducted in the mouse have been able to shape-up the picture of DC development. In the past, a distinction had been made between myeloid and lymphoid DC, considering migratory and resident DCs as part of the former group and plasmacytoid as part of the latter. As brilliantly reviewed by Liu and Nussenzweig (Liu and Nussenzweig 2010), the combined efforts of several groups and techniques, like DC ablation and Lutz ‘s culture methods (Lutz et al. 1999), made possible to find a common progenitor in the bone marrow, described as the common myeloid precursor (CMP). Studies have shown that another common progenitor, the common lymphoid precursor (CLP), has the potential to give rise to DC eventually, but given the larger abundance of CMP in the BM, this has been elected to be the general precursor (Traver et al. 2000). Thanks to the finding that DC progenitors express FMS-related tyrosine kinase 3 ligand (Flt3) (Karsunky et al. 2003) and that flt3-deficient mice have impaired DC levels (McKenna et al. 2000), a downstream monocyte macrophage and DC progenitor was isolated. It was called macrophage-DC progenitor (MDP) for its ability to develop into monocytes, macrophages and DCs (see Figure 15). Shortly after, a progenitor giving rise to DCs (both conventional ones and pDCs) but not monocytes was identified in the bone marrow by Onai and colleagues (Onai et al. 2007). As DCs are replaced by blood-borne precursors, the blood is where the next developmental step was searched and found: it was named pre-DC and was shown to be the DC blood precursor. Pre-DCs exit the BM to never return: they circulate in the blood and eventually enter secondary lymphoid tissues. Once more, it was the expression of the transcription factor Flt3 to shed a light on this population (Liu et al. 2009). Pre-DC is the last developmental step before differentiated DCs, as shown in Figure 15. The differentiation pathways of mouse DCs is now clear, and the final precursors of tissue DCs are pre-DCs found in the circulation.

Notably, all but the Langerhans DC subset are re-populated by the pre-DC precursor. A distinct LC precursor has been found, thanks to the observation that LCs resist irradiation and are not being repopulated by BM transfer (Ginhoux et al. 2006).

The LC precursor shares some characteristics with the MDP in that it is $CD45^+$ $CX3CR1^+$ (Chorro et al. 2009).

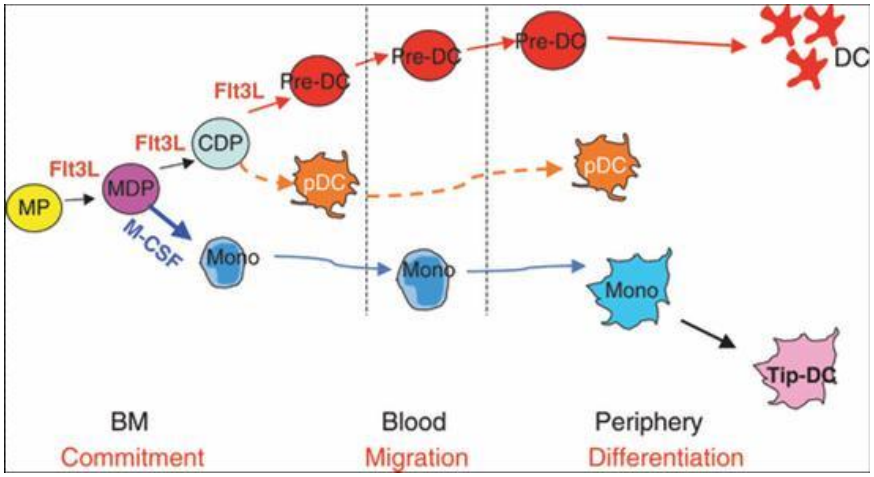



**Figure 15: Mouse DC origin, development and tissue distribution (Liu and Nussenzweig 2010).**

The homeostasis of DCs has been described by Liu and Nussenzweig as dependent on "three parameters: continuous input of pre-DC from the blood, limited DC division in situ and cell death" (Liu and Nussenzweig 2010). In particular pre-DCs divide for 10-14 days once exited the BM (del Hoyo et al. 2002) under the regulation of lymphotoxin-β and Flt3. Terminally differentiated DCs can also divide, and their half life varies between 3 and 25 days depending on the subset and the site.

The developmental path described is the one DCs follow in the steady state. During inflammation, monocytes can also develop into a specific subset of $Ly6C^-$ $CCR2^-$ $CD11b^{high}$ $CD4^-$ $CD8^-$ DC (Geissmann et al. 2003). The same monocytes will not turn into DC in absence of inflammation. A role for monocytes as precursors of conventional DCs in the steady state has been ruled out in various experiments both in humans and in mice. When activated monocytes are injected locally in the skin, they migrate to the local LN, acquire some of the typical DC markers and can stimulate T cells, but they do not express all of DC markers and low levels of CD11c (Randolph et al. 1999). Activated monocytes where found to express TNF and iNOS and where therefore named TiP, as shown in Figure 15 (Shortman and Naik 2007).

DCs are a pool of cells with common general characteristics but in which several subtypes with specific function, location and surface receptors are distinguishable. All but LCs derive from a blood pre-CD precursor, which proliferates to maintain the DC population stable. Resident DCs in lymphoid organs are different from migratory DCs and they collaborate to make priming the most efficient.

## 2.5.4 Antigen uptake

In the immature state, migratory DCs are located in peripheral tissues where they scavenge for pathogens through phagocytosis of soluble proteins, apoptotic cells and other material. Immature dendritic cells are in fact highly phagocytic cells which uptake antigen through 3 main routes: phagocytosis, macropinocytosis, and receptor-mediated internalization.

### 2.5.4.1 Phagocytosis

Phagocytosis is a mechanism used mainly by macrophages to internalize and dispose of killed infected cells and pathogens and to cross-present them to CD8 T cells (Desjardins et al. 2005). It allows internalization of relatively large particles, up to 3.0 μm (Roberts and Quastel 1963), and involves actin-dependent cytoskeleton rearrangement: extension of pseudopodia and formation of a phagosome. The triggering signal is ligation of cell surface receptors. The extra membrane required for extension of psedudopodia and for pahgosome formation is contributed by the membrane of intracellular vescicles which migrate to the plasma membrane and fuse with it, thus increasing its extension. GTPases of the Rho family are then recruited and phagosomes become phagolysosomes through fusion to endolysosomes: this step seems to be regulated at least in part by some endosome-located TLRs (Shiratsuchi et al. 2004). TLRs can in fact localize in the forming vesicle and get activated by its content (Underhill et al. 1999).

During maturation of DCs lysosomes have been shown to be remodelled and activated through increased acidification, in order to enhance antigen processing and presentation (Trombetta et al. 2003; Nayak et al. 2006).

#### 2.5.4.2 Macropinocytosis

Fluid phase endocytosis (pinocytosis) is the intake of soluble particles together with amounts of water, and it is used by many cells to engulf nutrients, growth factors and others particles. Macropinocytosis is a type of pinocytosis which involves actin-drive envagination of the plasma membrane with formation of large vacuoles (up to 2.5μm in diameter). Actin activation is mediated by, among others, activation of growth factor receptor signalling pathways (Lanzetti et al. 2004). Macropinocytosis is a non-selective mechanism. The internalisation is mediated by membrane ruffling and cytoskeleton modification and produces early endosomes that later fuse with endosomes. In immature DCs, on the contrary, this uptake mechanism is constitutively activated and contributes to the uptake of antigens destined to be presented on MHC molecules (Sallusto et al. 1995).

#### 2.5.4.3 Receptor-mediated phagocytosis

Receptor-mediated uptake is the main mechanism of internalization in most antigen presenting cells and it involves several types of membrane proteins. Once bound to antigens, the receptors are internalised through clathrin-coated pits or formation of vacuoles. The vacuole containing the antigen-receptor complex then fuses to primary endosomes, which become late endosomes and fuse to lysosomes where the proteases degrade uptaken antigens for loading onto MHC-II.

Among surface receptors in DCs, mannose-6-phospate (M6P) is one of the most represented. Once internalised, the M6P receptor is transported to the endosomal compartment and then recycled to the plasma membrane (Engering et al. 1997). Uptaken antigens are processed through two main pathways and subsequently presented into two different contexts: MHC-II, which are expressed mainly by APCs and are recognized by CD4 T cells, and exceptionally MHC-I, which are expressed on almost every cell type and are recognized by CD8 T cells.

A broad array of receptors is expressed on immature dendritic cells, accounting for DC ability of recognizing and internalizing as many different molecules and as possible. Among others are C-lectins, Fc and complement receptors (Bajtay et al. 2006), and Dectin-2 scavenger receptor. The most abundant receptor type on dendritic cells is represented by members of the C-type lectin family (Kanazawa 2007). C-type lectins are responsible for binding to self glycoproteins and being mediators of important cell-cell interactions in processes like maturation or migration of several cell types. Both type I and type II C-lectins, such as DEC-205 (CD205), CLEC-1 (C-type lectin receptor-1), DC immunoreceptor (DCIR) or CLEC-4 (Kanazawa et al. 2003), and dectin-2, DC-associated C-type lectin-2 (Ariizumi et al. 2000), are highly expressed on DC surface and are endocytosis mediators of considerable significance. They are able to recognize, bind and internalise upon binding most pathogen carbohydrate patterns and form multimeric complexes which signal intracellularly. In particular, intracellular signalling depends upon binding as each of them has a unique intracytoplasmic tail. DEC-205 binding, for instance, triggers its internalization, formation of clathrin-coated pits and directing to the lysosomal compartment. DEC-205 endocytic function is very efficient and it has been used to specifically target antigen to MHC-II in DCs (Mahnke et al. 2000).

DC-SIGN, dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (CD209), is another C-type lectin, specifically expressed on resident DCs of SLOs. For its ability to bind ICAM-2 and ICAM-3 (Geijtenbeek TB 2000) on epithelial and T cells (Geijtenbeek TB 2002), DC-SIGN has a role in intercellular adhesion It is nonetheless also a receptor for several pathogens and is able to mediate their internalization (Geijtenbeek TB 2003). DC-SIGN can also interfere with signalling from other molecules, e.g. TLRs, upon pathogen recognition and can downmodulate DC activation.

Furthermore, immature dendritic cells express the CD36 scavenger receptor, a molecule able to recognize lipids, like long-chain fatty acids, and altered lipoproteins, whose binding induces inflammatory molecule release and is able to modulate DC function (Urban et al. 2001). Its role is complex as it acts both as a receptor for recognition of apoptotic cells and as a positive regulator of dendritic cell maturation, as co-receptor for TLR2/6 in microbial recognition.

Another receptor involved in the uptake of apoptotic and necrotic cells is DNGR-1 or Clec9A, especially expressed by $CD8\alpha^+$ DCs, which recognizes and binds molecules extracellularly exposed on necrotic cells (Sancho et al. 2009).

The receptors involved in Phagocytosis, like DC-SIGN, Clec9, CD36, are down-regulated during dendritic cell maturation, so that the antigens presented in the SLOs mirror the peripheral tissue antigenic content. This downregulation demonstrates that the phagocytic function is only the first stage of DC functional complexity and it demonstrates the many functional and phenotypical changes these cells undergo.

### 2.5.5 Antigen processing and presentation

Antigens uptaken by DCs through the different routes described in 2.5.4 are directed to the intracellular sites of degradation. Proteins and material phagocytised through different routes are processed into lysosomal compartments and then presented on the cell surface in the context of either class I or class II MHC molecules. The two MHC molecules share the same genetic locus, as they originated from a common ancestor gene by duplication, but have different distribution in tissues and cell types. MHC-II is expressed by immune cells like macrophages, B cells and dendritic cells while MHC-I is expressed by all cell types except for red blood cells. MHC biogenesis occurs with a strict compartmentalization: mostly exogenous antigens are loaded on class II molecules through the endocytic pathway and only endogenous and intracellular infection-derived peptides are presented on MHC-I molecules. This accounts, at the cellular level, for the selective activation of CD8 T cells as they can bind MHC-I through CD8, in the case of virally cells for example, and for the activation of naïve CD4 helper T cells and direction to a $T_H1$ or $T_H2$ type of response through MHC-II, for example in the case of extracellular pathogens. Exceptions are possible and the presentation of exogenous antigens in the context of MHC-I molecules can be achieved through a mechanism called cross-presentation (Ackerman et al. 2003).

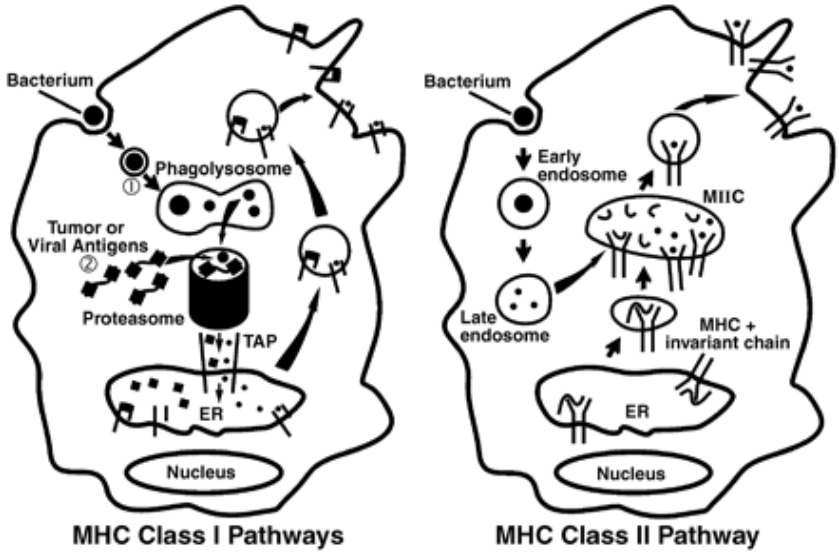



**Figure 16: Antigen degradation and presentation pathways: MHC-I (2) and cross-presentation (1) routes on the left; MHC-II route on the right (Lipscomb and Masten 2002).**

The intracellular route that leads to the formation and localization of the MHCs determines which antigens are presented by each of the two molecule, with the exception of endogenous molecules presented on MHC-I molecules in DCs.

### 2.5.5.1 Major histocompatibility complex-II

MHC molecules are surface receptors able to bind processed peptides deriving from phagocytised or endogenous antigens. MHCs are encoded on chromosome 6 in humans and on chromosome 17 in mice. In humans, the MHC-II is encoded by 3 different genes which are polymorphic and express one α- and one β-chain (to be combined) each: HLA-DR, HLA-DQ and HLA-DP. In mice, the gene complex contains two loci, A and E, each of which encodes α- and β-chain polypeptides. This gene complex is referred to as Ir (immune response) as the genes encoded determine the level of immune responsiveness to specific antigens of different mouse strains. The products of the A and E loci are therefore often called I-A and I-E.

Antigens from the endocytic pathway, i.e. the ones that have been internalized, bind predominantly to MHC class II molecules, although many reports show endogenously

synthesized antigens on MHC class II molecules (van Niel et al. 2008). Internalized extracellular proteins are transported through the acidified endocytic vesicles and localize in either endosomes or phagosomes, subsequently fused to lysosomes. In lysosomes, internalized proteins are processed into peptides and then transferred to the MHC class II compartment (MIIC), where degradation into smaller peptides and loading on to a class II MHC takes place. MIIC have a high content in both lysosomal-associated membrane protein (LAMP), Cathepsins (Cresswell 2005), and class II molecules, and the enzymatic processing that takes place produces the cleaving of proteins into peptides of 10 to 20 residues in length. The MHC molecules are formed in the Endoplasmic Reticulum (ER), where α- and β- chains, already bound to each other, are associated with the invariant chain (Ii). Ii stabilizes the complex by occupying its free cleft and by binding to the vesicle membrane through the insertion of its class II-associated invariant chain peptide (CLIP) portion. The cytoplasmic tail of Ii contains a motif for delivery and retention of the αβ-Ii complex to the endocytic route. Once the MHC-containing vesicle fuses with the MIIC, containing the processed peptide coming from the endocytic pathway, the invariant chain and its CLIP are removed from the αβ-Ii in order to give room to the processed antigens. The removal is achieved through degradation of the Ii itself, which is processed in various steps involving activity of cathepsins. This process is fundamental for the release of MHC complexes from the endosomal compartment. The retention signal is in fact contained in the cytosolic tail of the Ii chain and it has to be clipped off in order to get translocation and insertion in the cell membrane. In DCs, one of the steps of degradation of the CLIP is carried out by Cathepsin S (Shi et al. 1999). Subsequently, the low pH favouring an "open" conformation of the MHC dimer, the activity of a chaperone acting as a peptide-CLIP exchanger (HLA-DM in humans and H2-DM in mice), and the proteolytic elimination of the region flanking the CLIP, collaborate to the removal of the remaining peptide. Once the whole Ii is eliminated, internalized antigens can be loaded. The MHC-II molecule, which is a dimer, is stabilized again by peptide binding. The stable complex is ready to be delivered to the cell surface.

The half life of pMHCs depends upon the affinity of MHC-antigen interaction (Nelson et al. 1994): the MHC molecule itself is otherwise highly unstable and rapidly degraded, especially in immature DCs (Colledge et al. 2002; Wilson et al. 2004). In these cells, MHC-II complexes are transiently expressed on the plasma

membrane and are rapidly reendocytosed and delivered to lysosomes (Villadangos et al. 2001). MHC-II loading is a delicate mechanism which receives regulatory signals from several sources and adjusts very finely to the cell stage. The complexes are sequestered in MIIC during the immature stage of a DC life and they are transported to the cell surface, through tubular endosomal structures, when the cell receives danger signals and its maturation is triggered. Interestingly, immature DCs have shown to express empty MHC-II molecules on their surface, which seem to be loaded with peptides processed by secreted proteases from extracellular antigen (Santambrogio et al. 1999; Potolicchio et al. 2005). From a T cell response point of view, the number of MHC loaded molecules displayed on the dendritic cell surface at any given time is of a great importance for the regulation of activation and potency, and so is their half-life. Permanence of MHC complexes on the surface is dictated by a complex balance between loading plus delivery to plasma membrane, and kinetics of removal and destruction.

MHC-II half-life at DC surface has been estimated in ~5-10 hrs (Eberl et al. 1993). Regulation of MHC-II is mediated by transcription factors, like TGF-β and by chromatin modification. The transcriptional co-activator CIITA specifically regulates MHC-II levels in DCs. CIITA promotes MHC-II transcription when DCs are immature and but not when DCs are mature. CIITA promoter, CIITApI, is in fact silenced during maturation (Smith et al. 2011), MHC-II levels are further regulated by IL-10 in immature DCs and CD83 in mature ones (Thibodeau et al. 2008).

#### 2.5.5.2 Major histocompatibility complex-I

MHC-I molecules are expressed in most cell types. They are mainly responsible for the maintenance of peripheral tolerance and for activation of CD8 T cells responses against cancer cells and intracellular pathogens. In humans, the class I gene complex contains three major loci, B, C and A. In mice, there are two loci, K and D. Each of these loci encodes for a polypeptide, the α-chain, which contains antigenic determinants and is polymorphic. The α-chain associates with β-2 microglobulin or β-chain, which is encoded by a gene outside the MHC complex. Without the β-2 microglobulin, the class I molecule is not expressed on the cell surface. The MHC-I expression and loading, and the processing of endogenous antigen destined to this

molecules are separated from that of MHC-II. Nonetheless, antigens from the endocytic pathway can be presented on MHC-I thus favouring priming of CD8 T cells, hence named “cross-priming” (Bevan, 1976): this will be the subject of the next chapter.

The antigens loaded onto MHC-I via the default pathway are derived from either protein at the end of their half-life, or misfolded, or from the so called DRiPs (defective ribosomal products). DRiPs are the results of defective translation or transcription and need to be degraded to prevent aggregation: this happens to between 30-70% of all the proteins made (Schubert et al. 2000). The discovery of DRiPs clarified how viral proteins uploading onto MHC-I is achieved rather quickly after infection, faster than the expected half-life of the proteins. MHC-I molecules in fact present also antigens from intracellular pathogens and internalized extracellular antigens through cross-presentation.

Intracellular proteins ready to be degraded are tagged with a polyubiquitin chain to be transported to the proteasome where they will be processed. Antigens are cleaved into peptides by nuclear or cytoplasmic proteases which form a proteasome. Once formed, the peptides, which are between 8 and 16 amino acids, are transferred to the ER by the specialized transporter protein called TAP, transporter associated with antigen processing (Owen and Pease 1999). TAP also mediates contact with tapasin, the MHC chaperone, acting as a platform for the correct binding of the peptide to the MHC chains. In the ER, the MHC heterodimer formed by a polymorphic heavy chain and a light chain called (β2-microglobulin) are assembled and stabilised by a number of chaperons (the chaperon calreticulin, the transmembrane protein tapasin, TAP itself and the protein disulfide isomerase ERp57). The loading happens under the control of a loading complex composed of several ER resident chaperons, including tapasin, calnexin and calreticulin (Cresswell et al. 1999; Cresswell et al. 1999). Once associated to peptides, MHC class I molecules are rapidly transferred through the Golgi apparatus to the plasma membrane.

Notably, there is growing interest on differences in the proteasome composition between non-immune cells and antigen presenting cells. The proteasome common to all cell types is called 26S, because formed by a 20S core barrel and two 10S caps (Unno et. al 2002). Macagno and colleagues showed the existence of a special proteasome in APCs, called immunoproteasome. This special proteasome has been

shown to be made of 3 special subunits out of the 14 which form the 20S barrel. These 3 subunits (called β1i, β2i, β5i) are IFN-γ inducible homologues of the normal, constitutively expressed subunits and are expressed exclusively in immune cells, together with the "common" one (Macagno et al. 2004). The immunoproteasome has been reported to have a special processing activity resulting in formation of peptides different from the ones processed by common proteasome, having the immonoproteasome distinct proteolytic specificities (Toes et al. 2001). The reason for this different processing machinery is probably associated with the maintenance of tolerance. Self-peptides normally processed and presented on MHC-I in non-immune cell types are not meant to stimulate an immune response. Antigens processed and presented on dendritic and antigen-presenting cells that are deriving from infected or malignant cells instead should be able to induce, together with other signals, a specific response: a different processing could help allowing the differentiation of antigen presentation and T-cell activation specificity in this cell type. On the other hand, tumours have been shown to escape immune responses by producing peptides trough the immunoproteasone against which CTL responses are not induced (Chapatte et al. 2006). Also a thymic-specific proteasome has been reported in the epithelial cells of the thymus (Sijts and Kloetzel 2011).
These 3 different proteasomes have been accounted for the specificity of degradation of antigens in immunogenic peptides and the last two in particular are stimulated to work at a faster rate under IFNγ and stress signals.

#### 2.5.5.3 Antigen cross-presentation

When internalised antigens are efficiently presented on MHC-I, the process is called cross-presentation. Cross-priming is the ability of DCs cross-presenting exogenous antigen in order to achieve activation of CD8 T cells. Data reports that cross-presentation and cross-priming are more efficient with cell-associated antigens than with the corresponding soluble protein (Li et al. 2001). During infections, cross-presentation and presentation ability are likely to depend on the kinetic of viral invasion and spreading. The nature of the virus and its lytic or non-lytic characteristics might be of importance as well. Also, during intracellular infections MHC-I expression can be upregulated by interferon stimulation, in viral infections, or by tumour necrosis factors, in microbial ones.

Cross-presentation of antigen was discovered by Bevan in 1976: while studying immunization with fully allogenic antigens he found out that minor histocompatibility antigens could be transferred between cells for presentation on APCs and stimulate a CTL response (Bevan 1976). Burgdorf and colleagues, moreover showed that cross-presentation, which depends on TAP, is induced by TLR4 engagement and it is kept separated from endogenous antigen presentation on MHC-I (Burgdorf et al. 2008). In Keller *et al*. the authors use VPL-encapsulated gp33 in presence and absence of RNA to show how TLR stimulation enhances cross-priming of DCs. They demonstrate that signalling through MyD-88 is not necessary for cross-presentation but fundamental, together with type-I interferon production and signalling, for T cell priming (Keller et al. 2009). Moreover, engagement of type-I interferon receptor on the DCs enhances their ability to activate T cells during cross-presentation due to the expression of subunits forming the immunoproteasome (see above). Other than the presence of the immunoproteasome, two more factors seem to be involved in improving effectiveness of cross-presentation in DCs: reduced lysosomal proteolysis and expression of protease inhibitors. These two characteristics specifically described in DC, prove that these cells have an intracellular endocytic pathway adapted for antigen processing rather than degradation (reviewed in Lin et al. 2008)

Two models have been described to explain how endocytosed exogenous antigens are presented on MHC-I: the first is called “cytosolic pathway” and the second is termed “alternative vacuolar pathway”.

The first model proposes that antigens uptaken and internalized into phagosomes, are translocated to the cytosolic compartment of the DC where they are process by the proteasome and then transported to the ER: in the ER they are loaded onto MHC-I and finally transported to the plasma membrane by budding vesicles (Rock and Shen 2005). Data has shown the involvement of Sec61 in traslocation of antigens from the phagosome to the cytosol for processing (Ackerman et al. 2002). Transport of peptides to the ER from the cytosos after processing has been proposed to be TAP-dependent. An alternative model has been described: it involves budding of vescicles loaded with the entire MHC-I loading machinery from the ER which would fuse with pahgosomes having proteasomes for antigen processing associated: nonetheless, this model has not been clarified yet.

The second, less understood, model describes a TAP-independent pathway which involves degradation of endocytosed antigens in the lumen of the phagosome

mediated by cathepsin activity and consecutive loading of the peptides on MHC-I molecules in the same phagosomal vacuole (Rock and Shen 2005).

Cross-presentation can be seen as an "emergency" mechanism allowing efficient immune responses to intracellular pathogens that do not infect DCs and to tumour cell-derived antigens. On the other hand, cross-presentation has a key role in inducing tolerance to self-antigens not synthesised within DCs. Cross-presentation has been demonstrated to depend on high antigen availability and protein stability (Kurts et al. 1998).

### 2.5.6 Pathogen and danger sensing

Antigen scavenging and presentation is not the only function of immature DCs: the presence of several specific surface receptors allows DCs to sense an ongoing pathogen invasion (Matzinger 1994). The ability of DCs to recognise pathogen-associated patterns is unique to the innate immune system and it is fundamental for the initiation of adaptive responses. Pathogen recognition is coupled to intracellular signalling and results in regulation of antigen uptake, MHC presentation, DC maturation and expression of specific cytokines and coreceptors. DCs express Fc receptors, pattern recognition receptors (PRRs), scavsenger receptors, cytokine receptors and complement receptors CR3 and CR4 (Reis e Sousa et al. 1993). This wide array of surface molecules allows the recognition of classical signals of inflammation, like inflammatory cytokines, together with other danger signals. Inflammation and danger signals are fundamental for DC maturation and for the initiation of immune responses.

PRRs are all able to recognize pathogen-associated molecular patterns (PAMPs) and are constitutively expressed by macrophages, DCs and epithelial and fibroblast cells, but also in some T and B cells. Several families of PRRs have been discovered, some of which are cytosolic: the Toll-like receptors (TLRs), the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs). RLRs detect viral pathogens and

are expressed by most cells, while NLRs detect intracellular pathogen and stress signals. Due to their location, stimulation of these last two classes of receptors in most cases happens in infected cells. TLRs, instead, are expressed on the plasma membrane or on the membrane of endosomes and lysosomes.

TLRs have specific ligands and are non-redundant. They can be divided in subfamilies depending on the molecules they recognise and their location. On the plasma membrane are expressed: TLR4 which recognises LPS (Medzhitov et al. 1997) and viral proteins; TLR5 which recognises flagellin; TLR1/TLR2 and TLR2/TLR6 which recognise bacterial lipoproteins and peptidoglycans (TLR2 alone also recognises viral proteins, micobacteria and bacterial lipids). The recognition specificity of endosomal/lysosomal TLRs is more focused on nucleic acids: TLR3 recognises double-stranded RNA (dsRNA), TLR7 and TLR8 recognise single-stranded RNA (ssRNA), and TLR9 recognises double-stranded DNA. TLRs signal through dimerisation, recruitment of MyD88 (Takeda et al. 2003), the intracellular TIR (Toll/IL-1R homology domain and phosphorylation and degradation of IkB. When stimulated in DCs, TLRs have been shown to be potent inducers of adaptive immunity. Medzithov reported that TLR signalling is able to increase the phagocytic ability of DCs and also improve endosomal maturation, thus facilitating antigen association and presentation in MHC context (Blander and Medzhitov 2006). Stimulation of TLRs is now being tested in clinical trails as adjuvant.

During viral infection TLR3, TLR4, TLR7 and TLR9 in particular assume an important role in DCs, as they induce type I IFN secretion and proinflammatory signals. Their stimulation also leads to DC maturation and upregulation of costimulatory molecules (Durand et al. 2004). TLR3 is a strong inducer of Type I IFN after dsRNA recognition. TLR3 is localized on endosomal membrane thus suggesting that viral entry route may determine the magnitude of its contribution to DC activation and secretion of IFN. Nonetheless, since newly-synthesized dsRNA and dsRNA from released viral genomes are not accessible to TLR3, production of type I IFN can also occur in a TLR3-independent manner through stimulation of cytoplasmic RIG-1, retinoic-acid-inducible protein-1 (Yoneyama et al. 2004). TLR7 and TLR9 are highly expressed in pDCs and in this cell type they induce IFN-α secretion when stimulated. Among other receptors recognising pathogen-associated

patterns are the C-type lectins Dectin-1 and Dectin-2: they recognise β-glucan and mannan, respectively, from fungal cell walls (Iwasaki and Medzhitov 2010).

Danger signals differ from pathogen-associated signals as they are induced by self proteins whose availability or presence in the intracellular space indicates cellular stress or necrosis. Certain self proteins are in fact released when stress or necrosis occur in cells: among others are heat shock proteins, (Arnold-Schild et al. 1999), the high mobility group box-1 or HMGB-1 (Rovere-Querini et al. 2004), and intracellular molecules which are exposed externally on the plasma membrane like the ligand for CD36. Also, danger signals can derive from matrix degradation products such as hyalurotnan, cellular cytokines and cell surface ligands related to cell damage and necrosis such as TNF-α, IL-1, and CD40L. CD40L is very important in this context as the stimulation of CD40 is needed for maturation of DCs to occur (Cella et al. 1997).

Danger signals and the recognition of pathogen-associated patterns are necessary for proper maturation of DCs and for the secretion of pro-inflammatory mediators and upregulation of coreceptors. The activated receptors induce different intracellular signalling and thus regulate maturation of DCs, representing the first stage of differentiation of an immune response.

### 2.5.7 Dendritic cell migration to lymph nodes

Upon receiving danger and inflammatory signals from the microenvironment, the maturation program is triggered in DCs. Maturation induces internalisation of endocytic receptors, down-regulation of the antigen processing machinery and of the synthesis rate of MHC-I complexes (Rescigno et al. 1998) and an increase in the half-life of MHC-II complexes on the plasma membrane. Additionally, maturation induces upregulation of receptors for homing to LNs like RAGE (Manfredi et al. 2008), chemokine receptors like CCR7, and adhesion molecules like caderins, together with co-stimulatory molecules like CD80, CD86 and CD54. The secretion of cytokines is

also increased: in particular IL-2, which enhances and sustains T cell survival and proliferation. Finally DCs undergo cytoskeleton reorganisation, as mobility needs to be increased, and the cells round-up losing their dendritic projections.

Migration out of the infected tissue is led by the newly expressed chemotactic receptors. DCs follow chemotactic signals to the LNs and spleen where they will mediate presentation of antigen to T cells. The entry route and localisation in SLOs will be analysed in more detail below (Chapter 2.5.9). CCR7 is the main responsible for leading migration of DCs to the LN and also their positioning deeper into the cortex. CCL19 and CCL21 are instead involved in getting DCs to cross the subcapsular sinus floor of the LNs (see below). Notably, mature DCs cannot enter the thymus, where immature DCs and precursor have instead free access. Limitation of DC access to the thymus seems to be aimed at maintaining peripheral tolerance; mature DCs are not allowed to enter the thymus to avoid deletion of T cells recognising foreign antigens (Bonasio and von Andrian 2006). Both immature and mature DCs can enter the BM, which can function as a secondary lymphoid organ (Di Rosa and Pabst 2005).

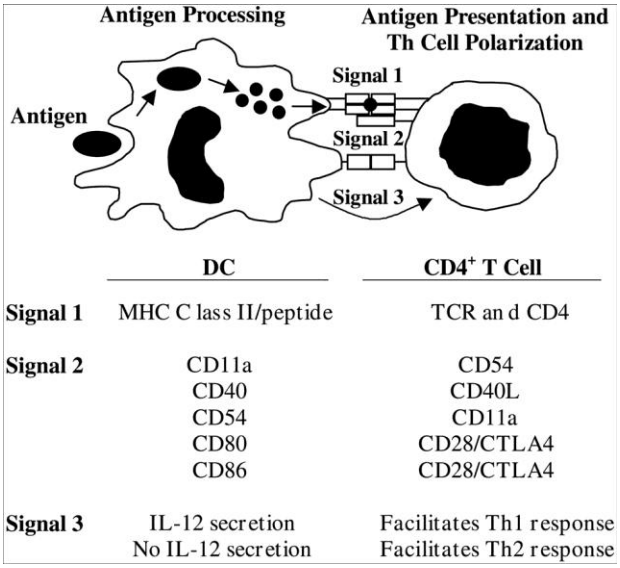
DCs reach the LN after skin challenge in 16-18 hrs, but soluble antigen in the blood can be presented after about 4 hr. Soluble antigen in fact travels to the LN itself, where it can be uptaken by resident DCs and presented right away as described below (Kissenpfennig et al. 2005).

### 2.5.8 Presentation and priming: co-stimuli and cytokines

Presentation of antigens and priming of CD8 T cells occurs in SLOs, where DCs migrate after maturation led by receptors like CCR7. The importance of DCs as initiators of the immune response is in the capacity of these cells to convey multiple signals, thus being able to tune and modulate CD8 T cell activation. Three signals,

according to the Danger Theory (Matzinger 1994), must be conveyed to a T cell for full activation to be achieved: antigen in MHC context, costimulation and inflammatory signals. Only DCs can deliver these 3 signals to a T cell through their MHC molecules, B7-1 and B7-2 receptors and cytokine production.

The clustering between a DC and T cell is mediated by the interaction between MHC and coreceptors, and by adhesion molecules, like integrins and proteins of the immunoglobulin superfamily LFA-2 and ICAM-1. The role of costimulation has been reviewed for CD8 T cells in the corresponding section of this manuscript (see 2.2.4.1), and it is now reviewed describing some of the main DC costimulatory receptors in this chapter.




| | DC | CD4⁺ T Cell |
|---|---|---|
| **Signal 1** | MHC C lass II/peptide | TCR an d CD4 |
| **Signal 2** | CD11a<br>CD40<br>CD54<br>CD80<br>CD86 | CD54<br>CD40L<br>CD11a<br>CD28/CTLA4<br>CD28/CTLA4 |
| **Signal 3** | IL-12 secretion<br>No IL-12 secretion | Facilitates Th1 response<br>Facilitates Th2 response |

**Figure 17: Scheme of the three danger signals needed for T cell activation – the figure illustrates the signals for CD4 T cells, which, apart from MHC-II, are the same as in CD8 T cells (Lipscomb and Masten 2002).**

DCs are able, through production of cytokines and transmission of costimulatory signals, to instruct T cell activation. CD28 and its ligands B7-1 (CD80) and B7-2 (CD86) were the first costimulatory molecules to be discovered (see Table 1). CD28 role in stimulating proliferation, antigen sensitivity and survival and in decreasing activation threshold and doubling time has been widely demonstrated both *in vivo* and *in vitro* (Harding et al. 1992). CD80 and CD86 are expressed in DCs upon TRL

stimulation and maturation. These receptors bind CD28 but can also bind CTLA-4 which, especially on $T_{regs}$, has an inhibitory role (Grohmann et al. 2002). CD86 is constitutively expressed at low levels and rapidly upregulated upon maturation, whereas CD80 expression is inducible. Turley and colleagues showed that B7 costimulatory molecules are loaded in the endosomes where MHC-II are loaded and are transferred to the plasma membrane together with the MHC (Turley et al. 2000). Specific depletion of CD28 costimulation induced apoptosis in antigen-specific cells, which resulted to be Fas- and CD4-independent (Dolfi et al. 2011).

A new B7 family member, which acts as a costimulating receptor, was identified by Chapoval and colleagues and named B7-H3 (Chapoval et al. 2001). This membrane protein is expressed on immature and mature human myeloid DCs and it is induced by LPS and IFN-γ (but suppressed by IL-4) in mouse DCs. Studies in KO mice support an inhibitory role for B7-H3 in polarised $T_H1$ responses (Suh et al. 2003) but its T cell receptor has not been identified yet. B7-H3 is also released by human DCs and can be found in the serum. Another member of the B7 family has been recently discovered and called B7-H4. Its ligand is not known and it is induced on B and T cells, DC, some NK and monocyte lineages in humans, therefore its role is not of too much interest for the scope of this project (Prasad et al. 2003).

During DC-T cell interaction, the ligation of coreceptors signals back to the DC as well sustaining further maturation. CD40 interaction with CD40L on T cells is required for IL-12 production, which is important for CD4 T cell responses (Cella et al. 1996). DCs also receive additional maturation signals from CD40L, RANK/TRANCE, 4–1BB, and OX40 ligand molecules, which induce the release of chemokines such as IL-8, fractalkine, and macrophage derived chemokines that attract lymphocytes (Tang and Cyster 1999). 4–1BB ligand expression and interaction with its receptor on T cells not only induces CD8 T cell proliferation but also production of IFN-γ (Shuford et al. 1997).

| DC | CD8 T Cell |
|---|---|
| *B7 family and receptors* | |
| B7-1 (CD80) | CD28, CTLA-4 (CD152) |
| B7-2 (CD86) | CD28, CTLA-4 (CD152) |
| B7RP-1 | ICOS |
| PD-L1 (B7-H1) | PD-1 |
| PD-L2 | PD-1 |
| B7-H3 | Not known |
| *TNF family ligand and receptors* | |
| CD40 | CD40L (CD154) |
| OX40L | OX40 (CD134) |
| 4-1BBL | 4-1BB (CD137) |
| TRANCE (RANK) | TRANCE (RANK-L) |
| CD27 | CD27L (CD70) |
| CD30L (CD153) | CD30 |
| *Miscellaneous* | |
| ICAM-1 (CD54) | LFA-1 (CD11a/CD18) |
| DC-SIGN (CD209) | ICAM-3 (CD50), ICAM-2 (CD102) |
| SLAM(CD150) | SLAM (CD150) |
| CD58 | CD2 |

**Table 1: Costimulatory molecules on DCs and receptors on T cells (Lipscomb and Masten 2002).**

Engagement of RANK, receptor activator of NFkB, a member of the TNFR family, on DCs by its ligand RANKL/TRANCE expressed on activated T cells, stimulates the secretion of inflammatory and $T_H1$ cytokines like IL-1, IL-6, and IL-12. Once triggered, RANK signalling also sustains DC survival through the upregulation of the antiapoptotic molecule Bcl-XL (Wong et al. 1997).

Costimulation also occurs by means of activation downregulation. B7RP-1 (or ICOS-L), the ligand for ICOS, is important mostly for T cell instruction of B cell activation (Yoshinaga et al. 1999). ICOS is upregulated on CD4 T and CD8 T

following activation and is present on effector and memory T cells. ICOS expression is stimulated on T cells by both TCR and CD28 signals and its interaction with the ligand provokes its downregulation, as a mean to control immune responses (McAdam et al. 2001). Another DC costimulatory molecule with negative regulatory effect on immune response is PD-L1 (programmed death ligand 1, also called B7-HI) which binds to PD-1 receptor on T cells, downregulating T cell activity (Liang et al. 2003). PD-L1 is constitutively expressed on DCs. What determines the final activation status of the CD8 T cell is therefore the result of stimulatory signals transmitted by coreceptors like CD28 and ICOS and inhibitory ones from PD-1 and CTLA-4.

DCs have been shown to be able to prime T cells responses outside lymphoid sites during influenza infection: this priming can for example take place in structures called induced BALT (iBALT) (Moyron-Quiroz et al. 2004; Moyron-Quiroz et al. 2006). In a paper published last year by Dolfi and colleagues, it was demonstrated that costimulation is needed in CTL responses at peripheral sites during the advanced phases of the response, as depletion of DCs at day 6 p.i. resulted in impaired CTL expansion. These results are important for determining the need for sustained versus limited antigen presentation in the stimulation of optimal CTL responses. This will be object of further discussion below.

### 2.5.9 Secondary lymphoid organs

When describing the lymphatic system, the organs generating immune cells are considered “primary”, like the thymus and the bone marrow, while those where the immune responses are initiated are deemed “secondary lymphoid organs”. Spleen and LNs are therefore considered secondary lymphoid organs together with tonsils, Peyer's patches, and the mucosa-associated lymphoid tissue (MALT). They have a very similar structure for lymphoid purposes which will be reviewed here for the purposes of my research.

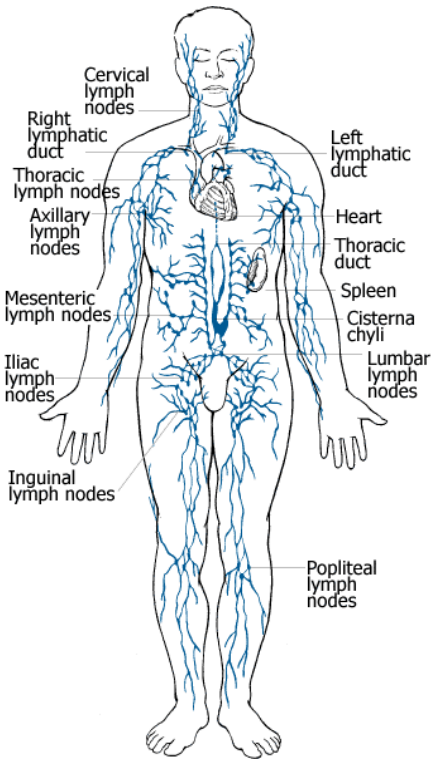



**Figure 18: Lymphatic system and LN distribution in the human body (http://www.gorhams.dk/html/the_lymphatic_system.html).**

LNs are spread across the body, usually at the junction of 2 or more lymphatic main vessels and receive the lymph drained from the neighbouring organs (Figure 18). The spleen acts as a blood filter, and due to this activity it also acts as a lymphoid organ as it houses T and B cell conglomerates which are ready to proliferate and activate when pathogens are sense during blood "filtration".

### 2.5.9.1 Lymph node structure and function

Lymph nodes are filter structures which sit at the junction of lymphatic vessels collecting the lymph out of peripheral tissues and operate as a check point (von Andrian and Mempel 2003). There are 450 identifiable LNs in humans (Figure 18) and 22 in mice (Figure 19) (Van den Broeck et al. 2006). These small spheroid organs have the key function of facilitating encounters between pathogen-derived antigen and the cellular compartment of the adaptive immune system.

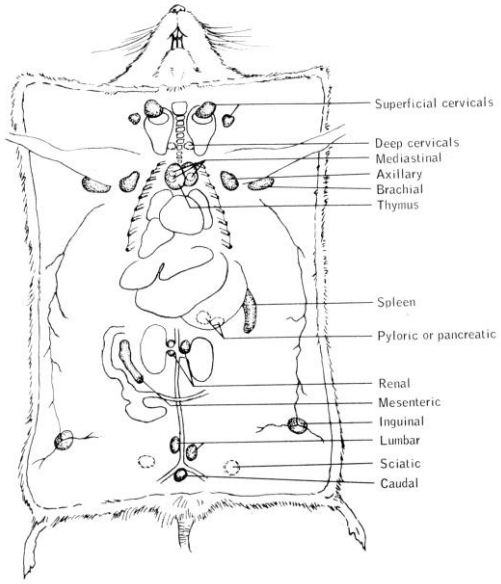



**Figure 19: LN location in mouse (Dunn 1954).**

The LN structure quite amazingly explains the reason for having several subsets of DCs and for a distinction between resident and migratory ones. Each lymph node is made of a capsule, the most external section, a cortex, where mainly B cells reside, and a medulla, which is the most internal area and where the T cells transiently reside (see Figure 20): B and T cells are therefore separated in the parenchyma of the organ.

Each LN receives several afferent lymphatic vessels and has one efferent vessel. LNs can contain one or more functional and anatomical units called lobules, each of which receives a single afferent lymphatic vessel and is formed by a capsule-surrounded follicle in the cortex and a deep cortical unit (DCU) in the medullary section. The lymph enters through the subcapsular sinus, flows down the sides of the lobule through the transverse sinuses and then flows into the medullary sinuses. In the peripheral DCU and in the interfollicular cortex run arterioles, high endothelial venules and paracortical sinuses. Arterioles and venules are then conveyed in the medullary cords, which form the efferent vessels.

Each lobule contains a dense reticular meshwork, composed of stellate, spindle shaped or elongated FRCs (Clark 1962), which provide a three dimensional scaffold for lymphocytes, APCs and macrophages to interact (Figure 20 and Figure 21). Each

cortical follicle, delimited by cortex, is home to B lymphocytes and allows them to interact with special DCs called follicular dendritic cells (FDCs).

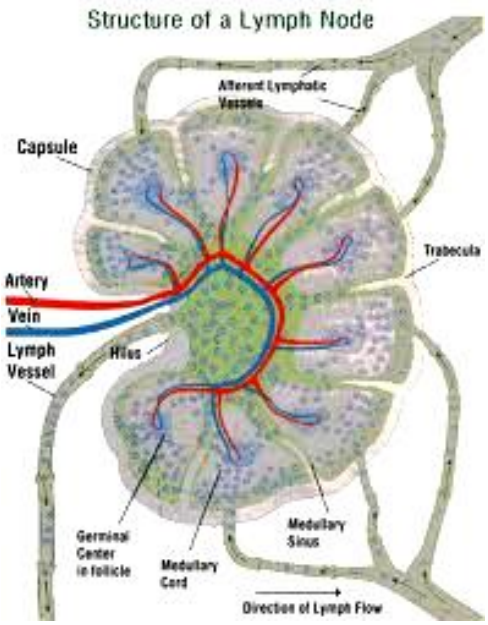



**Figure 20: Structure of a lymph node: identification of lobule units, medullary and cortical regions and afferent and efferent vessells (http://www.acm.uiuc.edu/sigbio/project/updated-lymphatic/lymph7.html).**

Stimulated B cells proliferate within the follicles forming distinctive germinal centres and the follicles are then referred to as secondary follicles. In the medullary section, T lymphocytes home to the deep cortical unit (DCU) where they interact with dendritic cells. T lymphocytes home to the paracortex and interfollicular cortex to survey DCs. Because each afferent lymphatic collects lymph from a different drainage field, each lobule is potentially exposed to a different set of antigens, APCs and inflammatory mediators (Sainte-Marie et al. 1982). Activated T lymphocytes proliferate in the DCU but do not form a germinal-centre-like structure.

Surrounding each lobule are the lymphatic sinuses, delimited by a trilaminar membrane formed by a layer of sinusal FRCs, a layer of lobular FRCs and a layer of basal lamina between them (Kaldjian et al. 2001). This thin membrane prevents lymph, cells and particulates from passively entering the lobules. Inflammatory

mediators such as MCP-1 and IL-8, secreted by innate cells upon TLR stimulation, are transported to the local lymph node in the lymph.

There are a few main points to be considered for our purposes. i) T cells are segregated in the T cell area and have high motility, driven by CCL19 and CCL21 chemokines. ii) A population of resident DCs inhabits the sinus and continuously migrates between this and the T cell area. iii) LNs receive lymph from afferent lymphatic vessels but the soluble particles which are in the lymph never directly get into contact with T cells, they rather go through ducts called conduits. iv) DCs in the T cell area seem to be responsible for uptaking antigens that flow through the LN: they can uptake soluble particles by extruding their dendrites into the lumen of the conduits. DCs in the T cell area can therefore receive information from the periphery via cytokines and other inflammatory mediators released in periphery and reaching the LN via the lymph (Sixt et al. 2005).

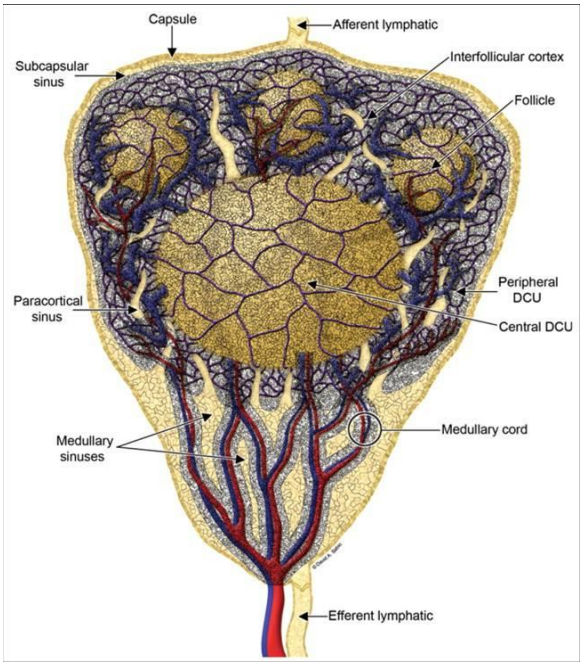



**Figure 21: Lobule structure (Willard-Mack 2006).**

The location of resident DCs resonates with a role for these cells in activating naïve T cells and modulating the developing CTL response. In addition, migratory DCs can be found in the spleen in inflammatory conditions. These cells can reach the LN from

the periphery when inflammatory stimula cause their maturation. Only mature DCs can cross the subcapsular sinus. Migratory DCs usually reach the T cell area first, showing high motility that slowly disappear as these cells join the network of resident DCs. Resident DCs have a much more limited motility but boast very contractile dendrites (Lindquist et al. 2004). The cortex structure is maintained in place by FRCs which create a network along which naïve T cells migrate constantly. It has been shown how this portion of the parenchyma almost totally excludes liquid and consists of 95% naïve T cell and 5% FRC. The reticular system has a role in facilitating encounters between naïve T cells and DCs, and in assist resident DCs in making contact with migratory ones, as this process needs to be efficient and quick.

As naive lymphocytes recirculate in the circulating and lymphatic systems uninterrupted, the body's pool of lymphocytes turns over 10 to 48 times every 24 hours. In particular, lymphocytes enter the LN continuously via the high endothelial venules (HEV) and exit via specialized sinuses called paracortical sinuses. Once inside, they move around in a narrow perivenular space between the endothelial basement membrane and the surrounding pericytic FRCs called the perivenular channel (Gretz et al. 1997). The perivenular channel receives inflammatory mediators from the conduit system. Lymphocytes spend 10 to 100 minutes in this space and inflammatory signals received in this area influence their subsequent behaviour. Lymphocytes then move out into the corridors of the paracortical cords by crawling along FRCs. T lymphocytes interact with dendritic cells positioned within the cords while B cells move through the cords to the follicles where they interact with FDC). The migration is guaranteed and maintained by chemokines (mainly CCL19 and CCL21) expressed by the FRC network. FRCs themselves can uptake, and transfer to the conduit system through transcytosis, both soluble antigens and inflammatory mediators (Anderson and Shaw 1993). T cells are also attracted by existing CD4 T cell-DC interactions which result in the release of cytokines and chemokines to attract CD8 T cells.

CXCL9 and CXCL10, CXCR3 ligands, are induced in a TNF-dependent way in the lumen of high endothelial venules in correspondence with the initial inflammatory status of peripheral tissues and expression of CCL3 and CCL4 in the lumen of HEV enhances early recruitment of DCs and pDCs to the LN (Yoneyama et al. 2004). These chemokines also attract T cells which are $CXCR3^{+}$, like CTLs and memory

cells, which are thought to be the component of the initial increase of cellularity in the LNs. CTLs and memory cells can kill resident DCs when activated. This mechanism seems nonsense, but may make the main contribution to the resolution of immune responses and was analysed in Chapter 2.4.

The LN architecture and its FRC system are very efficient at making sure low-frequency antigen-specific T cells are able to get in contact with antigen-bearing mature DCs quickly and efficiently. Also, it allows soluble signals to reach the LN in a matter of seconds from their release in the periphery. Naïve lymphocytes can spend from hours to days in a LN, looking for antigen (Gowans and Knight 1964). During their wandering around, sliding on each other to make contact, naïve lymphocytes interact with thousands of DCs and FRCs. Most lymphocytes do not bind cognate antigen and will eventually leave the lobule via the paracortical sinuses and recirculate to another lymph node to continue their search for antigen.

The DC population of the LN is quite complex. Five populations have been identified (Henri et al. 2001). As well as the $CD4^+$ $CD8\alpha^-$ DEC-$205^-$, the $CD4^-$ $CD8\alpha^-$ DEC-$205^-$ and the $CD4^-$ $CD8\alpha^+$ DEC-$205^+$ subsets, there are two additional DC subsets that lack CD4 and express only moderate levels of CD8α, but clearly express DEC-205 and high levels of MHC class II. One subset expresses moderate levels of DEC-205 and may represent interstitial tissue-derived DC, monocyte-derived DC, or an activated version of the splenic $CD4^-$ $CD8\alpha^-$ DC (described in 2.5.9.2). The second, more distinctive subtype, expresses higher levels of DEC-205 and appears to represent a mature form of LC. Both of these subsets are among the DCs that migrate out of the skin into the draining LNs. The migration pattern was described by Kamath and colleagues by catching DCs that emerge from skin explants before locating in LN and tracking skin-derived DCs after painting the skin with a fluorescent dye (Kamath et al. 2000). These cells cannot be found in the spleen due to the absence of afferent lymphatic in this organ. In lymph nodes, $CD8\alpha^+$ and $CD8\alpha^-$ DC each represent 20% of the total DC population. Within the LN, both $CD8\alpha^+$ and $CD8\alpha^-$ DC display a half-life of 4.5 days. Analyses of BrdU-labelling kinetics of LCs and dermal DCs within the per-LNs of C57BL/6 mice revealed that migratory cDCs have a slow turnover with an half-life of > 21 days for LCs and of 12 days for dermal DCs (Kamath et al. 2002).

The LN structure allows for maximal chance of encounter between a mature, antigen-loaded DC and a cognate CD8 T cell. This is also due to the continuous flow of naïve T cells and of migratory DCs contributing to supplying antigen from the periphery.

#### 2.5.9.2 The spleen as a secondary lymphoid organ

The spleen is a bean-shaped organ primarily acting as a filter for the blood. The blood reaches the organ though the afferent splenic arteries and leaves the circulation to enter the red pulp via the central arterioles, where it percolates to its cords via a system lacking endothelial lining but full of macrophages and lymphocytes. On the way to the afferent venules of the red pulp, the blood encounters the white pulp, sheets of lymphoid tissue (which contains T and B cells, but also DCs and macrophages) organized around the branching arterial vessels. The spleen therefore not only works as a filter to retain old stiffening erythrocytes and recycle the haems, but also as a secondary lymphoid tissue where naïve lymphocytes are able to interact with antigen-loaded dendritic cells and start an immune response. The external section of the spleen is called marginal zone (MZ) and its architecture is dictated, as explained below, by the function. Deeper than the marginal zone sit the germinal centers (B cell follicules) and the T-cell areas (Figure 22).

In the same way as in the LN the stromal component is fundamental not just for the tridimensional structure of the organ, but also for the organization and interaction of its cellular components, the non-lymphoid component of the spleen defines its architecture and function and controls the homeostasis of the T and B cell component. The structure of the spleen allows for maximized odds of encounter between an antigen-specific naïve lymphocytes and mature antigen-carrying DCs. A dense network of FRCs guides lymphocyte to travel inside the marginal zone first and the white pulp afterwards. These cells allow for close interaction between the resident DCs and the migrating T cells in the same way as they do in the LNs. Also mirroring the LN conduit system is a tubular network that permits the quick transport of solutes, cytokines and chemokines between the sheets of the lymphoid tissue to facilitate the diffusion of antigens.

Expression of CCL19 and CCL21 chemokines by FCRs in the T-cell zone makes sure entering T–lymphocytes find their way to the T-cell zone after accessing the organ via the terminal arterioles which feed the MZ. Lymphocytes then travel along narrow bridging channels that connect the MZ to the T-cell area, before extravasating into the white pulp. Once homed to the white pulp, they remain in the organ for a few hours during which they migrate within the T-cell zone directed and sustained by chemotactic signals released by the networking FRCs. During this time, they interact with resident DCs which might or might not present antigen and costimulatory molecules.

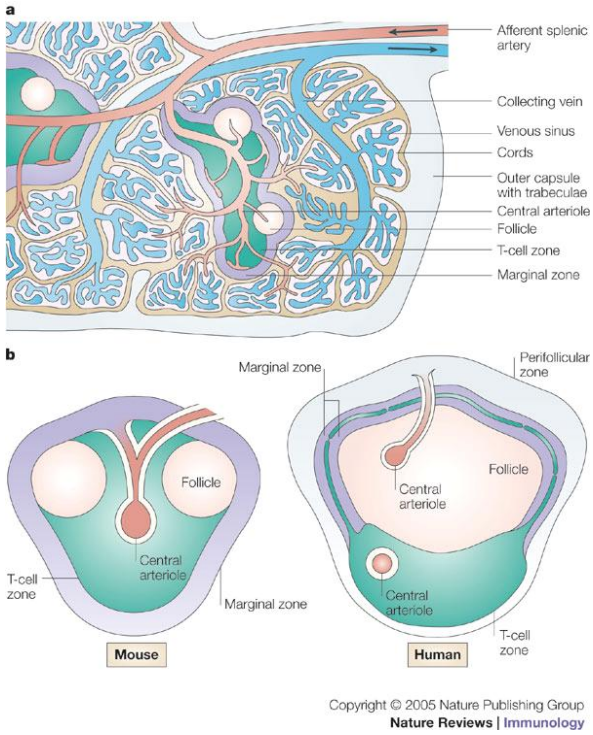



**Figure 22: Spleen structure (a) and differences between mouse and human spleen, b (Mebius and Kraal 2005).**

The spleen has three populations of resident DCs: $CD8\alpha^+$ DC, $CD8\alpha^-$ DC $CD4^+$ and $CD8\alpha^-$ $CD4^-$, of which the last is the biggest in number (Vremec et al. 2000). As described above, resident DCs developmentally arise from pre-DCs and proliferate in the spleen, regulated by lymphotoxin-β (Kabashima et al. 2005). Proliferation occurs at a fast rate, as the half-life of these cells here is about 3 days (Kamath et al. 2000).

Among the 3 spleen-resident conventional DC populations, $CD8\alpha^+$ DCs have the fastest turnover, with a half-life of 1.5-2 days. $CD8\alpha^-$ DCs have a slower turnover with a half-life of 3 days. Mattei and colleagues showed that type I IFN regulates DC turnover *in vivo* (Mattei et al. 2009).

$CD8\alpha^+$ DCs are mainly located within the T cell areas and marginal zone, whereas $CD8\alpha^-$ DC are preferentially located in antigen-capture areas, i.e. in the spleen red pulp and bridging areas and MZ and in the per-LN subsinusal layer beneath the subcapsular sinus (Idoyaga et al. 2009). Mature DCs move from the marginal zone to the inner pulp where they interact with antigen-specific T cells.

It is still unclear to what extent migratory DCs contribute to the spleen resident population. In the spleen there is no specifically directed trafficking of migratory DCs from the lymphatic, but migratory DCs can still reach the organ through the circulation and enter the marginal zone. When they enter the spleen through the circulation, DCs go through the Marginal zone first as well. CD47 has been involved in homing of DCs to the spleen, as in $CD47^{-/-}$ mice there is a substantial reduction of DCs in the MZ. Precursors of DCs at any developmental and differentiation stage also circulate freely and are retained in the spleen.

### 2.5.10 $CD8\alpha^+$ dendritic cells

Mouse DCs are an extremely complex population made up by several subtypes which reside in peripheral lymphoid and non-lymphoid tissues. Among all subsets the $CD8\alpha^+$ DCs (hereafter named CD8α DCs) are the cells that have been shown to be more efficient at cross-presenting antigen in MHC-I context to CD8 T cells. This subset is also mainly responsible for activation of CD8 memory responses. For this reason it will be the object of a more detailed description in this chapter.

The CD8α DC subset is characterised by the expression of CD8α as a homodimer, α-α as opposed to α-β which is found on CD8 T cells, and specific langerins and C-type lectins. The subset is identified as $CD8\alpha^+$ $CD11c^{high}$ $CD205^{high}$ $CD24^+$ $Clec9A^+$ $CD11b^{low}$ and $MHC\text{-}II^{high}$ when mature. CD8α DC is the subset mainly responsible

for the priming of CD8 T cell responses, particularly during viral infections (Belz et al. 2004). When pMHC complexes were analysed in splenic dendritic cells presenting soluble or cell-associated OVA, $CD8\alpha^+$ $CD11b^-$ dendritic cells resulted more efficient for MHC class I cross presentation (den Haan et al. 2000; Pooley et al. 2001) and $CD8\alpha^-$ $CD11b^+$ dendritic cells for MHC class II–restricted presentation (Chung et al. 2007).

The high cross-priming efficiency of $CD8\alpha^+$ DCs has been correlated with their ability to internalize dying cell-associated antigens (Iyoda et al. 2002) through the expression of specific scavenger and death recognition receptors. Clec9A (or DNGR-1) is a C-type lectin involved in the uptake of dead cells like DEC-205 (see 2.5.4.3). Clec9A is specifically expressed in pDCs in humans (Huysamen et al. 2008), and in CD8α DCs and pDCs in mouse, where it acts as a monomer. Clec9A recognises a self-component internal molecule that becomes exposed only when membrane integrity is compromised, this has recently been described to be actin (Sancho et al. 2009). Interestingly, several groups reported how antibody-conjugated targeting of antigen to this receptor resulted in an enhancement of antibody and CD4 T cell responses in both humans and mice (Caminschi et al. 2008; Idoyaga et al. 2001). Expression of CD36 is also characteristic of this DC subtype. CD36 (see 2.5.4.3) is another surface molecule involved in the recognition and endocytosis of dead cells. The presence of molecules like Clec9 and CD36 on this DC subset strongly suggests its involvement in the phagocytic uptake of dead or dying cells. Experimental evidence supporting this theory includes the verification that antigens uptaken through these receptors are efficiently processed and presented on MHC-I and MHC-II complexes (Inaba et al. 1998). The ability to specifically phagocytose apoptotic cells correlates with the need to prime and develop CD8 T cell responses against intracellular pathogens like viruses which do not infect DCs and against altered self-proteins derived from cancer cells. Moreover, through this mechanism DCs can prolong antigen presentation past their life. The uptake of an apoptotic DC by another DC would allow the presentation on the phagocytic cell of the antigens processed and presented by the dying cell. The ability of these apoptosis receptors to send internalised antigens to the cross-presentation pathways has been exploited to specifically deliver antigens to the cross-priming processing pathway, for example targeting of C-type lectin receptors such as DEC-205 (Dudziak et al. 2007) and the recently described DC, NK lectin group receptor-1, DNGR-1 (Sancho et al. 2008).

The specific composition of the TLR repertoire expressed by the CD8α DCs also supports the specificity of this subset for CD8 T cell priming during viral infections. The specific TLRs expressed indicate a role for CD8α DCs in sensing viral infections, for example through TLR3, which recognises dsRNA and drives DC maturation when engaged. TLR3 is expressed in early endosomes in immature DCs and migrates to $LAMP1^+$ endosomes when stimulated with short sequences of dsRNA, as showed by Jenilek and colleagues. They also demonstrated that the potency of DC activation was directly correlated to the size of the dsRNA (Schulz et al. 2005; Jelinek et al. 2011). Interestingly, TLR7 is not expressed in this subset while it is abundant in pDCs.

Analysis of gene-expression profiles suggests that CD8α DCs, in contrast to $CD8\alpha^-$ DCs, are endowed with an antigen-processing machinery specifically required for cross-presentation (Iyoda et al. 2002; Dudziak et al. 2007). The presence of antigen-specific machinery has been shown to be important for cross-presentation more than antigen capture levels, which are comparable in the two subsets (Schnorrer et al. 2006). Having shown how $CD8\alpha^+$ DCs compared to $CD8\alpha^-$ DCs are more efficient at cross-presenting and priming CD8 T cells, further studies focussed on the mechanism that make this possible. The suggestion that a specific subset of proteases might be present in the former, therefore making the cells more efficient at processing antigens to be loaded on MHC-I, has been tested. Two more studies, by Dudziak and by Kurts, have recently shown that quantitative differences in MHC-I and MHC-II processing machinery-related protein might exist in different DC subsets: for example CD8α DCs were shown to be enriched in proteines involved in MHC-I presentation like Tap1, Tap2, calreticulin, calnexin, Sec61, ERp57, ERAAP, as well as cystatin B and C, which is in line with their bias towards CD8 T cell priming (Dudziak et al. 2007; Burgdorf et al. 2008).

These results are also consistent with the localisation and predominance of this subtype in SLOs. The CD8α DC subset resides in the spleen and LNs, but not in peripheral tissues. How these cells enter in contact with antigens other than soluble ones travelling through the lymphatic system or dead cells in the same condition was studied. It has been proposed that CD8α DCs acquire antigen from mature migratory dendritic cells which enter the LNs of infected mice through the blood stream (Allan et al. 2006). For example, priming of HSV-specific CD8 T cells after skin inoculation

of HSV has been shown to be mediated by CD8α DC and not dermal/Langerhans or epidermal (skin-derived) ones. Nonetheless, the latter are present at the site of priming in the draining lymph-node, suggesting a role in transporting the antigen from peripheral tissues to resident CD8α DC to allow for specific and strong cross-presentation. Proof of this mechanism was found by the same group when, by blocking migration out of the skin of dermal and Langerhans cells, T cell priming was prevented (Allan et al. 2006). Also, in 2004, Belz and colleagues carried out some experiments in which, through intra nasal instillation of a CFSE solution and infection with a flu virus, they investigated the origin of the DCs involved in presentation of MHC-I restricted antigen. Results showed that CD8α DCs did not derive from the lungs, while another subset indirectly involved in cross-priming was. The latter was identified as $CD8\alpha^-$ $CD11b^-$ DC and was shown to be lung-derived. These results prompted the scientists to wonder how the $CD8\alpha^+$ DC population could acquire antigen if it had not met the pathogen in the lung. The same group demonstrated *in vitro* that the $CD8\alpha^-$ $CD11b^-$ DC population alone cannot prime CD8 T cells but, when an external CD8α DC population is added to the culture, CD8 T cell proliferation is obtained (Belz et al. 2004). They could therefore explain how migratory tissue-derived DCs can function as shuttles for peripheral antigens which would otherwise not reach the site of CD8 T cell priming.

One further function of CD8α DCs seems to be to act as early shuttles of intracellular or blood-borne pathogens like Listeria and Toxoplasma from the MZ to the T cell area of the spleen (Figure 23). This role is in line with the theory depicting CD8α DCs as highly endocytic machines for the uptake of soluble antigens and apoptotic cells locally in the LNs and spleen. A model has been proposed by Neuenhahn and Busch in 2007 whereby this subset would be mainly located in the T cell area but with an outpost in the Marginal zone where it patrols the blood for antigens to be taken to the T cell area (Neuenhahn and Busch 2007).

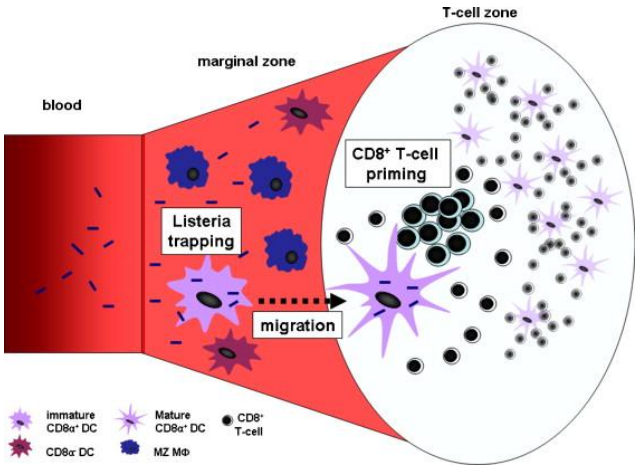



**Figure 23: Shuttle function of CD8α DCs for blood-born pathogens inside the spleen (Neuenhahn and Busch 2007).**

Production of high levels of IL-12p70 has been demonstrated when CD8α DCs are stimulated in an inflammatory environment, for example when CD40 stimulation is conveyed by CD4 T cells in presence of cytokines like IL-4, GM-CSF, IFN-γ, and engaged through a TLR molecule recognising a pathogen-associated pattern (Schulz et al. 2000). The CD8α subset is therefore capable of inducing a very strong CD8 T cell response: for this reason its regulation, both in the steady state and in the mature state has to be rigorous.

In humans, the identity of this subset has been long elusive, but the counterpart of murine CD8α DCs has now been identified in humans in BDCA-3$^+$ (CD141) cells. The first study to indicate these cells as possible candidate was a gene chip analysis of the transcriptome done by Robbins and colleagues in 2008 (Robbins et al. 2008). This same subset has been more recently shown to be highly efficient at cross-presenting to CD8 T cells and to express high levels of XCR1 in 2 of 4 studies published in 2010 although the latter was only confirmed with PCR (Crozat et al. 2010; Jongbloed et al. 2010). These cells also express Batf-3 and IRF-8, TLR3 but not TLR9 which is expressed on the murine DCs, and produce IL-12 when stimulated. Furthermore, they have been shown to be able to phagocyte dead cells. As described by Villadangos and Shortman (Villadangos and Shortman 2010), the studies to search for a homologue of the CD8α DC subset had been concentrated on SLOs like spleen and tonsils as the cells develop into immature DCs once accessed the organs themselves. The problem faced by scientists in defining the exact

correspondence of these cells to CD8α DCs regards the poor accessibility of LNs in humans due to ethical and logistical issues. Further functional studies can therefore only be conducted in *in vitro* systems.

CD8α DCs have to date been described to be highly involved in cross-presentation of soluble and intracellular antigens to CD8 T cells and to aided by migratory DC in this task. They are the most efficient DC subset in cross-presentation and have specific membrane receptors which mediate uptake and phagocytosis of apoptotic cells.

### 2.5.11 Plasmacytoid dendritic cells

Plasmacytoid DCs were first identified in human blood at the end of the 1950s by three groups, but their existence was not confirmed in mouse and linked to the human counterpart until the mid 1990s (Galibert et al. 2001). Human pDCs are distinguished by their phenotype, defined as $CD4^{+}$, $CD45RA^{+}$, $CD123^{+}$ (IL-3Rα), (MHC-II) HLA-$DR^{low}$, $CD11c^{-}$, $BDCA2^{+}$ and $BDCA\text{-}4^{+}$. Rarely found in the spleen in the steady state, this subset resides mainly in secondary lymphoid organs and in the blood. Mouse pDCs are $CD11c^{int}$ $B220^{+}$ Ly-$6C^{hi}$, express TLR7 and TLR9 but not TLR2, TLR3, TLR4, or TLR5 being therefore unable to recognise bacterial products like LPS. They recognise viral DNA through TLR7 and TLR9 and produce type-I IFN as a response: this induces cDC proliferation and increases their ability to cross-present. They also stimulate CTL and NK cytotoxic activity functioning as a link between innate and adaptive immunity. Plasmacytoid DCs are localised in SLOs, and other peripheral organs like lung, skin and liver.

The precursor for pDCs is believed to be of lymphoid origin despite experiments suggesting that there might be a common progenitor to myeloid cDCs and pDCs (Liu and Nussenzweig 2010). This precursor is immobilized from the BM by GM-CSF and stimulated to develop and differentiate into immature pDCs by Flt3 both in humans and mice (Arpinati et al. 2000). Flt3-deficient mice in fact have lower

numbers of pDCs while transgenic mice overexpressing Flt3 have elevated numbers of pDCs (Brawand et al. 2002). Moreover, Oldstone's lab showed in 2004 how persistent viral infection with LCMV CL13 is able to reprogram pDCs into cDCs (Zuniga et al. 2004). In the immature state, pDCs have a half-life of about 2 weeks (O'Keeffe et al. 2002).

Plasmacytoid DCs were originally called IPC (interferon-producing cell) as their role is mainly associated with viral and intracellular bacterial infections, which they help to clear mainly through the secretion of type I IFNs. They sense viral nucleic acid through the endosome-located TLR7 and TLR9: TLR7 senses ssRNA and TLR9 senses dsDNA and CpG oligonucleotides, synthetic mimickers of bacterial DNA which is rich in CpG regions (Iwasaki and Medzhitov 2010). TLR ligation in early endosomes triggers the production of type I IFN through the phosphorylation and translocation of IRF7 to the nucleus. Activation of the TLR in the late endosomes instead triggers maturation of the pDCs, cytokine production and at high levels, antigen presentation. Plasmacytoid DCs can produce, other than type I IFN, cytokines like IL-6, IL-12, TNF-α and chemokines like MIP-1α, MIP-1β, and RANTES. IL-6 and both type-I IFNs in particular can induce B cell maturation to antibody-producing plasma cells (Poeck et al. 2004). The production of these cytokines suggests that pDCs are involved in the regulation of inflammation, thus providing a link between adaptive and innate immunity. Type-I IFNs and IL-12 stimulate cytotoxic activity from NK cells and INF-γ production (Krug et al. 2004), and can stimulate macrophages and their release of inflammatory molecules.

In steady-state conditions pDCs are particularly abundant in the extra nodular compartment of LNs, around the HEV and in mucosal-associated lymphoid tissues, but they can accumulate in infected tissues when inflammatory signals are released, due to expression of CXCR3. CXCR3 binds chemokines CXCL9 and CXCL10 and its expression seems to be required for migration of pDCs to LNs in inflammatory conditions (Cella et al. 1999). Since pDCs are present in the blood it is also thought that these cells can travel to the LNs in the steady state through the HEV: they express CCR7 for constitutive migration to the LN through the lymphatic system. Nonetheless, pDCs are more frequently found in lymphoid tissues during infections. They are therefore thought to be important in bringing the necessary costimulation signals for activation during systemic infections, for prolonged *in loco* antigen-

dependent CTL stimulation and for priming of responses against pathogens with tropism for lymphoid tissues. Also, various studies demonstrated how the contribution of pDCs in interferon production might be only secondary when other cell types are also stimulated (as alveolar macrophages for example). This will be discussed further in the Discussion Chapter.

The role of pDCs as antigen-presenting cells has been long debated. They are poor inducers of T cell stimulation since they display low levels of costimulatory molecules, have scarce endocytic capacity and do not have an efficient antigen processing and presentation machinery. Plasmacytoid DCs express both MHC-I and MHC-II but have a different regulation system of the complexes' half-life compared to cDCs. In particular, the promoter of the transcription factor regulating MHC-II transcription machinery, CIITA, in pDC is not silenced upon maturation of the cell. The direct consequence is that pDCs continuously recycle MHC-II to the surface, presenting antigens they encounter but not retaining presentation of antigens previously encountered. Low expression of two cathepsins involved in antigen processing (Cathepsin S and Cathepsin L) has been found in pDCs (Fiebiger et al. 2001): therefore, pDCs cannot be as efficient as cDCs at presenting antigen at a lymphoid site. Nonetheless, pDCs are apt at presenting antigens in very high systemic antigen concentration or when antigen is available locally at the site of pDC-T interaction. Based on the latter assumption, and also due to their capacity of migrating to the infected tissue, pDCs might be involved in presenting antigens at the site of infection to sustain the T cell response, other than providing costimulatory signals as described above. This happens for example in malaria immunity (Cockburn et al. 2010). Moreover, stimulation with CpG-rich oligos has been shown to induce antigen presentation in pDCs, which can promote a Th1-polarised proliferation of CD4 T cells (Boonstra et al. 2003). Recent studies conducted by Pucchio and colleagues, also suggest that human pDCs could, when activated with influenza virus, induce proliferation of T cells, both CD8, therefore demonstrating ability to cross-present, and CD4 T cells (Di Pucchio et al. 2008).

Lastly, studies in humans and in mouse have suggested that pDC might be inducers of $T_{regs}$: freshly isolated antigen-pulsed immature pDCs can only stimulate a poor proliferation of T cells but, in presence of IL-2, they have shown to confer T cells a

regulatory function (Boonstra et al. 2003; Moseman et al. 2004). This tolerogenic role has been suggested to be location-dependent. More studies are needed in this regard.

Plasmacytoid DCs have a different developmental path compared to other migratory DCs. They have a longer half-life and are high producers of type I interferon, a fundamental tool in immune responses against viruses. They have an indirect role in the priming of CD8 T cells as they are not good antigen presenting cells unless highly stimulated. Their role is mainly that of supplier of costimulatory signals and cytokines.

## 2.6 SERPINB9: of humans and mice

Spi6 (SerpinB9) is a murine serine protease inhibitor (serpin) that has been shown to inhibit intracellular neutrophil elastase (Zhang et al. 2007) and to be a physiological inhibitor of granzyme B (Sun et al. 1997; Zhang et al. 2006). Spi6 is expressed in CD8 T cells, where it has a protective function against self-inflicted apoptosis, and in DCs, where its expression is upregulated specifically after maturation, its role is still controversial. Spi6 has a human homologue, PI-9 (SERPINB9) (Sun et al. 1996; Sun et al. 1997).

### 2.6.1 Protein structure, location, function

Spi6 is composed of nine α-helices, three β-sheets and a flexible reactive centre loop (RCL). The RCL structure is essential for its function, as it contains a sequence that resembles the natural substrate of the protease targeted (Wei et al. 1994). The serpin is held in a metastatic state until it reaches the target, with the RCL exposed as "bait" for the protease. As the protease binds to the serpin and cleaves it, it undergoes a conformational change that locks the protease in place and keeps it bound irreversibly with a covalent link (Huntington et al. 2000). The stoichiometry of this reaction is 1 : 1, therefore a tight regulation of the levels of Spi6 is crucial for its proper function. Once locked in place, GrB cannot be released and the conformational change of the protease makes the complex highly susceptible to proteolysis (Huntington et al. 2000) and stable when run on a Western blot (Phillips et al. 2004). Inhibition of the ubiquitin-proteasome machinery in fact showed accumulation of PI-9/GrB complexes in YT cells (Hirst et al. 2003).

### 2.6.2 Serpinb9/Spi6 in CD8 T cells

Spi6 in mice is expressed at low levels in naive CD8 T cells and is significantly upregulated following activation and maturation to CTL and memory cells. In both cases, Spi6 expression has been proven to correlate with that of GrB and to have a protective function against the action of GrB that leaks from the granules and could trigger apoptosis in the CTL itself. In the same study, Ashton-Rickardt and colleagues also demonstrated that Spi6 has a role in maintaining cytotoxic granule architecture in place, as detailed in Figure 24 (Zhang et al. 2006).

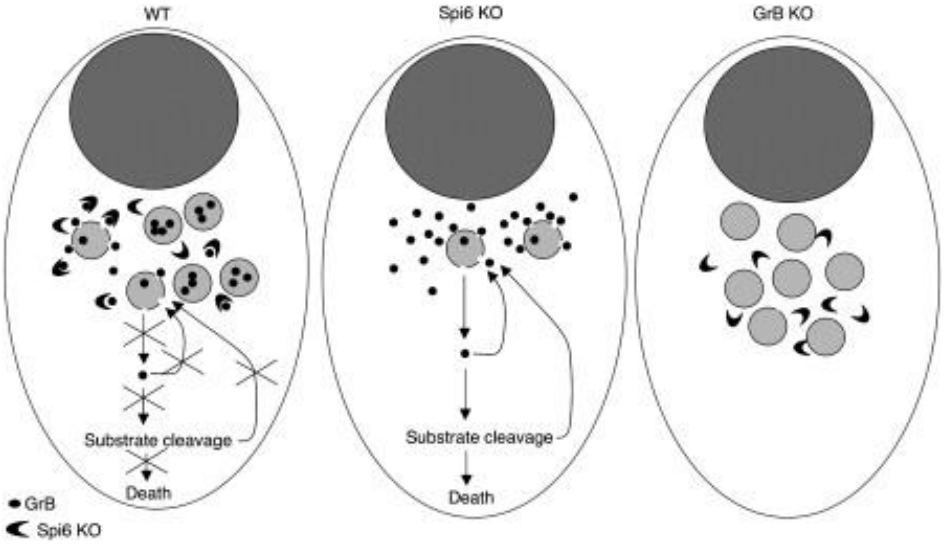



**Figure 24: Spi6 role in maintaining cytotoxic granule architecture. Spi6 binds GrB leaking in the cytosol from the granules in WT CTLs thus inhibiting its pro-apoptotic activity. In absence of Spi6 the number of granules in CTLs is defective and GrB activity is increased intracellularly (Zhang et al. 2006).**

A transgenic mouse expressing Spi6 under the human D2 promoter (Zhumabekov et al. 1995) was created to study Spi6 role in the development of memory cells. Overexpression of the serpin in these cells promoted long-term survival during the memory phase therefore increasing the size of the memory pool after LCMV infection, not affecting the size of CTL expansion (Phillips et al. 2004). Notably, other experiments conducted later in absence of Spi6, showed that the CTL clonal burst in these mice is impaired but the memory pool is comparable to WT mice, thus

demonstrating that the size of the memory pool is likely not to be directly correlated to Spi6 (Zhang et al. 2007). The proposed explanation for this is that memory precursors might express low levels of GrB therefore not needing to upregulate the anti-apoptotic molecule Spi6. In this model, Spi6 is not needed for memory formation. Notably, Spi6 levels in memory CD8 T cells have been shown to be 10 times higher than in naïve CD8 T cells (Bots et al. 2005). This can be explained by the property of memory cells to be able to rapidly re-activate effector functions. In this context, Zhang and colleagues showed that Spi6 absence in memory cells causes impaired re-expansion when memory cells are stimulated (Zhang et al. 2007).

This serpin is also expressed in NK cells, where it protects from misdirected GrB action as reported just last year by my colleagues (Ansari et al. 2010). It is also upregulated in DC during maturation induced by both CD40L and LPS, although studies on both PI-9 in humans and on Spi6 in mouse reported low levels of Spi6 mRNA also in immature DCs (Medema et al. 2001).

A recent report by El Haddad and colleagues in collaboration with our group has also shown expression of Spi6 in mesenchimal stem cells mirroring the human homologue expression (El Haddad et al. 2011). Notably, in a study by Medema and colleagues, Spi6 over-expression could protect tumour cell lines from CTL-induced apoptosis (Medema et al. 2001).

### 2.6.3 SERPINB9/PI9

PI (proteinase inhibitor)-9 is the human homologue of Spi6 and, like Spi6, can irreversibly inactivate GrB (Sun et al. 1996). Studies with PI-9 have shown its localisation is not limited to the cytoplasm as it has also been identified in the nucleus (Bird et al. 2001). It is expressed in CD8 T lymphocytes upon maturation with ConA/PMA, which mimics TCR triggering, but not with IL-2 alone, and in NK cells upon activation (Hirst et al. 2003). Its function in these cells is that of an anti-apoptotic molecule, as it is for the murine homologue (Figure 25). It is also expressed

in DC upon maturation (Bladergroen et al. 2001), in endothelial and mesothelial cells *(*Buzza et al. 2001*),* mast cells (Bladergroen et al. 2005) and at immune-privileged sites (Hirst et al. 2001). Studies have been conducted to investigate the modulation of the protein and reported that PI-9 has a slow turnover rate. It is upregulated by IL-2 and LPS in generic monocyte population but not in lymphocytes, and this effect is inhibited by blocking NF-kB (Classen et al. 2006). PI-9 has been shown to have an estrogen-responsive unit downstream of its promoter and to be expressed in an estrogen-dependent way in hepatocytes (Kanamori et al. 2000). Similarly, PI-9 is overexpressed in the estrogen-responsive cells of breast cancer (Jiang et al. 2006) .

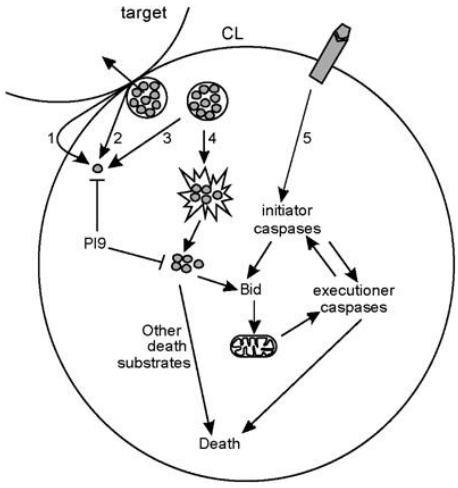



**Figure 25: Mechanism of CTL protection from apoptosis mediated by PI-9. PI-9 inhibits small amounts of GrB that might be endocytosed after degranulation (1), GrB leaking from loaded granules (2), GrB that might escape loading into granules during synthesis and storage processes. When GrB concentration is higher, PI-9 is not able to control its cytotoxic action and apoptosis is triggered (4). PI-9 has not apoptosis inhibition ability when this is triggered by death receptors (Kaiserman and Bird 2010).**

Overexpression of PI-9 has been shown to protect CTL-induced killing via the indirect pathway, involving GrB and perforin, of HeLa cells *in vitro* (Bird et al. 1998; Cunningham et al. 2007). This anti-apoptotic expression of PI-9 has been investigated in hepatocytes, where it has been found to be upregulated in a IFN-γ, TNF-α-dependent manner, for protection from CTLs and as a shield against cellular

immunity in estrogen-sensitive tumours (Kanamori et al. 2000; Barrie et al. 2004; Jiang et al. 2006; Stout-Delgado et al. 2007).

In a paper from 2006 Kaiserman and colleagues describe differences in the RCL sequence, in the kinetics and in the affinity to GrB for PI-9 compared to Spi6. The anti-apoptotic function of serpins was thus shown to be species-specific (Kaiserman et al. 2006; Kaiserman and Bird 2010).

PI-9 expression seems to be correlated to the need of both effector and stromal cells to defend themselves against inflammation and GrB-induced tissue damage, thus making the molecule a mediator of protection from apoptosis. PI-9 is not only expressed by NKs, CTLs and T regulatory cells but also by endothelial and mesothelial cells and at immuno-privileged sites (testis and placenta). The hypothesis of a protective function is also supported by the observation that PI-9 is upregulated in infected hepatocytes (Stout-Delgado et al. 2007) and in tumour cells, where its function is involved in escape from the immune response (Medema et al. 2001a). Studies on PI-9 upregulation by tumors to evade the immune system have been conducted mainly on Hodgkin lymphoma, small-cell lung cancer and prostate cancer (Bladergroen et al. 2002; Ray et al. 2011). Other studies reported the presence of extracellular PI-9 in the serum of cancer patients and in highly inflammatory diseases. As PI-9 has no secretory sequence, a role for extracellular PI-9 is highly unlikely and its serum levels are thought to be due to release by necrotic cells (Kaiserman and Bird 2010).

The inhibition of lytic enzymes is, as expected, a mechanism also exploited by viruses to evade the immune response: a number of viruses encode viral granzyme inhibitors. The pox virus, for example, is able to inhibit GrB by binding it with its cytokine response modifier gene-A (CrmA), which also binds and inhibits caspase-1 and -8 (Quan et al. 1995). The parinfluenza virus 3 instead degrades GrB mRNA with an unknown mechanism. In humans, adenovirus inhibits GrB through its Ad5-100k, which binds GrB and gets cleaved at a very slow rate. This is expressed in very high concentration in infected cells so that GrB action is completely absorbed by it (Andrade et al. 2001).

Since inhibition of GrB is desirable in a number of cases, where for example the expression of GrB is at the basis of disease pathogenesis, like in autoimmune diseases

(Casciola-Rosen et al. 1999), synthetic inhibitors of GrB have been produced. They include derivatives of isocoumarin, peptide chloromethyl ketones, and peptide phosphonates. As these compounds lack specificity, new compounds are being studied that have a specific 1,2,3-triazole moiety which confers more specificity (Willoughby et al. 2002)

An interesting report was that of Mharus and colleagues' in 2004: GrM was reported to be able to inhibit PI-9 activity when released extracellularly cleaving its RCL in an *in vitro* study about PI-9 expression in tumours. However, the study involved pretreatment of the cells with GrM before degranulation of CTLs, therefore not showing direct competition between GrM and GrB for binding to PI-9, not allowing to verify whether the affinity of GrM for PI-9 is higher than that of PI-9 for GrB, which would have meant this granzyme can efficiently bind PI-9 before it inhibits GrB and therefore can inhibit its activity (Mahrus et al. 2004). The road to a synthetic optimal GrB inhibitor is therefore still long.

## 2.7 Lymphocytic Choriomeningitis Virus

The Lymphocytic Choriomeningitis Virus (LCMV) is an enveloped single strand RNA virus, member of the *Arenaviridae* family which has now been widely accepted as a model for the study of CD8 T cell immune responses. Its name was coined in 1934 by Charles Armstrong who had isolated it from humans for the first time in 1933. Nevertheless, the common reservoir and host is the common *Mus musculus* (mouse).

In mice, the virus is transmitted vertically to the litter though intrauterine infection and it is spread among mice via saliva, faeces, nasal mucus, milk, semen, urine. When in contact with these, humans can become infected and the virus is especially dangerous when caught by pregnant women as it can be passed on to the foetus. Studied and characterised by Oldstone in 1986 (about), the virus can cause a readily resolved acute infection in low doses in an immunocompetent mouse, while it can cause chronic infection if the mouse is immunodeficient. An immunosuppressive strain called Clone 13 (CL13) has been characterized as able to induce chronic infections in immunocompetent mice.

The genome of LCMV is composed by 2 segments of RNA: S and L. S (short) encodes, at its 5' end, for a precursor of the glycoprotein (GP), post-transcriptionally cleaved into GP-1 (amino acids [aa] 1–264) and GP-2 (aa 265–486), and for nucleoprotein (NP) genes at the 3' end. GP-1 and GP-2 form tetramers and are responsible for receptor-binding to the target cell (Borrow and Oldstone 1994), while NP associates with the viral RNA to form the nucleocapsid (NC) used by the viral polymerase as a template. L (long) encodes for the viral polymerase (L) and Z genes, a small RING finger protein (Pedersen 1971). The association of NC and the polymerase forms the so-called viral nucleoriboprotein (RNP) which is the smallest actively infective unit of LCMV and any Arenaviridae virus.

### 2.7.1 The Armstrong strain of Lymphocytic Choriomeningitis Virus

The virus is available for research purposes in several derived strains, classified by their capacity to establish an acute versus a chronic infection in mouse. The strains causing an acute non-cytolytic infection are LCMV-Armstrong (Arm), -WE/Arm reassortant, -Traub and –DOC, all of which induce a strong CD8 T cell response. LCMV Armstrong 53b (LCMV Arm) is a clone triple-plaque purified of ARM CA 1371: the virus is non-cytolytic and in immune-competent mice it provokes a strong CTL-dominated immune response which resolves itself in about 8-10 days. MHC-II, IFN-γ genes and CD4 T cells are not required for its clearance, therefore making infection with LCMV Arm a good model for studies on CD8 T cells responses and CTL-mediated viral clearance (Matloubian et al. 1994). The critical importance of the CTL response is evident in perforin-deficient LCMV-infected mice, which cannot clear even a low dose of non-immunosuppressive virus (Kagi et al. 1994; Zhou et al. 2002; Fuller and Zajac 2003).

LCMV is detectable in the blood of an intact B6 mouse from 24h p.i. and it reaches its peak by day 3-4. LCMV acute infection causes the upregulation of RANTES, MIP-1α and β, IP-10, MCP-1 and of the receptors CCR1, CCR2, CCR5 and CXCR3 (Thomsen et al. 2003). Intracranial inoculation of the virus in adult immunocompetent mice leads to a lethal meningitis, from which its name derives (Hotchin 1962).

### 2.7.2 The Clone 13 strain of Lymphocytic Choriomeningitis Virus

The LCMV Armstrong Clone 13 (LCMV CL13) strain can cause chronic infections. A triple plaque purified from the spleen of an adult BALB/WEHI mouse infected neonatally with LCMV Arm, CL13 has 5 nucleotide changes out of the 10.600 nucleotides which compose the RNA genome of the virus compared to Arm. Of the 5 changes, only 2 have a significant impact on its viral activity. The first one is in the L segment, the Lysine (K) in Arm to Glutamine (Q) in CL 13 at aa 1079, while

insignificant ones are at positions 1268, 3267, 6192 (Salvato et al. 1991). The second significant change is on the S segment and results in 1 aa difference as well: in position 856 there is a change from A to G, meaning the aa 260 (of GP-1) is a Phenylalanine (small aliphatic) in Arm and a Leucine (bulky aliphatic) in CL13. The second, irrelevant, change on the S segment is in position 1298 (again A to G) and doesn't result in any amino acid change (Salvato et al. 1988). Worth noticing is that the site of major CTL recognition is unchanged in the two strains, meaning that the antigen-specific CD8 T cells arisen by an Arm infection can also recognise CL13 virus (Salvato et al. 1991). This is a major point for my study as we will see later.

#### 2.7.2.1 CL13 causes chronic infection

There is in CL13-infected mice but a minor depletion of T cells as LCMV infects a very low level of lymphocytes (Borrow et al. 1991). Depletion of antigen-specific CTLs directly induced by CL13 is therefore not at the basis of the chronic infection established by CL13. At the origin of persistent infection is, instead, the specific affinity of CL13 for α-distroglycan (α-DG), a surface molecule highly expressed by DC in the white pulp (interdigitating dendritic cells), which makes DCs the cell type the most susceptible to CL13 infection. Alpha-DG has a role in cell assembly and organisation of the basement membranes as it is a highly versatile cellular receptor for proteins of the extracellular matrix (ECM). Alpha-DG was identified as the receptor for LCMV in 1998 (Cao et al. 1998) and is a receptor for several other arenaviruses. Since Arm and CL13 only differ in their GP, the discovery of this receptor opened the discussion on the importance of the receptor binding in the dynamics of infection of the two viral strains and on how this point mutation was influencing this binding to the receptor. Studies before this discovery had already indicated that LCMV Arm and CL13 were showing a coincident pattern of infection in the spleen at day 1 p.i., but by day 3 this pattern had differentiated. Arm-infected mice showed viral nucleic acids within the red pulp while in CL13-infected these were localised within the white pulp. In the same piece of work, it was demonstrated that CL13 infects more DCs (about 60%) than Arm does (about 15% or less). CL13 achieves immunosuppression using these cells as a conduit for infection. The Armstrong strain, was confirmed, replicates in the red pulp of the spleen, while CL13 replicates in DCs in the T-cell areas. Finally, they concluded that "Immune

suppression induced by LCMV clone 13 is associated with a CD8-dependent loss of interdigitating dendritic cells from periarteriolar lymphoid sheaths in the spleen and, functionally, with a deficit in the ability of splenocytes from infected mice to stimulate the proliferation of naive T cells in a primary mixed lymphocyte reaction. Dendritic cells are not depleted in immunocompetent Armstrong-infected mice" (Borrow et al. 1995). As further confirmation, only antibodies against GP-1 exhibit neutralizing activity (Borrow and Oldstone 1992). Sevilla and colleagues conclusively showed that the point mutation in CL13 strain augments the affinity for the α-DG receptor (Sevilla et al. 2000). In particular, this α-DG is highly expressed by splenic $CD11c^+$ $DEC\text{-}205^+$ DC: the CL13 strain infects and replicates in DCs at a high rate, in particular DCs of the white pulp, causing immunosuppression (Sevilla, et al. 2000) and/or direct viral effect on DC function (Sevilla et al. 2000; Smelt et al. 2001). In both splenic and LN DCs, CL13 infection results in low levels of MHC-I, MHC-II and costimulatory molecules and in failure to migrate. The inability to stimulate T cells is a consequence of this lack of migration. Immunosuppressive strains also infect BM precursors therefore blocking development into new DCs (Sevilla et al. 2000). DC loss in CL13 infection is clearly CD8-dependent. When the antibody NLDC145 (anti-DEC-205), which recognises long-lived DCs, is used to stain CL13 chronically infected mice, no staining is obtained, thus indicating that these cells are depleted from the spleen. High doses of immunosuppressive virus initially induce a very strong CTL response which is effective but not long lasting and that progressively turns into exhaustion (Zhou et al. 2004).

## 2.8 Dendritic cell priming of viral responses: Rationale of the study and Spi6 role

The project focuses on the function of Spi6 during DC priming of CD8 responses in viral infections. More specifically, it investigates Spi6 ability to protect mature antigen-presenting DCs from GrB-induced apoptosis during the process. This chapter contextualizes CD8 T cells and DCs describing the interactions and dynamics between these two cell types during a viral infection, including the CTL-mediated killing of DCs during priming. It also describes the work supporting the need for a prolonged antigen presentation to achieve optimal CTL activation and it includes previous data supporting the hypothesis and the rationale of the study.

### 2.8.1 Naïve CD8 T cell and dendritic cell interaction

Interaction between DC and T cells is of primary importance, as it can shape the immune response. The strength and magnitude of the CTL response are crucial in viral infections to achieve clearance of the pathogen. A sufficient amount of specific $CD8^+$ T cells needs to be activated to produce a clonal burst quantitatively and qualitatively adequate to successfully address the infection. Several APCs can partially activate $CD8^+$ T cells, but DCs only (Jung et al. 2002) are able to present exogenously captured antigens on MHC class I molecules through cross-presentation (Heath et al. 2004). Cells presented with an antigen in an MHC context alone will not be activated but, on the contrary, become anergic. The Danger Theory (Matzinger 1994) states that the immune system cannot really distinguish between self and non-self by itself: discriminatory in this process are the type and strength of signals conveyed by professional APCs, inflammatory cytokines and danger molecules to a T cell during priming. For an effective activation of the maturation program of CD8 naïve T cells to CTLs, together with inflammation and danger signals, costimulatory signals have to be delivered to the T cell. Only professional APCs can deliver costimulatory signals through their B7 and other coreceptor molecules. This tripartite

signalling system sustains maturation and proliferation of the cell and triggers the production of IL-2 to ensure survival of the T cell.

#### 2.8.1.1 Short-term versus prolonged antigen presentation

DC maturation status, adequate survival and lifespan and kinetics of antigen presentation are critical to convey adequate signals to naïve $CD8^+$ cells (Kaech and Ahmed 2001). The duration of antigen stimulation needed for appropriate T cell priming has been a much debated issue. According to the original "Autopilot theory", a 2hr-contact is enough for full activation of CTLs, with development of cytotoxic functions in the same cell in the following 8 hrs (Bevan and Fink 2001), but other studies show the need of an additional 48-72 hrs to develop further effector functions (Curtsinger et al. 2003). Also, division of antigen-specific CTLs is observed in the LNs by 66-72 hrs (Hermans et al. 2000).

Several studies report the importance of long-term antigen presentation in primary immune responses to sustain and ensure the development of a proper CTL response through activation, differentiation and proliferation of naïve CD8 T cells (Usherwood et al. 1999; Belz et al. 2004). Prolonged presentation involves a steady presence and flux of antigen to the draining LNs: this is strongly correlated to DC availability. DC availability depends on DC survival and migration ability, which when optimal in mature DCs, allow for continuous recruitment of migratory DCs to the LN and antigen transfer to the resident ones.

The overall magnitude and duration of a CTL response are the result of a delicate balance between timing of migration, level of maturation, antigenic load and survival of DCs. To note, pre-DCs inhabit SLOs therefore participating to the replenishment of killed DCs. The dynamics of interaction between DCs and T cells are also dependent on the location in the SLO.

#### 2.8.1.2 Dynamics of dendritic cell-T cell interaction

After a naive CD8 T cell has entered the LN during an infection, the search for antigen takes place in 3 stages: during the first 8 hours the CD8 T cells have short contacts with many DCs, they upregulate CD44 and CD69 but are not properly

activated. Between 8 and 24 hours, the T cell-DC interactions become more stable, lasting up to 1 hr: the CD8 T cells have started the activation program and release IL-2 and IFN-γ but they do not divide yet. During the third phase, after approximately 24 hrs, T cells dissociate from DCs, proliferate and migrate out of the LN (Mempel et al. 2004). In the spleen, the pattern is similar: shortly after infection, CD8 T cells and DCs move to the subcapsular zone following an antigen gradient. In the subcapsular zone DCs can pick up antigen from resident infected macrophages which do not express the coreceptors needed for costimulation and which therefore cannot prime CD8 T cells responses (Mueller et al. 2007).

The first few hours are of key importance in directing the initiation of a CTL response, but its sustenance requires longer interaction. In a study with influenza virus infection, Belz and colleagues showed that antigen presentation still occurs several days after CTL maturation in primary infections, but rapidly decreases in secondary infections. Through CFSE labeling *in vivo*, they showed that ablation of DCs from day 3 post-infection severely impairs CD8 T cell expansion, and that DCs still migrate to LN after day 2 post-infection. The impaired response is therefore due to elimination of DCs in the LN rather than inefficient migration. In memory response, the same impairment in the response is seen when ablation occurs at day 3, but not from day 5 onwards. In the same paper it was also showed how in pfn deficient mice there is an accumulation of antigen-specific CTLs. Prolonged antigen presentation is thus required for optimal expansion of CTL primary response, but not in memory responses. Also, the magnitude of T cell expansion is not strictly dictated by antigen load and persistence (Belz et al. 2007).

#### 2.8.1.3 Dendritic cell positioning in the secondary lymphoid organs

Interactions between DCs and T cells and their location in the SLOs are tightly regulated and not left to chance. DCs enter LNs through the afferent lymphatic vessel and firstly line up around the HEV in a strategic position to maximise the encounter with T cells, which access the LNs coming from the blood. This was supported by Mempel's work showing that T cells make contact with APCs in the HEV. Once inside the LN, the cells move on to the T-cell cortex. One study, performed with a viral infection model as opposed to previous works where DCs were loaded, labeled

and injected in the mouse model, has conversely shown that T cells entering the LN in a naive status encounter activated antigen-loaded DCs in the subcapsular sinus or in the interfollicular area instead (Hickman et al. 2008). Shortly after infection, the T cells were shown to move to the peripheral regions of the LN.

In the spleen, similar results underlined the difference between a viral model and adoptive transfer of DCs. In mice infected with LCMV, the CD8 T cells were shown localise in the red pulp and not in the T cell areas like in naïve mice when DCs were loaded with antigen and injected in intact mice. It was also shown that downregulation of CCL21causes temporary abrogation of naïve T cell migration to the T cell zone and has an impact on DC migration as well (Mueller et al. 2007). The dynamics observed clearly demonstrate how the SLO environment changes between the steady and the inflammatory state, therefore making some *in vitro* models not suitable to test CD8 T cell priming.

Interestingly, growing evidence is supporting a model whereby, for infections like influenza virus or herpes simplex virus, CD8 T cells are activated and fully matured in the SLOs but need continuous stimulation in the peripheral infected tissues.

Studies showed that, after being primed and activated in the LN, CTLs reach the infected tissues where further *in situ* priming is required for optimal function and it is carried out by antigen-loaded $CD103^+$ DCs (Bedoui et al. 2009; McGill et al. 2009). CD103 is an integrin that marks migratory DCs and has been found to be expressed by LN-resident $CD8\alpha^+$ DCs. McGill & Legge reported CD8 T cell proliferation *in loco* sustained by migratory mature DCs (McGill et al. 2008). One more argument in favor of this model is the ability of naïve CD8 T cells to be successfully activated and proliferate after contact with antigen-loaded skin-derived or lung-derived $CD8\alpha^-$ DCs, as well as with $CD8\alpha^+$ LN-derived DCs (Belz et al. 2007). The need for further peripheral antigen presentation depends on virus type and infection modality.

#### 2.8.1.4 Collaboration between dendritic cell subsets: antigen transfer

A distinct mechanism allows for the transfer of antigen from migratory DCs to resident ones both in the LN and in the spleen, so to amplify the number of antigen-competent DC and therefore the chances of encountering the antigen-specific T cell

quickly (Carbone et al. 2004). Reports of migratory DCs instructing DCs resident in LN was also shown by Belz and Allan (Belz et al. 2004; Allan et al. 2006). Recent discoveries in the DC collaboration model describe a mechanism for antigen transfer called cross-dressing, where loaded MHC molecules are transmitted between DCs to activate memory CD8 responses (Figure 26).

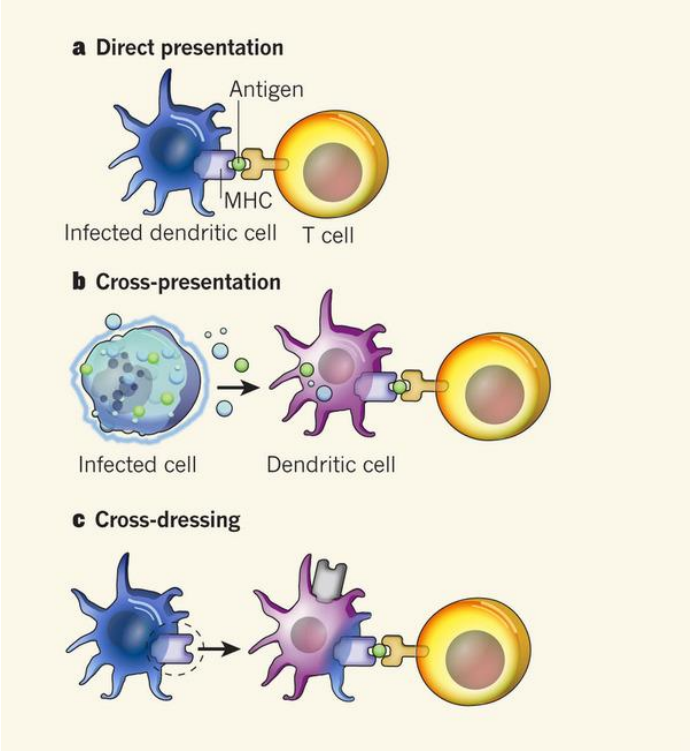



**Figure 26: CD8 presentation modalities: a) direct presentation; b) cross-presentation and c) cross-dressing (Yewdell and Dolan 2011).**

The process uses trogocytosis: the transfer of surface proteins or parts of plasma membrane between cells. Wakim and Bevan demonstrated that this mechanism is required *in vivo* for efficient memory CD8 responses, while it fails to promote and support maturation of naïve CD8 T cells. Exsosomes containing both pMHC-II and pMHC-I are released by DCs and uptaken by other DCs. When uptaken, the antigens loaded on MHCs are isolated and loaded on newly formed MHC molecules. Wakim and Bevan also showed that the cells involved in this cross-dressing are $CD8\alpha^-$ DCs (Wakim and Bevan 2011).

### 2.8.2 Dendritic cell survival

DCs regulate CTL responses through a feedback mechanism in place to avoid excessive CD8 T cell priming (Wong and Pamer 2003). This regulation is achieved by CTL-mediated killing of antigen-loaded DCs, allowing for the control of antigen availability. The higher the number of CTLs, the higher the number of DCs killed during priming and costimulation. DC levels, on which depend further CTL expansion and priming, are thus feeding back about CTL levels in a regulatory loop that keeps CTL expansion under control (Figure 27).

The mechanisms regulating DC survival are currently poorly understood. The studies which have addressed the correlation between DC levels and CTL expansion have done so via manipulating either antigen or DC availability in infection models. Stock and colleagues showed that elimination of the antigen source after the CTL response had started could negatively affect CTL expansion during a viral infection (Stock et al. 2004). In a similar study, when the bacterial load was cut down, earlier contraction of the immune response was seen (Porter and Harty 2006). Similar results were also obtained diminishing antigen presentation through the reduction of DC survival: deficient immune responses and lack of CTL expansion where observed as a result in LCMV infection (Yang et al. 2006). Accordingly, prolonging DC half-life by TRANCE treatment resulted in increased DC numbers reaching the lymph nodes and increased CD8 T cell priming (Josien et al. 2000) Also the co-transduction of Spi6 and viral DNA in a vaccine augmented the survival of DCs and the levels of the CD8 T cell response (Kim et al. 2003). The half-life of antigen-bearing DC may thus represent a crucial parameter for successful CD8 T cell priming.

The first to report that cytotoxic T cell-dependent killing of APCs can cause suppression of heterologous immune responses in intact wild-type mice was Zinkernagel in 1988 (Leist et al. 1988). E*x vivo* antigen-loaded DC rapidly disappear from lymph nodes after antigen-specific interaction with naïve CD4 T cells (Ingulli et al. 1997). These cells have been shown to be rapidly eliminated by CD8 T cells: antigen-loaded DCs are cleared in 20 hours by CTLs and in 72 hours by memory CD8 reactivated cells. One proof of the direct role of CTLs in limiting the survival of mature antigen-loaded DC is that the time course of acquisition of lytic effector function (more rapid for memory than for naïve cells) parallels the kinetics of

elimination of antigen-bearing dendritic cells (Hermans et al. 2000). A negative regulating loop working through the killing of DCs has also been observed in memory CD8 T cell responses (Wakim et al. 2008) to allow the containment and efficient resolution of CTL expansion (Wong and Pamer 2003).

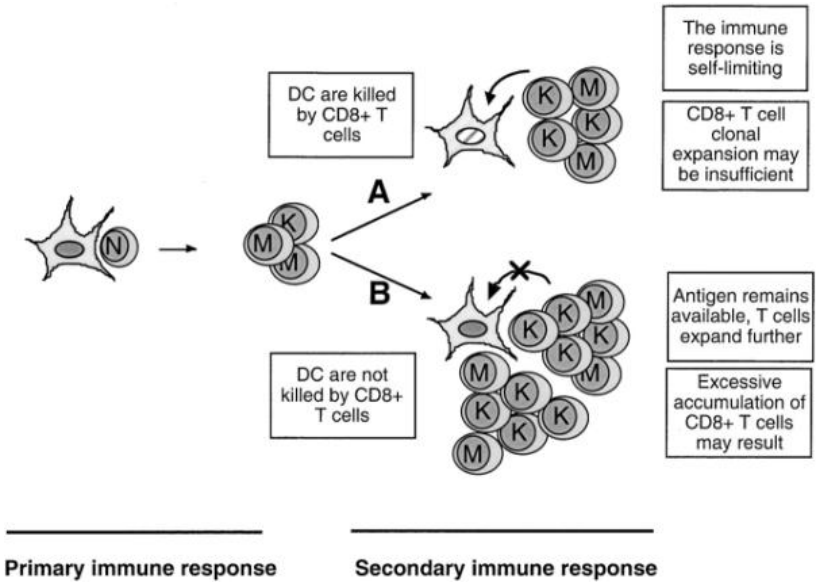



**Figure 27: Illustration of the negative feedback loop which regulates and limits the expansion of CD8 T cell responses (Ronchese and Hermans 2001).**

Guarda and colleagues studied how CTLs and central memory cells can access activated draining LNs, but not resting ones, as CXCR3 ligands are expressed on the lumen of HEV shortly after the beginning of a new response. Activated CTLs gain access to the activated LNs were they can kill DCs, therefore reducing the level of the ongoing primary response (Guarda et al. 2007).

DC elimination might be somewhat involved in the so-called “original antigenic sin” a mechanism that describes the inability of the adaptive response to produce a new CTL response to antigenic variants expressed by a virus after an initial CTL response has already been developed, by eliminating antigen-bearing DC in lymph nodes (Klenerman and Zinkernagel 1998). This is nonethelsee not at all clear and remains an hypothesis.

Interestingly, pfn KO mice cannot control LCMV replication. Mice with non-functional fasL and inactive Fas, on the contrary, can control LCMV infection (Walsh et al. 1994; Kagi et al. 1995). This suggests how the degranulation cytotoxic pathway is fundamental in this model and cytokines and Fas-FasL interaction alone are not sufficient to control the infection. In pfn KO mice there is a significant increase in IFN concentration, due to higher antigen presentation levels. In particular, DCs are not killed in pfn-deficient mice, demonstrating a major role for pfn in cytolytic activity of CTLs and also providing further testimony that DCs are target of GrB released by CTLs (Yang et al. 2006). Contrasting results were obtained by Lykens and colleagues when antigen presentation from pfn KO and WT mice was measured in a mixed leukocyte reaction. The results showed that responder cells (P14) were stimulated 5 times more by APC from pfn KO. No difference in the number of DC was seen in these mice, thus suggesting that these cells are just more efficient at presenting (Lykens et al. 2011).

Some studies support the theory that NK cells could be implicated in elimination of mature antigen-bearing DCs through release of perforin. Data has been acquired by Andrews and colleagues studying the effect of elimination of NKs and pfn in B6 (WT) MCMV-infected mice. They showed that the elimination of either NKs or pfn results in an increase in DC number during infection and that elimination of both results in no differences compared to an intact MVMV-infected B6 mouse (Andrews et al. 2010). It is important to notice that this viral infection is different from LCMV in that help by CD4 T cells and NK cells is required for the virus to be successfully cleared.

### 2.8.3 CD8 memory T cells and dendritic cells

In recall responses, the first and main signal for re-activation is antigen exposure in the context of DC priming. Therefore, an important factor in activation of CD8 memory cells is the survival and lifespan of DCs: when DCs are systemically depleted in mouse models the response to infection is drastically reduced (Zammit et al. 2005).

Reviewed in Bedoui *et al.* is the current model of memory activation involving DC-mediated antigen presentation (Bedoui et al. 2009). On this matter, Zammit and colleagues reported that the memory response to influenza is impaired in absence of $CD11c^+$ DCs (Zammit et al. 2005). Presentation via MHC-I from other hematopoietic or non-hematopoietic APCs is therefore not sufficient. In this respect, the ability described above of migratory DCs to present antigen to CTLs cells *in situ* is not applicable when talking about memory CD8 cells (Khanna et al. 2008). Belz and colleagues in fact reported that memory cells are more selective than naïve CD8 T cells in terms of priming requirements: only the lymphoid tissue resident subset of $CD8\alpha^+$ DC can fully activate memory cells. These have in fact limited ability to respond to tissue-derived antigen-loaded $CD8\alpha^-$ DCs but are instead primed efficiently by $CD8\alpha^+$ LN-resident DCs. This results show how the requirement for naïve T cells is less stringent, as some populations of $CD8\alpha^-$ tissue-derived DC are able to prime naïve CD8 T cells, as demonstrated in influenza virus infection for example (Belz et al. 2007).

As memory CD8 T cells require prolonged contact with antigen-loaded DCs (Iezzi et al. 1998), the latter are a target of degranulating memory cells. Secondary CD8 responses are clearly characterized by CD8-mediated loss of DCs (Ingulli et al. 1997; Wong and Pamer 2003; Belz et al. 2007; Guarda et al. 2007). Previous reports have shown how, despite DC number being a limiting factor during the early phases of CD8 T cell response (primary expansion), in memory responses this loss is not a limiting factor. Results by Belz's lab further reinforced the notion that memory cells mediate DC clearance in re-expansion, but in their hands data did not support such a mechanism in primary responses as the decrease in DC numbers seen was not proportional to the increase in CTLs (Belz et al. 2007). Interestingly, in contrast to primary immune responses, expansion of antigen-specific cells and viral clearance do not necessarily go together for secondary responses, as in some cases protection can be achieved without re-expansion of the existing pool.

## 2.8.4 Rationale of the study

Better survival of DCs is desirable: in chronic infections to re-awake the CD8 T cell response, in transplants to facilitate tolerance of the transplanted tissue and as a vaccination strategy. Spi6-alike molecules might provide DCs with better resistance to CTL-induced apoptosis, make them more fit to prime effective CTL response and even be modified to allow for tolerance induction.

Previous studies reported contrasting results regarding the role of Spi6 in protecting DCs from CTL-induced, GrB-dependent apoptosis.

### 2.8.4.1 Supporting data

The theory suggesting that CCR7 downregulation had a protective role for DCs in that it allowed to keep CTLs away from the LN was not satisfactory as, despite CCR7 downregulation, mature antigen-loaded DCs were shown to be still eliminated *in situ* (Hermans et al. 2000).

Evidence for Spi6 rendering mature DCs more resistant to cytotoxicity were reported in 2001 by Medema and colleagues. Murine cell line (D1) and murine BMDDCs were matured using LPS and CD40L, or $T_H1$ cells, and apoptosis in these cells was measured by DNA fragmentation levels after culture with CTLs for 6 hrs. The results showed that after maturation with LPS or anti-CD40, the D1 cell line was protected against CTL-induced apoptosis (residual killing was explained most likely by the fraction of DC which would not mature), while immature cells showed high levels of fragmentation. As DNA fragmentation is a late event in apoptosis, caspase activity was measured as well: CD40L-stimulated D1 cells did not show signs of caspase activation. The cell line and mouse BMDDCs were then analysed for Spi6 expression at mRNA level: immature cells showed some low expression, but CD40L-stimulated or LPS-stimulated ones had high expression of the protein. Spi6 levels were then measured *in vivo* in splenic murine $CD11c^+$ cells after an injection of anti-CD40 and in human monocyte-derived DCs: in both cases an increase of Spi6/PI-9 was seen after anti-CD40 stimulation. Spi6 expression and protection from CTL killing was then studied transducing D1 cells with a viral construct expressing Spi6:

these cells showed to be resistant to CTL killing even in an immature state. Interestingly, Medema and colleagues also showed that, although both $T_H1$ and $T_H2$ can induce maturation of DCs, only $T_H1$ cells can induce Spi6 expression in dendritic cells and make them insensitive to CTL killing in vitro, emphasizing the fact that protection is not directly correlated with maturation per se but with Spi6 expression. Finally, Spi6 levels were then measured by Western blot analysis in human monocyte-derived DC after LPS stimulation and were found to be high and stable for up to 96hr (Medema et al. 2001).

Further studies in humans were conducted by Hirst and colleagues, determining PI-9 levels by immunohistochemistry in human samples (Buzza et al. 2001). PI-9 levels were measured in different human cell types, included DCs, further strengthening the hypothesis that Spi6/PI-9 protects not only GrB-containing cells from self-inflicted apoptosis but also bystander and accessory cells from the effects of degranulation-deriving GrB. Quite interestingly both these populations showed quite high levels of PI-9 in the imamature/steady state. Through analysis with monoclonal antibody 7D8 (Buzza et al. 2001) Spi6 epression in blood-purified immature and, at higher levels, TNF-α-matured monocyte-derived DCs were also shown. Finally, lineage negative $CD3^-$ $CD19^-$ $CD11b^-$ $CD16^-$ DCs were isolated and PI-9 measured in Langerhans cell precursors (no expression) and pDCs (intermediate levels). This distribution suggests different needs in different cell subsets and is the reason for the analysis in different subset of mouse DCs which is achieved in this project.

#### 2.8.4.2 Controversial data

The role of Spi6/PI-9 as a protective agent against GrB-mediated apoptosis was undermined in 2008 when Ronchese's group published a study whose results contradicted Medema and Hirst's *in vitro* results (Andrew et al. 2008). The same group had previously reported how DCs are susceptible to CTL killing when presenting cognate antigen on MHC class I molecules (Yang et al. 2006). They wanted to verify whether anti-CD40 and/or LPS stimulation could make DCs resistant to CTL killing *in vivo*.

They investigated Spi6 levels in activated (LPS or CD40 stimulation) murine BMDDCs through detection of mRNA levels, showing upregulation after as early as

6 hrs, a peak at 24 hrs, and high levels until 48 hrs later in all conditions. Protein levels were then measured with an antibody against PI-9 in Western Blotting: to be noted is that, as previously discussed, the homology of Spi6 with the human protein is not 100%. BMDDCs were then cultured with activated effector cells from L318 mice and killing was measured by propidium iodide staining in presence of antigen. L318 mice carry a transgenic TCR specific for fragment 33-41 of the gp of LCMV (gp33) in association with H-2D$^{b}$ (Pircher et al. 1989). LPS-matured BMDDCs showed significant lower killing levels compared to the immature ones, like in previous results, but residual killing was seen. The residual killing was clearly due to the normal rate of death of cells in culture and to LPS pro-apoptotic action. A controversial point is the addition of peptide to the culture, which induces activated CTLs to degranulate independently from pMHC recognition.

One further step with respect to previous results was then taken, and DC survival in *vivo* was measured. Mature BMDDCs were labelled with fluorochrome for identification and injected into naïve or gp33-DC-immunised mice. The cells survived to some extent in naïve mice, while they failed to survive in immunized mice. Adoptive transfer of the CTLs used before was then conducted and DCs injected 24hrs later: draining LN analysis revealed that DCs were killed to the same extent whether they were mature or not. BMDDCs were then injected in mice that had been immunized with gp33-DCs 7 weeks earlier: analysis of draining LNs at 72hrs showed that LPS-matured and immature BMDDCs had been killed to same extent.

Data from this paper had therefore weakened Medema's findings about Spi6 role in DCs. Nonetheless, some considerations can be made regarding Andrew's experimental protocols.

The correlation between the measured percentages of injected DCs migrating to LN and their survival made by Andrew and colleagues was not fully clarified (Andrew et al. 2008). When recovering DC number in LNs, The group measured only the DCs that were able to migrate there. The LN analysis does not reflect the surviving population in this experimental setting, nor accounts for DC killing in the LN itself. Also, the system in Andrew's paper, due to the short duration of antigen stimulation,

does not mimic an infection. Moreover, LPS-matured DCs are expected to have a half-life shorter than 72 hrs, the timepoint chosen in Andrew's study (Ingulli et al. 1997; Hou and Van Parijs 2004).

The function of Spi6 in mature DCs does not involve making DCs completely immune from CTL killing. The absence of susceptibility to killing is seen only when overexpression of the protein is induced. In normal conditions, Spi6 regulates GrB-induced apoptosis levels, in my opinion based on the level of GrB and on the levels of CTLs. If Spi6 were to make DCs totally unsusceptible to CTL killing then controlling CTL response expansion would not be possible, as antigen-loaded DCs would sustain new priming and activation *ad infinitum*. This is valid both for memory and non memory system. The model used by Ronchese's group was not mimicking an infection model, nor measuring DC survival in an appropriate way. Finally, Spi6 has been seen at low levels in immature DCs, thus making these cells in certain conditions resistant to locally released GrB.

#### 2.8.4.3 Spi6 knock-out model and Spi6 requirement for cytotoxic T cell survival

Despite the critics that can be moved to the experimental model choice, the results obtained by Andrew *et al*. opened a controversy regarding the role of Spi6 in protecting mature DC against CTL killing. Their *in vivo* data was nonetheless limited to adoptive transfer of BMDDCs and comparison of mature to immature cells. The two major points that had not been studied were the levels of Spi6 in physiologically-matured DCs in an intact mouse and its role during DC priming of viral infections.

A KO mouse for Spi6 was created and characterized by Ashton-Rickardt's lab, in 2006, to complete functional studies and establish the physiological relevance of Spi6 action in CTL in an *in vivo* model (Zhang et al. 2006). Spi6 KO mice were obtained inserting a deletion in exon 7 which encodes 60% of Spi6 in mouse and includes the RCL. This mouse model was used in this project to study the role of physiologically-induced wild-type levels of the protein in DCs during the priming of an infection and the consequences of its absence.

LCMV Arm induces a primarily CD8-driven immune response, were CD4 T helper cells are not necessary for the successful resolution of the viral infection (Ahmed et

al. 1988) as anti-LCMV response is fully MHC-I dependent. Also, antibodies do not play a major role either in LCMV infection. Depletion of CD4 cells demonstrated that CD4 T cell help is dispensable in acute infection. In the same study was demonstrated how, on the contrary, CD8-depleted mice (β2-microglobulin KO mice) cannot resolve a LCMV Arm infection and become persistently infected (Matloubian et al. 1994). In Arm infection, CTL expansion is MHC-I-driven and therefore cross-presentation mediated: this is therefore a good model to study the survival of DC in priming of viral infections. The method was established in the lab and the Spi6 KO mouse strain gave me the opportunity to verify CD8 priming *in vivo* in a viral infection model.

LCMV was used in Zhang's work to infect Spi6 KO mice to study CTL response. A reduced number of antigen-specific CTLs in the cellular response to infection was recorded in Spi6 KO mice, as well as a higher percentage of apoptotic antigen-specific CTLs (see Figure 28).

Using a GrB KO Spi6 KO mouse to investigate the relevance of GrB in previous results, levels of CTL expansion and apoptosis were recorded and resulted comparable to wild-type. Zhang and colleagues concluded that, since the absence of GrB could restore the defect in the CTL response, Spi6 was involved at least to some extent in promoting the survival of effector CD8 T cells. By verifying the expansion levels of adoptively transferred LCMV-specific P14 cells and Spi6 KO P14 cells into a recipient injected with Arm, they also confirmed that the requirement for Spi6 in survival was cell-autonomous. P14 cells are transgenic for CD8 TCR which recognises the immunodominant gp33 antigen of LCMV (Pircher et al. 1990). The Spi6 KO P14 cells showed increased apoptosis thus demonstrating the cell-autonomous need for Spi6. Significantly higher GrB activity was measured in Spi6 KO CTLs compared to wild-type, and this was detected both in the cytotoxic granules and at the cytoplasmic level. Also, a loss of cytotoxic granules was measured with confocal immunofluorescece microscopy in Spi6 KO CTLs and a high level of GrB staining in the cytosol of these cells. These results led to the conclusion, already cited above, that Spi6 is responsible for ensuring the correct organization and integrity of cytotoxic granules, otherwise disrupted in a GrB-dependent way (Zhang et al. 2006).

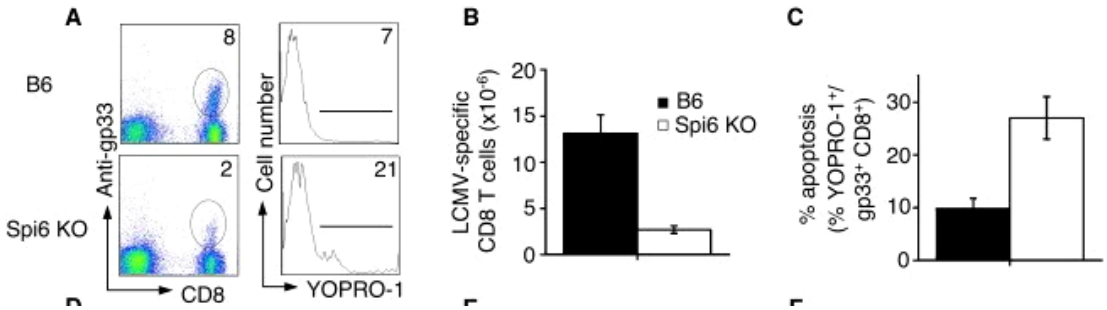



**Figure 28: Number of gp33$^{+}$ CD8$^{+}$ cells at day 8 after LCMV infection and levels of YOPRO-1 positive (apoptotic) cells (A) and levels of apoptosis in gp33-specific CD8 T cells (number of YOPRO-1-positive cells) at day 8 p.i. with LCMV (B and C) in Spi6 KO and WT (B6) mice (Zhang et al. 2006).**

In the same Spi6 KO mice, CD8 T cell loss was also measured during the contraction phase of the CTL response, and no significant difference was seen in the level of LCMV-specific CD8 T cells during the contraction phase. Spi6 is therefore required for the clonal burst of CTL responses but not for the contraction phase (Zhang et al. 2007).

Since DCs are crucial for proper expansion of the specific CTL population (Beltz et al. 2007) and previous reports did not agree on results, the causal connection between Spi6 upregulation in mature dendritic cells and their resistance to CTL-induced apoptosis needs to be further investigated.

### 2.8.4.4 Supporting *in vitro* data

*In vitro* data obtained in our lab before my arrival by Dr. Zhang showed that Spi6 absence in BMDDCs was correlated to increased susceptibility to CTL-induced granule-mediated death.

A JAM assay (Matzinger 1991) with matured and gp33-pulsed BMDDCs from Spi6 KO mice and CTL from a P14 mouse was performed. BMDDCs were grown according to the protocol commonly used (Lutz et al. 1999) for 9 days and with LPS (0.2 μg/ml) one more day. When harvested at day 10 (Figure 29), DCs were mature (78%), as shown by the upregulation of MHC-II (I-A$^{b}$). These same Spi6 KO-

derived BMDDCs were then cultured for 4 hrs with P14 CTLs obtained by 2 days in culture with IL-2 and gp33 antigen. In particular, the JAM assay was used to determine apoptosis levels in BMDDCs after culture with CTLs. Briefly, BMDDCs were labelled with $^{3}$H-Thymidine and pulsed with gp33 ($10^{-7}$M) for 1h and then incubated with P14 CTLs over a range of ratios in quadruplicate. The percentage specific lysis was determined after 4 hrs. $^{3}$H-Thymidine is incorporated during cell proliferation: comparison of the amount of $^{3}$H-Thymidine in the experimental wells (BMDDCs co-cultured with CTLs) compared to negative controls (BMDDCs alone) allows determination of the percentage of target cells that underwent apoptosis in the experimental well. The cells in which apoptosis is induced by CTLs will release $^{3}$H-Thymidine in the culture medium. When cells are harvested, $^{3}$H-tymidine from dead cells is washed away and the measured remaining radioactivity can be correlated to the amount of cells which survived (particularly to the DNA amount), therefore allowing the calculation of apoptosis levels (Matzinger 1991).

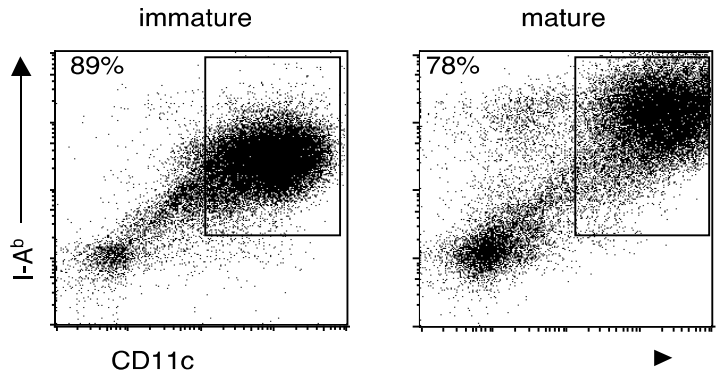



**Figure 29: BMDDC maturation status: CD11c and MHC-II (I-A$^b$) expression in immature BMDDC, left panel, and LPS-matured ones, right panel (by M. Zhang).**

The experiment showed that Spi6 KO BMDDCs are more susceptible to P14$^{+}$ CTL-mediated PCD, as described in Figure 30, than WT ones. As the JAM assay measures DNA fragmentation, which is a late event in apoptosis, specific lysis was then measured with the $^{51}$Cr-release assay (see Figure 31).

The next step was to demonstrate that the killing was granule-dependent and not triggered by Fas-FasL pathway. Figure 30 B shows the level of apoptosis measured in the same assay as above when CTLs are treated with concanamycinA (CMA), which inhibits granule-mediate cytolysis. In this case, apoptosis in gp33-pulsed BMDDC is abolished, indicating that the levels of apoptosis revealed before were specifically induced by a granule-dependent pathway.

Spi6 KO-derived BMDDC apoptosis was in this experiment specifically granule-mediated. Moreover, Figure 30 C shows that anti-Fas antibodies induce PCD both in Spi6 KO-derived and in B6-derived BMDDCs with no difference. This suggests Spi6 could have a role in protecting BMDDC from granule-mediated apoptosis pathway, but not from the Fas-activated killing pathway.

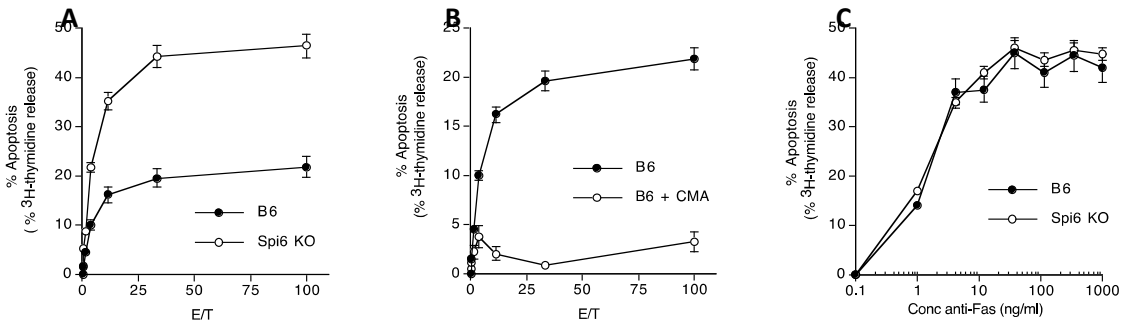



**Figure 30: Apoptosis of Spi6KO-derived BMDDC. A) Apoptosis levels measured via DNA fragmentation in gp33-pulsed BMDDC (target, T) after 4h culture with P14 CD8 T cells (effector, E). B) Apoptosis levels when CMA is added to the effector cells: CMA abolishes PCD in gp-33 pulsed BMDDC (T). C) BMDDCs were incubated with anti-Fas mab (JO2) for 16hr with cyclohexamide (by M. Zhang).**

In Figure 31, results from the measurement of apoptosis levels in a $^{51}$Cr-release assay are showed. Spi6 KO and B6-derived BMDDCs were labelled with $^{51}$Cr, pulsed with gp33 and mixed to CTLs. Lysis was measured as an indicator of apoptosis, and indicated that in Spi6 KO-derived BMDDCs is increased compared to wild type ones.

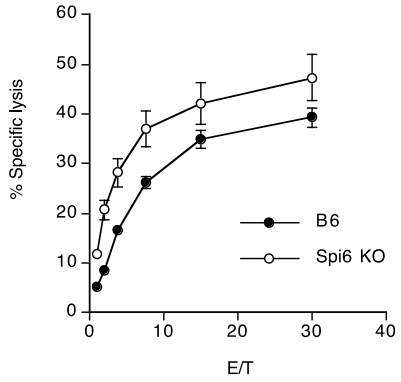



**Figure 31: BMDDC specific lysis, $^{51}$Cr release assay on LPS-matured Spi6 KO- and B6- (WT)-derived BMDDCs (by M. Zhang).**

On the basis of these data and previous reports, my hypothesis is that Spi6 can indirectly allow for a wider CTL response in two ways: promoting the survival of activated $CD8^+$ T cells through the inhibition of cytosolic GrB and conferring resistance of GrB-mediated apoptosis to DC. Moreover, Spi6 is responsible for optimal development of CD8 T cell responses to viral infections making DCs resistant to GrB-induced apoptosis.

## 2.8.5 Hypothesis

The project addresses the role of Spi6 in DC survival during CD8 T cell priming, both during primary effector responses and during secondary memory recall responses. Mature, antigen-loaded DC elimination by CTL has been shown to be perforin-dependent and consequently involve GrB action (Yang et al. 2006) and to be part of a negative feedback regulatory loop which keeps CTL responses in check. Notably, several reports have highlighted how sustained and prolonged priming, mediated by DCs both in secondary lymphoid organs and in peripheral tissues, is necessary for optimal expansion of effector antigen-specific cells in viral infections.

Spi6 expression in mature DCs might therefore play a very important role in protecting these cells from PCD, as previously reported with some controversy

(Andrew et al. 2008). Controversial results, obtained by several groups in a debate that has now been unsolved for ten years, clearly suggest that the function of Spi6 in mature DCs is at present not understood. This work addresses the issue through the use of an *in vivo* model of infection in which Spi6 expression is abolished: a Spi6 KO mouse. DCs are expected to depend on Spi6 for survival both during primary and secondary responses. I therefore investigated the role of this protein in the DC subsets responsible for priming and support of CD8 T cells antigen-specific responses to LCMV infection in both primary and secondary responses. The subsets of choice are $CD8\alpha^+$ DC and pDCs.

The hypothesis is that Spi6 KO DCs, priming wild-type non Spi6-deficient CD8 naïve (or memory) T cells in LCMV infection, will be eliminated through PDC to a higher extent than wild type DC. The CTL response is expected to be hampered in Spi6 KO mice due to defective priming. This assumption is based on previous results showing a direct correlation between DC number and T cell expansion levels, due to the specific cross-presentation-dependent nature of CD8 responses in LCMV infection.

This study aims:

To study whether Spi6 is required for the survival of dendritic cell exposed to CTL-released GrB;

To examine the expression of Spi6 in different murine DC subsets in vivo;

To determine whether Spi6 enhances DC survival when priming CD8 T cell responses against viruses;

To verify if CTL and $CD8^+$ memory cell expansion levels *in vivo* are affected by Spi6 absence in DC;

To confirm that Spi6 protects DCs from CTL killing by inhibiting GrB in CD8 T cells *in vivo*.

The following chapters will describe the materials and methods (Chapter 3) and the results obtained from the experiments performed to address the points above (Chapter 4), and a discussion of the results with an overview of future directions (Chapter 5)

# 3. MATERIALS and METHODS

## 3.1 Mice

C57BL/6J Spi6 KO mice (Phillips et al. 2004) and P14 mice (Zhang et al. 2006) were bred in house. Thy1.2$^{+}$ and Thy1.1$^{+}$ C57BL/6J wild-type and GrB KO mice (Heusel et al. 1994) were purchased from Jackson Lab. GrB KO P14 Thy1.1 mice were generated by inter-crossing and screening by PCR for the GrB allele (Heusel et al. 1994) and flow-cytometry for the P14 TCR (Vα2$^{+}$) and CD90.1 (Thy1.1) and CD90.2 (Thy1.2) in the blood (Zhang et al. 2006). P14 Thy1.1 mice were obtained by inter-crossing and screening for the P14 TCR (Vα2$^{+}$) in the blood. All animals were maintained in a pathogen-free environment at Imperial College London and all experiment were conducted in accordance to Home Office (UK) regulations.

### 3.1.1 Mouse genotyping

In order to uniquely identify the product of mice intercrossing, each pup's genotype was analysed between 4 and 6 weeks of age through antibody staining for leukocytes markers in the blood, or by DNA analysis.

For blood staining: 5µl of blood were obtained by bleeding the tail vein at about 6 weeks of age. The blood was mixed to EDTA 5M pH 8.0, then the antibody mix was added in 30µl of FACS buffer (PBS + 0.5% BSA from Sigma). The blood was incubated with the antibodies for 30 minutes in the dark, and then red cell lysis was performed with Red Blood Cell (RBC) Lysis Buffer (eBioscience). The samples were then washed with FACS buffer and resuspended in more FACS buffer for Flow Cytometry analysis. Blood staining was performed for the following mouse strains: for P14 mice, using PE-conjugated αV-β2 TCR antibody from eBioscience or PE-

conjugated gp33 tetramer from Proimmune. For Thy1.1 mice, identification of genotype was carried out using CD90.1-PacificBlue and CD90.2-PeCy7 from eBioscience.

**DNA analysis**: biopsies were taken from mouse ear according to a precise identification scheme based on the number of ear marks and on their position. DNA was extracted from the ear biopsies through a salting-out procedure. Briefly: biopsies were digested overnight with Lysis buffer (STE buffer: 0.1M tris pH 8.0 + 200mM NaCl + 5mM EDTA + 0.2% SDS in water) and Proteinase K (20 mg/ml) (Sigma P-2308) in a water bath at 55° C. Saturated NaCl (5M) was then added and samples mixed and incubated on ice for 15 minutes. Samples were then centrifuged for 40 minutes at 13,000rpm. The supernatant was then removed and placed in a new tube. 100% EtOH in equal amount was added to precipitate the DNA. The samples were then spun for 30 minutes at 13,000rpm. The supernatant was removed and 75% EtOH was added to the pellet to wash the salt away. Samples are resuspended and then centrifuged for 6 minutes. Supernatant is completely removed and the DNA is resuspended in TE buffer (10mM Tris pH 8.0 + 0.1mM EDTA in water) and allowed to dissolve in it overnight at 4° C. The following day the DNA is resuspended carefully in its buffer and concentration is determined by use of NanoDrop machine (Thermo Scientific).

**PCR**. Granzyme B and Spi6 genes were detected through Polyclonal chain reaction. DNA was then used for PCR reaction with primers specific for the Spi6 gene or the granzyme B gene.

The primers are:
Primer 8970 (Common): 5’ GGG GTA CAA GGT CAC AGA GC 3’
Primer 9596 (Wild type): 5’ CAG AAA CCA GGC CCA TTC TA 3’
Primer Reverse 7415 (Mutant reverse): 5’ GCC AGA GGC CAC TTG TGT AG 3’

PCR mix for Gzmb gene:

| REAGENT | VOLUME |
|---|---|
| $ddH_20$ | 17.22µl |
| TAQ 10X buffer | 3.6µl |
| dNTP mix 10mM | 0.72µl |
| $MgCl_2$ | 2.88µl |
| Primer 8970 | 1.8µl |
| Primer 9596 | 1.8µl |
| Primer 7415 | 1.8µl |
| TAQ polymerase | 0.18µl |
| DNA | 6µl |
| TOTAL | 30µl |

**Table 2: GrB PCR reaction mix**

The PCR conditions are:

1. 94° C for 3 min
2. 94° C for 30 sec
3. 62° C for 1 min
4. 72° C for 1 min
5. (Repeat steps 2-4 for 35 cycles)
6. 72° C for 2 min
7. 4° forever

The expected results:

Mutant = 217 bp

Heterozygote = 217 bp and 386 bp

Wild type = 386 bp

The Spi6 PCR mix was changed a number of time dues to problems in due course. The first PCR mix (Table 3) used the following primers:

F1 (wild-type forward): 5’ TGG TGG GCA GTA GAT TGT GCA TT 3’

R1 (common reverse): 5’ GAG GAC CCT GAT TAC AGA ACG 3’

And the following reaction parameters:

1. 94° C for 5 min
2. 94° C for 45 sec
3. 56° C for 45 sec
4. 72° C for 3 min
5. (Repeat steps 2-4 for 30 cycles)
6. 72° C for 10 min
7. 4° C forever

The readout was: WT = 2.5 kb

Spi6 KO (NEO deleted) = 800bp

Heterozygous = 2.5 kb and 800 bp

Spi6 PCR reaction mix:

| REAGENT | VOLUME |
|---|---|
| $ddH_2O$ | 28µl |
| Biolase TAQ 10X buffer | 5µl |
| dNTP mix 2.5mM | 4µl |
| $MgCl_2$ 50µM | 2µl |
| Primer F1 | 2.5µl |
| Primer R1 | 2.5µl |
| Biolase TAQ polymerase | 1µl |
| DNA | 5µl |
| TOTAL | 50µl |

**Table 3: First Spi6 PCR reaction mix**

This reaction had been tested before, but as problems arose with it and it was modified. It was substituted with 2 PCR to be done in sequence to identify both Heterozygous and WT if the WT band (2.5kb) was weak.

The first the PCR mix is in Table 4, the second in Table 5:

| REAGENT | VOLUME |
|---|---|
| $ddH_2O$ | 34.5µl |
| NEB TAQ 10X buffer | 5µl |
| dNTP mix 2.5mM | 4µl |
| $MgCl_2$ 50µM | (included in buffer) |
| Primer F1 | 0.5µl |
| Primer R1 | 0.5µl |
| NEB TAQ polymerase | 0.5µl |
| DNA | 5µl |
| TOTAL | 50µl |

**Table 4: New PCR reaction mix for Spi6**

The PCR conditions were the following:

1. 94° C for 4min
2. 94° C for 1min
3. 56° for 1min
4. 72° for 3min
5. repeat steps 2-4 for 35 cycles
6. 72° C for 7 min
7. 4° C forever

Second PCR mix for Spi6:

| REAGENT | VOLUME |
|---|---|
| $ddH_2O$ | 34.2µl |
| NEB TAQ 10X buffer | 5µl |
| dNTP mix 2.5mM | 4µl |
| $MgCl_2$ 50µM | (included in buffer) |
| Primer F2 | 0.5µl |
| Primer R1 | 0.5µl |
| NEB TAQ polymerase | 0.8µl |
| DNA | 5µl |
| TOTAL | 50µl |

**Table 5: Second reaction of new PCR reaction mix for Spi6**

F2 (mutant forward) of the second PCR mix (Table 5) was:
5’ TGG TGG GCA GTA GAT TGT GCA TT 3’

The PCR was run with the following conditions:

1. 94° C for 4 min
2. 94° C for 1 min
3. 58° for 1 min
4. 72° for 1 min
5. Repeat steps 2-4 for 35 cycles
6. 72° C for 7 min
7. 4° C forever

With these primers the Spi6 KO allele gives no band and the WT allele gives a 910bp band.

When the PCR would still not give any visible band for both the samples and the positive control, a Gradient PCR machine was used to modify the annealing time between 54° C and 59° C. This was performed both with the NEB and with a HOT START polymerase. When again no results were obtained, Q solution (Invitrogen) was added to the PCR mix and the annealing temperature kept at 58° C. Given the high cost of Q solution, Betaine (Sigma) was then purchased and added to the previous PCR mix (5µl).

The amplified DNA was then run in a 1% Agarose gel, stained with Ethidium Bromide and read under UV light (Trans-illuminator).

## 3.2 Cell culture, *in vitro* materials and methods

*In vitro* experiments were conducted with *ex vivo*-obtained and grown bone marrow-derived dendritic cells and with splenocyte-derived CD8 T cytotoxic T cells. The former were used to measure Spi6 expression and, cultured with the latter, to measure susceptibility of this cell type to CTL-dependent killing.

### 3.2.1 Cell culture reagents

All cultures were performed in complete media composed of Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen) supplemented with 10% heat-inactivated Fetal Calf Serum (Invitrogen), 5ml PenStrep-Glutamine 100X from Invitrogen (final concentration: Streptomycin 1ug/ml, Penicillin 1U/ml, .29mg/ml L-Glu), 1% ß-mercaptoethanol (Invitrogen) final concentration 0.1mM. Complete media was filtered through 0.2 μm pore membrane filters.

Collagenase type II was from Sigma and Collagenase D and DNaseI grade II from bovine pancreas was from Roche.

### 3.2.2 Bone marrow-derived dendritic cell origination

Dendritic cells were cultured from bone marrow progenitors according to Lutz protocol (Lutz et al. 1999). Briefly: bone marrow was flushed out of femoral bones taken from 6-8 weeks old mice. Cells were washed in PBS, counted and plated at $2x10^6$ in 10ml of complete media (as described above) in a 10cm diameter Petri dish. The culture is supplemented with murine recombinant-GM-CSF (200 U/ml i.e. 20 ng/m, from Peprotech) on the day of culture. At day 3 of culture, 10 more ml of fresh complete media are added, supplemented with new rmGM-CSF (20 ng/ml). At day 6 of culture, half of the supernatant (10ml) is replaced with fresh complete one and more rmGM-CSF (20 ng/ml) is added. The same is repeated at day 8, but the concentration of GM-CSF is dropped to 10ng/ml.

#### 3.2.2.1 Maturation of bone marrow-derived dendritic cells

To mature BMDDC for use in killing assay (CalceinAM) experiment and for adoptive transfer into B6 mice, LPS from E.Coli (Sigma), 1 ng/ml, and gp33 peptide (KAVYNFATM) from LCMV GP antigen, at $10^{-6}$ M, were supplemented at day 8 of culture. The control group, immature BMDDC, were instead supplemented on the same day with gp33 ($10^{-6}$ M) only. Both immature and mature BMDDC cell cultures were harvested and resuspended in fresh complete medium 24 hrs later.

Confirmation of BDMMC maturation was obtained through surface staining of the cells with the following antibodies: CD11c-PE, MHC-II (I-$A^b$)-FICT, CD86-APC (all from eBioscience).

### 3.2.3 Development of cytotoxic T cells in culture

To obtain CTL from P14 Thy1.1 mice for killing assays, CD8 cells were isolated from the spleen of these mice. A single cell suspension was obtained from processing the spleen with the use of Cell Strainers (from BD) in complete media (as above). Lympholyte (from Cedarlane), 2ml in 10ml of single cells suspension, was used to separate the lymphocyte layer through centrifugation for 15 min at 2,000rpm, with no brake. The obtained layer of leukocytes was then washed in PBS and resuspended in complete medium at $10^6$ cells/ml. Splenocytes were cultured in 12well-plates (2 ml/well) and supplemented with recombinant murine IL-2 (10 U/ml) and gp33 ($10^{-6}$ M) for 2-3 days, monitoring the formation of clumps of activated proliferating CTL to determine when the cells were mature and activated (Zhang et al. 2006). After about 48 hrs, clumps could be observed and the cells were harvested and CD8 cells were enriched through magnetic bead sorting. Specifically, cells were washed in cold MACS buffer (0.5% BSA + EDTA 2mM in PBS), resuspended in 9/10 MACS buffer and 1/10 beads with volumes depending on the number of cells (100µl of beads and 900µl of MACS buffer for $10^8$ cells) and incubated at 4° C for 15 minutes, then washed with 10ml of cold MACS buffer, and loaded onto the LS column in 2ml. The column was washed three times with additional MACS buffer and cells were eluted with the provided plunge in 5ml of cold MACS buffer, washed and resuspended according to the needs.

### 3.2.4 Intracellular staining for Spi6

Spi6 level analyses were carried out through intracellular staining of the protein performed with a primary antibody obtained from the serum of an immunized rabbit

(Aston-Rickardt lab) and a secondary, fluorocrome-conjugated, anti-rabbit antibody. Briefly: cells were first stained for surface markers to identify CD8 T cells versus pDC and CD8$\alpha^+$ DC in splenocytes as described below. Cells were then washed with FACS buffer and incubated with Cytofix/Cytoperm™ solution for 20 minutes at 4° C. Cells were then washed with Perm/Wash™ buffer (BD) and resuspended in Perm/Wash™ buffer containing primary antibody (or pre-immune serum as a control) at 1/1000 + goat serum 1/10. Cells were incubated at in the dark at RT for 40 minutes. They were then washed with Perm/Wash™ buffer and spun down. A second incubation with the secondary antibody as performed in Perm/Wash™ buffer + 1/1000 secondary antibody (APC-goat anti-rabbit from Jacksons immunobiology or PE-Cy7-goat anti-rabbit) + 1/50 goat serum (Zhang et al. 2007). Cells were incubated for 40 minutes at RT in the dark and successively resuspended in Perm/Wash™ buffer to be analysed.

### 3.2.5 Dendritic cell killing assay with Calcein, AM

Mature and immature BMDDC were generated as described above from Spi6 KO and WT mice. CTL from P14 Thy1.1 mice were obtained as described above from wild-type or GrB KO P14 Thy1.1 mice.

To measure CTL-induced lysis, gp33-pulsed LPS-matured BMDDC were also labelled with the cell-permeant non-fluorescent acetomethoxy derivate of calcein (Calcein, AM) according to the manufacturer's protocol (Invitrogen). Briefly: BMDDC were washed and resuspended at $5x10^6$ cells/ml. 50µg of desiccated Calcein, AM were diluted in 50µl sterile DMSO and, for a final concentration of 1µg/ml, 15µl are added to each millilitre of cells. Cells were incubated for 30 minutes at 37° C with occasional shaking and then washed three times in serum-free medium, counted and resuspended at the desired concentration.

P14 CTLs were obtained as described above and resuspended at the desired concentration ($10^6$ cells/ml). BMDDC were then mixed to P14 CTL in a V-shaped 96-well plate over a range of E/T ratios starting at 50:1 (with serial 1: 2 dilutions) in

200µl, in triplicates. As negative and positive controls, "Spontaneous release" (BMDDC target only in medium) and "maximum release" wells (BMDDCs in medium with 2% Triton X-100) were added for each BMDDC group in triplicates. After incubation at 37°C in 5% $CO_2$ for 4 or 8 hours, half of the supernatant was harvested and transferred into new 96-well flat-bottom plates (Costar 3596; Corning Inc). The release of fluorescent calcein into the supernatant was then measured with a dual-scanning microplate spectrofluorometer (Versamax microplate reader; excitation filter 485 ± 9 nm, band-pass filter 530 ± 9 nm). Percentage of BMDDC lysis was calculated with the same formula used for the $^{51}$Cr-release assay and presented as follows (Wang et al. 2004):

% cell lysis = (experimental wells − spontaneous release) / (maximum release − spontaneous release) X 100

The percentage specific lysis of RMA cells in the absence of gp33 was <10% and the spontaneous release was <10% of the maximum release.

## 3.3 In vivo analysis of dendritic cell survival and CD8 responses to LCMV infection

*In vitro* experiments were followed by the analysis of DC survival in an *in vivo* model of infection. Spi6 KO mice were adoptively transferred with gp33-specific CD8 T cells (P14 CD8 T cells) and infected with LCMV Arm for primary infection and subsequently with LCMV CL13 for secondary infections.

### 3.3.1 CD8 T cell isolation from the spleen of naïve mice

Naive Thy1.1$^+$ CD8$^+$ T cells were isolated from spleens of P14 Thy1.1 mice or GrB KO P14 Thy1.1 mice through bead enrichment (MACS beads, Miltenyi Biotech) with positive-selection LS columns for use in adoptive transfers and priming assays.

Briefly, spleens were processed to single cell suspension and Lympholyte was used to isolate lymphocytes from Red Blood Cells as above. Enriched lymphocytes were washed in PBS and mixed with anti-CD8 beads at the concentration advised by the manufacturer (100µl for $10^8$ cells) and incubated for 15 minutes on ice (or at $4^0$C). The cells were then washed in cold MACS buffer and added to LS columns for enrichment. Once enriched, the CD8 T cells were counted with Trypan Blue and resuspended in sterile PBS at $10^5$ cells/ml: $10^4$ cells (100µl) were injected i.v. into the recipient mice.

## 3.3.2 Dendritic cell isolation from the spleen of infected mice

Spleens of Spi6 KO and WT/B6 mice were cut into small fragments and digested with Collagenase D grade II (1 mg/ml) and DNaseI grade II (20 µg/ml) (both from Roche) in complete medium for 25 min at room temperature. EDTA (0.2mM) was successively added for another 5 min to disrupt DC-T cell interaction. Spleen fragments were then filtered through mesh (70µm) and centrifuged at 2,000 x g for 5 min at room temperature. Cells were re-suspended in MACS buffer and magnetically sorted with anti-CD11c beads (Miltenyi Biotec).

### 3.3.2.1 Enrichment of $CD11c^+$ cells

$CD11c^+$ enriched cells were used for Spi6 level analysis and for determination of CD8α DC and pDC numbers in the spleen of infected Spi6 KO and WT/B6 mice. They were also used for sorting of $CD8\alpha^+$ DC (see below).

Red blood cells were deleted from the splenocyte preparation with 1ml of RBC Lysis Buffer and washed in MACS buffer. Cells were then resuspended in 900µl of MACS buffer + 100µl of CD11c magnetic beads (100µl for $10^8$ cells) and incubated in the fridge for 15 minutes to allow binding to the CD11c surface molecule. The procedure for isolation of cells with the anti-CD11c beads was the same as described for CD8-beads. The cells obtained were then washed in MACS buffer and resuspended in MACS buffer (450µl MACS buffer + 50µl CD11c magnetic beads) for a second purification, to be performed in the same way as the first. The CD11c-

enriched obtained cells were counted with CV Acetic Acid and resuspended at desired concentration in complete RPMI medium.

The number of CD8α DC in each spleen was determined by determining the % of $CD11c^+$ $Ly6\text{-}C^{low}$ $CD8\alpha^+$ $CD4^-$ cells then multiplying by the number of enriched $CD11c^+$ cells. The number of pDC in each spleen was determined by multiplying the % of $CD11c^+$ $Ly6\text{-}C^+$ cells by the number of enriched $CD11c^+$ cells.

### 3.3.2.2 Sorting of splenic dendritic cells

$CD8\alpha^+$ $CD11c^{hi}$ $CD4^-$ cells were sorted from the pool of $CD11c^+$ cells enriched from the spleen of infected Spi6 KO and WT/B6 mice for *ex vivo* priming and expansion experiments. $CD8^+$ DCs from the spleens of infected mice were FACS-sorted after antibody staining (MoFlo; DakoCytomation).

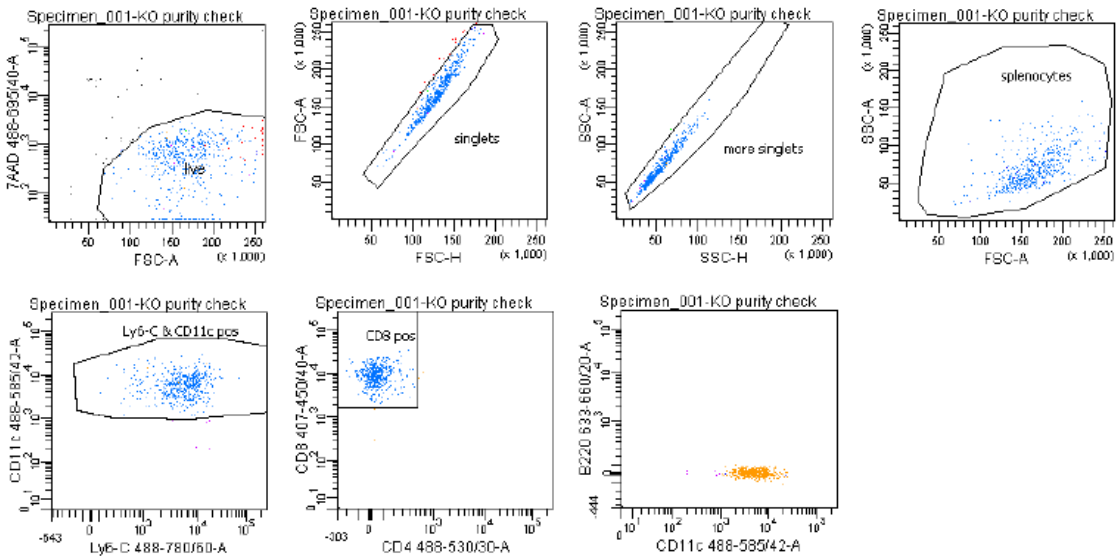



**Figure 32: Purity of CD8α DCs after FACS sorting using 7AAD, CD11c-PE, CD8-PAcBlue, CD4-FITC antibodies for surface markers.**

Briefly, $CD11c^+$ enriched cells (protocol as above) were stained for surface markers with CD11c-PE, CD4-FITC, Ly6C-PeCy7 and CD8-PacificBlue. $CD11c^{hi}$ $CD8^+$ $Ly6\text{-}C^{lo}$ $CD4^-$ cells (8% $CD11c^+$-enriched cells in wild-type, 3% in Spi6 KO) were purified by FACS sorting. The purity of live cells obtained through the sorting was about 90% and verified with a purity check (see Figure 32: >98% pure). Cells were

sorted in RPMI-1640 medium + 20% FCS, spun down and resuspended at $2.5x10^5$ cells/ml.

### 3.3.3 Cell counting

When it was needed to determine the total cell number in a specific organ, the cells obtained from that organ (spleen or LN) in a single-cell suspension were counted with counting Beads from Caltag. Briefly: Single cell suspensions from the spleen, or from pooled inguinal lymph nodes, of WT/B6 and Spi6 mice were obtained with the use of Cell Strainers. 10µl of cells were fixed with 50µl of Cytofix/Cytoperm™ solution (BD). FACS buffer (9 volumes) and 10µl of counting beads were then added to the cells. Cells were well shaken and mixed before being run into a Cyan ADP (Beckton Coulter) at high speed with a time limit of 1 minute. Total counts were obtained as follows:

Cells/µl = (Tot count live Leukocytes / (dim beads count + bright beads count)) * Number of Beads

Cells/ml= cells/µl * 1000

Tot cells/organ= cells/ml * X ml
(where X is the number of ml the total cells of that organ were resuspended in)

The number of beads is variable for each batch of Caltag Counting Beads. The same cell suspensions were stained with appropriate mAbs to detect surface expression of lymphocyte markers as described below. Mean cell counts were determined by multiplying the frequency of each cell type, determined by FACS, by the total number of cells in the organ(s) and are ± SEM ($n$ = 5 mice).

### 3.3.4 CD8 T cell priming by *ex vivo*-isolated dendritic cells

$CD11c^+$ $CD8\alpha^+$ DCs purified after sorting were used in a CD8 T cell priming assay to test their ability to present physiologically acquired antigens from LCMV and induce CTL proliferation.

#### 3.3.4.1 Priming assay with EdU incorporation

$CD11c^{hi}$ $CD8\alpha^+$ $CD4^-$ Dc, obtained by sorting as per above from the spleen of infected mice, were mixed with naïve P14 CD8 T cells, isolated from the spleen of P14 Thy1.1 mice as previously described. EdU (5μM) was added to the culture to measure the proliferation of CD8 T cells (Click-iT® EdU Invitrogen). EdU is a substitute to BrdU for direct measurement of newly synthesized DNA due to cell proliferation. EdU (5-ethynyl-2´-deoxyuridine) is a thymidine analogue and as such is incorporated into DNA when new DNA is synthesized due to proliferation. Detection of this analogue is based on click chemistry: a covalent reaction catalyzed by copper which happens between an azide and an alkyne. In particular, EdU can be detected in FlowCytometry thanks to the coupling of the azide with Alexa Fluor ® 488. FACS-purified CD8α DCs (0-5x$10^4$ per 96 U-bottom well/0.2ml) were incubated with bead sorted $CD8^+$ T cells from wild-type P14 mice (5x$10^4$ cells/well). Cells were diluted serially with a E/T starting ratio of 2: 1 DC to CD8 T cells and cultured at 37º C in 5% $CO_2$ for 60 hours. Harvested cells were washed, fixed and stained for EdU according to the manufacturer protocol. Briefly: cells were washed in FACS buffer and then 100μl of Click-iT® fixative was added for 15 minutes at RT. Cells were washed and suspended in of Click-iT® saponin-based permeabilization and wash reagent (100μl) and incubated for another 15 minutes at RT. Cells were spun down and resuspended in Click-iT® reaction cocktail (PBS, D-PBS, or TBS 438μl per sample + CuSO4 10μl per sample + Fluorescent dye azide 2.5μl per sample + Reaction Buffer Additive 50μl per sample ). After 30 minutes of incubation at RT and in the dark, cells were washed once with Click-iT® saponin-based permeabilization and wash reagent and then resuspended in the same reagent for FACS analysis. The percentage of $EdU^+$ cells was then determined by flow-cytometry according to manufacturer instructions.

### 3.3.5 Adoptive transfer of cells into wildtype or Spi6 knock-out mice

Cells were adoptively transferred into WT and Spi6 KO with the purpose of studying the CD8 gp33-specific T cell response. To repeat Andrew's experiment, mature BMDDCs from Spi6 KO and WT mice were first adoptively transferred into WT mice to study the ability of these cells to prime a CD8 T cell response. Once this was verified, DCs were studied in an *in vivo* model of viral infection. In order to obtain a mouse model in which CD8 T cells were competent for Spi6 and not subject to GrB autocrine action, WT P14 CD8 T cells or P14 CD8 memory cells were adoptively transferred into Spi6 and WT mice.

#### 3.3.5.1 Adoptive transfer of bone marrow-derived dendritc cells

Mature dendritic cells (BMDDCs) to be adoptively transferred into Spi6 KO and B6 recipients were obtained through culture with GM-CSF of bone marrow cells as described above. BMDDCs were harvested at day 10 and resuspended at $5x10^6$ cells/ml in sterile PBS: $5x10^5$ mature Spi6 KO/WT BMDDCs were injected into B6 mice i.v. trough the tail vein.

#### 3.3.5.2 Adoptive transfer of naïve $P14^+$ $Thy1.1^+$ CD8 T cells

Naïve $CD8^+$ cells were purified (>90%) from the spleens of P14 ($Thy1.1^+$) or GrB KO P14 mice ($Thy1.1^+$) by positive sort with anti-CD8 magnetic beads (Miltenyi Biotec) as described above. Enriched CD8 T cells were resuspended at $5x10^4$ cells/ml in sterile PBS and adoptively transferred ($5x10^3$) by i.v. injection into WT ($Thy1.2^+$) or Spi6 KO ($Thy1.2^+$) mice.

#### 3.3.5.3 Adoptive transfer of memory $P14^+$ $Thy1.1^+$ CD8 T cells

For the adoptive transfer of memory cells into Spi6 KO/WT mice, naïve CD8 T cells from a P14 Thy1.1 mouse were first isolated (according to the protocol above) and adoptively transferred into a WT/B6 mouse. The mouse was then infected with LCMV Arm and let recover. At 40 days post-infection, memory $Thy1.1^+$ $CD8^+$ T

cells were isolated from the spleen with anti-Thy1.1 magnetic bead enrichment (Miltenyi Biotec) with positive selection-LS columns. The protocol for magnetic beads enrichment was the same as for isolation of CD8 naïve T cells.

Enriched memory cells were resuspended in sterile PBS and injected in Spi6 KO/B6 mice ($10^3$, $10^4$ or $10^5$ cells total).

## 3.3.6 LCMV infection

Two variants of LCMV were used to infect the mouse models after adoptive transfer of P14 CD8 T cells. LCMV Armstrong (Arm) is non-cytolytic and it is easily eliminated by immunocompetent mice in 8-10 days. LCMV CL13 causes chronic infection when injected in immunocompetent naïve mice. In my study the latter was used to stimulate a re-expansion of memory cells, therefore being eliminated before being able to establish a chronic infection. Notably, LCMV infection of a H-2D$^b$ mouse results in 3 H-2D$^b$-restricted immunodominant epitopes, each against one of the proteins NP, GP1, and GP2. The epitope against NP is NP396–404 (FQPQNGQFI), the epitope from GP1 is GP33–41 or GP33-43 (KAVYNFATC/GI) and the epitope for GP2 is GP276–286 (SGVENPGGYCL) (Gairin et al. 1995). The GP33 epitope is both H-2Db and H-2Kb restricted (Hudrisier et al. 1997): gp33 stimulates both T cell clones but each clone recognises only one of the two GP33 antigens, epitope 33-41, which is the shrorter of the two.

### 3.3.6.1 LCMV Armstrong

LCMV Arm was obtained from Ray Welsh. Intra-peritoneal injection of $10^5$ PFU of LCMV Arm was performed in mice under anaesthesia with Fluravent.

### 3.3.6.2 LCMV Clone 13

For memory cell experiments, LCMV Armstrong recipients were re-infected after 40 days with intravenous injection of clone 13 variant of LCMV Armstrong, obtained from Annette Oxenius ($10^6$ PFU). Mice were placed in a heated environment (heath

box) with a maximum temperature of $30^0$ C, to allow for vein dilatation which facilitates i.v. injections.

The same PFU were injected into Spi6 KO/B6 mice after adoptive transfer of memory cells.

## 3.4 Viral titre analysis

In order to verify the correlation between CTL response expansion and ability of the cells to efficiently clear the infection, viral titres were analysed through measurement of viral RNA. Total RNA was extracted from the spleen of infected mice at day 6 post (primary) infection, or at day 5 post (secondary) infection.

### 3.4.1 Total RNA extraction

To measure the level of LCMV in infected mice, a quantitative PCR method was used as described by McCauslan and Crotty (McCausland and Crotty 2008). Whole spleens from infected mice were conserved in RNAlater RNA Stabilization (Quiagen) weighed and a known amount disrupted using a glass mortar and pestle in buffer RTL Plus (QIAGEN) followed by homogenisation using a QIAshredder homogenizer (QIAGEN).

Once the tissue was homogenized, RNA was extracted according to the RNeasy Plus Handbook (QIAGEN). The flowthrough was then transferred to a gDNA eliminator column placed in a 2ml tube, and centrifuged for 30 seconds at 10,000rpm. 600µl of 70% EtOH was added to the flowthrough solution, which was then transferred to an RNeasy spin column (including any precipitate). The column was centrifuged for 15 seconds at 10,000rpm. 700µl of RW1 buffer was then added to the RNeasy column, and spun for 15 seconds at 10,000rpm. The flowthrough was then discarded and 500µl of RPE buffer added to the RNeasy column. The column was

spun down, the flowthrough discarded and the column placed on a new tube (clean). 40µl of RNase-free water were added for elution of the total RNA. To obtain the RNA the column was spun at 10,000rpm for 1 minute to recover total RNA.

### 3.4.2 Reverse transcription

The RNA concentration was then measured on Nanodrop and 2.5µg of RNA are used for cDNA transcription. cDNA was synthesized with the SuperScript III reverse transcriptase from Invitrogen according to the manufacturer instructions (Invitrogen) 1µl dNTP + 1µl GSP + dd$H_2O$ (RNase free) to 13µl total. The sample was heated to 65º C for 5 minutes and then incubated on ice for 1min. 4µl of 5X buffer + 1ul DTT + 1µl RNaseOUT + 1ul Transcriptase were added and the sample was incubated at 50º C for 30-60 minutes if random primers,or at 55º C for the same time if primers were gene-specific. The transcriptase was then inactivated by heating at 70º C for 15 minutes. At the end of this process, complementary RNA was eliminated through incubation with *E.Coli* RNase H, 1 µl, at 37º C for 20 minutes.

### 3.4.3 Real Time-PCR

After the first strand cDNA synthesis was performed, the cDNA was used as the template for real-time PCR using primers specific for the glycoprotein (GP forward and reverse primers) and nucleoprotein (NP2 forward and reverse primers) of LCMV previously described (McCausland and Crotty 2008).

Primers:

Forward GP: GP-R, GCAACTGCTGTGTTCCCGAAAC

GP-F, CATTCACCTGGACTTTGTCAGACTC

Forward NP: NP2-R, CAGACCTTGGCTTGCTTTACACAG

NP2-F, CAGAAATGTTGATGCTGGACTGC

Using iTaq SYBR Green Supermix with ROX (Bio-Rad, USA), plated in 96-well plate format and run on a GeneAmp 5700 (ABI, Redwood City, CA). Amplification was done for 40 cycles, with each cycle consisting of two steps: 95º C for 15 seconds; 60º C, 30 seconds. All QPCR samples were run in duplicate. A standard curve with linearized plasmids encoding LCMV GP and NP genes was used to calculate the number of copies of LCMV genome per milligram of spleen.

## 3.5 Flow cytometry analysis

The fluorescently labelled monoclonal antibodies anti-CD86- (allophycocyanin [APC]-labeled), anti-I-$A^b$- (fluorescein isothiocyanate [FITC]-labelled), anti-CD11c- (R-phycoerythrin [PE]-labeled), anti-CD8-APC, anti-CD90.1-AlexaFluor450 were purchased from eBioscience. The following fluorescently labelled antibodies were purchased from BD Pharmingen: Ly-6C-PE-Cy7, CD4-FITC. The gp33 peptide [KAVYNFATM] /H-$2D^b$-APC tetramer was purchased from Becton Dickinson (Zhang et al. 2006). Lymph-node cells, splenocytes and BMDDC were prepared and stained with tetramers and mAb as before (Zhang et al. 2006). DC were subjected to ICS with rabbit anti-Spi6 antiserum (1/1000 dilution) or rabbit pre-immune serum (1/1000 dilution) then goat anti-rabbit IgG-APC (1/100 dilution; Jacksons ImmunoResearch). Stained cells were acquired on a Cyan ADP machine (Beckman Coulter) and analyzed with FlowJo software (TreeStar Inc.).

## 3.6 Statistical analysis

Calculation of means, standard deviations and *P*-values were carried out using MS-Excel™ and GraphPad Prism. Statistical analysis was conducted with GraphPad Prism. Normal standard distribution was assumed for all data: Student's t test was used for all data sets

# 4. RESULTS

Spi6 is an intracellular serpin (serine protease inhibitor) which is expressed by CTLs and NK cells upon activation and by DCs upon maturation. Mimicking a GrB target, it irreversibly binds to the enzyme, causing inhibition of GrB and leading to degradation of the Spi6/GrB complex. The protective function of Spi6 in mature DCs against ectopic GrB, released by CTLs during priming, has been proven in *in vitro* systems. When overexpressed in DCs, Spi6 has been demonstrated to protect these cells from CTL-inducted apoptosis (Medema et al. 2001). In another study, Hirst and colleagues showed upregulation of the human homologue PI-9 in mature DCs and suggested a correlation between the presence of ectopic GrB during CTL priming and the upregulation of PI-9 (Hirst et al. 2003).

The model suggesting that Spi6 could confer protection against CTL-induced apoptosis has been challenged by an *in vivo* study. Showing that mature bone marrow-derived dendritic cells (BMDDC) injected subcutaneously in a mouse could be retrieved in the draining LN in the same number as immature ones, Andrew and colleagues suggested that mature and immature BMDDCs have an equal degree of apoptosis-susceptibility (Andrew et al. 2008). My hypothesis proposes that Spi6 confers protection to DCs against CTL-delivered GrB-mediated apoptosis during priming of CD8 T cell responses *in vivo*.

As a first step it was decided to confirm previous *in vitro* results obtained by my colleague Dr. Zhang. It was therefore shown that Spi6 is upregulated in WT mature DCs and absent in Spi6 KO-derived ones, and that Spi6 KO BMDDC susceptibility to CTL-directed apoptosis is GrB-dependent (Chapter 4.1). The ability of these cells to stimulate a CD8 T cell response *in vivo* in a naïve and/or memory mouse was subsequently tested (Chapter 4.2). Expression of Spi6 was then measured in splenic CD8α DC and pDC subsets *in vivo*, and their numbers measured at various stages post-infection (Chapter 4.3). The functionality of the CD11c CD8α DC subset, measured as ability to prime CD8 T cell responses *ex vivo*, was also verified in Spi6 KO-derived DCs versus WT ones (Chapter 4.3.4). After clarifying the functional

status of dendritic cells in Spi6 KO mice and WT mice, the levels of antigen-specific CD8 T cell response were measured both in primary and secondary responses (Chapter 4.4). The secondary response was further investigated through analysis of re-expansion levels of known memory pools in Spi6 KO and WT mice (Chapter 4.4.3). GrB involvement in the shaping of these responses was investigated in relationship to DC survival and CD8 T cell response expansion. To conclude, CTL responses in these mice were tested for their ability to efficiently clear viral infections (Chapter 4.7).

## 4.1 Spi6 protects bone marrow-derived dendritic cells from GrB-mediated apoptosis *in vitro*

Spi6 had, up till now, been measured in DCs using human anti-PI-9 antibody staining and/or PCR for mRNA levels in both humans and mice. These methods are not accurate for the measurement of this protein in mice as the human PI-9 is structurally different from the mouse one and RNA levels are not always representative of levels of intracellular protein, as in the case of GrB (Fehniger et al. 2007). Prof. Ashton-Rickardt's group had previously developed a polyclonal anti-Spi6 rabbit serum that allowed a more specific identification of the mouse protein (Zhang et al. 2007). This serum was used to verify the intracellular expression of Spi6 in BMDDC from WT (B6) and Spi6 KO mice and correlate this expression to protection from CTL-induced apoptosis. The need to identify the role of physiological levels of Spi6 in DC protection against CTL-killing is the aim of the first set of experiments.

### 4.1.1 Spi6 is expressed at low levels in immature bone marrow-derived dendritic cells and it is up regulated upon maturation

Spi6 expression had been measured in a dendritic cell line, D1, by determining mRNA levels (Medema et al. 2001) or using anti-PI-9 antibody (Andrew et al. 2008), but nothing had been reported on the measurement of intracellular protein levels. Rabbit anti-Spi6 serum was used to carry out intracellular staining for Spi6 on BMDDCs, in order to assess if Spi6 is up-regulated at the protein level upon LPS-induced maturation. BMDDCs were generated from C57BL/6 mice (referred to as WT hereafter) and from Spi6 KO mice as previously described by Lutz *et al.* (Lutz et al. 1999). After 8 days in culture with GM-CSF, BMDDCs show an immature phenotype, with expression of the DC-specific marker CD11c, low levels of the antigen-presenting molecule MHC-II (I-$A^b$) and low levels of the maturation marker CD86, as shown in Figure 33 A.

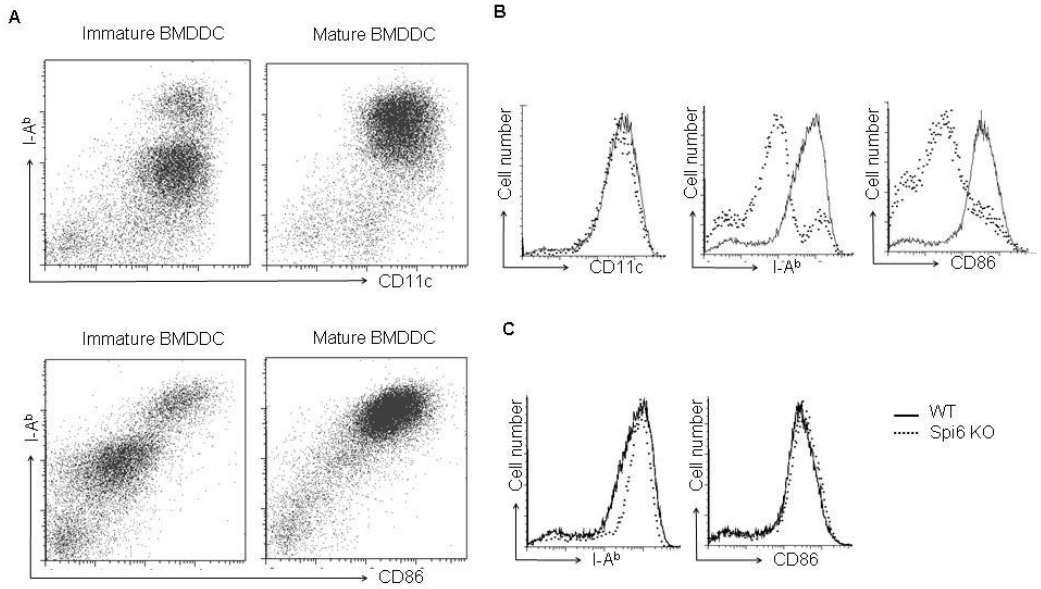



**Figure 33: LPS maturation of BMDDCs (48 hrs). A) Surface expression of CD11c, I-$A^b$ and CD86 on WT BMDDC before and after LPS stimulation (1 ng/ml). B) Expression of CD11c, I-$A^b$ and CD86 on gp33-pulsed LPS-stimulated (bold line) versus gp33-only pulsed cells (dotted line). C) Expression of markers on WT- (bold line) and Spi6 KO-derived (dotted line) BMDDCs after LPS treatment.**

To induce maturation, these cells were stimulated with LPS (1 ng/ml) for 24 hrs. After LPS stimulation, BMDDCs up-regulate expression of CD11c, MHC-II and CD86, becoming $CD11c^{hi}$ $I\text{-}A^{b}$ $CD86^{high}$ (see Figure 33 A), which indicate a mature phenotype. As expected, BMDDCs cultured in absence of LPS after 24 hrs still displayed an immature phenotype ($MHC\text{-}II^{low}$ $CD86^{low}$ Figure 33 B, dotted line). BMDDC from Spi6 KO showed no defects in maturation, as shown by the levels of maturation markers, which are comparable to WT (Figure 33 C).

Intracellular expression of Spi6 in WT and Spi6 KO BMDDCs, was carried out using polyclonal rabbit anti Spi6-serum. The results for mature BMDDCs (bold line) and immature ones (dotted line) are shown in Figure 34. As expected, mature WT BMDDCs up-regulate Spi6. Also, the levels of Spi6 in immature BMDDCs are lower than in mature cells, both in WT and Spi6 KO-derived cells, but higher than expression in pre-immune serum (solid grey area). This is likely due to cross-reactivity with other serpins, or to basal low expression of Spi6 in these cells. In Spi6 KO cells no up-regulation following maturation is seen.

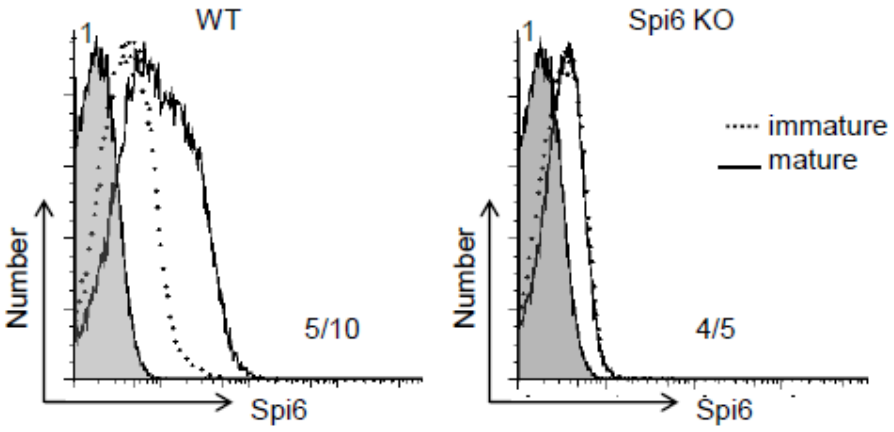



**Figure 34: Spi6 expression levels in immature and LPS-matured WT and Spi6 KO BMDDCs. Intracellular Spi6 was measured using polyclonal rabbit anti-Spi6 serum. Solid grey histogram: negative control (intracellular staining with pre-immune serum). Numbers within histograms indicate mean fluorescence intensity for Spi6 in the negative control (top left of histogram), and in the immature/mature DCs (bottom right).**

By directly comparing intracellular staining for Spi6 in WT immature versus immature Spi6 KO BMDDCs (Figure 35) and on WT LPS-matured versus their Spi6 KO counterparts, the up-regulation of Spi6 in mature cells can be better appreciated.

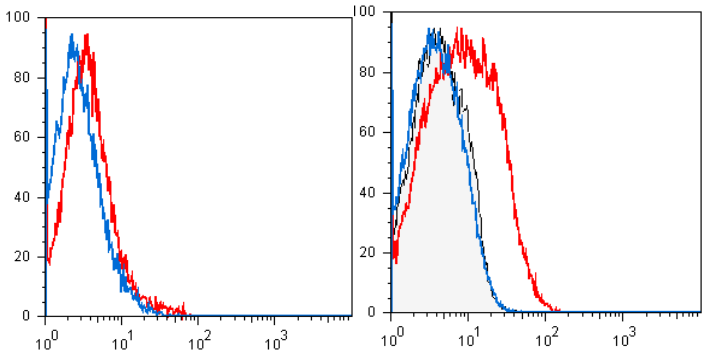



**Figure 35: Spi6 expression (intracellular staining) in immature (left panel) and mature (right panel) BMDDCs: red histogram WT, blue histogram, Spi6 KO cells.**

LPS stimulation of WT BMDDC for 48 hrs therefore results in up-regulation of Spi6 expression and concomitant high protein levels. As expected, neither immature nor mature Spi6 KO-derived BMDDCs express Spi6. Our results show that Spi6 is present and up-regulated in the cytoplasm of LPS-matured WT BMDDCs. This data complement observations from previous work by M. Zhang, which reported how Spi6 KO mice are susceptible to CTL killing, in supporting a protecting role for Spi6 in mature DCs.

### 4.1.2 Spi6 protects mature dendritic cells from granzyme B-mediated killing *in vitro*

Medema and colleagues demonstrated how overexpression of Spi6 protects DCs from CTL-mediated killing (Medema et al. 2001): however, early studies in our lab demonstrated the correlation between physiological levels of Spi6 and protection from CTL-killing (by M. Zhang). Having ascertained that Spi6 is up-regulated upon

maturation in WT BMDDCs *in vitro*, the next step was to confirm early data showing how lack of Spi6 leads to increased susceptibility of DCs to CTL-mediated killing *in vitro,* and investigate whether this was due to GrB-specific killing.

$^{51}$Cr-release killing assay have been used in our lab to verify DC susceptibility to CTL-induced programmed cell death (PCD) in addition to DNA fragmentation analysis, as described above (2.8.4.4). I sought to recapitulate these experiments albeit using a different read-out reagent. Immature and LPS-matured Spi6 KO- and WT-derived BMDDCs were used as target of CTLs from P14 mice (effector cells) in an assay where calcein acetoxymethylester (AM) served as an indicator of cell death. CalceinAM is a non-fluorescent cell-permeable compound that undergoes hydrolysis by intracellular esterases in the cytoplasm. In this process it is converted into a cell-impermeable, strongly fluorescent anion (calcein), which is retained by live cells. BMDDCs were generated after 8 days of culture with GM-CSF, then pulsed with gp33 only, or pulsed with gp33 and stimulated with LPS, for 24 hrs, harvested and loaded with calceinAM. Once loaded, BMDDCs were used as CTL target: BMDDCs (targets) were co-cultured at different ratios with fully activated P14 CTLs (effectors). P14 CTLs are TCR-Tg for LCMV-gp33. After 4 hrs, the percentage of killing was evaluated by measuring the levels of calcein in the supernatant. Once entered the cell and gained access to the cytosol, calceinAM is irreversibly modified and it can no longer permeate the cell and be released. The levels of calcein present in the supernatant after 4 hrs of co-culture with P14 CTLs are therefore due to membrane damage and correlate with apoptosis of BMDDCs.

We first tested whether there were any differences in survival between immature and mature WT DCs. We found that immature DCs, which express low levels of Spi6 and do not present antigen, undergo less apoptosis at 4 hrs than mature DCs, which express higher levels of Spi6 and are able to cross-present antigen to CTLs (Figure 36). Nonetheless, the difference between immature and mature killing was not significant in these experiments, where the ratio was 25 to 1. In the same way, the difference between killing of WT and Spi6 KO BMDDCs was not significant.

The difference in killing, although not significant, in this case was probably not directly due to Spi6 expression, but to the ability of mature DCs to induce activation

and degranulation of CTLs, as previously shown (Andrew et al. 2008). Immature DCs are not equipped to present antigen as the recycling rate of MHC-I and MHC-II is very high, thus leading to unstable antigen presentation capacity, in addition to low co-stimulatory molecule expression (Smith et al. 2011). Mature DCs can induce CTL differentiation and concomitant production of cytotoxic mediators. The results in Figure 36 confirm that mature DCs are preferential targets of GrB lysis.

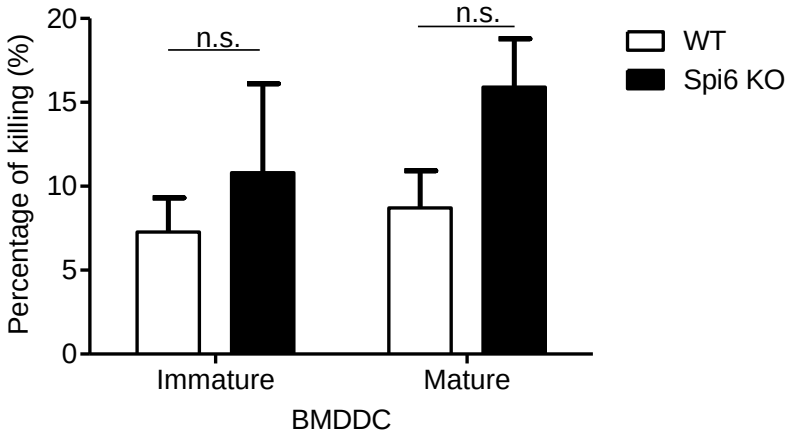



**Figure 36: Mature BMDDCs are more susceptible to granzyme B-mediated killing compared to immature BMDDCs. The plot shows percentage of killing of BMDDCs (WT and KO) by P14 effectors at 25 : 1 ratio, measured by calceinAM release in cultures 4 hrs post-stimulation. All mean values are ±SEM and are representative of three independent experiments.**

Killing of mature BMDDCs was further analysed using different effector (P14 CTL) to target (WT or Spi6 KO BMDDC) ratios. Results obtained show that percentages of target killing are proportional to effector cell numbers and more prominent in Spi6 KO-derived BMDDCs (Figure 37). The difference in the level of apoptosis confirms that mature DCs from WT mice are less susceptible to CTL-induced apoptosis compared to Spi6 KO-derived LPS-matured DCs. Also, they show that at high ratios DCs are killed at the same level.

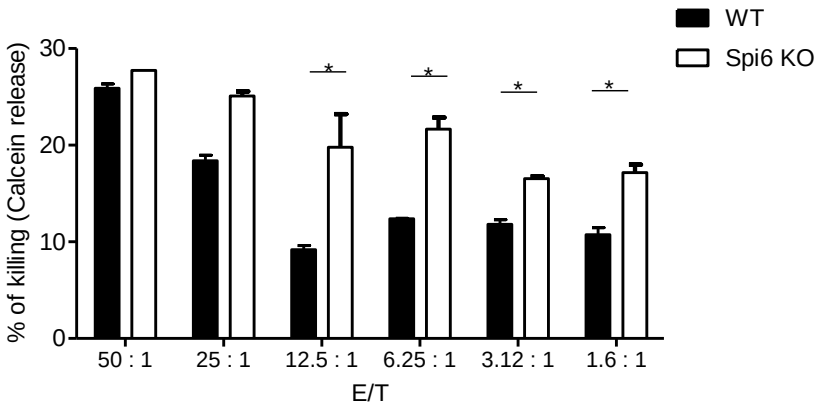



**Figure 37: Lack of Spi6 confers susceptibility to CTL-mediated apoptosis. Plot shows percentage killing of LPS-matured WT and Spi6 KO BMDDCs (targets) by P14 CTLs (effectors) at different ratios, measured by calcein release in culture supernatants 4 hrs post-stimulation. All mean values are ±SEM and are representative of three independent experiments; *$p < 0.05$.**

### 4.1.3 Susceptibility of bone marrow-derived dendritic cells to CTL-mediated death is abrogated in absence of granzyme B

As Spi6 specifically inhibits granzyme B, elimination of this mediator of cytotoxicity is predicted to counteract the protective function of Spi6. To verify that the apoptosis levels measured in the previous experiment were due to GrB activity and not to triggering of the Fas-FasL pathway, the same calceinAM-based assay was performed using P14 CTLs from GrB KO mice.

Using the same experimental conditions as above, we found that GrB KO P14 CTLs do not induce apoptosis in WT and Spi6 KO cells after 4 hrs of stimulation (Figure 38). This result is consistent with the hypothesis that DC apoptosis is due to GrB-dependent CTL killing.

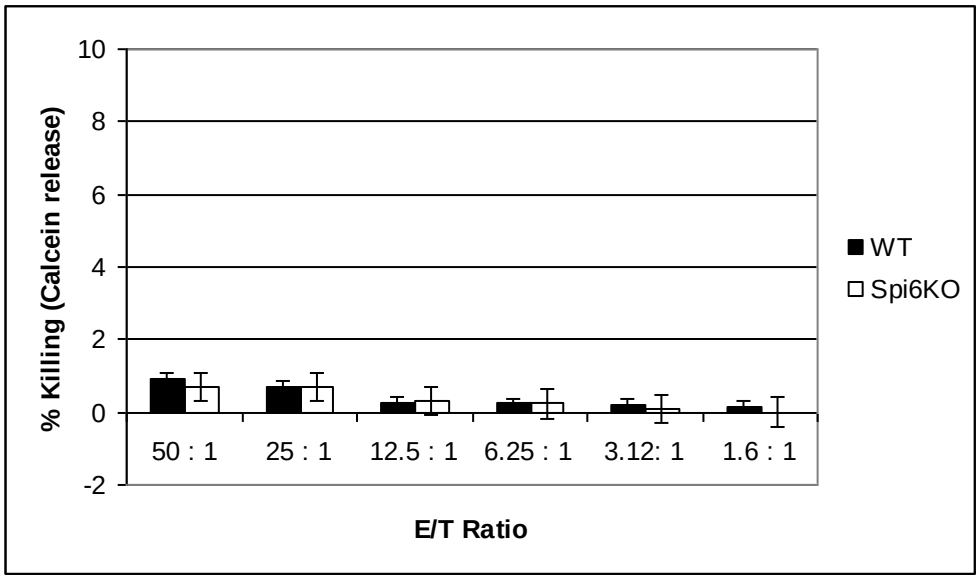



**Figure 38: Early apoptosis in WT and Spi6KO BMDDCs is mediated by granzyme B. The plot shows percentage of killing of LPS-matured WT and Spi6 KO BMDDCs by GrB KO P14 CTLs at different effector/target ratios, measured by calcein release in culture 4 hrs post-stimulation. All average values are ±SEM of triplicate measures and representative of three independent experiments.**

We decided to verify whether the absence of killing at 4 hrs was due to the absence of GrB, which is an early apoptosis inducer, and to demonstrate that alternative apoptotic pathways are activated at later stages. Apoptosis levels of WT and Spi6 KO BMDDCs were thus determined at a later time-point, 8 hrs. After 8 hrs of culture with GrB KO CTLs, apoptosis was measured for both WT and Spi6O BMDDcs but no significant difference in apoptosis levels was observed between the two (Figure 39).

These results suggest that GrB acts by inducing apoptosis within the first 4 hrs of interaction between CTLs and DCs. They also confirm that CTL priming by mature DCs confers the mature DCs susceptibility to GrB-mediated apoptosis.

Moreover, from these results we can hypothesise that other mediators can contribute to DC apoptosis, like Fas-FasL and other granzymes stored in cytotoxic granules, but these have slower kinetics and are therefore not the primary cause of DC killing. Spi6 up-regulation and inhibition of GrB function in DCs is therefore required to protect these cells from CTL- mediated killing.

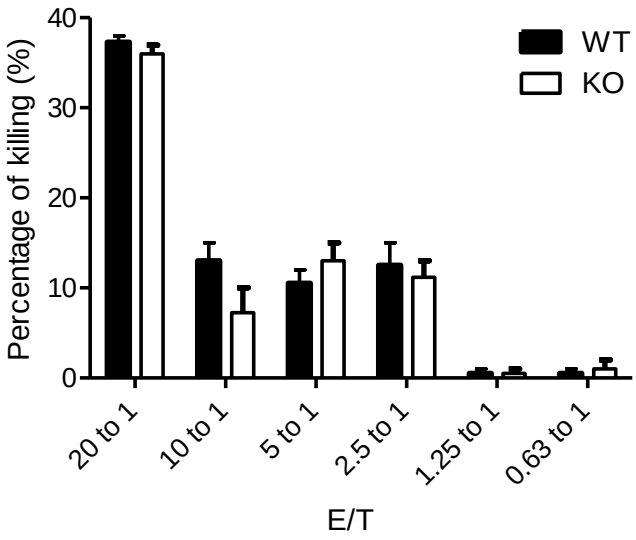



**Figure 39: Protection of BMDDCs from apoptosis is independent of Spi6 at later time-points. Plot shows percentage killing of BMDDCs (WT and Spi6 KO) by GrB KO P14 CTLs measured by calcein release after 8 hrs. All mean values are ±SEM of triplicate measure and representative of three independent experiments.**

## 4.2 Priming capacity of antigen-loaded mature bone marrow-derived dendritic cells

CD8 T cell responses can be stimulated *in vivo* by adoptively transferring antigen-loaded mature dendritic cells into naïve or antigen-boosted mice (Ludewig et al. 2001). Using the protocol adapted by Ludewig and colleagues, the ability of LPS-matured antigen-loaded WT and Spi6 KO-derived BMDDCs to promote CTL responses in naïve and antigen-experienced mice was studied. This also allowed the indirect study of Spi6 function in DC priming of CTL responses *in vivo.*

BMDDCs from Spi6 KO and WT mice were used to stimulate gp33-specific immune responses in WT mice. BMDDCs were generated *in vitro* with GM-CSF from WT and Spi6 KO mice for 8 days and matured with LPS and gp33, as described. BMDDCs were loaded with gp33 which is one of the two immunodominant LCMV

epitopes and which was also used in the *in vitro* CTL killing assay. MHC-I-specific gp33 peptide tetramers (H-2D$^b$) were used to detect antigen-specific CTLs. Tetramers are a complex formed of four identical MHC-I complexes. Their development was originally reported by Altman and colleagues in 1996 to allow enumeration of antigen-specific CTLs in a system (Altman et al. 1996). Tetramers recognise specific MHC-restricted TCR: in this study, gp33 tetramers conjugated with the fluorescent dye PE (phycoerythrin) were used.

In this chapter will be described how BMDDCs pulsed with LCMV gp33 were used to prime CTLs by adoptively transferring them into WT mice intravenously. The ability of DCs to stimulate a CD8 response by cross-presenting the soluble antigen was verified by measuring CD8 T cell expansion.

### 4.2.1 CD8 T cell response in naïve mice after adoptive transfer of antigen-loaded bone marrow-derived dendritic cells

BMDDCs were grown from the bone marrow of WT and Spi6 KO mice and matured with LPS as described above. Cells were then harvested and injected ($5x10^5$ cells) i.v. into the tail vein of WT mice. Mice were bled at day 4 to check the development of a CD8 T cell response and sacrificed at day 7 post-injection. Blood, inguinal LNs and spleen were analysed for gp33-specific CTL response.

Gp33-loaded mature BMDDCs adoptively transferred into a WT mouse were able to stimulate CTL-responses. The analysis of gp33-specific CTL levels in the spleen and inguinal LNs (in Figure 40) showed that both WT and Spi6 KO BMDDCs were able to induce CD8 T cell responses.

However, no difference was seen in the numbers of tetramer-positive CD8 T cells in both organs. In addition, no difference was measured in the gp33-specific CTL levels in the blood on days 4 and 7 post- transfer (Figure 41).

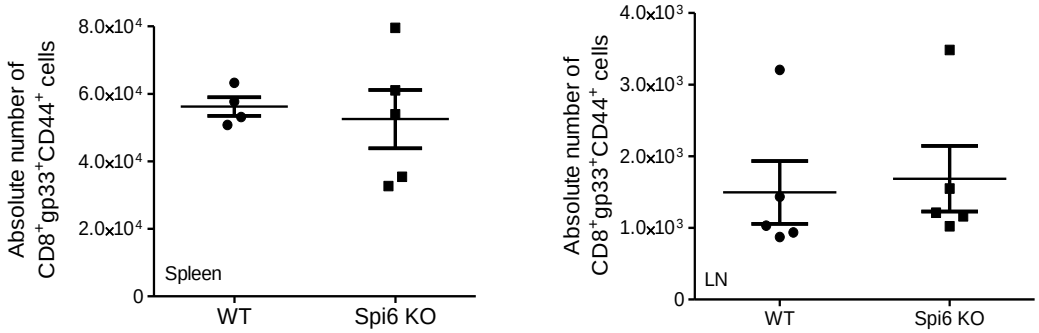



**Figure 40: Absolute numbers of CD8$^+$ gp33$^+$ CD44$^+$ T cells in the spleen (left) and inguinal LNs (right) of naïve WT mice adoptively transferred with WT or Spi6 KO BMDDCs, on day 7 post injection. Cells were quantified by MHC-I tetramer staining (gp33 tetramer). All mean values (middle horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 4-5).**

Nonetheless, since this model does not mimic a viral infection but rather an immunisation with limited antigen presentation, these antigen-specific CTLs may be induced by an autopilot mechanism. Several groups have in fact demonstrated how LPS-matured BMDDCs are quickly eliminated once injected into a mouse, thus not being able to sustain a prolonged antigen presentation (Hou and Van Parijs 2004).

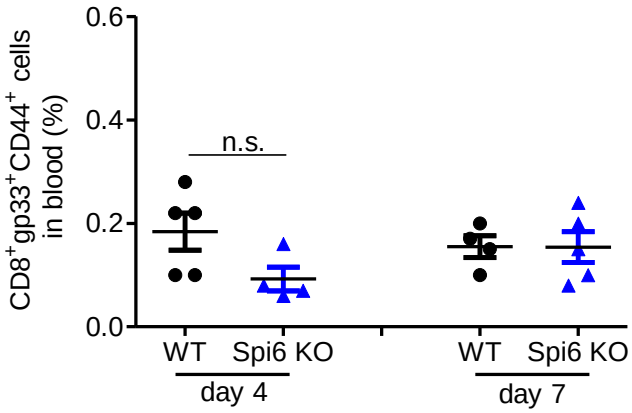



**Figure 41: Frequency of gp33-specific activated CD8 T cells in the blood of naïve WT mice adoptively transferred with Spi6 KO or WT BMDDC on day 4 (left) and 7 (right) post-transfer. All mean values (middle horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 4-5), n.s. Not Significant.**

### 4.2.2 CD8 T cell responses in antigen-experienced (memory) mice after adoptive transfer of mature bone marrow-derived dendritic cells

To verify the ability of in vitro matured BMDDCs to stimulate a secondary immune response, gp33-loaded BMDDCs were then adoptively transferred into antigen-experienced (memory) mice. Memory mice (WT) had been infected with LCMV Armstrong at least 40 days prior transfer, to allow for development of memory gp33-specific CD8 T cells. In this scenario, gp33-loaded mature BMDDCs could present LCMV gp33 antigen to cognate memory CTLs, thereby stimulating their activation and re-expansion. Data obtained is shown in Figure 42: no difference was seen in the number of responding CD8 T cells in the spleen 5 days after adoptive transfer of WT or Spi6 KO BMDDCs.

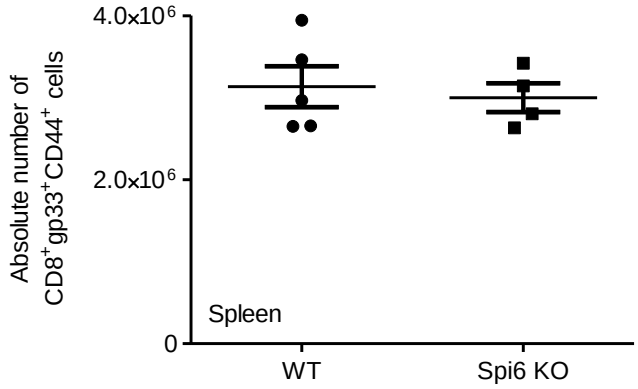



**Figure 42: Absolute numbers of gp33-specific CD8 T cells in WT memory mice (spleen) after adoptive transfer of BMDDCs generated from WT or Spi6 KO mice. All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 4-5).**

A significant difference, on the contrary, was seen in the inguinal LNs (Figure 43).

This difference may reflect the effect of the specific route of administration and may be due to the distribution of BMDDCs introduced into the circulation. It might also be explained in terms of the different memory T cell subsets present in the LN and spleen of memory mice, which might differently depend on Spi6-upregulating DCs for proper priming.

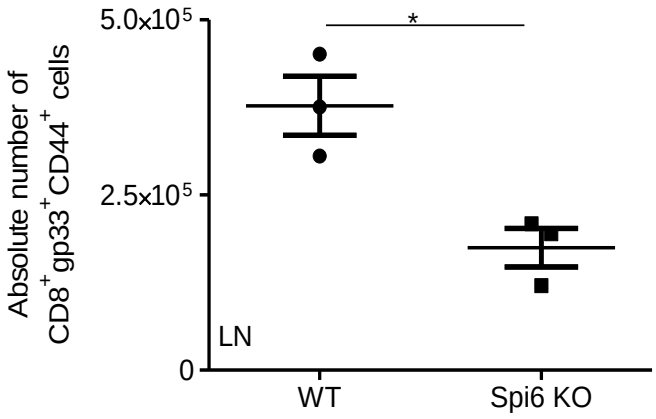



**Figure 43: Absolute numbers of $CD8^+$ $gp33^+$ $CD44^+$ T cells in the lymph nodes of WT memory mice 5 days after adoptive transfer of LPS-matured, Spi6 KO or WT BMDDCs. All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n*= 3), *$p < 0.05$.**

In the blood, levels of CTLs were comparable at all the time-points that were analysed in both naïve and memory mice (Figure 44). Donor-derived gp33-specific CD8 T cells in blood were measured as percentage of the total lymphocytes and not as absolute numbers due to the variability of the blood samples.

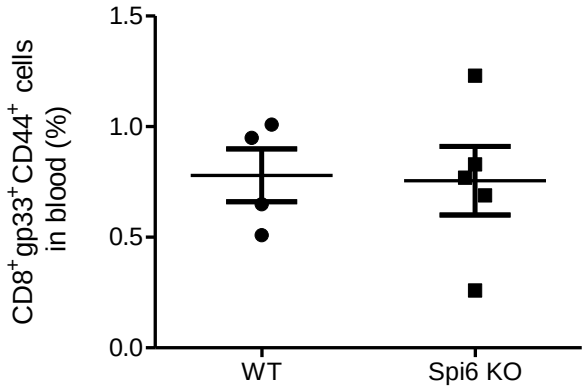



**Figure 44: Percentage of $gp33^+$ $CD8^+$ $CD44^+$ T cells in the blood of memory WT mice, 5 days after adoptive transfer of LPS-matured, Spi6 KO or WT BMDDCs. All mean values are ± SEM and are representative of three independent experiments (*n* = 4-5).**

Adoptive transfer of antigen-loaded LPS-matured BMDDCs in our hands induces a gp33-specific response and no difference is seen in the levels of these CD8 T cells in

the spleen. A difference is, on the contrary, seen when measuring absolute numbers of gp33-specific CD8 T cells in the inguinal LNs.

## 4.3 Dendritic cells *in vivo*: lessons from the spleen

Our observations in the previous section suggest that BMDDCs generated from WT or Spi6 KO mice are equally able to stimulate CTL responses in naïve and memory mice. We therefore sought to verify if Spi6 could protect endogenous DCs from CTL killing in an *in vivo* infection model.

LCMV, as already described, is a non-cytolytic, MHC-I dependent virus. Its infection is efficiently cleared in 8-10 days in immunocompetent mice and it has two immunodominant H-2D$^{b}$–restricted epitopes: gp33 (glycoprotein 33-41) and np396 (nucleoprotein 396-404). P14 mice (Kaech and Ahmed 2001) are transgenic mice whose entire T cell repertoire has been manipulated to recognise the gp33 antigen in the context of MHC-I. They are widely used to study CD8 T cell dynamics: in particular CTL primary responses, development of the memory pool and re-expansion of memory CD8 T cells. The LCMV infection model was therefore chosen to study CTL responses in Spi6 KO and WT mice.

Various groups have previously demonstrated that DCs are the sole antigen-presenting cells able to prime CD8 T cell responses in LCMV infection (Belz et al. 2004). Mouse dendritic cells can be subdivided in several populations depending on tissue distribution, presentation and stimulation capacity, and lifespan. In the spleen, mainly three types of DCs, called resident conventional DCs (cDCs), can be identified and distinguished by the expression of CD8α and CD4: CD8α$^{+}$ DCs, CD8α$^{-}$ CD4$^{+}$ DCs and CD8α$^{-}$ CD4$^{-}$ DC. In inflammatory conditions, monocyte-derived and peripheral antigen-loaded mature DCs can also enter the spleen. For instance, the CD11c$^{int}$ B220$^{+}$ Ly-6C$^{+}$ migratory plasmacytoid DCs gain access to the spleen from the circulation and locate to the marginal zone. In the LNs, similarly, one CD8α$^{+}$ and

two CD8α$^-$ populations of cDCs are present, together with a population of migratory DCs circulating from peripheral tissues. During inflammation, Langerhans cells and dermal DCs migrate to the LN, where they pass on antigens to resident CD8α$^+$ and CD8α$^-$ DCs to allow these to prime CD4 and CD8 T cells. The SLO-residing populations have been studied for their properties to capture and present self and non-self antigens and the current model supports a main role for CD8α$^+$ DCs in priming CD8 T cell responses supported by their phagocytosis receptors, their antigen-processing machinery and their location in the spleen and LNs (see 2.5.10).

CD8α$^+$ DCs and pDCs were thus chosen among these subsets for their specific role in viral infections. CD8α DCs have been shown to be responsible in particular for cross-presenting LCMV-derived antigens in Armstrong infection (Belz et al. 2004; see 2.5.10) and pDCs are responsible for interferon production which enhances viral clearance (see 2.5.11).

In immunocompetent mice, the dynamics of infection have been described as follows: by 24 hrs CD8α$^+$ DCs stimulate naïve T cells: by day 3 apoptosis of CD11c$^+$ CD8α$^+$ is started and by day 4 there is an increase in the number of pDCs and production of IFN (Montoya et al. 2005). These results by Montoya and colleagues were the basis on which the following experiments were designed.

### 4.3.1 Expression of Spi6 in splenic dendritic cells

We first set up a primary study to develop a protocol for the identification of DC subsets in the spleen of our mouse model and study Spi6 expression, and we tested it using intact WT mice. The WT mice were infected with LCMV Armstrong intra-peritoneally (i.p.; $10^5$ PFU) and the spleen was analysed on various days p.i. to verify the development of CD8α DC and pDC populations in terms of numbers and Spi6 expression. CD8α DC and pDC subsets were identified among CD11c$^+$ cells isolated from the spleen using flow cytometry (Figure 45).

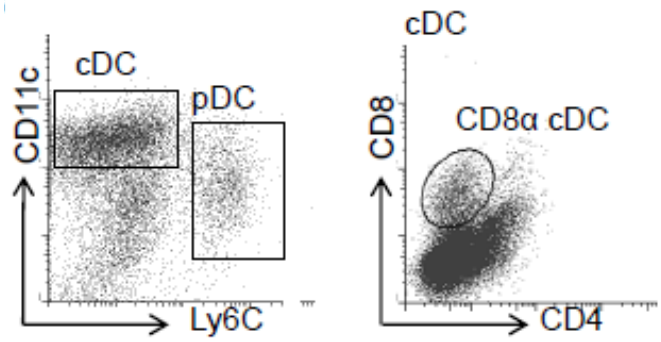



**Figure 45: Gating strategy of mouse splenic DCs using FACS analysis of antibody staining for surface markers. Conventional DCs are defined as CD11c$^{++}$ Ly6C$^{low}$, pDCs are CD11c$^{+}$ Ly6C$^{hi}$.**

Total cell numbers were obtained using Caltag counting beads and used to calculate the absolute numbers of each subset (i.e. the total number of those cells in the organ). CD8α DCs were identified as CD8α$^{+}$ CD4$^{-}$ among conventional DCs (identified as CD11c$^{hi}$ Ly6C$^{low}$), and pDCs were identified as CD11c$^{int}$ Ly6C$^{hi}$, according to the identification used by Sancho and colleagues (Sancho et al. 2008).

Analysis of DC numbers showed that the total number of cDCs increased by day 2 post-infection and decreased by day 4, probably due to killing by activated CTLs, followed by a further increase on days 6-8 post-infection (Figure 46). These results are nonethelss only indicative as only 2 samples were tested.

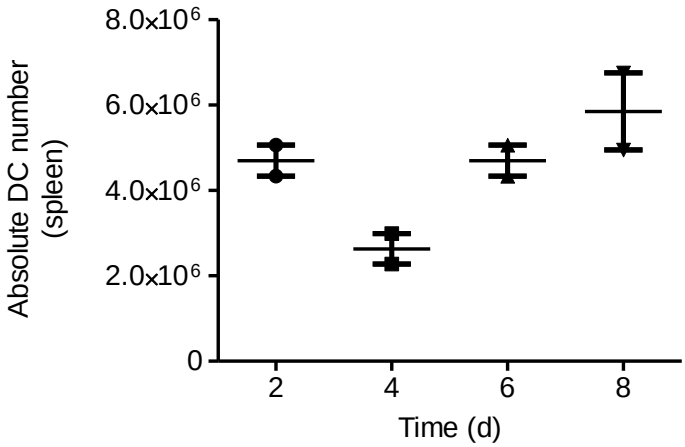



**Figure 46: Absolute numbers of cDCs (CD11c$^{hi}$ Ly6C$^{low}$) in the spleen of LCMV Arm-infected intact WT mice at time-points indicated. All mean values (middle horizontal line) are ± SEM (*n* = 2).**

Interestingly, when CD8α DC and pDC subpopulations were analysed individually, CD8α DC decreased at day 4 p.i. only to increase again from day 6 p.i. Plasmacytoid DCs, on the other hand, decreased on day 2 p.i., and were at undetectable levels in the spleen at day 6 p.i. Also, results indicated that numbers of CD8α DCs were at their lowest at day 4 post-infection (Figure 47).

Since Spi6 KO mice lack Spi6 in CD8 T cells, high levels of apoptosis are induced in CTLs when these cells mature to the effector status. Responses to viral challenge in these mice are thus are impaired as the expansion phase fail to reach the levels seen in WT mice (Zhang et al. 2006). The Spi6 KO intact mouse is therefore not a good model to study DC killing by CTLs. To study the effect of GrB in a Spi6-competent pool of CTLs, naïve WT P14 CD8 T cells were adoptively transferred into WT and Spi6 KO mice.

The immunodominant epitopes for LCMV, as previously mentioned, are gp33 and np396. This study will mostly concentrate on CD8 responses to gp33 because of the availability of transgenic mice expressing a TCR specific for this antigen, LCMV gp33 (gp33-41) in a 2-HD$^{b}$-restricted context. This mouse strain is called P14.

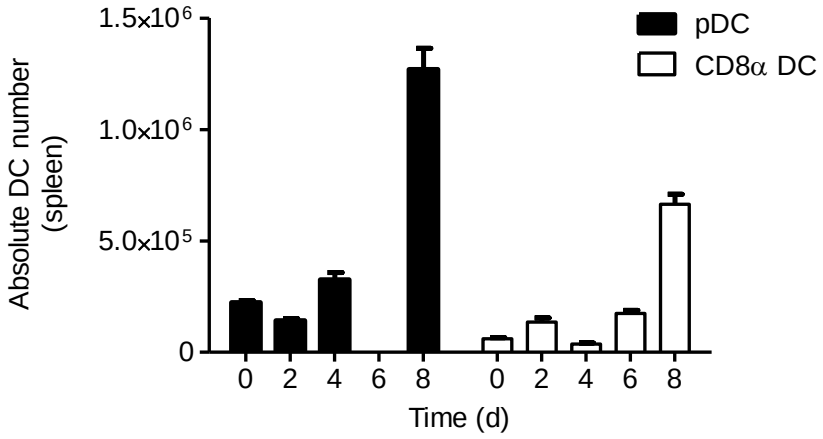



**Figure 47: Absolute number of CD8α DCs and pDCs in the spleen of an Arm-infected intact WT mouse, at indicated days post-infection All mean values are ± SEM (*n* = 2).**

Congenically marked naïve P14 CD8 T cells (Thy1.1$^{+}$) were transferred into WT and Spi6 KO (both Thy1.2$^{+}$) mice and both test groups were infected with LCMV

Arm. Mice were sacrificed on days 2, 4, and 8 post-infection and analysis of DC subpopulations in the spleen was carried out as described above (Figure 48). The adoptively transferred P14 cells are, in this model, primed by endogenous antigen-loaded DCs, matured by physiological inflammatory stimuli and signals triggered by viral recognition. DC numbers were studied in the spleen of these mice, as isolation of DCs from the LN is more challenging due to lower cellularity.

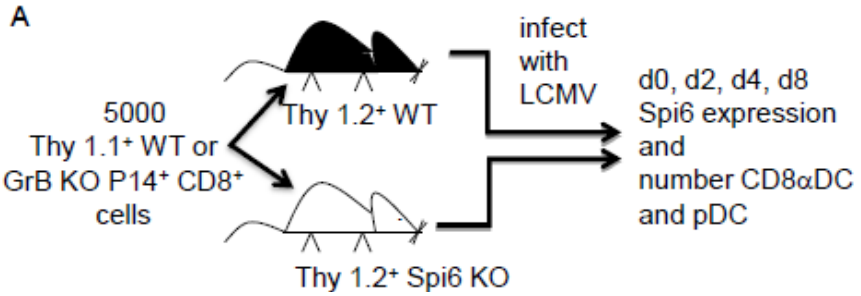



**Figure 48: Protocol for study of DC responses in Spi6 KO and WT mice after acute LCMV infection. Naïve Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into WT and Spi6 KO mice, which were subsequently infected with LCMV Arm. Analysis of DC subsets was carried out on day 0 (pre-infection), 2, 4 and 8 post-infection.**

Intracellular expression of Spi6 was analysed in the CD8α DC and pDC subsets in order to correlate expression of this protein and survival of each subset. As expected, CD11c$^+$ cells from Spi6 KO mice did not show expression of Spi6 (Figure 49, dotted lines). In WT cells, on the other hand, Spi6 was up-regulated at various stages. Spi6 was up-regulated in WT CD8α$^+$ DC from day 2 and until day 8. Spi6 levels in pDCs increased from day 4 in both Spi6 KO and WT mice, thus being not significantly different at any time-point (Figure 49).

Analyses of the mean fluorescence intensity (MFI) of the intracellular stain for Spi6 in in CD8α DCs showed a significant difference in the expression of Spi6 between Spi6 KO and WT mice from day 2 onwards, while no difference in Spi expression was observed in pDCs.

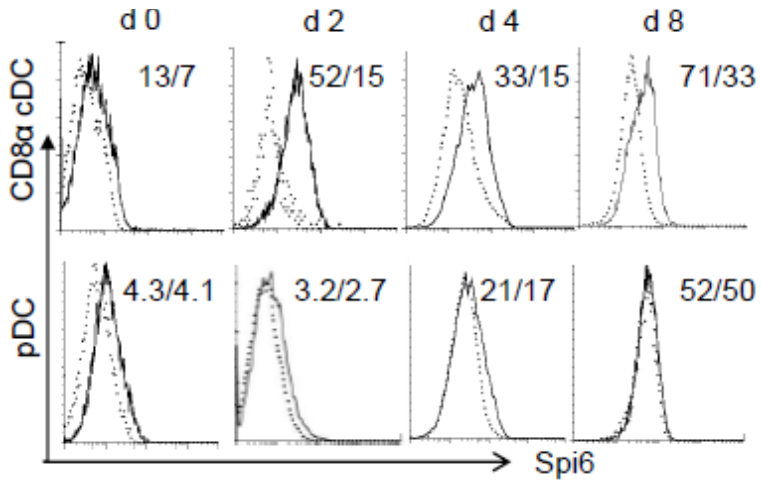



**Figure 49: Spi6 expression in CD8α DCs and pDCs in Arm-infected WT (bold line) and Spi6 KO mice (dotted line) adoptively transferred with WT P14 cells at various days post-infection. Numbers represent mean fluorescence intensity (MFI) obtained with intracellular staining of Spi6 in WT/Spi6 KO DCs. Values are representative of three independent experiments (*n* = 5-6).**

Expression of Spi6 in pDCs was nonetheless increased to some extent in both WT and Spi6 KO pDCs from day 6, suggesting that upregulation of other serpins, which cross-react with the polyclonal anti-Spi6 serum, might take place (Figure 50).

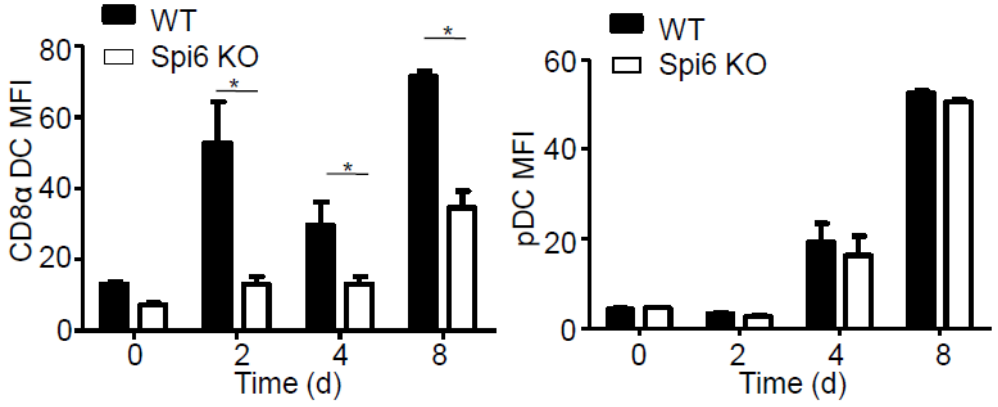



**Figure 50: Mean fluorescence intensity (MFI) intensity for the intracellular staining of Spi6 in WT (black) and Spi6 KO (white) mice at indicated days post-infection. The left panel shows the expression of Spi6 in CD8α DCs and the right one shows Spi6 expression in pDCs in the spleen of LCMV Arm-infected mice. Both WT and Spi6 KO mice were adoptively transferred with P14 Thy1.1$^{+}$ CD8 naïve cells before infection according to the protocol. All mean values are ±SEM and are representative of three independent experiments (*n* = 5-6), * *p* < 0.05**

### 4.3.2 The absolute number of CD8α$^+$ dendritic cells is reduced in the spleen of Spi6 KO mice when challenged with LCMV

Upregulation of Spi6 in mature WT DCs from day 2 post-infection indicates a need for these cells to protect themselves against GrB activity; cells lacking this protein are expected to be more susceptible to CTL-induced apoptosis. Absolute numbers of CD8α DCs and pDCs were thus measured to verify if Spi6 expression in CD8α$^+$ DC or pDC contributed to the survival of these cells. Reduced numbers of live DCs were seen in the spleen of Spi6 KO mice during the first phase of the response (Figure 511). On day 4 p.i., a 75% reduction ($p$ = 0.040) in the number of CD8α DCs was measured in Spi6 KO mice compared to WT. As CD8α DC is the subpopulation responsible for presentation of LCMV antigen to naïve CD8 T cells (Belz et al. 2004), Spi6 is thought to be required by these cells to avoid apoptosis triggering by GrB locally released by CD8 T cells during/after priming.

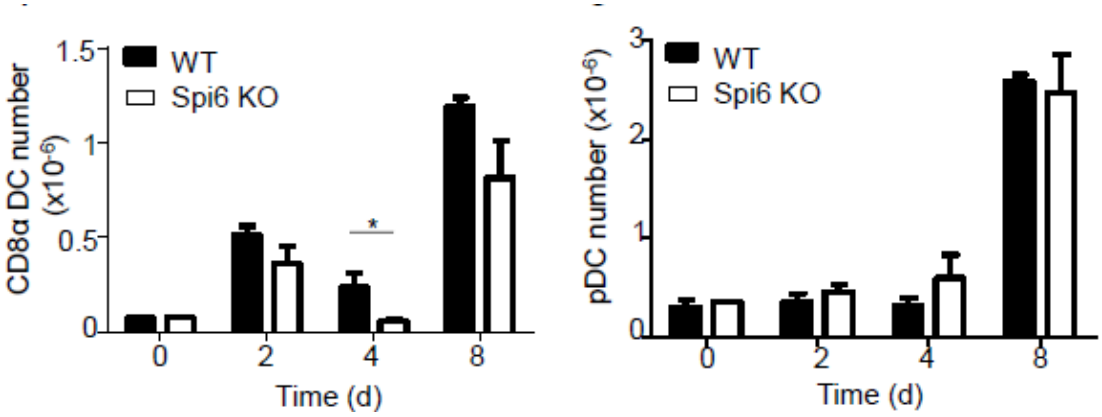



**Figure 51: Absolute numbers of CD8α DC (left) and pDC (right) in WT (black) and Spi6 KO (white) mice infected with LCMV Arm and adoptively transferred with P14 CD8 T cells, at indicated days p.i. All mean values are ±SEM and are representative of three independent experiments ($n$ = 5-6), * $p$ < 0.05.**

Interestingly, we did not observe any difference in the number of pDCs in the spleen of infected mice at all time-points studied. This data is consistent with the lack of Spi6 up-regulation in pDCs at any time during infection. Up-regulation of Spi6 is in fact expected to occur only in cells that are susceptible to lysis by GrB, which pDCs due to their non-presenting function are not. These results showing Spi6 upregulation

in CD8α DCs and absence of upregulation in pDCs support the hypothesis that Spi6 is important for survival of DC involved in CTL priming.

Alternatively, absence of Spi6 in pDCs might mean that levels of these cells do not need to be regulated, suggesting that they do not take part in regulating CTL expansion (Fuchsberger et al. 2005). It also further supports the hypothesis that pDCs do not actively participate in antigen presentation during LCMV infection.

### 4.3.3 CD8α dendritic cells in Spi6 KO mice are restored to wild-type levels in absence of granzyme B

Spi6 inhibits GrB activity, thereby preventing it from triggering intracellular apoptotic pathways. To verify whether reduced DC levels in Spi6 KO mice were directly dependent on GrB-triggered apoptosis, GrB was subtracted from the system. GrB absence was expected to restore DC levels to WT. In order to obtain GrB removal, CD8 T cells from GrB KO mice were used, on the basis that the majority of the GrB in the system would come from donor CTL.

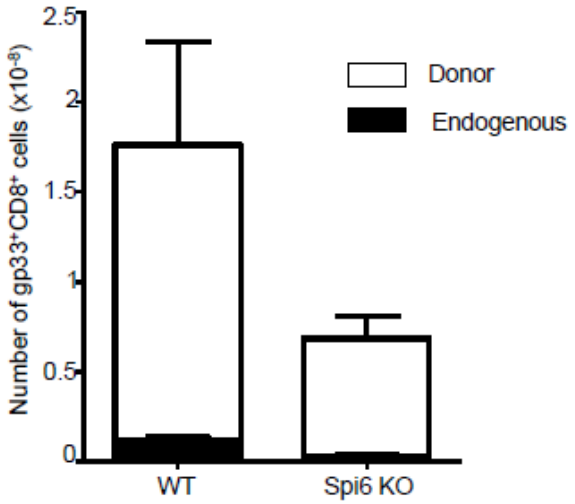



**Figure 52: Endogenous (black) versus adoptively transferred donor (white) gp33-specific CTL (absolute numbers) in the spleen of infected mice during primary response (day 8 post-infection). CD8 T cells are stained with Gp33, CD44, Thy1.1 and Thy1.2 antibodies to distinguish donor Thy1.1 from endogenous Thy1.2 antisgen-specific CD8 T cells. All mean values are ±SEM and are representative of three independent experiments (*n* = 5-6).**

To verify that adoptive transfer of GrB KO P14 T cells effectively resulted in elimination of GrB, the composition of the CTL response to gp33 in our model was analysed. It was verified that gp33-specific CD8 T donor cells (Thy1.1) were accounting for more than 90% of the gp33-specific response and were on average 14- to 17-fold more than the endogenous cells (Thy1.2) when measured at day 8 p.i. (see Figure 52). Therefore, it could be assumed that 90% of the GrB in the system was provided by degranulating donor-derived P14 CTLs and that, eliminating this source, the remaining amount of GrB in the system would have been negligible.

GrB KO P14 Thy1.1 cells were adoptively transferred into Spi6 KO and WT mice and the levels of CD8α DCs were measured at day 4 post-infection. This time point was chosen as analysis of CD8α DC levels had showed the biggest difference between WT and Spi6 KO at day 4 p.i. thus suggesting Spi6 function to be critical at this specific time point for DC survival. To isolate, identify and enumerate the two populations, the same protocol was used as above (Figure 48).

When priming occurred in absence of GrB, CD8α DC levels in the spleen of Spi6 KO mice were found to be rescued to WT levels, while higher numbers of pDCs were measured in Spi6 KO mice compared to WT (Figure 53 and Figure 54).

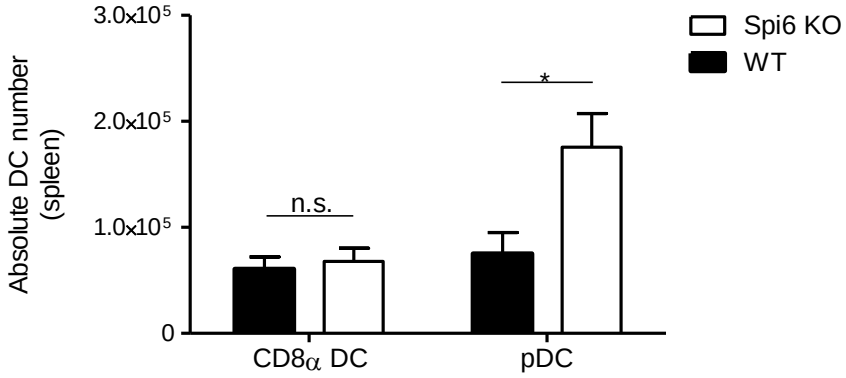



**Figure 53: CD8α DCs are rescued to WT levels after infection when GrB KO P14 CD8 T cells are adoptively transferred into Spi6 KO mice. Absolute numbers of CD8α DC and pDC in the spleen of LCMV Arm-infected mice adoptively transferred with GrB KO P14 CD8 T cells are shown. All mean values are ±SEM and are representative of three independent experiments (*n* = 3-4), **p* = 0.036.**

Figure 54 shows how the adoptive transfer of GrB KO P14 CD8 T cells into Spi6 KO mice results in the levels of DCs, at day 4 post-infection, being restored to the same level of WT mice. These results strongly indicate that DCs are eliminated by cognate CTLs in a GrB-dependent way during the initial phases of infections and that this is regulated by Spi6 expression in DCs.

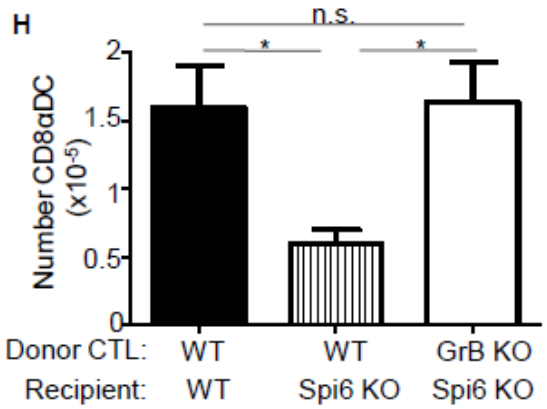



**Figure 54: CD8α DC levels are rescued when priming occurs in absence of GrB in spleen. Absolute numbers of CD8α DC in the spleen of WT and Spi6 KO mice adoptively transferred with WT or GrB KO P14 cells and infected with LCMV Arm. All mean values are ±SEM and are representative of three independent experiments (*n* = 3-4), * *p* < 0.05; n.s. Not Significant.**

It can be concluded that, during viral infections, DCs priming antigen-specific CD8 T cells are a target of GrB released by CD8 T cells themselves.

During LCMV infection in our Spi6 KO model, DC levels are especially affected at day 4 post-infection, showing significantly decreased numbers of CD8α DCs compared to WT, coincidentally with the acquisition of effector functions by primed CTLs.

### 4.3.4 Dendritic cells from Spi6 KO mice are functionally equivalent to wild-type

To date, no data has been produced on the effect of Spi6 on DC function. Therefore, once established that the number of CD8α DC is impaired in Spi6 KO mice when infected with LCMV, the functionality of these cells was assessed, to verify if the

lack of Spi6 had any consequence in presenting and priming functions. As CD8α DCs are the ones involved in antigen presentation to CD8 T cells, these cells were chosen for the functional study.

Following the same protocol as in the previous experiments, WT P14 cells were adoptively transferred into WT and Spi6 KO mice and the mice were then infected with LCMV Arm. Once again, day 4 post-infection was chosen as the preferred time-point, due to the difference measured in CD8α DC levels. At day 4 p.i. the mice were sacrificed and CD8α DC isolated from the spleen to test their ability to induce CTL maturation and proliferation (protocol shown in Figure 55).

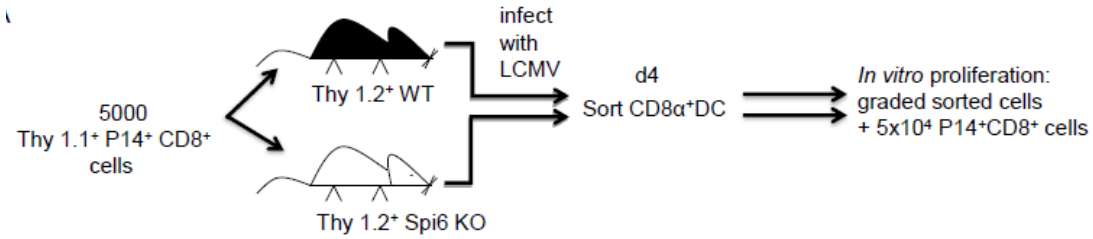



**Figure 55: Protocol for functional study of DCs from WT and Spi6 KO mice. WT and Spi6 KO mice are adoptively transferred with P14 CD8 T cells and infected with LCMV Arm. CD8α DCs are sorted from the spleen of the mice at day 4 p.i. and mixed to naïve P14 CD8 T cells for 60 hrs in vitro.**

$CD11c^+$ cells were isolated through enzymatic digestion followed by FACS sorting of $CD8\alpha^+$ DCs, and used in a proliferation assay where an incorporating base, EdU, was used to measure proliferation. EdU is used for proliferation assays and preferred to Thymidine incorporation due to its non-radioactive nature.

$CD8\alpha^+$ DCs were sorted using the same surface markers used for identification of $CD11c^{hi}$ $CD8\alpha^+$ $CD4^-$. These cells were used to prime naïve CD8 T cells from P14 mice. Naïve CD8 P14 cells ($5x10^4$) were mixed to titrated $CD8\alpha^+$ DC and EdU (5 μg/ml) and, according to a previously described protocol, cells were harvested after 60 hrs (Belz et al. 2004). EdU incorporation was analysed with flow cytometry, as shown in Figure 56. Analysis of the percentage of cells in active proliferation status after 60 hrs of culture with WT (left panel) or Spi6 KO (right panel) CD8α DCs shows no difference in the ability of sorted DCs to prime CD8 T cells.

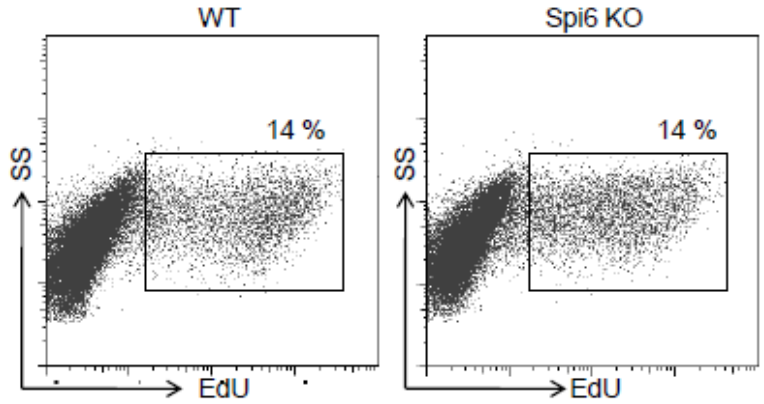



**Figure 56: EdU staining for proliferating CD8 T cells measured after 60 hrs in culture with sorted CD8α DCs at a 2 to 1 ratio, from infected WT and Spi6 KO mice adoptively transferred with P14 CD8 T cells. Data are representative of three independent experiments.**

The results show a proliferation rate of 14% of the total number of CD8 P14 T cells when half the number of CD8α DCs (target) are used to prime CD8 T cells (effector). The level of proliferation measured at a ratio of 2 : 1 was more than 10-fold the levels of basal proliferation. These data demonstrate that Spi6 KO-derived DCs are functionally not impaired. Also, titration of the CTL to DC ratio was performed and the proliferation measured showed that CD8 T cell proliferation is dependent on the levels of DC (Figure 57)

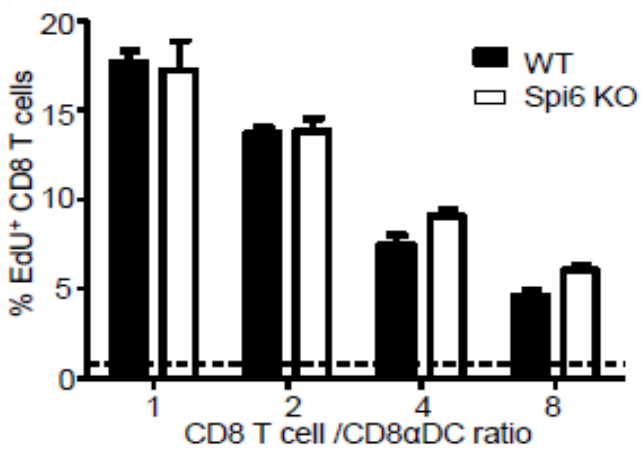



**Figure 57: Percentage of $EdU^+$ cells at different P14 CD8 T cells to CD8α DC ratios. The dotted line represents basal proliferation. Positive control cultures that were pulsed with gp33 peptide at a 1:1 ratio gave 20% $EdU^+$ cells and negative control cultures ($5x10^4$ P14 CD8 T cells alone/well) gave 0.7% $EdU^+$ cells. All mean values are ±SEM and are representative of three independent experiments.**

## 4.4 Dendritic cell priming of CD8 T cell responses *in vivo*

It has been hypothesised that Spi6 would have a role in DC priming of primary and secondary $CD8^+$ responses. To demonstrate it, an *in viv*o infection system in which DCs lack Spi6 but CD8 T cells do not was used. As the current theory hypothesizes that the CTL burst is dependent on DC priming and given that sustained antigen presentation is required for optimal CTL expansion (van Montfoort et al. 2009), it was investigated whether the impaired survival of CD8α DCs seen in previous experiments was mirrored by a deficit in the CTL response. A positive response to this question would also support the theory that a negative feedback loop is in place to regulate CTL expansion levels: the higher the killing of DC, the more the CTL burst is reduced.

### 4.4.1 Expansion of LCMV-specific adoptively transferred cytotoxic T cells is defective in Spi6 KO mice

Antigen-specific cytotoxic CD8 T cell levels were analysed in adoptively transferred mice to test whether a defect in CD8α DC population in the spleen of the mice resulted in defective CTL expansion.

Spi6 KO and control mice (WT mice), both Thy1.2, were injected with $5x10^3$ $CD8^+$ P14 $Thy1.1^+$ cells and subsequently infected with LCMV Armstrong i.p. Half of the mice for each test group were analysed at day 8 post infection, the peak of CTL expansion, while the other half were re-infected at day 35 post infection, to allow for memory cell development (see Figure 58 for protocol). Mice were bled at regular intervals until day 8 post-infection and then sacrificed to analyse P14 CTL response in the spleen and inguinal LNs. Inguinal LNs were chosen due to their position relative the i.p. infection.

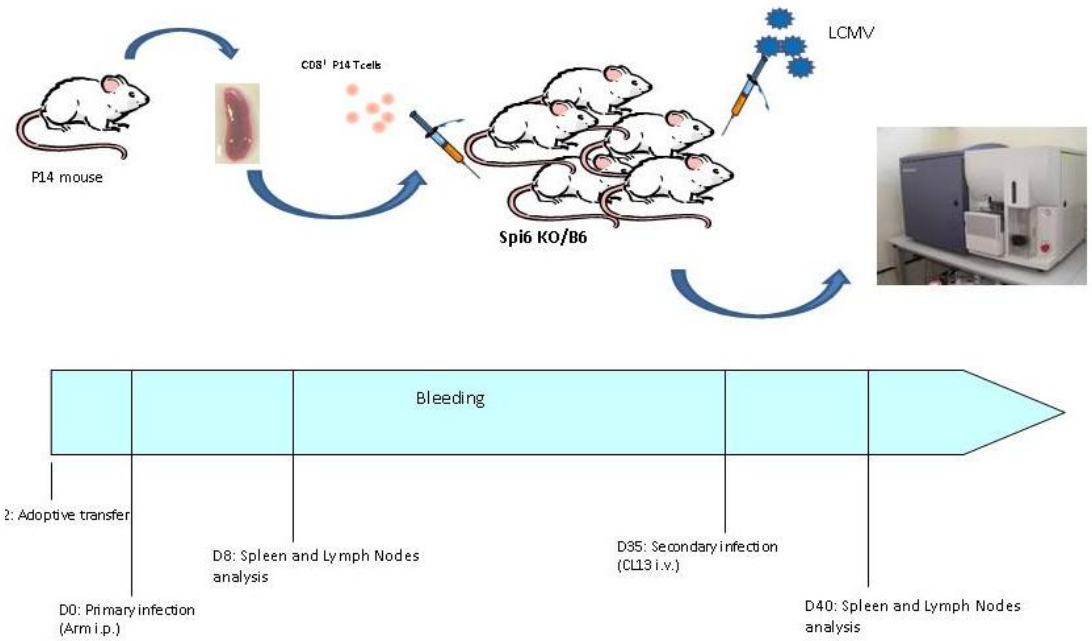



**Figure 58: Experimental protocol for *in vivo* analysis of CTL responses to LCMV Arm. Two groups of mice (n=5-6) were adoptively transferred with $5x10^3$ P14 Thy1.1 CD8 T cells and infected 2 days later with Arm (i.p.). Mice were bled from day 2 and half of each test group was sacrificed at day 8 p.i., while the other half was bled and memory cell formation allowed. After 40 days at least, the mice (n=5-6) were infected with LCMV CL13 i.v. and sacrificed 5 days p.i.**

CTLs were identified in whole spleen preparations, whole LN preparations and red blood cell-depleted blood preparation, by surface staining with gp33-tetramers, anti-CD8, anti-CD44 and anti-Thy1.1 (CD90.1) antibodies.

The gating strategy for individuation of donor-derived gp33-specific CD8 T cells is shown in Figure 59. $CD8^+$ T cells are first identified among lymphocytes. $Gp33^+$ $Thy1.1^+$ cells are then isolated from the $CD8^+$ T cell population. This gating strategy allows for identification of autologous CD8 T cells which recognise gp33 as well: these cells are $CD8^+$ $gp33^+$ $Thy1.1^-$ (see Figure 59).

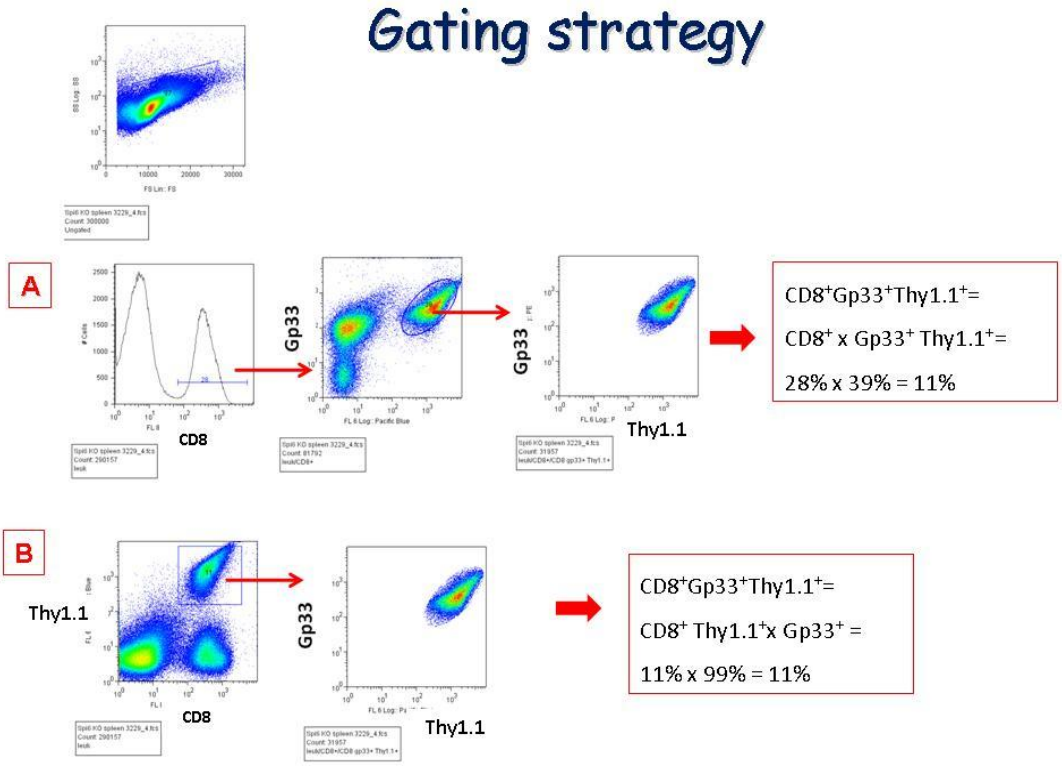



**Figure 59: Gating strategy for CD8 P14 donor-derived cells. Gp33 tetramers are used to identify P14-derived cells and Thy1.1 and Thy1.2 are used to distinguish donor-derived from autologous CD8 T cells.**

The population identified as donor-derived Thy1.1+ gp33+ CD8 T cells with FACS analysis of surface markers is shown in more detail in Figure 60.

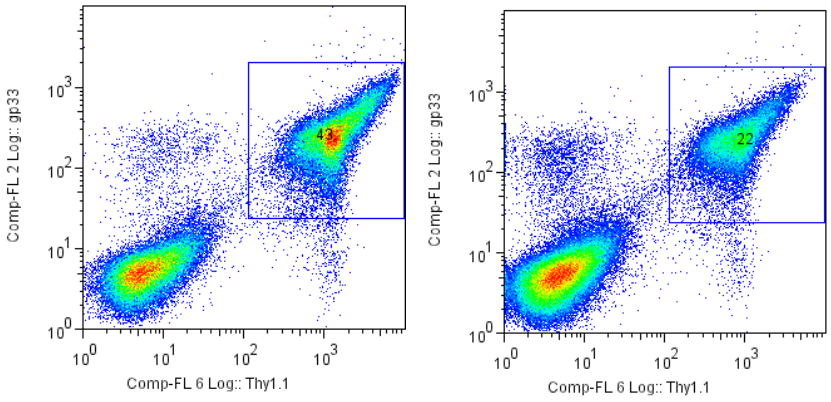



**Figure 60: Representative FACS plot for the identification of donor P14 cells as Gp33$^{+}$ Thy1.1$^{+}$ among CD8 T cells. Spi6KO cells on the left panel and WT cells in the right panel.**

The dynamics of the gp33-specific CD8 response in the spleen was followed from day 2 to day 8 at regular intervals. Data reported in Figure 61 show a significant difference from day 6 p.i., as expected from the findings that DC survival is impaired at day 4. The number represented total gp33-specific $CD44^+$ $CD8^+$ cells in the spleen of Spi6 KO and WT mice at indicated days post-infection (Figure 61).

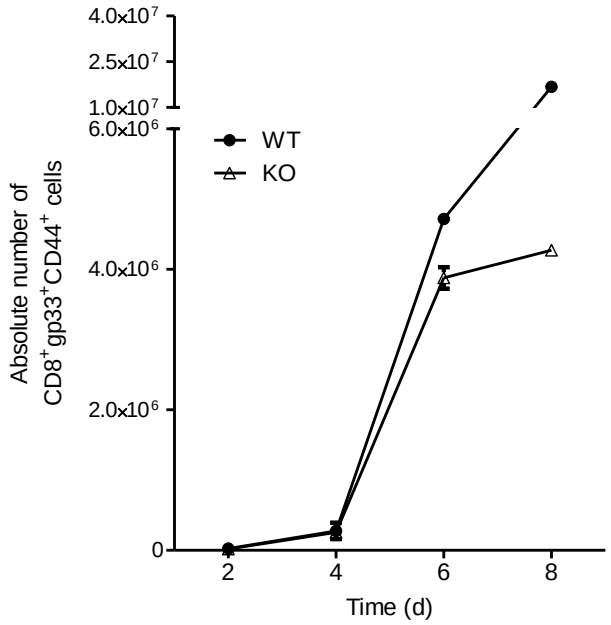



**Figure 61: Total gp33-specific CD8 cell levels in WT and Spi6 KO mice adoptively transferred with P14 CD8 T cells after Arm infection. All mean values are ±SEM and are representative of two independent experiments (*n*= 3). Error bars are covered by the data point in some cases.**

The results obtained from mice bled and analysed for gp33-specific $CD8^+$ $Thy1.1^+$ cells indicated that CTL expansion in Spi6 KO mice is defective at day 8 post-infection compared to WT. The levels of donor-derived $gp33^+$ $CD8^+$ cells in Spi6 KO mice were seen to be decreased by 2-fold ($p$ = 0.01) in the spleen and by 8-fold ($p$ = 0.003) in the lymph nodes, compared to WT mice (Figure 62).

These results are in agreement with the measurement of a 15-fold higher donor gp33-specific CTL response compared to the endogenous one reported above in Figure 52.

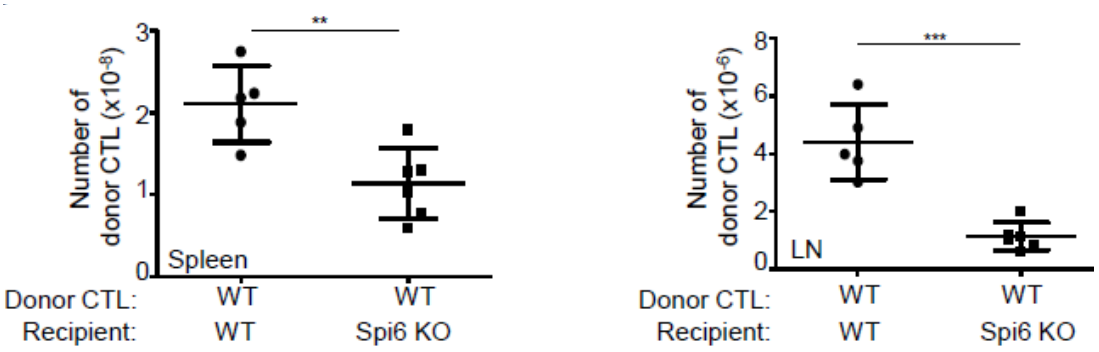



**Figure 62: Donor-derived CTL gp33-specific response in WT and Spi6 KO mice at day 8 pi. Both Spi6 KO and WT mice (recipient) were adoptively transferred with P14 CD8 T cells (donor CTL). All mean values are ±SEM and are representative of three independent experiments (*n*= 5-6), ***$p$ = 0.01; **$p$ = 0.01.**

Data showed that CD8 T cell primary response is defective in absence of Spi6. The experimental conditions also allow the conclusion that the deficit in the CTL expansion is independent of Spi6 role in CD8 T cells themselves The shortfall in antigen-specific CTLs from day 6 p.i. observed in Spi6KO mouse is not cell-autonomous On the basis of the results obtained from the analysis of splenic DC levels during LCMV infection after adoptive transfer of gp33 P14 CD8 T cells, the decrease in the levels of live CD8α DCs in the spleen of Spi6 KO mice resulted in a lower CTL response.

### 4.4.2 Gp33-specific donor CD8 T cell memory pool in adoptively transferred mice

Since elimination of DCs by memory cells has also been reported as being part of a feedback mechanism for the control of the re-expansion of these cells, it was decided to investigate whether Spi6 deficiency had a role in secondary responses as well.

The development and the levels of the donor-derived memory pool were studied in both Spi6 KO and WT mice from day 8 to day 40 post-infection. Mice were bled at regular intervals in order to follow the development of the memory cells. The percentage of donor-derived gp33-specific CD8 T cells in the blood is recorded in

Figure 63 and shows that, despite a difference in the effector pool between Spi6 KO and WT mice at the peak of the secondary response in the spleen and LN, the levels in the blood were comparable. Also, no difference in the percentage of antigen-specific CD8 T memory cells was seen in the blood of Spi6 KO compared to WT mice during the contraction phase and up to day 40 post-infection. Notably, after re-infection with CL13 LCMV, WT donor-derived gp33-specific memory cells re-expend to higher levels compared to Spi6 KO ones.

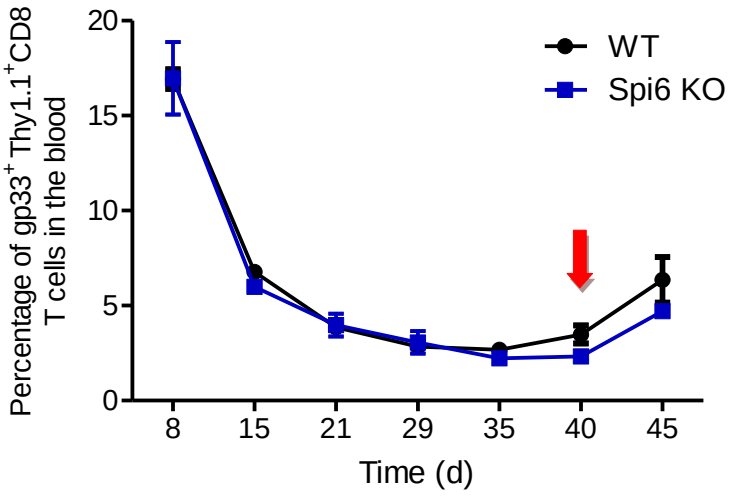



**Figure 63: Blood levels of donor-derived gp33-specific CTLs shown as percentage of gp33$^+$ Thy1.1$^+$ CD8 T cells in the blood at the indicated time points after infection. Secondary infection, with CL13 strain of LCMV, was performed at day 40 (see arrow). All mean values are ±SEM and are representative of three independent experiments (*n* = 5-6).**

To better describe the gp33-specific donor-derived memory pool, analysis of spleen and inguinal LNs was carried out on memory mice, on day 40 post-infection. Absolute numbers of memory cells were measured as before (CD8$^+$ gp33$^+$ Thy1.1$^+$ cells in whole spleen and whole LNs). Results in Figure 64 show that levels of donor memory cells (Thy1.1$^+$) in the spleen of Spi6 KO mice were 2-fold higher than level of these cells in WT, while in the inguinal LNs no difference was seen (Figure 65).

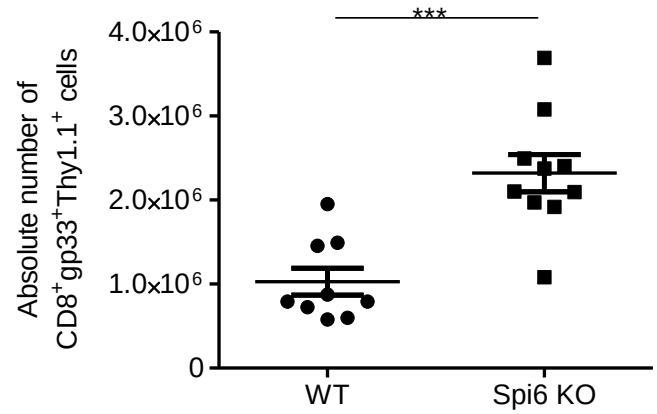



**Figure 64: Absolute numbers of donor-derived P14 CD8 memory cell pool (gp33-specific Thy1.1$^+$ CD8 T cells) in the spleen of WT and Spi6 KO mice >40 days post-infection. All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 5-6), ****p* = 0.0002.**

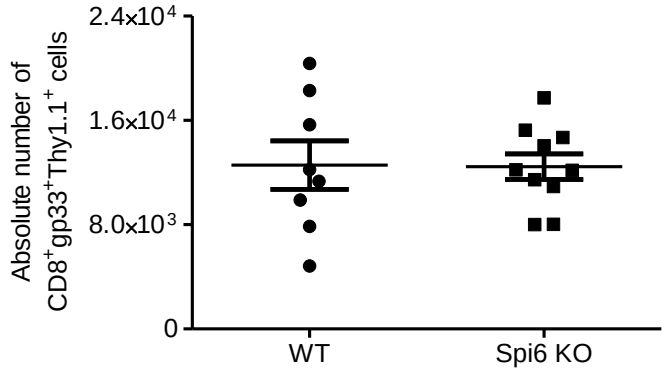



**Figure 65: Absolute numbers of donor derived P14 CD8 memory cell pool (gp33-specific Thy1.1$^+$ CD8 T cells) in the inguinal LN of WT and Spi6 KO mice >40 days post-infection. All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 5-6).**

These results are consistent with previous studies from Zhang and colleagues that showed how, despite a defective primary responses that fails to peak around day 8 p.i. memory in Spi6 KO mice develops to levels comparable to WT after LCMV Arm infection (Zhang et al. 2007)

### 4.4.3 Re-expansion of LCMV-specific adoptively transferred cytotoxic T cells is defective in memory Spi6 knock-out mice

In a typical CD8 T cell response, memory CD8 cells start developing during the CTL contraction phase, and a stable memory pool can be clearly identified 35 days after the infection. To verify whether Spi6 absence could interfere with memory cell re-expansion, Spi6 KO and WT mice were re-infected with LCMV CL13 i.v. ($10^6$ PFU) 40 days after the primary infection. The CL13 LCMV variant strain can cause chronic infection in immunocompetent naïve mice. Nonetheless, as reported by Nolz and Harty in 2011, who adoptively transferred antigen-specific memory cells into naïve mice (Nolz and Harty 2011), antigen-specific memory cells can prevent chronic infection and efficiently clear the virus in 5-6 days.

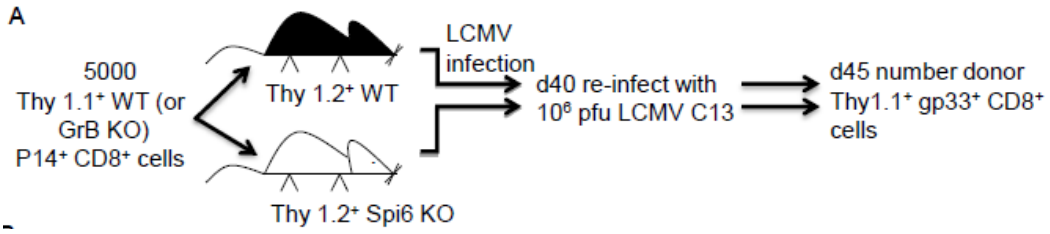



**Figure 66: Secondary response experiment protocol. WT and Spi6 KO mice were adoptively transferred with 5000 WT or GrB KO P14 naïve CD8 T cells and infected with LCMV Arm. After 40 days from primary infection, the mice were infected with LCMV CL13. Donor-derived gp33$^+$ Thy1.1$^+$ CD8 T cells were measured in the spleen and LNs 5 days after the secondary infection.**

Infection with CL13 was performed as, due to the high levels of donor-derived memory cells, a high titre of an aggressive virus was needed to stimulate memory cell proliferation and avoid elimination of the virus without secondary spread. The measurement of blood levels of gp33-specific memory cells confirmed that CL13 infection involved a re-expansion of the memory pool. The chart in Figure 63 shows how specific gp33$^+$ Thy1.1$^+$ CD8 T cell levels increase from day 40 (the day of secondary infection) to day 45 (day 5 after secondary infection).

To measure memory response levels, mice were bled and spleen and inguinal lymph nodes were analysed as before: the total number of cells was counted, and lymphocytes stained for CD8, gp33 tetramer, and Thy1.1. As shown in Figure 67, in

the spleen of Spi6 KO mice the level of donor cells after re-expansion was 2-fold lower than in WT mice (*p* = 0.0014) and in the LNs this was 4-fold lower (*p* = 0.0006). When looking at these results in the light of the pre-existing memory pool (Figure 64 and Figure 65), this means a 4-fold expansion for the memory cells occurred in the WT mice.

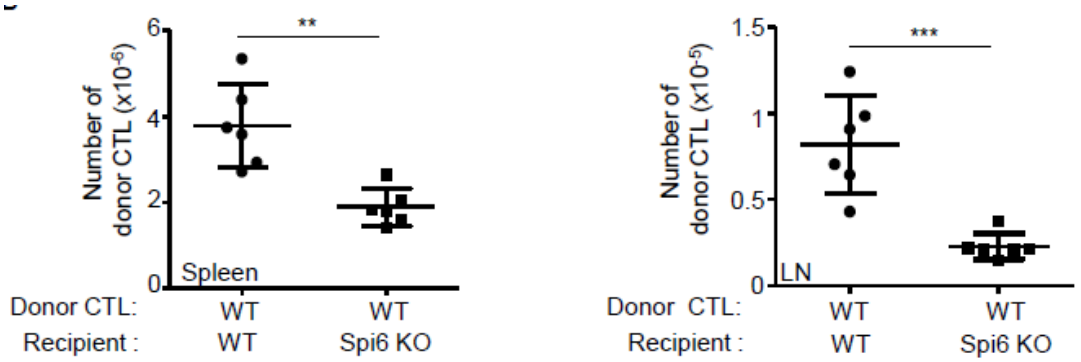



**Figure 67: Levels of donor-derived gp33-specific memory cells after re-expansion. Donor cells are gp33$^{+}$ Thy1.1$^{+}$ CD8$^{+}$. All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 5-6), ***p* = 0.0014, ****p* = 0.0006.**

These results suggest that Spi6 is needed for re-expansion of memory responses. This is likely to be due to a specific need memory cells have for DC priming of re-expansion, in absence of which they cannot be activated to re-acquire effector functions. Memory cell re-expansion is therefore dependent on Spi6 and the defect is not ascribable to a CD8 T cell autocrine mechanism.

## 4.5 Expansion of antigen-specific CTL response in absence of granzyme B

Spi6 is a GrB inhibitor and it therefore has an anti-apoptotic role in CTLs and in DCs. To prove that the deficit in the DC number and CTL response levels seen is due to the lack of Spi6 in DCs, it was once again decided to eliminate the target of Spi6 from the system.

Having established that the gp-33 response was mainly due to donor cells and therefore the contribution of endogenous GrB was negligible, GrB KO P14 T cells were used once more to study the dependence of the CTL response on this cytotoxic molecule in WT and Spi6 KO mice. Since in an adoptively transferred mouse, GrB absence could restore the levels of DC to normal (see 4.3.3) it was decided to verify if the rescue of DC levels was accompanied by a rescue of the antigen-specific CTL expansion.

### 4.5.1 Cytotoxic T cell expansion defect is rescued by granzyme B absence in primary responses against LCMV

P14 Thy1.1$^+$ mice were bred in our facilities with GrB KO mice to obtain Thy1.1 P14 GrB KO mice. GrB KO P14 CD8 naïve T cells were isolated from the spleen of these mice in order to be adoptively transferred to WT and Spi6 KO mice with the same protocol as before. WT and Spi6 KO mice were adoptively transferred with a low number of either WT P14 Thy1.1$^+$ CD8 naïve T cells or GrB KO P14 Thy1.1$^+$ CD8 naïve T cells ones and successively infected with LCMV Arm i.p. Analysis of spleen and LNs was carried out at day 8 post-infection: donor-derived gp33-specific CTLs were measured.

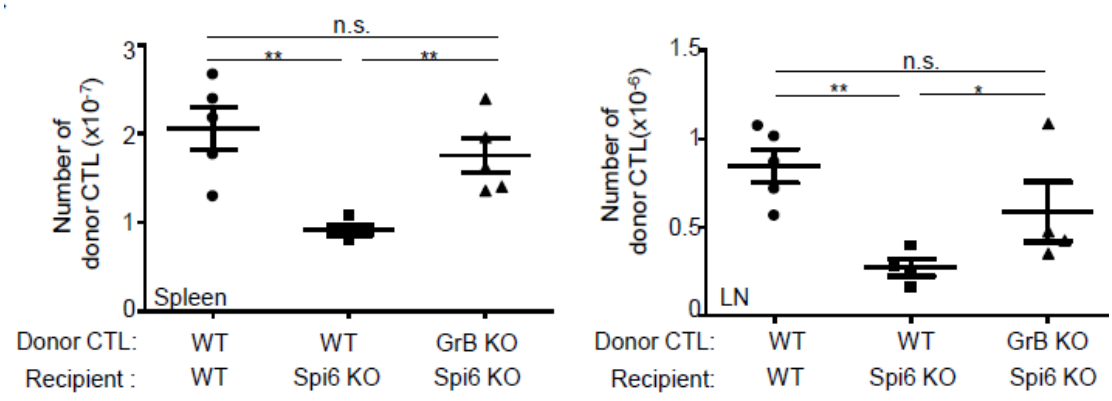



**Figure 68: Rescue of CTL deficit in Spi6 KO mice. WT mice were adoptively transferred with GrB KO P14 Thy1.1 CTL and their levels after infection (day 8 p.i.) were compared to adoptive transfer of WT and GrB KO P14 CD8 T cells in Spi6 KO mice. All mean values (horizontal bar) are ±SEM and are representative of 3 independent experiments (*n*= 5-6): **$p < 0.005$; *$p < 0.5$.**

Results showed CTL expansion restored to WT level in Spi6 KO mice adoptively transferred with GrB KO P14 T cells both in the spleen and in the inguinal LNs (Figure 68). Therefore when GrB was eliminated from the system, the deficit in the primary response was rescued. This suggested a direct correlation between the absence of GrB and the absence of Spi6.

### 4.5.2 Cytotoxic T cell expansion defect is rescued by granzyme B absence in recall responses

Having shown that GrB deletion could rescue CTL levels to normal in Spi6 KO mice, we wanted to test if GrB was also responsible for the re-expansion defect in memory responses. The contribution of the donor-derived CTL to the antigen-specific re-expansion was measured and it was once more verified that donor cells contributed for more than 90% being 12.7-fold higher than endogenous in WT and 17.8-fold in Spi6 KO mice, as shown in Figure 69.

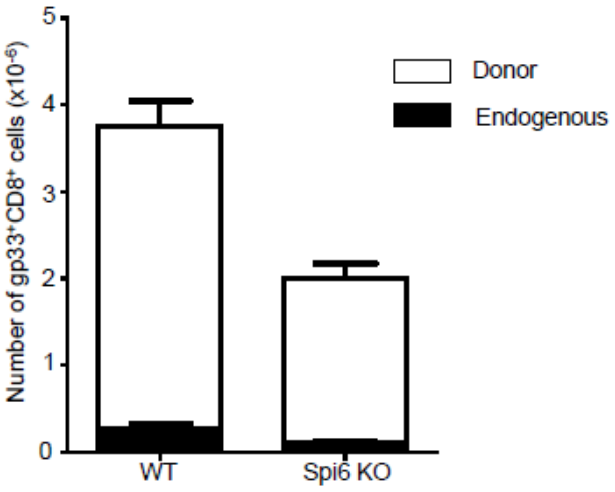



**Figure 69: Endogenous (black) VS adoptive transferred donor (white) CTL levels in the spleen of CL13-infected memory mice at day 5 post-secondary infection. Donor cells are Thy1.1, endogenous cells are Thy1.2. All mean values are ±SEM and are representative of three independent experiments (*n* = 5-6).**

To note, the contribution of endogenous GrB derived from np396-specific endogenous cells has not been studied and it would be an interesting issue to investigate. Nonetheless, my results show how in the system because the exogenous response is overwhelming, the rescue is still achieved.

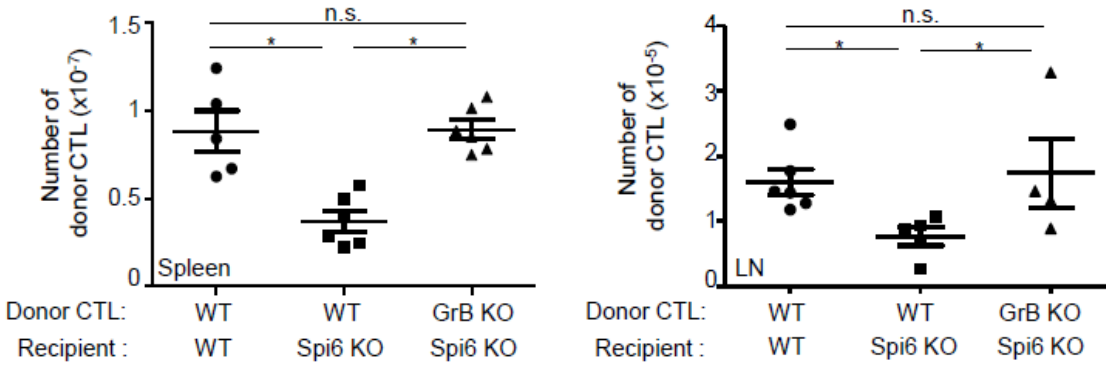



**Figure 70: Rescue of gp33-specific donor CTL response in Spi6 KO mice adoptively transferred with GrB KO P14 CD8 T Cells. Number of donor-derived gp33-specific CD8 T cells at day 5 post-secondary infection in the spleen (left) and in the inguinal LN (right) of Spi6 KO or WT mice (recipient) adoptively transferred with P14 CD8 T cells or GrB P14 CD8 T cells (donor CTL). All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 5-6), *$p < 0.05$.**

The deficit seen in secondary response in Spi6 KO mice was restored when GrB KO P14 CD8 T cells were adoptively transferred to Spi6 KO mice Figure 70). The absence of GrB in donor-derived CD8 T cells only was therefore shown to be enough to restore the levels of gp33-specific CTL response.

The difference in the CTL levels at the peak of the response between Spi6 KO and WT mice is therefore dependent on GrB, suggesting a role for Spi6 and once more, the effect is not CD8-autonomous. Despite the different requirements for memory re-expansion compared to primary responses, including short priming, limited to the early phases of the response, Spi6 is therefore required protecting DCs from GrB-mediated killing in memory priming and for the priming of optimal memory CD8 responses.

## 4.6 Re-expansion of adoptively transferred memory CD8 T cells in CL13-challenged mice

An experiment was designed to test whether the difference in the levels of the secondary response expansion, seen in the previous experiments, was due to a difference in the number of the specific memory cells in the two groups.

Although the levels of memory gp33-specific CD8 T cells had been verified in WT and Spi6 KO mice after 40 days, further experiments were carried out to verify that the secondary response defect was not due to a substantial difference in the existing memory pools at the time of infection.

To exclude the possibility that Spi6 might play some other role in memory development, memory cells were developed in WT mice and adoptively transferred into naïve Spi6 KO and WT mice. These mice were then infected with LCMV CL13 to analyse DC ability to prime these memory cells. P14 Thy1.1$^+$ CD8 naïve T cells were first transferred into WT mice, which were infected with Arm i.p. as in the previous protocol. After 40 days, these memory P14 Thy1.1 cells were isolated from the WT mice with Thy1.1-specific magnetic bead enrichment and adoptively transferred into WT and Spi6 KO mice. The mice were then infected with LCMV CL13 and sacrificed for spleen and LN analysis 5 days post infection (see Figure 71).

In particular, the P14 CD8$^+$ Thy1.1$^+$ memory cells were adoptively transferred in different amounts (respectively $10^4$, $10^3$ and $10^2$ cells) into WT and Spi6 KO mice in order to investigate whether memory re-expansion was based on a dose-response effect. This would determine if high numbers of memory cells have significance in re-expansion dynamics.

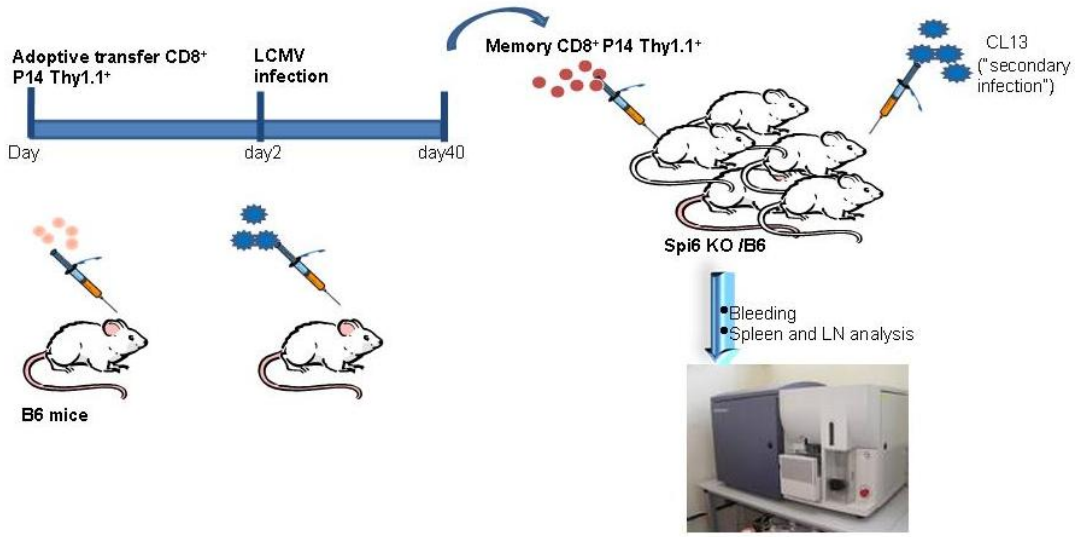



**Figure 71: Experimental protocol. P14 Thy1.1 CD8 naïve T cells are adoptively transferred into a WT mouse, which is infected with LCMV Arm. After 40 days, memory cells are isolated with anti-Thy1.1 magnetic beads and adoptively transferred into WT and Spi6 KO naïve mice. These are infected with LCMV CL13 and blood spleen and LN analysed 5 days p.i.**

The results in Figure 72 show the numbers of $CD8^+$ $gp33^+$ $Thy1.1^+$ donor cells in the spleen of Spi6 KO and WT mice 5 days after secondary infection with LCMV CL13. The levels of the CD8 T cell response correlate with the number of transferred memory cells in the spleen in both WT and Spi6 KO mice. A significant difference in the response levels is seen between the mice injected with $10^2$ and $10^3$ cells or $10^4$ cells. No difference is seen between the re-expansion levels in WT and Spi6 KO mice adoptively transferred with the same number of gp33-specific memory CD8 T cells.

The results obtained by donor-derived CD8 T cell analysis in the LNs are different. A deficit in the re-expansion levels is seen in Spi6 KO mice compared to WT: our hypothesis is that the difference in the subset composition between the spleen and the LN, and in the memory cell population, might play a part in the difference seen in levels of memory cell proliferation. Results from the LNs suggest that the levels of expansion correlate with the number of adoptively transferred CD8 memory cells only in the Spi6 KO mice. In the WT mice levels of $CD8^+$ $gp33^+$ $Thy1.1^+$ cells post secondary-challenge seem to reach a plateau, as despite the difference in the number of adoptively transferred memory cells, these were similar (see Figure 72).

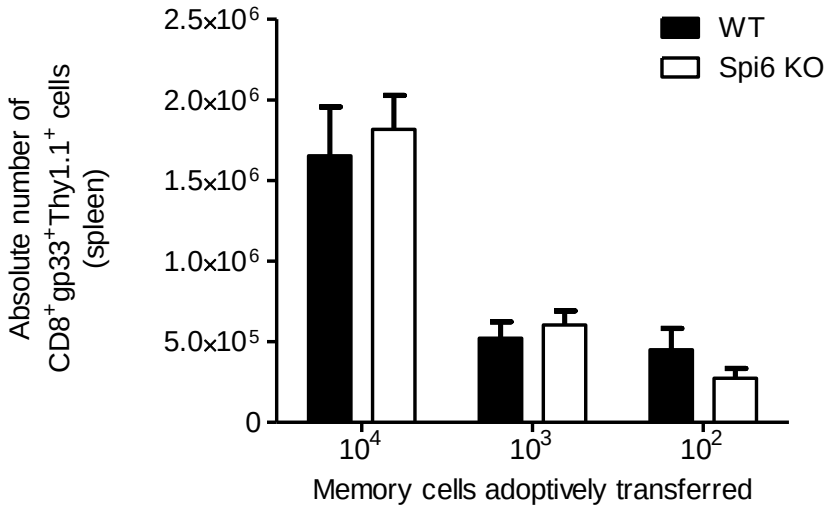



**Figure 72: Re-expansion levels of adoptively transferred Thy1.1$^+$ gp33$^+$ P14$^+$ CD8 memory T cells in the spleen at day 5 post-infection with LCMV CL13, absolute numbers. The difference is non significant between WT and Spi6 KO in any of the sub-groups. All mean values are ±SEM and are representative of two independent experiments (*n* = 5-6).**

In the LN the levels of memory cells in the Spi6 KO mice were significantly lower than in the WT, showing a deficit in the re-expansion ability of these cells in absence of Spi6 (see Figure 73).

The difference in the levels of memory re-expansion between the spleen and the LN might be linked to the specific "preference" of CL13 to infect DC, which is, as see above, due to its high affinity for α-distroglycan, expressed in high levels by splenic interdigitating DCs (Salvato et al. 1991). CL13 infection of splenic DC might in fact result in higher killing of these cells as they are recognised as target by activated memory cells, thus eliminating the protective function of Spi6 in WT cells. Possibly, this difference could also be due to the different ability of central and effector cells to migrate to the LN and the spleen, and their different requirements in terms of priming. The LN is mainly accessible to central memory cells which are activated specifically by resident CD8α DC. This might explain why Spi6 KO mice have defective memory cell levels in the LN: the DCs in this tissue are dependent on Spi6 for survival when activating memory cells. Once primed, these cells will egress the LNs to reach the infected tissue. In the case of CL13, the infected tissue is the spleen, as just discussed, due to CL13 high preference for interdigitating DC. This would

mean that DC are not only killed by ectopic release of GrB due to priming, but also by targeted release of GrB and other killing pathways specifically aimed at these infected cells to eliminate the pathogen.

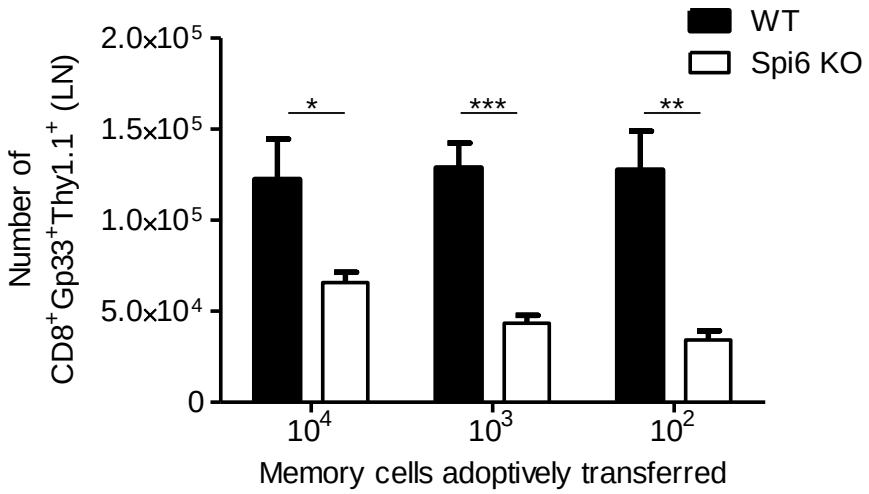



**Figure 73: Re-expansion levels of adoptively transferred Thy1.1$^+$ gp33$^+$ P14$^+$ CD8 T cells in the LN at day 5 post-infection with LCMV CL13, absolute numbers. All mean values are ±SEM and are representative of two independent experiments ($n$ = 5-6), *p < 0.05; **$p$ < 0.005; ***$p$ < 0.0005.**

Therefore in the same conditions of infection (same viral titre) and of initial CD8 memory cell pool (same number of CD8$^+$ gp33$^+$ Thy1.1$^+$ memory cells), Spi6 KO mice have a deficient secondary response expansion in the LN.

From this set of experiments can be concluded that Spi6 controls the expansion of both primary and secondary CD8 T cell responses, independent of its role in CD8 themselves. In the light of its ability to protect DC from CTL-induced damage, findings are consistent with a role for Spi6 in protecting DC from CTL *in vivo*.

## 4.7 Donor CD8 T cells response functionality: LCMV clearance

LCMV Arm viral infection is efficiently cleared by immunocompetent mice within 8-10 days with a physiological antigen-specific precursor frequency of about $10\text{-}10^3$ cells. Having obtained results supporting the theory that Spi6 protects DCs from CTL killing and that its absence indirectly affects CTL responses, I wanted to verify the consequences of the deficient expansion seen in Spi6 KO mice in terms of viral clearance. Viral levels were measured in the spleen of infected mice at day 6 post-infection. Viral peak is reached in B6 (WT) intact mice by day 3-4, therefore making day 6 a good time point to establish the efficiency of clearance by adoptively transferred CD8 cells. Due to the high precursor frequency of antigen-specific CD8 T cells in this model, viral clearance was expected to be reached ahead of times compared to an intact WT mouse. Also, a time-point at which viral levels could be detected before being too low for RT-PCR sensitivity was needed.

LCMV major proteins are glycoprotein (GP) and nucleoprotein (NP). Among the two, Np is the most abundant protein in infected cells. It forms the capside containing the viral RNA (Buchmeier et al. 1978) and it is required for RNA replication as it encapsidates nascent viral RNA. Moreover its levels are controlling the switch of the polymerase activity from transcription to replication (Blumberg et al. 1981).

### 4.7.1 Viral clearance of primary cytotoxic T cell response

WT and Spi6 KO mice were adoptively transferred and infected as above with P14 CD8 naïve T cells and infected with LCMV Arm (Figure 58). At day 6 p.i., the spleens were harvested and total RNA was extracted to measure viral titre through viral RNA analysis (see Chapter 3.4). Results for the two major viral proteins, gp and np, are shown in Figure 74 and clearly indicate a clearance defect in Spi6 KO mice which results in 100-fold more virus in these mice at day 6 p.i., compared to WT.

This indicates that higher titres of LCMV are present in Spi6 KO mice at day 6 p.i., compared to WT. Deficient primary response expansion is therefore associated with a significant defect in clearance of LCMV Arm.

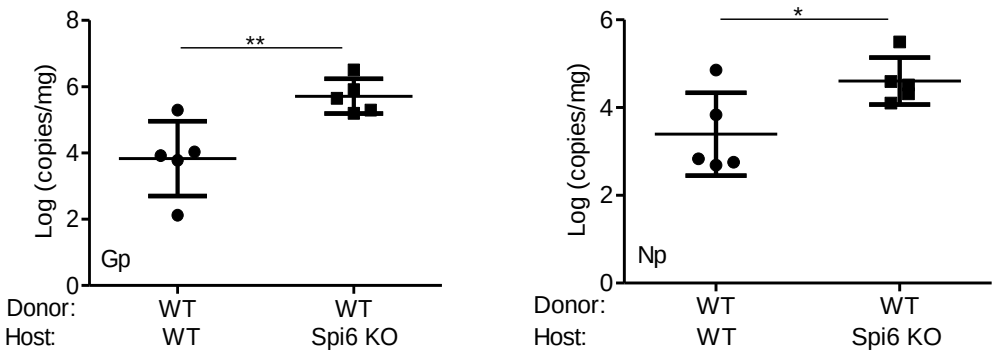



**Figure 74: Viral GP (left) and NP (right) levels in the spleen of day 6 infected mice obtained measuring viral mRNA levels in the spleen of infected mice with RT-PCR. All mean values (horizontal bar) are ±SEM and are representative of two independent experiments (*n* = 5-6), **p* < 0.05, ***p* < 0.005.**

## 4.7.2 Viral clearance of secondary cytotoxic T cell response

Viral load was also measured after memory responses, at day 5 post re-infection. The results show a very low viral presence, indicating the ability of the antigen-specific memory pool to efficiently fight the infection (Figure 75) even in an Spi6 KO environment as reported in previous studies (Zhang et al. 2007). A difference in the levels of NP was revealed, while GP levels were the same for Spi6 KO and WT (Figure 76). This difference suggests that clearance of LCMV CL13 is delayed in Spi6 KO mice, as np is a regulator of viral RNA transcription and its levels indicate that transcription in Spi6 KO mice is till in place at day 5 p.i.

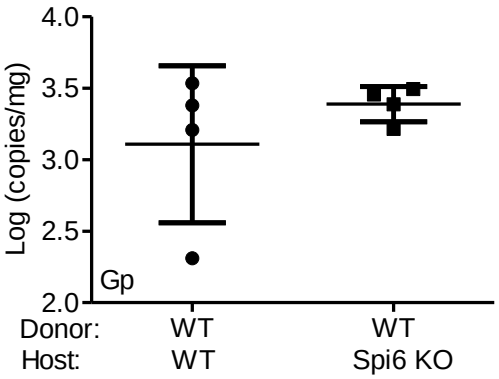



**Figure 75: Viral titre (Gp levels) at day 5 post secondary infection. All mean values (horizontal bar) are ±SEM and are representative of three independent experiments (*n* = 4).**

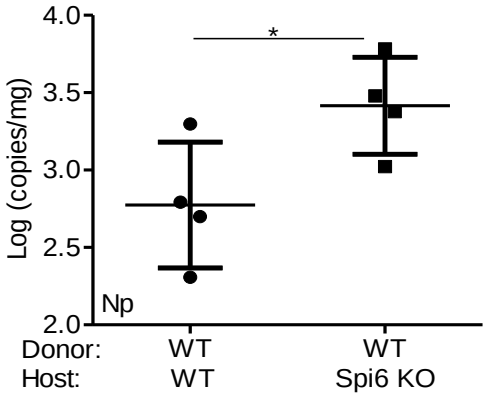



**Figure 76: Viral titre (Np levels) at day 5 post secondary infection. All mean values (horizontal bar) are ±SEM and are representative of two independent experiments (*n* = 4), **p* < 0.05.**

Based on these results that show better clearance in WT mice compared to Spi6 KO ones despite the WT status of the effector cells, it can be concluded that Spi6 is required for the priming of functionally relevant CTL responses by DCs *in vivo*.

# 5. DISCUSSION

## 5.1 Introduction

A big percentage of infectious diseases, which constitute a great social and economical burden other than of course a public walfare problem throughout the world, are caused by viruses. Chronic viral diseases, on the other hand, affect an ever-growing portion of the Western and Developing Countries adult population. Advanced vaccination approaches, preventive and therapeutic, targeted at ameliorating specific responses against viruses are desirable to prevent and treat infectious diseases, cancer of viral origin and chronic viral diseases. Compared to vaccines generating antibody protection or humoral responses, CD8 T cell immunity has been more challenging to achieve, therefore being an area where much improvement is needed. Also, CD8 T cell responses are desirable due to their efficient and rapid methodology of clearing the body from infected and unwanted tumorigenic cells through induction of a self-contained death called apoptosis. Dendritic cells are a main target of new strategies aimed at eliciting CD8 T cell primary and memory responses (Butler et al. 2011; Nolz and Harty 2011).

Clearance of viral infections *in vivo* is mainly mediated by cytotoxic CD8 T cells, which are specifically activated by dendritic cells among the professional antigen presenting cells. DCs are highly phagocytic cells which reside in peripheral tissues and are able to sense inflammation and pathogen-associated patterns. In inflammatory conditions, DCs undergo a maturation program which allows them to exit peripheral tissue and reach secondary lymphoid organs. Here, naïve CD8 T cell receive activation signals responsible for the regulation and extent of the immune response to

be mounted by the mature DCs. Antigen-specific CD8 naïve T cells activated by mature DCs, undergo maturation to cytotoxic T cells and proliferate at high rate. CTLs will then migrate out of the lymphoid tissue to reach the site of infection and kill infected cells. CTLs can kill infected cell via contact-dependent apoptosis induction, which can be achieved in two ways: the release of cytotoxic molecules by degranulation and/or Fas-FasL ligation. The importance of the granule-mediated pathway emerges when considering that perforin deficient mice develop spontaneous lymphomas and have impaired ability to clear many viruses. On the other hand, Fas-FasL-defective mice have lymphoproliferative disorders. The hypothesis is therefore for the death receptor pathway to be involved in maintenance of lymphocyte homeostasis, while the cytotoxic molecule-release pathways is involved in clearance of virally infected cells and tumours. After successful clearance of the virus, the antigen-specific CTL population is reduced by clonal contraction, of about 9/10 to form a pool of resting CD8 T cells able to quickly reactivate effector functions and undergoing homeostatic proliferation. These cells are called memory cells: they survive without antigenic stimulation and their re-activation is dependent on antigen presentation by antigen-loaded DC (see Chapter 2.3).

Due to the key role of DCs in stimulating both primary and secondary CD8 T cell responses, understanding the mechanisms that regulate their maturation, survival and migration will allow better design of vaccination and therapeutic strategy. This project in particular investigated the function of a protein with antiapoptotic properties expressed by DCs. DC survival is of particular importance since it has been involved in a feedback mechanism which regulates/controls the CD8 response expansion levels through the killing of antigen-loaded DCs (Wong and Pamer 2003; Yang et al. 2006). The molecules regulating balance between life and death in DCs will thus have an impact on CTL responses themselves. In particular, the feedback regulatory mechanism has been shown to depend on perforin, as in pfn KO mice DCs are not eliminated to WT levels (Yang et al. 2006). Granzyme B is a cytotoxic molecule released by CTL as part of the granule exocytosis pathway (Heusel et al. 1994). GrB can enter the target cell, where it triggers apoptotic pathways, only in presence of perforin (Kagi et al. 1996). GrB is also involved in the negative feedback loop described above as it has been suggested to be involved in DC killing by CTL

(Ashton-Rickardt 2010). GrB has lately been proposed as an immunomodulator molecule also due to its expression in T regulatory cells (Cao et al. 2007).

Serine protease inhibitor (Spi)-6 is an intracellular protease responsible for GrB inactivation. It was first described in CD8 T cells, where it is expressed upon activation, and NK cells. Spi6 protects CD8 T cells from GrB-dependent self-induced apoptosis and is responsible for the integrity of its cytotoxic granules (Zhang et al. 2006). Spi6 is also expressed by immature DC at low levels and it is upregulated upon maturation (Medema et al. 2001). Studies conducted *in vitro*, demonstrated that overexpression of Spi6 can protect mature DCs from CTL-mediated apoptosis in both humans and mice (Medema et al. 2001; Hirst et al. 2003). Unpublished data from our lab also shows that DC lacking Spi6 are more susceptible to CTL-induced PCD. Nonetheless, in an *in vivo* study, Andrew and colleagues results contradict Medema and Zhang's data on Spi6 requirement for DC survival, showing no protection in LPS-matured antigen-loaded BMDDCs when injected into a previously boosted mouse (Andrew et al. 2008). Notably, these data were obtained relying on mRNA level measurements to establish Spi6 protein levels. RNA measurement is not a good strategy to evaluate protein levels as the mRNA might be translated at a later time and therefore not mirror the amount of protein present at that stage in the cell. This project addresses the controversy regarding Spi6 protective role in DCs and aims to further test the consequences of Spi6 absence in mature DCs when priming CTL responses after viral challenge *in vivo*.

The hypothesis tested in this project was that Spi6 could prevent CTL-induced apoptosis in DCs during the priming of both naïve and memory CD8 T cells by inhibiting GrB. Based on the model describing a negative feedback loop regulating the expansion of CTL responses, the hypothesis also predicts a defective expansion of antigen-specific CD8 T cells in absence of Spi6 in DCs. The confirmation of this hypothesis supports Spi6 involvement in the negative feedback mechanism that regulates CTL expansion as a regulator of the susceptibility of DCs to CTL-mediated killing.

Protection of DCs from CTL-mediated death is desirable in the design of vaccination strategies aimed at eliciting CD8 T cell-mediated responses. Also, DC enhanced survival would be desirable as part of therapeutic strategies aimed at the reawakening of the CTL response in chronic infections.

## 5.2 Spi6 protects dendritic cells from granzyme B-mediated apoptosis *in vitro*

Medema's and Hirst's groups showed how Spi6 is expressed at low levels in immature DCs and upregulated in mature DCs both in humans and mice (REF). Medema had also demonstrated that overexpression of Spi6 could protect DCs from CTL-mediated killing in vitro (Medema et al. 2001), which was confirmed by Andrew and colleagues (Andrew et al. 2008). I*n vitro* data obtained in my lab by M. Zhang showed increased Spi6 KO BMDDC susceptibility to CTL-killing compared to WT ones, and correlated it to degranulation of CTLs rather than involvement of the Fas-FasL pathway. These results all support a role for Spi6 in protecting DC from CTL-mediated killing, and represent the basis on which this project was developed.

Since previous *in vitro* results are the basis for the hypothesis regarding Spi6 anti-apoptotic role in DC, some of the *in vitro* experiments previously performed by my colleague Dr. Zhang were repeated. The aim was to confirm previous results and specifically show that Spi6 KO BMDDC susceptibility to CTL-mediated apoptosis was GrB-dependent. Spi6 KO- and WT-derived BMDDCs were therefore cultured with CTL in presence or absence of GrB obtaining protection from apoptosis in mature $Spi6^{+}$ cells only (WT cells). On the other hand, the elimination of GrB from CTL granules could abolish DC apoptosis both in presence and in absence of Spi6 at 4 hrs (see Figure 38). These two pieces of data suggest that CTL-derived GrB triggers

apoptosis pathways in DCs, and also confirming Yang results obtained in pfn KO mice, where no DC killing was seen (Yhang et al. 2006). Notably, in the same experiment involving GrB KO P14 CTLs, apoptosis was induced after 8 hrs to the same extent in both Spi6 KO and WT BMDDCs. This suggests triggering of apoptosis pathway by involvement of the death receptor pathway. GrB has been described as the quickest mediator of apoptosis (Martin et al. 1996) out of the granule.

In the context of in vitro CTL-induced antigen-specific killing, granzyme B is the mediator of apoptosis seen in mature BMDDC culture. Spi6 upregulation in mature DCs is key for protection of these cells from apoptosis.

## 5.3 LCMV infection model and adoptive transfer of Spi6-competent CD8 T cells

The physiological relevance of Spi6 upregulation in mature DCs is nonetheless not clear, as in vitro experiments showed a protection from CTL-mediated killing, while in vivo results did not. In order to analyse Spi6 function, its levels were measured in physiologically matured splenic DCs in an infected *in vivo* model. The viral model of choice, LCMV, stimulates a MHC-I-driven immune response and is widely used to study CD8-specific T cell responses. In WT (B6) mice, the virus peaks at day 3-4 post-infection and CTLs expand up to 10-fold from the original antigen-specific repertoire, creating a CD8 T cell response which reached its peak at day 8 and can clear the virus usually by day 10 p.i. At day 8 p.i., in the spleen of a WT mouse, the antigen-specific CD8 T cell response is composed as follows: 33% by np396-specific CTL, 25% by gp33-specific CTL, 5% by other minor epitopes (Murali-Krishna et al. 1998). Previous data from Ashton-Rickardt's group demonstrated how the CD8 T cell responses, to both viral and bacterial infection, in Spi6 KO mice have a defective

expansion phase but normal levels of memory cells (Zhang et al. 2004). In particular, antigen-specific CTLs show high levels of apoptosis in these mice thus failing to accumulate and form a peak around day 8 post-infection as is seen in WT mice after LCMV challenge. Apoptosis levels are mainly GrB-mediated, as Ashton-Rickardt demonstrated by staining for GrB (Zhang et al. 2006). Therefore, to ensure the development of a proper CD8 T cell response to the virus, the adoptive transfer of congenically marked naïve P14 CD8 T cells was employed. The adoptive transfer allowed/ensured the study of Spi6 function in DCs when antigen-specific CD8 T cells are Spi6 competent and thus do not die of self-inflicted PCD.

The adoptive transfer of naïve P14 T cells was designed to minimize non-physiological effects due to the high number of gp-33 specific precursors. A study by Badovinac and colleagues reports that CTL responses deriving from the adoptive transfer of 60-6x$10^3$ TCR-specific cells are likely not to disturb the physiological development of endogenous responses. This was found despite the frequency of transgenic cells being about $10^2$-fold higher than the number found in an intact mouse. In fact, after adoptive transfer, only about 30-40% of transferred transgenic cells will successfully colonise the host (Blattman et al. 2002; Badovinac et al. 2007). The expansion of 50 transgenic T cells has in fact been shown to reach up to 400,000 times the original number, while when 5x$10^5$ transgenic cells are adoptively transferred into a mouse, an increase of 4- to 400-fold is observed. They also showed that a maximum level of expansion is supported by the mouse organism (Kemp et al. 2004). Regarding the development of memory, anticipated CTL peak and higher frequencies of $CD127^+$ $CD62L^+$ cells during the contraction phase, seen with high numbers of transgenic cells, could be avoided with the adoptive transfer of up to 6x$10^3$ transgenic cells (Badovinac et al. 2007). The aim was to avoid any modification of the physiological development of the response and the number of cells to adoptively transfer was therefore chosen accordingly. On this basis, 5x$10^3$ P14 CD8 T cells were adoptively transferred into Spi6 KO and WT mice and showed a physiological CTL peak at day 8 p.i., confirming Badovinac's results. The dynamics of the endogenous and donor antigen-specific T cells were studied and found to be in accordance to physiological expansion in intact mice.

CD4 help is generally needed for the successful expansion of memory cells (Behrens et al. 2004), but some antigens are considered Th- independent: LCMV is one of these. This happens both as the virus can infect dendritic cells and because of the inflammatory status they provoke in the host (Lanzavecchia 1998). More studies confirmed the requirement for CD4 help for an optimal memory expansion, although in absence of these cells during the boost did not make much difference in the expansion levels (Shedlock and Shen 2003). In LCMV Arm infection, on the contrary, the elimination of CD4 T cells did not correlate to any defect in CD8 expansion levels or function (Matloubian et al. 1994).

In order to study Spi6 role *in vivo*, a viral model of infection was used to achieve physiological maturation of DCs and study the priming of CD8 antigen-specific T cells in presence or absence of Spi6. As Spi6 absence has a negative impact on CTL survival, as reported by previous studies, transgenic CD8 T cells specific for a viral antigen, were adoptively transferred into these mice (both the WT and the KO). This approach allowed the study of the effect of CTL release of GrB on DCs in the presence or absence of Spi6 in these cells.

## 5.4 CD8α dendritic cells and plasmacytoid dendritic cells in LCMV infection

Due to the variety of dendritic cell subpopulations in mice, there was a need for focussing on specific subsets to study the expression of Spi6 in the relevant *in vivo* context. For this reason, the protein levels were studied in splenic DCs with a specific focus on two particular subsets: $CD8\alpha^+$ DCs (hereafter defined as CD8α DCs) and pDCs.

### 5.4.1 CD8α dendritic cells in LCMV acute infection

Belz and colleagues observed that, during LCMV Arm infection, resident CD8α DC in the spleen and LN are the main vector in presenting antigens to naïve CD8 T cells (Belz et al. 2004). Because of CD8α DC central role in cross-priming to CD8 T cells it was decided to focus the study of Spi6 expression and function on the CD8α DC population of the spleen. Further data supporting the central role of CD8α DCs in cross-presentation of LCMV antigens include the specific TLR repertoire expressed by CD8α DC which allows them to sense viral DNA and RNA. Also, CD8α DCs express an antigen-processing machinery attaining better cross-presentation compared to $CD8\alpha^-$ subsets (see 2.5.10). Since CD8α DCs are spleen and LN residential cells, it was unclear how these cells could present antigen deriving from peripheral infected tissue. A mechanism that allows for the transfer of antigen from migratory DCs to resident CD8α DCs, both in the LN and in the spleen, has been uncovered. It aims at amplifying the antigen level in the SLOs and increasing the odds for cognate antigen recognition by CD8 T cells (Carbone et al. 2004; Allan et al. 2006). A phenomenon with similar effects termed cross-dressing has also been described, whereby loaded MHC molecules are passed on between DCs to allow more efficient activation of naïve and memory CD8 response (Wakim and Bevan 2011).

The measurement of Spi6 levels in the CD8α DC subset shows that these cells upregulate Spi6 at day 2 post-infection (see Figure 51). As the upregulation of Spi6 indicates that CD8α DCs are target of GrB themselves against GrB action, these findings further support the hypothesis that CD8α DC cross-present LCMV Arm antigens to CD8 T cells. Spi6 expression in CD8α DCs is then maintained at high levels until the resolution of the infection, suggesting that prolonged DC involvement, antigen presentation and costimulation, is required for the effective development of antigen-specific CTLs.

The finding of high levels of Spi6 in DCs is indicative of a requirement for protection from CTL-mediated apoptosis. Nonetheless, the analysis of Spi6 levels alone does not explain if the protein effectively protects the cells. To further/ investigate Spi6 ability to preserve DCs after exposure to CTL-released GrB *in vivo*, DC numbers were measured at various time points in LCMV Arm-infected WT and Spi6 KO mice after adoptive transfer of WT and GrB KO P14 CD8 T cells. The time

points were chosen on the basis of the dynamics of infection that have been described in immunocompetent mice. By 24 hrs after LCMV Arm infection, $CD8\alpha^+$ DCs can stimulate naïve T cells and by day 3 apoptosis of $CD11c^+$ $CD8\alpha^+$ can be seen. Measurement of the apoptosis levels through surface marker staining indicates an increase in apoptotic cell levels from day 3, but a more detailed analysis revealed that this staining had included phagocytosed CD8 T cells undergoing apoptosis. As described above in fact (see 2.5.10), CD8α DCs are highly equipped to uptake dying and apoptotic cells.

My results, showing a lower peak in DC numbers at day 4 post-infection, support Montoya's work that shows increased apoptosis in DCs from day 4 post LCMV-infection (Montoya et al. 2005). They also support the work by Bahl and colleagues, who reported a decline in DC number in the spleen of WT LCMV-infected mice after day 3 p.i. (Bahl et al. 2010).

In my hands, absence of Spi6 results in higher susceptibility to apoptosis in CD8α DCs from day 4 p.i., as demonstrated by a significant decrease in the number of these cells in Spi6 KO mice compared to WT. Apoptosis levels were particularly induced in these cells in a GrB-dependent way by CTLs. This is supported by the correction obtained in CD8α DC numbers o WT level when eliminating GrB from the granules of antigen-specific CTLs.

It is also important to also establish whether Spi6 absence could impair DC function. To investigate if Spi6 absence had any functional consequence on DCs, these cells were isolated from Arm-infected mice and used to stimulate antigen-specific CD8 T cell proliferation. The results obtained showed no functional difference between Spi6 KO DCs and WT ones. These results favour the hypothesis that any defect in CTL response is to be ascribed to diminished survival of antigen-presenting DC, not impaired functionality.

On the basis of these results showing upregulation of Spi6 in CD8α DC in mice and demonstrating the dependence of these cells on Spi6 for protection from CTL-mediated killing, the expression of Spi6 is expected to be upregulated in the correspondent subset in humans which represents the desired target of adjuvant strategies to improve CTL responses.

### 5.4.2 Plasmacytoid dendritic cells in LCMV acute infection

According to results from Montoya, Colonna and Dalod, upon viral infection, pDCs seem to be able to induce the activation of components of both the innate and the adaptive response, depending on the virus strain. Studies with LCMV infections have given unclear results regarding the degree of involvement of pDCs in eliciting CTL function and maturation of DCs. pDCs are DCs specialised in the systemic release of type I IFN. This is induced by the triggering of pathogen-sensing, PRR, pathways induced particularly by TLR7 and TLR9 stimulation. These two TLRs are expressed in endocytic vesicles in the pDCs and are activated by high levels of RNA and unmetilated CpG-rich DNA. They can also express IL-12 and IL-6. Type I IFNs contribute to mediate DC maturation (Montoya et al. 2002) and IL-12 has a stimulatory effect on CD8 T and NK cell-mediated release of cytotoxic molecules. Interestingly, in murine cytomegalovirus (MCMV) infection, whose target is mainly is splenic DCs, pDCs production of IFNα/β is needed for accumulation of this cell type in the spleen and has an adjuvant effect on cross-presentation as it enhances MHC-I expression and support CD8α DC maturation in particular (Dalod et al. 2003). In humans, patients with chronic infections like HCV (Kanto et al. 2004) and HIV (Fitzgerald-Bocarsly and Jacobs 2010) have diminished pDC circulating levels. In these infections, type I IFN levels are also low despite stimulation of TLRs. Plasmacytoid DC role in LCMV infection has been studied by Colonna and colleagues who used a BDCA-1-DTR mouse to study pDC role in LCMV Arm infection. The BDCA-1-DTR mouse has the peculiarity of allowing for the selective elimination of pDCs by DT administration. A DT receptor is coupled with the promoter for BDCA-1 making all the cells that express DTR sensitive to the toxin. BDCA-1 is the marker for pDCs in humans and its promoter is present in the same position and role in mice. Comparable CTL responses, cytokine levels and viral clearance were seen in the pDC-deleted mouse and the WT counterpart. In LCMV infection, cleared effectively both in the pDC-defective mouse and in the WT, the source of interferon needed for a strong CTL antiviral response is likely to be redundant. The authors suggest that pDC production of IFN might be required in weak CTL responses (Barchet et al. 2005). A different study suggests instead that LCMV itself inhibits pDC capacity of secreting type I IFNs and pDC numbers decrease until day 4-5, to then increase until day 10 p.i (Zuniga et al. 2008).

Nonetheless, Montoya and colleagues' study established that an increase in the number of pDC and in the production of type I interferon occurs early after LCMV infection and it is mirrored by a decrease in the number of cDCs. IFNs peak at day 3 post-infection to decline steadily (Montoya et al. 2005; Lee et al. 2009) and pDC numbers are shown to increase in the first days of the response. In the same report, susceptibility of pDC to PCD was also measured and no increased apoptosis was measured at day 3 p.i. compared to day 1. Interestingly $CD11c^+$ total cells were also tested for apoptosis susceptibility and increased apoptotic cells were measured at day 3 p.i. Finally the turnover rate of pDC was established and found to be slower compared to $CD11c^+$ DCs (Montoya et al. 2005).

Findings are contrasting to date and have been mainly carried out over the very initial stages of the response, as type I interferons are typically released early and transiently. The study of pDC "evolution" during the development of CD8 T responses to LCMV is therefore of particular interest. In order to verify whether pDCs are involved in the presentation of antigen to CTLs during the expansion phase, the number of pDCs and intracellular expression of Spi6 were measured in the spleen of infected mice. In my hands, pDC do not upregulate Spi6 at any point during LCMV Arm infection. These data support the theory that pDCs are not involved in antigen presentation to CTL in LCMV Arm infections, as suggested by studies showing their poor antigen-presenting properties (Barchet et al. 2005). These results suggest that no protection against GrB is required for pDCs to properly deploy their functions. Nonetheless, the lack of Spi6 does not rule out the possibility that pDC contribute to LCMV clearance through IFN secretion or other cell-contact independent mechanisms. The high level of cross-staining seen from day 5 p.i. onwards in particular indicates that other serpins are upregulated instead, and are needed in these cells. My data regarding the number of pDCs in the spleen of infected mice mirror both Colonna's and Montoya's results: pDCs decrease from day 1 to day 3-4 p.i. and subsequently increase till day 8 p.i.

CD8α DC and pDCs were chosen as the subsets in which to study Spi6 expression due to their specific role during LCMV Arm infection. CD8α DCs in particular are

the subset responsible for cross-presenting CMV-derived antigens to CD8 T cells, and pDCs support CD8α DC maturation and cross-presentation activity and aid the cytotoxic activities of CTLs. The analysis of Spi6 expression revealed upregulation in the former subset early after infection and maintenance of the expression levels until day 8 p.i., while pDCs did not show to express Spi6. CD8α DC levels are impaired in Spi6 KO mice, despite the levels of aiding pDC are not different from WT levels. It is therefore expected that CD8α DC numbers are affected by the lack of SPi6, which makes them more susceptible to GrB-mediate apoptosis, and not by the lack of maturation signals deriving from pDCs.

## 5.5 Importance of sustained antigen presentation by dendritic cells during primary responses

Having shown that the survival of DCs is impaired in absence of Spi6 but their functionality is preserved it was of interest to investigate whether the diminished number of DCs affected CD8 T cell responses to the pathogen. In order to determine whether primary and memory CD8 T cell expansion levels *in vivo* are affected by Spi6 absence in DCs, the levels of donor CTL were measured in Arm-infected P14 CD8-adoptively transferred WT and Spi6 KO mice.

According to the antigen persistence theory, described by VanMoonfort, the presence of antigen and its continuous presentation are needed for optimal expansion of antigen-specific CTLs in viral and bacterial infections. In particular, Von Monfoort supports the need for sustained antigen stimulation throughout the expansion phase of CD8 responses (Jusforgues-Saklani et al. 2008; van Montfoort et al. 2009). This theory contrasts with the previously formulated theory of the “autopilot”. The autopilot theory asserts that a short-term initial contact between a CD8 T cell and a

DC is enough to trigger activation, proliferation and effector functions in the CD8 T cell. This process is not negate by the antigen persistence theory, but rather included in it in that the initial contact is certainly key for activation and proliferation but it is not enough to sustain a maximal expansion. Curstinger and colleagues had suggested how continuous antigen presentation and costimulation during CD8 T cell priming resulted in a larger cell number and higher lytic activity. They showed in particular that cytolytic activity requires 40hr of antigen presence to be fully developed in CTL (Curtsinger et al. 2003). More recently, Van Montfoort's work demonstrated the presence of antigen stored in intracellular compartment in DCs and that DCs could present antigen on MHC-I complexes despite their quick turnover rate of 24 hrs (van Montfoort et al. 2009). Dolfi and colleagues also showed in a recent report how the CD8α DC subset in particular is required to sustain viral-specific responses during the effector phase in order to have maximal expansion levels (Dolfi et al. 2011). Dynamics of DC-T cell interaction are also being studied. John and colleagues described short-time contacts happening between T cells and several DCs in a normal, non-inflammatory status in the LNs, while during pathogen invasion, the contacts between DCs and T cells become longer and less frequent (John et al. 2009). The results described, taken together, depict a model in which: initial DC-T cell interaction are quite sustained, DCs can store antigen in intracellular compartment for cross-presentation, DC-mediated presentation of antigen is required during the whole expansion phase, and CD8α DCs are not just required for initial cross-presentation of viral antigens to CD8 T cells but are also required to cross-present the viral antigens at later stages during the effector response. sustained antigen presentation is therefore required for efficient CD8 T cell activation not only in the early phases of an infection, but during the expansion phase as well.

According to the antigen persistence theory, DCs mediate antigen presentation to CD8 T cells throughout the expansion phase also thanks to their antigen. The results obtained through priming of CTLs by adoptively transferred BMDDCs should be seen in light of van Monfoort's model of prolonged antigen presentation. The experimental model involving BMDDC adoptive transfer described in Chapter 4.2 specifically addresses this issue. BMDDCs injected into a host have an average survival rate of 3 days due to their half-life, maturation stage, and LPS pro-apoptotic action (Hou and Van Parijs 2004). They will therefore be able only to contribute to

the initial phase of a CD8 T cell response. As these cells are eliminated by day 3 (as shown in Belz et al. 2004), they are not be able to sustain further CTL expansion. Also, due to the nature of the experiment, the source of foreign antigen in this experiment was limited to the peptides presented on injected mature BMDDCs. This model therefore replicates a toxin invasion rather than a viral infection.

The adoptive transfer of antigen-pulsed ex vivo-matured BMDDC does not mirror a viral infection as, in the case of an infection, the system provides continuous source of antigen which is continuously presented to CTL to sustain the expansion. As shown by Pham and colleagues (see Figure 77), DC immunisation alone, or accompanied by early or late inflammation elicits a transient effector proliferation and earlier CTL peak and formation of memory. DC immunisation needs to be accompanied by constant and concomitant inflammation to induce a physiological expansion and formation of memory comparable to (viral) infections. LPS-matured DCs are both killed by activated CTL and by the apoptosis process induced by LPS itself (Lutz et al. 1999; Heath et al. 2004*).*

When considering the number of DCs retrieved at the draining LN as a measure of surviving DCs, Andrew and colleagues failed to discriminate between death induced by CTL action and death induced by LPS action. For this reason, Andrew's experiment represents a non-physiological condition and its conclusions regarding Spi6 protection cannot be extended to physiological context. In LCMV-infected mice, the CD8 T cells localise in the red pulp and not in the T cell areas like in the naïve mice. Abrogation of T cell naïve migration to the T cell zone is reconnected to the downregulation of CCL21, and this impacts DC migration as well (Mueller et al. 2007). These dynamics demonstrate how the SLO environment changes between the steady state and infection, therefore making some in vitro models not suitable to test priming. Among these is Andrew's model.

Andrew and colleagues had, moreover, compared the survival of mature versus immature DCs, not the effect of Spi6 absence in mature DCs versus the wild-type situation. The main difference resides in the ability of mature DCs to prime CD8 T cells, while immature DCs cannot do so. Immature cells will therefore not be target of CTL-mediated killing in the same way as mature antigen-loaded cells are, thus

making the study of Spi6 protective function in the latter not comparable to the function in the former.

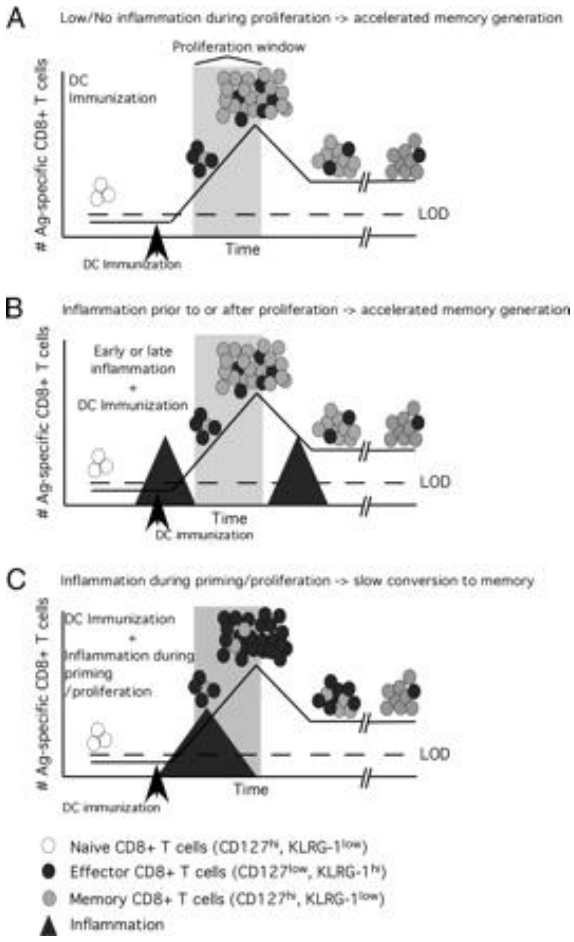



**Figure 77: DC immunisation in inflammatory context shaping the composition of the effector and memory pool (Pham et al. 2009).**

The same experiment was thus repeated as in Andrew and colleagues to collect preliminary data, with some modifications. Mature DC functionality and survival were in fact measured in presence and absence of Spi6. Quite interestingly, it has been reported that injected BMDDC, when mature, accumulate in the spleen when injected i.v., therefore stimulating re-expansion of both CD8 central memory and T effector memory cells, and in the LN when injected i.p. (Huck et al. 2008) Gp33-specific CTL responses were measured in the spleen and inguinal LN of the mice.

No difference in the ability of BMDDCs injected into naïve mice to induce a CD8 T cell response was measured. The hypothesis is that the lack of difference in expansion

levels at the early stage of a CD8 T cell response is due to the absence of GrB. In this experiment in fact, DCs only take part to the initial CD8 T cell priming and no prolonged antigen presentation occurs at a later stage as DC are quickly removed. Notably, staining of blood levels of gp33-specific CTLs suggests the peak of the CTL expansion is around day 4, rather than at day 7, when CD8 cell levels are declining (see Figure 61). We can deduce that Spi6 expression is required in DCs not for the initial priming, but at later stages of the CTL expansion. This is further confirmed by the fact that DC elimination is seen from day 4 post-infection in LCMV Arm-infected mice. It also shows that in vivo, DCs lacking Spi6 are able to prime CD8 as efficiently as WT DCs. Consistently with previous reports, this work confirms that a short stimulation/brief exposure to antigen is sufficient to promote naïve CD8 T cell proliferation and multiple rounds of cell division, but it is not sufficient to induce an effective anti-viral response. In particular, injection of gp33-pulsed BMDDC represents an immunization rather than mimicking a viral infection. The CTL response generated by this immunization is 10-folds lower than the one generated by viral infection.

Due to the need of prolonged antigen presentation during CD8 T cell responses, and based on these preliminary results, deficient DC levels caused by Spi6 absence were expected to affect antigen-specific CTL responses in two ways. Premature death induced by CTL, will cause shorter DC life-span, thus accounting for less naïve antigen-specific CD8 T cells being primed. Moreover, higher death levels of DCs will impact the negative feedback regulatory loop which is in place to control CTL expansion levels. The negative feedback loop is a regulatory mechanism which depends on DC numbers (and therefore their survival) to regulate CTL expansion levels. The theory is based on the assumption that CTLs kill antigen-loaded DCs during primary CD8 T cell responses. When a higher number of CTL is activated, a higher level of DC killing will occur, due to CTL-released GrB action on these cells. The increased DC death levels will then feedback negatively on further CTL activation and priming. The antigen persistence theory supports indirectly this model. In fact it describes the need for prolonged antigen-presentation, therefore involving prolonged DC-T cell interaction and exposure of DC to CTL-killing, this prolonged exposure to GrB-loaded CTLs is at the basis of the regulatory feedback mechanism, as just seen above. The two theories taken together form a model whereby CTLs can

induce DC apoptosis during the prolonged antigen presentation required for an optimal expansion of effector cells. DC survival is therefore threatened by the prolonged priming need of CD8 T cell responses.

To measure the effect of deficient DC levels mice on CTL expansion in Spi6 KO, the dynamics of CTL expansion and the levels of donor-derived antigen-specific CD8 T cells at day 8 post-infection were analysed. The results showed a difference in the CTL levels, starting from day 6 p.i. and continuing to day 8 p.i., in the spleen of Spi6 KO mice compared to WT. The antigen-specific CD8 T cell levels in the Spi6 KO mouse were lower. The defect in CTL levels was particularly significant at day 8 p.i. and resulted in impaired viral clearance in the Spi6 KO mice when measure at day 6 p.i (See Figure 74). The defective CTL expansion and the inability of clear the viral infection to WT levels point towards the importance of guaranteeing a proper full expansion of CD8 T cells in viral response, indicating this is fundamental for the efficient clearance of the pathogen.

These results confirm that antigen persistence during CD8 T response is important to shape the CTL expansion, and also prove that Spi6 is important in protecting antigen-loaded DCs from CTL killing during the expansion phase. The defect in CTL expansion seen in Spi6 KO mice can be correlated to Spi6 absence in DCs: the experimental design in fact excludes any autocrine effect of Spi6. The difference seen in the P14-adoptive transfer experiment between Spi6 KO and WT mice is due to the absence of Spi6 in DCs which makes these cells more susceptible to CTL-mediated killing thus bringing about a lack of sustained presentation during the expansion phase. *"*The results, showing hampered CTL expansion in P14 adoptively transferred LCMV-infected Spi6 KO compared to WT mice, and showing no difference in CTL expansion levels between mice SPI6 KO- and WT-BMDDC transferred, can be explained in the light of the prolonged antigen stimulation requirement and the negative feedback loop that regulates CTL expansion*"*(Lovo et al. 2012).

Other studies correlate the survival of DCs to the expansion of CTL response in vivo (Loyer et al. 1999; Hermans et al. 2000), showing how pfn-dependent killing of DCs regulate priming of CD8 T cell responses. These results taken together further support the regulatory role played by mature DC levels in shaping CD8 T cell responses.

## 5.6 Dendritic cells in secondary response priming

Secondary immune responses arise from the re-activation of memory cells, whose characteristics are quick triggering of degranulation, proliferation and a lower threshold for activation. Memory CD8 T cells are formed during primary responses and survive in absence of antigen due to their ability to undergo homeostatic proliferation. At day 40 after the primary infection, CD8 T memory cells can be measured in the spleen and LN. These cells require lower costimulation and are activated in presence of low levels of antigen. Despite the lower threshold, memory CD8 T cells have specific activation requirements. Recent studies have shown that CD8α DCs but not other DC subsets can prime re-expansion of memory CD8 cells.

The killing of DCs as a self-containment mechanism for limiting the expansion of the immune response has been shown by Wakim and colleagues in memory re-expansion (Wakim et al. 2008). On the observation that DCs are target of degranulation-mediated apoptosis when priming memory CD8 responses, it was hypothesized that Spi6 expression could sustain DC survival during the priming of memory cells. Memory CD8 T cells are loaded with cytotoxic granules and can degranulate very quickly upon recognition of antigen in MHC context. During the expansion of secondary responses, DC survival was thus expected to be impaired in Spi6 KO mice due to the lack protection against granule-released GrB. This was expected to have a consequence on the ability of DC to prime memory responses and therefore to result in deficient memory cell expansion. In order to study Spi6 requirement in priming of memory responses, CTL re-expansion levels were tested/analysed after secondary infection.

As a means to study Spi6 role in memory responses priming, the same experimental protocol as above was used: Spi6 KO and WT mice were adoptively transferred with P14 CD8 T cells (Thy1.1) and infected with LCMV Arm. In this experiment, mice were not sacrificed at day 8 p.i. The test group was instead monitored for donor-derived P14 cells in the blood and re-infected with LCMV CL13 40 days after the primary infection. This strain was used because, due to the high number of adoptively transferred P14 CTL and to the efficiency of CD8 T cell responses in eliminating

Arm, a high titre and more virulent strain was needed to ensure re-expansion of the memory cells and therefore make sure DC were involved in the process.

As described above (see 2.7.2.1), CL13 infects DCs with much higher affinity than Arm, due to an aminoacid change in its glycoprotein sequence. CL13 in particular has a high tropism for interdigitating DCs in the spleen, which causes low expression levels of MHC-I and MHC-II and costimulatory molecules in these cells and ultimately a failure in T cell stimulation long-term. CL13 leads to chronic infection in immunocompetent naïve mice, but it has been shown that in memory mice CL13 can be easily cleared (Borrow et al. 1995).

Cross-presentation is not as important for CD8 T cell priming after CL13 infection, as DCs themselves are infected and will therefore present viral antigens on MHC-I even if they are poor cross-presenters. More DC subsets might therefore be involved in activating CD8 memory cells in this case. During LCMV CL13 infection, the spleen represents the infected tissue, and due to its memory cell composition which includes both effector memory and central memory cells, DCs are expected to be eliminated via two mechanisms. The first one is GrB-mediated killing during priming, and the second is direct, targeted killing by effector memory cells (Marzo et al. 2007), as DCs are as infected cells. DC will be subject to both local and indirect release of GrB due to priming, and targeted release of granzymes and triggering of the death receptors.

The results obtained by donor-derived antigen-specific CD8 T cell analysis at 5 days post-infection show that CL13 induces a re-expansion of memory donor (Thy1.1) cells when injected >40 days after p.i. (see Figure 63). This expansion is very contained in Spi6 KO mice and it is defective compared to WT. The difference in re-expansion levels does not depend on different levels of existing CD8 memory cells in the two test groups. No difference was in fact seen in the LN of Spi6 KO and WT mice when donor-derived CD8 memory cell were measured at day 40 p.i. A difference could be seen in the spleen, where donor-derived CD8 memory cell levels were higher in Spi6 KO mice compared to WT. This is consistent with the results shown by Zhang and colleagues regarding memory formation in Spi6 KO mice: the defective peak in the primary response is corrected by homeostatic mechanisms that increase the development of the memory pool (Zhang et al. 2007). As the memory T

cell pool levels in Spi6 KO mice were higher than in WT mice, the deficit in the re-expansion levels in Spi6 KO mice is even more considerable. The failure of Gp33-specific memory cell in Spi6 KO mice to expand to WT levels is possibly due to defective priming and lack of proper co-stimulation.

To further confirm the findings obtained by measuring the memory pool at day 40 p.i., the correlation between memory pool size and expansion levels was investigated. Equal numbers of memory cells congenically marked were adoptively transferred into naïve Spi6 KO and WT mice before infection with LCMV CL13. It was further addressed whether the difference seen in the re-expansion levels was due to a difference in the memory pool size and functionality. When the same amount of P14 CD8 T memory cells were injected in naïve Spi6 KO and WT mice, re-expansion levels in Spi6 KO mice were, when compared to the WT, deficient in the LN, but not in the spleen (see Figure 72 and 73). The data collected therefore concurs with previous findings describing DC killing by memory cells and describing memory development in Spi6 KO mice, but also suggest that at the LN level a different requirement for Spi6 might exist.

Secondary memory cells have been shown to display different characteristics from primary ones. They in fact fail to express CD62L, thus not being able to access the LN, and exhibit sustain expression of GrB (Masopust et al. 2006). Also, in the LN five populations of DC can be found: two migratory ones, Lagerhans and dermal DCs, and three resident ones, $CD8\alpha^+$, $CD8\alpha^-$ $CD4^+$ $CD8\alpha^-$ $CD4^-$ DCs. In this organ, the LN, in Spi6 KO mice, the levels of secondary expansion of CD8 gp33-specific memory cells were dependent on the number of donor gp33-specific memory cells adoptively transferred. In the WT mice, on the other hand, the CD8 gp33-specific donor memory cell expansion levels seem to reach a plateau, irrespectively of the number of memory cells adoptively transferred. One hypothesis might be that, even at low number, memory cells adoptively transferred are in too high number compared to a physiological situation, and the mature antigen-bearing resident DCs are only able to prime a limited number of them. As CD8α DCs are responsible for the priming of CD8 memory cells in the LN, and since these cells have a much lower activation threshold and quicker degranulation dynamics than CTLs, it can be inferred that CD8α DCs in Spi6 KO mice are killed to a much higher extent than in WT mice. This

higher level of killing would explain the lower levels of memory CD8 re-expansion, consistent to the impairment in memory cell re-expansion observed in CL13 infection -secondary response- experiments (see Figure 67). This suggests a role for Spi6 in protecting CD8α DCs in priming of secondary responses in the LN is therefore. The same results also show that the size of the memory cell pool influences the re-expansion in the spleen but not in the LNs. The hypothesis is that DCs in the LN are only eliminated through indirect GrB released shortly after priming.

Additionally, in the spleen, during the priming of secondary responses, the antigen-loaded CD8α DCs, due to the expression of gp33 in MHC-I context and their proximity to the memory cells during priming, are targeted by degranulating effector memory cells both directly and indirectly. One explanation as to why CD8α DCs in the spleen are killed to the same extent in the WT and Spi6 KO could be that the anti-apoptotic effect of Spi6 is not sufficient to counteract high levels of GrB and the triggering of other apoptotic pathways. The levels of GrB and the contribution of other apoptosis pathways are likely to render Spi6 unable to prevent DC killing in this context.

## 5.7 Spi6 inhibition of granzyme B

The results reported in chapter 4.4 illustrate that, in absence of Spi6, the donor-derived CTLs are not able to expand to WT levels. The defect is not cell autonomous, as the adoptively transferred naïve CD8 T cells are from WT mice. To verify that the defect was due to the action of GrB, as Spi6 is a physiological inhibitor of GrB, elimination of GrB from the system was sought. The absence of GrB was expected to result in rescue of the defect in CTL expansion, in both primary and memory responses. This verification was important to correlate CTL expansion levels with Spi6 absence. It also intended to verify if Spi6 function as a GrB inhibitor was specifically the cause for CTL defects in the KO mice. Confirmation of the

fundamental function of Spi6 in shielding DCs from GrB -mediated CTL killing was obtained with the elimination of GrB from antigen-specific CTLs and was linked to CTLs' ability to proliferate.

The contribution of endogenous versus donor gp33-specific CD8 response was measured to verify that elimination of donor-derived GrB would account for reducing the total GrB of more than 90% in the system. Endogenous levels of anti-gp33 CTL were negligible in my model both during the primary and the secondary CD8 T cell response. As shown in Figure 52 and Figure 69, due to the adoptive transfer of transgenic TCR cells, the donor gp33-specific response is dominated by $Thy1.1^{+}$ cells, which are on average about 15-fold higher than the endogenous Thy1.2 cells.

GrB KO CTLs, which were used in order to eliminate GrB from the system, are not impaired in their killing functions (Zajac et al. 2003). CTLs in pfn KO mice, on the other hand, have been shown to be deficient in their ability to clear LCMV infection (Kagi et al. 1994; Walsh et al. 1994). In GrB KO mice, the kinetics of viral clearance is delayed compared to WT but substantially similar, with complete clearance reached by 8-10 days p.i. when LCMV Arm is used for infection i.p. (Fiigure 78). On the other hand, pfn KO CTL have impaired killing functions due to the inability to deliver the content of granules to the cytosol of the target cell (Kagi et al. 1994) and they accumulate without being able to clear the infection (Matloubian et al. 1999; Stepp et al. 2000). The accumulation of these cells further supports the existence of a feedback regulatory loop driven by DC elimination. Pfn deficient CTL cannot induce GrB-dependent apoptosis in DCs, thus failing to eliminate them.

The levels of antigen-specific CTL in the spleen have been shown to be the same as WT in LCMV-infected mice (Zajac et al. 2003). Moreover, from a functional point of view GrB KO CD8 T cells are equally able to induce apoptosis in target cells as WT ones in a $^{51}$Cr-release assay. This was also reproduced by the results reported above in CalceinAM assays (see Figure 39). In my hands, the only difference between GrB KO and WT CTL is the time-lapse between target-effector contact and apoptosis onset: GrB KO CTLs are less able to induce rapid (within 4hr) DNA fragmentation.

This is due to the longer dynamics of apoptosis induced by other granzymes release in the IS together with GrB, like GrA.

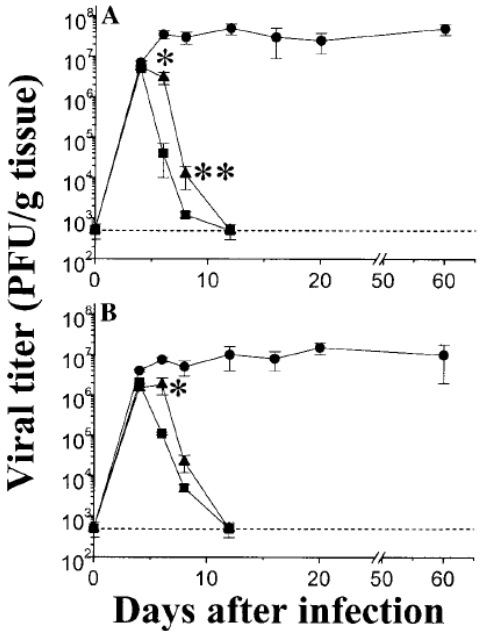



**Figure 78: Viral titre in GrB KO mice (triangles) infected with LCMV compared to viral titres in WT mice (squares) and pfn KO (circles) at various time points after infection (Zajac et al. 2003).**

On this basis, the adoptive transfer of GrB KO P14 was chosen as to validate GrB role in DC survival, thus maintaining the ability of these cells to eliminate antigen-loaded DC and to clear the virus. In agreement to the initial hypothesis, the elimination of GrB in Spi6 KO mice restored CTL expansion to WT levels.

The results from the correction of CTL deficient expansion in secondary response in particular further support the hypothesis that a feedback regulation loop might exist in secondary responses as well.

## 5.8 Concluding remarks

The function of Spi6 in DCs during priming of viral responses was studied in this project using LCMV infection as a model. The controversy raised by Andrew and colleagues, regarding the ability of Spi6 to protect DCs from GrB-induced apoptosis *in vivo*, was addressed using a Spi6 KO mouse. The results described in this manuscript showed a higher susceptibility to GrB-mediated death in CD8α DCs lacking Spi6 during priming of CD8 responses, therefore suggesting a physiological role for Spi6 in protecting DC from apoptosis.

Andrew's results after the adoptive transfer of mature BMMDC, can be looked at in the context of an initial priming of CTL response because manipulated BMDDCs, matured with LPS stimulation, have a relatively short half-life once injected into a naïve mouse. These results show how the initial priming phase does not require Spi6 for DC survival, while prolonged antigen presentation, which happens in the case of viral and bacterial infection, requires Spi6 to allow for the occurring of a negative feedback mechanism regulating CTL levels based on the levels of DC killing. These data taken together reinforce the theory of Spi6 role in protecting DCs during priming, which is especially important from day 3 p.i. onwards. Spi6 protection granted to DC depends on the amount of GrB in the system, thus allowing for DC killing when CTLs are in high numbers. This is in line with the theory that describes the existence of a negative feedback loop mechanism, which states that DC are killed by CTL therefore sending a feedback signal that allows for diminished further priming. Moreover, Fas-FasL engagement can mediate DC killing, but it is not the main mechanism in physiological conditions. The final proof is that Spi6 KO DCs are killed to a higher extent than WT, but these are still subject to GrB action and therefore a fraction of them is still killed in physiological conditions.

Preservation of DC by pharmacological inhibition of GrB would enhance not only the priming of naïve CD8 T cells but also the boosting of vaccine responses by presentation to $T_{EM}$ cells. In the analysis of CTL responses the deficit in the expansion level is therefore well documented as being due to CD8α decreased levels.

The results presented in this manuscript further reinforce the antigen persistence hypothesis and also the negative feedback regulating loop, showing that DC killing can modulate the levels of CTL expansion. The Spi6 role is to be seen in this context, as allowing for proper and efficient expansion of viral responses through its role in DCs and CTLs.

Given the results obtained in this project, the dispute about Spi6 role in protecting DC and allowing for a negative regulation feedback to control expansion of CTL response is seen in a specific context: the function of Spi6 does not, in fact, consist in making DCs completely unsusceptible to GrB-mediated apoptosis, but in maintaining a balance between life and death which depends on the CTL levels and therefore on local and systemic GrB levels.

Experiments with Vaccine virus and LCV and others showed how parenchymal cells may have a role in antigens presentation to CD8 cells as T memory's cardinal feat has historically been those of having a lower threshold for activation, rapid proliferative capacity, less stringent requirement for costimulatory molecules than naïve CD8. These cells might be studied for Spi6 expression to further analyze their role in CD8 cell response sustainment.

## 5.9 Future directions

Granzymes are fundamental players in cellular immunity, being the mediators of infected cell killing and the weapon to rely on for tumour elimination. On the other hand, granzymes have been implicated in the pathogenesis of several diseases, transplant rejection and inflammation.

Among inflammatory and autoimmune diseases in which granzymes play a key role are graft versus host disease, (Graubert et al. 1997), rheumatoid arthritis (Goldbach-Mansky et al. 2005), Crohn's disease (Jenkins et al. 2000) and systemic lupus erythematosus (Blanco et al. 2005). The pathogenic function of granzymes has been in particular associated with tissue damage and, in humans, PI-9 has been measured in the serum of patients with the above-mentioned pathologies, at the sites of inflammation in association with non-lymphoid cells, as mentioned above (2.6.1), and at immune-privileged sites, where it is likely to have a protective role against misdelivered GrB or uncontrolled CTL activity (Bladergroen et al. 2001).

Transplant acute rejection is also mediated by GrB released by degranulating CTLs: renal transplant recipients have been shown to present subclinical rejection in correlation with high levels of PI-9 (Rowshani et al. 2004), while acute rejection is associated with low levels of this GrB-inhibitor.

The use of recombinant GrB inhibitors might be relevant in these cases and would be potentially interesting as a supportive therapy to reduce inflammation. Nonetheless, recombinant protein therapeutics are unfortunately a non-ideal therapeutic strategy as, due to their pharmacokinetics, the bioavailability is reduced when given *per os*, and i.v. route of administration imply a complex patient management system, higher costs and immunity problems.

On the other hand, PI-9 serum levels have been shown to correlate to tumour aggressiveness in advanced stages. PI-9 has also been shown to be expressed in tumours on their membrane but not on adjacent non-transformed tissue. Inhibition of PI-9 is desirable to overcome the protection from CD8 T cell human tumours achieved by overexpression of PI-9, as these use the serpin inhibitor to escape the immune response. The release of extracellular inhibitors of GrB has also been exploited by tumours to achieve immune escape, thus opening a route for new studies. A therapeutic approach that uses inhibition of Spi6 to overcome Spi6 protective function in these tumours would nonetheless have bring along the disadvantage of inhibiting Spi6 from CTL as well. An approach that takes into account the regulation of Spi6 or that is designed as a 2-step method, so to first downregulate Spi6 in cancer cell and successively induce CTL responses, might be more appropriate in this case.

As the results in this project support, inhibition of GrB is correlated with preservation of DCs, which in turn enhances not only priming of naïve CD8 T cells but also the boosting of secondary responses by presentation to $T_{EM}$ cells. DNA vaccines are a good putative mean to induce CTL responses, both primary and secondary, although the results currently achieved show the achievement of only short-lived CD8 T cell responses (Hassett et al. 2000). Increase efficacy of DNA vaccines could be obtained by increasing viability of DCs through protection from CTL killing (Radcliffe et al. 2007).

A main goal in the development of vaccines is the induction of protective antibodies and CTL responses, especially against highly mutating virus like HIV and HCV and parasites like malaria. $CD8\alpha^+$ DC, for their capacity to elicit both CD8 T cell and $T_H1$ responses, are considered at present the key tool for vaccine development. Among various methods, protein vaccines have been shown to be good candidates but they are poorly immunogenic to T cells. At present, the use of proteins must therefore either be in conjunction with attenuated viral vectors or other vectors, or with adjuvant whose role is fundamental. The recent publication of several reports showing antibody-mediated delivery of antigen to CD8α DC to induce potent antibody and CTL responses is an example of how the use and manipulation of DC might be the right way to go (Boscardin et al. 2006; Caminschi et al. 2009). The receptors involved in this process in particular are DEC-205, Clec9A, and α-langerin. The delivery of proteins in conjunction with monoclonal antibodies directly targeted to DCs has been shown to increase by almost 100 fold the efficiency of antigen presentation (Idoyaga et al. 2011).

Other studies showed the advantage of prolonging DC survival in DNA vaccines (Kim et al. 2003; Porter and Harty 2006). One strategy might be to target antigens to DCs via the scavenger receptors mentioned above (like Clec9A) and link them to Spi6 to promote survival of DCs. The use of Spi6 as an adjuvant, in order to achieve enhanced DC survival in these particular formulations has been explored by Kim and colleagues in a very elegant approach (Kim et al. 2004). A DNA vaccine encoding for Spi6 and a human papilloma virus E7 were injected intradermally in mice and linked to molecules targeting MHC-II loading pathway. They observed increased CD8 and CD4 antigen-specific responses and tumour resistance. The use of a viral vector encoding for Spi6 could also be exploited to achieve inhibition of GrB in those

autoimmune and inflammatory diseases mentioned above where GrB has a pathogenic role. The development of small molecule might be another valid alternative for which the differences between the human and the murine proteins will have to be kept into account.

Definition of localisation, migratory ability and specific function of each DC subtype is also of primary importance due to these cells growing application in vaccine design and therapeutic strategies. The ability to understand which DC subset more efficiently stimulates which T cell type and how this can be achieved is of fundamental importance in designing protocols to manipulate DC to be used in therapy or vaccines. DC manipulation would in fact allow for activation or tolerization of the T cell of interest. For example, in the absence of inflammatory signals DCs can tolerize T cells: this mechanism is thought to be of main importance for maintenance of peripheral self-tolerance (Lutz and Kurts 2009) Not just the environment but also the subset determines the outcome of a DC-T cell interaction. The current paradigm describes for example contrasting function for cDCs: $CD8\alpha^-$ DCs are thought to mainly participate in the induction of $T_H2$-biased responses, while $CD8\alpha^+$ ones are key to the induction of CD8 T and $T_H1$ responses (Maldonado-Lopez et al. 1999). These two subsets in fact differ in the specific surface receptor pool, in the PAMP receptors, in the ability to respond to inflammatory signals and to produce cytokines. $CD8\alpha^+$ DCs produce much higher levels of IL-12p70 and can produce a small amount of IFN-α and IFN-β, while $CD8\alpha^-$ DCs produce mostly IFN-γ. $CD8^+CD4^-$ DC seem, instead, to be poor producers of any of these cytokines (Hochrein et al. 2001). Moreover, the coreceptor molecules expressed by each subset are different both qualitatively and quantitatively, therefore accounting for different costimulatory signals delivered to T cells and specific cytokine production by the activated T cell itself (Winkel et al. 1997).

One further point on which to focus research efforts is the study of how DCs circulate and how they reach the SLOs. Studies to fully understand how DCs circulate both in a mature and immature state and how they reach target tissue, be them lymphoid or not, are of growing importance since antigen-loaded DC have been used as immune response stimuli as anti-cancer vaccine (Steinman and Banchereau 2007). One of the most studied routes is i.v. injection, which results in DC accumulation,

both in humans and in mice, mainly in lungs and liver, and to a lower extent to spleen and BM. A study by Cavanagh and colleagues demonstrated that a small number of DCs can in fact access the BM, where they can prime memory cells when antigen-loaded. This is very important for DC-based vaccination strategies and central memory activation (Cavanagh et al. 2005).

The development of GrB inhibitors and of DNA vaccinations aimed at prolonging DC survival might prove useful in the designing of drugs or therapeutic strategies aimed at the treatment of a variety of conditions, from autoimmune diseases to transplant rejection to cancer.

# 6. REFERENCES


Ackerman, A.L., A. Giodini and P. Cresswell (2002). “A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells.” Immunity **25**: 607-617.

Ackerman, A. L., C. Kyritsis, R. Tampe and P. Cresswell (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**(22): 12889-12894.

Acuto, O. and F. Michel (2003). "CD28-mediated co-stimulation: a quantitative support for TCR signalling." Nat Rev Immunol **3**(12): 939-951.

Ahmed, R., M. J. Bevan, S. L. Reiner and D. T. Fearon (2009). "The precursors of memory: models and controversies." Nat Rev Immunol **9**(9): 662-668.

Ahmed, R., L. D. Butler and L. Bhatti (1988). "T4+ T helper cell function in vivo: differential requirement for induction of antiviral cytotoxic T-cell and antibody responses." J Virol **62**(6): 2102-2106.

Ahmed, R. and D. Gray (1996). "Immunological memory and protective immunity: understanding their relation." Science **272**(5258): 54-60.

Akira, S. (2011). "Innate immunity and adjuvants." Philos Trans R Soc Lond B Biol Sci **366**(1579): 2748-2755.

Allan, R. S., J. Waithman, S. Bedoui, C. M. Jones, J. A. Villadangos, Y. Zhan, A. M. Lew, K. Shortman, W. R. Heath and F. R. Carbone (2006). "Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming." Immunity **25**(1): 153-162.

Alnemri, E. S., D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry, W. W. Wong and J. Yuan (1996). "Human ICE/CED-3 protease nomenclature." Cell **87**(2): 171.

Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael and M. M. Davis (1996). "Phenotypic analysis of antigen-specific T lymphocytes." Science **274**(5284): 94-96.

Anderson, A. O. and S. Shaw (1993). "T cell adhesion to endothelium: the FRC conduit system and other anatomic and molecular features which facilitate the adhesion cascade in lymph node." Semin Immunol **5**(4): 271-282.

Anderson, G. and E. J. Jenkinson (2001). "Lymphostromal interactions in thymic development and function." Nat Rev Immunol **1**(1): 31-40.

Andrade, F., H. G. Bull, N. A. Thornberry, G. W. Ketner, L. A. Casciola-Rosen and A. Rosen (2001). "Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death." Immunity **14**(6): 751-761.

Andrade, F., E. Fellows, D. E. Jenne, A. Rosen and C. S. Young (2007). "Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition." EMBO J **26**(8): 2148-2157.

Andrew, K. A., H. M. Simkins, S. Witzel, R. Perret, J. Hudson, I. F. Hermans, D. S. Ritchie, J. Yang and F. Ronchese (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**(12): 8356-8362.

Andrews, D. M., M. J. Estcourt, C. E. Andoniou, M. E. Wikstrom, A. Khong, V. Voigt, P. Fleming, H. Tabarias, G. R. Hill, R. G. van der Most, A. A. Scalzo, M. J. Smyth and M. A. Degli-Esposti (2010). "Innate immunity defines the capacity of antiviral T cells to limit persistent infection." J Exp Med **207**(6): 1333-1343.

Andrin, C., M. J. Pinkoski, K. Burns, E. A. Atkinson, O. Krahenbuhl, D. Hudig, S. A. Fraser, U. Winkler, J. Tschopp, M. Opas, R. C. Bleackley and M. Michalak (1998). "Interaction between a Ca2+-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules." Biochemistry **37**(29): 10386-10394.

Ansari, A. W., J. N. Temblay, S. H. Alyahya and P. G. Ashton-Rickardt (2010). "Serine protease inhibitor 6 protects iNKT cells from self-inflicted damage." J Immunol **185**(2): 877-883.

Araki, K., A. P. Turner, V. O. Shaffer, S. Gangappa, S. A. Keller, M. F. Bachmann, C. P. Larsen and R. Ahmed (2009). "mTOR regulates memory CD8 T-cell differentiation." Nature **460**(7251): 108-112.

Ariizumi, K., G. L. Shen, S. Shikano, R. Ritter, 3rd, P. Zukas, D. Edelbaum, A. Morita and A. Takashima (2000). "Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms." J Biol Chem **275**(16): 11957-11963.

Arnold-Schild, D., D. Hanau, D. Spehner, C. Schmid, H. G. Rammensee, H. de la Salle and H. Schild (1999). "Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells." J Immunol **162**(7): 3757-3760.

Arpinati, M., C. L. Green, S. Heimfeld, J. E. Heuser and C. Anasetti (2000). "Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells." Blood **95**(8): 2484-2490.

Ashton-Rickardt, P. G. (2010). "Serine protease inhibitors and cytotoxic T lymphocytes." Immunol Rev **235**(1): 147-158.

Ashton-Rickardt, P. G. and S. Tonegawa (1994). "A differential-avidity model for T-cell selection." Immunol Today **15**(8): 362-366.

Badovinac, V. P., J. S. Haring and J. T. Harty (2007). "Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection." Immunity **26**(6): 827-841.

Badovinac, V. P. and J. T. Harty (2007). "Manipulating the rate of memory CD8+ T cell generation after acute infection." J Immunol **179**(1): 53-63.

Badovinac, V. P., K. A. Messingham, A. Jabbari, J. S. Haring and J. T. Harty (2005). "Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination." Nat Med **11**(7): 748-756.

Badovinac, V. P., B. B. Porter and J. T. Harty (2002). "Programmed contraction of CD8(+) T cells after infection." Nat Immunol **3**(7): 619-626.

Bahl, K., A. Huebner, R. J. Davis and R. M. Welsh (2010). "Analysis of apoptosis of memory T cells and dendritic cells during the early stages of viral infection or exposure to toll-like receptor agonists." J Virol **84**(10): 4866-4877.

Bajtay, Z., E. Csomor, N. Sandor and A. Erdei (2006). "Expression and role of Fc- and complement-receptors on human dendritic cells." Immunol Lett **104**(1-2): 46-52.

Bankovich, A. J., L. R. Shiow and J. G. Cyster (2010). "CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4." J Biol Chem **285**(29): 22328-22337.

Bannard, O., M. Kraman and D. Fearon (2009). "Pathways of memory CD8+ T-cell development." Eur J Immunol **39**(8): 2083-2087.

Bannard, O., M. Kraman and D. T. Fearon (2009). "Secondary replicative function of CD8+ T cells that had developed an effector phenotype." Science **323**(5913): 505-509.

Barber, D. L., E. J. Wherry and R. Ahmed (2003). "Cutting edge: rapid in vivo killing by memory CD8 T cells." J Immunol **171**(1): 27-31.

Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman and R. Ahmed (2006). "Restoring function in exhausted CD8 T cells during chronic viral infection." Nature **439**(7077): 682-687.

Barchet, W., M. Cella and M. Colonna (2005). "Plasmacytoid dendritic cells--virus experts of innate immunity." Semin Immunol **17**(4): 253-261.

Barrie, M. B., H. W. Stout, M. S. Abougergi, B. C. Miller and D. L. Thiele (2004). "Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6." J Immunol **172**(10): 6453-6459.

Bedoui, S., P. G. Whitney, J. Waithman, L. Eidsmo, L. Wakim, I. Caminschi, R. S. Allan, M. Wojtasiak, K. Shortman, F. R. Carbone, A. G. Brooks and W. R. Heath (2009). "Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells." Nat Immunol **10**(5): 488-495.

Behrens, G., M. Li, C. M. Smith, G. T. Belz, J. Mintern, F. R. Carbone and W. R. Heath (2004). "Helper T cells, dendritic cells and CTL Immunity." Immunol Cell Biol **82**(1): 84-90.

Bellone, M., G. Iezzi, A. Martin-Fontecha, L. Rivolta, A. A. Manfredi, M. P. Protti, M. Freschi, P. Dellabona, G. Casorati and C. Rugarli (1997). "Rejection of a nonimmunogenic melanoma by vaccination with natural melanoma peptides on engineered antigen-presenting cells." J Immunol **158**(2): 783-789.

Belz, G., C. Smith, M. Bharadwaj, A. Rice and D. Jackson (2004). "DCs as targets for vaccine design." Cytotherapy **6**(2): 88-98.

Belz, G. T., S. Bedoui, F. Kupresanin, F. R. Carbone and W. R. Heath (2007). "Minimal activation of memory CD8+ T cell by tissue-derived dendritic cells favors the stimulation of naive CD8+ T cells." Nat Immunol **8**(10): 1060-1066.

Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone and W. R. Heath (2004). "Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses." J Immunol **172**(4): 1996-2000.

Belz, G. T., C. M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F. R. Carbone and W. R. Heath (2004). "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**(23): 8670-8675.

Belz, G. T., L. Zhang, M. D. Lay, F. Kupresanin and M. P. Davenport (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**(15): 6341-6346.

Beresford, P. J., D. Zhang, D. Y. Oh, Z. Fan, E. L. Greer, M. L. Russo, M. Jaju and J. Lieberman (2001). "Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks." J Biol Chem **276**(46): 43285-43293.

Berke, G. (1995). "The CTL's kiss of death." Cell **81**(1): 9-12.

Beuneu, H., F. Lemaitre, J. Deguine, H. D. Moreau, I. Bouvier, Z. Garcia, M. L. Albert and P. Bousso (2010). "Visualizing the functional diversification of CD8+ T cell responses in lymph nodes." Immunity **33**(3): 412-423.

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**(5): 1283-1288.

Bevan, M. J. and P. J. Fink (2001). "The CD8 response on autopilot." Nat Immunol **2**(5): 381-382.

Bird, C. H., E. J. Blink, C. E. Hirst, M. S. Buzza, P. M. Steele, J. Sun, D. A. Jans and P. I. Bird (2001). "Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires a nonconventional nuclear import pathway and the export factor Crm1." Mol Cell Biol **21**(16): 5396-5407.

Bird, C. H., J. Sun, K. Ung, D. Karambalis, J. C. Whisstock, J. A. Trapani and P. I. Bird (2005). "Cationic sites on granzyme B contribute to cytotoxicity by promoting its uptake into target cells." Mol Cell Biol **25**(17): 7854-7867.

Bird, C. H., V. R. Sutton, J. Sun, C. E. Hirst, A. Novak, S. Kumar, J. A. Trapani and P. I. Bird (1998). "Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway." Mol Cell Biol **18**(11): 6387-6398.

Bladergroen, B. A., C. J. Meijer, R. L. ten Berge, C. E. Hack, J. J. Muris, D. F. Dukers, A. Chott, Y. Kazama, J. J. Oudejans, O. van Berkum and J. A. Kummer (2002). "Expression of the granzyme B inhibitor, protease inhibitor 9, by tumor

cells in patients with non-Hodgkin and Hodgkin lymphoma: a novel protective mechanism for tumor cells to circumvent the immune system?" Blood **99**(1): 232-237.

Bladergroen, B. A., M. C. Strik, N. Bovenschen, O. van Berkum, G. L. Scheffer, C. J. Meijer, C. E. Hack and J. A. Kummer (2001). "The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites." Journal of Immunology **166**(5): 3218-3225.

Bladergroen, B. A., M. C. Strik, A. M. Wolbink, D. Wouters, R. Broekhuizen, J. A. Kummer and C. E. Hack (2005). "The granzyme B inhibitor proteinase inhibitor 9 (PI9) is expressed by human mast cells." Eur J Immunol **35**(4): 1175-1183.

Blanco, P., V. Pitard, J. F. Viallard, J. L. Taupin, J. L. Pellegrin and J. F. Moreau (2005). "Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus." Arthritis Rheum **52**(1): 201-211.

Blander, J. M. and R. Medzhitov (2006). "Toll-dependent selection of microbial antigens for presentation by dendritic cells." Nature **440**(7085): 808-812.

Blattman, J. N., R. Antia, D. J. Sourdive, X. Wang, S. M. Kaech, K. Murali-Krishna, J. D. Altman and R. Ahmed (2002). "Estimating the precursor frequency of naive antigen-specific CD8 T cells." J Exp Med **195**(5): 657-664.

Blattman, J. N., J. M. Grayson, E. J. Wherry, S. M. Kaech, K. A. Smith and R. Ahmed (2003). "Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo." Nat Med **9**(5): 540-547.

Blumberg, B. M., M. Leppert and D. Kolakofsky (1981). "Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication." Cell **23**(3): 837-845.

Bonasio, R. and U. H. von Andrian (2006). "Generation, migration and function of circulating dendritic cells." Curr Opin Immunol **18**(4): 503-511.

Boonstra, A., C. Asselin-Paturel, M. Gilliet, C. Crain, G. Trinchieri, Y. J. Liu and A. O'Garra (2003). "Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation." J Exp Med **197**(1): 101-109.

Borrow, P., C. F. Evans and M. B. Oldstone (1995). "Virus-induced immunosuppression: immune system-mediated destruction of virus-infected

dendritic cells results in generalized immune suppression." J Virol **69**(2): 1059-1070.

Borrow, P. and M. B. Oldstone (1992). "Characterization of lymphocytic choriomeningitis virus-binding protein(s): a candidate cellular receptor for the virus." J Virol **66**(12): 7270-7281.

Borrow, P. and M. B. Oldstone (1994). "Mechanism of lymphocytic choriomeningitis virus entry into cells." Virology **198**(1): 1-9.

Borrow, P., A. Tishon and M. B. Oldstone (1991). "Infection of lymphocytes by a virus that aborts cytotoxic T lymphocyte activity and establishes persistent infection." J Exp Med **174**(1): 203-212.

Boscardin, S. B., J. C. Hafalla, R. F. Masilamani, A. O. Kamphorst, H. A. Zebroski, U. Rai, A. Morrot, F. Zavala, R. M. Steinman, R. S. Nussenzweig and M. C. Nussenzweig (2006). "Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses." J Exp Med **203**(3): 599-606.

Bossi, G. and G. M. Griffiths (2005). "CTL secretory lysosomes: biogenesis and secretion of a harmful organelle." Semin Immunol **17**(1): 87-94.

Bots, M., I. G. Kolfschoten, S. A. Bres, M. T. Rademaker, G. M. de Roo, M. Kruse, K. L. Franken, M. Hahne, C. J. Froelich, C. J. Melief, R. Offringa and J. P. Medema (2005). "SPI-CI and SPI-6 cooperate in the protection from effector cell-mediated cytotoxicity." Blood **105**(3): 1153-1161.

Brawand, P., D. R. Fitzpatrick, B. W. Greenfield, K. Brasel, C. R. Maliszewski and T. De Smedt (2002). "Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs." J Immunol **169**(12): 6711-6719.

Brocker, T., M. Riedinger and K. Karjalainen (1997). "Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo." J Exp Med **185**(3): 541-550.

Buchmeier, M. J., J. H. Elder and M. B. Oldstone (1978). "Protein structure of lymphocytic choriomeningitis virus: identification of the virus structural and cell associated polypeptides." Virology **89**(1): 133-145.

Burgdorf, S., C. Scholz, A. Kautz, R. Tampe and C. Kurts (2008). "Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation." Nat Immunol **9**(5): 558-566.

Burkhardt, J. K., E. Carrizosa and M. H. Shaffer (2008). "The actin cytoskeleton in T cell activation." Annu Rev Immunol **26**: 233-259.

Butler, N. S., J. C. Nolz and J. T. Harty (2011). "Immunologic considerations for generating memory CD8 T cells through vaccination." Cell Microbiol **13**(7): 925-933.

Buzza, M. S., C. E. Hirst, C. H. Bird, P. Hosking, J. McKendrick and P. I. Bird (2001). "The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses." Cell Immunol **210**(1): 21-29.

Buzza, M. S., L. Zamurs, J. Sun, C. H. Bird, A. I. Smith, J. A. Trapani, C. J. Froelich, E. C. Nice and P. I. Bird (2005). "Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin." Journal of Biological Chemistry **280**(25): 23549-23558.

Caminschi, I., M. H. Lahoud and K. Shortman (2009). "Enhancing immune responses by targeting antigen to DC." Eur J Immunol **39**(4): 931-938.

Cao, W., M. D. Henry, P. Borrow, H. Yamada, J. H. Elder, E. V. Ravkov, S. T. Nichol, R. W. Compans, K. P. Campbell and M. B. Oldstone (1998). "Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus." Science **282**(5396): 2079-2081.

Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms and T. J. Ley (2007). "Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance." Immunity **27**(4): 635-646.

Carbone, F. R., G. T. Belz and W. R. Heath (2004). "Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity." Trends Immunol **25**(12): 655-658.

Casciola-Rosen, L., F. Andrade, D. Ulanet, W. B. Wong and A. Rosen (1999). "Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity." J Exp Med **190**(6): 815-826.

Cavanagh, L. L., R. Bonasio, I. B. Mazo, C. Halin, G. Cheng, A. W. van der Velden, A. Cariappa, C. Chase, P. Russell, M. N. Starnbach, P. A. Koni, S. Pillai, W. Weninger and U. H. von Andrian (2005). "Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells." Nat Immunol **6**(10): 1029-1037.

Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia and M. Colonna (1999). "Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon." Nat Med **5**(8): 919-923.

Cella, M., F. Sallusto and A. Lanzavecchia (1997). "Origin, maturation and antigen presenting function of dendritic cells." Curr Opin Immunol **9**(1): 10-16.

Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia and G. Alber (1996). "Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation." J Exp Med **184**(2): 747-752.

Chan, A. C., M. Iwashima, C. W. Turck and A. Weiss (1992). "ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain." Cell **71**(4): 649-662.

Chang, J. T., M. L. Ciocca, I. Kinjyo, V. R. Palanivel, C. E. McClurkin, C. S. Dejong, E. C. Mooney, J. S. Kim, N. C. Steinel, J. Oliaro, C. C. Yin, B. I. Florea, H. S. Overkleeft, L. J. Berg, S. M. Russell, G. A. Koretzky, M. S. Jordan and S. L. Reiner (2011). "Asymmetric proteasome segregation as a mechanism for unequal partitioning of the transcription factor T-bet during T lymphocyte division." Immunity **34**(4): 492-504.

Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger and S. L. Reiner (2007). "Asymmetric T lymphocyte division in the initiation of adaptive immune responses." Science **315**(5819): 1687-1691.

Chapatte, L., M. Ayyoub, S. Morel, A. L. Peitrequin, N. Levy, C. Servis, B. J. Van den Eynde, D. Valmori and F. Levy (2006). "Processing of tumor-associated antigen by the proteasomes of dendritic cells controls in vivo T-cell responses." Cancer Res **66**(10): 5461-5468.

Chapoval, A. I., J. Ni, J. S. Lau, R. A. Wilcox, D. B. Flies, D. Liu, H. Dong, G. L. Sica, G. Zhu, K. Tamada and L. Chen (2001). "B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production." Nat Immunol **2**(3): 269-274.

Chorro, L., A. Sarde, M. Li, K. J. Woollard, P. Chambon, B. Malissen, A. Kissenpfennig, J. B. Barbaroux, R. Groves and F. Geissmann (2009). "Langerhans cell (LC) proliferation mediates neonatal development, homeostasis,

and inflammation-associated expansion of the epidermal LC network." J Exp Med **206**(13): 3089-3100.

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

Chung, Y., J. H. Chang, B. S. Kim, J. M. Lee, H. Y. Kim and C. Y. Kang (2007). "Anatomic location defines antigen presentation by dendritic cells to T cells in response to intravenous soluble antigens." Eur J Immunol **37**(6): 1453-1462.

Clark, S. L., Jr. (1962). "The reticulum of lymph nodes in mice studied with the electron microscope." Am J Anat **110**: 217-257.

Classen, C. F., P. I. Bird and K. M. Debatin (2006). "Modulation of the granzyme B inhibitor proteinase inhibitor 9 (PI-9) by activation of lymphocytes and monocytes in vitro and by Epstein-Barr virus and bacterial infection." Clin Exp Immunol **143**(3): 534-542.

Cockburn, I. A., Y. C. Chen, M. G. Overstreet, J. R. Lees, N. van Rooijen, D. L. Farber and F. Zavala (2010). "Prolonged antigen presentation is required for optimal CD8+ T cell responses against malaria liver stage parasites." PLoS Pathog **6**(5): e1000877.

Colledge, L., C. L. Bennett, P. A. Reay and C. C. Blackburn (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**(11): 3246-3255.

Cresswell, P. (2005). "Antigen processing and presentation." Immunol Rev **207**: 5-7.

Cresswell, P., B. Arunachalam, N. Bangia, T. Dick, G. Diedrich, E. Hughes and M. Maric (1999). "Thiol oxidation and reduction in MHC-restricted antigen processing and presentation." Immunol Res **19**(2-3): 191-200.

Cresswell, P., N. Bangia, T. Dick and G. Diedrich (1999). "The nature of the MHC class I peptide loading complex." Immunol Rev **172**: 21-28.

Crozat, K., R. Guiton, V. Contreras, V. Feuillet, C. A. Dutertre, E. Ventre, T. P. Vu Manh, T. Baranek, A. K. Storset, J. Marvel, P. Boudinot, A. Hosmalin, I. Schwartz-Cornil and M. Dalod (2010). "The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells." J Exp Med **207**(6): 1283-1292.

Cullen, S. P., C. Adrain, A. U. Luthi, P. J. Duriez and S. J. Martin (2007). "Human and murine granzyme B exhibit divergent substrate preferences." Journal of Cell Biology **176**(4): 435-444.

Cullen, S. P. and S. J. Martin (2008). "Mechanisms of granule-dependent killing." Cell Death Differ **15**(2): 251-262.

Cunningham, T. D., X. Jiang and D. J. Shapiro (2007). "Expression of high levels of human proteinase inhibitor 9 blocks both perforin/granzyme and Fas/Fas ligand-mediated cytotoxicity." Cell Immunol **245**(1): 32-41.

Curtsinger, J. M., C. M. Johnson and M. F. Mescher (2003). "CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine." J Immunol **171**(10): 5165-5171.

Cyster, J. G. (2005). "Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs." Annu Rev Immunol **23**: 127-159.

Cyster, J. G. and S. R. Schwab (2012). "Sphingosine-1-Phosphate and Lymphocyte Egress from Lymphoid Organs." Annu Rev Immunol.

Dalod, M., T. Hamilton, R. Salomon, T. P. Salazar-Mather, S. C. Henry, J. D. Hamilton and C. A. Biron (2003). "Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta." J Exp Med **197**(7): 885-898.

Debrick, J. E., P. A. Campbell and U. D. Staerz (1991). "Macrophages as accessory cells for class I MHC-restricted immune responses." J Immunol **147**(9): 2846-2851.

del Hoyo, G. M., P. Martin, H. H. Vargas, S. Ruiz, C. F. Arias and C. Ardavin (2002). "Characterization of a common precursor population for dendritic cells." Nature **415**(6875): 1043-1047.

den Haan, J. M., S. M. Lehar and M. J. Bevan (2000). "CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo." J Exp Med **192**(12): 1685-1696.

Deng, A., S. Chen, Q. Li, S. C. Lyu, C. Clayberger and A. M. Krensky (2005). "Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator." J Immunol **174**(9): 5243-5248.

Desjardins, M., M. Houde and E. Gagnon (2005). "Phagocytosis: the convoluted way from nutrition to adaptive immunity." Immunol Rev **207**: 158-165.

Di Pucchio, T., B. Chatterjee, A. Smed-Sorensen, S. Clayton, A. Palazzo, M. Montes, Y. Xue, I. Mellman, J. Banchereau and J. E. Connolly (2008). "Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I." Nat Immunol **9**(5): 551-557.

Di Rosa, F. and R. Pabst (2005). "The bone marrow: a nest for migratory memory T cells." Trends Immunol **26**(7): 360-366.

Dolfi, D. V., P. A. Duttagupta, A. C. Boesteanu, Y. M. Mueller, C. H. Oliai, A. B. Borowski and P. D. Katsikis (2011). "Dendritic cells and CD28 costimulation are required to sustain virus-specific CD8+ T cell responses during the effector phase in vivo." J Immunol **186**(8): 4599-4608.

Dudziak, D., A. O. Kamphorst, G. F. Heidkamp, V. R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H. W. Lee, C. G. Park, R. M. Steinman and M. C. Nussenzweig (2007). "Differential antigen processing by dendritic cell subsets in vivo." Science **315**(5808): 107-111.

Duke, R. C., P. M. Persechini, S. Chang, C. C. Liu, J. J. Cohen and J. D. Young (1989). "Purified perforin induces target cell lysis but not DNA fragmentation." J Exp Med **170**(4): 1451-1456.

Dunn, T. B. (1954). "Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms." J Natl Cancer Inst **14**(6): 1281-1433.

Durand, V., S. Y. Wong, D. F. Tough and A. Le Bon (2004). "Shaping of adaptive immune responses to soluble proteins by TLR agonists: a role for IFN-alpha/beta." Immunol Cell Biol **82**(6): 596-602.

Dzionek, A., A. Fuchs, P. Schmidt, S. Cremer, M. Zysk, S. Miltenyi, D. W. Buck and J. Schmitz (2000). "BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood." J Immunol **165**(11): 6037-6046.

Eberl, G., A. Sabbatini, C. Servis, P. Romero, J. L. Maryanski and G. Corradin (1993). "MHC class I H-2Kd-restricted antigenic peptides: additional constraints for the binding motif." Int Immunol **5**(11): 1489-1492.

Ebner, S., Z. Ehammer, S. Holzmann, P. Schwingshackl, M. Forstner, P. Stoitzner, G. M. Huemer, P. Fritsch and N. Romani (2004). "Expression of C-type lectin

receptors by subsets of dendritic cells in human skin." Int Immunol **16**(6): 877-887.

El Haddad, N., D. Heathcote, R. Moore, S. Yang, J. Azzi, B. Mfarrej, M. Atkinson, M. H. Sayegh, J. S. Lee, P. G. Ashton-Rickardt and R. Abdi (2011). "Mesenchymal stem cells express serine protease inhibitor to evade the host immune response." Blood **117**(4): 1176-1183.

Engering, A. J., M. Cella, D. Fluitsma, M. Brockhaus, E. C. Hoefsmit, A. Lanzavecchia and J. Pieters (1997). "The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells." Eur J Immunol **27**(9): 2417-2425.

Ernst, B., D. S. Lee, J. M. Chang, J. Sprent and C. D. Surh (1999). "The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery." Immunity **11**(2): 173-181.

Fan, Z., P. J. Beresford, D. Y. Oh, D. Zhang and J. Lieberman (2003). "Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor." Cell **112**(5): 659-672.

Farber, D. L. (2000). "T cell memory: heterogeneity and mechanisms." Clin Immunol **95**(3): 173-181.

Favier, B., N. J. Burroughs, L. Wedderburn and S. Valitutti (2001). "TCR dynamics on the surface of living T cells." Int Immunol **13**(12): 1525-1532.

Fehniger, T. A., S. F. Cai, X. Cao, A. J. Bredemeyer, R. M. Presti, A. R. French and T. J. Ley (2007). "Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs." Immunity **26**(6): 798-811.

Fiebiger, E., P. Meraner, E. Weber, I. F. Fang, G. Stingl, H. Ploegh and D. Maurer (2001). "Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells." J Exp Med **193**(8): 881-892.

Fitzgerald-Bocarsly, P. and E. S. Jacobs (2010). "Plasmacytoid dendritic cells in HIV infection: striking a delicate balance." J Leukoc Biol **87**(4): 609-620.

Frauwirth, K. A., J. L. Riley, M. H. Harris, R. V. Parry, J. C. Rathmell, D. R. Plas, R. L. Elstrom, C. H. June and C. B. Thompson (2002). "The CD28 signaling pathway regulates glucose metabolism." Immunity **16**(6): 769-777.

Fuchsberger, M., H. Hochrein and M. O'Keeffe (2005). "Activation of plasmacytoid dendritic cells." Immunol Cell Biol **83**(5): 571-577.

Fuller, M. J. and A. J. Zajac (2003). "Ablation of CD8 and CD4 T cell responses by high viral loads." J Immunol **170**(1): 477-486.

Gairin, J. E., H. Mazarguil, D. Hudrisier and M. B. Oldstone (1995). "Optimal lymphocytic choriomeningitis virus sequences restricted by H-2Db major histocompatibility complex class I molecules and presented to cytotoxic T lymphocytes." J Virol **69**(4): 2297-2305.

Galibert, L., C. R. Maliszewski and S. Vandenabeele (2001). "Plasmacytoid monocytes/T cells: a dendritic cell lineage?" Semin Immunol **13**(5): 283-289.

Gascoigne, N. R. (2008). "Do T cells need endogenous peptides for activation?" Nat Rev Immunol **8**(11): 895-900.

Geijtenbeek TB, E. A., Van Kooyk Y (2002). "DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology." J Leukoc Biol **71**(6): 921-931.

Geijtenbeek TB, T. R., van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, Figdor CG (2000). "Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses." Cell **100**(5): 575-585.

Geijtenbeek TB, v. K. Y. (2003). "DC-SIGN: a novel HIV receptor on DCs that mediates HIV-1 transmission." Curr Top Microbiol Immunol **276**: 31-54.

Geissmann, F., S. Jung and D. R. Littman (2003). "Blood monocytes consist of two principal subsets with distinct migratory properties." Immunity **19**(1): 71-82.

Gerlach, C., J. W. van Heijst and T. N. Schumacher (2011). "The descent of memory T cells." Ann N Y Acad Sci **1217**: 139-153.

Gerlach, C., J. W. van Heijst, E. Swart, D. Sie, N. Armstrong, R. M. Kerkhoven, D. Zehn, M. J. Bevan, K. Schepers and T. N. Schumacher (2010). "One naive T cell, multiple fates in CD8+ T cell differentiation." J Exp Med **207**(6): 1235-1246.

Ginhoux, F., F. Tacke, V. Angeli, M. Bogunovic, M. Loubeau, X. M. Dai, E. R. Stanley, G. J. Randolph and M. Merad (2006). "Langerhans cells arise from monocytes in vivo." Nat Immunol **7**(3): 265-273.

Goldbach-Mansky, R., S. Suson, R. Wesley, C. E. Hack, H. S. El-Gabalawy and P. P. Tak (2005). "Raised granzyme B levels are associated with erosions in patients

with early rheumatoid factor positive rheumatoid arthritis." Ann Rheum Dis **64**(5): 715-721.

Gondek, D. C., L. F. Lu, S. A. Quezada, S. Sakaguchi and R. J. Noelle (2005). "Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism." J Immunol **174**(4): 1783-1786.

Gowans, J. L. and E. J. Knight (1964). "The Route of Re-Circulation of Lymphocytes in the Rat." Proc R Soc Lond B Biol Sci **159**: 257-282.

Graubert, T. A., J. F. DiPersio, J. H. Russell and T. J. Ley (1997). "Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation." J Clin Invest **100**(4): 904-911.

Gretz, J. E., A. O. Anderson and S. Shaw (1997). "Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex." Immunol Rev **156**: 11-24.

Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti and P. Puccetti (2002). "CTLA-4-Ig regulates tryptophan catabolism in vivo." Nat Immunol **3**(11): 1097-1101.

Groom, J. R. and A. D. Luster (2011). "CXCR3 in T cell function." Exp Cell Res **317**(5): 620-631.

Groom, J. R. and A. D. Luster (2011). "CXCR3 ligands: redundant, collaborative and antagonistic functions." Immunol Cell Biol **89**(2): 207-215.

Grujic, M., T. Braga, A. Lukinius, M. L. Eloranta, S. D. Knight, G. Pejler and M. Abrink (2005). "Serglycin-deficient cytotoxic T lymphocytes display defective secretory granule maturation and granzyme B storage." J Biol Chem **280**(39): 33411-33418.

Guarda, G., M. Hons, S. F. Soriano, A. Y. Huang, R. Polley, A. Martin-Fontecha, J. V. Stein, R. N. Germain, A. Lanzavecchia and F. Sallusto (2007). "L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells." Nat Immunol **8**(7): 743-752.

Hand, T. W., M. Morre and S. M. Kaech (2007). "Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection." Proc Natl Acad Sci U S A **104**(28): 11730-11735.

Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet and J. P. Allison (1992). "CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones." Nature **356**(6370): 607-609.

Haring, J. S., G. A. Corbin and J. T. Harty (2005). "Dynamic regulation of IFN-gamma signaling in antigen-specific CD8+ T cells responding to infection." J Immunol **174**(11): 6791-6802.

Hart, D. N. (1997). "Dendritic cells: unique leukocyte populations which control the primary immune response." Blood **90**(9): 3245-3287.

Harty, J. T. and V. P. Badovinac (2008). "Shaping and reshaping CD8+ T-cell memory." Nat Rev Immunol **8**(2): 107-119.

Hassett, D. E., M. K. Slifka, J. Zhang and J. L. Whitton (2000). "Direct ex vivo kinetic and phenotypic analyses of CD8(+) T-cell responses induced by DNA immunization." J Virol **74**(18): 8286-8291.

Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone and J. A. Villadangos (2004). "Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens." Immunological Reviews **199**: 9-26.

Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone and J. A. Villadangos (2004). "Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens." Immunol Rev **199**: 9-26.

Heath, W. R. and F. R. Carbone (2009). "Dendritic cell subsets in primary and secondary T cell responses at body surfaces." Nat Immunol **10**(12): 1237-1244.

Henri, S., D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman and K. Shortman (2001). "The dendritic cell populations of mouse lymph nodes." J Immunol **167**(2): 741-748.

Heredia, A., A. Amoroso, C. Davis, N. Le, E. Reardon, J. K. Dominique, E. Klingebiel, R. C. Gallo and R. R. Redfield (2003). "Rapamycin causes down-regulation of CCR5 and accumulation of anti-HIV beta-chemokines: an approach to suppress R5 strains of HIV-1." Proc Natl Acad Sci U S A **100**(18): 10411-10416.

Hermans, I. F., D. S. Ritchie, J. Yang, J. M. Roberts and F. Ronchese (2000). "CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity." Journal of Immunology **164**(6): 3095-3101.

Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell and T. J. Ley (1994). "Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells." Cell **76**(6): 977-987.

Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell and T. J. Ley (1994). "Cytotoxic Lymphocytes Require Granzyme B for the Rapid Induction of DNA Fragmentation and Apoptosis in Allogeneic Target Cells." Cell **76**: 977-987.

Hickman, H. D., K. Takeda, C. N. Skon, F. R. Murray, S. E. Hensley, J. Loomis, G. N. Barber, J. R. Bennink and J. W. Yewdell (2008). "Direct priming of antiviral CD8+ T cells in the peripheral interfollicular region of lymph nodes." Nat Immunol **9**(2): 155-165.

Hirst, C. E., M. S. Buzza, C. H. Bird, H. S. Warren, P. U. Cameron, M. Zhang, P. G. Ashton-Rickardt and P. I. Bird (2003). "The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency." Journal of Immunology **170**(2): 805-815.

Hirst, C. E., M. S. Buzza, V. R. Sutton, J. A. Trapani, K. L. Loveland and P. I. Bird (2001). "Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction." Mol Hum Reprod **7**(12): 1133-1142.

Hochrein, H., K. Shortman, D. Vremec, B. Scott, P. Hertzog and M. O'Keeffe (2001). "Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets." J Immunol **166**(9): 5448-5455.

Hotchin, J. (1962). "The biology of lymphocytic choriomeningitis infection: virus-induced immune disease." Cold Spring Harb Symp Quant Biol **27**: 479-499.

Hou, Q., T. Zhao, H. Zhang, H. Lu, Q. Zhang, L. Sun and Z. Fan (2008). "Granzyme H induces apoptosis of target tumor cells characterized by DNA fragmentation and Bid-dependent mitochondrial damage." Mol Immunol **45**(4): 1044-1055.

Hou, W. S. and L. Van Parijs (2004). "A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells." Nat Immunol **5**(6): 583-589.

Hoves, S., V. R. Sutton, N. M. Haynes, E. D. Hawkins, D. Fernandez Ruiz, N. Baschuk, K. A. Sedelies, M. Schnurr, J. Stagg, D. M. Andrews, J. A. Villadangos and J. A. Trapani (2011). "A critical role for granzymes in antigen cross-presentation through regulating phagocytosis of killed tumor cells." J Immunol **187**(3): 1166-1175.

Hu, Q., C. R. Bazemore Walker, C. Girao, J. T. Opferman, J. Sun, J. Shabanowitz, D. F. Hunt and P. G. Ashton-Rickardt (1997). "Specific recognition of thymic self-peptides induces the positive selection of cytotoxic T lymphocytes." Immunity **7**(2): 221-231.

Huang, J. H., L. I. Cardenas-Navia, C. C. Caldwell, T. J. Plumb, C. G. Radu, P. N. Rocha, T. Wilder, J. S. Bromberg, B. N. Cronstein, M. Sitkovsky, M. W. Dewhirst and M. L. Dustin (2007). "Requirements for T lymphocyte migration in explanted lymph nodes." J Immunol **178**(12): 7747-7755.

Huck, S. P., S. C. Tang, K. A. Andrew, J. Yang, J. L. Harper and F. Ronchese (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**(1): 63-71.

Hudrisier, D., M. B. Oldstone and J. E. Gairin (1997). "The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D(b) and H-2K(b) molecules." Virology **234**(1): 62-73.

Huntington, J. A., R. J. Read and R. W. Carrell (2000). "Structure of a serpin-protease complex shows inhibition by deformation." Nature **407**(6806): 923-926.

Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos and R. A. Kroczek (1999). "ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28." Nature **397**(6716): 263-266.

Huysamen, C., J. A. Willment, K. M. Dennehy and G. D. Brown (2008). "CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes." J Biol Chem **283**(24): 16693-16701.

Idoyaga, J., A. Lubkin, C. Fiorese, M. H. Lahoud, I. Caminschi, Y. Huang, A. Rodriguez, B. E. Clausen, C. G. Park, C. Trumpfheller and R. M. Steinman (2011). "Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A." Proc Natl Acad Sci U S A **108**(6): 2384-2389.

Idoyaga, J., N. Suda, K. Suda, C. G. Park and R. M. Steinman (2009). "Antibody to Langerin/CD207 localizes large numbers of CD8alpha+ dendritic cells to the marginal zone of mouse spleen." Proc Natl Acad Sci U S A **106**(5): 1524-1529.

Iezzi, G., K. Karjalainen and A. Lanzavecchia (1998). "The duration of antigenic stimulation determines the fate of naive and effector T cells." Immunity **8**(1): 89-95.

Imboden, J. B. and J. D. Stobo (1985). "Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores." J Exp Med **161**(3): 446-456.

Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, M. Albert, N. Bhardwaj, I. Mellman and R. M. Steinman (1998). "Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells." J Exp Med **188**(11): 2163-2173.

Ingulli, E., A. Mondino, A. Khoruts and M. K. Jenkins (1997). "In vivo detection of dendritic cell antigen presentation to CD4(+) T cells." J Exp Med **185**(12): 2133-2141.

Irmler, M., S. Hertig, H. R. MacDonald, R. Sadoul, J. D. Becherer, A. Proudfoot, R. Solari and J. Tschopp (1995). "Granzyme A is an interleukin 1 beta-converting enzyme." J Exp Med **181**(5): 1917-1922.

Isaaz, S., K. Baetz, K. Olsen, E. Podack and G. M. Griffiths (1995). "Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway." Eur J Immunol **25**(4): 1071-1079.

Issekutz, A. C. and T. B. Issekutz (2002). "The role of E-selectin, P-selectin, and very late activation antigen-4 in T lymphocyte migration to dermal inflammation." J Immunol **168**(4): 1934-1939.

Iwasaki, A. and R. Medzhitov (2010). "Regulation of adaptive immunity by the innate immune system." Science **327**(5963): 291-295.

Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Akiyama, Y. Maeda, K. Takahara, R. M. Steinman and K. Inaba (2002). "The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo." J Exp Med **195**(10): 1289-1302.

Jacobs, S. R., C. E. Herman, N. J. Maciver, J. A. Wofford, H. L. Wieman, J. J. Hammen and J. C. Rathmell (2008). "Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways." J Immunol **180**(7): 4476-4486.

Janeway, C. A., Jr. (1992). "The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation." Annu Rev Immunol **10**: 645-674.

Jelinek, I., J. N. Leonard, G. E. Price, K. N. Brown, A. Meyer-Manlapat, P. K. Goldsmith, Y. Wang, D. Venzon, S. L. Epstein and D. M. Segal (2011). "TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection." J Immunol **186**(4): 2422-2429.

Jenkins, D., R. Seth, J. A. Kummer, B. B. Scott, C. J. Hawkey and R. A. Robins (2000). "Differential levels of granzyme B, regulatory cytokines, and apoptosis in Crohn's disease and ulcerative colitis at first presentation." J Pathol **190**(2): 184-189.

Jiang, J., L. L. Lau and H. Shen (2003). "Selective depletion of nonspecific T cells during the early stage of immune responses to infection." J Immunol **171**(8): 4352-4358.

Jiang, X., B. A. Orr, D. M. Kranz and D. J. Shapiro (2006). "Estrogen induction of the granzyme B inhibitor, proteinase inhibitor 9, protects cells against apoptosis mediated by cytotoxic T lymphocytes and natural killer cells." Endocrinology **147**(3): 1419-1426.

John, B., T. H. Harris, E. D. Tait, E. H. Wilson, B. Gregg, L. G. Ng, P. Mrass, D. S. Roos, F. Dzierszinski, W. Weninger and C. A. Hunter (2009). "Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii." PLoS Pathog **5**(7): e1000505.

Jongbloed, S. L., A. J. Kassianos, K. J. McDonald, G. J. Clark, X. Ju, C. E. Angel, C. J. Chen, P. R. Dunbar, R. B. Wadley, V. Jeet, A. J. Vulink, D. N. Hart and K. J. Radford (2010). "Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens." J Exp Med **207**(6): 1247-1260.

Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin and S. M. Kaech (2007). "Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor." Immunity **27**(2): 281-295.

Josien, R., H. L. Li, E. Ingulli, S. Sarma, B. R. Wong, M. Vologodskaia, R. M. Steinman and Y. Choi (2000). "TRANCE, a tumor necrosis factor family

member, enhances the longevity and adjuvant properties of dendritic cells in vivo." J Exp Med **191**(3): 495-502.

Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E. G. Pamer, D. R. Littman and R. A. Lang (2002). "In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens." Immunity **17**(2): 211-220.

Jusforgues-Saklani, H., M. Uhl, N. Blachere, F. Lemaitre, O. Lantz, P. Bousso, D. Braun, J. J. Moon and M. L. Albert (2008). "Antigen persistence is required for dendritic cell licensing and CD8+ T cell cross-priming." J Immunol **181**(5): 3067-3076.

Kabashima, K., T. A. Banks, K. M. Ansel, T. T. Lu, C. F. Ware and J. G. Cyster (2005). "Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells." Immunity **22**(4): 439-450.

Kaech, S. M. and R. Ahmed (2001). "Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells.[see comment]." Nature Immunology **2**(5): 415-422.

Kaech, S. M., S. Hemby, E. Kersh and R. Ahmed (2002). "Molecular and functional profiling of memory CD8 T cell differentiation." Cell **111**(6): 837-851.

Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh and R. Ahmed (2003). "Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells." Nature Immunology **4**(12): 1191-1198.

Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel and H. Hengartner (1994). "Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice." Nature **369**(6475): 31-37.

Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel and H. Hengartner (1995). "Lymphocyte-mediated cytotoxicity in vitro and in vivo: mechanisms and significance." Immunol Rev **146**: 95-115.

Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel and H. Hengartner (1996). "Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo." Annu Rev Immunol **14**: 207-232.

Kagi, D., B. Odermatt, P. S. Ohashi, R. M. Zinkernagel and H. Hengartner (1996). "Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity." J Exp Med **183**(5): 2143-2152.

Kaiserman, D., C. H. Bird, J. Sun, A. Matthews, K. Ung, J. C. Whisstock, P. E. Thompson, J. A. Trapani and P. I. Bird (2006). "The major human and mouse granzymes are structurally and functionally divergent." J Cell Biol **175**(4): 619-630.

Kaiserman, D. and P. I. Bird (2010). "Control of granzymes by serpins." Cell Death Differ **17**(4): 586-595.

Kaldjian, E. P., J. E. Gretz, A. O. Anderson, Y. Shi and S. Shaw (2001). "Spatial and molecular organization of lymph node T cell cortex: a labyrinthine cavity bounded by an epithelium-like monolayer of fibroblastic reticular cells anchored to basement membrane-like extracellular matrix." Int Immunol **13**(10): 1243-1253.

Kalia, V., S. Sarkar, S. Subramaniam, W. N. Haining, K. A. Smith and R. Ahmed (2010). "Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo." Immunity **32**(1): 91-103.

Kallies, A. (2008). "Distinct regulation of effector and memory T-cell differentiation." Immunol Cell Biol **86**(4): 325-332.

Kamath, A. T., S. Henri, F. Battye, D. F. Tough and K. Shortman (2002). "Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs." Blood **100**(5): 1734-1741.

Kamath, A. T., J. Pooley, M. A. O'Keeffe, D. Vremec, Y. Zhan, A. M. Lew, A. D'Amico, L. Wu, D. F. Tough and K. Shortman (2000). "The development, maturation, and turnover rate of mouse spleen dendritic cell populations." J Immunol **165**(12): 6762-6770.

Kanamori, H., S. Krieg, C. Mao, V. A. Di Pippo, S. Wang, D. A. Zajchowski and D. J. Shapiro (2000). "Proteinase inhibitor 9, an inhibitor of granzyme B-mediated apoptosis, is a primary estrogen-inducible gene in human liver cells." J Biol Chem **275**(8): 5867-5873.

Kanazawa, N. (2007). "Dendritic cell immunoreceptors: C-type lectin receptors for pattern-recognition and signaling on antigen-presenting cells." J Dermatol Sci **45**(2): 77-86.

Kanazawa, N., K. Tashiro, K. Inaba and Y. Miyachi (2003). "Dendritic cell immunoactivating receptor, a novel C-type lectin immunoreceptor, acts as an activating receptor through association with Fc receptor gamma chain." J Biol Chem **278**(35): 32645-32652.

Kanto, T., M. Inoue, H. Miyatake, A. Sato, M. Sakakibara, T. Yakushijin, C. Oki, I. Itose, N. Hiramatsu, T. Takehara, A. Kasahara and N. Hayashi (2004). "Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection." J Infect Dis **190**(11): 1919-1926.

Kaplan, D. H. (2010). "In vivo function of Langerhans cells and dermal dendritic cells." Trends Immunol **31**(12): 446-451.

Karsunky, H., M. Merad, A. Cozzio, I. L. Weissman and M. G. Manz (2003). "Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo." J Exp Med **198**(2): 305-313.

Kaspar, A. A., S. Okada, J. Kumar, F. R. Poulain, K. A. Drouvalakis, A. Kelekar, D. A. Hanson, R. M. Kluck, Y. Hitoshi, D. E. Johnson, C. J. Froelich, C. B. Thompson, D. D. Newmeyer, A. Anel, C. Clayberger and A. M. Krensky (2001). "A distinct pathway of cell-mediated apoptosis initiated by granulysin." J Immunol **167**(1): 350-356.

Keefe, D., L. Shi, S. Feske, R. Massol, F. Navarro, T. Kirchhausen and J. Lieberman (2005). "Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis." Immunity **23**(3): 249-262.

Keller, S. A., K. Schwarz, V. Manolova, C. E. von Allmen, M. G. Kinzler, M. Bauer, S. Muntwiler, P. Saudan and M. F. Bachmann (2009). "Innate signaling regulates cross-priming at the level of DC licensing and not antigen presentation." Eur J Immunol **40**(1): 103-112.

Kemp, R. A., T. J. Powell, D. W. Dwyer and R. W. Dutton (2004). "Cutting edge: regulation of CD8+ T cell effector population size." J Immunol **173**(5): 2923-2927.

Khanna, K. M., C. C. Aguila, J. M. Redman, J. E. Suarez-Ramirez, L. Lefrancois and L. S. Cauley (2008). "In situ imaging reveals different responses by naive and memory CD8 T cells to late antigen presentation by lymph node DC after influenza virus infection." Eur J Immunol **38**(12): 3304-3315.

Kieper, W. C., M. Prlic, C. S. Schmidt, M. F. Mescher and S. C. Jameson (2001). "Il-12 enhances CD8 T cell homeostatic expansion." J Immunol **166**(9): 5515-5521.

Kim, T. W., C. F. Hung, D. Boyd, J. Juang, L. He, J. W. Kim, J. M. Hardwick and T. C. Wu (2003). "Enhancing DNA vaccine potency by combining a strategy to prolong dendritic cell life with intracellular targeting strategies." J Immunol **171**(6): 2970-2976.

Kim, T. W., C. F. Hung, D. A. Boyd, L. He, C. T. Lin, D. Kaiserman, P. I. Bird and T. C. Wu (2004). "Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6." Cancer Res **64**(1): 400-405.

Kissenpfennig, A., S. Henri, B. Dubois, C. Laplace-Builhe, P. Perrin, N. Romani, C. H. Tripp, P. Douillard, L. Leserman, D. Kaiserlian, S. Saeland, J. Davoust and B. Malissen (2005). "Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells." Immunity **22**(5): 643-654.

Klein, L., M. Hinterberger, G. Wirnsberger and B. Kyewski (2009). "Antigen presentation in the thymus for positive selection and central tolerance induction." Nat Rev Immunol **9**(12): 833-844.

Klenerman, P. and R. M. Zinkernagel (1998). "Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes." Nature **394**(6692): 482-485.

Klonowski, K. D., K. J. Williams, A. L. Marzo and L. Lefrancois (2006). "Cutting edge: IL-7-independent regulation of IL-7 receptor alpha expression and memory CD8 T cell development." J Immunol **177**(7): 4247-4251.

Kohlmeier, J. E., W. W. Reiley, G. Perona-Wright, M. L. Freeman, E. J. Yager, L. M. Connor, E. L. Brincks, T. Cookenham, A. D. Roberts, C. E. Burkum, S. Sell, G. M. Winslow, M. A. Blackman, M. Mohrs and D. L. Woodland (2011). "Inflammatory chemokine receptors regulate CD8(+) T cell contraction and memory generation following infection." J Exp Med **208**(8): 1621-1634.

Krensky, A. M. and C. Clayberger (2009). "Biology and clinical relevance of granulysin." Tissue Antigens **73**(3): 193-198.

Krug, A., A. R. French, W. Barchet, J. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama and M. Colonna (2004). "TLR9-dependent

recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function." Immunity **21**(1): 107-119.

Kummer, J. A., A. M. Kamp, F. Citarella, A. J. Horrevoets and C. E. Hack (1996). "Expression of human recombinant granzyme A zymogen and its activation by the cysteine proteinase cathepsin C." J Biol Chem **271**(16): 9281-9286.

Kupfer, A., G. Dennert and S. J. Singer (1983). "Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets." Proc Natl Acad Sci U S A **80**(23): 7224-7228.

Kupfer, A., S. L. Swain and S. J. Singer (1987). "The specific direct interaction of helper T cells and antigen-presenting B cells. II. Reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells." J Exp Med **165**(6): 1565-1580.

Kurts, C., M. Cannarile, I. Klebba and T. Brocker (2001). "Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo." J Immunol **166**(3): 1439-1442.

Kurts, C., J. F. Miller, R. M. Subramaniam, F. R. Carbone and W. R. Heath (1998). "Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction." J Exp Med **188**(2): 409-414.

Kyewski, B. and J. Derbinski (2004). "Self-representation in the thymus: an extended view." Nat Rev Immunol **4**(9): 688-698.

Lanzavecchia, A. (1998). "Immunology. Licence to kill." Nature **393**(6684): 413-414.

Lanzavecchia, A. and F. Sallusto (2002). "Progressive differentiation and selection of the fittest in the immune response." Nat Rev Immunol **2**(12): 982-987.

Lanzetti, L., Palamidessi, A., Areces, L., Scita, G. and Di Fiore, P. P. (2004). "Rab5 is a signalling GTPase involved in actin remodelling by receptor tyrosine kinases". Nature 429, 309-314.

Lau, L. L., B. D. Jamieson, T. Somasundaram and R. Ahmed (1994). "Cytotoxic T-cell memory without antigen." Nature **369**(6482): 648-652.

Lauw, F. N., A. J. Simpson, C. E. Hack, J. M. Prins, A. M. Wolbink, S. J. van Deventer, W. Chaowagul, N. J. White and T. van Der Poll (2000). "Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria." J Infect Dis **182**(1): 206-213.

Lee, L. N., S. Burke, M. Montoya and P. Borrow (2009). "Multiple mechanisms contribute to impairment of type 1 interferon production during chronic lymphocytic choriomeningitis virus infection of mice." J Immunol **182**(11): 7178-7189.

Lefrancois, L. and J. J. Obar (2010). "Once a killer, always a killer: from cytotoxic T cell to memory cell." Immunol Rev **235**(1): 206-218.

Leist, T.P., E. Ruedi, and R.M. Zinkernagel (1988). "Virus-triggered immune suppression in mice caused by virus-specific cytotoxic T cells." J Exp Med **167**(5):1749–1754.

Li, M., G. M. Davey, R. M. Sutherland, C. Kurts, A. M. Lew, C. Hirst, F. R. Carbone and W. R. Heath (2001). "Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo." J Immunol **166**(10): 6099-6103.

Liang, S. C., Y. E. Latchman, J. E. Buhlmann, M. F. Tomczak, B. H. Horwitz, G. J. Freeman and A. H. Sharpe (2003). "Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses." Eur J Immunol **33**(10): 2706-2716.

Lin, M. Y. Zhan, J.A. Villadangos and A.M. Lew (2008). "The cell biology of cross-presentation and the role of dendritic cell subsets." Imm and Cell Biol **86**: 353-362.

Lindquist, R. L., G. Shakhar, D. Dudziak, H. Wardemann, T. Eisenreich, M. L. Dustin and M. C. Nussenzweig (2004). "Visualizing dendritic cell networks in vivo." Nat Immunol **5**(12): 1243-1250.

Link, A., T. K. Vogt, S. Favre, M. R. Britschgi, H. Acha-Orbea, B. Hinz, J. G. Cyster and S. A. Luther (2007). "Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells." Nat Immunol **8**(11): 1255-1265.

Lipscomb, M. F. and B. J. Masten (2002). "Dendritic cells: immune regulators in health and disease." Physiol Rev **82**(1): 97-130.

Liu, C. C., C. M. Walsh and J. D. Young (1995). "Perforin: structure and function." Immunol Today **16**(4): 194-201.

Liu, K. and M. C. Nussenzweig (2010). "Origin and development of dendritic cells." Immunol Rev **234**(1): 45-54.

Liu, K., G. D. Victora, T. A. Schwickert, P. Guermonprez, M. M. Meredith, K. Yao, F. F. Chu, G. J. Randolph, A. Y. Rudensky and M. Nussenzweig (2009). "In vivo

analysis of dendritic cell development and homeostasis." Science **324**(5925): 392-397.

Lo, C. G., Y. Xu, R. L. Proia and J. G. Cyster (2005). "Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit." J Exp Med **201**(2): 291-301.

Lopez-Bravo, M. and C. Ardavin (2008). "In vivo induction of immune responses to pathogens by conventional dendritic cells." Immunity **29**(3): 343-351.

Lovo, E., M. Zhang, L. Wang and P. G. Ashton-Rickardt (2012). "Serine protease inhibitor 6 is required to protect dendritic cells from the kiss of death." J Immunol **188**(3): 1057-1063.

Loyer, V., P. Fontaine, S. Pion, F. Hetu, D. C. Roy and C. Perreault (1999). "The in vivo fate of APCs displaying minor H antigen and/or MHC differences is regulated by CTLs specific for immunodominant class I-associated epitopes." J Immunol **163**(12): 6462-6467.

Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani and G. Schuler (1999). "An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow." Journal of Immunological Methods **223**(1): 77-92.

Lutz, M. B., N. Kukutsch, A. L. J. Ogilvie, S. Robner, F. Koch, N. Romani and G. Schuler (1999). "An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow." Journal of Immunological Methods **223**: 77-92.

Lutz, M. B. and C. Kurts (2009). "Induction of peripheral CD4+ T-cell tolerance and CD8+ T-cell cross-tolerance by dendritic cells." Eur J Immunol **39**(9): 2325-2330.

Lykens, J. E., C. E. Terrell, E. E. Zoller, K. Risma and M. B. Jordan (2011). "Perforin is a critical physiologic regulator of T-cell activation." Blood **118**(3): 618-626.

Macagno, A., M. Gilliet, F. Sallusto, A. Lanzavecchia, F.O. Nestle, and M. Groettrup. (1999). "Dendritic cells up-regulate immunoproteasomes and the proteasome regulator PA28 during maturation.". Eur J Immunol. **29**:4037–4042.

MacDonald, G., L. Shi, C. Vande Velde, J. Lieberman and A. H. Greenberg (1999). "Mitochondria-dependent and -independent regulation of Granzyme B-induced apoptosis." J Exp Med **189**(1): 131-144.

Macintyre, A. N., D. Finlay, G. Preston, L. V. Sinclair, C. M. Waugh, P. Tamas, C. Feijoo, K. Okkenhaug and D. A. Cantrell (2011). "Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism." Immunity **34**(2): 224-236.

Maciver, N. J., S. R. Jacobs, H. L. Wieman, J. A. Wofford, J. L. Coloff and J. C. Rathmell (2008). "Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival." J Leukoc Biol **84**(4): 949-957.

Mahnke, K., M. Guo, S. Lee, H. Sepulveda, S. L. Swain, M. Nussenzweig and R. M. Steinman (2000). "The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments." J Cell Biol **151**(3): 673-684.

Mahrus, S., W. Kisiel and C. S. Craik (2004). "Granzyme M is a regulatory protease that inactivates proteinase inhibitor 9, an endogenous inhibitor of granzyme B." J Biol Chem **279**(52): 54275-54282.

Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain and M. Moser (1999). "CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo." J Exp Med **189**(3): 587-592.

Manfredi, A. A., A. Capobianco, A. Esposito, F. De Cobelli, T. Canu, A. Monno, A. Raucci, F. Sanvito, C. Doglioni, P. P. Nawroth, A. Bierhaus, M. E. Bianchi, P. Rovere-Querini and A. Del Maschio (2008). "Maturing dendritic cells depend on RAGE for in vivo homing to lymph nodes." J Immunol **180**(4): 2270-2275.

Manjunath, N., P. Shankar, J. Wan, W. Weninger, M. A. Crowley, K. Hieshima, T. A. Springer, X. Fan, H. Shen, J. Lieberman and U. H. von Andrian (2001). "Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes." J Clin Invest **108**(6): 871-878.

Martin, S. J., G. P. Amarante-Mendes, L. Shi, T. H. Chuang, C. A. Casiano, G. A. O'Brien, P. Fitzgerald, E. M. Tan, G. M. Bokoch, A. H. Greenberg and D. R. Green (1996). "The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism." EMBO J **15**(10): 2407-2416.

Martinvalet, D., P. Zhu and J. Lieberman (2005). "Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis." Immunity **22**(3): 355-370.

Marzo, A. L., H. Yagita and L. Lefrancois (2007). "Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells." J Immunol **179**(1): 36-40.

Masopust, D., S. J. Ha, V. Vezys and R. Ahmed (2006). "Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination." J Immunol **177**(2): 831-839.

Masson, F., A. M. Mount, N. S. Wilson and G. T. Belz (2008). "Dendritic cells: driving the differentiation programme of T cells in viral infections." Immunology & Cell Biology **86**(4): 333-342.

Matloubian, M., R. J. Concepcion and R. Ahmed (1994). "CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection." J Virol **68**(12): 8056-8063.

Matloubian, M., M. Suresh, A. Glass, M. Galvan, K. Chow, J. K. Whitmire, C. M. Walsh, W. R. Clark and R. Ahmed (1999). "A role for perforin in downregulating T-cell responses during chronic viral infection." J Virol **73**(3): 2527-2536.

Mattei, F., L. Bracci, D. F. Tough, F. Belardelli and G. Schiavoni (2009). "Type I IFN regulate DC turnover in vivo." Eur J Immunol **39**(7): 1807-1818.

Matzinger, P. (1991). "The JAM test. A simple assay for DNA fragmentation and cell death." Journal of Immunological Methods **145**(1-2): 185-192.

Matzinger, P. (1994). "Tolerance, danger, and the extended family." Annu Rev Immunol **12**: 991-1045.

McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman and A. H. Sharpe (2001). "ICOS is critical for CD40-mediated antibody class switching." Nature **409**(6816): 102-105.

McCaughtry, T. M., M. S. Wilken and K. A. Hogquist (2007). "Thymic emigration revisited." J Exp Med **204**(11): 2513-2520.

McCausland, M. M. and S. Crotty (2008). "Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo." J Virol Methods **147**(1): 167-176.

McGill, J., J. W. Heusel and K. L. Legge (2009). "Innate immune control and regulation of influenza virus infections." J Leukoc Biol **86**(4): 803-812.

McGill, J., N. Van Rooijen and K. L. Legge (2008). "Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs." J Exp Med **205**(7): 1635-1646.

McKenna, H. J., K. L. Stocking, R. E. Miller, K. Brasel, T. De Smedt, E. Maraskovsky, C. R. Maliszewski, D. H. Lynch, J. Smith, B. Pulendran, E. R. Roux, M. Teepe, S. D. Lyman and J. J. Peschon (2000). "Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells." Blood **95**(11): 3489-3497.

Mebius, R. E. and G. Kraal (2005). "Structure and function of the spleen." Nat Rev Immunol **5**(8): 606-616.

Medema, J. P., J. de Jong, L. T. Peltenburg, E. M. Verdegaal, A. Gorter, S. A. Bres, K. L. Franken, M. Hahne, J. P. Albar, C. J. Melief and R. Offringa (2001). "Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors." Proceedings of the National Academy of Sciences of the United States of America **98**(20): 11515-11520.

Medema, J. P., D. H. Schuurhuis, D. Rea, J. van Tongeren, J. de Jong, S. A. Bres, S. Laban, R. E. Toes, M. Toebes, T. N. Schumacher, B. A. Bladergroen, F. Ossendorp, J. A. Kummer, C. J. Melief and R. Offringa (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.[see comment]." Journal of Experimental Medicine **194**(5): 657-667.

Medzhitov, R., P. Preston-Hurlburt and C. A. Janeway, Jr. (1997). "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity." Nature **388**(6640): 394-397.

Mempel, T. R., S. E. Henrickson and U. H. Von Andrian (2004). "T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases." Nature **427**(6970): 154-159.

Menasche, G., S. Kliche, N. Bezman and B. Schraven (2007). "Regulation of T-cell antigen receptor-mediated inside-out signaling by cytosolic adapter proteins and Rap1 effector molecules." Immunol Rev **218**: 82-91.

Metkar, S. S., B. Wang, M. Aguilar-Santelises, S. M. Raja, L. Uhlin-Hansen, E. Podack, J. A. Trapani and C. J. Froelich (2002). "Cytotoxic cell granule-mediated

apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation." Immunity **16**(3): 417-428.

Michalek, R. D. and J. C. Rathmell (2010). "The metabolic life and times of a T-cell." Immunol Rev **236**: 190-202.

Miles, J. J., D. C. Douek and D. A. Price (2011). "Bias in the alphabeta T-cell repertoire: implications for disease pathogenesis and vaccination." Immunol Cell Biol **89**(3): 375-387.

Montoya, M., M. J. Edwards, D. M. Reid and P. Borrow (2005). "Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type 1 IFN." J Immunol **174**(4): 1851-1861.

Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow and D. F. Tough (2002). "Type I interferons produced by dendritic cells promote their phenotypic and functional activation." Blood **99**(9): 3263-3271.

Moseman, E. A., X. Liang, A. J. Dawson, A. Panoskaltsis-Mortari, A. M. Krieg, Y. J. Liu, B. R. Blazar and W. Chen (2004). "Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells." J Immunol **173**(7): 4433-4442.

Motyka, B., G. Korbutt, M. J. Pinkoski, J. A. Heibein, A. Caputo, M. Hobman, M. Barry, I. Shostak, T. Sawchuk, C. F. Holmes, J. Gauldie and R. C. Bleackley (2000). "Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis." Cell **103**(3): 491-500.

Moyron-Quiroz, J. E., J. Rangel-Moreno, L. Hartson, K. Kusser, M. P. Tighe, K. D. Klonowski, L. Lefrancois, L. S. Cauley, A. G. Harmsen, F. E. Lund and T. D. Randall (2006). "Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs." Immunity **25**(4): 643-654.

Moyron-Quiroz, J. E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund and T. D. Randall (2004). "Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity." Nat Med **10**(9): 927-934.

Mueller, S. N., K. A. Hosiawa-Meagher, B. T. Konieczny, B. M. Sullivan, M. F. Bachmann, R. M. Locksley, R. Ahmed and M. Matloubian (2007). "Regulation of

homeostatic chemokine expression and cell trafficking during immune responses." Science **317**(5838): 670-674.

Murali-Krishna, K., J. D. Altman, M. Suresh, D. Sourdive, A. Zajac and R. Ahmed (1998). "In vivo dynamics of anti-viral CD8 T cell responses to different epitopes. An evaluation of bystander activation in primary and secondary responses to viral infection." Adv Exp Med Biol **452**: 123-142.

Nansen, A., O. Marker, C. Bartholdy and A. R. Thomsen (2000). "CCR2+ and CCR5+ CD8+ T cells increase during viral infection and migrate to sites of infection." Eur J Immunol **30**(7): 1797-1806.

Nayak, J. V., D. A. Hokey, A. Larregina, Y. He, R. D. Salter, S. C. Watkins and L. D. Falo, Jr. (2006). "Phagocytosis induces lysosome remodeling and regulated presentation of particulate antigens by activated dendritic cells." J Immunol **177**(12): 8493-8503.

Nelson, C. A., S. J. Petzold and E. R. Unanue (1994). "Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell." Nature **371**(6494): 250-252.

Neuenhahn, M. and D. H. Busch (2007). "Unique functions of splenic CD8alpha+ dendritic cells during infection with intracellular pathogens." Immunol Lett **114**(2): 66-72.

Nolz, J. C. and J. T. Harty (2011). "Protective capacity of memory CD8+ T cells is dictated by antigen exposure history and nature of the infection." Immunity **34**(5): 781-793.

Nolz, J. C. and J. T. Harty (2011). "Strategies and implications for prime-boost vaccination to generate memory CD8 T cells." Adv Exp Med Biol **780**: 69-83.

Nussbaum, A. K. and J. L. Whitton (2004). "The contraction phase of virus-specific CD8+ T cells is unaffected by a pan-caspase inhibitor." J Immunol **173**(11): 6611-6618.

O'Keeffe, M., H. Hochrein, D. Vremec, I. Caminschi, J. L. Miller, E. M. Anders, L. Wu, M. H. Lahoud, S. Henri, B. Scott, P. Hertzog, L. Tatarczuch and K. Shortman (2002). "Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus." J Exp Med **196**(10): 1307-1319.

Okada, T. and J. G. Cyster (2007). "CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node." J Immunol **178**(5): 2973-2978.

Oliaro, J., V. Van Ham, F. Sacirbegovic, A. Pasam, Z. Bomzon, K. Pham, M. J. Ludford-Menting, N. J. Waterhouse, M. Bots, E. D. Hawkins, S. V. Watt, L. A. Cluse, C. J. Clarke, D. J. Izon, J. T. Chang, N. Thompson, M. Gu, R. W. Johnstone, M. J. Smyth, P. O. Humbert, S. L. Reiner and S. M. Russell (2010). "Asymmetric cell division of T cells upon antigen presentation uses multiple conserved mechanisms." J Immunol **185**(1): 367-375.

Onai, N., A. Obata-Onai, M. A. Schmid, T. Ohteki, D. Jarrossay and M. G. Manz (2007). "Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow." Nat Immunol **8**(11): 1207-1216.

Opferman, J. T., B. T. Ober and P. G. Ashton-Rickardt (1999). "Linear differentiation of cytotoxic effectors into memory T lymphocytes." Science **283**(5408): 1745-1748.

Opferman, J. T., B. T. Ober, R. Narayanan and P. G. Ashton-Rickardt (2001). "Suicide induced by cytolytic activity controls the differentiation of memory CD8(+) T lymphocytes." Int Immunol **13**(4): 411-419.

Owen, B. A. and L. R. Pease (1999). "TAP association influences the conformation of nascent MHC class I molecules." J Immunol **162**(8): 4677-4684.

Pappu, R., S. R. Schwab, I. Cornelissen, J. P. Pereira, J. B. Regard, Y. Xu, E. Camerer, Y. W. Zheng, Y. Huang, J. G. Cyster and S. R. Coughlin (2007). "Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate." Science **316**(5822): 295-298.

Pearce, E. L. and H. Shen (2007). "Generation of CD8 T cell memory is regulated by IL-12." J Immunol **179**(4): 2074-2081.

Pedersen, I. R. (1971). "Lymphocytic choriomeningitis virus RNAs." Nat New Biol **234**(47): 112-114.

Pellegrini, M., G. Belz, P. Bouillet and A. Strasser (2003). "Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim." Proc Natl Acad Sci U S A **100**(24): 14175-14180.

Petschner, F., C. Zimmerman, A. Strasser, D. Grillot, G. Nunez and H. Pircher (1998). "Constitutive expression of Bcl-xL or Bcl-2 prevents peptide antigen-induced T cell deletion but does not influence T cell homeostasis after a viral infection." Eur J Immunol **28**(2): 560-569.

Pham, N. L., V. P. Badovinac and J. T. Harty (2009). "A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation." J Immunol **183**(4): 2337-2348.

Phillips, T., J. T. Opferman, R. Shah, N. Liu, C. J. Froelich and P. G. Ashton-Rickardt (2004). "A role for the granzyme B inhibitor serine protease inhibitor 6 in CD8+ memory cell homeostasis." J Immunol **173**(6): 3801-3809.

Pipkin, M. E., A. Rao and M. G. Lichtenheld (2010). "The transcriptional control of the perforin locus." Immunol Rev **235**(1): 55-72.

Pircher, H., K. Burki, R. Lang, H. Hengartner and R. M. Zinkernagel (1989). "Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen." Nature **342**(6249): 559-561.

Pircher, H., D. Moskophidis, U. Rohrer, K. Burki, H. Hengartner and R. M. Zinkernagel (1990). "Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo." Nature **346**(6285): 629-633.

Poeck, H., M. Wagner, J. Battiany, S. Rothenfusser, D. Wellisch, V. Hornung, B. Jahrsdorfer, T. Giese, S. Endres and G. Hartmann (2004). "Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help." Blood **103**(8): 3058-3064.

Pooley, J. L., W. R. Heath and K. Shortman (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**(9): 5327-5330.

Porter, B. B. and J. T. Harty (2006). "The onset of CD8+-T-cell contraction is influenced by the peak of Listeria monocytogenes infection and antigen display." Infect Immun **74**(3): 1528-1536.

Potolicchio, I., S. Chitta, X. Xu, D. Fonseca, G. Crisi, V. Horejsi, J. L. Strominger, L. J. Stern, G. Raposo and L. Santambrogio (2005). "Conformational variation of surface class II MHC proteins during myeloid dendritic cell differentiation accompanies structural changes in lysosomal MIIC." J Immunol **175**(8): 4935-4947.

Prasad, D. V., S. Richards, X. M. Mai and C. Dong (2003). "B7S1, a novel B7 family member that negatively regulates T cell activation." Immunity **18**(6): 863-873.

Prlic, M. and M. J. Bevan (2008). "Exploring regulatory mechanisms of CD8+ T cell contraction." Proc Natl Acad Sci U S A **105**(43): 16689-16694.

Prlic, M., G. Hernandez-Hoyos and M. J. Bevan (2006). "Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response." J Exp Med **203**(9): 2135-2143.

Quan, L. T., A. Caputo, R. C. Bleackley, D. J. Pickup and G. S. Salvesen (1995). "Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A." J Biol Chem **270**(18): 10377-10379.

Radcliffe, J. N., J. S. Roddick, F. K. Stevenson and S. M. Thirdborough (2007). "Prolonged antigen expression following DNA vaccination impairs effector CD8+ T cell function and memory development." J Immunol **179**(12): 8313-8321.

Raja, S. M., S. S. Metkar, S. Honing, B. Wang, W. A. Russin, N. H. Pipalia, C. Menaa, M. Belting, X. Cao, R. Dressel and C. J. Froelich (2005). "A novel mechanism for protein delivery: granzyme B undergoes electrostatic exchange from serglycin to target cells." J Biol Chem **280**(21): 20752-20761.

Randolph, G. J., K. Inaba, D. F. Robbiani, R. M. Steinman and W. A. Muller (1999). "Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo." Immunity **11**(6): 753-761.

Rao, R. R., Q. Li, K. Odunsi and P. A. Shrikant (2010). "The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin." Immunity **32**(1): 67-78.

Rathmell, J. C., M. G. Vander Heiden, M. H. Harris, K. A. Frauwirth and C. B. Thompson (2000). "In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability." Mol Cell **6**(3): 683-692.

Ray, M., D. R. Hostetter, C. R. Loeb, J. Simko and C. S. Craik (2011). "Inhibition of Granzyme B by PI-9 protects prostate cancer cells from apoptosis." Prostate.

Reis e Sousa, C., P. D. Stahl and J. M. Austyn (1993). "Phagocytosis of antigens by Langerhans cells in vitro." J Exp Med **178**(2): 509-519.

Rescigno, M., S. Citterio, C. Thery, M. Rittig, D. Medaglini, G. Pozzi, S. Amigorena and P. Ricciardi-Castagnoli (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**(9): 5229-5234.

Rissoan, M. C., T. Duhen, J. M. Bridon, N. Bendriss-Vermare, C. Peronne, B. de Saint Vis, F. Briere and E. E. Bates (2002). "Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells." Blood **100**(9): 3295-3303.

Robbins, S. H., T. Walzer, D. Dembele, C. Thibault, A. Defays, G. Bessou, H. Xu, E. Vivier, M. Sellars, P. Pierre, F. R. Sharp, S. Chan, P. Kastner and M. Dalod (2008). "Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling." Genome Biol **9**(1): R17.

Roberts, J. and J. H. Quastel (1963). "Particle Uptake by Polymorphonuclear Leucocytes and Ehrlich Ascites-Carcinoma Cells." Biochem J **89**: 150-156.

Rock, K. L. and L. Shen (2005). "Cross-presentation: underlying mechanisms and role in immune surveillance." Immunol Rev **207**: 166-183.

Ronchese, F. and I. F. Hermans (2001). "Killing of dendritic cells: a life cut short or a purposeful death?" J Exp Med **194**(5): F23-26.

Rossi, F. M., S. Y. Corbel, J. S. Merzaban, D. A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi and H. J. Ziltener (2005). "Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1." Nat Immunol **6**(6): 626-634.

Rovere-Querini, P., A. Capobianco, P. Scaffidi, B. Valentinis, F. Catalanotti, M. Giazzon, I. E. Dumitriu, S. Muller, M. Iannacone, C. Traversari, M. E. Bianchi and A. A. Manfredi (2004). "HMGB1 is an endogenous immune adjuvant released by necrotic cells." EMBO Rep **5**(8): 825-830.

Rowshani, A. T., S. Florquin, F. Bemelman, J. A. Kummer, C. E. Hack and I. J. Ten Berge (2004). "Hyperexpression of the granzyme B inhibitor PI-9 in human renal allografts: a potential mechanism for stable renal function in patients with subclinical rejection." Kidney Int **66**(4): 1417-1422.

Rowshani, A. T., M. C. Strik, R. Molenaar, S. L. Yong, A. M. Wolbink, F. J. Bemelman, C. E. Hack and I. J. Ten Berge (2005). "The granzyme B inhibitor SERPINB9 (protease inhibitor 9) circulates in blood and increases on primary cytomegalovirus infection after renal transplantation." Journal of Infectious Diseases **192**(11): 1908-1911.

Russell, J. H. and T. J. Ley (2002). "Lymphocyte-mediated cytotoxicity." Annu Rev Immunol **20**: 323-370.

Sainte-Marie, G., F. S. Peng and C. Belisle (1982). "Overall architecture and pattern of lymph flow in the rat lymph node." Am J Anat **164**(4): 275-309.

Sallusto, F., M. Cella, C. Danieli and A. Lanzavecchia (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**(2): 389-400.

Sallusto, F. and A. Lanzavecchia (2001). "Exploring pathways for memory T cell generation." J Clin Invest **108**(6): 805-806.

Sallusto, F., D. Lenig, R. Forster, M. Lipp and A. Lanzavecchia (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature **401**(6754): 708-712.

Salmond, R. J., J. Emery, K. Okkenhaug and R. Zamoyska (2009). "MAPK, phosphatidylinositol 3-kinase, and mammalian target of rapamycin pathways converge at the level of ribosomal protein S6 phosphorylation to control metabolic signaling in CD8 T cells." J Immunol **183**(11): 7388-7397.

Salvato, M., P. Borrow, E. Shimomaye and M. B. Oldstone (1991). "Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence." J Virol **65**(4): 1863-1869.

Salvato, M., E. Shimomaye, P. Southern and M. B. Oldstone (1988). "Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL+) small genomic segment and that of its variant, Clone 13 (CTL-)." Virology **164**(2): 517-522.

Sancho, D., O. P. Joffre, A. M. Keller, N. C. Rogers, D. Martinez, P. Hernanz-Falcon, I. Rosewell and C. Reis e Sousa (2009). "Identification of a dendritic cell receptor that couples sensing of necrosis to immunity." Nature **458**(7240): 899-903.

Sancho, D., D. Mourao-Sa, O. P. Joffre, O. Schulz, N. C. Rogers, D. J. Pennington, J. R. Carlyle and C. Reis e Sousa (2008). "Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin." J Clin Invest **118**(6): 2098-2110.

Sandau, M. M., J. E. Kohlmeier, D. L. Woodland and S. C. Jameson (2010). "IL-15 regulates both quantitative and qualitative features of the memory CD8 T cell pool." J Immunol **184**(1): 35-44.

Santambrogio, L., A. K. Sato, G. J. Carven, S. L. Belyanskaya, J. L. Strominger and L. J. Stern (1999). "Extracellular antigen processing and presentation by immature dendritic cells." Proc Natl Acad Sci U S A **96**(26): 15056-15061.

Sarkar, S., V. Kalia, W. N. Haining, B. T. Konieczny, S. Subramaniam and R. Ahmed (2008). "Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates." J Exp Med **205**(3): 625-640.

Schluns, K. S., W. C. Kieper, S. C. Jameson and L. Lefrancois (2000). "Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo." Nat Immunol **1**(5): 426-432.

Schnorrer, P., G. M. Behrens, N. S. Wilson, J. L. Pooley, C. M. Smith, D. El-Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, G. T. Belz, F. R. Carbone, K. Shortman, W. R. Heath and J. A. Villadangos (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**(28): 10729-10734.

Schoenberger, S. P., E. I. van der Voort, G. M. Krietemeijer, R. Offringa, C. J. Melief and R. E. Toes (1998). "Cross-priming of CTL responses in vivo does not require antigenic peptides in the endoplasmic reticulum of immunizing cells." J Immunol **161**(8): 3808-3812.

Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell and J. R. Bennink (2000). "Rapid degradation of a large fraction of newly synthesized proteins by proteasomes." Nature **404**(6779): 770-774.

Schulz, O., S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom and C. Reis e Sousa (2005). "Toll-like receptor 3 promotes cross-priming to virus-infected cells." Nature **433**(7028): 887-892.

Schulz, O., A. D. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher and C. Reis e Sousa (2000). "CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal." Immunity **13**(4): 453-462.

Schwarz, B. A. and A. Bhandoola (2006). "Trafficking from the bone marrow to the thymus: a prerequisite for thymopoiesis." Immunol Rev **209**: 47-57.

Scimone, M. L., T. W. Felbinger, I. B. Mazo, J. V. Stein, U. H. Von Andrian and W. Weninger (2004). "CXCL12 mediates CCR7-independent homing of central

memory cells, but not naive T cells, in peripheral lymph nodes." J Exp Med **199**(8): 1113-1120.

Sevilla, N., S. Kunz, A. Holz, H. Lewicki, D. Homann, H. Yamada, K. P. Campbell, J. C. de La Torre and M. B. Oldstone (2000). "Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells." J Exp Med **192**(9): 1249-1260.

Shedlock, D. J. and H. Shen (2003). "Requirement for CD4 T cell help in generating functional CD8 T cell memory." Science **300**(5617): 337-339.

Shi, G. P., J. A. Villadangos, G. Dranoff, C. Small, L. Gu, K. J. Haley, R. Riese, H. L. Ploegh and H. A. Chapman (1999). "Cathepsin S required for normal MHC class II peptide loading and germinal center development." Immunity **10**(2): 197-206.

Shiow, L. R., D. B. Rosen, N. Brdickova, Y. Xu, J. An, L. L. Lanier, J. G. Cyster and M. Matloubian (2006). "CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs." Nature **440**(7083): 540-544.

Shiratsuchi, A., I. Watanabe, O. Takeuchi, S. Akira and Y. Nakanishi (2004). "Inhibitory effect of Toll-like receptor 4 on fusion between phagosomes and endosomes/lysosomes in macrophages." J Immunol **172**(4): 2039-2047.

Shortman, K., M. Egerton, G. J. Spangrude and R. Scollay (1990). "The generation and fate of thymocytes." Semin Immunol **2**(1): 3-12.

Shortman, K. and Y. J. Liu (2002). "Mouse and human dendritic cell subtypes." Nat Rev Immunol **2**(3): 151-161.

Shortman, K. and S. H. Naik (2007). "Steady-state and inflammatory dendritic-cell development." Nat Rev Immunol **7**(1): 19-30.

Shresta, S., J. W. Heusel, D. M. Macivor, R. L. Wesselschmidt, J. H. Russell and T. J. Ley (1995). "Granzyme B plays a critical role in cytotoxic lymphocyte-induced apoptosis." Immunological Reviews **146**: 211-221.

Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, T. C. Pearson, J. A. Ledbetter, A. Aruffo and R. S. Mittler (1997). "4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses." J Exp Med **186**(1): 47-55.

Sigal, L. J., S. Crotty, R. Andino and K. L. Rock (1999). "Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen." Nature **398**(6722): 77-80.

Sijts, E. J. and P. M. Kloetzel (2011). "The role of the proteasome in the generation of MHC class I ligands and immune responses." Cell Mol Life Sci **68**(9): 1491-1502.

Singer, A., S. Adoro and J. H. Park (2008). "Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice." Nat Rev Immunol **8**(10): 788-801.

Sixt, M., N. Kanazawa, M. Selg, T. Samson, G. Roos, D. P. Reinhardt, R. Pabst, M. B. Lutz and L. Sorokin (2005). "The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node." Immunity **22**(1): 19-29.

Smelt, S. C., P. Borrow, S. Kunz, W. Cao, A. Tishon, H. Lewicki, K. P. Campbell and M. B. Oldstone (2001). "Differences in affinity of binding of lymphocytic choriomeningitis virus strains to the cellular receptor alpha-dystroglycan correlate with viral tropism and disease kinetics." J Virol **75**(1): 448-457.

Smith, M. A., G. Wright, J. Wu, P. Tailor, K. Ozato, X. Chen, S. Wei, J. F. Piskurich, J. P. Ting and K. L. Wright (2011). "Positive regulatory domain I (PRDM1) and IRF8/PU.1 counter-regulate MHC class II transactivator (CIITA) expression during dendritic cell maturation." J Biol Chem **286**(10): 7893-7904.

Smyth, M. J. and J. A. Trapani (1995). "Granzymes: exogenous proteinases that induce target cell apoptosis." Immunol Today **16**(4): 202-206.

Soares, H., H. Waechter, N. Glaichenhaus, E. Mougneau, H. Yagita, O. Mizenina, D. Dudziak, M. C. Nussenzweig and R. M. Steinman (2007). "A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo." J Exp Med **204**(5): 1095-1106.

Spaeny-Dekking, E. H., A. M. Kamp, C. J. Froelich and C. E. Hack (2000). "Extracellular granzyme A, complexed to proteoglycans, is protected against inactivation by protease inhibitors." Blood **95**(4): 1465-1472.

Sprent, J. and C. D. Surh (2002). "T cell memory." Annu Rev Immunol **20**: 551-579.

Steinman, R. M. and J. Banchereau (2007). "Taking dendritic cells into medicine." Nature **449**(7161): 419-426.

Steinman, R. M. and Z. A. Cohn (1973). "Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution." J Exp Med **137**(5): 1142-1162.

Stemberger, C., K. M. Huster, M. Koffler, F. Anderl, M. Schiemann, H. Wagner and D. H. Busch (2007). "A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets." Immunity **27**(6): 985-997.

Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, S. A. Porcelli, B. R. Bloom, A. M. Krensky and R. L. Modlin (1998). "An antimicrobial activity of cytolytic T cells mediated by granulysin." Science **282**(5386): 121-125.

Stepp, S. E., P. A. Mathew, M. Bennett, G. de Saint Basile and V. Kumar (2000). "Perforin: more than just an effector molecule." Immunol Today **21**(6): 254-256.

Stock, A. T., S. N. Mueller, A. L. van Lint, W. R. Heath and F. R. Carbone (2004). "Cutting edge: prolonged antigen presentation after herpes simplex virus-1 skin infection." J Immunol **173**(4): 2241-2244.

Stout-Delgado, H. W., Y. Getachew, B. C. Miller and D. L. Thiele (2007). "Intrahepatic lymphocyte expression of dipeptidyl peptidase I-processed granzyme B and perforin induces hepatocyte expression of serine proteinase inhibitor 6 (Serpinb9/SPI-6)." J Immunol **179**(10): 6561-6567.

Suh, W. K., B. U. Gajewska, H. Okada, M. A. Gronski, E. M. Bertram, W. Dawicki, G. S. Duncan, J. Bukczynski, S. Plyte, A. Elia, A. Wakeham, A. Itie, S. Chung, J. Da Costa, S. Arya, T. Horan, P. Campbell, K. Gaida, P. S. Ohashi, T. H. Watts, S. K. Yoshinaga, M. R. Bray, M. Jordana and T. W. Mak (2003). "The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses." Nat Immunol **4**(9): 899-906.

Sun, J., C. H. Bird, V. Sutton, L. McDonald, P. B. Coughlin, T. A. De Jong, J. A. Trapani and P. I. Bird (1996). "A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes." Journal of Biological Chemistry **271**(44): 27802-27809.

Sun, J., L. Ooms, C. H. Bird, V. R. Sutton, J. A. Trapani and P. I. Bird (1997). "A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9)." Journal of Biological Chemistry **272**(24): 15434-15441.

Sutton, V. R., J. E. Davis, M. Cancilla, R. W. Johnstone, A. A. Ruefli, K. Sedelies, K. A. Browne and J. A. Trapani (2000). "Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation." J Exp Med **192**(10): 1403-1414.

Takeda, K., T. Kaisho and S. Akira (2003). "Toll-like receptors." Annu Rev Immunol **21**: 335-376.

Talanian, R. V., X. Yang, J. Turbov, P. Seth, T. Ghayur, C. A. Casiano, K. Orth and C. J. Froelich (1997). "Granule-mediated killing: pathways for granzyme B-initiated apoptosis." Journal of Experimental Medicine **186**(8): 1323-1331.

Tang, H. L. and J. G. Cyster (1999). "Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells." Science **284**(5415): 819-822.

Thibodeau, J., M. C. Bourgeois-Daigneault, G. Huppe, J. Tremblay, A. Aumont, M. Houde, E. Bartee, A. Brunet, M. E. Gauvreau, A. de Gassart, E. Gatti, M. Baril, M. Cloutier, S. Bontron, K. Fruh, D. Lamarre and V. Steimle (2008). "Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes." Eur J Immunol **38**(5): 1225-1230.

Thomsen, A. R., A. Nansen, A. N. Madsen, C. Bartholdy and J. P. Christensen (2003). "Regulation of T cell migration during viral infection: role of adhesion molecules and chemokines." Immunol Lett **85**(2): 119-127.

Thomson, A. W., H. R. Turnquist and G. Raimondi (2009). "Immunoregulatory functions of mTOR inhibition." Nat Rev Immunol **9**(5): 324-337.

*T*oes. R.. E. M, A. K. Nussbaum, S. Degermann, M .Schirle, N.P. Emmerich, M. Kraft C. Laplace, A. Zwinderman, T.P. Dick, J. Müller, B. Schönfisch, C. Schmid, H.J. Fehling, S. Stevanovic, H.G. Rammensee, and H. Schild. (2001). "Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products." J Exp Med **194***:* 1–12

Tough, D. F. and J. Sprent (1994). "Turnover of naive- and memory-phenotype T cells." J Exp Med **179**(4): 1127-1135.

Trapani, J. A. (2011). "Granzymes, cytotoxic granules and cell death: the early work of Dr. Jurg Tschopp." Cell Death Differ.

Trapani, J. A., V. R. Sutton, K. Y. Thia, Y. Q. Li, C. J. Froelich, D. A. Jans, M. S. Sandrin and K. A. Browne (2003). "A clathrin/dynamin- and mannose-6-phosphate receptor-independent pathway for granzyme B-induced cell death." J Cell Biol **160**(2): 223-233.

Traver, D., K. Akashi, M. Manz, M. Merad, T. Miyamoto, E. G. Engleman and I. L. Weissman (2000). "Development of CD8alpha-positive dendritic cells from a common myeloid progenitor." Science **290**(5499): 2152-2154.

Trombetta, E. S., M. Ebersold, W. Garrett, M. Pypaert and I. Mellman (2003). "Activation of lysosomal function during dendritic cell maturation." Science **299**(5611): 1400-1403.

Turley, S. J., K. Inaba, W. S. Garrett, M. Ebersold, J. Unternaehrer, R. M. Steinman and I. Mellman (2000). "Transport of peptide-MHC class II complexes in developing dendritic cells." Science **288**(5465): 522-527.

Turner, S. J., G. Diaz, R. Cross and P. C. Doherty (2003). "Analysis of clonotype distribution and persistence for an influenza virus-specific CD8+ T cell response." Immunity **18**(4): 549-559.

Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti and A. Aderem (1999). "The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens." Nature **401**(6755): 811-815.

Unno, M., T. Mizushima, Y. Morimoto, Y. Tomisugi, K. Tanaka, N. Yasuoka, and T. Tsukihara. (2002). "The structure of the mammalian 20S proteasome at 2.75 Å resolution." J Biochem **131**(2):171-3.

Urban, B. C., N. Willcox and D. J. Roberts (2001). "A role for CD36 in the regulation of dendritic cell function." Proc Natl Acad Sci U S A **98**(15): 8750-8755.

Usherwood, E. J., T. L. Hogg and D. L. Woodland (1999). "Enumeration of antigen-presenting cells in mice infected with Sendai virus." J Immunol **162**(6): 3350-3355.

Van den Broeck, W., A. Derore and P. Simoens (2006). "Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice." J Immunol Methods **312**(1-2): 12-19.

van Montfoort, N., M. G. Camps, S. Khan, D. V. Filippov, J. J. Weterings, J. M. Griffith, H. J. Geuze, T. van Hall, J. S. Verbeek, C. J. Melief and F. Ossendorp (2009). "Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity." Proc Natl Acad Sci U S A **106**(16): 6730-6735.

van Niel, G., R. Wubbolts and W. Stoorvogel (2008). "Endosomal sorting of MHC class II determines antigen presentation by dendritic cells." Curr Opin Cell Biol **20**(4): 437-444.

Villadangos, J. A. and W. R. Heath (2005). "Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm." Semin Immunol **17**(4): 262-272.

Villadangos, J. A. and P. Schnorrer (2007). "Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo." Nat Rev Immunol **7**(7): 543-555.

Villadangos, J. A., M. Cardoso, R. J. Steptoe, D. van Berkel, J. Pooley, F. R. Carbone and K. Shortman (2001). "MHC Class II Expression Is Regulated in Dendritic Cells Independently of Invariant Chain Degradation". Immunity 14(6): 739-749.

Villadangos, J. A., P. Schnorrer and N. S. Wilson (2005). "Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces." Immunol Rev **207**: 191-205.

Villadangos, J. A. and K. Shortman (2010). "Found in translation: the human equivalent of mouse CD8+ dendritic cells." J Exp Med **207**(6): 1131-1134.

Villadangos, J. A. and L. Young (2008). "Antigen-presentation properties of plasmacytoid dendritic cells." Immunity **29**(3): 352-361.

von Andrian, U. H. and T. R. Mempel (2003). "Homing and cellular traffic in lymph nodes." Nat Rev Immunol **3**(11): 867-878.

Voskoboinik, I. and J. A. Trapani (2006). "Addressing the mysteries of perforin function." Immunol Cell Biol **84**(1): 66-71.

Vremec, D., J. Pooley, H. Hochrein, L. Wu and K. Shortman (2000). "CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen." J Immunol **164**(6): 2978-2986.

Wakim, L. M. and M. J. Bevan (2011). "Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection." Nature **471**(7340): 629-632.

Wakim, L. M., J. Waithman, N. van Rooijen, W. R. Heath and F. R. Carbone (2008). "Dendritic cell-induced memory T cell activation in nonlymphoid tissues." Science **319**(5860): 198-202.

Walsh, C. M., M. Matloubian, C. C. Liu, R. Ueda, C. G. Kurahara, J. L. Christensen, M. T. Huang, J. D. Young, R. Ahmed and W. R. Clark (1994). "Immune function in mice lacking the perforin gene." Proc Natl Acad Sci U S A **91**(23): 10854-10858.

Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson and J. A. Bluestone (1994). "CTLA-4 can function as a negative regulator of T cell activation." Immunity **1**(5): 405-413.

Wang, X., J. Cai, H. Zhong, S. A. Denham and P. I. Terasaki (2004). "Screening of high cytotoxic tumor killer cells using a sensitive adherent target detachment assay." J Immunol Methods **295**(1-2): 57-65.

Waterhouse, N. J., V. R. Sutton, K. A. Sedelies, A. Ciccone, M. Jenkins, S. J. Turner, P. I. Bird and J. A. Trapani (2006). "Cytotoxic T lymphocyte-induced killing in the absence of granzymes A and B is unique and distinct from both apoptosis and perforin-dependent lysis." J Cell Biol **173**(1): 133-144.

Watts, T. H. (2005). "TNF/TNFR family members in costimulation of T cell responses." Annu Rev Immunol **23**: 23-68.

Wei, A., H. Rubin, B. S. Cooperman and D. W. Christianson (1994). "Crystal structure of an uncleaved serpin reveals the conformation of an inhibitory reactive loop." Nature Structural Biology **1**(4): 251-258.

Wei, S. H., A. Ming-Lum, Y. Liu, D. Wallach, C. J. Ong, S. W. Chung, K. W. Moore and A. L. Mui (2006). "Proteasome-mediated proteolysis of the interleukin-10 receptor is important for signal downregulation." J Interferon Cytokine Res **26**(5): 281-290.

Weiss, A. and J. D. Stobo (1984). "Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line." J Exp Med **160**(5): 1284-1299.

Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian and R. Ahmed (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol **4**(3): 225-234.

Whitmire, J. K., B. Eam and J. L. Whitton (2008). "Tentative T cells: memory cells are quick to respond, but slow to divide." PLoS Pathog **4**(4): e1000041.

Willard-Mack, C. L. (2006). "Normal structure, function, and histology of lymph nodes." Toxicol Pathol **34**(5): 409-424.

Willoughby, C. A., H. G. Bull, M. Garcia-Calvo, J. Jiang, K. T. Chapman and N. A. Thornberry (2002). "Discovery of potent, selective human granzyme B inhibitors that inhibit CTL mediated apoptosis." Bioorg Med Chem Lett **12**(16): 2197-2200.

Wilson, N. S., D. El-Sukkari and J. A. Villadangos (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**(6): 2187-2195.

Winkel, K. D., V. Kronin, M. F. Krummel and K. Shortman (1997). "The nature of the signals regulating CD8 T cell proliferative responses to CD8alpha+ or CD8alpha- dendritic cells." Eur J Immunol **27**(12): 3350-3359.

Witt, C. M., S. Raychaudhuri, B. Schaefer, A. K. Chakraborty and E. A. Robey (2005). "Directed migration of positively selected thymocytes visualized in real time." PLoS Biol **3**(6): e160.

Wojciechowski, S., M. B. Jordan, Y. Zhu, J. White, A. J. Zajac and D. A. Hildeman (2006). "Bim mediates apoptosis of CD127(lo) effector T cells and limits T cell memory." Eur J Immunol **36**(7): 1694-1706.

Wong, B. R., R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman and Y. Choi (1997). "TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor." J Exp Med **186**(12): 2075-2080.

Wong, P. and E. G. Pamer (2003). "Feedback regulation of pathogen-specific T cell priming." Immunity **18**(4): 499-511.

Yajima, T., K. Yoshihara, K. Nakazato, S. Kumabe, S. Koyasu, S. Sad, H. Shen, H. Kuwano and Y. Yoshikai (2006). "IL-15 regulates CD8+ T cell contraction during primary infection." J Immunol **176**(1): 507-515.

Yang, J., S. P. Huck, R. S. McHugh, I. F. Hermans and F. Ronchese (2006). "Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo." Proceedings of the National Academy of Sciences of the United States of America **103**(1): 147-152.

Ye, W., L. M. Zheng, J. D. Young and C. C. Liu (1996). "The involvement of interleukin (IL)-15 in regulating the differentiation of granulated metrial gland cells in mouse pregnant uterus." J Exp Med **184**(6): 2405-2410.

Yewdell, J. W. and B. P. Dolan (2011). "Immunology: Cross-dressers turn on T cells." Nature **471**(7340): 581-582.

Yoneyama, H., K. Matsuno, Y. Zhang, T. Nishiwaki, M. Kitabatake, S. Ueha, S. Narumi, S. Morikawa, T. Ezaki, B. Lu, C. Gerard, S. Ishikawa and K. Matsushima (2004). "Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules." Int Immunol **16**(7): 915-928.

Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira and T. Fujita (2004). "The RNA helicase RIG-I has an essential

function in double-stranded RNA-induced innate antiviral responses." Nat Immunol **5**(7): 730-737.

Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak and G. Senaldi (1999). "T-cell co-stimulation through B7RP-1 and ICOS." Nature **402**(6763): 827-832.

Young, J. L., G. K. Sukhova, D. Foster, W. Kisiel, P. Libby and U. Schonbeck (2000). "The serpin proteinase inhibitor 9 is an endogenous inhibitor of interleukin 1beta-converting enzyme (caspase-1) activity in human vascular smooth muscle cells." J Exp Med **191**(9): 1535-1544.

Zajac, A. J., J. M. Dye and D. G. Quinn (2003). "Control of lymphocytic choriomeningitis virus infection in granzyme B deficient mice." Virology **305**(1): 1-9.

Zammit, D. J., L. S. Cauley, Q. M. Pham and L. Lefrancois (2005). "Dendritic cells maximize the memory CD8 T cell response to infection." Immunity **22**(5): 561-570.

Zhang, M., S. Byrne, N. Liu, Y. Wang, A. Oxenius and P. G. Ashton-Rickardt (2007). "Differential survival of cytotoxic T cells and memory cell precursors." J Immunol **178**(6): 3483-3491.

Zhang, M., S. M. Park, Y. Wang, R. Shah, N. Liu, A. E. Murmann, C. R. Wang, M. E. Peter and P. G. Ashton-Rickardt (2006). "Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules." Immunity **24**(4): 451-461.

Zhou, S., R. Ou, L. Huang and D. Moskophidis (2002). "Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection." J Virol **76**(2): 829-840.

Zhou, S., R. Ou, L. Huang, G. E. Price and D. Moskophidis (2004). "Differential tissue-specific regulation of antiviral CD8+ T-cell immune responses during chronic viral infection." J Virol **78**(7): 3578-3600.

Zhumabekov, T., P. Corbella, M. Tolaini and D. Kioussis (1995). "Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice." J Immunol Methods **185**(1): 133-140.

Ziegler-Heitbrock, L., P. Ancuta, S. Crowe, M. Dalod, V. Grau, D. N. Hart, P. J. Leenen, Y. J. Liu, G. MacPherson, G. J. Randolph, J. Scherberich, J. Schmitz, K. Shortman, S. Sozzani, H. Strobl, M. Zembala, J. M. Austyn and M. B. Lutz (2010). "Nomenclature of monocytes and dendritic cells in blood." Blood **116**(16): e74-80.

Zuniga, E. I., L. Y. Liou, L. Mack, M. Mendoza and M. B. Oldstone (2008). "Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections." Cell Host Microbe **4**(4): 374-386.

Zuniga, E. I., D. B. McGavern, J. L. Pruneda-Paz, C. Teng and M. B. Oldstone (2004). "Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection." Nat Immunol **5**(12): 1227-1234.

# APPENDIX

Lovo, E., M. Zhang, L. Wang and P. G. Ashton-Rickardt (2012). "Serine protease inhibitor 6 is required to protect dendritic cells from the kiss of death." J Immunol **188**(3): 1057-1063.

# Serine Protease Inhibitor 6 Is Required To Protect Dendritic Cells from the Kiss of Death

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**How dendritic cells (DC) present Ag to cytotoxic T cells (CTL) without themselves being killed through contact-mediated cytotoxicity (so-called kiss of death) has proved to be controversial. Using mice deficient in serine protease inhibitor 6 (Spi6), we show that Spi6 protects DC from the kiss of death by inhibiting granzyme B (GrB) delivered by CTL. Infection of Spi6 knockout mice with lymphocytic choriomeningitis virus revealed impaired survival of CD8α DC. The impaired survival of Spi6 knockout CD8α DC resulted in impaired priming and expansion of both primary and memory lymphocytic choriomeningitis virus-specific CTL, which could be corrected by GrB deficiency. The rescue in the clonal burst obtained by GrB elimination demonstrated that GrB was the physiological target through which Spi6 protected DC from CTL. We conclude that the negative regulation of DC priming of CD8 T lymphocyte immunity by CTL killing is mitigated by the physiological inhibition of GrB by Spi6.** ***The Journal of Immunology*****, 2012, 188: 1057–1063.**

The killing of infected cells by $CD8^+$ cytotoxic T cells (CTL) is critical for immunity to intracellular pathogens such as viruses (1). After the resolution of an acute viral infection, a subpopulation of CTL differentiates into memory CD8 T cells (2). Memory cells exhibit long-lived protection from subsequent infections by the same virus through robust secondary responses involving immediate cytotoxic function from effector memory ($T_{em}$) cells (3) and rapid expansion of central memory into new CTL effectors (3, 4). Dendritic cells (DC) are the physiological APCs that stimulate both naive CD8 T cells and memory CD8 T cells to differentiate and proliferate into CTL (5, 6). In mice, the $CD11c^+$ $CD8\alpha^+$ $CD205^+$ DC population can best acquire a wide variety of cellular Ags (including viral proteins) from infected and apoptotic cells, and present them on self class I MHC to cognate CD8 T cells in a process known as cross-presentation (7, 8). CD8αDC are also the subset specifically responsible for cross-presentation of lymphocytic choriomeningitis virus (LCMV) Ags to CD8 T cells (7).

Contact-dependent, lymphocyte-mediated cytotoxicity proceeds through two pathways. The first pathway is triggered by members of the TNF receptor family, of which Fas is the most important (1). The second involves the exocytosis of proteins present in CTL and NK cell granules (1). Exocytosis of perforin (1) facilitates the entry of serine proteases called granzymes, which trigger apoptosis in target cells (9). Granzymes A and B (GrB) are the most abundant granzymes in mice and humans, and are the best characterized (1). GrB activates the caspase-dependent pathways of apoptosis and like caspases, cleaves after aspartic-acid residues.

Given the effectiveness of the granule exocytosis pathway to deliver a kiss of death (1), it is not surprising that Ag-presenting DC are themselves killed by cognate CTL. Experiments with perforin-deficient CTL show that primary CTL eliminate DC that express cognate peptide-Ag/MHC (pMHC) as part of a negative-feedback mechanism that limits the expansion of the immune response to tumor (10) or virus (11). This is consistent with observations that the maximum primary clonal burst requires the continual presentation of Ag by DC to differentiated CTL over the course of several days in addition to the initial presentation for a few hours to cytotoxically inert naive CD8 T cells (12, 13). A negative regulatory loop working through the killing of DC has also been observed in the memory CD8 T cell response (14) to allow the containment and efficient resolution of CTL expansion from cytotoxic $T_{em}$ cells (15). However, the fact that DC are still highly effective at priming CTL expansion implies that they have mechanisms that protect them from the kiss of death.

The mouse *ser*ine *p*rotease *in*hibitor (serpin), serine protease inhibitor 6 (Spi6), is a potent inhibitor of GrB (16). Spi6 lacks signal secretory sequences, and thus it has been suggested that this endogenous inhibitor protects cells from CTL-induced damage by inhibiting GrB in the cytoplasm (1). The upregulation of Spi6 in DC upon maturation or through transgene expression results in the protection of DC from granule-mediated programmed cell death (PCD) in vitro (17). However, increased Spi6 expression in bone marrow-derived DC (BMDDC) is not sufficient to protect from direct killing by CTL in vivo (18). Therefore, whether protection of DC from GrB-mediated killing is a physiological mechanism for the control of CTL immunity remains to be determined. We show that Spi6 when upregulated in mature BMDDC is required to protect from GrB-mediated CTL killing. Importantly, we show a similar requirement for protection from GrB by Spi6 in the in vivo survival of CD8αDC after LCMV infection. The impaired

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Abbreviations used in this article: BMDDC, bone marrow-derived dendritic cell; CTL, cytotoxic T cell; DC, dendritic cell; EdU, 5-ethynyl-2′-deoxyuridine; GrB, granzyme B; ICS, intracellular staining; KO, knockout; LCMV, lymphocytic choriomeningitis virus; LN, lymph node; MFI, mean fluorescence intensity; PCD, programmed cell death; pDC, plasmacytoid DC; pMHC, cognate peptide-Ag/MHC; Spi6, serine protease inhibitor 6; $T_{em}$, effector memory.



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survival of Spi6 knockout (KO) CD8αDC resulted in defective expansion of primary CTL and secondary CTL from memory CD8 T cells. We conclude that the negative regulation of DC priming of CD8 T cells by CTL killing is mitigated by the physiological inhibition of GrB by Spi6.

## Materials and Methods

### *Mice*

Thy1.2$^+$ and Thy1.1$^+$ C57BL/6J wild-type and GrB KO mice (9) were purchased from The Jackson Laboratory. C57BL/6J Spi6 KO mice and P14 mice were bred inhouse (19). GrB KO P14 Thy1.1 mice were generated by intercrossing and screening by PCR for the GrB allele (9) and flow cytometry for the P14 TCR (Vα2$^+$) and CD90.1 in the blood (19). All animals were maintained in a pathogen-free environment at Imperial College London, and all experiments were conducted in accordance to Home Office (U.K.) regulations.

### *Flow cytometry*

The following fluorescently labeled mAbs were purchased from eBiosciences: anti–CD86- (allophycocyanin-labeled), anti–I-A$^b$- (FITC-labeled), anti–CD11c- (R-PE–labeled), anti–CD8-allophycocyanin, anti–CD90.1-Alexa Fluor 450. The following fluorescently labeled Abs were purchased from BD Pharmingen: Ly-6C-PE-Cy7, CD4-FITC. The gp33 peptide [KAVYNFATM]/H-2D$^b$–allophycocyanin tetramer was purchased from Becton Dickinson (19). Lymph node (LN) cells, splenocytes, and BMDDC were prepared and stained with tetramers and mAb as described previously (19). Intracellular staining (ICS) with anti–5-ethynyl-2′-deoxyuridine (EdU)-Alexa Flour 488 to detect EdU incorporation in dividing P14 CD8 cells was performed according the manufacturer's protocol (Invitrogen). DC were subjected to ICS with rabbit anti-Spi6 antiserum (1/1000 dilution) or rabbit preimmune serum (1/1000 dilution) (20), then goat anti-rabbit IgG-allophycocyanin (1/100 dilution; Jackson Immunoresearch). Stained cells were acquired on a Cyan ADP machine (Beckman Coulter) and analyzed with FlowJo (Tree Star).

### *CTL assays*

Bone marrow was flushed out of femoral bones taken from 6- to 8-wk-old mice and immature BMDDC generated by 8–10 d of culture as described in Lutz et al. (21). BMDDC were matured by culture for 24 h with LPS (1 μg/ml; Sigma). Spleen cells ($10^6$/ml) from wild-type or GrB KO P14 mice were cultured with LCMV gp33 peptide [KAVYNFATM] ($10^{-6}$ M) and IL-2 (10 U/ml) for 2 d to generate CTLs (19). BMDDC targets were pulsed with gp33 ($10^{-7}$ M) for 1 h, genomic DNA labeled with [$^3$H]thymidine, and then incubated with P14 CTL over a range of ratios in 12 replicates, to measure CTL-induced apoptosis. The percentage apoptosis was determined after 4 h as follows: % apoptosis = ($^3$H-labeled DNA retained in target without CTL) − ($^3$H-labeled DNA retained in target with CTL/$^3$H-labeled DNA retained in target without CTL) × 100 (22). Apoptosis was induced by treatment with anti-mouse Fas mAb (clone Jo2) and cycloheximide (BD Biosciences). BMDDC targets were pulsed with gp33 ($10^{-7}$ M) for 1 h and labeled with [$^{51}$Cr], then incubated with P14 CTL over a range of ratios in quadruplicate, to measure CTL-induced lysis. The percentage specific lysis was determined after 4 h as follows: % specific release = (specific release − spontaneous release)/(maximum release − spontaneous release) × 100. The percentage specific lysis of RMA cells in the absence of gp33 was <10%, and the spontaneous release was <10% of the maximum release (19). In addition, gp33-pulsed BMDDC were also labeled with the cell-permanent nonfluorescent acetomethoxy derivate of calcein (Calcein AM), according to the manufacturer's protocol (Invitrogen), then incubated with P14 CTL over a range of E:T ratios. After 4 or 8 h, BMDDC lysis was measured by the release of fluorescent calcein into the supernatant as follows: % specific release = (specific release − spontaneous release)/(maximum release − spontaneous release) × 100. The percentage specific lysis of RMA cells in the absence of gp33 was <10%, and the spontaneous release was <10% of the maximum release.

### *Adoptive transfer and LCMV infection*

Naive CD8$^+$ cells were purified (>90%) from the spleens of wild-type or GrB KO P14 mice (Thy1.1$^+$) by positively sorting with anti-CD8 magnetic beads (Miltenyi Biotec) and adoptively transferred ($5 \times 10^3$) by i.v. injection into wild-type or Spi6 KO (Thy1.2$^+$), and after 2 d infected with LCMV Armstrong ($2 \times 10^5$ PFU i.p.). For memory cell experiments, LCMV Armstrong recipients were reinfected after 40 d with clone 13 variant of LCMV Armstrong ($10^6$ PFU i.v.). A quantitative PCR method was used to measure the level of LCMV Armstrong from infected mice exactly as described by McCauslan and Crotty (23). In brief, total spleens were weighed, then a known amount was homogenized and total RNA recovered. First-strand cDNA synthesis was performed, then cDNA was used as the template for real-time PCR using primers specific for the glycoprotein (GP forward and reverse primers) and nucleoprotein (NP2 forward and reverse primers) of LCMV. A standard curve with linearized plasmids encoding LCMV GP and NP genes was used to calculate the number of copies of LCMV genome per milligram spleen.

### *Ex vivo DC analysis*

Spleens were cut into small fragments and digested with collagenase D grade II (1 mg/ml) and DNaseI grade II (20 μg/ml) (both from Roche) in RPMI 1640 medium + 10% FCS for 25 min at room temperature. EDTA (0.2 mM) was successively added for another 5 min to disrupt DC–T cell interaction. Spleen fragments were filtered through mesh (70 μm) and centrifuged at 2000 × *g* for 5 min at room temperature (7). Cells were resuspended in MACS buffer (2% BSA + 0.5 M EDTA in PBS) and magnetically sorted with anti-CD11c beads (according to Miltenyi Biotec protocol). The number of CD8α DC in each spleen was determined by determining the percentage of CD11c$^+$ Ly6C$^-$ CD8α$^+$ CD4$^-$ cells, then multiplying by the number of enriched CD11c$^+$ cells. The number of plasmacytoid DC (pDC) in each spleen was determined by multiplying the percentage of CD11c$^+$ Ly6C$^+$ cells by the number of enriched CD11c$^+$ cells. For ex vivo priming and expansion experiments, CD8α DC from the spleens of infected mice were FACS sorted after Ab staining (MoFlo; DakoCytomation). In brief, CD11c$^+$-enriched cells (protocol as described earlier) were stained for surface markers, and CD11c$^{2+}$CD8$^+$CD4$^-$ cells (8% CD11c$^+$-enriched cells in wild-type, 3% in Spi6 KO) were purified by FACS (>98% pure). FACS-purified CD8α DC ($0–5 \times 10^4$ per 96 U-bottom well/0.2 ml) were incubated with bead-sorted CD8$^+$ T cells from wild-type P14 mice ($5 \times 10^4$ cells/well). Positive control cultures that were pulsed with gp33 peptide gave 55% EdU$^+$ cells, and negative control cultures ($5 \times 10^4$ P14 CD8 T cells alone/well) gave 0.7% EdU$^+$ cells. Cells were pulsed with EdU (5 μM) for 2.5 d, and the percentage of EdU$^+$ cells was then determined by ICS with anti–EdU-Alexa Flour 488 and flow cytometry according to manufacturer instructions (Click-iT EdU; Invitrogen).

### *Statistics*

The significance of difference was measured using two-tailed Student *t* tests.

## Results

### *Spi6 is required to protect BMDDC from killing by GrB delivered by CTL*

We used Spi6 KO mice to determine whether Spi6 was required to protect DC from CTL killing in vitro (19). BMDDC from C57BL/6 mice (H-2$^b$) were generated, then "matured" by activation with culture in LPS, as evidenced by the upregulation of the CD86 and I-A$^b$ markers (21) (Supplemental Fig. 1). ICS with anti-Spi6 antiserum (20) revealed the intracellular expression of Spi6 after LPS activation of BMDDC from wild-type mice (Fig. 1*A*). Spi6 KO BMDDC showed an activated phenotype comparable with that of wild-type (Supplemental Fig. 1), but did not express Spi6 (Fig. 1*A*). We examined the sensitivity of matured BMDDC pulsed with the gp33 LCMV Ag peptide to killing by wild-type anti-LCMV CTL that express the P14 TCR specific for gp33/H-2D$^b$ (19). Spi6 KO BMDDC are more susceptible to PCD than wild-type BMDDC, as evidenced by DNA fragmentation measured after 4 h (Fig. 1*B*). Furthermore, the specific lysis of Spi6 KO BMDDC was consistently greater than wild-type, as evidenced by $Cr^{51}$ release from the cytoplasm (Fig. 1*C*). Our in vitro findings with BMDDC are in agreement with previous reports of increased expression of Spi6 upon maturation by LPS activation correlating with resistance to CTL killing (17, 18).

Treatment of CTLs with concanamycin A inhibits granule-mediated cytolysis but does not affect Fas-mediated cytotoxicity (24). P14 CTLs treated with concanamycin A did not induce the PCD of gp33-pulsed DC, indicating that the increased lysis and DNA fragmentation of Spi6 KO BMDDC was due to increased sensitivity to granule-mediated PCD and not Fas killing (Fig. 1*D*).

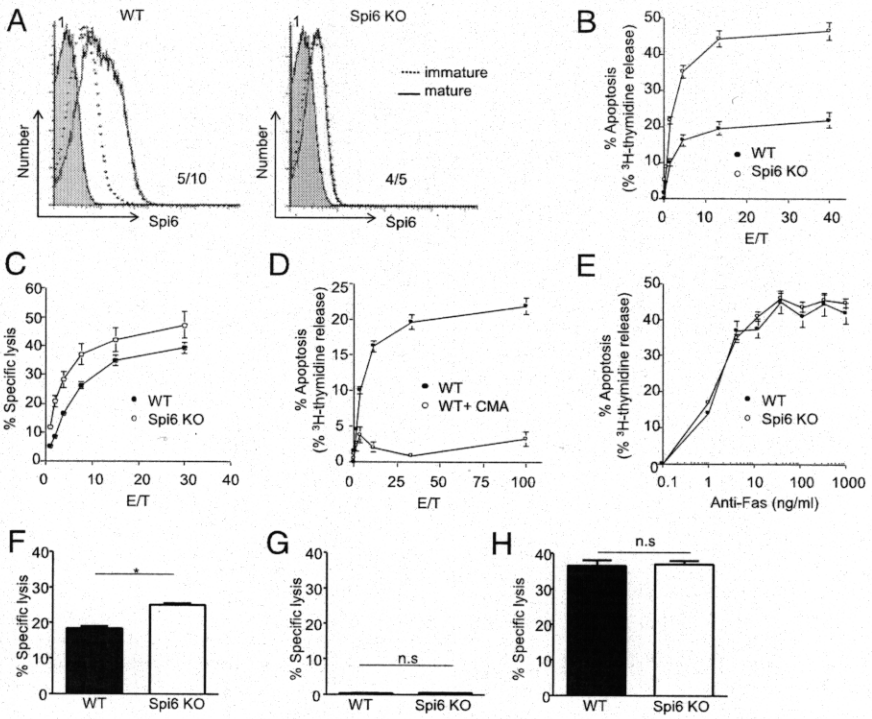


**FIGURE 1.** Spi6 upregulation in mature BMDDC is required to protect from CTL killing. *A*, Histograms for staining with rabbit preimmune serum (shaded histogram) and rabbit anti-Spi6 antiserum in immature (broken line) or LPS-matured BMDDC (solid line). MFI for preimmune serum (*top left*) and anti-Spi6 serum (*bottom right*). *B*, Percentage apoptosis based on [$^3$H]thymidine release from LPS-matured and gp33-pulsed BMDDC after 4-h culture with P14 CTL. *C*, Percentage specific lysis based on [$^{51}$Cr] release from BMDDC after 4-h culture with P14 CTL. *D*, Percentage apoptosis based on [$^3$H]thymidine. *E*, Percentage apoptosis based on [$^3$H]thymidine after treatment with anti-Fas mAb. *F*, Percentage specific lysis based on the release of calcein AM from BMDDC after 4-h culture with WT P14 CTL assay at E:T = 20 after 4 h. $*p < 0.05$. *G*, Percentage specific lysis based on release of calcein AM BMDDC after 4-h culture with GrB KO P14 CTL at E:T = 20. *H*, Percentage specific lysis based on release of calcein AM with GrB KO P14 CTL assay at E:T = 20 after 8 h. Values are median and ±SEM of triplicate measure and are representative of three independent experiments. n.s., Not significant.

This conclusion is supported by Fig. 1*E*, which shows that BMDDC from Spi6 KO mice are equally susceptible to PCD induced by anti-Fas Ab. Finally, we used P14 CTL from GrB KO mice (9, 19) to address the specific role of GrB in the susceptibility of Spi6 KO BMDDC to CTL killing. GrB KO P14 CTLs did not induce the lysis of gp33-pulsed DC after either 4 (Fig. 1*G*) or 8 h (Fig. 1*H*), indicating that the increased killing of Spi6 KO BMDDC was due to increased sensitivity to GrB. We conclude that Spi6 upregulation is required to protect BMDDC from GrB-mediated killing by CTL.

### *Protection from GrB by Spi6 determines the survival of CD8α DC in vivo*

Expression correlation studies have given contradictory findings on the role of Spi6 in controlling the negative feedback loop of CTL expansion based on the killing of DC (17, 18). To address this issue, we determined whether Spi6 was required for the protection of endogenous splenic DC from GrB delivered by CTL. Congenically marked (Thy1.1$^+$) wild-type P14 CD8 T cells were adoptively transferred to either wild-type or Spi6 KO mice (both Thy1.2$^+$), which were then infected with LCMV (Fig. 2*A*). After the infection of mice with LCMV, CD8α$^+$ DC acquire LCMV proteins from infected cells and cross-present them with class I MHC to cognate CD8 T cells (7). CD11c$^+$ cells were purified with magnetic beads from the spleens of infected mice; then CD8α$^+$ DC (CD11c$^+$ CD8α$^+$ CD4$^-$) and pDC (CD11c$^+$ Ly-6C$^+$) populations were identified by Ab staining and flow cytometry (Fig. 2*B*). We measured the expression of Spi6 in wild-type compared with Spi6 KO negative control DC by ICS with anti-Spi6 Ab (20). We observed the significant upregulation of Spi6 in wild-type CD8α$^+$ DC on days 2 (mean fluorescence intensity [MFI]: wild-type 3.3-fold higher than Spi6 KO; $p = 0.050$), 4 (MFI: wild-type 2-fold higher than Spi6 KO; $p = 0.047$), and 8 (MFI: wild-type 2-fold higher than Spi6 KO; $p = 0.017$) after LCMV infection (Fig. 2*C*, 2*D*). However, we did not observe any upregulation of Spi6 in wild-type pDC over the level of Spi6 KO cells (Fig. 2*E*).

We then measured the absolute number of CD8α$^+$ DC and pDC in recipient mice after LCMV infection. On day 4, we observed a 75% reduction ($p = 0.040$) in the number of CD8α$^+$ DC in Spi6 KO mice compared with wild-type recipients (Fig. 2*E*) but did not observe any difference in the number of pDC at any time point (Fig. 2*F*). The deficit in the level of CD8α$^+$ DC in Spi6 KO mice is consistent with the expression of Spi6 from day 2 onward. Wild-type pDC do not express Spi6 and are present in wild-type levels in Spi6 KO mice; however, the strong cross-reactivity with Spi6 antiserum (Fig. 2*E*) suggests that other serpins may play a role in pDC survival and function (1).

Staining for congenic markers revealed that >95% of the clonal burst of anti-gp33 CTL in Spi6 KO recipients was derived from adoptively transferred P14 CD8 T cells, resulting in a 20-fold excess of the number of donor over endogenous primary CTL (Supplemental Fig. 2). Therefore the replacement of wild-type donor with GrB KO cells in Spi6 KO recipients was an effective means to reduce the potential for GrB-mediated killing of targets by CTL (Fig. 2*A*).

We next determined whether the decrease in the level of viable CD8α$^+$ DC in Spi6 KO mice was due to increased CTL killing by donor P14 CD8 T cells. The adoptive transfer of GrB KO P14 CD8 T cells resulted in the rescue of the number of Spi6 KO CD8α$^+$ DC up to wild-type levels on day 4 after LCMV infection (Fig. 2*G*). Therefore, we conclude that Spi6 is required for the survival of CD8α$^+$ DC through protection from GrB delivered by CTL. The specific upregulation of Spi6 and corresponding requirement for survival in CD8α$^+$ DC compared with pDC is consistent with the role of CD8 αDC in cross-presentation of LCMV to CD8 T cells (7).

### *Functionality of Spi6 KO CD8α DC*

We next determined whether Spi6 was required for CD8α DC function. Equal numbers of FACS-purified CD8α$^+$ DC from recipient wild-type or Spi6 KO mice (Thy1.2$^+$) on day 4 after LCMV infection were cocultured with naive P14 CD8 responder

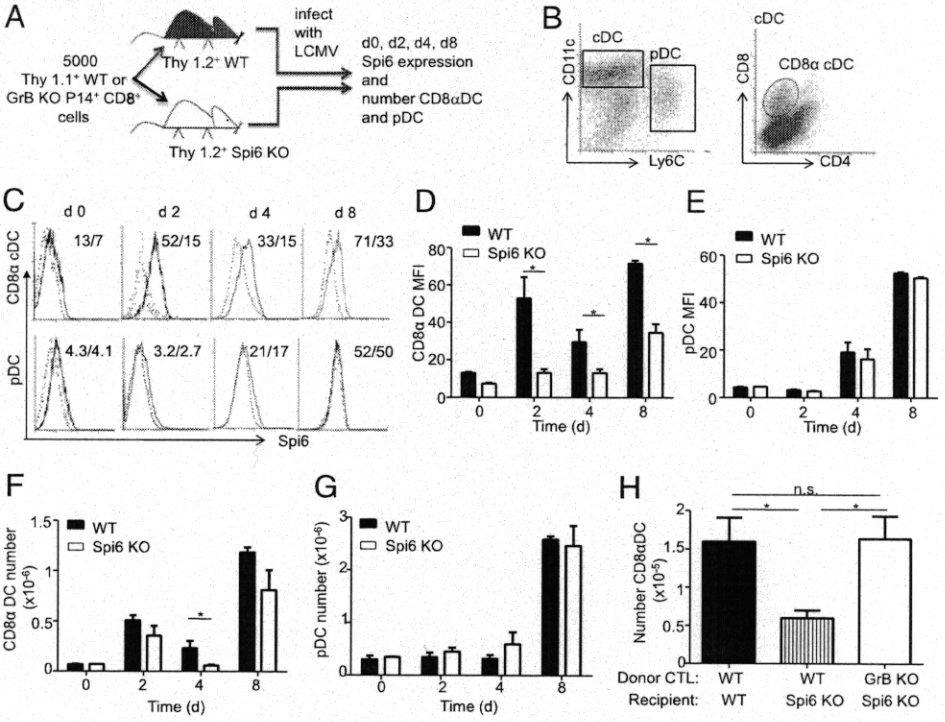


**FIGURE 2.** Spi6 upregulation in CD8αDC is required for survival. *A*, Experimental plan. *B*, Flow cytometry plots of staining for markers identifying DC subsets from CD11c$^+$ magnetic bead-sorted splenocytes. *Left plot* shows conventional DC (cDC) and pDC; *right plot* shows the markers for CD8αDC of gated cDC. *C*, Histograms for staining with anti-Spi6 antiserum on CD8αDC and pDC from the spleen after LCMV infection over time. Numbers represent MFI WT/Spi6 KO. Mean MFI values for ICS anti-Spi6 serum in CD8α DC (*C*) and pDC (*D*). Mean absolute number of CD8α DC (*E*) and pDC (*F*). *G*, Mean absolute number of CD8α DC in WT mice or Spi6 KO after adoptive transfer of either WT or GrB KO P14 CD8 T cells. All mean values are ±SEM ($n = 3$–6) and are representative of three independent experiments; *$p < 0.05$. n.s., Not significant.

T cells (Fig. 3*A*). P14 CD8 T cell division was measured by EdU incorporation into genomic DNA (25) (Fig. 3*B*). We found that the number of CD8α$^+$ DC from LCMV-infected mice determined the extent of proliferation of wild-type P14 CD8 T cells, as evidenced by the titration of percentage EdU$^+$ P14 CD8 T cell with the ratio of P14 CD8 T cell to CD8α$^+$ DC (Fig. 3*B*). However, we observed no difference in the percentage of EdU$^+$ P14 CD8 T cells after culture with Spi6 KO compared with wild-type CD8α$^+$ DC (Fig. 3*B*, 3*C*). Therefore, ex vivo Spi6 KO DC are not qualitatively impaired in their ability to prime the proliferation of cognate CD8 T cells. We conclude that although Spi6 is required for the survival of CD8α DC, it is not directly required for function.

### *Spi6 is required for the priming of primary CTL responses in vivo*

Given that the number of viable LCMV$^+$ CD8α$^+$ DC determines the proliferation of P14 CD8 T cells (Fig. 3*B*), we determined whether the impaired survival of Spi6 KO CD8α$^+$ DC resulted in impaired expansion of primary CTL in vivo. Wild-type P14 CD8 T cells (Thy1.1$^+$) were adoptively transferred to wild-type or Spi6 KO recipients (Thy1.2$^+$), and the number of donor-derived gp33$^+$ CD8$^+$ cells was determined in the clonal burst on day 8 by flow cytometry (Fig. 4*A*). We observed a 2-fold ($p = 0.01$) decrease in the level of donor-derived gp33$^+$ CD8$^+$ cells in the spleen and an 8-fold ($p = 0.003$) decrease in the LNs of Spi6 KO compared with wild-type recipients (Fig. 4*B*). Therefore, the decrease in the number of Spi6 KO CD8α$^+$ DC compared with wild-type resulted in impaired priming and expansion of anti-LCMV CTL. This defective expansion could be rescued when GrB KO P14 CD8 T cells were adoptively transferred, demonstrating that the GrB-dependent deficit in Spi6 KO CD8α$^+$ DC survival (Fig. 2*G*) also results in impaired CTL expansion (Fig. 4*C*). The defective expansion of wild-type anti-LCMV CTL in Spi6 KO compared with wild-type recipients led to a corresponding 100-fold increase in the level of LCMV in the spleen on day 6 postinfection (Supplemental Fig. 3). Therefore, Spi6 is required for the priming of functionally relevant CTL responses by DC in vivo.

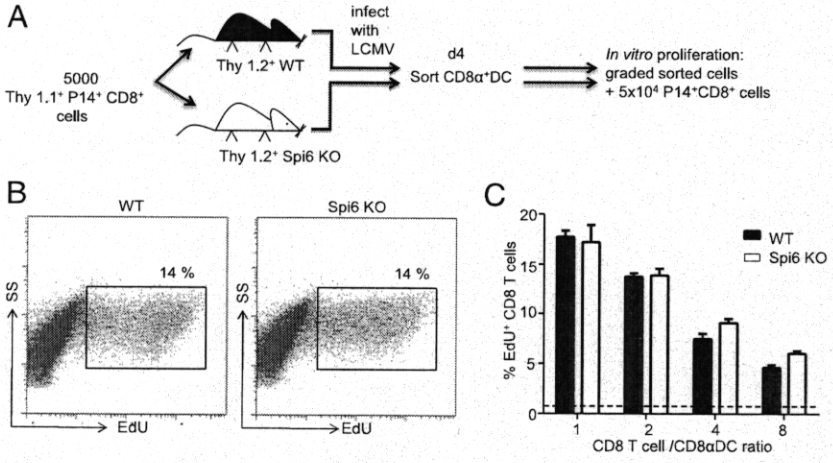


**FIGURE 3.** Functionality of Spi6 KO CD8αDC. *A*, Experimental plan. *B*, Flow cytometry plots for ICS with anti-EdU mAb on P14 CTL after 60 h of culture with CD8α DC FACS purified from LCMV-infected mice. Percentage EdU$^+$ is indicated next to gates. *C*, Mean percentage EdU$^+$ P14 CD8 T cells. Broken line indicates the background level of proliferation. All mean values are ±SEM ($n$ = 3–4) and are representative of three independent experiments; *$p < 0.05$.

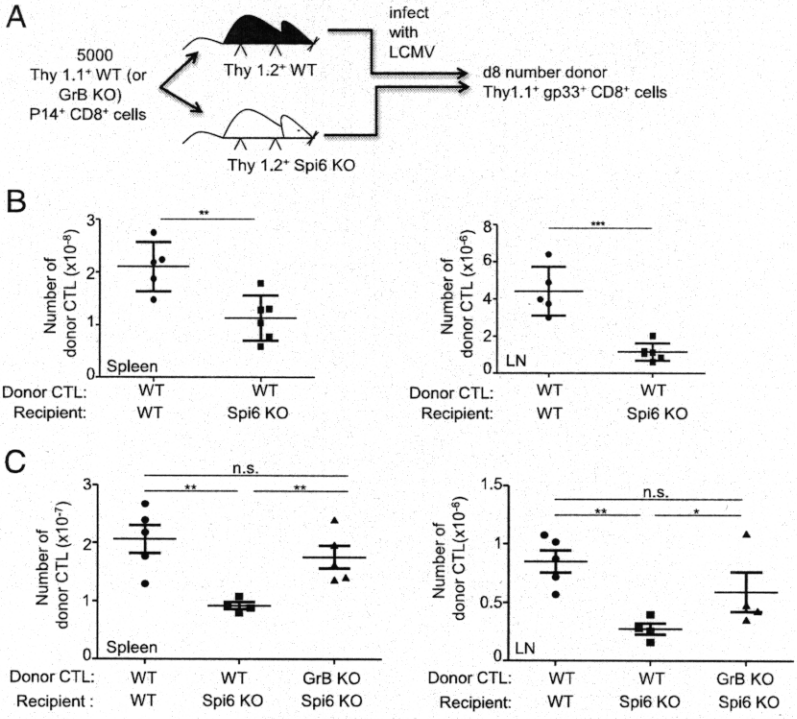


**FIGURE 4.** Defective expansion of primary CTL in Spi6 KO mice. *A*, Experimental plan. *B*, Mean absolute number of donor WT P14 CTL ($gp33^+Thy1.1^+CD8^+$) at day 8 after LCMV infection in the spleen and LNs of recipients. *C*, Mean absolute number of donor P14 CTL ($gp33^+Thy1.1^+CD8^+$) at day 8 after LCMV infection. All mean values (horizontal line) are ±SEM (vertical lines with bars; $n = 3$–6) and are representative of three independent experiments; $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. n.s., Not significant.

*Spi6 is required for the priming of memory CTL responses in vivo*

The elimination of DC by cytotoxic $T_{em}$ cells also is thought to provide a negative feedback mechanism for the control of secondary effectors (6, 14, 15, 26). Therefore, we determined whether Spi6 was required to protect $CD8\alpha^+$ DC from cytotoxic $T_{em}$ cells. First, we established the level of memory CD8 T cells in wild-type and Spi6 KO mice. On day 40 after primary LCMV infection, the level of donor memory gp33 CD8 T cells in the spleen of Spi6 KO mice was ~2-fold higher than wild-type, and in the LN the same as wild-type (Supplemental Fig. 4). This is consistent with previous observations in the spleens of intact Spi6 KO mice that homeostatic mechanisms correct diminished clonal burst by increasing development in the memory phase (20).

We then examined the expansion of secondary CTL from anti-LCMV memory CD8 T cells. Wild-type and Spi6 KO recipient mice were infected with LCMV, then on day 40 were reinfected with a high dose of the clone 13 variant of LCMV ($10^6$ PFU i.v.), and the re-expansion of $gp33^+$ $CD8^+$ cells was measured after 5 d (Fig. 5*A*) (4). We show in Fig. 5*B* that the number of donor

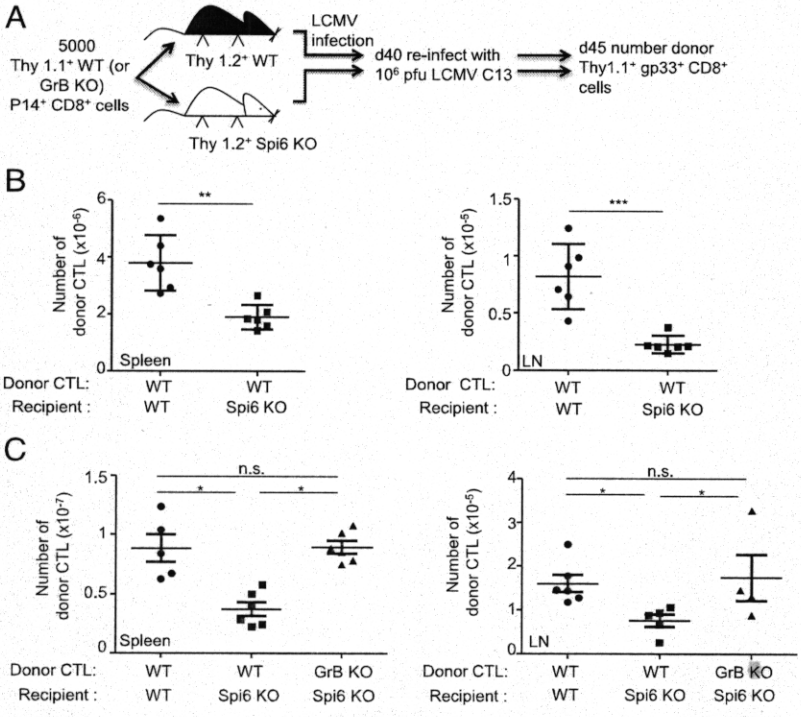


**FIGURE 5.** Defective expansion of secondary CTL in Spi6 KO mice. *A*, Experimental plan. *B*, Mean absolute number of donor WT P14 CTL ($gp33^+$ $Thy1.1^+CD8^+$) at day 5 after LCMV clone 13 reinfection in the spleen and LNs of recipients. *C*, Mean absolute number of donor P14 CTL. All mean values (horizontal line) are ±SEM (vertical lines with bars; $n = 3$–6) and are representative of three independent experiments; $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. n.s., Not significant.

secondary effectors was 2-fold lower in the spleen ($p = 0.0014$) and 4-fold lower in the LNs ($p = 0.0006$) of Spi6 KO compared with wild-type mice. This corresponds to defective expansion in number of anti-LCMV CTL in Spi6 KO mice upon reinfection over the level observed in day 40 memory (Supplemental Fig. 4), such that the 4-fold expansion in the number of donor $gp33^+$ $CD8^+$ cells observed in the spleens of wild-type mice upon reinfection was completely abolished in Spi6 KO mice (Fig. 5*B*, Supplemental Fig. 4). The defect in memory CTL expansion in Spi6 KO recipients could be corrected by GrB deficiency of the donor P14 CD8 T cells (Fig. 5*C*). We conclude that Spi6 is required for the priming of secondary effectors from memory CD8 T cells, and thus dampens the negative feedback control of effector cell expansion exerted by DC elimination.

## Discussion

Understanding the mechanisms that initiate and terminate CTL responses is central to our understanding immunity to intracellular pathogens and tumor cells. A negative feedback mechanism in which priming DC expressing pMHC are killed by the CTL they prime has been proposed for the control of the expansion of naive and memory CD8 T cells levels (11, 15). This model predicts that factors that control the viability of DC in the face of CTL killing will exert control over the expansion of CTL. However, expression correlation (17, 18) gives contradictory findings on the role of Spi6 in controlling this negative feedback loop at the level of DC viability. Collectively, our findings resolve the controversy on the role of Spi6 in protecting DC from CTL activity in vivo (17, 18). Using Spi6 KO mice, we demonstrate a nonredundant role for Spi6 in facilitating the priming and expansion of CTL through the protection of DC from the kiss of death.

Our in vitro finding that Spi6 KO BMDDC are susceptible to CTL killing is in agreement with previous reports of increased expression of Spi6 upon maturation by LPS activation correlating with resistance to CTL killing (17, 18). However, our in vivo findings differ from those of Andrew et al. (18), who showed that LPS-treated BMDDC ($Spi6^{hi}$) were no more resistant to killing by cognate CTL than non–LPS-treated BMDDC ($Spi6^{lo}$). However, an important point to consider is that Andrew et al.'s study (18) correlated the expression of Spi6 mRNA to in vivo survival, whereas our study directly interrogated the role of Spi6 in DC by using Spi6-deficient cells. Furthermore, whether LPS-treated BMDDC continue to express Spi6 after adoptive transfer was not tested, and it is well-known that LPS induces PCD in DC (8, 21), and so this may have counteracted any cytoprotection afforded by initial Spi6 upregulation.

A negative feedback loop in which priming DC expressing pMHC are killed by cognate CTL has been proposed for the control of the expansion of primary CTL (11, 15). Our results indicate that Spi6 is a physiological factor that controls the viability of DC, and thus regulates the negative feedback of CTL expansion. Spi6 was upregulated in CD8αDC, but not pDC, after LCMV infection, and in Spi6 KO mice, only the CD8αDC were diminished. $CD8\alpha^+$ DC are responsible for cross-presentation to anti-LCMV CD8 T cells, and so are in direct contact with $GrB^+$ cells, in contrast with pDC, which although they drive inflammation and support indirect CTL activation by secreting type I IFNs (8), are less efficient than $CD8\alpha^+$ DC at cross-presentation to CTL (27). In addition, our findings are consistent with reports that CD8αDC priming requires several days to generate the clonal burst of CTL in addition to the initial presentation for a few hours to noncytolytic naive CD8 T cells (12, 13).

The elimination of DC by cytotoxic $T_{em}$ cells also is thought to provide a negative feedback mechanism for the control of secondary effectors (6, 14, 15, 26). We observed that the expansion of wild-type, donor-derived secondary effectors was also impaired in Spi6 KO mice. Furthermore, the deficit could be corrected when GrB KO cells replaced wild-type donor CD8 T cells. We conclude that Spi6 is required for the priming of secondary effectors from memory CD8 T cells, and thus dampens the negative feedback control of effector cell expansion exerted by DC elimination by GrB.

The cytoprotective function we describe for Spi6 in DC can be placed in a wider context of protection from GrB by the intracellular serpin (1). Spi6 KO mice have revealed that Spi6 is also required to protect CTL (19) and invariant NK T cells (28) from their own GrB, as well as mesenchymal stem cells from CTL-delivered GrB (29). Spi6 and the human homolog Proteinase Inhibitor 9 (30, 31) also inhibit other proteases. For example, Spi6 can inhibit neutrophil elastase (20), and proteinase inhibitor 9 inhibits caspase 1 (32). However, the complete rescue of DC survival and expansion of anti-LCMV CTL by GrB deficiency argues that at least in this context GrB is the physiological target of Spi6.

GrB was first characterized as an effector molecule in the granule exocytosis pathway of killing (9). The discovery that CTL can be killed by their own GrB (19) or when it is delivered by a T regulatory cell (33) has led to the suggestion that perhaps GrB should also be viewed as a negative immunomodulator (1). The survival of DC during the expansion phase is required for potent T cell responses; therefore, our current findings would predict that preservation of DC by pharmacological inhibition of GrB would enhance not only priming of naive CD8 T cells but also the boosting of vaccine responses by presentation to $T_{em}$ cells.

## Acknowledgments

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## Disclosures

The authors have no financial conflicts of interest.

## References

1. Ashton-Rickardt, P. G. 2010. Serine protease inhibitors and cytotoxic T lymphocytes. *Immunol. Rev.* 235: 147–158.
2. Opferman, J. T., B. T. Ober, and P. G. Ashton-Rickardt. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science* 283: 1745–1748.
3. Sallusto, F., D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
4. Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature* 369: 648–652.
5. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, 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.
6. Zammit, D. J., L. S. Cauley, Q. M. Pham, and L. Lefrançois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* 22: 561–570.
7. Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath. 2005. CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J. Immunol.* 175: 196–200.
8. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199: 9–26.
9. Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell, and T. J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* 76: 977–987.
10. Hermans, I. F., D. S. Ritchie, J. Yang, J. M. Roberts, and F. Ronchese. 2000. CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. *J. Immunol.* 164: 3095–3101.
11. Yang, J., S. P. Huck, R. S. McHugh, I. F. Hermans, and F. Ronchese. 2006. Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 103: 147–152.

12. Curtsinger, J. M., C. M. Johnson, and M. F. Mescher. 2003. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J. Immunol.* 171: 5165–5171.
13. Jusforgues-Saklani, H., M. Uhl, N. Blachère, F. Lemaître, O. Lantz, P. Bousso, D. Braun, J. J. Moon, and M. L. Albert. 2008. Antigen persistence is required for dendritic cell licensing and CD8+ T cell cross-priming. *J. Immunol.* 181: 3067–3076.
14. Wakim, L. M., J. Waithman, N. van Rooijen, W. R. Heath, and F. R. Carbone. 2008. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science* 319: 198–202.
15. Wong, P., and E. G. Pamer. 2003. Feedback regulation of pathogen-specific T cell priming. *Immunity* 18: 499–511.
16. Sun, J., L. Ooms, C. H. Bird, V. R. Sutton, J. A. Trapani, and P. I. Bird. 1997. A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9). *J. Biol. Chem.* 272: 15434–15441.
17. Medema, J. P., D. H. Schuurhuis, D. Rea, J. van Tongeren, J. de Jong, S. A. Bres, S. Laban, R. E. M. Toes, M. Toebes, T. N. M. Schumacher, 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.
18. Andrew, K. A., H. M. Simkins, S. Witzel, R. Perret, J. Hudson, I. F. Hermans, D. S. Ritchie, J. Yang, and F. Ronchese. 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.
19. Zhang, M., S. M. Park, Y. Wang, R. Shah, N. Liu, A. E. Murmann, C. R. Wang, M. E. Peter, and P. G. Ashton-Rickardt. 2006. Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity* 24: 451–461.
20. Zhang, M., S. Byrne, N. Liu, Y. Wang, A. Oxenius, and P. G. Ashton-Rickardt. 2007. Differential survival of cytotoxic T cells and memory cell precursors. *J. Immunol.* 178: 3483–3491.
21. Lutz, M. B., N. Kukutsch, A. L. J. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
22. Matzinger, P. 1991. The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods* 145: 185–192.
23. McCausland, M. M., and S. Crotty. 2008. Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo. *J. Virol. Methods* 147: 167–176.
24. Kataoka, T., N. Shinohara, H. Takayama, K. Takaku, S. Kondo, S. Yonehara, and K. Nagai. 1996. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol.* 156: 3678–3686.
25. Yu, Y., A. Arora, W. Min, C. M. Roifman, and E. Grunebaum. 2009. EdU incorporation is an alternative non-radioactive assay to [(3)H]thymidine uptake for in vitro measurement of mice T-cell proliferations. *J. Immunol. Methods* 350: 29–35.
26. Wolint, P., M. R. Betts, R. A. Koup, and A. Oxenius. 2004. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. *J. Exp. Med.* 199: 925–936.
27. Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat. Rev. Immunol.* 7: 543–555.
28. Ansari, A. W., J. N. Temblay, S. H. Alyahya, and P. G. Ashton-Rickardt. 2010. Serine protease inhibitor 6 protects iNKT cells from self-inflicted damage. *J. Immunol.* 185: 877–883.
29. El Haddad, N., D. Heathcote, R. Moore, S. Yang, J. Azzi, B. Mfarrej, M. Atkinson, M. H. Sayegh, J. S. Lee, P. G. Ashton-Rickardt, and R. Abdi. 2011. Mesenchymal stem cells express serine protease inhibitor to evade the host immune response. *Blood* 117: 1176–1183.
30. Sprecher, C. A., K. A. Morgenstern, S. Mathewes, J. R. Dahlen, S. K. Schrader, D. C. Foster, and W. Kisiel. 1995. Molecular cloning, expression, and partial characterization of two novel members of the ovalbumin family of serine proteinase inhibitors. *J. Biol. Chem.* 270: 29854–29861.
31. Sun, J., C. H. Bird, V. Sutton, L. McDonald, P. B. Coughlin, T. A. De Jong, J. A. Trapani, and P. I. Bird. 1996. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. *J. Biol. Chem.* 271: 27802–27809.
32. Annand, R. R., J. R. Dahlen, C. A. Sprecher, P. De Dreu, D. C. Foster, J. A. Mankovich, R. V. Talanian, W. Kisiel, and D. A. Giegel. 1999. Caspase-1 (interleukin-1beta-converting enzyme) is inhibited by the human serpin analogue proteinase inhibitor 9. *Biochem. J.* 342: 655–665.
33. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27: 635–646.

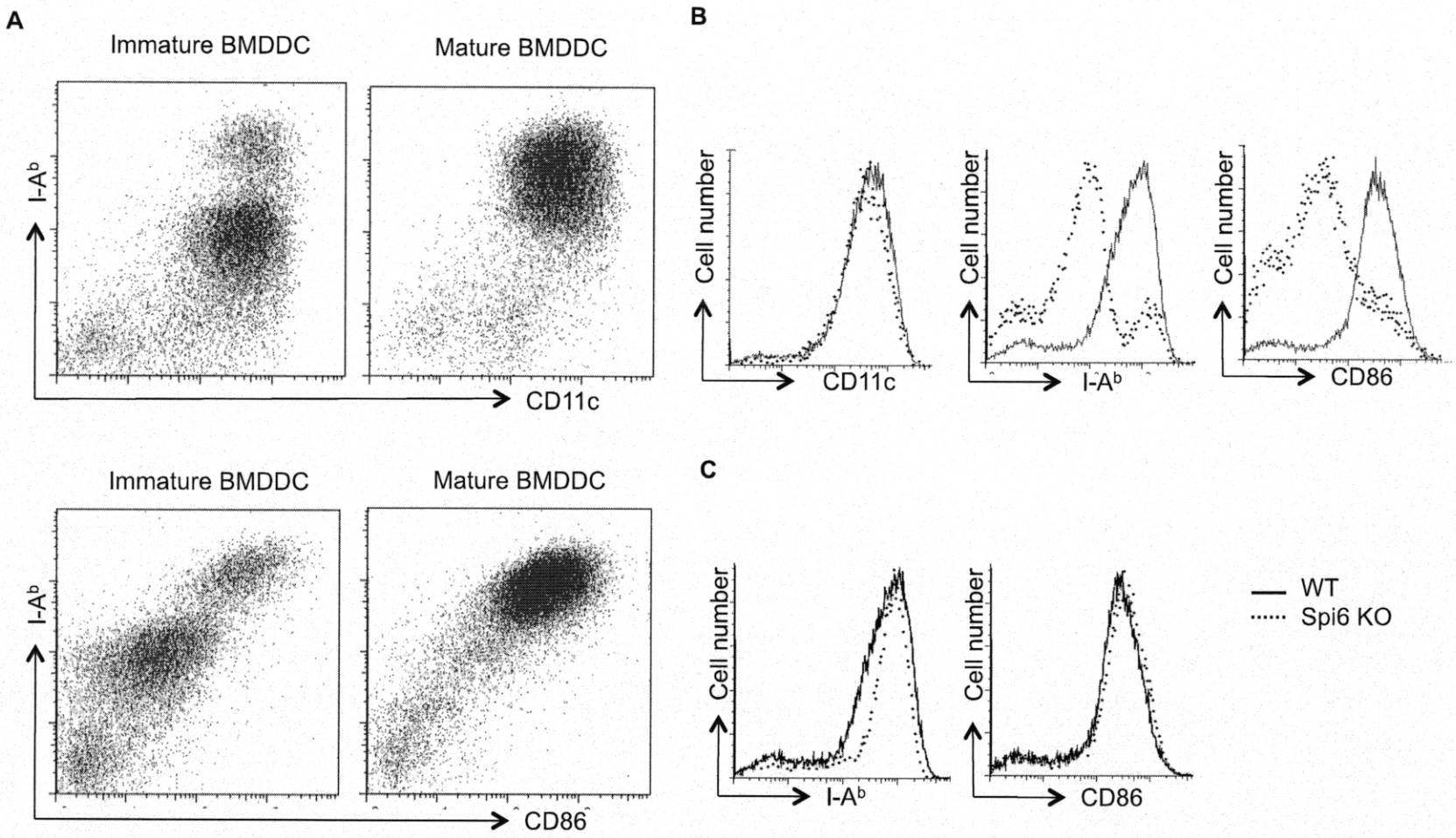


FIG. S1. Upregulation of MHC-II and CD86 in CD11c$^+$ LPS-matured BMDDC. A) Flow cytometry plots of staining for markers identifying BMDDCs pre- and post-LPS culture. B) histograms for staining with the same markers as before. C) Histograms for staining as before comparing maturation markers on WT- and Spi6 KO-derived BMDDCs

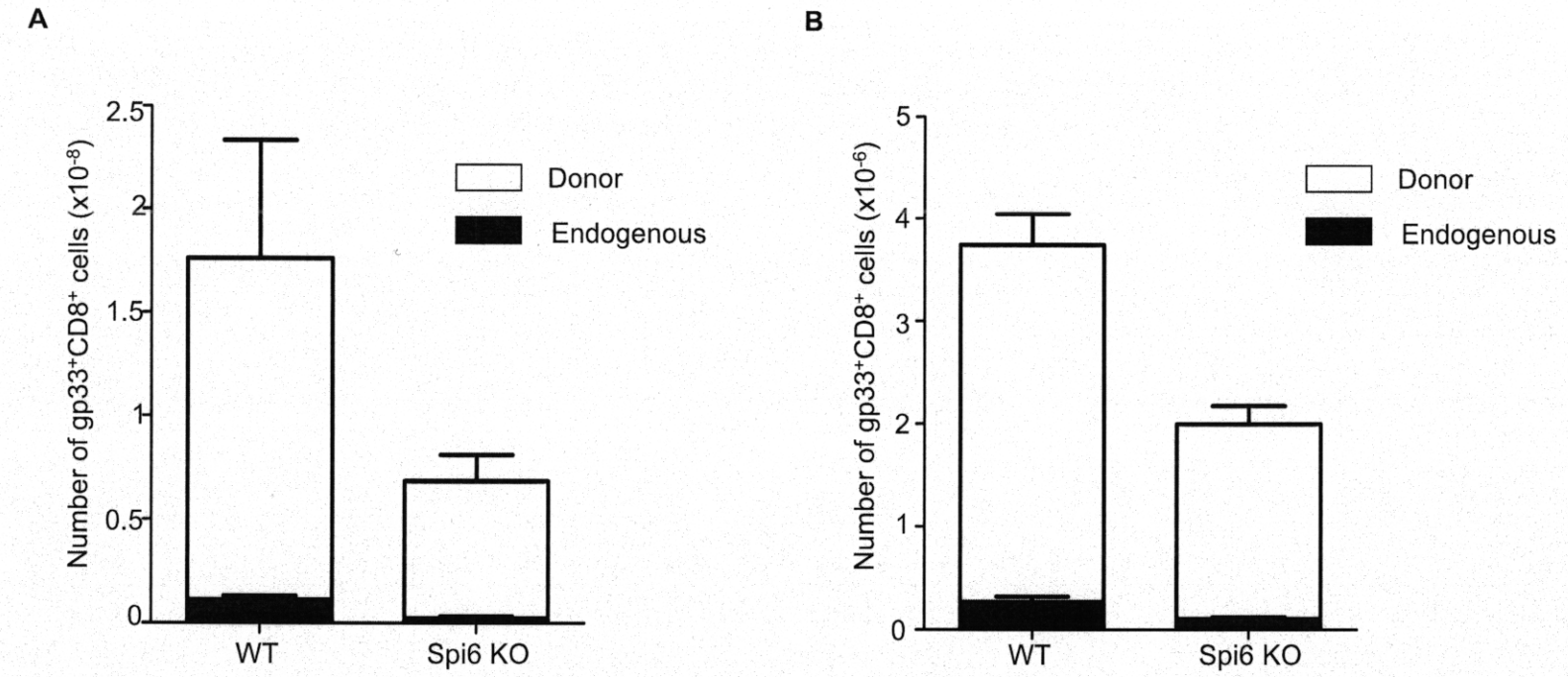


FIG S2. Absolute cell number of endogenous gp33$^{+}$CD8$^{+}$ T cell response versus donor. A) endogenous (black) versus donor (white) response in the spleen of WT and Spi6 KO mice during primary response to LCMV. Donor response is on average 15.2-fold higher than endogenous in WT and 23.8-fold in Spi6 KO mice. B) as A but in secondary response. Donor response is on average 12.7-fold higher than endogenous in WT and 17.8-fold in Spi6 KO mice.

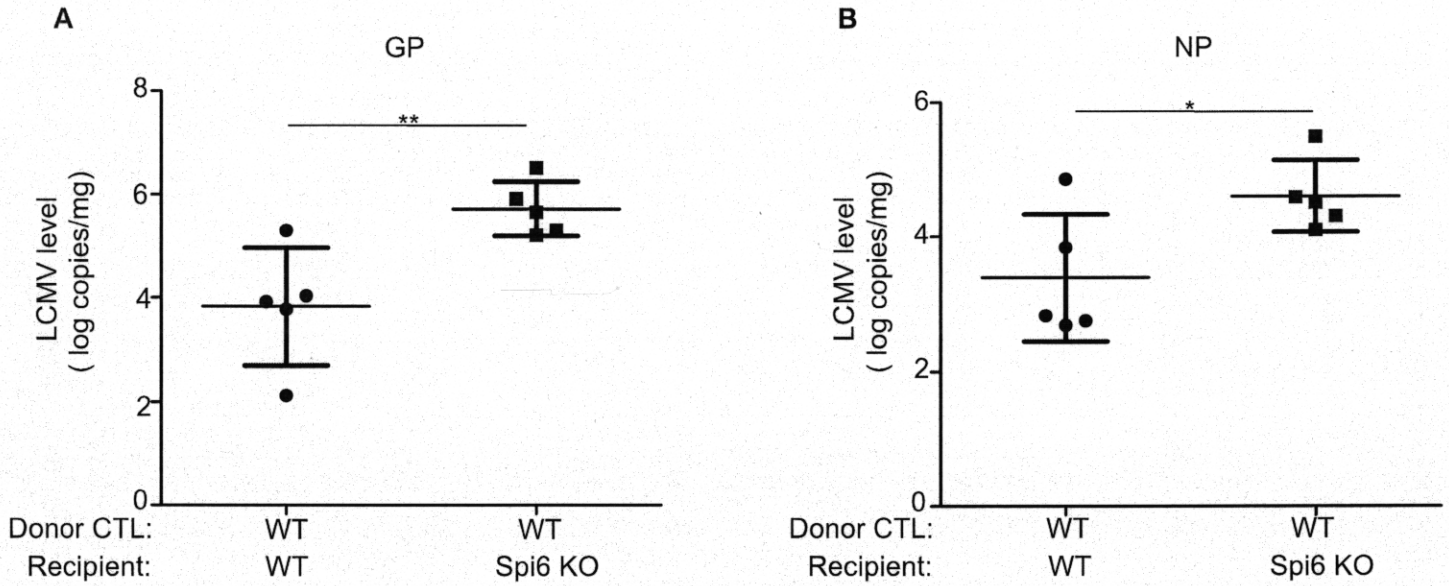


FIG S3. Level of LCMV Armstrong at day 6 post-infection. Levels of LCMV Arm in the spleen of Spi6 KO and B6 mice after adoptive transfer with P14 CD8 naive T cells and infection with 2x $10^5$ PFU LCMV Arm. The values are determined in spleen homogenates, by performing RT-PCR for viral Glycoprotein (GP) (A) and Nucleoprotein (NP) (B). The level of LCMV in each recipient mouse is indicated by a circle (WT) or square (Spi6 KO). The mean is indicated by horizontal line. * $p<0.05$, ** $p<0.01$.

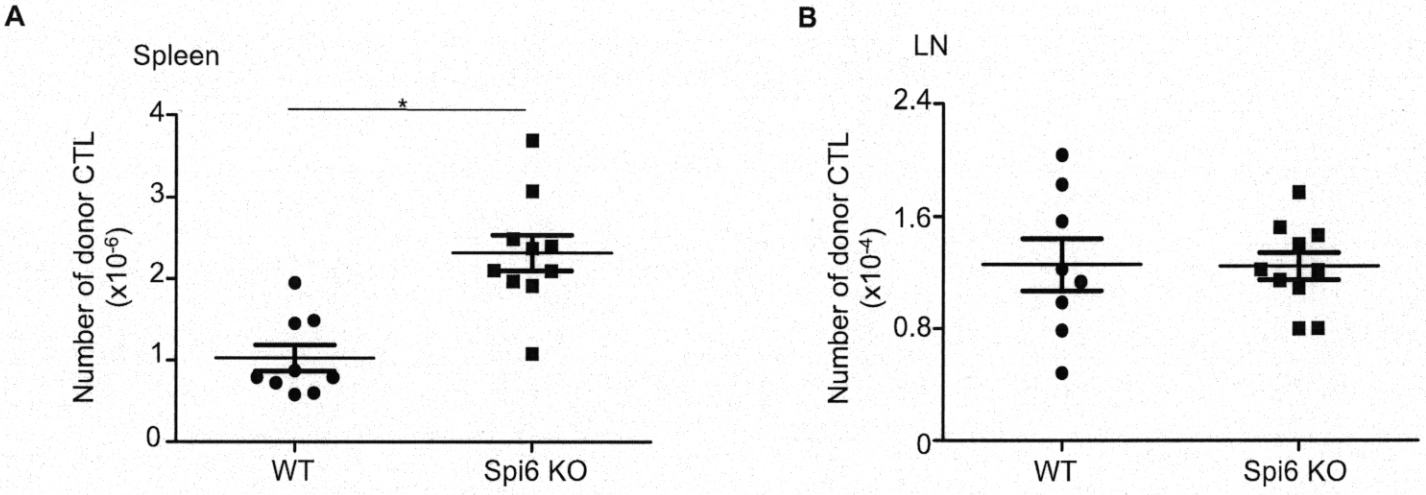


FIG. S4. Donor-derived memory cell ($gp33^+Thy1.1^+CD8^+$) absolute number 40 days after infection with LCMV. Absolute number of $gp33^+Thy1.1^+CD8^+$ T cells in the spleen (A) and LN (B) of WT and Spi6 KO mice.