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Curriculum: Immunology and Immunopathology

Different cell cycle stages characterize early and late phases of antigen-specific CD8 T cell response after vaccination

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Preface

After infection, or vaccination, naïve CD8 T cells are activated by antigen presenting cells (APCs) in secondary lymphoid organs. Activated antigen-specific CD8 T cells undergo a strong proliferation (so-called clonal expansion) and differentiate, generating a progeny composed by short-lived effectors and long-lived memory cells. Once antigen is eliminated, most cells die in the contraction phase and only few cells persist as memory CD8 T cells. These cells are able to respond to a second antigenic challenge in a more effective and faster way than in the primary response.

Several phases of T cell response are regulated by responding T cell entry and exit from cell cycle. Indeed, naïve T cells are quiescent cells in G_0 and the acute phase of response is characterized by their fast entry in cell cycle (from G_0 to G_1) and progression into subsequent phases till cell division (S-G_2/M). Several cell cycles characterize the clonal expansion of activated T cells. During the early phases of immune response, changes in cell cycle regulation of activated T cells could deeply affect the response, for example reduced clonal expansion could lead to decreased number of effector and memory cells. Moreover, it has been proposed that memory T cells are maintained over time by a fine balance between different cell cycle states, including a fine regulation of quiescence. According to this hypothesis quiescence represents an actively regulated process like other cell cycle phases and not a passive mechanism of cell persistence over time. Any perturbation of this balance could affect the ability to mount an efficient secondary response with consequent loss of protection.

In this scenario, our hypothesis is that a fine balance between different cell cycle phases regulates T cell response both in the acute and in the memory phase of response. Thus, in this project we aimed to investigate the kinetic of cell cycle phases of antigen-specific CD8 T cells responding to heterologous prime/boost vaccination in a mouse model.

By using a combination of DNA and Ki67 staining together with a novel strategy for analysis of flow cytometry data, we were able to discriminate antigen-specific CD8 T cells in G_0, in G_1 and in S-G_2/M phases of cell cycle. At early times after vaccination we found a previously missed population of cycling cells characterized by high Forward and Side Scatter (FSC-SSC) parameters. Cells with these characteristics are usually excluded from the analysis of normal lymphocytes ex vivo. By including them, we discovered an "extra" population of cycling antigen-specific CD8 T cell in spleen, lymph nodes and also in the blood which is not expected to be a site for antigen-responding CD8 T cells proliferation.

We found that antigen-specific CD8 T cells accumulated in lymph nodes and the bone marrow during memory phase. These cells switched to a quiescent phenotype and a few of them acquired a central memory phenotype at late times after priming. Interestingly, boosting when quiescent state was established resulted in a much higher frequency of antigen-specific CD8 T cells that persisted in different lymphoid organs, and accumulated in high numbers in the bone marrow.

Our results have implications for prior and future immunological studies in animal models and in humans. Indeed, our results will be instrumental to track CD8 T cell response in humans after infections or vaccination, as well as in cancers, and will improve the design of new therapeutic approaches to cancer and immune-mediated diseases.

Introduction

1. Adaptive and innate immunity

The immune system is composed by two main harms: the innate and the adaptive immunity.

The innate immune system comprises physical barriers; phagocytes; soluble mediators of inflammation; proteins of the complement cascade; and cells able to kill target cells. The cellular components of the innate immunity include Dendritic Cells (DCs), macrophages, mast cells, granulocytes, natural killer cells and some types of unconventional T cells (NKT cells and γδ T cells). The innate immunity is poorly specific and is induced by the recognition of molecules shared by different pathogens. Indeed, innate immune cells, for examples DCs, express innate receptors named Pattern Recognition Receptors (PRRs), which recognize microbe-derived molecules, named Pathogen Associated Molecular Patterns (PAMPs), or molecules related to tissue damage, named Damaged Associated Molecular Patterns (DAMPs). This process, also known as pathogen/danger sensing, activates a series of intracellular signals that culminate in the activation of innate immune cells with release of different pro-inflammatory and chemoattractant molecules (such as cytokines and chemokines). These factors promote microbe phagocytosis, and direct lysis of bacteria, as well as recruitment of other immune cells at the site of infection. Finally, the innate immune system activates the adaptive system by a process that involves the action of DCs.

The components of the adaptive immunity are lymphocytes and their soluble products (antibodies and cytokines). Lymphocytes are B and T cells and express membrane receptors that recognize molecules named antigens, usually derived from pathogens. Antigens include proteins, peptides, lipids, carbohydrates, nucleic acids and metabolites. B and T cell receptors recognize a specific portion of the antigen, the immune-dominant epitope. This process

activates a series of events leading to a highly specific response against the pathogen. Moreover, lymphocyte receptors are encoded by mature genes, which derive from gene segments through a process of somatic recombination during development. This process generates a wide range of receptor specificities (lymphocyte repertoire) and allows the recognition of a vast variety of antigens. Another peculiar feature of the adaptive immunity is the ability to “learn” from the first antigen exposure and to mount a more effective response in case of a subsequent challenge with the same antigen. This property is defined memory and is mediated by long-lived cells known as memory lymphocytes. The adaptive immunity is divided into humoral and cell-mediated immunity. B cells that produce and release antibodies mediate humoral immunity. Antibodies promote microbe phagocytosis, activate the complement cascade, and can also directly neutralize the antigen. Cell-mediated immunity involves the action of T cells. These cells recognize extracellular pathogens internalised by phagocytes, intracellular pathogens infecting host cells, and cancer cells. T cells can perform several effector functions including release of cytokines, that promote leukocyte recruitment and activation, and killing of infected or cancer cells.

Under physiological condition both innate and adaptive harms can control the immune system avoiding response against host tissues (self). This important function is named tolerance and is mediated by several mechanisms. For example, tolerance is ensured by the action of regulatory T cells that release immune-suppressive factors and inhibit other T cells by the expression of inhibitory receptors.

2. Induction of the immune response

The first encounter with a pathogen leads to the induction of the primary immune response. The innate response is the first line of host defence. The innate response is very rapid, acting within minutes after pathogen recognition, and is mediated by both cells and soluble molecules. As previously mentioned, innate immune cells are activated by ligands of PRRs. The adaptive response develops slowly, days after pathogen encounter, and elicits a potent and highly specific response. The induction of adaptive response involves antigen uptake and processing by specialised cells, named Antigen Presenting Cells (APCs) in peripheral tissues. DCs are the principal type of APCs involved in the activation of T cells. Indeed, after engulfment of antigens from environment, DCs process them into small peptides, which are then displayed on cell surface in combination with major histocompatibility complex molecules (MHC). Antigen-loaded DCs migrate to secondary lymphoid organs where they interact with T cells that have never met the antigen before, named naïve T cells. Antigen recognition by naïve T cells and other signals generated by DCs leads to the induction of a functional T cell response (priming) (Paul et al., 2013).

2.1 Role of Dendritic Cells

Dendritic Cells (DCs), so called because of their long cytoplasmatic processes that resemble those of neurons, are consider a bridge between the innate and adaptive immunity. Indeed, during immune response the main function of DCs is to migrate to secondary lymphoid organs and activate T cells.

In physiological conditions DCs are present as resting or *immature* DCs in lymphoid and non-lymphoid tissues, wherein they sample the environment for the possible presence of pathogens or damaged cells. Immature DCs are specialised in the antigen uptake and express high levels of PRRs, including Toll-like Receptors (TLRs), by which they can sense the presence of dangerous signals (pathogens, dead cells or tumors). Immature DCs also express low levels of MHC class II and co-stimulatory molecules (CD40, CD86, CD80). After infection, tissue damage or vaccination, immature DCs are activated and undergo a process defined as *maturation* (Figure 1). Activated or *mature* DCs start to process antigens, increase the expression of MHC class II and co-stimulatory molecules, and release cytokines and chemokines that orchestrate the immune response. Upregulation of lymphoid homing receptors and morphological changes mediate mature DC migration to the secondary lymphoid organs wherein they present the antigen to naïve T cells. Once activated, DCs die by apoptosis or necroptosis depending on the type of maturation stimuli (Banchereau et al., 2000*;* Reis & Sousa, 2006; Steinman at al., 2007; Merad & Manz, 2009; Zou et al., 2013).

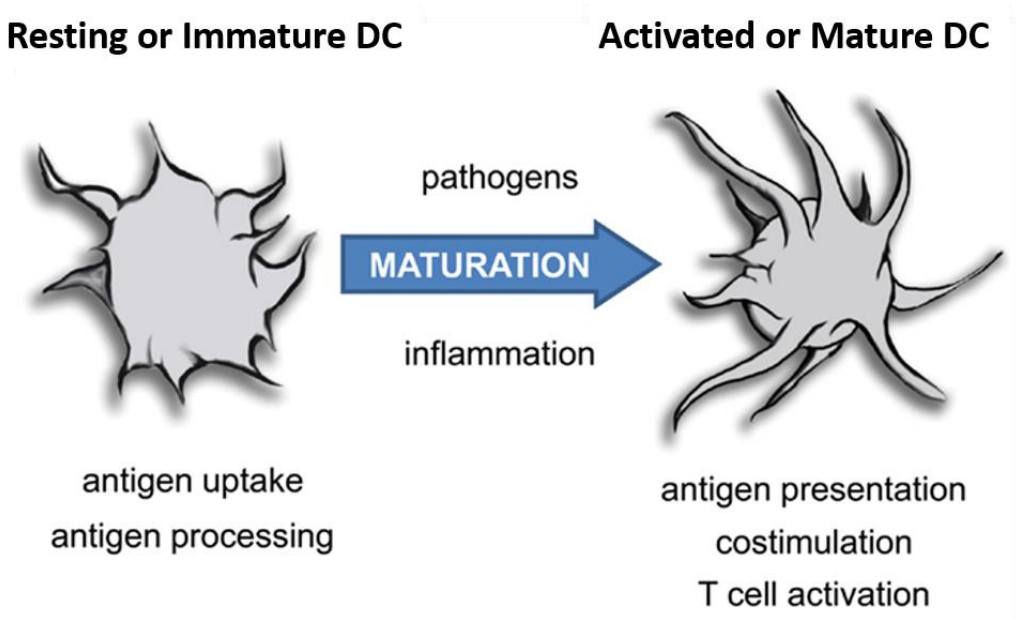

Figure 1: DC maturation

Regulation of DC homeostasis has an impact in immunity and tolerance. In healthy tissues DCs persist for less than a week before dying, with slightly diverse half-lives of different DC subpopulations. After pathogen sensing, DCs maturation occurs in few hours but migration to lymph nodes and T cell priming require more time. At this point the balance between DC survival and death is critical for a durable T cell response. Indeed, DCs must survive to ensure the complete T cell activation and die soon after priming to avoid immune over-activation. DC homeostasis is also critical for the maintenance of tolerance, for example in T cell development and selection in primary lymphoid organs. Indeed, T cell precursors are generated in the bone marrow and then migrate to the thymus to complete their differentiation in naïve T cells. Tolerogenic DCs are present in thymus where they present self-antigen to developing T cells, leading to the elimination of those autoreactive T cells that interact with strong affinity with MHC-self-antigen complex (thymic negative selection). In the thymus, death of tolerogenic DCs must be followed by replacement with newly generated DCs from the bone marrow to guarantee T cell selection and to avoid autoimmune response. However, some autoreactive T cells can escape the thymic selection.

DCs are critical also to avoid the activation of these self-reactive T cells in the periphery. Indeed, DCs can induce T cell death in absence of costimulation (anergy) and promote the generation of a T cell population involved in the immune suppression (inducible Regulatory T cells or T Regs) (Matzinger, 2002; Manicassamy and Pulendran, 2012).

To date many aspects of DC homeostasis are still unknown, for example the possible changes in DC life-span occurring after activation by different stimuli. In this context, our research group has recently demonstrated that Stem Cell Factor (SCF) mediated a pro-survival circuit for resting DCs, and that its receptor c-kit was down-modulated by activated DCs in response to some inflammatory stimuli (Poly I:C, CpG, TNF-α, IFN-β) but not others (LPS). These evidences suggest a possible role of SCF/c-kit axis in the control of DC homeostasis in response to different TLR ligands, with a likely impact on the regulation of T cell response (Barroeta Seijas et al., 2017; Simonetti et al., 2019).

2.2 T cells

T cells are a heterogeneous population of lymphocytes responsible for cell-mediated immunity against several pathogens and cancers. The expression of different co-receptors defines two main T cell subsets: CD4 and CD8 T cells. T cell phenotype, function and localization can be used to further divide them into different subpopulations.

CD4 T cells express the co-receptor CD4 and coordinate the immune response by interacting with other immune cells mainly by producing cytokines. These cells are also known as “helper” T cells (Th) for their essential role in the activation of antibody-mediated and cellular responses. Moreover, CD4 T cells control the immune response by inhibiting other immune cells thus preventing host damage, avoiding immune reaction against host cells (tolerance) and contributing to tissue repair. CD4 T cells are highly heterogeneous and can be divided into different subpopulations depending on the type of cytokines that they produce and the expression of specific transcription factors. The major CD4

T cell subpopulations are: Th1, Th2, Th17, Th9, regulatory T cells (T regs) and follicular T cells (TFH).

CD8 T cells express the co-receptor CD8 and are active against intracellular pathogens and cancer cells. After activation, CD8 T cells migrate to the site of infection or tumor growth and kill the target cells by membrane interaction and release of effector molecules. Due to their cytotoxic function, they are also known as Cytotoxic T Lymphocytes (CTLs).

T cells express the T cell receptor by which they recognize antigens bound to MHC molecules. Two classes of MHC molecules have been described: MHC-class I, and II. MHC-class I molecules are expressed by all nucleated cells, while MHC-class II molecules are expressed by APCs (i.e. DCs cells, macrophages and B cells). Extracellular antigens, derived from phagocytosis of extracellular pathogens, are associated to MHC-class II molecules and presented to CD4 T cells. Intracellular antigens, derived from intracellular pathogens infecting host cells, from tumor cells or damaged cells are associated to MHC-class I molecules and presented to CD8 T cells.

The T Cell Receptor (TCR) and the membrane proteins CD3 and ζ chains compose the T cell receptor complex, which is responsible for MHC-antigen complex recognition together with the co-receptor CD4 or CD8. Once TCR binds MHC-antigen complex, the co-receptors stabilize the binding and promote intracellular signal transduction by CD3 molecules.

The 90% of circulating T cells express a TCR composed by two polypeptides, α and β. The rest of the T cells in human blood (about 10%) express a TCR composed by γδ heterodimer. γδ T cells present a less diverse antigenic repertoire compared to that of αβ T cells and can recognize non-protein antigens such as lipid antigens. Recently studies have shown that this population plays a role in the context of immune response in non-lymphoid tissues. Indeed, γδ T cells are abundant in epithelial tissues and can protect against tuberculosis, malaria, and other infections.

2.3 T cell response

The first encounter with the antigen leads to the induction of a primary T cell response (Figure 2). This process involves DC-naïve T cell interaction in secondary lymphoid organs, as previously mentioned. Cell-cell contact generates a complex network of intracellular signals, which induces T cell activation (priming). This occurs about 7 days after exposure to antigen. During activation, antigen-specific T cells undergo a strong proliferation (so-called clonal expansion) and differentiate, generating a progeny composed by short-lived effectors and long-lived memory cells. Activated antigen-specific T cells egress from secondary lymphoid organs and reach the target organ where they kill antigen-positive cells, for example an infected organ or a tumor site. Following antigen clearance, most of the antigen-specific CD8 T cells (about 90%) die in the so-called contraction phase, only few cells persist and create the pool of the memory CD8 T cells.

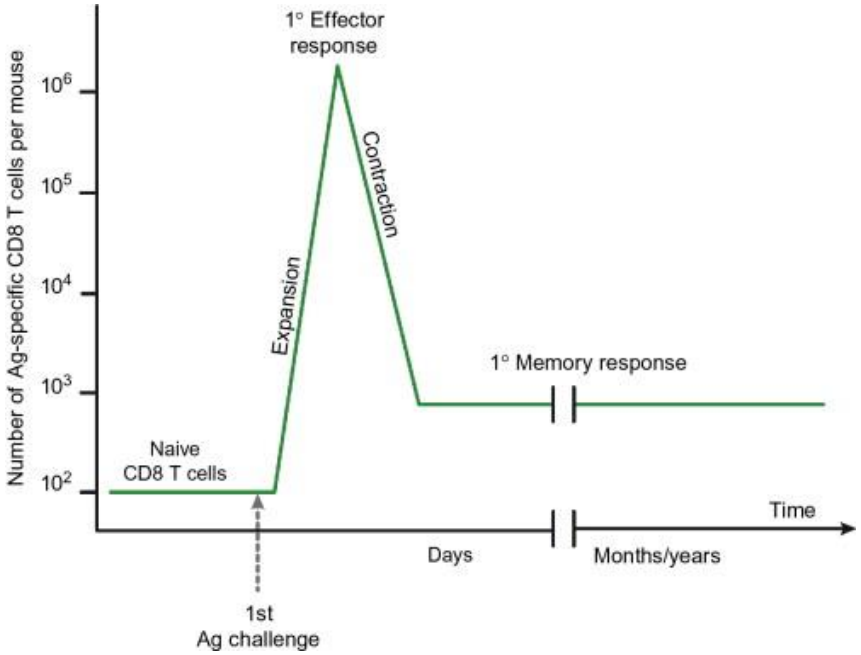

Figure 2: Primary antigen-specific T cell response

2.3.1 T cell activation

The first event of T cell activation is the interaction between naïve T cell and antigen-loaded DC that occurs in specific niches within the organ. Indeed, this process is regulated by local factors, such as chemokines that guide naïve CD8 T cells at the site of interaction with the APC (Castellino F et al., Nature, 2006). Three main signals are required for full T cell activation: antigen recognition (signal 1); co-stimulation (signal 2) and cytokines necessary for proliferation and differentiation (signal 3). Antigen recognition by TCR corresponds to signal 1. The interaction between the TCR and the MHC-peptide complex express by DCs leads to the generation of the immunological synapse, the so-called Supramolecolar Activation Cluster (SMAC), a specific cluster of membrane molecules, including adhesion molecules and intracellular signalling receptors, at the site of cell-cell contact between T cell and DC. Activation signal transduction spreads at the site of SMAC formation and is mediated by the TCR, the coreceptors CD4 or CD8, and CD3- ζ, which contains tyrosine phosphorylation motifs (ITAMs, Immunoreceptor Tyrosine-based Activation Motifs) in its cytoplasmic region. Following the TCR-MHC-peptide complex binding, these motifs are phosphorylated leading to the activation of three signalling pathways: calcium-calmodulin, Protein Kinase C (PKC) and Mitogen-Activated Protein (MAP) kinases pathway. This signalling culminates with the activation of transcription factors, such as NF-kB, AP-1 e NFAT, which induce the expression of proliferation and cell-survival associated genes (Abbas et al., 2013). However signal 1 alone is not sufficient to fully activate T cells, but there is need for signal 2, provided by costimulatory molecules express by DCs. These molecules are upregulated on the surface of the DCs only after the recognition of signals associated with pathogens or tissue damage, such as self-proteins and nucleic acids (DAMPS). The control exerted by signal 2 guarantees that the induction of T cell response occurs only in case of danger from infectious agents or cell damage and not in case of self-antigen recognition in healthy conditions. Indeed, when T cells recognize antigens in absence of costimulation they are not activated and undergo an inactivation process called anergy (Matzinger, 2002).

The main costimulatory molecules are CD80 (B7-1) and CD86 (B72), which can bind both activatory and inhibitory receptors present on T cell surface. The CD28 family receptors belong to the activatory receptor class and bind B7-1 and B7 -2 inducing a cascade of intracellular signals which, together with the signals coming from the TCR, induce the expression of anti-apoptotic proteins and the production of IL-2 by T cells, critical for their clonal expansion. In contrast, inhibitory receptors, for example Cytotoxic T Lymphocyte Antigen 4 (CTLA-4), compete with CD28 for the binding to costimulatory molecules and regulate the immune response by inhibiting T cell activation (Figure 3). Therefore, T cell activation is finely regulated by the levels of costimulatory molecules on the APCs membrane and by the type of receptors (activatory or inhibitory) expressed by the T cell (Abbas and Janeway, 2000; Alegre et al., 2001; Alegre et al., 2001).

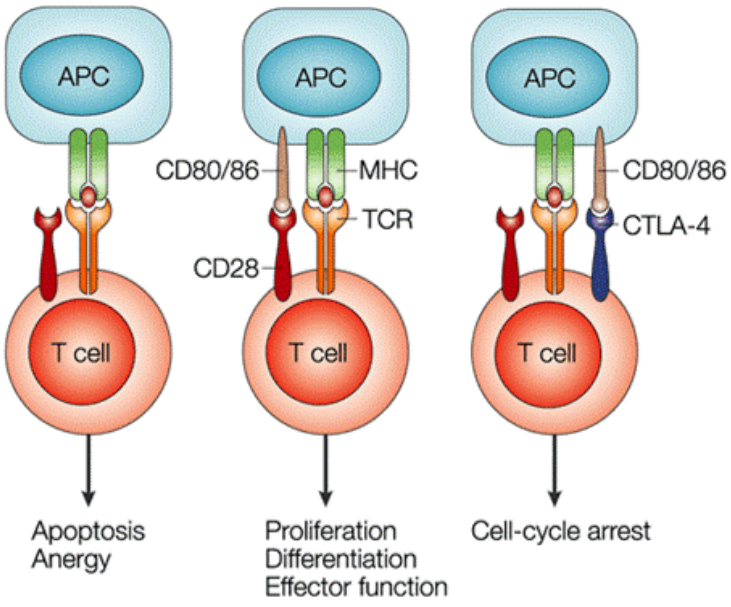

Figure 3 The levels of costimulatory molecules on the APC surface and their binding to activatory or inhibitory receptors control T cell activation (Alegre et al., 2001)

The cytokines released by APCs, or by other cells, provide signal 3. These cytokines drive T cells towards differentiation into different kinds of effector cells, thus regulating the type (or class) of adaptive immune response (Simin Goral, 2011).

Usually CD8 T cell activation requires help by CD4 T cells. These cells act by secreting cytokines and regulating DC function. Indeed, CD4 T cells release cytokines, such as IL-2, that sustain CD8 T cell proliferation and regulate their differentiation. Moreover, CD4 T cells express CD40 ligand (CD40L) that interacts with its receptor CD40 expressed by APCs leading to the upregulation of costimulatory molecules on APC surface. This function of CD4 T cells, called CD8 T cell licensing, promotes CD8 T cell activation (Bevan et al., 2004)

2.3.2 Clonal expansion

Once activated, antigen-specific CD8 T cells undergo extensive proliferation, called clonal expansion. This leads to considerable increase of the number of T cells capable of eliminating the antigen. Indeed, in a typical primary response the antigen-specific CD8 T cells expand increasing their number of about 100 times or more (Zhang and Bevan, 2011). Clonal expansion is driven by T cell-APCs interaction within specialised niches in secondary lymphoid organs and it is controlled by several factors (cytokines, antigen persistence, costimulation).

One of the principal cytokines regulating T cell expansion is IL-2 which is released mainly by CD4 T cells and can act on the producing cells (autocrine stimulation) or on other T cells (paracrine stimulation) (Zhang & Bevan, 2011).

Moreover, clonal expansion seems to be differentially regulated in CD4 and CD8 T cells. It has been demonstrated that efficient proliferation and differentiation of CD4 T cells require a persistent exposure to the antigen. On the contrary, CD8 T cells can undergo multiple rounds of cell division after a short antigen encounter (less than 2 hours), acquiring effector function and forming a pool of long-lived memory T cells (Bevan & Fink, 2001; van Stipdonk et al., 2001; Beverley, 2008)

Dysregulation of clonal expansion could deeply affect the quality of the T cell response. Indeed, reduced proliferation of activated T cells leads to decreased number of effector and memory cells.

2.3.3 Effector phase

Clonal expansion culminates with the differentiation of activated T cells into different subpopulations of differentiated effector T cells for example CD4 T cells can differentiate into Th1, Th2, Th17, T regs e TFH.

This process of differentiation is called Th polarization, and is mediated by different cytokines, which polarize activated CD4 T cells towards one subpopulation inhibiting the differentiation into the others. Once at the target site, Th cells are triggered by APCs expressing MHC II-antigen complex and perform their effector functions. These functions include the production of cytokines that activate innate and adaptive immune cells (i.e. B cells).

Th1 polarization occurs in response to phagocyte activation and is mainly induced by IL-12 and Interferon γ (IFN-γ). Th1 cells produce IFN-γ which increases the anti-microbial activity of macrophages, favouring the elimination of engulfed pathogens. They also induce B cells to produce antibodies for microbe phagocytosis (Sallusto e Lanzavecchia, 2002; Zhu et al., 2010).

CD4 T cells are polarized towards Th2 population, in the presence of IL-4 released by mast cells, eosinophils and other granulocytes responding to allergens or parasites. These cells release IL-4, IL-5 and IL-13, which altogether mediate eosinophil activation and stimulate B cells to produce IgE, thus inducing mast cells degranulation. Furthermore, Th2 cells induce macrophages to release mediators of tissue repair (Sallusto e Lanzavecchia, 2002; Zhu et al., 2010).

Th17 polarization is induced by pro-inflammatory cytokines, such as Trasforming Growth Factor β (TGF-β), IL-1 and IL-6 that are released in response to bacterial or fungal infections. The key cytokine released by Th17 cells is IL-17, which induces strong inflammation by stimulating the production of anti-microbial molecules and by recruiting neutrophils at the site of

inflammation. Because of their pro-inflammatory function, Th17 are often associated with the pathogenesis of several chronic inflammatory diseases (Zhu et al., 2010).

Tregs can be generated in secondary lymphoid organs when antigen recognition occurs without strong costimulation and in the presence of Trasforming Growth Factor β (TGF-β) (Sakaguchi et al, 2008; Ohkura et al., 2013). Their immune-suppressive function is mediated by several mechanisms, including the release of IL-10 and TGF-β, which jointly suppress the activation of several immune cells. Furthermore, Tregs express high levels of CTLA-4 which compete with CD28, thus blocking naïve T cells activation in secondary lymphoid organs (Sakaguchi et al, 2008; Ohkura et al., 2013).

TFH differentiation is regulated by IL-6 and IL-21. TFH cells are present in B cell follicles in secondary lymphoid organs where they release IL-21 and express costimulatory molecules mediating B cell activation (Zhu et al., 2010).

CD8 T cell differentiation into CTLs is characterized by the acquisition of their cytotoxic function. When CTLs migrate to the site of infection they specifically recognize infected or tumor cells, that express antigen complexed with MHC I molecules, and kill them via several mechanisms. CTL differentiation is mediated by pro-inflammatory cytokines (such as IL-12, IL-6, and IFN-γ) and by IL-2, which altogether induce the upregulation of specific transcription factors (T-bet and Eomes). These factors are necessary for the expression of chemokines that guide CTLs to the site of infection (such as CXCR3, CCR5), for IFN-γ and Tumor Necrosis Factor (TNF) production and for accumulation of cytoplasmatic granules which contain cytotoxic proteins (perforin and granzyme) (Zhang e Bevan, 2011).

Cytotoxicity is regulated by the formation of a secretory immunological synapse at the site of interaction between CTL and the target cell (Figure 4). This structure regulates cytotoxic protein release only in the synapse region without damage of healthy cells in the proximity (Jenkins and Griffiths, 2010; Stinchcombe and Griffiths, 2003).

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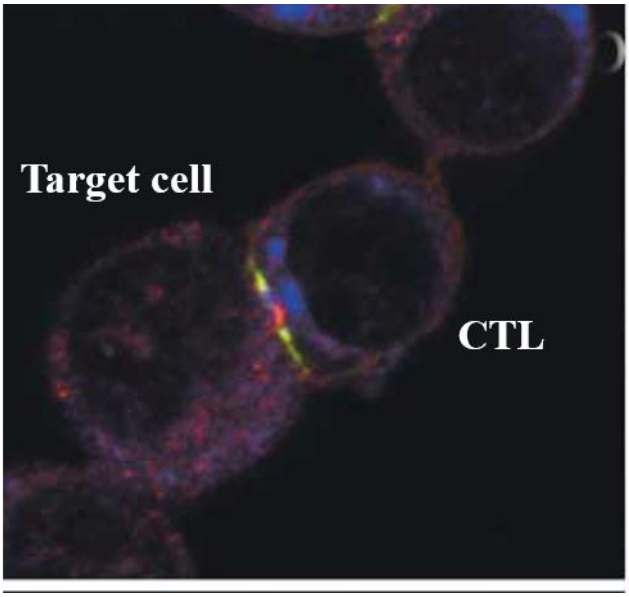

Figure 4: Formation of a secretory immunological synapse at the site of CTL-target cell contact (adapted from Stinchcombe et al., 2001)

There are two main mechanisms of CTL cytotoxicity mediated by direct cell-cell contact. The first mechanism involves the release of granules containing perforin and granzymes (Figure 5A). Perforin forms pores in the plasma membrane of the target cell and allows granzymes to enter the cytoplasm. Granzymes are serine proteases that, once in the cytoplasm, induce apoptosis by caspase 3 activation-dependent or -independent mechanisms. Granules contain also granulisin, which can induce target cell apoptosis or act on intracellular pathogens, but its mechanism of action is poorly defined. The second mechanism of cytotoxicity involves the induction of apoptosis by the recognition of the Fas death receptor on the target cell (Figure 5B). This receptor binds its ligand (FasL) present on CTL inducing the activation of caspase 8 and the consequent death of the target cell.

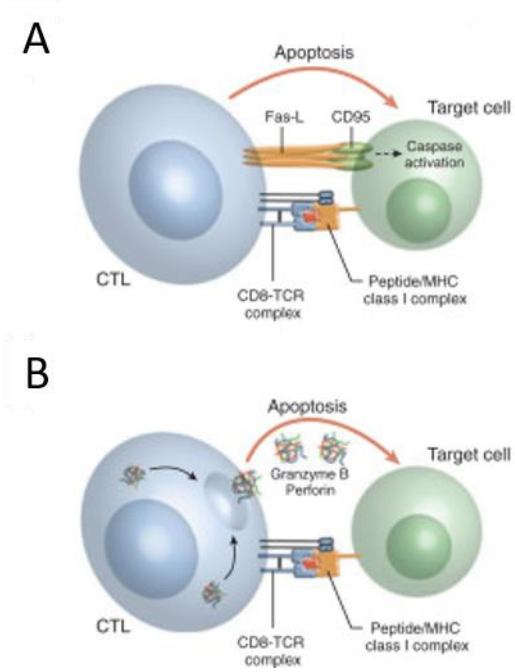

Figure 5: Mechanisms of CTL cytotoxicity (adapted by Andersen et al., 2006)

Furthermore, CTL produce some cytokines, such as IFN-γ and TNF that may contribute to their cytotoxic function. Indeed, IFN- γ increases the expression of MHC class I-peptide and Fas complexes on the target cell membrane, thus favouring recognition of target cells and their killing by CTL. TNF induces both upregulation of Fas and direct activation of caspases. IFN-γ and TNF also recruit and activate macrophages and neutrophils at the site of infection (Harty et al., 2000; Andersen et al., 2006).

2.3.4 Contraction phase

At the end of the primary response and following antigen clearance, most of the antigen-primed T cells die by apoptosis (about 90%) and only few cells survive and create the pool of memory T cells (about 5-10%). Effector T cell death is crucial for the control of the immune response. Indeed, the great number of activated T cells generated during the expansion phase must be reduced to prevent damage of healthy tissues. The factors that regulate contraction are mostly undefined. For years the beginning of this phase was commonly

associated with antigen clearance. To date it has been proposed that the contraction is programmed early during T cell response and evidences supporting the role of the antigen are under debate. Pro-inflammatory cytokines such IFN-γ seem to have a positive role in the regulation of the contraction phase, probably by regulating anti- and pro-apoptotic proteins (Harty & Badovinac, 2008; Garrod et al., 2012).

2.4 Migration of naïve and effector T cells

In physiological conditions naïve T cells continuously recirculate between blood and secondary lymphoid organs where they can encounter the antigen. Migration into secondary lymphoid organs is finely regulated, especially in lymph nodes. Naïve T cells homing to lymph nodes occurs through the High Endothelial Venules (HEVs) and is regulated by chemokines and adhesion molecules expressed by both T and endothelial cells. The homing process is divided into 4 phases: 1) rolling of naive T cells on the HEVs; 2) weak adhesion of naive T cells to HEVs; 3) stable adhesion; 4) extravasation of naïve T cells. Rolling is mediated by the binding of L-selectin (CD62L) expressed by naïve T cells to one of its sialomucine-type ligands, for example the Glycan-bearing Cell Adhesion Molecule 1 (GlyCAM-1), expressed by endothelial cells. For this reason, CD62L is commonly considered a marker of naïve T cell phenotype. Naïve T cells weakly adhere to HEVs through the expression of the membrane integrin Lymphocyte Function-associated Antigen 1 (LFA-1) (low affinity conformation) that binds to the Intercellular Adhesion Molecule expressed by the endothelium. This binding is reinforced by the interaction between the chemokines produced in the lymph nodes, CCL19 and CCL21, and their receptor CCR7 expressed by naïve T cells thus allowing the stable adhesion of the lymphocytes to the HEVs (LFA-1 switch to a high affinity conformation). At this point naïve T cells can cross the endothelium and reach the parafollicular areas of the lymph node (T areas) by chemotaxis. Also mature DCs express

CCR7 and localize in the T area, thus presenting the antigen to the entering naïve T cells (Bajenoff et al., 2007).

T cell retention in secondary lymphoid organs is controlled by the interaction between CD69 and Sfingosine-1-Phosphate (S1P) receptor, both expressed on T cell membrane. S1P is a lipid molecule that is more concentrated in the blood and in the lymph than in tissues (Takahama, 2006). When naïve T cells patrolling the lymph node do not encounter the antigen in this organ, S1P receptor expression is high leading to the egress of T cells from lymph nodes, following the S1P gradient. In contrast, in case of antigen encounter in the lymph node, activated T cells upregulate CD69, in response to cytokines such as IFN-I. CD69 inhibits the expression of the Sfingosine-1-Phosphate (S1P) receptor on T cell membrane, holding T cells in the lymph node until they complete differentiate into effectors, which can takes few days. Once differentiation is completed, effector T cells down-regulate CD69, re-express S1P receptor and migrate out of the lymph node along the S1P gradient. Furthermore, effector T cells reduce the expression of CD62L and express chemokines receptors that guide them to the site of infection.

After antigen encounter and T cell activation in the lymph nodes, effector T cells must migrate from secondary lymphoid organs to peripheral tissues where they can meet the antigen again and perform their effector functions. Migration into peripheral tissues involves the rolling of effector T cells on the peripheral blood vessels; the weak adhesion and then stable adhesion of the T cells to the endothelium, and the extravasation of the T cells into the infected or inflamed tissue. Notably, the molecules that mediate this process are different from those that allow naïve T cells to reach the lymph nodes and each effector T cell subpopulation expresses a set of adhesion molecules and chemokine receptors that regulates the migration in a specific tissue. For example, after viral or bacterial infections that induce a very strong immune response, cytokines produced by innate immune cells (e.g. IFN-γ produced by NK cells) induce the release of chemokines, mainly CXCL9 and CXCL10, and the expression of E and P selectins on endothelial cells. Virus-specific CTLs express glycoproteins,

for example the Cutaneous Lymphocyte Antigen 1 (CLA-1) that bind E and P selectins allowing the adhesion to the vascular endothelium at the infected skin. Furthermore, Virus-specific CTLs express high levels of CXCR3 receptors that direct them to the infection sites, by binding to CXCL9 and CXCL10. T cell entry into peripheral tissues is regulated by the expression of integrins, such as LFA-1 and Very Late Antigen 4 (VLA-4), on the membrane of T cells which bind to integrin receptors, for example ICAM-1 and VCAM-1 (Vascular Cell Adhesion Molecule 1), expressed by endothelial cells (Figure 6) (Iijima & Iwasaki, 2015).

After antigen clearance, antigen-specific T cells can come back to lymphoid organs, for example to lymph nodes, by upregulation of CD62L, and recirculate between these organs and blood, or can reside in the tissues never reaching the circulation again, for example by the upregulation of CD69.

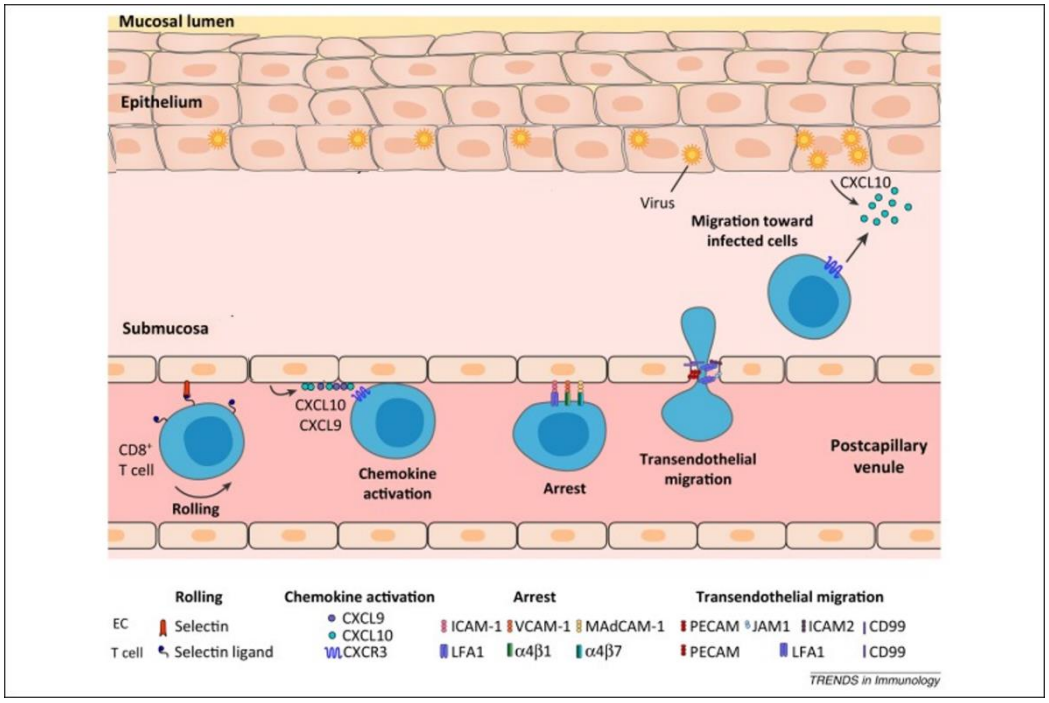

Figure 6: Effector CD8 T cell migration to infected tissues (adapted from Iijima et al., Tissue instruction for migration and retention of TRM cells, Trends Immunol 2015).

3. Memory and Vaccines

Immunological memory is one of the most important features of adaptive immunity. Indeed, memory is the capacity of the immune system to respond more efficiently and rapidly to a subsequent exposure to an antigen already encountered (secondary response).

A typical secondary T cell response is more powerful and rapid than the primary one. Figure 7 shows the kinetics of the primary and secondary CD8 T cell response. After the resolution of the primary response, memory phase is established (Stage 3). In the case of a new encounter with the same antigen (re-infection) the memory CD8 T cells can rapidly (in 1-3 days) trigger a secondary response and block the infection before the disease occurs (Stage 4) (Paul, 2013). Antigen-specific memory CD8 T cells are 1000 times more numerous than the naïve CD8 T cells and for this reason the secondary response has a power (magnitude) greater than the primary response (Figure 7) (Paul, 2013).

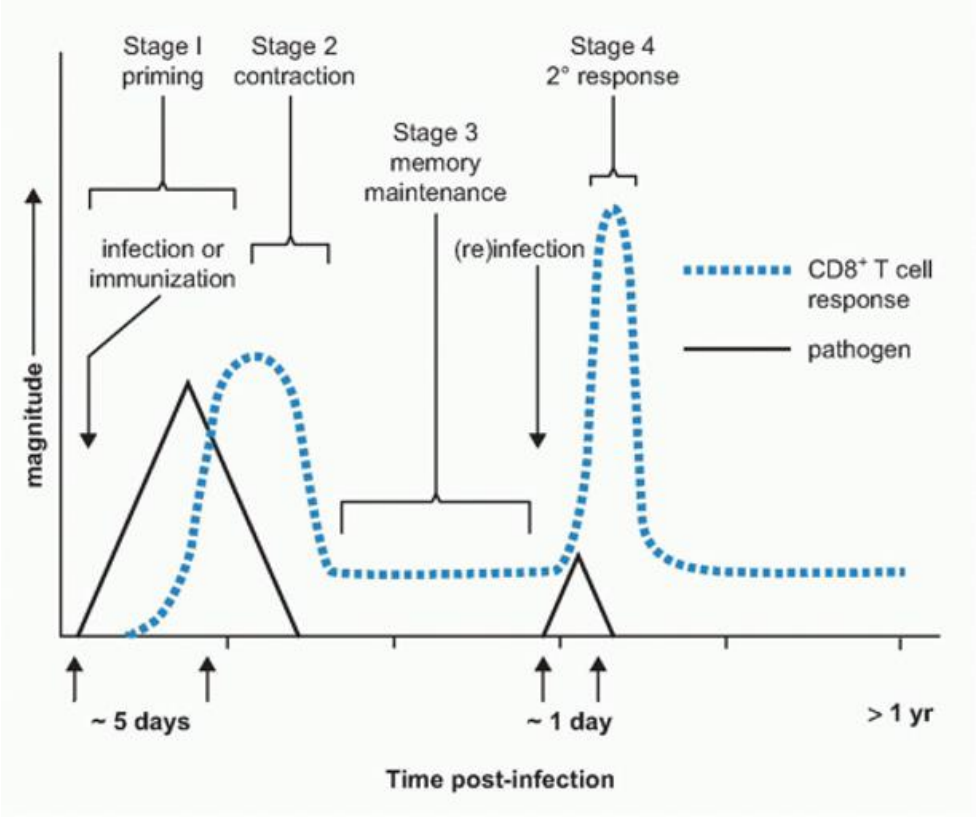

Figure 7: Kinetics of primary and secondary response (Paul, 2013).

The knowledge on immunological memory is certainly very ancient and originates by smart observations made when medical efforts were focused to contain or eradicate infectious diseases that plagued the population. An example is represented by one the most famous and historical vaccination attempt known as variolation. This practice, very common in China in the 10^{th} century, consisted in inoculating healthy subjects with material coming from the pustules of subjects suffering from smallpox (an infectious disease caused by Poxviruses) (Hume et al., 1940). This method was thought to protect from infection by the virus, but actually induced the development of a very serious and infectious form of smallpox. Therefore, it was necessary to develop less risky and more effective vaccination therapies. A very important advancement was obtained by Edward Jenner, who developed the smallpox vaccine in 1796. This vaccine consisted of materials coming from the pustules of the milkmaids infected with cowpox and was able to induce a very mild form of the disease inducing protection against human smallpox infection. Jenner hypothesized that a vaccine composed of organisms related to those that induce human disease could induce protective immunity. His work and the subsequent global scale vaccination enabled the complete eradication of smallpox in 1960 (Edward Jenner, 1798; De Micheli and Izaquirre-Avila., 2011).

Over the years, numerous vaccines have been produced, some of which are to date mandatory in childhood. Vaccines can consist of the whole organism (attenuated and inactivated vaccines), of purified antigens (subunit vaccines) or of immunogenic proteins produced in the laboratory, or nucleic acids such as DNA and RNA (engineered vaccines). Most of the vaccines need microbial products or other substances able to activate the innate response, thus allowing a complete induction of the adaptive response. These are called adjuvants. For example, subunit vaccines may be purely immunogenic and require addition of adjuvants to increase their immunogenic properties. Indeed, adjuvants do not have antigenic properties but act by enhancing DC function, for example by increasing MHC and costimulatory molecule expression (Abbas et al., 2013). Many of the current efforts are concentrated on the development of vaccines that

induce both a strong humoral response (production of neutralizing antibodies) capable of blocking the infection, and a strong cellular response with the production of memory CD8 T cells capable of eliminating the infected cells (Seder et al., 2000).

3.1 Memory T cells

Memory T cells are a heterogenous population of T cells that persist in the body after the resolution of the primary response, circulating through the blood flow or residing in the tissues. In humans, memory T cells are generated in response to several antigenic exposures leading to the creation of a memory pool characterized by different antigenic specificities (polyclonal population), many of which are generated in the first years of life in response to widespread pathogens, such as CMV (cytomegalovirus), and remain in the body throughout adulthood.

Monitoring antigen-specific CD8 T cells *ex vivo* can be crucial for the clinical evaluation of several diseases and for the design of new therapeutic approaches. T cell response can be evaluated by phenotypical analysis of T cell progeny at different times after antigen encounter. A series of membrane markers can be used to discriminate between naïve, effector and memory T cells. This is sometimes indicated as membrane memory phenotype. In humans naïve T cells express a 200kDa isoform of the common leukocyte antigen (CD45) called CD45RA, while memory and effector T cells express a 180kDa alternative RNA splicing isoform that miss the exon A and is called CD45R0. In mice, the main marker for memory T cells is the Hyaluronic acid receptor CD44, expressed at low levels by naïve T cells and at high-level by activated and memory cells. In addition, naïve and memory T cells express low levels of the IL-2 receptor (CD25), but high levels of the IL-7 receptor consisting of an α chain (CD127) and a γ chain (CD132, also called common γ chain as it is shared with other interleukin receptors). The expression of CD127 is essential for Il-7 responsiveness and memory T cell maintenance (Samji and Khanna; 2017).

However, membrane phenotype is transient and can be modulated by several factors in the microenvironment, and indeed it does not depict the presence of antigen-specific CD8 T cells in a specific phase of the response. Furthermore, T cells identified by memory phenotype are a heterogeneous mixture of cells with different antigen-specificity and they do not give information on antigen-specific responses. Indeed, qualitative and quantitative analysis of antigen-specific T cells population results much more instrumental to evaluate the properties of the T cell response. Technical improvements have been performed over the years to allow the identification and quantification of antigen-specific T cells. Most of these methods are flow cytometry-based methods, for example the staining of cells with multimers MHC-peptide conjugated with fluorochromes. These meothods are currently used in research laboratories, but their exploitation in the clinic is increasing. Unfortunately, the use of MHC-peptide multimers has several limitations in humans, for example multimers commercially available are only for some allelic variants of MHC molecules, thus MHC typing of human samples is necessary before starting to track an antigen-specific T cell response (Farber et al., 2014).

3.2 Memory T cell subsets

Different subpopulations of memory T cells have been identified based on membrane marker expression, proliferative potential, localization and function.

Memory T cells can be divided into two main subsets: Effector Memory (TEMs) and Central Memory T cells (TCMs). Both subsets are present in the blood. TEMs, that have low expression of CD62L, migrate to the site of infection, have low proliferative capacity but maintain great effector function. On the contrary TCMs express high levels of CD62L by which they are retained in lymphoid organs and seem to be implicated in the maintenance of memory pool by self-renewal proliferation (Sallusto et al., 1999). It is still unclear whether TEMs can differentiate into TCMs, and vice versa. Thus, despite these subsets are commonly used to track T cell responses, there is a need for further

investigation. The so-called TEMRA cells ($CD45RA^+$ TEMs) exhibit greater perforin production and therefore show greater cytotoxic capacity than TEM cells (Geginat et al., 2003). About 10 years ago, Restifo and collaborators have identified a new population of memory T cells that they called Stem Cell Memory T cells stem (TSCM). These cells express CD62L as TCMs, CD45RA as naïve T cells, and have great proliferative potential and self-renewal ability. For this reason, it has been proposed that TSCM could generate all the memory subpopulations (Gattinoni et al., 2011).

Recently, studies in mice have shown that a great proportion of memory T cells are non-circulating cells. These cells, called Resident Memory T cells (TRMs), reside in tissues (such as skin and lungs) after the end of the primary response and are responsible for the immunity at the barrier sites. The main feature of TRMs cells is that they can persist in the organ for long time never going back to the circulation. However, these cells are not fixed but can migrate inside the organ interacting with other immune cells or target cells during secondary response. Many efforts are concentrated to the characterization of these cells in different mouse and human tissues. TRM can be distinguished from circulating memory cells for the expression of CD69, a membrane molecule involved in blocking of S1P receptor and in the retention of these cells in tissues. In addition, some murine TRMs, such as those of the intestine or skin, express CD103, an integrin involved in the migration of T cells into mucosal sites. In humans, phenotypical identification of TRMs is still incomplete (Shenkel & Masopoust, Immunity, 2014; Jameson & Masopost, Immunity, 2018).

3.3 Models of memory T cell differentiation

Memory T cell differentiation is a very controversial topic and several models have been proposed to explain what are the factors that control memory cell generation during primary response. The two main models of memory T cell differentiation are the divergent and the linear mode (Figure 8).

The divergent differentiation model (Figure 8a) proposes that the differentiation of effector T cells and memory occurs independently. According to this model, after antigen encounter, naïve T cells can differentiate into either effector or memory cells (Kaech et al., 2002). Differentiation into one or the other population could be random or determined by specific signals.

In the linear differentiation models (Figure 8b) naïve T cells differentiate into memory cells passing through subsequent developmental stages, including the stage of effector T cells. Indeed, it has been proposed that the effector T cells that survive the contraction phase give rise first to TEMs and subsequently to TCMs. In this model TEMs and TCMs would represent two differentiation stages of the same cell and not different cellular populations (Bannard et al., 2009; Wherry et al., 2003).

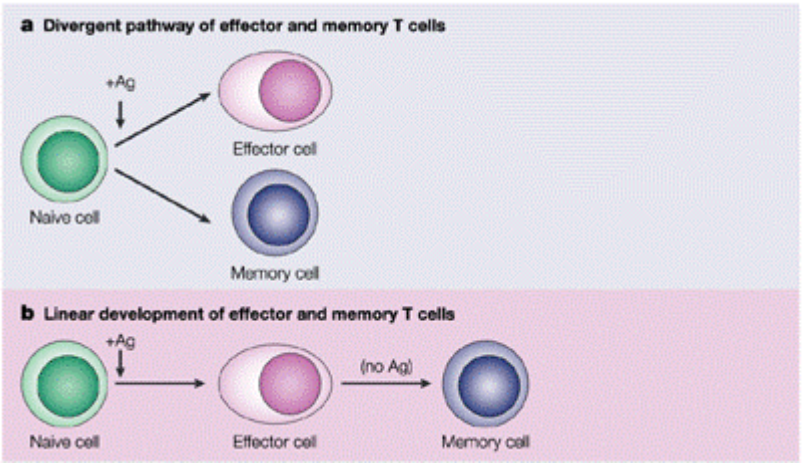

Figure 8: Models of memory T cells differentiation (Kaech et al., 2002).

3.4 New vaccination strategies to induce long-lasting protection

The generation of memory cells able to confer durable long-lasting protection is the main goal of vaccine development. Classical vaccination approaches induce a strong humoral response with a great production of neutralizing antibody, generation of memory B cells and memory CD4 cells required for effective B cells response. Although their success in the past, these approaches are actually ineffective against some human diseases for which no vaccine is still available, and which required the activation of a strong CD8 T cells response. Thanks to their effector function CD8 T cells can kill infected cells expressing antigens from intracellular pathogens (such as M. tuberculosis, HIV, P. berghei) or cancer cells expressing tumor antigens. This CD8 T cells capacity explain why their induction is considered necessary in the context of vaccines development against some incurable infectious diseases and cancer (Seder & Hill, 2000; Nolz & Harty, 2011).

One of the first hurdles in the induction of CD8 T cells response is to achieve strong and long-term response. Single immunization, called “prime”, is not sufficient to induce a robust and durable CD8 T cells response and so new vaccination approaches take advantage on the idea of a second immunization with the same antigen, called “boost”. This strategy exploits the fact that secondary CD8 response is more powerful and rapid than primary one and that antigen-specific CD8 T cells pool increases upon second antigenic challenge (Harty & Badovinac, 2008). One of the first prime-boost approaches developed is the so-called homologous prime-boost vaccination in which prime and boost immunizations are performed using the same vaccine expressing the target antigen. This vaccination is very effective for boosting humoral immunity but induces only a weak secondary CD8 T cells response, because of the induction of neutralizing antibodies after the first exposure to vaccine, which impairs antigen presentation to CD8 T cells upon second vaccine challenge (Nolz & Harty, 2011).

The limitation of homologous prime-boost has been overcome by the development of heterologous prime-boost vaccination strategy, based on the

combination of different deliveries expressing the same target antigen. Indeed, priming with a vaccine vector followed by boosting with a different vector expressing the same antigen increases the frequency of antigen-specific CD8 T cells much more than homologous approach and leads to the generation of CTLs which are able to kill also target cells that express low levels of antigen (high-avidity CTLs) (Estcourt et al., 2002). Vaccine design research is focused now to understand how to improve heterologous prime-boost protocols to develop vaccines for many incurable disease (HIV, malaria, cancer).

4. Memory maintenance

How memory T cells can be maintained for long time in the body is still under investigation. It has been proposed that memory maintenance could be mediated by CD4 T cells, by persisting antigen and by cytokines (Kaech e Wherry, 2007).

CD4 T cells mediate the complete activation of CD8 T cells (licensing) during primary T cell response and they also promote the generation of a sufficient number of memory CD8 T cells. IL-2 plays a key role during the expansion phase and could also induce the generation of memory cells with better capacity of reacting to subsequent antigen exposures. Nevertheless, studies in mice have obtained opposing results regarding the role of CD4 T cells in the maintenance of CD8 T cell memory (Kaech e Wherry, 2007; Sun et al., 2004).

For long time the antigen was considered as one of the most important signals for memory T cell maintenance. Indeed it was believed that memory T cells could persist over time only in the presence of residual amounts of antigen at the end of the primary response. It was also proposed that exposure to cross-reactive antigens in the environment was important for memory T cell maintenance. Indeed, cross-reactive antigens have some molecular similarity with the antigen that induced the primary response and are able to stimulate antigen-specific memory T cells. Although the antigen is not required to

maintain memory in controlled experimental conditions, its role cannot be excluded in humans. In the mouse, adoptive transfer experiments (transfer of cells from a donor mouse to a recipient mouse, usually intravenously) have shown that memory CD8+ T cells purified from mice immunized with Lymphocytic Choriomeningitis Virus (LCMV) persist for long time in LCMV-negative recipient mice that do not express MHC class I molecules (class I MHC knockout mice), thus lacking antigenic presentation. Furthermore, to exclude the possibility that antigen recognition could occur through MHC class I molecules expressed by the LCMV-specific CD8 T cells, a similar experiment was performed by isolating memory CD8 T cells that do not express MHC class I molecules from chimeric mice (generated by bone marrow transplantation from MHC class I knockout mice to previously irradiated healthy mice) and transferring these cells into MHC class I knockout mice. The results demonstrated that memory CD8 T cells can persist also in the absence of antigen, o MHC-I expression in the recipient mouse (Murali-Krishna et al., 1999).

Finally, cytokines certainly modulate memory maintenance. On one hand, IL-7 and IL-15 have a positive effect on memory and are key for the stability of the memory cell pool over time. Indeed, IL-15 knockout mice show a reduced number of circulating memory CD8 T cells and it has been shown that IL-7 promotes not only the development of T cells in the thymus but also memory CD8 T cell survival. Both of these cytokines bind to receptors that contain the common γ chain (CD132) and can support memory T cell proliferation and survival, for example by stimulating the synthesis of anti-apoptotic proteins (Harty and Badovinac, 2008; Carrio et al. , 2007; Parretta et al., 2005; Kennedy et al., 2000).

On the other hand, high amounts of inflammatory cytokines (for example IFN-γ and IL-12) result in a greater clonal expansion, and a greater cell death during the contraction phase, with consequent reduction of the memory T cell pool. Therefore, increase of inflammatory cytokines has a negative effect on the memory T cells generation and maintenance.

4.1 Role of the Bone Marrow in the maintenance of memory T cells

Bone marrow is the major site for the formation of mature blood cells (haematopoiesis) after birth. It is a highly vascularized primary lymphoid organ that contains cells of hematopoietic origin and heterogeneous populations of stromal cells. It is known that bone marrow cells organize themselves forming specific microenvironments, called "niches" in which cells release several factors and express molecules that contribute to bone marrow functions (for example hematopoietic stem cell (HSC) reside in specialized niches within the organ (Asada et al., 2017).

In addition to the well-known hematopoietic function, bone marrow is also considered an important site for the maintenance of both long-lived plasma cells and memory T cells (Di Rosa and Pabst, 2005; Palendira et al., 2008; Tokoyoda et al., 2009; Tokoyoda et al., 2010). Several studies reveal that upon primary immune response memory T cells preferentially home the bone marrow in which they can persist over time.

Several chemokines, including CXCL12, CCL3, CCL4 and CXCL16, and adhesion molecules regulate memory T cell homing into bone marrow. The principal chemokine involved in this process is CXCL12 that binds the receptor CXCR4 expressed by naïve and memory T cells (Di Rosa & Pabst 2005; Di Rosa, 2009). It has been proposed that some memory cells pass through the bone marrow recirculating with the blood flow, while some cells can stop inside specific bone marrow niches and persist for a period of time sustained by homeostatic proliferation (Figure 9). Memory TRM can be also present in the bone marrow but probably in niches that differ from those of circulating memory cells (Di Rosa and Gebhardt, 2016).

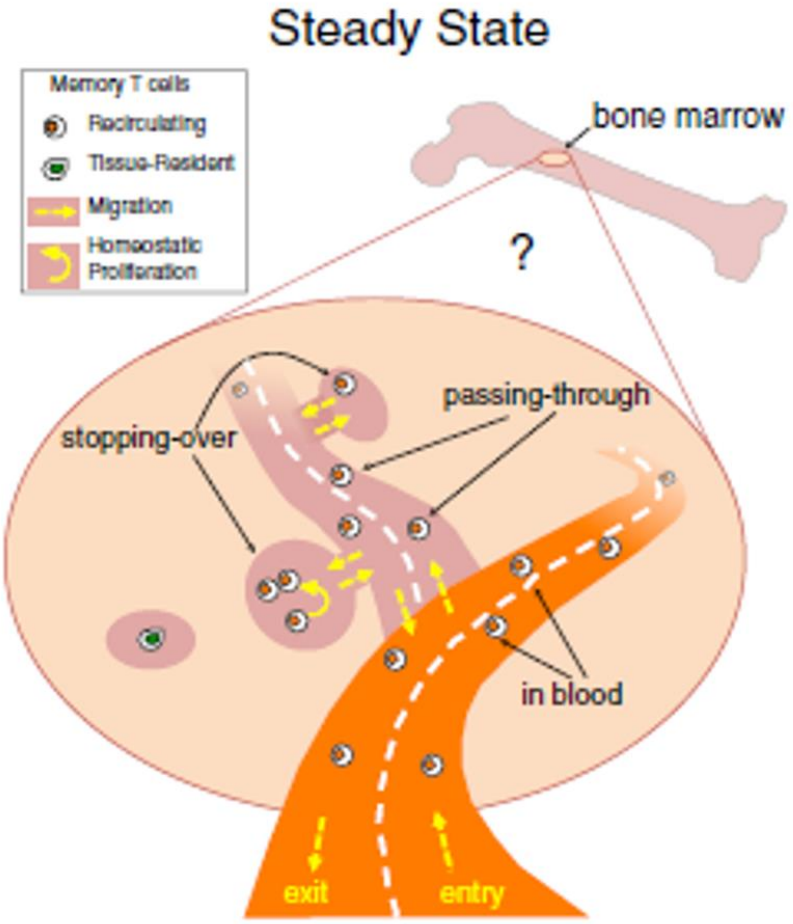

Figure 9: Fate of memory T cell inside the bone marrow (Di Rosa and Gebhardt, 2016)

Despite several studies, the cellular components of the bone marrow niches for memory T cells and how the organ microenvironment interacts with memory T cells (release of cytokines or cell-cell interactions) mediating their maintenance remain still unclear. It has been suggested that once in the bone marrow, memory T cells could interact with specific cytokines (such as IL-15 and IL-7) and factors released or expressed by bone marrow cells into specific niches that can regulate the proliferation, survival and activation state of memory T cells, similarly to the above mentioned haematopoietic niches. Indeed, within the bone marrow microenvironment memory T cells acquire phenotypic and functional characteristics that distinguish them from the blood and spleen counterpart (Di Rosa e Santoni, 2003; Di Rosa e Pabst, 2005; Zhang et al., 2006). For example, it has been shown that in response to IL-15 murine bone marrow memory CD8 T cells reduce the expression of CD127 (alpha chain of the IL-7 receptor) (Cassese et al., 2007; Quinci et al., 2012). Other studies showed that IL-2 and IL-15 can indirectly induce increase expression of GITR (glucocorticoid-induced TNFR-related protein) by memory T cells in the bone marrow but not in the spleen, and that GITR is required for memory T cells persistence in vivo (Snell et al., 2012). Bone marrow memory T cells also express CD69, possibly involved in the retention into the organ and and/or in the proliferation or survival of memory T cells (Sercan Alp et al., 2015).

4.2 Cell cycle niches for memory T cells

How memory T cells are maintained in the bone marrow is currently an issue of debate. For example, it is still debated whether the memory T cells in the bone marrow simply survive over time, or rather undergo a slow turn-over. In the first case, memory maintenance would be a static process, thus any interventions to obtain a long-term memory should act especially in the priming phase. In the second case memory maintenance would be more dynamic, as memory T cells would undergo a homeostatic proliferation that would support cell turnover by compensating loss due to cell death. It would therefore be possible to hypothesize to modulate memory maintenance more easily even after priming (Sercan Alp et al., 2015; Okhrimenko et al., 2014; Parretta et al., 2005; Becker et al., 2005).

In this context, F. Di Rosa has recently proposed that memory T cells are maintained by both slow proliferation and quiescence (Figure 10 and 11) (Di Rosa F, 2016). In this view quiescence is an actively regulated process like other cell cycle phases and not a passive mechanism of cell persistence over time. Quiescence would be essential for a prompt response to restimulation, in a similar way to what has been shown for hematopoietic stem cells.

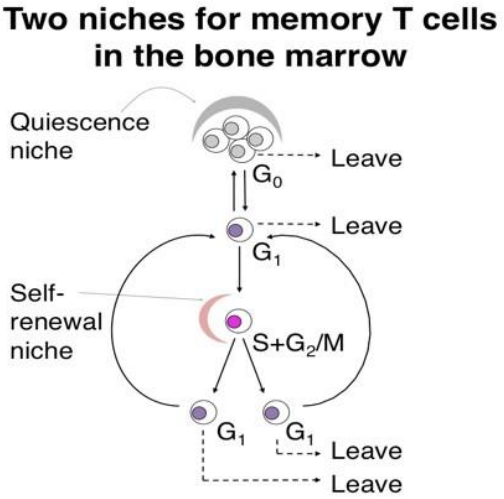

Figure 10: Two cell cycle niches sustain memory T cell maintenance in the bone marrow (Di Rosa, 2016)

Dysregulation of the cell cycle in the memory phase (either self-renewal or quiescence) could affect the ability of antigen-specific memory CD8 T cells to mount a secondary response (Figure 11A), with consequent loss of protection. On one hand, a loss of the slow proliferation occurring predominantly in the bone marrow could lead to decreased number of memory cells, reducing memory CD8 T cell pool over time. On the other hand, a deficiency in quiescence could impair the restimulation of the memory cells in secondary response (Figure 11B) (Di Rosa F, 2016).

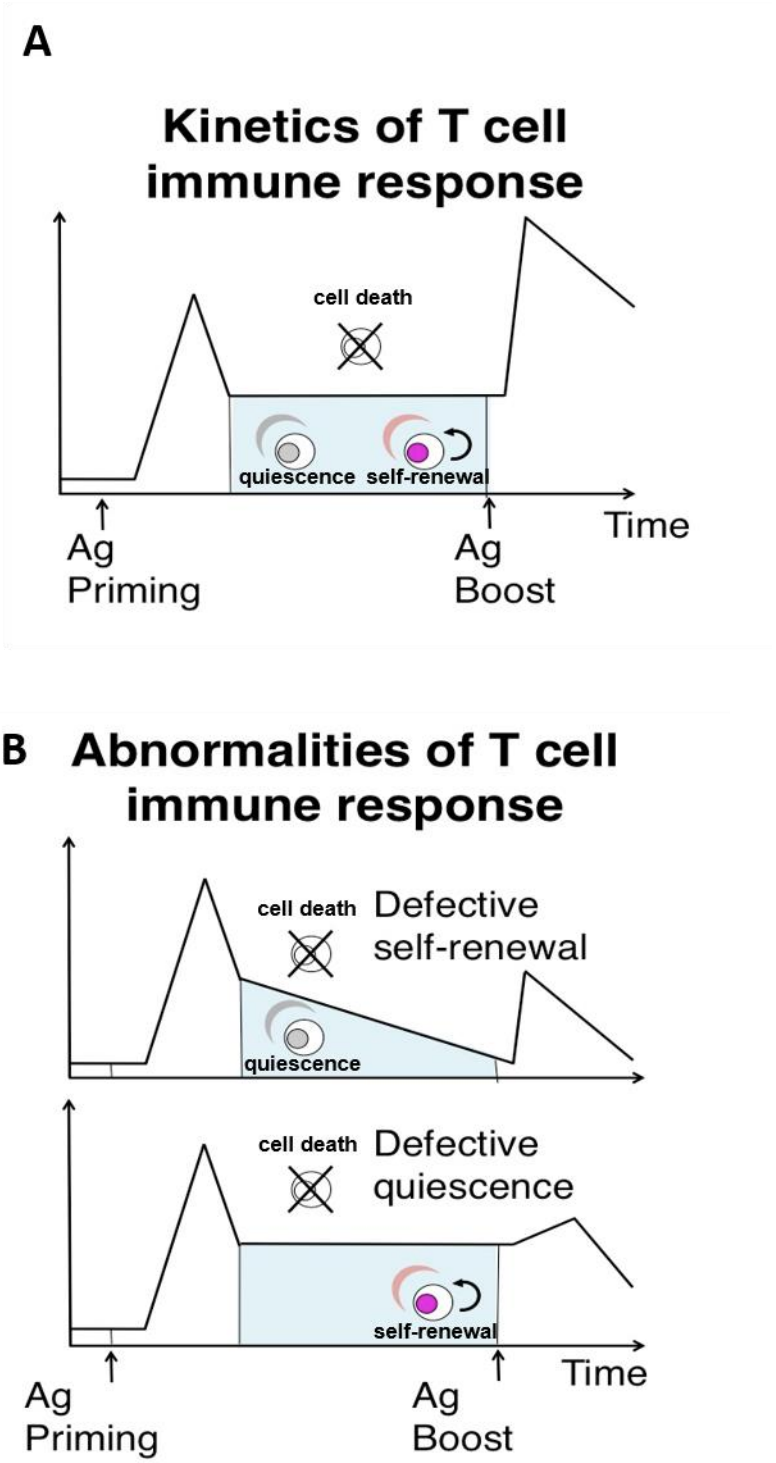

Figure 11: Self-renewal (also called slow proliferation) and quiescence regulate memory T cell maintenance and the effectiveness of secondary response (Di Rosa, 2016). See text for more details.

In this context, a better knowledge of how memory T cell quiescence is regulated could pave the way to modulating memory to infectious disease, and response to vaccination.

Purpose

Our working hypothesis is that a fine balance between different cell cycle phases regulates T cell response both in the acute and in the memory phase of response. We are particularly interested in investigation of quiescence, a currently neglected cell cycle phase of antigen-specific T cell response.

In this project we aimed to:

1) Investigate the kinetic of cell cycle entry and exit of antigen-specific CD8 T cells at early times after vaccination in a mouse model of heterologous prime/boost vaccination.
2) Address whether the kinetics of re-entry in a quiescence state is synchronized in spleen, lymph nodes, bone marrow and blood at late times after vaccination.
3) Correlate the quiescence state with the ability to mount secondary responses at different times after priming.

Materials and Methods

1. Adenoviral and MVA vectors

We used the HIV-1 protein gag as our model antigen. We used a replication-defective, ΔE1 ΔE2 ΔE3, ChAd3 vector encoding HIV-1 gag protein under HCMV promoter (ChAd3- gag) and a Modified Vaccinia Ankara encoding the HIV-1 gag protein under the control of vaccinia p7.5 promoter (MVA-gag). ChAd3-gag was generated as previously described (Colloca S, Barnes E, Folgori A, et al., 2012), amplified in Human Embryonic Kidney (HEK) 293 cells, purified by a two-step caesium chloride gradient ultracentrifugation, and titrated by real-time quantitative polymerase chain reaction (PCR). MVA-gag was generated by in vivo recombination in chicken embryo fibroblast (CEF) cells using Red-to-Green gene swapping method and flow cytometry-based cell sorting for isolation of recombinants (Di Lullo et al., 2009; Di Lullo et al., 2010), propagated in CEF cells, purified by centrifugation through sucrose cushion and quantified by plaque assay.

2. Vaccination

Six-week-old female BALB/c mice were housed and maintained at Plaisant animal facility (Castel Romano, Rome, Italy) according to institutional national and international laws and policies (UE Directive 2010/63/UE; Italian Legislative Decree 26/2014). Mice were primed by intramuscular injection (i.m.) of ChAd3-gag and some of them were analysed after priming only. Subgroups of primed mice were boosted once with MVA-gag i.m., at either day 30 (d30) (range 27-35) day (d) 60 (range 60-67) or d100 (range 95-109) post-prime and analysed at d44 post-boost (range 41-45). Vectors were administered at a dose of 10^7 viral particles (vp) for ChAd3-gag and 10^6 plaque-forming units (pfu) for MVAgag, in a volume of 50 μL per side (100 μL total).

3. Organs

Spleen, lymph nodes (LNs), bone marrow and blood were obtained at different times after either prime or boost, that is, d7, d10, d14 (early times) and d30, d60, d100 (late times) post prime; d3, d7 and d44 post-boost. At each time, the organs were collected from 3 vaccinated and 3 untreated mice, and cells from the 3 mice of each group were pooled. Blood was immediately put into heparin or EDTA blood collection tubes and further processed for analysis. Single cell suspensions from spleen and LNs (iliac and inguinal) were prepared by mechanical disruption and passage through cell strainers, as previously described (Quinci et al., 2012). Bone marrow from femur and tibia of the hind legs was obtained by centrifugation after cutting the bone ends.

4. Membrane staining

Single cell suspensions from spleen, LNs and bone marrow were incubated with Fixable Viability Dye conjugated with eFluor780 fluorochrome and background staining was blocked with anti-FcγR mAb (clone 2.4G2). Cells were then incubated for 15 minutes at 4°C with H-2k(d) AMQMLKETI APC-labelled Tetramer (Tetr-gag) (from NIH Tetramer Core Facility, Atlanta, USA) and PE-labelled Pentamer (Pent-gag) (from Proimmune, Oxford, UK) to stain for gag197-205(gag)-specific CD8 T cells. In the last step cells were incubated for further 15 minutes at 4°C with the following monoclonal antibodies (mAbs): anti-CD3 (clone 145-2C11), anti-CD8α (clone 53-6.7) and anti-CD62L (clone MEL-14) (from BD Biosciences; Biolegend; conjugated with PerCP-Cy5.5, Brilliant Violet (BV) 805; PE-Cy7). Blood samples were incubated for 30 minutes at RT with the above antibodies/reagents that were placed all together. After washing, blood cells were fixed with Cell Fix solution (BD Biosciences) and red cells were lysed with Pharm Lyse solution (BD Biosciences).

5. Intracellular staining for Ki67 and DNA

After membrane staining, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer (Affimetrix, eBioscience) over night. On the following day cells were incubated for 30 minutes at RT after addition of anti-Ki67 antibody (clone SolA-15) (from eBioscience, conjugated with fluorescein isothiocyanate, FITC, or AlexaFluor 700). In some experiments, samples were further incubated with 2µg/ml of Hoechst 33342 (LifeTechnologies) for 15 minutes at RT. After centrifugation at 400 g for 10 minutes, cells were resuspended in PBS and analysed by flow cytometry.

6. Flow cytometry analysis

Samples were analysed by LSRFortessa flow cytometer (BD Biosciences) using DIVA software. $CD3^{(-)}$ cells were gated out when acquiring spleen and bone marrow samples. Data were analysed using FlowJo software, v.10 (FlowJo, Ashland, OR, USA).

7. Estimates of total cell counts

The absolute numbers of gag-specific CD8 T cells were calculated based on their percentages determined by flow cytometry and on cell count estimates. Cells from spleen, LNs and bone marrow were counted by trypan blue exclusion, after lysis of red blood cells. Total counts of LNs, spleen and bone marrow cells were estimated in agreement with previous studies in C57BL/6 mice (Parretta et al., 2005). Mouse white blood cell (WBC) counts/µl and total blood volume were previously reported (Nemzek et al., 2001).

8. Statistical analysis

The vaccinated group was compared with its corresponding untreated group by performing a two-tailed unpaired Student t test with Welch's correction. A two tailed paired Student t test was used for comparison of N and R gates. Friedman test with Dunn's multiple comparison was used for comparison of multiple cell subsets within vaccinated mice. Statistical analysis was performed using Prism v.6.0f, GraphPad Software (La Jolla, CA, USA). Differences were considered significant when $*P \leq .05$. $**P \leq .01$. $***P \leq .001$.

Results

1. Antigen-specific CD8 T cells show myeloid-like morphology at early times after vaccination

To evaluate antigen-specific CD8 T cell expansion after vaccination, female BALB/c mice were vaccinated intramuscularly (im) against the model antigen, HIV-1 gag, using a recombinant chimpanzee-derived adenoviral vector (ChAd3-gag) and a Modified Virus Ankara (MVA-gag) for priming and boosting, respectively. Frequency and proliferation of gag-specific CD8 T cells were analysed at early times after prime (day (d) 7, 10 and d14) and boost (day (d) 3).

Single cell suspensions from lymph nodes (LNs), spleen (SP), bone marrow (BM) and whole blood of untreated and vaccinated mice were stained with dead cell dye for living cell determination and monoclonal antibodies (mAb) conjugated with fluorophores for CD8 T cell marker evaluation (anti-CD3; anti-CD8 and anti-CD62L mAbs). gag-specific CD8 T cells were discriminated by using two MHC-class I-peptide-multimers conjugated with fluorophores, and the cell cycle stages of gag-specific CD8 T cells were analysed using Hoechst 33342, a DNA dye, and anti-Ki67 mAb. Figure 12-A shows a schematic representation of the gating strategy for analysing gag-specific CD8 T cells by flow cytometry. Figure 12-B is an example of LN cell analysis at d3 post-boost. Steps 1-2 identify single cells by DNA analysis and live cells by dead cell marker exclusion. In Step 3 we used Forward Scatter-A (FSC-A) and Side Scatter-A (SSC-A) parameters to identify leukocyte populations. Usually, lymphocytes have low SSC-A and medium-low FSC-A and were discriminated by using the canonical “narrow”, whereas myeloid cells have high SSC-A, and are normally excluded from this lymphocyte gate (Yu et al., 2015; Gordon et al., 2017) (Figure 12B, Step 3, “narrow”). However, by performing back-gating analysis of gag-specific CD8 T cells from LNs of

vaccinated mice, we noticed that most of these lymphocytes had high scatter profile and were outside of the “narrow” (N) gate (Figure 13A). By performing SP cell analysis, we also found an unusual population of cells with high SSC-A that appeared only in the SPs of vaccinated mice. These cells contained a significant proportion of gag-specific CD8 T cells that were not included in the conventional N gate (Figure 13B). Thus, we enlarged our FSC-A/SSC-A gate performing a “relaxed” R gate at Step 3 (Figure 12A-B) before gating on CD8 T cells (Step 4) and antigen-specific T cells (Step 5). Indeed, by representing gag-specific CD8 T cells in FSC-A/SSC-A profiles we found that most of the antigen-specific CD8 T cells were out of the canonical N gate confirming that the R gate was appropriate for our analysis (Figure 12C).

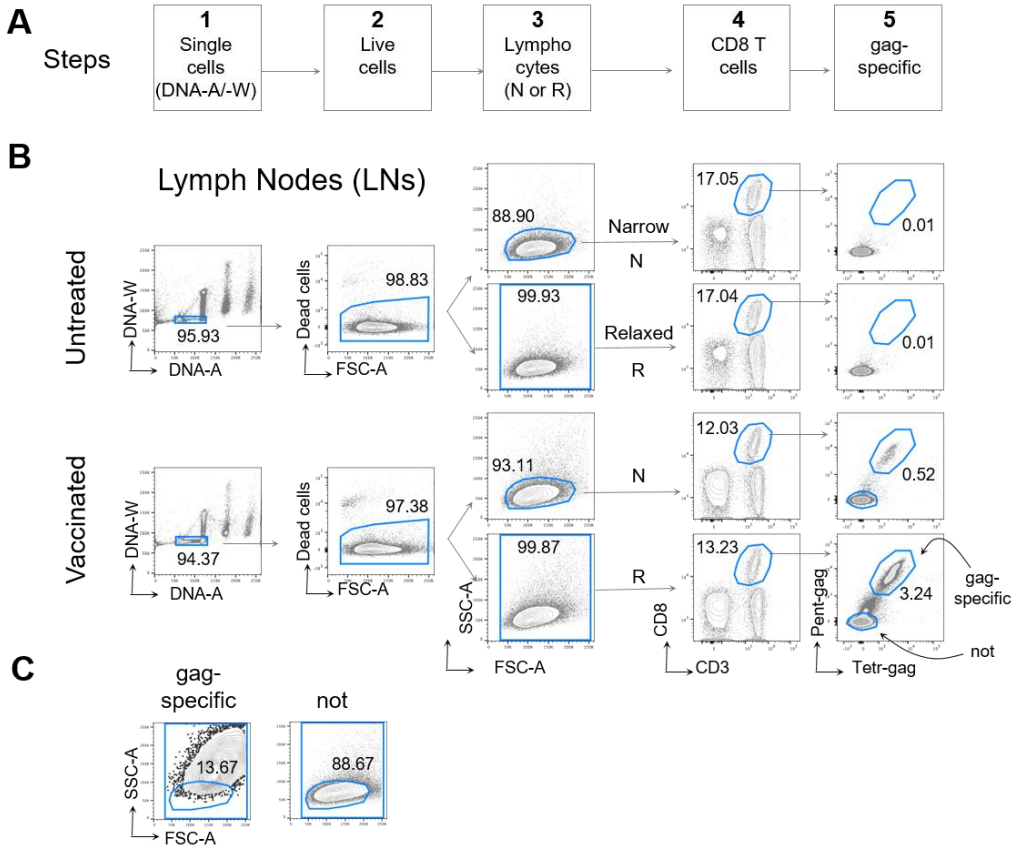

Figure 12: Comparison between Narrow (N) and Relaxed gating strategy for gag-specific CD8 T cell analysis. (A) shows a schematic representation of the gating strategy, (B) an example of LN cell analysis at d3 post-boost. Step 1 shows single cell discrimination by DNA content (DNA-A /W plot), Step 2 dead cell elimination by the eFluor780 Fixable Viability Dye, in Step 3 we used either the canonical N gate or our proposed R gate for lymphocyte analysis in FCS-A/SSC-A plot, at Step 4 we gated on CD3+ CD8+ cells, and at Step 5 we evaluated the percentages of gag197-205 peptide (gag)-specific cells among them, by combined staining with Pent-gag and Tetr-gag. The numbers represent the percentages of cells in the indicated regions. (C) is a typical FSC-A/SSC-A plot of gag-specific (left) and not gag-specific (right) CD8 T cells from LNs of vaccinated mice at d3 post-boost.

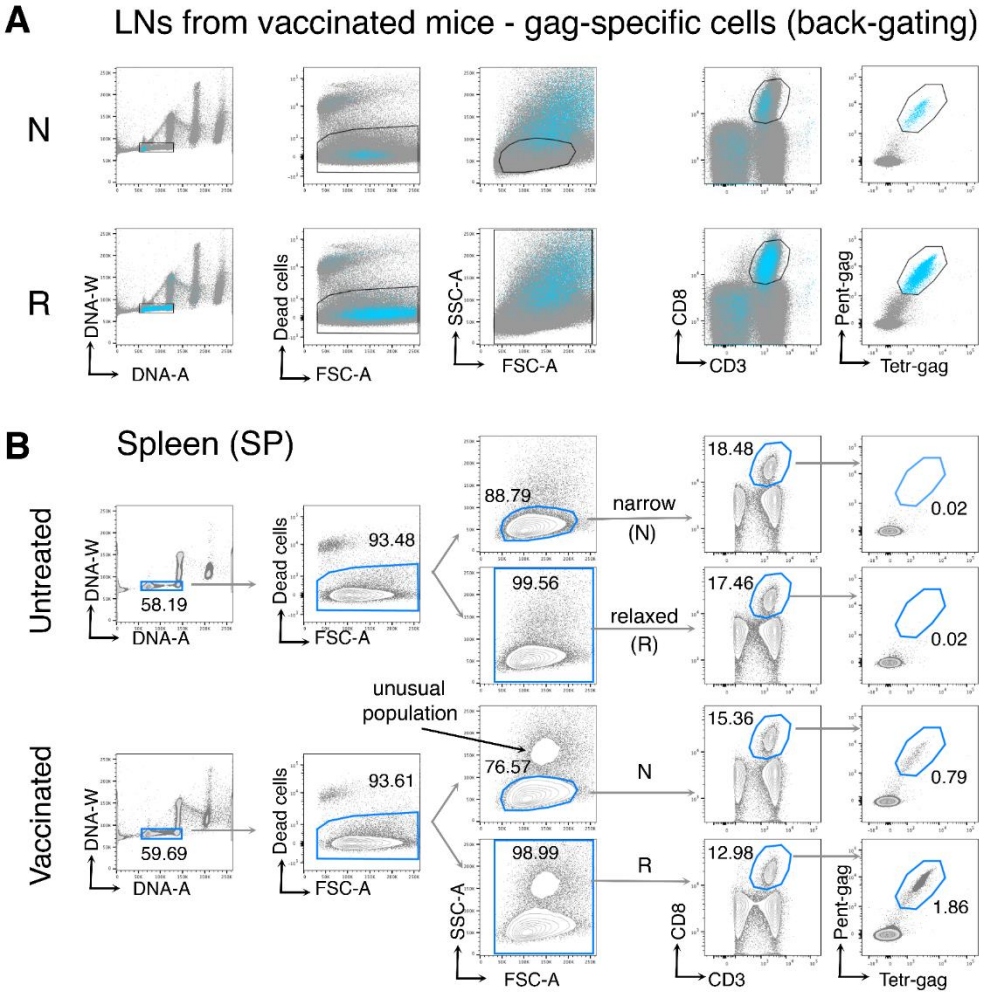

Figure 13:Flow cytometric analysis of gag-specific CD8 T cells in spleen and LNs at d3 post-boost. Spleen and LNs were analysed as in Figure 12. (A) shows a back-gating strategy of LN sample analysed using either the N gate (top) or the R gate (bottom), (B) an example of spleen cell analysis at d3 post-boost. Spleen was analysed with either the N or the R gate using the gating strategy described in Figure 12. CD3(-) cells were gated out when acquiring spleen samples. Black arrow indicates an anusual population in FCS-A/SSC-A plot that appeared only in the vaccinated spleen at d3 post-boost and that was excluded by the N gate

Although the R gating strategy is novel for standard ex vivo studies of lymphocytes, cells with high FSC-A and high SSC-A are often included when examining in vitro activated T cells (Aslan et al., 2017). Notably, in current ex vivo studies of lymphocytes, single cells are normally discriminated in the FSC-A/FSC-H gate (Figure 14A-B) and less frequently by a two-step gating strategy using FSC-H/ FSC-W and SSC-H/SSC-W plots. Our results show that a high

proportion of gag-specific CD8 T cells from LNs of vaccinated mice was outside of these gates (FSC-A/FSC-H; FSC-H/ FSC-W and SSC-H/SSC-W gates) (Figure 14C-D).

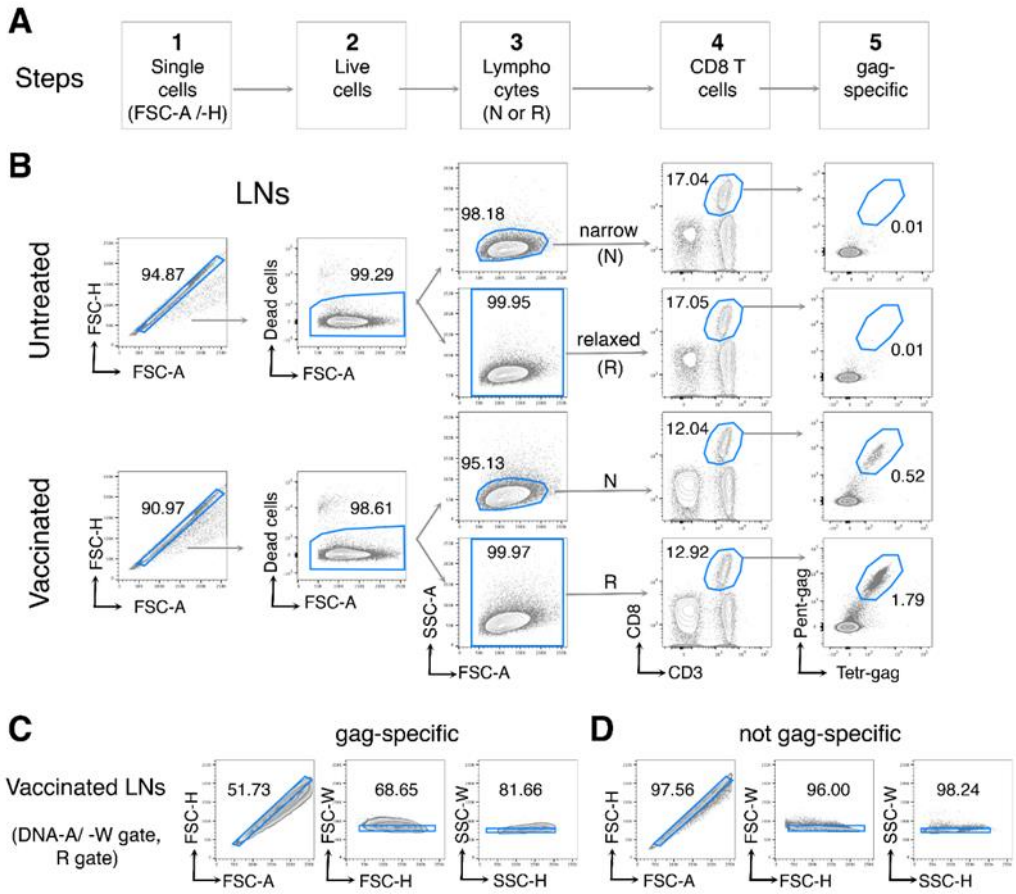

Figure 14 Single cell discrimination based on FSC and SSC parameters for gag-specific CD8 T cells at d3 post-boost. (A) shows a schematic representation of the gating strategy, (B) an example of LN cell analysis at d3 post-boost. Step 1 shows single cell discrimination by FSC (FSC-A /H plot), Step 2 dead cell elimination by the eFluor780 Fixable Viability Dye, in Step 3 we used either the canonical N gate or our proposed gate R gate for lymphocyte analysis in FCS-A/SSC-A plot, at Step 4 we gated on CD3+ CD8+ cells, and at Step 5 we evaluated the percentages of gag-specific cells among them, by combined staining with Pent-gag and Tetr-gag. The numbers represent the percentages of cells in the indicated regions. (C) and (D) show the FSC-H/W and SSC-H/W profiles of gag-specific (left) and not gag-specific (right) CD8 T cells from LNs of vaccinated mice at d3 post-boost, analysed with our R gate strategy (as in Figure 12).

2. Our new gating strategy increases the sensitivity of antigen-specific T cell ex vivo analysis at early times after vaccination

When we compared the N gate to the R gate strategy, we found a significant increase of gag-specific CD8 T cell frequency at d3 post-boost, with a two- to six-fold greater proportion of gag-specific CD8 T cells in the R gated population than in the N gated population in both LNs and SP (Figure 15A). By using the R gate strategy we were also able to increase the sensitivity of the analysis at early times post-prime when the frequency of gag-specific CD8 T cells was very low (less than 1%) in both LNs and SP, without increasing the background in the untreated mice (Figure 15B).

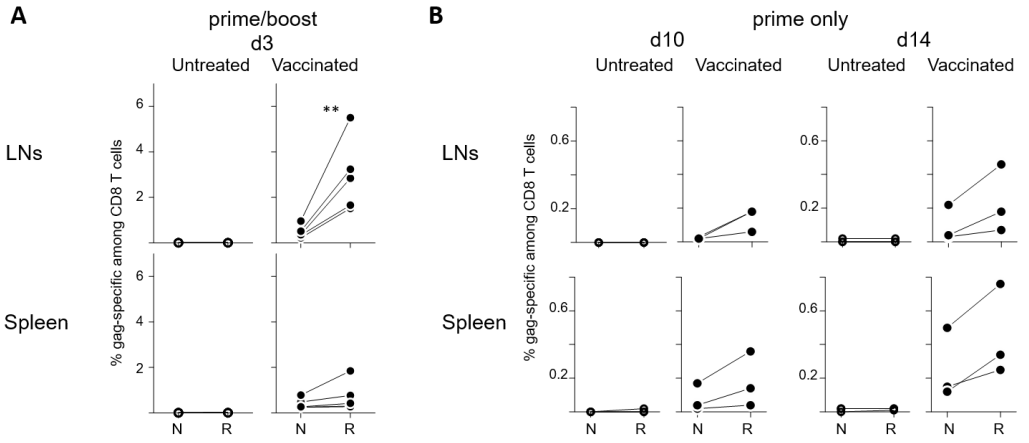

*Figure 15: Frequency of gag-specific CD8 T cells at early times after prime only and prime/boost: comparison of N and R gating strategy. Results were obtained in 5 prime/boost experiments (A) and 3 prime only experiments (B) with a total of 39 mice. Each symbol represents a pool of 3 mice. Statistically significant differences between N and R gates are indicated (**P ≤ 0.01). Differences in the frequency of gag-specific CD8 T cells between untreated and vaccinated mice were statistically significant both in LNs and spleen, using either R or N gating strategy (P ≤ 0.05).*

3. Cycling antigen-specific CD8 T cells can be fully detected by using R gate, but only partially by using N gate

In order to examine gag-specific CD8 T cells in all the phases of the cell cycle, we combined the Ki67 expression with DNA content analysis. This method allowed us to distinguish between gag-specific CD8 T cells in G_0, in G_1, and in S-G_2/M. Cell cycle analysis was performed by using either the N or the R gate (Figure 16). We observed a striking loss of proliferating gag-specific CD8 T cells by using the N gate strategy. Indeed, with the N gate the percentage of dividing cells in S-G_2/M was around 2% of the gag-specific cells in both LNs and SP. On the contrary, the R gate was able to detect all of the dividing cells revealing that these cells made up to 42% of the gag-specific cells in LNs and 26% in SP (Figure 16C-D). Indeed, the progression between the different cell cycle phases (from G_0 to G_1 and from G1 to S-G_2-M phase) occurred in parallel with a graded increase of FSC-A and SSC-A, thus proliferating cells showed extremely high FSC-A and SSC-A (Figure 16B). Results were also confirmed after a single immunization (prime only).

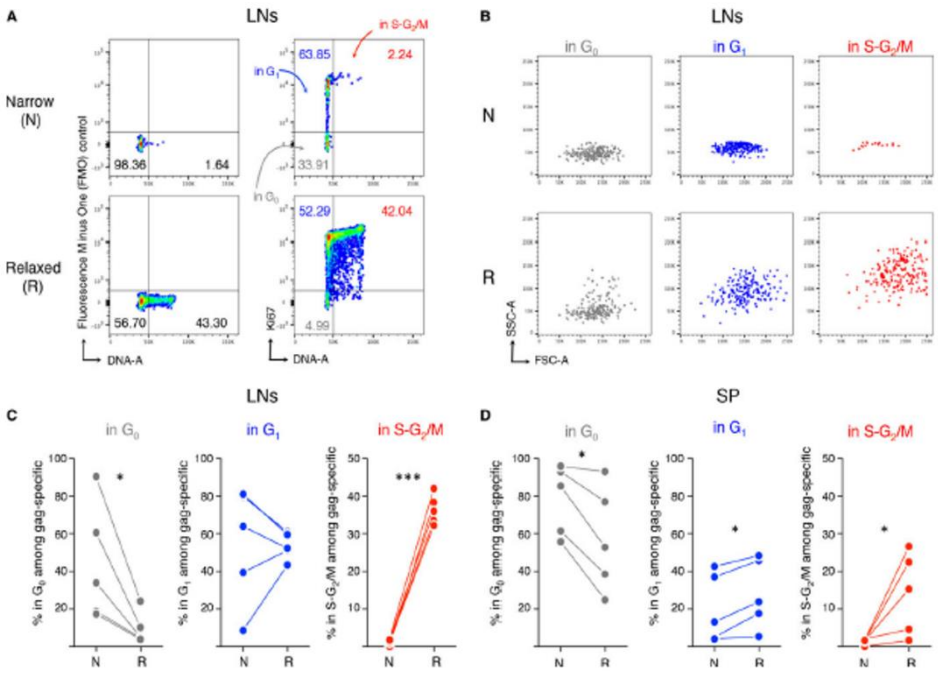

*Figure 16: Comparison between the narrow (N) and the relaxed (R) gating strategy to evaluate cell cycle of gag-specific CD8 T cells from LNs and spleen of vaccinated mice at d3 post-boost. Cell cycle of gag-specific CD8 T cells at d3 post-boost was analysed by Ki67 plus DNA staining, using either the N or the R gate as in Figure 12B. (A) shows a typical DNA/Ki67 staining profiles of LNs, after gating on gag-specific CD8 T cells. Fluorescence Minus One (FMO) controls (left) and Ki67 staining (right). Based on DNA and Ki67 staining, cells in the following phases of cell cycle were identified in the corresponding quadrant: cells in G_0 (Ki67-, 2n DNA), cells in G_1 (Ki67+, 2n DNA) and cells in S-G_2/M (Ki67+, 2n<DNA<4n). The numbers represent the percentages of cells in the corresponding quadrant. (B) shows a typical FSC-A/SSC-A plots of LN gag-specific CD8 T cells in G_0, G_1 and S-G_2/M, gated as in A. (C) and (D) show a summary of the percentages of gag-specific CD8 T cells in G_0, in G_1 and in S-G_2/M phases in LNs (C) and spleen (D). Results were obtained from 5 boost experiments with a total of 15 vaccinated mice. Each symbol represents a pool of 3 mice. Statistically significant differences are indicated (*P ≤ 0.05; ***P ≤ 0.001).*

4. Cycling antigen-specific CD8 T cells circulate in the blood at early times after vaccination

In the blood the R gate detected about a third of the gag-specific CD8 T cells that were lost by using the N gate (data not shown), confirming that our strategy was appropriate also for analysis in this organ. We then analysed the kinetic of the response in blood at d3, d7 and d44 post-boost. The frequency of gag-specific CD8 T cells with the R gate was on an average 2% at d3, 36% at d7% and 13% at d44 post-boost (Figure 17A-C). As previously described (Quinn et al., 2013) gag-specific CD8 T cells down-modulated the lymphoid homing marker CD62L (Figure 17B).

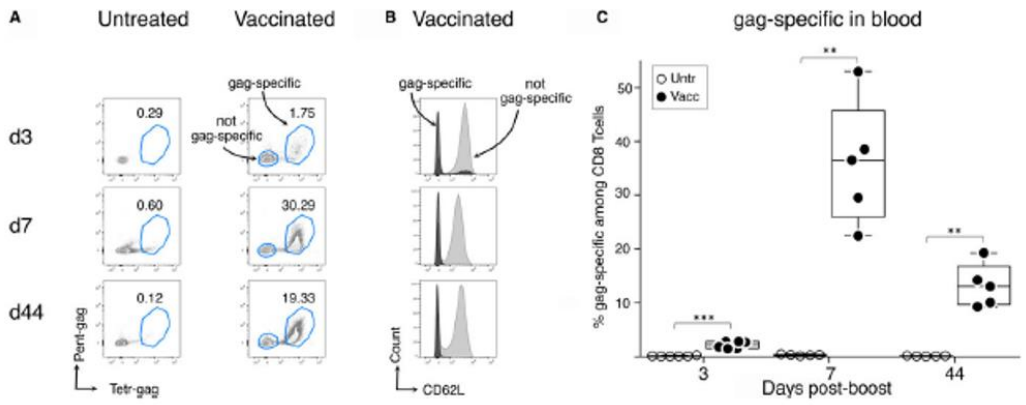

*Figure 17: Analysis of gag-specific CD8 T cell frequency in the blood of vaccinated mice at d3, d7 and d44 post-boost, using R gate. Blood was obtained from untreated and vaccinated mice at d3, d7 and d44 post-boost, and gag-specific CD8 T cells were analysed in 5 steps as in Figure 12A and B, using the R gate at Step 3. (A) shows typical Pent-gag and Tetr-gag staining profiles of CD8 T cells from untreated (left panels) and vaccinated mice (right panels). The numbers represent the percentages of gag-specific cells in the indicated region, after gating on CD3+CD8+cells. (B) shows typical examples of CD62L membrane expression by gag-specific (black histograms) and not gag-specific (grey histograms) CD8 T cells from vaccinated mice, gated as in A. (C) shows a summary of gag-specific CD8 T cell frequencies in the blood of untreated and vaccinated mice. The Figure summarizes results obtained in 6 prime/boost experiments with a total of 60 mice. Each symbol represents a pool of 3 mice. Statistically significant differences between vaccinated and untreated mice are indicated at each time of analysis (**P ≤ 0.01; ***P ≤ 0.001)*

When we evaluated the cell cycle stages of blood gag-specific CD8 T cells we surprisingly found a well-defined population of gag-specific CD8 T cells in S-G_2/M phase at d3 post-boost. This population can be detected only by using the R gate (Figure 18). We also observed dividing antigen-specific CD8 T cells in blood after prime only vaccination. Cells in S-G_2/M could be detected at both d10 and d14 post-prime only by using the R gate (data not shown).

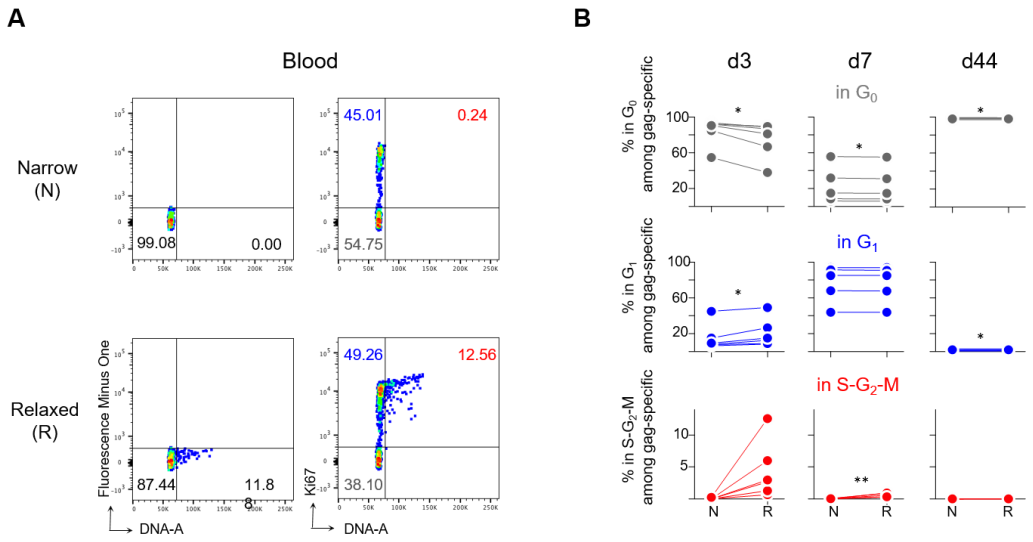

*Figure 18: Comparison between the narrow (N) and the relaxed (R) gating strategy to evaluate cell cycle of gag-specific CD8 T cells from blood of vaccinated mice at d3 post-boost. Cell cycle of gag-specific CD8 T cells at d3 post-boost was analysed by Ki67 plus DNA staining, using either the N or the R gate as in Figure 12B. (A) shows a typical DNA/Ki67 staining profile of blood, after gating on gag-specific CD8 T cells as in Figure 16A. Fluorescence Minus One (FMO) controls (left) and Ki67 staining (right). The numbers represent the percentages of cells in the corresponding quadrant. (B) shows a summary of the percentages of gag-specific CD8 T cells in G_0, in G_1 and in S-G_2/M phases in blood using either the N or the R gate. Results were obtained from 5 boost experiments with a total of 15 vaccinated mice. Each symbol represents a pool of 3 mice. Statistically significant differences are indicated (*P ≤ 0.05; ***P ≤ 0.001).*

Dividing gag-specific CD8 T cells (those in S-G_2/M) were high at d3 (up to 13%) and less evident at d7 (0.3%-0.9%) post-boost. At d7 up to 94 % of gag-specific CD8 T cells were Ki67+, suggesting that Ki67+ cells (non-G_0) persist in blood after actively proliferating cells disappear (Figure 19). At d44, almost all gag-specific CD8 T cells were in G_0 (Figure 19), suggesting that they had mostly switched to a resting memory state.

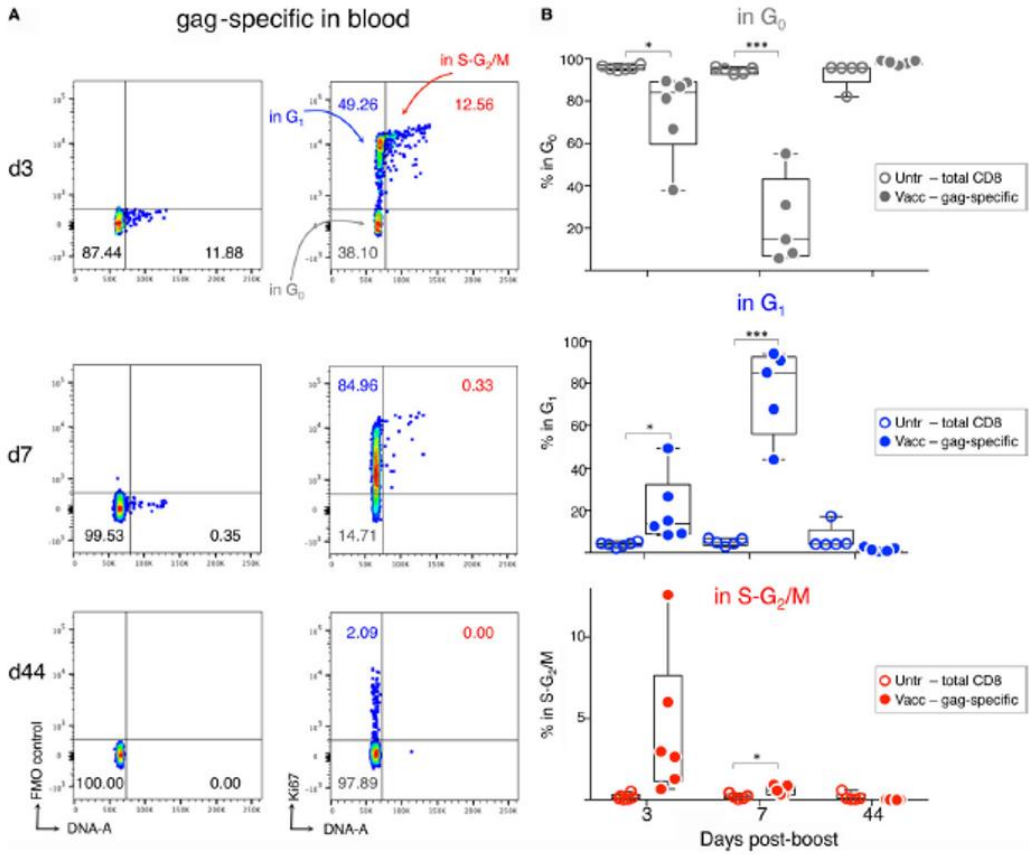

*Figure 19: Cell cycle analysis of gag-specific CD8 T cells in the blood at d3, d7 and d44 post-boost, using R gate. Blood sample were analysed as in Figure 16A. (A) shows typical DNA/Ki67 staining profiles of vaccinated mice, after gating on gag-specific CD8 T cells as in Figure 12A. Fluorescence Minus One (FMO) controls (left) and Ki67 staining (right) are shown, as indicated; the numbers represent the percentages of cells in the corresponding quadrant. (B) shows a summary of the percentages of gag-specific CD8 T cells in G_0 (top), in G_1(middle) and in S-G_2/M (bottom) in the blood of vaccinated mice, compared with corresponding percentages among blood CD8 T cells from untreated controls. The Figure summarizes results obtained in 6 prime/boost experiments with a total of 60 mice. Each symbol represents a pool of 3 mice. Statistically significant differences between vaccinated and untreated mice are indicated at each time of analysis (**P ≤ 0.01; ***P ≤ 0.001)*

5. Specific enrichment of antigen-specific cells in the cycling non-naïve CD8 T cell population in the blood

Although we detected dividing gag-specific CD8 T cells in the blood, the percentage of cells in S-G_2/M among the total CD8 T cells in blood was always very low (about 0.5% or less). We hypothesized that the few dividing CD8 T cells in blood could be enriched in recently activated antigen-specific CD8 T cells in expansion. Thus, we focussed on the expanding CD8 T cells (those with increased DNA content) that were negative for CD62L (CD62L$^{(-)}$ cells), a marker that is generally down-regulated upon activation (see Figure 17B). We evaluated the frequency of gag-specific cells among the CD62L$^{(-)}$ in S-G_2/M (dividing non-naïve cells) CD8 T cells (Figure 20). At d3 post-boost, the average percentage of gag-specific cells among this population was 15-fold higher than among total CD8 T cells (Figure 20C) and up to 70% (Figure 20B). This proportion was higher than the percentage of gag-specific cells measured in CD62L$^{(-)}$ (non-naïve cells) or in CD62L$^{(-)}$ Ki67^{+} (non-G_0 non-naïve cells) CD8 T cell populations (Figure 20B-C). By d7, gag-specific cell percentage was about 40% in the dividing non-naïve and 84% in the non-G_0 non-naïve population. It should be noted that at d7 post-boost CD8 T cells contained a high percentage of gag-specific cells (around 36%) (Figure 17A). Furthermore, at d7 most of the gag-specific CD8 T cells were not proliferating anymore and accumulated in a post-expansion G_1 (Figure 19). By d44, gag-specific cells were decreased in all the populations, though less evidently in the CD62L$^{(-)}$ population (Figure 20C). Similar results were obtained after a single immunization, at both d10 and d14 post-prime.

One implication of our results is that the dividing CD62L$^{(-)}$CD8 T cells in blood could potentially be a valuable source of live antigen-specific proliferating CD8 T cells at early times of response, considering that CD62L is a cell

membrane molecule, and DNA can be visualized using vital dyes. This will be investigated in future studies.

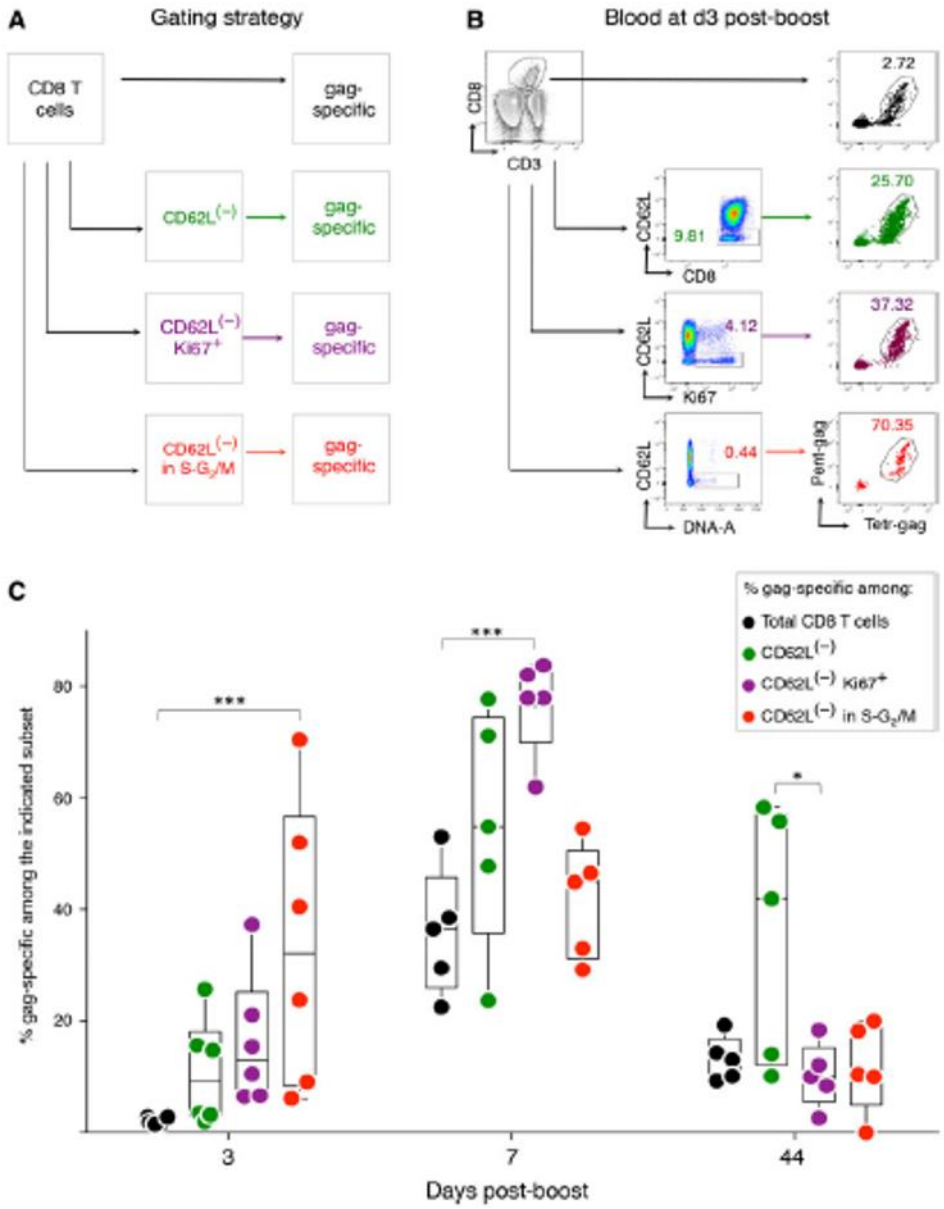

*Figure 20: Specific enrichment of gag-specific CD8 T cells within a population of CD62L$^{(-)}$ CD8 T cells in S-G$_2$/M in the blood of vaccinated mice at d3 post-boost. Blood samples were analysed at d3, d7 and d44 post-boost as in Figures 16 and 18, using the R gate. The frequency of gag-specific CD8 T cells among the following cell populations was determined: total CD8 T cells; CD62L$^{(-)}$ CD8 T cells; Ki67^{+}CD62L$^{(-)}$ CD8 T cells and CD62L$^{(-)}$ CD8 T cells in S-G$_2$/M. (A) shows the gating strategy, (B) an example of flow cytometry profiles at d3 post-boost. Numbers represent the percentages of cells in the indicated regions. (C) show a summary of the results. The Figure summarizes results obtained in 6 prime/boost experiments with a total of 30 vaccinated mice. Each symbol represents a pool of 3 mice. Statistically significant differences are indicated at each time of analysis (*P ≤ 0.05; ***P ≤ 0.001).*

6. Cycling antigen-specific CD8 T cells drastically drop at day 30 after prime

We then focussed on the late times (memory phase) of the primary T cell response, to better investigate exit of gag-specific CD8 T cells form cell cycle and entry into quiescence. Female BALB/c mice were primed i.m. with Chad3-gag and gag-specific CD8 T cells were analysed at d30, d60 and d100 post-prime.

First of all, we evaluated whether our gating strategy was as essential to detect antigen-specific CD8 T cells at late times as it was at early times after priming. We thus examined side by side d10 (early times) and d30 after priming (late times, at the transition from acute to memory phases). Single cell suspensions from LNs, SP, BM and blood were stained as previously described to identify gag-specific CD8 T cells. Cell cycle stages of gag-specific CD8 T were evaluated by staining with Hoechst 33342 dye and anti-Ki67 mAb. For these experiments, we compared our gating strategy, using DNA for single cell discrimination at Step 1, and a relaxed (R) gate for FSC-A/ SSC-A at Step 3 (Figure 21A-B), with an alternative gating strategy in which the canonical FSC-A/FSC-H gate for single cell discrimination was used at Step 1 and the R gate at Step 3 (Figure 21C-D). The alternative gating strategy for LN cell analysis at d10 post-prime resulted in a 5-fold decrease of gag-specific CD8 T cell frequency, as shown in Figure 21D, confirming that responding antigen-specific CD8 T cells were excluded from the usual FSC-A/FSC-H gate at early times after immunization (see also Figure 14C-D). In contrast, no differences were found in the frequency of gag-specific CD8 T cells by using one or the other gating strategy at d30 post-prime (Figure 22). This observation suggests that at late times after immunization antigen-specific CD8 T cells are not any more excluded using the canonical FSC-A/FSC-H gate for single cell discrimination.

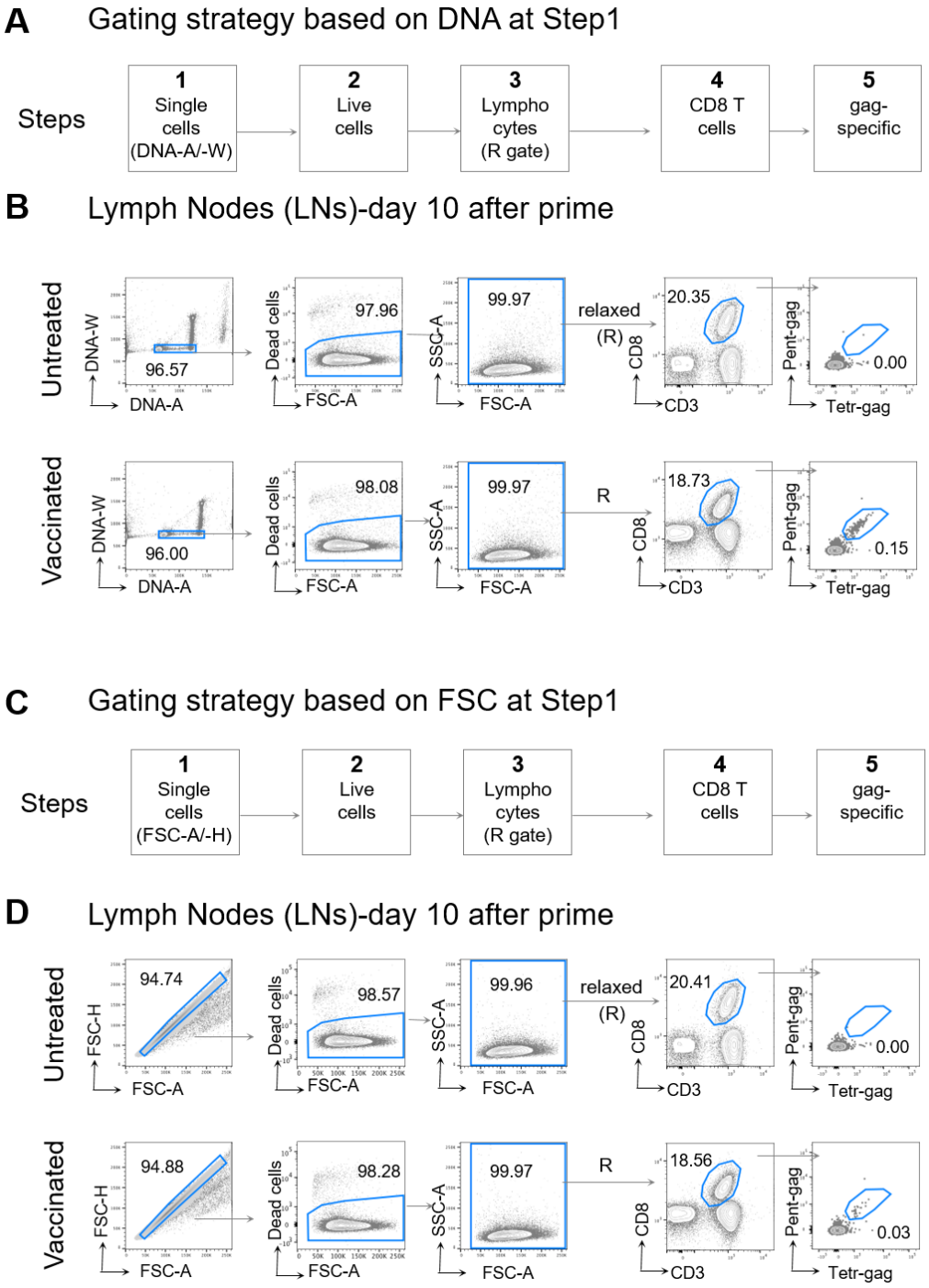

Figure 21: Comparison between DNA-A/W and FSC-A/H single cell discrimination gating strategy for gag-specific CD8 T cell analysis at d10 post-prime. (A) and (C) show a schematic representation of the R gating strategy with either DNA or FSC for single cell discrimination at step 1, respectively. (B) and (D) show corresponding examples of LN cell analysis at d10 post-boost. The numbers represent the percentages of cells in the indicated regions.

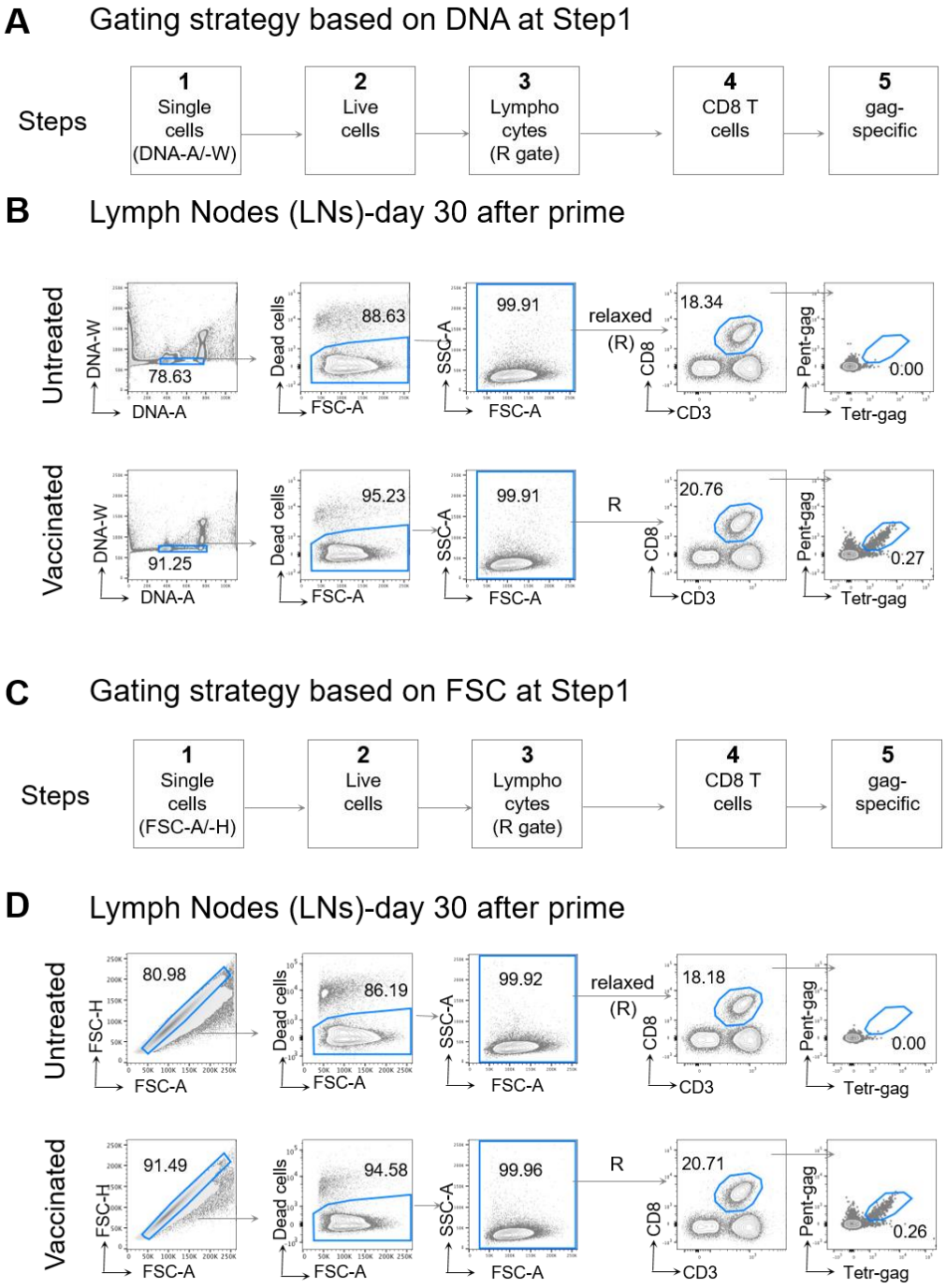

Figure 22: Comparison between DNA-A/W and FSC-A/H single cell discrimination gating strategy for gag-specific CD8 T cell analysis at d30 post-prime. (A) and (C) show a schematic representation of the R gating strategy with either DNA or FSC for single cell discrimination at step 1, respectively. (B) and (D) show corresponding examples of LN cell analysis at d10 post-boost. The numbers represent the percentages of cells in the indicated regions.

We also compared results of cell cycle analysis of gag-specific CD8 T cells using either the DNA-A/DNA-W gate or the FSC-A/FSC-H gate, at d10 and d30 after priming. In these experiments, we examined cell cycle by either a combination of Ki67 and DNA staining (discrimination of 3 sets of cells: those in G_0, in G_1 and in S-G_2-M) or Ki67 expression only (discrimination of 2 sets of cells: those in G_0, and those in G_1-S-G_2-M) (Table 1).

Furthermore, we included also analysis of the bone marrow, in consideration of its well-established role in the memory phase of the response (Table 1). Notably, we observed that the bone marrow played a key role in the response not only at d30, but also at d10, suggesting that gag-specific CD8 T cells homed to this organ early after i.m. vaccination.

At d10 post-prime, most of the dividing gag-specific CD8 T cells were lost by using FSC-A/FSC-H gate strategy, as expected. We observed that cells in S-G_2/M were reduced 30-fold in LNs (from 33.56% to 0.00%), 7-fold in SP (from 11.11% to 1.52%), and 2-fold in the BM (from 28.57% to 14.29%) using single cell discrimination based on FSC. On the other hand, exclusion of cells in S-G_2-M by FSC-A/FSC-H gate resulted in an overestimation of cells in G_0 in LNs and BM.

At d30 post-prime the magnitude of the clonal expansion was strongly reduced, and only few gag-specific CD8 T cells were still in S-G_2/M in the organs (2.66% in LNs, 1.49% in SP and 1.53% in BM). We found that the percentages of cells in G_0, in G_1 and in S-G_2/M (see columns of Ki67/ DNA analysis), and the percentages of cells in G_0 and in G_1-S-G_2-M cells (see columns of Ki67 only analysis) were very similar when we compared the DNA-A/DNA-W and the FSC-A/FSC-H gate strategy, with only a slightly reduction of cells in S-G_2-M by using FSC-A/FSC-H gate.

Taken together, these results suggest that the use of DNA-A/DNA-W gate together with the R gate strategy is essential during the acute phase of the response when antigen-responding cells are actively cycling. In contrast, at late times after immunization, when antigen-responding cells go back to a quiescent state, FSC-A/FSC-H gating strategy is an appropriate method for antigen-

specific CD8 T cell analysis. Furthermore, since only 1-2% of cells are in S-G_2/M at d30, DNA analysis is not as essential at d30 as it is at early times, and Ki67 staining only is a valuable approach to measure the quiescence phase of gag-specific CD8 T cells.

Thus, we used Ki67 staining only in combination with the FSC-A/FSC-H gating strategy to analyse the late times of the gag-specific CD8 T cell response, from d30 to d100 after priming.

			DNA/Ki67 analysis			Ki67 only analysis	
Day after prime	**Organ**	**Strategy**	**%G_0**	**%G_1**	**%S-G_2-M**	**%G_0**	**%G_1-S-G_2-M**
10	LN	DNA	2,01	64,43	33,56	2,01	97,99
	LN	FSC	5,26	94,74	0,00	5,26	94,74
30	LN	DNA	75,06	22,28	2,66	75,06	24,94
	LN	FSC	76,35	22,41	1,23	76,35	23,65
10	SP	DNA	5,19	83,70	11,11	5,19	94,81
	SP	FSC	5,08	93,40	1,52	5,08	94,92
30	SP	DNA	68,36	30,15	1,49	68,36	31,64
	SP	FSC	68,66	30,45	0,90	68,66	31,34
10	BM	DNA	17,14	54,29	28,57	17,14	82,86
	BM	FSC	25,00	60,71	14,29	25,00	75,00
30	BM	DNA	79,08	19,39	1,53	79,08	20,92
	BM	FSC	78,17	19,80	2,03	78,17	21,83

Table 1: Cells from lymph nodes (LNs), spleen (SP) and bone marrow (BM) of primed mice at either d10 and d30 post-primed were analysed as in Figure 21 and Figure 22. Cell cycle analysis was performed as in Figure 5, using the R gate. In the "DNA/Ki67 analysis" column, the table shows the average percentages of cells in G_0, in G_1, in S-G_2/M among gag-specific CD8 T cells from LNs, spleen (SP) and bone marrow (BM) of vaccinated mice. In the "Ki67 analysis" columns, the table shows the average percentages of cells in G_0, and in G_1-S-G_2/M among gag-specific CD8 T cells obtained by evaluating only Ki67 expression. Cells were analysed by using either DNA or FSC to discriminate single cells at Step 1 of the gating strategy as shown in Figure 21 and 22. The table summarize the results obtained in 4 prime experiments with a total of 24 mice.

7. Quiescent antigen-specific CD8 T cells accumulate at late times after prime

We measured the frequency of gag-specific CD8 T cells and their expression of Ki67 to discriminate between cells in G_0 (Ki67 negative cells) and cells in G_1-S-G_2-M (Ki67 positive cells) at d30, d60 and d100 after priming. At these time points, gag-specific CD8 T cell frequency was lower in SP and LNs compared with BM and Blood. Notably, from d30 to d100 gag-specific CD8 T cell frequency showed a tendency to increase in BM and decrease in the Blood (Figure 23A). These results confirm that antigen-specific CD8 T cells home to the BM at late times after immunization.

At d30 gag-specific CD8 T cells were not expanding anymore (few cells were in S-G_2-M phase, see Figure 22). About half of them was still in G_1 and the other half was already back to a quiescent state (G_0). Furthermore, the percentage of gag-specific CD8 T cells expressing Ki67 (i.e. in G_1-S-G_2-M) showed a tendency to be higher in SP and LNs (up to 45%) than in BM and Blood (up to 32%), although there was some variation among experiments (Figure 23B-left panels). The percentage of Ki67positive cells declined at d60 and d100. At d100 post-prime about 98% of the gag-specific CD8 T cells were in G_0 in all the organs (Figure 23B-right panels).

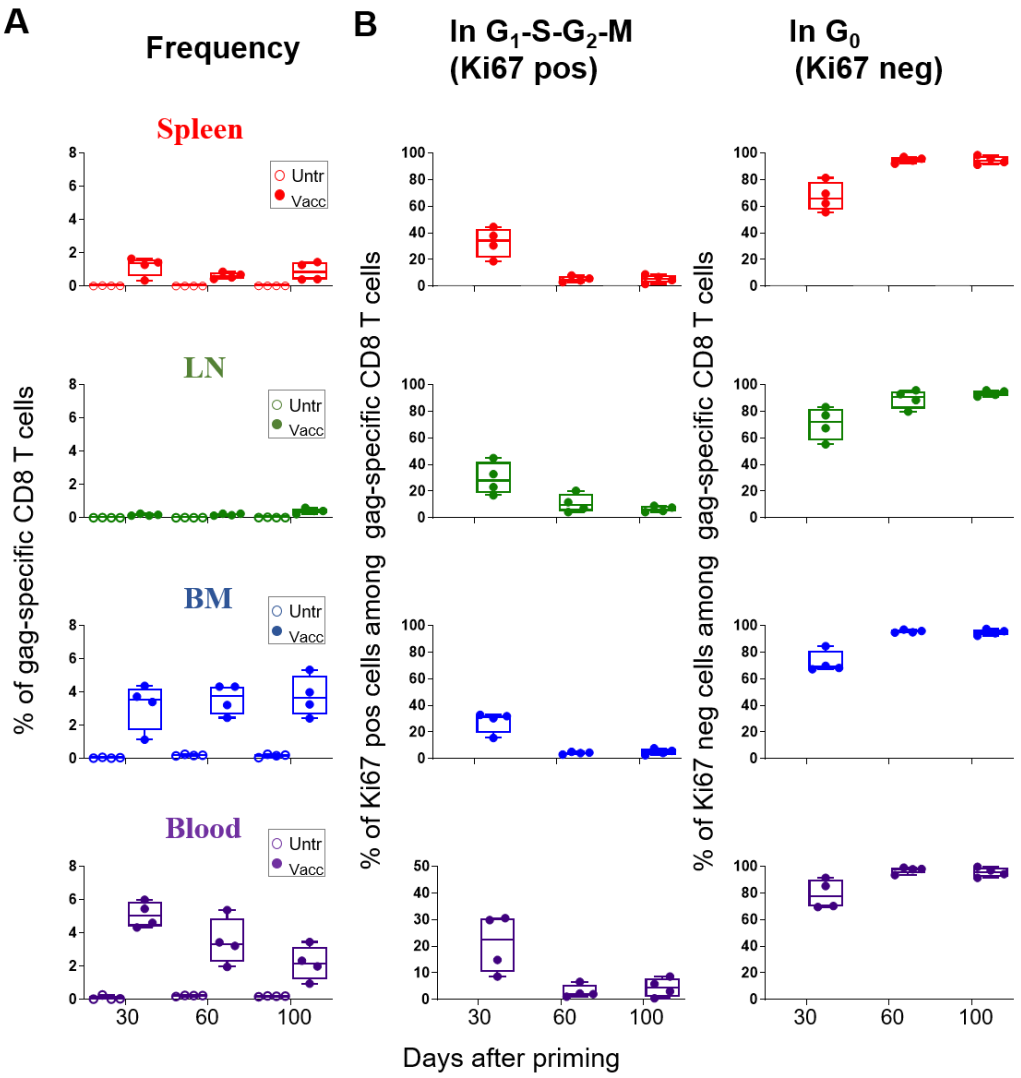

Figure 23: Frequency and Ki67 expression of gag-specific CD8 T cells at late times after prime. Spleen, LNs, bone marrow (BM) and blood were obtained from untreated and vaccinated mice d30, d60 and d100 after prime, and gag-specific CD8 T cells were analysed in 5 steps as in Figure 22C and D, using FSC at Step 1 and the R gate at Step 3. Ki67 expression by gag-specific CD8 T cells was evaluated. (A) shows a summary of gag-specific CD8 T cell frequencies in the organs of untreated and vaccinated mice. (B) shows the percentages of Ki67 positive cells (left panels) and Ki67 negative cells (right panels) among gag-specific CD8 T cells at different times after prime. Results were obtained in 4 prime experiments with a total of 72 mice. Each symbol represents a pool of 3 mice.

8. Boosting at d100 is more effective than boosting at d30 and d60 after prime

We then compared the frequency of antigen-specific CD8 T cells generated by boosting at different times post-prime. A large group of BALB/c mice was primed at d0. Then, individual subgroups were boosted a single time at 3 different time points, i.e. at d30, d60 or d100 post-prime. Frequency of gag-specific CD8 T cells was evaluated at d44 after each boost, to measure a combination of expansion and survival of gag-specific CD8 T cells stimulated in the secondary response.

The frequency of gag-specific CD8 T cells was always higher in the BM than in the other organs, suggesting an important role of the BM in the secondary response, independently of the time of boost (Table 2). Notably, gag-specific CD8 T cell frequency gradually increased after boosting at d30, at d60, and d100. Indeed, boosting at d30 was less efficient than boosting at d60, which showed intermediate values of gag-specific CD8 T cell frequency, while boosting at d100 resulted in the best boosting response (Table 2).

Taken together these results suggest that boosting at d100 is more effective than boosting at earlier times after prime.

	Frequency of gag-specific CD8 T cells at d44 post-boost
Boost at d30 post-prime	
SP	3.96
LN	0.47
BM	9.57
Blood	14.10
Boost at d60 post-prime	
SP	5.19
LN	1.19
BM	13.05
Blood	12.64
Boost at d100 post-prime	
SP	11.67
LN	3.08
BM	27.26
Blood	14.90

Table 2: Analysis of gag-specific CD8 T cell frequency in lymph nodes (LNs), spleen (SP), bone marrow (BM) and blood of vaccinated mice at d44 after three different boosts. A large group of BALB/c mice was primed at d0. Then, individual subgroups were boosted a single time at 3 different time points, i.e. at d30, d60 or d100 post-prime. The frequency of gag-specific CD8 T cells was evaluated at d44 post-boost using FSC based single cell discrimination at Step 1 and the R gate strategy at Step 3 as in Figure 21 and 22 C-D. The table shows the average percentages of 3 prime/boost experiments with a total of 18 mice.

9. Antigen-specific CD8 T cell numbers in SP, LNs, BM and blood are different at d30 and d100 post-prime

We then evaluated the contribution to secondary response of gag-specific CD8 T cells found in SP, LNs, BM and blood. For these experiments, we compared d30 and d100 post-prime, as boosting in these two times resulted in the worst and the best secondary response, respectively.

We calculated the absolute numbers of gag-specific CD8 T cells in SP, LNs, BM and Blood of BALB/c mice at d30 and d100 post-prime. To calculate these numbers, we estimated the absolute number of total cells contained in the examined organs (Table 3), in agreement with previous studies by other groups (Benner et al., 1981; Hunt 1987), and with previous estimates obtained in C57BL/6 mice by our team (Parretta et al., 2005). Thus, for LNs and BM we considered 74 and 336 million of cells in the total body of the mouse, respectively. For the spleen of BALB/c mice we considered 185 million cells, a number about 2-times higher than that of the C57BL/6 mouse spleen (Parretta et al., 2005). For white blood cell (WBCs) count in the mouse whole blood we referred to Nemzek et al., Inflamm Res, 2001, in which the total blood volume was considered around 2 ml and the total number of WBC 8 x 10^6 cells (Table 3).

We then estimated the absolute numbers of gag-specific CD8 T cells in SP, LNs, BM and blood, using the absolute number of total cells contained in the examined organs, the percentage of CD8 T cells and the frequency of gag-specific CD8 T cells obtained by flow cytometry experiments.

Examined Organ	Average Cell Count (x10^6)	Estimated Cell Yield from the Examined Organ (%)	Total Organ	Estimated Percentage of the Total Organ cointained in the Examined Organ (%)	Estimated Total Cell Number (x10^6)
Spleen	166.24	90	Spleen	100	184.72
LNs (inguinal and iliac)	3.87	75	Total LNs	7	73.76
BM from 2 femurs and 2 tibiaes	33.27	55	Total BM	18	336.04
Blood	-	-		-	8.00

Table 3: Organs were collected, and single-cell suspensions were obtained from spleen, inguinal and iliac lymph nodes (LNs) and bone marrow (BM) obtained from femur and tibia of the two hind legs. Cells were counted by trypan blue exclusion, after lysis of RBCs. Average numbers of cell count were obtained from 8 experiments. Our estimates of LNs and BM total cell counts are in agreement with those of Hunt and Benner. For White Blood Cell estimate we referred to Nemzek et al., 2001. The table summarizes the results obtained in 8 groups of experiments

At both d30 and d100 post-prime gag-specific CD8 T cells were more abundant in the SP compared with the other organs. However, while in the SP and in the Blood gag-specific CD8 T cells were reduced, they increased in LNs and in BM between d30 and d100 (Figure 24B). By analysing the number of G_0 cells among gag-specific CD8 T cells we found the same trend (Figure 24D), while cells in G_1-S-G_2-M were always lower at d100 than at d30 in all the organs (Figure 24C). To determine the distribution of different memory CD8 T cell populations at d30 and d100 post-prime, we calculated the number of central CD62L$^{(+)}$ and effector CD62L$^{(-)}$ memory cells among gag-specific CD8 T cells (Figure 24E-F). The majority of gag-specific CD8 T cells were effector memory at both d30 and d100. However in the SP and in the Blood effector memory gag-specific CD8 T cells were reduced between d30 and d100 (Figure 24F). These cells possibly switched their phenotype to central memory cells. Indeed, central memory cells increased between d30 and d100 in all the organs (Figure 24E). Taken together these results suggest that the major contribution to the gag-specific CD8 T cell response was in the SP at both d30 and d100, however at

d100 cells were redistributed from SP and Blood to LNs and BM. These cells were mostly quiescent and still maintained an effector memory phenotype though central memory cells showed a tendency to increase at this time.

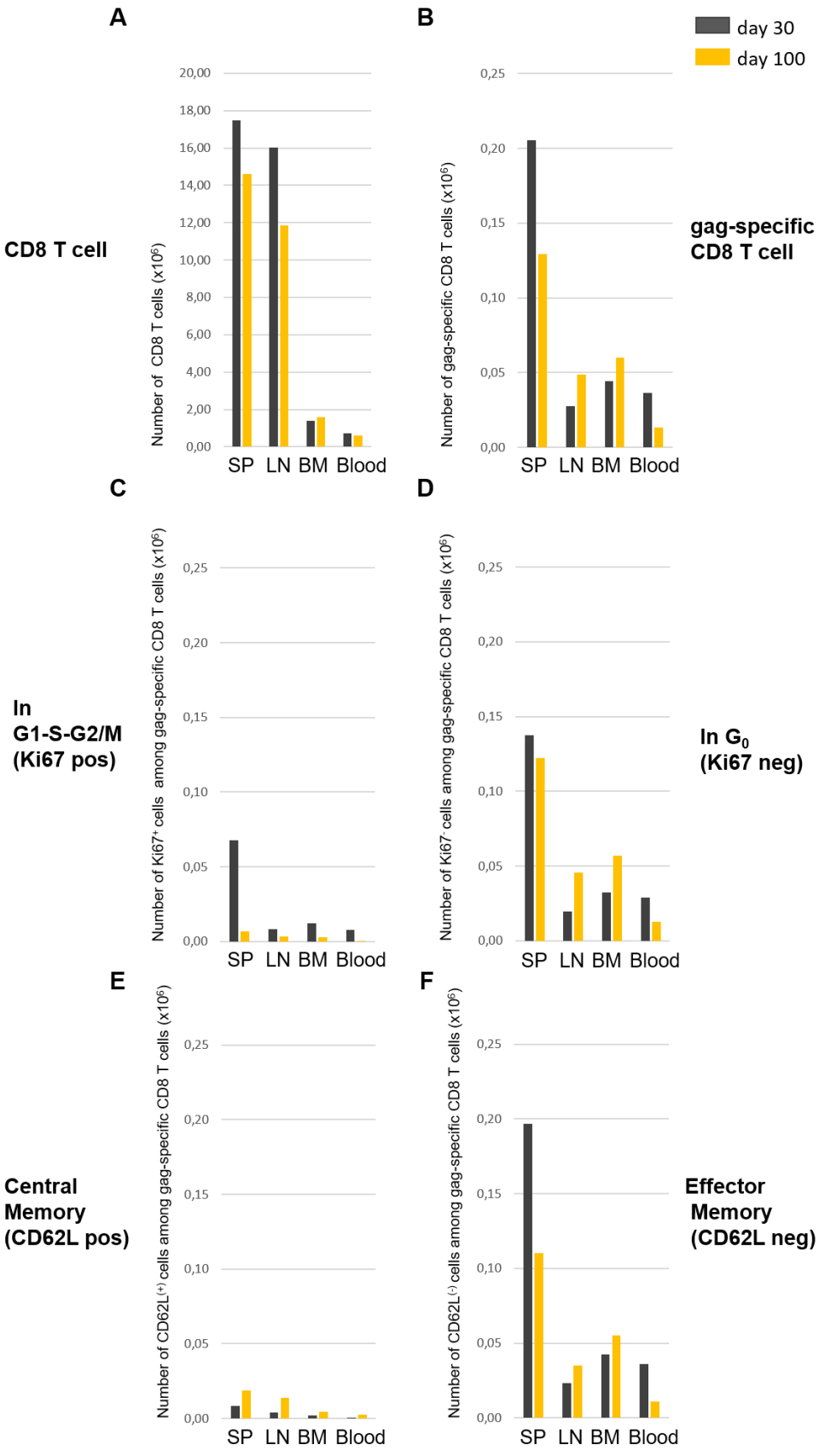
A
B
day 30
day 100
CD8 T cell
Number of CD8 T cells (x10⁶)
20,00
18,00
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
SP LN BM Blood
gag-specific CD8 T cell
Number of gag-specific CD8 T cells (x10⁶)
0,25
0,20
0,15
0,10
0,05
0,00
SP LN BM Blood
C
D
In G1-S-G2/M (Ki67 pos)
Number of Ki67⁺ cells among gag-specific CD8 T cells (x10⁶)
In G0 (Ki67 neg)
Number of Ki67⁻ cells among gag-specific CD8 T cells (x10⁶)
E
F
Central Memory (CD62L pos)
Number of CD62L⁽⁺⁾ cells among gag-specific CD8 T cells (x10⁶)
Effector Memory (CD62L neg)
Number of CD62L⁽⁻⁾ cells among gag-specific CD8 T cells (x10⁶)

Figure 24: Absolute numbers of gag-specific CD8 T cells at d30 and d100 post-prime. The average total number of gag-specific CD8 cells contained in spleen, LNs, BM, and blood was determined by using flow cytometry data and estimated total cell number in each organ (see Table 3). The histograms show the absolute number of CD8 T cells (A); gag-specific CD8 T cells (B);gag-specific CD8 T cells in G1-S-G2/M (C); gag-specific CD8 T cells in G0 (D); central (E) and effector (F) memory gag-specific CD8 T cells (E); at d30 (black bars) and d100 (yellow bars) after prime. The figure summarizes results obtained in 8 prime experiments for a total of 48 mice. Numbers represent millions of cells ($x10^6$).

Discussion

In the first part of the project we analysed the kinetic of the clonal expansion at early times after antigenic stimulation in vaccinated mice. We set up a more sensitive technical approach based on a novel gating strategy for flow cytometry data analysis and a Ki67/DNA staining method for cell cycle evaluation that is not commonly used in CD8 T cell studies, but often used for evaluation of hematopoietic stem cells. We found that actively responding antigen-specific CD8 T cells showed increased FSC-A and unusually high SSC-A, resembling those of myeloid cells. These unique morphological parameters can be likely due to biological changes occurring during the transition from G_1 to S-G_2/M phases, for example changes in mitochondria, chromatin condensation, organelle distribution etc (Darzynkiewicz et al., 1981; Nusse et al., 1990). Cells with high FSC-A and high SSC-A are usually excluded from the analysis of normal lymphocytes ex vivo, even though cells with these characteristics are often included when examining in vitro activated T cells (Aslan et al., 2017).

By using our novel approach, we revealed that proliferating antigen-specific T cells circulate in the blood of vaccinated normal mice at early times after boost in a prime/boost vaccination protocol with ChAd3-gag prime and MVA-gag boost. This result challenges the traditional view by which clonal expansion is initiated and completed within lymphoid niches in tissues (lymphoid organs, sometimes BM or extra-lymphoid tissues (Jones et al., 2016; Siracusa et al., 2018) and suggests a rapid entry of dividing antigen-specific CD8 T cells into the blood circulation. The possibility that clonally expanding CD8 T cells recirculate was neglected so far possibly due to the currently used methods for the analysis of proliferating antigen-specific CD8 T cells. Indeed, T cell clonal expansion has been assessed so far by a few methods, mostly based on the use of proliferating cell specific-labelling dyes (e.g. BrdU, CFSE) and of antibodies specific for the nuclear marker Ki67. These methods do not allow

evaluating whether the labelled cells found in one organ proliferated locally or rather migrated in this organ during or after division (van Stipdonk et al., 2001, Nguyen et al., 2003). Furthermore, Ki67 expression is usually considered a marker of dividing cells while it actually labels cells that are not in the G_0 (or quiescent) phase of cell cycle. Thus, Ki67 does not distinguish cycling cells (in S-G_2/M phases) from those in G_1, which may remain for long time in G_1, or even revert to G_0 without dividing (Di Rosa, 2016).

In our study, proliferating gag-specific CD8 T cells in S-G_2/M were found in the blood of normal mice also after a single dose of ChAd3-gag, although they were fewer than after a boost with MVA-gag, as a result of a lower number of total gag-specific cells. Our results suggest that blood might represent an extremely valuable window for analysis of T cell proliferative response, as well as a source of antigen-activated cycling CD8 T cells also in humans. Considering that almost all the immunological studies in humans use blood samples, our novel sensitive approach can be used to avoid incorrect conclusions and improve the knowledge of CD8 T cell response in several pathological conditions. For example, in both melanoma and non-small-cell-lung cancer (NSCLC) patients it has been shown that unleashed anti-tumor CD8 T cells increase Ki67 expression after treatment with immune checkpoint inhibitors (ICI) (Kamphorst AO et al., 2017; Wieland A et al., 2018). These findings suggest that upon ICI treatment anti-tumor CD8 T cells undergo a proliferative expansion simultaneously to their re-activation. However, Ki67 expression does not discriminate between cells in different cell cycle states and is not a marker of dividing cells. In this case our cell cycle analysis, applied to anti-tumor CD8 T cells, can be an instrumental method to test the efficacy of the therapy by a sensitive evaluation of post-treatment expansion. Thus, we suggest that the widespread use of our technical approach might prevent incomplete data analysis and/or biased interpretations

Finally our results suggest that at early times after stimulation human blood might be the source of enriched populations of recently activated CD8 T cells, proliferating in response to vaccines, infections, transplantation and

cancers, that could be studied, cloned and used therapeutically, even without knowing the antigen to which they are responding, or as a way of searching for that antigen.

The second part of the project was focussed on the analysis of antigen-specific CD8 T cells after a single dose of the priming vaccine (ChAd3-gag) and the comparison of boosts (with MVA-gag) performed at different times after prime. We found that the percentage of antigen-specific CD8 T cells increased in the bone marrow at late times after priming (memory phase), confirming the role of this organ as a reservoir for memory cells. Antigen-specific T cells were still in cell cycle at d30 post-prime (about 50% in G_1, and 1-2% in S-G_2/M). The vast majority of them (>98%) switched to a quiescent phenotype (G_0) at d60 and d100. Interestingly, boosting at d100 resulted in a much higher frequency of antigen-specific CD8 T cells that persisted at d44 after boost.

Furthermore, we found that antigen-specific CD8 T cell numbers diminished in the spleen and in the blood and accumulated in LNs and bone marrow at late time after prime. Quiescent antigen-specific CD8 T cells showed a similar trend, as well as effector memory cells which probably reverted into central memory cells. Indeed, at late times central memory antigen-specific CD8 T cells increased of all the organs.

We suggest that a fine balance between a quiescent pool (almost all cells) and a slow-proliferative pool (very few cells) of memory T cells is established at late times after prime, after d30. Our results could have important implications in human diseases. For example, in the context of vaccine design, an unsolved issue is how to shorten the time between the first (prime) and the second immunization (boost). This is important especially in case of sudden epidemics (such as Ebola) or in highly progressive diseases (Sallusto F et al., 2010). In this context, identification of molecular or cellular correlates of effective secondary response could be instrumental to choose the best times for boosting. It is also important to evaluate the function of cells expanded by boost performed at different times.

Thus, we plan to: 1) perform RNA sequencing analysis of antigen-specific CD8 T cells at late times after prime to identify key molecules that differentiate cells at d30 and d100 post-prime; 2) to compare the functional activity of antigen-specific CD8 T cells obtained after boost at either d30 or d100 post-prime, using in vivo killing assay.

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