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SCUOLA DI DOTTORATO IN BIOSCIENZE E BIOTECNOLOGIE

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XXV ciclo

TESI di DOTTORATO

A new TLR2 agonist is able to promote cross-presentation

COORDINATORE

Ch.mo Prof. Cesare Montecucco

Università degli Studi di Padova

DOTTORANDO

Melissa Santone

SUPERVISORE

Dott.ssa Cecilia Buonsanti

Novartis Vaccine and Diagnostics, Siena

Summary

Cross-presentation is the process by which professional Antigen Presenting Cells (APCs) are able to load peptides from an extracellular processed protein into class I molecules, triggering a $CD8^+$ T cells response. How to increase the efficiency of this process is thus an area of great interest for the development of antitumor and antiviral vaccines which are required to stimulate a cytotoxic T cell response. One promising strategy is the use of adjuvants, molecules that are added to vaccines in order to modulate the immune response and increase protection. The discovery that some compounds with adjuvant function are recognized by Toll like receptors (TLRs), receptors involved in pathogen-associated molecular patterns recognition, drove the interest in developing a novel family of vaccine adjuvant based on triggering the TLR pathways. The aim of my PhD project is to investigate if the novel compound, agonist of TLR2 (SMIP2-1), can enhance cross-presentation of soluble antigens to $CD8^+$ T cells. SMIP2-1 is a novel synthetic lipopeptide, identified through two cell-based high throughput screens performed at the Genomic Institute of Novartis Research Foundation, and is currently under evaluation as vaccine adjuvant by Novartis Vaccines. Using mice reconstituted with Ovalbumin (OVA) specific OT-I TCR transgenic cells, as well as unreconstituted wild type mice, immunized with OVA and SMIP2.1, we found that addition of our compound to the vaccine formulation increases $CD8^+$ T cell proliferation, cytokines production, and cytotoxic activity, along with specific antibody production. Moreover, using an OVA expressing tumor model, we show that the CTLs induced by the SMIP2.1 formulated vaccine are able to inhibit tumor growth *in vivo*. One mechanism by which SMIP2.1 could enhance the immunogenicity of antigens is its ability to increase antigen deposition in the draining lymph node of the site of injection, and the amount of APC that take up the antigen, as we demonstrated by confocal

analysis. We then identified which cells populations can cross-present *in vitro* and we found that both $CD8\alpha^+$ and $CD8\alpha^-$ DCs are able to cross-present when stimulated with SMIP2.1 In order to evaluate the effect of SMIP2.1 on human cells, we used a flow cytometry assay to detect activation of $CD8^+$ T cells isolated from human PBMC of Cytomegalovirus seropositive donors. We found that stimulation with SMIP2.1 is able to greatly increase the capacity of human APC, pulsed *in vitro* with the pp65 CMV protein, to activate CMV-specific $CD8^+$ T cells. Over all our data demonstrate that vaccination with exogenous antigen formulated with SMIP2.1 is a successful strategy to induce a cytotoxic T cell response along with antibody production.

Sommario

Si definisce “Cross-presentazione” il meccanismo attraverso il quale le Cellule Presentanti l’Antigene (APCs) sono capaci di catturare, processare e caricare sul complesso MHC di classe I peptidi derivanti da un antigene extracellulare, inducendo una risposta citotossica da parte dei linfociti T $CD8^+$. La risposta citotossica è di particolare importanza per lo sviluppo di vaccini antitumorali e antivirali, per i quali è necessaria un’attivazione delle cellule T $CD8^+$. Come indurre la cross-presentazione ha quindi stimolato grande interesse da parte delle industrie farmaceutiche per lo sviluppo di questi vaccini. Una strategia promettente è l’utilizzo di adiuvanti, molecole aggiunte ai vaccini per modulare la risposta immunitaria e aumentare la protezione. La scoperta che composti con funzioni adiuvanti legassero i recettori di tipo Toll (TLRs), recettori coinvolti nel riconoscimento di motivi molecolari comuni a molti microrganismi patogeni, ha sollevato l’interesse verso lo sviluppo di una nuova famiglia di adiuvanti basati sull’attivazione dei pathways dei TLRs. Lo scopo del mio progetto di Dottorato è investigare se un nuovo composto, agonista del TLR2 (SMIP2.1), può aumentare la cross-presentazione di un antigene esogeno alle cellule T $CD8^+$. SMIP2-1 è un lipopeptide di sintesi, identificato attraverso uno screening effettuato al Genomic Institute of Novartis Research Foundation, che Novartis Vaccines intende utilizzare come adiuvante. Utilizzando Ovalbumina (OVA) come antigene esogeno, abbiamo osservato che l’immunizzazione con OVA + SMIP2.1, sia di topi transgenici con un TCR specifico per OVA che di topi *wild type*, aumenta la risposta T $CD8^+$ in termini di proliferazione, produzione di citochine e attività citotossica, rispetto a topi vaccinati solo con l’antigene. Inoltre, SMIP2.1 induce un’ottima risposta anticorpale contro l’antigene, caratteristica fondamentale di un adiuvante. Utilizzando cellule tumorali murine che esprimono OVA, abbiamo osservato che la risposta citotossica indotta dall’agonista del

TLR2 è capace di inibire la crescita tumorale *in vivo*. Un meccanismo attraverso cui SMIP2.1 aumenta la risposta immunitaria potrebbe essere la sua abilità di aumentare la deposizione dell'antigene nei linfonodi drenanti sul sito di iniezione e l'uptake dell'antigene da parte delle APCs, come dimostrato dalle osservazioni al microscopio confocale. Abbiamo poi analizzato quale popolazione cellulare può cross-presentare *in vitro*, scoprendo che sia le cellule dendritiche $CD8\alpha^+$ e $CD8\alpha^-$ sono capaci di cross-presentare dopo stimolazione con SMIP2.1.

Per valutare l'effetto dell'agonista del TLR2 su cellule umane, abbiamo usato un'analisi al citofluorimetro per misurare l'attivazione delle cellule T $CD8^+$ isolate dai PBMC di pazienti positivi per il Citomegalovirus. Abbiamo trovato che SMIP2.1 aumenta la capacità delle APC umane, stimolate *in vitro* con la proteina pp65 del CMV, di attivare una popolazione T $CD8^+$ specifica per il CMV.

In conclusione, i nostri dati dimostrano che la vaccinazione con una proteina esogena formulata con SMIP2.1 è una promettente strategia per indurre sia una risposta citotossica che una risposta anticorpale.

Contents

1. Introduction

1.1 Overview of the immune system

We are constantly exposed to infectious agents and, in most cases, we resist these infections by means of our immune system that is able to mount an immune response against the pathogens that otherwise would cause infection. The mammalian immune system is comprised of two branches: innate and adaptive immunity. The innate immune system is our first line of defense against invading organisms while the adaptive immune system acts as a second line of defense and also affords protection against re-exposure to the same pathogen. Although both the innate and adaptive immune systems work to protect against invading organisms, they play different roles. The adaptive immune system requires some time to react to an invading organism, whereas the innate immune system includes defenses that, for the most part, are constitutively present and ready to be mobilized upon infection. Second, the adaptive immune system is antigen specific and reacts only with the organism that induced the response. In contrast, the innate system is not antigen specific and reacts equally well to a variety of organisms. Moreover, the adaptive immune system demonstrates immunological memory: remembering to have met a pathogen, it is able to react more rapidly to its following exposure. In contrast, the innate immune system does not demonstrate immunological memory.

Although these two arms of the immune system have distinct functions, there is interplay between these systems.

1.1.1 The innate immunity

The innate immunity is the first line of defense against pathogenic microbial invasion. In vertebrates, the skin and other epithelial surfaces, including those lining the lung and gut, provide a physical barrier between the inside of the body and the outside world. Microorganisms can occasionally pass through the epithelial barriers. It is then up to the immune system to identify and destroy them, without harming the host.

Cells of the innate immune system sense infection with a variety of receptors called Pattern Recognition Receptors (PRRs), which are innate immune receptors extremely conserved throughout evolution. These receptors are able to bind and recognize few, highly conserved structures expressed by a microorganisms but not found in higher organisms, referred as Pathogen-Associated Molecular Patterns (PAMPs). For this reason the innate immune response is not specific in terms of antigen recognition.

Neutrophils, eosinophils, basophils, macrophages/monocytes, dendritic cells (DCs), and natural killer (NK) cells are the cellular components of the innate immune system. Phagocytic cells as macrophages, monocytes, DCs and neutrophils express PRRs. The recognition of PAMPs on the surface of pathogens by PRRs induces the engulfment of the pathogen in a phagosome where a combination of degradative enzymes, antimicrobial peptides, and reactive oxygen species kills the invading microorganism.

NK cells are other important effector cells of the innate immune response. They recognize microbes and cells infected by viruses or intracellular bacteria in an antigen-non-specific manner. They function by secreting cytokines, mainly IFN-γ, which activates macrophages and other cells. NK cells can also destroy microbes or virus-infected cells by producing perforins and inducing the apoptosis of infected cells.

Other important components of innate immunity are soluble molecules, such as cytokines and chemokines, produced by cells of the innate system and active on cells of both the innate and adaptive immunity. Chemokines release favors the leukocyte mobility from the lymph nodes to the site of infection through the endothelial barriers. Cytokines promote the phagocytic ability of macrophages and neutrophils, the cytotoxic activity of NK cells and the differentiation and activation of T and B lymphocytes. A group of cytokines playing a crucial function in the innate immune response is represented by the interferon family. Interferons function as immunomodulators, as well as an inducer of an antiviral state in cells (Christensen and Thomsen, 2009; Rasmussen et al., 2009).

Despite the lack of specificity, innate immunity is highly effective and in many cases sufficient to eliminate the invading agent. In other cases the innate immune response cannot cope with infection but through different mechanisms triggers the initiation of an adaptive immune response.

1.1.1.1 Toll-like receptors

Toll-like receptors (TLRs) are among the best studied PRRs (Franchi et al., 2009) (Takeuchi and Akira, 2009). These components of the innate immune response recognize pathogen-derived molecules, ranging from bacterial and yeast cell wall components to viral and bacterial nucleic acids including lipopolysaccharide (LPS) of gram-negative bacteria, peptidoglycan of gram-positive bacteria, flagellin, lipopeptides, non-methylated CpG DNA. The protein Toll, German slang for ‘‘fantastic,’’ was first derived from the Toll gene of Drosophila, a maternal-effect gene that plays a central role in the establishment of dorsal–ventral patterning during embryonic development (Belvin and Anderson, 1996). Moreover, Toll is required for Drosophila response to fungi being part of a peptidoglycan sensing cascade which ends in the production of antifungal peptides by cells of the fat body (Crotta

et al.; De Gregorio et al., 2002; Lemaitre et al., 1996).The high homology between the intracellular regions of Drosophila Toll and mammalian IL-1R, as well as the similarity in the signaling pathways triggered by these two receptors, both of which result in NF-kB activation, led to the hypothesis that Toll could also have a function in the mammalian immune response (Hoffmann, 2003; Lemaitre, 2004).

TLRs engagement leads to the induction of various genes that function in host defense, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules. TLRs are type I transmembrane proteins with an ectodomain containing leucine-rich repeats that mediate the recognition of PAMPs, a transmembrane domain and an intracellular region which contain the Toll-Interleukin 1(IL-1) receptor (TIR) domain required for downstream signal transduction. The TIR domain interacts with cytoplasmatic adaptor molecules that initiate intracellular signaling. There are four such adaptors in mammalian TLRs, whose activation influences which signals will be activated by TLRs: MyD88, TIRAP (also called MAL), TICAM1 (also called TRIF), and TICAM2 (also called TRAM) (Figure 1). MyD88 associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NF-κB which induces expression of inflammatory cytokines. With the exception of TLR3, which signals exclusively via TICAM1, all TLRs utilize a MyD88-dependent pathway resulting in the production of TNF-α, IL-1, IL-6, and other cytokines dependent on NF-κB. MyD88 also trigger mitogen-activated protein (MAP) kinase cascades that lead to activation of AP-1, cyclic AMP (cAMP) response element-binding protein (CREB) and the transcription factor interferon regulatory factor 7 (IRF7), leading to the induction of type I interferons (IFNs), which are especially potent anti-viral cytokines. TIRAP, the second TIR

domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. Third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway leading to activation of IRF-3 to induce type I IFNs. TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

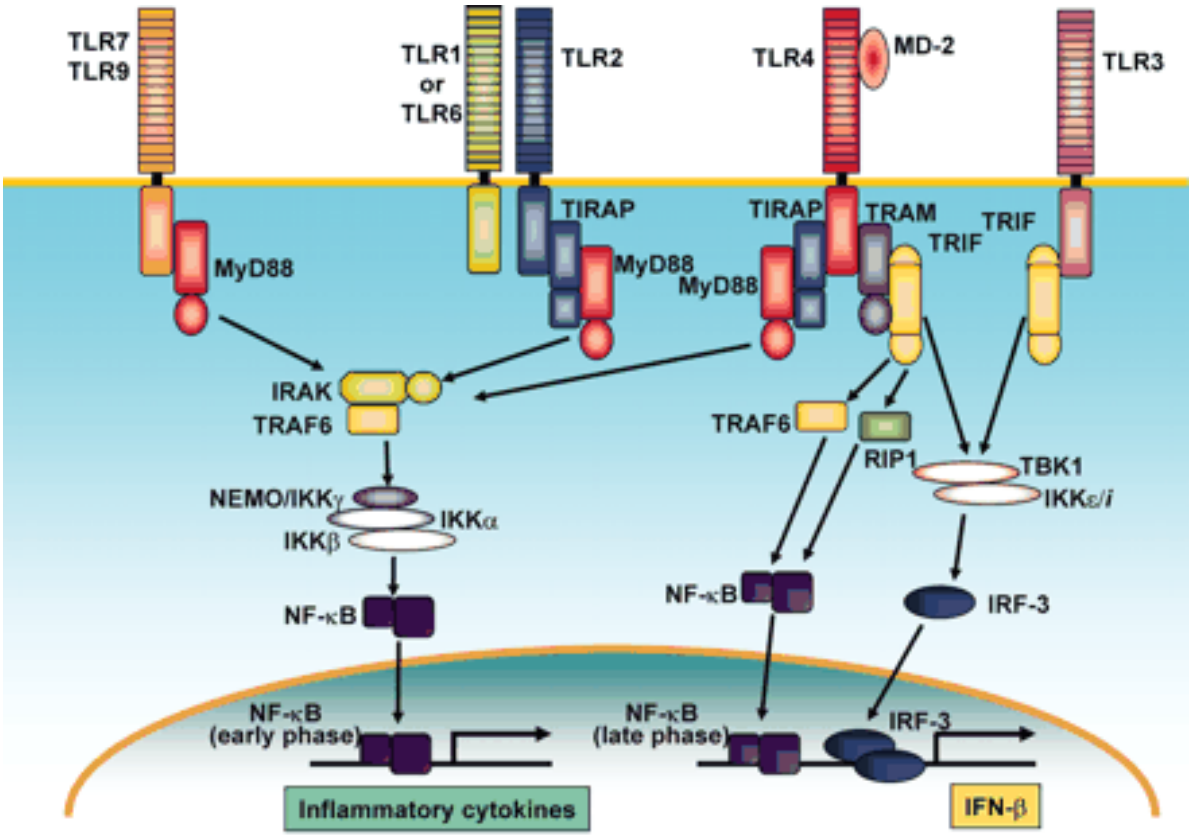


Figure1 TLR signaling pathway (Takeda and Akira, 2005)

Most classes of TLRs are found in immune cells such as polymorphonuclear neutrophils, monocytes/macrophages, DCs, NK cells, T and B cells, where they trigger an immediate response against pathogens. However, accumulating evidence indicates that a number of

TLRs are widely expressed also in non-immune cells, such as endothelial cells, epithelial cells, skin keratinocytes, and fibroblasts (Pegu et al., 2008).

Humans express ten functional TLRs (TLR1 to TLR10), whereas twelve TLRs (TLR1 to TLR9 and TLR11 to TLR13) have been identified in mice. Ligands have been determined for all TLRs except for human TLR10, mouse TLR12 and mouse TLR13.

Studies with mice deficient for each TLR have demonstrated that they have distinct functions in terms of PAMP recognition and immune responses (Abe et al.). The intracellular localization of some members of the TLR family is believed to be important to restrict ligand accessibility and therefore avoid recognition of self-molecules such as endogenous nucleic acids. On the basis of their respective PAMP ligands and their cellular localization TLRs can be divided into two groups (Figure 2):

- One group is composed of TLRs which are expressed on cell surface and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins. This group includes TLR1, TLR2, TLR4, TLR5 and TLR6. TLR2 can heterodimerize with TLR1 or TLR6 to recognize respectively triacyl- or diacyl-lipopeptides which are present on the surface of many bacteria. TLR4 is specialized in responding to lipopolysaccharide (LPS) expressed by Gram-negative bacteria, while flagellin, a protein of the bacterial flagella, is recognized by TLR5.
- The second group is composed of TLRs which are expressed exclusively in intracellular vesicles such as the endosplasmatic reticulum (ER), endosome, lysosome and endolysosomes, where they recognize microbial nucleic acids (Abe et al.). It is composed of TLR3, TLR7, TLR8 and TLR9. TLR3 binds double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA while TLR9 signaling is triggered by binding to CpG-rich sequences which are abundant in microbial DNA.

Expression of TLRs is quite different among cell types. For example TLR2 and TLR4 are expressed on various immune cells including macrophages, DCs, B cells, granulocytes, NK cells and T cells. They are also expressed on non-immune cells such as fibroblasts and epithelial cells. TLR7 and TLR9 are largely expressed in the immune cells and in particular are predominantly expressed in plasmacytoid DCs.

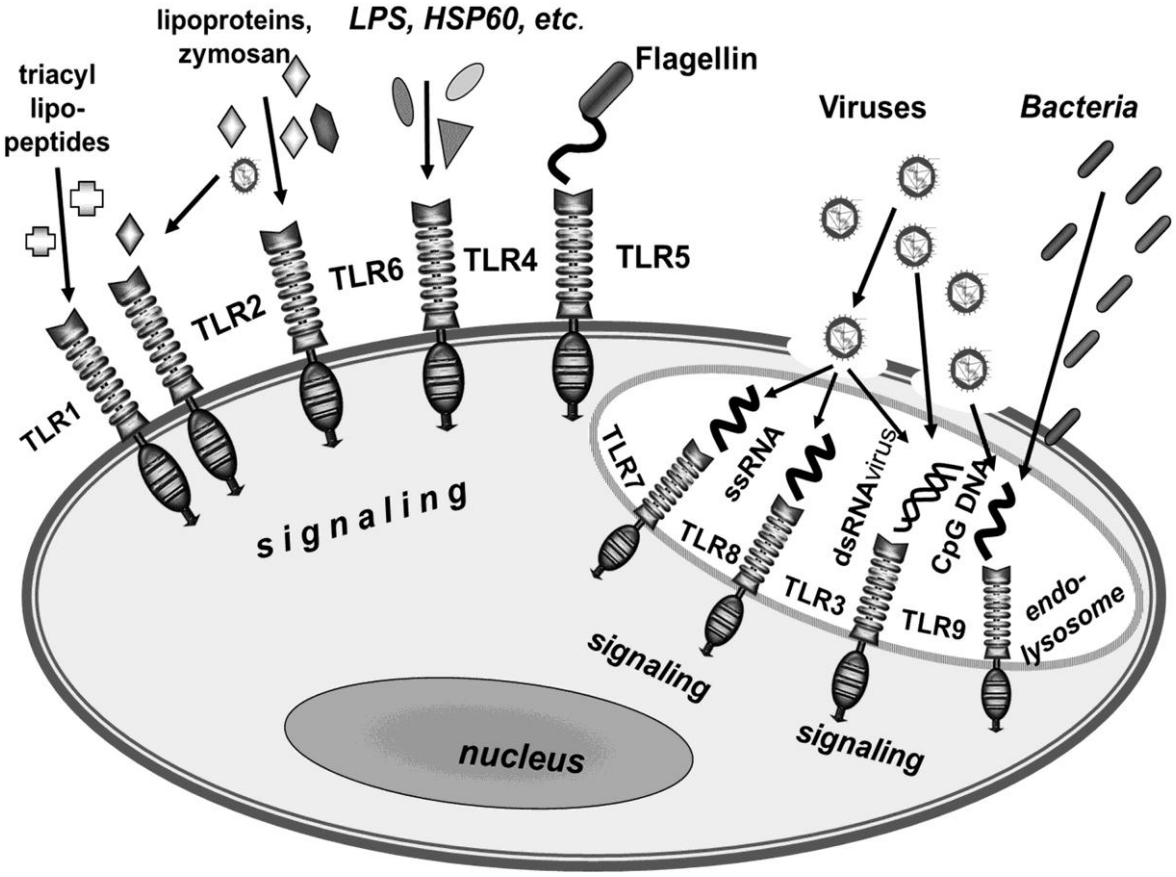


Figure 2. Surface (TLR1, 2, 4, 5, and 6) and intracellular (TLR3, 7, 8, and 9) localization of TLRs in a cell of the innate immune system. Only a few molecules out of a large variety of PAMPs are shown (Land, 2012).

1.1.1.2 Toll-like receptor 2

TLR2 interacts with a broad and structurally diverse range of ligands, including molecules expressed by microbes and fungi (Abe et al., 2007). Among them there are lipopeptides, peptidoglycan and lipoteichoic acid from bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi and the hemagglutinin protein from viruses. Ligand specificity as well as signal transduction ability of TLR2 is determined by its heterodimeric interactions with TLR1 and TLR6 (Ozinsky et al., 2000). Specifically, the TLR2-TLR1 heterodimer recognizes triacylated lipopeptides from Gram-negative bacteria, whereas the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram-positive bacteria and mycoplasma. Since TLR2 is expressed on many different cell types (including dendritic cells, macrophages and lymphocytes) the mechanisms by which bacterial lipopeptides activate the immune system are diverse. Pre-clinical testing indicates that lipopeptides co-administered with or physically linked to Ag can induce DC maturation leading to the up-regulation of co-stimulatory signals and Ag-presenting molecules (e.g. MHC class II, CD80, CD83, IFNα, IL-12) (Hertz et al., 2001), stimulate macrophages to release cytokines (e.g. TNFα, IL-1, IL-6) (Muhlradt et al., 1997), promote the maturation and activation of B cells leading to increased production of Ag-specific IgG and IgM Abs (Borsutzky et al., 2005) and boost the generation of antigen-specific $CD8^+$ T cell (CTL) responses (Jackson et al., 2004) (Borsutzky et al., 2006).

1.1.2 The adaptive immunity

The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens to be activated. Adaptive immune responses are carried out by white blood cells called lymphocytes. There are two major lymphocytes types, B cells and T cells

which differ in their functionality but use structurally similar receptors to specifically recognize antigenic determinants. Differences in T-cell and B-cell receptors are generated in precursor cells through rearrangement of their respective B- and T-cell receptor gene segments and through a process of "somatic hypermutation". This mechanism allows a small number of genes to generate a vast number of different antigen-specific receptors, which are then uniquely expressed on each individual B or T lymphocyte. This allows the body to have cells that can specifically target most of the pathogens that an organism might encounter during its lifetime.

- **B cells.** The antigen-recognition molecules of B cells are the immunoglobulins (Ig) that exist in membrane-anchored or secreted forms. The membrane-bound form, known as the B-cell receptor (BCR), is expressed on the cell surface and work as receptor for the antigen. When a B cell recognizes its cognate antigen thought the BCR, the B cell proliferates and differentiates into terminally differentiated B cells, called plasma cells, able to secrete immunoglobulin of the same antigen specificity as antibody. The secretion of antibodies, key mediators of humoral immune response, has two separate functions. One is to bind specifically molecules from the pathogens, leading to their neutralization; the other is to recruit other cells, e.g. phagocytes, to destroy the pathogen once the antibody is bound to it (a process known as opsonophagocytosis). There are different types of antibodies, known as IgM, IgA, IgG, IgD and IgE. Each immunoglobulin differs in its biological proprieties and in the capability to bind different antigens. All B cells initially express and synthetize IgM, but may switch to other isotypes upon gene rearrangement, a mechanism known as isotype-switching. Switched isotypes are antibodies that maintain their specific antigen-recognition capacity but differ in other domains of the molecule that determine their functionality. IgA antibodies are usually present in different mucosal body fluids and are important in

the local infection. IgG are the most abundant immunoglobulin that are present in the serum and can be divided in 4 different classes in humans: IgG1, IgG2, IgG3 and IgG4. IgD are present on the surface of mature but not immature B lymphocytes. They bind the antigen and stimulate the cells to grow and differentiate in plasma cells. IgE are important against parasites infection, and in the autoimmune response.

- **T cells.** T-cell receptors (TCRs), the antigen-recognition molecules of T cells, are related to immunoglobulins in their protein structure but they are made only of membrane-bound polypeptide chains. Unlike the BCR, the TCR does not recognize and bind the antigen directly, but recognizes short, linear peptide fragments of protein antigens, bound to transmembrane glycoproteins known as Major Histocompatibility Complex (MHC) molecules on the surface of cells. There are two classes of MHC molecule, called MHC class I and MHC class II, which differ in their structure, expression pattern and source of peptides that they carry to the cell surface. MHC class I molecules are expressed on all nucleated cells: they collect peptides derived from proteins synthesized in the cytosol and are thus able to display protein fragments of intracellular pathogens on the cell surface. MHC class II molecules are present only on APCs, such as DCs, B lymphocytes and macrophages. APCs are able to capture, process and present antigenic peptides derived from extracellular antigen.

  Once reached the cell surface with their peptide cargo, the two classes of MHC molecule are recognized by different functional classes of T cells: helper, cytotoxic and regulatory T cells.

  - **Helper T cells.** Antigenic peptides bound to MHC class II molecules are recognized by T lymphocytes marked by the cell-surface protein CD4. $CD4^+$ T cells are called T helper (Th) cells. When a TCR expressed on a T lymphocyte recognizes the antigen presented on MHC II molecules on the surface of an APC, it starts an

activation cascade within the cell. The T cell initiates its clonal expansion, to give rise to a number of identical progeny cells, and starts to produce cytokines. These cytokines and other signals can activate additional immune cells (e.g. give help to antigen-specific B cells to produce antibodies against the antigen). Based on the types of pathogen that should be fought, T helper cells can differentiate in 3 main subtypes, which secrete different cytokines depending on the type of immune response required to eliminate the pathogen: Th1, Th2 or Th17. Th1 responses are involved in the cellular or cytotoxic immune response needed to eliminate intracellular bacteria or viruses. Th2 responses are important for the humoral immune response against extracellular organisms. In general the Th2 response is specific against large extracellular parasites or soluble antigens. The stimulation of a Th response causes B cell activation and therefore a humoral response with antibody production. Th17 fights for example fungal infections.

- **Cytotoxic T cells.** MHC I molecules are recognized by T lymphocytes bearing the cell-surface protein CD8. $CD8^+$ T cells are cytotoxic T cells (CTL) able to kill somatic cells that are infected with cytosolic pathogens like viruses, intracytoplasmic bacteria, and protozoa, but also damaged or dysfunctional cells like tumor cells. Cytotoxic T lymphocytes are able to release a panel of molecules, like two families of proteases: perforins and granzymes. Perforins form pores in the target cells' plasma membrane allowing granzymes to enter the target cell and activate a series of proteases that eventually induce apoptosis of the infected cells. A second mechanism to induce apoptosis involves the recognition between the surface molecule FAS ligand, expressed on activated $CD8^+$ T cells, to Fas molecules, expressed on the target cells. The binding induces a signaling cascade leading to apoptosis of the target cell. In addition, cytotoxic T lymphocytes are able to lead the

activation of different cellular pathways, including the activation of NF-κB, which is an important factor implicated in the response to different stimuli including stress, cytokines, inflammation, etc. The main result of NF-κB activation is the production of cytokines and chemokines that activate and recruit immune cells to the site of infection.

- **Regulatory T cells.** Although the existence of cells able to suppress an immune response has been long postulated, their identification and characterization have only been recently established. Regulatory T cells (T_{Reg}) constitutively express a key transcription factor, known as forkhead box P3 (FOXP3), that is required for their development, maintenance and function (Hori et al., 2003). The major function of regulatory T lymphocytes is the maintenance of peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases by blocking the activity of effector, helper and APC cells. There are a lot of possible mechanisms used by T_{Reg} cells to mediate suppression that remain to be fully elucidated (Vignali et al., 2008)**.**

1.1.3 Antigen presentation mechanisms

The initial step in the presentation of an extracellular antigen is the binding and the internalization of the antigen by APCs. There are different ways by which APCs can bind the antigen. For example, they express receptors that recognize mannose residues present in bacterial walls and mediate the internalization of bacteria. Macrophages also express specific receptors for the Fc portions of the antibodies that enhance the internalization of antigens with attached antibodies. After their internalization, antigen localizes in intracellular vesicles called endosomes that will then fuse with phagosomes, characterized

by an acidic pH and the presence of proteolytic enzymes. Enzymatic degradation of the endocytosed antigen generates peptides that can bind the peptide-binding motif of MHC II molecules. These molecules are synthetized in the endoplasmic reticulum with an associated short protein called invariant chain that, occupying the binding cleft, prevents the aspecific binding of self-peptides to MHC II molecules. Vesicles transporting class II molecules out of the ER meet and fuse the phagosome containing the processed antigen. The proteolitic enzymes in the endocytic vesicles remove the invariant chain so that the cleft becomes accessible to peptides generated by proteolysis of exogenous antigens. The complex MHC II molecules-peptide is then displayed on the cell surface of APC where is recognized by specific $CD4^+$ T cells.

MHC class I-presented peptides are derived from intracellular proteins. Foreign antigens in the cytosol may be proteins derived from viruses or other microbes that infect nucleated cells and synthetize their own proteins in the cytosol of host cells, or they can derive from mutated genes in tumor cells. These proteins are degraded by the proteasome, a large multiprotein enzyme complex, with proteolitic activites, found in the cytoplasm of cells. Peptides derived from proteasome degradation are transported through the transporter associated with antigen processing (TAP) in the endoplasmic reticulum, where newly MHC class I molecules are synthesized. The MHC class I complex binds the peptide; the peptide/MHC complex is transported through the Golgi apparatus to the cell surface by exocityc vesicles. Once expressed on the cell surface, the peptide/MHC I complex may be recognized by antigen specific $CD8^+$ T cells.

1.1.4 Cross-presentation

Since the mid-80s, it was accepted that peptide derived from degradation of endogenous protein in the cytosol of APCs were loaded into MHC class I molecules , while MHC class II molecules presented peptides derived from exogenous antigens degraded within the endocytic route. The MHC class I presentation of endogenous antigen by professional APC to elicit an immune response is also called direct presentation. If only the direct pathway exists for MHC class I, then how can cytotoxic T lymphocyte (CTL) responses be elicited against tumor cells of non-haematopoietic origin or against viruses that do not infect professional APC?

This enigma was resolved when Bevan showed that CTL responses could also be elicited against antigens derived from an exogenous source (Bevan, 1976). He showed that minor histocompatibility antigens could be transferred from donor cells to host APCs and termed the resultant T-cell priming 'cross-priming'. Thus, the term 'cross-presentation' has been used to encompass when presentation of an exogenous antigen results in activation (cross-priming) or tolerization (cross-tolerance) of $CD8^+$ T cells.

Cross-presentation is the mechanism by which peptides derived from intracellular phagocytosed antigens can be presented by APC on MHC class I molecules, leading to $CD8^+$ T cells activation (Bevan, 1976) (Rock and Shen, 2005). The mechanisms underlying cross-presentation are not so clearly understood; however different pathways have been proposed.

- TAP-independent pathway: Antigens could be degraded by endosomal proteases, for example cathepsins, and subsequently be loaded onto MHC I molecules within endosomes (Figure 3a). This model should not require the transporter associated with antigen processing (TAP), and has been referred to as TAP-independent pathway. However, most studies examining the cell biology of cross-presentation reported a

requirement of TAP and the proteasome (Burgdorf et al., 2007) (Ackerman et al., 2006).

- TAP-dependent pathway: The canonical model states that antigen uptake into endosomes is followed by translocation of internalized antigens from endosomes to the cytosol of the APCs (Figure 3b). In the cytosol, proteins are degraded by the proteasome complex, and TAP shuttles the resultant peptides into the endoplasmic reticulum (ER) for MHC class I loading before being transported into the lumen of the (ER) for presentation at the cell surface. This model, also referred as TAP-dependent pathway, is further supported by data showing that cross-presentation is increased by the inhibition of lysosomal acidification with the drug chloroquine, indicating that these peptides were generated in non-lysosomal compartments (Bertholet et al., 2006).
- TAP-dependent within endosomes pathway: Third, antigen-derived peptides might be reimported from the cytoplasm into early endosomes, and MHC I loading might occur there (Figure 3c). This of course would require the presence of the MHC I-loading machinery in these endosomes.

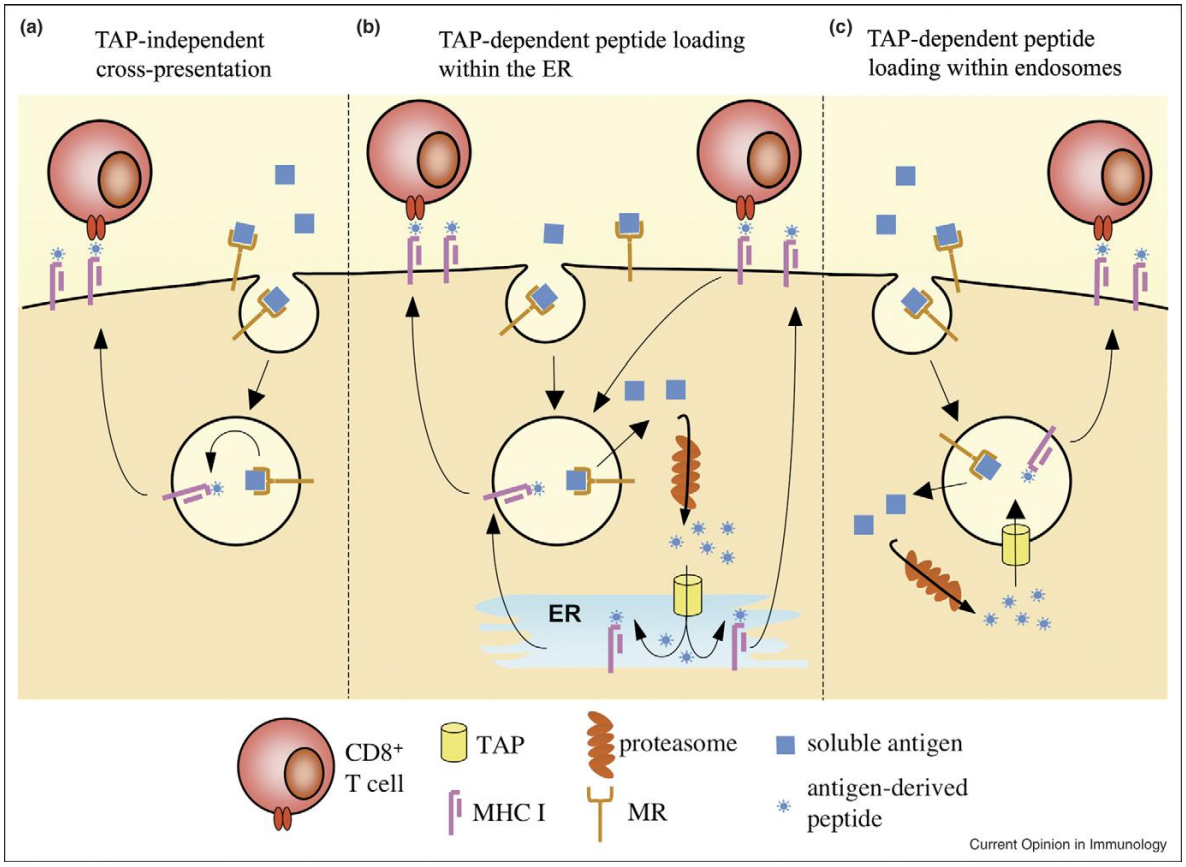


Figure 3: Putative intracellular mechanisms of cross-presentation. (Burgdorf and Kurts, 2008)

1.2 Vaccines

1.2.1 Principle of vaccination

Vaccination is one of the most successful public health interventions in modern history and has dramatically reduced damage and death caused by infectious diseases. Vaccines are biological products that mimic an infectious agent without causing the disease. The goal of vaccination is to generate a strong immune response of sufficient duration and magnitude to the administered antigen in order to stimulate an immunological memory to prevent infection and reduce the related pathology. Although classically represented by attenuated or killed microorganisms, modern vaccines more often comprise pathogen-derived components or recombinant proteins that represent safer and cost-saving formulations. However, a series of clinical and experimental observations have clearly illustrated the reduced immunogenicity of subcellular or subunit-based vaccines when compared with inactivated/killed whole organisms. The weak immunogenicity of soluble proteins, lacking the inherent danger-signature often associated with a pathogen, appears to be related to their inability to induce DCs maturation representing the limiting step in the development of efficient vaccines. The challenge for modern vaccinology is therefore to be able to elicit *in vivo* all the required steps that lead to immune activation. This limitation could be overcome by the use of adjuvants.

1.2.2 Adjuvants

Successful vaccines should contain not only a protective antigen, but also a good adjuvant (Coban et al., 2007). The term "adjuvant" derives from the Latin "*adjuvare"*, meaning to help. The purpose of adjuvants in vaccine formulations is to help the function of the immune system so that a coadministered microorganism or protein or polysaccharide becomes more immunogenic. Thus adjuvants can be defined as molecules acting

independently of an antigen in order to directly activate, increase and/or modulate innate and/or adaptive immune cells. They can promote humoral or cellular immunity, influence the cytokine polarity of T-helper cell responses, and promote a local or systemic immune response. Adjuvants can also help inducing stronger immune responses with fewer injections, and consequently improving both the feasibility and success rate of large-scale population vaccine campaigns in developing countries (Rappuoli, 2007). Vaccine adjuvants are represented by different classes of compounds such as microbial products, mineral salts, emulsions, microparticles, and liposomes. The mechanism of action of many adjuvants has been long investigated. Recent studies suggest that the formation of Ag depots, that prolong the persistence of Ag in vivo, and the activation of inflammation by various innate cells are components dictating the success of an adjuvant (Coban et al., 2007) (Seubert et al., 2008) (Lambrecht et al., 2009).

Adjuvants can be classified according to their component sources, physiochemical properties or mechanisms of action. Two classes of adjuvants commonly found in modern vaccines include immunopotentiators and delivery systems.

Immunopotentiators directly act on the immune system to increase responses to antigens. Examples include: TLR ligands, cytokines, glycolipids that alter antigen processing, saponins and bacterial exotoxins, all of them able to stimulate immune responses.

Delivery systems have the ability of presenting vaccine antigens to the immune system in an optimal manner, including controlled release of the antigen in order to increase the specific immune response. Indeed, this class of adjuvants is able to modulate antigen persistency or antigen uptake by different populations of APCs. They can also serve to deliver the immunopotentiators described previously. Examples include: mineral salts, emulsions, liposomes, virosomes (nanoparticles made of viral proteins such as influenza

hemagglutinin and phospholipids), biodegradable polymer microspheres and so-called immune stimulating complexes (i.e. ISCOM, ISCOMATRIXTM^{TM}).

Of the many experimental adjuvants currently available, only aluminum-based salt and squalene oil water emulsions adjuvants have been licensed for addition to human vaccine in the USA. Recently then, vaccine formulations containing Monophosphoryl lipid A (MPL), a TLR4 agonist, were approved for human use (Mbow et al.; Mosca et al., 2008) (Rappuoli et al., 2011). Aluminium based adjuvants (alum) have a good safe profile and increase the immune response to many vaccine antigens, such as that against tetanus, diphtheria and pertussis (Lindblad, 2004). Alum is generally effective at generating a strong antibody production to an antigen and it induces a Th2-type of immune response. The mechanisms of action of alum involve inflammation and recruitment of antigen-presenting cells, retention of antigen at the injection site, stimulation of antigen uptake, DCs maturation and T-cell activation (Coban et al., 2007). Although the efficacy of alum as adjuvant has been proved in a large number of vaccines, some limitations exist. For example, alum has failed to induce a satisfactory increase of the immune response in some vaccines, such as the influenza vaccine.

MF59 is squalene oil water emulsions adjuvant. It is a Novartis proprietary adjuvant that was licensed in Europe for an influenza vaccine for the elderly (Fluad®). MF59 is a safe and strong adjuvant able to increase the immunogenic response to a large panel of antigens. Similarly to alum, MF59 treatment induces production of cytokines and chemokines and a strong cell recruitment of immune cells into the injection site.

A current challenge is to identify vaccine adjuvants of various classes (cytokines, toll-like receptor ligands, etc.) with specific immune-modulating properties in order to tailor the immune response to certain pathological situations. Identification and development of new adjuvants is necessary because the small number of currently approved vaccine adjuvants

do not always elicit the desired protective immune response against different target pathogens (Mbow et al.).

1.2.2.1 TLRs agonists as adjuvants

In the past decade, most attention in the vaccine field has been placed on innate adjuvants that trigger pattern recognition receptors, such as TLRs. Triggering TLR stimulates the production of proinflammatory cytokines/chemokines and type I IFNs that increase the host's ability to eliminate the pathogen. This innate immune response also supports the subsequent development of adaptive immunity, and thus can accelerate and enhance the induction of vaccine-specific responses. A large number of synthetic or natural TLR ligands have been explored as adjuvants in pre-clinical or clinical studies. Indeed, TLRs are the major pathogen sensors that modulate the host innate and adaptive immune systems and targeting TLR pathways represents a smart strategy to develop therapeutic vaccines. The role of TLRs as adjuvants is traditionally based on promoting DC maturation and antigen presentation. However, T cells also express functional TLRs and TLR signaling can directly modulate T cell function either as costimulatory or survival signaling. Clinical trials involving TLRs 2, 3, 4, 7/8 and 9 (Steinhagen et al., 2011) (Lahiri et al., 2008) (van Duin et al., 2006) support the broad conclusion that TLR ligands can be safe and effective vaccine adjuvants, with vaccines already licensed in the US, Europe and Argentina containing such ligands.

At present, two improved adult HBV and HPV vaccines that use the TLR4 agonist MPL as the adjuvant have been approved (Rappuoli et al., 2011). MPL is a non-toxic derivative of the lipopolysaccharide (LPS) of *Salmonella minnesota,* and is a potent simulator of Th1 responses. The effects of MPL as adjuvant include cytokine production, antigen

presentation and migration of APC to T cell area of draining lymph nodes, allowing for an efficient priming of naïve T cells (Boland et al., 2004).

Another TLR's agonist adjuvant is represented by the synthetic oligodeoxynucleotide containing unmethylated CpG motifs (CpG-ODN), used in both preclinical and clinical studies (Cooper et al., 2004b) (Cooper et al., 2004a). CpG-ODN acts through TLR9 expressed by human plasmacytoid dendritic cells and B cells, inducing cellular activation and secretion of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-12, but also IFN-α and IFN-γ. This TLR9 agonist is an extremely efficient inducer of Th1 immunity, humoral and CTL responses and it is also able to mediate protection against infectious disease and allergic disorders.

The TLR7/8 pathway, specialized in recognition of viral single stranded RNA molecules, has demonstrated promising pre-clinical results as a target for potential vaccine adjuvant. Small molecule agonists of TLR7, such as Imiquimod, or TLR7/8, such as Resiquimod, have been shown to improve the immunogenicity of a variety of anticancer and antiviral vaccines if adequately formulated or directly conjugated to protein antigens. In particular, Imiquimod is licensed as a topical therapy for treatment of HPV and basal cell carcinoma (Schulze et al., 2005) (Lebwohl et al., 2004). The exact mechanism of action of Imiquimod is unknown but it is thought that its activity as a TLR7 agonist leads to the expression of different cytokines such as IL-1, IL-6, IL-12, IFN-α and TNF-α; in turn, these cytokines stimulate or enhance both the innate immune system and the cell-mediated immune response, enhance migration of Langerhans' cells from the dermis to regional lymph nodes, in addition to the induction of apoptosis in basal cell carcinoma.

Multiple TLR2 ligands have undergone clinical testing. The most extensively studied was Pam3Cys linked to outer surface protein A of Borrelia burgdorferi that causes Lyme

disease (Steere et al., 1998). The vaccine, that in clinical trials induced a good Abs protection, was licensed by the FDA in 1998 for general use.

1.2.2.2 TLR2 agonist SMIP2.1 as adjuvant

The discovery that some compounds with adjuvant function are recognized by TLRs drove the interest of Novartis Vaccines in developing a novel family of vaccine adjuvants based on triggering the TLR pathways. In the 2007 Novartis Vaccines and Diagnostics has started a program with the goal to synthesize Small Molecules Immune Potentiators (SMIPs), synthetic compound with a chemical structure similar to TLRs ligands, to be tested as vaccine adjuvants.

Using HEK293 cells expressing TLRs as well as luciferase reporter driven by the promoter of the NF-kB transcription factor, in my group it was identified a new scaffold of chemical compounds, the activity of which was TLR2-dependent. Among them, SMIP2.1 was selected for further characterization. SMIP2.1 is tryacilated lipopeptide, well-know TLR2 agonist (Figure 4). SMIP2.1 showed a strong activation, TLR2 dependent, of innate immune cells, such as monocytes and dendritic cells *in vitro*. It was furthermore evaluated its possible use as adjuvant in vaccine formulations. My group showed that *in vivo* the lipopeptide was able to enhance the specific IgG titers against coadministered protein antigens (H1N1 Solomon Flu and Tetanus Toxoid), clearly demonstrating the adjuvant activity of our molecule (Unpublished data).

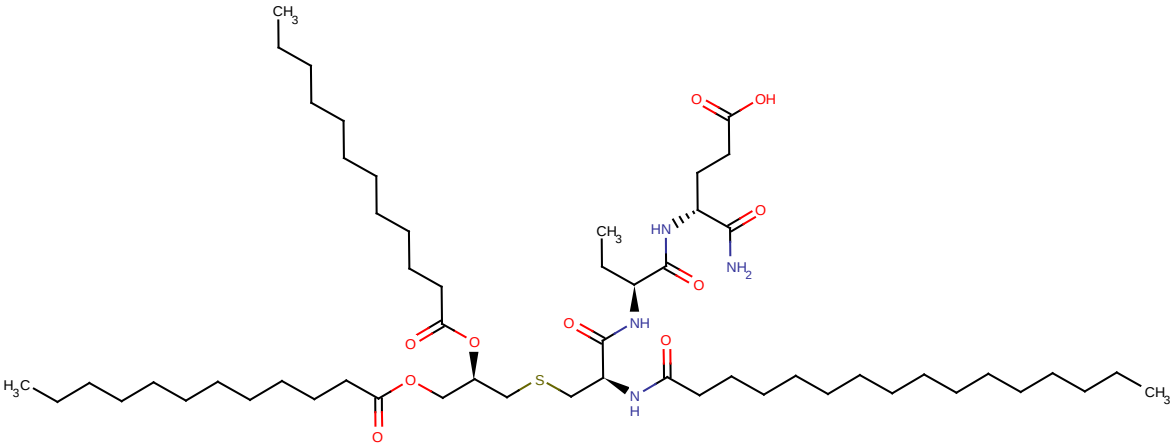


Figure 4. Structure of the SMIP2.1 (IUPAC Name = (4R)-4-[(2S)-2-[(2R)-3-{[(2R)-2,3-bis(dodecanoyloxy)propyl]sulfanyl}-2-hexadecanamidopropanamido]butanamido]-4-carbamoylbutanoic acid).

2. Aim of the research

Efficient MHC presentation of vaccine proteins by APC is a prerequisite for induction of a protective immune response. Purified proteins, which are the component in most of the new vaccine, are usually presented by APCs mainly in class II MHC, since class I presentation of extracellular antigens is generally not very efficient. This results in an optional stimulation of $CD4^+$ T cells but a poor $CD8^+$ T cells priming, which is desirable for a vaccine against virus, intracellular bacteria or cancer. Cross-presentation is the process by which professional APCs are able to load peptides from a processed extracellular protein antigen into the class I molecules, triggering a CTL response. Adjuvants that specifically activate this pathway in the APCs are expected to improve the efficacy of vaccines for which a CTL response is of paramount importance. The discovery of immunization approaches that elicit a robust $CD8^+$ T cell response by activation of the cross-presentation pathway along with a strong antibody response would represent a step forward in the development of vaccines against viral infections and tumors.

Engagement of TLRs can increase cross-presentation, albeit the mechanism that underlies this phenomenon is still not clear. The ability of TLRs to recognize pathogens and modulate the host's innate and adaptive immune system, and the discovery that some compounds with adjuvant function are recognized by TLRs, drove the interest in developing a novel family of vaccine adjuvant based on triggering the TLR pathway.

The aim of my PhD project is to investigate if the novel compound, agonist of TLR2 (SMIP2-1), can enhance cross-presentation of soluble antigens to $CD8^+$ T cells. SMIP2-1 is a novel synthetic lipopeptide, identified through two cell-based high throughput screens

performed at the Genomic Institute of Novartis Research Foundation, and is currently under evaluation as vaccine adjuvant by Novartis Vaccines. Previous *in vitro* and *in vivo* experiments demonstrated that this new compound can induce activation of the innate immune system via a TLR2-dependent mechanism, inducing maturation of antigen presenting cells and eliciting a strong antibody response against Flu and tetanus toxoid antigens (unpublished data).

Both *in vitro* and *in vivo* data show that SMIP2-1 can enhance $CD8^+$ T cells activation, along with a robust $CD4^+$ T cell response, a feature that can be exploited in the design of an effective adjuvant for antitumor and antiviral vaccines.

3. Materials and methods

3.1 Mice and immunization

Female, 8 weeks old C57Bl/6, OT-I and B6-Ly5.2 (Charles River) mice were used for immunogenicity studies approved by the institutional review committees. Animals were immunized intra-muscularly in both quadriceps muscle with 50µl dose per leg with Dulbecco's Phosphate Buffered Saline (DPBS 1x, GIBCO) alone, 10 or 25 µg of OVA protein (EndoGradeTM Ovalbumin, Hyglos) dissolved in DPBS and OVA in the presence of SMIP2.1 or Pam3CSK4. SMIP2.1 was used at 10 or 100 ug/dose/mouse. Pam3CSK4 was purchased from Invivogen (tlrl-pms) and used at 10ug/dose/mouse. Six to ten mice per group were used in different experiments. Mice were immunized at days 0, 21 and 35. Blood samples for antibody titers analysis were collected 2 weeks after the second and the third immunization, and serum prepared by centrifugation. For $K^b/OVA_{257–264}$ tetramer analysis, blood was collected 1 week after each immunization in a tube with heparin.

3.2 Cell Culture

All cultured cells were grown at 37°C in a humidified environment containing 5% CO_2. Mouse cells from lymph nodes and spleens were cultured in complete RPMI 1640 (GIBCO) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine (GIBCO), 2×10^{-5}M 2-mercaptoethanol (Sigma) and 5% heat-inactivated fetal calf serum (Hyclone).

Human PBMC were isolated from buffy coats of healthy donors using Ficoll gradient and cultured in complete RPMI 1640 (GIBCO) supplemented with 100 U/ml penicillin, 100

µg/ml streptomycin, 2mM glutamine (GIBCO), 1% of non-essential amino acids (GIBCO) and 5% heat-inactivated AB human serum (Euroclone).

3.3 OT-I Tcell proliferation *in vitro*: [^{3}H]Thymidine incorporation

CD11c DCs were isolated from spleens of C57Bl6 mice by cell sorting using FACS Aria sorter. Sorted DCs were plated at 25.000 cells/well in a 96 well plate and loaded with medium alone or with medium containing OVA (10ug/ml) or OVA + SMIP2.1 (10uM) for 4 hours. Cells were then washed twice with PBS and incubated in a ratio 1:5 with $CD8^+$ T cells, purified form spleen and total lymph nodes of OT-I mice using a Miltenyi $CD8a^+$ T cell isolation kit,. Cells were pulsed with [^{3}H]Thymidine (Amersham Biosciences) at 0.5µCi/well for the last 18h of a 72h-culture, then harvested onto filter plates (Packard Instruments). [^{3}H]Thymidine uptake was determined using a Top Count NXT β counter (Packard Instruments).

3.4 *In vivo* proliferation of OVA-specific T cells

$CD8^+$ T cells were immunomagnetically separated from spleen and total LN of OT-I mice, accordingly to manufacturer instructions (Miltenyi Biotec). The efficiency of enrichment was routinely 85−95%, as determined by flow cytometry. Cells were incubated with CFSE at 0,5 M for 15min at RT in the dark and CFSE staining was quenched by the addition of 5 ml of FBS. Cells were washed exstensively with PBS and 1×10^6 CFSE-labeled OT-I cells were injected intravenously into Ly5 mice (100ul/mouse). The day after, mice were immunized as indicated. Proliferation of adoptively transferred OT-I cells in the inguinal lymph nodes was quantified 48 h after the immunization by determining the number of

$CFSE^{low}$ OT-I cells by flow cytometry. Total cells were stained with CD3 PE, anti CD8 V500, CD44 APC (BD Pharmingen) and CD45.2 Alexa 700 (eBioscience).

3.5 Tetramer analysis of OVA-specific $CD8^+$ T cells

Peripheral blood samples, collected from the saphenous vein at the time points indicated, were stained with APC-conjugated $K^b/OVA_{257–264}$ tetramer reagent (Beckman Coulter), combined with surface staining using anti-CD8 PE Texas Red, anti CD3 PerCP Cy5.5, anti CD4 V500, anti-CD44 V450 (BD Pharmingen) for 30min at RT. Samples were lysed and fixed (iTAG MHC tetramer lyse and fixative (Beckman Coulter). Cells were analyzed at the cytofluorimeter collecting a minimum of $2{,}5x10^5$ events.

3.6 *In Vivo* Killing Assay

A single-cell suspension of splenocytes from naïve B6-Ly5.2 was prepared, and red cells were lysed. Cells were split into two aliquots and labeled with either 1 μM or 0.1 μM CFSE Cells were then resuspended at a concentration of 1×10^7 cells/ml in cRPMI containing 10 μg/ml $OVA_{257–264}$ peptide ($CFSE^{high}$) or an irrelevant control peptide derived from HCMV pp65protein ($CFSE^{low}$) and incubated for 30 min at 37 °C. Unbound peptide was removed by three washes in PBS, and labeled cells were resuspended at a 1:1 ratio in PBS (50×10^7 cells/ml). 100 μl of cell suspension was delivered intravenously into the tail vein of C57Bl/6 mice that had been immunized 7 days before as described above. Twenty-four hours after target cell implant, mice were euthanized and splenocytes preparations were stained with CD45.2 Alexa 700 and assayed for the presence of CFSE-labeled cells by flow cytometry. The percentage of specific lysis of fluorescent target cells in each group was calculated according to the formula: [1-(mean of CFSE ratio of mice immunized with

adjuvant / mean of CFSE ratio of mice immunized with PBS)] x 100, as described by Ingulli (Ingulli, 2007).

3.7 Determination of Antigen-Specific Antibody titers by ELISA

Anti-Ovalbumin specific total immunogloblulin G (IgG) antibodies were measured by ELISA performed on sera, using a standard reference serum. Maxisorp plates (Nunc) were coated over-night at 4°C with 50 µg/ml of OVA in carbonate buffer, washed with PBS 0,05% Tween-20 and blocked for 1 h at 37°C with 100 µl of PBS 1% BSA. Serum samples and serum standard were serially diluted in PBS + 1% BSA + 0,05% Tween-20 and transferred into OVA coated-blocked plates. Antigen-specific IgG, were detected with alkaline phosphatase-conjugated goat anti-mouse IgG, (Southern Biotech). Plates were washed and the substrate 4-Nitrophenyl phosphate disodium salt hexahydrate (SIGMA) added Color reaction was stopped by addition of H_2SO4 and measured with SpectraMax (Molecular Devices) reader by determining OD at 450 nm. Antibody titers were calculated using a 4-parameter interpolation fit as the reciprocal of the dilution of each serum sample.

3.8 E.G7 tumor challenge experiments

C57BL/6 mice were immunized two times as indicated. 7 days after the second immunization, mice were subcutaneously inoculated in the right flank with $2{,}5 \times 10^5$ E.G7-OVA cells (ATCC) in 100µl of PBS. Mice were monitored every 2-3 days. Tumor bearing-mice were euthanized when mice became moribund in accordance with Animal Care guidelines.

3.9 Confocal analysis of LNs cryosections

OCT-embedded LN was sectioned transversely at 8 □m, mounted onto Superfrost glass slides, and quickly air dried. Slides were fixed in a 4% formaldehyde solution on ice and blocked with PBS + 3% BSA + 1% saponin. Slides were then stained with CD169 FITC (Serotec) and CD45R. Slides were mounted with ProLong gold antifade reagent (Invitrogen), and imaged on a LSM 710 microscope (Zeiss).

3.10 Donors

Buffy coats from healthy donors were obtained from the Blood Transfusion Section, Empoli Hospital. Informed consent was obtained before all blood donations. The study protocol was approved by the Novartis Research Center ethical committee and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

3.11 Stimulation with CMV peptide for activation of antigen-specific $CD8^+$ T cells

PBMC isolated from healthy blood donors were assayed for the positivity to CMV using CMV-specific tetramers (Bekman Coulter) and then stimulated with a mix of peptides derived from pp65 protein (JPT Peptide Technologies) at 3ug/ml for each peptide. Cells were co-cultured for 14 days, replacing fresh medium when required and adding IL-2 at days 4, 7, 10. At day 14, autologous PBMC were plated in a 96 well plate at 500.000 cells/well in complete medium and loaded with pp65 protein (Miltenyi Biotec) at 50 µg/ml with or without SMIP2.1 at 15 µM. After 2 hours, cells were washed twice and co-cultured over-night with the expanded CMV specific $CD8^+$ T cells population in a ratio 1:1 in the presence of 5 µg/ml secretion inhibitor BFA (Sigma). Cells were then fixed, stained for intracellular cytokine production with CD3 V450, CD8 APC, IFN□ PerCP-Cy5.5 and

CMV-specific tetramer PE and analyzed by flow cytometry. The magnitude of IFN□ production in the effector OT-I T cells was used as a measure of the level of Ag cross-presentation by DCs.

3.12 Antibodies and flow cytometry

Cells were stained with combinations of the following antibodies: Ly6C-FITC, CD11b-PE-Cy7, CD11c-APC, F4/80 PacificBlue, CD11c-APC-AlexaFluor750 (all from eBioscience). Flow cytometry was performed on FacsCanto or FACS LSRII instruments using DIVA software (Becton Dickinson) and data were analyzed using Flowjo software (Treestar Inc.).

4. Results

4.1 SMIP2.1 induces cross-presentation *in vivo*

In order to test the potential of SMIP2.1to induce cross-presentation of soluble antigen *in vivo*, we assessed its effects on antigen-induced expansion of $CD8^+$ T cells. We used TCR transgenic OT-I $CD8^+$ T cells that recognize an OVA-derived peptide and that have been extensively used in many *in vivo* and *in vitro* experimental settings to investigate cross-presentation (Hogquist et al., 1994). We compared the efficacy of our compound with the TLR2 ligand benchmark N-Palmitoyl-S-cysteinyl-seryl-4lysyl (Pam3CSK4). In a first experiment, we adoptively transferred CFSE-labeled OT-I $CD8^+$ T cells in congenic Ly5 mice and the day after we immunized the recipient mice with PBS alone, OVA, OVA + SMIP2.1 or OVA + Pam3CSK4. We determined OT-I proliferation in draining lymph nodes after 48 h by flow cytometry using a mix of antibodies as described in Fig.1.

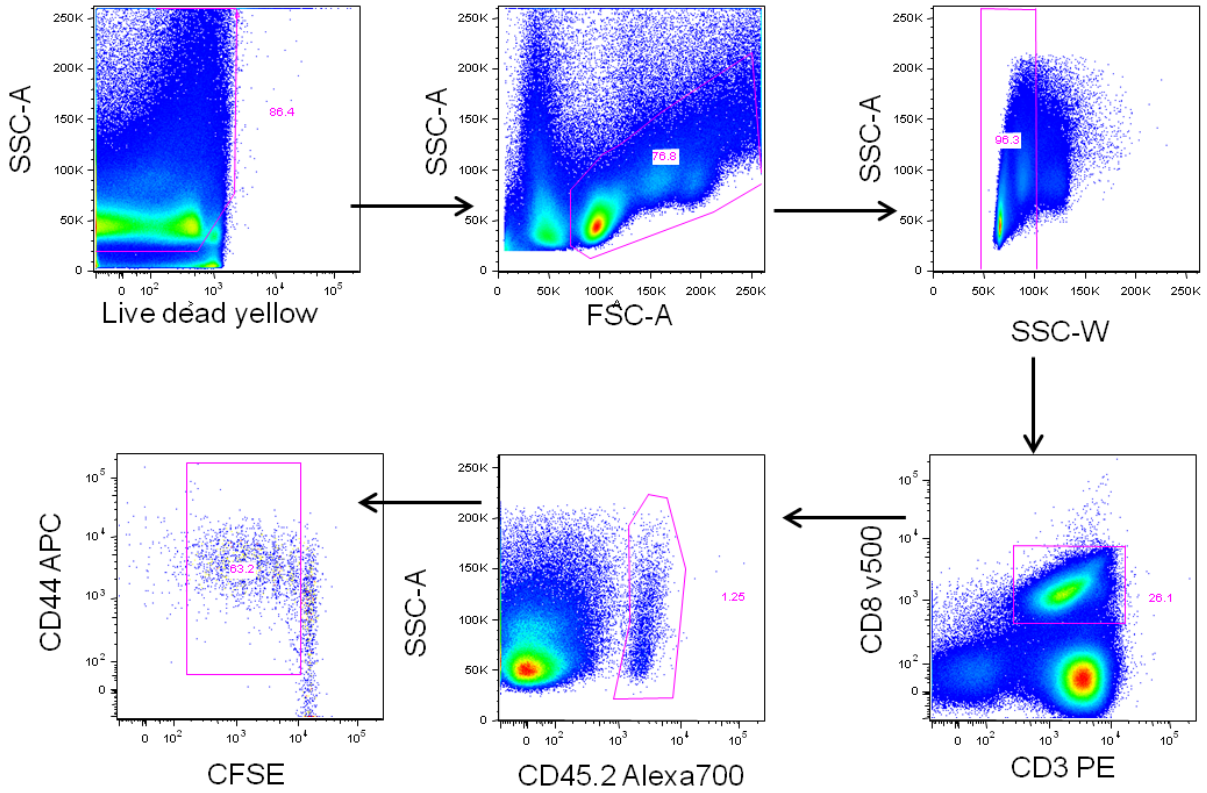
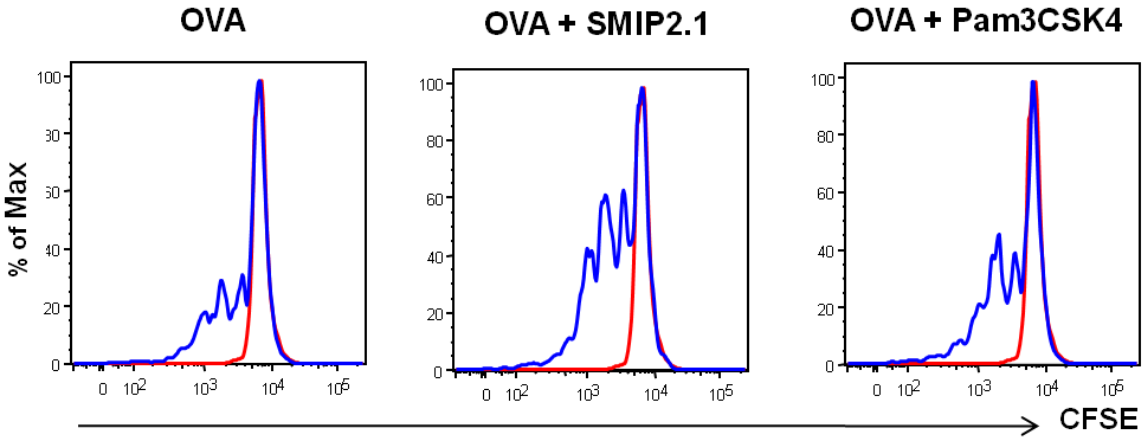


Figure 1. Gating strategy for defining proliferating CD8$^+$ T cell population in the adoptive transfer experiment. Cells isolated from draining LN have been stained with the indicated antibodies (CD3 PE, CD8 V500, CD45.2 Alexa 700, CD44 APC) to identify the adoptively transferred proliferating CD8$^+$ T cell population (CFSE^{low} CD8$^+$ T cells). The inset numbers represent the percentage of the gated cells in the respective gating step. FCS-A, forward scatter; SSC-A, side scatter; SSC-W, width scatter.

The magnitude of OT-I CD8$^+$ T cells expansion was used as a measure of the level of OVA cross-presentation. Analysis of CFSE dilution revealed an increased proliferation of OT-I cells in mice immunized with the protein in the presence of SMIP2.1 (48,9% dividing OT-I cells in the OVA + SMIP2.1 condition *vs* 24,5% in the not adjuvanted OVA condition) **(Fig2A and B)**. We observed a comparable CD8$^+$ T cells expansion in mice immunized with OVA + Pam3CSK4 (43,8% dividing OT-I cells in the OVA + Pam3CSK4 condition *vs* 24,5% in the not adjuvanted OVA condition).

A



B

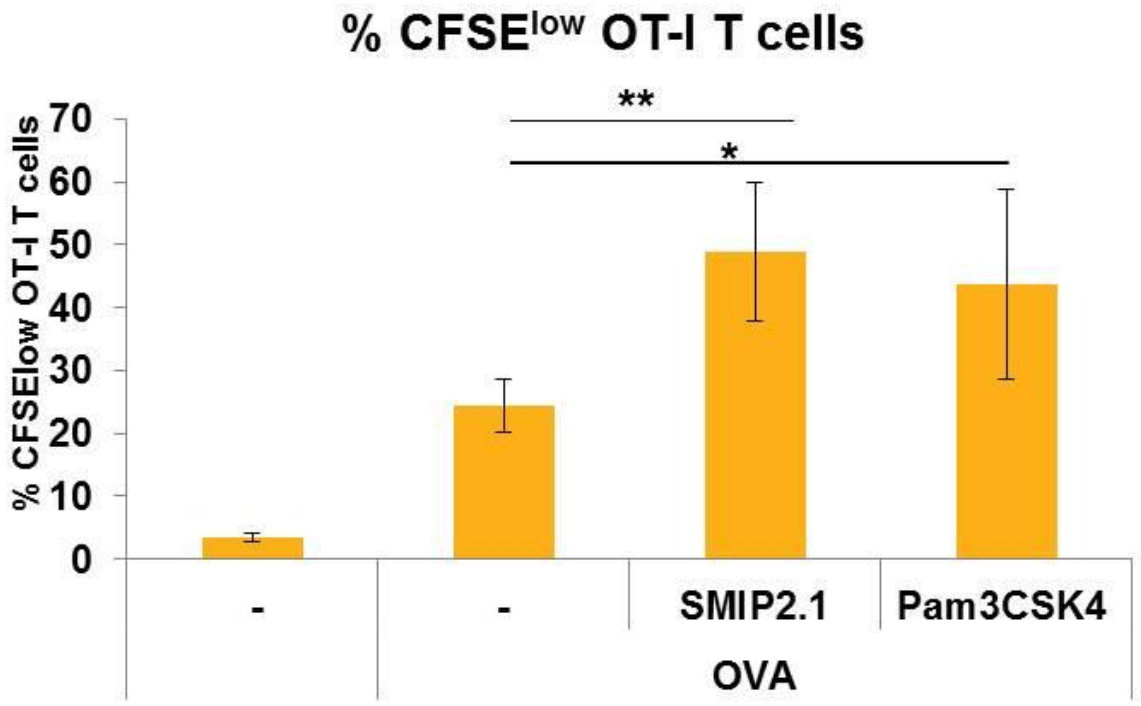
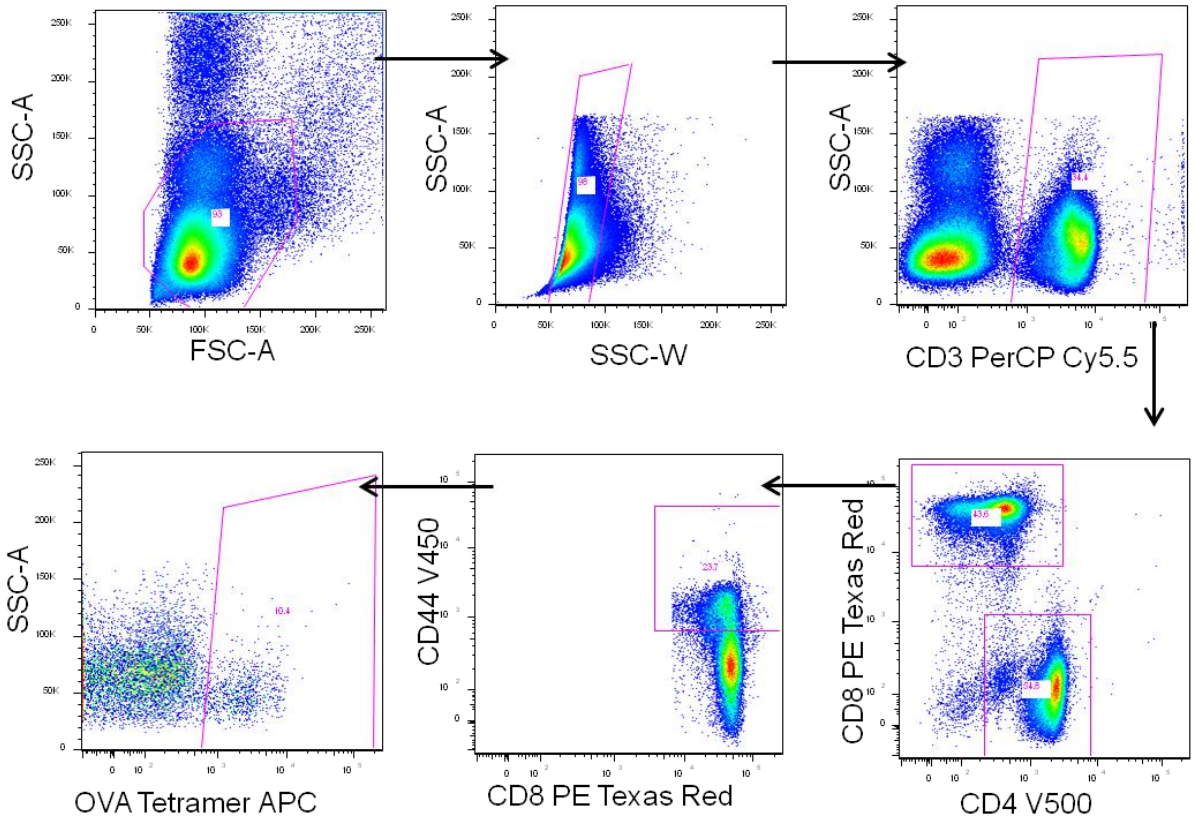


Figure 2. SMIP2.1 induces cross-presentation *in vivo.* Congenic Ly5 mice, injected in the tail vein with CFSE-labeled OT-I $CD8^+$ T cells, were immunized with PBS alone, OVA (10 µg/mouse), OVA + SMIP2.1 (10 µg/mouse), OVA + Pam3CSK4 (10 µg/mouse). ***A,*** One representative plot for each group is shown to compare proliferation of adoptively transferred OTI cell in PBS immunized mice (red curve) to OVA, OVA + SMIP2.1 and OVA + Pam3CSK4. ***B,*** The graph shows the percentage of proliferating $CD8^+$ T cells (mean ± SD). Representative data of three independent experiments are shown.

We then tested the ability of SMIP2.1 to induce cross-presentation in a more physiological vaccination animal model. We immunized C57Bl/6 mice at days 1, 21, 35 with PBS alone, OVA, OVA + SMIP2.1 or OVA + Pam3CSK4. We collected blood samples, sera and

spleens for further analysis. Priming of CTLs, induced by cross-presentation of OVA protein to naive $CD8^+$ T cells, was evaluated measuring the percentage of $CD8^+$ T cells positive for the K^b-$OVA_{257-264}$ tetramer, in the peripheral blood 7 days after the 1^{st} and the 2^{nd} immunization **(Fig3A)**. No expansion of Ag-specific $CD8^+$ T cells was observed in the absence of TLR2 agonists. Addition of SMIP2.1 or Pam3CSK4 strongly enhanced the frequency of OVA primed $CD8^+$ T cells and the magnitude of the response increased significantly after the second immunization **(Fig3B)**. SMIP2.1 proved to be more potent than the benchmark Pam3CSK4.

A



B

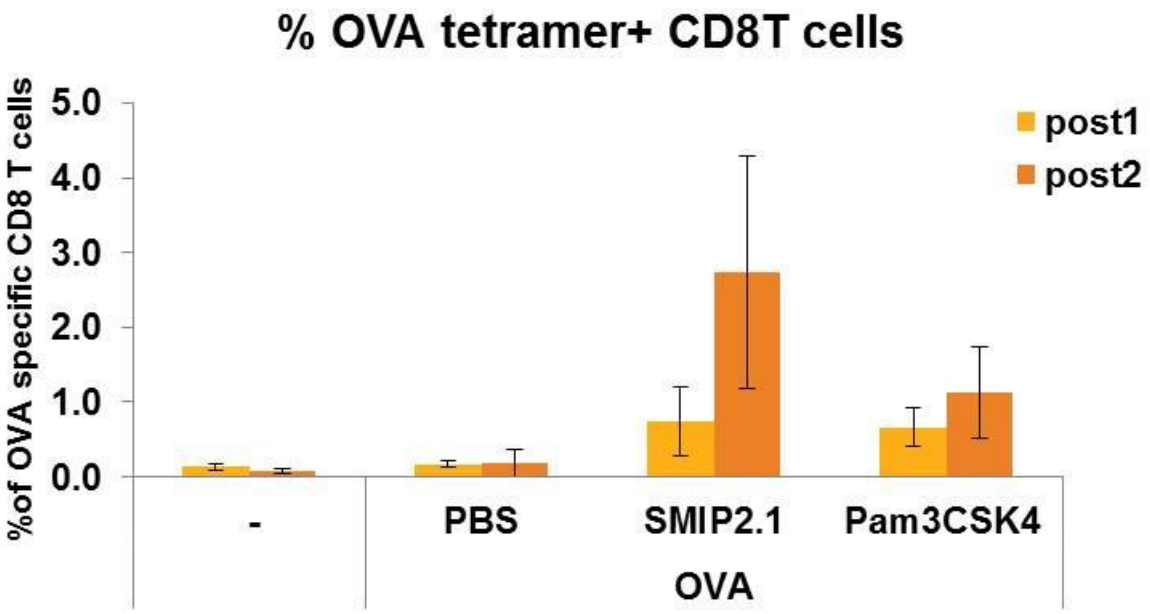


Figure 3. SMIP2.1 induces cross-presentation *in vivo.* C57Bl/6 mice were immunized twice with PBS alone, OVA (25 µg/mouse), OVA + SMIP2.1 (10 µg/mouse) or OVA + Pam3CSK4 (10 µg/mouse) and bled from the tail vein 7d days post 1st and 2nd immunization. Blood cells were stained with K^b /$OVA_{257-264}$ tetramer to measure the frequency of OVA specific $CD8^+$ T cells. ***A,*** Gating strategy used for defining frequency of OVA specific $CD8^+$ T cells. Blood cells have been stained with the indicated antibodies (CD3 PerCP Cy5.5, CD8 PE Texas Red, CD4 V500, CD44 V450). ***B,*** The histogram show the percentage of OVA specific $CD8^+$ T cells. Data shown is representative of two different experiments.

7 days post 3rd immunization, mice were sacrificed and splenocytes were re-stimulated *ex vivo* with PBS alone or with $OVA_{257-264}$ peptide. We compared the ability of splenic $CD8^+$ T cells to respond to specific antigenic stimuli by measuring intracellular IFN□ and TNF□

production by flow cytometry staining **(Fig4)**. We showed that splenic $CD8^+$ T cells of mice immunized with SMIP2.1 or Pam3CSK4 were able to induce a strong pro inflammatory cytokines production when re-stimulated *in vitro*. Taken together, these data demonstrate that SMIP2.1 was able to expand the number of functionally active $CD8^+$ T cells even more efficiently than Pam3CSK4, as shown by the increased frequency of $CD8^+$ T cells and pro-inflammatory cytokine production.

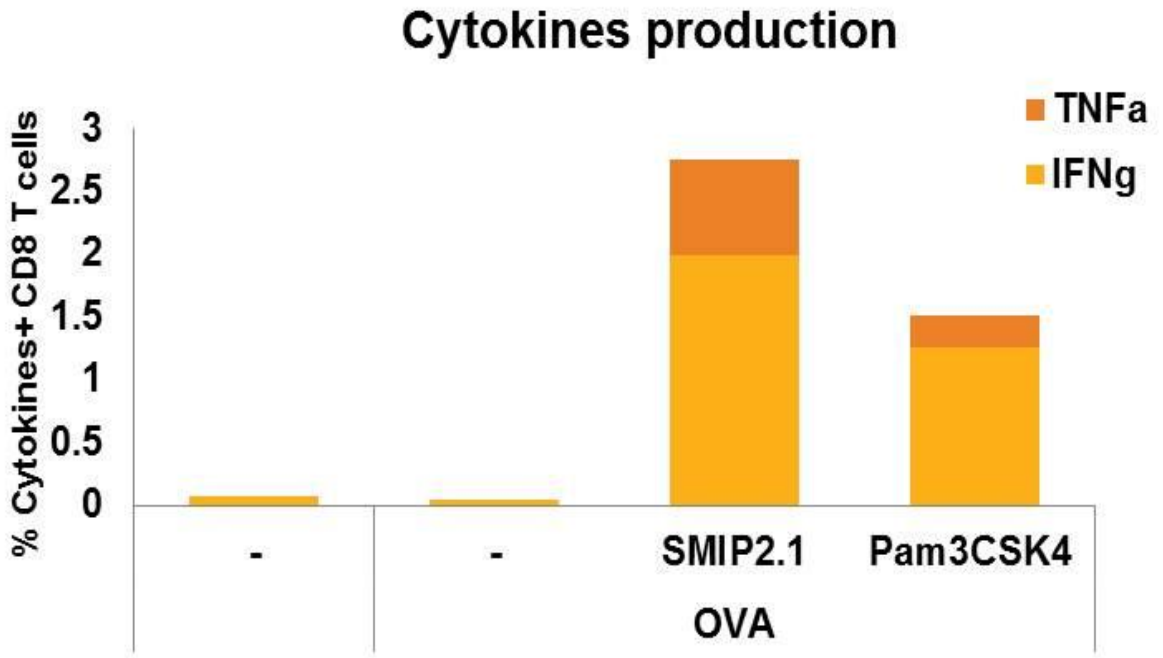


Figure 4. SMIP2.1 induces cross-presentation *in vivo.* C57Bl/6 mice were immunized twice with PBS alone, OVA (25 µg/mouse), OVA + SMIP2.1 (10 µg/mouse) or OVA + Pam3CSK4 (10 µg/mouse). Spleens of C57Bl/6 immunized mice were collected 7 days after the 3rd immunization and spelonocytes were pulsed with $OVA_{SIINFEKL}$ peptide (3 µg/ml) or with PBS alone, as negative control, for 6h. Cells were then fixed and stained for IFNγ and TNFα.

SMIP2.1 has been previously tested *in vivo* as adjuvant in two vaccines against tetanus and influenza in mouse. In both systems, the compound was able to significantly increase the

antibody titer against the vaccine antigen (Manuscript in preparation). Also in OVA immunized mice, we demonstrated that SMIP2.1 is more potent than Pam3CSK4 inducing high OVA specific total IgG titer, measured in the serum two weeks after the 3rd immunization **(Fig5)**.

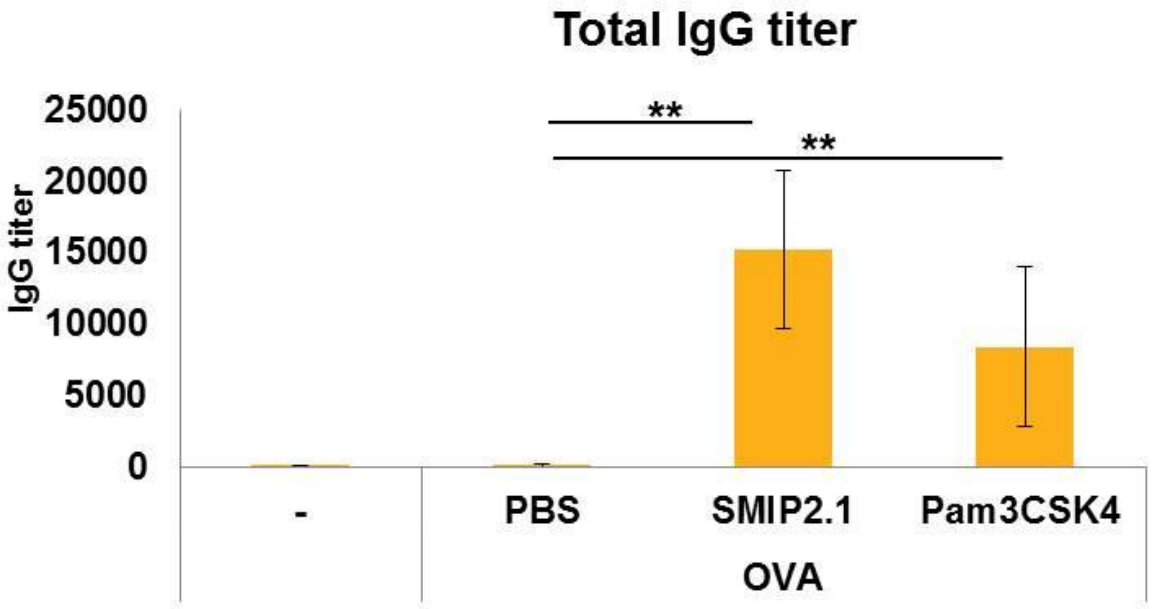


Figure 5. SMIP2.1 boosts antibody production. Sera from immunized mice were collected 2 weeks after the 3rd immunization and total IgG titer was measured by ELISA. Data are representative of 2 different experiments.

4.2 SMIP2.1 induces antigen-specific CTL activity *in vivo*

We then evaluated if antigen-specific $CD8^+$ T cells, induced by immunization with OVA protein plus SMIP2.1, were functional in terms of cytolytic activity. To test the CTL activity, we used an *in vivo* cytotoxicity assay wherein C57Bl/6 mice were immunized with

PBS alone, OVA, OVA + SMIP2.1 or OVA + Pam3CSK4. Seven days after the immunization, we injected mice i.v. with CFSE labeled splenocytes, isolated from a congenic Ly5 mouse, pulsed with either $OVA_{SIINFEKL}$ peptide or an irrelevant control peptide and mixed in a ratio 1:1 prior to injection. The SIINFEKL-loaded splenocytes were labeled with 1 μM CFSE and are identified as the $CFSE^{high}$ target population, while the control splenocytes, labeled with an irrelevant peptide, were labeled with 0.1μM CFSE and are identified as the $CFSE^{low}$ cell population. Twenty-four hours after target cell implant, we sacrificed mice and assayed the splenocytes for the presence of CFSE-labeled cells by flow cytometry. The ratio between the two cell populations correlates with cytolitic activity **(Fig6)**.

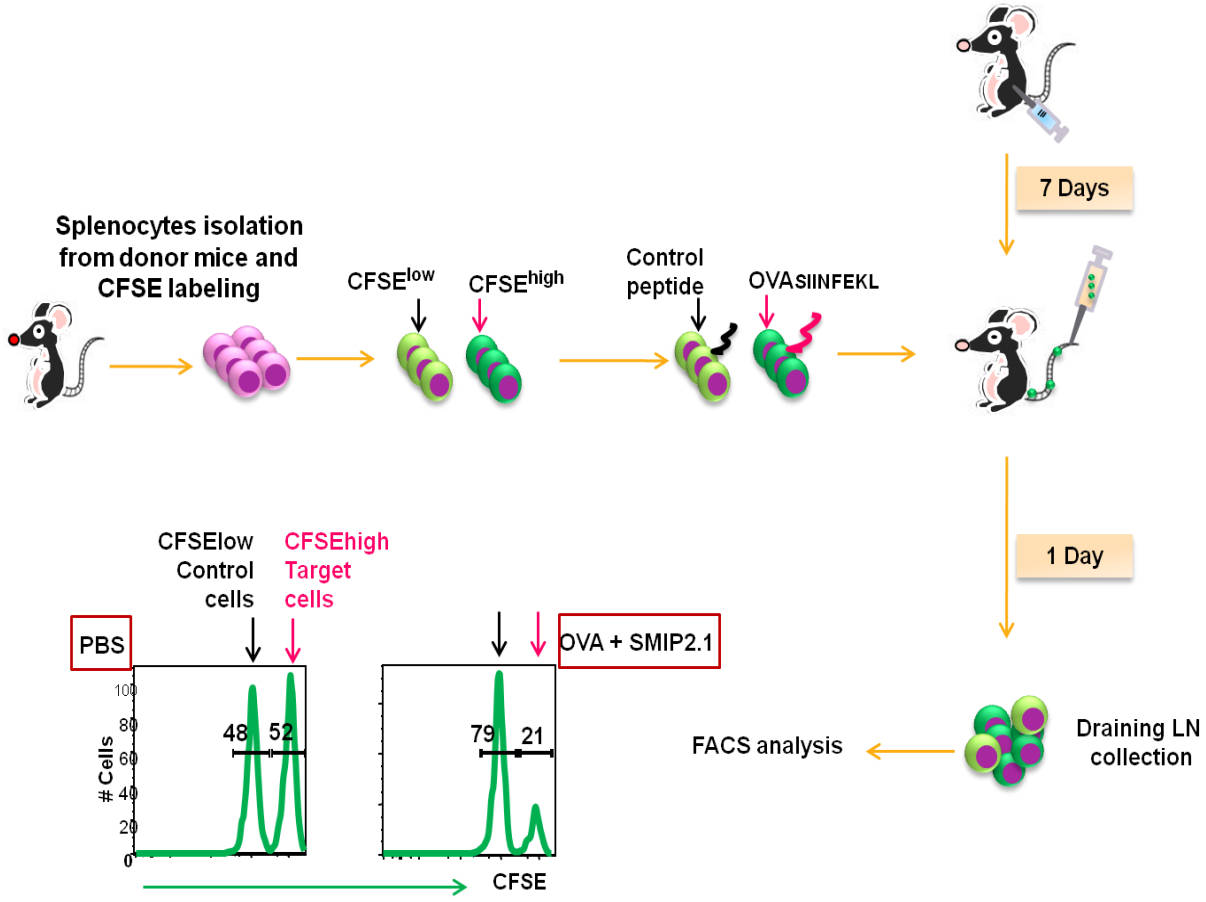
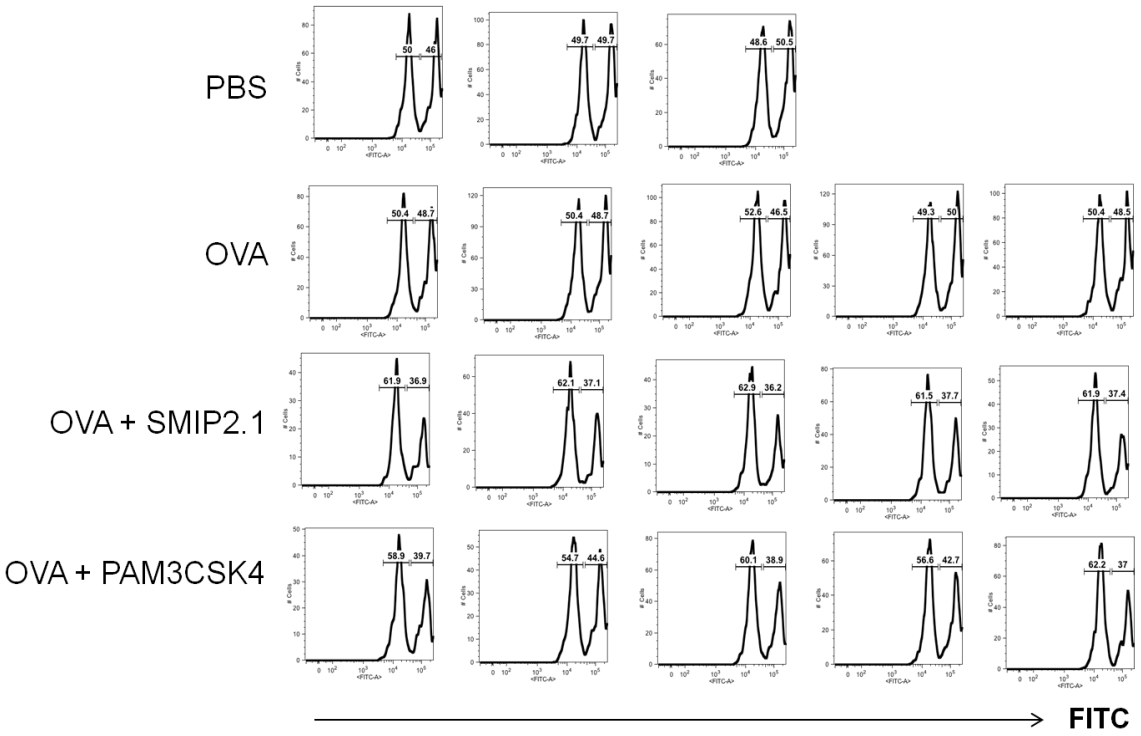


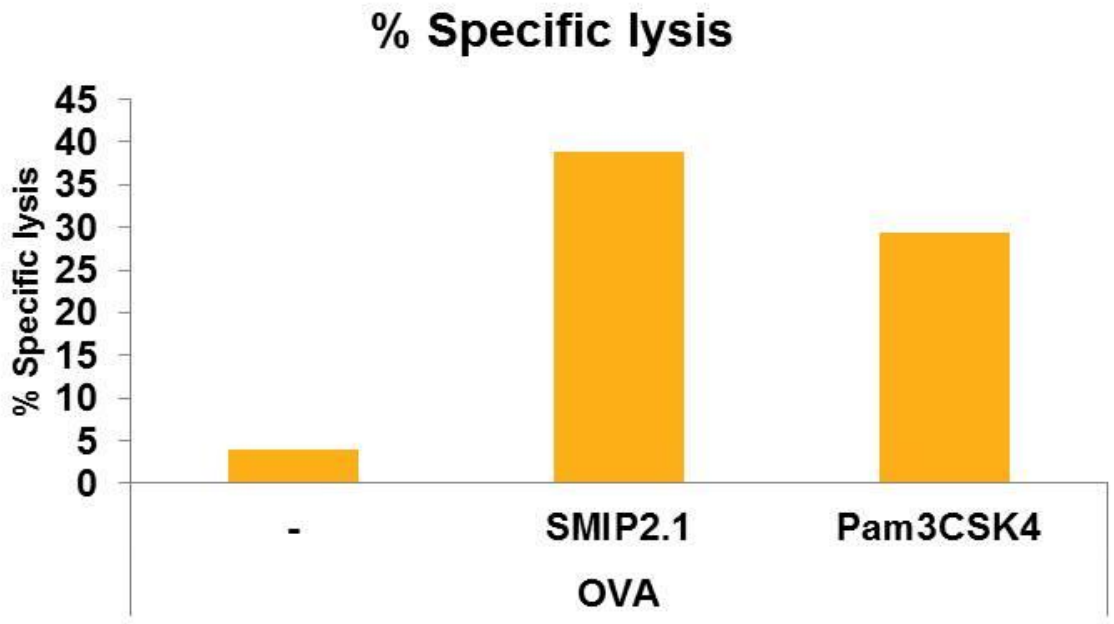
Figure 6. Experimental scheme describing the cytotoxic assay *in vivo*. Seven days after the immunization, C57Bl/6 mice were injected i.v. with CFSE labeled splenocytes, isolated from a donor mouse, pulsed with either $OVA_{SIINFEKL}$ peptide or an irrelevant control peptide and mixed in a ratio 1:1 prior to injection. The SIINFEKL-loaded splenocytes were labeled with 1 μM CFSE ($CFSE^{high}$ target population), while the control splenocytes, labeled with an irrelevant peptide, were labeled with 0.1μM CFSE ($CFSE^{low}$ control population). Twenty-four hours after target cell implant, we sacrificed mice and assayed the splenocytes for the presence of CFSE-labeled cells populations by flow cytometry.

The killing activity towards peptide coated target cells is strongly increased when mice are immunized with OVA plus TLR2 agonists compared to mice immunized with OVA alone **(Fig7A and B)** and SMIP2.1 is even more efficient than Pam3CSK4. The OVA-specific CTL activity correlates with the expansion of OVA specific $CD8^+$ lymphocyte population, as demonstrated by the percentage of $OVA_{SIINFEKL}$ tetramer positive $CD8^+$ T cells in peripheral blood in mice immunized with adjuvanted OVA **(Fig7C)**.

A


PBS
OVA
OVA + SMIP2.1
OVA + PAM3CSK4
50 46
49.7 49.7
48.6 50.5
50.4 48.7
50.4 48.7
52.6 46.5
49.3 50
50.4 48.5
61.9 36.9
62.1 37.1
62.9 36.2
61.5 37.7
61.9 37.4
58.9 39.7
54.7 44.6
60.1 38.9
56.6 42.7
62.2 37
FITC

B


% Specific lysis
% Specific lysis
45
40
35
30
25
20
15
10
5
0
-
SMIP2.1
Pam3CSK4
OVA

C

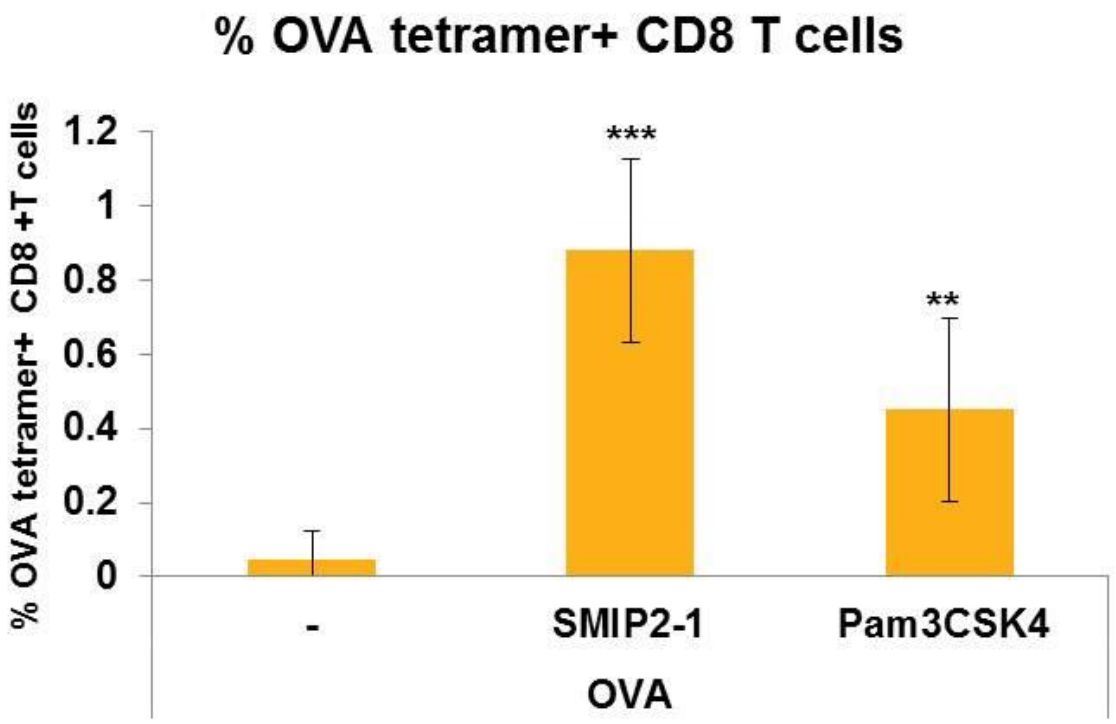


Figure 7. SMIP2.1 induces antigen-specific CTL activity *in vivo.* C57Bl/6 mice were immunized with PBS alone, OVA (25 µg/mouse), OVA + SMIP2.1 (10 µg/mouse) or OVA + Pam3CSK4 (10 µg/mouse). After seven days, mice were implanted i.v. with syngeneic splenocytes loaded with two different concentrations of CFSE and pulsed with either $OVA_{SIINFEKL}$ peptide ($CFSE^{high}$) or an irrelevant control peptide ($CFSE^{low}$) in a ratio 1:1. Twenty-four hours later, CTL response was assessed in draining LN measuring the presence of $CFSE^{high}$ target cells by flow cytometry. **A,** In the panel are reported the histograms showing the CFSE content for each mouse (3 or 5 mice/group) in OVA, OVA + SMIP2.1 and OVA + Pam3CSK4 immunized groups. **B,** The graph shows the percentage of specific lysis of fluorescent target cells in OVA, OVA + SMIP2.1 and OVA + Pam3CSK4 immunized groups calculated as described in M&M. **C,** A peripheral blood sample was obtained from mice prior to cells infusion and cells were stained with K^b /$OVA_{257-264}$ tetramer to

measure the frequency of OVA specific $CD8^+$ T cells. The percentage of K^b /$OVA_{257-264}$ $tetramer^+$ $CD8^+$ T cells in mice immunized with PBS was subtracted to the other groups.

We next assessed if $CD8^+$ T cells elicited by immunization with OVA plus SMIP2.1 were able to recognize and kill tumor cells, evaluating the antitumor effect of SMIP2.1 in mice bearing E.G7-OVA tumors. Mice were immunized twice with PBS alone, OVA, OVA + Pam3CSK4 10 µg/mouse or OVA + SMIP2.1 at 10 µg/mouse. 7 days after the second immunization, mice were subcutaneously inoculated with OVA-expressing E.G7 tumor cells and tumor growth was monitored. 47 days after E.G7-OVA tumor cells inoculation, the 80% of non-vaccinated and OVA-immunized mice died (20% of survival). In mice immunized with OVA + TLR2 agonists the rate of survival was considerably increased. In mice immunized with OVA + Pam3CSK4, 5 mice out of 10 developed tumor (50% of survival) while in mice immunized with OVA + SMIP2.1 at 10 µg/ml only 3 out of 10 mice developed tumor and were sacrificed (70% of survival) (**Fig8**). We can then conclude that in this prophylactic setting immunization with OVA + SMIP2.1 results in a strong tumor growth inhibition.

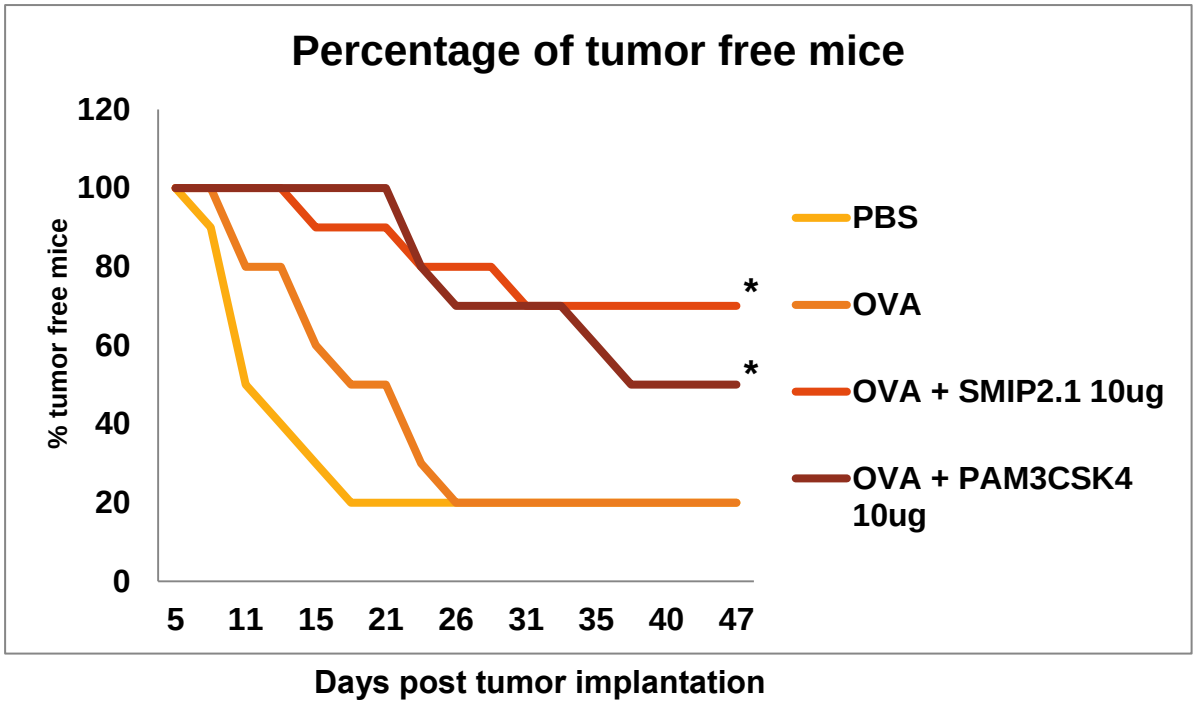


Figure 8. SMIP2.1 induces antigen-specific CTL activity *in vivo*. C57Bl/6 mice were immunized twice with PBS alone, OVA (25 µg/mouse), OVA ± SMIP2.1 (10 µg/mouse) or OVA + Pam3CSK4 (10 µg/mouse). Seven days after the second immunization, mice were implanted s.c. with OVA-expressing E.G7 tumor cells and mice were monitored for tumor growth. The graph shows the percentage of tumor-free mice 47 days after tumor cells implantation. Mice were euthanized when moribund. Representative data of three independent experiments are shown.

4.3 SMIP2.1 induces antigen deposition

The mechanisms by which adjuvants enhance immunogenicity of the antigens have been investigated for several years. Some papers show that they increase Ag depot, prolonging the persistence of antigen *in vivo* (Seubert et al., 2008) (Schijns and Tangeras, 2005). To explore the mechanism by which TLR2 agonist works as a vaccine adjuvant, we examined the possibility that SMIP2.1 increases the antigen depot in lymphoid organs. We

immunized mice with fluorescent-labeled OVA in the presence or not of SMIP2.1 and we collected the draining LN 24 hours after the immunization to detect the antigen deposition. We analyzed the tissues by confocal microscopy and found that mice immunized with OVA plus SMIP2.1 show a slightly increase in the deposition of the OVA antigen compared to mice immunized with OVA alone **(Fig9).** Therefore, also SMIP2.1 increases the amount of captured antigen.

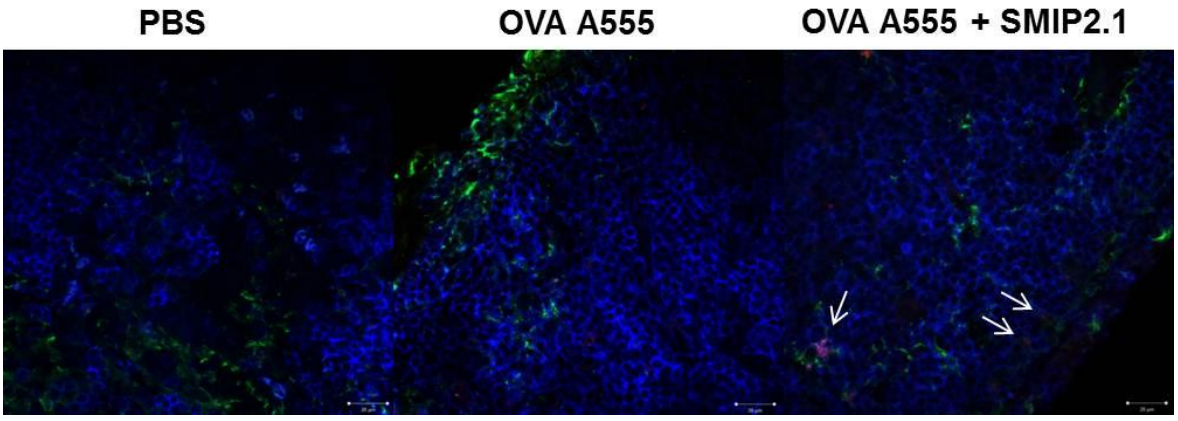


Figure 9. SMIP2.1 increases Ag deposition in the draining LN. C57Bl/6 mice were immunized with PBS alone, OVA A555 (25 µg/mouse) ± SMIP2.1 (100 µg/mouse) and draining LN were collected 24h later. 8µm thick cryosections of draining LNs were stained for CD169 (green) and CD45R (blue) and observed under a confocal microscope. The picture (magnitude 40x) shows the OVA antigen deposition (red) only in mice immunized with OVA + SMIP2.1. Bar represents 20 µm.

We then analyzed if SMIP2.1 has an effect in the induction of antigen uptake by a specific cell type. To this aim, we collected draining LNs from fluorescent-OVA immunized mice and we analyzed antigen content in different cell types by FACS using CD11b, CD11c,

MHC II, CD8α, F4/80, Ly6C cell markers to identify CD8α$^-$ DCs (Cd11b^{high}, CD11c$^+$, CD8α$^-$, MHC II$^+$), CD8α$^+$ DCs (Cd11b^{high}, CD11c$^+$, CD8α$^+$, MHC II$^+$), macrophages (Cd11b$^+$, F4/80^{high}), inflammatory monocytes (Cd11b^{high}, CD11c$^-$, Ly6C^{high}) and B cells (Cd11b$^-$, CD11c$^+$, MHC II$^+$). In agreement with the confocal analysis, SMIP2.1 induces an increase in OVA uptake and this is evident in all cell subtypes analyzed **(Fig10).**

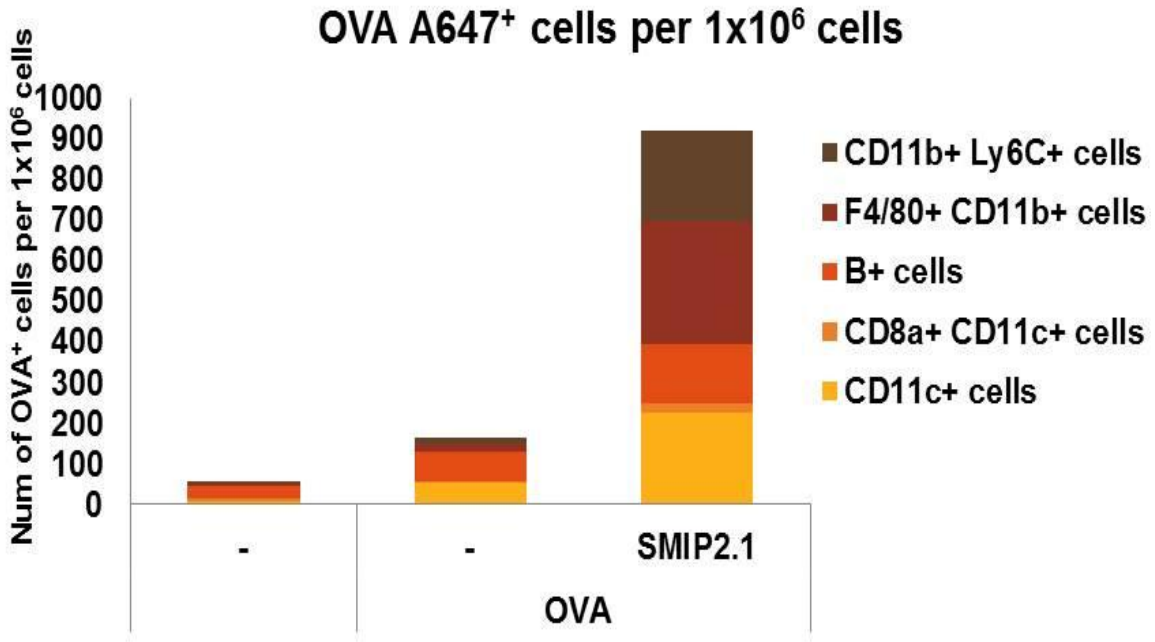


Figure 10. SMIP2.1 increases Ag deposition in the draining LN. Groups of 3 mice were immunized with PBS alone, OVA A647 (25 µg/mouse) ± SMIP2.1 (100 µg/mouse). Draining LN were collected 24h later and analyzed in pool by FACS to identify specific cells types and Ag-content. The graph shows the number of OVA A647$^+$ cells per 1x10^6 total cells. The data shown are representative of two independent experiments.

4.4 SMIP2.1 induces cross-presentation *in vitro*

In mice, two subpopulations of dendritic cells are discriminated by the expression of the CD8α marker, with different functions. $CD8\alpha^+$ $CD11c^+$ DCs may have the unique ability to cross-present (Belz et al., 2004) (Shortman and Heath, 2010) (Heath and Carbone, 2001) whereas in humans, cross-presenting DC are $CD141^+$ (1977)($BDCA3^+$). However, there are evidence showing that both $CD8\alpha^+$ and $CD8\alpha^-$ $CD11c^+$ are able to present exogenous antigen to $CD8^+$ T lymphocytes with equivalent efficacy when opportunely stimulated (den Haan and Bevan, 2002) (Moron et al., 2002). Since immunization with TLR2 agonist increases the antigen uptake by both $CD8\alpha^+$ $CD11c^+$ and $CD8\alpha^-$ $CD11c^+$ dendritic cells, we assessed the potential of SMIP2.1 to enhance cross-presentation ability of both $CD11c^+$ DCs subpopulations, evaluating its effect on antigen-induced expansion of $CD8^+$ T cells *in vitro*.

$CD8\alpha^+$ $CD11c^+$ and $CD8\alpha^-$ $CD11c^+$ dendritic cells were purified by cell sorting from the spleen of a C57Bl/6 mouse and loaded with soluble low endotoxin OVA protein, in the presence or not of SMIP2.1, for 4 hours. After washing, DCs were co-cultured for 72 hours with OT-I $CD8^+$ T cells, that were previously immunomagnetically isolated from the spleen and total lymph nodes of OT-I transgenic mice. As assessed by thymidine incorporation, both DC populations pulsed with OVA and SMIP2.1 induced a greater OT-I proliferation as compared to DCs pulsed with OVA alone **(Fig11)**.

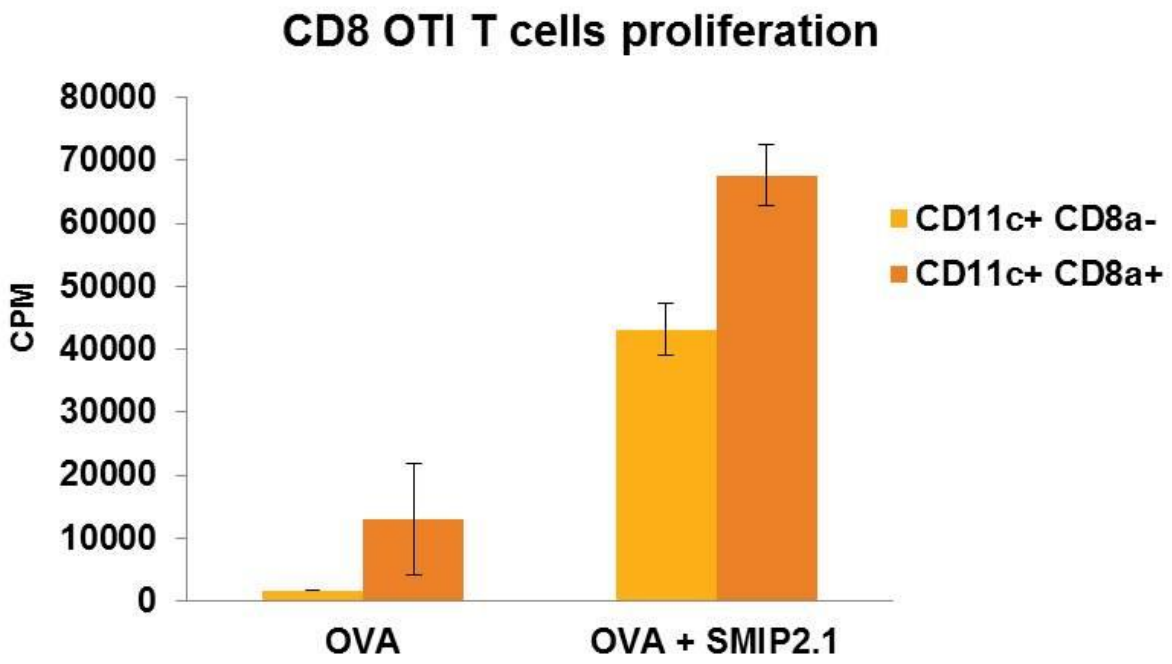


Figure 11. *In vitro* proliferation of OT-I cells to OVA protein. $CD8\alpha^+$ $CD11c^+$ and $CD8\alpha^-$ $CD11c^+$ DCs were purified by cell sorting from the spleen of C57Bl/6 mice and cultured with medium alone, OVA (10 µg/ml) ± SMIP2.1 (10 µM) for 4 hours. After washing, DCs were co-cultured for 60 hours with purified OT-I $CD8^+$ T cells. ^3H thymidine (0,5µCi/well) was added and proliferation of $CD8^+$ OTI T cells was checked after 12 h by liquid scintillation counting. Data indicate counts per min (CMP) and expressed as mean ± SEM of triplicate wells. Values of CPM from cells stimulated with medium alone were subtracted from the other conditions.

4.5 In human experiment

To test if SMIP2.1 is able to induce cross-presentation also in human cells, we co-cultured PBMCs isolated from a HCMV seropositive donor with autologous $CD8^+$ T cells recognizing a mix of peptides from the HCMV protein pp65. CMV is an herpes simplex virus and $CD8^+$ T cell response to CMV antigens is detectable in most CMV-seropositive

donors (Harari et al., 2004). We set up a cell-based *in vitro* model in which we evaluate the efficiency of cross-priming with the purified recombinant protein pp65 in human peripheral blood mononuclear cells (PBMC), using a flow cytometry assay to detect intracellular cytokine production by $CD8^+$ T cells.

PBMC from CMV seropositive donors were isolated by a density gradient centrifugation in Ficoll and stimulated with a library of pp65 peptides to expand the CMV-specific memory $CD8^+$ T cell population, as identified by tetramer staining **(Fig12)**.

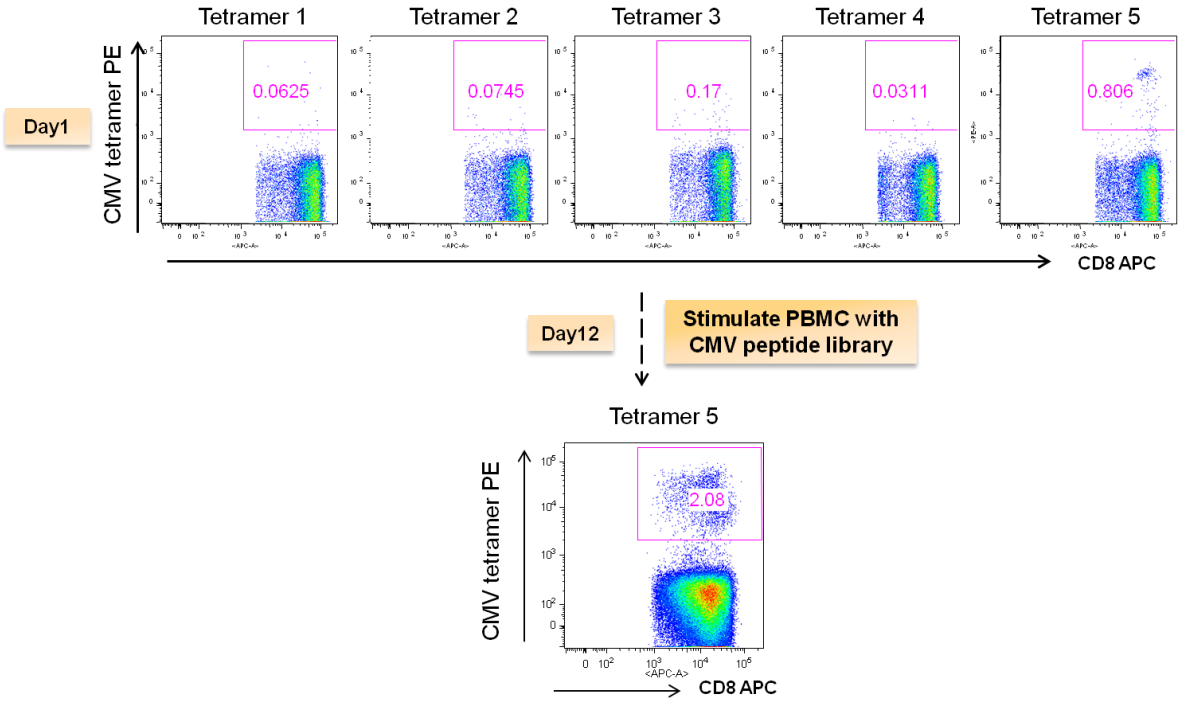


Figure 12. CMV tetramer staining on human peripheral blood: example. PBMC from a CMV seropositive donor were stained with 5 CMV tetramers, able to recognize different TCR specific for 5 immunodominant peptides of the CMV pp65 protein. The inset numbers represent the percentage of the CMV $tetramer^+$ cells for the different tetramers. In the example shown, the donor has the 0,806% of $CD8^+$ T cells specific for the CMV tetramer 5. PBMC were stimulated with a library of pp65 peptides to expand the CMV-specific memory $CD8^+$ T cell population, as demonstrated by

tetramer staining 12 days later showing an increase in the percentage of CMV tetramer 5 specific $CD8^+$ T cells (2.08 vs 0,8%).

The CMV-specific $CD8^+$ T cells were then restimulated with autologous APC that were previously loaded with pp65 CMV protein with or without SMIP2.1. After 4 hours of stimulation, cells were fixed, permeabilized and stained with CD3, CD8, IFNγ antibodies and CMV-tetramers to detect IFNμ production by CMV-specific $CD8^+$ T cells.

In the experiments shown in **Figure 13**, APCs pulsed with pp65 antigen and stimulated with SMIP2.1 were found to cross-present the protein more efficiently than APCs pulsed with pp65 antigen alone, as it is shown by their ability to stimulate a higher frequency of IFNμ producing CMV-specific $CD8^+$ T cells.

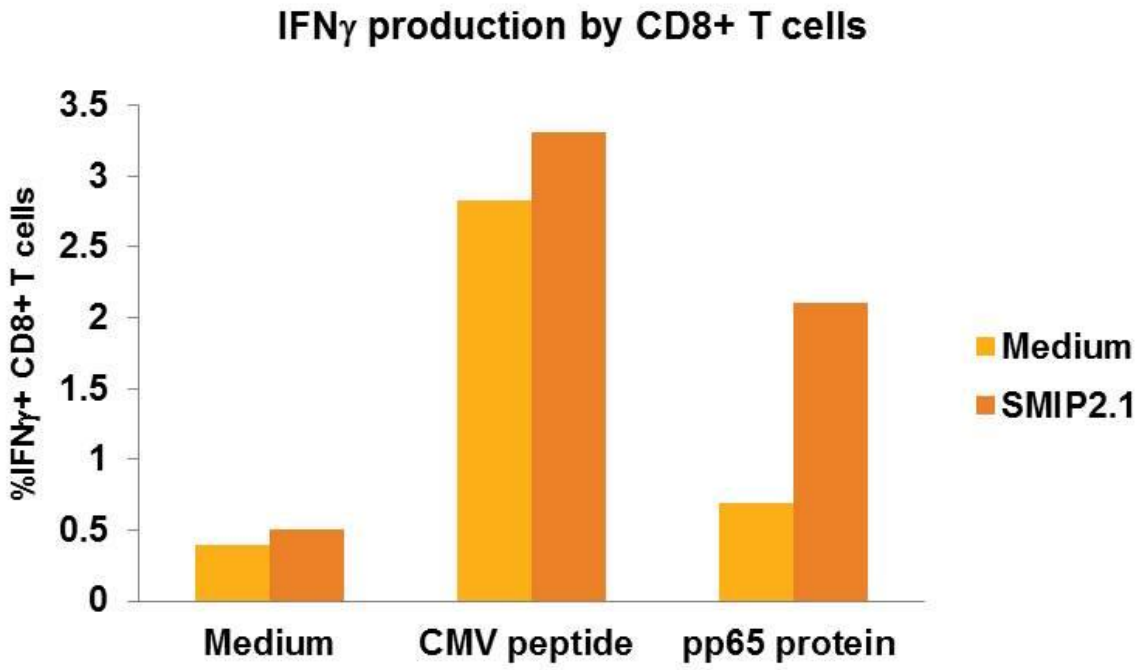


Figure 13. SMIP2.1 induces cross-presentation *in vitro* in human cells. PBMC isolated from a CMV seropositive donor were pulsed *in vitro* as indicated for 2 hours, washed and co-cultured for 4 hours with an expanded CMV specific $CD8^+$ T cell population. Production of IFNγ by $CD8^+$ T cells was quantified by intracellular cytokine staining assay and is shown as the percent of $CD3^+$, $CD8^+$, $IFN\gamma^+$ pp65 CMV $tetramer^+$ $CD8^+$ T cells.

5. Discussion

The failure to stimulate a CTL response is a major impediment to the development of subunit vaccines. Exogenous proteins in vaccines formulation are poor inducers of $CD8^+$ T cell immunity, as they are captured by APCs and presented to $CD4^+$ T cells, leading to a good humoral response but poor cellular immunity. The discovery of TLRs can modulate the host innate and adaptive immune response has triggered interest in studying this class of receptors to identify potential vaccine adjuvants and therapeutic agents.

In this study, we demonstrate that a soluble protein can be introduced into the class I MHC Ag-restricted processing and presentation pathway by the use of the SMIP2.1 adjuvant in order to induce a $CD8^+$ T cell response. SMIP2.1 is a tryacilated lipopeptide, well-know TLR2 agonist, identified by two High-Throughtput Screenings performed at the Genomic Institute of the Novartis Research Foundation in San Diego. This compound is under evaluation as vaccine adjuvant by Novartis.

Using Ovalbumin (OVA) as model antigen, our findings show that mice immunized with OVA protein and TLR2 adjuvant greatly increase OVA-specific $CD8^+$ T cell priming. Moreover, using an OVA expressing tumor model, we show that the CTL response induced by the SMIP2.1 adjuvanted vaccine is able to inhibit tumor growth. This evidence suggests that SMIP2.1 could be extremely useful as a component in subunit vaccines, especially when a CTL response is required. Different papers have previously shown that TLR2 agonists act as antitumor agents (Ingale et al., 2007) (Garay et al., 2007) (Brandau and Suttmann, 2007) (Murata, 2008) but they might also promote tumor growth in some tumor models (Kim et al., 2009) (Huang et al., 2007). Others have also described a role for Toll like receptor-2 to abrogate regulatory T cell function (Piccioli et al., 2007) (Sutmuller et al.,

2006). In this work, we clearly show that SMIP2.1 is an inhibitor of tumor growth, and, even if we have shown a direct effect on the cross-priming of $CD8^+$ T cells, as well as directly enhance $CD4^+$ and $CD8^+$ T cells function (Unpublished data) we still haven't investigated if it has a direct effect on regulatory T cells, as demonstrated by Zhang and co-workers for BCG (Zhang et al., 2011).

Mechanisms by which adjuvants could enhance the immunogenicity of Ags remain unclear and under debate. Some studies implicate the formation of Ag depots as the underlying cause. These findings have led to the proposal that adjuvants enhance the immune response through prolonging the persistence of Ag in vivo. Moreover, Ag aggregation could affect the pathway and efficiency of uptake by APCs. We have shown that SMIP2.1 increases Ag deposition in the draining lymph node of the site of injection, and the amount of Ag presenting cells that have taken up the antigen. Due to the observation that after immunization with adjuvanted vaccine not only DCs but also other cell populations in the draining LN increase their ability to take up the antigen, we wondered if this increased uptake could result in enhanced cross-presentation ability. Different papers show in mice that only $CD8\alpha^+$ $CD11c^+$ DCs are especially able to cross-present. The superior ability of $CD8\alpha^+$ DCs in cross-presenting cell-bound antigens may be reflected by their unique ability to phagocyte dead cells (Schulz and Reis e Sousa, 2002) (Iyoda et al., 2002). However, they are also able to cross-present soluble antigens. Since the $CD8\alpha^+$ DC population represents only the 20% of the conventional DCs in the mouse spleen (Shortman and Heath, 2010), the possibility to induce an MHC I-mediated presentation to $CD8^+$ T cells in $CD8\alpha^-$ DC represents a big challenge. If rightly stimulated, also $CD8\alpha^-$ DC can

cross present and our data show that stimulation with SMIP2.1 activates cross-presentation in $CD8\alpha^-$ DCs subsets.

Cross-presentation has been extensively described in different animal models, while very little reports have been published on human cells. Sub-populations of human DCs have been targeted through C-like lectin receptors with the aim to increase their ability to cross-present (Schreibelt et al., 2012) (Bonifaz et al., 2004) (Idoyaga et al., 2011) and recently, human $CD141^+$ DCs have been identified as the human counterpart of the murine $CD8\alpha^+$ DCs, the most effective in antigen cross-presentation (Bachem et al., 2010) (Poulin et al., 2010) (Jongbloed et al., 2010). In this report, we show that an adjuvant is able to increase cross-presentation in human PBMCs, without targeting neither a specific subpopulation of DCs, nor using a conjugated antigen, corroborating the mouse data in which we do see enhanced cross-presentation ability in different cell populations. However, additional studies are required to identify which cell type is specifically targeted by SMIP2.1 in order to enhance cross-presentation.

In conclusion, in this work we showed that SMIP2.1 induces a strong CTL response after only one immunization, confirming that the compound is potent adjuvant for priming peptide-specific CTL responses. Furthermore, only small amount of the lipopeptide is needed to cause robust humoral and cell-mediated immune response (10 nmoli lipopeptide per animal) thus avoiding possible toxic effects associated with the use of an adjuvant.

Although TLR ligands are effective at promoting vaccine efficacy, a better immune response is generated when both TLR ligand and Ag are covalently (Ozinsky et al., 2000) (Prajeeth et al., 2010) (Shirota et al., 2001) or electrostatically (Chua et al., 2011) linked. However, these approaches have several drawbacks in the large-scale production of

vaccines. Antigen and lipopeptides conjugation can be chemically challenging because they are dissolved in aqueous and lipid phases, respectively. Moreover, both techniques might have stability problems. Jackson's group has recently shown that electrostatic ligation of soluble proteins to the TLR2 agonist dipalmitoyl-S-glyceryl-cysteine (Pam2Cys), a diacetylated lipopeptide, elicits a good $CD8^+$ T cell response. Since our compound is a triacetylated lipopeptide, we used the triacetylated lipopeptide Pam3CSK4 as TLR2 benchmark. We showed that Pam3CSK4 is able to induce a good immune response without the need to be coupled to the antigen. Using HEK293 stable transfected with TLR2 as well as primary immune cells (unpublished data), we observed that TLR2 benchmark Pam3CSK4 and SMIP2.1 have a similar potency as TLR2 activators but, when we compared the ability of the TLR2 agonists to cross-present *in vivo*, we observed that our compound works better that Pam3CSK4 in inducing antibody and CTL response.

This study show that SMIP2.1 can generate antigen specific CTL, along with a robust $CD4^+$ T cells activation, a feature that can be exploited in the design of an effective adjuvant for antitumor and antiviral vaccine.

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