Route of immunization and timing for adjuvant delivery : critical factors for efficient T cell cross-priming

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Thèse de doctorat de l'Université Pierre et Marie CURIE

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École doctorale Physiologie et Physiopathologie (ED 394)

Présentée par

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VOIE D'IMMUNISATION ET SÉQUENCE D'ADMINISTRATION DE L'ANTIGÈNE ET DE L'ADJUVANT: FACTEURS CRITIQUES POUR UNE RÉPONSE LYMPHOCYTAIRE T EFFICACE

Soutenue le 4 juillet 2012 devant le jury composé de:

Adrien SIX	Président
Danila VALMORI	Rapporteur
Jose A. VILLADANGOS	Rapporteur
Sebastian AMIGORENA	Examinateur
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Abstract

Most successful vaccines currently in use are based on the generation of protective antibodies. However, $CD8^+$ T cell responses are crucial in the defense against several infectious agents, including HIV or plasmodium, as well as for the treatment of cancer or chronic diseases. Thus, the development of vaccine strategies capable of eliciting robust $CD8^+$ T cell responses is absolutely needed. Antigen cross-presentation is known to be an important mechanism for the activation of antigen-specific $CD8^+$ T cells, and it has been shown that multiple parameters contribute to the efficiency of cross-priming. We examined two of them in detail: the route of immunization and the timing of adjuvant delivery.

Our first priority was to develop and optimize the tetramer-based enrichment strategy and combined its advantages with additional approaches, which allowed us to perform an in-depth study of the kinetics, phenotype and functionality of the endogenous $CD8^+$ T cell response. These approaches permitted us to work within a model reflecting "physiologic" conditions in terms of initial precursor T cell frequency.

We applied these methods to investigate the impact of the route of immunization on $CD8^+$ T cell cross-priming. By comparing different strategies of immunization, we report that local delivery of cell-associated antigen results in delayed cross-priming due to the increased time required for antigen capture and presentation. In comparison, delivery of systemically disseminated antigen resulted in rapid T cell priming. Surprisingly, local injection of cell-associated antigen, while slower to mount a functional response, resulted in the differentiation of a more robust, polyfunctional effector T cell population and an enhanced secondary response. However, the overall diversity and avidity of the responding antigen-specific T cells did not appear to be affected by the route of immunization. Factors such as inflammation induced at the site of injection, dendritic cell (DC) subsets involved in antigen uptake and presentation, or the persistence of antigen may all contribute to the differences observed.

We were next interested in evaluating the combination of cell-associated antigen with the delivery of poly I:C, an adjuvant known to induce the production of type I interferons (IFN). We observed an immunization-route-specific effect regarding the timing of innate immune stimulation and identified the optimal time window for adjuvant administration in order to maximize the boosting effects on $CD8^+$ T cell cross-priming. We characterized in detail several effects of poly I:C, as well as type I IFN, exerted on immune cells, such as the induction of DC maturation and recruitment in lymphoid organs, but also disappearance of the $CD8\alpha^+$ DC subset, providing the basis for our hypotheses as to why adjuvant treatment may lead to either the inhibition or enhancement of cross-priming depending on the timing of delivery.

Together, these studies highlight the importance of working within conditions that reflect the "physiologic" conditions of human vaccination. We demonstrated in a fundamental model, that it is crucial to consider the timing and persistence of antigen presentation, and to coordinate this kinetic with the timing for adjuvant delivery in order to elicit a potent cell-mediated immune response. Similar approaches to those used here in an established experimental model of cross-presentation may also be applied to assess the efficiency of combinatorial therapies and sequence of administration of several treatments in complex human diseases such as cancer or chronic viral diseases.

Keywords: cross-priming, cell-associated antigen, $CD8^+$ T cell, route of immunization, timing of adjuvant delivery, pleiotropic roles of type I interferons, vaccination

Résumé

La mise au point de vaccins efficaces est généralement basée sur le développement d'une réponse anticorps. Néanmoins, la protection contre certains agents infectieux ainsi que le traitement de maladies chroniques ou de cancers nécessite l'induction d'une réponse cellulaire. Le développement de vaccins étant capables d'induire une réponse T CD8 efficace est donc essentiel. La présentation croisée de l'antigène est importante pour l'activation de lymphocytes T CD8 spécifiques. Il a été démontré que de nombreux facteurs participent au développement d'une réponse lymphocytaire T efficace. Nous nous sommes intéressés à deux d'entre eux: la voie d'immunisation, et la séquence d'aministration de l'antigène et d'un adjuvant.

Dans un premier temps, nous avons développé une technique d'enrichissement des lymphocytes T CD8 spécifiques d'un antigène. Nous l'avons combinée avec d'autres approches, telles que le marquage intracellulaire de cytokines ou l'immunoscope, ce qui a permis une étude précise de la réponse T CD8 endogène. Cette stratégie rend possible l'analyse de la réponse lymphocytaire dans des conditions où la fréquence initiale de précurseurs T spécifiques de l'antigène correspond aux conditions physiologiques rencontrées lors d'essais cliniques de vaccination.

Nous avons utilisé cette approche pour étudier l'influence de la voie d'immunisation sur l'efficacité de la réponse lymphocytaire T CD8. Nous avons observé que l'injection intradermique d'un antigène cellulaire induit une réponse T CD8 plus tardive, comparée à une administration par voie systémique. Cependant, la réponse T CD8 induite par une injection locale de l'antigène est plus efficace, avec de nombreux lymphocytes capables de sécréter plusieurs cytokines. Alors que la fonctionalité des lymphocytes T CD8 spécifiques de l'antigène dépend de la voie d'immunisation, leur diversité et leur avidité ne sont pas régulées par ce paramètre. L'inflammation induite par l'injection, les types de cellules dendritiques (DC) impliqués dans la prise en charge et la présentation de l'antigène, ainsi que la persistance de l'antigène sont des facteurs qui pourraient expliquer les différences observées.

Nous avons ensuite évalué l'administration d'un adjuvant – le poly I:C connu pour induire la production d'interférons (IFN) de type I – en parallèle de celle de l'antigène. Nous avons montré que le moment optimal d'administration de l'adjuvant dépend de la voie d'immunisation. De plus, il existe une durée limitée durant laquelle l'adjuvant induit des effets positifs sur l'activation des lymphocytes T CD8. Nous avons identifié plusieurs effets du poly I:C et des IFN de type I sur les cellules du système immunitaire, et plus particulièrement les DC, tels que l'induction de leur maturation , de leur migration dans les ganglions lymphatiques, ou la disparition des DCs CD8α^+. Ces observations nous ont permis de comprendre comment un même adjuvant pouvait avoir des effets opposés en fonction du moment où il était administré.

Nous avons montré l'importance de travailler dans des conditions "physiologiques" et d'étudier la cinétique de la réponse immunitaire de façon à coordonner l'administration de l'adjuvant et de l'antigène. Le même style d'approche peut être appliqué pour évaluer l'efficacité de la combinaison de différent traitements et déterminer leur séquence optimale d'administration dans le cadre de traitement de maladies chroniques ou de cancers.

Mots clés: présentation croisée, antigène cellulaire, lymphocytes T CD8, voie d'immunisation, séquence d'administration de l'adjuvant, interférons de type I, vaccination

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Abbreviations

APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guérin
BM-chimera	Bone-Marrow chimera
cDC	Conventional DC
CDR3	Complementary-determining region 3
CFU	Colony-forming unit
CLR	C-type lectin-like receptor
CTL	Cytotoxic T lymphocyte
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic Cell
dsRNA	double-stranded RNA
DTR	Diphteria Toxin Receptor
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
HSV-TK	Thymidine kinase isolated from HSV
IAP	Inhibitor of Apoptosis
i.d. or ID	Intradermal
IFN	Interferon
IFNAR	Interpheron alpha receptor
IL	Interleukin
i.p.	Intraperitoneal

ISG	Interferon-Stimulated Genes
i.v. or IV	Intravenous
LCMV	Lymphocytic choriomeningitis virus
MDSC	Myeloid-Derived Suppressor Cell
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibiliy Complex
MPEC	Memory Precursor Effector Cell
NI	Non-Immunized
NK cells	Natural Killer cells
NLR	NOD-Like Receptors
OVA	Ovalbumin
PAMP	Pathogen-Associated Molecular Patterns
pDC	Plasmacytoid DC
PDGF	Platelet-Derived Growth Factor
PE	Phycoerythrin
Poly I:C	Polyinosinic- polycytidylic acid
PRR	Pathogen Recognition Receptor
RLR	RIG-I-Like Receptor
ROS	Reactive Oxygen Species
s.c.	Subcutaneous
SFC	Spot Forming Cell
SLEC	Short-Lived Effector Cell
ssRNA	single-stranded RNA
TAA	Tumor-associated antigen
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
TCR	T Cell Receptor

Th	T helper cell
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
YFP	Yellow Fluorescent Protein

Chapter 1: General Introduction

The practice of vaccination began, based on empirical observations, about 200 years ago. Edward Jenner first observed that milkmaids showing symptoms of cowpox appeared to be less susceptible to later infection by smallpox, suggesting that they were protected from smallpox by previous exposure to a similar disease. It was only at the end of the 19th century that the rationale behind vaccination was first appreciated, with the discovery that immune serum contains molecules that can neutralize toxins or bacteria. Methods to inactivate whole bacteria and passage of pathogens in alternative animal hosts, resulting in the selection of less virulent strains were used to develop vaccines against various agents, such as typhoid, cholera and tuberculosis. In the 20th century, new techniques of virus growth in cell culture and genetic engineering allowed for the development of new vaccines (Plotkin and Plotkin, 2011). It became clear that, in many cases, the presence of neutralizing antibodies correlated with the observed protection against the pathogen. The aim then became to render the infectious agents less virulent but still immunogenic. While the presence of antibodies was considered to be the most important characteristic of protection, two vaccines (the Bacille Calmette-Guérin- BCG, and the varicella zoster virus vaccine) were shown to be dependent on T cell immunity.

Indeed, while most of the successful vaccines developed thus far are based on the generation of protective antibodies, it is also crucial to develop vaccines capable of eliciting protective T-cell responses. Vaccines that have been developed successfully have the advantage that they target agents that have limited antigen diversity and thus, can be treated by antibodies (**Figure 1**). However, there remain many diseases for which efficient vaccines are not yet available because of extensive antigen variability and/or the requirement for T cell immunity (Rappuoli and Aderem, 2011). Viruses like HIV that evolve and mutate quickly, or parasites such as Plasmodium that are found in different forms display a variety of antigenic compositions that change frequently. Consequently, neutralizing antibodies are not efficient to mediate potent protection against these pathogens. Moreover, T cell immunity has been shown to be crucial to controlling HIV and Mycobacterium tuberculosis infection. New approaches are thus required to develop vaccines that target these difficult-to-control pathogens.

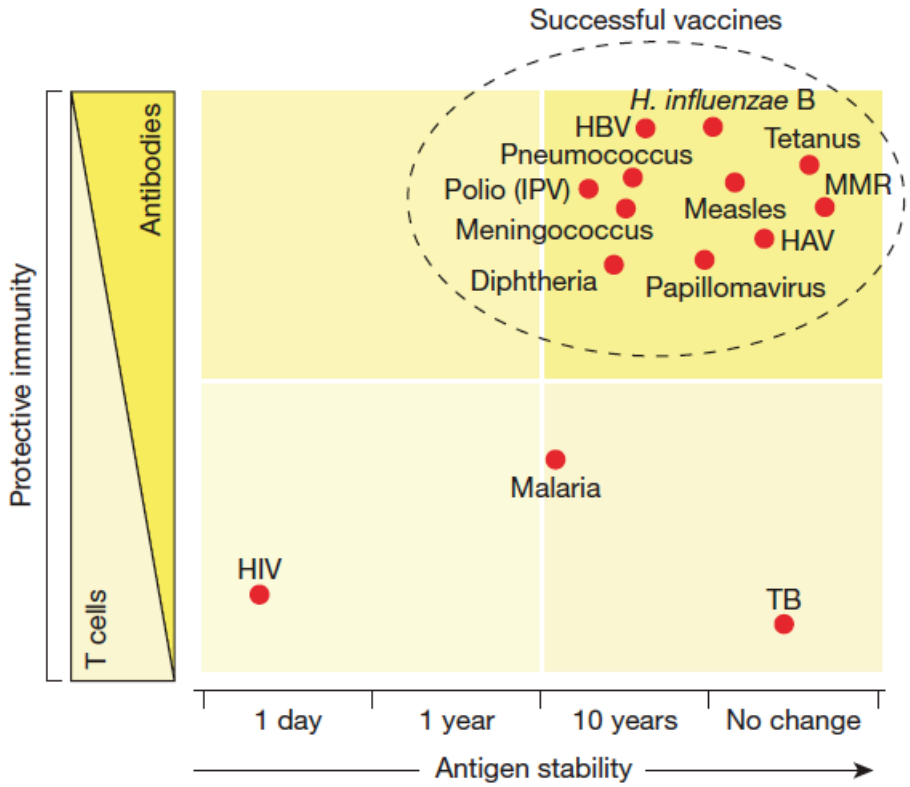


Figure 1. Successful vaccines and challenging infectious diseases. Pathogens are classified depending on the requirement of T cell-immunity and/or humoral response for their clearance, as well as on their antigen stability. TB, Tuberculosis. Figure from *Rappuoli et al., 2011*.

In contrast to the many successful vaccines developed against infectious diseases, few vaccines exist that provide protection against cancer. The concept of cancer immunotherapy first appeared at the end of the 19th century, when Coley observed a tumor reduction upon the injection of bacterial products into the tumor and applied this strategy to the treatment of inoperable sarcomas. Many studies have been performed to further understand the interactions between tumor cells, their microenvironment and, in particular, immune cells. The identification of tumor-associated antigens, the discovery of dendritic cells (DCs), as well as the central role of cytotoxic CD8^{+} T cells in tumor clearance had raised expectations for the development and successful use of tumor vaccines in the clinic. Unfortunately, several mechanisms of tumor-mediated immunosuppression have also been identified, making this goal increasingly complex. Additionaly, cancer vaccines need to be therapeutic, rather than prophylactic, which creates additional complexity when trying to elicit an immune response while also combating tumor-associated immune regulation. Numerous approaches have thus far failed to provide reproducible clinical efficiency despite the development of a measurable anti-tumor response in patients in some cases (Lesterhuis et al., 2011).

For the treatment of cancer, as well as for chronic diseases or infectious agents such as HIV or plasmodium, efficient vaccines eliciting robust T cell responses are urgently needed. This is

why further, detailed understanding of the factors regulating T cell responses as well as the precise action mechanisms of vaccines and other agents is essential. In parallel, efficient experimental strategies to accurately evaluate the antigen-specific $CD8^+$ T cell response must be developed in order to compare different methods of treatment administration, and to determine the efficiency of novel approaches.

In this introduction, I will first present the mechanism of dendritic cell-mediated antigen presentation and the development of antigen-specific T cell responses. Next, I will review how this process can be modulated by the use of different forms of antigen or adjuvants to boost the resulting T cell response. To conclude, I will describe the existing techniques to study these questions in experimental mouse models, detailing their advantages and limitations in a context where it is important to remain as close as possible to physiologic conditions, such that the results can be applied to questions of human immunity.

I. ANTIGEN PRESENTATION BY DENDRITIC CELLS

A. Mechanisms of antigen presentation

Upon the initiation of an immune response, antigen is identified and taken up by antigen-presenting cells (APCs) that proceed to process and present it to specific T cells, resulting in antigen-specific T cell-mediated immunity. The classical view of antigen presentation is divided into two mechanisms: (i) antigens acquired from the extracellular environment (exogenous antigens) are processed and presented on MHC-II molecules and induce the activation of $CD4^+$ T cells; (ii) antigens originating from the endogenous proteins (either self protein or viral proteins expressed upon infection) are presented on MHC-I molecules, leading to the activation of $CD8^+$ cytotoxic T cells. However, a third mechanism has also been described: the cross-presentation pathway, in which extracellular antigens are presented on MHC-I molecules, allowing for the development of a specific $CD8^+$ T cell response. This pathway is now known to be critical for the development of a cytotoxic T cell response against viruses that do not directly infect APCs or against tumors derived from non-APCs. *In vitro* many cells are able to cross-present antigen (DCs, macrophages, B cells); however DCs are the main antigen cross-presenting cell *in vivo* (Heath et al., 2004; Kurts et al., 2001). A detailed overview of these mechanisms will be discussed below.

1) Presentation of intracellular antigen on MHC-I molecules

MHC-I molecules are expressed by all nucleated cells and allow for the presentation of intracellularly synthetized peptides on their surface. Basically, endogenously synthetized proteins are degraded by the proteasome or the immunoproteasome in the cytosol and transferred into the endoplasmic reticulum (ER) through the TAP transporter (Buckwalter and Albert, 2009). They are then loaded on MHC-I molecules, which transit through the Golgi complex to reach the cell surface (**Figure 2, pathway 1, red arrows**).

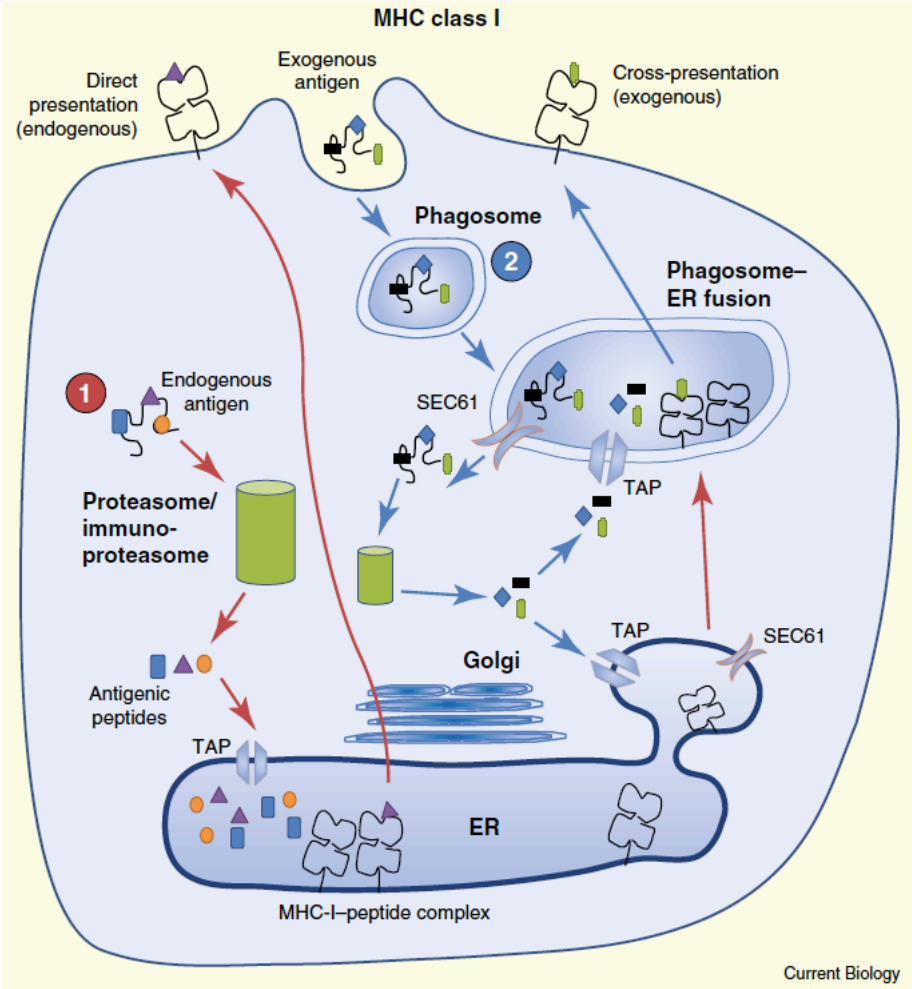


Figure 2. Generation of MHC-I-peptide complexes from endogenous and exogenous antigen. The pathway 1 (red arrows) corresponds to the direct presentation of endogenously synthetized peptides. The pathway 2 (blue arrow) represents the cross-presentation of exogenous antigens. Figure from *Buckwalter et al., 2009*.

2) Presentation of extracellular antigens

In order to be presented, extracellular antigens must first be taken up from the outside environment by the APC. Different endocytosis pathways will be used for this process, depending on the nature of antigen of interest. Particulate antigens, such as cell-associated antigen, are taken up by phagocytosis whereas receptor-mediated endocytosis or pinocytosis

are required for the uptake of soluble antigens. Once the antigen is inside the cell, it can be processed and the resulting peptides are presented on MHC-I or MHC-II molecules.

(a) Antigen uptake

(i) Diversity of engulfment receptors

Extracellular antigens must be detected and engulfed by cells prior to their processing and presentation. A diverse repertoire of receptors exists to modulate the engulfment process and the pattern of expression of these receptors varies from one APC subset to another. As examples, scavenger receptors, mannose receptor, DEC-205, Langherin, Fc receptors, and Dectin-1 can all mediate antigen uptake. The engulfment receptor choice is critical, as depending on the receptor engaged, the outcome of the immune response may vary (Burgdorf and Kurts, 2008).

(ii) The particular case of dying cells

Upon the death of a cell from any cause, the cellular remnants must be removed quickly to preserve tissue homeostasis. Depending on the mechanism of cell death involved, this clearance is accompanied by tolerance and tissue repair or activation of an immune response. The dying cells advertise their presence through the release of "find-me" signals such as sphingosine-1-phosphate or the nucleotides ATP or UTP (Ravichandran, 2011). These molecules promote the recruitment of phagocytes and enhance their activity. Phagocytes identify the dying cells via the expression of "eat-me" signals. For example, phosphatidylserine is a lipid that is present in the inner leaflet of the plasma membrane in live cells. When a cell undergoes apoptosis, this lipid is exposed on the outer membrane and can then act as an "eat-me" signal for phagocytes surveying the local environment. In addition, changes in charge and/or glycosylation can be detected on the surface of dying cells. "Eat-me" signals are most often recognized by scavenger receptors or complement receptors… In the case of phosphatidylserine expression, the TIM molecule family can act as receptors for phagocytosis (Miyanishi et al., 2007). Specifically, TIM4 is exclusively expressed on APCs and mediates the phagocytosis of apoptotic cells. However, there exists a variety of TIM receptors that allow for the detection of dying cells and result in functionally different outcomes, particularly immune activation or tolerance, depending on the cell type and TIM molecule engaged (Freeman et al., 2010). Another recently characterized receptor for dying cells is Clec9A, which has been demonstrated to interact with necrotic cell (Sancho et al., 2009). This receptor recognizes actin which is normally contained intracellularly and that is exposed in dying cells upon necrosis (Ahrens et al., 2012; Zhang et al., 2012).

(b) Presentation of extracellular antigens on MHC-II

While MHC-I molecules are expressed by all nucleated cells, only few cell types express MHC-II molecules (B cells, thymic epithelial cells, macrophages and DCs) and can thus present extracellular antigens to $CD4^+$ T cells.

Following engulfment, the exogenous antigen is partially degraded and the resulting peptides are transferred to the MHC-II compartment where they compete with the Class II associated invariant chain peptide (CLIP) for binding to the pre-assembled MHC-II molecule. Then MHC molecules loaded with peptides are stabilized and transported to the cell surface (**Figure 3, pathway 1, orange arrows**). Degradation of the engulfed antigen is dependent on the acidic pH in endosomes and phagosomes, which allows the activation of lysosomal proteases required for the digestion of antigens and their loading on MHC-II molecules. Of note, endogenous peptides originating from endosomal proteins, proteins from plasma membrane, or from cytosolic components transferred into endosomes via autophagy can also be presented on MHC-II (Buckwalter and Albert, 2009) (**Figure 3, pathway 2, green arrows**).

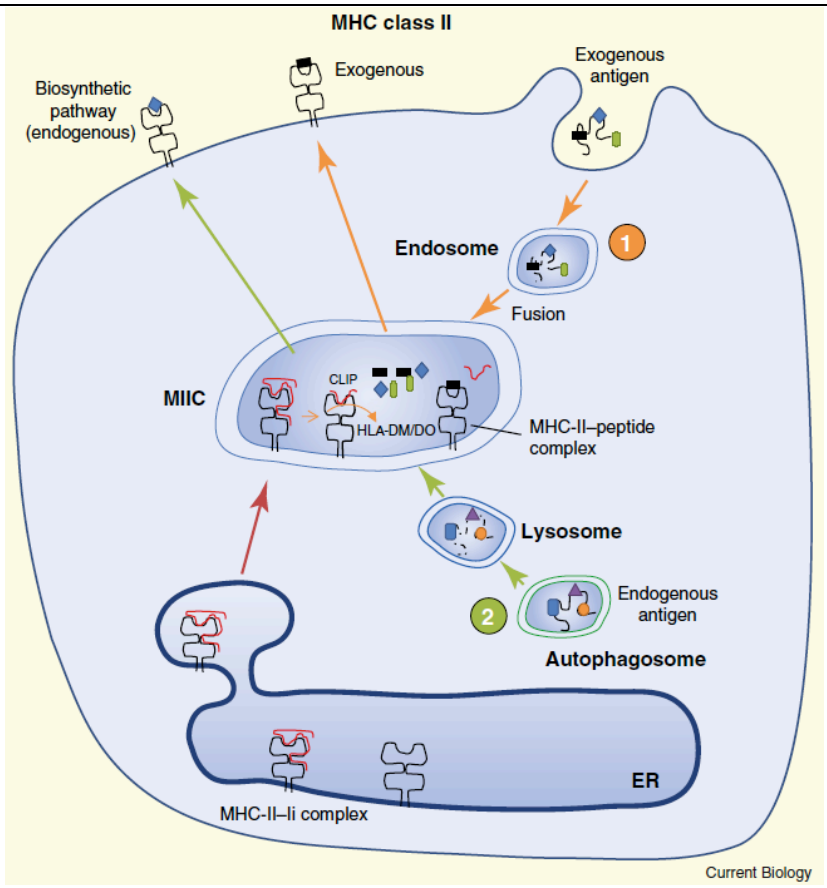


Figure 3. Generation of MHC-II-peptide complexes from endogenous and exogenous antigen. The pathway 1 (orange arrows) represents the presentation of exogenous antigen on MHC-II. The pathway 2 (green arrows) corresponds to the presentation of endogenous antigen on MHC-II molecules. Figure from *Buckwalter et al., 2009*.

(c) Cross-presentation of extracellular antigens on MHC-I

(i) Cross-presentation pathway

Exogenous antigens can also be presented on MHC-I molecules allowing for the activation of cytotoxic T cells. Upon endocytosis, the antigen is digested by lysosomal enzymes, similar to the mechanism outlined above, but then is presented on MHC-I molecules (**Figure 2, pathway 2, blue arrows**). It is not clear whether it is a completely independent pathway, or if it is a combination of endocytosis initially, followed by transition to a pathway similar to that of endogenous presentation on MHC-I. Three different mechanisms have been proposed for the pathway of cross-presentation based on the observation that it is possible to detect antigen and the corresponding MHC-I-peptide complexes at the same time and in the same compartment (Amigorena and Savina, 2010). First, the "vacuolar pathway" suggests that the antigen is processed by lysosomal proteases and loaded on MHC-I molecules entirely within the phagosome. This has been termed TAP-independent cross-presentation because the antigen would not go through the endoplasmic reticulum (ER). However, it has been shown in several studies that TAP and the proteasome, found in the cytosol, are required for optimal cross-presentation (Guermonprez et al., 2003; Ackerman et al., 2006). Thus, "the cytosolic pathway" was proposed (TAP-dependent cross-presentation): initial antigen processing begins in the phagosome and the antigen is then transferred into the cytosol where it is completely processed by the proteasome. The next step remains unclear: either the resulting peptides are imported into the ER through TAP transporter, loaded on MHC-I molecule there and then follow the classical route for MHC-I presentation at the cell surface; or the peptides are re-imported into the phagosome that has recruited ER components (TAP, MHC-I…) after the processing by the proteasome. In this latter scenario, peptides are loaded on MHC molecules inside the phagosome.

(ii) DC specialization for cross-presentation

Compared to other phagocytes, DCs have increased capacity to cross-present antigens. These cells have adapted their endocytic and phagocytic pathways to favor antigen cross-presentation. A key question is how DCs degrade enough antigen to generate relevant peptides that will be loaded onto MHC-I, but avoid the complete degradation of antigen into peptides and amino acids. In neutrophils and macrophages, the pH in phagosomes and endosomes drops rapidly and remains acidic for several hours following antigen uptake, inducing the activation of the lysosomal proteases that have been delivered to these compartments. This activation is critical for antigen degradation (Savina and Amigorena, 2007). However, these kinds of general mechanisms may destroy the relevant epitopes needed

for presentation on MHC molecules necessary for specific T cell activation. Thus, the DC phagocytic pathway is slightly different to ensure efficient antigen cross-presentation. First, DCs express reduced levels of lysosomal proteases as compared to macrophages (Delamarre et al., 2005) and the recruitment of these enzymes to the phagosome is also limited (Lennon-Dumenil et al., 2002). Consequently, the overall antigen degradation is low compared to other APCs. Moreover the pH in the phagosome is differently regulated in DCs. The proton pump V-ATPase that contributes to the acidification of the compartment and the optimal activation of proteases is not completely assembled in lysosomes of immature DCs (Trombetta et al., 2003). Additionally, in DCs, the NADPH-oxidase NOX2 is recruited upon phagocytosis to endosomes and phagosomes (Savina et al., 2006). This oxidase generates reactive-oxygen species (ROS) that induce the consumption of protons and limit the acidification. This reduced proteolytic activity and relatively higher pH result in limited antigen degradation and favor antigen cross-presentation.

(d) Differential regulation of MHC-I versus MHC-II presentation depending on the nature of antigen

Antigen can be identified and taken up by phagocytes in different forms: particulate (for example, cell–associated antigen) or soluble (for example, protein). While phagocytosis is the primary mode of uptake in the case of particulate antigen, soluble antigen will often be engulfed by receptor-mediated endocytosis. These pathways are regulated independently and it may influence whether antigen is presented in the context of MHC-I or MHC-II.

(i) Particulate antigen

In the case of a particulate antigen taken up by a DC such as a cell-associated antigen, the conditions of low proteolytic activity and high pH favor cross-presentation, but this benefit is transient. With time, ROS production stops and results in the acidification of the phagosome. Proteases from the lysosome are increasingly activated and go on to degrade antigens to generate peptides for MHC-II loading. Thus, the type of antigen presentation favored is time-dependent: in the initial period post-antigen uptake, the conditions are optimal for cross-presentation on MHC-I, however, later it changes to favor the presentation on MHC-II (Burgdorf and Kurts, 2008).

(ii) Soluble antigen

The pathways involved in processing and presenting soluble antigens are notably different. First, they are taken up by either receptor-mediated endocytosis or pinocytosis, rather than phagocytosis. Depending on the type of receptors engaged, soluble antigens will be directed

into one of two types of endosomes that can mature with different kinetics: one population matures rapidly, favoring acidification, antigen degradation and presentation on MHC-II, whereas the other group is characterized by more stable pH and enzymatic activity, leading to more efficient cross-presentation on MHC-I. For example, antigens that are endocytosed through mannose receptor are directed to the stable endosomes and result in cross-presentation, whereas the endocytosis through scavenger receptors, DC-SIGN, or pinocytosis targets antigen to the acidic endosome compartment and results in presentation on MHC-II (Burgdorf et al., 2007). Some receptors such as DEC-205 have the ability to promote both MHC-I and MHC-II presentations (Mahnke et al., 2000; Dudziak et al., 2007).

3) DC activation

In the steady state, DCs are immature, displaying a high ability for phagocytosis, and high levels of surface expression of a large pattern of engulfment receptors, but a low capacity to activate naïve T cells (Wilson et al., 2003). During antigen processing and presentation, the phenotype of DCs matures, also dependent on the other signals they have received (Steinman, 2003). Signals helping DCs to distinguish what is 'self' and 'non-self' are particularly critical to allow their maturation and the development of an appropriate response. In inflammatory conditions, these cells mature and acquire a phenotype allowing for the activation of antigen-specific T cells. While immature DCs have a strong capacity for phagocytosis and a low expression of MHC-II molecules on their surface, these two characteristics are inverted during maturation, with a severe decrease in phagocytosis capacity and an increase in the MHC-II surface expression observed. In parallel, DCs increase their expression of costimulatory molecules and cytokines required for T cell activation. Moreover, the maturing migratory DCs upregulate the expression of the chemokine receptor CCR7, allowing for interaction with the gradient of chemokines CCL19 and CCL21 released by cells from the stroma and high endothelial veinules in the lymph nodes. At this point, the DCs modify their morphology and migrate to lymphoid organs where they will interact with T cells (Verdijk et al., 2004) (**Figure 4**).

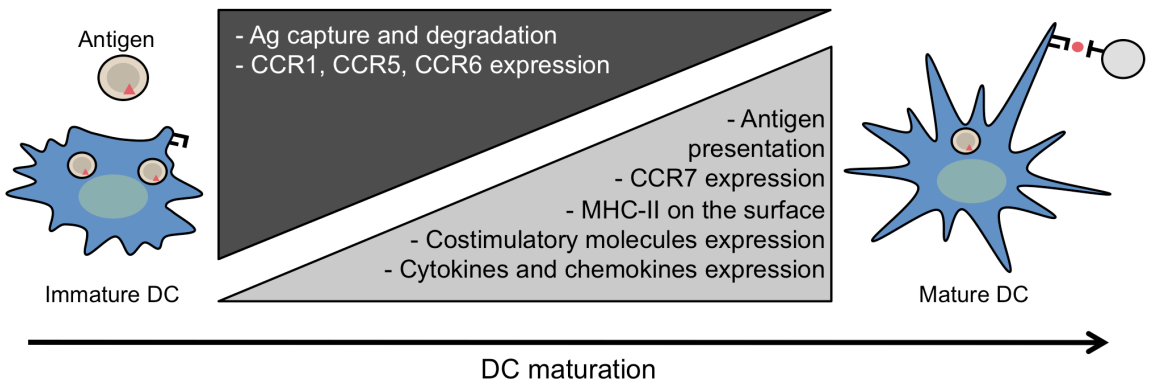


Figure 4. DC maturation.

Several events have been described that are necessary to license DCs and allow for efficient priming of T cells. Several key mechanisms involved in this process will be described below.

(a) Pathogen-Associated Molecular Patterns (PAMPs) and Pathogen Recognition Receptors (PRRs)

In 1989, Charles Janeway introduced the idea that pathogens are detected by the immune system via Pathogen Recognition Receptors (PRRs), which would interact with invariant microbial components, termed Pathogen-Associated Molecular Patterns (PAMPs) (Janeway, 1989). PAMPs include a diverse group of molecules: proteins, lipids, carbohydrates and nucleic acids derived from microbes and act as ligands for PRRs. APCs remain quiescent until they recognize PAMPs via their constitutively expressed PRRs and, following this, they acquire and process antigen and mature such that they can activate specific T cells. This interaction between PAMPs and PRRs acts as the signal that allows APCs to discriminate between the "infectious non-self" and the "non-infectious self". Of the PRRs, the Toll-like receptors (TLRs) were the first identified. Subsequently, other PRRs have been described including the cytosolic NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), and the cell-surface C-type lectins receptors (CLRs). Many cell types, including hematopoietic, but also non-hematopoietic cells, express PRRs and can activate signaling pathways upon PAMP recognition.

PRR		PAMP	Species	References
Signals detected by TLRs				
Cell surface TLRs	TLR4	LPS	Bacteria	*Akira, 2006*
		Envelope proteins	Virus	
	TLR2	Lipopeptides, peptidoglycan lipoteichoic acid	Bacteria	*Akira, 2006*
		Lipoarabinomannan	Mycobacteria	
		Zymosan	Fungi	
		Hemmaglutinin protein	Virus	
	TLR1/2	Triacylated lipopeptides	Bacteria/ mycoplasma	*Jin, 2007* *Kang, 2009*
	TLR2/6	Diacylated lipopeptides		
	TLR5	Flagellin	Bacteria	*Akira, 2006*
	TLR1	Uropathogenic bacterial components?	Bacteria	*Zhang, 2004* *Yarovinsky, 2005*
		Profiling-like molecules	*Toxoplasma gondii*	
Nucleic acids sensing TLRs	TLR3	dsRNA	Virus	*Akira, 2006* *Kawai 2008*
	TLR7	ssRNA	Virus	
	TLR8	ssRNA	Virus	
	TLR9	CpG DNA motifs	Bacteria/ virus	*Akira, 2006* *Kawai 2008* *Coban, 2010*
		Crystal hemozin	*Plasmodium*	

Table 1. Diversity of Toll-like receptors. dsRNA, double-stranded RNA, ssRNA, single-stranded RNA.

(i) Toll-Like Receptors

TLRs are transmembrane receptors that can be found in various cellular compartments (**Table 1**). This family of receptors can be divided into two groups based on expression location and ligand specificity. TLR1, 2, 4, 5, 6 and 11 are expressed on the cell surface and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins. TLR3, 7, 8 and 9 recognize nucleic acids and are localized in intracellular compartments such as endosomes and lysosomes. Studies of mice deficient for the different TLRs have

demonstrated that they are highly specific for their cognate ligand and, upon engagement, different TLRs induce a variety of signaling pathways (**Figure 5**). While TLR3 and TLR4 signal through the adaptor molecule TRIF and trigger the production of type I IFN and inflammatory cytokines, TLR1, 2, 4, 5 and 6 induce only inflammatory cytokines via signaling through Myd88-dependent pathways (Kawai and Akira, 2010). TLR 7 and 9 also signal via Myd88.

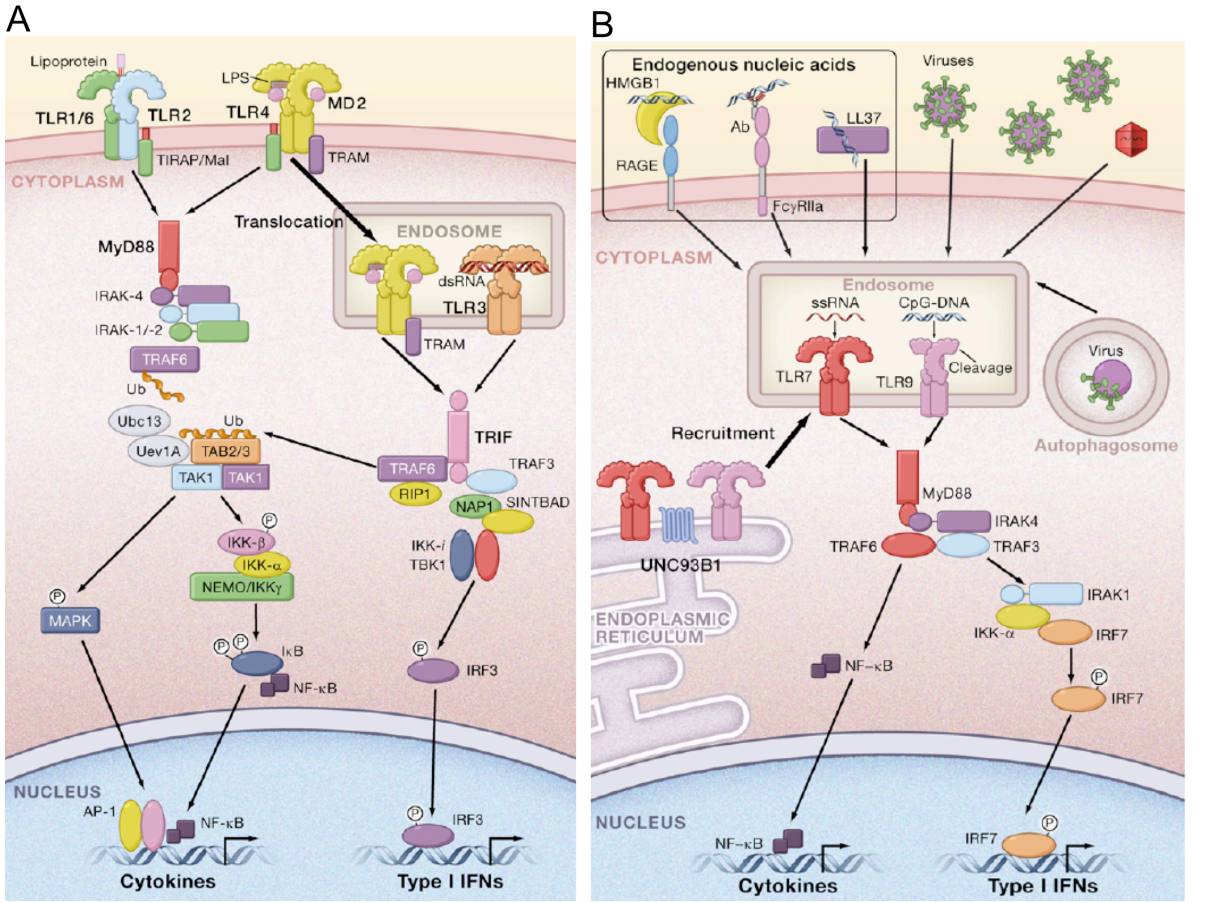


Figure 5. Signaling pathways downstream of TLRs. (A) Myd88-dependent pathway downstream of TLR1/6, 2 and 4 and TRIF-dependent pathway activated by TLR3 and 4. **(B)** Myd88-dependent pathway downstream of TLR7 and 9. Adapted from *Takeuchi et al., 2010*.

(ii) RIG-I-Like Receptors

The RIG-I-like receptors are critical for the detection of infecting viruses. Three cytoplasmic receptors form this family. RIG-I, Mda5 and LPG2 are specific for viral RNA species (**Table 2**). RIG-I, in particular, detects short double-stranded RNAs only found during the replication of RNA viruses, while Mda5 recognizes longer nucleic acid chains.

(iii) Nod-Like Receptors

The NOD-like receptor family consists of more than 20 unique members that are all cytosolic (**Table 2**). Among them, NOD1 and NOD2 recognize the degradation products of bacterial

wall components. NLRP3 (NALP3) responds to various stimuli as part of the recently characterized inflammasome complex, which results in the cleavage of pro-IL-1β and pro-IL-18 via the activation of Caspase 1.

PRR	PAMP	Species	References
Signals detected by RLRs			
RIG-I	RNA (5'-PPP ssRNA, short dsRNA)	Virus	*Yoneyama, 2009*
MDA-5	dsRNA	Virus	*Yarovinsky, 2005*
LGP2	RNA	Virus	
Signals detected by NLRs			
NOD1/ NLRC1	iE-DAP	Bacteria	*Ting, 2008*
NOD2/ NLRC2	MDP	Bacteria	
NALP3/ NLRP3	MDP	Bacteria	
	RNA	Bacteria/ Virus	
	Toxins	Bacteria	
NALP1/ NLRP1	Anthrax lethal toxin	Bacteria	
IPAF/ NLRC4	Flagellin	Bacteria	
NAIP5	Flagellin	Bacteria	
Signals detected by CLRs			
Dectin-1	Beta-glucan	Fungi	*Takeuchi, 2010*
Dectin-2	Beta-glucan	Fungi	
MINCLE	SAP130	Fungi	

Table 2. Diversity of RLRs, NLRs, and CLRs. ssRNA, single stranded RNA; dsRNA, double stranded RNA; iE-DAP, dipeptide present in bacterial peptidoglycan; MDP, muramyl dipeptide.

(iv) CLRs

CLRs are transmembrane receptors that recognize carbohydrates from microorganisms (**Table 2**). As an example, Dectin-1 and Dectin-2 detect β-glucans from fungi. Clec9A is the prototypic member of this family. It is expressed on $CD8\alpha^+$ DCs and is responsible for recognizing necrotic cells (Takeuchi and Akira, 2010).

Each of these PRRs may allow for the distinction between "self" and "non-self" by the recognition of specialized microbial components. However, in 1994, Polly Matzinger proposed "the danger model", another theory that describes the signals that activate APCs and explains how and when the immune system decides to mount an inflammatory response (Matzinger, 1994). In Matzinger's model, the immune system would be more concerned with damage than foreigness and, as a result, would be more activated by alarm signals from injured tissues than by the recognition of non-self components. Both Janeway's and Matzinger's models assume that APCs can be activated by signals from their environment but Janeway restricted these signals to the detection of PAMPs, whereas Matzinger hypothesized the involvement of more general danger signals, both exogenous molecules from pathogens, or endogenous signals released from injured tissues of the host. For instance, danger signals can be molecules derived from a pathogen (PAMP) but also RNA, heat shock proteins, hydrophobic molecules or components of the extracellular matrix. Under normal conditions, these components are not detectable by the immune system but after damage they can be released in the microenvironment and activate immune cells.

(b) Damage-Associated Molecular Patterns (DAMPs)

In 1999, Gallucci and colleagues demonstrated that DCs can be activated by mechanical stimulation without the addition of any exogenous molecules (Gallucci et al., 1999). Concurrently, necrosis was described as a cell death mechanism producing generally more danger signals than apoptosis, which was considered as a controlled and less damage-signal inducing form of cell death. Thus began the field of investigation into Damage-Associated Molecular Patterns (DAMPs) (Chen and Nunez, 2010) (**Table 3**). Among the first molecular damage signals described were heat-shock proteins, which were shown to activate dendritic cells when they were released from dying cells (Basu et al., 2000). The DNA-binding protein High-Mobility Group Box 1 protein (HMGB1) was also discovered to be an important mediator of damage signaling, since it induces inflammation. Furthermore, it is involved in the differential damage signaling activities of necrotic versus apopotic cells. Other molecules such as extracellular ATP or uric acid have also been shown to be released after injury and have the ability to activate the immune system. Interestingly, some of these DAMPs can also be detected by PRRs such as TLRs (Ohashi et al., 2000). Another pathway discovered only recently, the inflammasome, acts as a molecular platform capable of converging danger stimuli including both PAMPs and DAMPs (Schroder and Tschopp, 2010). The activation of this complex triggers the release of inflammatory cytokines such as IL-1β and IL-18.

Damage signal	Sensor	Associated pathology	References
Signals associated with release of intracellular content			
HMGB1	TLR2/4/9 RAGE CD24	Cellular injury	*Yu, 2006* *Chen, 2009*
Heat-Shock Proteins	TLR2/4 CD14/40/91 CD24	Cellular Injury	*Vabulas, 2001* *Basu,2001* *Quintana, 2005*
SAP130	CLEC4E	Cellular Injury	*Brown, 2008*
S100 proteins	RAGE	Cellular injury	*Hofmann, 1999*
Mitochondrial formyl peptides	FRP1	Cellular Injury	*Zhang, 2010*
ATP	NLRP3 Inflammasome	Cellular Injury	*Mariathasan, 2006*
Low intracellular K+	NLRP Inflammasome	Membrane permeabilization	*Gurcel, 2006*
Signals associated with "dangerous" processes			
RNA	TLR3	Cellular Injury Viral infection	*Cavassani, 2008* *Kariko, 2004*
DNA	TLR9 AIM2 Inflammasome	Cellular Injury Viral infection	*Burckstummer, 2009* *Hornung, 2009*
Hyaluronan, Versican, Bliglycan, Heparan Sulfate	TLR2/4 CD44	ECM degradation	*Scheibner, 2006* *Schaefer, 2005* *Kim, 2009*
Excessive actin polymerization	Flightless-I?	Bacterial and viral cell invasion	*Li, 2008*
Signals associated with "dangerous" products			
Uric acid , CPPD, Cholesterol crystals	NLRP3 Inflammasome CD36	Gout, Atherosclerosis	*Martinon, 2006* *Stewart, 2010*
beta-Amyloid	RAGE NLRP3 Inflammasome CD36	Amyloid plaque formation	*Yan, 1996* *Halle, 2008*
Silicate, Asbestos	NLRP3 Inflammasome	Intestitial fibrosis	*Dostert, 2008*

Table 3. Diversity of DAMPs. ECM, extracellular matrix.

B. Characteristics of the different subsets of dendritic cells

1) Heterogeneity of DCs

Phenotypically and functionally, all DCs are not equal. Different DC subsets have been described based on their surface marker expression profile, tissue localization and specialization in antigen presentation (**Table 4**). The first distinction can be made between conventional DC (cDCs) and plasmacytoid DC (pDCs) populations that can be observed and

characterized in mice as well as in humans. pDCs are strongly implicated in innate immunity due to their capacity to secrete high amounts of type I Interferons (IFN) and, in some studies, demonstrated ability to present antigen (Villadangos and Young, 2008). However, for the remainder of this introduction, I will focus on cDCs. Collectively, cDCs is an heterogeneous group of DCs that can be divided into the broad groupings of lymphoid tissue-resident DCs and peripheral, migratory DCs (Heath and Carbone, 2009). In the spleen, 3 further cDC subsets have been described based on CD8 and CD4 expression: the $CD8\alpha^+$ DCs, the $CD8\alpha^-$ DCs and the double negative DCs (Vremec et al., 2000). However in draining lymph nodes, other subsets can be observed that correspond to the migratory DC populations that reside in tissue and have the ability to migrate to lymphoid organs upon activation. Using the skin-draining lymph node as an example, 3 additional subsets are found: the Langherans cells that are normally found in the epidermis, the classical dermal $CD11b^+$ $CD103^-$ DCs, and the dermal $CD11b^{low}$ $CD103^+$ DCs (Ginhoux et al., 2007). Interestingly the 2 subsets $CD11b^+$ $CD103^-$ DCs, and the $CD11b^{low}$ $CD103^+$ DCs are also found in other peripheral tissues such as the lung (Sung et al., 2006) and the gut (Annacker et al., 2005) where they seem to have the same specialized function, regulating the immune response. In contrast, the Langherans cells are specific to the skin. An additional subset that is not present at steady state, but that appears under conditions of inflammation is the inflammatory monocyte-derived DCs.

	Inflammatory conditions	Steady state				
		Resident-DCs		Skin-derived DCs		
	Mono-DCs	**CD8α$^+$**	**CD8α$^-$**	**CD11b$^+$**	**CD103$^+$**	**LC**
Surface markers						
CD11b	+	-	+	+	-	+
CD103		-	-	-	+	-
Langerin	-	+	-	-	++	++
Engulfment receptors						
DEC205		+	-	+	++	++
DC-SIGN			+			
Dectin-1		-	+			
Mannose Receptor		+				
Clec9A		+	-		+	
PRR expression						
TLR3		+	-		**(*)**	
TLR7		-	+			
TLR9		+	+			
Specialization						
Cross-presentation		++			++	
Presentation on MHC-II			++	++		
Implication on secondary responses	++					

Table 4. Mouse DC subsets. Mono-DC, Monocyte-derived DC; LC, Langherans cell. **(*)** This subset was shown to respond to poly I:C but TLR3 was not identified in these cells.

2) Specialization

(a) Ability to present antigen

The different cDC subsets have been described to have various abilities for antigen uptake and presentation. The CD8α$^+$ DCs showed a superior capacity to take up dying cells (Iyoda et al., 2002), which is correlated with their relatively increased expression of some receptors known for clearance of dead cells, such as Clec9A. Moreover this subset has been demonstrated to better cross-present antigen compared to the CD8α$^-$ DC subset from the

spleen (den Haan et al., 2000). In contrast, the latter subset was shown to specialize in MHC-II antigen presentation (Dudziak et al., 2007). The same kind of dichotomy is true for migratory DCs. $CD103^+$ DCs performed more antigen cross-presentation while $CD11b^+$ DCs are better for triggering CD4 help and humoral response stimulation (Bedoui et al., 2009).

(b) Cooperation between different DC subsets

Resident DCs in the spleen screen the blood for pathogens but also phagocytose materials that are drained directly from the periphery through the lymphatic conduits. Migratory DCs migrate to lymphoid organs upon activation where they can present antigen to specific T cells. However, once they have arrived, they can transfer antigen to resident DCs that will present antigen (Allan et al., 2006). Consequently the same antigen can be presented by different DC subsets, with different specialization for antigen presentation (Belz et al., 2004).

(c) Ability to respond to different signals

Interestingly, DCs subsets display different patterns of PRR expression, resulting in differential sensitivity to danger signals. In the spleen, the $CD8\alpha^+$ DCs are the only subset to express TLR3, but not TLR7, while the $CD8\alpha^-$ subset is characterized by the opposite phenotype (Edwards et al., 2003). These observations have important implications for modulation of the immune response stimulated by PAMPs and for considering the appropriate choice of adjuvants, which will be discussed later in this thesis.

3) Parallel between resident $CD8\alpha^+$ DCs and migratory $CD103^+$ DCs

To study the role of $CD8\alpha^+$ DC subset *in vivo*, a mouse line lacking the transcription factor Batf3 was developed. In these mice, this subset is missing and this knock-out has been correlated with the absence of a protective $CD8^+$ T cell response upon experimental West Nile virus infection, as well as an inability to reject a syngeneic tumor (Hildner et al., 2008). These results clearly demonstrated the crucial role of $CD8\alpha^+$ DCs in cross-presentation.

Interestingly, the skin-resident $CD103^+$ DCs are also absent in this mouse. These two subsets share several common characteristics such as the expression of DEC205 or CD24, their cross-presentation efficiency of both soluble and cell-associated antigens (den Haan et al., 2000) (del Rio et al., 2007), and their ability to respond to the TLR3 ligand poly I:C (Schulz et al., 2005; Sung et al., 2006). However, there are also differences: TLR3 was not detected in $CD103^+$ DCs and this subset does not seem as efficient as $CD8\alpha^+$ DCs in promoting memory $CD8^+$ T cell responses. While they share several similarities, these 2 subsets are not identical and, therefore, it was surprising that Batf3 would be critical for both DC populations.

Although this question remains unclear, this result may be due to a common precursor that can give rise either to CD8α$^+$ DC or CD103$^+$ DC depending on its tissue localization.

4) Heterogeneity of human DCs

While many DC subsets have been described in the context of experimental mouse models, the same extensive identification and characterization has not yet been possible in humans, mainly due to the restricted materials available for human study – especially blood. Recently, a group studying DCs from the thymus identified different DC subsets in this organ (Vandenabeele et al., 2001). A subset of human DCs expressing Clec9A, similar to the CD8α$^+$ DC subset in the mouse, was identified: it corresponds to BDCA3$^+$ DC subset in the blood (Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). Further investigations are required to validate that these DCs have the same functional properties as CD8α$^+$ mouse DCs. But further dissection of the different subsets of human DCs will be critical for the purpose of improving human vaccination (Caminschi et al., 2008).

II. DEVELOPMENT OF A CD8$^+$ T CELL RESPONSE

In a naïve mouse, CD8$^+$ T cells that are specific for a variety of antigens are present. They become activated upon encounter with APCs presenting the specific cognate antigen. Signals from the microenvironment are required to mount an efficient T cell response. Upon activation, T cells expand in order to clear the antigen burden and then the excess specific T cells die to maintain the homeostasis of the T cell population. Nevertheless, some antigen-specific T cells will persist as memory T cells (**Figure 6**).

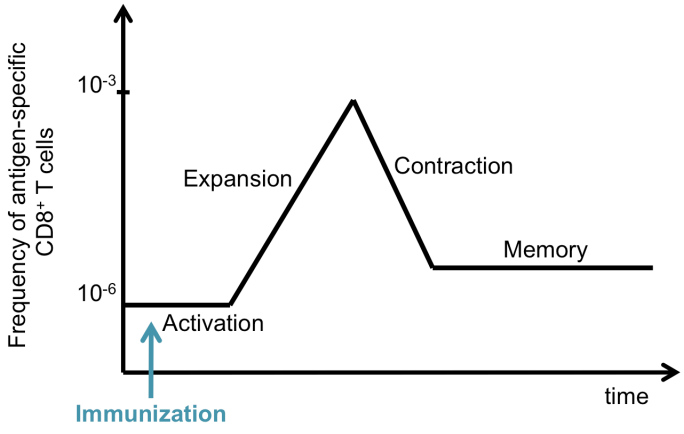


Figure 6. The different phases of a T cell response

A. Naïve T cells: diversity of the repertoire

Mature $CD8^+$ T lymphocytes bear T cell receptors (TCR) that are specific for a MCH class I molecule bound to a unique peptide. This TCR is a heterodimer of αβ chains. Each gene is composed of segments that were joined by somatic recombination during lymphocyte development (**Figure 7**). The Vβ chain is composed of 3 different segments Vβ, Dβ and Jβ. During the rearrangement process, nucleotides are randomly added or removed at junction sites creating variability between different TCRβ chains. This region of high variability is called CDR3 (Complementary-determining Region 3) and is thought to carry the specificity for the specific epitope. The recombination of these 3 segments constitutes the variable region and is associated with a constant region Cβ. The same mechanism is responsible for the formation of α chain except that there is only recombination between two segments for the variable region: Vα and Jα. Then the α and β chains associate to form the TCR. The random variability created during somatic recombination is responsible for the high diversity of TCRs present in an organism. Interestingly there is also extensive variability between the T cell repertoire of different individuals (Bousso et al., 1998). In the endogenous repertoire of mice, the number of T cells specific for a given antigen ranges from 10 to 1000 cells per individual animal (Casrouge et al., 2000).

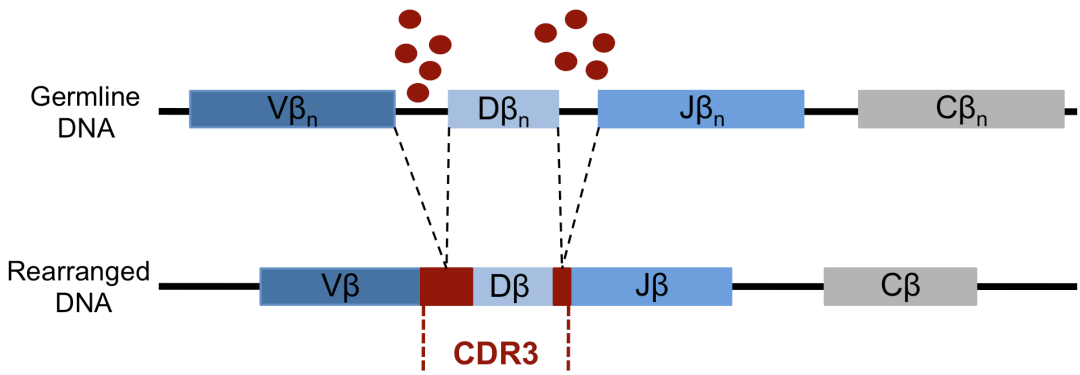


Figure 7. Somatic recombination leading to rearranged DNA coding for the TCRβ chain. Three segments V, D and J are associated to constitute the variable part of the chain. Additional nucleotides are removed or added at the junctions, introducing variability in the sequence.

B. T cell activation by dendritic cells

Naïve T cells are activated after following their encounter with an antigen-presenting cell in the secondary lymphoid organs. Several signals are required for complete activation and differentiation of the $CD8^+$ T cells (**Figure 8**).

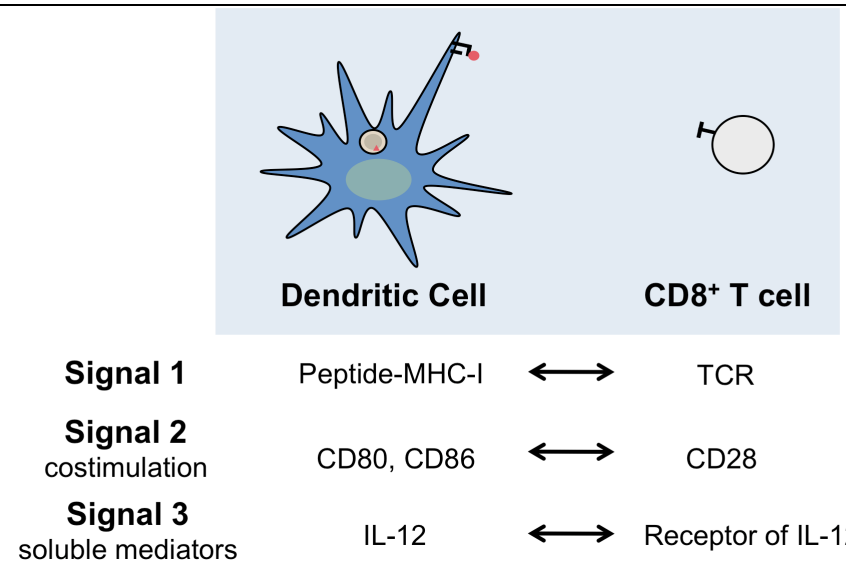


Figure 8. Three signals model for the activation of antigen-specific T cells by DCs.

1) Signal 1: interaction between the TCR and the peptide-MHC-I complex

The first signal required for T cell activation is the interaction between the peptide-MHC-I complex at the DC surface and the TCR on an antigen-specific T cell. The density of peptide-MHC complexes at the surface of DC and their affinity for the TCR both regulate the outcome of the T cell activation. For instance, using a peptide with a better affinity *in vitro* promotes the proliferation of specific T cells despite stimulating with a lower concentration of antigen (Hemmer et al., 1998). Concerning the number of peptide-MHC complexes, it has been shown that increasing the density of peptide-MHC complexes induces the proliferation of high affinity, but also lower affinity T cells (Rees et al., 1999). Zehn and colleagues developed an elegant model to study the impact of the T cell affinity for antigen on the T cell response, using altered peptide ligands that bind equivalently the MHC molecule but have differing abilities to interact with specific TCR-transgenic T cells (Zehn et al., 2009). They showed that weak interactions are sufficient to activate naïve T cells but that strong interaction is required to induce a sustained and robust T cell response. More recently, it has been shown that TCR affinity for the peptide-MHC complex is critically important. By comparing weak stimulations resulting from either a decreased antigen affinity or a decreased density of peptide-MHC complexes, Gottschalk and colleagues demonstrated that, although both induce T cell proliferation, the condition with the highest affinity induces a better responsiveness of T cells to IL-2 and sustained interaction between DCs and T cells (Gottschalk et al., 2012).

2) Signal 2: co-stimulatory signals

Upon activation after antigen uptake, DCs upregulate their expression of costimulatory molecules CD80 and CD86. These molecules interact with CD28 on the surface of T cells, providing the second signal, a necessary component for T cell activation. Nevertheless, those two signals alone are not sufficient. A third signal is also necessary for complete activation and differentiation of antigen-specific T cells (Mescher et al., 2006).

3) Signal 3

(a) Nature of signal 3

Activation of DCs also triggers the secretion of inflammatory cytokines that are required for efficient T cell activation. Curtsinger and colleagues demonstrated that T cells stimulated with antigen and B7 molecules are able to proliferate but do not develop cytolytic functions and are not activated upon secondary rechallenge, unless a third signal has been provided in the form of IL-12. Thus, this third signal allows the switch from induction of tolerance to complete priming of T cells (Curtsinger et al., 2003). IL-12 is not the only possible "signal 3" since the injection of antigen and adjuvant into IL-12-deficient mice does trigger a cytolytic T cell response (Schmidt and Mescher, 1999). Type I IFN has also been demonstrated to have the ability to act as "signal 3" (Curtsinger et al., 2005).

(b) Action of cytokines

Agarwal and colleagues studied the regulation of gene expression by IL-12 and type I IFN in $CD8^+$ T cells activated by antigen and co-stimulatory signals (Agarwal et al., 2009). They showed that many genes implicated in effector function and memory development were regulated by these cytokines and that this regulation was at least, in part, modulated by epigenetic modifications. For instance, histone acetylation triggers chromatin relaxation and thus favors gene expression, whereas DNA methylation induces a physical constraint to transcription factor binding and represses gene transcription. By acting on these regulation mechanisms at crucial loci, IL-12 and type I IFN influence the outcome of the $CD8^+$ T cell response. Interestingly, while these two cytokines regulate many genes in common, there are also genes for which the expression is modulated only by one, but not the other, of these two cytokines (Curtsinger and Mescher, 2010). That could explain the dependence of different models on IL-12, but not type I IFN, or vice-versa. Moreover that also indicates that depending on the "signal 3" context, T cell differentiation and memory formation may be differentially regulated.

(c) Source of cytokines and efficiency of signal 3

(i) Requirement for detection of antigen and TLR ligand by the same APC

IL-12 or type I IFN have been described as inflammatory cytokines able to provide signal 3 for the optimal activation of DCs. *In vitro*, they have been shown to activate DCs, leading to DC maturation and the ability to activate T cells. *In vivo*, they are released into the extracellular milieu by various cell types upon PAMP recognition. Indeed, many cells, both hematopoietic and non-hematopoietic, express PRRs and are capable of secreting cytokines upon PAMP detection. Thus it was not clear whether these cytokines are secreted by cells other than the DCs that have phagocytosed antigen and, perhaps, act to promote an inflammatory response as bystanders; or whether PAMPs have to be sensed directly by DCs that engulf antigen in order to induce its full activation. This question was first addressed in the context of $CD4^+$ T cell activation by using mixed BM-chimeras in which half of the APCs were able to present a specific antigen but cannot respond to LPS, and the other half could respond to LPS but did not present antigen, and compared them with control chimeras where all APCs were able to do both. They showed that DCs activated directly by LPS or indirectly through cytokines released by DCs that have sensed LPS matured. However, only DCs that have been directly activated by LPS were able to induce a functional $CD4^+$ T cell response (Sporri and Reis e Sousa, 2005). $CD4^+$ T cells activated by indirectly stimulated DCs proliferated but did not differentiate further into effector cells. The same results were next obtained for $CD8^+$ T cell responses using a similar approach of mixed BM-chimeras in which half of the APCs can present antigenic peptide but cannot respond directly to CpG because they are TLR9-deficient, while the other half can respond to CpG but cannot present peptide because they are H-$2K^b$-deficient (Kratky et al., 2011). Both APC populations displayed a mature phenotype but only DCs that were stimulated directly through TLR9 engagement by CpG could induce a functional $CD8^+$ T cell response. Bystander DCs can thus induce maturation without complete activation of APCs. One critical caveat in the last study was that it was performed using short peptide as antigen, so endocytosis was not required; the results may not agree in a system where the antigen required phagocytosis for processing and presentation. In another study, Nolte et al. were interested by the impact of inflammatory signals coming from tissues only. This question was addressed in the context of $CD4^+$ T cell activation in BM-chimeras where only tissue could respond to LPS stimulation. They demonstrated that the cytokines released by tissue could induce systemic inflammation but are not sufficient to promote the maturation of DCs (Nolte et al., 2007). Together, these data demonstrate that inflammation participates in the immune response, but is not sufficient in

itself to initiate the response. DCs must sense the PAMPs directly. This initially seems in contradiction with previous work showing that cytokines such as IL-12 or type I IFN could be sufficient (Curtsinger and Mescher, 2010). It is possible that a PAMP contamination of the cytokines used in the previous studies could explain this difference. The requirement for direct PAMP detection allows the DC to evaluate the nature of the agent causing the inflammation in order to modulate an appropriate response. It is also possible that other kinds of signals such as some DAMPs have the same ability to activate DCs to initiate an immune response. DCs that have matured by bystander signals but are not fully activated may have a regulatory role in the immune response (Joffre et al., 2009). While inflammation by itself does not appear sufficient to initiate the response, it is important to note that inflammatory cytokines produced by bystander cells are required in some cases to promote efficient priming. For instance, Longhi and colleagues demonstrated that type I IFN production from stromal and hematopoietic origin are required for the priming of $CD4^+$ T cells (Longhi et al., 2009).

(ii) Requirement for detection of antigen and TLR ligand in the same phagosome

Blander and Medzhitov went further and proposed a model to distinguish self from non-self at the subcellular level of phagosome through TLR engagement (Blander and Medzhitov, 2006). Indeed phagocytosis is critical for two different, but related mechanisms: (i) the removal of apoptotic cells to maintain tissue homeostasis, a process that must be done without inducing any inflammatory response; and (ii) the engulfment of pathogens, a function in host defense that will be associated with stimulating an immune response. Blander et al. have demonstrated that in macrophages, TLR ligands must be associated with antigen and internalized in the same phagosome to trigger antigen processing and presentation on MHC-II molecule (Blander and Medzhitov, 2004). By contrast, apoptotic cells are also phagocytosed but are not associated with TLR ligand engagement and thus, a tolerogenic response and degradation of this cargo is induced. They suggested that TLR signaling induces a different phagosomal maturation pathway with an enhanced kinetic and the generation of MHC-II molecules ready to present antigen. These results remain controversial, as another group has demonstrated that phagosome maturation occurs independently of TLR signaling (Yates and Russell, 2005).

4) <u>What happens when a signal is missing? Tolerance vs priming</u>

It was initially proposed that signal 1 alone induces tolerance while the addition of signal 2 provides for DC maturation and, therefore allows T cell priming (Steinman and Nussenzweig,

2002). However it has been observed that mature DCs are not always sufficient to prime T cells (Albert et al., 2001; Fujii et al., 2004). The critical checkpoint between tolerance and priming seems to be the presence of a signal 3. *In vitro* experiments with artificial APCs demonstrated that antigen and costimulation signals trigger T cell expansion but without effector function and memory development. In this case, the T cells that persist are tolerant. In contrast, if a third signal is provided, T cell expansion is accompanied by complete effector function development and memory T cell differentiation (Curtsinger et al., 1999). This has been confirmed by *in vivo* experiment with peptide immunization. Injection of peptide alone triggers tolerance, whereas it is capable of inducing T cell priming when delivered in combination with IL-12, playing the role of adjuvant and providing the third signal (Schmidt and Mescher, 1999).

5) Other factors regulating T cell activation

This model with 3 signals required for the proper induction of a T cell response represents the established scientific dogma. In addition, several other factors and signals have been described that further modulate this process.

(a) Antigen persistence

$CD4^+$ and $CD8^+$ T cells are both able to proliferate even after a short antigenic stimulation. However, these two populations seem not to require the same duration of antigenic stimulation for optimal priming.

It has been demonstrated that $CD4^+$ T cells need a long antigenic stimulation for optimal priming and differentiation. Short exposure to antigen allows for low-level proliferation of $CD4^+$ T cells but a longer stimulation increases this proliferation. These cells stop proliferating as soon as the antigen dose decreases under a certain threshold. Moreover, continuous stimulation throughout the expansion phase is required for the optimal differentiation (Obst et al., 2005).

By contrast, $CD8^+$ T cells can completely differentiate even after a very short interaction with APCs. *In vitro*, $CD8^+$ T cells stimulated for only a short period of time can proliferate (van Stipdonk et al., 2001). In a model of infection by *Listeria monocytogenes*, it has been demonstrated that $CD8^+$ T cells can proliferate in spite of the removal of bacteria after the use of antibiotic (Mercado et al., 2000). Bevan and Fink proposed an "autopilot" model for the development of $CD8^+$ T cells, meaning that a CD8-specific transcriptional program is engaged after the initial antigen encounter and that differentiation occurs even in the absence of sustained antigen stimulation (Bevan and Fink, 2001). However this model has been

challenged by data obtained in different models (Storni et al., 2003). While the duration of antigenic stimulation does not alter the functionality of $CD8^+$ T cells, it is an important parameter regulating the magnitude of the response (Prlic et al., 2006). Usharauli et al. demonstrated finally that the duration of antigenic stimulation affects the differentiation of $CD8^+$ T cells (Usharauli and Kamala, 2008). Our laboratory has also contributed to this field, demonstrating that persistence of cell-associated antigen is required for the efficient cross-priming of $CD8^+$ T cells (Jusforgues-Saklani et al., 2008).

(b) CD4 T cell help

$CD8^+$ T cells can be directly activated by antigen-presenting DCs. However, it was demonstrated that additional help from $CD4^+$ T cells is often required. Bennett and colleagues showed that the APC had to interact with the antigen-specific $CD4^+$ T cell and $CD8^+$ T cell for efficient priming (Bennett et al., 1997). Moreover CD4 help was no longer required when anti-CD40 antibodies were injected into immunized mice (Bennett et al., 1998). Indeed antigen-specific $CD4^+$ T cells interact with DCs and, consequently express CD40L that interacts with CD40 on DC surface. This interaction "licenses" DCs to prime $CD8^+$ T cells by the induction of IL-12 secretion and the upregulation of co-stimulatory molecules (Filatenkov et al., 2005). Thus, $CD4^+$ T cells can provide signal 3 indirectly through the licensing of DCs. This model requires the encounter between three cell types that could occur simultaneously (DC - $CD4^+$ T cell - $CD8^+$ T cell) or more probably following 2 successive steps (first, DC - $CD4^+$ T cell, and then DC - $CD8^+$ T cell). While CD4 help is not always required for primary response, it does appear to be essential for an efficient secondary response (Janssen et al., 2003).

(c) Other signals regulating T cell activation

Other signals have been described that add further detail and understanding to this 3-signal model of T cell priming. Notably, other cells than $CD4^+$ T cells can provide help. In fact, pDCs have been shown to provide help in promoting immunity against viral infections rather than $CD4^+$ T cells (Kuwajima et al., 2006; Yoneyama et al., 2005). pDCs released cytokines in the extracellular milieu that favored T cell priming. Moreover, they upregulated rapidly CD40L, permitting DC licensing. It has also been demonstrated that NKT cells can interact with DCs, stimulating the release of chemokines attracting effector $CD8^+$ T cells (Semmling et al., 2010). These chemokines lead T cells toward antigen-presenting DCs and consequently, the probability that these two cell types interact is increased. This phenomenon has been

called signal 0, as it occurs prior to the critical T cell-DC encounter (Bousso and Albert, 2010).

C. T cell expansion and differentiation

Upon activation, T cells undergo proliferation and differentiation to form a diverse population of cells with various functionalities and abilities to convert into memory cells.

1) Metabolic demands of activated T cells

Activated T cells increase in size and quickly divide up to 20 times. This requires significant changes to their metabolism in order to support this high proliferation rate. Indeed naïve T cells are in a quiescent state where catabolism is predominant. They use autophagy to generate the molecules required for energy and basal protein synthesis. Upon activation, the metabolic demand increases dramatically and the cells switch from catabolism to anabolism. The cells now employ glycolysis to generate energy despite high amounts of oxygen. This state of oxidative glycolysis is called the "Warburg effect" and has also been described for cancer cells. ATP production by aerobic glycolysis is much less efficient than by oxidative phosphorylation, but this metabolic pathway also allows for the generation of molecules to build new cellular components (Pearce, 2010). That could be the explanation as to why T cells use this pathway. Additionally, the cells increase their expression of nutrient transporters. Together these changes facilitate the massive proliferation that is initiated following T cell activation. Following the expansion phase, cellular metabolism returns again to a quiescent state in memory cells.

2) Heterogeneity of the T cell population

Circulating T cells exist as a diverse, heterologous population. The parameters responsible for the diversification are not yet known. However it is thought that the different quantitative and qualitative signals received by the naïve T cell during the first steps of activation (signals 1, 2, 3) can significantly impact the diversity of the global T cell population. Additionally, the role of the tissue microenvironment in promoting T cell diversity during the course of the immune response remains to be completely understood.

(a) T cell diversity

(i) T cell affinity

Upon encountering an antigen-presenting DC, T cells that are specific for the epitope presented on MHC-I will proliferate. The strengh of the signal, the environmental conditions and the number of different epitopes presented by DCs will all influence the number of T cell clones that will proliferate.

(ii) Memory potential

Multiple subsets of effector $CD8^+$ T cells have been identified based on their ability to become memory cells. After antigen clearance, the effector T cell population undergoes a dramatic decrease in numbers, known as the "contraction phase", mediated mostly by apoptotic cell death. 5-10% of antigen-specific T cells survive this transition and make up the memory T cell pool. This latter subset is characterized by stem-cell-like properties such as slowed cell cycling, longer survival, telomerase expression and the ability of self-renewal. The initial events during T cell activation and signals from the microenvironment during stimulation impact the differentiation of effector T cells toward a terminally differentiated cytotoxic phenotype or toward a memory phenotype. First, the Short-Lived Effectors Cells (SLECs) are characterized by high expression of KLRG1 and low expression of IL-7Rα. These cells exhibit stronger effector function potential. In contrast, the Memory Precursor Effector Cells (MPECs) are $KLRG1^{low}$ and IL-$7R\alpha^{high}$ and are more likely to become memory cells (Joshi et al., 2007). Thes distinctions are not absolute, as both subset populations are highly heterogenous. A more accurate depiction of effector $CD8^+$ T cell population heterogeneity would be represented by a continuum with the MPEC phenotype at one extreme, the SLEC at the other extreme, and intermediately differentiated effector cells between these two set phenotypes (**Figure 9**) (Cui and Kaech, 2010). Several transcription factors have been described that control T cell differentiation. An expression gradient of T-bet has been identified as a critical cell fate decision factor between terminal effector *versus* memory differentiation and is highly expressed in SLECs (Joshi et al., 2007). In contrast, Eomesodermin (Eomes) acts either in synergy or in opposition with T-bet to favor memory differentiation (Intlekofer et al., 2007; Intlekofer et al., 2005). Other factors such as Blimp-1, Bcl-6 or Id2 have also a role in this mechanism (Cui and Kaech, 2010). Inflammatory signals such as IL-12 can regulate these transcription factors and thus influence the outcome of T cell differentiation in the context of an inflammatory response (Takemoto et al., 2006).

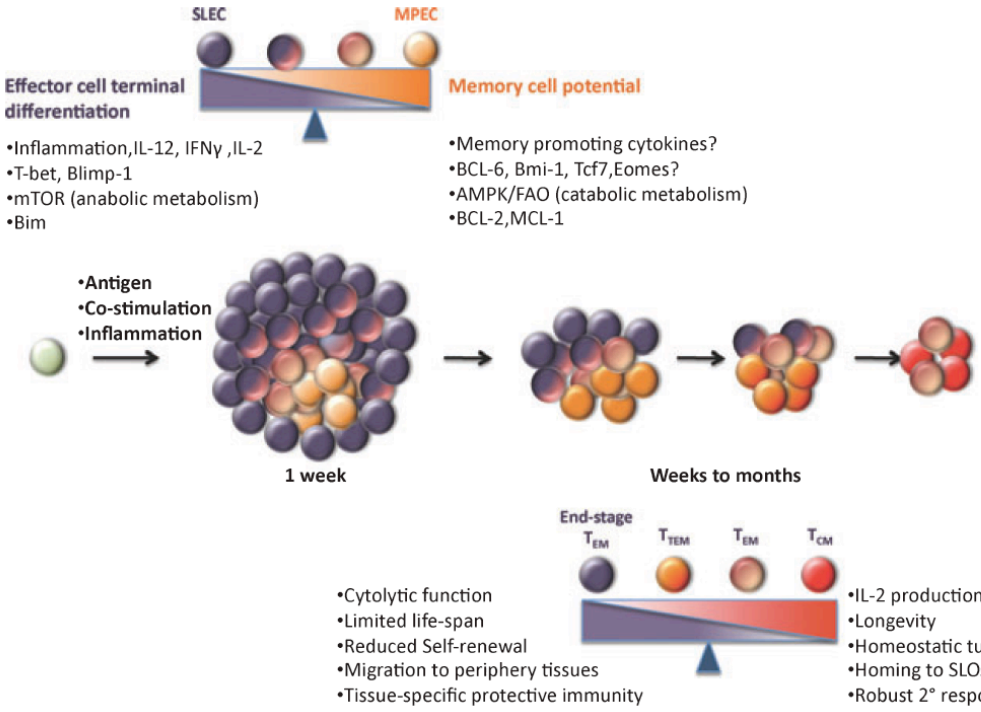


Figure 9. Effector and memory T cell differentiation upon activation. SLO, secondary lymphoid organ, SLEC, Short-lived effector cell, MPEC, Memory precursor effector cell, T_{EM}, effector memory T cell, T_{CM}, central memory T cell. Figure from *Cui et al. 2010.*

(iii) T cell quality

The quality of the T cell response was first characterized by measuring its magnitude, represented by the frequency of antigen-specific T cells and their ability to secrete IFNγ following antigen-specific restimulation using techniques such as IFNγ-ELISPOT. However, the use of only a few parameters to define the quality of the response and its ability to confer a protection is often not sufficient (Seder et al., 2008). For instance, the magnitude of the HIV-specific $CD8^+$ T cell response alone does not predict the progression of the disease (Gea-Banacloche et al., 2000). However, by comparing the T cell responses in patients that are long-term non-progressors to those of progressors, it has been shown that T cell function is not equivalent between these two groups, and that these differences could explain the range of disease progression and outcome phenotypes in these two patient groups. In general, all the effector T cells are not functionally identical. While some T cells secrete only one cytokine such as IFNγ, others are able to secrete several cytokines once activated. These polyfunctional T cells have been demonstrated to be critical for mounting an efficient response in multiple disease models.

Practically speaking, T cells can be characterized both by their surface markers expression and also by their various functions, including the ability to secrete cytokines, cytolytic activity or proliferative capacity. The development of more advanced techniques such as

multiparameter flow cytometry and intracellular staining, or large genomic screening allow for the analysis of an increased number of defined parameters with which characterize antigen-specific T cells. Historically, IFNγ is the most studied cytokine secreted by T cells as it has been shown to have an important role in the clearance of several infections. Tumor Necrosis Factor (TNF) that has also been extensively surveyed due to its implication in the killing of various pathogens. Finally, IL-2 is often measured in experimental systems due to its known role in inducing the proliferation of T cells through both an autocrine and paracrine manner, as well as its role in promoting memory T cell differentiation. Combining the analysis of these three cytokines allows for a more precise characterization of the T cell population under examination. Additionally, the expression of granzyme and perforin can be analyzed to study the cytolytic ability of T cells. Secretion of chemokines can also be assessed and may reflect the role of the T cell in the orchestration of the inflammatory response. Interestingly the presence of multifunctional T cells has been correlated to a better protection against infection (Almeida et al., 2007; Darrah et al., 2007). Specifically, the simultaneous production of IFNγ and TNFα by the same T cell has been shown to result in the enhanced killing of *Leishmania major* as compared to T cells that only produce one of these cytokines (Bogdan et al., 1990). The fact that multifunctional T cells are associated with a better response can be explained both by their ability to combine several functions, as well as the fact that polyfunctional T cells also secrete more cytokines on a per-cell basis, highlighted by the higher median fluorescent intensity (MFI) of this population (Seder et al., 2008).

(b) When does diversification occur?

A first approach taken to address the question of the timing of T cell diversification was to follow the development of a T cell response starting from a single naïve T cell. Initially, Stemberger and colleagues adoptively transferred a single naïve OT-I T cell and showed that the different subsets of effector and memory cells can be obtained from this single cell (Stemberger et al., 2007). Another group obtain similar results using a biological bar-coding system, which makes each T cell clone traceable *in vivo* (Gerlach et al., 2010). Together, these data support a model of progressive diversification starting from just a few cells that differentiate into various effector cells, which lose their ability to convert into memory cells over time. In contrast, another study suggests that the CD8 T cell clonal heterogeneity could be induced at the first cell division. The formation of the immunological synapse between the DC and the engaged T cell generates an asymmetry in the location of protein degradation machinery, resulting in different amounts of the transcription factor T-bet passed along to the two daughter cells, leading to differential functional evolution in that generation (Chang et al.,

2011). Finally, a study using the reporter mouse strain Ifnγ-YFP, which allows for the visualization and detection of cells that express IFNγ show that even at the first cell division, there exists already a high degree of variation in the expression of IFNγ on a per cell basis. This suggests that a T cell is part of a fixed lineage and that its fate is imposed before the first division (Beuneu et al., 2010).

D. T cell contraction and memory T cell formation

Following successful pathogen or antigen clearance, many effector T cells will die in order to reestablish homeostatic, steady state levels of circulating immune cells.

1) T cell apoptosis during contraction

As part of this contraction phase, most of the effector cells will undergo apoptotic cell death. This mechanism occurs through two distinct pathways (Strasser, 2005): (i) the extrinsic pathway defined by TNF Receptor family engagement by their cognate ligands (TNFα, Fas, TRAIL), or (ii) the intrinsic pathway, which is mediated by the disruption of the mitochondrial membrane due to cellular stress and the release of components into the cytoplasm. Signaling through both pathways results in the activation of Caspase 3 and 7 and eventual apoptotic cell death. Yet, a small subset of effector cells survives this extreme population contraction and these are the cells that constitute the memory T cell pool.

2) Conversion to memory T cells

(a) Metabolic switch

The T cells that survive to contraction will return to a resting quiescent state by switching back to a catabolic metabolism, which allows for cell survival following the growth factor withdrawal during the contraction phase. For example mTOR is a regulator of cell metabolism integrating signals from microenvironment and its inhibtion by rapamycin treatment promotes differentiation of effector T cells into memory cells (Araki et al., 2009). Physiologically one could imagine that following antigen clearance, the microenvironment is poor in nutrients and growth factors used up during the expansion phase, and that it would be beneficial for this environment to favors the differentiation of memory T cells.

(b) Subsets of memory cells

All memory cells share the same stem cell-like phenotype. Memory cells are characterized by their long life, in that they persist for long time even in the absence of antigen. During their

differentiation to the memory cell phenotype they acquire extended survival functionality, the expression of telomerase, and the ability to self-renew. IL-7, IL-15 and IL-21 are known to be important in the maintenance of this phenotype: IL-7 promotes survival, while IL-15 appears to be involved in self-renewal (Cui and Kaech, 2010).

However, this population is also extremely heterogeneous (**Figure 9**). The effector memory cells (T_{EM}) subset express low levels of CD62L and CCR7, receptors involved in lymph node trafficking, and therefore reside mainly in non-lymphoid peripheral tissues. These cells rapidly upregulate their effector functions upon secondary challenge but have a reduced proliferative capacity. In contrast, the central memory T cells (T_{CM}) express high levels of CD62L and CCR7 allowing for their localization in lymphoid tissues, and have a capacity of rapid and robust proliferation upon re-stimulation. Thus, there is a complementarity between the characteristics of these two subsets. Interestingly, signals from the environment can also modulate the differentiation of individual cells into the T_{EM} or T_{CM} subset. For instance, IL-15 is produced upon type I IFN stimulation and presented to T cells by DCs and macrophages. When IL-15 is presented to T cells by macrophages, it induces both T_{EM} and T_{CM}, yet the same cytokine presented by DCs favors T_{CM} differentiation (Mortier et al., 2009).

E. Control of the T cell response

1) Negative feedback

The immune response has to be carefully regulated in order to avoid an overactivation of the immune response, which can result in tissue damage as well as autoimmunity. Negative feedback loops allow for the control of inflammation and the maintenance of homeostasis. For example, antigen-specific T cells express CD28, which interacts with the co-stimulatory molecules CD80, and CD86 on the surface of DCs, resulting in T cell activation. Upon activation, T cells upregulate other receptors such as CTLA-4 that also interact with CD80 and CD86, and will compete with CD28 for binding and, therefore, negatively regulate T cell stimulation (Bour-Jordan et al., 2011). In this way, the T cell response is controlled.

2) Regulatory T cells

In order to control the generation of self-reactive T cells, the majority of those cells that are specific for self-antigens are deleted in the thymus during T cell development, in a process known as central tolerance. However, some of them will escape this deletion and can be found circulating in periphery. These cells generally have a relatively low antigen affinity and are controlled by regulatory T cells. They are thus not activated by self-antigens. This T cell-

based immune suppression is important for avoiding autoimmune responses that could develop as a side effect of inflammation. Unfortunately, these self-regulatory systems can also be manipulated and inhibit an efficient immune response or therapeutic intervention; often this occurs during cancer development, when the tumor itself promotes the action of regulatory T cells, as well as the secretion of immunosuppressive cytokines such as TGFβ or IL-10, which then limits the development of an anti-tumor $CD8^+$ T cell response (Vanneman and Dranoff, 2012).

III. HOW TO MODULATE $CD8^+$ T CELL CROSS-PRIMING?

Understanding the basis of antigen cross-presentation and $CD8^+$ T cell priming is essential to be able to modulate the immune response and develop effective treatments against disease. As previously mentioned, the majority of successful vaccines developed thus far trigger a humoral, antibody-based response rather than cell-mediated immunity. However, in many cases it is the $CD8^+$ T cell immunity that has been shown to be critical for controlling diseases for which efficient vaccines have not yet been developed, such as HIV, malaria or tuberculosis (Rappuoli and Aderem, 2011). Cell-mediated immunity is also crucial to fight cancer or chronic viral infections. Consequently, developing vaccines that trigger T cell immunity will be absolutely required to improve current therapies for a wide range of pathologies. To explore this concept further, I will review the possible ways to modulate $CD8^+$ T cell cross-priming: first, how different forms of antigen can be used to elicit various T cell responses and second, the use of adjuvants, and especially type I IFN-inducers, to boost an antigen-specific T cell response.

A. Stimulation of the immune response with diverse forms of antigen

A variety of antigens can be used to induce an immune response. Each of them displays particular characteristics regarding their uptake, processing, presentation by APCs and their resulting immunogenicity. Depending on the desired immune response, the form of antigen must be carefully chosen and, in some cases, additional reagents such as adjuvant should be administered in parallel to attain maximal protective immunity (**Table 5**).

1) Peptide and protein-based vaccines

Tumor-associated antigens (TAA) are proteins that are only expressed by or expressed at a much higher level by tumor cells. Since the identification and cloning of the first human TAA gene, peptide vaccination has been considered a promising approach for cancer treatment. The first studies were performed with peptides 8 to 10 aminoacids in length that would bind directly to MHC molecules without the requirement for endocytosis and/or intracellular processing. Aichele et al. initially demonstrated that injection of a peptide encoded by LCMV elicited a robust anti-viral T cell response in mice (Aichele et al., 1990). However, later data demonstrated that injection of short peptides induced tolerance rather than immunity (Toes et al., 1996). This approach clearly did not give the robust T cell response against antigen that had been expected. Further characterization of the response to injected peptides revealed that those peptides that elicited efficient responses actually contained overlapping epitopes for cytotoxic and helper T cells (Fayolle et al., 1991).

Based on these data, longer peptides, physically linking several epitopes (CTL as well as T-helper epitopes) were then developed (Perez et al., 2010). Injection of these chimeric peptides displays several advantages as compared to the previous short peptide injection strategy. Due to their length, they cannot directly bind MHC molecules and, therefore, must be engulfed and processed prior to presentation. This implies that the long peptides are taken up by professional APCs, which would limit presentation on MHC-I expressed by non-specialized APCs known to trigger short cytotoxic T cell response or even tolerance, which could explain the inefficiency of the short peptide model. Moreover, the requirement for engulfment and processing inside APCs allows a longer period of antigen persistence (Melief and van der Burg, 2008). The presence of several epitopes in the same vaccine permits activation of antigen-specific $CD4^+$ and $CD8^+$ T cells concurrently, and limits the phenomenon of tumor escape. Experimental mouse models and clinical studies using either naturally linked epitopes, or artificially linked epitopes have given promising results (Shirai et al., 1994; Zeng et al., 2002).

	Soluble antigens				Particulate antigens		DC-based vaccines
	8-10mer peptides	Long peptides	Proteins	Nucleic acids	Whole tumor cells	T cell-associated antigen	
Expression required	-	-	-	+	-	-	-
Endocytosis/ Phagocytosis	-	E	E	P	P	P	-
Processing required	-	+	+	+	+	+	-
Dissemination-Ability to reach lymphoid organs	+	+	+	-	-	+	-
Advantages	Rapid antigen presentation	Multi-epitopes	Multi-epitopes	Multi-epitopes	No need to know epitopes	Migrate to lymphoid organs	Ability to present antigen
Limitations	- One single epitope - Do not target only APCs - Adjuvant required	Adjuvant required	Adjuvant required	Delivery	Immuno-genicity	Immuno-genicity	Targeting to lymphoid organs

Table 5. Comparison of different forms of antigen for vaccine purposes. E, endocytosis; P, phagocytosis.

2) Nucleic acid-based vaccines

DNA or RNA have also been tested as antigen for eliciting anti-tumor and anti-viral responses. Injection of nucleic acids should work at two levels: first, these components encode for a specific antigen, but they can also act as ligands for PRRs resulting in the additional induction of an inflammatory response.

DNA vaccines consist of bacterial plasmids in which specific sequences encoding antigens have been inserted. They are injected intramuscularly or intradermally where the DNA will be taken up and antigen will be expressed in transfected cells. In most cases, the transfected cells are not APCs and have to be phagocytosed by DCs, allowing cross-presentation on MHC-I molecules. Alternatively, DCs may be directly transfected inducing direct antigen presentation (Rice et al., 2008). Interestingly, other sequences such as leader sequences that

will direct antigen to various intracellular compartments or sequences coding for immunomodulatory molecules can be added to these vaccines. The main limitation of this approach is the inefficiency of the vaccine delivery method. Intramuscular injections such as those optimized for DNA vaccine delivery in mice did not show the same efficiency in humans, perhaps due to the different volume used for injection. Viral vectors have been tested for their efficiency as DNA vaccine delivery systems with better success, but reactivity against the vector itself limited their efficacy overall. An electroporation method seems promising thus far, as it has been shown to enhance antigen expression upon intramuscular injection (Ahlen et al., 2007).

Despite similar limitations concerning their delivery, RNA vaccines have also been tested. It was observed that mRNA injection into the muscle induces protein expression in mice (Wolff et al., 1990). Additionally, it was demonstrated that intradermal injection of antigen-encoding RNA, together with an adjuvant, had a positive impact on the anti-tumor immune response (Weide et al., 2009). In order to optimize injection procedures, stabilization of RNA with protamine has been used to protect the molecule from RNases and promote persistence. Similar to DNA transfection, following mRNA injection, transfected cells are engulfed by DCs that cross-present antigen, unless the DCs themselves were directly transfected. Interestingly, Fotin-Mleczek and colleagues combined the same mRNAs in two different forms to induce a potent immune response in a mouse model of tumor vaccination: a non-complexed free RNA known to have a high translation efficiency and a stabilized protamine-complexed mRNA, which stimulated TLR7 (Fotin-Mleczek et al., 2011). This combination triggered a humoral and cellular response and allowed the immune system to mount a response despite the tumor-induced immune suppression.

3) Cell-based vaccines

Cellular vaccines have also been developed in the context of cancer immunotherapy. The overall aim of cancer vaccines is to induce an anti-tumor $CD8^+$ T cell response against the TAAs. Several cell-based strategies have been tested to achieve that goal, including using the tumor cells directly as a source of antigen, or injecting DCs that have been previously loaded with the tumor antigen of interest. Additionally, other cell types have been examined for their feasibility as antigen delivery vehicles.

(a) Whole tumor-cell vaccines

The main benefit of using tumor cells as a source of antigen is that it does not require detailed prior knowledge of the immunodominant antigen responsible for promoting a protective

immune response. Moreover, using entire tumor cells will allow for multiple epitopes to be processed and presented, in the hope that a broadly reactive response will be increasingly protective. When tested, the vaccine has been comprised of autologous cells originating from a resected fragment of the patient's own tumor. For example, M-Vax is composed of autologous irradiated tumor cells that have been modified with a hapten (dinitrophenyl) in order to improve its immunogenicity, and then mixed with BCG as an adjuvant. This vaccine has been tested intradermally for the treatment of melanoma (Berd, 2004). A limitation of this vaccine strategy is the difficulty in obtaining sufficient numbers of cells for vaccination.

Another well-characterized approach is the use of the allogeneic whole cell vaccines. These are cellular "cocktails" composed of several cancer cell lines mixed together. One advantage is that logistically, it is far easier to generate a mix of cell lines as compared to extracting autologous tumor cells for a vaccine. Furthermore, the different cell lines can be generated from different stages of tumor growth and development, thus representing a broader antigenic profile. This is an important point to consider as the antigenic repertoire of a tumor evolves over time, especially when metastases appear: the mixing of different cell lines increases the variety of TAAs exposed to the immune system and, in this way, can counteract immune escape. In particular, the melanoma vaccine compound Melacine is made of lyophilized melanoma lysates from two melanoma cell lines mixed with a complex adjuvant (Detox PC) (Sondak and Sosman, 2003). Similarly, the prostate treatment vaccine Onyvax-P is composed of three irradiated prostate cancer cell lines, which are administered with BCG intradermally to increase immune control in prostate cancer patients (Schlom et al., 2007).

While this kind of vaccines appears promising from a theoretical standpoint, the success of these therapies remains limited thus far. One explanation is that tumor-associated antigens are usually only weakly antigenic and more often they induce tolerance rather than immunity. Several directions must be explored in order to improve their efficiency; in particular the choice of adjuvant used to overcome immune suppression often observed in cancer patients (Copier and Dalgleish, 2010). Additionally, as the precise antigens are not known in this context, it is difficult to study their efficiency specifically. Thus an overall better understanding of immune response induced by these vaccines and the discovery of novel biomarkers helping to define their efficiency is greatly needed.

(b) DC-based vaccines

To elicit the most efficient response, a cancer vaccine will have to deliver the antigen to the lymphoid tissue in an immunostimulatory context. As described previously, DCs are known to be the most potent APC with an increased ability to present and cross-present antigen as

compared to other APC populations. Moreover, DCs also express high levels of costimulatory molecules and immunostimulatory cytokines upon activation that are required for an effective T cell response. Consequently many trials have been performed to investigate the capability of these cells to mount an anti-tumor immune response when given as a vaccine. Together, these studies have demonstrated that DCs pulsed with tumor antigens can induce a protective immunity in mouse models (Fuertes et al., 2011), and clinical trials are currently ongoing to evaluate the feasibility of multiple DC-based vaccines in humans (Palucka and Banchereau, 2012).

The first human DC-based vaccine trials were performed using cDCs that have been isolated or expanded *ex vivo* and then loaded with the antigen of interest. DCs can either be loaded with the specific peptide or the protein, or they can be transfected with the nucleic acid encoding the specific epitope for presentation (Palucka et al., 2005). The prostate cancer DC-based vaccine, sipuleucel-T, is the only DC-based vaccine that has received the approval of the FDA. Sipuleucel-T is composed of autologous mononuclear cells cultured with a fusion protein containing a common prostate cancer antigen, the prostatic acid phosphatase (PAP), linked to GM-CSF (Higano et al., 2009). The main limitation of this approach is that the sequence of the antigen and the epitope must be well defined. Another approach without this caveat is to load DCs with dying cells or tumor lysates in order to promote an anti-tumor response based on the processing and presentation of unknown tumor antigens. In this case, the immunodominant epitope(s) is/are likely present but does not require detailed knowledge on the part of the investigator (Palucka et al., 2006).

There are, however, limitations with this approach as well. First, the majority of DCs injected remains at the injection site upon administration and fail to migrate to the lymph node. This sequestration could result in DC dedifferentiation due to extended time in the injected site tissue microenvironment; a potential consequence of this is the induction of immune tolerance by these cells, rather than activation. Second, there is a concern that DCs are terminally differentiated cells. Consequently, they cannot be expanded to large numbers *ex vivo* and the life span of these cells upon injection is only about 24-48 hours. This limited time frame results in poor antigen delivery to the lymphoid tissue, where optimal T cell priming occurs. Several studies have been performed to address and, hopefully, circumvent these potential problems using strategies such as using different routes of vaccination and, in particular, the use of intranodal injection (Aarntzen et al., 2008). Multiple observations made during these studies have allowed for the optimization of DC-based vaccine development. It has been shown that upon uptake, the antigen must be transferred to endogenous DCs in order for the

vaccine to be efficient (Kleindienst and Brocker, 2003). Moreover, selective ablation of endogenous DCs or the injection of dying, loaded DCs, rather than live cells, are enough to abrogate the effects of a vaccine (Petersen et al., 2011). This indicates that the injected DCs have to migrate away from the injection site and transfer antigen to resident DCs to promote an efficient response. In parallel, knowledge about DC subsets has been expanded and improved. The murine resident CD8α^+ DC subset has been identified as the most competent for cross-presentation (den Haan et al., 2000). The human equivalent has recently been identified and characterized by its expression of the receptor Clec9A (Crozat et al., 2010; Poulin et al., 2010; Zhang et al., 2012). This subset is likely implicated in the engulfment of antigens that enter the lymphoid tissue, as well as antigen that may be transferred from peripheral, migratory DCs. From these data it made sense to enhance the delivery of antigen directly to the CD8α^+ DC subset, hoping that this strategy would help to avoid the problems of injecting *ex vivo* generated DCs. These antigens are targeted to the CD8α^+ DC subset via conjugation to antibodies specific of CD8α^+ DC surface receptors such as DEC-205 or DC-SIGN, both members of the C-type lectin receptor family (Bonifaz et al., 2002). This approach has great interest in the field of cancer vaccines, because it can be developed on a large scale. The existing limitation is that it must be combined with an adjuvant to trigger T cell priming (Bonifaz et al., 2004).

(c) T cells used as cell-associated antigens

Initially, because of their role in promoting an effective immune response, DCs appeared to be the best candidate for the development of a cellular vaccine. However it is possible that other cell types may be used as antigen delivery vehicles. Following interesting observations during a clinical study, T cells appeared to be a potential vehicle to deliver antigen in vivo. In this trial, during allogeneic bone marrow transplantation, lymphocytes were infused into patients. However, these cells had been genetically modified to express the herpes simplex virus thymidine kinase (HSV-TK) “suicide gene”, as a security mechanism in case of an autoreactive response against these transferred cells (Bonini et al., 1997). In this case, it was shown that patients developed anti-HSV-TK CD4$^+$ and CD8$^+$ T cell responses that promoted the elimination of the therapeutically transferred T cells; furthermore, memory T cells targeting HSV-TK were generated (Berger et al., 2006). Due to this unexpected response, T cells were then considered as a potential source of antigen. More than just an antigen delivery vehicle in the context of vaccination, T cells also have some advantages as compared to DCs: (i) these cells efficiently migrate to the lymphoid tissues allowing for the optimal localization of antigen for phagocytosis by resident DCs and induction of a T cell response; (ii) T cells can

be loaded with tumor peptides or genetically modified to express the whole tumor antigen; and (iii) these cells can be expanded to large numbers *ex vivo* allowing for the injection of sufficient cell-associated antigen into patients. For instance, Russo and colleagues injected T cells modified to express the tyrosinase-related protein 2 (TRP-2), a TAA specific for melanoma, into B16F10 tumor-bearing mice and observed a protective immunity (Russo et al., 2007). They convincingly demonstrated that $CD8\alpha^+$ DCs phagocytosed genetically modified T cells, matured and cross-presented antigen. Thus the response observed was not due to direct presentation of antigen by T cells. This promising mouse study was followed by an initial clinical trial (Fontana et al., 2009).

B. Stimulation of the immune response by adjuvants

Adjuvants are compounds that are injected in parallel with antigens to "help" the immune response by increasing the immunonogenicity of the antigen through a variety of mechanisms. Initially, the effects of adjuvants were discovered empirically: they were used to enhance the adaptive immune response and the effectiveness was evaluated by the antibody titers generated and level of protection that was conferred. Traditionally, adjuvants were used to boost the immune response against a specific antigen and, therefore, allowed for the use of lower antigen doses to confer protection. In recent years, another aspect of adjuvant function has been thoroughly explored and developed: the ability of adjuvant to modulate the quality and outcome of the T cell response. The combination of adjuvants and antigen, in some cases, will result in the generation of immunity that would not be induced by the antigen alone (Coffman et al., 2010). For instance, using a specific adjuvant injection strategy, it is possible to manipulate the balance between the Th1 *versus* Th2 response, or CD4 *versus* CD8 responsiveness; moreover, particular adjuvants can be selected to induce the generation of memory cells or decrease the time required for the development of an adaptive immune response. Although the first adjuvants were identified empirically, today there is a growing body of work that further characterizes the signaling pathways implicated in adjuvant activity. This increased understanding will allow for more targeted and specific modulation of the immune response during vaccination, as well as identify and support the development of the next generation of adjuvants.

1) Adjuvants used in therapies

(a) Adjuvants developed empirically

Despite extensive investigation, it is still not understood how adjuvants currently used in experimental models and/or in clinical applications function to modulate the immune response. In particular, the most common adjuvants used are Complete Freund's adjuvant, oil-in-water emulsions, saponin-based adjuvants or aluminum salts. It was initially thought that these molecules only act as passive depot or delivery vehicles when formulated in combination with antigen. However, recent data suggest that they have also an impact on innate immune response by inducing cellular damage at the site of injection, leading to necrotic cell death and release of DAMPs, promoting increased antigen uptake, or stimulating the inflammasome pathway (Maraskovsky et al., 2009; Marrack et al., 2009).

(b) New adjuvants

While the functional mechanisms of empirically discovered adjuvants are still not completely understood, new adjuvants have been developed more recently based on our knowledge about what is needed to stimulate the innate immune response. Specifically, most PRRs are potential targets for adjuvant stimulation, and the choice of receptors to engage depends on how the immune response should be modulated to obtain the desired extent of activation. Natural and synthetic agonists of various PRRs, especially TLRs, have been developed as adjuvants.

2) Adjuvants inducing type I IFN production

(a) Type I IFN

Type I IFN refers to a family of highly related cytokines that have and promote antimicrobial activity. Isaacs and Lindenmann discovered type I IFN 50 years ago, based on their ability to "interfere" with virus replication and spread (Isaacs and Lindenmann, 1957). Three families of IFN molecules with antiviral properties have since been identified (Pestka et al., 2004). Type I IFN consist of seven classes: IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ and IFNτ. By contrast, type II IFN corresponds to a single protein, IFNγ. Type III IFN was identified only recently and this family is composed of three IFNλ proteins.

Here I will focus only on type I IFN molecules. While they were initially identified due to antiviral properties, they also can act to defend against other pathogens such as bacteria, parasites, fungi and their microbially-derived products (Bogdan et al., 2004). Several

molecules that are known to induce type I IFN production, including TLR and RLR ligands, are currently being tested for their effectiveness and feasibility as candidate adjuvants.

(b) TLR3 and RLR ligands

Doubled stranded RNA (dsRNA) is a potent activator of innate immune cell activity following engagement with endosomal TLR3, as well as cytosolic ribonucleic acid helicases RIG-I and Mda5 (Alexopoulou et al., 2001; Kato et al., 2006). Poly I:C is a synthetic dsRNA analog that is known to be detected by TLR3 and Mda5. In this case, the size of this ligand is critical, as long dsRNA is required for optimal activation of Mda5. Several derivatives of poly I:C have been developed to limit toxicity and modify the downstream biological effects (Nicodemus and Berek, 2010). Poly ICLC corresponds to poly I:C complexed with poly-L-lysine and carboxymethylcellulose, allowing for a prolonged effect *in vivo*. This complexe is actively used in clinical trials under the brand name Hiltonol®. Ampligen® (polyI: polyC$_{12}$U) is another modified dsRNA obtained by the addition of uridine in the sequence leading to occasional base pair mismatches and a more rapid metabolism *in vivo*, which serves to limit its toxicity. Ampligen appears to act only through TLR3. TLR3 expression, and therefore stimulation, is not uniform and is likely different in specific tissue microenvironments. TLR3 is expressed in some subsets of cDCs, macrophages, and NK cells as well as non-immune cells such as fibroblasts and epithelial cells. Additionally, RLRs are expressed in different cell types, although the RLR expression profile has not yet been detailed thoroughly. The signaling pathways triggering type I IFN production upon TLR3 or RLR engagement are different and will be described later in this introduction. TLR3 stimulation has been demonstrated to act on DCs by stimulating IL-12 and type I IFN secretion, as well as upregulating antigen presentation (Schulz et al., 2005). Mda5 appears to be more involved in the response of non-hematopoietic cells by stimulating their production of type I IFN (Longhi et al., 2009). The activation of both TLR3 and Mda5 pathways by poly I:C serves to strongly enhance the Th1 and $CD8^+$ T cell response, better than either one of the two pathways alone. Thus far, the combination of TLR3 action directly on DCs and bystander Mda5 action mainly on non-hematopoietic cells appears to be the best way to boost the $CD8^+$ T cell response.

(c) TLR7 and TLR8 ligands

TLR7 and TLR8 are also endosomal PRRs, responsible for detecting single stranded RNAs (Diebold et al., 2004). These PAMPs are not particularly stable because they are quickly degraded by RNases in the environment. Thus, they do not make for good adjuvants unless they are modified or formulated with another component that confers an increased stability.

Some synthetic compounds such as imidazoquinolines and adenosine and guanosine analogs, first developed as type I IFN inducers are known to act as ligands for TLR7 and TLR8 (Hemmi et al., 2002). Although they bind similar ligands, TLR7/8 are differentially expressed *in vivo*: TLR7 is found in B cells, neutrophils and pDCs in both human and mice, and also in macrophages and CD8α^- DC in mice; TLR8 is expressed in DCs in human but does not seem functional in mice (Iwasaki and Medzhitov, 2010). Use of TLR7 and TLR8 ligands has been demonstrated to activate DCs and pDCs by upregulating costimulatory molecules and inducing the production of type I IFN and IL-12.

(d) TLR9 ligands

TLR9 is also found in the endosome and is capable of recognizing DNA, preferentially modified by the CpG dinucleotide (Blasius and Beutler, 2010). Synthetic 18-25 oligodeoxynucleotides with CpG motifs have been developed commercially as an adjuvant and have been tested as either a soluble molecule or formulated as nanoparticles. TLR9 has a much more restricted expression profile than the other PRRs previously discussed: it is only expressed in pDCs and in B cells in humans and mice, and also seen in cDCs in mice only (Campbell et al., 2009). CpG has been shown to both enhance humoral immunity and favor Th1 response. CpG primarily activates pDCs. Their stimulation usually results in the release of large amounts of type I IFN, as pDCs are understood to be "professional" producers of these cytokines.

3) Signaling pathways implicated in type I IFN production

Several signaling pathways are responsible for the production of type I IFN depending on the receptor that has been engaged. While ligation of TLR3 and TLR4 ligands triggers a TRIF-dependent pathway, other molecules such as TLR9 ligands induce a Myd88-dependent pathway. In most cells, activation of either of these pathways leads to the phosphorylation of the transcription factor IRF3 that will interact with other molecules to form the enhanceosome. The enhanceosome then promotes the expression of IFNβ and IFNα4 is then induced. Secreted type I IFN can participate in an autocrine amplification loop through IFNAR signaling. Type I IFN bind their receptor inducing the phosphorylation of IRF7, which stimulates further type I IFN production (**Figure 10**). Interestingly, the mechanism of type I IFN production in pDCs is slightly different. They constitutively express IRF7, thus any inflammatory stimuli directly triggers IRF7 phosphorylation and rapid regulation of type I IFN production without the requirement for an amplification loop (Honda et al., 2006).

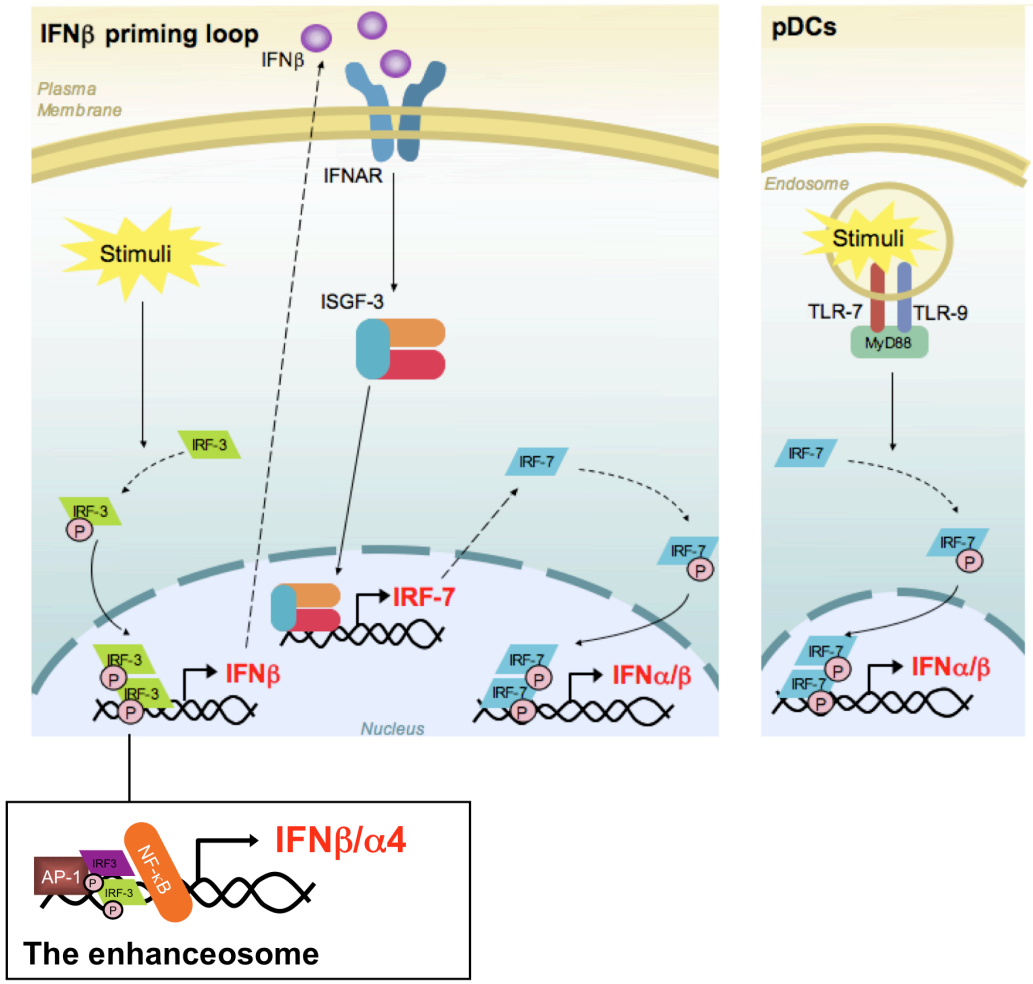


Figure 10. Pathways leading to type I IFN production. Original design *from J. Decalf.*

4) Effects of type I IFN

As mentioned previously, type I IFN were first discovered for their antiviral properties; they limit viral replication by inhibiting protein translation, viral polymerase activity and by promoting RNA degradation and/or cell apoptosis. Importantly, these cytokines are also known for their ability to modulate the function of immune cells. They can act directly, via signaling through the IFN-alpha receptor (IFNAR), or indirectly by inducing the secretion of chemokines that recruit cells to the site of immunization or cytokines that regulate cell functions, or activate bystander cells.

(a) Signaling pathways activated by type I IFN

In spite of the high number of type I IFN isoforms, they bind to and signal through the same IFNAR receptor, although they do appear to differ in the binding affinity for the receptor. The receptor is composed of two subunits, IFNAR1 and IFNAR2, and is expressed in most tissues. The main signaling pathway described downstream of IFNAR is the Janus Activated Kinase /Signal Transducer and Activation of Transcription (JAK/STAT) pathway (**Figure 11,**

A). IFNAR is constituvely associated with Tyrosine Kinase 2 (Tyk2) and JAK1, which are activated by transphosphorylation upon IFN interaction with the receptor. These activated kinases then phosphorylate IFNAR on tyrosine residues that serve as docking sites for STAT molecules. STATs become substrates for phosphorylation when they are recruited to the docking sites on the receptor. Once phosphorylated, the STATs dimerize and migrate to the nucleus where they will regulate the expression of Interferon-Stimulated Genes (ISGs). Although STAT1 and 2 are the most common mediators of the type I IFN response, other STAT molecules can be recruited and mediate signaling depending on the immunization conditions and cell type. Moreover, signaling via particular STAT molecules can result in differential downstream effects that have been implicated in the pleiotropic roles of type I IFN. This point will be further discussed later in this thesis.

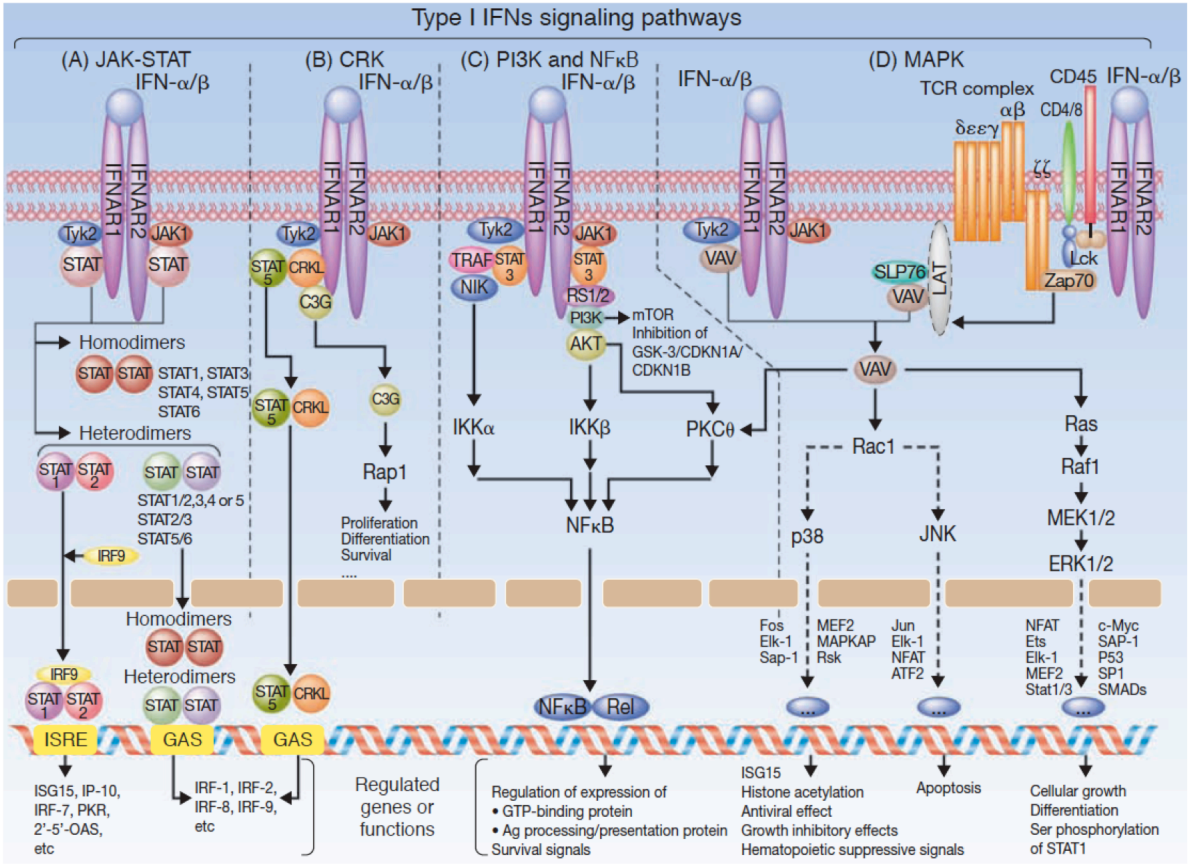


Figure 11. Signaling pathways activated by type I IFN. Figure from *Hervas-Stubb et al., 2011.*

IFNAR can activate more than the JAK/ STAT pathway (Hervas-Stubbs et al., 2011); the Mitogen Activated Protein Kinase (MAPK) and the phosphoinositide-3-kinase (PI3K) pathways can also be activated. Cross-talk between these different pathways has been observed (**Figure 11, B, C, D**).

(b) Effects of type I IFN on dendritic cells

(i) DC lifespan

Regulated apoptosis of DCs controls the magnitude of an immune response by limiting antigen presentation to specific T cells and is modulated by both extrinsic and T-cell mediated signals (Kushwah and Hu, 2010). Under steady state conditions, immature DCs express high levels of the anti-apoptotic molecule Bcl-2 and their half-life is approximately 1.5 to 3 days. Upon activation, DCs downregulate Bcl-2 expression allowing for increased apoptosis and eventually leading to their terminal differentiation and death. Type I IFN are capable of regulating DC turnover *in vivo*. DC turnover is more rapid in WT than in IFNAR$^{-/-}$ mice and injection of a type I IFN inducer enhances the turnover of DCs in WT mice further (Mattei et al., 2009). Specifically, it was recently demonstrated that poly I:C injection induces transient DC activation followed by a marked reduction in the number of CD8α^{+} cDCs due to the apoptotic cell death (Fuertes Marraco et al., 2011). To distinguish whether this decrease was due to DC migration or death, they sorted DCs a few hours post-injection and examined the induction of apoptosis. They demonstrated that poly I:C modulates the expression of pro- and anti-apoptotic genes and that these differential patterns are dependent on type I IFN signaling. In fact, the initiation of apoptosis is not specific for poly I:C, as the same effect was observed upon treatment with other adjuvants. A caveat to this work is that it was performed following the injection of a TLR ligand known to induce IFN only – without the administration of antigen. Interestingly, in a model combining administration of cell-associated antigen with type I IFN, it was shown that type I IFN sustain the survival of antigen-bearing DCs whereas it induces the apoptosis of bystander DCs by regulating pro- and anti-apoptotic genes (Lorenzi et al., 2011).

(ii) DC maturation

Following type I IFN administration, mouse cDCs undergo both a phenotypic and functional maturation upon type I IFN exposure (Montoya et al., 2002). Moreover, type I IFN appear to participate in increasing the efficiency of cross-priming of CD8^{+} T cells. Le Bon and colleagues showed this phenomena was due to a direct action of type I IFN on the DCs by comparing cross-priming ability of WT versus IFNAR$^{-/-}$ DCs (Le Bon et al., 2003). Furthermore, these cytokines allow a higher amount of peptide-MHC-I complexes and maturation markers on the DC surface (Lorenzi et al., 2011).

Similarly, immature human cDCs treated with type I IFN upregulate MHC, as well as costimulatory molecules and have been shown to be better inducers of effective CD8^{+} T cell

responses. Building upon these conclusions, type I IFN are now commonly used for DC immunotherapy to induce DC differentiation and maturation (Santini et al., 2000).

(iii) Persistence of antigen

Lorenzi and colleagues have analyzed the effects of type I IFN on cross-presentation by using UV irradiation-induced apoptotic EG7 thymoma cells in combination with type I IFN, both *in vitro* and *in vivo* (Lorenzi et al., 2011). They demonstrated that type I IFN do not actually affect the uptake of antigen by DCs but, rather, enhance the retention of engulfed antigen inside of DCs, probably in phagosomal compartments. Moreover, they showed that this was most likely due to the regulation of phagosomal pH by type I IFN (**Figure 12**).

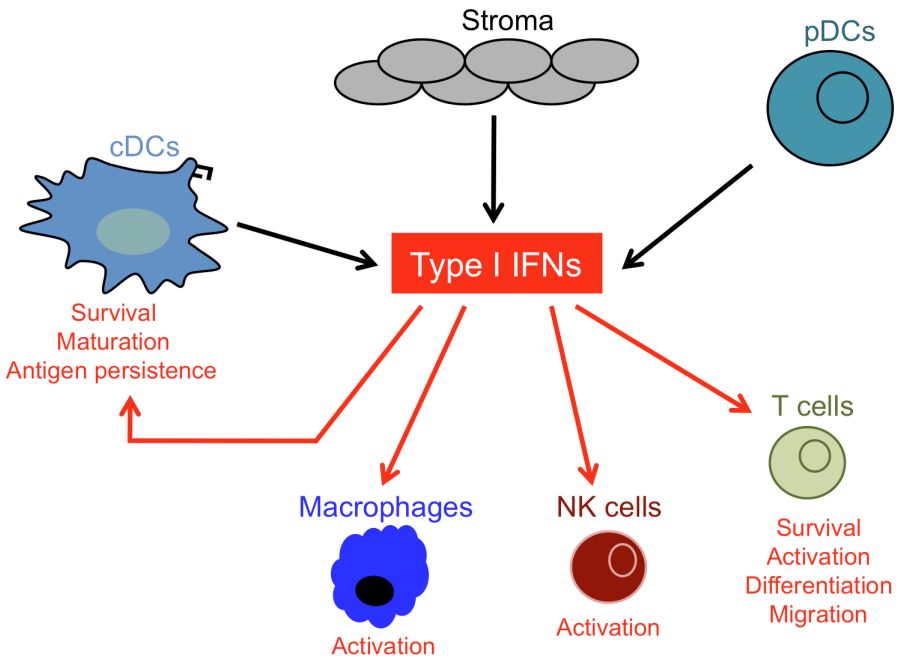


Figure 12. Multiple sources and multiple targets of type I IFN.

(c) Effects of type I IFN on T cells

Type I IFN not only modulate the DC life cycle and responsiveness, but also appear to play a critical role in many events surrounding T cell survival, activation and responsiveness (**Figure 12**).

(i) T cell survival

It has been shown that type I IFN act to promote the survival of WT but not IFNAR-deficient T cells, indicating that IFN signaling is critical in this process (Marrack et al., 1999). This function for these cytokines is thought to help in avoiding the rapid death of antigen-specific T cells upon antigenic stimulation.

(ii) T cell migration and recirculation

It has been shown that type I IFN are capable of modulating T and B cell recirculation by inducing the redistribution of lymphocytes throughout the body. Treatment with type I IFN triggers a transient blood lymphopenia, which occurs through the direct stimulation of lymphocytes (Kamphuis et al., 2006). Type I IFN also improve T cell priming by facilitating the encounter between antigen-presenting DCs and T cells in the draining lymph node. To accomplish this, type I IFN have been shown to induce the retention of T cells in the lymph node by downregulating the expression of sphingosine-1-phosphate (Shiow et al., 2006), increasing the probability that DCs will encounter the antigen- specific T cells.

(iii) $CD8^+$ T cell activation and differentiation

Type I IFN have been described to act as a potential "signal 3" in the activation cascade needed for $CD8^+$ T cell stimulation by antigen-presenting DCs (Curtsinger et al., 2005). While Le Bon et al. initially showed that the enhancement of $CD8^+$ T cell cross-priming is exclusively due to a direct action of type I IFN on DCs, they further demonstrated a few years later that IFN are additionally acting at the level of the $CD8^+$ T cell. To do this, they used a mixed BM-chimera model where the T cell population was deficient for IFNAR. In these animals, they observed an overall reduction in effective priming (Le Bon et al., 2006). These results were confirmed by another group using the transfer of antigen-specific $CD8^+$ T cells into either WT or $IFNAR^{-/-}$ mice to show that the T cells are actually the direct targets of type I IFN and that these cytokines have a direct impact on both T cell clonal expansion and memory formation (Kolumam et al., 2005; Thompson et al., 2006).

(iv) $CD4^+$ T cell differentiation

Type I IFN also modulate the differentiation of $CD4^+$ T cells. They have been shown to favor Th1 differentiation, when signaling in combination with other cytokines such as IL-12. By contrast, they inhibit T cell differentiation into the Th2 or Th17 subsets, which have been implicated in allergies and inflammatory responses (Huber and Farrar, 2011).

(d) Effects of type I IFN on other immune cells

Type I IFN have also been implicated in the regulation of other leukocytes (**Figure 12**). In particular, type I IFN are known to activate NK cells by enhancing their cytolytic activity and production of IFNγ. Moreover they also induce the accumulation of proliferating NK cells via induction of the main NK-regulatory cytokine, IL-15 (Nguyen et al., 2002). Beyond lymphocytes, type I IFN is also known to boost macrophage activity (Bogdan et al., 2004).

5) Pleiotropic roles of type I IFN

(a) Impact on the global immune response

Type I IFN are currently approved for use as the treatment of several diseases, including solid and hematologic cancers, multiple sclerosis, as well as chronic viral hepatitis. In patients chronically infected with HCV, it is known that endogenous type I IFN are produced, but the infection is not cleared. However, treatment with exogenous IFNα can lead to the resolution of infection in approximately 50% of patients (Mihm et al., 2004). These data strongly suggest that there is a differential effect of endogenous versus exogenous injected IFN on viral clearance. Of note, for this treatment regimen, IFNα is stabilized by its conjugation to polyethylene glycol, which confers an increased half-life upon injection and also a lag in clearance from the patient's system. In contrast, IFNβ is used for the treatment of multiple sclerosis patients, in order to inhibit their autoreactive immune response. These two examples of the contrasting effects of type I IFN treatments in clinical settings suggest the vast complexicity of its action.

Studies examining the secondary side effects of IFNα treatment in cancer patients have demonstrated that this treatment may have further differential effects on the immune response (Gogas et al., 2006). Several autoimmune disorders induced by treatment have been described. DC-derived monocytes obtained following IFNα treatment have been shown to be fully matured, able to engulf apoptotic bodies and capable of trigerring an anti-tumor T cell response. One explanation for the development of autoimmune disorders during the course of IFNα treatment could be that DCs are stimulated and take up apoptotic bodies derived from normal host cells and present self-antigens, leading to a robust anti-self immune response (Rizza et al., 2010).

Pleitropic functions of type I IFN have also been described for infectious diseases (Decker et al., 2005). Type I IFN favor infection by *Listeria monocytogenes* or *Chlamydia muridarum,* probably by sensitizing effector cells to death (Qiu et al., 2008). However, in the case of infection by *Streptococcus pneumoniae* or *Salmonella typhimurim*, type I IFN protect the host by enhancing antibody production or inducing IFNγ production, respectively.

(b) Factors influencing type I IFN action

The pleiotropic effects of type I IFN observed at the systemic level are not at all well understood. Nevertheless, recent studies have offered some new insight into the parameters that regulate the function(s) of type I IFN at cellular and molecular level, which may be able to explain these differential functional observations. Importantly, it has been observed that

several different environmental conditions appear to play a large role in modulating the action of type I IFN.

(i) Type I IFN dose

Differential effects of type I IFN have been described to correlate with the dosing amount. High levels of type I IFN, such as those produced during viral infections, inhibit IL-12 secretion by DCs whereas lower levels are required for the production of the bioactive IL-12 heterodimer (Gautier et al., 2005). To explain these results, the following model was proposed: the level of IFN produced corresponds to the severity of the infection and thus, is capable of modulating the outcome of the response including the induction of differential mechanisms/functions necessary to combat a wide range of pathogens (Biron, 2001).

(ii) Location of type I IFN secretion

While pDCs are known for their ability to secrete high amounts of type I IFN, every cell of the body is capable of generating type I IFN upon stimulation. Moreover, each cell type expresses a specific panel of PRRs and, based on this profile, are more or less responsive to signals that result in type I IFN production. As previously described, inflammatory signals derived from hematopoietic or stromal cells are not functionally equivalent. This could be the case for type I IFN. Stromal production of type I IFN has been shown to be critical in the control of some infections (Schilte et al., 2010), whereas other immune responses are solely dependent on IFN production by pDCs.

(iii) Timing of type I IFN production and the activation state of target cells

Previous studies performed in our lab have demonstrated that IFNα/β has mutually exclusive effects on human DCs depending on their maturation state. Immature DCs exposed to IFNα/β were impaired in their ability to cross-present antigen and activate $CD8^+$ T cells, whereas IFNα/β stimulation of mature DCs results in enhanced T cell activation. The contradictory actions of type I IFN in this example appear to depend on the timing of stimulation as compared to the state of DC maturation (Longman et al., 2007). It has been shown that these differential effects are due to a molecular switch between the use of STAT1 and STAT4 in the IFNAR signaling pathway. These results are in line with a previous study showing that adjuvant delivery prior to immunization impairs subsequent priming, perhaps due to the modulation of APC maturation state by the adjuvant (Wilson et al., 2006). Nagai and colleagues also showed opposite effects of type I IFN on DCs and T helper cell differentiation that was dependent on the timing of IFN administration. The presence of IFN

during DC maturation enhances their ability to induce Th1 differentiation, whereas IFN stimulation of mature DCs during primary T cell stimulation promotes the generation of IL-10-secreting T cells (Nagai et al., 2003). Similar contrasting effects have also been observed for NK cells. At steady state, NK cells expressed a high basal level of STAT4, which allows for their rapid activation and IFNγ secretion upon type I IFN stimulation. However, following activation, there is a switch from STAT4 to STAT1, resulting in the downregulation of IFNγ production (Miyagi et al., 2007).

IV. STRATEGIES FOR ASSESSMENT OF ANTIGEN-SPECIFIC T CELLS IN VIVO

The study of $CD8^+$ T cell responses in mouse models requires a technical approach that will allow for the study of the T cell populations during the different phases of the response. As illustrated in **Figure 13**, the number of antigen-specific T cells varies considerably over the course of an immune response and it remains challenging to detect these cells, particularly in naïve animals, as well as in the first days following immunization. Effective techniques must be highly sensitive in order to create a complete picture of the T cell response to a specific antigen.

Additionally, the experimental model used to test a given antigen or a vaccination strategy must be carefully considered prior to starting the study. Factors such as the conditions of immunization, quality of the microenvironment, T cell precursor frequency, antigen dose, antigen form and administration route, may all affect the regulation of the subsequent response. These conditions should be chosen to reflect the physiologic situation as closely as possible in order to accurately test the efficiency of a vaccine.

A. Available techniques to study the T cell response in vivo

Several techniques are currently used to detect antigen-specific T cells *in vivo*. One common limitation to most of these methods is their sensitivity; in many cases, the limits of detection of these assays do not allow for the detection of rare T cell populations. Additional approaches have been developed to circumvent this problem.

1) Current techniques

(a) Limiting dilutions analysis

For many years, limiting dilution analysis was the standard method used to determine the frequency of antigen-specific T cells in a particular model. The main limitations of this approach were the requirement for exogenous stimulation and expansion, which introduced potential bias and significant inter-assay variability.

(b) Tetramer staining

The generation of MHC-I tetrameric complexes, originally described by Altman and Davis, represents what has been proven to be a major technical advance for the study of the antigen-specific T cell responses (Altman et al., 1996). MHC tetramers are reagents that carry 4 MHC class I-peptide complexes and, thus, have the ability to interact with multiple TCRs at the surface of a single $CD8^+$ T cell. Fluorescent labeling of tetramers has allowed the identification of antigen-specific T lymphocytes based on the avidity of their TCR. By combining this method with other antibody-based staining protocols (surface markers or intracellular cytokines), it is now possible to phenotypically and functionally characterize the antigen-specific T cell response. Nevertheless, there remains one important technical limitation: the limit of detection is relatively high – for tetramer staining, only one cell in 10^4 can be observed, which does not permit the direct detection of rare antigen-specific populations such as circulating naïve antigen-specific T cells (**Figure 13**).

(c) ELISPOT and intracellular staining

Both ELISPOT and intracellular staining techniques allow the functional characterization of the T cell populations of interest. These assays are based on the ability of antigen-specific T cells to secrete cytokines upon short, *in vitro* restimulation with cognate peptide. Such approaches are capable of distinguishing lymphocytes possessing the capacity to secrete a given cytokine at the time of the assay, indicating the extend of prior T cell priming; however this response corresponds only to a fraction of the antigen-specific population.

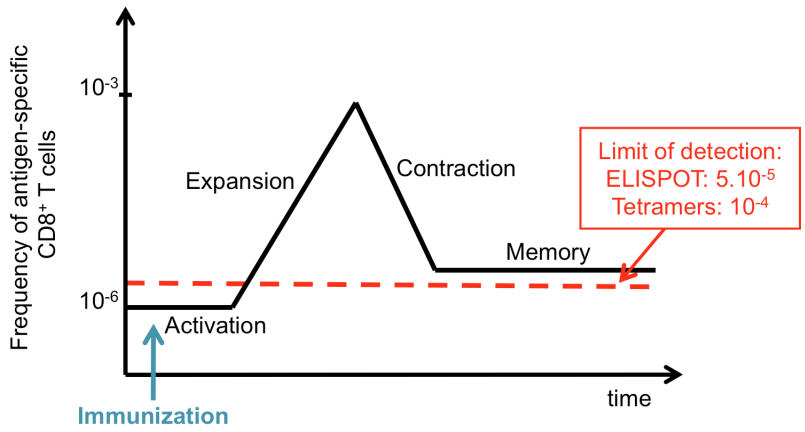


Figure 13. Limit of detection of established techniques for studying the T cell response *in vivo*.

(d) Adoptive transfer of TCR-transgenic CD8 T cells

Some antigens induce a low level T cell response, which is not consistently detectable by the techniques just described. Another approach was developed to study the T cells that are specific for a given epitope, even in the case that the response is not robust. To address this and develop a technique that would allow for these instances, mice that were transgenic for a given TCR were generated. In these mice, the vast majority of the $CD8^+$ T cells are specific for the same epitope. These mice were then crossed to Rag-deficient mice, ensuring that all T cells were specific for this epitope. In this way, various numbers of antigen-specific T cells can be transferred into naïve mice, allowing for easier detection by artificially increasing the precursor frequency. The transfer of TCR-transgenic T cells has been extensively used to model the endogenous response in combination with tetramer and intracellular staining. Another benefit of this approach is the possibility to label transgenic T cells prior to their transfer in order to follow them *in vivo*. Moreover, it is also possible to transfer TCR-transgenic T cells that are deleted for specific genes to further dissect the mechanisms of the T cell response.

Some years ago, several groups demonstrated that adoptive transfer experiments using large numbers of specific T cells can actually modify the outcome of the immune response under examination (Marzo et al., 2005). It was then realized and acknowledged that the transfer of non-physiologic numbers of TCR-transgenic T cells does not accurately mimic the endogenous response, which poses a severe limitation on the interpretation of studies using this strategy. Specifically, Badovinac and colleagues performed experiments transferring graded numbers of TCR-transgenic T cells and showed that high initial precursor frequencies actually limited the expansion of effector cells (Badovinac et al., 2007). They concluded that

a limited amount of transgenic cells must be transferred in order to interpret the data as a mimic of the endogenous repertoire.

2) Recent improvement: tetramer-based enrichment

To overcome the problem of the low T cell frequency for a given specificity in the endogenous repertoire, Moon and colleagues developed a new technique using tetramer-based enrichment to detect rare $CD4^+$ T cells of a given specificity (Moon et al., 2007). To use this technique, the cells are first stained with MHC-I-peptide tetramers and then enriched, using magnetic beads. In this way, it is possible to enrich tetramer-positive T cells more than 100-fold. This strategy successfully managed to detect antigen-specific T cells in a naïve mouse and has been adapted and optimized for the study of $CD8^+$ T cells *in vivo* (Obar et al., 2008).

B. Conditions of immunization

As briefly introduced previously, the outcome of a T cell response may be regulated by the conditions of immunization. The characteristics of the antigen, the administration methods, and the inflammatory context in which the injection is performed are all parameters that could modify the T cell response. These potential points of variation should be anticipated and carefully considered when developing an experimental system in order to be as close as possible to the physiologic and clinical conditions one is attempting to model.

1) Antigen form and dose

As described previously, different forms of antigen are used in vaccinology, as well as in experimental animal models. The time needed to elicit an effector T cell response upon antigen injection depends on several factors including the nature of antigen and/or the context of immunization. For instance, antigen presentation occurs rapidly after the injection of 8-10 mer peptide vaccines because processing is not required. On the contrary, there is a considerably longer delay when injecting a cell-associated antigen that has to be phagocytosed and processed prior to presentation. Moreover, it is possible to target vaccines to different DC subsets by complexing the antigen with specific antibodies such as DEC-205, the administration of the vaccine in a specific location, or via its expression as part of viral vectors that infects a particular cell type of interest. Knowing that DC subsets have different abilities to present antigen on MHC-I or –II and, as a result, trigger various T cell or B cell responses, the choices of antigen form and delivery technique/location are of major

importance and must be performed in a manner that will optimize the immune outcome required to cure the disease of interest.

Additionally, dosing of antigen is also critical when considering how to modulate an immune response upon vaccination. Immunization with a very high dose of antigen will result with the site of injection being overloaded with antigen leading to the recruitment of DCs that are normally not implicated in a more "physiologic" response. Other parameters of the response may be affected due to this large dose of antigen. Additionally, this parameter is of specific importance when considering the translation of experimental animal model data to therapeutic development in humans. A good example is the case of vaccination with cell-associated antigen such as tumor cells. While it is easy to use this strategy in mouse models due to the availability of reagents, obtaining the quantities of this antigen in clinical setting necessary to obtain the desired result may not be reasonable or realistic (Copier and Dalgleish, 2010).

2) Context of immunization

Depending on the conditions of immunization, the outcome of the immune response may be varied. This variability is explained whith several lines of reason that should be considered when models are developed to study the T cell response (**Figure 14**).

(a) Probability that DCs encounter the antigen

Clearly, the dose of antigen regulates the probability that DCs will encounter antigen. The route of immunization also impacts this probability. For instance, an intravenous injection delivers antigen directly to bloodstream, which is screened for antigen in the spleen. In this case, the antigen rapidly travels to a lymphoid organ where a large number of resident DCs are prepared for antigen encounter. On the contrary, an antigen delivered to the skin via intradermal injection is delivered in a location where there are other subsets of DCs. In this case, the antigen either migrates by itself to the draining lymph node, is drained by the lymph, or is taken up by skin-resident DCs that will transport it to the lymph node. Delivery of antigen to a mucosal surface reflects yet another unique environment that requires its own specific factors to regulate the uptake of antigen. It is important to note that the number of DCs available in different sites of immunization is highly variable and, consequently, the probability that the antigens will encounter the appropriate DC, and the time required to facilitate antigen presentation in lymphoid organs differs greatly depending on the route of immunization. Moreover, the DC subsets present at various injection sites are not of the same quality and will present antigens with various efficiencies. The delivery of antigen to specific tissue locations may also trigger the formation of an antigen depot influencing the persistence

of antigen, a factor also known to modulate immune responses. Due to the combination of these factors, previous studies have demonstrated that treatment efficiency depends on the form of antigen and the route of immunization (Abadie et al., 2009).

(b) Probability that antigen-presenting DCs encounter antigen-specific T cells

Once the antigen is processed and presented on the DC surface, these cells will next interact with antigen-specific T cells. This meeting occurs in lymphoid organs where T cells and DCs are present at very high numbers. This concentration of cells increases the probability that antigen-presenting DCs encounter the correct antigen-specific T cells. As described earlier, the frequency of antigen-specific T cells can be artificially increased in experimental systems via adoptive transfer, allowing for a more rapid encounter between the two cell types. Other circumstances such as a prior exposure to this antigen and presence of memory antigen-specific T cells can also influence the rate of the subsequent response to the same antigen.

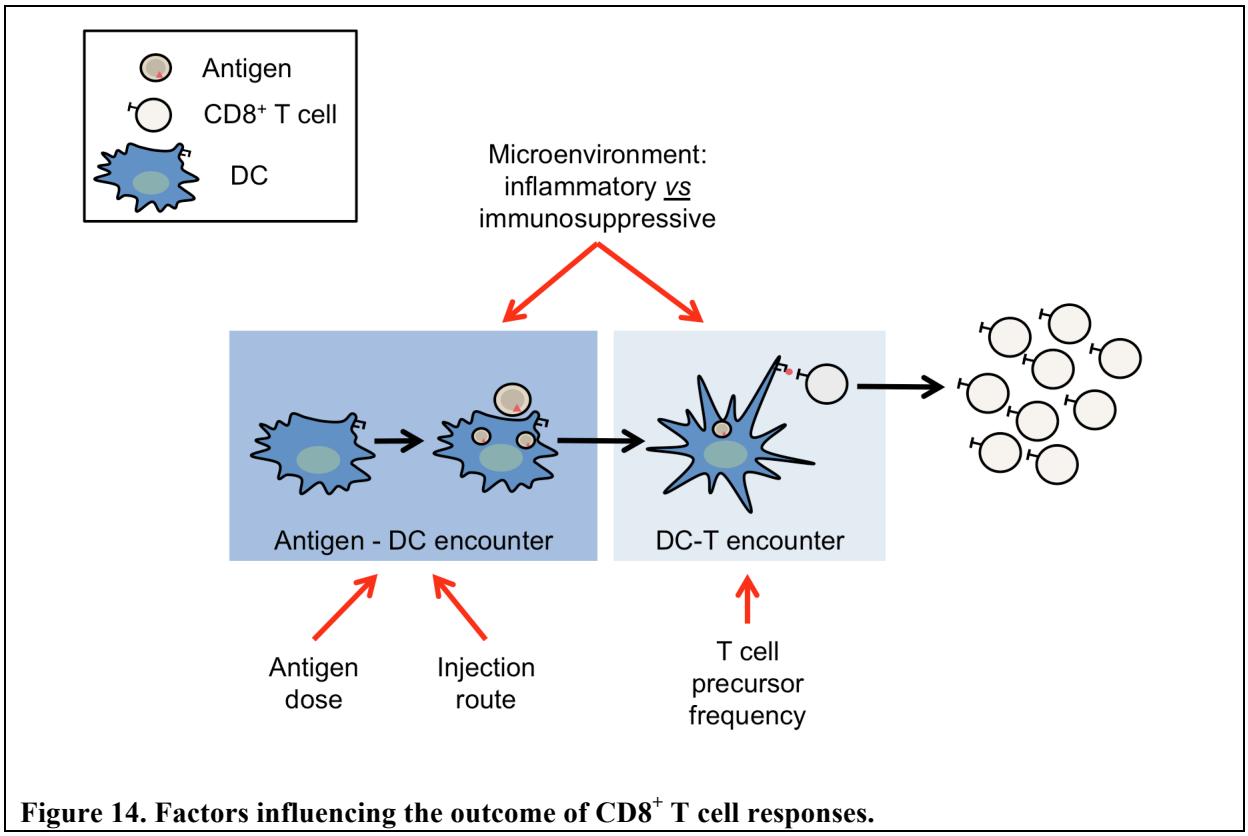


Figure 14. Factors influencing the outcome of CD8$^+$ T cell responses.

(c) Microenvironment

Although the probability that the different actors of the immune response interact together in a steady state has already been discussed, the microenvironment is often modified by infection, tumor growth or adjuvant administration resulting in altered inflammatory or

immunosuppressive conditions. The conditions are highly likely to impact the outcome of the immune response.

(i) Inflammatory conditions

A parallel infection or adjuvant administration can induce an inflammatory microenvironment at the time of vaccination. Consequently, the immune response induced following injection may qualitatively different because of the impact of existing inflammatory signals on local immune and/or stromal cells (Wilson et al., 2006). Similarly, patients that have a chronic disease might respond differently to vaccination because their immune system is being continually stimulated, which critically can lead to exhaustion of the immune system (Frebel et al., 2010).

(ii) Immunosuppressive conditions

The investigation of cancer vaccines represents an interesting and informative perspective that exemplifies the impact of pre-existing conditions on the effectiveness of immune response. Many well-characterized mouse studies have investigated prophylactic vaccination, which refers to the vaccination of the animal against tumor antigens prior to tumor challenge. Yet, in most physiologic cases, the tumor will already be present at the time point that the patient would be treated with cancer vaccine. Thus, the focus needs to be on the development of therapeutic vaccination strategies. The presence of a tumor makes a dramatic difference, as tumors are known to induce an immunosuppressive microenvironment, as well as have a limited access to immune cells (Lesterhuis et al., 2011). Both factors would critically impact the response to a vaccine. To circumvent the impact of the tumor on the effectiveness of the immune response, patients can be treated at very early stages when the immune system is still competent or therapies can be modified by combining the vaccine with other treatments that will inhibit tumor-mediated immunosuppression.

(iii) Combination of treatments

To optimize treatment efficiency, several approaches are often combined: for example, adjuvant mixed with peptide vaccine, chemotherapy or radiotherapy administered in parallel of cancer vaccine. These additional treatments modify the microenvironment and may enhance the immune response despite tumor-mediated immune suppression. Importantly, the timing for these different treaments remains to be tested, as it has been observed that targeted therapies applied before or after the administration of a cancer vaccine do not have the same impact on disease progression (Vanneman and Dranoff, 2012). Similarly, the injection of adjuvant prior to immunization has been shown to completely inhibit subsequent T cell

response, despite the substantial evidence that the optimal use of these components will significantly boost the immune response (Wilson et al., 2006).

In this section, I have reviewed the many parameters that can influence the outcome of an immune response. These factors must all be carefully considered and examined when developing new vaccines. To efficiently address this, models that represent physiologic conditions in humans are needed, as well as the development of technologies and tools that will allow for the detailed examination of a physiological immune response, even at low levels. Indeed, as currently studied, some parameters that influence the extent of an immune response, such as adjuvant dose, T cell precursor frequency and route of administration are artificially modified in mouse models. While this can facilitate the study of the immune response and limit the variability in the results, it was just reviewed here how these manipulations can impact the outcome of the response. Thus, it is imperative to keep these parameters as close as possible to human physiology. In order to study the immune response in these "physiologic" conditions, new techniques are continually being developed, such as the tetramer-based enrichment assay. As each technique presents its own limitations, the difficulty lies in choosing the compromise between the physiologic relevance of the model and the ability to study the T cell response with the tools available.

The $CD8^+$ T cell response is a critical component of the adaptive immune system. These cells are considered particularly important for the host response to microorganisms and cells undergoing malignant transformation. In order to carry out their effector function efficiently, these cells must be activated by DCs, presenting MHC-I-peptide complexes. For many infections and most tumors, an indirect presentation pathway (referred to as cross-priming) is utilized for the loading of antigen onto the MHC-I of DCs. Although an effective $CD8^+$ T cell response is critical for the control of many diseases, including tumor growth, chronic viral infection and other intracellular pathogens, vaccines capable of eliciting protective $CD8^+$ T cells have not yet been developed. In order to accomplish this goal, much work has been performed to further understand the mechanisms of cross-priming and targeting this pathway for the purpose of novel vaccination strategies. For instance, Fontana and colleagues have conducted clinical studies utilizing a cell-associated antigen - peripheral blood lymphocytes genetically modified to express tumor antigens - as a strategy for inducing tumor immunity in cancer patients (Fontana et al., 2009).

Our laboratory has been interested in defining optimal strategies to cross-prime $CD8^+$ T cells after the delivery of cell-associated antigen. However, in the development of experimental models, it has been important to prioritize establishing conditions that reflect the physiologic situation present during the vaccination of humans, particularly in terms of antigen-specific T cell frequency. In order to do this, we expanded upon a recently described tetramer-based enrichment assay that allows for the detection of low numbers of antigen-specific T cells. This strategy allowed us to work within relatively physiologic conditions, specifically in terms of T cell precursor frequency, in order to investigate the impact of two important parameters that must be taken into consideration by investigators interested in initiating adaptive immune responses during vaccination - the route of vaccination and the use of adjuvants.

I. IMPACT OF THE ROUTE OF IMMUNIZATION ON $CD8^+$ T CELL CROSS-PRIMING

To mimic the administration of cell-associated antigen and study the efficacy of the resulting $CD8^+$ T cell cross-priming, we used a well-characterized model of cross-presentation. Donor

splenocytes derived from K^{bm1}mOva mice were inoculated into WT recipients. These cells express a membrane-bound ovalbumin as an antigen and display a mutated K^b molecule ensuring cross-presentation and not direct presentation of antigen.

To avoid the requirement for TCR-transgenic T cells, we optimized the previously described technique of tetramer-based enrichment to detect low numbers of endogenous antigen-specific T cells (Moon et al., 2007). We combined this strategy with additional techniques, such as intracellular staining or immunoscope, in order to perform an in-depth phenotypic and functional analysis of the tetramer-positive T cell population. This approach was then applied to compare the efficiency of cross-priming following systemic dissemination of cell-associated antigen upon intravenous injection or local administration by intradermal injection. Antigen-specific T cells generated from these two types of injection were compared for their proliferative capacity, quantity, polyfunctionality, re-stimulation properties, diversity and affinity. Furthermore, we also studied the persistence of cell-associated antigen and antigen cross-presentation in order to explain the differential immune responses observed between the two routes of immunization.

II. DEFINING THE OPTIMAL TIMING OF ADJUVANT DELIVERY

While adjuvants have been shown to be useful for enhancing the response to an antigen, it has been observed that adjuvant delivery prior to immunization can actually result in inhibitory effects (Wilson et al., 2006).

Following the results obtained by comparing the two routes of immunization, particularly in regards to the kinetics of the T cell response, we were interested to ask whether the route of immunization impacts the optimal timing of adjuvant delivery. Poly I:C was used as adjuvant, as it is known to induce type I IFN secretion. The adjuvant was delivered at various time points prior to, during or after immunization with cell-associated antigen. We observed various effects of adjuvant on antigen presentation, priming and the resulting $CD8^+$ T cell response, depending on the timing of administration. In an attempt to further dissect the mechanism of these time-dependent differential effects, we investigated the role of type I IFN in these phenomena. Specifically we examined which cells type I IFN are acting on, in order to modulate T cell cross-priming.

Chapter 2: Impact of the route of immunization on CD8 T cell cross-priming

Overall, my project aimed to expand upon the newly described tetramer-based enrichment strategy in order to study parameters influencing the outcome of $CD8^+$ T cell responses. We specifically chose to examine our hypotheses in the context of a well-characterized model of cross-presentation as this pathway is known to be crucial for eliciting robust $CD8^+$ T cell response and has already been targeted for purposes of vaccination.

Specifically, our experimental model of cross-presentation was designed as follows: live, H-$2K^{bm1}$ splenocytes expressing a membrane-bound Ovalbumin (K^{bm1}mOva splenocytes) were injected into recipient mice. The SIINFEKL peptide is known to be the immunodominant peptide originating from the Ovalbumin protein and is presented on the MHC-I molecule H-$2K^b$. Consequently, the $CD8^+$ T cell response directed against Ovalbumin was followed over time by using the MHC-I-peptide 'K^b-SIINFEKL' tetramer. The naïve precursor frequency of T cells interacting with K^b-SIINFEKL complexe was determined by tetramer-based enrichment: between 100 and 200 specific $CD8^+$ T cells can be found in naïve mice. Compared to other epitopes from several antigens that have been studied, this represents a rather low frequency, as the number of epitope-specific T cells for a given antigen can vary from 50 to 1000 cells in naïve mice (Obar et al., 2008).

Importantly, in this model of antigen, the expression of the H-$2K^{bm1}$ molecule, which contains a mutation that inhibits direct peptide presentation, ensures that all $CD8^+$ T cell responses were induced by cross-presentation (**Figure 15**).

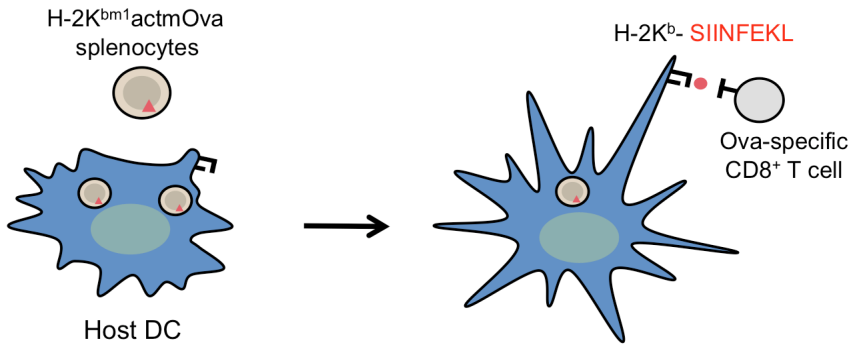


Figure 15. Model of cross-presentation.

Interestingly, despite the expression of a mutated K^b molecule by the injected splenocytes, this MHC molecule K^{bm1} can still be detected by the anti-K^b antibody (clone AF6-88.5) (**Figure 16A**). However, this mutation no longer allows for the binding and presentation of the Ovalbumin-derived SIINFEKL peptide on K^{bm1} molecule (**Figure 16B**). While $\beta 2m^{-/-}$

splenocytes are targets for NK cells, as they completely lack MHC-I molecule surface expression, this is not the case for H-2K^{bm1} splenocytes on which the defective MHC is still expressed (**Figure 16C**). To note, the K^{bm1} molecule differs from the K^{b} molecule by seven nucleotides, resulting in only three different amino acids differences (Schulze et al., 1983).

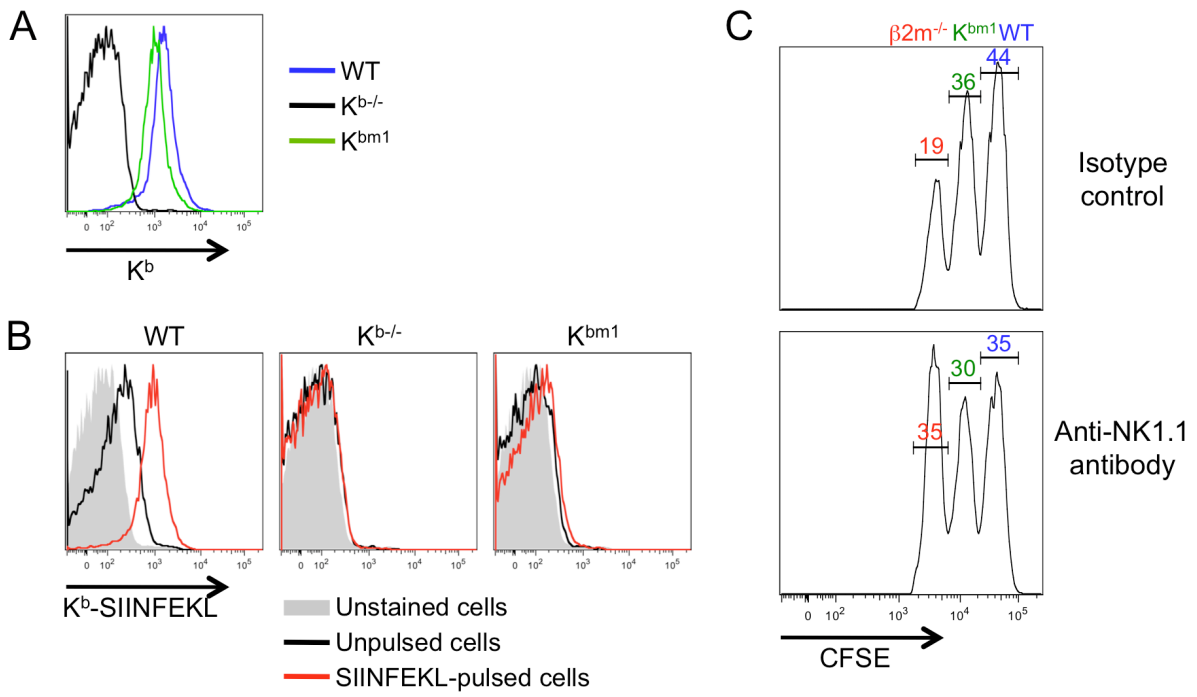


Figure 16. Characterization of K^{bm1}mOva splenocytes. **(A)** WT, K$^{b-/-}$ or K^{bm1}mOva splenocytes were stained for K^{b} using a monoclonal antibody, clone AF6-88.5. **(B)** The same splenocytes were incubated with SIINFEKL peptide for 1 hour and then whashed and stained with an anti-K^{b}-SIINFEKL antibody. **(C)** C57BL/6 mice were injected with anti-NK1.1 antibody or the isotype control the day prior to, and the day of immunization. Mice were immunized with a mixture of β2m$^{-/-}$, K^{bm1} and WT Ova-expressing splenocytes labeled with 3 different concentrations of CFSE. 16 hours post-immunization, the spleen was harvested and the proportions of injected cells still present was evaluated. ***(C)*** *This experiment was performed by H. Saklani.*

Using this model, we were first interested in examining the impact of the route of immunization on CD8^{+} T cell cross-priming. Several injection routes have been used in a variety of experimental research models and, when we compared datas based on the different routes of injection, we observed some striking differences in the induction of effective immune responses. For example, the injection of male splenocytes, depleted for CD11c^{+} cells to avoid direct presentation, into female recipients was able to trigger efficient cross-priming directed against male antigen only if they were delivered i.d.; no response was observed if the cells were injected i.v. (**Figure 17**).

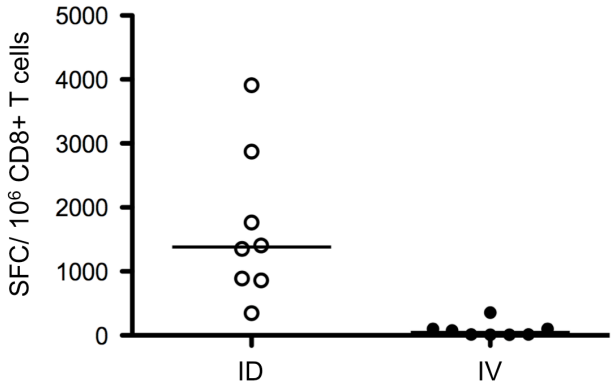


Figure 17. Cross-priming efficiency is dependent on the route of immunization in the HY model. C57BL/6 female mice were immunized i.d. or i.v. with $5x10^6$ male splenocytes depleted for $CD11c^+$ cells. On day 12 for i.d. immunization, and day 8 for i.v. immunization, the spleen and draining lymph node were harvested and an IFNγ ELISPOT was performed. SFC, Spot forming cell.

In our model, we compared the influence of different routes of immunization on the development of a $CD8^+$ T cell response: specifically, the intravenous (i.v.) route, which results in a systemic dissemination of antigen *versus* the intradermal (i.d.) route, leading to a restricted local antigen dissemination.

We initiated our studies by developing and optimizing the tetramer-based enrichment strategy for our cross-presentation model and testing it in combination with intracellular staining techniques to incorporate the functional characterization of the stimulated T cell response. Once these protocols were established, we compared the immune responses induced by the administration of cell-associated antigen either i.d., or i.v. Uniquely, we chose a relatively low dose of antigen for our immunization, as compared to what has been used in previous studies with this technique (LPS + peptide) (Moon et al., 2007) in order to be relatively similar to physiologic conditions relevant for interpretation of our results in the context of human vaccination. Given this low dose of antigen, enrichment of tetramer-positive cells was required to obtain a significant number of cells to study.

I. DEVELOPMENT OF TETRAMER-BASED ENRICHMENT STRATEGY

A. Tetramer-based enrichment

The tetramer-based enrichment is a technique that was first developed by Moon and colleagues to study the naïve antigen-specific $CD4^+$ T cell repertoire in mice (Moon et al., 2007). It has since been adapted for $CD8^+$ T cells characterization (Obar et al., 2008). Specifically, the spleen and 14 lymph nodes from a mouse are harvested, representing approximately 200 million cells. These cells are stained with peptide-MHC-I tetramers labeled with Phycoerythrin (PE) and then incubated with anti-PE magnetic beads (**Figure 18**). This cell-antibody-bead suspension is passed over a magnetic column, which allows for a 100-fold enrichment of tetramer-positive cells.

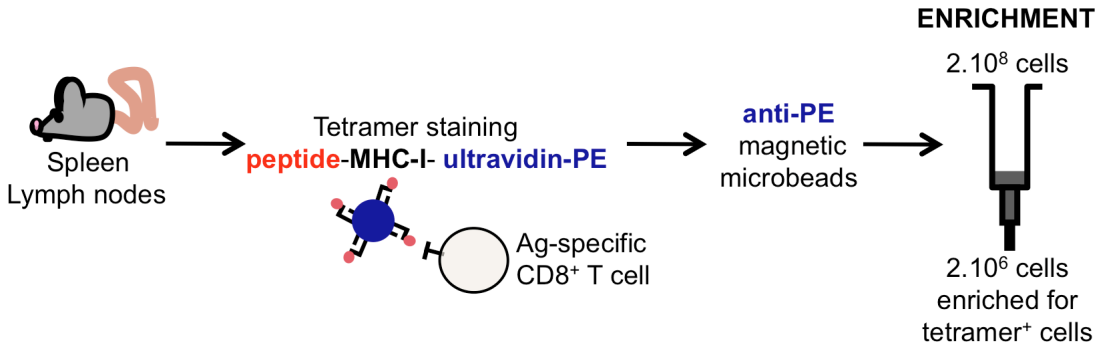


Figure 18. Tetramer-based enrichment strategy.

The main aim of this technique is to enrich for the totality of antigen-specific T cells and, consequently there remained many cells in the enriched fraction that are not of interest. To eclude these cells, a specific gating strategy is used for flow cytometry analysis to focus on tetramer-positive cells. First, using SSC-W and a dead cell marker, the doublets and the dead cells are eliminated. Then a dump channel is used to remove all the cells that are not of interest based on their unique surface marker profile (DC, Macrophages, B cells, NK cells, $CD4^+$ T cells…). Finally CD3 and CD8 antibodies are used to detect $CD8^+$ T cells and observe the tetramer-positive cells (**Figure 19A**). This technique was initially developed to investigate and characterize naïve T cells. In the context of our studies, we chose to apply it in a model using a modest dose of antigen and then investigating the immune response in the first few days post-immunization. These parameters permitted us to work within reasonably "physiologic" conditions in terms of initial T cell precursor frequency, but also in terms of

conditions of immunization. This lower dose of antigen will lead to a less robust response and, therefore, an enrichment step is required to be able to study the antigen-specific T cells.

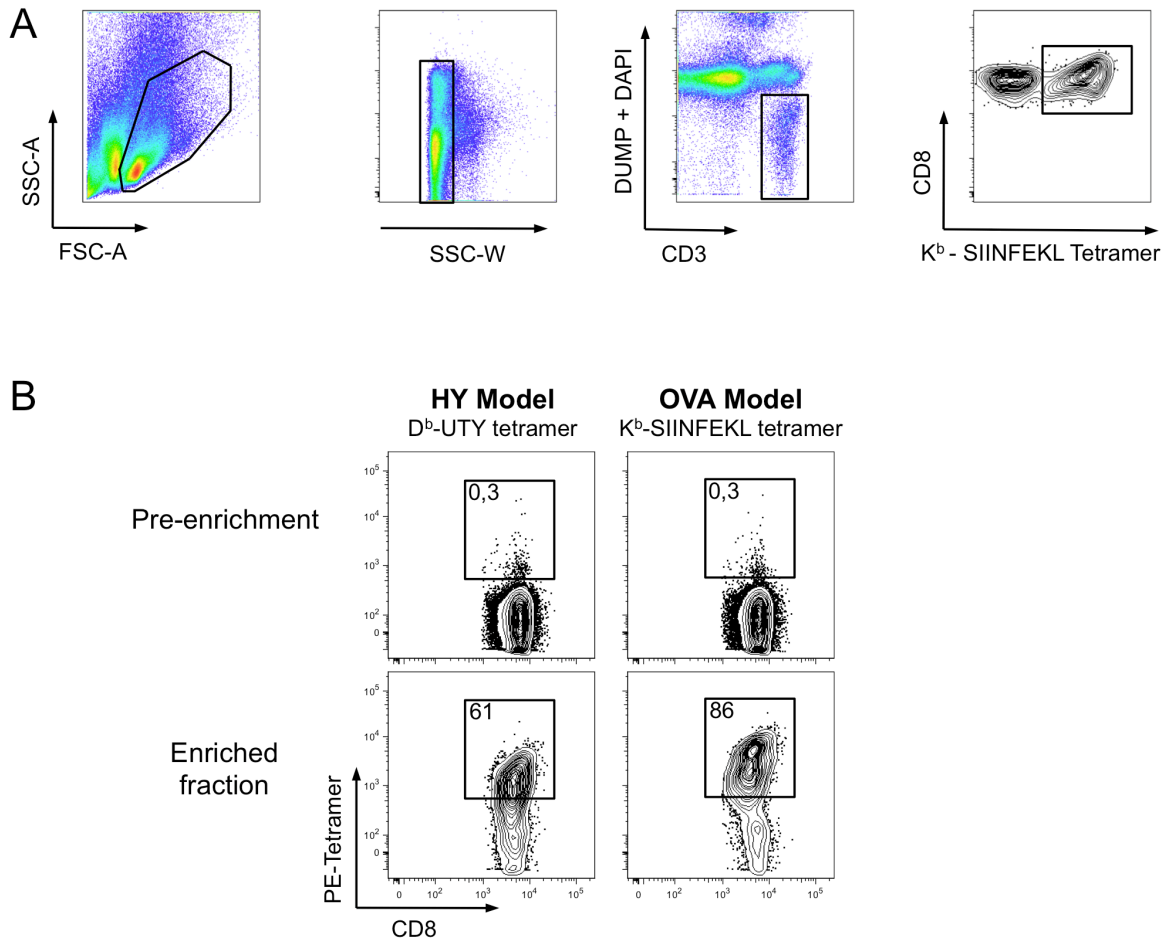


Figure 19. Tetramer-based enrichment. (A) The gating strategy used is detailed. Single cells were selected using SSC-W. Then cells were stained with a mixture of antibodies for lineage markers and DAPI to exclude cells that are not of interest (DUMP gate). $CD3^+$ cells were selected and CD8 and K^b-SIINFEKL tetramer labeling were used to detect antigen-specific $CD8^+$ T cells. **(B)** C57BL/6 mice were immunized i.d., either with male splenocytes (HY model) or K^{bm1}mOva splenocytes (Ova model). On day 11, the spleen and lymph nodes were harvested and enrichment was performed using D^b-UTY (HY model) or K^b-SIINFEKL (Ova model) tetramers. The tetramer staining on cells prior to enrichment and on the enriched fraction is shown. The numbers indicated represent the percentages of tetramer-positive cells among $CD8^+$ T cells for each sample. Of note, approximately 350 000 cells were acquired for both samples: this corresponds to the total enriched fraction, but it reflects only a part of the pre-enrichment cell suspension.

To validate this technique in our hands, we tested it in two different models, HY and Ovalbumin, using the respective peptide-MHC-I tetramers. We compared what was obtained with and without enrichment, both in the enriched and in the flow-through fraction. Without enrichment, it is extremely difficult to detect tetramer-positive cells for mainly two reasons: first, it was not possible to collect the total pre-enrichment cell suspension for flow cytometry analysis as it is comprised of approximately 200 million cells. Additionally K^b-SIINFEKL tetramers are known for their high background staining, seen in the intermediately stained

population observed in the pre-enrichment fraction (Obar et al., 2008). By contrast, the tetramer-positive cell population represents a substantial number of cells following enrichment (**Figure 19B**).

B. Combination with intracellular staining

The tetramer-based enrichment strategy allowed us to detect antigen-specific T cells, enumerate them and characterize them phenotypically by looking at surface marker expression. However, we were not able to obtain any information about the functionality of these cells. To address this, we developed a protocol that combined the tetramer-based enrichment with intracellular staining (**Figure 20**). Unfortunately, several technical hurdles became immediately apparent, most importantly that these cells needed restimulation in order to secrete cytokines; however, this restimulation also appeared to induce TCR downregulation, inhibiting enrichment with tetramers.

In order to find a solution to this problem, we initially developed a protocol using *in vivo* restimulation of the cells. Three hours prior to organ harvest, mice were injected with CpG formulated with DOTAP combined with SIINFEKL peptide. After the restimulation period and harvest, we performed tetramer-based enrichment as previously described, followed by an intracellular staining (**Figure 20A, Protocol 1**). *In vivo*, the restimulation effect was strong enough to allow for the observation of IFNγ production in, and tetramer-positive cells at the same time (**Figure 20B**).

Nonetheless, when we attempted to examine the production of other cytokines, we were not able to detect a signal, perhaps due to the lower extent of restimulation *in vivo*. We tried again to optimize a protocol with *ex vivo* restimulation with peptide-pulsed splenocytes. This resulted in a robust restimulation that led to a loss of tetramer staining at this step. Our final protocol involved first performing the enrichment step, followed by the restimulation with peptide-pulsed splenocytes for several hours before applying the established staining panels (**Figure 20A, Protocol 2**). Although we lost the positive tetramer staining, we knew from the initial trial (**Protocol 1**) that IFNγ-positive cells are also tetramer-positive cells and, in this way, we were able to investigate additional cytokines that are secreted by these cells.

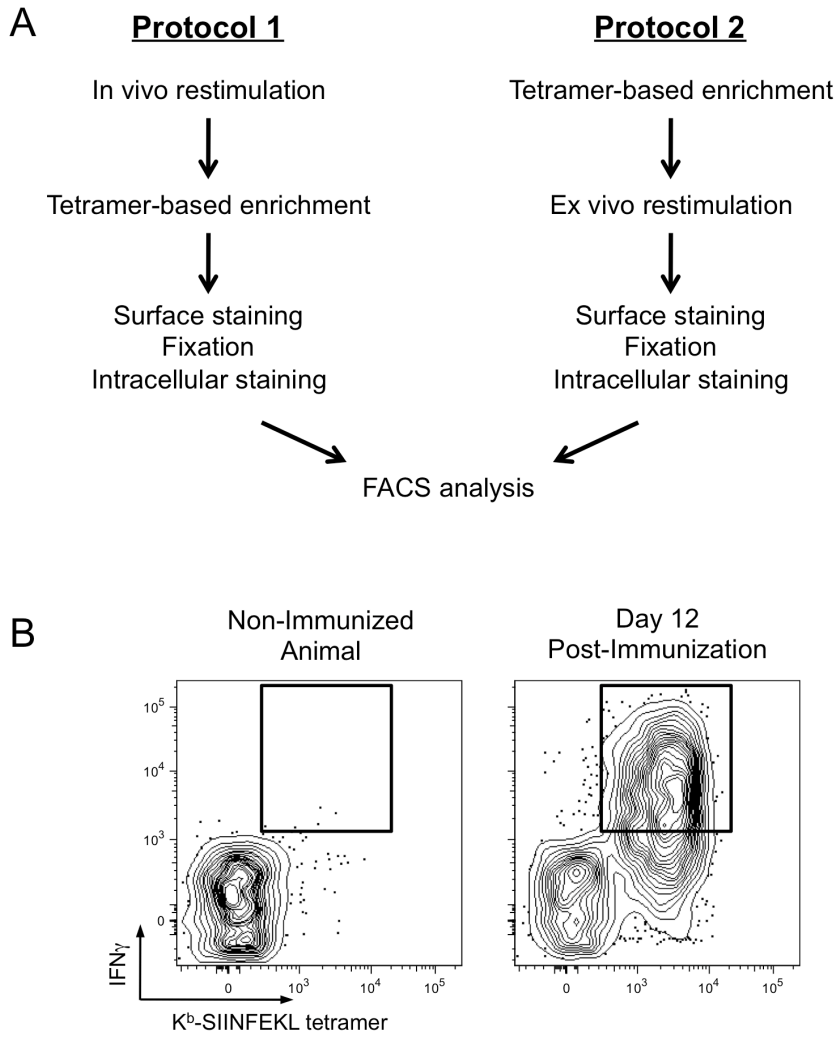


Figure 20. Combination of tetramer-based enrichment with intracellular staining. (A) The 2 protocols were developed as described. **(B)** C57BL/6 mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. On day 12, spleen and lymph nodes were harvested and an enrichment combined with intracellular staining for IFNγ (protocol 1) was performed. $CD8^+$ T cells are shown.

II. CHARACTERIZATION OF THE T CELL RESPONSE

Once the tetramer-enrichment technique was developed and validated in our hands, it was used to characterize the $CD8^+$ T cell response in our model of cross-presentation to examine the impact of route of immunization, i.d. or i.v., on the establishment of the T cell response.

A. Kinetic of $CD8^+$ T cell response

To determine the optimal conditions for achieving cross-priming, we compared the effects of immunizing with a local versus systemic dissemination of cell-associated antigen. C57BL/6 mice were injected i.d. or i.v. with live splenocytes from H-$2K^{bm1}$ mice engineered to express a membrane-bound form of chicken ovalbumin in all tissues (referred to as K^{bm1}mOva). Use of membrane-associated Ovalbumin (mOva) ensured that our model was not confounded by secreted protein captured by endocytosis; and an altered K^b molecule (known as K^{bm1}) ensured a role for host presenting cells in the cross-priming of $CD8^+$ T cells. In order to precisely monitor the priming of the endogenous T cell repertoire, we utilized K^b-SIINFEKL tetramer-based enrichment, thus allowing precise enumeration and phenotypic analysis of Ovalbumin peptide-specific T cells at early time points after immunization. Accumulation of tetramer-positive cells could be observed as early as day 5 for i.v. immunization (**Figure 21A**), with cells showing downregulation of CD62L (**Figure 21B**) and expression of CD25 (**Figure 21C**). In contrast, the kinetics of T cell priming was delayed when cell-associated antigen was delivered via the i.d. route. In the latter condition, accumulation of Ova-specific $CD8^+$ T cells was not observed until day 7 post-immunization. For both routes of immunization, antigen-specific T cells accumulated over time, with day 9-12 being the peak of the response (**Figure 21A**). These data demonstrated that the local delivery of cell-associated antigen results in delayed T cell cross-priming.

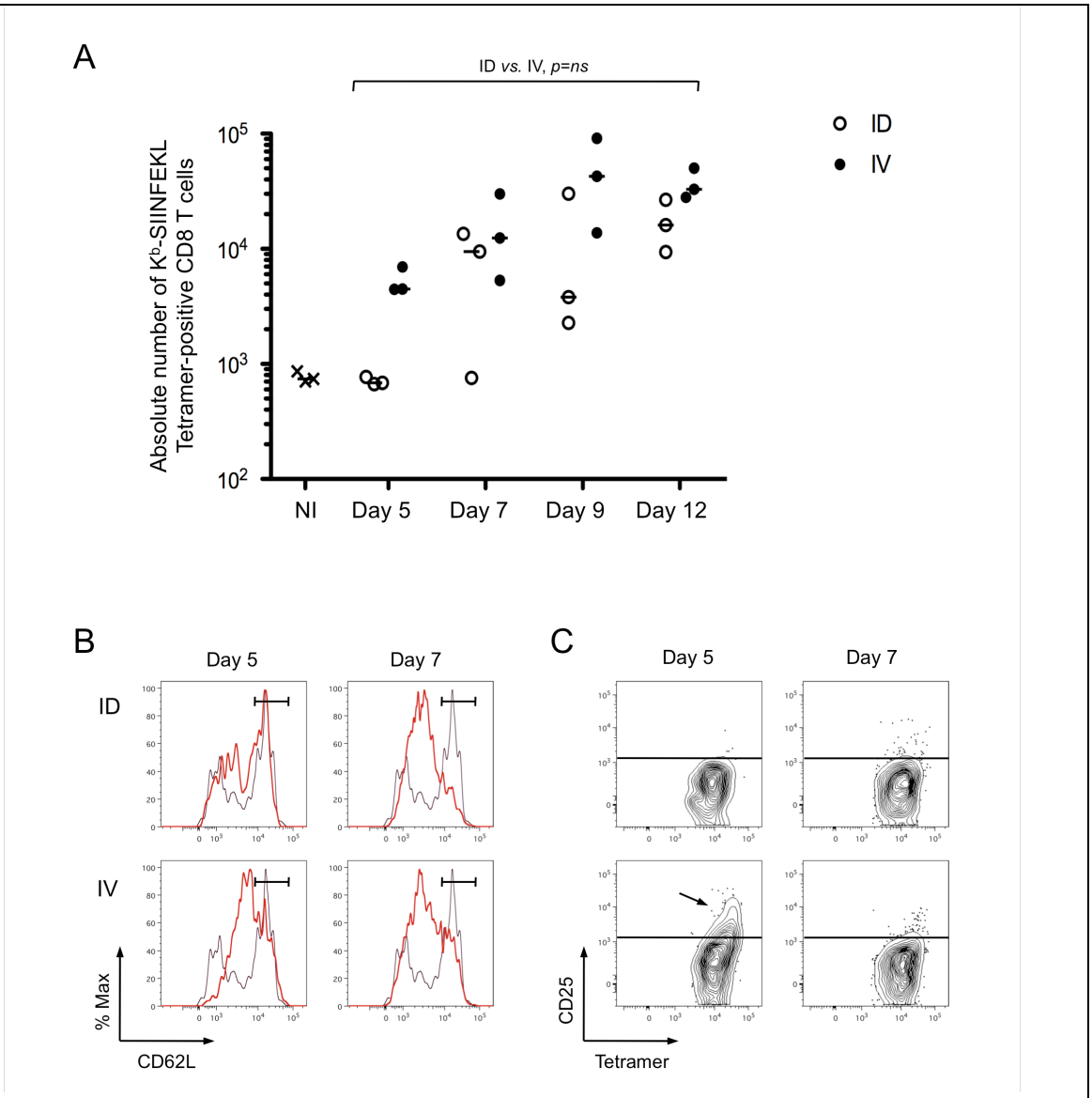


Figure 21. Route of immunization influences the kinetic of T cell response. Mice were immunized intradermally (ID) or intravenously (IV) with $5x10^5$ K^{bm1}mOva splenocytes. On days 5, 7, 9, 12, 14 macroscopic lymph nodes and spleen were harvested and a K^b-SIINFEKL tetramer-based enrichment was performed. **(A)** Absolute numbers of Ova-specific $CD8^+$ T cells at each of the time points were determined. Data points indicate a single mouse. Results are representative of four independent experiments. The distributions according to the two immunization routes were not significantly different over time employing a general linear modeling analysis. CD62L **(B)** and CD25 **(C)** stainings are shown.

While prior studies suggest that the precursor frequency of Ova-specific T cells is similar across individual C57BL/6 mice (Obar et al., 2008), it is true that each mouse possesses distinct T cell repertoires (Bousso et al., 1998). In addition, we wanted to confirm that the delayed priming was not a result of inability to access high affinity Ova-specific T cells. Thus we employed the strategy of adoptive transfer of low numbers (10^3) of monoclonal OT-I T cells (Badovinac et al., 2007), transferred one day prior to immunization. On day 5, tetramer-based enrichment was performed using a combination of anti-CD45.1 antibody and K^b-SIINFEKL tetramer, permitting for the simultaneous assessment of the transferred $CD45.1^+$

OT-I T cells and endogenous Ova-specific T cells. As shown, only the i.v. immunization resulted in the early priming of Ova-specific T cells. Representative plots are shown, indicating that both the OT-I and the endogenous T cells behaved similarly, and that responses were comparable to those observed in animals that had not received OT-I (**Figure 22**). Analysis of later time points supported the conclusion that priming is delayed when mice are immunized via the i.d. route (data not shown). Furthermore, we demonstrated that T cell precursor frequency influences the kinetics of priming. Transfer of 10^6 OT-I prior to immunization, in contrast to low transfer conditions, resulted in the robust and rapid expansion of Ova-specific T cells in both i.v. and i.d. conditions (**Figure 22**). Also evident, the transferred cells outcompeted the endogenous repertoire. These data indicate that there exists a qualitative difference between i.v. and i.d. immunization, which is masked when using adoptive transfer of high numbers of monoclonal T cells. This highlights also the necessity of new techniques such as tetramer-based enrichment to address this kind of questions.

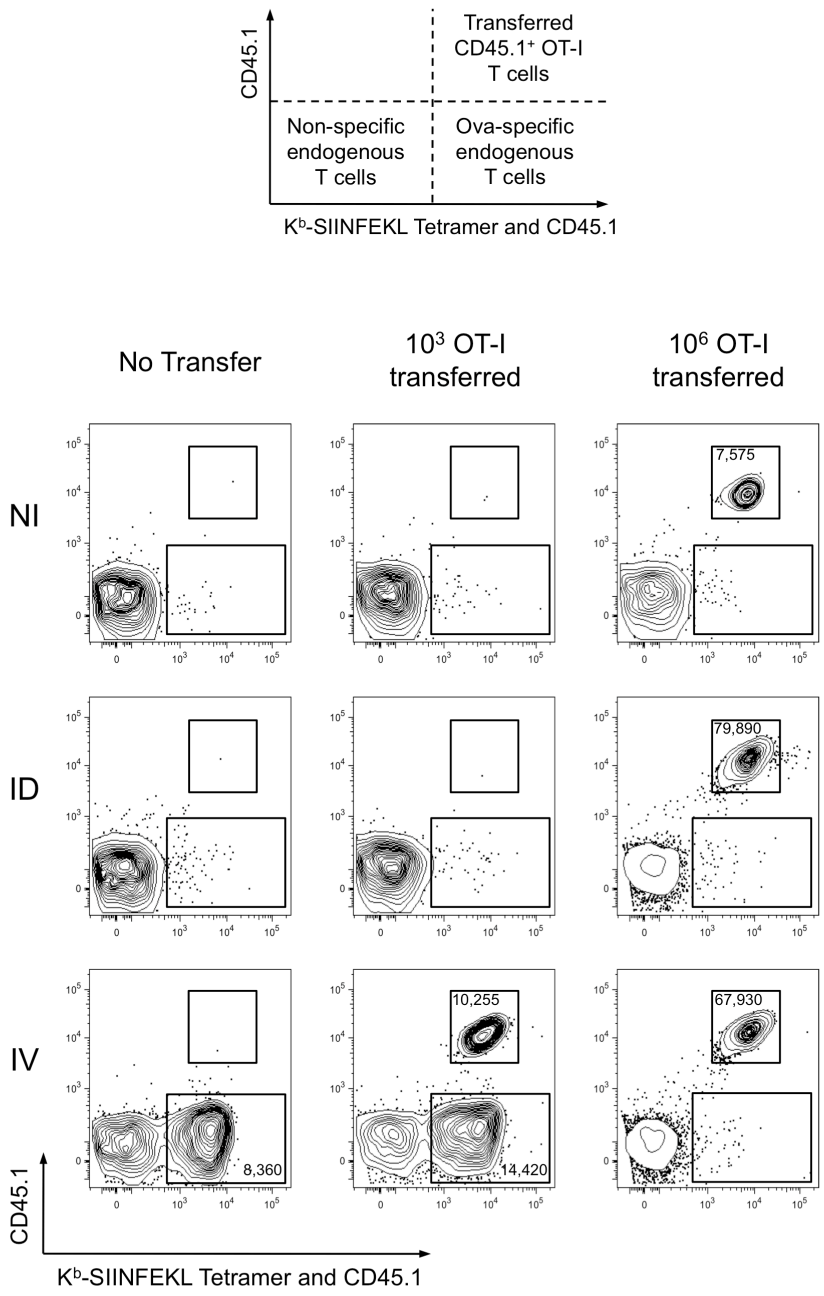


Figure 22. The requirements for efficient cross-priming may be skewed by high T cell precursor frequency. 10^3 or 10^6 CD45.1 OT-I splenocytes were transferred into CD45.2 recipients prior to immunization. Use of congenic markers allowed simultaneous assessment of transferred and endogenous Ova-specific T cells (schematic representation). Mice were immunized with $5x10^5$ K^{bm1}mOva splenocytes. On day 5 post-immunization, enrichment was performed using both K^b-SIINFEKL tetramer and CD45.1 antibody to distinguish endogenous tetramer-positive cells and OT-I. Live $CD3^+$ $CD8^+$ $DUMP^-$ cells are shown. The upper region highlights the transferred OT-I and the lower region marks the endogenous Ova-reactiva $CD8^+$ T cells. Absolute cell numbers are indicated for the respective cell populations. Plots were selected from an experiment with three mice per group. Data are representative of three independent experiments.

B. Functionality of antigen-specific CD8$^+$ T cells

To further define the impact of early dissemination of antigen (i.v. immunization) as compared to a local antigen administration (i.d. immunization), we monitored T cell effector functions. First, we performed an *in vivo* cytotoxicity assay to determine if the expanded T cells possessed cytolytic effector function. At different time points following immunization, mice received targets cells pulsed with SIINFEKL peptide and specific killing was determined (**Figure 23**). We observed a rapid induction of CTL activity after i.v. immunization that began to wane by day 12. Consistent with the delayed expansion after local immunization, we observed a stronger response on Day 12 following i.d. immunization. While both routes of immunization elicit CTL induction, this assay system does not provide *per* cell information about effector activity.

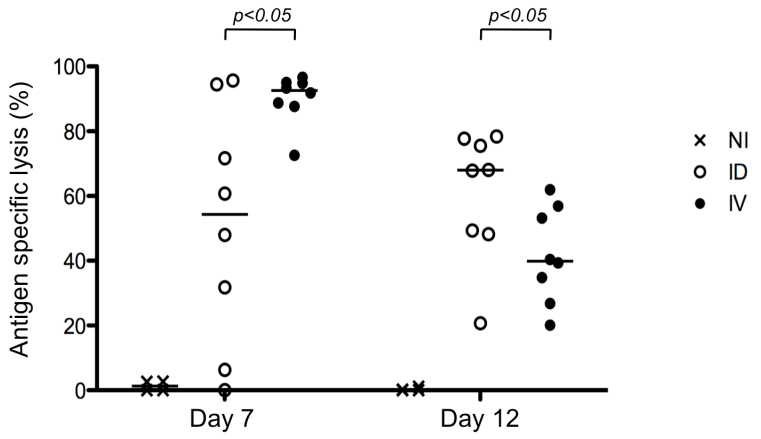
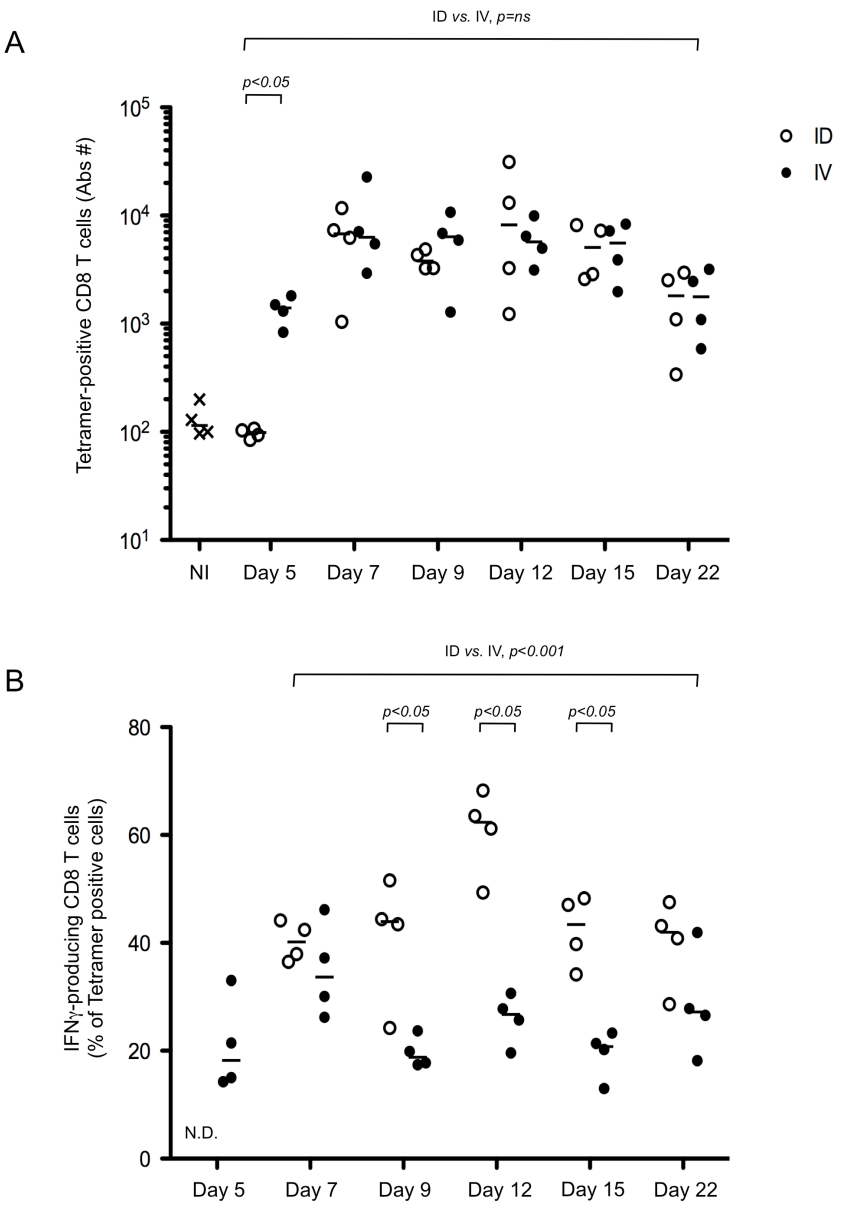


Figure 23. Both i.v. and i.d. immunization result in CTL induction. Mice were immunized i.d. or i.v. with 5x10^5 K^{bm1}mOva splenocytes. At day 7 or 12 post-immunization, an *in vivo* cytotoxicity assay was performed. Antigen-specific killing is reported. *p*-values were calculated using a Mann-Whitney test (comparing ID versus IV). NI, non-immunized mice, shown here to indicate baseline killing.

To achieve such an analysis, we combined tetramer-based enrichment with intracellular staining. Using this approach, it was possible to determine the absolute number of tetramer-positive CD8$^+$ T cells (**Figure 24A**); as well as the percentage of those cells producing IFNγ (**Figure 24B**). Of note, the absolute number of cells observed in this experiment is lower than those reported in **Figure 21A**, a consequence of performing intracellular cytokine staining, which requires additional washing and fixation steps. By day 7, the number of Ova-specific T cells was similar for the two route of immunization with the contraction phase beginning after day 15. Consistent with the delayed T cell expansion and cytotoxicity test, IFNγ production following i.v. immunization peaked at day 7, as compared to the i.d. route where the peak response was on day 12. Remarkably, comparing the peak responses indicated that 25-45% of

the Ova-specific T cells were producing IFNγ after i.v. injection; whereas 50-70% of the cells were effector $CD8^+$ T cells at the peak of the i.d. response (**Figure 24B**). Representative FACS plots highlight that not only did we achieve a higher percentage of IFNγ-producing cells with i.d. injection, but also, on a per cell basis, many of the effector T cells were making 10-fold more cytokine as compared to those isolated after i.v. immunization (**Figure 24C**). This was also evident using a population-based analysis – as shown, the geometric mean fluorescent intensity (MFI) of tetramer-positive cells was significantly higher in the i.d. condition on days 9-15 (**Figure 24D**). While intradermal immunization is delayed, it cross-primes $CD8^+$ T cells with greater effector function.


A
ID vs. IV, p=ns
p<0.05
Tetramer-positive CD8 T cells (Abs #)
ID
IV
NI
Day 5
Day 7
Day 9
Day 12
Day 15
Day 22
B
ID vs. IV, p<0.001
p<0.05
p<0.05
p<0.05
IFNγ-producing CD8 T cells
(% of Tetramer positive cells)
N.D.

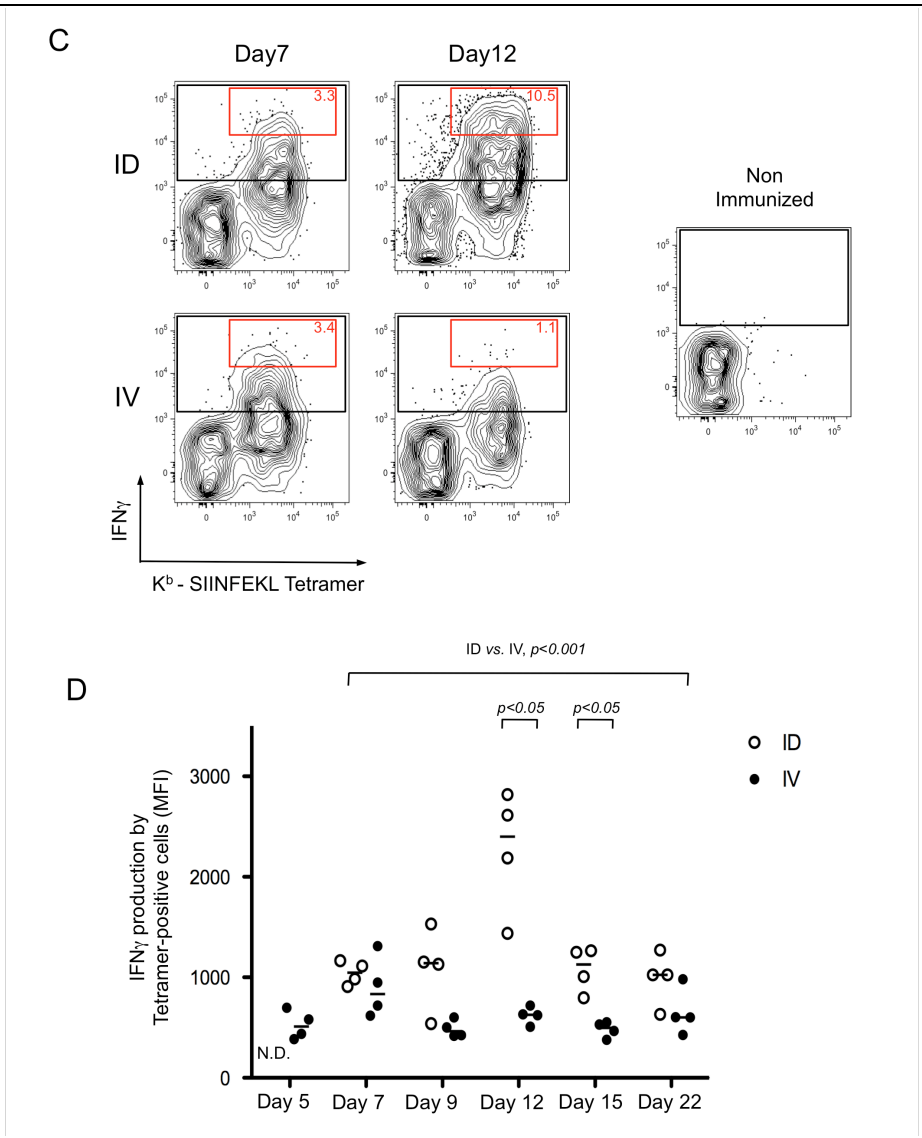


Figure 24. Intradermal immunization results in a more robust differentiation of effector CD8$^+$ T cells. (A-D) Mice were immunized i.d. or i.v. with 5x10^5 K^{bm1}mOva splenocytes. Three hours prior to the defined time point, mice were re-stimulated in vivo by injecting 5μg of CpG/DOTAP formulated as a mixture with 1μg SIINFEKL peptide. K^b-SIINFEKL tetramer-based enrichment combined with an intracellular staining for IFNγ was performed. The absolute number of tetramer-positive cells is reported **(A)**; and the percentage of IFNγ-producing cells among the population of tetramer-positive cells was determined **(B)**. Representative plots of enriched tetramer-positive cells and the respective IFNγ production, per cell, is shown. Data from live CD3$^+$ CD8$^+$ DUMP$^-$ cells are shown. The red gate highlights the tetramer-positive cells with the higher IFNγ staining and the numbers correspond to the percentage of these cells among the tetramer-positive cells population **(C)**. To represent the respective per cell production of IFNγ, tetramer-positive cells were gated and the geometric mean fluorescent intensity (MFI) is shown **(D)**. Data points indicate a single mouse. N.D., not determined, due to low absolute numbers of cells. Results are representative of two independent experiments. Individual pairings of ID versus IV were assessed by Mann-Whitney test and *p*-values are shown. The global distributions were also evaluated using time as a continuous variable (general linear modeling) **(A,B,D).**

C. Quality of the T cell response

Next, we were interested in characterizing the quality of the T cell response. Prior studies have indicated that cells producing high levels of IFNγ have the unique capacity to secrete multiple cytokines, leading to their being referred to as polyfunctional T cells (Seder et al., 2008). In our model, we evaluated the simultaneous production of IFNγ, IL-2 and TNFα. Mice were primed using the strategies discussed in **Figure 21** and *ex vivo* restimulation of the tetramer-enriched fraction was performed prior to intracellular staining. As anticipated, the cells producing high levels of IFNγ also expressed TNFα and IL-2 (**Figure 25A**, nb. IL-2 producing cells are shown in red). The response was evaluated throughout the kinetics of T cell priming (**Figure 25A**), and for purposes of comparing i.d. *vs* i.v. immunization, we focused on the peak of the response: Day 7 for i.v. immunization; and Day 12 for i.d. immunization. The percentages of $IFN\gamma^+$ cells producing the 3 cytokines – IFNγ, IL-2 and TNFα – was significantly higher after i.d. immunization (**Figure 25B**). The converse is also true – the percentage of cells producing only IFNγ was higher following i.v. immunization (**Figure 25C**). Thus, we conclude that cross-priming via the i.d. route establishes a stronger, more polyfunctional response.

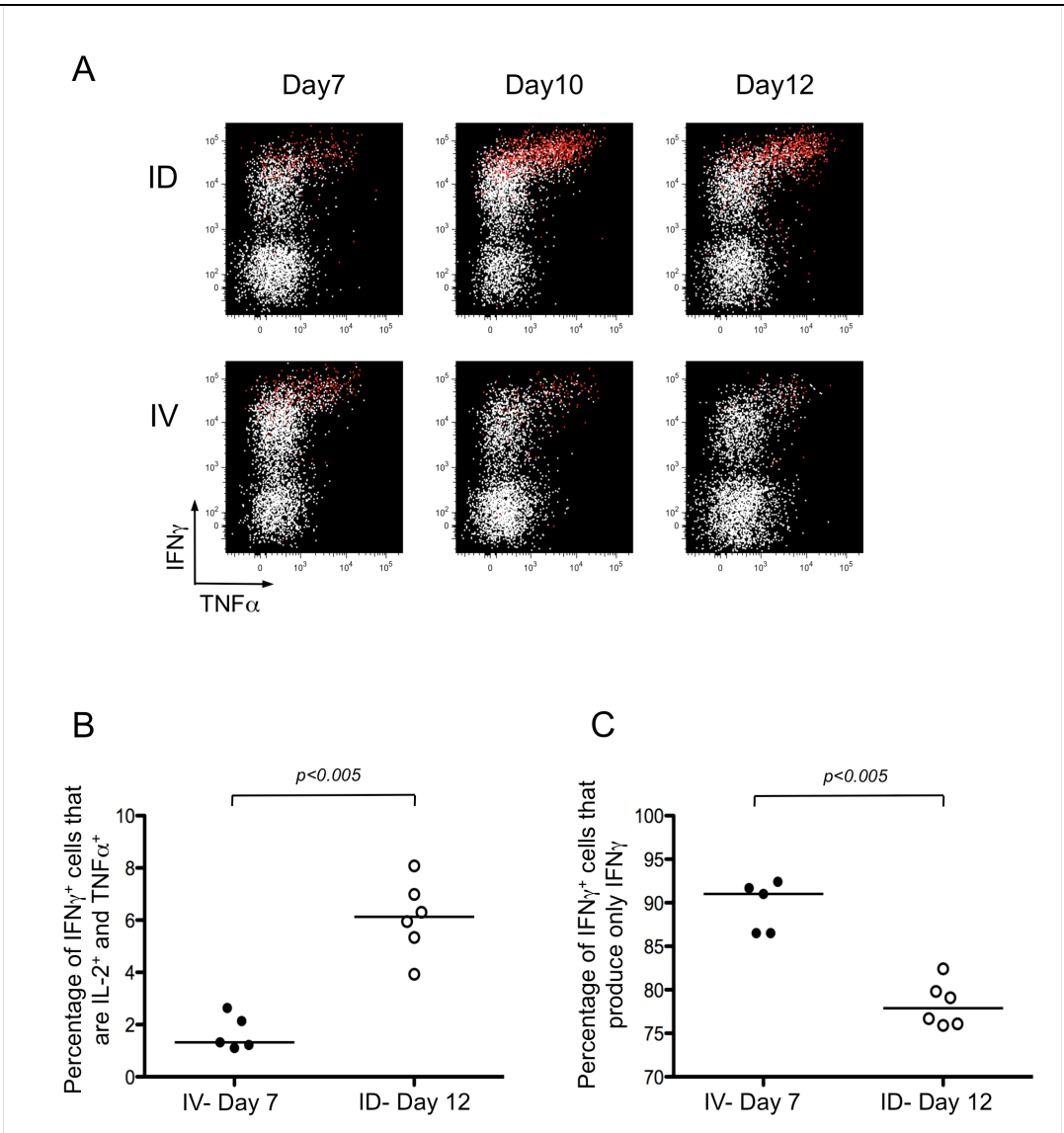


Figure 25. Intradermal immunization induces polyfunctional T cells. (A-C) Mice were immunized i.d. or i.v. with $5x10^5$ K^{bm1}mOva splenocytes. At the different time points, lymph nodes and spleen were harvested and a K^b-SIINFEKL tetramer-based enrichment was performed. The enriched fraction was incubated for 4h with SIINFEKL-pulsed splenocytes, followed by surface and intracellular staining. **(A)** Data from live $CD3^+$ $CD8^+$ T cells are shown. Cells producing IL-2 are highlighted in red. The percentage of $IFN\gamma^+$ cells that produce either the three cytokines – IFNγ, IL-2 and TNFα **(B)** or only one cytokine – IFNγ **(C)** were calculated. *p*-values were calculated using a Mann-Whitney test.

D. Memory potential of antigen-specific $CD8^+$ T cells

Following from the T cell functionality results, we tested whether the route of immunization impacts also secondary responses. As previously, mice were immunized i.d. or i.v., and 34 days later the same animals were re-stimulated by i.v. administration of a second dose of $5x10^5$ K^{bm1}mOva splenocytes (**Figure 26A**). IFNγ-producing Ova-specific $CD8^+$ T cells were enumerated (**Figure 26B**). While the absolute number of tetramer-positive cells was similar in both conditions, we detected a higher percentage of IFNγ-producing cells when the first immunization was performed via the i.d. route (**Figure 26C**). We wanted to confirm these

data in an infectious model. Mice were immunized i.d. or i.v. with K^{bm1}mOva splenocytes and challenged 1 month later with Ova-expressing *Listeria monocytogenes*. Unfortunately we were not able to repeat the data obtained previously in the rechallenge with splenocytes (data not shown). This is most likely due to relatively small differences between the two routes of immunization as compared to the strength of Listeria infection.

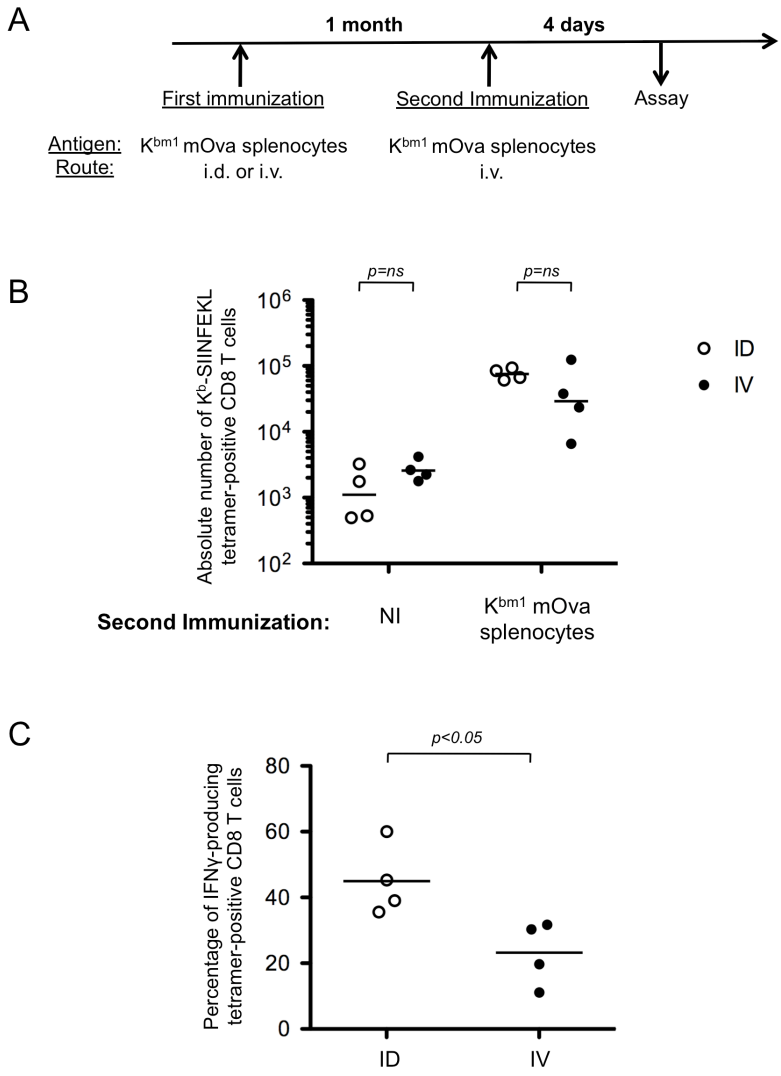


Figure 26. Robustness of intradermal immunization results in better memory. (A-C) Mice were immunized i.d. or i.v. with $5x10^5$ K^{bm1}mOva splenocytes. One month post-immunization, mice were challenged i.v. with $5x10^5$ K^{bm1}mOva splenocytes. On day 4 of the secondary response, mice were re-stimulated *in vivo* by injecting CpG and SIINFEKL, as described above. Fifteen lymph nodes and the spleen were harvested and K^b-SIINFEKL tetramer-based enrichment combined with intracellular IFNγ staining was performed. The experimental scheme is shown **(A)**. The absolute number of tetramer-positive cells is reported **(B)**; and the percentage of IFNγ-producing cells among the population of tetramer-positive cells was determined **(C)**. Data points indicate a single mouse. Results are representative of 2 independent experiments. NI, non-immunized animals during secondary restimulation. *p*-values were calculated using a Mann-Whitney test.

E. Diversity of antigen-specific CD8$^+$ T cells

1) TCR diversity

One potential caveat for the differences observed is that the rate and means of antigen dissemination might influence the diversity of the responding T cell population, with possible consequences for the relative avidity for MHC-peptide complexe (Catron et al., 2006; Zehn et al., 2009). Our hypothesis was that the access to antigen may not be the same depending on the route of immunization. Upon i.v. immunization, antigen is distributed systemically; there is no competition between T cells for access to antigen, leading to the possibility that even low affinity T cells may be activated. In contrast, i.d. immunization might result in a more restricted tissue dissemination making antigen access more challenging, and consequently, only the T cells with the highest antigen affinity will be activated (**Figure 27A**). The alternative hypothesis is that, irregardless of the route of immunization, T cells will have similar access to antigen-presenting cells. If this is the case, the same diversity of antigen-specific T cells would be found after both i.d. or i.v. immunization (**Figure 27B**).

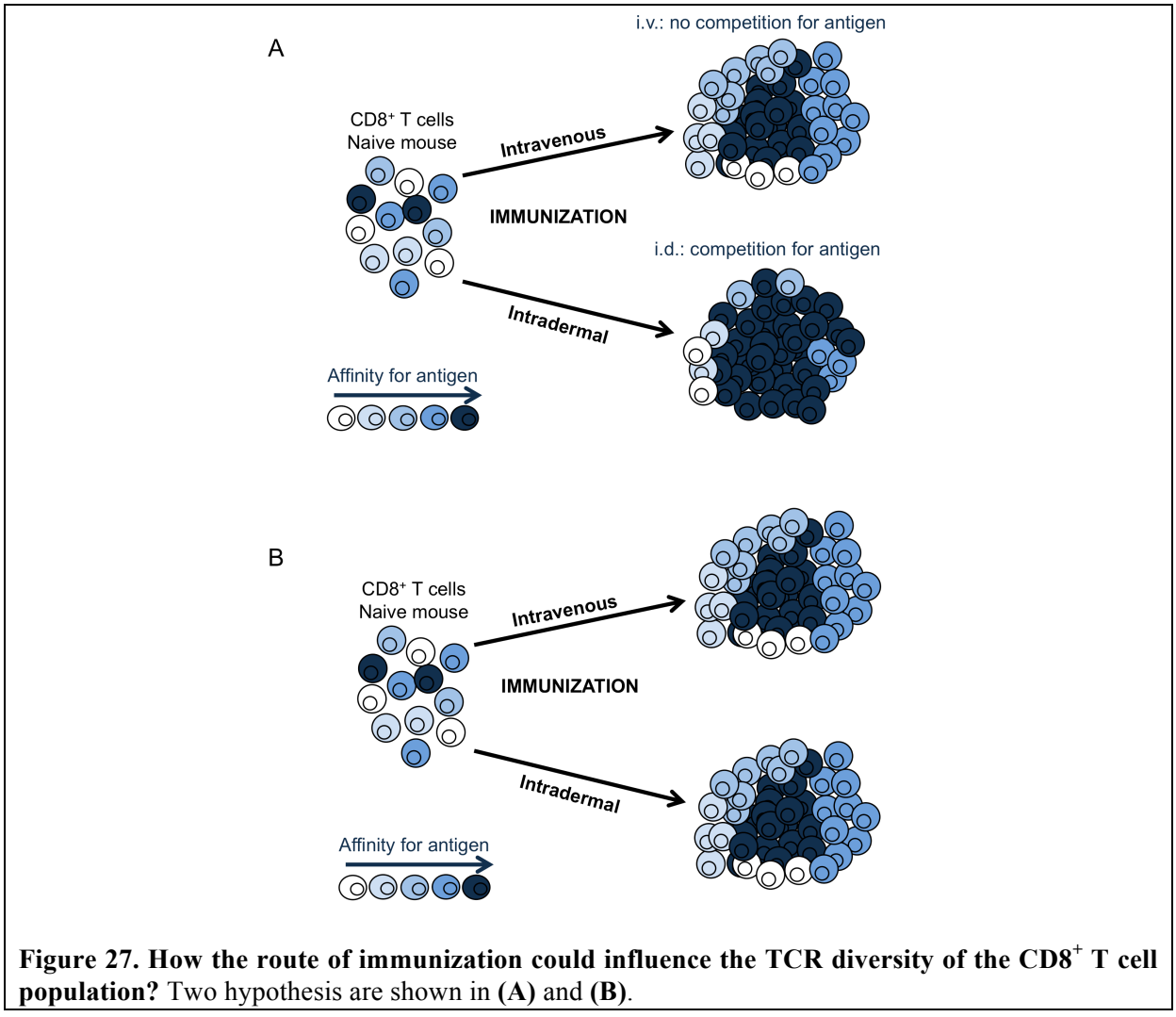


Figure 27. How the route of immunization could influence the TCR diversity of the CD8$^+$ T cell population? Two hypothesis are shown in **(A)** and **(B)**.

To test these hypothesis, Ova tetramer-positive $CD8^+$ T cells were sorted by FACS, followed by TCR gene amplification and characterization of the distribution of Vβ-Jβ CDR3 lengths present. This method, called immunoscope, accurately evaluates TCR diversity (Pannetier et al., 1993) (**Figure 28**). Specifically, antigen-specific T cells were sorted based on tetramer staining upon enrichment. RNA was extracted and cDNA was synthetized. The different Vβ germline genes can be clustered in 24 families according to their level of homology. PCR reactions using specific primers for each Vβ family and one for Cβ segment were performed. This gave the frequency of the different Vβ families in the T cell pool. Then, a second step was performed to obtain further information: a nested primer specific for the constant region was used for an elongation step. These products were then analyzed to obtain an immunoscope profile with the length of the CDR3 region.

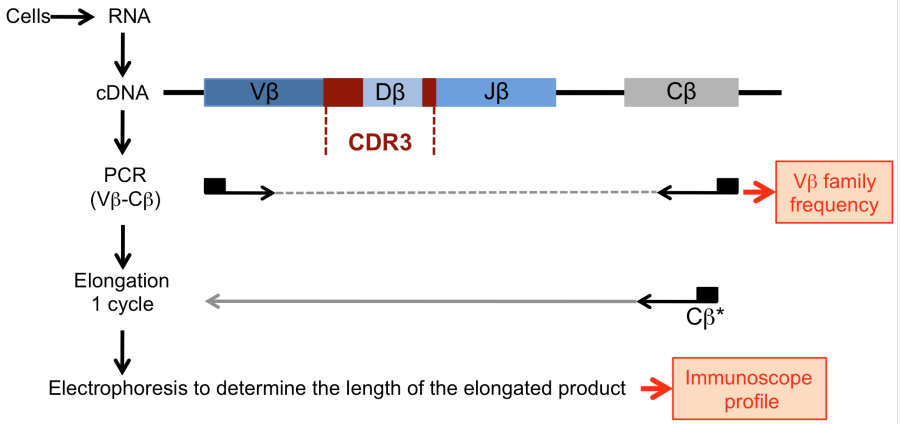


Figure 28. Immunoscope allows the determination of the Vβ family diversity as well as the length of the CDR3 region.

In our experiment, $5x10^3$ cells per mouse, isolated from 5 mice per group, were pooled for the analysis. As a control, we purified twenty-five thousand bulk $CD3^+$ $CD8^+$ T cells from a non-immunized animal. 22 Vβ families were detected in both the non-immunized and immunized animals. Data are represented as a profile of the Vβ-Jβ products obtained, plotted in arbitrary intensity units as a function of the size of the DNA fragment (Pannetier et al., 1993). As expected, analysis of the expanded antigen-specific cells in immunized animals showed a non-Gaussian distribution of the peaks as compared to the naïve bulk $CD8^+$ population (**Figure 29A**). Notably, the Vβ 12.1 and 13.1 families were highly represented in the immunized animals, consistent with prior reports (Dillon et al., 1994). (Please note the change in nomenclature – the populations found here correspond with Vβ 5 and Vβ 8 respectively). To determine the diversity of the T cell responses the number of distinct peaks detected in all immunoscope profiles were determined (**Figure 29B**). As shown, the number of peaks was

significantly reduced in immunized mice with comparable results between the i.v. and i.d. conditions.

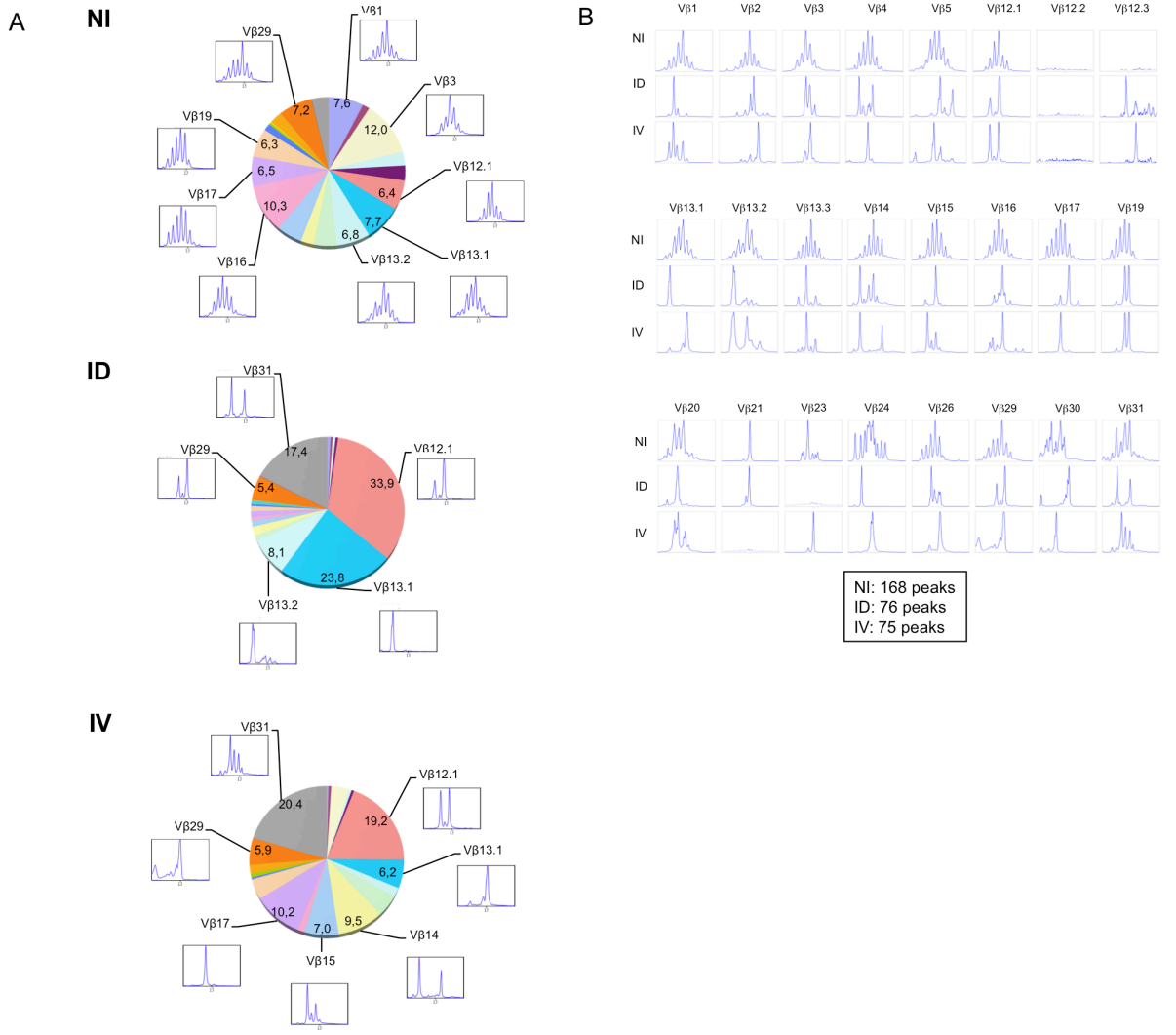


Figure 29. Route of immunization does not influence T cell diversity. (A,B) Mice were immunized i.d. or i.v. with $5x10^5$ K^{bm1}mOva splenocytes. On day 9, tetramer-based enrichment was performed followed by FACS sorting. For the non-immunized (NI) condition, bulk tetramer-negative $CD8^+$ T cells were sorted. For immunized animals, cells were sorted from individual mice and 5,000 cells per animal were pooled to obtain 25,000 cells per condition. Immunoscope analysis was performed to define the length of the CDR3 loop in the 24 Vβ families (IMGT nomenclature). **(A)** Each color represents a distinct Vβ family. The numbers correspond to the estimated percentage of total population. The immunoscope profile is presented for families that represent more than 5% of the total population. **(B)** The immunoscope profile is shown for each Vβ family. The total number of peaks is indicated for each condition.

Given that these results were obtained from pooled mice, there exists the possibility that differences were homogenized and thus not detected; we therefore repeated the experiment using tetramer-positive cells purified from individual animals. Vβ families represented in the primed responses are shown (**Figure 30A, B**), and the number of peaks per mouse is plotted (**Figure 30C**).

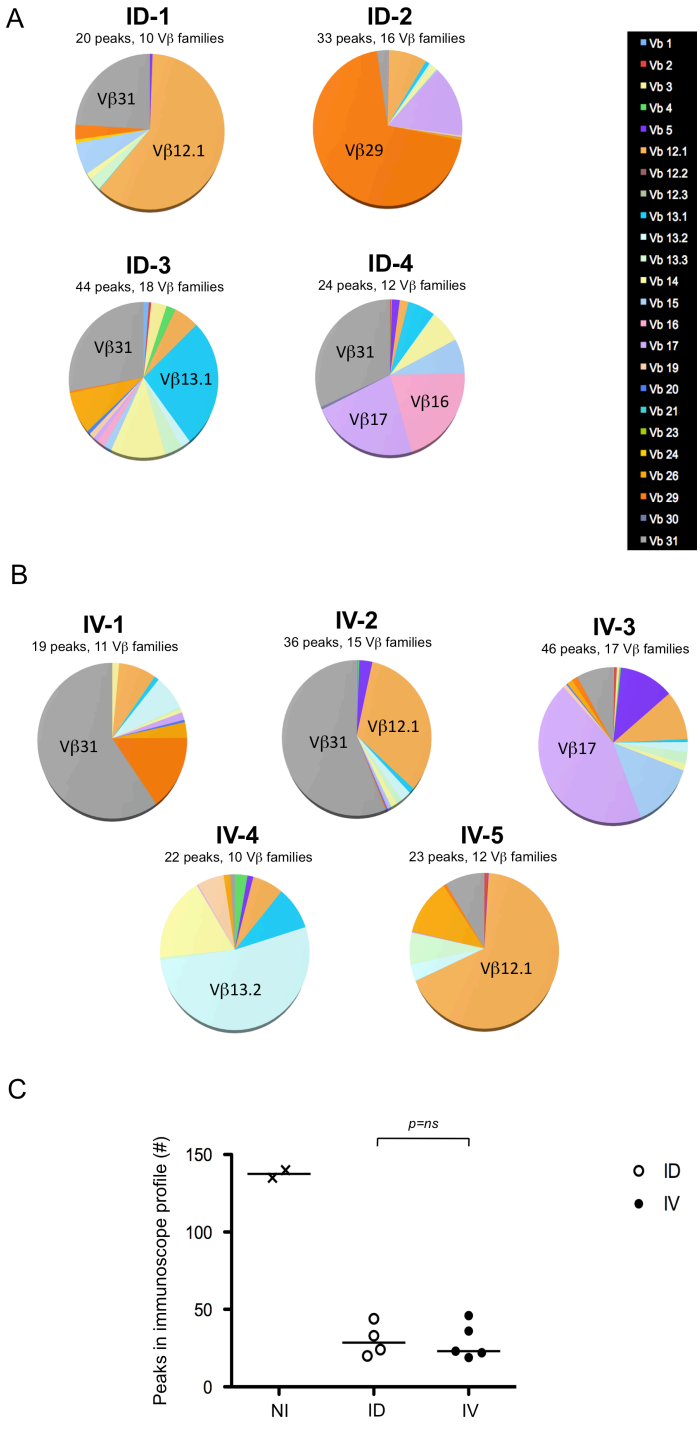


Figure 30. Single mouse analysis confirms that route of immunization does not influence T cell diversity. Mice were immunized i.d. **(A)** or i.v. **(B)** with $5x10^5 K^{bm1}$mOva splenocytes. On day 9, 15 macroscopic lymph nodes and the spleen were harvested and a K^b-SIINFEKL tetramer-based enrichment was performed for each mouse. $CD8^+$ K^b-SIINFEKL tetramer-positive cells were sorted. Immunoscope was performed on cells sorted from individual mice (3,000- 5,000 cells sorted per mouse) to define the length of the CDR3 loop in the 24 Vβ families. **(A, B)** Each color represents a distinct Vβ family. **(C)** The total number of peaks detected in all Vβ profiles was enumerated and represented. Data points indicate a single mouse. Statistical analysis comparing ID and IV was assessed by Mann-Whitney test. NI, non-immunized mice, shown to indicate baseline diversity of TCR.

2) T cell avidity

We evaluated the avidity of the responding T cells by determining their ability to produce IFNγ after re-stimulation with limiting concentrations of SIINFEKL peptide. Responses were in the linear range for peptide concentrations 10^{-13} – 10^{-9}, after which maximal IFNγ production was achieved. No differences were observed when comparing T cells isolated from mice that had been primed via the i.d. *versus* i.v. route (**Figure 31**).

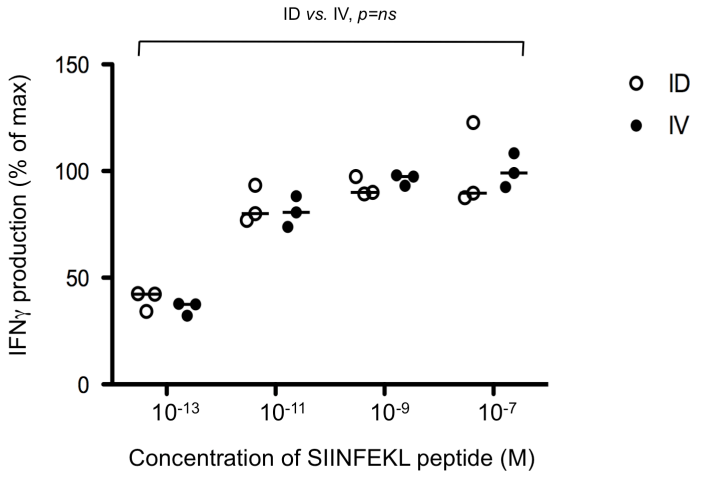


Figure 31. Route of immunization does not influence T cell avidity. Mice were immunized i.d. or i.v. with $5x10^5$ K^{bm1}mOva splenocytes. On day 8 post-immunization, the draining lymph node and the spleen of five mice were pooled. $CD8^+$ T cells were purified and analyzed by IFNγ ELISPOT. SIINFEKL peptide-pulsed DCs were used to re-stimulate $CD8^+$ T cells. SIINFEKL peptide concentrations are indicated. Results are represented as the percentage of maximal IFNγ production. Data points indicate replicates for each condition. Results are representative of two independent experiments. Percentage of maximum IFNγ production was analyzed using peptide concentration as a continuous variable (general linear modeling).

Based on these data we concluded that neither the diversity nor the avidity of the Ova-specific $CD8^+$ T cells was influenced by the route of antigen delivery.

III. DOES THE EFFICIENCY OF CROSS-PRIMING DEPEND ON ANTIGEN PERSISTENCE?

The dissemination of antigen is different depending on the route of immunization. We wanted to additionally explore whether the route of immunization would also affect antigen persistence and, if so, whether this effect could explain the enhanced priming efficiency following i.d. immunization.

First, we determined the relationship between antigen dissemination and antigen presentation by host accessory cells. We applied multiple experimental approaches to address this question: both looking at the persistence of the MHC-peptide complexe on APCs, as well as directly evaluating the proportion of injected cells that remain at different time points following immunization.

A. Persistence of antigen cross-presentation

First, we assessed the persistence of antigen cross-presentation by evaluating the presence of K^b-SIINFEKL complexes in different lymphoid organs. In order to compare i.d. injection performed on the flank and i.v. immunization, we looked in the spleen and in the inguinal lymph node, as it is the draining lymph node for i.d. injection, and in the opposite inguinal lymph node as a non draining lymph node control. Mice were immunized with K^{bm1}mOva splenocytes and, at different time points, CFSE-labeled $CD45.1^+$ OT-I splenocytes were transferred as a means of assessing cross-presentation by host APCs (**Figure 32**).

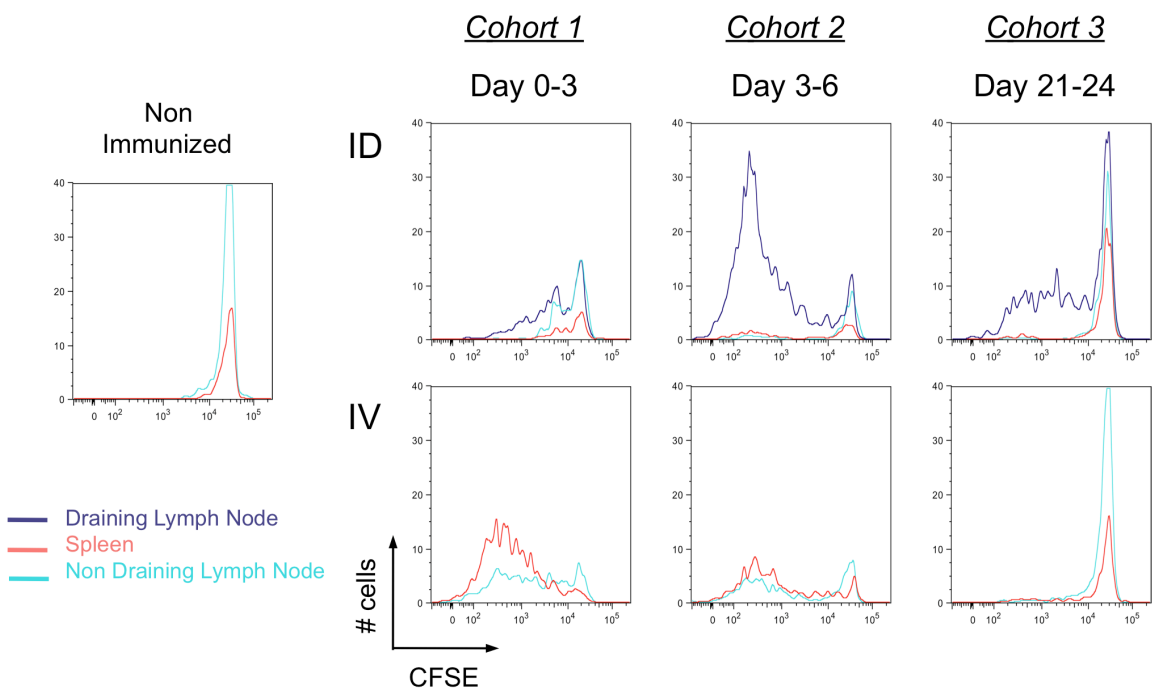


Figure 32. Delayed but persistent antigen presentation in the local draining lymph node after intradermal immunization. Mice were immunized i.d. or i.v. with $5x10^5$ K^{bm1}mOva splenocytes. On days 0, 3, or 21, $5x10^6$ CD45.1 CFSE-labeled OT-I splenocytes were adoptively transferred into immunized recipients. Three days later, the spleen, draining lymph node and a non-draining lymph node were harvested and the dilution of CFSE staining of OT-I was determined. Results are representative of three independent experiments.

OT-I transferred prior to immunization and analyzed three days later showed significant dilution of CFSE, indicating that cell-associated antigen injected via the i.v. route had already been cross-presented in spleen and lymph nodes (**Figure 32, cohort 1**). Given that up to 7 cell divisions could be observed and that the first cell division is thought to require more than 24 hours post-engagement by host DCs, we suggest that cross-presentation must have occurred immediately following immunization. Antigen presentation persisted from days 3-6 as the second cohort of OT-I also showed dilution of CFSE (**Figure 32, cohort 2**). In contrast to the i.v. condition, the i.d. immunization demonstrated only minimal OT-I divisions in the first cohort of transferred cells. By day 3-6, the response had increased and significant OT-I proliferation could be observed in the draining lymph node, with minor responses in the spleen. These data confirmed the local *versus* systemic dissemination of antigen via the two routes and helped to explain the delayed kinetics of T cell priming after i.d. immunization. Unexpectedly, the transfer of a third cohort of OT-I at 21 days post-immunization indicated that, when delivered via the i.d. route, antigen was still being presented within the draining lymph node (**Figure 32, cohort 3**). This was not observed in the i.v. condition, suggesting the absence of APCs presenting SIINFEKL peptide. Based on these findings, we concluded that the localized administration of cell-associated antigen impacts the timing of cross-presentation. While i.d. immunization is slightly slower to mount a response due to the need for antigen to be captured and cross-presented in local lymphoid organs, the sustained presentation of MHC-I-petide complexes could influence and amplify effector and memory responses.

B. Persistence of antigen

The persistence of MHC-I-peptide complexes obtained by performing CFSE dilution experiments suggests potentially interesting conclusions regarding the influence of antigen persistence on cross-presentation, but it is not a direct measure of the persistence of antigen. To determine the amount of injected splenocytes that persist over time, we examined the injected cells using bioluminescence. To note, only remaining live cells are detectable by this technique. Luciferase-expressing splenocytes isolated from transgenic animals were injected into wild-type recipients. Due to strain constraints, we were not able to perform this experiment in the model previously used. Rather, luciferase-expressing FvB male mice were used as a source of donor splenocytes, harboring minor histocompatibility differences with the female recipients. Thus, we used the HY model instead of the Ovalbumin model and, in this case, the cross-presentation is not ensured as these cells are not H-2K^{bm1}. Nevertheless

this model worked well to investigate the behaviour of injected splenocytes upon i.d. or i.v. injection. Cells delivered via the i.d. route remained primarily localized within the injection site (**Figure 33A**). Kinetic studies suggested persistence of donor cells in the skin for more than 13 days after i.d. injection (**Figure 33B**). Furthermore, we observed live injected splenocytes in the draining lymph node of i.d.-immunized mice and in the spleen of i.v.-immunized animals, indicating that there remains intact cell-associated antigen several days after immunization, irregardless of the delivery route (**Figure 33C, D**); however, there exists clear differences in splenocyte persistence between i.d. and i.v. immunization.

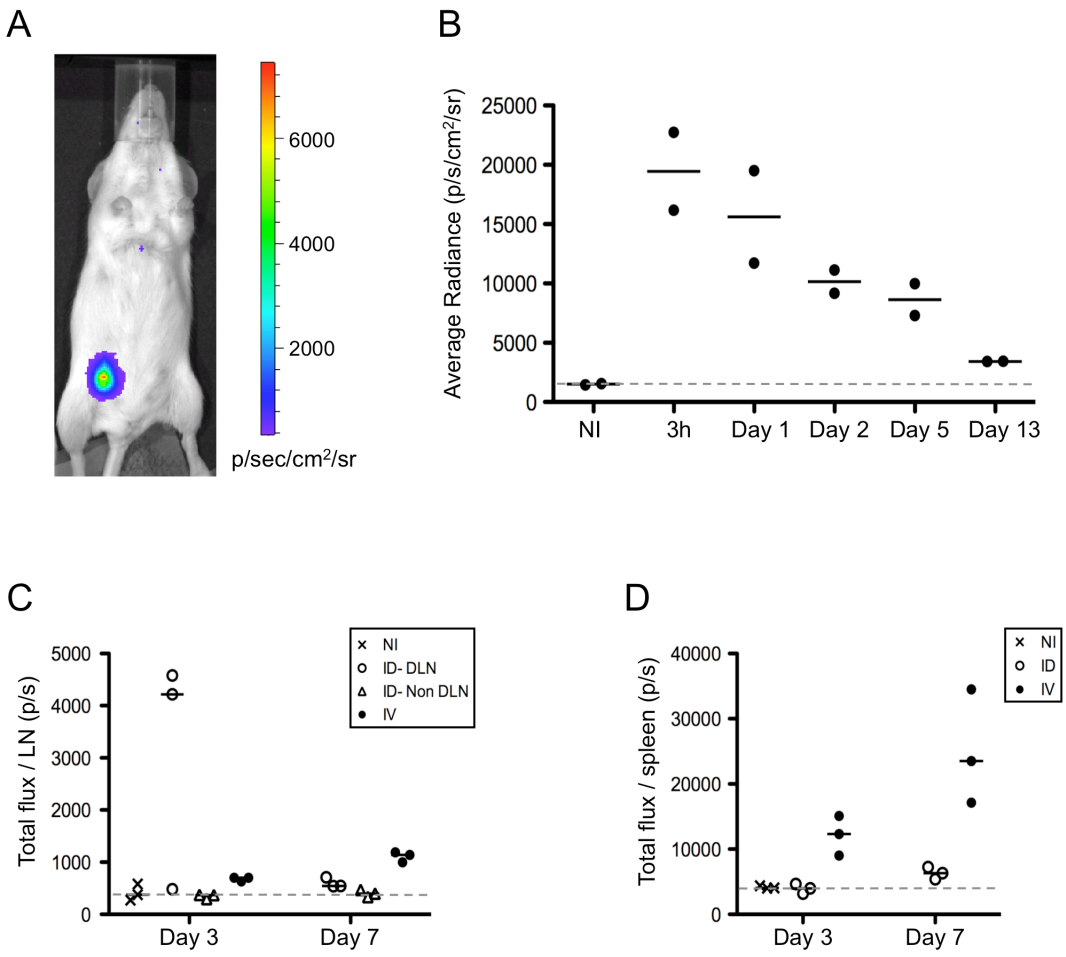


Figure 33. Intradermal injection of splenocytes results in a local depot of donor cells. FVB/N female recipients were immunized i.d. or i.v. with $5x10^6$ FVB/N-luciferase$^+$ male splenocytes. **(A,B)** Following immunization, mice injected i.d. were evaluated at 3h and then on day 1, 2, 5, and 13. Prior to imaging, mice were injected i.p. with D-luciferin. **(A)** Representative bioluminescence analysis performed at 3 h is shown. **(B)** Kinetic analysis of the bioluminescent signal is shown. **(C,D)** On days 3 and 7, the LNs **(C)** and the spleen **(D)** from mice immunized i.d. or i.v. were harvested and placed in wells containing PBS and D-luciferin to determine the total bioluminescent signal from each organ.

We employed additional strategies to study the persistence of antigen in our specific model. In particular, we looked for remaining antigen in the skin at the site of i.d. immunization. DNA was extracted from these tissues and a PCR reaction for the Ovalbumin gene was

performed as a read-out for antigen persistence. To the contrary of our bioluminescence results, this approach allowed for the detection of all cells, dead or alive. Surprisingly, Ovalbumin DNA was detected only few days after i.d. immunization (**Figure 34**). Although, these were only preliminary results, they reinforced the need for a strategy to examine antigen persistence in our model.

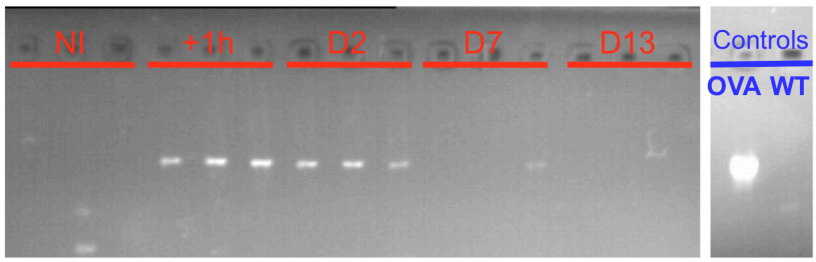


Figure 34. Persistence of antigen evaluated by PCR. Mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. The site of injection was removed at 1h, and on days 2, 7 and 13 post-immunization. The skin was digested and DNA was extracted. A PCR reaction was performed to detect Ovalbumin DNA. This experiment was done with 3 mice per group. NI, Non-immunized mice. These are preliminary data.

We decided to compare the persistence of H-$2K^{bm1}$mOva splenocytes with WT mOva splenocytes. To do this, we injected CD45.2 splenocytes CD45.1 recipient mice i.v. and examined their persistence over time by flow cytometry. We observed that H-$2K^{bm1}$mOva splenocytes disappeared faster than their WT counterparts indicating that they are targeted *in vivo* (**Figure 35**), unless we showed that they were not killed by NK cells (**Figure 16C**).

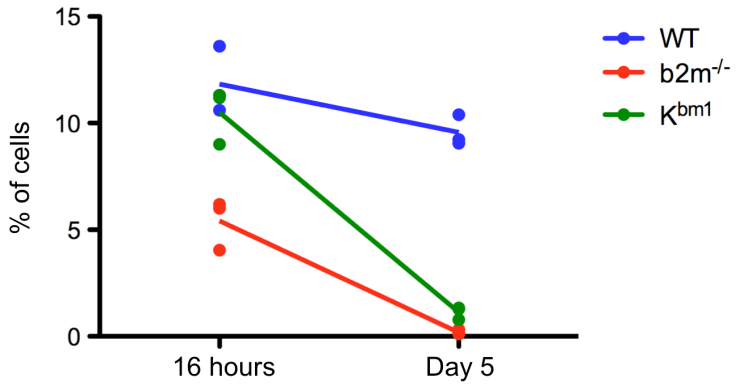


Figure 35. K^{bm1}mOva splenocytes disappear faster than their WT counterparts. Mice were immunized i.v. with a mixture of Ova-expressing splenocytes that are either WT, $\beta 2m^{-/-}$ or K^{bm1} and labeled with 3 different concentrations of CFSE. At 16 h, or on day 5, the spleen was harvested and the percentage of injected splenocytes that were remaining was evaluated. *This experiment was performed by H. Saklani.*

C. Inhibition of cross-presentation with anti-K^b-SIINFEKL antibody

Given the differences we observed for the persistence of antigen upon i.d. or i.v. immunization (**Figures 32 and 33**), we hypothesized that this may explain why we obtained an enhanced T cell response after local antigen delivery. It was possible that i.d. immunization resulted in a local antigen depot that would allow for the delayed release of antigen from the site of injection and, consequently, an extended period of antigen presentation in lymphoid organs and a better $CD8^+$ T cell response. This model was also in line with previous results obtained in our lab showing that persistence of antigen is required to have effective cross-priming (Jusforgues-Saklani et al., 2008).

To test this hypothesis, we injected mice with antibodies directed against the MHC-I-peptide complexe: H-$2K^b$-SIINFEKL. In this way the antibody will bind and mask antigen, blocking its presentation to T cells. This antibody has been previously characterized and used to block antigen presentation *in vivo* (Obar and Lefrancois, 2010). If our hypothesis was true, we expected to obtain the same level of response for i.d.-immunized mice that were injected with blocking antibody one week after immunization, and for i.v.-immunized animals. Yet this was not the result observed: the injection of the blocking antibody did not appear to impact the T cell response at all (**Figure 36A, B**). From these data, it is unclear whether our hypothesis was incorrect, or if the antibody is not efficient at blocking persistent cross-presentation. Indeed, it is difficult to address this question and validate its effect on peptide presentation, as it does not block OT-I proliferation in a control experiment (**Figure 36C**). Another strategy could be used to address this question: the injection of diphteria toxin receptor-expressing splenocytes could be performed, followed by their removal upon treatment with diphteria toxin. The ability to manipulate the presence of the antigen depot would allow for the investigation of the impact of antigen persistence on T cell priming over time.

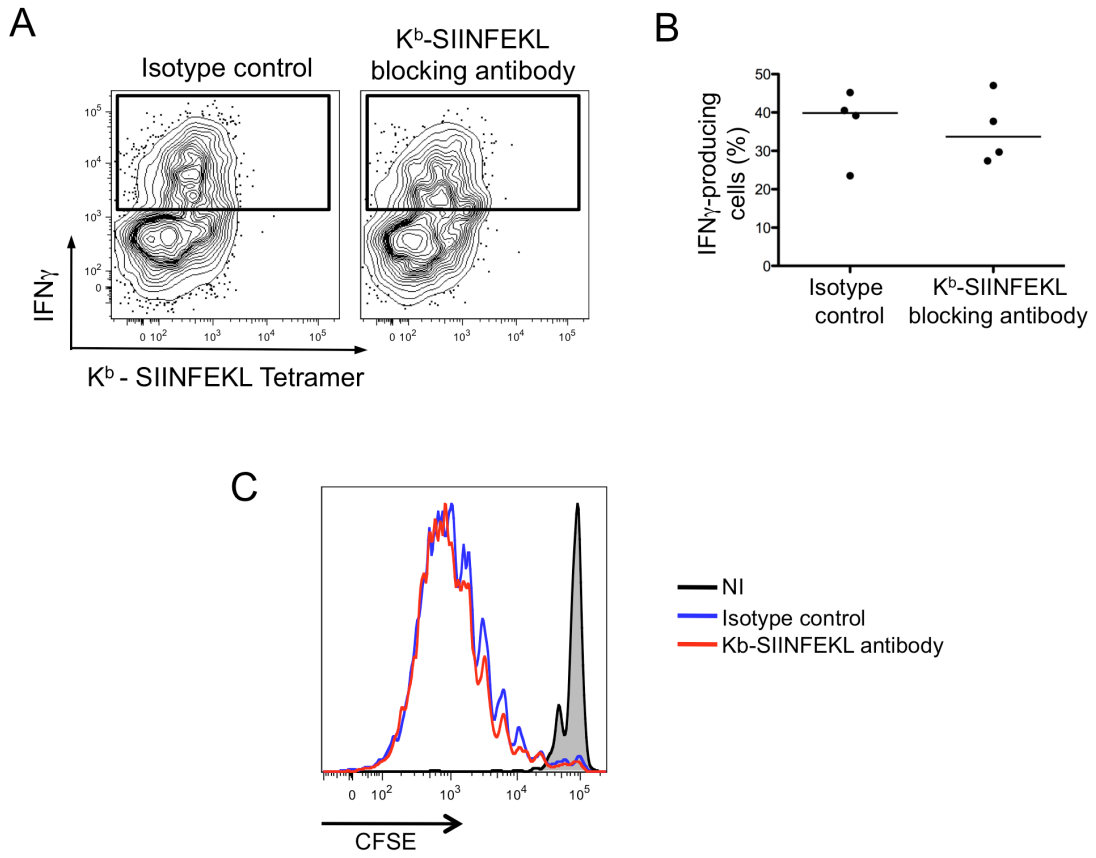


Figure 36. Administration of K^b-SIINFEKL antibody does not impact IFNγ production after i.d. immunization. (A,B) Mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. They received anti-K^b-SIINFEKL antibody or isotype control on days 7, 8 and 9. On day 10, the spleen and lymph nodes were harvested for tetramer-based enrichment followed by *ex vivo* restimulation and intracellular staining for IFNγ. **(A)** Representative FACS plots of $CD8^+$ T cells were shown. Percentages of $CD8^+$ T cells making IFNγ were evaluated **(B)**. **(C)** To test the ability of the antibody to block antigen presentation, mice were immunized i.v. with $5x10^5$ K^{bm1}mOva splenocytes. On day 2, they received the blocking antibody or the isotype control. On day 3 post-immunization, $5x10^6$ CD45.1 CFSE-labeled OT-I were transferred and the CFSE dilution was evaluated 3 days later.

In this section we demonstrated that the quality of the T cell response depends on the route of immunization. As expected, systemically disseminated antigen resulted in rapid cross-presentation, which correlated with the early differentiation of antigen-specific effector T cells. This was in contrast to locally administered antigen, which showed delayed cross-presentation and expansion of responding T cells. Although initially delayed, the T cell response upon i.d. injection was much more robust and polyfunctional that that seen following i.v. injection. Interestingly, the magnitude of T cell expansion was similar for both routes of immunization. Thus, we can conclude that the route of immunization impacts T cell quality but not primary expansion. These differences were not observed upon transfer of large numbers of TCR-transgenic T cells, which highlights the importance of the in-depth

examination of vaccine candidates using the endogenous repertoire as a read out for successful priming. The tetramer-based enrichment strategy was essential for this study as the absolute numbers of antigen-specific T cells generated after immunization with this low dose of antigen are extremely few in number. Indeed, cytokine profiling or TCR analysis can only be performed after enrichment such that there are a sufficient population of specific T cells to study. We additionally hypothesized that the differential antigen persistence observed with the two types of immunization may be responsible for the more efficient cross-priming seen upon i.d. immunization; however, a more detailed examination of this mechanism will be required before a more definitive conclusion about this point can be formed.

Chapter 3: Defining optimal timing for adjuvant delivery

In instances where microbial associated molecular patterns are absent (e.g., cell-associated antigen), it is common practice to formulate the vaccine with an adjuvant. While adjuvants have been shown to be useful for enhancing the response to antigen, it has also been observed that DC maturation prior to immunization can have the opposite effect – the inhibition of subsequent T cell priming (Wilson et al., 2006). Following from our results indicating delayed cross-presentation after i.d. immunization (**Figure 32**), we predicted that the optimal timing of adjuvant delivery will depend on the route of immunization.

I. THE OPTIMAL TIMING FOR ADJUVANT DELIVERY IS DEPENDENT ON THE ROUTE OF IMMUNIZATION

Our prediction was that the optimal timing for adjuvant delivery may be earlier for i.v. immunization as compared to i.d. immunization, due to the different kinetic of cross-presentation observed in **Figure 32**. To test this, mice were again immunized with K^{bm1}mOva splenocytes i.d. or i.v. Poly I:C was used as an adjuvant and administered at different time points relative to immunization with antigen. The absolute number of antigen-specific T cells was determined at the respective time of peak response (day 7 for i.v. and day 9 for i.d. immunization). When poly I:C was injected one day prior, or the day of i.v. immunization with the K^{bm1}mOva cells, T cell priming was greatly reduced (**Figure 37A**). Strikingly, injection of poly I:C one day after i.v. immunization enhanced T cell priming. For the i.d. route, poly I:C injection one day prior to, the day of, or even one day after immunization, resulted in the inhibition of T cell priming (**Figure 37B**). It was necessary to wait until three days post-immunization to inject poly I:C in order to observe an enhancement of T cell priming (**Figure 37B**). Following from the results presented in **Figure 32**, we suggest that antigen capture has to occur prior to adjuvant detection by DCs and that one day of antigen capture is sufficient to permit T cell priming after i.v. immunization, but that additional time is required for antigen capture after i.d. immunization. This hypothesis could explain why delayed adjuvant delivery is optimal for i.d. immunization.

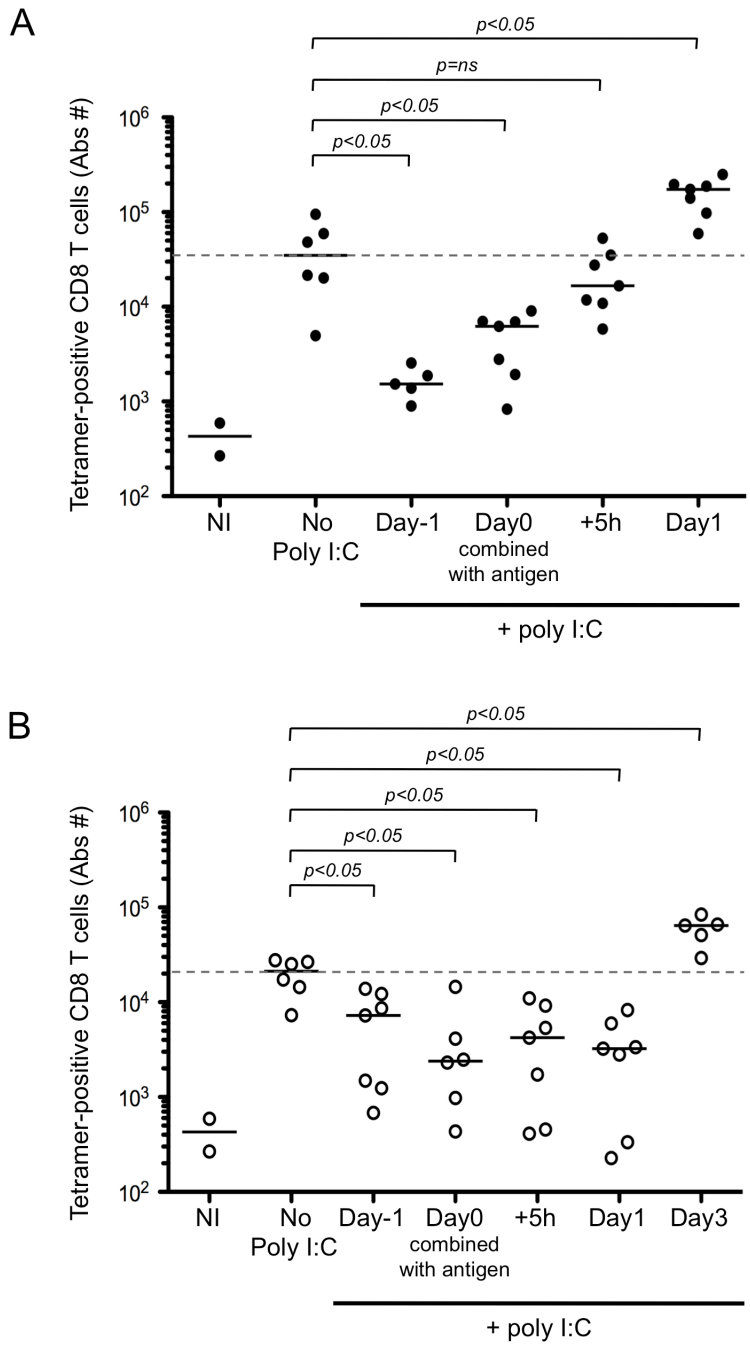


Figure 37. Optimal timing of adjuvant delivery is dependent on the route of immunization. (A,B) Mice were immunized i.d. or i.v. with $5x10^5$ K^{bm1}mOva splenocytes and received 100μg of poly I:C at the indicated time points. For mice immunized i.v., they received poly I:C i.v. either: 1 day before immunization, the day of immunization combined with antigen, 5h, or 1 day post-immunization. The spleen and 15 macroscopic lymph nodes were harvested on day 7, which corresponds to the peak of the $CD8^+$ T cell response. K^b-SIINFEKL tetramer-based enrichment was performed and the absolute numbers of tetramer-positive $CD8^+$ T cells is reported **(A)**. For mice immunized i.d., they received poly I:C at the same time points and one additional group was added, 3 days post-immunization. Poly I:C was administered i.v., except for the mice injected on day 0 with poly I:C formulated with the antigen. Analysis was performed on day 9 post-immunization, again corresponding with peak $CD8^+$ T cell response **(B)**. *p*-values were calculated using a Mann-Whitney test, comparing in a two-way test, adjuvant condition to no poly I:C treatment. Dotted lines correspond to the median number of responding cells in the absence of poly I:C. NI, non-immunized mice are shown to indicate baseline responses.

To confirm that early delivery of adjuvant inhibited priming due to a failure to capture and present cell-associated antigen, we utilized the adoptively transferred CFSE-labeled OT-I system. Administration of poly I:C one day after i.d. immunization completely blocked OT-I proliferation and IFNγ production (**Figure 38**). If instead we waited until three days post-immunization to administer the poly I:C, this blockade was no longer observed and, in fact, a greater percentage of OT-I showed maximal cell division and effector function (**Figure 38, arrow**). Based on these results, we initiated a study to further detail the mechanisms underlying these differential effects of adjuvant on cross-priming efficacy depending on the timing of adjuvant delivery.

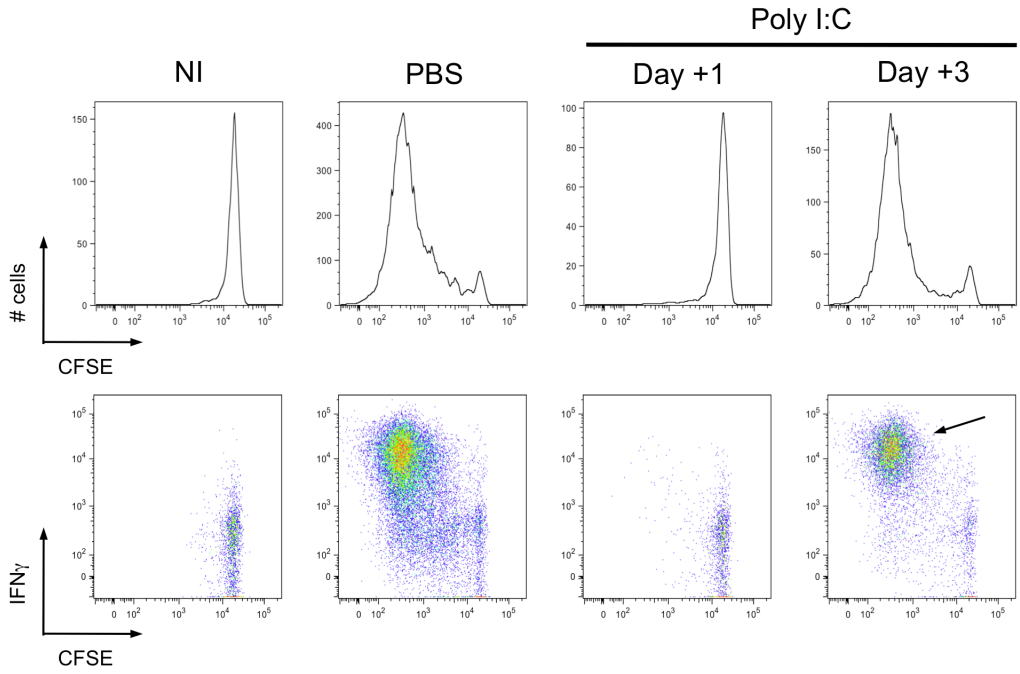


Figure 38. Opposite effects of adjuvant depending on the timing of administration. Mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. On day 1 or day 3 post-immunization, 50µg of poly I:C or PBS was injected i.v. On day 3 post-immunization, $5x10^6$ $CD45.1^+$ CFSE-labeled OT-I splenocytes were transferred i.v. Three days later the draining lymph node was harvested and the dilution of CFSE staining of OT-I was determined, represented by histograms. Intracellular staining for IFNγ was performed at the same time, shown in the corresponding FACS plots. $CD3^+$ $CD8^+$ $CD45.1^+$ cells were gated for the analysis shown. Data are representative of three independent experiments. NI, non-immunized mice.

II. MECHANISMS RESPONSIBLE FOR THE OPPOSITE EFFECTS OF POLY I:C ON CROSS-PRIMING

Whichever the route of immunization, we can conclude that an early poly I:C injection inhibits subsequent cross-priming, while a late administration significantly boosts the T cell response. Following these data, the aim was then to understand what are the mechanims underlying the differential effects of poly I:C treatment that are dependent on the timing of administration. To address this question, we worked in our established model presented in Chapter 2, but focused on only one route of immunization: the intradermal route; and two time points for poly I:C administration: 7 hours post-immunization, which leads to inhibition of cross-priming, and 3 days post-immunization that results in an enhancement of the T cell response (**Figure 39**). As poly I:C is a ligand for both TLR3 and Mda5 and is known to trigger type I IFN production, we were also curious to know whether the effects of poly I:C that we observed are dependent on type I IFN production. Tools were developed to examine these questions in detail.

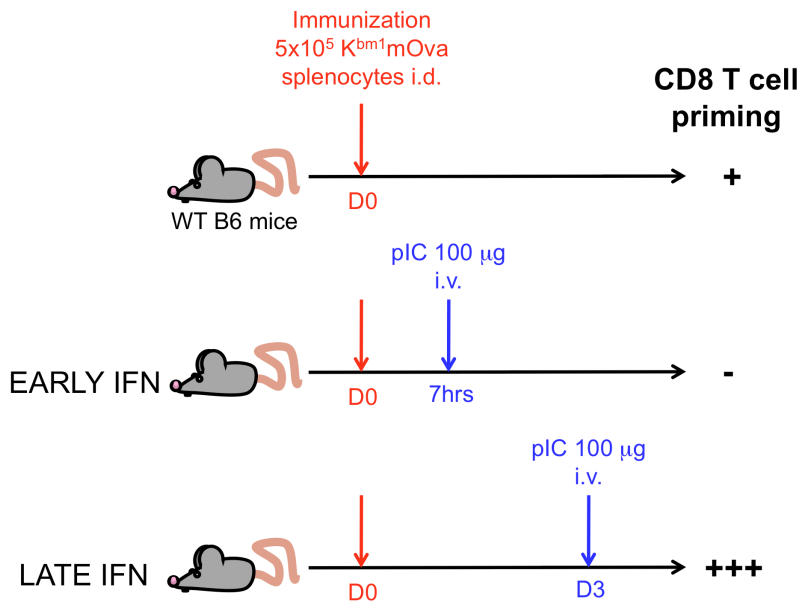


Figure 39. Model used to dissect the mechanisms underlying the opposite effects of poly I:C on cross-priming depending on the timing of delivery.

A. Mice available to study the effects of type I IFN on cross-priming

Different mouse lines useful for addressing these questions were available for use in our lab. IFNAR1-deficient mice (IFNAR$^{-/-}$ mice) are deficient for one subunit of the receptor for type I IFN. While these mice are not able to respond to type I IFN, a significant amount of type I

IFN is generated and found in the circulation after poly I:C injection (**Figure 40**). Furthermore we had access to mice deficient for two major transcription factors, involved in the expression of type I IFN: IRF3 and IRF7. These mice (IRF3/7 DKO) are unable to produce type I IFN upon poly I:C injection (**Figure 40**). However it is important to note that they are able to produce these cytokines after injection with other stimuli (Daffis et al., 2009). Both the IFNAR$^{-/-}$ and the IRF3/7 DKO were compared to WT mice, which are able to produce and to respond to type I IFN, for their ability to trigger a CD8^{+} T cell response.

As our main interest was to look at antigen-specific CD8^{+} T cells and the impact of type I IFN on the outcome of the T cell response, we developed TCR-transgenic OT-I T cells that are also IFNAR-deficient in order to have the capability to study the direct effect of type I IFN on specific T cells.

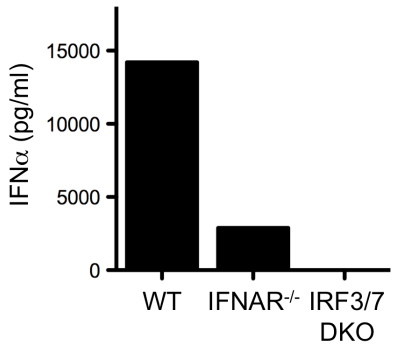


Figure 40. Type I IFN production upon poly I:C stimulation. WT, IFNAR$^{-/-}$ and IRF3/7 DKO mice received 100μg of poly I:C i.v. 6 hours later, plasma was collected and circulating IFNα concentration was measured.

B. Inhibition of cross-priming upon early type I IFN production

We started by studying the mechanisms responsible for the inhibition of cross-priming upon early delivery of poly I:C. Our goals were to determine which cells are the targets of poly I:C, specifically focusing on the two main cell types implicated in cross-priming: DCs and CD8^{+} T cells; whether the effects of poly I:C are type I IFN-dependent and, if that is the case, what are the precise actions of type I IFN on its targets.

1) Type I IFN inhibit cross-priming through direct action on cross-presentation

Initially, we wanted to examine if the inhibition of cross-priming observed upon early poly I:C injection was type I IFN-dependent. We started by testing the role of type I IFN in cross-presentation. WT or IFNAR$^{-/-}$ recipient mice were immunized with K^{bm1}mOva splenocytes

and received poly I:C 7 hours post-immunization. This time point was known from previous work to induce an inhibition of priming (**Figure 39**). On day 3 post-immunization, CFSE-labeled antigen-specific OT-I T cells were transferred into the mice and 3 days later, OT-I division was assessed by looking at CFSE dilution. If DCs presenting antigen were present, OT-I would proliferate. As shown in **Figure 41A**, OT-I transferred into WT recipients did not divide in the non-immunized animals whereas a robust proliferation can be observed in immunized mice. As expected, inhibition of OT-I division in immunized mice was conferred by poly I:C treatment 7 hours post-immunization. In contrast, the same level of OT-I proliferation was observed in IFNAR$^{-/-}$ recipients that had been immunized, irregardless of their treatment with poly I:C (**Figure 41B**). These results demonstrated that in WT recipients there are not DCs available to cross-present antigen after poly I:C injection and this phenomena is type I IFN-dependent.

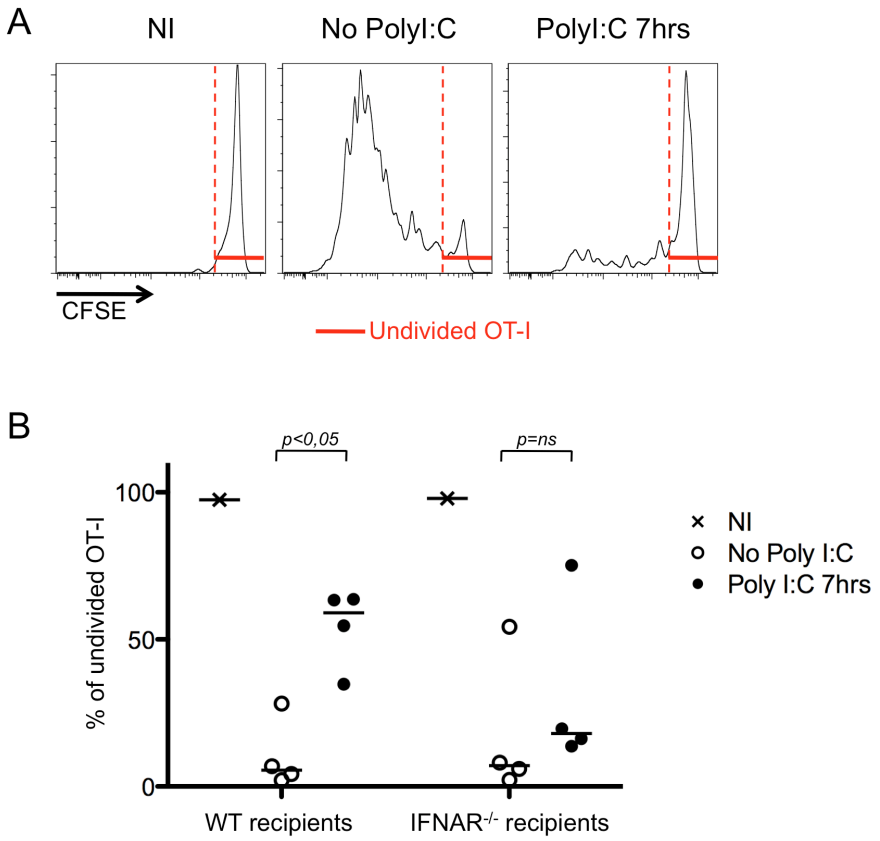


Figure 41. Inhibition of cross-presentation by poly I:C is type I IFN-dependent. WT and IFNAR$^{-/-}$ mice were immunized i.d. with 5x10^5 K^{bm1}mOva splenocytes and received 100µg of poly I:C 7 hours later. On day 3, 5x10^6 CD45.1$^+$ CFSE-labeled OT-I were transferred and CFSE dilution was evaluated 3 days later. Representative results of CFSE dilution of CD8$^+$ OT-I cells obtained from WT mice are shown **(A)**. The percentage of undivided OT-I was determined and plotted **(B)** for WT and IFNAR$^{-/-}$ mice. *p*-values were calculated using a Mann-Whitney test. NI, non-immunized mice.

2) Type I IFN act on cDCs but cell-intrinsic production is not required

As type I IFN are produced by several cell types following poly I:C treatment, particularly the cDCs themselves, we asked whether the cell-intrinsic production of these cytokines by cDCs was required to inhibit cross-presentation. Bone marrow chimeras were generated by transferring IRF3/7 DKO bone marrow into WT recipients, allowing for type I IFN production from only the stromal cells in these mice. While IRF3/7 DKO mice do not produce any type I IFN upon poly I:C stimulation, a significant level of these cytokines can be detected in these chimeras, albeit at lower levels than in their WT counterparts (**Figure 42A**). As previously described, the transfer of CFSE-labeled OT-I was performed to assess the presence of antigen-presenting DCs following adjuvant treatment. As expected, cross-presentation occured in IRF3/7 DKO mice after poly I:C injection. Interestingly, cross-presentation was completely abrogated in the BM-chimeras suggesting that the indirect, stromal production of type I IFN was sufficient to inhibit cross-presentation (**Figure 42B**).

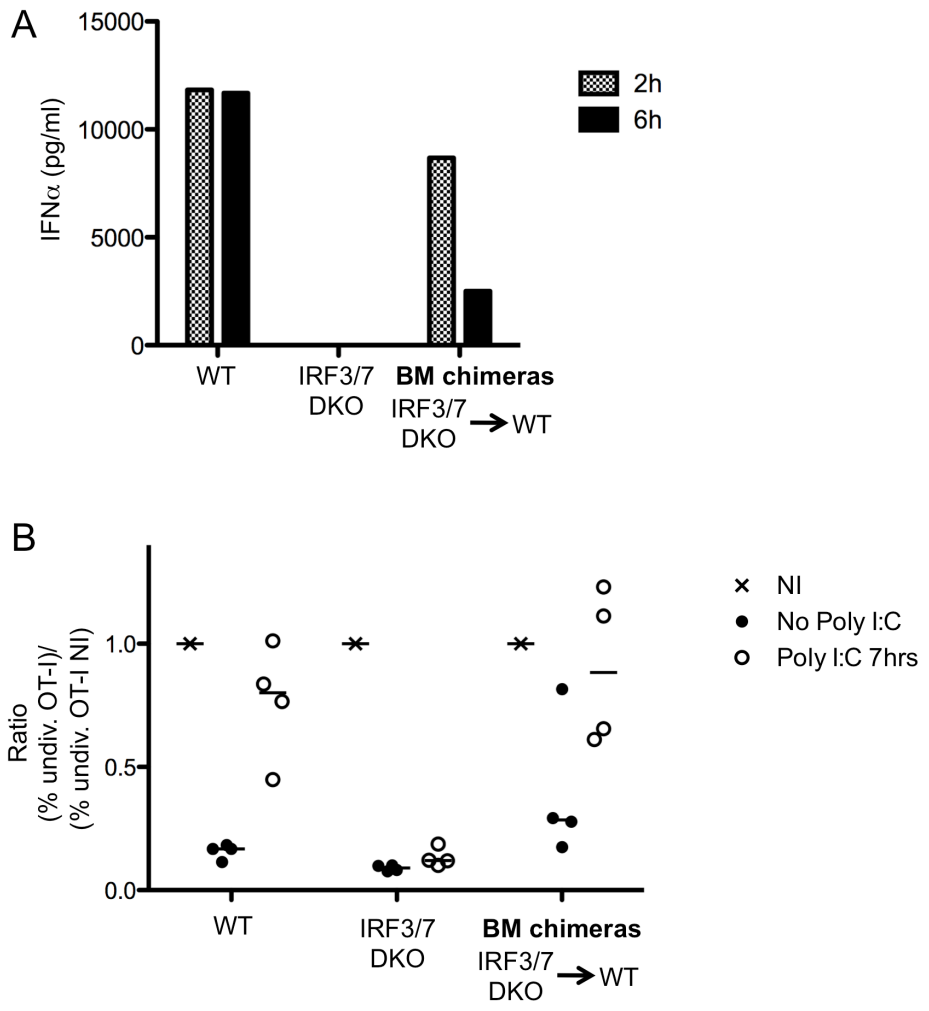


Figure 42. Inhibition of cross-presentation does not require cell-intrinsic production of type I IFN by cDCs. (A) WT, IRF3/7 DKO and (IRF3/7 DKO into WT) bone-marrow chimeras were injected with 100μg of poly I:C. At 2 and 6 hours post-stimulation, mice were bled to determine the plasma concentration of IFNα. **(B)** Mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes and received 100μg of poly I:C at 7 hours. Three days later, $5x10^6$ $CD45.1^+$ CFSE-labeled OT-I splenocytes were transferred i.v. The percentage of undivided OT-I was determined 3 days later and normalized for each mouse strain by comparing it with the percentage of undivided OT-I for the non-immunized animals of the same background. NI, non-immunized mice.

3) Early poly I:C affects DC numbers and phenotype

We demonstrated that the inhibition of cross-priming upon early delivery of poly I:C was type I IFN-dependent and characterized by an absence of cross-presenting DCs. To more precisely determine the action of poly I:C on host DCs, we performed an *in vivo* kinetic study, enumerating and phenotyping DC populations in the spleen and lymph nodes of WT mice. We focused on $CD8\alpha^+$ DCs and $CD103^+$ DCs, as these two subsets are known to respond to the TLR3 ligand poly I:C and are required for antigen cross-presentation (del Rio et al., 2007;

den Haan et al., 2000). Following poly I:C injection, we observed a striking decrease in the total number of splenic CD8α^+ DCs (**Figure 43A**). Analysis of the remaining cells indicated that activation markers CD86 and MHC-II are upregulated within 15h post-injection, indicating that maturation is a rapid process (**Figure 43B**). In contrast to the spleen, DC numbers in lymph nodes increased after poly I:C injection; and again the cells demonstrated a mature phenotype within 1 day of poly I:C administration (**Figure 43B**). Clearly, poly I:C impacted survival and/or migration of DCs in the lymphoid organs. This effect may be responsible for the inhibition of cross-priming if it results in the loss of DCs available at the site of injection, prepared to take up antigen. This rapid DC maturation could also contribute to the inhibition of subsequent priming by downregulating antigen engulfment, which is a characteristic feature of immature DCs.

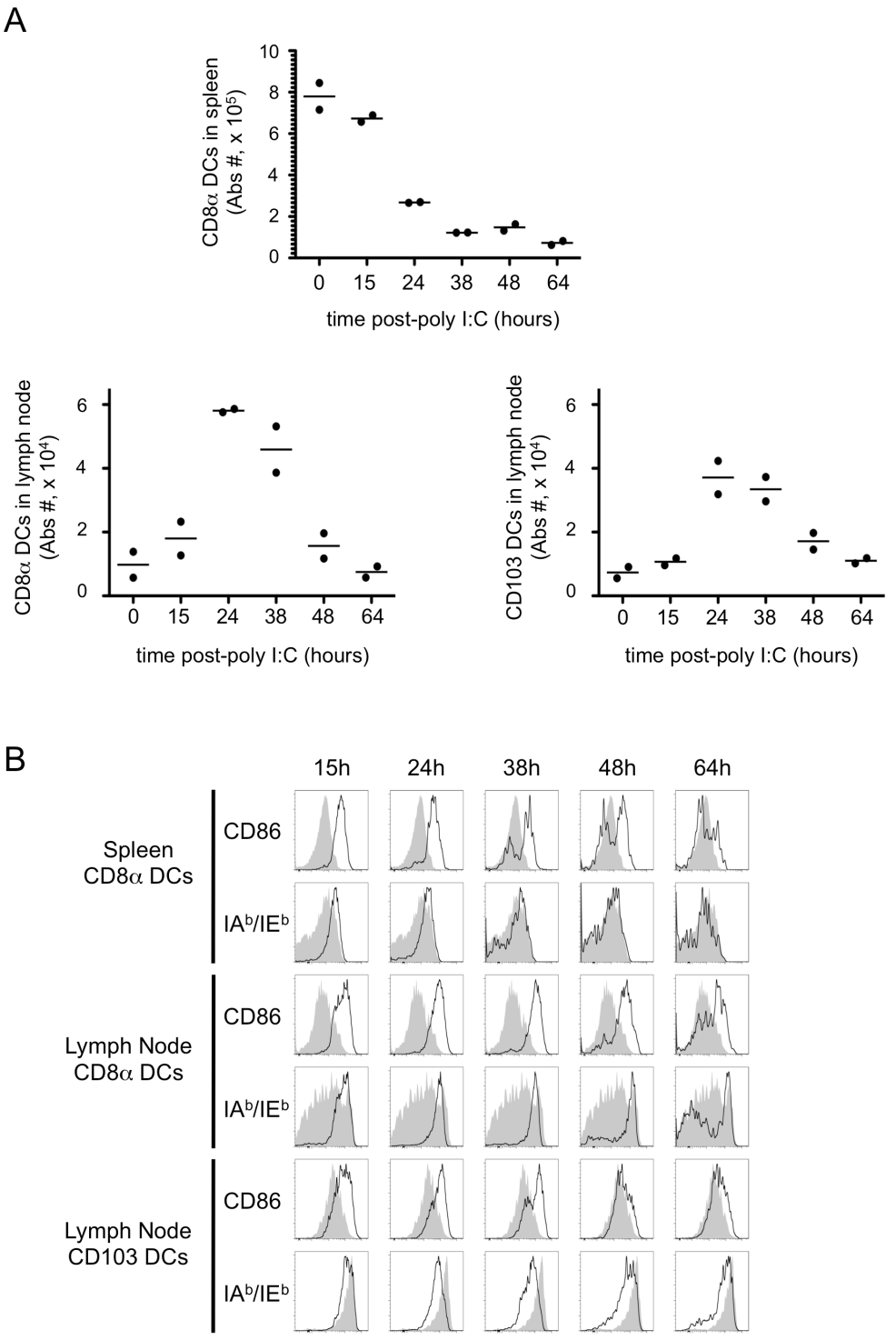


Figure 43. Poly I:C induces rapid DC maturation. C57BL/6 mice were injected i.v. with 100µg of poly I:C. At the indicated time points, the spleen and a lymph node were harvested. The total number of $CD8\alpha^+$ DCs and $CD103^+$ DCs per organ **(A)** and the expression of CD86 and IA^b/IE^b **(B)** were determined. In **(B)** the gray histograms indicate the level of expression in untreated animals and the black line correspons to poly I:C-injected mice.

4) Early poly I:C treatment affects antigen uptake

In the previously described experiment, only poly I:C was injected without antigen. To mimic physiologic vaccination conditions, it made sense to add the antigen back to the experimental protocol to examine how poly I:C affects antigen uptake and presentation by the DCs. To study the behaviour of injected antigenic splenocytes, the cells were stained with PKH26 dye prior to injection, allowing for their detection in the draining lymph node and even inside DCs upon phagocytosis by flow cytometry. Due to technical limitations, we needed to inject 10 times more splenocytes to ensure detection. WT CD45.1 recipients were immunized with $5x10^6$ PKH-26-labeled CD45.2 K^{bm1}mOva splenocytes and received poly I:C 7 hours later. On day 2, the draining lymph node was collected, collagenase digested and analyzed for labeled splenocytes as well as for the detection of splenocytes engulfment by different DC subsets. Many more PKH-26-labeled DCs were detected in immunized mice as compared to mice that received the combination of poly I:C and splenocytes (**Figure 44A**). As previously described, the $CD8\alpha^+$ DC subset appeared to be reduced in the latter condition and furthermore, no $CD8\alpha^+$ DCs labeled for PKH26 (indicating antigen engulfment) were detected. We also examined the draining lymph node for injected splenocytes. Lymphocytes are the main cell population present in the splenocyte injected preparation and furthermore, these cells are able to migrate and thus, reach the draining lymph node on their own. Interestingly, we found PKH26-labeled B and T cells in all immunized animals, irregardless of poly I:C treatment (**Figure 44B**). These data demonstrated that while early poly I:C treatment inhibits antigen uptake by DCs, injected splenocytes can still migrate to the draining lymph node even upon poly I:C injection.

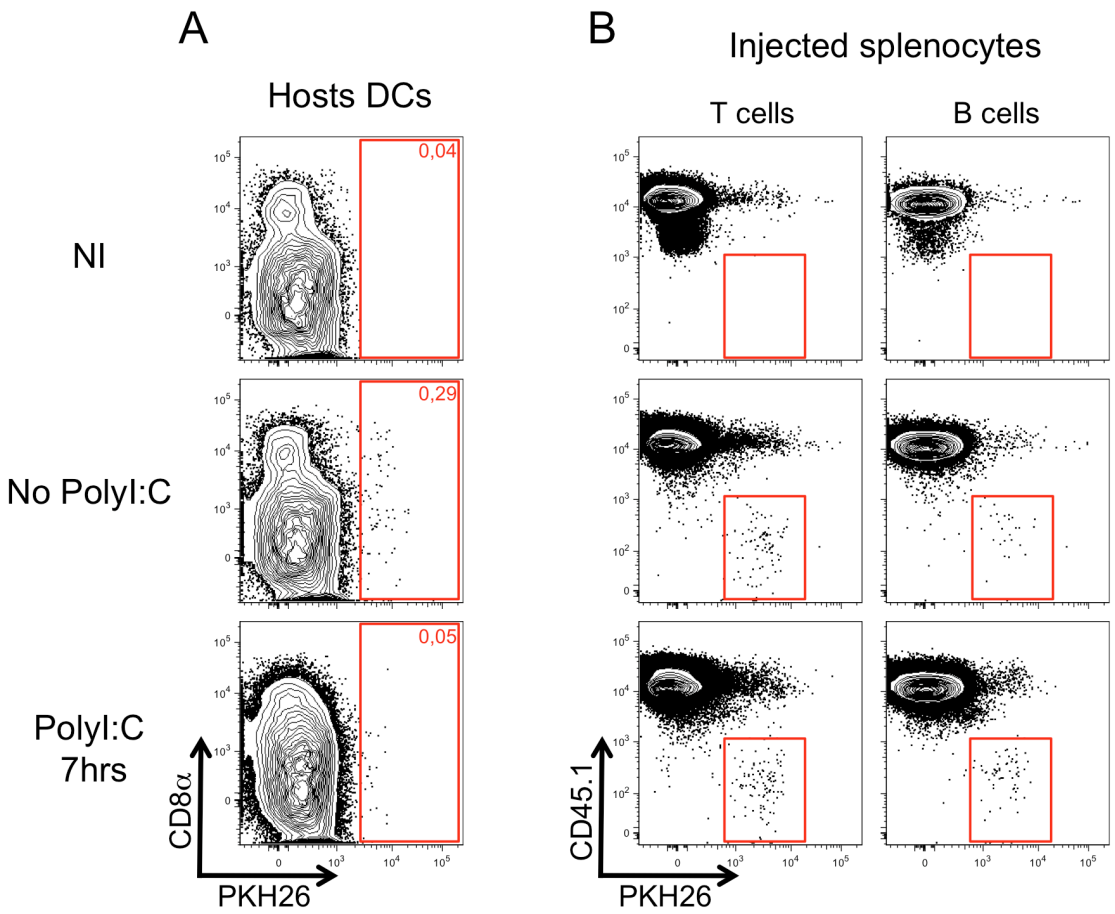


Figure 44. Early poly I:C affects antigen uptake by cDCs. C57BL/6 CD45.1$^+$ mice were immunized i.d. with 5x10^6 PKH-26-labeled CD45.2$^+$ K^{bm1}mOva splenocytes. 7 hours later, mice received 100µg of poly I:C. On day 2, the draining lymph nodes of several mice were pooled and an enrichmend for CD11c$^+$ cells was performed. Flow cytometry was performed on the enriched fraction to analyze DC subsets. CD11c$^+$ cells are shown **(A)**. In parallel, another flow cytometry analysis was performed on the 'flow-through' fraction to detect the injected lymphocytes that reached the draining lymph node independent of APCs. CD45.1 and PKH-26 staining were shown for CD3$^+$ (T cells) and CD19$^+$ cells (B cells) **(B)**.

5) Does early poly I:C treatment act directly on antigen-specific T cells?

We convincingly demonstrated that poly I:C acts on DCs. However, type I IFN have also been shown to act on T cells directly (Le Bon et al., 2006). To address whether early poly I:C treatment has a direct effect on antigen-specific T cells in our model, we immunized WT recipients with K^{bm1}mOva splenocytes, followed by an injection of poly I:C 7 hours later. On day 3 post-immunization, CFSE-labeled WT and IFNAR$^{-/-}$ OT-I were transferred and three days later, the dilution of the CFSE marker was analyzed to examine the proliferative capacity of the different T cell populations. As shown in **Figure 45**, the two populations of OT-I cells displayed the same behavior, suggesting that poly I:C does not differentially regulate WT and IFNAR$^{-/-}$ OT-I (**Figure 45**). However, one caveat to this experiment is that the OT-I cells

were transferred only three days after immunization and thus were not present during the peak of type I IFN production (**Figure 40**). Therefore, the impact of the defect in IFN-responsiveness of the IFNAR$^{-/-}$ OT-I cells may be underestimated here. These results should be further confirmed by performing the same experiment, with the OT-I transfer made prior to adjuvant delivery.

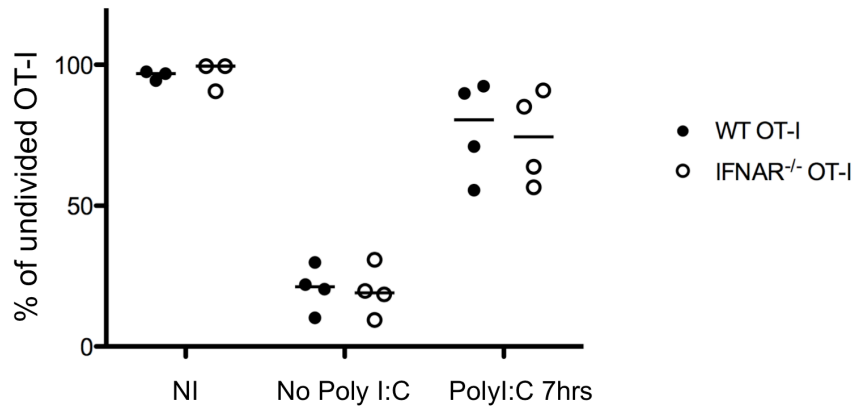


Figure 45. Type I IFN do not act on T cells transferred three days post-immunization. C57BL/6 CD45.1/1 mice were immunized with 5x10^5 K^{bm1}mOva splenocytes. Mice received 100µg of poly I:C 7 hours post-immunization. On day 3, WT CD45.1/2 and IFNAR$^{-/-}$ CD45.2/2 CFSE-labeled OT-I splenocytes were transferred. Three days later, the proliferation of the two populations of CD8$^+$ OT-I was assessed. The percentage of undivided OT-I was shown for each population.

C. Enhancement of cross-priming after late type I IFN production

Using the same model, we performed similar experiments to further understand the mechanisms underlying the enhancement of cross-priming upon late adjuvant delivery.

1) <u>Enhancement of cross-priming upon late poly I:C delivery is type I IFN-dependent</u>

The T cell response was studied in WT, IFNAR$^{-/-}$ and IRF3/7DKO mice to determine whether the enhancement of cross-priming upon late poly I:C administration is dependent on type I IFN signaling. These three mouse lines were immunized i.d. with K^{bm1}mOva splenocytes and received poly I:C three days later. On day 10, cells from the spleen and the draining lymph node were harvested to perform an IFNγ-ELISPOT. As expected, a higher number of CD8$^+$ T cells secrete IFNγ in WT mice that received poly I:C than those that received antigen alone. This enhancement was not observed for IFNAR$^{-/-}$ or IRF3/7 DKO mice confirming that late poly I:C treatment boosts cross-priming in a type I IFN-dependent manner (**Figure 46**).

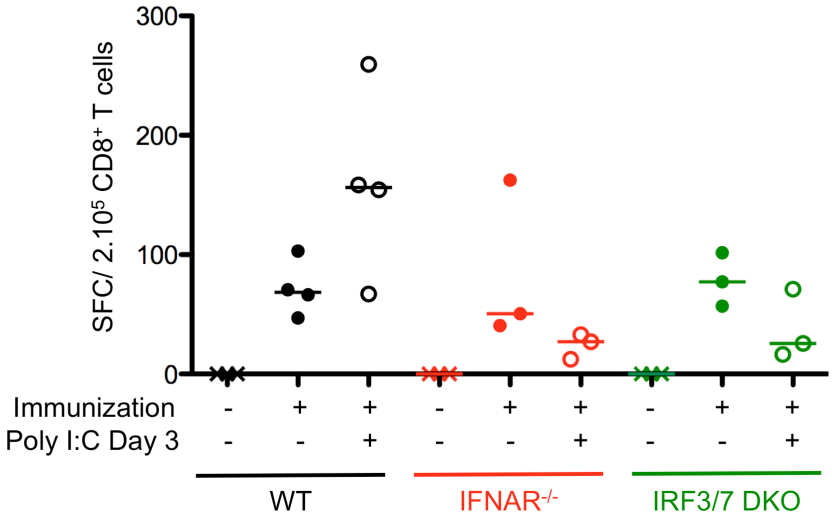


Figure 46. Enhancement of cross-priming upon late poly I:C delivery is type I IFN-dependent. WT, IFNAR$^{-/-}$ and IRF3/7DKO mice were immunized i.d. with 5x10^5 K^{bm1}mOva splenocytes. Three days later, 100µg of poly I:C was administered. On day 10, the spleen and the draining lymph node were harvested and pooled. CD8$^+$ T cells were purified to perform an IFNγ-Elispot. SFC, Spot forming cell.

2) Late type I IFN enhance cross-priming at the level of antigen uptake and/or presentation

As we observed that type I IFN are responsible for the enhancement of the response, we were interested in dissecting the mechanisms of this phenomenon and, in particular, determining the targets of these cytokines. CD45.1/2 WT and CD45.2/2 IFNAR$^{-/-}$ OT-I were transferred into CD45.1/1 WT recipients. These mice were then immunized with K^{bm1}mOva splenocytes and injected with poly I:C three days later. Seven days post-immunization, OT-I cells were enriched with an anti-CD45.2 antibody for further characterization (**Figure 47A**). We observed that the percentage of IFNγ-producing cells increased upon poly I:C treatment in WT as well as in IFNAR$^{-/-}$ OT-I populations (**Figure 47B**). Similar increases in expression were obtained by comparing the MFI of the IFNγ signal in the two populations (**Figure 47C**). Of note, IL-2 and TNFα production by these cells was also evaluated (data not shown). No clear differences were observed for these cytokines either - the cells producing the highest amount of IFNγ were able to secrete IL-2 and TNFα. From these data, we concluded that type I IFN act at the level of antigen uptake and/or presentation. However we also observed that the basal levels and the magnitude of the increase was not the same between WT and IFNAR$^{-/-}$ populations. Consequently, it is possible that these differences could be explained by the direct action of type I IFN on T cells.

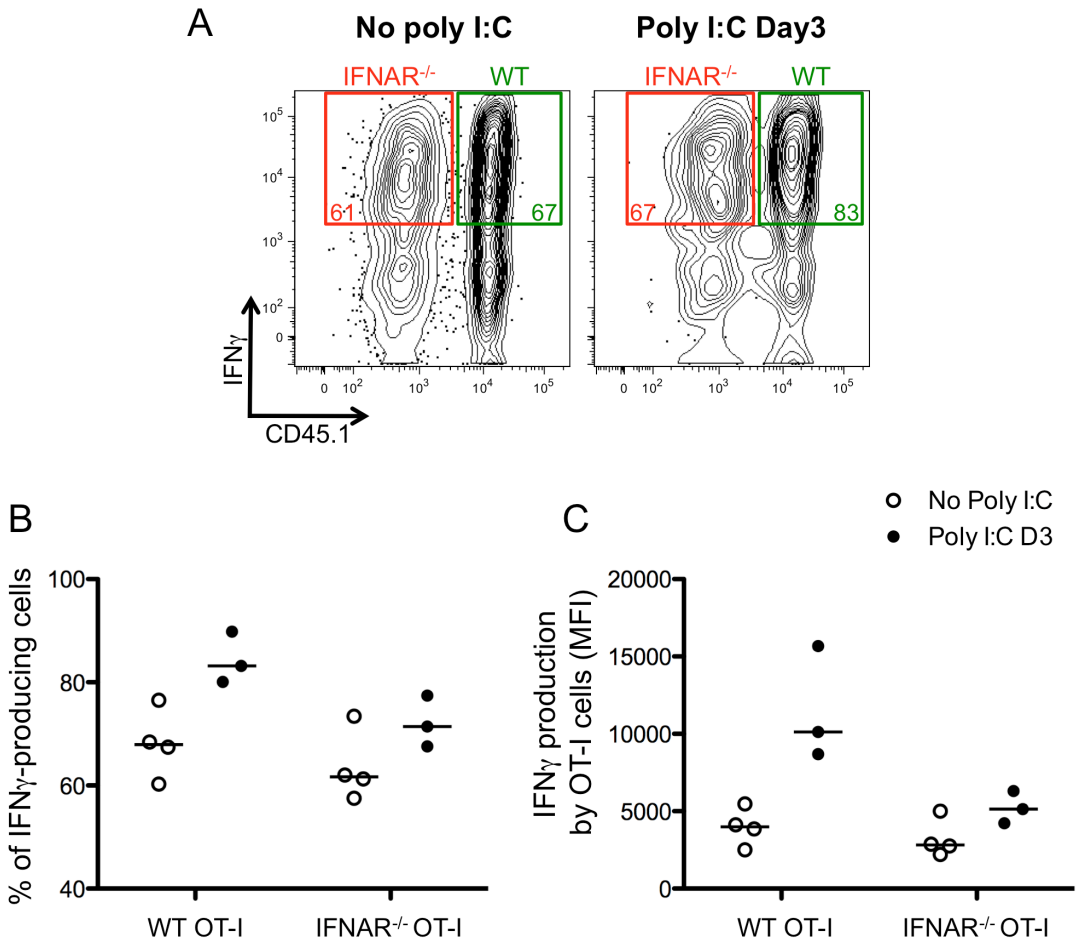


Figure 47. Type I IFN enhance T cell cross-priming at the level of cross-presentation. $5x10^3$ CD45.1/2 WT OT-I and $5x10^3$ CD45.2/2 IFNAR$^{-/-}$ OT-I were transferred into WT CD45.1/1 recipients. The following day, mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. They received 100µg of poly I:C on day 3. On day 7, enrichment was performed using a CD45.2 antibody to detect the 2 populations of OT-I. After *ex vivo* restimulation, IFNγ intracellular staining was performed. **(A)** Representative FACS plots are depicted (live DUMP$^-$ CD3$^+$ CD8$^+$ CD45.2$^+$ cells are shown) with the gates corresponding to IFNAR$^{-/-}$ OT-I and WT OT-I producing IFNγ (red and green gate respectively). The numbers correspond to the percentage of OT-I in each of the gates. The percentages of IFNγ-producing OT-I **(B)** as well as the geometric fluorescent mean for IFNγ **(C)** are reported.

3) Does late type I IFN act directly on antigen-specific CD8$^+$ T cells?

The same experimental approach was then taken using IFNAR$^{-/-}$ rather than WT recipients. WT and IFNAR$^{-/-}$ OT-I cells were transferred, mice were immunized and received poly I:C on day 3. Only WT OT-I cells were able to respond to type I IFN in these mice. An increase in the percentage of IFNγ–producing cells (**Figure 48A**) or in the MFI (**Figure 48B**) for IFNAR$^{-/-}$ OT-I cells was not observed. In the case of WT OT-I cells, there was only a slight increase in the MFI, but overall there was not substantial difference based on poly I:C treatment. Moreover we observed a strong basal level of IFNγ signal in the WT OT-I cells (**Figure 48B**).

This high background may prevent the visualization of a small increase in these conditions. Repeating this experiment using alternative restimulation strategies, or analysis at earlier time points is required to validate these results.

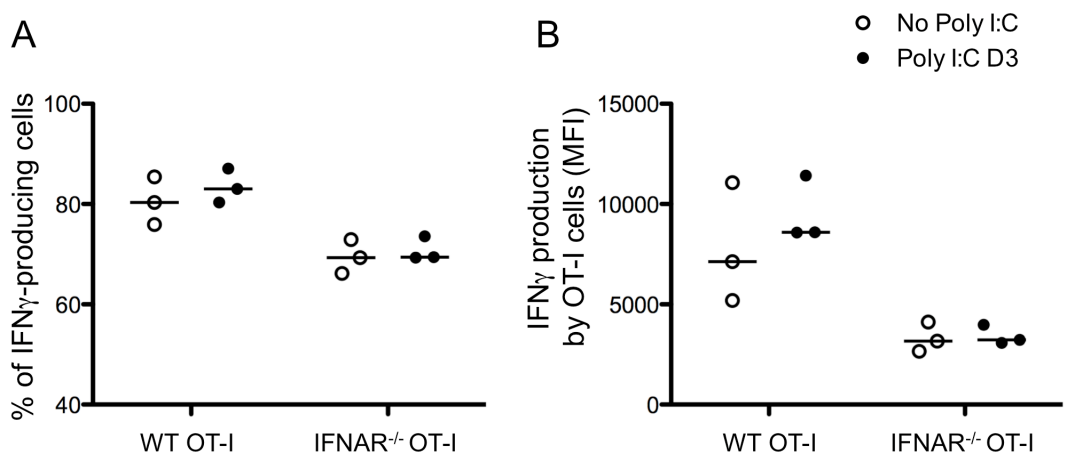


Figure 48. Type I IFN has a limited action on antigen-specific T cells. $5x10^3$ CD45.1/2 WT OT-I and $5x10^3$ CD45.2/2 IFNAR$^{-/-}$ OT-I were transferred into IFNAR$^{-/-}$ CD45.1/1 recipients. The day after, mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. They received 100µg of poly I:C on day 3. On day 7, enrichment was performed using a CD45.2 antibody to detect the 2 populations of OT-I. After *ex vivo* restimulation, IFNγ intracellular staining was done. The percentages of WT and IFNAR$^{-/-}$ CD8$^+$ OT-I cells producing IFNγ **(A)** as well as the geometric fluorescent mean for IFNγ **(B)** are reported.

Together these data demonstrate that the timing-dependent differential effects of poly I:C are at least partially type I IFN-dependent. This adjuvant clearly acts at the level of antigen-presenting DCs by modulating their survival and/or migration, maturation state, ability to cross-present antigen and even the persistence of antigen inside them. Type I IFN might also play a critical role at the level of responding T cells but additional work will be needed to confirm this.

III. HOW TO CHOOSE THE OPTIMAL TIMING FOR ADJUVANT DELIVERY?

Our data demonstrate that the optimal timing for adjuvant delivery is dependent on the route of immunization. We first showed that the early administration of adjuvant abrogates subsequent cross-priming (**I**). These results were followed by a more detailed study that attempted to understand the mechanisms underlying the differential effects of poly I:C and

the type I IFN that are produced upon adjuvant delivery on immune response. These findings remain preliminary and additional work is needed to confirm our results. Nevertheless, we convincingly demonstrated that poly I:C acts at the level of antigen uptake and cross-presentation by cDCs (**II**). When adjuvant is delivered early, it leads to DC maturation, resulting in the downregulation of antigen uptake; DC migration to lymph nodes, which removes DCs that engulf antigen from the site of injection; and/or DC death, which also may explain the disappearance of cross-presenting DCs. The combination of these effects creates an environment that is no longer optimal for cross-presentation. In contrast, if adjuvant stimulation occurred with a short delay, once DCs have engulfed antigen, the beneficial effects of adjuvant on maturation and migration to the lymph nodes could act to effectively promote cross-priming.

Following from these results, we returned to our comparison between the two routes of immunization and optimal timing of adjuvant delivery. Poly I:C acts on antigen uptake, DC maturation and antigen presentation, and it is clear that the kinetics of these steps vary depending on the route of immunization (**Figure 32**). As adjuvant delivery should be well coordinated with antigen uptake and presentation in order to enhance these processes, this may explain why the optimal timing for its delivery is delayed upon i.d. immunization. Taken these data together, we propose the following model to explain the differential impact of adjuvant delivery, with regards to the route of immunization (**Figure 49**). Systemic dissemination (represented by i.v. injection) of cell-associated antigen allows for capture and cross-presentation within one day. Within this kinetic, administration of poly I:C on day 1 post-immunization serves to stimulate cross-presenting DCs and enhances priming (**Figure 49A**). In contrast, localized delivery of cell-associated antigen, as seen with i.d. immunization, requires three days for efficient antigen uptake and presentation. Consequently, administration of poly I:C on day 1 results in “pre-mature” DCs, which are unable to cross-present cell-associated antigen (**Figure 49B**). If instead, adjuvant administration is performed on day 3 post-immunization, there has been sufficient time for uptake and cross-presentation and the pro-maturation/pro-migration effects of the adjuvant result in the enhancement of cross-priming (**Figure 49C**).

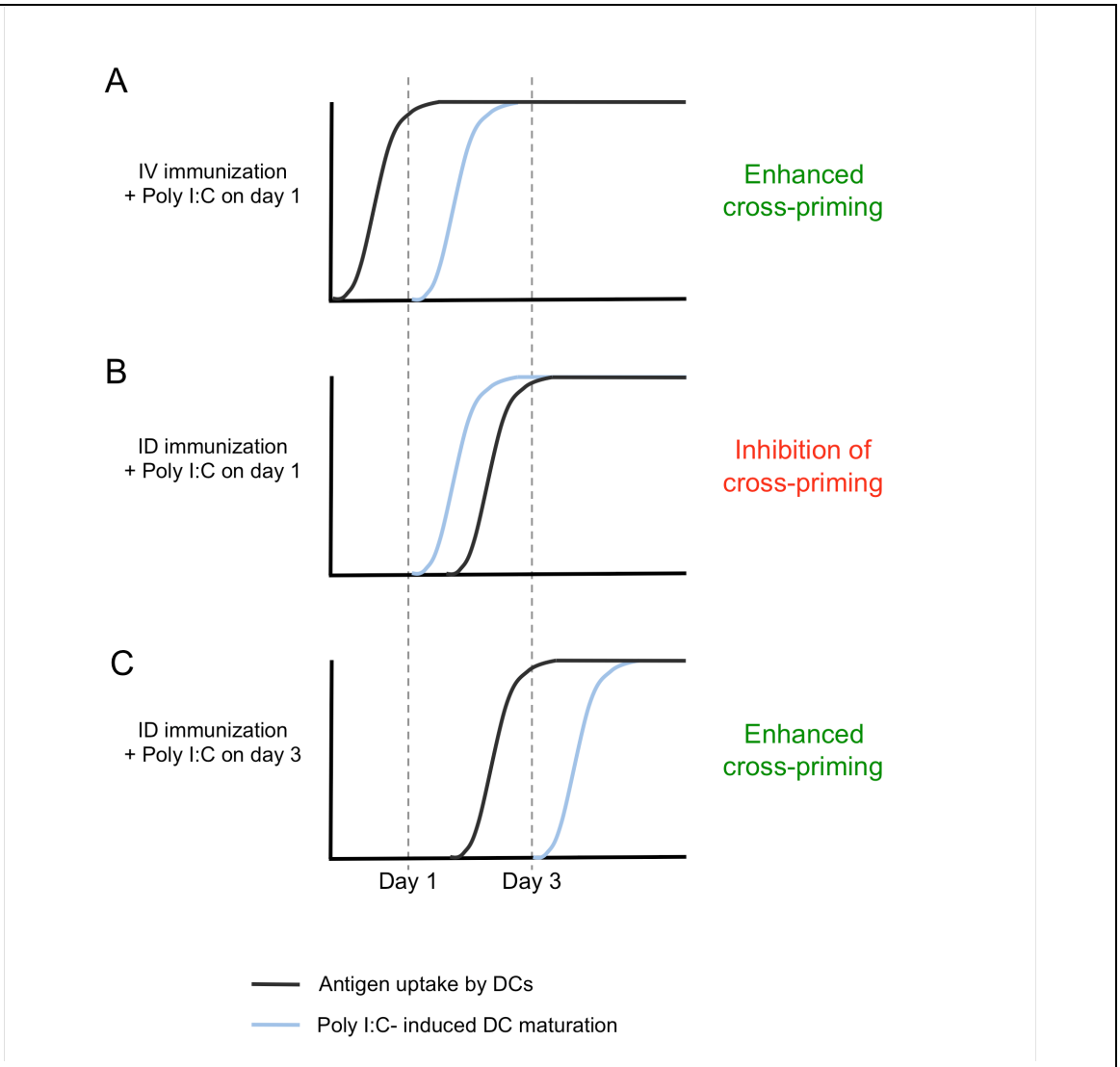


Figure 49. Proposed model to explain the differential actions of poly I:C depending on the timing of delivery. The proposed timing of antigen uptake (black line) and the kinetic of DC maturation upon poly I:C injection (blue line) are represented for three different conditions **(A,B,C)**.

To validate our hypothesis in an infectious model, we evaluated the timing of adjuvant delivery and its impact on immune responses in the context of *Listeria* infection. Mice were immunized i.d. with K^{bm1}mOva splenocytes, and poly I:C was either co-administered on the day of immunization or given 3 days post-immunization. On day 9, mice were challenged with Ova-expressing *Listeria monocytogenes* and two days later, the bacterial load was determined in the spleen (**Figure 50A**) and in the liver (**Figure 50B**). We observed that immunization with K^{bm1}mOva splenocytes alone conferred partial protection to *Listeria* challenge. If mice received poly I:C on the day of immunization, this basal level of protection was completely abrogated. In contrast, the protection was significantly improved when poly I:C was administered 3 days after immunization. Indeed, the delayed adjuvant delivery enhanced priming and resulted in a 2-to-3 log reduction in bacterial load.

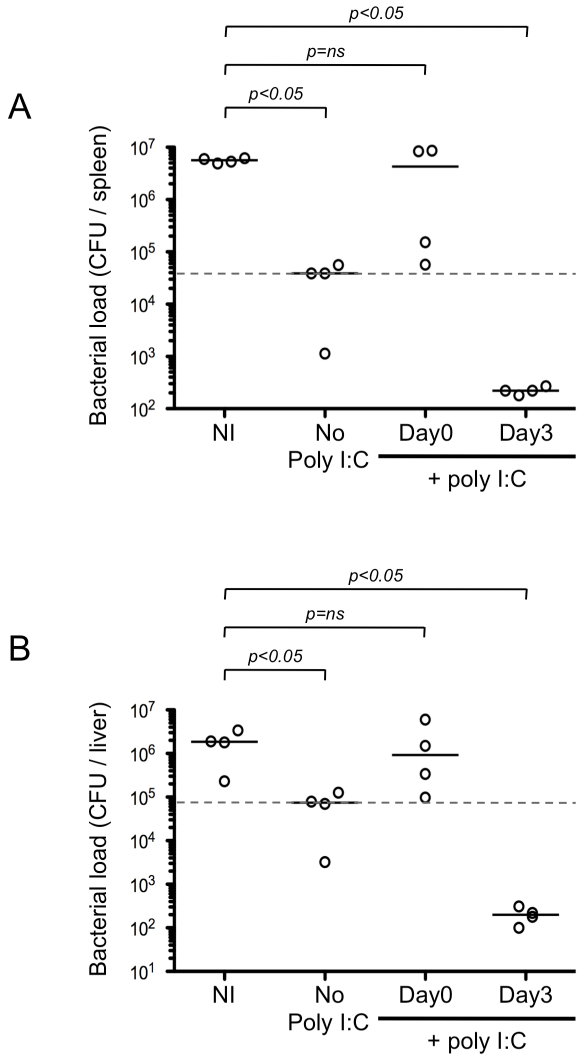


Figure 50. The differential effects of adjuvant impact protection against *Listeria*. Mice were immunized i.d. with $5x10^5$ K^{bm1}mOva splenocytes. Poly I:C was administered either the day of immunization or 3 days later. On day 9 post-immunization, mice were challenged with $5x10^5$ CFU of Ova-expressing *Listeria*. Two days later, the spleen **(A)** and the liver **(B)** were harvested and bacterial load per organ was determined. NI, non-immunized mice. Dotted lines correspond to median CFU in the absence of poly I:C. Mann-Whitney test *p*-values were calculated, comparing immunization condition to the NI control.

In this chapter, we have demonstrated that the optimal timing for adjuvant delivery is critically dependent on the route of immunization. Our data strongly suggest that this effect is due to the different kinetics of antigen uptake and presentation upon i.d. or i.v. antigen delivery. Specifically, the timing of antigen capture and T cell engagement has a profound impact on the appropriate timing for adjuvant treatment. For optimal vaccination, there is interest and need to optimize the coordination between innate and adaptive immune responses; our data suggest that a careful optimization of adjuvant and antigen administration will be required.

Chapter 4: General discussion

$CD8^+$ T cell responses are crucial for the defense against many microorganisms and cells undergoing malignant transformation. Consequently, in-depth study of $CD8^+$ T cell response after immunization is critical for understanding the mechanisms underlying the impact of multiple parameters known to influence immune response, in order to improve current vaccination strategies. We initiated our studies by developing a tetramer-based enrichment technique that allowed us to carefully analyze the $CD8^+$ T cell response, both phenotypically and functionally. We then applied these techniques to the in-depth examination of two parameters that impact immune response: the route of immunization and the timing of adjuvant delivery. In this section, I will first discuss the technical improvement that tetramer-based enrichment provides for the study of $CD8^+$ T cells. I will then return to a detailed discussion of our results, including several hypotheses that could explain why the intradermal immunization of cell-associated antigen generated a more robust immune response than intravenous delivery. Finally, I will discuss the results observed regarding the timing of adjuvant delivery and the optimization of treatments that contains a combination of adjuvant and antigen. I will extend these considerations to other types of antigen, as well as discuss the combination of several existing vaccine strategies and the issues raised in terms of the timing and sequence of administration.

I. TETRAMER-BASED ENRICHMENT: A POWERFUL TOOL

A. Advantages and limitations

1) Sensitivity of the technique

The development of tetramer-based enrichment allows for the detection of rare antigen-specific $CD4^+$ and $CD8^+$ T cells (Moon et al., 2007; Obar et al., 2008). This technique allows for the study of specific T cells in naïve mice and during the first steps of T cell activation upon immunization without transferring high numbers of TCR-transgenic T cells. The ability to detect low numbers of circulating antigen-specific T cells also makes possible to study the T cell response following a relatively weak antigenic stimulation, which allows for the use of reduced antigen dose that more accurately reflects a strategy that could be adapted for human vaccination.

In our studies, the comparison of the intradermal or intravenous routes of immunization permitted us to determine the outcome of an immune response stimulated by a local *versus* systemic dissemination of antigen. As expected, systemically disseminated antigen resulted in

rapid cross-presentation; although delayed slightly, the i.d. immunization induced more robust, polyfunctional T cells (**Figures 24B and 25**). Overall, however, the magnitude of the T cell response was the same for the two routes of immunization, as demonstrated by tetramer-based enrichment of antigen-specific T cells (**Figure 24A**). Strikingly, the differences in T cell responses between i.d. or i.v. immunization were not obtained after transferring TCR-transgenic T cells. These opposing data highlight the importance of examining the successful priming of the endogenous repertoire when performing in-depth studies of vaccine candidates for the most accurate estimation of what may occur in patients. The increased limit of detection using tetramer-based enrichment strategy allowed us to distinguish successful priming of the endogenous repertoire after i.v. immunization, and an enhanced priming effect after i.d. immunization.

In this model we chose to administer a relatively low dose of cell-associated antigen. As shown in **Figure 21**, we obtained only between 20-50,000 tetramer-positive T cells at the peak of the response, which may appear to be low. However, this reflects antigen delivered alone, without the addition of any adjuvant. If we compare these results with those obtained following injection with the recombinant Ovalbumin protein, which represents the same antigen in another form, the recombinant protein cannot elicit effective T cell priming unless formulated with an adjuvant such as poly I:C or CpG (Durand et al., 2004). Correspondingly, the addition of poly I:C enhanced the absolute numbers of tetramer-positive cells generated upon immunization in our model as well (**Figure 37**). Although the frequency of tetramer-positive cells is low following immunization, this response is sufficient to induce efficient priming as demonstrated in the data generated by cytotoxicity assay (**Figure 23**) and the reduction of the bacterial load after Listeria challenge (**Figure 50**). This tetramer-based enrichment strategy allowed us to carefully study a T cell response that is too low to be dissected with previously established techniques, but that is sufficient to induce a substantial response against the specific antigen. Similar approaches with alternate antigens may help to optimize the doses of antigen used in experimental mouse models, that may be easier to translate into human therapies.

2) Applications for human immunology

Experimental models that more accurately reflect physiologic conditions are required to test potential vaccine candidates. Moreover, it is also necessary to develop efficient, novel technical assays to more completely study the endogenous T cell response in humans as well as compare the efficacy of different treatments. It is now well accepted that analysis of only one cytokine, such as IFNγ, in order to assess the efficiency of a T cell response is not

sufficient (Seder et al., 2008). It has become clear that, more than the quantity of antigen-specific T cells that are generated upon immunization, it is their functional qualities that are reflective of their responsiveness and need to be analyzed.

$CD8^+$ T cell responses directed against a pathogen or elicited by efficient vaccines are usually robust enough to allow for the detection and characterization of antigen-specific T cells *ex vivo* without performing an enrichment. However, in some cases, such as for the study of naïve T cells or low frequency populations, an enrichment step may be necessary to sufficiently dissect a functionally effective immune response. It is important to note that tetramer-based enrichment can be also applied to the study of T cell populations in humans. For this purpose, the tetramer-based enrichment strategy has been developed and optimized for the study of human samples in our lab (Alanio et al., 2010). This method allowed for the detection and characterization of antigen-specific, naive T cells in the blood of healthy donors. Interestingly, it was shown that specific precursor frequencies are conserved between human donors, as it has been observed in mice. Using similar techniques, one group has demonstrated that in chronically HCV-infected patients, immunodominance is correlated to the naïve, HCV-specific precursor T cell frequency (Schmidt et al., 2011).

3) Limitations

Despite the clear advantages of the tetramer-based enrichment system, there are also limitations, mostly technical in nature. The protocol for enrichment and staining of T cells is extensive and time consuming, thus fewer samples can be processed concurrently. Although this approach allows for the characterization of smaller populations, there is a limit to the number of conditions that can feasibly be compared in the same experiment. Moreover, in the case that an experiment requires the use of transgenic cells or to follow CFSE-labeled specific cells *in vivo*, it remains extremely difficult to combine the techniques and follow both the transferred cells and the endogenous repertoire. Nevertheless, using the tetramer-based enrichment to follow transferred cells only, does allow the transfer of fewer transgenic T cells, which maintains "physiologic" conditions in many cases.

Additional limitations exist when trying to compare other approaches with results about endogenous repertoire obtained after enrichment. For example, techniques such as *in vivo* imaging are not yet sensitive enough to look at the low number of cells that can be detected by tetramers and still require the transfer of high amounts of TCR-transgenic T cells in order to have a consistent read-out. These concerns must be taken into account when combining different approaches to address a question.

To conclude, tetramer-based enrichment is a powerful tool allowing for the study of the endogenous T cell response in relatively physiologic conditions, including the investigation of antigen-specific precursor frequency. Because of this, it is particularly useful for the validation of models reflecting physiologic conditions. However, other equally sensitive tools are needed to combine with enrichment that will enable the study of other aspects of the immune response and can be limited to the investigation of the endogenous repertoire. There must always be a careful balance when a model of study is chosen: the consideration of its physiologic relevance, but also the tools available to answer the question in the most efficient and consistent way.

B. Future directions

1) Other antigenic models

As discussed previously, it is often difficult to develop a new technique with the most physiologic model because of the technical difficulties. This was the reason why we initially chose to use the Ovalbumin model, for which many tools have been developed: a model of cross-presentation with the injection of K^{bm1}mOva splenocytes, the TCR-transgenic OT-I ($CD8^+$) and OT-II ($CD4^+$) T cells, and the anti-K^b-SIINFEKL antibody. Furthermore, much has been already done to characterize the T cell response against Ovalbumin, which facilitated and validated our approach and results. In these conditions we chose a known model but we still adapted the conditions to more accurately mimic physiologic antigen challenge by limiting the number of antigenic cells injected, thus diminishing overall stimulation. Using this model allowed us to further optimize the technical protocol, as well as demonstrate the efficacy of the technique and define the limit of detection in our hands. Moreover, the efficiency and sensitivity of this method permitted us to show the critical differences between routes of immunization and timing of adjuvant delivery; and importantly, that results obtained upon transfer of TCR-transgenic T cells do not always reflect what is happening with the endogenous repertoire. Now that the technique has been tested and its validity confirmed, these same questions should be explored further in more physiologic models such as viral infection or tumor immunity in order to gather information that can be applied to model to human vaccination studies.

2) Improve the physiologic relevance of our model

As it exists now, our model can be easily adapted to work within more relevant, physiologic conditions and provide further informations about how to effectively combine antigen and

adjuvant. Specifically, we made an effort to use a relatively low dose of antigen ($5x10^5$ splenocytes injected/mouse), but the dose of adjuvant was quite high (100μg of poly I:C/mouse). Poly I:C is known to be toxic at high concentrations and this dose could not be injected into humans for this reason (Nicodemus and Berek, 2010). Further experiments should be performed to titrate the immunostimulatory capacity of lower doses of adjuvant. In parallel, the administration of antigen physically linked with adjuvant, which is known to reduce toxicity and, involves lower doses of adjuvant, can also be examined.

3) Other recently developed techniques

Not only did tetramer-based enrichment allowed for this new approach to study T cell responses, but we were able to combine this strategy with other established techniques such as immunoscope or intracellular cytokine staining to gain a much more in-depth understanding of the function and specificity of the antigen-specific T cells isolated by enrichment. The combination of these techniques provides the opportunity to extend T cell analysis to the study of rare populations and even further to single cell analysis, through the combination of enrichment and single cell PCR. Several additional techniques have been recently developed to permit an in-depth study of T cells.

(a) Transfer of single cell

The transfer of TCR-transgenic T cells has provided many details about antigen-specific T cell responses, mainly because these populations could be followed over time upon transfer using congenic surface markers. One of the main limitations of these types of studies was that the data obtained were reflective of a bulk population, not a single cell. It is now well established that an antigen-specific T cell population is composed of a diverse heterogeneous mix of cells. To really gain insight into the molecular events of T cell priming and activation, information at the single cell level is required. Stemberger et al. developed a specialized injection system, which allowed them to transfer just a single cell. To do this, they purified $CD8^+$ OT-I T cells, diluted them, and applied them to a glass slide. Under the microscope, they were able to aspirate a single cell into the tip of a glass microinjection needle. This single cell was directly transferred intraperitoneally into a recipient mouse (Stemberger et al., 2007). This work showed the feasibility of the approach and allowed them to demonstrate that a single transferred T cell has polyfunctional potential and can develop into several effector and memory subsets.

(b) Barcoding technology

An elegant strategy of cellular barcoding has recently been developed to analyze the kinship between different T cell populations (Schumacher et al., 2010). A retroviral plasmid library was generated in which each individual virus carried a unique molecular "barcode". T cells were infected and, thus, labeled by retroviral transduction and then reintroduced into mice. After immunization and the resulting differentiation of transferred T cells, different cell subsets were sorted, DNA was isolated and the overlap of barcodes between different functional subsets was analyzed. Using this technique, lineage relationships could be analyzed between T cell subsets. The power of cellular barcoding has already been harnessed to answer two long-standing questions. First, it was used to determine whether populations of T cells found in a specific location or those that share a common functional activity, come from a common progenitor (Schepers et al., 2008). Following this study, van Heijst and colleagues made use of this approach to determine the number of precursors that are recruited to form a given effector T cell population depending on the conditions of immunization (van Heijst et al., 2009).

(c) Combicolor approach

The consistent improvement of flow cytometry technology has also provided new tools by which to study T cell responses. It is now possible to combine many fluorophores in the same experiment. While one tetramer labeled by one colour was used before to characterize a T cell response, it is now possible to simultaneously detect multiple different antigen-specific T cells with several tetramers within the same sample. In particular, Hadrup and colleagues developed a novel combinatorial method, in which each specific T cell is labeled with a mix of identical tetramers conjugated to different fluorophores. Each antigen-specificity is labeled and identified by a unique fluorophore combination. In this way, 15 specificities can be detected concurrently by using 4 different fluorophores (Hadrup et al., 2009). Depending on the frequency of the cells of interest, cells can be stained directly or upon tetramer enrichment.

Although previous studies that characterized the T cell response were performed by examining cells at the population level, the trend is now to study rare populations and characterize the response at the single cell level by using the multiple new techniques described here, either alone or in combination, including tetramer-based enrichment. Indeed,

it has been demonstrated that the quality of the response at a cell-by-cell level is crucial to predict the outcome of a response.

II. IMPACT OF THE ROUTE OF IMMUNIZATION ON $CD8^+$ T CELL CROSS-PRIMING

A. The route of immunization impacts the efficiency of $CD8^+$ T cell cross-priming but not the diversity of antigen-specific T cells

Using our model of cross-presentation we identified both common characteristics and differences between the $CD8^+$ T cell responses generated after local *versus* systemic administration of cell-associated antigen.

1) Kinetic of the response

Our first question was to examine whether the route of immunization affected the kinetics of establishing a productive T cell response. We initially observed a faster kinetic upon i.v. immunization as compared to i.d. immunization (**Figure 21**). This result was expected because antigen was delivered directly in the blood, which is screened for antigen in the spleen. In this way, the antigen should be rapidly taken up by APCs and presented to T cells. In contrast, antigen injected i.d. first must reach the draining lymph node, either by itself or after engulfment by migratory DCs prior to T cell activation. This need for more time to arrive at the location of optimal T cell priming could explain the difference in the kinetic of the immune response.

2) Quality of the response

More surprisingly we demonstrated that while delayed, the local i.d. immunization leads to a more robust cross-priming, resulting in a higher percentage of IFNγ-producing cells, as well as multifunctional T cells, and a higher IFNγ production on a per cell basis (**Figures 24 and 25**). Based on prior patient studies and experimental models of HIV, *Leishmania major* and *Mycobacterium tuberculosis*, the T cell quality appears to be important for an efficient host response and eventual control of the infectious agent (Almeida et al., 2007; Darrah et al., 2007; Precopio et al., 2007). Therefore, we were able to conclude that local immunization leads to a better, overall response. Interestingly, the tetramer-based enrichment technique allowed us to perform this in-depth study and identify the small differences between the two conditions studied. Despite the enhanced $CD8^+$ T cell cross-priming obtained with i.d.

immunization, the i.v. immunization also induced good priming. Notably, the magnitude of the T cell response is the same for the two routes, only the quality in the T cells elicited differs (**Figure 24A**).

3) Diversity of antigen-specific T cells

When examining the extent of T cell clone diversity after i.d. or i.v. immunization, we expected to see differences due to the differential access to antigen depending on the route of immunization (**Figure 27A**). Surprisingly, we did not observed any differences (**Figures 29 and 30**). This could be explained by the dissemination of antigen upon i.d. immunization: in the first days, the antigen remains localized, with presentation only in the draining lymph node, but antigen presentation was also detected in the spleen at later time points, even if it was limited. In this way, many T cells may have access to antigen. Another explanation is that the T cells were rapidly recruited and able to be activated regardless of the site of immunization. These results are in accordance with the work of Van Heijst and colleagues. Using the previously detailed barcoding system to follow distinct T cells, they stimulated mice with a variety of pathogens via several different routes and demonstrated that the number of diverse T cells recruited is similar (van Heijst et al., 2009). Their data also showed that naïve T cell recruitment is constant but does not reflect the efficiency of the subsequent response.

B. Why does local immunization result in more robust T cell cross-priming?

We demonstrated that, while delayed, local delivery by i.d. injection resulted in efficient cross-presentation of cell-associated antigen and induced a better multifunctional T cell response than systemic (i.v.) injection. While the difference in the response kinetic is clear, it is more difficult to understand how a local immunization led to a qualitatively better response. Following are several potential hypotheses that may explain these observations.

1) Nature of injected splenocytes

Our model was based on the injection of cell-associated antigen, specifically, live K^{bm1}mOva splenocytes. These cells express a membrane-bound form of ovalbumin, which is known to not be secreted as a soluble antigen *in vivo*. Moreover, these cells express a mutated form of the MHC-I molecule K^b, such that the antigen cannot be directly presented and, therefore,

host DCs are required for cross-presentation. These cells have features that may influence the differential outcome of T cell response after i.d. or i.v. immunization.

(a) Preparation of splenocytes

In our model we injected total splenocytes after the lysis of red blood cells. The injected cells are alive and are comprised of a complex mixture of cell types, including lymphocytes that retain the ability to migrate, but also DCs, macrophages and other cells that cannot move without additional activation. Flow cytometry analysis of injected splenocytes indicated that the mixture was made up of mainly lymphocytes. Once such a mixture was injected via the different routes examined, they may have behaved differently. Upon retroorbital i.v. injection, cells are delivered directly in the blood. A portion of them will be engulfed by macrophages in the lungs, while the rest circulate to the spleen where the blood is screened for antigen. In contrast, i.d. immunization delivers antigen directly to a tissue where there are physical constraints limiting its dissemination, and an antigen depot will be formed. Only cells that are able to migrate on their own power (i.e. lymphocytes) will be capable of reaching lymphoid organs. All others will require phagocytosis by skin-resident DCs to be transported to the draining lymph node.

(b) How H-2K^{bm1} cells are killed?

As described in the Chapter 2, H-2K^{bm1} splenocytes disappeared more rapidly than their WT counterparts upon i.v. immunization (**Figure 35**). However these cells are not targets for NK cells (**Figure 16C**) and therefore are not cleared as quickly as β2m$^{-/-}$ cells (**Figure 35**). Although a few other teams have previously used this model of cross-presentation *in vivo*, the mechanism of splenocyte clearance remains unknown. We hypothesize that they are recognized and cleared by other cells such as macrophages but this must be confirmed by further investigation. The removal of different cell populations using depleting antibodies or clodronate may be a useful approach to answer this question. Another potential way to understand the mechanisms behind this cell death would be to systematically inhibit specific cell death pathways, such as treatment with z-VAD drug to inhibit apoptosis.

Further understanding of the way injected splenocytes are eliminated and by which cell it is mediated is important and a possible point to explain differences between the two routes of immunization. Thus far, we have discussed the cell-intrinsic properties of the cell-associated antigen that may be implicated in the differences observed. It is also important to consider the context in which these cells were delivered.

2) In which circumstances does splenocyte engulfment occur?

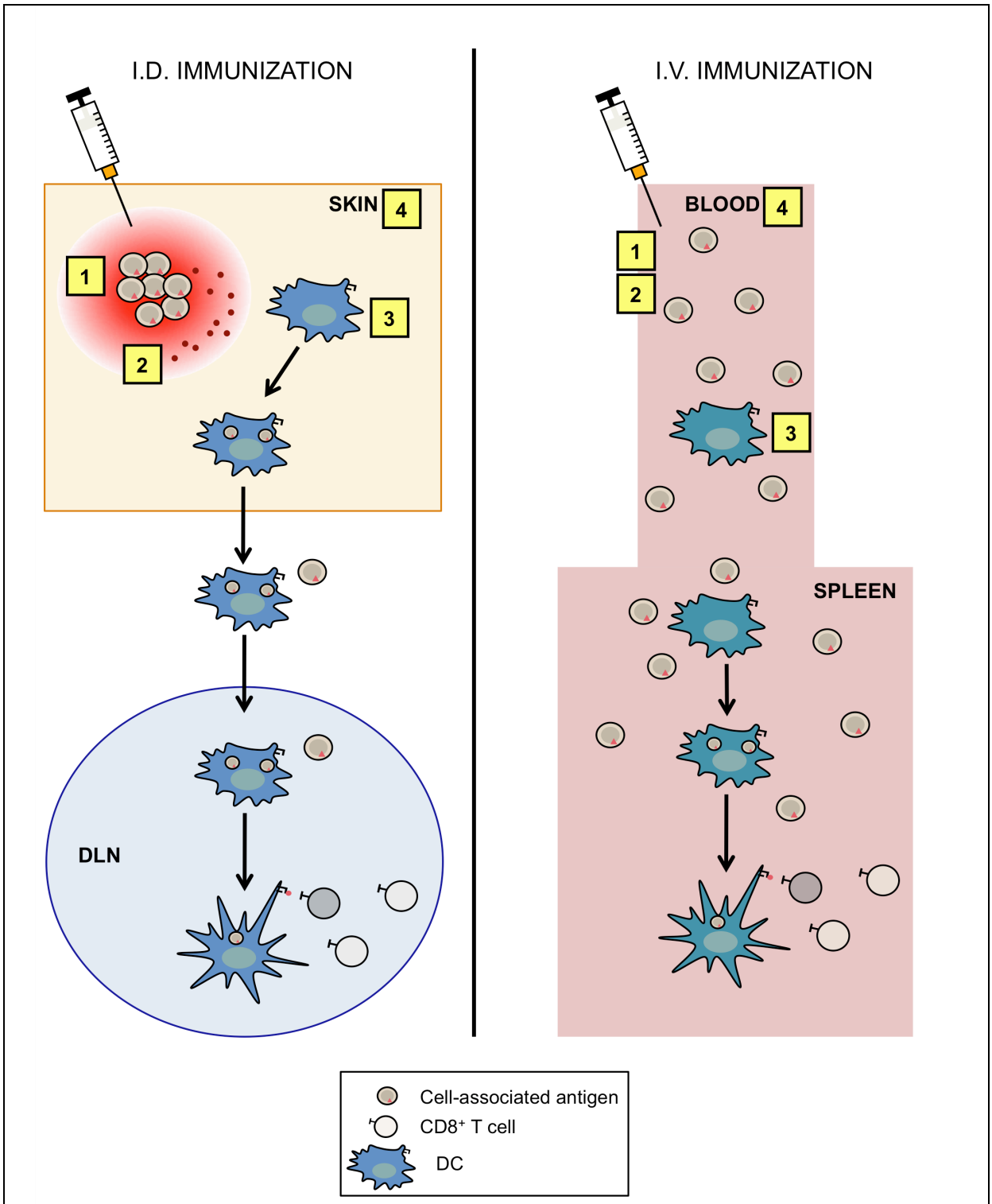


Figure 51. Intradermal versus intravenous injections: conditions of immunization. Differences between are highlighted in yellow: formation (i.d.) or absence (i.v.) of an antigen depot **(1)**, damage associated with injection (i.d.) or not (i.v.) **(2)**, different DC subsets involved **(3)**, different levels of danger depending on where antigen is delivered **(4)**.

(a) Depot formation after i.d. immunization

When we injected bioluminescent splenocytes *in vivo*, we observed that they formed an antigen depot afer i.d. immunization at the site of injection, that persisted for several days (**Figure 33**). After i.v. immunization, such a depot was not observed. Previous studies have already demonstrated that the presence of a depot facilitates the development of a better immune response. Moreover, the antigen depot is a well-known characteristic of empirically developed adjuvants such as oil-in-water emulsions or aluminum salts, which may partially explain their effectiveness (Coffman et al., 2010). Finally proteins formulated with this kind of adjuvant or a protein anchored to a cell membrane are not dramatically different – a protein antigen in combination with lipids in both cases – with either cellular membrane or adjuvant, allowing for the formation of a depot. This depot formation and resulting antigen persistence could explain why a more robust cross-priming is observed upon i.d. immunization **(Figure 51, (1)).**

(b) Tissue damage induced after i.d. immunization

Inflammation can be induced at the site of injection **(Figure 51, (2))**: however, i.v. and i.d. immunizations would probably not trigger the same tissue damage and inflammation. As it is being delivered directly to the bloodstream, an intravenous injection should not induce as much inflammation. This is completely different for an intradermal immunization, in which the full volume of injection is pushed into an organized tissue, which is characterized by its structural integrity. Injection will lead to damage of the local tissues and capillaries, including the necrotic cell death of neighbouring cells. This type of damage would induce the release of danger signals, activation of immune cells within the skin and the initiation of a local inflammatory response. In fact, we can observe the skin visibly distending during i.d. injection. Interestingly adjuvants previously described as favoring the formation of a depot were recently "re-discovered", as they were thought to maybe play a role also in inflammasome activation or induction of necrosis (Coffman et al., 2010). Because of these points, the i.d. route can be considered as a more inflammatory than i.v. injection. This observation can also be confirmed by the results obtained in DNA vaccination trials. Although exciting data were obtained in mouse models of DNA vaccination, the results in humans were disappointing. One justification of this inconsistency was the volume of injection used (Rice et al., 2008). The volume used in mice was relatively high probably allowing for increased transfection of host cells and, potentially, more damage at the site of infection leading to increased inflammatory responses, as discussed here. Unfortunately, the proportional volume was far too large to be used in humans and was reduced for clinical

trials. This lesser volume would not have had the side effects such as increased transfection and increased danger signals that have lead to a very promising and productive vaccination strategy in mice.

(c) Implication of different DC subsets depending on the route of immunization

As described in the introduction, several DC subsets have been identified in mice, both in lymphoid organs and in peripheral tissues (**Table 4**). Depending on the route of immunization, the injected splenocytes would not necessarily come into contact with the same DCs **(Figure 51, (3))**. As each subset has particular characteristics and levels of antigen presentation, we propose that the DCs encountered upon immunization could greatly impact on the subsequent T cell response. $CD8\alpha^+$ DCs are most likely the main subset involved in cross-presentation following i.v. immunization, as they are specialized for cross-presentation. $CD8\alpha^+$ but also $CD103^+$ may participate in antigen cross-presentation after i.d. immunization. Uptake of antigen by other DC subsets may modulate the response. Additionally, other APCs may also be implicated. As an example, $CD169^+$ macrophages found at the entry of lymph nodes have been shown to have a role in antigen uptake (Asano et al., 2011). To examine the different APCs involved in antigen uptake in more detail, experiments using the injection of dye-labeled splenocytes, such as with PKH26 as described in Chapter 3 (**Figure 44**), may be useful to identify cell subsets implicated in transfer and cross-presentation of antigen in both injection conditions.

Abadie and colleagues compared the response stimulated by intradermal and intramuscular routes of injection for Modified Vaccinia virus Ankara (MVA). MVA is not able to replicate and propagate, but can infect most APCs, including monocytes, macrophages, DCs, and B cells. They also demonstrated that the quality of the T cell response is better after i.d. immunization and showed that the APC subsets involved in antigen uptake, transfer and presentation differed depending on the route of immunization. Moreover, these different APC populations, originally imprinted at the site of immunization, have the capacity to further shape the quality of the T cell response (Abadie et al., 2009).

(d) Level of danger represented by a local versus systemic immunization

In the case of an active pathogenic infection, the host will definitely not react in the same manner regardless of the detection of antigen locally or systemically. The detection of antigen in the blood is characteristic of a systemic microbial dissemination, meaning that it represents a considerable threat and is targeted for rapid removal. In contrast, the local detection of

antigen is not viewed by the immune system as this dangerous and clearance is less urgent. For instance, monocytes from the blood respond vigorously to LPS alone in circulation, even in the absence of other signals. Inversely, tissue-resident macrophages activate the inflammasome pathway only following the detection of multiple signals of an active infection, only one of which is the presence of LPS (Blander and Sander, 2012). The same perspective can be considered in our model for the injected splenocytes **(Figure 51, (4))**. Cell-associated antigen might be removed rapidly upon i.v. immunization by macrophages, explaining the reduced persistence of antigen and less efficient cross-priming: antigen was removed quickly, so the T cell response does not need to be maintained. However, following i.d. immunization, local immune cells are less efficient at clearing the injected antigen because the level of threat is lower; the antigen persists and there remains continual stimulation to maintain T cell activation and differentiation.

Each of these points highlights potential differences between the mechanims and the context of intradermal *versus* intravenous immunization and could be responsible for the differential outcomes observed for $CD8^+$ T cell response.

3) A role for antigen persistence?

In our model we observed that cross-presentation persisted longer in the draining lymph node after i.d. immunization that what was seen following i.v. immunization (**Figure 32**). This persistence alone could explain the enhanced polyfunctional T cell response (**Figure 25**), as well as a more robust secondary T cell response (**Figure 26**).

As described previously, the requirement for antigen persistence for the generation of an efficient $CD8^+$ T cell response is quite controversial. It has been initially shown in both an *in vitro* model (van Stipdonk et al., 2001) or an *in vivo* model, in which antigen was removed by antibiotics (Mercado et al., 2000), that a brief antigenic stimulation is sufficient to trigger a cell autonomous program of $CD8^+$ T differentiation. However, since the proposal of this "autopilot model" by Bevan and Fink, highlighting that $CD8^+$ T cells require only a short stimulation for a complete differentiation, other studies have further defined the model. Usharauli and colleagues demonstrated *in vitro* that duration of antigen stimulation matters: brief antigen stimulation induced the generation of effector $CD8^+$ T cells with low cytotoxicity and high IL-2 production, whereas a sustained stimulation generated effector cells with the opposite phenotype that convert quickly into memory-like $CD8^+$ T cells (Usharauli and Kamala, 2008). In another study, Prlic et al. controlled antigen persistence *in vivo* by using CD11c-DTR mice and by removing $CD11c^+$ cells via diphteria toxin injection.

They also showed differences in the corresponding responses, but in a different manner: in their hands, the magnitude and the secondary response of the $CD8^+$ T cells were affected by the duration of antigenic stimulation, but not the functionality of effector cells (Prlic et al., 2006). In another model, the authors blocked antigen presentation by using anti-K^b-SIINFEKL MHC-peptide complexe antibody in order to assess the effect of modulating the duration of MHC-peptide complexe signaling on T cell responsiveness. They demonstrated that altering this interaction affected both T cell expansion and the differentiation into memory T cells (Obar and Lefrancois, 2010). Interestingly, different and sometimes even opposite results have been reported in answer to these questions, seemingly dependent on the model used to modulate antigen persistence.

Based on our results about persistence of antigen and its relation to cross-presentation, we can consider, for our comparisons, the i.v. condition as a brief stimulation and the i.d. condition as sustained antigen stimulation (**Figure 32, cohort 3**). We observed a similar expansion of T cells in both conditions, but a qualitatively better primary response and a more robust secondary response after a sustained stimulation. These results are in accordance with previous papers showing that the duration of antigenic stimulation can impact $CD8^+$ T cell differentiation.

However, different and even opposite results have also been described depending on the model used to modulate antigen persistence. Another approach used in our lab to modulate persistence of antigen has also led to controversial results. Previous work in our lab, comparing cross-priming after immunization with WT or $\beta2m^{-/-}$ splenocytes demonstrated that persistence of antigen is crucial for efficient priming. Indeed $\beta2m^{-/-}$ are rapidly killed by NK cells, resulting in their removal. Cross-priming is much more efficient after immunization with WT than $\beta2m^{-/-}$ splenocytes, suggesting that the ability of the WT cells to stay in the tissue contributed to the increased efficiency of cross-priming. Furthermore, efficient cross-priming was restored in the $\beta2m^{-/-}$ immunization conditions, but only if NK cells were depleted, allowing for the $\beta2m^{-/-}$ cells to persist (Jusforgues-Saklani et al., 2008). In contrast, Krebs and colleagues demonstrated the opposite effects of NK killing in an Ovalbumin model. They immunized mice with $K^{b-/-}$mOva splenocytes that are also targets for NK cells. However, in this setting, they observed more efficient priming when NK cells were present, suggesting a role for NK cells in the killing of cell-associated antigen and providing antigen to APCs (Krebs et al., 2009).

From the results described here we can conclude that the model used to study antigen persistence is a critical factor to take into account when studying the modulation of T cell

priming: *in vitro* versus *in vivo* study, the antigenic model used, the role of NK cells, the removal of DCs via CD11c-DTR mice treated with diphteria toxin *versus* the inhibition blocking of antigen presentation with blocking antibodies. Each of these approaches modulate antigen persistence, but at a different level, and may also impact other aspects of immune response at the same time.

Our model would clearly benefit from further understanding regarding how H-2K^{bm1}mOva cells are killed after injection, as this would allow us to better control persistence of antigen and, as a result, understand whether persistence of live cell-associated antigen is a crucial parameter for the robustness of the response.

In parallel, the use of splenocytes expressing the receptor for the diphteria toxin as the cell-associated antigen may help us to address this question. It would permit the removal of antigen at different time points post-immunization by injecting diphteria toxin and investigate the quality of the subsequent response in these differing conditions. The aim here would be to block antigen persistence upon i.d. immunization and observe whether we obtain a response similar to what we observed after i.v. immunization. This idea was the aim of our experiments using the K^b-SIINFEKL antibody to block antigen presentation. The opposite approach could also be investigated and would involve mimicking antigen persistence after i.v. immunization (by several successive antigen injections for instance) and examine whether we are able to improve the quality of the resulting T cell response.

4) <u>A role for CD4 help?</u>

CD4 help has been shown crucial for the initiation of a completely functional CD8$^+$ T cell response (Bennett et al., 1997), especially for effective cross-priming. However it has been demonstrated in a tumor model that CD4 help may or may not be required depending on the route of immunization. Bour and colleagues compared i.d. and i.p. injection of tumor cells and observed that an anti-tumor CD8$^+$ T cell response developed without CD4$^+$ help after i.d. immunization, whereas help was required after i.p. immunization (Bour et al., 1998). We investigated the necessity of CD4$^+$ help using a variety of different approaches in our model: in particular we transferred low numbers of TCR-transgenic specific OT-II CD4$^+$ T cells. We also tried to stain endogenous antigen-specific CD4+ T cells using MHC-II-peptide tetramers. However, we were not able to detect specific cells in a reproducible way. The role of these cells need to be further investigated.

5) Different T cell activation depending on the timing of activation?

Our immunoscope data (**Figures 29 and 30**), as well as the work from Van Heijst (van Heijst et al., 2009), suggest that the route of immunization will not impact T cell recruitment and or T cell activation because the recruitment occurs rapidly regarless the site of immunization. However other studies have investigated the timing of T cell recruitment to lymphoid organs upon immunization and demonstrated that this timing plays a role in the differential activation of these cells after immunization. Specifically, they compared naïve T cells that are already present in the draining lymphoid organ at the time of immunization to the "latecomer" T cells that are recruited later to the lymphoid tissue after immunization. Catron and colleagues studied $CD4^+$ T cell responses after i.d. immunization of antigen and isolated the lymphoid resident T cells from the 'latecomers" using treatment with an anti-CD62L antibody to prevent latecomer T cell entry into lymph node (Catron et al., 2006). They showed that the latecomer cells divided less due to a lower density of peptide-MHC-II complexes on DC surfaces and competition with T cells that already divided and, thus, became central-memory cells. D'Souza et al. did similar studies for $CD8^+$ T cells after viral infection. They compared activation and differentiation of low numbers of specific T cells that were transferred at the time of, or several days after infection. They observed that the timing of recruitment had a substantial impact on the differentiation program of T cells (D'Souza and Hedrick, 2006). These studies can be directly compared to the work reported here. Upon i.d. immunization, antigen cross-presentation remains localized mainly in the skin-draining lymph node. Some naïve T cells are resident in this lymphoid organ at the time of injection. Other, recirculating naïve T cells will be attracted via inflammatory signals and will enter this lymph node over the course of a prolonged period of time, thereby resulting in the non-synchronous activation of antigen-specific T cells. It is possible that T cells that arrive later may receive less stimulatory signals and are incompetition with specific T cells that have already divided. This differential activation based on timing of recruitment and inter-cellular competition for stimulatory signals may explain the extensive diversity of functional T cells generated after i.d. immunization. In contrast, i.v. immunization induces a systemic dissemination of antigen and allows for a more simultaneous activation of antigen-specific T cells, resulting in a relatively functionally homogenous T cell population.

C. Clinical applications

Our studies strongly highlight the importance of considering the route of immunization and persistence of antigen presentation in the design of clinical trials and vaccine strategies.

Moreover, the data clearly suggest that intradermal injection is the optimal strategy for achieving robust $CD8^+$ T cell cross-priming. Although initially the model of Ovalbumin-expressing splenocytes appears to be very distant from physiologically relevant conditions, this form of cell-associated antigen is being used in clinics. Russo and Fontana have conducted pre-clinical and clinical studies utilizing peripheral blood lymphocytes genetically modified to express tumor antigens as a strategy for inducing tumor immunity in cancer patients (Russo et al., 2007; Fontana et al., 2009). In their treatment protocols, patients received five bi-weekly, i.v. infusions of escalating doses of $5x10^8$ total lymphocytes on average (range: $2\text{-}7x10^8$). Their clinical trial was not initially designed with the goal of assessing efficacy; nonetheless, it was possible to observe clinical responses in 3/10 patients, which correlated to the priming of Mage-3 specific $CD8^+$ T cells. These preliminary studies highlight the feasibility of utilizing cell-associated antigen as a means of immunizing patients.

Additionally, the use of T cells as antigen vehicles for vaccination has recently been described (Bear et al., 2011). These cells were able to migrate by themselves to lymphoid organs after immunization where the activation of antigen-specific T cells by DCs will occur. This mechanism provides an advantage, as compared to the injection of antigen-loaded DCs, which may remain at the site of injection and limit vaccination efficiency. Since one crucial limitation of existing cell-based vaccines is the access to lymphoid organs, it may appear that the i.v. route may be optimal since antigen would be disseminated systemically. In fact, this route has been chosen by Fontana and colleagues for their clinical trial (Fontana et al., 2009). However, our data suggest that they may obtain more efficient anti-tumor responses by immunizing patients via the i.d. route. Furthermore, in a practical sense, local immunizations are more straightforward and easier to perform on patients, as compared to i.v. inoculation.

While T cells may appear to have some advantages when acting as antigen vehicles, as compared to DCs, there remains the limitation that, *in vivo*, T cells do not express high levels of costimulatory molecules and inflammatory cytokines. This means that DCs would still have to be activated in parallel with the vaccination. It may be possible to accomplish this by genetically modifying T cells to express molecules that are able to induce DC maturation, in addition to carrying the antigen. TLR ligands, such as flagellin, which can be easily artificially expressed in T cells might be a good candidate to use for this approach, as well as CD40 ligand.

III. COMBINATION OF ANTIGEN WITH ADJUVANT

A. The timing of adjuvant delivery should be coordinated with antigen processing and presentation

1) Effects of poly I:C and type I IFN in our model

Adjuvants and more specifically type I IFN can impact $CD8^+$ T cell cross-priming in a variety of ways. In our specific model we demonstrated that the optimal timing of adjuvant delivery is immunization route-dependent (**Figure 37**). Moreover, for the optimal benefit, administration should be closely coordinated with the timing of antigen uptake and presentation, as delivery that occured too early inhibited subsequent priming while late delivery enhanced it (**Figure 38**). These effects of poly I:C were shown to be type I IFN-dependent (**Figures 41 and 46**). Specifically, we observed that poly I:C and type I IFN act at several levels. From these results, we propose a model for adjuvant action and timing of delivery that illustrates how these diverse effects can impact cross-priming, based on the kinetic of antigen uptake, processing and presentation in both a positive or negative fashion. **Figure 52** recapitulates these points in different experimental conditions: **(A)** absence of adjuvant, **(B)** early adjuvant delivery, **(C)** late adjuvant delivery.

In our model of immunization with cell-associated antigen, in combination with poly I:C adjuvant delivery, we observed:

- A substantial recruitment of DCs into the draining lymph node (**Figure 43A**). If these cells have already phagocytosed antigen, this recruitment can be considered to have a positive effect for cross-priming, as it may increase the amount of antigen reaching the draining lymph node. In contrast, if antigen has not yet been engulfed, then this flow of DCs to lymph node results in the removal of them from the skin, rendering them no longer capable of phagocytosing antigen (**Figure 52, (1)**).
- In parallel, poly I:C treatment induced DC maturation (**Figure 43B**). Again, this process will potentiate cross-priming if the antigen has already been phagocytosed, by improving the ability of DCs to activate T cells. However, if these maturation signals arrive before antigen engulfment, DC maturation would lead to downregulation of antigen capture ability and inhibition of cross-presentation (**Figure 52, (2)**).
- We observed the disappearance of $CD8\alpha^+$ DCs upon poly I:C stimulation (**Figures 43 and 44**), which may be due to death of this cell subset. As these cells are

known to be critical for effective cross-presentation, their disappearance also has the potential to inhibit cross-priming. Further discussion of this point will be continued below.

- Although the direct action of type I IFN on T cells has been described previously, further investigation of that mechanism is needed in our model, as our current results are inconclusive (**Figures 45 and 48**). However, preliminary date (not shown) using *in vivo* imaging confirms previous results suggesting that poly I:C treatment induces the retention of T cells in lymphoid organs (Shiow et al., 2006) (**Figure 52, (3)**).
- No effects of adjuvant delivery on the injected antigenic cells were observed in our experiments. Specifically, it does not seem to impact their migration into the draining lymph nodes. However, it is important to note that the live splenocyte preparation is theorically sensitive to adjuvant. This potential caveat needs further investigation.

Some compelling data from previously published studies could be used to complete our model, especially at the cellular level. In mice, Lorenzi et al. showed that type I IFN did not affect antigen uptake but induced an increase in the retention of engulfed antigen in subcellular compartments. This could be due to the regulation of phagosomal pH by type I IFN (Lorenzi et al., 2011). Interestingly, other intracellular organelles required for cross-presentation are also modulated by poly I:C treatment, including the lipid bodies that are lipid storage organelles required for cross-presentation, although we do not yet know in which process they are involved. In particular, poly I:C promotes the accumulation of lipid bodies, especially in $CD8\alpha^+$ DCs which favors cross-presentation (Bougneres et al., 2009). Additionally, a study using human DCs treated with type I IFN demonstrated enhanced cross-presentation due to the promotion of antigen survival as well as directed targeting of the antigen to the cross-presentation pathway, rather than direct presentation on MHC-II molecules (Spadaro et al., 2012). Finally the induction of $CD8\alpha^+$ DC death by poly I:C treatment may also contribute to our observations (Fuertes Marraco et al., 2011). All these studies characterizing effects of type I IFN at the cellular level further support our observations of the positive effects of adjuvant on cross-priming when administered with the proper timing. Interestingly, we observed in our model that early adjuvant delivery inhibited T cell priming but also that substantially delayed delivery (i.e. on day 3 post-immunization for the i.v. route for instance, data not shown) did not boost the T cell response at all, as

compared to the antigen administered alone. Although the adjuvant theorically could still promote the immune response further, the lack of this effect is most likely due to being past the time point when type I IFN has already acted to promote antigen survival and processing.

Interesting results have also been obtained regarding the effects of poly I:C and type I IFN effects at the level of $CD8^+$ T cells. Several groups have demonstrated a direct effect of type I IFN on T cells (Le Bon et al., 2006; Kolumam et al., 2005). Additionally, Marshall and colleagues studied the effects of type I IFN released after viral infection on bystander T cells that are not specific for the viral antigens. They demonstrated that type I IFN sensitize bystander T cells, leading to enhanced effector functions, such as IFNγ secretion, upon stimulation with their cognate antigen. Similar effects were observed after poly I:C administration and prior to immunization with antigen. It was shown that these effects were also due to IFN, acting indirectly on bystander T cells (Marshall et al., 2010). In a follow-up study, the same group observed that IFN released upon viral infection or poly I:C treatment resulted in a transient immunosuppression and inhibition of T cell proliferation depending on the timing of its stimulation and production. Likewise, IFN was able to act as a stimulatory adjuvant when bystander T cells were exposed to the inflammatory milieu and cognate antigen at the beginning of type I IFN production, whereas an immunosuppressive effect on T cell proliferation was observed when T cells encountered the antigen after prior exposure to type I IFN (Marshall et al., 2011). These mechanisms may also play a role in our model and contribute to the pleiotropic roles of poly I:C and downstream type I IFN production on cross-priming.

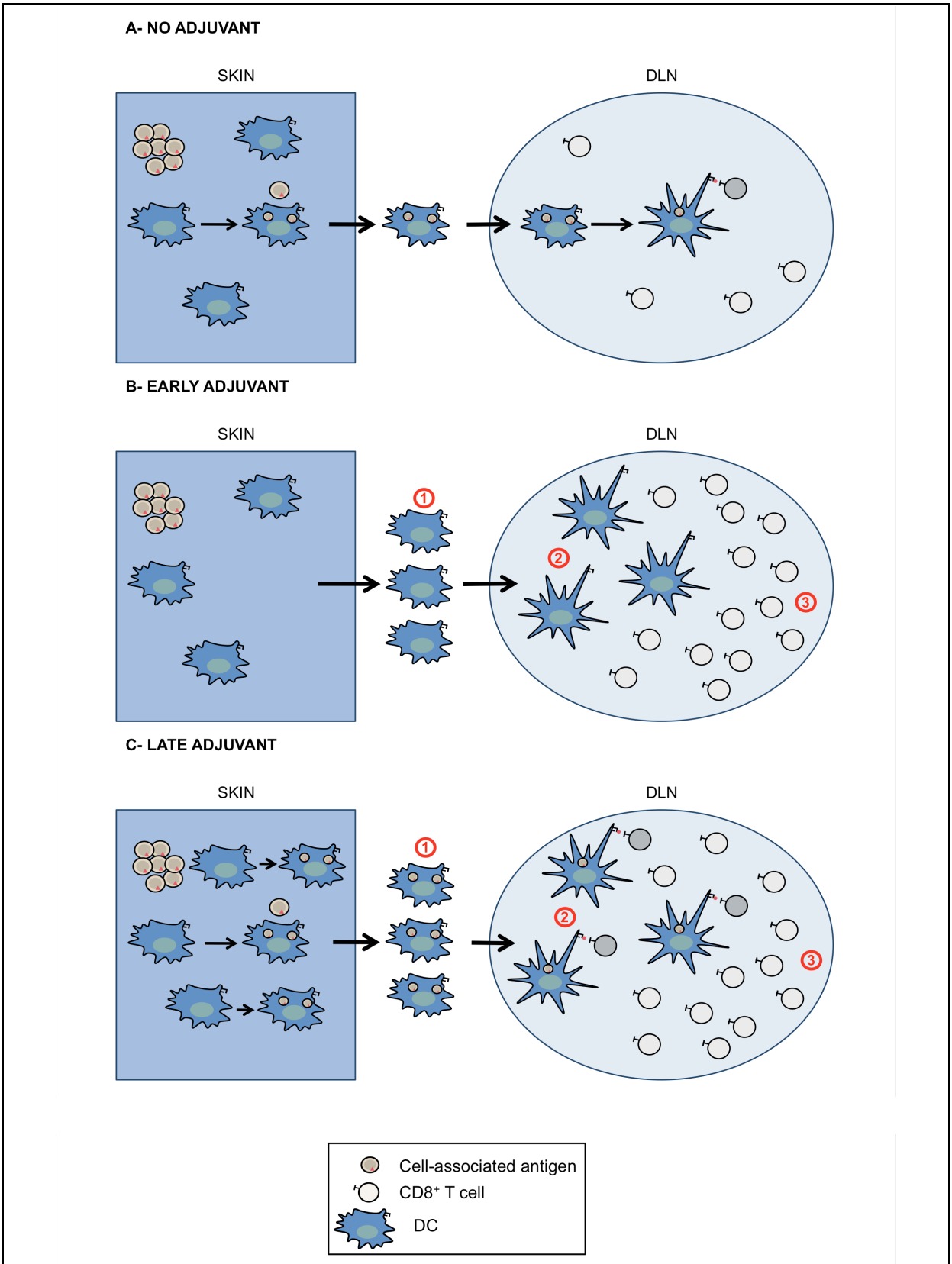


Figure 52. Differential effects of adjuvant depending on the timing of delivery. (A) Cross-priming occurs after immunization with a cell-associated antigen in the absence of adjuvant. **(B,C)** Poly I:C was administered either early **(B)** or late **(C)** after immunization. These time points correspond to conditions where antigen was already engulfed by DCs **(C)** or prior to antigen uptake **(B)**. Some effects of poly I:C are highlighted by red numbers: **(1)** DC recruitment in lymph node, **(2)** DC maturation and **(3)** T cell retention in lymph node. DLN, draining lymph node. The grey T cells correspond to activated T cells.

2) Is poly I:C action on cells similar regardless of the timing of delivery?

The effects of poly I:C described here were similar for both treatment time points: recruitment of immune cells into the lymph node, DC maturation and death. The only observable difference between the two delivery time points was whether antigen phagocytosis had already occured. This question alone may be the explanation for such dramatically different effects. There are two hypotheses that could both explain the differential effects based on adjuvant timing. First, whathever the time point, adjuvant treatment induces DC maturation, terminal differentiation and death **(Figure 53A, B hypothesis 1)**. If antigen was not phagocytosed yet, the stimulated DCs will no longer be able to capture it; if antigen has already been engulfed, then the adjuvant stimulates DC maturation at an optimal time point, allowing for T cell activation, prior to DC death.

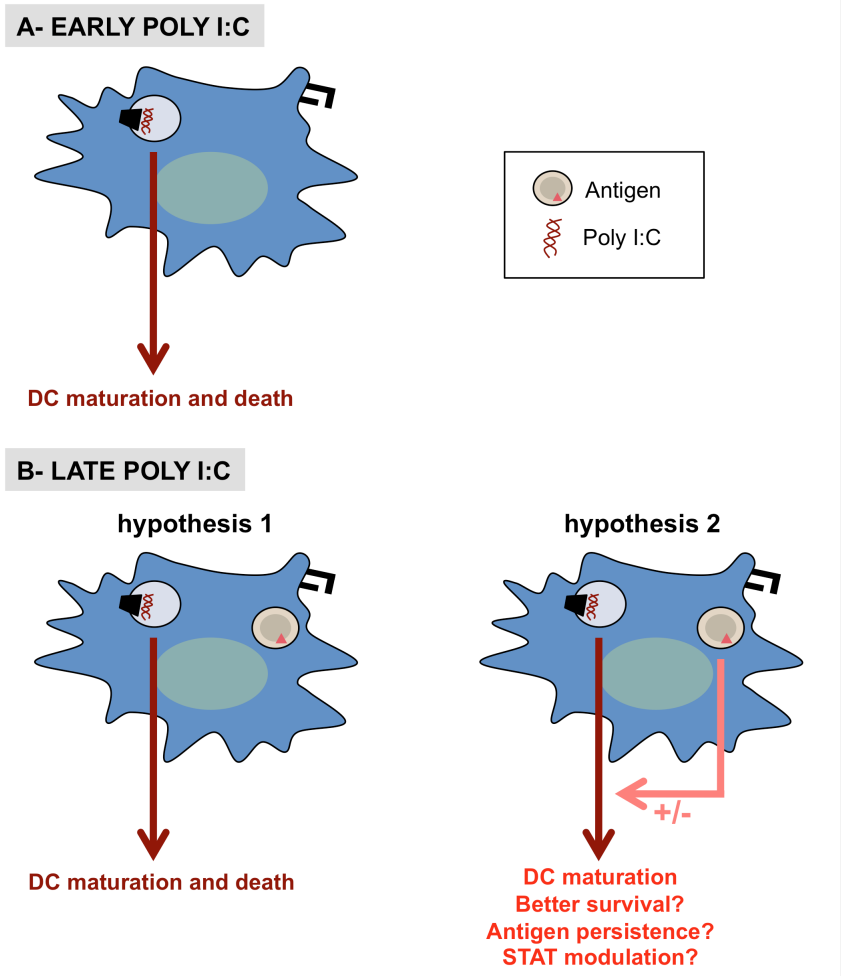


Figure 53. Two hypothesis to explain effects of poly I:C on DCs. (A) When poly I:C is delivered prior to antigen uptake, it induces DC maturation and death. **(B)** When poly I:C is detected after antigen uptake, either poly I:C acts in a similar way **(hypothesis 1)**; or the fact that antigen was already taken up modulates poly I:C action on DCs **(hypothesis 2)**.

However, another potential explanation can be envisioned. Perhaps poly I:C acts differently on DCs depending on the timing of delivery, and this switch in poly I:C effector function is

regulated at the level of antigen engulfment When adjuvant is delivered prior to antigen uptake, it induces DC maturation and death **(Figure 53A)**. Yet, if antigen has already been engulfed, poly I:C may activate the DC differently, inducing, for instance, a different survival program, enhanced persistence of antigen inside the DC, or longer interactions with antigen-specific T cells **(Figure 53B, hypothesis 2)**.

Differentiating between these two hypotheses is difficult to address *in vivo* because, while many DCs will sense poly I:C, only few of them will phagocytose antigen (**Figure 44**). In order to address this on a per cell basis, the aim would be to develop an *in vitro* model that could compare DCs sensing only poly I:C and DCs that detect both antigen and poly I:C. In order to do this, bone-marrow-derived DCs could be cultivated with Flt3-ligand to obtain $CD8\alpha^+$-like DCs. Liposomes containing phosphatidylserine may be used to mimic dying cells. In this way, we could be able to identify and compare the adjuvant treated versus adjuvant + antigen treated populations of DCs by looking at their maturation state, or doing RNA expression analysis to study regulation of cell survival genes. The transcription factors STAT would be viable targets for analysis, as the STAT molecules responsible for transmitting type I IFN signaling have already been shown to differ depending on the DC maturation state (Longman et al., 2007).

3) Poly I:C versus type I IFN effects

In our model, we used poly I:C as a type I IFN-inducer. We investigated the specific effects of type I IFN by using $IFNAR^{-/-}$ and IRF3/7 DKO mice as recipients, as well as $IFNAR^{-/-}$ OT-I responding T cells. However, we cannot exclude a concurrent effect of poly I:C on the cellular targets.

(a) Cross-talk between TLR3 and type I IFN pathways

$CD8\alpha^+$ DCs are the only DC subset that expresses TLR3. We observed a disappearance of these cells upon poly I:C injection. Interestingly, Hasan and colleagues studied the survival of human DCs after treatment with several different TLR ligands: TLR3 ligands that signal through TRIF, TLR5 ligands signaling through Myd88 and TLR4 ligands that can signal through both pathways. They showed that TLR5 ligands induced DC survival, while treatment with TRL3 or TLR4 ligands, poly I:C and LPS respectively, trigerred DC death. Importantly, DC survival was restored if the type I IFN pathway was blocked by anti-IFNAR neutralizing antibodies (Hasan et al., 2007). These results demonstrated that TLR signaling can impact the DC life cycle depending on which intracellular pathway is engaged and that this phenomenon can be modulated by type I IFN.

(b) Programmed necrosis

CD8α$^+$ DCs disappeared upon poly I:C delivery and TRIF signaling may be implicated in this phenomenon (Hasan et al., 2007). A new pathway of cell death has been recently described: the programmed necrosis that is implicated upon death receptor engagement or following TLR3 or TLR4 stimulation in cases where apoptosis was blocked by treatment with the caspase inhibitor z-VAD (Green, 2011). Notably it has been shown that programmed necrosis was responsible for macrophage death upon poly I:C treatment (He et al., 2011) through TRIF signaling and downstream activation of a receptor-interacting kinase 3 (RIPK3)-dependent pathway. This appears to occur independently of type I IFN production. The same pathway may be engaged in CD8α$^+$ DCs and induce their death. Additionally, we have to consider the potential pleiotropic role of poly I:C, not only the effects of type I IFN effects, on cross-priming (**Figure 54**).

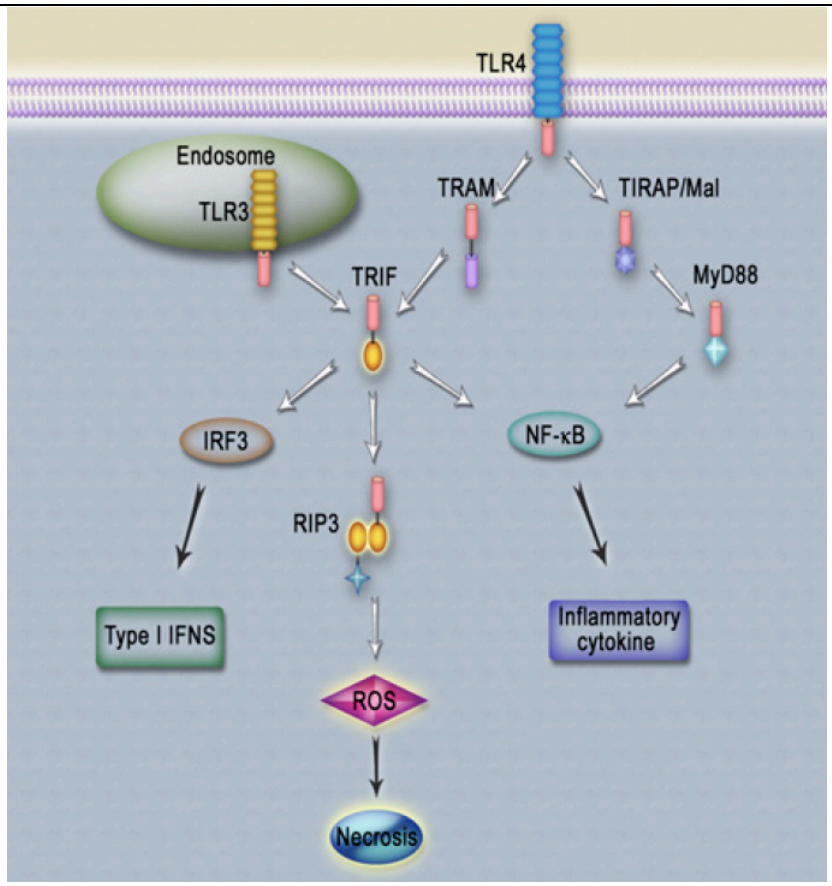


Figure 54. Signaling pathways downstream of TLR3 and TLR4. TLR3 engages TRIF: several independent pathways are then trigerred: type I IFN production, programmed necrosis, as well as inflammatory cytokine production. In contrast, TLR4 ligand induces TRIF- but also Myd88-dependent pathways. Figure from *He et al., 2011*.

(c) Use of other adjuvants

Poly I:C was chosen as the adjuvant for this study based on its ability to induce type I IFN expression and secretion. Other adjuvants such as CpG, poly A:U, or TLR7 agonists could be used to induce type I IFN production as well. The interest in using such reagents would be that they induce type I IFN production, but via a different pathway than poly I:C. This could allow for the distinction between actions promoted by type I IFN and the direct impact of poly I:C on different target genes.

B. The optimal timing for adjuvant delivery depends on the nature of antigen

For our model we focused on the study of cell-associated antigen and cross-presentation, however, other types of antigen can be used for vaccination as described in the introduction. Based on the implications of our results, we can attempt to predict what would be the optimal timing for adjuvant delivery depending on the nature of antigen. Indeed, more than proposing to consistently delay adjuvant administration after immunization, we believe that adjuvant must be sensed by APCs at a critical time point, simultaneously with, or just after antigen uptake. In our model, the cell-associated antigen used requires a relatively long time to be engulfed, processed and presented, and this delay between antigen exposure and productive T cell response allowed us to demonstrate that the timing of adjuvant delivery should be perfectly coordinated with this kinetic. It is important to note that each of these different steps can take up to several days, especially after local immunization. However, with another form of antigen, such as soluble antigen, or peptide that can bind directly to the MHC complex, the same steps are either not required or occur far more rapidly and the optimal timing for adjuvant delivery will have to be adjusted accordingly. Using our experience will cell-associated antigen and our knowledge about the presentation of different types of antigen, we tried to predict the optimal timing of adjuvant delivery for each kind of antigen. These data are summarized in **Table 6**. Our model used a cell-associated antigen, the antigenic form that has the longest lag time between engulfment and time to presentation. Thus, we were able to dissect the action(s) of the adjuvant treatment at different time points. Vaccination with a protein antigen requires a similar set of steps except that it will disseminate more rapidly as it is soluble, reducing the overall duration of the process. Consequently, the optimal timing for adjuvant administration in the case of soluble antigen should be closer to the time of initial immunization. Finally, vaccination with a peptide that is capable of direct binding and presentation by the MHC complex would trigger an extremely rapid presentation kinetic and

we can suppose that co-administration with adjuvant would most likely be the optimal formulation in this case.

1) Peptide vaccine

In agreement with our predictions, it is well established that adjuvant co-administered with short peptides enhances the specific response. Indeed, uptake and processing of this antigen is not the rate-limiting steps as peptide interacts directly with MHC molecule. Thus, it is reasonable to assume that co-administration of antigen and adjuvant would be optimal (**Table 6**).

2) Protein vaccine

Protein or synthetic long peptides require endocytosis and processing prior to presentation. This slight delay suggests that adjuvant should be delivered a short time after immunization. An interesting comparison can be made concerning vaccination with the NY-ESO protein and adjuvant for the treatment of melanoma patients. In a previous study, Nair and colleagues demonstrated that injection of immature DCs into skin pretreated with the TLR7 agonist, imiquimod, allowed a better activation of anti-tumor response compared to delivery of *ex vivo* mature DCs into untreated skin (Nair et al., 2003). Following from these results, a clinical trial was performed using NY-ESO protein injected locally in imiquimod-pretreated skin of melanoma patients. However, in this case, only a limited $CD4^+$ T cell response and no $CD8^+$ T cell responses were observed (Adams et al., 2008). In contrast, the same antigen injected simultaneously with another adjuvant, CpG and formulated with Montanide gave much better results (Valmori et al., 2007). This difference in the efficiency of the immune response may be due to the different timing of adjuvant application. Pretreatment of skin with adjuvant prior to immunization does not appear as the optimal timing for adjuvant delivery.

In the case of synthetic long peptides, there are ongoing trials that are examining the feasibility of specific epitopes linked to TLR ligands, in order to favor the simultaneous detection of antigen and the danger signal adjuvant.

Additionally, recent reports have highlighted that uptake of soluble antigens through pinocytosis or receptor-mediated phagocytosis can be independent of DC maturation in some cases (Drutman and Trombetta, 2010; Platt et al., 2010). Thus, some of the mechanisms of adjuvant-mediated inhibition for cell-associated antigen (i.e. the inverse relationship between DC maturation and antigen uptake ability) may not apply to mounting an immune response to

soluble antigen and, therefore, the timing of adjuvant delivery may have less dramatic consequences.

As a conclusion for protein antigen, we suggest that (**Table 6**):

- If adjuvant is linked to the specific protein, they will be detected simultaneously by DCs, allowing an optimal maturation of DC and presentation of antigen.
- If adjuvant is not physically linked with the protein of interest, it has to be delivered either at the time of, or immediately following immunization, unless the protein can be still taken up by already mature DCs.

3) Nucleic-acid based vaccine

As previously described, nucleic acids can also act as effective vaccine antigens. To examine the effect of adjuvant on their efficiency, Carralot et al. tested an RNA vaccine delivered intradermally using GM-CSF as an adjuvant in a mouse model. GM-CSF is known for increasing the density of DC in the skin and consequently improving overall priming. Interestingly, the delivery of GM-CSF either 1 day prior to, or the day of immunization, did not alter the response, as compared to RNA alone, whereas it induced an enhanced Th1 response when it was administered one day after RNA vaccination (Carralot et al., 2004). This effect may be explained by the time required for transfection and antigen expression prior to its uptake by DCs. Based on these observations, this functional delay in GM-CSF delivery was kept in place during human clinical trials (Weide et al., 2009).

In another study, RNA was injected intranodally. This route of immunization was demonstrated to induce direct RNA uptake by macropinocytosis by lymph-node resident DCs. In this case, they showed that administration of adjuvant prior to immunization abrogated subsequent response (Diken et al., 2011). Here again, the delayed adjuvant delivery after immunization appears to be optimal.

4) Whole tumor cell vaccine

While the use of whole tumor cells for vaccination is intriguing because it does not require the identification of TAAs for therapeutic development, these cells are usually poorly immunogenic and their combination with an adjuvant is essential.

McBride and colleagues compared the efficiency of injecting irradiated Ova-expressing EL4 tumor cells, either electroporated with poly I:C or mixed with soluble poly I:C as a model cancer vaccine. They demonstrated that cell-associated dsRNA enhanced cross-priming, while mixing with its soluble countepart did not modify the response as compared to

irradiated cells alone (McBride et al., 2006). One explanation may be that when dsRNA is associated with the antigen, it can be sensed by DCs simultaneously with antigen uptake and this process ensures optimal adjuvant action. In contrast, when irradiated cells were injected s.c. with soluble poly I:C, the dsRNA adjuvant might have disseminated faster than cells and was detected first by DCs. Similarly, it was demonstrated that CpG-conjugated apoptotic tumor cells induce a better anti-tumor response than the same cells formulated with free CpG (Shirota and Klinman, 2011). The authors hypothesize that direct conjugation of antigen with CpG enhances antigen uptake through DNA receptors, but it may also ensure that DC does not sense soluble CpG prior to antigen.

Generally, Blander and Sander point out in a recent review that the detection of PAMPs, either soluble or linked to a pathogen, influences the development of the subsequent response (Blander and Sander, 2012). Most studies reviewed had been performed with soluble PAMPs that could be found at a distance from the site of infection, while detection of PAMPs linked to a pathogen is a direct sign of local pathogen presence that requires a microbicidal response. They suggested that phagocytosis of the pathogen must occurr in parallel of PAMP sensing in order to activate the appropriate response. This explanation could also explain the results of the previous two studies described. When TLR ligands are free, it is sensed but there is not associated antigen and, therefore, no need for an active immune response.

To conclude for cell-associated antigen, we propose that (**Table 6**):

- Either the antigen is physically linked to the adjuvant, allowing a simultaneous detection of both by DCs.
- Or, the adjuvant is free, and must be delivered with a delay after immunization in order to ensure antigen uptake prior to adjuvant sensing by DCs.

Antigen	Peptide (8-10aa)	Soluble antigen: protein, long peptide, immune complexe		Cell-associated antigen	
Requirement for antigen uptake and processing	-	+		+	
Adjuvant: soluble or linked to the antigen	Soluble	Linked	Soluble	Linked	Soluble
Optimal timing for adjuvant delivery	Co-administration	Co-administration	Delayed or co-administration	Co-administration	Delayed administration
Explanation	No requirement for antigen uptake and processing	Antigen and adjuvant detected in the same time	The delayed administration avoids DC maturation prior to antigen uptake If the antigen can still be taken up by mature DCs, co-administration may be optimal	Antigen and adjuvant detected in the same time	The delayed administration avoids DC maturation prior to antigen uptake

Table 6. Combination of antigen with adjuvant. Compiled from our data and previous studies, we predict the optimal timing for adjuvant delivery.

5) Conclusion

Together, these results highlight the need for an increased and deeper understanding of the mechanisms of antigen uptake, processing and presentation in order to adapt the timing for optimal adjuvant delivery. Each form of antigen likely requires a different adjuvant delivery schedule. Cell-associated antigen requires the longest time between immunization and presentation, and therefore a delay should be considered when deciding upon an adjuvant delivery schedule. Although the presentation requires the same processing as cell-associated antigen, vaccination with a soluble protein antigen that disseminates quickly will result in a more rapide presentation and reduce the overall duration of the process. Consequently, the optimal timing for adjuvant administration will be closer to the initial timing of immunization. Finally, vaccination with a peptide, that directly interacts with the MHC molecules triggers a rapid presentation, and, therefore, we propose that co-administration with adjuvant would provide the optimal effect.

To conclude, we propose that antigen needs to be detected and taken up at the same time as adjuvant is sensed, but not necessarily co-administered. Most importantly, depending on the time required for antigen to be disseminated, processed and detected, the timing of adjuvant delivery must be adjusted in order to obtain optimal boosting of the subsequent immune response.

(a) Efficiency of adjuvant physically linked with antigen

Despite differences in antigen formulation, it is clear that antigen physically linked with its adjuvant generally gives very good results, as the two molecules are most likely sensed at the same time by APCs (Wille-Reece et al., 2005; Huleatt et al., 2007). This effect was highlighted in the work of Nierkens and colleagues in which they studied the adjuvant effect of CpG in a tumor model. B16-OVA tumor-bearing mice were treated with cryoablation, which provided an instant antigen source for DCs and this was combined with injections of CpG at various time points surrounding the antigen injection. They observed that the strongest adjuvant effect was obtained when CpG was administered concurrently with cryoablation (Nierkens et al., 2008). As cryoablation leads to the generation of soluble, as well as cell-associated antigen, they chose to study soluble Ovalbumin to mimic antigen quickly released after cryoablation. In these conditions, they demonstrated that the most efficient response correlated with a colocalization between soluble antigen and CpG in the same cellular compartment. This is also in accordance with studies demonstrating that antigen and danger signals have to be detected by the same cell to induce an optimal response (Kratky et al., 2011; Sporri and Reis e Sousa, 2005). Moreover, Blander and Medzhitov even suggest that antigen and danger signals have to be delivered into the same compartment (Blander and Medzhitov, 2006) in order to observe efficient priming, although these results remain controversial (Yates and Russell, 2005).

Lastly, an additional advantage of adjuvant that is physically linked to its antigen is that the amount of adjuvant necessary is lower, as it is directly targeted to the effector APC. In conditions using lower adjuvant doses, less side effects of adjuvant were observed (Nicodemus and Berek, 2010).

(b) Importance of non-hematopoietic cells

In several studies, it has been observed that the most robust response was obtained when co-detection of adjuvant and antigen was made by the same APCs. However, in some cases, the non-hematopoietic cells were also demonstrated to be crucial for the adjuvant effect of a molecule. Longhi et al. studied the adjuvant effect of poly I:C on the $CD4^+$ T cell response and they demonstrated in bone-marrow chimeras that type I IFN production by hematopoietic cells, as well as stromal cells, was required for the most effective T cell response (Longhi et al., 2009).

(c) Similar pleiotropic roles of type I IFN on macrophages depending on the timing of application

In our studies, we have focused on adjuvant and type I IFN action on DCs. The same IFN-mediated differential effects were also observed for other cell types. In a model of macrophage infection by *Leishmania major*, it was demonstrated that type I IFN could also have conflicting effects on macrophage activity and parasite clearance (Mattner et al., 2000). In this model, when macrophages detect parasite and IFN at the same time, macrophages are activated and the expression of the inducible nitric oxide synthase (iNOS) is increased, leading to *Leishmania* killing. If IFN are detected later than *Leishmania,* it also favors parasite clearance. However, if macrophages detect IFN prior to *Leishmania* exposure, this activation inhibits the expression of iNOS and, in turn, restricts *Leishmania* killing (**Figure 55**). Since IFN are produced directly by macrophages, the authors proposed a model in which this mechanism acts as a type of negative feedback loop, providing for the desensitization of neighbouring macrophages if they had not yet been infected.

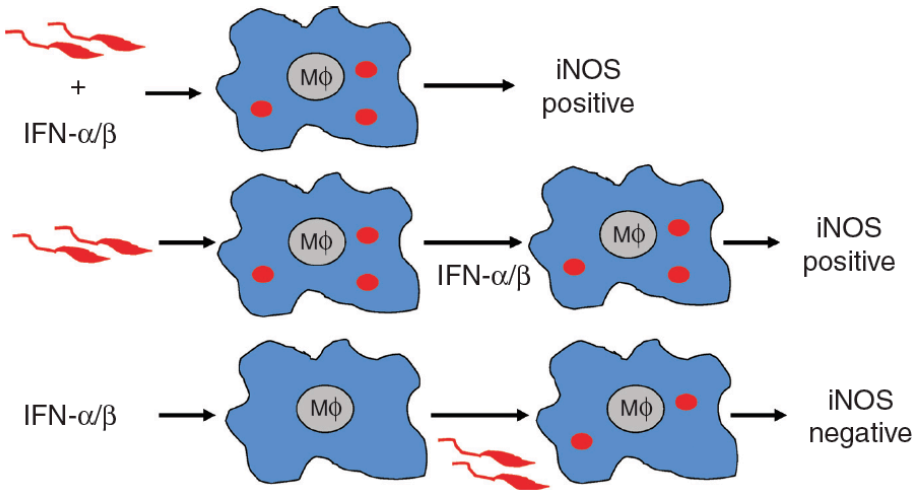


Figure 55. Opposite effects of type I IFN on macrophage activity depending on the sequence of the stimuli. *Leishmania major* (in red) and type I IFN are administered in 3 different sequences. iNOS, inducible nitric oxide synthase. Figure from *Bogdan et al., 2004*.

C. Comparison with other TLR ligands

Previous studies have already pointed out that the timing of adjuvant delivery should be carefully determined. A deeper understanding of the mechanisms of adjuvant action is required to both improve vaccination and further understand seemingly conflicting results.

Stimulation with TLR ligands has been shown to enhance T cell priming (Schulz et al., 2005). However in certain contexts, the opposite effects can be observed (Wilson et al., 2006). For

example, in our model we observed both the positive and negative effects of adjuvant depending on the timing of its delivery.

While we proposed a delayed adjuvant delivery to overcome this complication, other studies demonstrated that co-administration with antigen may lead to a more robust priming. West et al. showed in an *in vitro* model that co-administration of LPS with Ovalbumin immune complexes enhanced antigen uptake and presentation by remodeling the actin cytoskeleton. In contrast, delayed delivery of adjuvant after antigen exposure did not promote the same effect (West et al., 2004). Importantly, this effect is only transient and overall, a down-regulation of antigen capture was observed upon adjuvant exposure, similar to what has been described in our work and by other teams (Wilson et al., 2006; Weck et al., 2007). This transient enhancement of antigen uptake has been demonstrated to be dependent on TLR-signaling.

1) Optimal timing depends on the TLR ligand

The downregulation of antigen uptake observed upon adjuvant delivery was first thought to be due to DC maturation and be dependent on TLR trigerring. However, it appears to also depend on the type of TLR ligand used for stimulation, despite the fact that all of them were shown to induce DC maturation *in vivo* (Schwarz et al., 2003). Weck and colleagues demonstrated that DCs matured after stimulation with TLR3 or TL4 ligands were not able to take up apoptotic cells efficiently, as compared to DCs matured with TLR7/8 or TLR2 (Weck et al., 2007). This dichotomy parallels the intracellular pathways engaged by TLR ligands. TLR7/8 and TLR2 trigger a Myd88-dependent pathway, whereas TLR3 induces a TRIF-dependent pathway. TLR4 can trigger both pathways (**Figure 5**). Consequently, it has been suggested that TLR3 and TLR4 ligands could be co-administered as adjuvants for antigens that do not need to be engulfed, such as peptide, whereas TLR7/8 or TLR2 ligands would be used for antigens that required phagocytosis to be presented. Another possibility to work around these heterogeneous functions is to delay the administration of TLR3 or TLR4 ligand as previously discussed.

2) Optimal timing depends on receptor engaged

Recently, Tirapu et al. focused on the inhibition of antigen uptake following poly I:C delivery and demonstrated that it was not actually due to TLR trigerring (Tirapu et al., 2009). Instead, there was a competition between poly I:C and antigen to interact with scavenger receptors. Interestingly, while poly I:C stimulation reduced uptake through scavenger receptor, it did not affect endocytosis by mannose receptor, which is known to be crucial for Ovalbumin

endocytosis. Compared to the study performed by Weck and colleagues, the reduction of antigen uptake in this study was not associated with less efficient priming. Weck at al. studied uptake of apoptotic cells by human DCs whereas Tirapu et al. performed their experiments with mouse DCs and soluble Ovalbumin. These different systems and antigens may explain the functional variations observed, specifically the fact that soluble antigen and dying cells are not engulfed via the same pathways, which are also differentially regulated by adjuvant treatment (Burgdorf et al., 2007).

These differences highlight the importance of considering several critical parameters when combining an antigen and an adjuvant for therapy: (i) the antigen type, (ii) the receptor(s) and the pathway(s) engaged by the antigen, (iii) the adjuvant type and (iv) the receptor(s) and the pathway(s) engaged by the adjuvant. The understanding of antigen presentation pathways, as well as intracellular pathways trigerred by the adjuvant of interest, are necessary to optimize vaccination strategies.

D. Applications in clinical studies

1) Translation into human treatments

The use of adjuvant is important in order to boost immune responses. However the pattern of TLR expression is not always similar between mice and humans. The best example is CpG, the ligand for TLR9. This receptor is expressed by cDCs, pDCs and B cells in mice, but only on pDCs and B cells in humans (Kadowaki et al., 2001). From what has been discussed regarding the potential requirement for DCs to detect antigen and adjuvant in the same time, the different expression patterns might constitute a huge difference in overall responsiveness. This may explain why some treatments with CpG that provided nice results in mice, were just not as good in humans (Schmidt, 2007). These differences are crucial and must be taken in account when developing an experimental mouse model to address questions of cross-presentation and antigen/adjuvant delivery.

2) Manipulating DCs for vaccination

(a) Use of IFN-treated DCs

Treatment with type I IFN represents a strategy for inducing the maturation of DCs *ex vivo* prior to their administration into patients, an approach often taken in the case of DC-based vaccines. Interestingly, treatment with different cytokines *ex vivo* skewed the differentiation

of monocytes into DCs with varying phenotypes and functions. Activation of monocytes with IL-4, IFNα, TNF or IL-15 gave rise to different populations of DCs with diverse phenotypes. That could explain the different efficiencies of these cells in mounting $CD8^+$ T cell responses (Paquette et al., 1998; Dubsky et al., 2007).

(b) Side effects of type I IFN treatment on DCs

Not all type I IFN effects on DCs promote a well-regulated immune response. Autoimmune disorders have been observed in melanoma patients undergoing treatment with type I IFN. One hypothesis is that IFN induced *in vivo* maturation of DCs that have taken up self-antigen and this led to an autoimmune response (Rizza et al., 2010).

(c) Targeting DCs with specific antigens

It was previously introduced that the type of antigen and thus the type of receptor implicated for antigen uptake plays a role in the efficiency of the subsequent immune response. One promising approach for vaccination development is to target antigens to a specific subset of DCs by coupling them with antibodies directed against DC-specific cell-surface molecules. The $CD8\alpha^+$ DC subset has been the most studied thus far, due to its specialization for cross-priming. Antibodies coupled to Ovalbumin and targeting different receptors, DEC-205, Clec9A and Clec12A were tested. Interestingly it was observed that the outcome of the response and the requirement for adjuvant depends on which receptor was targeted. DEC-205 and Clec9A are effective targets to promote cytotoxic T cell responses, while Clec12A was shown to be inefficient (Lahoud et al., 2011). Addition of adjuvant is always required to induce a $CD8^+$ T cell response. In contrast, a potent humoral response can be obtained upon targeting of antigen to Clec9A in the absence of adjuvant (**Figure 56**). Since Clec9A expression is restricted to $CD8\alpha^+$ DCs and pDCs, this observation may be due to a longer persistence of antigen coupled to antibody in the blood because fewer cells are available that can endocytosed it, allowing for a sustained presentation on MHC-II.

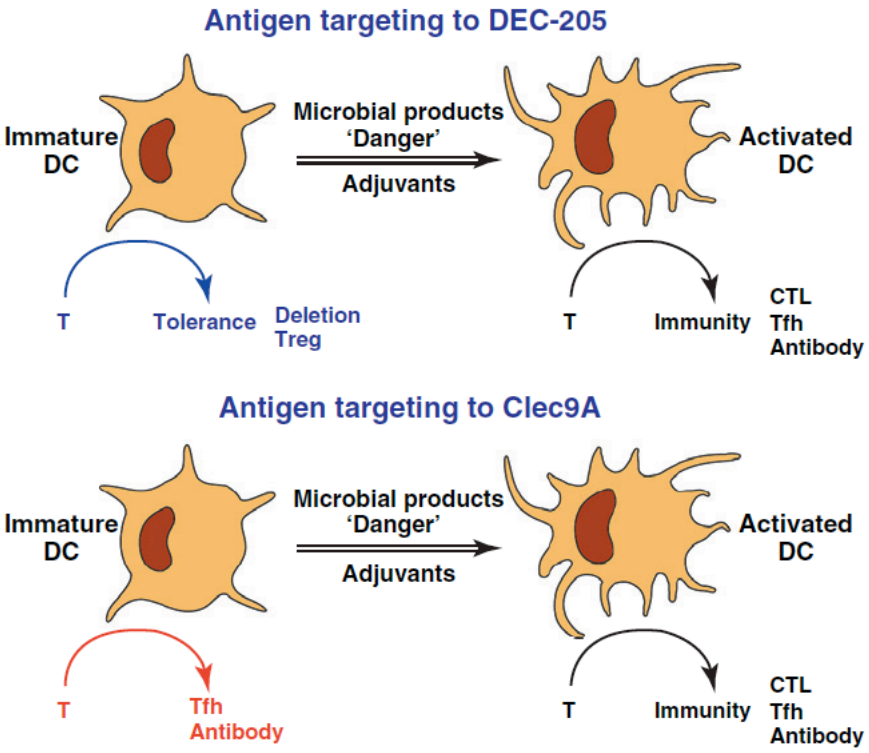


Figure 56. Immune responses generated after targeting of antigen to the same DC subset via different receptors. Tfh, follicular helper T cells. Figure from *Caminschi et al., 2012*.

3) Context of vaccination

Patients that are chronically infected with Hepatitis C virus (HCV) do not display type I IFN production in the liver, which is the organ targeted by the virus. Yet a significant amount of type I IFN was found circulating in the blood of these patients (Mihm et al., 2004). This raises questions regarding the maturation state of DCs, as well as the reactivity of other cell types in this system, when therapeutic vaccination is considered for treatment. Indeed, several trials are currently underway to test vaccine candidates. As an example, IC41 which is a synthetic peptide containing several $CD8^+$ and $CD4^+$ epitopes formulated with the adjuvant poly-L-arginine was tested in chronic HCV patients. The effectiveness of this antigen in combination with the topical application of the TLR7 ligand imiquimod was also examined (Klade et al., 2012). The results obtained from the trial were encouraging, although not as good as expected. In the light of our results regarding the timing of type I IFN production, we can identify several caveats in this type of approach. Indeed, if DCs were already completely matured or impaired in their function prior to vaccination, we may have observed an inefficient response against the virus (Ryan and O'Farrelly, 2011). Most likely, several approaches must be combined in this case to create an environment that allows for the development of a response against the peptide vaccine. Moreover, adding yet another type I IFN-inducer as an adjuvant (such as imiquimod) in chronic HCV patients will probably not

have a boosting effect, due to the desensitization of certain cell types to IFNα (Francois-Newton et al., 2011). This desensitization may explain why imiquimod did not improve the efficiency of the vaccine during the trial period.

4) Combination of several approaches: example of cancer treatments

In the previous section we discussed the combination between antigen and adjuvant as a single therapy. Now we will consider the combination between more than two different reagents as they may be combined and used in the treatment of cancer. Several approaches have been developed in order to further understand the mechanisms of tumor growth, the anti-tumor response and the development of therapies to enhance tumor clearance. First, the tumor can be targeted, either for killing or for modifications that might render it more immunogenic. Secondly, the immune system could be boosted against specific TAAs in order to obtain an efficient response even if the initial stimulation by tumor antigens was suboptimal. Finally, the immunosuppressive environment created by the tumor could be altered such that the development of an effective inflammatory response was allowed (Lesterhuis et al., 2011).

(a) Treatments targeting directly tumor cells

Initial chemotherapy treatments targeting tumors involved the use of broadly acting compounds that induced the rapid death of dividing cells. With time, the signaling pathways involved in cell survival and tumor growth were identified and more specific agents blocking unique steps in these pathways were developed to stop tumor progression and promote tumor cell death. This approach was minimally successful, demonstrating some progress in inducing tumor regression, but resistance mechanisms often developed and the treatment benefits did not extend to long-term survival (Vanneman and Dranoff, 2012). However, these treatments are interesting for further study because they induced death in at least a fraction of tumor cells, facilitating the effect of cytotoxic T cells. These types of treatments also trigger the release of dying cell debris and danger signals that can be taken up by DCs, such as ATP or HMGB1 that will result in immune cell activation.

(b) Development of an efficient anti-tumor immune response

Another approach to fight cancer is to boost the immune response in order to render it more efficient against specific tumor antigens. As discussed previously, different types of antigen can be used for vaccination (**Table 5**). The aim is to use a more immunogenic antigen in order to mount an effective T cell response directed against tumor. Drugs modulating the steps of immune response could be combined with the vaccine treatment to obtain the most efficient T

cell response. As an example, one could combine a vaccine with any of many molecules that regulate antigen uptake, processing and presentation by DCs, maturation of DCs, or differentiation of T cells into effector and memory cells. Furthermore, antibodies that target activating receptors on T cells such as OX40 or 4-1BB can also be used to boost T cell activation.

(c) Circumvent immunosuppressive context

Immunosuppressive mechanisms are required to maintain self-tolerance as well as critical for preventing tissue damage during an immune response. Inhibitory molecules such as CTLA4 or PD-1 are expressed by and act to control activated T cells. CTLA4 competes with the co-stimulatory molecule CD28 for the interaction with CD80 and CD86 on DCs, thus preventing extended activation of T cells. PD-1 interacts with PD-1 ligands that are expressed by myeloid cells in inflamed tissues, resulting in T cell exhaustion. These feedback loops provide a critical regulation step within the immune response. Interestingly, PD-1 ligands are also expressed by most tumor cells and many of these physiological mechanims of immunosuppression are found in the tumor microenvironment resulting in the limitation of the development of anti-tumor responses. Furthermore, numerous regulatory T cells as well as MDSCs are found in tumors.

The high levels of regulatory T cells also express CTLA4 and PD-1. These receptors appear to increase suppressive regulatory T cell activity, whereas they act to inhibit effector T cell activity. Clinical trials have been conducted to test antibodies targeting CTLA4 or PD-1 in the context of cancer, and promising results were obtained. Anti-CTLA4 antibodies have even received FDA approval for use in the clinic to improve anti-tumor responses. Anti-PD-1 and PD-1-ligand antibodies are currently undergoing further testing and, thus far display less toxicity than those antibodies directed against CTLA4. Additional blocking antibodies directed against other molecules involved in immunosuppressive mechanisms are also undergoing investigation (Lesterhuis et al., 2011).

This strategy can be efficient at overcoming the immunosuppressive actions of tumor cells. Nevertheless, it works only if an anti-tumor response has already been established. Otherwise, blocking antibodies will have to be combined with a vaccination strategy.

(d) Combination of several approaches and timing of delivery

As reviewed above, multiple strategies have been developed to treat cancer: non specific chemotherapy that kills dividing cells and thus limits tumor expansion; vaccination to induce an effective anti-tumor T cell response, given with or without adjuvant to boost this response;

and inhibitory drugs to limit immunosuppressive conditions in tumor microenvironment. Each of these strategies individually revealed only a limited efficiency, but promising results were obtained when several of them were given in combination (Dougan et al., 2010, Balachandran et al., 2011, Hodi et al., 2008). Interactions between the different approaches should be studied further to determine the best strategy(ies) to treat cancer. Bioavailability of the different molecules or vaccines, step of the immune response that is targeted and tumor growth, are all factors that should be taken into consideration to determine the optimal dose, sequence and timing for the application of the different treatment strategies.

(i) Multiple effects of a given reagent

Each of these strategies was developed to target one single element of the tumor development. Often side effects are observed and also must be considered when treatment combinations are planned. The response to a given vaccine may involve the contribution of unknown mechanims that could be modified by a second therapy given concurrently. As we observed in our model, a given cytokine, type I IFN, has pleiotropic roles on both immune and non-immune cells; a given adjuvant, poly I:C, is responsible for its direct effect through TLR and RLR engagement but also provides a second wave of activation through type I IFN (**Figures 52 and 54**). Interestingly, molecules developed to target signaling pathways implicated in tumor survival and growth have been demonstrated to also be involved in the activation and differentiation of immune cells (Vanneman and Dranoff, 2012). For example, cetuximab is an anti-tumor agent composed of neutralizing antibodies directed against the EGF receptor. It blocks the growth signaling pathway in tumor cells; however it has also been demonstrated to facilitate the uptake of tumor cells by inducing the formation of immune complexes (Correale et al., 2012). IAP inhibitors that sensitize tumor cells to apoptosis actually enhance T and NK cell function (Fesik, 2005; Dougan et al., 2010). This dual activity illustrates the need for an in-depth understanding of the various actions of a given molecule on tumor cells, but also in the context of the immune response, such that optimal administration conditions can be determined and potential combinatorial side effects can be identified and predicted.

(ii) Opposite effects of reagents depending on the timing of administration

We observed in our model that early delivery of poly I:C inhibited subsequent priming while, if delivered a few days later, it boosted the same response (**Figure 37**). The same functionally opposing effects have been observed for other molecules depending on the timing of administration. Sunitinib is an inhibitor of tyrosine kinase receptors such as VEGF or PDGF receptors and this inhibition results in limiting tumor growth. Moreover it also acts on

immune cells, reducing the immunosuppressive action of regulatory T cells and MDSCs. Farsaci et al. tested combinations of sunitinib with a vaccine in a mouse tumor model (Farsaci et al., 2012). Co-administration of sunitinib with vaccine, or sequential injection of vaccine and then sunitinib did not show an anti-tumor benefit compared to sunitinib alone. In contrast, when vaccine was administered after sunitinib treatment, it induced a better anti-tumor response. The possible explanation is that sunitinib triggered a less immunosuppressive environment, favoring the development of a more robust immune response once vaccine was administered.

(iii) Determine optimal sequence and timing to combine several treatments

From our knowledge regarding mechanism, processing and interactions between the different molecules, we would eventually be able to predict what may be the optimal timing and treatment strategy for their administration and combination (**Figure 57**). Although, for this, a much more thorough understanding of their multiple actions on tumors and on the immune system is absolutely necessary. Once established and confirmed by experimental model data, these predictions would then have to be tested in pre-clinical trials.

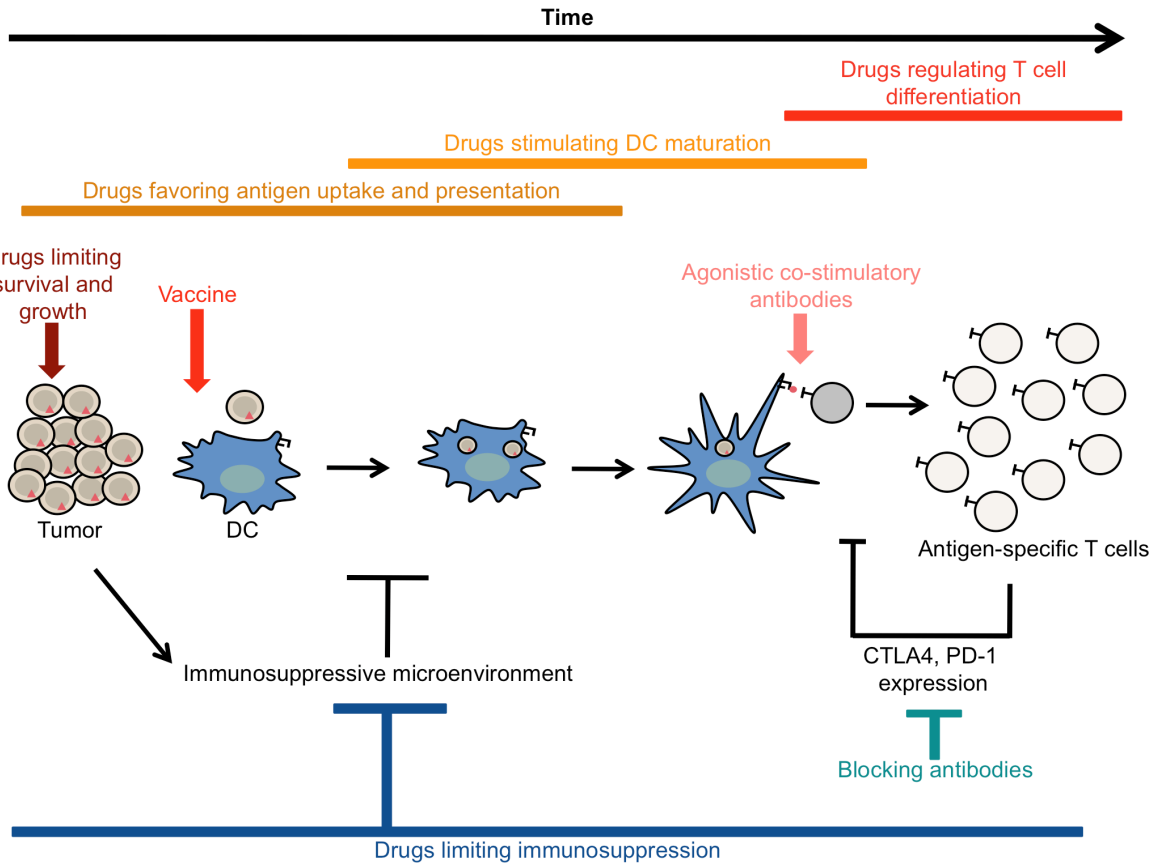


Figure 57. Combination and sequence of administration of different approaches for cancer therapy. From the known actions of the different therapeutic agents, we propose a model to choose the optimal timing of delivery. Arrows and lines represent the optimal time window for each treatment depending on the step it is supposed to act.

Although, we chose to describe how to apply our data and model to the development of anti-cancer therapy, the same strategies may be applied to other diseases where different approaches and combinations might be required to optimize current patient treatment.

IV. CONCLUSION

In conclusion, we have used a model of cell-associated antigen to perform a thorough, careful anaysis of the $CD8^+$ T cell response after cross-presentation. The development of the tetramer-based enrichment strategy and its combination with other approaches such as intracellular cytokine staining or immunoscope allowed us to perform an in-depth study of the kinetics, phenotype and functionality of the endogenous $CD8^+$ T cell response.

We applied these strategies to study first the impact of the route of immunization on $CD8^+$ T cell cross-priming. We compared the efficiency and effectiveness of local *versus* systemic

delivery of cell-associated antigen. As expected, i.v. immunization led to a more rapid priming. Surprisingly, while delayed kinetically, i.d. immunization triggered a more robust and polyfunctional primary T cell response and a better secondary response. In contrast, the route of immunization did not impact the diversity or the avidity of the population of responding T cells. Factors such as inflammation induced at the site of injection, subsets of DC implicated, or persistence of antigen may all contribute to the differences observed.

To follow the characterization of the endogenous $CD8^+$ T cell response, we were interested in assessing the effectiveness of the combination of adjuvant with our antigen and, in particular, focus on understanding the optimal timing for delivery, as the kinetics of antigen presentation were dependent on the route of immunization. We demonstrated that the optimal timing of adjuvant delivery was route of immunization-dependent and that there was an optimal time window for adjuvant application to observe positive effects on cross-priming. We identified some effects of poly I:C as well as type I IFN on DCs and T cells allowing us to propose a model explaining why the timing of adjuvant delivery is crucial for optimal priming. Our study in a fundamental model that combined antigen and adjuvant highlights multiple factors that are important to consider when several treatments are combined. The same kind of approaches used here in an established experimental model of cross-presentation may also be used to compare combinatorial therapies and sequence of administration of several treatments in complexe diseases such as cancer or chronic viral diseases.

Material and Methods

I. MICE

A. Mouse strains

Name	Background	Source
WT	C57BL/6	Charles River
IFNAR1$^{-/-}$	C57BL/6	Lab
IRF3$^{-/-}$ IRF7$^{-/-}$	C57BL/6	M. Diamond
OT-I	C57BL/6	Jackson Laboratory
CD45.1/2 OT-I	C57BL/6	Lab
IFNAR1$^{-/-}$ CD45.2/2 OT-I	C57BL/6	Lab
WT CD45.1/1	C57BL/6	Jackson Laboratory
IFNAR1$^{-/-}$ CD45.1/1	C57BL/6	Lab
K^{bm1}mOVA	C57BL/6	S. Schoenberger
β2m$^{-/-}$ mOVA	C57BL/6	Jackson Laboratory
WT mOVA	C57BL/6	Jackson Laboratory
K$^{b-/-}$	C57BL/6	Taconic
WT	FVB/N	Charles River
β-actin luciferase	FVB/N	C. Contag

Table 7. Mouse strains used

B. Bone-Marrow chimeras

6 week-old mice to be used as BM-chimeras hosts were irradiated (Cesium source). The appropriate duration of treatment was calculated based on the time necessary to receive an irradiation dose of 1250rad. Mice used as bone marrow donors were all less than 6 months of age. Donor mice were euthanized and bones were harvested. Bone marrow cell suspensions were prepared in PBS and at least 5 million total leukocytes were transferred i.v. per mouse. Six weeks later, the extent of chimerism was determined in the blood by evaluating the staining for the congenic markers CD45.1 and CD45.2 by flow cytometry.

C. Injection strategies

1) Immunization

Splenocytes isolated from K^{bm1}mOva mice were used for immunization. The spleen was harvested, mashed and red blood cells were lyzed by adding 2.5mL NH_4Cl 1.66% and incubating at 37°C for 5 minutes. Cells were washed with PBS and counted. $5x10^5$ (or $5x10^6$ depending on the experiment) cells in a volume of 100µl were injected intradermally (i.d.) or intravenously (i.v.). The intradermal injection was performed in the right flank with the inguinal lymph node being the draining lymph node. Retro-orbital injection was used to deliver antigen intravenously.

2) TCR-transgenic T cell transfer

For OT-I transfer, bulk splenocytes were isolated from OT-I mice. After red blood cell lysis, 10^3, 10^6 or $5x10^6$ splenocytes were transferred i.v. in a volume of 100µl.

3) Adjuvant delivery

100µg of Poly I:C (high molecular weight product from Invivogen) was injected i.v. in a final volume of 100µl.

4) Antibody injection

The antibody binding the MHC-I-peptide complexe H-$2K^b$-SIINFEKL was isolated from the hybridoma 25-D1.16 and obtained from National Cell Culture Center. An isotype control (MOPC-21) was injected into control mice.

II. CHARACTERIZATION OF THE T CELL RESPONSE

A. Tetramer-based enrichment

See manuscript 2 for further information

1) Tetramer preparation

Monomers were prepared by Fabrice Lemaître using a modified version of the methods previously described (Altman et al., 1996), and tetramerization was performed prior to use, using PE-Streptavidin (Invitrogen), added for 1 hour at 25°C.

2) Magnetic enrichment

Leukocytes were harvested from 15 lymph nodes (2 inguinal, 2 axillary, 2 brachial, 4 cervical- deep and superficial, 2 peri-aortic, and the mesenteric chain) and the spleen. Organs were mashed and cells transferred in a tube after filtering the cell suspension with a 70µm cell strainer. Cells were Fc-blocked with anti-CD16/CD32 antibody and stained with PE-labeled K^b-SIINFEKL tetramers in PBS containing 2% FCS and 0.1% of Sodium azide for 30min at 4°C. This was followed by an incubation with anti-PE magnetic microbeads (Miltenyi Biotech). Cells were passed over a magnetic LS column to enrich tetramer-positive cells. Bound cells were eluted ("enriched fraction"). A 5µl aliquot was collected for precise counting of the bound fraction using the Accucheck beads (Invitrogen).

3) Flow Cytometry

Cells were stained with a mixture of antibodies (anti-CD11c, CD11b, CD4, NK1.1, F4/80, B220, CD3, CD8) to exclude cells that are not of interest (DUMP gate) and focus the subsequent analysis on $CD8^+$ T cells (**Table 8**). Prior to analysis, DAPI (Invitrogen) was added to label dead cells. Cells were analyzed using a FACS Canto II (BD Biosciences). Live, non-clumped, $CD3^+$ $CD8^+$ tetramer-positive cells were gated. The percentage of tetramer-positive cells in each sample was multiplied by the total number of cells in the enriched fraction in order to obtain the absolute number of tetramer-positive $CD8^+$ T cells.

Antigen	Clone	Isotype	Fluorochrome	Company
cd3ε	145-2C11	Hamster IgGl, κ	PerCP–Cy5.5	BD Pharmingen
CD4	RM4-5	Rat IgG2a, κ	Pacific blue	BD Pharmingen
CD8α	53-6.7	Rat IgG2a, κ	Alexa fluor 700	BD Pharmingen
CD8α	53-6.7	Rat IgG2a, κ	PerCP–Cy5.5	BD Pharmingen
CD8β	H35-17.2	Rat IgG2b, κ	APC	eBioscience
CDIIb	MI/70	Rat IgG2b, κ	eFluor 450	eBioscience
CDIIc	N418	Hamster IgG	eFluor 450	eBioscience
CDIIc	HL3	Hamster IgGl, λ	APC	BD Pharmingen
CD45.1	A20	Mouse IgG2a, κ	PE	BD Pharmingen
CD45.1	A20	Mouse IgG2a, κ	APC	BD Pharmingen
CD45.1	A20	Mouse IgG2a, κ	Pacific blue	Biolegend
CD86	GL1	Rat IgG2a, κ	FITC	BD Pharmingen
CD103	M290	Rat IgG2a, κ	PE	BD Pharmingen
NK1.1	PK136	Mouse IgG2a, κ	Pacific blue	Biolegend
B220	RA3-6B2	Rat IgG2a, κ	Pacific blue	BD Pharmingen
F4/80	BM8	Rat IgG2a, κ	eFluor 450	eBioscience
IA/IE	M5/114.15.2	Rat IgG2b, κ	Alexa fluor 700	eBioscience
IFNγ	XMG1.2	Rat IgGl, κ	APC	BD Pharmingen
IL-2	JES6-5H4	Rat IgG2b	Alexa fluor 488	BD Pharmingen
TNFα	MP6-XT22	Rat IgGl	PE–Cy7	BD Pharmingen

Table 8. Antibodies used for flow cytometry experiments

4) Combination of tetramer-based enrichment with intracellular staining

For *in vivo* restimulation, mice were injected with 5μg of CpG ODN2216/ DOTAP formulated as a mixture with 1μg SIINFEKL peptide 3 hours prior to leukocyte harvest. CpG was purchased from Invivogen, DOTAP from Roche, and the SIINFEKL peptide from Polypeptide group. Next, the tetramer-based enrichment protocol was performed with the addition of Brefedin-A during each incubation step. After the elution step, enriched cells were stained with Aqua as a dead cell marker, incubated with surface staining antibodies and fixed. Next, cells were permeabilized and stained with anti-IFNγ as per the manufacturer's instructions. Intracellular cytokine staining was performed using the Cytofix/ Cytoperm/Brefeldin-A kit (BD Biosciences). For *ex vivo* restimulation, the tetramer-based enrichment was performed first, and the eluted fraction was incubated 4h with SIINFEKL-pulsed splenocytes at 37°C. Then cells were stained intracellularly for IFNγ, IL-2 and TNFα (**Table 8**) as per the manufacturer's instructions (BD Biosciences).

B. Antibody-based enrichment

Of note, a similar enrichment strategy was used to enrich TCR-transgenic CD45.1/2 WT OT-I and CD45.2/2 IFNAR$^{-/-}$ OT-I that were transferred into CD45.1/1 WT recipient mice using a anti-CD45.2-PE staining of leukocytes rather than PE-labeled K^{b}-SIINFEKL tetramers.

C. IFNγ ELISPOT

At different time points following immunization, the spleen and the draining lymph node were harvested and CD8^{+} T cells were purified using anti-CD8 microbeads and MS columns (Miltenyi Biotech). IFNγ ELISPOT was performed as previously described (Blachere et al., 2006). The Elispot plate evaluation was performed in a blinded fashion by an independent evaluation service (Zellnet Consulting) using an automated ELISPOT reader (Carl Zeiss).

D. Cytotoxicity in vivo

CD45.1 splenocytes were prepared. Half of them were stained with 0.5μM carboxyfluorescein diacetate succinimidyl ester (CSFE) using the Vybrant cell tracer kit from Invitrogen, and pulsed with SIINFEKL peptide. The other half were stained with 5μM CFSE and left unpulsed. At different time points following immunization, mice received i.v. 5x10^{6} of cells from each of these pools. 15 hours later, spleen was harvested and cells were stained

with an anti-CD45.1 antibody. The lysis of injected splenocytes was determined using the CFSE staining and the percentage of specific lysis was calculated.

E. Immunoscope

These experiments were done in collaboration with Annick Lim and Brigitte Lemercier.

K^b-SIINFEKL tetramer-positive $CD8^+$ T cells were sorted using a FACS Aria-II. Total RNA was prepared from sorted T cells using the Total RNA Miniprep kit (Sigma), and cDNA was synthetized using the SuperScript™ II Reverse Transcriptase (Invitrogen). The different Vβ germline gens can be clustered in 24 families according to their level of homology (IMGT nomenclature). For quantitative repertoire, PCR reactions were carried out by combining a reverse primer and a specific fluorophore-labeled probe for the constant region (MGB-TaqMan probe) with one of 24 primers covering the different Vβ chains (**Table 9**). Real-time PCR reactions were subsequently carried out with a final concentrations of 400nmol/L of each oligonucleotide primer, 200nmol/L of the fluorogenic probe, and FastStart master Mix (Roche). Thermal cycling conditions comprised Taq DNA Polymerase activation at 95°C for 10min, then subjected to 40 cycles of denaturation at 95°C for 15sec, annealing and extension at 60°C for 1min. For all these different reactions, real tim quantitative PCR was then performed on an ABI-7300 system (Applied Biosystems). The relative usage of each Vβ family was calculated according to the formula:

$$U(VB_y) = \sum_{x=1}^{x=24} 2^{(C_t(x)-C_t(y))}$$

$C_t(x)$ is the fluorescent threshold cycle number measured for the Vβy family. For immunoscope profiles, products were then subjected to run-off reactions with a nested fluorescent primer specific for the constant region (**Table 9**: FAM-primer)- run for a total of 3 cycles. The fluorescent products were separated and analyzed using an ABI-PRISM 3730 DNA analyzer. The size and intensity of each band were analyzed with "Immunoscope Software" (Pannetier et al., 1993), which has been adapted to the capillary sequencer. Fluorescence intensities were plotted in arbitrary units on the y axis, and CDR3 lenghts (in amino acids) on the x axis.

Vβ1	TCACTGATACGGAGCTGAGGC
Vβ2	GCCTCAAGTCGCTTCCAACCTC
Vβ3	CACTCTGAAAATCCAACCCAC
Vβ4	ATCAAGTCTGTAGAGCCGGAGGA
Vβ5	CTGAATGCCCAGACAGCTCCAAGC
Vβ12.1	AAGGTGGAGAGAGACAAAGGATTC
Vβ12.2	CATTATGATAAAATGGAGAGAGAT
Vβ12.3	AGAAAGGAAACCTGCCTGGTT
Vβ13.3	CATTACTCATATGTCGCTGAC
Vβ13.2	TTCATATGGTGCTGGCAGCACT
Vβ13.1	TGCTGGCAACCTTCGAATAGGA
Vβ14	AGGCCTAAAGGAACTAACTCCAC
Vβ15	GATGGTGGGGCTTTCAAGGATC
Vβ16	GCACTCAACTCTGAAGATCCAGAGC
Vβ17	TCTCTCTACATTGGCTCTGCAGGC
Vβ19	CTCTCACTGTGACATCTGCC
Vβ20	CCCATCAGTCATCCCAACTTATCC
Vβ21	CTGCTAAGAAACCATGTACCA
Vβ23	TCTGCAGCCTGGGAATCAGAA
Vβ24	AGTGTTCCTCGAACTCACAG
Vβ26	ACCTTGCAGCCTAGAAATTCAGT
Vβ29	TACAGGGTCTCACGGAAGAAGC
Vβ30	CAGCCGGCCAAACCTAACATTCTC
Vβ31	ACGACCAATTCATCCTAAGCAC
Reverse primer	GGTAGCCTTTTGTTTGTTTGCAA
MGB–Taqman probe	AGCCATCAAAAGCA
Fam-primer	CTTGGGTGGAGTCACATTTCTC

Table 9. Sequences of the primers used for immunoscope analysis

III. CHARACTERIZATION OF DENDRITIC CELLS

A. DC phenotype

Spleen and lymph nodes were digested with Collagenase D (Roche) and Dnase (Invitrogen). Cell suspensions were stained for CD11c, CD11b, CD8a, CD103, CD86, IA^b/IE^b and analyzed by flow cytometry (**Table 8**). An aliquot was collected to determine the absolute number of cells per organ using the Accucheck beads from Invitrogen

B. Visualization of antigen engulfed by DCs

To visualize injected cell-associated antigen, K^{bm1}mOva splenocytes were labeled with the PKH26 dye (Sigma) prior to immunization. This staining allowed for the detection of cells that have phagocytosed injected splenocytes. The draining lymph nodes from several mice

were pooled for our analysis. $CD11c^+$ cells were enriched using CD11c magnetic beads (Miltenyi Biotech), and the enriched fraction was analyzed by flow cytometry to identify the different subsets of DCs present. In parallel, the flow-through fraction was stained to identify the different populations of cells stained with the PKH26 dye.

IV. CHARACTERIZATION OF ANTIGEN PERSISTENCE

A. Persistence of antigen cross-presentation

CD45.1 OT-I splenocytes were isolated and stained using 5μM carboxyfluorescein diacetate succinimidyl ester (CSFE) in PBS. After washing with ice-cold PBS, $5x10^6$ OT-I splenocytes were injected i.v. into immunized mice. Three days later the draining and non-draining lymph nodes, and the spleen were harvested. Organs were processed independently and cells were labeled with CD8b and CD45.1 antibodies allowing for the identification of the transferred CD8 OT-I T cells and the determination of CFSE intensity.

B. Persistence of antigen

1) Bioluminescence

FVB/N female mice were immunized with $5x10^6$ FVB/N-$luciferase^+$ male splenocytes. For in vivo imaging, mice were injected at a given time point with 3mg of D-luciferin (Synchem), followed by isoflurane inhalation to keep animals sedated during acquisition. Images from mice were acquired over 10 minutes and the bioluminescent signal was expressed in photon/ s/cm^2/steradian. For ex vivo imaging, organs were harvested and placed in wells containing PBS and D-luciferin to determine the total bioluminescent signal from each organ; the bioluminescence is expressed as the total flux/organ in photons/s. Bioluminescence imaging was performed by using an IVIS Lumina II system (Caliper Life Sciences). Quantification of the light emission was analyzed using Living Image Software version 3.1 (Xenogen Corporation).

2) PCR

To investigate the presence of remaining DNA from injected splenocytes, mice were tattooed around the site of injection prior to immunization. At defined time points, the skin from the site of immunization was harvested and DNA was extracted using the QIAamp DNA Mini Kit from Qiagen. PCR was performed on DNA using the primers specific for the ovalbumin gene:

- forward TCCATCGGCGCAGCAAGCATGGAA
- reverse ATGTCTAGAAGGGGAAACACATCTGCC

3) Flow cytometry

To analyze remaining injected splenocytes at different time points post-immunization, CD45.1 mice were immunized with a mixture of 3 types of CD45.2 splenocytes labeled with 3 different concentrations of CFSE: WTmOVA 5µM CFSE, K^{bm1}mOVA 1,5µM CFSE and $\beta2m^{-/-}$mOVA 0,5µM CFSE. At different time points post-immunization the spleen and lymph nodes were harvested and cells were labeled for CD45.2. The 3 populations of injected splenocytes were identified based on the differing degrees of CFSE staining.

V. CYTOKINE ANALYSIS

Plasma samples were harvested and conserved at -80°C for analysis. Mouse IFNa level was quantified by ELISA following manufacturer's instructions (PBL biomedical).

VI. LISTERIA INFECTION

The Ovalbumin-expressing *Listeria monocytogenes* is a kind gift from N. Glaichenhaus. Mice were infected i.v. with $5x10^5$ colony forming units (CFU). Two days later, the spleen and the liver were harvested and homogenized in 0.2% NP-40 in water, and serial dilutions were plated to determine the CFU per organ.

VII. STATISTICAL ANALYSIS

These analyses were performed in collaboration with Sandrine Leroy

Data is plotted with bars representing median values. Non-parametric (two-tailed) Mann-Whitney tests were used to compare the distributions between two conditions. In some instances, selective comparisons between two groups within a multi-parameter experiment were also performed using a non-parametric Mann-Whitney test. Continuous measurements were studied over time or according to the peptide concentration unsing general linear modeling. Statistical analysis was performed using Stata 11 software (StataCorp, College Station, TX USA) and Prism 5 (Graphpad Software Inc., La Jolla, CA USA).

Manuscripts

Manuscript 1

Immunization route dictates cross-priming efficiency and impacts the optimal timing of adjuvant delivery

Isabelle Bouvier, Hélène Jusforgues-Saklani, Annick Lim, Fabrice Lemaître, Brigitte Lemercier, Charlotte Auriau, Marie-Anne Nicola, Sandrine Leroy, Helen K. Law, Antonio Bandeira, James J. Moon, Philippe Bousso and Matthew L. Albert

Frontiers in Immunology, December 2011

Manuscript 2

Tracking antigen-specific $CD8^+$ T cells using MHC Class I multimers

Cécile Alanio, **Isabelle Bouvier**, Hélène Jusforgues-Saklani and Matthew L. Albert

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Immunization route dictates cross-priming efficiency and impacts the optimal timing of adjuvant delivery

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Delivery of cell-associated antigen represents an important strategy for vaccination. While many experimental models have been developed in order to define the critical parameters for efficient cross-priming, few have utilized quantitative methods that permit the study of the endogenous repertoire. Comparing different strategies of immunization, we report that local delivery of cell-associated antigen results in delayed T cell cross-priming due to the increased time required for antigen capture and presentation. In comparison, delivery of disseminated antigen resulted in rapid T cell priming. Surprisingly, local injection of cell-associated antigen, while slower, resulted in the differentiation of a more robust, polyfunctional, effector response. We also evaluated the combination of cell-associated antigen with poly I:C delivery and observed an immunization route-specific effect regarding the optimal timing of innate immune stimulation. These studies highlight the importance of considering the timing and persistence of antigen presentation, and suggest that intradermal injection with delayed adjuvant delivery is the optimal strategy for achieving $CD8^+$ T cell cross-priming.

Keywords: dentritic cells, cross-priming, polyfunctional T cells, adjuvant delivery

INTRODUCTION

CD8 T cell responses are key components of the adaptive immune system. These cells are considered particularly important in the host response to microorganisms and cells undergoing malignant transformation (Heemels and Ploegh, 1995). To carry out their effector function, they must first be activated by dendritic cells (DCs) presenting MHC I/peptide complexes (Mellman and Steinman, 2001). In instances of direct infection of DCs, antigen presentation via the endogenous pathway may account for $CD8^+$ T cell priming; however, for many infections and most tumors, an indirect pathway (referred to as cross-priming) is utilized for the loading of antigen onto the MHC I of DCs (Albert et al., 1998; Albert, 2004). The cross-priming pathway has also been targeted for purposes of prophylactic and therapeutic vaccination (Amigorena, 2000; Palucka et al., 2006; Mitchell et al., 2007; Weide et al., 2008). While of potential value in therapeutic strategies, there is a need to optimize strategies for antigen and adjuvant delivery, taking care that conditions mimic those present during treatment of humans (Russo et al., 2007; Fontana et al., 2009). Herein, we investigate the impact of different routes of immunization when employing cell-associated antigen for cross-priming by host DC.

Over the last 10 years, it has been shown that several factors participate in efficient cross-priming: (i) the presence of high affinity $CD8^+$ T cells (Zehn et al., 2009); (ii) $CD4^+$ T cell help, acting to "license" DCs via CD40L/CD40 engagement, along with other activation stimuli (Bennett et al., 1997, 1998; Ridge et al., 1998; Schoenberger et al., 1998; Albert et al., 2001); (iii) DC maturation, often achieved by delivery of adjuvant (Longhi et al., 2009; Tewari et al., 2010; Flynn et al., 2011); (iv) sufficient antigen capture, thus allowing for high occupancy of MHC I (Buckwalter and Srivastava, 2008); and (v) the persistence of cell-associated antigen, which achieves sustained presentation and TCR stimulation (Prlic et al., 2006; Jusforgues-Saklani et al., 2008). While several of these parameters have been well characterized, experimental models typically do not reflect the conditions present during vaccination of humans. In much of the *in vivo* experimental work, strategies have been taken to increase the probability of initial encounter between antigen-specific T cells and DCs presenting their cognate antigen. For example, adoptive transfer has been used to artificially increase the precursor frequency of monoclonal, antigen responsive T cells (Kearney et al., 1994; Kurts et al., 1996; den Haan et al., 2000). The trend, however,

is moving toward physiologic situations with low cell precursor frequency of responding T cells, and recent data has conclusively demonstrated that all phases of T cell activation are influenced by artificially increasing the precursor frequency: they are easier to activate, they expand more rapidly and typically result in greater memory cell differentiation (Marzo et al., 2005; Badovinac et al., 2007; van Heijst et al., 2009). Newly described assays have made it possible to measure low numbers of antigen-specific T cells in naïve mice or during the first days following immunization (Moon et al., 2007; Obar et al., 2008). Nonetheless, consideration has not been given to the artificial dosing of antigen used in these studies (e.g., LPS + peptide), which remain supra-threshold and do not accurately reflect typical vaccination protocols where antigen is limited. Moreover, the question of cross-priming polyfunctional T cells has not been fully evaluated, and again, optimization of vaccine delivery may help enhance therapeutic strategies aimed at the clearance of chronic infection or malignancies.

We report that following injection of cell-associated antigen, targeting of cross-presenting antigen presenting cells (APCs) for the generation of MHC I/peptide complexes is a limiting factor during the priming of the endogenous repertoire. Strikingly, due to the kinetics of antigen capture, local delivery of antigen resulted in a delayed yet ultimately more robust effector T cell activation as compared to systemic delivery of antigen. Our findings also have important implications for the formulation of vaccines combined with adjuvants, thus providing insight into how to best prime an effector $CD8^+$ T cell response.

RESULTS

LOCAL DELIVERY OF CELL-ASSOCIATED ANTIGEN RESULTS IN DELAYED T CELL CROSS-PRIMING

To determine optimal conditions for achieving cross-priming, we compared the effects of immunizing with a local versus systemic dissemination of cell-associated antigen. C57BL/6 mice were injected intradermally (i.d.) or intravenously (i.v.) with splenocytes from H-2 K^{bm1} mice engineered to express a membrane-bound form of chicken ovalbumin in all tissues (referred to as K^{bm1}mOva). Use of membrane associated Ova (mOva) ensured that our model was not confounded by secreted protein captured by endocytosis (Nierkens et al., 2008); and an altered K^b molecule (known as K^{bm1}) ensured a role for host APCs in the cross-priming of $CD8^+$ T cells. In order to precisely monitor the priming of the endogenous T cell repertoire, we utilized K^b–SIINFEKL tetramer-based enrichment, thus allowing precise enumeration and phenotypic analysis of Ovalbumin peptide-specific T cells at early time points after immunization (gating strategy shown in **Figure 1A**). Accumulation of tetramer-positive cells could be observed as early as day 5 for i.v. immunization (**Figure 1B**), with cells showing downregulation of CD62L and expression of CD25 (data not depicted). In contrast, the kinetics of T cell priming was delayed when cell-associated antigen was delivered via the i.d. route. In the latter condition, accumulation of Ova-specific $CD8^+$ T cells was not observed until day 7 post-immunization. For both routes of immunization, antigen-specific T cells accumulated over time, with day 9–12 being the peak of the response (**Figure 1B**).

While prior studies suggest that the precursor frequency of Ova-specific T cells is similar across individual C57BL/6 mice (Obar et al., 2008), it is true that each mouse possesses distinct T cell repertoires (Bousso et al., 1998). In addition, we wanted to confirm that the delayed priming was not a result of the inability to access high affinity Ova-specific T cells. Thus we employed the strategy of adoptive transfer of low numbers (10^3) of monoclonal OT-I cells (Badovinac et al., 2007), transferred 1 day prior to immunization. On day 5, tetramer-based enrichment was performed using a combination of anti-CD45.1 and K^b–SIINFEKL tetramer, thus permitting simultaneous assessment of the transferred $CD45.1^+$ OT-I T cells and endogenous Ova-specific T cells. As shown, only the i.v. immunization resulted in the early priming of Ova-specific T cells. Representative plots are shown, indicating that both the OT-I and the endogenous T cells behaved similarly, and that responses were comparable to those observed in animals that had not received OT-I (**Figure 1C**). Analysis of later time points supported the conclusion that priming is delayed when mice are immunized via the i.d. route (data not shown). Furthermore, we demonstrated that T cell precursor frequency influences the kinetics of priming. Transfer of 10^6 OT-I prior to immunization, in contrast to low transfer conditions, resulted in the robust and rapid expansion of Ova-specific T cells in both i.v. and i.d. conditions (**Figure 1C**). Also evident, the transferred cells outcompeted the endogenous repertoire. These data indicate that there exists a qualitative difference between i.v. and i.d. immunization, which is masked when using adoptive transfer of high numbers of monoclonal T cells.

INTRADERMAL IMMUNIZATION CROSS-PRIMES $CD8^+$ T CELLS WITH GREATER EFFECTOR FUNCTION

To further define the impact of early dissemination of antigen (i.v. immunization) as compared to the establishment of an antigen depot (i.d. immunization), we monitored T cell effector functions. First, we performed an *in vivo* cytotoxicity assay to determine if the expanded T cells possessed cytolytic effector function. At different time points following immunization, mice received targets cells pulsed with SIINFEKL peptide and specific killing was determined (**Figure 2**). We observed a rapid induction of CTL activity after i.v. immunization that began to wane by day 12. Consistent with the delayed expansion after local immunization, we observed a stronger response on Day 12 following i.d. immunization. While both routes of immunization elicit CTL induction, this assay system does not provide *per* cell information about effector activity. To achieve such an analysis, we combined tetramer-based enrichment with intracellular staining. Using this approach, it was possible to determine the absolute number of tetramer-positive $CD8^+$ T cells (**Figure 3A**); as well as the percentage of those cells producing IFNγ (**Figure 3B**). Of note, the absolute number of cells observed in this experiment is lower than those reported in **Figure 1B**, a consequence of performing intracellular cytokine stain, which requires additional washing and fixation steps. By day 7, the number of Ova-specific T cells was similar for the two routes of immunization, with the contraction phase beginning after day 15.

Consistent with the delayed T cell expansion and cytotoxicity test, IFNγ production following i.v. immunization peaked at day

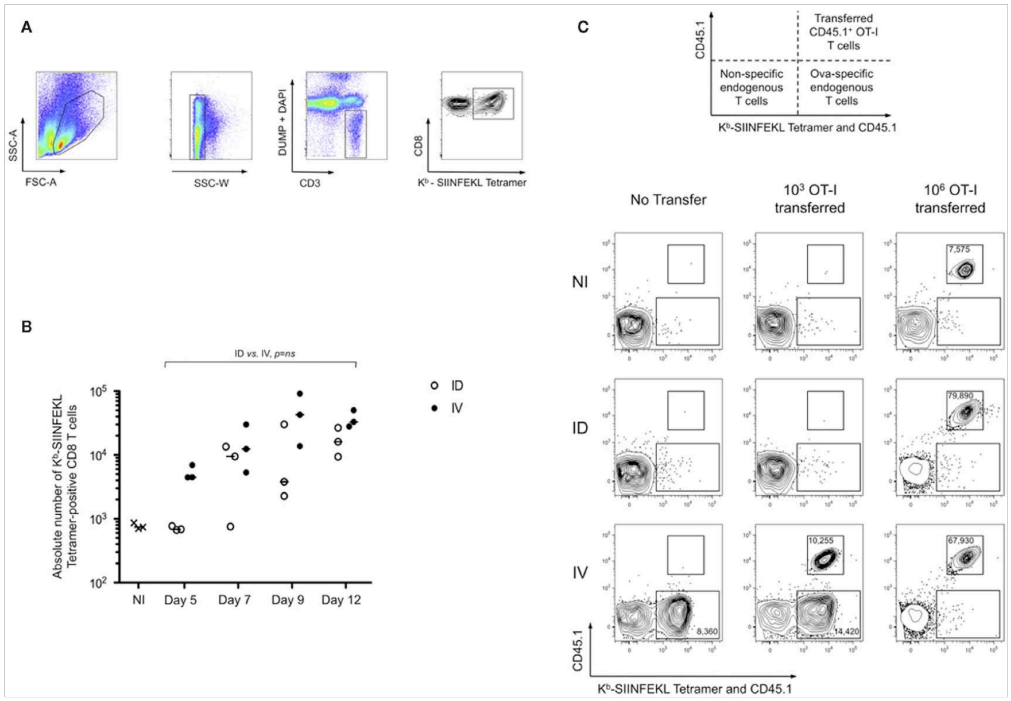

FIGURE 1 | Route of immunization influences the timing of peak T cell cross-priming. (A,B) Mice were immunized intradermally (ID) or intravenously (IV) with 5×10^5 K^{bm1} mOva splenocytes. On days 5, 7, 9, and 12, 15 macroscopic lymph nodes and the spleen were harvested and a K^b–SIINFEKL tetramer-based enrichment was performed. The gating strategy used for tetramer-based enrichment described in the methods is shown **(A)**. Single cells were selected using SSC-W. Then cells were stained with a mixture of antibodies to lineage markers and DAPI to exclude cells that are not of interest (DUMP gate). $CD3^+$ cells were selected and CD8 and K^b–SIINFEKL tetramer labeling were used to detect antigen-specific $CD8^+$ T cells. **(B)** Absolute numbers of Ova-specific CD8 T cells at each of the time points were determined. Data points indicate a single mouse. Results are representative of four independent experiments. The distributions according to the two immunization routes were not significantly different over time employing a general linear modeling analysis. **(C)** To evaluate the skewing of cross-priming responses by adoptive transfer of monoclonal T cells, 10^3 or 10^6 CD45.1 OT-I splenocytes were transferred into CD45.2 recipients prior to immunization. Use of congenic markers allowed simultaneous assessment of transferred and endogenous Ova-specific T cells (schematic representation). On day 5 post-immunization enrichment was performed using both K^b–SIINFEKL tetramer and CD45.1 antibody to distinguish endogenous tetramer-positive cells and OT-I cells. Live $CD3^+$ $CD8^+$ $DUMP^-$ cells are shown. The upper region highlights the transferred OT-I and the lower region marks the endogenous Ova-reactive $CD8^+$ T cells. Absolute cell numbers are indicated for the respective cell populations. Plots were selected from an experiment with three mice per group; Data are representative of three independent experiments.

7, as compared to the i.d. route where the peak response was on day 12. Remarkably, comparing the peak responses indicated that 25–45% of the Ova-specific T cells were producing IFNγ after i.v. injection; whereas 50–70% of the cells were effector $CD8^+$ T cells at the peak of the i.d. response (**Figure 3B**). Representative FACS plots highlight that not only did we achieve a higher percentage of IFNγ producing cells, but also, on a per cell basis, many of the effector T cells were making 10-fold more cytokine as compared to those isolated after i.v. immunization (**Figure 3C**, red gate day 12). This was also evident using a population-based analysis – as shown, the geometric mean fluorescent intensity (MFI) of tetramer-positive cells was significantly higher in the i.d. condition on days 9–15 (**Figure 3D**).

Next, we were interested in characterizing the quality of the T cell response. Prior studies have indicated that cells producing high levels of IFNγ have the unique capacity to secrete multiple cytokines, leading to their being referred to as polyfunctional T cells (Seder et al., 2008). In our model system, we evaluated the simultaneous production of IFNγ, IL-2, and TNFα. Mice were primed using the strategies discussed in **Figure 1** and *ex vivo* restimulation of the tetramer-enriched fraction was performed prior to intracellular staining. As anticipated, the cells producing high levels of IFNγ also expressed TNFα and IL-2 (**Figure 4A**, IL-2 producing cells are shown in red). The response was evaluated throughout the kinetics of T cell priming (**Figure 4A**), and for purposes of comparing i.d. versus i.v. immunization, we focused

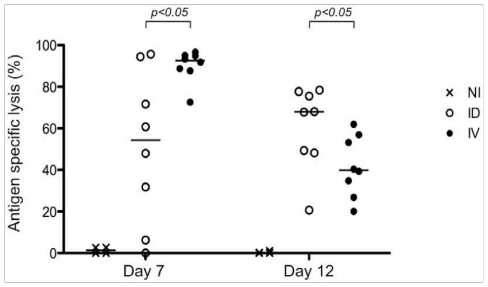

FIGURE 2 | Both i.v. and i.d. immunization result in CTL induction. Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1}mOva splenocytes. At day 7 or 12 post-immunization, an *in vivo* cytotoxicity assay was performed. Antigen-specific killing is reported. *p*-Values were calculated using a Mann–Whitney test (comparing ID versus IV). NI, non-immunized mice, shown here to indicate baseline killing responses.

on the peak of the response: Day 7 for i.v. immunization; and Day 12 for i.d. immunization. The percentages of $IFN\gamma^+$ cells producing the three cytokines – IFNγ, IL-2, and TNFα – was significantly higher after i.d. immunization (**Figure 4B**). The converse was also true – the percentage of cells producing only IFNγ was higher following i.v. immunization (**Figure 4C**). Thus, we conclude that cross-priming via the i.d. route establishes a stronger, polyfunctional response.

LOCAL IMMUNIZATION DOES NOT IMPACT THE DIVERSITY OR THE AVIDITY OF THE T CELL RESPONSE

One potential caveat for the differences observed is that the rate and means of antigen dissemination might influence the diversity of the responding T cell population, with possible consequence on the relative avidity for MHC/peptide complexes (Catron et al., 2006; Zehn et al., 2009). To test this possibility, Ova tetramer-positive $CD8^+$ T cells were FACS sorted, followed by TCR gene amplification and characterization of the distribution of Vβ–Jβ CDR3 length. This method accurately evaluates TCR diversity. 5×10^3 cells per mouse, isolated from five mice per group, were pooled for the analysis. As a control, we purified 25,000 bulk $CD3^+$ $CD8^+$ T cells from a non-immunized animal. Twenty-two Vβ families were detected in both the non-immunized and immunized animals. Data are represented as a profile of the Vβ–Jβ products obtained, plotted in arbitrary intensity units as a function of the size of the DNA fragment (Pannetier et al., 1993). As expected, analysis of the expanded antigen-specific cells in immunized animals showed a non-Gaussian distribution of the peaks as compared to the naïve bulk $CD8^+$ population (**Figure 5A**). Notably, the Vβ 12.1 and 13.1 families were highly represented in the immunized animals, consistent with prior reports (Dillon et al., 1994). (Please note the change in nomenclature – the populations found here correspond with Vβ 5 and Vβ 8, respectively). To determine the diversity of the T cell responses, the number of distinct peaks detected in all immunoscope profiles were determined (**Figure A1** in Appendix). As shown, the number of peaks was significantly reduced in immunized mice with comparable results in the i.v. and i.d. conditions. Given that these results were obtained from pooled mice, there exists the possibility that differences were homogenized and thus not detected; we therefore repeated the experiment using tetramer-positive cells purified from individual animals. Vβ families represented in the primed responses are shown (**Figure A2** in Appendix), and the number of peaks per mouse is plotted (**Figure 5B**).

Next, we evaluated the avidity of the responding T cells by determining their ability to produce IFNγ after restimulation with limiting concentrations of SIINFEKL peptide. Responses were in the linear range for peptide concentrations 10^{-13}–10^{-9}, after which maximal IFNγ production was achieved. No differences were observed when comparing T cells isolated from mice that had been primed via the i.d. versus i.v. route (**Figure 5C**). Based on these data, we concluded that neither the diversity nor the avidity of the Ova-specific $CD8^+$ T cells was influenced by the route of antigen delivery.

INTRADERMAL IMMUNIZATION RESULTS IN DELAYED BUT PERSISTENT ANTIGEN CROSS-PRESENTATION

To further evaluate the differences observed, we determined the relationship between antigen dissemination and antigen presentation by host accessory cells. First, we assessed the establishment of an antigen depot following i.d. immunization. Luciferase-expressing splenocytes isolated from transgenic animals were injected into wild-type recipients. Due to the strain constraints, FvB male mice were used as a source of donor splenocytes, harboring minor histocompatibility differences with the female recipients. Cells delivered via the i.d. route remained primarily localized within the injection site (**Figure A3A** in Appendix). Kinetic studies suggested persistence of donor cells for greater than 13 days (**Figure A3B** in Appendix). Moreover we observed live injected splenocytes in the draining lymph node of i.d. immunized mice and in the spleen of i.v. immunized animals, indicating that there remains intact cell-associated antigen several days after immunization (**Figures A3C,D**).

Functional studies were used to confirm these findings. As described above, mice were immunized with K^{bm1}mOva splenocytes and at different time points, CFSE-labeled $CD45.1^+$ OT-I splenocytes were transferred as a means of assessing cross-presentation by host APCs (**Figure 6**). OT-I transferred prior to immunization and analyzed 3 days later showed significant dilution of CFSE, indicating that cell-associated antigen injected via the i.v. route had already been cross-presented in spleen and lymph nodes (**Figure 6**, cohort 1). Given that up to seven cell divisions could be observed and that the first cell division is thought to require >24 h post-engagement by host DCs (Celli et al., 2005), we suggest that cross-presentation must have occurred immediately following immunization. Antigen presentation persisted from days 3–6 as the second cohort of OT-I also showed dilution of CFSE (**Figure 6**, cohort 2). In contrast to the i.v. condition, for i.d. immunization only minimal OT-I divisions were observed for the first cohort of transferred cells. By day 3–6, the response increased and significant OT-I proliferation could be observed in the draining lymph node, with minor responses in the spleen. These data confirm the local versus systemic dissemination of antigen via the two

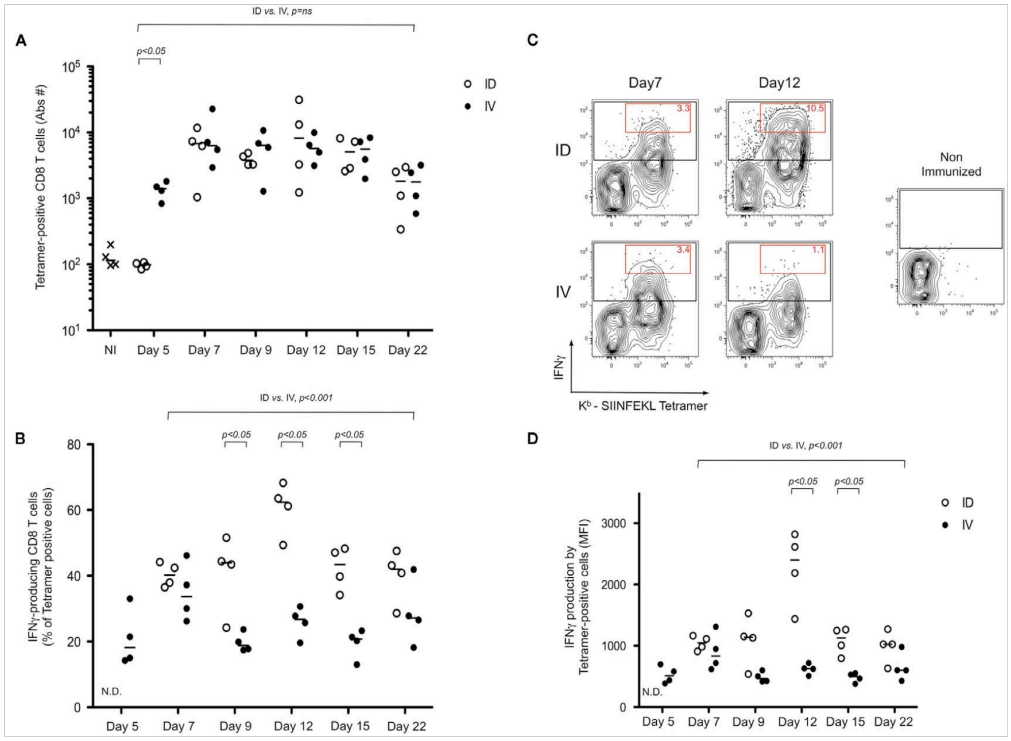

FIGURE 3 | Intradermal immunization results in a more robust differentiation of effector CD8⁺ T cells. (A–D) Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1} mOva splenocytes. Three hours prior to the defined time point, mice were re-stimulated *in vivo* by injecting 5 μg of CpG/DOTAP formulated as a mixture with 1 μg SIINFEKL peptide. K^b–SIINFEKL tetramer-based enrichment combined with an intracellular staining for IFNγ was performed. The absolute number of tetramer-positive cells is reported **(A)**; and the percentage of IFNγ-producing cells among the population of tetramer-positive cells was determined **(B)**. Representative plots of enriched tetramer-positive cells and the respective IFNγ production, per cell, is shown. Data from live, $CD3^+$ $CD8^+$ $DUMP^-$ cells are shown. The red gate highlights the tetramer-positive cells with the higher IFNγ staining and the numbers correspond to the percentage of these cells among the tetramer-positive cells population **(C)**. To represent the respective per cell production of IFNγ, tetramer-positive cells were gated and the geometric mean fluorescent intensity (MFI) is shown **(D)**. Data points indicate a single mouse. N.D., not determined, due to low absolute numbers of cells. Results are representative of two independent experiments. Individual pairings of ID versus IV were assessed by Mann–Whitney test and *p*-values are shown **(A,B,D)**. The global distributions were also evaluated using time as a continuous variable (general linear modeling) **(A,B,D)**.

routes, and helps to explain the delayed kinetics of T cell priming after i.d. immunization.

Unexpectedly, the transfer of a third cohort of OT-I at 21 days post-immunization indicated that when delivered via the i.d. route, antigen was still being presented within the draining lymph node (**Figure 6**, cohort 3). This was not observed in the i.v. condition, suggesting the absence of APCs presenting Ova-peptide. Based on these findings, we conclude that the localized administration of cell-associated antigen impacts the timing of cross-presentation. While i.d. immunization is slightly slower due to the need for antigen to be captured and cross-presented in local lymphoid organs, the sustained presentation of MHC I/peptide complexes could influence effector and memory response.

ADJUVANT DELIVERY MUST OCCUR AFTER ANTIGEN CAPTURE

In instances where microbial associated molecular patterns are absent (e.g., cell-associated antigen), it is common practice to formulate the vaccine with an adjuvant. Following from the result of delayed cross-presentation after i.d. immunization (**Figure 6**, cohort 1), we predicted that the optimal timing of adjuvant delivery will depend on the route of immunization. While adjuvants have been shown to be useful for enhancing the response to an antigen, our hypothesis is based on the observation that DC maturation prior to immunization can have the opposite effect – inhibiting T cell priming due to a failure to phagocytose cell-associated antigen (Wilson et al., 2006). To test our prediction, mice were stimulated using poly I:C, injected at different

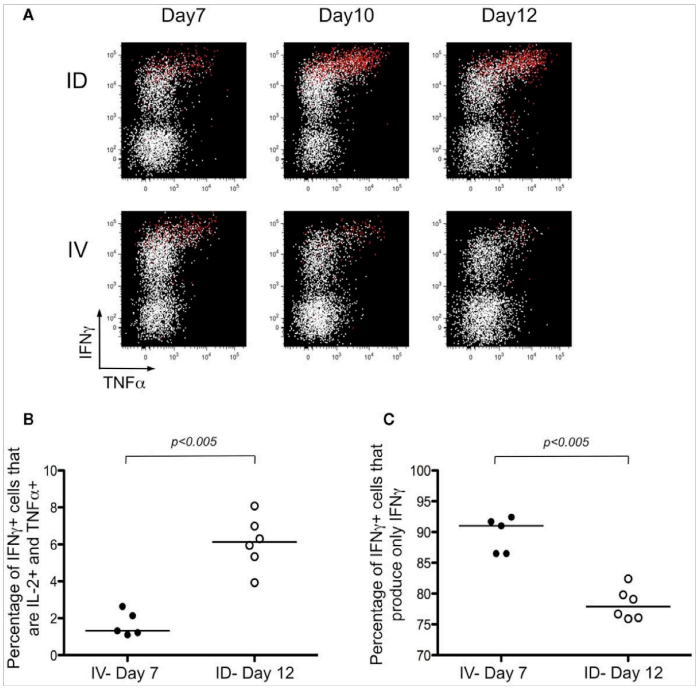

FIGURE 4 | Intradermal immunization induces polyfunctional T cells. **(A–C)** Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1}mOva splenocytes. At the different time points, lymph nodes and spleen were harvested and a K^b–SIINFEKL tetramer-based enrichment was performed. The enriched fraction was incubated for 4 h with SIINFEKL-pulsed splenocytes, followed by surface and intracellular staining. **(A)** Data from live, $CD3^+$, $CD8^+$ T cells are shown. Cells producing IL-2 are highlighted in red. The percentages of $IFN\gamma^+$ cells that produce either the three cytokines – IFNγ, IL-2, and TNFα **(B)** or only one cytokine – IFNγ **(C)** were calculated. *p*-values were calculated using a Mann–Whitney test.

time relative to immunization with antigen. The absolute number of antigen-specific T cells was determined at the respective time of peak response (day 7 for i.v. and day 9 for i.d. immunization). When poly I:C was injected 1 day prior, or the day of i.v. immunization with the K^{bm1}mOva cells, T cell priming was greatly reduced (**Figure 7A**). Strikingly, injection of poly I:C 1 day after immunization enhanced T cell priming for the i.v. route. For the i.d. immunization, poly I:C injection 1 day prior to, the day of, or even 1 day after immunization, resulted in inhibited T cell priming (**Figure 7B**). As shown, it was necessary to wait until day 3 post-immunization to inject poly I:C in order to observe an enhancement of T cell priming (**Figure 7B**). Following from the results in **Figures 6** and **7**, we suggest that 1 day of antigen capture is sufficient to permit T cell priming after i.v. but that additional time is required for antigen capture after i.d. immunization.

To confirm that early delivery of adjuvant inhibited priming due to a failure to capture and present cell-associated antigen, we again utilized adoptively transferred CFSE-labeled OT-I as a read-out. Administration of poly I:C 1 day after i.d. immunization completely blocked OT-I proliferation and IFNγ production (**Figure 7C**). If instead we waited until day 3 post-immunization to administer the poly I:C, we no longer observed a blockade and in fact a greater percentage of OT-I showed maximal cell division and effector function (**Figure 7C**, arrow). To examine precisely the action of poly I:C on host DCs, we performed an *in vivo* kinetic study, enumerating and phenotyping DC populations in the spleen and lymph nodes. We focused on $CD8\alpha^+$ DCs and $CD103^+$ DCs, as these two subsets are known to express TLR3 and have been shown to be required for antigen cross-presentation (Edelson et al., 2010). Following poly I:C injection, we observed a striking decrease in the total number of splenic $CD8\alpha^+$ DCs (**Figure 8A**). Analysis of the remaining cells indicated that CD86 and MHC-II molecules are upregulated within 15 h of injection, indicating that maturation is a rapid process (**Figure 8B**). In contrast to the spleen, DC number in lymph nodes increased after poly I:C injection; and again the cells demonstrated a mature phenotype within 1 day of poly I:C administration (**Figures 8A,B**).

Comparing the timing of poly I:C induced DC maturation (**Figures 8A,B**), with the kinetics of antigen cross-presentation (**Figure 6**), we propose a model to explain the differential impact

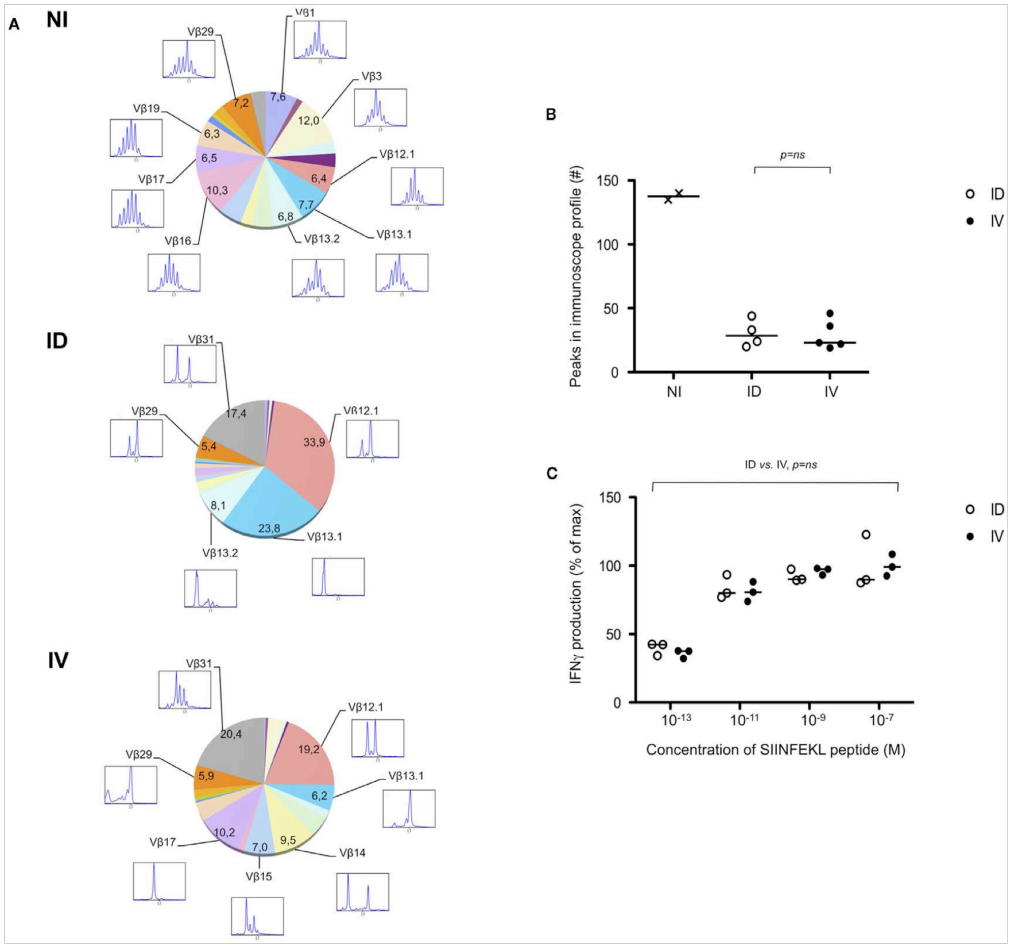

FIGURE 5 | Route of immunization does not influence T cell diversity. (A) Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1} mOva splenocytes. On day 9, tetramer-based enrichment was performed followed by FACS sorting. For the non-immunized (NI) condition, bulk tetramer negative $CD8^+$ T cells were sorted. For immunized animals, cells were sorted from individual mice and 5,000 cells per animal were pooled to obtain 25,000 cells per condition. Immunoscope analysis was performed to define the length of the CDR3 loop in the 24 Vβ families (IMGT nomenclature). Each color represents a distinct Vβ family. The numbers correspond to the estimated percentage of total population. The immunoscope profile is presented for families that represent more than 5% of the total population. **(B)** Immunoscope was performed on cells sorted from individual mice (3,000–5,000 cells sorted/mouse). The total number of peaks detected on all the Vβ profiles was enumerated and represented. Data points indicate a single mouse. Vβ family representation for each animal is shown in **Figure A2** in Appendix. Statistical analysis comparing ID and IV was assessed by Mann–Whitney *U*-test and *p*-values are shown. NI, non-immunized mice, and is shown to indicate baseline diversity of TCR. **(C)** To assess functional avidity of the responding T cells, the draining lymph node and spleen of five mice were pooled, day 8 post-immunization. $CD8^+$ T cells were purified and analyzed by IFNγ ELISPOT. SIINFEKL peptide pulsed DCs were used to re-stimulate $CD8^+$ T cells. SIINFEKL peptide concentrations are indicated. Results are represented as the percentage of maximal IFNγ production. Data points indicate a replicates for each condition. Results are representative of two independent experiments. Percentage of maximum IFN-γ production was analyzed using peptide concentration as a continuous variable (general linear modeling).

of adjuvant delivery, with regards to the route of immunization. Systemic dissemination of cell-associated antigen allows for capture and cross-presentation within 1 day. As such, administration of poly I:C on day 1 serves to stimulate cross-presenting DCs

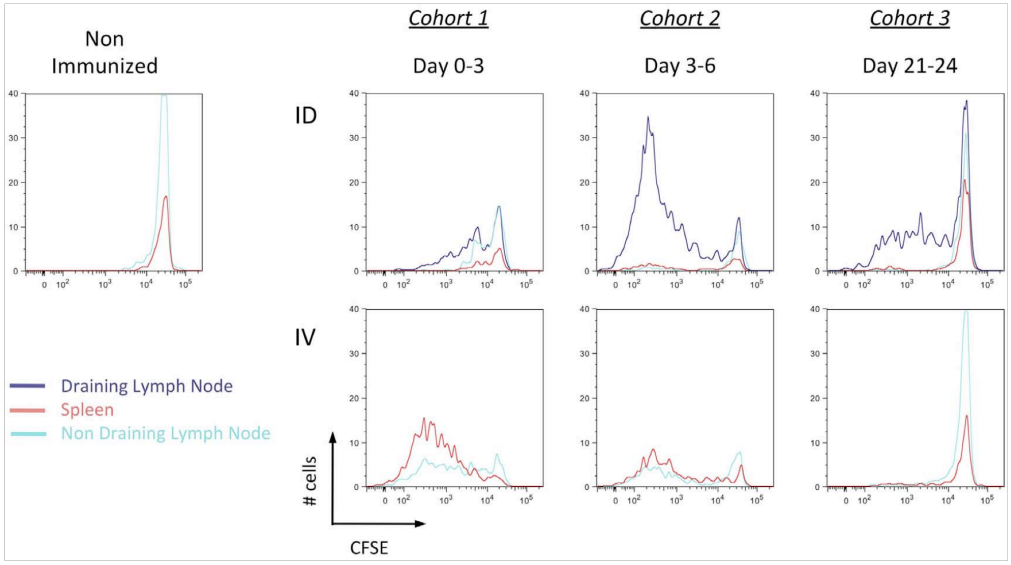

FIGURE 6 | Delayed but persistent antigen presentation in the local draining lymph node after intradermal immunization. Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1}mOva splenocytes. On days 0, 3, or 21, 5×10^6 CD45.1 CFSE-labeled OT-I splenocytes were adoptively transferred into immunized recipients. Three days later, the spleen, draining lymph node and a non-draining lymph node were harvested and the dilution of CFSE staining of OT-I was determined. Results are representative of three independent experiments.

and enhance priming (**Figure 8C**). In contrast, localized delivery of cell-associated antigen requires 3 days for antigen uptake and presentation. Consequently, administration of poly I:C on day 1 results in early maturation of DCs, which are unable to cross-present cell-associated antigen (**Figure 8D**). If instead, adjuvant administration is performed on day 3 it is possible to achieve the beneficial effects of DC maturation, and enhancement of cross-priming is achieved (**Figure 8E**).

EARLY EXPOSURE TO POLY I:C INHIBITS CROSS-PRIMING AND BLOCKS PROTECTIVE IMMUNITY TO *LISTERIA*

To test our model, we evaluated the timing of adjuvant delivery using an infectious model. Mice were immunized i.d. with K^{bm1}mOva splenocytes, and poly I:C was either co-administered on the day of immunization or given 3 days post-immunization. On day 9, mice were challenged with Ova-expressing *Listeria* and 2 days later, the bacterial load was determined in the spleen (**Figure 9A**) and in the liver (**Figure 9B**). We observed that immunization with K^{bm1}mOva splenocytes alone conferred partial protection to *Listeria* challenge. If mice received poly I:C on the day of immunization, this basal level of protection was completely abrogated. In contrast, the protection was significantly improved when poly I:C was administered 3 days after immunization. Indeed, the optimization of adjuvant delivery enhanced priming and resulted in a 2–3 log reduction in bacterial load.

In sum, our study reinforces the need to understand the basis of therapeutic and prophylactic vaccination strategies, taking care to appropriately time the administration of adjuvant in order to effectively coordinate innate and adaptive immune response.

DISCUSSION

ROBUST CROSS-PRIMING AFTER INTRADERMAL IMMUNIZATION

There is considerable interest in the development of vaccine strategies for the priming of $CD8^+$ T cell responses. Stymieing the development of strategies that can be translated to humans is the fact that most experimental models utilize adoptive transfer of T cells and/or delivery of extremely high doses of antigen. Recent advances have solved the problem of detecting rare antigen-specific cells within the endogenous repertoire. Most notably, Moon et al. (2007) combined tetramer labeling and magnetic bead-based enrichment, which permitted the enumeration of T cells with a precursor frequency of 10^{-7} (equivalent to ~10 cells per mouse). This approach has now been applied for the study of both $CD4^+$ and $CD8^+$ T cells, however in these studies the priming conditions used trigger maximal activation of the endogenous repertoire. In our study, we have utilized tetramer-based enrichment to evaluate vaccination strategies that more closely reflect what is done for immunotherapy in humans. Specifically, we evaluated the efficiency of $CD8^+$ T cell cross-priming using cell-associated antigen, testing two important parameters that face investigators interested in initiating adaptive

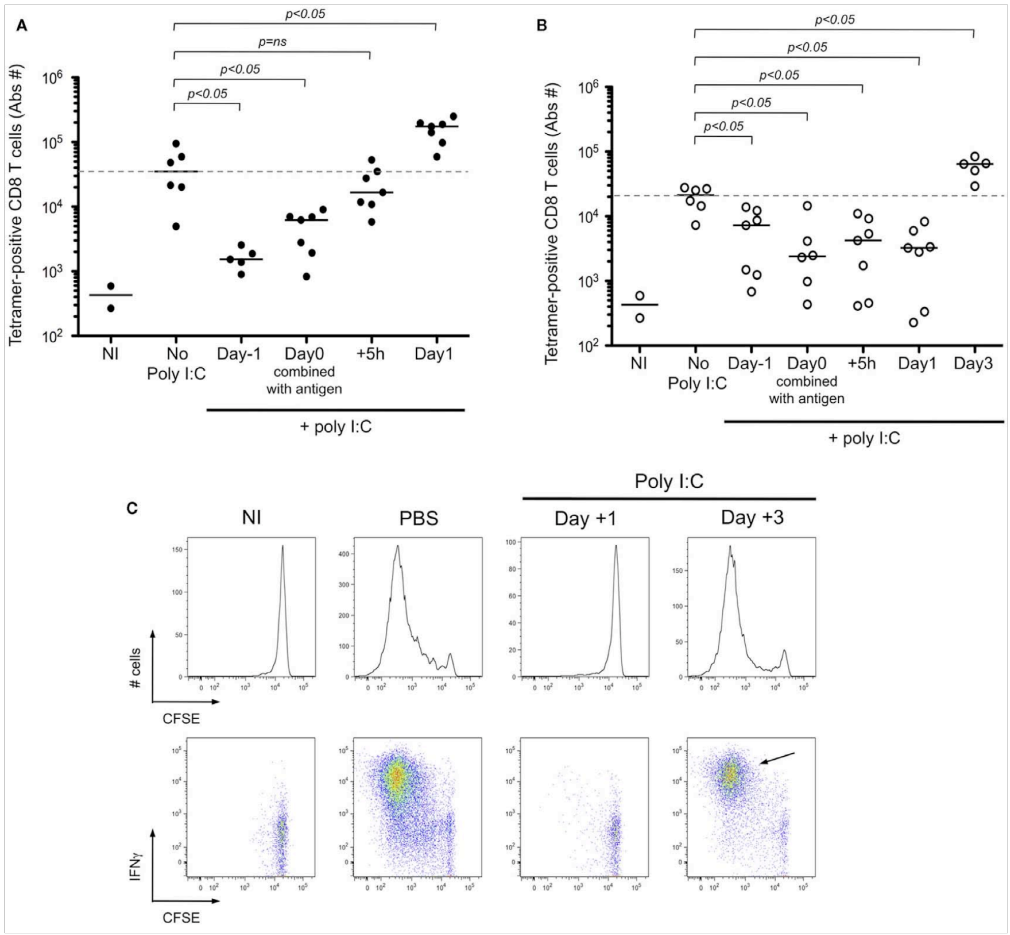

FIGURE 7 | Adjuvant delivery must occur after antigen capture in order to achieve CD8⁺ T cell priming. (A,B) Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1}mOva splenocytes and received 100 μg of poly I:C at indicated time points. For mice immunized i.v., they received poly I:C i.v. either: 1 day before immunization; the day of immunization combined with antigen; 5 h or 1 day post-immunization. The spleen and 15 macroscopic lymph nodes were harvested on day 7, which corresponds to the peak of the CD8+ T cell response. K^b–SIINFEKL tetramer-based enrichment was performed and the absolute numbers of tetramer-positive CD8+ T cells is reported **(A)**. For mice immunized i.d., they received poly I:C at the same time points and one additional group was added, 3 days post-immunization. Poly I:C was administrated i.v. except for the mice injected on day 0 with poly I:C formulated with the antigen. Analysis was performed on day 9 post-immunization, again corresponding with peak CD8+ T cell response **(B)**. *p*-values were calculated using a Mann–Whitney test, comparing in a two-way test, adjuvant condition to no poly I:C treatment. Dotted lines correspond to median number of responding cells in the absence of poly I:C. NI, non-immunized mice are shown to indicate baseline responses. **(C)** Mice were immunized i.d. with 5×10^5 K^{bm1}mOva splenocytes. On day 1 or day 3 post-immunization, 50 μg of Poly I:C or PBS was injected i.v. On day 3 post-immunization, 5×10^6 CFSE-labeled CD45.1 OT-I splenocytes were transferred i.v. Three days later the draining lymph node was harvested and the dilution of CFSE staining of OT-I was determined, represented by the histograms. Intracellular staining for IFNγ was performed at the same time, shown in the corresponding FACS plots. $CD3^+$ $CD8^+$ $CD45.1^+$ cells were gated for the analysis shown. Data are representative of three independent experiments. NI, non-immunized mice.

immune responses – the route of vaccination and the use of adjuvants. Importantly, there already exist therapeutic vaccines that closely reflect the model system studied herein (Fontana et al., 2009).

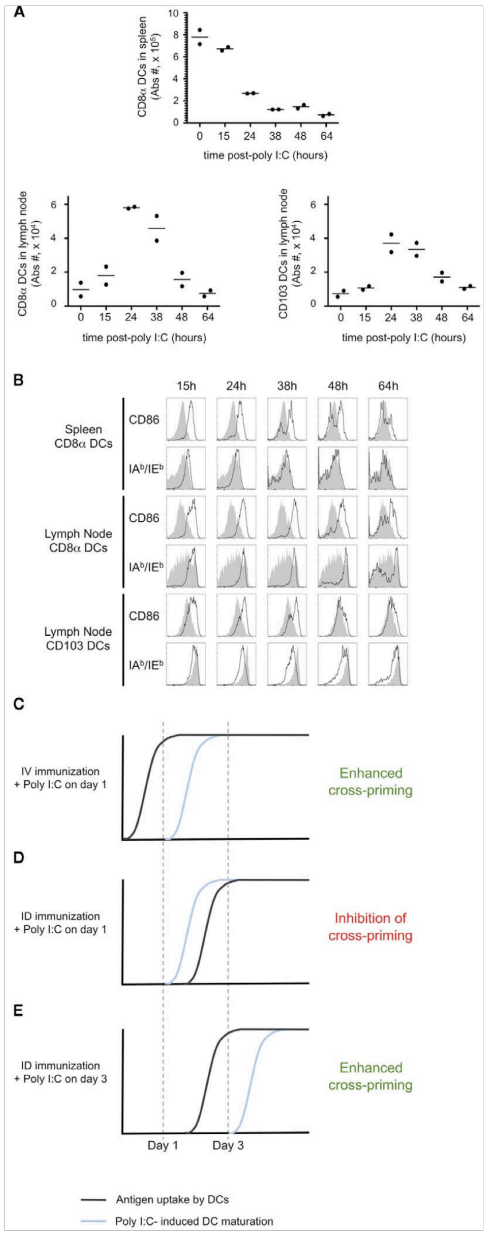

FIGURE 8 | Continued

FIGURE 8 | Poly I:C induces rapid DC maturation. Mice were injected i.v. with 100 μg of Poly I:C. At defined time points, the spleen and a lymph node were harvested. The total numbers of CD8α$^+$ DCs and CD103$^+$ DCs per organ **(A)** and the expression of CD86 and IA^b/IE^b **(B)** were determined. In **(B)** the gray histograms indicate the level of expression in untreated animals and the black line corresponds to poly I:C-injected mice. **(C–E)** Proposed model to explain the different effects of Poly I:C depending on the timing of delivery. The proposed timing of antigen uptake (black line) and the kinetic of DC maturation upon Poly I:C injection (blue line) are represented for three different conditions.

We chose to administer donor splenocytes derived from K^{bm1}mOva mice as the source of antigen: this ensured the need for antigen transfer to host DCs; excluded the possibility that secreted antigen or peptide exchange could account for the generation of MHC I/peptide complexes; and obviated the requirement for a danger signal as live cells expressing a mutated K^b are efficient sources of antigen for cross-priming (Krebs et al., 2009). While we support a role for phagocytosis of donor cells as a means of antigen transfer, an alternative possibility is the spread of antigen via exosomes produced by living cells (Wolfers et al., 2001). In our study, comparison of the intradermal and intravenous routes permitted us to determine the outcome of local versus systemic dissemination of antigen. As expected, systemically disseminated antigen resulted in rapid cross-presentation (**Figures 1** and **6**, cohort 1), which correlated with early differentiation of effector antigen-specific T cells (**Figures 2** and **3**). This was in contrast to locally administered antigen, which showed delayed cross-presentation and expansion of responding T cells.

Although delayed, one of the interesting features of locally administered antigen is that it acted as an antigen depot (**Figure A3** in Appendix). Our data indicates that persistent antigen cross-presentation by host DCs (**Figure 6**, cohort 3) correlates and likely is the mechanism for inducing a more robust priming of polyfunctional effector CD8$^+$ T cells (**Figures 3** and **4**). Interestingly, the magnitude of the T cell response following i.v. immunization was similar to that of the i.d. route. Thus, we conclude that the route of immunization impacted T cell quality but not primary expansion, highlighting the importance of providing in-depth study of vaccine candidates using the endogenous repertoire as a read-out for successful priming. Based on prior patient studies and experimental models of HIV, *Leishmania major* and *Mycobacteria tuberculosis* the T cell quality appears important for efficient host response and control of the infectious agent (Almeida et al., 2007; Darrah et al., 2007; Precopio et al., 2007).

TIMING OF ADJUVANT DELIVERY HAS A PROFOUND IMPACT ON CROSS-PRIMING EFFICIENCY

Concerning the timing of cross-priming via the i.d. and i.v routes, we do not argue that the observed differences are not simply academic, nor do we consider that achieving T cell cross-priming 2 days earlier is going to improve vaccination strategies. Instead, it is our contention that the timing of antigen capture and T cell engagement has a profound impact on the appropriate timing for adjuvant delivery. Clearly, there is interest to coordinate

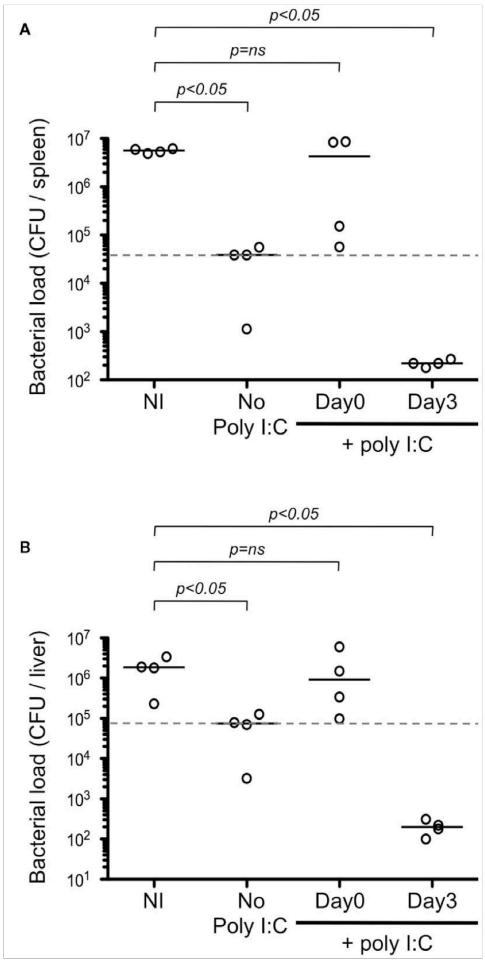

FIGURE 9 | The differential effects of adjuvant impact protection against *Listeria*. Mice were immunized i.d. with 5×10^5 K^{bm1}mOva splenocytes. Poly I:C was administered either the day of immunization or 3 days later. On day 9 post-immunization, mice were challenged with 5×10^5 CFU of Ova-expressing *Listeria*. Two days later, the spleen **(A)** and the liver **(B)** were harvested and bacterial load per organ was determined. NI, non-immunized mice. Dotted lines correspond to median CFU in the absence of poly I:C. Mann–Whitney test *p*-values were calculated, comparing immunization condition to the NI control.

both innate and adaptive responses, but a careful evaluation of how to optimally administer adjuvant and antigen is required. In our studies we chose to evaluate poly I:C, a synthetic double-stranded RNA (dsRNA) that engages endosomal TLR3 and MDA/5 on stromal cells (Longhi et al., 2009). It can induce IFNα/β and IL-12p70 by DCs and has been reported to be a superior adjuvant for T cell priming (Longhi et al., 2009). In addition, the similar expression pattern of host sensors in mice versus human make poly I:C a more attractive adjuvant for study in experimental models as compared to CpG (Rehli, 2002). Poly I:C has been tested as a direct therapeutic agent in the setting of viral infection and cancer; and has also been used as an *ex vivo* maturation agent for DC adoptive cell therapy trials. Several formulations of poly I:C are under late stage testing, including Ampligen (HemispheRx) and Hiltonol (Oncovir, Inc.) (Nicodemus and Berek, 2010; Rosenfeld et al., 2010; Flynn et al., 2011; Okada et al., 2011).

While poly I:C is considered a proinflammatory adjuvant, previously studies have also reported that pre-treatment of animals with poly I:C inhibited antigen cross-presentation (Wilson et al., 2006). The contrasting action of poly I:C remains poorly understood and the mechanism of action governing these polar phenomena has not been explored. Herein, we demonstrated that administration of poly I:C 1 day post-intravenous immunization resulted in enhanced cross-priming, however the same timing of administration resulted in a blockade for intradermal injection (**Figure 7A**). Consistent with the need for DCs to capture antigen prior to adjuvant administration, poly I:C given on day three enhanced the cross-priming of $CD8^+$ T cells following local antigen delivery (**Figure 7B**). We showed that poly I:C induces DC activation as established by upregulation of CD86 and MHC-II expression 1 day after administration (**Figure 8**). Together, these data establish that in order to enhance cross-priming poly I:C must be delivered at a time point after the host DCs have captured the injected cell-associated antigen (**Figures 8C–E**).

Results of recent clinical trials that combine delivery of antigen and adjuvant indicate the importance of defining the optimal time of innate immune stimulation. Using the same NY-ESO-1 protein preparation, delivered locally in the skin, it was observed that co-administration of adjuvant permitted efficient cross-priming, whereas pre-conditioning of the injection site diminished the ability to stimulate antigen-specific T cells (Valmori et al., 2007; Adams et al., 2008). There have also been studies showing that injection of RNA vaccine in combination with innate stimulation is not always the best strategy to achieve efficient priming. First, Carralot and colleagues showed that the delivery of GM–CSF 24 h after RNA injection enhanced T cell priming (Carralot et al., 2004). Importantly, this adjuvant effect was not observed when GM–CSF was delivered in combination with RNA. Moreover, in a follow-up study from Diken et al., it was shown in experiments comparable to ours that subcutaneous delivery of 20 μg poly I:C, 1 day prior to RNA injection intranodally, abrogated the uptake of RNA vaccine (Diken et al., 2011). Taken together with the observations we have made in mice, we suggest that there is a trade-off between stimulating innate receptors in immature DCs for purposes of triggering an inflammatory response and the resulting decrease in antigen capture that is due to the induction of DC maturation. One option might be the use of agonists that bind receptors selectively expressed on mature DCs (e.g., CD40L; Lanzavecchia, 1998).

OPTIMAL T CELL PRIMING BY INTRADERMAL INJECTION

Our studies highlight the importance of considering the timing and persistence of antigen presentation, and suggest intradermal injection with delayed adjuvant delivery to be the optimal strategy for achieving $CD8^+$ T cell cross-priming. While many studies of $CD8^+$ T cell priming conclude with a remark about how important their findings are for predicting efficient means of vaccinating humans, our efforts have a true possibility to be translated into practice. For example, Russo and Fontana have conducted pre-clinical and clinical studies utilizing peripheral blood lymphocytes genetically modified to express tumor antigens as a strategy for inducing tumor immunity in cancer patients (Russo et al., 2007; Fontana et al., 2009). In their treatment protocols, patients received five bi-weekly, i.v. infusions of escalating numbers of autologous lymphocytes, reaching doses of 5×10^8 total lymphocytes infused (range: $2–7 \times 10^8$). Their clinical trial was not designed to assess efficacy; nonetheless, it was possible to observe clinical responses in 3/10 patients, which correlated with priming of Mage-3 specific $CD8^+$ T cells (Fontana et al., 2009). These studies, as well as others, highlight the feasibility of utilizing cell-associated antigen as a means of immunizing patients. It also points to the need for relevant mouse models aimed at optimizing strategies for achieving robust $CD8^+$ T cell cross-priming.

In sum, we demonstrate that, while slower, local injection of cell-associated antigen resulted in the differentiation of a polyfunctional effector cell response during the T cell priming. Our studies also highlight the importance of considering the timing and persistence of antigen presentation, and suggest intradermal injection with delayed adjuvant delivery to be the optimal strategy for achieving $CD8^+$ T cell cross-priming. While we hope this study will impact vaccine design for prophylaxis and therapy, it is clear that in the latter situation additional investigations will be required in order to overcome intrinsic suppressive and/or regulatory mechanisms that limit the success of immunotherapy strategies.

MATERIALS AND METHODS

MICE

C57BL/6J wild-type mice were obtained from Charles River. $Ptprc^aPepc^b$/BoyJ (CD45.1) and Tg(TcraTcrb) 1100Mjb (OT-I $Rag^{+/+}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice expressing membrane-bound full-length Ova under an actin promoter were a gift from Dr. M. Jenkins (University of Minnesota, USA) and the cross onto the H-$2K^{bm1}$ line was performed by Dr. S. Schoenberger (LIAI, USA). All mice were maintained and bred in a SPF helicobacter-negative facility, and used under approved protocols. In all experiments, 6- to 12-week-old mice were used.

REAGENTS

Antibodies for FACS analysis were obtained from BD Biosciences, Biolegend, or eBiosciences (**Table A1** in Appendix). Antibodies used in the IFNγ–ELISPOT assays were purchased from Mabtech. The Ovalbumin H-$2K^b$ epitope SIINFEKL peptide was obtained from Polypeptide Group. Monomers were prepared using a modified version of that described (Altman et al., 1996) and tetramerization was performed prior to use, using PE–Streptavidin (Invitrogen), added for 1 h at 25°C. Intracellular cytokine staining was done using the Cytofix/Cytoperm/Brefeldin-A kit (BD Biosciences). Poly I:C and CpG ODN2216 were purchased from Invivogen. DOTAP was obtained from Roche. Labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE) was performed using the Vybrant cell tracer kit from Invitrogen. To label dead cells, DAPI or Aqua-Live/Dead Fixable Dead Cell Stain kits from Invitrogen were used after tetramer-based enrichment.

INJECTIONS

Splenocytes used for immunization were isolated from K^{bm1}mOva mice. 5×10^5 cells in a volume of 100 µl were injected intradermally (i.d.) or intravenously (i.v.). The intradermal injection was performed in the right flank with the inguinal lymph node being the draining lymph node. For OT-I transfer, bulk splenocytes were isolated from CD45.1 OT-I mice. 10^3, 10^6, or 5×10^6 splenocytes were transferred i.v. depending on the experiment. For injection of Poly I:C, 100 µg of Poly I:C was injected i.v. in a final volume of 100 µl. For *in vivo* restimulation before intracellular staining, 5 µg of CpG is diluted in PBS and DOTAP; this was then formulated with 1 µg of SIINFEKL peptide and injected i.v. in a volume of 100 µl.

TETRAMER-BASED ENRICHMENT

Leukocytes were harvested from 15 lymph nodes and the spleen. Cells were Fc-Blocked with anti-CD16/CD32 antibody and stained with PE-labeled K^b–SIINFEKL tetramers in PBS containing 2% FCS and 0.1% of Sodium Azide for 30 min at 4°C. It was followed by an incubation with anti-PE magnetic microbeads (Miltenyi). Cells were passed over a magnetic LS column to enrich tetramer-positive cells. Bound cells were eluted ("enriched" fraction). Five microliter aliquot was collected for precise counting of the bound fraction. Cells were stained with a mixture of antibodies (CD11c, CD11b, CD4, NK1.1, F4/80, B220, CD3, and CD8) to exclude cells (DUMP gate) and focus on $CD8^+$ T cells (see **Figure 1A**). Prior to analysis, DAPI was added to mark dead cells. Cells were analyzed using a FACS Canto II (BD Biosciences). Live, non-clumped, $CD3^+$ $CD8^+$ tetramer-positive cells were gated. The percentage of tetramer-positive cells was multiplied by the total number of cells in the enriched fraction to obtain the total number of tetramer-positive $CD8^+$ T cells.

TETRAMER-BASED ENRICHMENT COMBINED WITH INTRACELLULAR STAINING

For *in vivo* restimulation, mice were injected with 5 µg of CpG/DOTAP formulated as a mixture with 1 µg SIINFEKL peptide 3 h prior to leukocyte harvest. Next, the tetramer-based enrichment was performed with the addition of Brefeldin-A during each incubation step. After the elution step, enriched cells were stained with Aqua as a dead cell marker, incubated with surface staining antibodies and fixed. Next, cells were permeabilized and

stained using anti-IFNγ as per the manufacturer's instructions (BD Biosciences). For *ex vivo* restimulation, the tetramer-based enrichment was performed first and the eluted fraction was incubated 4 h with SIINFEKL-pulsed splenocytes at 37°C. Then cells were stained for IFNγ, IL-2, and TNFα as per the manufacturer's instructions (BD Biosciences).

DETERMINING PERSISTENCE OF H-2K^b–SIINFEKL/MHC–PEPTIDE COMPLEXES

CD45.1 OT-I splenocytes were isolated and stained using 5 μM CFSE in PBS. After washing with ice-cold PBS 5×10^6 OT-I splenocytes were injected i.v. into immunized mice. Three days later the draining and non-draining lymph nodes, and the spleen were harvested. Organs were processed independently and cells were labeled with CD8β and CD45.1 antibodies allowing for the identification of the transferred CD8 OT-I T cells and the determination of CFSE intensity.

IFNγ ELISPOT

Spleen and the draining lymph node were harvested and CD8$^+$ T cells were purified using anti-CD8 microbeads and MS columns (Miltenyi). IFNγ ELISPOT assays were performed as previously described (Blachere et al., 2006). The ELISPOT plate evaluation was performed in a blinded fashion by an independent evaluation service (Zellnet Consulting) using an automated ELISPOT reader (Carl Zeiss).

IMMUNOSCOPE

K^b–SIINFEKL tetramer-positive CD8$^+$ T cells were sorted using a FACS Aria-II. Total RNA was prepared from sorted T cells using the Total RNA Miniprep kit (Sigma), and cDNA was synthesized using the SuperScript™II Reverse Transcriptase (Invitrogen). The different Vβ germline genes can be clustered in 24 families according to their level of homology (IMGT nomenclature). For quantitative repertoire, PCR reactions were carried out by combining a reverse primer and a specific fluorophore-labeled probe for the constant region (MGB–TaqMan probe) with 1 of 24 primers covering the different Vβ chains (**Table A2** in Appendix). Real-time PCR reactions were subsequently carried out with a final concentrations of 400 nmol/L of each oligonucleotide primer, 200 nmol/L of the fluorogenic probe, and FastStart master Mix (Roche). Thermal cycling conditions comprised Taq DNA Polymerase activation at 95°C for 10 min, then subjected to 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. For all these different reactions, real-time quantitative PCR was then performed on an ABI-7300 system (Applied Biosystems). The relative usage of each Vβ family was calculated according to the formula:

$$U\left(V\beta\gamma\right) = \sum_{x=1}^{x=24} 2^{\left(C_t(x) - C_t(y)\right)}$$

$C_t(x)$ is the fluorescent threshold cycle number measured for the Vβγ family. For immunoscope profiles, products were then subjected to run-off reactions with a nested fluorescent primer specific for the constant region (**Table A2** in Appendix: Fam-primer) – run for a total of three cycles. The fluorescent products were separated and analyzed using an ABI-PRISM 3730 DNA analyzer. The size and intensity of each band were analyzed with "Immunoscope software" (Pannetier et al., 1993), which has been adapted to the capillary sequencer. Fluorescence intensities were plotted in arbitrary units on the *y*-axis, and CDR3 lengths (in amino acids) on the *x*-axis.

CYTOTOXICITY *IN VIVO*

At different time points following the immunization, mice were injected i.v. with 5×10^6 CD45.1 splenocytes stained with 0.5 μM CFSE and pulsed with SIINFEKL peptide, and 5×10^6 CD45.1 splenocytes stained with 5 μM CFSE and left unpulsed. Fifteen hours later, spleen was harvested and cells were stained with an anti-CD45.1 antibody. The lysis of injected splenocytes was determined using the CFSE staining and the percentage of specific lysis was calculated.

DC PHENOTYPE

Spleen and lymph node were digested with Collagenase D (Roche) and Dnase (Invitrogen). Cells were stained for CD11c, CD11b, CD8α, CD103, CD86, IA^b/IE^b, and analyzed by flow cytometry. An aliquot was used to determine the absolute number of cells per organ.

LISTERIA INFECTION

The Ovalbumin-expressing *Listeria* is a kind gift from N. Glaichenhaus. Mice were infected i.v. with 5×10^5 colony forming units (CFU). Two days later, the spleen and the liver were harvested and mashed in NP-40 0.2% in water, and serial dilutions were plated to determine the CFU per organ.

STATISTICAL ANALYSIS

Data was plotted with bars representing median value. We used non-parametric (two-tailed) Mann–Whitney test to compare the distributions between two conditions. In some instances, selective comparisons between two groups within a multi-parameter experiment were also performed using non-parametric Mann–Whitney test. Continuous measurements were studied over time or according to the peptide concentration using general linear modeling. Statistical analysis was performed using Stata 11 software (StataCorp, College Station, TX, USA) and Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

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APPENDIX

Table A1 | Antibodies used for flow cytometry experiments.

Antigen	Clone	Isotype	Fluorochrome	Company
cd3ε	145-2C11	Hamster IgGl, κ	PerCP–Cy5.5	BD Pharmingen
CD4	RM4-5	Rat IgG2a, κ	Pacific blue	BD Pharmingen
CD8α	53-6.7	Rat IgG2a, κ	Alexa fluor 700	BD Pharmingen
CD8α	53-6.7	Rat IgG2a, κ	PerCP–Cy5.5	BD Pharmingen
CD8β	H35-17.2	Rat IgG2b, κ	APC	eBioscience
CDIIb	MI/70	Rat IgG2b, κ	eFluor 450	eBioscience
CDIIc	N418	Hamster IgG	eFluor 450	eBioscience
CDIIc	HL3	Hamster IgGl, λ	APC	BD Pharmingen
CD45.1	A20	Mouse IgG2a, κ	PE	BD Pharmingen
CD45.1	A20	Mouse IgG2a, κ	APC	BD Pharmingen
CD45.1	A20	Mouse IgG2a, κ	Pacific blue	Biolegend
CD86	GL1	Rat IgG2a, κ	FITC	BD Pharmingen
CD103	M290	Rat IgG2a, κ	PE	BD Pharmingen
NK1.1	PK136	Mouse IgG2a, κ	Pacific blue	Biolegend
B220	RA3-6B2	Rat IgG2a, κ	Pacific blue	BD Pharmingen
F4/80	BM8	Rat IgG2a, κ	eFluor 450	eBioscience
IA/IE	M5/114.15.2	Rat IgG2b, κ	Alexa fluor 700	eBioscience
IFNγ	XMG1.2	Rat IgGl, κ	APC	BD Pharmingen
IL-2	JES6-5H4	Rat IgG2b	Alexa fluor 488	BD Pharmingen
TNFα	MP6-XT22	Rat IgGl	PE–Cy7	BD Pharmingen

Table A2 | Sequences of the primers used for the immunoscope analysis.

Vβ1	TCACTGATACGGAGCTGAGGC
Vβ2	GCCTCAAGTCGCTTCCAACCTC
Vβ3	CACTCTGAAAATCCAACCCAC
Vβ4	ATCAAGTCTGTAGAGCCGGAGGA
Vβ5	CTGAATGCCCAGACAGCTCCAAGC
Vβ12.1	AAGGTGGAGAGAGACAAAGGATTC
Vβ12.2	CATTATGATAAAATGGAGAGAGAT
Vβ12.3	AGAAAGGAAACCTGCCTGGTT
Vβ13.3	CATTACTCATATGTCGCTGAC
Vβ13.2	TTCATATGGTGCTGGCAGCACT
Vβ13.1	TGCTGGCAACCTTCGAATAGGA
Vβ14	AGGCCTAAAGGAACTAACTCCAC
Vβ15	GATGGTGGGGCTTTCAAGGATC
Vβ16	GCACTCAACTCTGAAGATCCAGAGC
Vβ17	TCTCTCTACATTGGCTCTGCAGGC
Vβ19	CTCTCACTGTGACATCTGCC
Vβ20	CCCATCAGTCATCCCAACTTATCC
Vβ21	CTGCTAAGAAACCATGTACCA
Vβ23	TCTGCAGCCTGGGAATCAGAA
Vβ24	AGTGTTCCTCGAACTCACAG
Vβ26	ACCTTGCAGCCTAGAAATTCAGT
Vβ29	TACAGGGTCTCACGGAAGAAGC
Vβ30	CAGCCGGCCAAACCTAACATTCTC
Vβ31	ACGACCAATTCATCCTAAGCAC
Reverse primer	GGTAGCCTTTTGTTTGTTTGCAA
MGB–Taqman probe	AGCCATCAAAAGCA
Fam-primer	CTTGGGTGGAGTCACATTTCTC

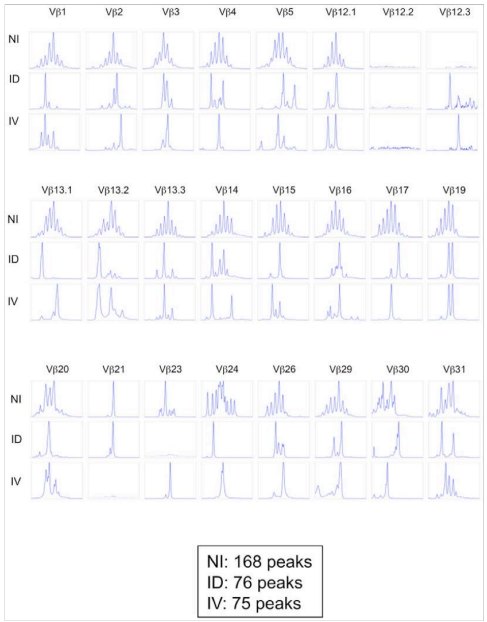

FIGURE A1 | Immunoscope profiles from mice immunized i.d. or i.v. Mice were immunized i.d. or i.v. with 5×10^5 K^{bm1}mOva splenocytes. On day 9, 15 macroscopic lymph nodes and the spleen were harvested and a K^b–SIINFEKL tetramer-based enrichment was performed for each mouse. CD8 K^b–SIINFEKL tetramer-positive cells were sorted and pooled to obtain 25,000 cells per condition (corresponding to five mice per group). For the non-immunized condition (NI), CD8+ T cells were sorted. An immunoscope was performed to detect the 24 Vβ families (IMGT nomenclature). The immunoscope profile was shown for each Vβ family. The total number of peaks is indicated for each condition. Of note the values indicated in this Figure are higher than those reported in Figure 5B as the former represent pooled mice.

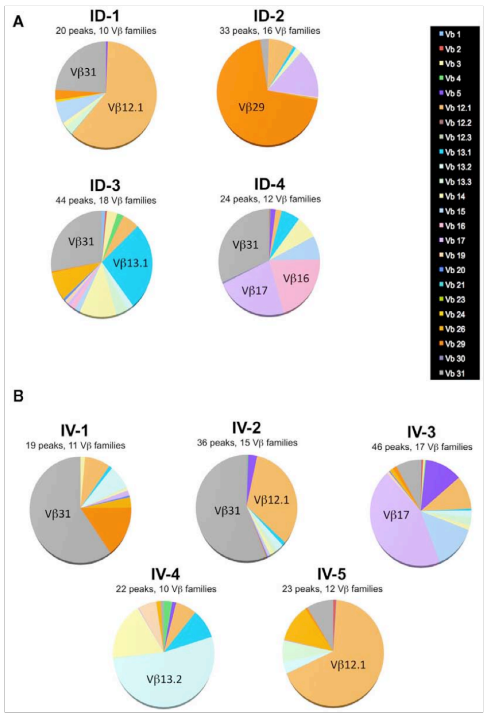

FIGURE A2 | Single mouse analysis confirms that route of immunization does not influence T cell diversity. Mice were immunized i.d. **(A)** or i.v. **(B)** with 5×10^5 K^{bm1}mOva splenocytes. On day 9, 15 macroscopic lymph nodes and the spleen were harvested and a K^b–SIINFEKL tetramer-based enrichment was performed for each mouse. CD8 K^b–SIINFEKL tetramer-positive cells were sorted. Immunoscope was performed on cells sorted from individual mice (3,000–5,000 cells sorted per mouse) to define the length of the CDR3 loop in the 24 Vβ families (IMGT nomenclature). Each color represents a distinct Vβ family.

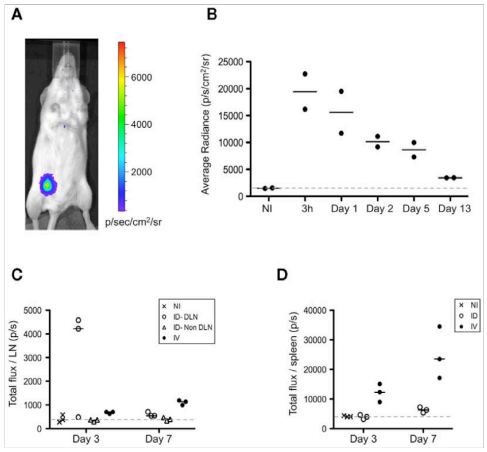

FIGURE A3 | Intradermal injection of splenocytes results in a local depot of donor cells. (A,B) FVB/N female recipients were immunized i.d. or i.v. with 5×10^6 FVB/N-luciferase$^+$ male splenocytes. **(A,B)** Following immunization, mice injected i.d. were evaluated at 3 h and then on day 1, 2, 5, and 13. Prior to imaging, mice were injected i.p. with 3 mg of D-luciferin (Synchem), followed by isoflurane inhalation to keep animals sedated during analysis. Bioluminescence imaging was performed by using an IVIS Lumina II system (Caliper Life Sciences). Images from mice were acquired over 10 min. Quantification of the light emission was analyzed using Living Image Software version 3.1 (Xenogen Corporation), expressed in photons/s/cm^2/steradian. **(A)** Representative bioluminescence analysis performed at 3 h is shown. **(B)** Kinetic analysis of the bioluminescent signal is shown. **(C,D)** On days 3 and 7, the LNs **(C)** and the spleen **(D)** from mice immunized i.d. or i.v. were harvested and placed in wells containing PBS and D-luciferin to determine the total bioluminescent signal from each organ. The bioluminescence is expressed as the total flux/organ in photons/s. DLN, draining lymph node.

Tracking antigen-specific CD8$^+$ T cells using MHC Class I multimers

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Running title: MHC Class I multimers

Summary

The tracking of epitope-specific T cells is a useful approach for the study of adaptive immune responses. This protocol describes how Major Histocompatibility Complex Class I (MHC-I) multimers can be used to stain, enrich and enumerate (rare) populations of $CD8^+$ T cells specific for a given antigen. It provides the detailed steps for multimer labeling, magnetic enrichment and cytometric analysis. Additionally, it provides informations for multiplexing experiments in order to achieve simultaneous detection of multiple antigenic specificities, and strategies for coupling the protocol with functional assays (e.g., intracellular cytokine staining). Future developments in cytometric systems (e.g., mass spectroscopy-based cytometry) and gene expression studies (e.g., single cell PCR) will extend these approaches and provide an unprecedented assessment of the immune repertoire.

Key Words: $CD8^+$ T cells, MHC Class I multimers, Antigen-specific T cells, T-cell receptor

1. Introduction

Mature $CD8^+$ T lymphocytes bear αβ T cell receptors (TCR) that are specific for a major histocompatibility complex (MHC) class I molecule bound to a unique peptide. A major goal in the study of adaptive immune responses is to understand the developmental progression of antigen-specific T cells from naive precursors to activated effector cells and long-lived memory cells [1,2]. Prior to 1996, limiting dilution analysis was the standard method for estimating the frequency of antigen specific T cells. The major limitation of this approach is the requirement for exogenous stimulation and expansion, introducing potential bias and significant inter-assay variability. Notably, cloning efficiency is typically <40%, suggesting that the assay necessarily underestimates precursor frequencies [3]. Other techniques such as ELISpot or intracellular cytokine staining (ICS) are based on the ability of antigen-specific T cells to secrete cytokines upon short *in vitro* restimulation with the cognate peptide [4,5]. Such approaches identify lymphocytes possessing the capacity to secrete a given cytokine at the time of the assay, however this represents only a fraction of the antigen-specific population(s).

The generation of MHC class I tetrameric or multimeric complexes (referred to herein as MHC multimers), originally described by Altman and Davis [6,7], represents a major technical advance for the study of T cell repertoires. MHC multimers are reagents that carry multiple MHC Class I / peptide (MHC-I / pep) complexes, and thus have the ability to interact with multiple TCRs on a single $CD8^+$ T cell (**Figure 1**). Fluorescent-labeling of MHC multimers permits identification of antigen-specific T lymphocytes based on the avidity of their TCR, independent of their functional or differentiation state. This technology has been recently reviewed by Davis, Altman and Newell [8]. Using MHC multimers, it is now possible to directly track and quantitate antigen-specific T cells during the course of immunization [9]. And by co-staining with antibodies directed against phenotypic cell surface proteins, one can define subsets of cells of interest based on their activation or differentiation state, or chemokine receptors expression [10]. MHC multimer technology has also been successfully coupled to conventional functional assays (e.g., CFSE dilution, ICS), and specific T cells can be sorted for ELISpot, cytotoxicity, gene expression studies or for generating long-term cultures [11]. Multimers are also widely used in the immune monitoring of T cell responses following therapeutic or prophylactic vaccination. Finally, the recent availability of GMP-grade multimers is enabling the *ex vivo* expansion of T cells for immunotherapy [12-15].

Several key improvements have been reported since the initial description of multimer technology [16,8,17]. First, as multimerization is the key to overcoming the relatively low intrinsic affinity of TCR/MHC interaction, MHC-I multimers now exist as tetramers, pentamers and dextramers (with the latter containing >10 MHC I / pep complexes). Monomer production has also been substantially optimized. Most notable is the work of Schumacher and colleagues, who demonstrated the possibility to generate high-throughput production of MHC I / pep complexes using a photo-destructible peptide that permits an exchange reaction with peptides of interest [18]. Additionally, the implementation of a dump channel, dual tetramer labeling and multiplexing have all helped establish a robust foundation for translating this technology into the clinics [18-21]. Nonetheless, there exist remaining technical limitations: staining methods, analysis protocols, validation and data sharing have to be standardized [22]; and the limit of detection for standard multimer assays is 10^{-4}, which does not allows for direct detection of rare antigen-specific populations such as naive ones [23].

In order to improve the limit of detection, MHC multimer staining has recently been combined with magnetic bead enrichment [24], a concept initially developed in mice for assessment of $CD4^+$ T and $CD8^+$ T cells [25-27]. Following from these studies, our lab, as well as others, developed a similar enrichment protocol for human $CD8^+$ T cells (**Figure 2**) [23,28]. Efforts have been made to standardize the procedure - herein described in details - and to optimize any details in order to achieve sufficient sensitivity to allow detection of naive antigen-specific T cells from human peripheral blood. This protocol permits up to 100-fold increased detection of antigen-specific populations, allowing assessment of populations with frequencies as low as 10^{-6}. As such, it is now possible to characterize the naive T cell repertoire, opening up new opportunities for defining how T cells are selected, as well as to investigate aspects of their homeostasis [29]. These approaches may also serve as powerful strategies for tracking rare antigen-experienced self-, tumor-, transplant- or microbe-specific T cells, either in mice or in humans, in turn providing insight into parameters that shape immune T-cell responses. This unit describes our method for labeling antigen-specific $CD8^+$ T cells obtained from mice or from human peripheral blood with MHC class I multimers, for enriching and enumerating them, and eventually multiplexing the assay and/or coupling it to intracellular cytokine staining (ICS) procedure. Future developments in cytometric systems (e.g., mass spectroscopy-based cytometry) and gene expression studies (e.g., single cell PCR) will further extend these approaches and provide an unprecedented look at the immune repertoire [8,11].

2. Materials

2.1. Common reagents

1. Fresh or frozen sample (for mice, see **Section 3.1;** for humans, prepare PBMCs according to standard procedures (see **Note 1**))
2. For humans studies only: fluorescently labeled mAb specific for MHC class I molecules of interest, suitable for flow cytometry (for example anti-human HLA A2 antibody, BD Biosciences), and corresponding isotype (see **Note 2**)
3. 60mm-Petri dish
4. Falcon 15mL tube
5. 5mL FACS tubes
6. FcR blocking reagents
7. Anti-PE microbeads (see **Note 3**)
8. MACS separation columns, magnets, stands (see **Note 4**)
9. BD Falcon Cell Strainer 70µm
10. More than 5-colors flow cytometer, ideally with possibility to cell sorting

2.2 Buffers

1. PBS
2. PBS-2%FCS
3. Human Pulldown Buffer (HPB; ~50 mL for one enrichment sample): PBS 1X, 5% of Human Serum Albumin 20% (final concentration 1%), 5% Citrate Dextrose Anticoagulant (see **Note 5**)
4. Mice Pulldown Buffer (MPB): PBS 1X, 2% FCS, 0.001% Sodium Azide
5. Mice Pulldown Buffer (MPB) without azide
6. Mice R-10 buffer: RMPI, 10% Fetal Calf Serum, 10mM HEPES, 1x Non Essential Amino-Acids, 1mM Sodium Pyruvate, 60nM 2-Mercaptoethanol, 20 ng/mL Gentamycin

2.3. Flow cytometry

1. PE- and/or APC- labeled MHC class I multimers (see **Note 6 and 7**)
2. For multiplexing experiments (determination of multiple specificities in one single tube), biotinylated monomers (see **Note 8**) and streptavidin coupled to fluorochrome or reporter of interest (PE-, APC-, PE-Cy7-, APC-Cy7-, Qdots-streptavidin, 1mg/mL)
3. Cocktail of fluorescently labeled mAb that are known to be expressed on cells you wish to exclude from analysis (e.g., monocytes, B cells, and NK cells) (see **Note 9**). These mAb should be coupled to a common fluorochrome, for example Pacific Blue,

thus giving a positive signal in one fluorescent channel, which will be referred to as the « dump channel » in our gating strategy (see **Section 3.5** and **Figure 5A**)
4. Viability marker that will specifically stain dead cells (e.g., DAPI Nucleic Acid Stain, Invitrogen) (see **Note 10**)
5. Fluorescently labeled mAbs including at least an anti-CD8 antibody. Others will be chosen depending on the desired phenotypic characterization of target T cells (see **Note 11**)
6. For Intracellular Cytokine Staining (ICS) in mice: CpG formulated with DOTAP, and specific peptide for *in vivo* restimulation
7. For ICS, BDCytofix/Cytoperm Fixation/Permeabilization solution kit with BD GolgiPlug containing Brefeldin A (BD biosciences)
8. For ICS, LIVE/DEAD fixable dead cell stain kit such as Aqua (Invitrogen) (see **Note 12**)

3. Methods

Please note that we describe in this section both mice and human protocols, which suppose the reader to be careful to specific human or mice reagents and buffers.

For human, you will start with HLA typing (**Section 3.2**), then stain with multimer(s) (**Section 3.3**), optionally pursue by enrichment (**Section 3.4**), and finally acquire your samples on flow cytometer (**Section 3.5**) and evaluate precursor frequency (**Section 3.6**).

For mice, you will start with mice dissection (**Section 3.1**), then stain with multimer(s) (**Section 3.3**), and optionally pursue the experiment by enrichment (**Section 3.4**), and/or intracellular cytokine staining (**Section 3.7**). In all cases you will acquire your samples on flow cytometer (**Section 3.5**), and evaluate precursor frequency (**Section 3.6**).

3.1 Mice dissection

1. Harvest 15 lymph nodes (2 inguinal, 2 axillary, 2 brachial, 4 cervical- deep and superficial, 2 peri-aortic, and the mesenteric chain) and the spleen in a 60mm-Petri dish containing 2 mL of Mice R-10 buffer.
2. Mash the organs and transfer the cells into a Falcon 15mL tube after filtering the cell suspension with a 70µm cell strainer.
3. Wash the well with 3x1 mL Mice R-10 to recover the maximum of cells.

4. Add 10 mL of Mouse Pulldown Buffer (MPB), count them, spin down at 300g for 5 min at 4°C, and go to **Section 3.3**.

3.2 HLA typing (human)

1. Generic haplotyping of the sample can be easily achieved by flow cytometry, and is sufficient for most multimer uses.
2. Count PBMCs and resuspend in PBS at 10^7 cells/mL.
3. Dispense 2x50 µL of this solution into 5mL FACS tubes. The remaining cells will be spinned down (300g, 5 min, 4°) and used for multimer staining (**Section 3.3**).
4. Add either isotype or anti-HLA antibody titrated to the optimal concentration to each FACS tube (optimal Cf=1/400 in our hands, meaning that you put 1 µL of a solution diluted 1/8 in 50 µL staining volume).
5. Incubate for 15 min at 4°C in the dark.
6. Wash cells once at 300g for 5 min at 4°C, and resuspend in 100 µL of PBS.
7. Acquire these 100 µL in flow cytometry (**Figure 3**).

3.3 Multimer staining

1. Use cells prepared on **Section 3.1** (mice) or **3.2** (human). Resuspend cells in cold Pulldown Buffer (MPB for mice or HBP for human, hereafter referred as PB), and dispense defined numbers of cells in Falcon 15mL tubes (one for each specificity) (see **Note 13**).
2. Wash once in PB (300g, 5min, 4°), and resuspend each sample in 90 µL cold PB.
3. Add 10 µL of FcR Blocking Reagent to each tube. Vortex.
4. Incubate 10 min at 4°C.
5. Add PE MHCI multimer and APC MHCI multimer of the same specificity at the appropriate concentration (see **Notes 14** and **15,** and **Figure 4**).
6. If needed, it is possible to multiplex the experiment (*i.e.* determine multiple specificities - up to 25 - within one single tube) by preparing each specific multimer with a unique combination of two different colors [21]. In the case you want to simultaneously enrich your target populations with antiPE microbeads, one of these two colors will have to be PE (see **Note 16** and **Figure 6A**).
7. Vortex gently and incubate 30 min at 4°C (see **Note 17**).
8. Wash once in 2 mL PB, spinning at 300g for 5 min at 4°C.
9. If you stop here, transfer your cells into 5mL FACS tubes, spin, resuspend in 90 µL of PBS-2%FCS, and proceed directly to flow cytometry analysis on **Section 3.5**. Otherwise, you can follow the procedure by enrichment (**Section 3.4**).

3.4 Enrichment

1. To start the enrichment protocol, resuspend labeled cells obtained in **Section 3.3** (step 8) in 400 µL PB.
2. Take a 10 µL aliquot of labeled cells, and place it into 5mL FACS tubes. Complete with 90 µL with PBS-2%FCS. This gives you your « Pre-enriched » fraction.
3. To the cells used for enrichment, add 100 µL of anti-PE microbeads (see **Note 18**).
4. Vortex and incubate for 20 min at 4°C in the dark.
5. Wash twice in 2 mL cold PB, spinning cells at 300g for 5 min at 4°C.
6. During the washing step, prepare MACS columns (one per Falcon 15mL tube) on a magnet support (see **Note 4**). Rinse each column with PB (discard elution). Label Falcon 15 ml tubes for collecting the flow through fraction.
7. Resuspend each sample in 1 mL PB, and load the column.

   It is important to filter cells just prior to loading on the column in order to remove any clumped cells.

8. Wait until the sample has completely passed through the column bed.
9. Add 1mL of PB to the initial Falcon 15mL tube (wash step to get every last cell).
10. Load column with this fraction.
11. Wait until the sample has completely passed through.
12. Collect first flow-through fraction and load it on the same column a second time (again, an effort to capture all multimer labeled cells).
13. Again, wait until the sample has completely passed through the column bed.
14. Wash the column with 3x1 or 2x3 mL of PB (for MS and LS columns respectively).
15. Wait until the sample has completely passed through: the collective liquid in the collection tube (flow through fraction) is your « Depleted fraction ».
16. Remove one column at a time. Place it in a corresponding labeled Falcon 15mL tube. Add 2-5 mL (for MS and LS columns respectively) of PB to the upper fraction of the column. Push the plunger using steady pressure.
17. Gently remove the plunger.
18. Add again 2-5 mL of PB to the upper fraction of the column.
19. Push the plunger. The collective liquid (total volume is 4-10 mL) is considered the « Enriched fraction ».
20. Spin Depleted and Enriched fractions at 300g for 5 min at 4°C.
21. For the Depleted fraction (see **Note 19**):
    a) Resuspend in 1 mL of PBS-2%FCS.
    b) Aliquot 90 µL in one 5ml FACS tube and add Ab mix.
    c) Incubate 20 min at 4°C in the dark.
    d) Wash in 3 mL PBS-2%FCS at 300g for 5 min at 4°C.
    e) Resuspend in 300 µL PBS-2%FCS.
22. For the Enriched fraction, you can either continue with ICS (proceed to **Section 3.7**) or prepare your samples for flow cytometry analysis on **Section 3.5**:
    a) Resuspend in 90 µL PBS-2%FCS.
    b) Add your Ab mix directly into the Falcon 15mL tube.
    c) Incubate 20 min at at 4°C in the dark.
    d) Add 1 mL of PBS-2%FCS; transfer to 5mL FACS tubes.
    e) Add 1 mL of PBS-2%FCS to the initial Falcon 15mL tube.

f) Transfer this 1 mL to the same 5mL FACS tubes.
g) Spin at 300g for 5 min at 4°C.
h) Resuspend in 300 µL PBS-2%FCS.

(examples for mice and human data are provided in **Figure 5B**)

3.5 Flow cytometry

1. If you came directly from **Section 3.3**, add your mAb mix and incubate 20 min at 4°C in the dark (see **Note 20**)

   Wash in 3 mL PBS-2%FCS at 300g for 5 min at 4°C.

   Resuspend in 300 µL PBS-2%FCS

2. If you have pursued with enrichment on Section 3.4, your Depleted and Enriched fractions are now ready to be analyzed.
3. Add DAPI to each sample just prior to acquisition (Cf=1/5000; 3 µL of solution 1/50 in 300 µL of cells)
4. Set stopping gate at 2 000 000 events on Single cells ($SSC\text{-}A^{low}SSC\text{-}W^{low}$)
5. Importantly, acquire all samples for Enriched fraction (add PBS twice) (see **Note 21**)
6. Gating strategy: SSC-A *vs*. SSC-W to exclude doublets; Dump *vs*. CD3 to isolate viable pure CD3; CD3 *vs*. CD8 to gate on $CD3^+CD8^+$; Multimer-PE *vs*. CD8 gated on CD3 to have background evaluation; Multimer-PE *vs*. CD8 gated on CD8 to have percentages; any further phenotypic analysis on Multimer-PE^+ cells (**Figure 5A**)
7. If you enriched multiple specificities, you will gate on $CD8^+PE^+$ multimer positive cells, then discriminate antigen specificity from another by gating on double positive T cells: $PE^+color\text{-}A^+$ will be T cells with specificity A, $PE^+color\text{-}B^+$ will be T cells with specificity B, … (see **Note 22** and **Figure 6B**)

3.6 Precursor frequency

1. To determine the size of the epitope-specific populations within each sample, we recommend a precise calculation, initially proposed by Moon *et al* [26].
2. The absolute number of total CD8 T cells within any sample is determined using the following equation: absolute number of $CD8^+$ T cells = (number of $CD8^+$ T cells acquired in the pre-enriched sample) x [(total number of PBMCs in the pre-enriched sample) / (total number of cells acquired in the single cell gate in the pre-enriched sample)].

3. The absolute number of multimer-positive T cells is the number of multimer-positive cells within the « single, live, non-dump $CD3^+CD8^+$ » T-cell gate present in the enriched fraction. (see **Note 23**)
4. The frequency of circulating multimer-positive cells is defined as the absolute number of multimer-positive T cells / absolute number of $CD8^+$ T cells.

3.7 Intracellular Cytokine Staining (optimized for mice)

1. Restimulation of cells is performed *in vivo*. Three hours prior to leukocyte harvest, inject mice intravenously with 5µg of CpG/DOTAP formulated as a mixture with 1µg specific peptide (e.g. SIINFEKL peptide in the Ovalbumin model).
2. Perform the staining and enrichment as described in **Sections 3.3 and 3.4** with addition of BD GolgiPlug containing Brefeldin A during multimer and beads incubation steps (final concentration 1/1000).
3. After elution from the column, resuspend enriched cells in MPB without azide, add Aqua fluorescent reactive dye (final concentration 1/1000) to stain dead cells (see **Note 12**) and incubate 30 min at 4°C in the dark.
4. Spin 5 min at 300g at 4°C in 3 mL MPB without azide.
5. Resuspend cells in 100 µL MPB without azide; add the mix of antibodies for surface staining, and incubate 20 min at 4°C in the dark.
6. Wash with 3 mL of MPB without azide and resuspend thoroughly cells with 250 µL of Cytofix/Cytoperm reagent. Vortex and incubate 20 minutes at 4°C.
7. Wash with 1 mL of 1xPerm/wash buffer.
8. Incubate cells for 30 min at 4°C with anti-IFNγ antibody diluted in Perm/Wash buffer.
9. Wash cells once with Perm/Wash buffer and once with MPB without azide.
10. Resuspend in 300 µL PBS-2%FCS and acquire sample in flow cytometry (**Figure 7**).

4. Notes

1. Although the protocol described here focuses on antigen-specific T cells harvested from human peripheral blood and from mice, similar procedures can be applied to other non-human samples [25-27] and to other tissues (e.g., tumor infiltrating lymphocytes, TILs). For human peripheral blood, prepare PBMCs using Ficoll separation. For tissue-based applications, we recommend including a CD45 staining in one of the channels in order to segregate CD45-positive hematopoietic cells, and decrease noise in the assay.

2. Although the protocol described here focuses on HLA A2 individuals as example, it can be applied to any HLA specificity without modification. Moreover, enrichment protocols would also be applicable for $CD4^+$ T cell, NK-T and γδT cell populations, using respective multimer reagents.

3. Although the protocol described here is based on the combination of PE-labeled multimers and anti-PE microbeads, you can similarly stain with APC-labeled multimers and enrich with anti-APC microbeads.

4. When establishing the assay on human samples, we found a better recovery of rare specific T cells when using MS columns, regardless of the number of loaded PBMC used ($1x10^7$-$4x10^8$ starting cell populations tested). Exceptions concern TILs, for which you need to use LS columns in order to avoid clumps and blockage of the column. Similarly, for mouse experiments, LS columns are recommended due to potential of stromal tissue from lymph nodes and spleen to clog the columns.

5. This recipe was chosen based on our experience in the lab. Other conventional sorting buffers can be used, but may result in slightly different background signals. Note that sodium azide should be omitted if planning to cultivate the cells or perform functional assays.

6. Concerning MHC multimers, there are two options. Commercial vendors exist and will sell off-the-shelf reagents as well as generate custom materials. Providers include Beckman Coulter, ProImmune and Immudex. The alternative and recommended option is to prepare your own monomers, thus facilitating multimerization with streptavidin coupled to your desired fluorescent

tag. This approach allows you to work with the same multimer labeled in different colors, thus improving specificity of the assay (see **Note 14**) and permits multiplexing different specificities in the same tube (see **Notes 16 and 22**). Note that regardless of the source, high-quality monomers are important, with monomer purity impacting multimerization. Moreover, the choice of streptavidin reagent is critical, and it is recommended to purchase high-quality streptavidin conjugated to bright fluorochromes.

7. When using MHC multimers, it is recommended to choose an appropriate method to validate the specificity of tetramer-stained cells. Positive controls will be multimers targeting abundant populations of CD8 T cells. For human, EBV $BMLF1_{280-288}$ or Influenza A-$M1_{58-66}$ can be used as a positive control. For mice, the strategy will be to stain splenocytes from a TCR-transgenic mouse with the corresponding multimer (CD8 OT-I T cells that are specific for H2-K^b-SIINFEKL complexes stained with H2-K^b-SIINFEKL multimer for instance). It will help you to establish the assay and ensure that enrichment is sufficient for detection of rare cells. Concerning naïve cells in humans, $MART1_{26-35(Leu27)}$ is a good choice, as it will also be a useful reference for establishing gating parameters for naïve *vs*. memory populations [30]. Negative control tetramers can be employed to help establish the assay, although there is the potential to observe $CD8^+$ T cells with the capacity to bind any MHCI, including self-antigens. An alternative option is the evaluation of background staining based on the non-specific labeling of $CD4^+$ T cells. That said, recent work has suggested that even this interaction might be of physiologic relevance [28]. More definitive controls are also important, such as assessment of TCR CDR3-variable region usage skewing, peptide-induced TCR downregulation and, after cell sorting, TCR sequences analysis or TCR genes transfer into immortalized cell lines to show that the specificity can be reconstituted [8].

8. Biotinylated monomers may be stored for months at -80°C. Stability testing is recommended. In contrast, multimers are less stable, should be stored at 4ºC and ideally should be used within 4 weeks. Best is even to multimerize the amount you will need for each experiment one day before.

9. The use of a « Dump channel » is essential as it excludes cells that bind non-selectively to the MHC multimer reagents. Its composition has to be reviewed in the context of the experimental aims. For

example, CD56 is useful for exclusion of NK cells in human samples when evaluating naive cell repertoires, but should be used with caution when studying human memory or activated T cell populations as some cells express CD56 and would thus be lost in the gating strategy. Similarly, it can be useful to add anti-CD33 and anti-CD34 antibodies when studying T cell populations present in bone-marrow populations. As indicated above, we deliberately do not included CD4 as we use the staining of this population as an assessment of background, but this can be added when multiple free channels are needed for complex multicolor experiments. In the same way, the Dump channel must to be chosen carefully for mouse experiments: some markers, such as CD11c, may in fact upregulate on activated T cells.

10. Addition of a viability marker is necessary in order to avoid non-specific background staining on dead cells. While this may be omitted in some instances in which fresh blood is utilized, it should be noted that the enrichment columns have an affinity for dead cells. Ideally, select a viability dye in the same channel as the Dump channel – thus keeping the maximum number of channels free for phenotypic characterization.

11. At least four fluorescent channels are necessary for careful assessment of enriched T cells: (i) a Dump channel; (ii) CD3 staining for gating on T cells; (iii) CD8 staining for gating of $CD8^+$ T cells; and the multimer-conjugated label for the specificity of interest. This can be extended when differently labeled multimers are included in the same experiment (for reducing non-specific binding in the assay and/or for multiplexing enrichment). Anti-CD3 can eventually be omitted if you really need a maximum of free channels for phenotypic characterization. Additional channels that are available will depend upon the technical specifications of the cytometer and antibodies will be chosen depending on the experimental questions being evaluated. Note that it is crucial to stain with multimers prior to washing and staining with other Abs, especially for CD8 and CD4, as some clones have been shown to influence multimer staining [31].

12. As cells will be fixed to perform intracellular staining, DAPI cannot be used as a viability marker. We therefore recommend the use of a fixable live/dead cell marker such as Aqua. Note that Aqua labeling has to be done in azide-free buffer.

13. The starting number of cells is a critical point. It is required in order to calculate precursor frequency; and in most instances it is the determinant of the limit of sensitivity for the assay. For example, if you suppose your population to be around 10^{-6} (meaning 1 cell into 10^6 CD8), you will need to start from at least 10^7 cells to maximize the possibility of achieving a well-defined multimer-positive population. Of note, during the enrichment procedure, cell loss is in the range of 10 – 30%.

14. Staining cells with two multimers sharing the same specificity but labeled in different colors permits a further decrease in the non-specific binders [21]. We and others strongly recommend to include dual labeling, especially if your aim is to detect ultra-rare populations of cells of variable avidity. Of course, if you want to pursue with enrichment with anti-PE microbeads, one of the two colors need to be PE. Otherwise, you can use any fluorochrome combination (up to 25) [21], as soon as you titrate both streptavidin and multimers before use, and be cautious with settings and compensations.

15. Concentration of multimers is another important parameter to consider. Optimal concentrations must be defined for each multimer by titrating on specific cell lines. In general, we recommend working at high concentrations, *i.e.* 10-20nM = 3-10μg/mL final concentration (NB: PE Tetramers $\underline{\sim}$ 500kDa; APC Tetramers $\underline{\sim}$ 350kDa). Of note, the receptor density of TCR on responding T cells is also critical. While not thoroughly validated, it may be of interest to evaluate exposure to the protein tyrosine kinase inhibitor dasatinib prior to staining and/or enrichment as a means of enhancing tetramer binding [31].

16. For example, if you aim to stain and enrich T cells specific for CMV, Influenza A, and MART1 specific populations at the same time, you will put in the same tube, at the same incubation step, the following multimers: PE-CMV, PE-Cy7-CMV, PE-Flu, APC-Flu, PE-MART1, APC-Cy7-MART1 (see **Note 22** and **Figure 6A**).

17. Temperature is a critical parameter in multimer staining. It is advisable to assess the effects of temperature (and time) for each individual system. In our assays, MHC I staining is performed at 4°

for 30 min. For staining at room temperature, be cautious with respect to internalization of multimers – which might interfere with the enrichment procedure.

18. Please note that the number of beads used here is not adjusted to the number of targeted cells but rather fixed at a high level, sufficient for enrichment of rare or common populations.

19. We recommend to systematically analyze the Depleted fraction, at least during assay optimization in order to evaluate cell loss during the procedure (usually 10-30% in our hands). .

20. All reagents should be titrated before use.

21. To ensure you can detect rare specific T-cell populations, it is important to start with sufficient number of cells (see **Note 13**), and acquire sufficient number of events [32].

22. In the example provided above (see **Note 16** and **Figure 6**), after gating on $CD8^+$ PE-multimer-positive T cells (which will contain the four T cell specificities), CMV-specific T cells may be segregated based on PE-Cy7 positivity; Flu-specific cells will be stained with APC; and MART1-specific T cells will be APC-Cy7 labeled. With the increased number of available fluorescent channels (e.g., 18-parameter cytometer), one can theoretically combine up to 10 enriched specificities at the same time, the difficulty being the optimization of compensation settings. The quality of the enrichment procedure, and capacity of detecting rare events will in fact also depend on ones experience using the cytometer.

23. This calculation is based on the hypothesis that you recover all epitope-specific T cells while acquiring your Enriched sample and should therefore be considered as the lower limit of precursor frequency. However, this requires rigorous adherence to the protocol, and in particular the standardization of wash steps, and consistent acquisition of the Enriched sample. Mixing studies with known input numbers of monoclonal TCR transgenic cells in wild-type congenic mix, or T cell clones into HLA-mismatched PBMCs may be used to evaluate efficiency and establish in-house criteria [25,23].

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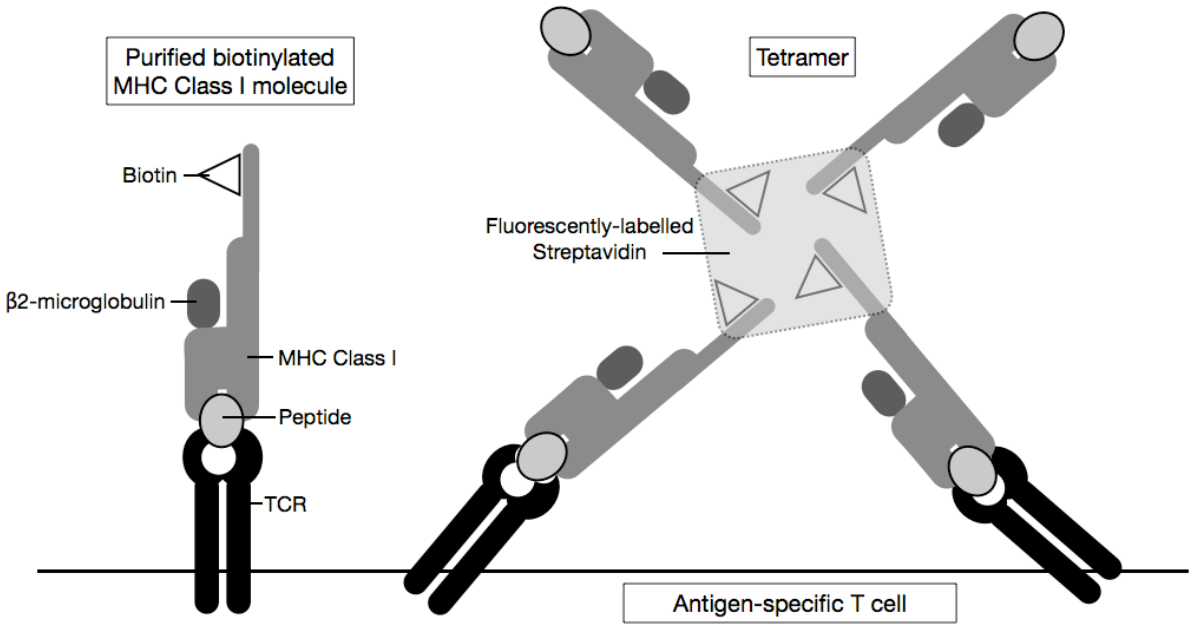

Fig.1. Schematic representation of purified biotinylated MHC Class I molecule (left) and multimer (right) (adapted from Klenerman *et al.* (**9**)).

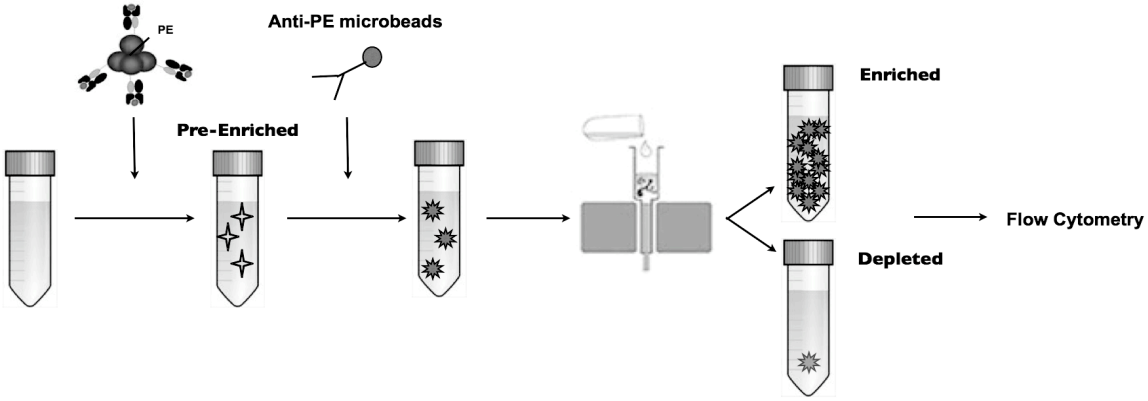

Fig.2. Schematic representation of the enrichment procedure. Starting from cellular suspension, cells are stained with PE-labeled multimer, then incubated with antiPE-microbeads before loading on a MACS column. Flow through is the Depleted fraction. By removing the column, you then have access to your Enriched fraction, containing increased numbers of multimer-positive cells.

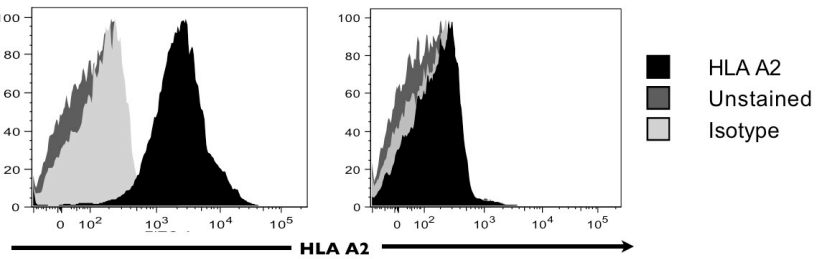

Fig.3. Flow cytometry based-HLA typing (Human). PBMCs are prepared as usual, then incubated with either isotype or anti-HLA antibody of interest titrated to the optimal concentration. Histograms represent data obtained from one HLA-A2 positive (left) and one HLA-A2 negative (right) blood donors.

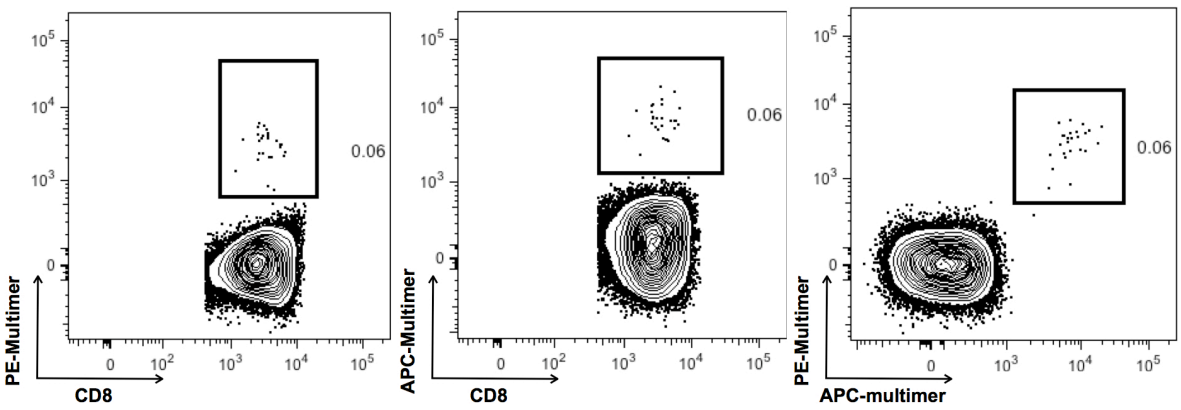

Fig.4. Representative example of single and double multimer staining. PBMCs from one healthy donor were stained with Influenza A-Matrix1$_{58\text{-}66}$ MHCI multimer labeled either in PE (left), APC (middle), or both (right). Plots are gated into global CD8^{+} population using the gating strategy described in Section 3.5-Figure 5A.

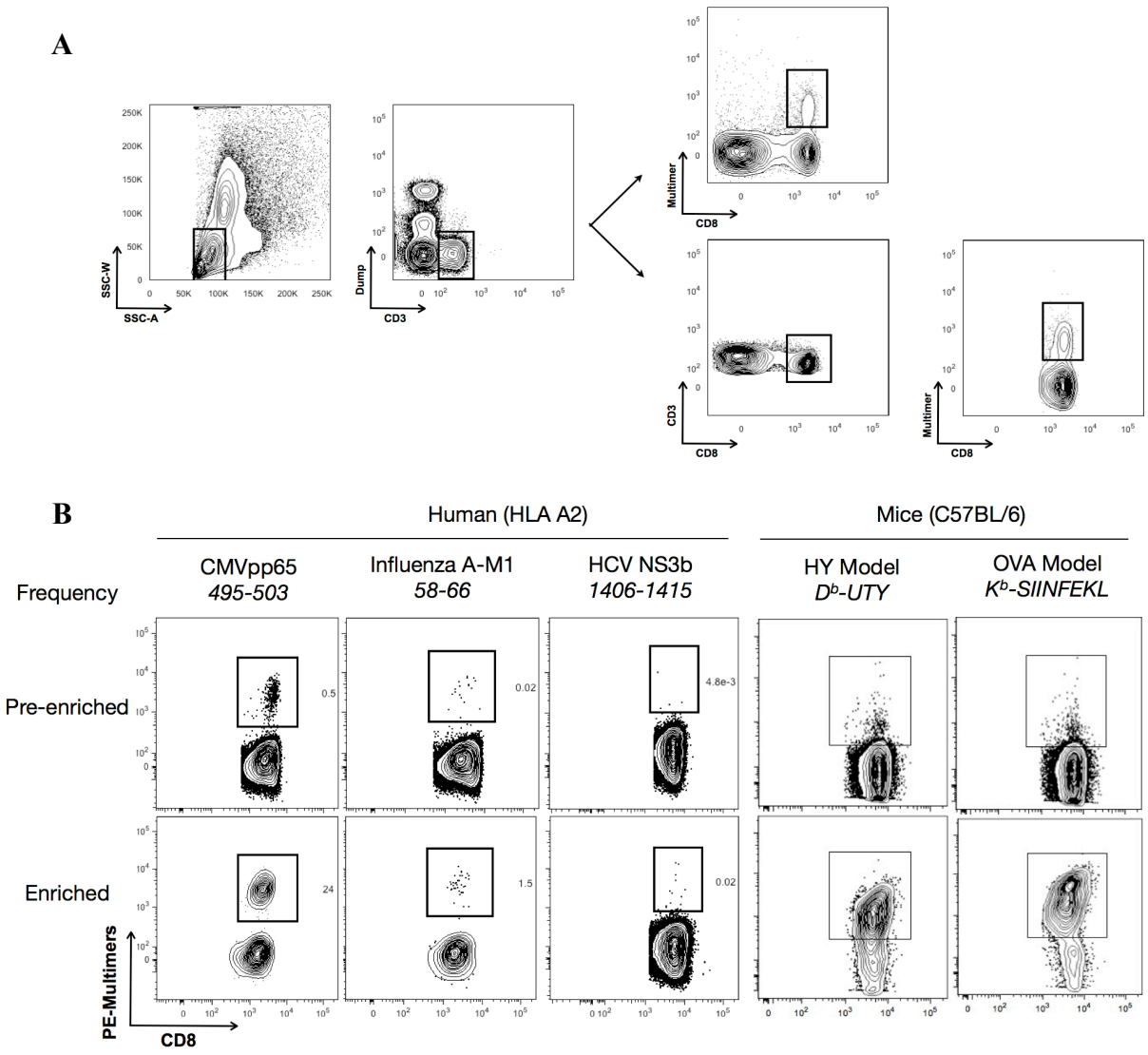

Fig.5. Gating strategy and Enrichment. (**A**) Example of the gating strategy applied to an Influenza A-Matrix $1_{58\text{-}66}$ enriched human sample. SSC-A^{low}/SSC-W^{low} initial gating permit to exclude doublets, then Dump *vs*. CD3 contour plot permits to isolate viable pure CD3 for further analysis. Upper line shows evaluation of background on a Multimer-PE vs CD8 contour plot still gated on the total CD3 population. Bottom line illustrates $CD3^+CD8^+$ selection, then finally evaluation of Multimer-PE^+ cells percentages within $CD8^+$ T cells. (**B**) Left plots: PBMCs from a healthy donor have been incubated with CMV, Flu or HCV MHCI multimers, then enriched as described in the protocol. Right plots : C57BL/6 mice were immunized intradermally, either with male (HY model) or K^{bm1}mOva (Ova model) splenocytes. On day 11, the spleen and lymph nodes were harvested and enrichment was performed as described in the protocol, using D^b-UTY (HY model) or K^b-SIINFEKL (Ova model) multimers.

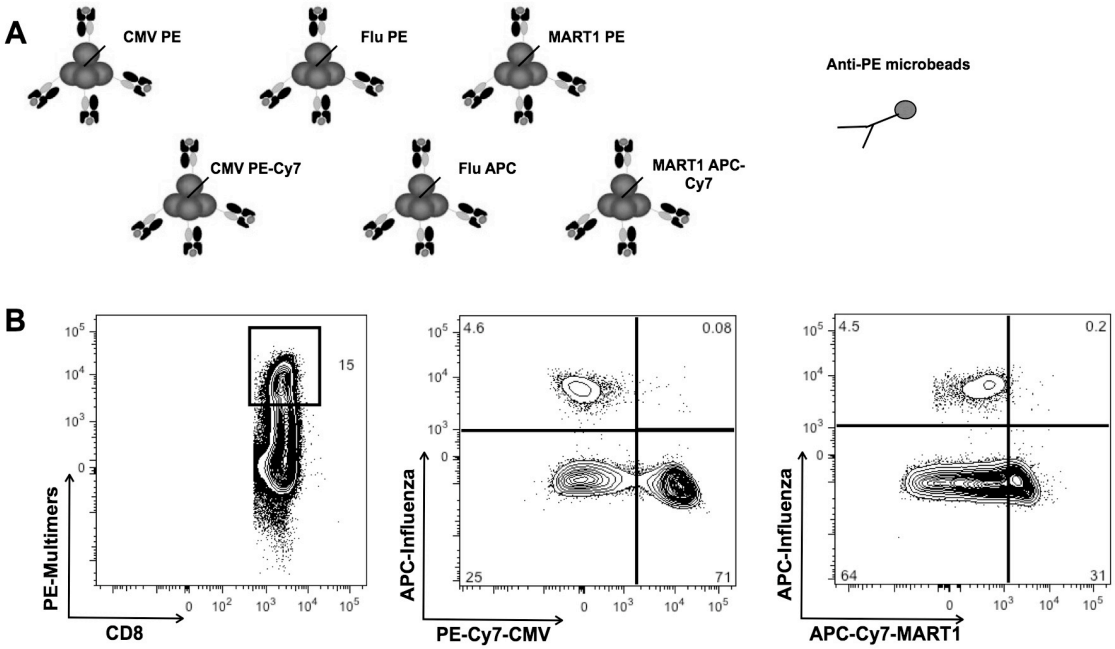

Fig.6. Multi-enrichment. **A.** PBMCs from a healthy donor are incubated with a cocktail of MHCI multimers. Each specificity (CMV, Flu, MART1) is labeled with PE and with another color (PE-Cy7, APC, APC-Cy7 respectively). Enrichment is performed with anti-PE microbeads as described in Section 3.4. **B.** After applying the gating strategy described in Section 3.5 and Figure 5A, $CD8^{+}PE^{+}$ cells are gated (left plot). Each specificity is then identified within $CD8^{+}PE^{+}$ population using the second color readout (middle and right plots).

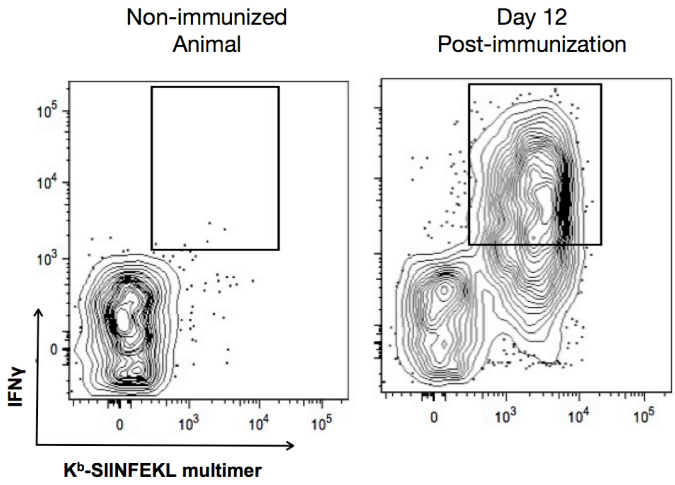

Fig.7. Intracellular cytokine staining on mouse samples. C57BL/6 mice were immunized intradermally with K^{bm1}mOva splenocytes. On day 12, mice were injected intravenously with CpG/DOTAP and SIINFEKL peptide. 3 hours later, the spleen and lymph nodes were harvested and Ova-specific T cells were enriched with K^b-SIINFEKL multimer, fixed and stained intracellularly for IFNγ as described in the protocol.

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Abstract: Most successful vaccines currently in use are based on the generation of protective antibodies. However, $CD8^+$ T cell responses are crucial in the defense against several infectious agents, as well as for the treatment of cancer or chronic diseases. Thus, the development of vaccine strategies capable of eliciting robust $CD8^+$ T cell responses is absolutely needed. Antigen cross-presentation is known to be an important mechanism for the activation of antigen-specific $CD8^+$ T cells, and it has been shown that multiple parameters contribute to the efficiency of cross-priming. We examined two of them in detail: the route of immunization and the timing of adjuvant delivery. Our first priority was to develop and optimize the tetramer-based enrichment strategy, which allowed us to perform an in-depth study of the endogenous $CD8^+$ T cell response. This approach permitted us to work within a model reflecting "physiologic" conditions in terms of initial precursor T cell frequency. We applied these methods to investigate the impact of the route of immunization on $CD8^+$ T cell cross-priming. By comparing different strategies of immunization, we report that local delivery of cell-associated antigen results in delayed cross-priming due to the increased time required for antigen capture and presentation. In comparison, delivery of systemically disseminated antigen resulted in rapid T cell priming. Surprisingly, local injection of cell-associated antigen, while slower to mount a functional response, resulted in the differentiation of a more robust, polyfunctional effector T cell population and an enhanced secondary response. However, the diversity of the responding antigen-specific T cells did not appear to be affected by the route of immunization. We were next interested in evaluating the combination of cell-associated antigen with the delivery of poly I:C, an adjuvant known to induce the production of type I interferons (IFN). We observed an immunization-route-specific effect regarding the timing of innate immune stimulation and identified the optimal time window for adjuvant administration in order to maximize the boosting effects on $CD8^+$ T cell cross-priming. We characterized in detail several effects of poly I:C, as well as type I IFN, exerted on immune cells, and especially on cDCs, providing the basis for our hypotheses as to why adjuvant treatment may lead to either the inhibition or enhancement of cross-priming depending on the timing of delivery.

Résumé: La mise au point de vaccins efficaces est généralement basée sur le développement d'une réponse anticorps. Néanmoins, la protection contre certains agents infectieux ainsi que le traitement de maladies chroniques ou de cancers nécessite l'induction d'une réponse cellulaire. Le développement de vaccins induisant une réponse T CD8 efficace est donc essentiel. La présentation croisée de l'antigène est importante pour l'activation de lymphocytes T CD8 spécifiques. Il a été démontré que de nombreux facteurs participent au développement d'une réponse lymphocytaire T efficace. Nous nous sommes intéressés à deux d'entre eux: la voie d'immunisation et la séquence d'aministration de l'antigène et d'un adjuvant. Dans un premier temps, nous avons développé une technique d'enrichissement des lymphocytes T CD8 spécifiques d'un antigène, ce qui a permis une étude précise de la réponse T CD8 endogène. Cette stratégie rend possible l'analyse de la réponse lymphocytaire dans des conditions où la fréquence initiale de précurseurs T spécifiques de l'antigène correspond aux conditions physiologiques rencontrées lors d'essais cliniques de vaccination. Nous avons utilisé cette approche pour étudier l'influence de la voie d'immunisation sur l'efficacité de la réponse lymphocytaire T CD8. Nous avons observé que l'injection intradermique d'un antigène cellulaire induit une réponse T CD8 plus tardive, comparée à une administration par voie systémique. Cependant, la réponse T CD8 induite par une injection locale de l'antigène est plus efficace, avec de nombreux lymphocytes capables de sécréter plusieurs cytokines. Alors que la fonctionalité des lymphocytes T CD8 spécifiques de l'antigène dépend de la voie d'immunisation, leur diversité et leur avidité ne sont pas régulées par ce paramètre. Nous avons ensuite évalué l'administration d'un adjuvant – le poly I:C connu pour induire la production d'interférons (IFN) de type I – en parallèle de celle de l'antigène. Nous avons montré que le moment optimal d'administration de l'adjuvant dépend de la voie d'immunisation. De plus, il existe une durée limitée durant laquelle l'adjuvant induit des effets positifs sur l'activation des lymphocytes T CD8. Nous avons identifié plusieurs effets du poly I:C et des IFN de type I sur les cellules du système immunitaire, et plus particulièrement les DCs. Ces observations nous ont permis de comprendre comment un même adjuvant pouvait avoir des effets opposés en fonction du moment où il était administré.