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Selection of an antibody library identifies a pathway to induce immunity by targeting CD36 on steady state CD8α^+ dendritic cells

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ABSTRACT

Due to their prominent role in the orchestration of a broad range of immune responses, dendritic cells (DCs) have emerged in the past decade as central target for cancer immunotherapy. Recent advance in the knowledge of DCs functions and subset specialization led to the design of novel immunotherapeutic approaches based on the possibility to target DCs directly *in vivo*, thus avoiding their *ex vivo* manipulation.

A promising strategy is to use antibodies to target antigens to cell-surface molecules expressed by DCs, in order to increase T-cell mediated immune responses. The studies performed so far, have revealed that the efficacy of *in vivo* DC vaccination depend on several factors including the specific DC subset targeted, their maturation status and the nature and biological properties of the receptor targeted. Therefore, the identification of the most appropriate ligand/receptor pairing it is a requisite to improve the modality of delivery tumor Ags to DCs.

To this aim, we screened a library of Ab fragments on mouse DCs to isolate new potential antibodies capable of targeting DCs *in vivo* and able to induce T-cell mediated immune responses against specific antigens. In this study, we provide the proof of principle that the phage display technology can be successfully used to isolate internalizing antibodies on mouse DCs. We further develop such technology by engineering the selected molecules to create antigen fusion proteins to use in vaccination protocols.

In particular, we focus on a high affinity Ab against CD36, a multiligand scavenger receptor primarily expressed by the $CD8\alpha^+$ subset of conventional DCs. We characterize the antigen presenting properties of this receptor which help to delineate a novel function of CD36 in adaptive immunity. We show that targeting CD36 on DCs results in the delivery of exogenous Ags to both the MHC class-I and MHC class-II processing pathways. In addition, immunization with the recombinant anti CD36-Ag fusion Ab induces the robust activation of naïve $CD4^+$ and $CD8^+$ Ag specific T lymphocytes and the differentiation of primed $CD8^+$ T cells into long term effector CTLs. Finally, we demonstrate that *in vivo* targeting of $CD8\alpha^+$ DCs with anti-CD36 Ab elicits humoral and cell mediated protection from the growth of an Ag specific tumor. Collectively, these results identify CD36 as an appropriate receptor to better elucidate the properties of the lymphoid organ resident $CD8^+$ DCs and indicate it as a novel potential target for cancer immunotherapy.

1 INTRODUCTION

1.1 Dendritic cells and the control of immunity

Optimal defense against invading pathogens requires specialized classes of non-specific and antigen-specific immune responses, generally referred as the innate and the adaptive components of immunity. Innate immunity or non-specific host defenses that exist prior to exposure to an antigen, specifically detects key features of an infectious process consisting in a limited set of conserved molecular patterns (PAMPs) that are unique to the microbial world and invariant among entire classes of pathogens (1). PAMPs recognition by immune system has two major effects. First, it triggers effector cells of inflammation (macrophages and neutrophils) that limit the infection at the site of pathogen entry. Second, it activates antigen presenting cells (APC) to trigger adaptive immunity, which increases antigenic specificity and generates immunological memory.

Central to this process are dendritic cells (DCs), a heterogeneous family of leukocytes that integrate innate information and convey it to lymphocytes. Upon direct recognition of PAMPs through specific pattern recognition receptors (PRRs) or indirect sensing of infection through inflammatory cytokine or other endogenous "danger" signals, DCs enter an integrated developmental program that triggers their differentiation into immunogenic APCs capable of priming and sustaining the expansion of naïve T cells. Among the "professional" APCs, DCs have a preeminent role in the initiation and modulation of the immune response. Their unique distribution and capacity to accumulate within the T cells area of lymphoid organs optimize antigen capture and clonal selection of rare $CD4^+$ and $CD8^+$ T cells. Their inherent greater efficiency for antigen presentation allows small numbers of DCs and low levels of antigen to induce strong T cell responses (2). In addition, DCs have the unique capacity to translate the environmental signals into specific classes of adaptive immune responses by polarizing T cells development. Finally, DCs are not only implicated in the induction of immunity, but also play an essential role in the generation of both central and peripheral T cells tolerance by inducing deletion, anergy or regulation of T lymphocytes.

1.1.1 The "Langerhans cells Paradigm"

Dendritic cells are described as following a life cycle that was modeled according to observations of studies carried out in the 1970s and 1980s by Steinman and colleagues on DCs found in the epidermis (Langerhans cells) and on DCs purified from the spleen (3). This model, generally referred as "Langerhans cells paradigm", proposes that DCs can exist in two functional

states: immature and mature. In the periphery, immature DCs efficiently take-up self and non self antigens but are quite inefficient in presenting it to T cells. Only upon encounter of pathogens and mediators of inflammations, DCs enter an integrated developmental program called maturation. During this process, they downregulate their endocytic capacity, activate the antigen processing machinery that generates complex of MHC molecules with peptides derived from the internalized antigens and increase the expression of T cell co-stimulatory molecules. In parallel, modifications in the pattern of chemokine receptors and adhesion molecules and changes in the cytoskeleton organization cause the mobilization from the periphery to secondary lymphoid organs where the antigen are efficiently presented to T cells thus initiating adaptive immune responses.

Important findings of the last few years have revealed a far greater complexity of the DCs system of APCs with the consequent need to add few modifications to this original DCs life cycle paradigm. The analysis of the DCs found in the thymus, spleen and lymph nodes has revealed a considerable heterogeneity and has led to the definition of several DCs subtypes, each with a particular location and specialized function in the immune system (4). It is now clear that only a fraction of the DCs found in lymph nodes are derived from cells previously resident in the peripheral tissues. In a steady state condition (i.e in absence of infections or inflammatory stimuli), half of the DCs found in lymph nodes and most of the DCs in the spleen seem to be lymphoid tissue resident cells, with an immature phenotype and great efficiency in antigen uptake and processing (5, 6). Therefore, these lymphoid-organ-resident DCs do not conform to the Langerhans cells paradigm, since they develop from bone marrow precursors within the lymphoid organs and without previously trafficking through peripheral tissues (7).

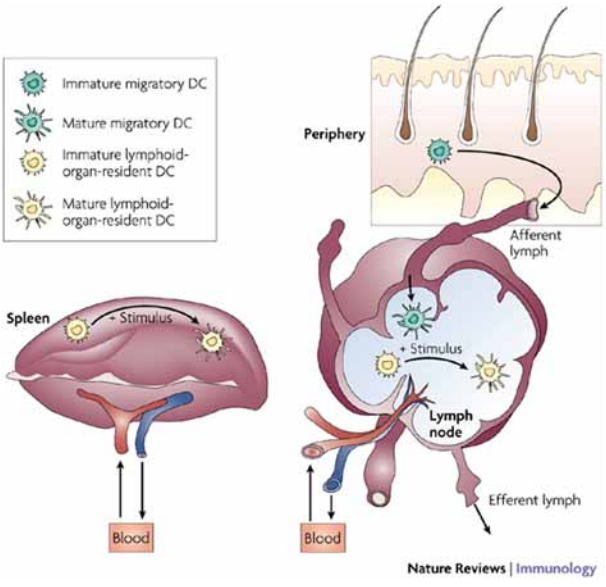


Figure I. Different life cycles of migratory and resident dendritic cells. Migratory DCs have an immature phenotype in peripheral tissue where they survey the environment and endocytose extracellular material. They migrate through the afferent lymph to the local lymph nodes where they acquire a mature phenotype. Resident DCs, present in lymph nodes and spleen, spend their entire lifespan in an immature state unless they encounter pathogen products or inflammatory signals, which cause their maturation in situ.

1.2 Dendritic cells general features

1.2.1 Mechanisms of Antigen uptake and pathogen recognition

Dendritic cells have evolved several features that greatly enhance their capacity as APCs. Among these, the property of immature DCs to efficiently take up particles and microbes by phagocytosis and the ability to form large pinocytic vesicles to sample extracellular fluids and solutes in a process referred to as macropinocytosis (8). Moreover, DCs express a great variety of receptors involved in antigen recognition and uptake by mechanisms of receptor mediated endocytosis. Endocytosis is a regulated process in DCs, being highly active in immature DCs and downmodulated upon cells maturation (9). This assures the efficient sampling of the environment in the periphery and concomitantly limits the range of antigens that the cells will be able to present after living the peripheral tissues. The downregulation of internalization is achieved by decreasing the expression of the cell receptors involved in antigen uptake and by downmodulating both macropinocytic and phagocytic activities.

Phagocytosis

The uptake of large particulate antigens (>0,5μm) by phagocytosis is the prevalent form of antigen uptake in vivo for both pathogen-derived and endogenous antigens (8). It is an active and highly regulated process initiated by the engagement of specific cell surface receptors, generally involved also in clathrin mediated endocytosis, that trigger a signaling cascade mediated by the Rho-family GTPases (Rho, Rac and Cdc42). The final outcome is the extensive reorganization of the actin cytoskeleton with the formation of cell-surface extensions that zipper up around the pathogen to engulf it (10). The nature of the particle to be ingested and the receptors that recognizes it determine the mode of protrusive activity and the signaling pathways that are activated. In the case of FcγR mediated phagocytosis, the cells extends pseudopods that engulf the particle and subsequently fuse to form a phagosome. This process requires the activation of Cdc42 for the pseudopods extension and Rac in pseudopods fusion and phagosome closure. In contrast, phagocytosis mediated by complement receptor is controlled by Rho, do not induce pseudopod formation but requires the coordinated action of chemokines and integrin ligation to form the phagocytic cup (11).

Phagocytosis is essential in host defense against infections: immature DCs were reported to phagocytose almost any kind of bacteria, both $GRAM^+$ and $GRAM^-$ and mycobacteria, yeast cells

and parasites (12). Phagocysis is also crucial for clearing apoptotic cells, both at sites of inflammation and tissue damage and during development. This process is mediated by the recognition of specific molecules that are normally absent on live cells such as calreticulin and phosphatidylserine, amino sugars and lysophospholipids and involves various types of scavenger receptors, complement receptors and integrins on DCs. The nature of the cargo determines the set of receptors involved in its recognition and uptake, the signaling cascade that is activated upon receptor binding and ultimately regulates the efficiency of processing/presentation and the immunogenicity of the internalized material. For instance, phagocytosis of microbial cells and of apoptotic bodies rely on the same phagocytic machinery, but the cellular responses that accompany these forms of phagocytosis are different, being respectively inflammatory with the production of tumor necrosis factors, IL-1and IL-6 in case of pathogens and anti-inflammatory in case of uptake of apoptotic material (13).

Macropinocytosis

Macropinocytosis contributes to bulk fluid-phase uptake via the formation of membrane protrusions that collapse onto and fuse with the plasma membrane generating large endocytic vesicles (micropinosomes) that allow the sampling of large volumes of extracellular milieu (10). The signaling cascades that induce macropinocytosis involve the Rho-family of GTPases and especially Cdc42, that trigger the actin-dependent formation of the membrane ruffles (11). In immature DCs macropinocytosis is constitutive and like phagocytosis, is downmodulated upon cells maturation. The impact of antigen uptake via macropinocytosis is vivo is not clear since pathogens release few soluble antigens, with most proteins integrated in membranes, cell walls or cytoplasmic compartments that would need to be taken up as particles. Conversely, it has a role in the capture of soluble antigens that become available upon intravenous, intraperitoneal and intradermal injections (8). The degree of internalization of fluid-phase antigens is proportional to their concentration in the medium. The efficiency of endocytosis is increased by non-specific binding of solutes to the cell membrane (adsorptive pinocytosis) and even more by the capture of soluble antigens through specific high affinity receptors (receptor mediated endocytosis) that are concentrated into specialized endocytic transport vesicles (10).

Receptor mediated endocytosis

Uptake of extracellular material through receptor mediated endocytosis greatly increases the efficiency of antigen capture thus allowing DCs to present antigens found at picomolar and nanomolar concentrations (2). This process is initiated by signal cascades triggered by specific endocytic receptors present in specialized regions of the plasma membrane. The nature of the receptors engaged determines the mechanism of internalization that can be mediated by clathrin-coated vesicles, by caveolae or can be both clathrin- and caveolae- independent.

DCs express a wide range of endocytic receptors that belongs to different families. Receptors for the Fc portion of immunoglobulin (FcRs) and complement receptors (CRs) are involved in the uptake of particles that are opsonized by immunoglobulins or complement factors. A second class of endocytic receptors comprises scavenger receptors (SRs) and C-type lectin family receptors (CLRs) that directly recognize specific structures on both self-antigens and pathogens and are generally referred to as innate immunity receptors. A large number of these endocytic molecules are selectively expressed by subpopulations of DCs thus contributing to the functional diversity among different DCs.

Fcγ and complement receptors

Mouse immature DCs express different types of FcRs such as FcγRI, FcγRIIb, FcγRIII and FcγRIV and the complement receptors CR3 and CR4. Both FcRs and CRs may be functionally considered as antigen receptors due to their ability to bind and to mediate the uptake of immune complexes and complement-opsonized particles. The FcγRs differs in terms of antibody isotype specificity and binding affinity with FcγRI binding with the highest affinity exclusively monomeric IgG2a and FcγRIV binding with intermediate affinity IgG2a and IgG2b but not IgG1, whereas both FcγRIIb and FcγRIII binds immune complexes of all IgG isotypes (14). Antigens bound to soluble Ig can follow different fates depending on the cell type that captures them. Among APCs, DCs are unique for their capacity to efficiently deliver antigens internalized via FcγRs to the antigen processing machinery which results in the formation of both MHC class I and MHC class II-peptide complexes (15).

CR3 binds a promiscuous range of ligands, both of host and microbial origin. It was shown to be involved in the uptake of complement opsonized apoptotic cells and also in the direct recognition of pathogens such as *M. tuberculosis* and yeast-derived zymosan (16).

C-type lectin receptors

Immature DCs differentially express a broad variety of C-type lectin receptors (CLRs) that are involved in the recognition of a wide range of carbohydrate structures in antigens and act as endocytic or phagocytic receptors depending on the size of the antigen. The most characterized examples of DCs CLRs include the mannose receptor, DEC205, DC-SIGN, Dectin-1 and -2 and Langerin. They are generally classified as type I or type II receptors depending on the orientation of their N terminus, which is extracellular or cytoplasmic respectively, and on the number of carbohydrate recognition domains (CRDs) present in their extracellular portion. The cytoplasmic domains of the CLRs are diverse and contain several conserved motifs that are important for antigen uptake and determine the intracellular route followed by the antigen upon receptor-mediated internalization. For instance, most CLRs (DEC205, DC-SIGN, BDCA-2 and Dectin-1) contain a tri-acidic cluster that target internalized antigens to lysosomes and MHC class II^+ late endosomes, whereas the MR constitutively delivers antigens to early endosomes and rapidly cycle back to the cell surface (17).

Several CLRs expressed by immature DCs are capable of binding pathogens through the recognition of specific carbohydrate structures present on the cell wall component. Some CLRs have overlapping carbohydrate specificity and recognize a wide variety of carbohydrate structures such as mannose- and fucose-containing carbohydrates, yet others have a unique recognition profile dictated by a specific branching of the mannose structures (17). However, little is known about the antigen specificity of CLRs expressed by DCs and their functions are not yet documented. Only in few cases specific pathogens have been associated to CLRs. For example, the mannose receptor is able to bind yeast, human immunodeficiency virus and *Micobacterium tuberculosis*, whereas DC-SIGN binds a large variety of pathogens, including viruses (HIV, HCV, CMV, Ebola and Dengue virus), *Candida albicans*, bacteria (*Micobacterium tuberculosis*, *Helicobacter pylori* and *Klebsiella pneumonae*) and parasites (*Leishmania pifanoi* and *Schistosoma mansoni*) (18). Dectin-1 recognizes conserved molecular pattern (β1,3-glucans) in fungal pathogens (*P.carinii, C.albicans* and *A.fumigatus*) and triggers a signaling pathway that induces the activation of adaptive immunity (19). To date this is the only documented case of host protection mediated by a CLR. In fact, although DC-SIGN and MR mediate the uptake of pathogens, this rather represents a mechanism exploited by the pathogen itself to favor its survival and to escape immune activation (18). Therefore, further analysis will be required to clarify the role of CLRs in protection from invading pathogens (20).

In addition, some CLRs were shown to mediate the uptake of host derived molecules. For example, MR-deficient mice were shown to have increased plasma concentration of endogenous

glycoproteins, such as lysosomal hydrolases (21) and lutotropin (22) confirming an important role for this CLR in the clearance of self antigens.

Scavenger receptors

A third class of endocytic receptors expressed by DCs is the family of scavenger receptors (SRs). SRs encompass a broad range of cell-surface glycoproteins that differ greatly in their structure but are, by definition, all capable to mediate the uptake of selected polyanionic ligands, including modified low-density lipoproteins (LDL). The main feature of SRs is their ability to recognize multiple ligands, both microbial and host derived, and to carry out a multiplicity of cellular functions (ranging from anti-microbial immunity, antigen presentation and cell adhesion to atherosclerosis and Alzheimer's disease). However, while SR expression and function has been extensively investigated for macrophages, the role of these molecules on DCs is only beginning to be unraveled. Three SRs belonging to different classes, defined on the basis of their molecular structure, are expressed by DCs: Scavenger Receptor A (SR-A) (23), CD36 (24-26) and Lox-1 (27).

SR-A is a trimeric type II glycoprotein containing in the extracellular portion an α helical coiled coil, a collagenous and a C-terminal cysteine-rich domain. Its cytoplasmic tail instead, contains a potential protein kinase C interaction site, but not an internalization motif. SR-A was shown to bind several components of both $GRAM^-$ and $GRAM^+$ bacteria (lipid A and lipoteichoic acid respectively). Indeed, its role in microbial clearance has been confirmed in two studies performed on SR-A deficient mice, where the absence of the receptor resulted in increased bacterial loads of *Listeria monocytogenes* and *Staphylococcus aureus* (28). Recently, *Amiel et al* showed that $SR\text{-}A^{-/-}$ BMDCs are impaired in their ability to phagocytose and accumulate *E.coli* bacteria and that the internalization requires lipid raft structures (29). Similarly to other SRs, SR-A recognizes also endogenous ligands and modified host molecules expressed on aged/apoptotic cells, with a possible role in tissue homeostasis (30). Finally, *Berwin et al* identified SR-A as a predominant endocytic receptor for the ER chaperones gp96 and calreticulin and demonstrate that SR-A can direct gp96-OVA derived peptide complex into an MHC class I presentation pathway (31). However, SR-A is not required for antitumor immunity generated by HSP-based vaccines, rather its lack restored the immunogenicity of poorly immunogenic tumors and enhanced the tumor specific immune response, suggesting a role of SR-A in immune suppression and in the maintenance of host immune homeostasis (32).

LOX-1 is a 48-50 kDa type II membrane glycoprotein with a C type lectin-like domain whose antibacterial potential was revealed by binding studies performed with both $GRAM^+$ and $GRAM^-$ bacteria (*S. aureus* and *E.coli*). LOX-1 was also identified as a receptor for *Klebsiella*

pneumoniae OmpA, a major component of the outer membrane of *Enterobacteriaceae*. Specifically, it was shown to act in a sequential and coordinate way with TLR-2 to mediate OmpA internalization and DCs activation (33). *Delneste et al* first reported LOX-1 as a receptor for Hsp70 on human DCs and showed its involvement in Hsp70 mediated antigen cross-presentation in vivo thus suggesting a possible role for LOX-1 in the recognition of cellular damage (27).

CD36 belongs to the class B SR family. It is a double-spanning N-linked glycosylated protein of 53 kDa thought to be associated with lipid rafts. The predicted structure orients most of the protein extracellularly, except for two short (9-13 amino acids) cytoplasmic tails (34). The C-terminal cytoplasmic domain was shown to have a role in the localization of CD36 in the plasma membrane and in the uptake of long-chain fatty acid (35) and oxidized LDL (OxLDL) (36). Binding of OxLDL to CD36 leads to endocytosis through a lipid raft pathway that is both clathrin and caveolin independent (37).

As most of SRs, CD36 is a multiligand receptor shown to play a role in diverse cellular processes including foam cell formation, fatty acid transport, suppression of angiogenesis, cell-matrix interaction (34). CD36 is one of the first DCs receptors shown to be involved in the uptake of apoptotic cells (24), probably through interactions with oxidized phosphatidylserine (38) and with thrombosponsdin-1 (39) present on the membrane of apoptotic cells.

CD36 has also been implicated in the innate immune response to $GRAM^+$ bacteria such *S. aureus* . *Hoebe et al* first demonstrated the selective and nonredundant role of CD36 as sensor for microbial diacylglycerides such as macrophage-activating lipopetide 2 and lipoteichoic acid (LTA) (40). Upon binding to bacterial LTA, CD36 forms a cluster with the heterodimer TLR2/TLR6 within lipid raft, followed by the internalization of the complex and its targeting to the Golgi apparatus (41). The C-terminal cytoplasmic residues of CD36 were shown to be essential to trigger phagocytic engulfment and specifically, the tyrosine 463 was implicated in signaling to the actin cytoskeleton (42). These data collectively indicated that CD36 acts as a facilitator or co-receptor for diacylglyceride recognition through the TLR2/6 complex.

1.2.2 Mechanisms of Antigen presentation and cross-presentation

After their entry into DCs, antigens are degraded into small immunogenic epitopes that associate with the major histocompatibility complex molecules (MHC) and are transported to plasma membrane where they eventually trigger the activation of naïve T lymphocytes.
Specifically, the activation of $CD8^+$ and $CD4^+$ T lymphocytes requires recognition by the T cell receptor (TCR) of fragments of antigens (peptides) associated with MHC class I and MHC class II molecules, respectively. The antigen processing pathways that lead to the formation of peptide-MHC complexes, rely on ancient proteolytic mechanisms (proteasome, lysosomes) that in DCs were geared towards optimal T cells stimulation (8).
Initial studies on antigen presentation defined a strict compartmentalization of MHC class I and II biogenensis, with MHC class II molecules encountering exogenous antigen in the endocytic pathway and MHC class I loading with endogenous antigens in the endoplasmic reticulum (ER). Although still valid, recent evidence have proved that both boundaries can be crossed and so MHC-II molecules can present intracellular antigens not coming from extracellular space and MHC-I can present peptides derived from exogenous antigens, phenomenon generally referred to as cross-presentation.

MHC class I–restricted antigen presentation

DCs process and present on MHC class I molecules proteins coming from various sources including cytosolic proteins (either endogenous or viral), alternative translation products and defective ribosomal products (DRiPs), protein retrotranslocated to the cytosol from the ER (i.e. secretory and membrane proteins) and internalized proteins transferred to the cytosol and imported into the ER (8). Independently of the route of entry, most of the peptides to be loaded on MHC-I molecules are generated by the proteasome and further trimmed by cytosolic or ER resident peptidases. The proteasome has a critical role in initiating the processing and in most cases, releases precursor peptides that have the correct C terminus for MHC-I anchoring and an N-terminal extension that requires further degradation. The efficiency and rate of epitope generation by the proteasome are increased in response to an inflammatory environment (i.e. IFN-γ) by expression of facultative subunits (referred to as immunosubunits). The proteasome has also been implicated in splicing of peptides to create epitopes from sequences that are not contiguous in the original protein (43, 44). The N-terminal trimming occurs in both cytosol and ER by the tripeptidyl peptidase II (TPPII) and ER-associated aminopeptidase (ERAAP) respectively (45). Once the peptide products

are shuttled into the ER via the transporter associate with antigen processing (TAP) and customized by ERAAP, the final MHC-I peptide complex (pMHC-I) is formed and eventually displayed on the cell surface (46).

Cross-presentation of exogenous antigens

Dendritic cells have evolved a specialized machinery that allows the entry of exogenous antigens into the MHC class I pathway of antigen presentation (47). This unique capacity, generally referred to as "cross presentation," is a major mechanism for the immune surveillance of tissues, allowing the immune system to monitor parenchymal cells for the presence of foreign antigens.

Several types of antigens have been reported to be cross-presented: soluble proteins, immune complexes, pathogens and cellular antigens, independently of the mechanism of internalization (macropinocytosis, phagocytosis or receptor mediated endocytosis) (48). However, the mechanisms by which APCs transfer internalized antigens to the MHC class I loading pathway are not well understood (49). In most of the cases, proteasomal digestion and transport into ER by TAP seem to be required, implicating a mechanism to allow the release of the antigen from the endocytic structures to the cytosol. However, the cellular and molecular bases of endosomal egress into the cytosol remain unknown (49). Reports have suggested that a potential mechanism for cross-presentation relies on the provision of MHC-I loading components to the phagosome by membranes derived from the ER, resulting in ER-phagosome complexes self-sufficient for cross-presentation (50, 51). Although the relative contribution of the ER versus the plasma membrane as source of membrane for phagocytosis is still a matter of debate (49), recent data by the Cresswell group have shown that soluble antigens can gain access to the lumen of the ER after internalization (52) and that the ER protein retrotranslocation machinery (ERAD) is involved during cross-presentation (53).

Among APCs, DCs have the highest cross-presentation efficiency. One of the mechanisms that accounts for this specialization is the limited degradation of internalized antigens in the endocytic compartments, which may increase the opportunity for the internalized antigens to reach the cytosol (54). Indeed, experimental reduction of lysosomal proteolysis by choloquine was shown to increase the efficiency of cross-presentation (55). Moreover, recent findings by *Savina et al* propose the NADPH oxidase NOX2 as a key regulator of the DC phagosome pH. Recruitment of NOX2 to immature DC phagosomes causes their active alkalinization and is required for efficient antigen cross-presentation to $CD8^+$ T cells (56), further supporting the evidence for a unique lysosomal compartment in DCs.

The uniqueness of DCs with respect to antigen handling reflects the essential role that these cells play in priming in vivo CTL responses to exogenous antigens. In an elegant study, Jung and colleagues demonstrated that the *in vivo* depletion of DCs abrogates priming of $CD8^+$ T cells by exogenous cell-associated antigens and results in the failure to mount a CTL response to infection with the intracellular bacterium *Listeria monocytogenes* and the malaria parasite *Plasmodium yoelii* (57). In addition to intracellular bacterial infection, cross-presentation have an essential role in responses to those viruses, e.g. cytopatic strains or ones with immune evasion molecules, that impair the ability of directly infected DCs to function, or to tissue specific viruses that do no infect DCs (58-61). Finally, cross-presentation is also implicated in the maintenance of both central and peripheral tolerance to self antigens eventually leading to the deletion or anergy of self reacting CTLs (62).

MHC class II–restricted antigen presentation

Antigens loaded on the MHC-II are typically exogenous proteins that are internalized by DCs through different mechanisms of endocytosis or endogenous proteins that reside in the secretory system. Moreover, analysis of the peptides eluted from the MHC-II molecules has revealed that also antigens topologically isolated from the endosomal system (i.e. cytosolic and nuclear proteins) can gain access to MHC-II class antigen presentation pathway (63). Recent studies have implicated autophagy as likely source of peptide derived from various cytosolic and nuclear antigens (64). Schmid and colleagues have recently shown that macroautophagy is a constitutively active pathway in all MHC-II positive cells and that autophagosome continuously fuse with multivesicular MHC-II loading compartments. They also show that targeting antigens to autophagosomes leads to enhanced MHC-II presentation and $CD4^+$ T cells stimulation (63).

De novo synthesized MHC-II αβ dimers assemble in the ER with the chaperon invariant chain (Ii), which inserts its class II associated peptide (CLIP) portion within the peptide binding site thus stabilizing and protecting the MHC-II dimer from other polypeptides. The MHC-II αβIi complexes are transported to early endosomes and further on to late endosomes and lysosomes. Along this pathway, the Ii is progressively processed by various proteases (AEP, cathepsins B and S) and finally displaced to allow the binding of the antigenic peptide, in a reaction favored by the acidic pH and by the chaperone protein HLA-DM (8). MHC-II molecules can bind polypeptide precursors that are trimmed by various proteases along the endocytic route, so that the final MHC-II peptide complex eventually reaches the plasma membrane.

Recent findings by Blander *et al* have demonstrated how the nature of the cargo internalized by phagocytosis and thus the type of receptors engaged during this process are able to influence the efficiency of presentation on MHC class II molecules. They show how the generation of peptide-MHC class II complexes is controlled by Toll-like receptors (TLRs) by regulating the progressive proteolysis of the Ii to CLIP in a strictly phagosome-autonomous manner. This mechanism allows DCs to distinguish between different forms of antigens as self or non-self at the subcellular level and ensures that only antigens that concomitantly engage TLRs are preferentially delivered to the pool of MHC-II molecules transported to the plasma membrane (13).

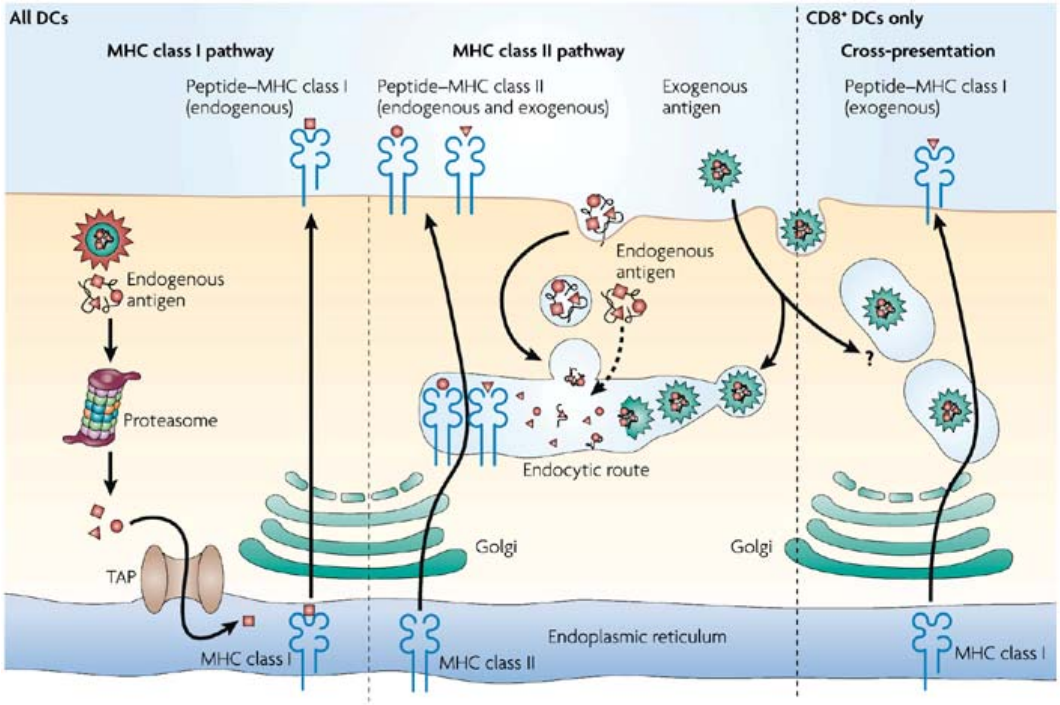


Figure II. The antigen-presentation pathway in dendritic cells. MHC class I molecules present peptides that are primarily derived from endogenously synthesized proteins of either self of pathogen origin. These proteins are degraded into peptides by the proteasome and transported through TAP molecules into the endoplasmic reticulum for loading on MHC class I molecules. MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment, and also endogenous components, such as plasma membrane proteins and cytosolic proteins that access the endosomes by autophagy.
DCs have the unique ability to present peptides derived from exogenous proteins on MHC class I molecules (cross-presentation), although the mechanism involved in this pathway is still poorly understood. The bifurcated arrow indicates that the MHC class II and MHC class I cross-presentation pathway may "compete" for exogenous antigens, or that the endocytic mechanism involved in the internalization of a given antigen may determine whether it is preferentially delivered to the MHC class II pathway or the MHC class I cross-presentation pathway.

1.2.3 Induction of Dendritic cells activation and signal transduction

Only upon direct recognition of pathogens associated pattern (PAMPs) or sensing of inflammatory signals, DCs are activated to enter the program of maturation that endows the cells with the ability to stimulate naïve T lymphocytes. DCs response to microbial/inflammatory signals is mediated by different classes of surface and intracellular receptors whose engagement triggers signal cascades that lead to the production of inflammatory cytokines, to the up-regulation of co-stimulatory molecules and to the altered expression of chemokine receptors profile. Mature DCs have an increased antigen-presenting capacity and migrate to the draining lymph nodes where they interact with and stimulate antigen specific T cell. DCs maturation is therefore a critical link between innate and adaptive immunity (65).

Toll like receptor family

One of the best characterized classes of pattern recognition receptors (PRRs) that directly contribute to the inflammatory response to pathogens is the Toll-like receptor family (TLRs). Mammalian TLRs are a family of at least 12 transmembrane proteins that collectively recognize lipids, carbohydrate, peptide and nucleic-acid structures broadly expressed by different groups of micro-organisms. Some TLRs (TLRs 1,2,4,5 and 6) are expressed at the cell surface and seem to specialize in the recognition of mainly bacterial products, whereas others (TLRs 3,7,8, and 9) are expressed on the membrane of endocytic vesicles or other intracellular organelles and are specialized in detection of viral nucleic acids (66). TLRs differ from each other in ligand specificities, expression patterns and presumably in the target genes that they can induce.

TLR4 plays an essential role in the recognition of LPS, a major component of $GRAM^-$ bacteria, whereas TLR2 is critical to the recognition of bacteria peptidoglycan and lipoproteins, but it is also able to recognize components from fungi (zymosan) and parasites (glycoinositolphospholipids from Trypanosoma Cruzi). TLR2 promiscuity in ligand recognition derives from its ability to cooperate with other TLRs (TLR1 and TLR6) by formation of different heterodimers which confer discrimination among different microbial components (67). Further complexity is added by recent data showing the involvement of non-TLRs in the presentation of some PAMPs to TLR2. For instance, the scavenger rector CD36 acts as facilitator to mediate the interaction of microbial diacylglicerydes to the heterodimer TLR2/6 (40). Moreover, the potential interaction of TLR2 with a member of CLRs is suggested by data from *Wang et al*. showing the critical role of TLR2 in the initiation of the adaptive immune response to the polysaccharide A (PSA) from *Bacteroides fragilis*

(68). Other TLRs are involved in the recognition of microbial proteins such as flagellin by TLR5 or apicomplexan profilins by TLR11 (69).

TLRs have also an essential role in the antiviral immune responses due to their ability to induce type I IFNs, a family of cytokines specialized to coordinate immunity to viruses and other intracellular infections (70). For instance, TLR9 and TLR7 which represent a structurally related subfamily, are involved in the recognition of unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs present in bacteria and viral DNA, or ribonucleic acid homologs (imiquimod and resiquimod) and synthetic ssRNA rich in guanosine/uridine respectively. A further member, TLR3, responds to dsRNA and to its synthetic analog poly-inosinic acid-cytidylic acid (poly(I)•poly(C)) (71).

Upon recognition of their ligands, TLRs transduce signals through two pathways involving distinct adaptor proteins containing Toll/IL-1R (TIR) domains. One of these adaptors, MyD88, is utilized by all of the known TLRs except TLR3. MyD88 is recruited to the receptor-ligand complex in an interaction that is mediated its C-terminal TIR domain. Through its N-terminal domain the adaptor engages the kinase IRAK which is activated by phosphorylation a then associates with TRAF6, leading to the activation of two distinct signalling pathways: JNK and NF-kB (72).

TLR3 and TLR4 are able to activate NF-kB in a MyD88 independent way that involves the adaptor molecule TRIF and other signalling molecules such as RIP1 and TRAF6. Activation of TLR3 and 4, through the adaptor TRIF, induces also the production of type I IFN. TRIF associates with the kinases TBK1 and IKKi which mediate the phosphorylation and the nuclear translocation of IFN-regulatory factor 3 (IRF3) (73). Also TLR7, 8 and 9 elicit type I IFN induction, especially IFN-α, through MyD88, in a pathway that involve the kinase IRAK and the IFN-regulatory factor 7 (IRF7) (73)

Collectively, activation of these signalling cascades induce the expression of a variety of host defence genes that include inflammatory cytokines (IL-12, TNFα and IL-6) and type I interferon cytokines, the up-regulation of co-stimulatory molecules (CD40, CD80 and CD86) and the altered expression of chemokines receptors (CCR2, CCR 5 and CCR7) (65).

TLRs ligands are also able to signal an array of responses that affect the membrane vacuolar system, the cytoskeleton and the machinery involved in antigen processing and presentation (74). One of the earliest responses triggered by TLRs ligands in DCs is the transient increase in macropynocytosis (74) and phagocytosis (75) probably through the activation of signalling pathways that regulate the actin cytoskeleton (74). Moreover, the simultaneous exposure of the antigen with LPS, the ligand of TLR4, enhances antigen presentation on MHC class I molecules (74) and favor peptide-MHC class II generation within TLR-signalling phagosomes (13). Finally, LPS signals a shut-down of peptide-

MHC class II endocytosis, thus extending the half-life of assembled peptide-MHC complexes at the plasma membrane (76).

Fc receptor family

Some classes of PRRs involved in antigen recognition and uptake are also able to activate a downstream signalling cascade that leads to DCs maturation. Among these, the well characterized class of Fcγ receptors. The potent immunoregulatory functions of immune complexes (ICs) rely on two different classes of DCs FcγRs: the activatory FcγRIII and FcγRIV and the inhibitory receptor FcγRIIb that transduce their signals via immunoreceptor tyrosine-based activation (ITAM) and inhibitory motifs (ITIM), respectively (14).

FcγRIIb is a single-chain receptor that carries an ITIM motif in its cytoplasmic domain. Its expression on DCs controls ICs mediated DCs maturation, as DCs derived from mice deficient for FcγRIIb have an enhanced potential to generate Ag-specific T cells responses (77). FcγRIIb may function as a regulator of DCs activation by setting the threshold that prevents spontaneous maturation of DCs and thus promoting steady-state tolerance (14).

The activating FcγRs transmit their signals through a common accessory γ chain that carries the ITAM motif and that is essential for their cell surface expression. DCs maturation is triggered by FcγRs engagement in a signalling pathway that leads to tyrosine phosphorylation of the ITAM motif by members of the Src-kinase family and subsequent recruitment of members of the Syk-kinase family (78).

The paired expression of activating and inhibitory FcγRs on the same DCs is the key for the generation of balanced immune responses, where the final outcome will depend on the expression levels of activating and inhibitory receptors, on their relative affinity for IgGs and on the isotype of the antibodies produced by the humoral response.

C-type lectin receptor family

Among C-type lectin receptors, Dectin-1 is the first example of PRR that can mediate its own intracellular signals thus coupling PAMP recognition to the induction of adaptive immunity. Dectin-1 contains an ITAM-like motif in its cytoplasmic tail which resembles sequences found in the activating FcγRs and similarly to them, upon receptor engagement, it is phosphorylated by Src-kinases and subsequently it recruits Syk-kinases (79). The tyrosine kinase Syk activates downstream signalling components including the transcription factor NF-kB in a pathway that also involves the

CARMA1-related adaptor protein CARD9. Dectin-1 signalling in DCs results in maturation and production of a distinct combination of cytokines, including IL-2, IL-10, TNF and IL-23 which strongly biased T helper cell differentiation to a T helper 17 fate (19).

Intracellular pattern recognition receptors

In addition to membrane associated PRRs, DCs expresses cytosolic receptors involved in the recognition of bacteria and viruses that gain access into the cytoplasm and are able to induce the production of cytokines and cell activation. These cytosolic receptors are grouped in two main family: the NLRs (nucleotide-binding oligomerization domain (NOD)-like receptor family) which comprises at least 23 members that are either NOD receptors or NALPs; and the RLRs (RIG-I like receptors), a family of receptors that have an RNA-helicase domain joined to two caspase-recruitment domains (CARDs) which include RIG-I (the retinoic-acid inducible gene I) and MDA5 (the melanoma-differentiation-associated gene 5) (80). NLRs are involved in the recognition of bacterial peptidoglycans from both $GRAM^+$ and $GRAM^-$ bacteria and drive the activation of MAP kinases and NF-kB in a signalling pathway similar to that triggered by TLRs. In addition to microbial products, some NALP proteins can also sense the presence of host danger signals such as low concentrations of intracellular potassium, monosodium urate and calcium pyrophosphate crystals and drive the formation of a complex called "inflammasome". This complex has an important role in the activation of pro-inflammatory caspase such as caspase-1 which in turn leads to the processing and the release of the active form of the inflammatory cytokine IL-1β. RLRs are cytoplasmic sensor of virally derived dsRNA and similarly to anti-viral TLRs trigger the activation of NF-kB and transcription factors of the IRF family (IRF3 and IRF7), which cooperate in the induction of antiviral IFN type I (81).

Microorganisms are thus able to simultaneously trigger a complex set of PRRs, both within and outside the TLRs family. It is becoming clear that the combined activation of these different receptors can result in complementary, synergistic or agonistic effects especially in terms of cytokine production (81). Not only the combination, but also the order in which the stimuli are delivered over a defined period of time is crucial to determine the overall outcome of the response. DCs activation can be viewed as a dynamic system that continuously adjust transcriptional activity depending on the detection of different stimuli and enables the integration of multiple signals in a defined temporal window in order to trigger the most appropriate immune response (82)

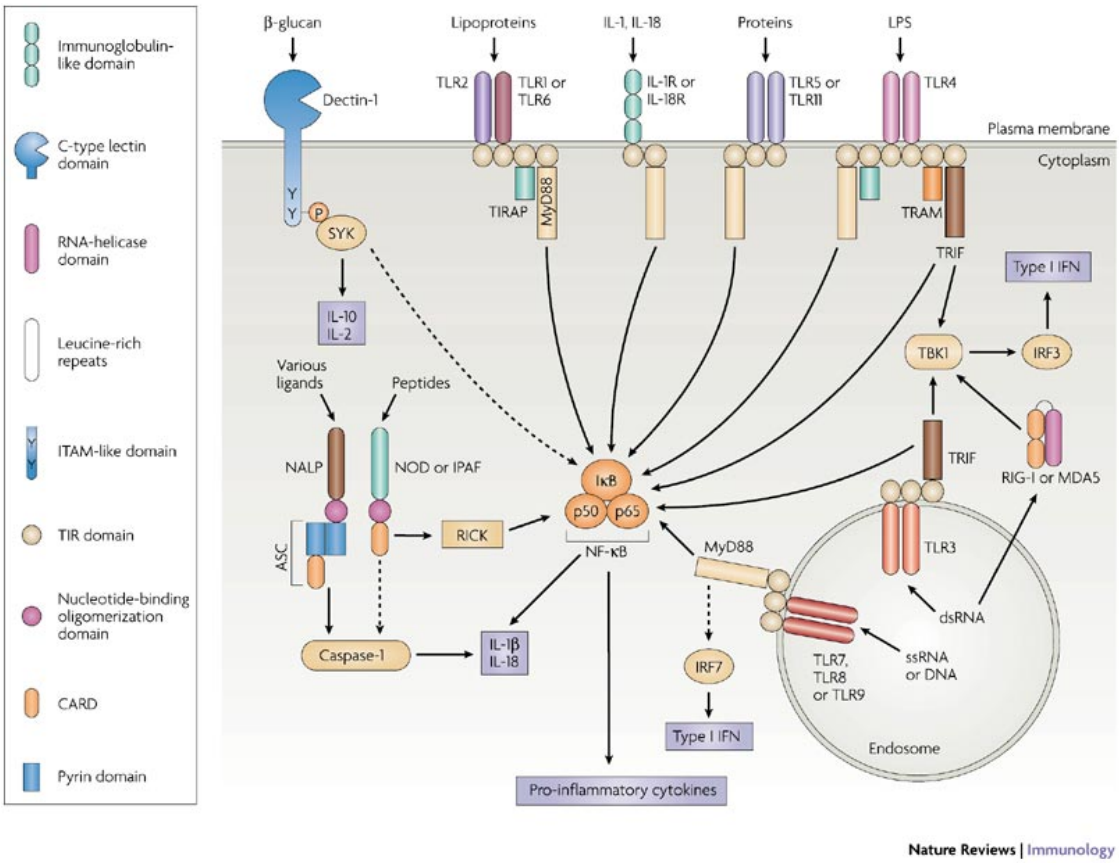


Figure III. Schematic representation of the main signaling pathways of the PRR families. Only the adaptor molecules and the main signaling pathways that differentiate the different classes of PRRs are shown. In reality, the pathways that are activated by the different receptors are multiple. TLRs (with the exception of TLR3), IL-1R and IL-18R induce NF-kB dependent cytokine production through a pathway involving the adaptor MyD88. However, TLRs signaling may also involve MAP kinases and phosphatidylinositol 3-kinase. TLR3 and TLR4 uses a MyD88-independent signaling pathway that involves the adaptor molecule TRIF and induces type I IFNs production. Dectin-1 (a β-glucan) receptor is shown as an example of various cell-surface PRRs, that like FcRs, signal via their ITAM-like motif to activate the kinase SYK. The NLRs family of intracellular PRRs signal through complex pathways that result in the activation of caspase-1 in case of NALP, or in the induction of NF-kB in case of NOD proteins. The RLRs (RIG-I and MDA5) activate NF-kB, but also induce type I IFNs production via IRF3 and IRF7.

1.3 T cells stimulation by Dendritic cells

1.3.1 T cells immunity

Several unique properties contribute to the prominent role of DCs in the initiation and modulation of adaptive immune responses: the great efficiency in antigen uptake and processing, the unique capacity of sensing and integrating different environmental signals and the ability to migrate and to localize in T cells rich areas of secondary lymphoid organs. Is in this definite area of lymph nodes that antigen specific immune responses initiate by engagement of the T cell receptor (TCR) with the cognate peptide-MHC complex displayed by the DCs. TCR triggering determine the antigen-specificity of the response and represents the fist of the three signals delivered by DCs that dictates the fate of naïve T cells. The second signal is referred to as T cell co-stimulation and is mediated by the engagement of both positive (CD28) and negative (CTLA-4 and PD-1) co-stimulating receptors on T cells by DCs. The fine balance between these positive and negative signals controls the initiation of protective immunity. If the DC is not fully activated, due to the lack of inflammatory stimuli, negative signals prevail leading to T cell inactivation by anergy or deletion and the induction of a state of tolerance (83). Finally, T cells sense and integrate signals, mediated by different cytokines and chemokines (referred to as signals 3) that are produced by DCs and whose nature strictly depends on the conditions under which DCs are primed. The set of secreted cytokines determine the extent of T cells proliferation, the survival and the ability to develop effector functions and specifically the differentiation into effector cytotoxic T cells or the acquisition of a T_H1, T_H2, T_H17 of T regulatory phenotype in case of $CD8^+$ and $CD4^+$ T lymphocytes respectively.

1.3.2 Dendritic cell maturation and T cell priming

Several studies initiated almost a decade ago, have provided the direct evidence that DCs play a crucial role not only in the induction of adaptive immunity, but also in the maintenance of peripheral tolerance to self and nonpathogenic environmental antigens. Pioneering work by *Kurts et al*. initially showed how the constitutive cross-presentation of ovalbumin, expressed as a transgene in several organs (pancreatic islets, kidney tubular cells, thymus and testis) of RIP-mOVA mice by a bone-marrow derived APCs, induced the proliferation of adoptively transferred Ag specific $CD8^+$ T cells (OT-I) in the lymph nodes draining these organs. However, this initial OT-I activation was followed by the progressive removal of the autoreactive T cells via a deletional mechanism that

ultimately led to an immune tolerant state (84). Further studies by Steinman group implicated DCs as the APCs responsible for tolerance induction. They showed that in a steady state condition (i.e. absence of inflammation), direct targeting of DCs *in situ* through DEC205 induced the efficient presentation of the peptide HEL (fused to anti-DEC antibody) on MHC class II molecules and the extensive proliferation of adoptively transferred HEL-specific $CD4^+$ T cells. However, the activated T cell were eventually deleted unless an additional DCs activation signal, such as CD40 ligation, was provided (85). A second study by the same group extended these findings on Ag specific $CD8^+$ T cells. Targeting DCs via an anti-DEC-205 Ab coupled to OVA induced the efficient cross-presentation of the Ag and the extensive proliferation of adoptively transferred OT-I cells. However, by 10-12 days, the stimulated T cells underwent a deletional mechanism, being entirely absent from lymph nodes, spleen and blood and were unresponsive upon rechallange with OVA Ag, thus proving the induction of peripheral tolerance. Similarly to the previous study, the co-administration of an agonistic anti-CD40 antibody as an adjuvant, activated DCs *in situ* and produced a strong CTLs response and memory (86).

The finding that cross-presentation of different forms of Ags, either soluble proteins (85, 86) or dying cells (87) by steady state DCs is an essential mechanism to guarantee tolerance to self, was further extended to the classical pathway of MHC class I presentation by works performed in van den Broek laboratory. *Probst et al*. used a transgenic mouse model (DIETER) that allows the controlled expression and presentation of lymphocytic choriomeningitis virus (LCMV)-derived CTL epitopes exclusively by DCs. They observed that activated DCs (by systemic administration of anti-CD40 Ab), efficiently primed naïve endogenous CTL to proliferate and to differentiate into protective effectors, whereas resting DCs induced Ag specific tolerance, that could not be broken by a subsequent infection with LCMV (88).

Collectively, all these data demonstrate that DCs maturation state is the critical switch that provides signals for effector T cell development and memory, diverting T cells from anergy or deletion to protective immunity (89).

Several properties of mature DCs could account for their capacity to efficiently prime T lymphocytes and to induce their differentiation into long-lived functional memory. Among these, the increased expression of co-stimulatory, MHC and adhesion molecules which are crucial in controlling the stability and the duration of the DC-T cell contact (90) so that the duration of the TCR signaling may influence the functional outcome of T cells activation (91). In addition, mature DCs secrete immune enhancing cytokines like IL-12 and type I/type II IFNs that favor the differentiation of T lymphocytes into effector cells and T cells survival. On the contrary, immature DCs induce peripheral T cell tolerance through combined mechanisms of antigen specific T cells deletion and functional inactivation or by generation of regulatory T cells. However, the *in vivo* relative contribution of these mechanisms remains still elusive and may largely depend on the specific experimental setting and model analyzed. For instance, delivery of antigens to steady state DCs via DEC205 leads to T cells tolerance by both deletional mechanisms (85, 86) and induction of $CD4^+/CD25^+$ regulatory T cells (92). Alternatively, presentation of cellular antigens expressed by resting DCs results in the depletion of antigen specific T cells upon engagement of the T cells co-inhibitory molecules PD-1 and CTLA-4 (83). Finally, a population of $CD103^+$ DCs found in the mesenteric LNs is able to promote the conversion of naïve T cells into $Foxp3^+$ T reg cells upon oral administration of the antigen, in a mechanism dependent on TGFβ and retinoic acid (93).

1.3.3 Dendritic cell "licensing" by $CD4^+$ T cells

Dendritic cells play a prominent role in the generation of CTL responses not merely by presenting antigens, but in supplying critical co-stimulatory and differentiation-inducing mediators that guide the development of lymphocyte responses. Experimental evidences from the late of 1990s led to a model of DCs licensing, in which antigen-specific $CD4^+$ T cells interacted with DCs via CD40/CD40L interactions and conferred upon DCs the ability to prime CTL responses (94, 95). Whether such $CD4^+$ T cells help is essential for the induction of primary CTL responses or in the generation of functional memory $CD8^+$ T cells it is still an open issue and a matter of intense study.
The generation of a primary $CD8^+$ T cells response has been shown to be independent from the cognate T cells help in various experimental settings of acute infection such as *Listeria monocytogenes* and lymphocytic choriomeningitis virus (96-98). However, the situation changes during chronic infections, HSV infection, or in noninflammatory conditions where the antigen specific $CD8^+$ T cell response is greatly impaired in the absence of $CD4^+$ T cells (99, 100). A possible explanation is that the $CD4^+$ T dependency of primary CTL responses is inversely correlated with the level of inflammation and other danger signals present during initial priming. In

this context, the recognition of PAMPs by TLRs and other PRRs may lead to a DCs activation status that allows to bypass the need for $CD4^+$ T cells help (95).

A second critical issue regards the requirement of $CD4^+$ T cell help in generating functional $CD8^+$ T cell memory. In this setting, many studies have clearly demonstrated that even if the primary CTL response to pathogens like *Listeria monocytogenes* and lymphocytic choriomeningitis virus fully develops in a T_H independent manner, the resulting memory $CD8^+$ T cell population is poorly functional and unable to undergo a second round of clonal expansion following re-encounter with the antigen (96-98). The general accepted view is that $CD4^+$ T cell help makes the initial $CD8^+$ response bigger and programs the differentiation of responding $CD8^+$ T cells into long lived, protective memory. However, exactly what $CD4^+$ T cell provide to maintain $CD8^+$ T cell memory remains a mystery (95). Janssen and colleagues showed that the un-helped $CD8^+$ T cells undergo death, which is mediated by TNF-related apoptosis-inducing ligand (TRAIL) upon antigen restimulation (101). The regulation of *Trail* expression by CTLs may thus be one of the mechanisms by which $CD4^+$ T cells influence the generation of $CD8^+$ T cell memory.

A critical point is whether different stimulatory signals can bypass the requirement for $CD4^+$ T cell help in the induction of fully efficient memory CTLs. In a recent study, *Hervas-Stubbs et al*. evaluated whether signals generated by TLR engagement may be sufficient to cross-prime effector/memory CTLs independently of $CD4^+$ T cells help. This was achieved by immunizing mice with synthetic microsphere covalently linked to the OVA class I peptide together with different TRL ligands. This work showed that the requirement for T-cell help to trigger effector CTLs can be bypassed by TLR3-L (poly(I:C)) and TLR9-L (CpG mixed to a lipid formulation), but not with TLR2/4/7-L, although they induced DCs maturation and IL-12 secretion. The production of type I IFNs was necessary to induce a full CTL response but not sufficient alone, since it required the concert action of other proinflammatory cytokines. Finally, the "helpless" CTLs induced by TRL3-L differentiated into functional memory CTLs able to proliferate upon secondary immunization, thus proving that signals derived from TLR triggering can bypass the requirement of $CD4^+$ T cells help (102). Further studies will be required to assess whether the engagement of other activating receptors on DCs would be sufficient to trigger signaling cascades resulting in generation of functional memory.

1.3.4 Dendritic cells control of T-cell polarization

Dendritic cells have a central role in the orchestration of various forms of immunity and tolerance. They can modify and adapt the T-cell response to the type of invading pathogen by developing into functional DC phenotypes that initiate either T_H1, T_H2, T_H17 or T regulatory responses. It is becoming increasingly clear that the way in which DCs bias the development of T_H cell subsets is related to the engagement of specific receptors that are involved in DC maturation. The integration of the signals triggered by multiple PRRs upon antigen encounter and the factors produced in the surrounding tissues by infected cells, lead to the polarized maturation of DCs and the subsequent production of specific molecules, primarily cytokines, that promote T_H1, T_H2, T_H17 or regulatory T cell development.

In general, T_H development is characterized by the presence of reiterative feedback mechanisms that propagate the early lineage decision once initiated (103), i.e. the cytokines produced by a given helper T cell subset often serve as potent inducers of the differentiation of that subset, as well as negative regulators of the other subsets. Cytokines regulate T_H differentiation by repressing or inducing the transcription of genes encoding for key transcription factors which in turn, activate specific cytokines receptors genes and thus define the growth factor preference of each mature helper T cell lineage (104). If the identification of the key transcription factors that regulate the development of specific T_H subsets has shed light on the signaling pathways that sustain T_H cells differentiation once initiated, on the other side, the identity of the DCs PRRs whose engagement drive the polarized maturation of DCs is far from being complete.

T_H1 cells polarization evolved to enhance clearance of a broad spectrum of intracellular bacteria, viruses and some parasites. Engagement TLR3, 4, 7 and 9 on DCs by these pathogens results in the production of both types of IFNs and IL-12, which play a crucial role in inducing T_H1 cells differentiation and in the cell proliferation and survival of committed T_H1 cells respectively. T_H1 differentiation is coupled to the sequential action of IFNγ and IL-12 through a signalling pathway that involves STAT1 and the transcription factor T-bet (103), which represent the master regulator of T_H1 cells (105).

Marked T_H2 responses are induced by infection with parasistic helminths and various allergens. Although phosphorylcholine-containing glycoproteins and polylactosamine sugars from different parasites have been shown to induce the maturation of DCs that polarize a T_H2 type of response, the PRRs responsible for this effect still remain to be established. The key cytokine involved in T_H2 differentiation is IL-4 that initiate a signaling pathway via STAT6 and leads to the upregulation of the transcription factor GATA-3. This master regulator of T_H2 differentiation

induces the transcription of Il4, Il5 and Il13 genes while suppressing the factors critical to T_H1 pathway.

T_H17 cells are a third class of effector $CD4^+$ T lymphocytes that has been implicated in numerous autoimmune and inflammatory conditions including arthritis, multiple sclerosis, psoriasis and inflammatory bowel diseases and are characterized by production of a distinct profile of effector cytokines, including IL-17 and IL-6 (104). They probably have evolved to enhance the host clearance of a range of pathogens distinct from those targeted by T_H1 and T_H2 (103). Indeed, IL-17 has been linked to resistance to infection by extracellular bacteria such as *Klebsiella pneumoniae* as well as by fungi such as *Candida albicans* (106). Although the PRRs able to induce a DCs phenotype that initiate a T_H17 response are largely unknown, a recent work by Reis e Sousa group showed how the dectin-1-Syk-CARD9 signaling pathway promotes DCs maturation and the secretion of proinflammatory cytokines such as IL-6, IL-2, TNF and IL-23 that strongly bias T_H differentiation to T_H17 fate. Indeed, large amount of IL-17 were produced by $CD4^+$ spleen cells from mice infected with *C. albicans* thus linking the induction of T_H17 responses to fungal infection. The authors also showed that T_H17 differentiation is dependent on the presence of T regulatory cells and requires TGFβ (19). These data are consistent with reports suggesting that TGFβ and IL-6 act in the first step of T_H17 development, by activating the key transcription factor RORγt, and that IL-23 acts secondarily probably to expand the committed T_H17 effectors or maintain their function (104).

Finally, exposure of DCs to cytokines like IL-10 and TGFβ can induce the differentiation of $CD4^+$ T cells into a distinct regulatory subsets characterized by the ability to suppress adaptive T cell responses and prevent autoimmunity. Regulatory T cells that develop from naïve $CD4^+$ T cell precursor in the periphery are called adaptive Treg and differ from the naturally occurring regulatory T cells that develop intrathymically. At least two types of adaptive Treg have been described. Treg1 cells develop under the control of IL-10-conditioned DCs, do not express the transcription factor Foxp3 and are marked by the production of high amount of IL-10. Treg2 instead, are induced from naive precursors under the influence of TGFβ, express $Foxp3^+$ and have suppressive activities indistinguishable from naturally occurring Treg (104). Indeed, proinflammatory cytokines such as IL-6 and IL-1 that are released by TLR-matured DCs, are able to subvert Treg suppression, but at the same time they also reverse the Treg anergic state thus permitting their proliferation while retaining the suppressive functions once proinflammatory cytokines production ceases (103). Moreover, many pathogens such as *Mycobacteria* species which bind to DC-SIGN, *Schistosoma mansoni* and *Yersinia* species to TLR2 or *Plasmodium falciparum* infected erythrocytes to CD36 and CD52, have been described to prime DCs to support the development of regulatory T cells as a mechanism of immune evasion (107).

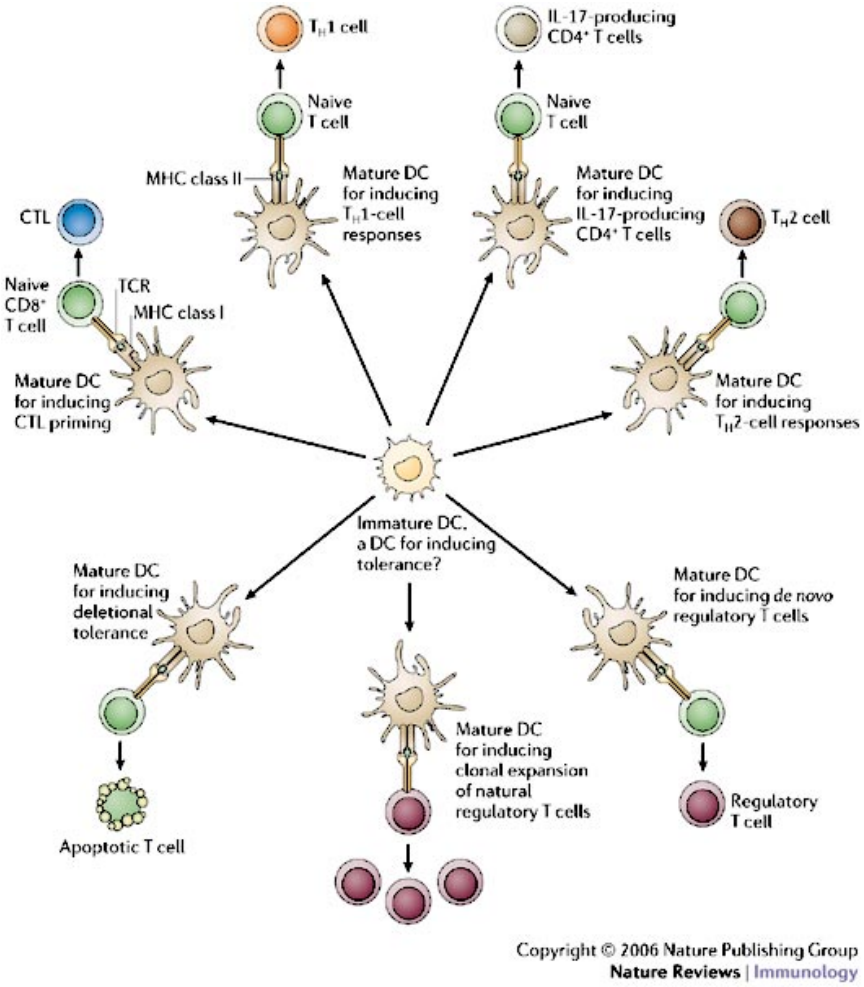


Figure IV. Dendritic cells effector function. Immature DCs can give rise to multiple types of "effector" DC that instruct distinct T-cell fates, including immunity, tolerance and immune deviation. Maturation refers to the changes that accompany immature DC transition to a given effector state in response to environmental signals of exogenous (microbial) or endogenous (cytokines, hormones, dying cells) origin. The quality of these signals determines the choice of effector DC, although ontogeny can also have a role, predisposing certain DC subtypes towards particular effector states. Some maturation signals can promote the generation of tolerogenic DCs. This does not negate the possibility that some immature DCs have an intrinsic tolerogenic function.

1.4 Dendritic cells subsets and T cells priming

1.4.1 Dendritic cells subsets

The phenotypic and functional analyses of the DCs found in the thymus, spleen and lymph nodes have revealed a considerable heterogeneity among the DCs population, such that different DCs subsets have emerged and described as following a life cycle that differed from the original "Langerhans cell paradigm" (3). Although all DCs are capable of antigen uptake, processing and presentation to naïve T cells, the DCs subtypes differ in hematopoietic origin, location, migratory pathways, immunological functions and dependence on infection or inflammatory stimuli for their generation (7).

A first criteria to classify DCs is to distinguish among conventional DCs (cDCs) that have a dendritic form and exhibit DCs functions in steady state, from precursors of DCs (pre-DCs), that require further development to acquire DCs phenotype and full DCs function. Although the development of some pre-DCs can occur in steady state, in many cases the further development of pre-DCs requires a microbial or inflammatory stimulus (7). An example of pre-DCs are the plasmacytoid DCs that circulate through the blood and lymphoid tissues and only acquire the typical DCs morphology upon activation. Also monocytes can serve as source of pre-DCs and differentiate into functional DCs upon infection or inflammatory conditions (108, 109).

Among conventional DCs, two main categories can be distinguished based on the path they follow to access the lymphoid organs: migratory DCs and lymphoid-tissue resident DCs. Migratory DCs comprise epidermal Langerhans cells and interstitial or dermal DCs. The two subsets can be readily distinguished by the selective expression of the C-type lectin langerin on the surface of epidermal LCs and by LC-specific intracellular organelles known as Birbeck granules (110). Migratory DCs follow a life cycle described by the Langerhans cell paradigm serving as antigen sampling sentinels in the peripheral tissues and trafficking to the lymph nodes in response to danger signals. Migratory DCs display a mature phenotype in LNs also in steady state conditions, indicating that their migration and maturation proceed constitutively and independently of pathogens and are probably triggered by inflammatory compounds that are constitutively released by peripheral tissues (3).

Lymphoid-tissue resident DCs or blood-derived DCs constitute half of the lymph node DCs (the second half being migratory DCs) and all the DCs found in the spleen and thymus (6). They can be further subdivided into three subsets depending on the expression of CD4 and CD8 markers: $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$ and $CD4^{-}CD8^{-}$ cells. Resident DCs do not conform to the Langerhans cells

paradigm, since they develop from bone marrow precursors directly in secondary lymphoid organs without previously trafficking through peripheral tissues. In contrast to migratory DCs, blood-derived DCs maintain an immature phenotype in steady state, but can be induced to mature by inoculation of inflammatory stimuli. Thus the function of the immature lymphoid organ DCs may be to promote tolerance in steady state while maintaining their capacity to respond to infections reaching those organs (6).

Table I. Mouse dendritic-cell subsets

Features	Lymphoid-organ-resident DC subsets			Migratory DC subsets		Monocyte derived
	$CD4^+$ DCs	$CD8^+$ DCs	DN DCs	Interstitial DCs	Langerhans cells	
Location						
Spleen	Yes	Yes	Yes	No	No	Sites of inflammation
Subcutaneous lymph nodes	Yes	Yes	Yes	Yes	Yes	
Visceral lymph nodes	Yes	Yes	Yes	Yes	No	
Thymus	Yes	Yes	Yes	No*	No	
Surface markers						
CD11c	+++	+++	+++	+++	+++	+++
CD4	+	–	–	–	–	–
CD8	–	++	–	–	–/+	–
CD205	–	++	–/+	+	+++	–/+
CD11b	++	–	++	++‡	++	++
Langerin	–	+	–	–	+++	–
CD24	+	++	+	ND	ND	ND
SIRPα	+	–	+	+	+	ND
Functional features in the steady state						
Maturity	Immature	Immature	Immature	Mature	Mature	N/A
Co-stimulatory§	+	+	+	++	++	N/A
Antigen processing and presentation‖	+++	+++	+++	+/–	+/–	N/A
MHC class II	++	++	++	+++	+++	N/A
In vitro *equivalent*						
	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus GM-CSF, TNF and TGFβ	Bone-marrow precursors plus GM-CSF, TNF and TGFβ	Bone-marrow, spleen or blood precursors plus GM-CSF

1.4.2 Dendritic cells subset and antigen-presenting functions

Since the initial characterization of different DCs population less than a decade ago, a great effort has been spent trying to elucidate the role of various DC subsets in the initiation of adaptive immune responses or in the maintenance of a tolerogenic state under normal circumstances. A scenario where the coordinate action of the different DCs subtypes, each with a specific function, operates to mount effective responses against specific pathogens or to prevent such responses in the absence of danger can be envisaged.

The possibility to isolate highly pure populations of DCs from different lymphoid organs taking advantage of specific lineage markers and the generation of transgenic mice that allows the tracking (111) or the conditional ablation of specific DCs types in vivo (57) have allowed to investigate the relative efficiency of DCs in priming T cell responses and their differential contribution to in vivo immune responses.

Although all DCs types express high levels of MHC class I and MHC class II molecules, especially when mature, and are able to generate peptide-MHC complexes, an increasing amount of data is indicating a different ability of the various DCs subset to incorporate peptides, derived from a given antigen, into their presentation pathway and thus a different capacity to activate $CD4^+$ or $CD8^+$ T lymphocytes.

Plasmacytoid DCs (pDCs) are a principal subset of DCs in both human and mouse with a unique ability to sense and respond to viral infections mainly by secreting large amounts of type I IFNs. Differently from conventional DCs, pDCs constitutively express the IFN regulatory factor 7 (IRF-7) that allows the rapid secretion of vast amount of IFN-α in a signaling pathway initiated by TLR7 and TLR9 engagement. Due to their poor phagocytic activity and thus inefficiency to present antigens (112), pDCs were generally considered to have a role in boosting the ability of conventional DCs to mature and stimulate T cells in viral infections, rather than playing a direct role in antigen presentation. However, recent findings by *Sapoznikov et al* indicate that pDCs are indeed able to initiate productive $CD4^+$ T cell responses in lymph nodes upon antigen challenge. Taking advantage of mice conditionally depleted of $CD11c^{hi}$ cDCs but retaining a normal pool of pDCs, the authors demonstrate how the ability of this specific subset to trigger antigen specific $CD4^+$ T cell responses is organ dependent, being limited to lymph nodes and absent in spleen. The unique priming of $CD4^+$, but not $CD8^+$, T cell responses by pDCs could result from exclusive presentation of exogenous antigen on MHC class II, but not MHC class I molecules and would predict that pathogens that specifically activate pDCs will elicit exclusively humoral but not citotoxic immunity (113).

Migratory DCs present in the epidermis (Langerhans cells) and in the dermis (interstitial DCs) are characterized by the expression of many PRRs and endocytic receptors and are able to efficiently mediate the uptake of macromolecules and a broad range of microorganisms (114). Residing in most peripheral tissues at site of interface with the environment, migratory DCs constitutively take up antigens and traffic to lymph nodes where they interact with both $CD4^+$ and $CD8^+$ T lymphocytes. Indeed, cutaneous DCs express upon activation high levels of MHC class I and class II molecules and are able to efficiently prime both T helper and cytotoxic T lymphocytes. Conversely, their ability to cross-present soluble or cell-associated antigens is less defined. In a mouse model of autoimmunity, where the OVA class I peptide is expressed under the control of the human keratin 14 promoter (K14-OVAp mice), Langerhans cells have been demonstrated to cross-present this highly expressed antigen in the skin draining LNs and to induce the expansion of antigen specific cytotoxic T cells endowed with effector functions (115). A recent study by Romani group showed that Langerhans cells in epidermal skin explants were able to cross-present soluble and cell-associated antigens in a TAP-dependent manner. Interestingly, the in vitro comparison of the relative efficiency of Langerhans cells and $CD8\alpha^+$ resident DCs to present an exogenous antigen on MHC class II or cross-present it on MHC class I molecule was different, with $CD8\alpha^+$ spleen cells being more potent in inducing $CD8^+$ T cells and Langerhans cells better in stimulating $CD4^+$ T cells (116).
Whether migratory DCs are the main DCs subset involved in the presentation of peripheral-tissue antigens or antigens derived from pathogens that infect the skin, lungs or gut, to both $CD4^+$ and $CD8^+$ T cells is still a matter of controversy. Although migratory DCs have an essential role in carrying peripheral antigens to the draining lymph nodes, recent studies using various models of viral infection have highlighted the dominance of the non-migrating, lymph node-resident $CD8\alpha^+$ DCs subset in priming CTL responses thus implicating the transfer of peripheral antigens from migratory to resident DCs upon entry in the T cell areas of the lymph nodes (59, 61, 117).
Villadangos et al propose a model where the relative contribution of the two subsets to the presentation of peripheral antigens depends on the pathogen capacity to infect DCs or impair their antigen presenting functions and on the number of antigen-carrying migratory DCs that reach the lymph node (3).

The DCs that reside in the lymph nodes and in the spleen are ideally located to monitor the blood for the presence of infectious agents that spread through circulation and thus should play a major role in the induction of immune response against blood-borne pathogens. The fact that intravenous inoculation of inflammatory compounds such as LPS or CpG induce the activation of conventional DCs, their migration to the T cell areas of spleen and LNs, the acquisition of a mature

phenotype and the secretion of large amount of IL-12, support the hypothesis that cDCs indeed play a major role in immunosurveillance. On the other hand, if peripheral tolerance is mediated by immature DCs, the large number of blood derived DCs contained in the steady state in the lymphoid organs would provide a strong tolerogenic environment for naïve T cells (118). Due to their crucial role in the control of immunity, the definition of the in vivo relevance of the various subsets of lymphoid organ resident DCs in terms of antigen presenting functions has been subject of intense study.

Both in vitro and in vivo analysis of the capacity of blood-derived DCs subsets to present exogenous antigens on MHC class II or cross-present them on MHC class I have revealed a functional dichotomy between the $CD8\alpha^+$ and $CD8\alpha^-$ (comprising both $CD4^+$ and double negative DCs) subsets of resident DCs. Specifically, it emerges that the $CD8\alpha^+$ DCs are by far the most efficient at cross-presenting cellular (119, 120), soluble (121, 122) or latex bead-associated antigens (122). Indeed, the ability of $CD8\alpha^+$ DCs to cross-present cellular associated antigens is attributable to their selective capacity to endocytose dying cells in culture and in vivo (120, 123).

Furhtermore, $CD8\alpha^+$ DCs have also been identified as the major subset involved in the MHC class I presentation of antigens from viruses (HSV1, influenza and vaccinia virus) and cytosolic bacterium *L. monocytogenes* (61, 124). By contrast, $CD8\alpha^-$ DCs seem to be more efficient than $CD8\alpha^+$ DCs at presenting exogenous antigens on MHC class II molecules, especially in the case of phagocytosed antigens (i.e antigens associated to latex beads) (122) and soluble antigens, although less evident (120-122).

The $CD8\alpha^+$ and $CD8\alpha^-$ subsets of DCs differentially express multiple putative antigen receptors belonging to the C-type lectin family. The DEC-205 and mannose receptors are primarily expressed by the $CD8\alpha^+$ DCs (125, 126), whereas dectin-1, DCIR2, FIRE and CIRE are specific for the $CD8\alpha^-$ DCs (127-129). Targeting of soluble antigens coupled to antibodies against these various receptors has shed light on the in vivo role of the two DCs subtypes and allowed to characterize the type of immune response (humoral vs cytotoxic) preferentially triggered by these DCs subsets. Collectively, these studies highlight the greatest efficiency of $CD8\alpha^+$ DCs to activate CTLs and the preferential induction of $CD4^+$T cells and humoral responses by the $CD8\alpha^-$ DCs (127-129).

As suggested by *Dudziak et al*, a possible explanation of this dichotomy is the differential expression of various components of the MHC class I and MHC class II machinery by the $CD8\alpha^+$ and $CD8\alpha^-$ subsets respectively (127). Alternatively, the MHC class I and class II pathways are fully operational in both subsets, but in addition, the $CD8\alpha^+$ DCs possess a specialized machinery for cross-presentation which is largely absent in the $CD8\alpha^-$ subtype (3). A recent work by *Burgdorf et al* demonstrate how the $CD8\alpha^+$ DCs are able to preferentially deliver exogenous antigens to either the

MHC class I or class II pathways depending on the mechanism of antigen uptake. Specifically, mannose receptor mediated endocytosis results in cross-presentation of ovalbumin, whereas pinocytosis conveys OVA to lysosomes for MHC class II-restricted presentation (126). It is thus plausible that cross-presentation requires the delivery of antigens to a specialized compartment that is found only in the $CD8\alpha^+$ subset of DCs (3).

The better understanding of the in vivo functions and of the interplay among DCs type will indeed provide new possible strategies to potentiate the immune response against poorly immunogenic pathogens, to shift the type of immune response according to the infectious agents and finally to prevent or delay the onset of autoimmune pathologies.

Table II. Uptake, MHC class I and MHC class II presentation of several model antigens by $CD8^+$ and $CD8^-$ dendritic cells.

Antigen	$CD8^+$ DCs			$CD8^-$ DCs		
	Uptake	MHC class I	MHC class II	Uptake	MHC class I	MHC class II
Endogenous						
	N/A	++	++	N/A	++	++
Exogenous: phagocytosed						
Cells	++	++	+	+/–	–	+/–
Beads	++	++	+/–	++	–	++
Exogenous: pinocytosed (soluble)						
	++	++	++	++	+/-	++
Exogenous: receptor-mediated endocytosed						
CD205	++	++	+	Not expressed	N/A	N/A
DCIR2	Not expressed	N/A	N/A	++	–	++
Dectin-1	Not expressed	N/A	N/A	++	–	++
TLR11	++	?	++	Not expressed	N/A	N/A
FcR	++	++	+	++	++	++

1.5 Dendritic cells based cancer vaccines

Due to their central role in the orchestration of all elements of the immune system, dendritic cells have emerged in the past few decades as a fundamental target and tool for cancer immunotherapy.

Current approaches are based on the immunization of cancer patients with autologous, patient-derived DCs loaded with tumor antigens *ex vivo*. Indeed, numerous studies in mice have shown that DCs loaded with tumor antigens can induce therapeutical and protective antitumor immunity (130). Furthermore, the immunogenicity of antigens delivered by DCs has also been shown in patients with cancer or chronic infections, thus providing the proof of principle that using DCs as vaccines can work (131).

In the recent years, strategies have been developed to generate large numbers of clinical-grade DCs from various types of progenitors cells. Moreover, several protocols have been designed to load different formulations of antigens on DCs and to provide the cells with the activation stimuli necessary to optimize their antigen presenting potential and T cells stimulatory capacities.

Despite the limited rate of objective tumor regressions that has been observed in clinical studies so far, the increasing knowledge of DCs functions in vivo and the discovery of the factors that contribute to the tumor-induced immunosuppression will indeed provide the rational for new combined DCs vaccination protocols with an enhanced clinical efficacy.

1.5.1 *Ex vivo* generation of immunocompetent DCs

A first critical parameter for the production of DC vaccines is the number of DCs that need to be isolated/generated, keeping in mind that cancer patients may present alterations in the number, phenotype and functionality of DCs isolated from peripheral blood or generated from monocytes precursors (132).

DCs (both myeloid-derived and plasmacytoid) can be isolated directly *ex vivo* from the blood of patients, either through positive selection using DCs-specific markers or by depletion of contaminating cells or by a combination of both. Despite the fact that Flt3-L (fms-related tyrosine kinase 3 ligand) can be used to mobilize blood DC, yields are generally too low to obtain sufficient numbers of DCs for vaccination.

Human DCs can be generated in culture from $CD34^+$ hematopoietic progenitors, which can be isolated from blood or bone marrow by positive selection and differentiated into DCs by addition of

different cytokine mixtures (e.g. GM-CSF and TNFα or GM-CSF and IL4/IL13) for 2 weeks. Purities are variable and rather low, with the contaminating cells being mainly granulocytes (132).
The most commonly used cells type for DCs generation is the peripheral blood monocytes that can be differentiated into immature "myeloid-type" DC by a 5-7 day culture in the presence of GM-CSF and IL4 or GM-CSF/IL13 and type I IFNs (132). Alternatively, several rapid, two-days "fast-DC" protocols have been developed that generate DCs able to stimulate T cells responses in vitro as effectively as DCs generated by standard protocols (133).

For cancer vaccination, the goal is to generate *ex vivo* a population of antigen-loaded DCs that stimulate robust and long-lasting $CD4^+$ and $CD8^+$ T cell responses in patients with cancer. This imply the setting up of protocols that reproduce *ex vivo* the development of immunocompetent DCs and the process of DCs activation.
DCs need to be generated in vitro such that they undergo optimal maturation (i.e. expression of co-stimulatory molecules, lymph node migratory capacity and proper cytokine secretion) and become responsive to licencing stimuli when they reach the lymph node and encounter cognate T cells. The most widely used maturation protocol for human monocytes-derived DCs consist of a cytokines cocktail including TNF, IL1β, IL6 and PGE_2, also known as monocyte-conditioned medium (MCM) (134). However, a recent phase III clinical trial failed to demonstrate that vaccination with peptide loaded, MCM-matured DCs was more efficient than standard decarbazine chemotherapy in stage IV melanoma patients (135). Extensive controversy exists about which stimulus might be optimal. A recent work by Sporri and Reis e Sousa has shown that optimal activation of DCs requires TLRs signaling (136) in combination to inflammatory cytokines. A promising maturation protocol that includes TNF, IL1β, PolyI:C, IFNα and IFNγ, induced the differentiation of DCs that retained lymph node migratory capacity, responsiveness to IL12 and were able to generate potent CTL responses (137). An alternative is to provide DCs with a maturation stimulus directly in situ by injecting immature DCs into sites that have been pre-treated with adjuvants (TLRs ligand) to induce local inflammatory reaction. Clinical trials are ongoing to assess the therapeutic efficacy of this simplified method of DC vaccination (137).

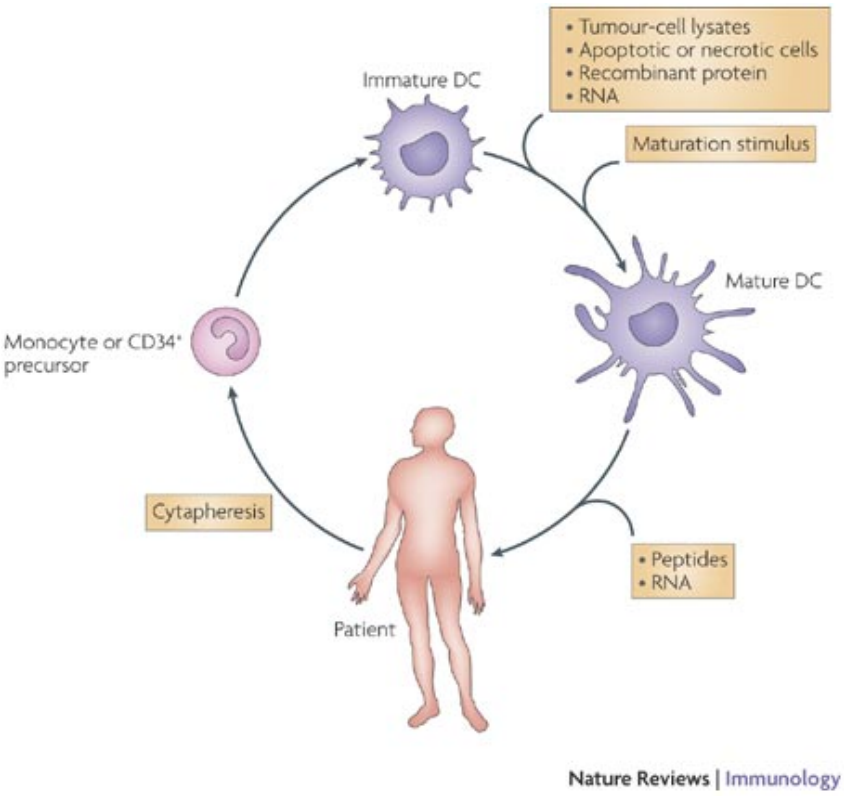


Figure V. DC-based vaccines using ex vivo loaded DCs to induce immunity. Most of the DC-based vaccines currently explored in clinical trials consist of mature antigen-loaded autologous DCs that are administered to patients with the intention of inducing antigen – specific T- and B-cell responses. The DCs used for these studies are derived from monocytes of CD34+ precursors that are isolated from patients blood by cytapheresis. These cells are cultured in the presence of various cytokine mixture to produce immature DCs, and loaded with antigen either before or following DC maturation.

1.5.2 Loading dendritic cells with tumor antigens

The ideal target for cancer immunotherapy would be a tumor associated antigen (TAA) which is exclusively expressed in tumor cells and not in normal tissues, to avoid the potential induction of autoimmunity. In addition to the type of antigen used, the modality of antigen loading on DCs will have a great impact on the its ability to access the MHC class I and MHC class II presentation pathways and thereby will influence the type of T cells response. Antigens can be added exogenously in the form of short peptides, whole proteins, tumor lysates or apoptotic debris or complexed with antibody. Alternatively, DCs can be engineered to synthesize it endogenously by transfection with mRNA encoding the antigen.

The most commonly used protocol for loading Ags onto DCs is pulsing with short synthetic peptides corresponding to the MHC class I and MHC class II epitopes of TAAs. Apart the obvious advantages, important drawbacks are the need to determine the MHC haplotype of the patient, the paucity of known tumor specific peptides and the limited persistence of the peptide-MHC complex on the surface of the DCs.

TAAs like carcinoembryonic antigen (CEA), prostate specific antigen (PSA) or antigens expressed in virtually every type of cancer ("universal" antigens) such as survivin and telomerase reverse transcriptase (TERT) can be loaded on DCs as whole proteins, allowing the derived peptides to be channelled into both MHC class I and class II presentation pathways independently of the patient MHC haplotype. However, a major limit of this methodology is the access to clinical grade reagents.
Methods that take advantage of the complete protein content of the tumor cell and thus broaden the induced immune response, have been developed, including loading the DCs with tumor lysate or tumor-derived exosomes, engulfment of apoptotic/necrotic cells by DCs and fusion of DCs with tumor cells. However, the need to isolate a high number of tumor cells, the complex modality of antigen preparation and the safety concerns, represent the major drawback of these vaccination strategies.
A genetic approach based on the electroporation of DCs with mRNAs that encode for tumor derived antigens or mRNA amplified from whole tumors associates the ease of isolating the genetic material to the opportunity to induce a broad patient specific immune response against both known and unknown TAAs. One main drawback is that antigen is mainly channelled to the MHC class I presentation pathway, thus limiting the generation of effective $CD4^+$ T cells responses.
Advancing knowledge on the intracellular routing of antigens allows for the rational design of effective vaccines by manipulating the antigens to induce optimal T-cells priming. Antigens can be engineered and fused to targeting signals for different intracellular compartments, so that rechannelling of a cytoplasmic antigen into the MHC class II presentation pathway can be achieved by fusing it to the lysosomal targeting signal (138). Alternatively, fusogenic peptides that are based on the N-terminal sequence of the influenza virus haemagglutinin protein can be applied to enhance cytoplasmic delivery of proteins thus possibly stimulating antigen cross-presentation (139).
Besides the form of the antigen, other important factors to be considered are the amount of antigen loaded on the DCs, the efficiency of loading, the length of time the antigen will persist and be presented, and the potential adverse effects of the loading technique (137).

1.5.3 Clinical trials with DC-based vaccines

A substantial number of clinical trials on different cancers, using dendritic cells has been carried out over the last decade. Results are difficult to compare due to the variability in the type and activation state of the DCs used, the antigen loading methods, the use of different immunomonitoring methods and non-objective clinical criteria and the overall study design (132). In most of the trials, a fraction of patients exhibited immune responses against the vaccinating antigen, but durable clinical responses were exceedingly rare. However, these studies have generally be pursued in patients with late stage cancer with poor prognosis and the patients probably suffer from immunosuppression as a results of a large tumor burden and prior radiation therapy or chemotherapy (140). So, at the moment the single observation that can be made is that DC vaccination is safe and well tolerated, as no or mild adverse effects have been reported only in a small number of patients (137).

So far, the published studies highlight some critical aspects that have to be considered for the future design of DCs-based vaccination strategies. Among these, the necessity to use properly activated DCs by the combination of multiple maturation stimuli and the use of antigen loading approaches that favor the generation of both peptide-MHC class I and peptide-MHC class II complexes.

Another variable possibly influencing the effectiveness of DC vaccination is the route of vaccine administration. Preliminary results from clinical trials indicate that intravenous injection primarily induces humoral immune responses, whereas intradermal and intranodal injection mediate the induction of T_H1 immunity (141). Conversely, subcutaneously injected DCs fail to migrate to the lymph node (142). This is probably due to the fact that most of the DCs sense other DCs in the vaccination mixture and fail to contact the microenviroment. Therefore, administration of DCs by multineedle devices may results in better dispersal, contact with the microenviroment and thus increased DCs migration (137).

Finally, the boosting frequency may affect the efficacy of DC vaccination. Current protocols vary among weekly, biweekly and monthly intervals. However, recent mouse studies suggest that more frequent vaccinations are superior, especially in generating a long-lasting response (137).

1.5.4 Future perspectives: *in vivo* dendritic cell targeting

The optimization of antigens delivery to dendritic cells is a crucial factor to improve the efficiency of current DCs-based vaccination protocols.

The discovery that the uptake of extracellular material through receptor mediated endocytosis greatly increases the efficiency of antigen capture, allowing DCs to present antigens found at picomolar and nanomolar concentration, led to the hypothesis that targeted delivery of antigens to dendritic cell surface molecules might increase T cell mediated immune responses. Indeed, uptake of immune complexes through Fcγ receptors results in DCs maturation and antigen cross-presentation to T cells (15). Moreover, immunization of mice with tumor antigen in the form of immune complexes was shown to induce antigen specific T cells responses and tumor protection (143).

The identification of receptors that are more or less specifically expressed by DCs and the availability of antibodies that are directed against these receptors or the knowledge of their natural ligands, led to the development of vaccination strategies based on targeting antigens to DCs in vivo. The major advantage is the relative easiness of large-scale production of GMP-grade vaccines and the opportunity to target natural DCs subsets leading to the activation of the cells within their natural environment.

So far, these targeting studies have revealed that the efficacy of in vivo DC vaccination depends on various factors including the expression pattern and biological properties of the specific receptor and the maturation or activation status of the DCs (140).

Initial studies in mouse models, identified the C-type lectin family receptor DEC205 as the ideal candidate to target DCs in vivo (85, 86). DEC205 was found to be highly expressed by DCs within the T cells area of lymphoid tissues and subcutaneous injection of the anti-DEC205 mAb NLDC145 was shown to specifically stain $CD11c^{+}MHCII^{+}$ DCs in lymphoid tissues (85). Further studies then demonstrated that in vivo delivery of various antigens coupled to the anti-DEC205 Ab greatly increased the efficiency of antigen presentation on both MHC class I and class II molecules (85, 86, 144), so that targeting of activated DCs via DEC205 resulted in improved T cells vaccination and led to the induction of an anti-tumoral response (145, 146).

Apart DEC205, various members of the CLRs family such as CIRE and FIRE, dectin-1 and DCIR-2 were shown to increase the efficiency of antigen presentation and induce Ab response in mouse models of DCs targeting (127-129). Of note, the type of response was qualitatively different from that triggered by DEC205, providing evidence that in vivo targeting of various receptors on different DCs subsets has different immunological outcomes both in terms of type of T cells response elicited and the requirement of additional adjuvant stimulation.

Other studies performed in mice and focusing on different classes of endocytic receptors such as scavenger receptors (Lox-1) (27), TNF-receptor superfamily (CD40) (147) and integrins (CD11c) (148) further proved that antibody mediated targeting of APCs is a promising strategy to elicit Ag-specific immune responses and induce protection in various tumor models. Moreover, not only the antibodies directed against specific endocytic receptors, but also the natural receptor ligands can be engineered to carry model antigens and thus be employed in DCs targeting strategies. Specifically, fusion of antigens to TLR2 (149) or TLR4 (150) ligands, or recombinant proteins encoding chemokines (MIP3α/CCL20) fused to established tumor antigens (151, 152) were shown to be effective in inducing antigen-specific $CD8^+$ T cells responses and elicited protective anti-tumor immunity.

Despite basic similarities between mouse and human DCs, it remains to be assessed whether the human homologues of the previously described receptors do play similar roles in vivo. Moreover, it remains difficult to directly compare human DC populations with mouse DC subtypes owing to differences in cell surface marker expression and to the fact that only few studies have analyzed human DC populations freshly isolated from tissues (140).

The DCs targeting approaches that are most likely to enter the clinic in the near future target DC-SIGN/CD209, DEC205/CD205 and the mannose receptor/CD206 (140). Although some reports have compared the distribution of these CLRs in different human tissues, it remains unclear whether DC-SIGN is the most DC-specific receptor or whether CD205 is unique in its distribution in the T cell area of lymphoid organs (153, 154). Indeed, several studies reported the effective induction of tumor specific antigen T cells responses by targeting human DCs via DC-SIGN and mannose receptor (155-157). Interestingly, a recent work by He and colleagues extended these in vitro observations to an animal model of transgenic mice expressing the human MR/CD206 (hMR Tg). Immunization of Tg mice with a recombinant anti-hMR mAb B11 fused to OVA, resulted in the adjuvant-dependent induction of effector T cells and antibody response able to protect the mice in tumor graft experiments (158).

In a comparative study performed on human DCs, targeting CD205 was shown to be superior to DC-SIGN and to CD206 in inducing proliferation and IFNγ production from antigen specific $CD8^+$ T cells, supporting the hypothesis that CD205 receptor better allows access to the cross-presentation pathway (159). However, the identification of the most successful targeting candidate will indeed require a clear picture of the distribution of these receptor in various lymphoid organs and a better understanding of the in vivo functions of the different DCs types.

Table III. Pros and cons of *in vivo* targeting versus *ex vivo* loading

	***In vivo* targeting**	***Ex vivo* loading**
Pros	Off the shelf use : • One product • Lower costs at large-scale production • One specialized GMP (good manufacturing practice) manufacturer • One procedure for product control • Equal product quality among clinical centres • Accessible to a large number of patients • Clinical interventions limited to vaccinations Optimal antigen delivery within the natural environment: • Antigens can be targeted to multiple DC subsets by targeting multiple receptors • DCs are reached and activated within the natural environment and at multiple sites	Highly controlled maturation and activation: • DCs can be properly stimulated *ex vivo* and maturation status is checked before administration High specificity: • Only the *ex vivo* cultured DCs are reached
Cons	Poor control of maturation and activation: • DCs activated and matured *in vivo*, stimuli need to be administered systemically or incorporated into the targeting vector Limited specificity: • Most receptors are not specific for a single cell type	Tailor made: • Labour-intensive procedure for each individual patient • High costs, mainly independent of the number of procedures • Multiple procedures for product control at different sites • Product quality differs per production site, procedure and patient • Accessible to a limited number of patients • Requires cytapheresis Limitations to DC subsets and *in vivo* distribution: • Limited to DC subsets that can be isolated in sufficient numbers or cultured *in vitro* • Poor distribution of DCs injected at high concentrations at specific sites

2 MATERIALS AND METHODS

Mice- Female C57BL/6 (B6) mice (6-7 weeks old) were purchased from Harlan (Milan, Italy). OVA specific, TCR transgenic OT-I and OT-II mice were purchased from the Jackson Laboratories. CD45.1 congenic C57BL/6 were bred to OT-I mice to obtain OT-I/CD45.1. Mice were housed at the ICGEB animal house.

Cells- DCs were isolated from single cell suspensions of lymph nodes or spleen treated with 400U/ml collagenase D (Roche) for 30 min followed by enrichment (depletion of B, T, NK) or positive selection by $CD11c^+$ beads (Miltenyi Biotec).
Langherhans and dermal DCs were isolated after washing C57Bl/6 ears once in 96% ethanol and three times in PBS. Dorsal and ventral part of the ears were separated and digested in a solution of PBS, 3% FCS, 0.5% Tripsin, for 45 min at 37°C. Epidermal and dermal layers were separated and cultured o/n in 24-well plates (one ear per well) in complete RPMI medium. Layers were discard and cells were recovered from supernatant.
BMDCs were differentiated from BM precursors obtained from the femur of C57Bl/6 mice using either Fms-like tyrosine kinase 3 ligand (Flt3L) or GM-CSF. DCs were used at day 10 and purity of $CD11c^+$ was higher than 90%.
OT-II and OT-I cells were isolated from total lymph nodes suspension either by positive or negative selection using MACS® isolation kit.

Cloning of 2.4G2 and DEC205 antibodies- RNA was prepared from 2.4G2 and NLDC-145 hybridoma cells (~$5x10^6$) using the RNeasy Mini Kit (Qiagen). Upon standard RT-PCR reaction, the DNA fragment encoding for variable regions of rat anti-DEC-205 and anti-2.4G2 IgG antibodies were amplified using degenerated primers: mVHB and mCHγ for the VH region; and mCk2 and mVkB2 for the VL region. Upon cloning in *pUC19* vector and sequencing, VL and VH regions were re-amplified using VL and VH families specific primers containing restriction enzyme sites (XhoI/NheI and BssHII/SalI for VH and VL, respectively) to allow subsequent cloning in *pDAN3* phagemid vector.
Primers used:

mVHB: 5' aggt$^{ca}_{gc}a^{a}_{g}$ctgcag$^{c}_{g}$agtc$^{a}_{t}$gg 3'

mCHγ: 5' ggccagtggatagac 3'

mCk_2: 5' tggatacagttggtgcagc 3'

$mVkB_2$: 5' gatattgtgatgacccagtctcca 3'

BssHII-VL $_{2.4G2}$: 5' tatgcgcgcatgccgatgttcagatgacccagtct 3'
SalI-$VL_{2.4G2}$: 5' ataggtcgacccttttgatttccagcttggt 3'
XhoI-$VL_{2.4G2}$: 5' tatctcgagcaaaggtgaggtgcagctggtggagtcg 3'
NheI-$VH_{2.4G2}$: 5' tatgctagctccggaggagacagtgactgaaact 3'

BssHII-VL_{DEC205}: 5' tatgcgcgcatgccgatattgtgatgacccagtct 3'
SalI-VL_{DEC205}: 5' ataggtcgacccttttcaattccagcttggt 3'
XhoI-VL_{DEC205}: 5' tatctcgagcaaaggtgaggtgaaactgttggaat 3'
NheI-VH_{DEC205}: 5' tatgctagctccggaggagactgtgaccatgact 3'

Cloning of recombinant scFv-OVA$_{257-264}$ in pDAN3 bacterial vector- The *pDAN3* phagemid vector was modified by insertion of a linker between the sequences coding for SV5 and H6 tag, containing the restriction enzyme site AgeI and NsiI. An oligonucleotide containing the sequence coding for OVA class I peptide was then subcloned in the modified *pDAN3* vector.
Oligo used:
Linker AgeI-NsiI UP:
5' ctagcggcaaaccaatcccaaacccactgctgggcctggataccggtatctttatgcatcccaccatcaccatcactaggc 3'
Linker AgeI-NsiI DOWN:
5' ggccgcctagtgatggtgatggtgatgcataaagataccggtatccaggcccagcagtgggtttgggattggtttgccg 3'
OVA UP: 5' ccggtcttgagcagcttgagagtataatcaactttgaaaaactgactgaatggaccagtgatgca 3'
OVA DOWN: 5' tcactggtccattcagtcagtttttcaaagttgattatactctcaagctgctcaaga 3'

Cloning of recombinant scFv-OVA$_{257-264}$ in pcDNA3 vector- The *pDAN3-DEC205-OVA$_{257-264}$* vector was modified by insertion of an oligo containing the restriction enzyme site ApaLI upstream the sequence coding for VL_{DEC205}. The modified *pDAN3* vector was then digested ApaLI/NotI. The fragment containing the entire sequence coding for DEC205 antibody fused to $OVA_{257-264}$ was subcloned in *pUT-SecIg* vector downstream of the signal sequence of human IgG1. *pUT-SecIg-DEC205-OVA* was finally digested HindIII/NotI and the fragment containing the scFv-OVA fusion was subcloned in *pcDNA3* (Invitrogen).
Oligo sequence:
BAT UP: 5' cgcgcgtgcactcggatattgtgctgacc 3'
BAT DOWN: 5' gggtcagcacaatatccgagtgcacg 3'
A similar procedure was used for the cloning of *pcDNA3-2.4G2-OVA$_{257-264}$* construct.

Cloning of the scFvs isolated from the screening into pcDNA3 vector- The DNA sequence coding for the selected scFvs was isolated by digestion of *pDAN3* vector with the restriction enzymes BssHII/NheI and subsequently cloned in the *pcDNA3secIless-mEshortDcyto* vector (available in the lab). The new scFv containing vector was then digested HindIII/BspEI and the isolated scFv subcloned into *pcDNA3-OVA*$_{257\text{-}264}$ vector to produce the final scFv-OVA$_{257\text{-}264}$ fusion. The sequence coding for scFv2E5-hγCH3 protein was obtained by digestion of *pcDNA3-scFv2E5-OVA*$_{257\text{-}264}$ vector with the restriction enzymes BspEI/NotI and subsequent cloning of the DNA fragment coding for third constant domain of human IgG1 (γ1-CH3).

Cloning of the scFv/SIP full-length OVA fusion constructs- The *pcDNA3Fc-OVA* vector containing the sequence coding for ovalbumin (available in the lab) was modified by insertion of a linker containing the restriction enzyme NheI upstream the starting codon of OVA. The modified vector was digested with the restriction enzymes HindIII/XhoI to isolate the full-length sequence of OVA, which was then subcloned into *pcDNA3* vector. The sequence coding for the various scFvs was isolated by digestion with the enzymes HindIII/NheI and then cloned upstream of OVA to generate *pcDNA3-scFv-OVA* vector.
Oligo used:
Linker NheI UP: 5' agcttaatgctatgctagcggcggtac 3'
Linker NheI DOWN: 5' cgccgctagcatagcatta 3'

To produce *pcDNA3-SIP-OVA* constructs the *pcDNA3Fc-OVA* vector was modified by insertion of a linker containing the restriction enzyme BamHI upstream the starting codon of OVA. The modified vector was digested with the restriction enzymes BamHI/XhoI to isolate the full-length sequence of OVA, which was then subcloned into *pcDNA3*-BCL1CH3ΔStop vector (available in the lab) to generate *pcDNA3-SIP-OVA* vector.
Oligo used:
Linker BamHI UP: 5' agcttaatgctatggatccggcggtac 3'
Linker BamHI DOWN: 5' cgccggatccatagcatta 3'

Rescuing phagemid libraries- The inculum size should be 10x library size in number of bacteria at the start, but should not exceed 0.05 O.D.600nm. The inoculum, generally 30μl of concentrated TG1 bacteria, was transferred into 100ml of 2YT, 100μg/ml ampicillin, 2% glucose and grown with shaking (270rpm) for 1.5-2.5h at 37°C, to an O.D.600nm of 0.5. Bacteria were next infected with an appropriate amount of helper phage (20:1 phage/bacteria ratio) for 30 at 37°C with occasional

agitation. After the infection event, cells were pelleted for 10min at 4000 rpm and the supernatant removed. The bacterial pellet was resuspended in 100ml of 2YT, 100μg/ml ampicillin, 25μg/ml kanamycin and further incubated with shaking (270rpm) o/n at 30°C. The culture was spinned at 4000rpm to pellet the bacteria. Phage in the supernatant were precipitated by adding 1/5 of the volume of PEG solution (20% Polyethylene glycol 6000, 2.5M NaCl) and left on ice for 1h. Phages were pelleted by spinning for 15 min, 4000rpm at 4°C. The pellet was finally resuspended in 1 ml of PBS and stored at 4°C. The standard yield is about 2-10 x 10^{12} from a 25 ml of culture.

Phage Display Screening- Phages were prepared as previously described. Whole cell panning was performed by incubating 10^{13} cfu phage, pre-blocked in PBS/5% Milk, and 6 $\times 10^{6}$ DCs, pre-blocked in PBS/1% Milk, for 2h at 4°C. After 2 washings in ice cold PBS/1% Milk followed by 2 washings in PBS, cells were resuspended in IMDM and incubated at 37°C for 30 min to allow phage internalization. Extracellular phages were inactivated with Subtilisin type VIII (3mg/ml) diluted in Buffer B (HBSS(-), 20mM Tris,2mM EDTA, pH8) for 30 min. Cells were washed and lysed in 1ml of triethylamine (100mM) for 8 min at RT. The lysate was neutralized with 0.5ml of Tris-HCl 1M, pH7.4. Internalized phages were recovered by infecting exponentially growing *E.Coli* (TG1) cells. Samples were then processed for quantitation of phage titer.

BstN1 fingerprinting- Fingerprinting were carried out on variable regions amplified from individual clones using the primers:

Fd seq= 5' GAATTTTCTGTATGAGG 3'

M13rev= 5' AGCGGATAACAATTTCACACA 3'

After amplification, each sample was digested for 2-3h at 60°C by incubation with a mix containing BstNI restriction enzyme. Digested samples were loaded on 2% Agarose TBE gel.

FACS and Immunofluorescence Analysis Using Phages- Phages (10^{12} cfu) were blocked in PBS/5%Milk and allowed to bind to BMDCs 1 h at 4 °C. For FACS analysis, phage binding was detected by anti-M13 IgG mAb (Amersham Pharmacia) and FITC-conjugated anti-mouse IgG (KPL). For confocal analysis, cells were plated on poly-L-lysine treated coverslips and incubated either at 4°C or 37°C for 30 min. After PBS washings, cells were fixed in 4% PFA and permeabilized with PBS/0.1% Triton. The coverslips were saturated with PBS/1%BSA (30 min at RT). Phage particles were detected with rabbit anti-fD Bacteriophage antibody (Sigma) and FITC-conjugated swine anti-rabbit IgG (DAKO). Samples were analyzed by confocal microscopy (Axiovert; Carl Zeiss).

Biotinylation of membrane proteins and Receptor Immunoprecipitation- 10^7 BMDCs, WEHI or BO9 T cells were surface-biotinylated (Amersham ECL protein biotinylation system) following manufacturer directions. Briefly, cells were washed in PBS and resuspended in 1ml of biotinylation buffer. One µl of biotinylation reagent was next added and cells were incubated for 30 min at 4°C on orbital shaker. After being washed with cold PBS, cells were lysed in TNN buffer (50mM Tris, pH8, 250mM NaCl, 0,5% NP-40) containing protease inhibitors. Cell debrids were spun out (12000 rpm, 15 min) and supernatants were precleared with anti-SV5tag mAb (160) (10µg), and 200µl of 50% protein A-Sepharose CL-4B beads (Amersham Biosciences) for 2 h at 4 °C. Biotinylated proteins were IP with scFv-2E5 or scFv-ctrl (20µg/ml) for 2 h at 4 °C, followed by addition of 5µg of anti-SV5tag mAb and 50µl of 50% protein A-Sepharose CL-4B. Beads were washed three times in ice-cold TNN buffer before SDS-PAGE analysis. Upon transfer on PVDF membrane (Millipore), presence of biotinylated protein was revealed by HRP conjugated-streptavidin (Amersham).

In-gel digestion and Mass Spectrometry- In-gel digestion was performed essentially as described in (161). Briefly, protein gel pieces were excised destained and tryptically digested with porcine trypsin. After incubation overnight (12–16 h), gel pieces were centrifuged at 14,000 rpm for 5 min, then extracted once with 5% formic acid/50% acetonitrile, dried by vacuum centrifugation, and stored at —20°C until ready for analysis. Mass spectrometry data were acquired working in reflectron mode with a 4800 MALDI TOF/TOF. A 0.5 uL aliquot of the peptide solution was mixed with 0.5 uL a-cyano-4- hydroxycinnamic acid matrix (Sigma, St. Louis, MO, USA) and subjected to MALDI analysis. MS data were subjected to database searching using Mascot (Matrix Science, UK) against Swissprot. Up to one missed tryptic cleavage and optional methionine oxidation and carbamidomethylation was considered. Mass accuracy was limited to 80 ppm.

Cloning of murine CD36 cDNA- Total RNA was prepared from $WEHI^{231}$ B cells (~$5x10^6$) using the RNeasy Mini Kit (Qiagen).. Full-length CD36 cDNA was produced using the specific primers:
CD36 HindIII= 5’ AAAGCTTGCACGGGAGAATGGGCTGT 3’,
CD36 XhoI= 5’ CCTCGAGACTACTTATTTTCCATTCTTGGATTT 3’.
CD36 cDNA was subcloned *pcDNA3* vector (Invitrogen) using HindIII and XhoI as restriction enzyme sites and then transiently expressed in 293T cells after calcium-phosphate transfection.

HEK293T cell transient transfection with calcium phosphate- Cells were plated in 6-well Petri dishes. Fresh medium was added 4h before transfection. 5µg of plasmidic DNA was resuspended in 50µl of 0.1X TE (10mM Tris, 1mM EDTA). Mix A was prepared by addition of 169µl of deionized water, 5µl of $CaCl_2$ 2M and drop by drop plasmidic DNA and 26µl of $CaCl_2$ 2M. The mix A was added drop by drop to 250µl of 2X HBS (280mM NaCl, 10mM KCl, 1.5mM NaH_2PO_4, 12mM dextrose and 50mM Hepes). Total mix was added to cells drop by drop. Cells were incubated o/n at 37°C and then medium was replaced. 2 days later, cell and/or supernatants were collected and analyzed.

Expression of scFv-OVA recombinant proteins in bacteria- *pDAN3-scFv-OVA* plasmids were used to transform the E.coli strain HB2151. Bacteria were grown in 2YT ampicillin medium at 37°C to 0.7 O.D.600nm Upon induction with 0.5mM IPTG, bacteria were incubated for 5h at 30°C. The scFv periplasmic fraction was prepared by osmotic shock. Pelleted bacteria were resuspended in PPB buffer (200mg/ml sucrose, 1mM EDTA, 30mM TrisHCl pH 8) and left in ice for 20 min. After centrifugation the supernatant was collected and the cells resuspended in 5mM $MgSO_4$ buffer for 20 min. The solution was centrifuged and both supernatants pooled and dialyzed against PBS.

PolyH6 tag protein purification-Recombinant scFv-OVA proteins were purified by affinity chromatography using polyH_6 tag purification system (Ni-NTA His Bind Resin, Novagen) following manufacturer procedure. Briefly, scFv-OVA proteins, either obtained from periplasmic extract of HB2151 E,coli cells or from supernatants of 293T transfected cells, were dialized o/n against a solution of 30mM Tris/HCl, 300mM NaCl, pH8. PBS washed Ni-NTA resin was next added to the dialized proteins and incubated for 1,5h at 4°C on orbital shaker. After binding, samples were centrifuged 2 min at 3000 rpm and the supernatant discard. Samples were washed once with a solution of 30mM Tris/HCl; 100mM NaCl, 20mM Imidazol, pH8. Finally, proteins were eluted from the resin by incubation with a solution of 30mM Tris/HCl; 100mM NaCl, 250mM Imidazol, pH8. Eluted proteins were then dialized o/n against PBS.

SIP-CD36-OVA Internalization Assay- DCs were incubated with SIP-CD36-OVA for 1h at 4°C, washed and then either kept at 4°C or incubated at 37°C for 15, 30 or 60 min. For FACS analysis: the level of SIP-CD36-OVA remaining at the cell surface was detected using rabbit anti-OVA and FITC-conjugated goat anti-rabbit IgG (KPL). The median fluorescent values of OVA^+ cells was determined and used to calculate the % remaining $scFv^{CD36}$-OVA at the surface, with 1 h 4°C incubated cells taken as 100%. For confocal analysis: after binding at 4°C for 1h, cells were washed,

plated on poly-L-lysine treated coverslips and incubated either at 4°C or 37°C for 30 min. Membranes were counterstained with 5μg/ml cholera toxin subunit B-FITC washed, fixed in 4% PFA and permeabilized with 0.02% saponin. ScFvCD36-OVA was detected with Alexa Fluor 594-$F(ab')_2$ goat anti-human IgG (Molecular Probes).

In vitro T cell Proliferation Assay - DCs were pulsed with graded doses of scFv/SIP-OVA proteins, soluble OVA (Worthington biochemical Corp., Lakewood, NJ, USA) or $OVA_{257-263}$ peptide for 4h at 37°C. After washing, 1×10^5 OT-I or OT-II cells were co-cultured with Ag pulsed DCs in round bottom 96-well plate (1:5 DC/T cell ratio). After 2d, [^{3}H]thymidine (1μCi; Amersham Biosciences) was added for 18 h, and incorporation was measured by liquid scintillation counting after collection of cells on a glass fibre filter with and automatic cell harvester (Tomtec, Calif., USA). Alternatively, CFSE labeled (1μM) OT-I cells were added to pulsed DCs (1:2 DC/T cell ratio) and proliferation evaluated 3 d later as CFSE dilution.

Adoptive Transfer and T Cell Proliferation Responses- OT-I or OT-II cells were labeled with 7μM CFSE according to manufacturer instructions. C57BL/6 mice were i.v injected with 1-2 $x10^6$ OT-I or OT-II cells followed by injection in the footpad of scFv/SIP-OVA proteins. 3 d later, lymph nodes cell suspensions were stained for CD8 or CD4 respectively and CFSE dilution evaluated by FACS. To evaluate long term OT-I persistance, $2x10^6$ OT-I/CD45.1 were injected i.v. into recipient CD45.2 host, immunized with scFv-OVA proteins. 12 d later, spleen and lymph node cells suspension were stained with anti-CD8 and anti-CD45.1 to evaluate OT-I cells number.

In Vivo Citotoxicity Assay- C57Bl/6 mice were s.c immunized with 0,1μg of recombinant SIP-CD36-OVA or SIP-Ctrl-OVA proteins. After 12 days, mice were i.v injected with target spleenocytes, prepared as follow: naïve syngenic spleenocytes were pulsed with $OVA_{257-264}$ peptide (5μM) (1h at 37°C), washed and labeled with high concentration (5μM) of CFSE. The non-pulsed control spleenocytes were labeled with low concentration (0,5μM) CFSE. Both $CFSE^{high}$ and $CFSE^{low}$-labeled cells were mixed at 1:1 ratio (7×10^6 cells of each population) and injected i.v. into mice. The number of CFSE positive cells remaining in the spleen and lymph nodes after 15h was determined by FACS.

Intracellular IFNγ staining- Intracellular IFNγ production by primed $CD8^+$ T cells was evaluated using bulk splenocytes, lymph node cells or blood cells cultured for 5h with 1μM of SIINFEKL peptide or medium alone in the presence of brefeldin A (7μg/ml)(BD Biosciences). Cells were stained with anti-mouse CD8 Ab and anti-CD45.1 Ab for 25 min at 4°C. After fixation with 2% PFA, cells were stained for intracellular IFNγ in PermWash solution (BD Biosciences) for 30 min at 4 °C.

Anti-OVA Ab ELISA- 96-well Maxisorp ELISA plates (Nunc) were coated o/n with 3μg/ml OVA. Plates were washed and blocked in PBS/1%BSA/0.1%Tween20. Sera collected from the clotted blood of immunized mice were serially diluted 1/100 to 1/12,800 in blocking solution and incubated 1h at RT. Plates were washed and Ab binding was detected using HRP-conjugated anti mouse IgG antibody (KPL) followed by tetramethylbenzidine peroxidase substrate (Sigma).

Tumor rejection experiments- Mice were injected in the footpad on day 0 and 7 with 0,5μg of SIP-CD36-OVA (6 mice), SIP-Ctrl-OVA (6 mice) or left untreated (4 mice). 7 d after the second immunization, each mouse was challenged s.c with $2x10^5$ EG7-OVA cells (ATCC: CRL-2113). Tumor size was measured with a caliper ruler 14, 21 and 28 days after the challenge. Average size is expressed in cubic millimeters using the formula $V=(length \times width^2)/2$. Significance of protection was evaluated using a two-tailed Student *t* test.

3 RESULTS

3.1 Screening of a scFv phage library on mouse Dendritic Cells

3.1.1 The Phage display technology

The phage display technology allows the presentation of large combinatorial libraries of peptide or proteins on the surface of filamentous phage. The ligand is displayed on the surface of the phage by fusing its gene to the phage genome, thus providing a physical linkage between the phenotype and the genotype. In case of antibodies libraries, the displayed proteins are generally fused to the N-terminus of the phage coat protein pIII.

To reduce the interference of the foreign moiety with phage assembly and infectivity, a phagemid display system was developed. A phagemid is a plasmid that encodes for the recombinant pIII-fusion protein and contains the packaging signal of filamentous phage. It produces large amount of the recombinant displayed proteins, but it is unable to assemble progeny phage, unless the bacteria carrying the phagemid also contain a helper phage, which supplies the other protein required for virion assembly.

Antibody phage display is accomplished by fusing the coding sequence of the antibody variable (V) regions to the pIII phage protein. A single-chain variable fragment (scFv) is a single polypeptide chain that contains one light chain variable region (VL) joined to one heavy chain variable region (VH) by a flexible linker. The linker, usually 15-18aa long, allows the association of the VL and VH to form the antigen-binding site. Functional scFv are expressed on the surface of the infective particles while the encoding genes reside within the phage. The linkage between antibody genotype and phenotype allows the enrichment of antigen specific phage antibodies.

Antibody library are screened and enriched for antigen-specific clones by a technique known as bio-panning, in which scFv-phage particles are incubated with the antigen of interest. When the target antigen is difficult to express and purify (i.e. membrane proteins) or it is still uncharacterized (i.e. novel cell markers), the panning can be performed directly on cell surface or tissue sections. Whole cell panning is useful for the identification of antigen that are only expressed on specific cell subsets (162) and it has the advantage of focussing on accessible and biologically relevant epitopes (i.e. surface molecule that trigger internalization of the bound ligand) (163-166).

3.1.2 Setting up of the screening procedure

In this work, we took advantage of the phage display technology to isolate antibodies directed against DCs surface molecules. We decided to apply a whole cell panning procedure to specifically select antibodies able to trigger the internalization of the bound DCs receptor.

To take advantage of the phage display technology in targeting endocytic receptors, phage vectors should be internalized upon receptor binding, capable of being rescued from cells and amplified for subsequent rounds of selection. To set up the conditions to selectively recover internalized phages when mixed to a pool of not internalizing ones, we initially engineered two phagemid vectors displaying antibody fragments (scFv) that recognize known DCs endocytic receptors.

We chose two antibodies directed against well characterized families of endocytic receptors: the Fc gamma receptor family and the C-type lectin family of receptors. More specifically, we selected the mAb 2.4G2 that recognizes mouse FcγRIIb and FcγRIII (167, 168) and the mAb NLDC-145 directed against the receptor DEC-205 (169).

The variable regions of 2.4G2 and NLDC-145 were amplified and engineered in the scFv format. Both scFvs were subsequently subcloned in the phagemid vector *pDAN3* to allow the display on phage particles as fusion with the pIII coat protein.

pDAN3 vector also enables the production of the scFv as soluble protein when expressed in a "nonsuppressor" strain of *E.Coli* cells (i.e.HB2151). These cells recognize an amber stop codon inserted between the scFv gene and the gene coding for pIII, thus allowing the expression of the scFv without the pIII protein (Fig.1). Such soluble protein is secreted into the periplasmic space of the bacteria and can be isolated by means of a His_6 tag fused to the expressed scFv (170).

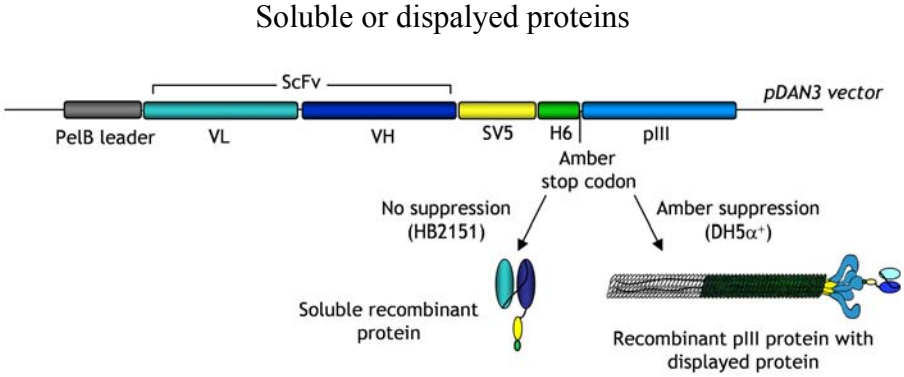


Figure 1. Schematic representation of the phagemid vector *pDAN3*. Single-chain antibody fragments can be either displayed on the surface of phage particles as fusion with the coat protein pIII, or produces as soluble proteins when expressed in "nonsuppressor" E.coli strains.

Binding and Internalization of scFv2.4G2 and scFvDEC205 by BM-DCs

Phage particle displaying scFv2.4G2, scFvDEC-205 or a control scFv (scFvCtrl) directed against an irrelevant Ag, were produced and quantified following standard protocols. To evaluate the efficiency of display of the fusion pIII-scFv proteins, we performed Western Blot analysis using a mAb against the SV5 tag fused to the expressed scFv. The expected size for the recombinant pIII-scFv protein is around 105kDa, while for the pIII alone is around 75kDa. As shown in Fig.7, all the pIII-scFv fusions were properly displayed on the fd phage.

The selected scFv were also expressed in the "nonsuppressor" *E.Coli* strain HB2151 as soluble proteins and subsequently purified from periplasmic extracts by means of the His_6 tag. ScFv proteins of the expected size (32kDa) were revealed by Western Blot analysis (Fig.2).

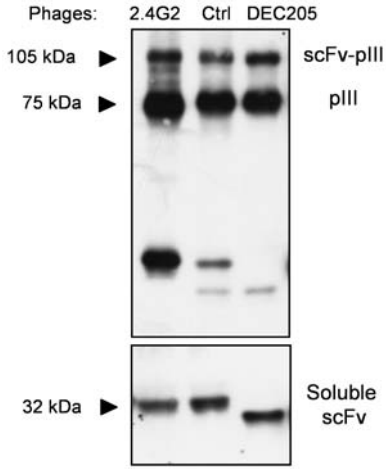


Figure 2. Production of phage particles displaying specific scFv proteins. Western blot analysis of scFv-2.4G2, scFv-DEC205 and scFv-Ctrl displayed on phage (upper panel) or produced as soluble proteins (lower panel), detected with anti-SV5 mAb.

To assess the functionality of scFv2.4G2 and scFvDEC-205, we performed a flow cytometry analysis on bone marrow derived DCs. As shown in Fig.3a and 3b, both scFv2.4G2 and scFvDEC205 displayed on fd phage retain their binding ability to FcγRs and DEC-205 receptor respectively, whereas no binding is observed with scFvCtrl-phage.

To assess whether BMDCs internalize scFv2.4G2 phagemid through receptor mediated endocytosis, we performed a series of analysis using both flow cytometry and confocal fluorescence microscopy. In a first set of experiments, BMDCs were incubated with scFv2.4G2-phage or scFvCtrl-phage at 4°C to allow binding and after extensive washing, cells were either kept at 4°C or incubated at 37°C. 30 minutes internalization time was chosen to allow the active endocytosis of the bound receptor and at the same time to prevent the inactivation of the internalized phage in the acidic cellular compartments (171). Upon incubation of DCs at 37°C, we observed a reduction in scFv2.4G2-phage staining indicating the effective internalization of the phagemid. As expected, no binding on DCs was detected with scFvCtrl-phage (Fig. 3c). To exclude the possibility that the

decrease in surface staining was due to dissociation of scFv2.4G2 from FcγR rather than specific internalization, we analyzed the cells by confocal fluorescence microscopy. Upon phage binding and internalization, cells were permeabilized and stained with the anti-fd bacteriophage Ab. Intracellular staining was detected only in cells treated with phages displaying the scFv2.4G2 and not the scFvCtrl (Fig. 3d).

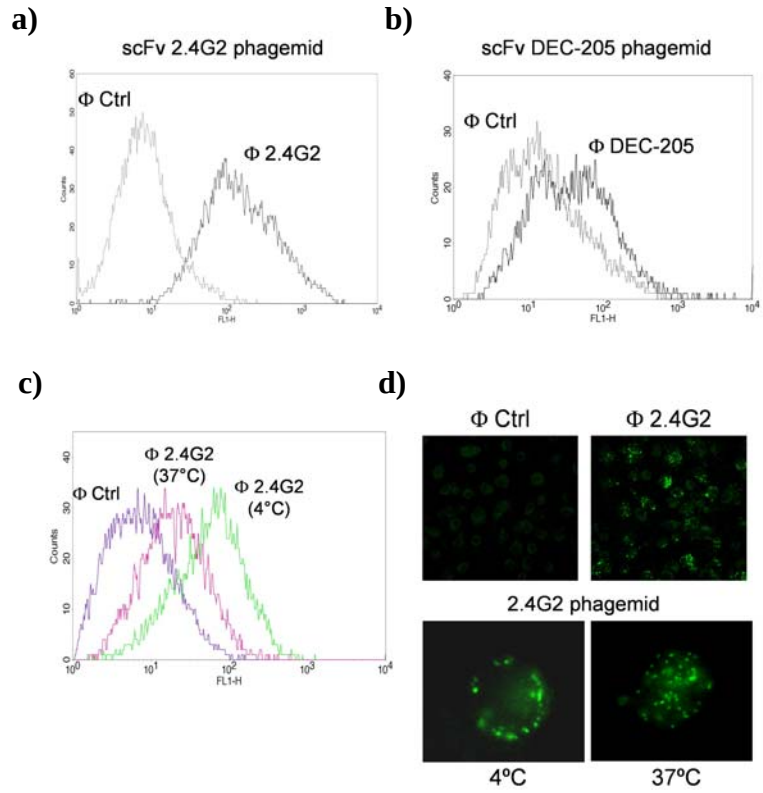


Figure 3. Functionality of scFv-2.4G2 and scFv-DEC205 phagemid. Binding of recombinant scFv-2.4G2 and scFv-DEC205 phages to BMDCs detected with an anti-phage mAb **(a-b)**; FACS **(c)** and confocal **(d)** analysis of scFv-2.4G2 phage internalization revealed with anti-phage antibodies.

Recovery of internalized scFv 2.4G2 phagemid from BMDCs lysates

Selection of internalized phages from a phage library requires the optimization of the screening in order to increase the enrichment of specifically internalized phages over non-internalized ones. Whereas nonspecifically or weakly bound phages can be removed by several PBS washings, in case of phages bound to cell surface molecules that do not undergo internalization a stronger treatment is required. At first, we tested whether washing BMDCs with a low pH glycine buffer may favor the dissociation of scFv 2.4G2-phagemid bound to the cells surface. Despite this procedure has been previously applied for the selection of internalizing antibodies from phage library (164, 172), we found that the low pH buffer severely damaged BMDCs, shown by the cell profile in FACS analysis in (Fig. 4a). In a second set of experiments, we tested the protease subtilisin to specifically inactivate extracellular phages. Subtilisin has been shown to proteolyze the

phage protein pIII, thus leaving the phage particles non-infectious (173). We evaluated subtilisin activity on scFv2.4G2 phagemid bound to BMDCs. Cells were either treated or not with a solution containing the protease for 30 min, before lysis. The phage titre was then evaluated by infection of *E.Coli* cells. Subtilisin treatment is effective since it reduced the phage titre of the treated sample at less than 2% respect to the non treated one. Viability of the cells upon subtilisin incubation was evaluated by FACS using propidium iodide to stain dead cells. We did not observe a difference in the percentage of cell death between protease treated or non treated samples (Fig. 4b).

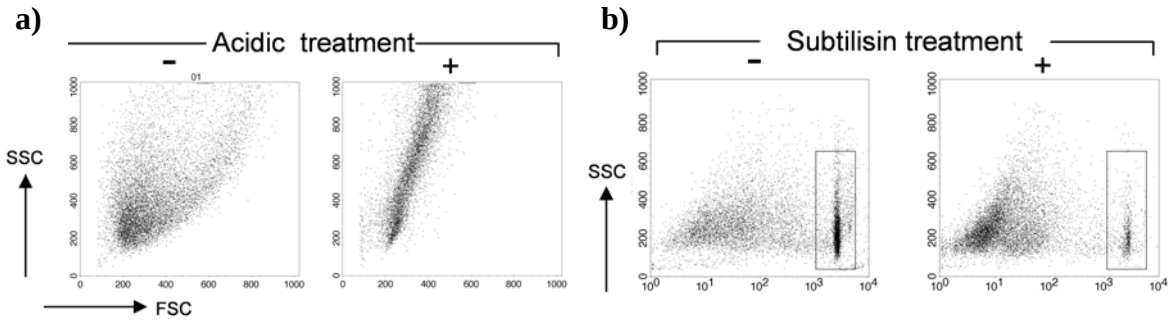


Figure 4. Effects of acidic/subtilisin treatment on BMDCs viability. FACS profile of BMDCs incubated with a low pH glycine buffer **(a)**; propidium iodide staining of BMDCs treated with subtilisin **(b)**.

Based on these preliminary results we moved to test the conditions that allow recovery of internalized scFv2.4G2 when mixed to a pool of irrelevant phages (scFvCtrl). BMDCs were incubated with a mix of scFv2.4G2 and scFvCtrl phage at 1/100 or 1/1000 ratio. Upon internalization and subtilisin treatment, cells were lysed and used to infect *E.coli* cells. To evaluate the enrichment of scFv2.4G2 against scFvCtrl phage, we performed a DNA fingerprinting analysis. DNA from single clones derived from *E.Coli* infections was subjected to PCR using a specific set of primers to amplify the scFv region. The amplified DNA was then treated with the restriction enzyme BstN1 to obtain a pattern of digested fragments that is sequence specific and thus allows to distinguish different scFv. The experiment was repeated several times and analysis of the recovered clones indicated that we could achieved a 20 to 100 fold enrichment of the scFv2.4G2 for a single round of selection (Fig. 5).

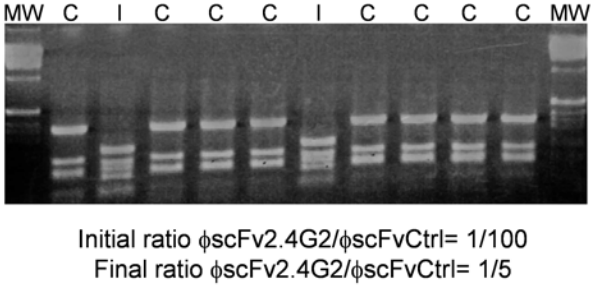


Figure 5. Enrichment of internalizing phage particles. Representative fingerprinting pattern obtained after phage internalization assay. I: scFv-2.4G2phage; C: scFv-Ctrl phage.

3.1.3 Selection of internalizing phages by panning on BM-DCs

Having defined the conditions for the internalization and the recovery of a model endocytosed phage, we next set out the screening to identify internalizing scFv phages from a highly diverse human scFv-phage library (>10^{10} independent clones) (174). The diversity of the library was confirmed by BstN1 DNA fingerprinting. More than 80% of clones contained a full length scFv and they all were found to be different (Fig. 6).

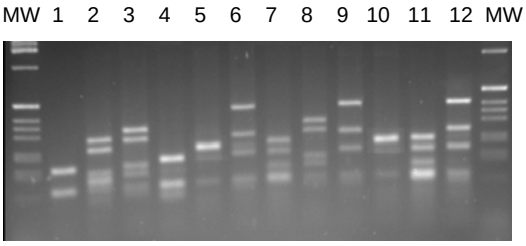


Figure 6. ScFv phage library diversity. Fingerprinting pattern of 12 randomly picked clones from the human scFv-phage library used in the screening.

We decided to carry out a first screening on in vitro differentiated BMDCs, in order to easily obtain a large amount of cells. We used immature DCs since upon maturation they have been shown to downregulate the antigen internalization capacity (9).Both DCs (10^7 cells) and the phage preparation (~10^{13}cfu) were pre-blocked with a solution of PBS and skimmed milk and were allowed to bind at 4°C for 2h. The stringency and number of the washings was increased in each round of selection to favor the enrichment of clones with higher affinity. Upon internalization, cells were treated with subtilisin, washed and finally lysed in TEA. Cell lysates were used to infect *E.coli* cells to recover and amplify phages for the next round of selection. A total of three rounds of panning were performed (Fig. 7).

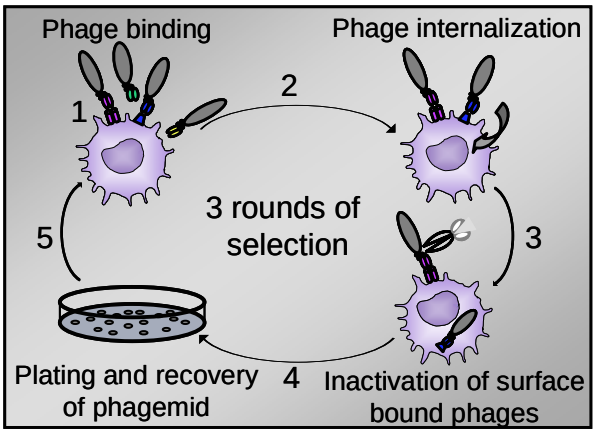


Figure 7. Schematic representation of the phage panning procedure. Phages were allowed to bind to DCs and to be internalized by incubation at 37°C. Not internalized phages were inactivated by subtilisin treatment. DCs were lysed to recover internalized phages by infection of E.Coli cells. Rescued phagemids were used for the next round of selection.

To initially evaluate the enrichment for scFv-phages recognizing DCs surface antigens, we analyzed the pool of clones recovered after each round of selection by flow cytometry. The polyclonal scFv-phage population from the third round of panning, compared to the pools of the first and second round, showed significantly increased binding on DCs indicating the presence of BMDCs specific scFv-phages (Fig. 8a). We next evaluated the binding of the three pools of phages to a different cell type: the B cell line A20. Although a slight increase in surface staining is detected, there is no significant enrichment of binders in the phage pool from the third round compared to the first, thus validating the specificity of the selection (Fig. 8b).

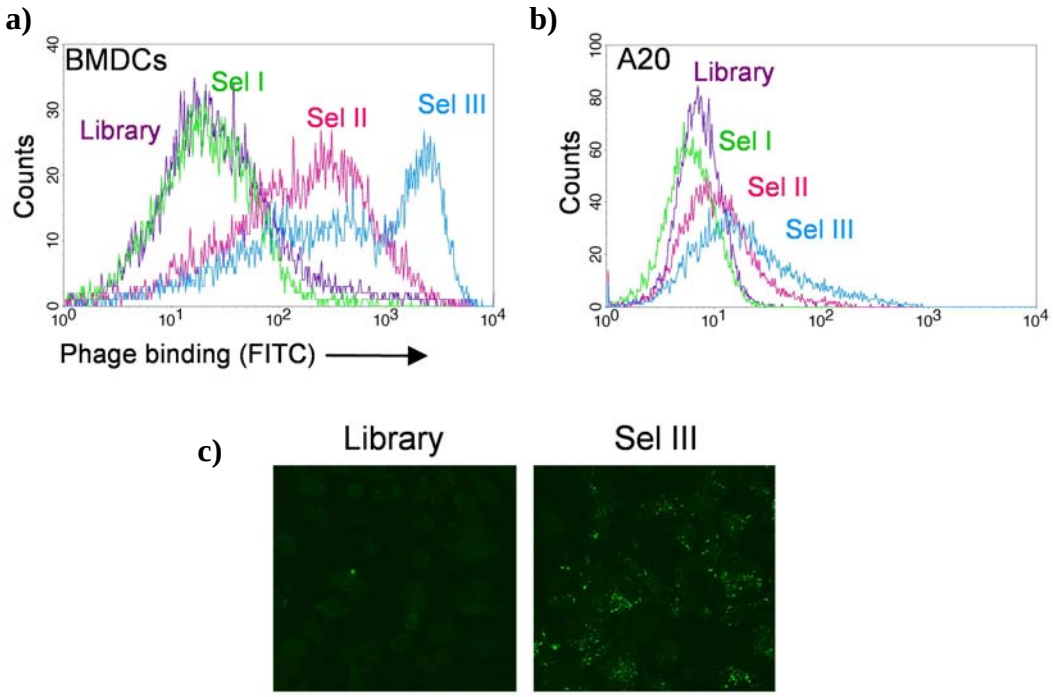


Figure 8. Evaluation of the phage panning procedure. Flow cytometry analysis on DCs **(a)** and A20 B cells **(b)** to monitor the enrichment of binders in the polyclonal phage preparations from each round of selection. **(c)** Internalization of phage particles by DCs incubated with the original library or the pool of phages from the third round of panning (sel III), using the anti-pIII Ab.

To determine whether the selected scFv-phages were internalized, we performed immunofluorescence analysis using confocal fluorescence microscopy. Intracellular staining was revealed upon incubation at 37°C of DCs with the polyclonal scFv-phage population from the third round of panning suggesting that most of the isolated ligands were targeting endocytic receptors. As expected, no staining was detected upon incubation of the cells with the un-screened scFv-phage library (Fig. 8c).

Characterization of selected scFvs

In light of the binding data from the flow cytometry, we next randomly picked 40 individual phage clones from the third round of panning and further analyzed them by DNA finger-printing and DNA sequencing. Ninety percent of the clones contained a full length scFv. We could identify 7 different fingerprint patterns with one being mostly represented: scFv-2 which recurred with a frequency of 60% (Fig. 9). The clones were sequenced and the VH and VL family obtained were assessed by screening against the VBASE database. Notably, all the clones but one (scFv-4), which contained rearrangements and was not functional, carried a VH belonging to the VH 1 family and a VL to the Vλ 1 family. The length of the CDRs H3 was rather short (ranging from 9 to 11 aa) and similar in all the clones, with differences only in the first 4-6 aa of the CDR3 region (Table IV).

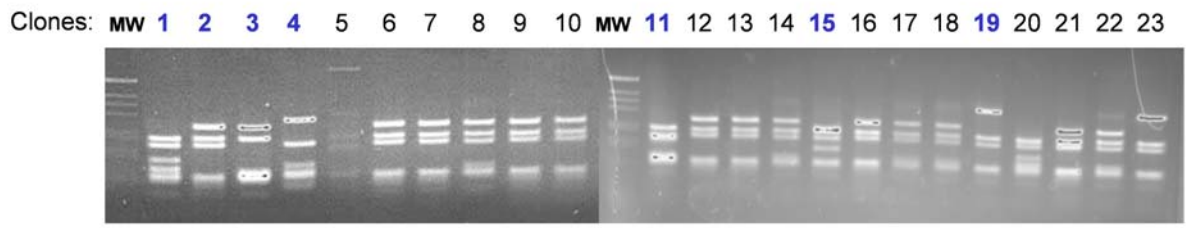


Figure 9. Diversity of the pool of scFv clones isolated after three rounds of selection. BstN1 fingerprinting of randomly picked single clones from the third round of selection. ScFv clones with a different fingerprinting profile are indicated in blu.

Table IV. Analysis of the families and CDR3 regions of clones isolated from the phage selection.

VH segments				
	VH family	VH CDR3	CDR 3 length	JH
Clone 1	VH 1	AGDTDAFDI	9	JH3a
Clone 2	VH 1	MEQQHDAFDI	10	JH3a
Clone 3	VH 1	GSYYGDAFDI	10	JH3a
Clone 11	VH 1	EVGASPDAFDI	11	JH3a
Clone 15	VH 1	GGGASDAFDI	10	JH3a
Clone 19	VH 1	SSGYYDAFDI	10	JH3a

VL segments				
	VL family	VL CDR3	CDR 3 length	JL
Clone 1	Vλ1	AWDDSLSGY	9	Jλ1
Clone 2	Vλ 1	SWDDSLSGY	9	Jλ1
Clone 3	Vλ 1	SWDDSLTGY	9	Jλ1
Clone 11	Vλ 1	TWDDSLDGY	9	Jλ1
Clone 15	Vλ 1	AWDDSLNGY	9	Jλ1
Clone 19	Vλ 1	VWDDDLNGY	9	Jλ1

We next assessed the quality of the clones at the phenotypic level by Western Blot analysis. As shown in Fig. 10a, most the clones were well displayed on the phagemid and were expressed as soluble recombinant scFv proteins. FACS and immunofluorescence analysis on BMDCs were next performed to evaluate the binding and the internalization of each clone. All the displayed scFvs isolated from the screening were able to bind to DCs and showed a similar staining profile, moreover, they were efficiently internalized (Fig. 10b-c). We have therefore provided the proof of principle that internalizing antibodies can be selected by direct panning on DCs.

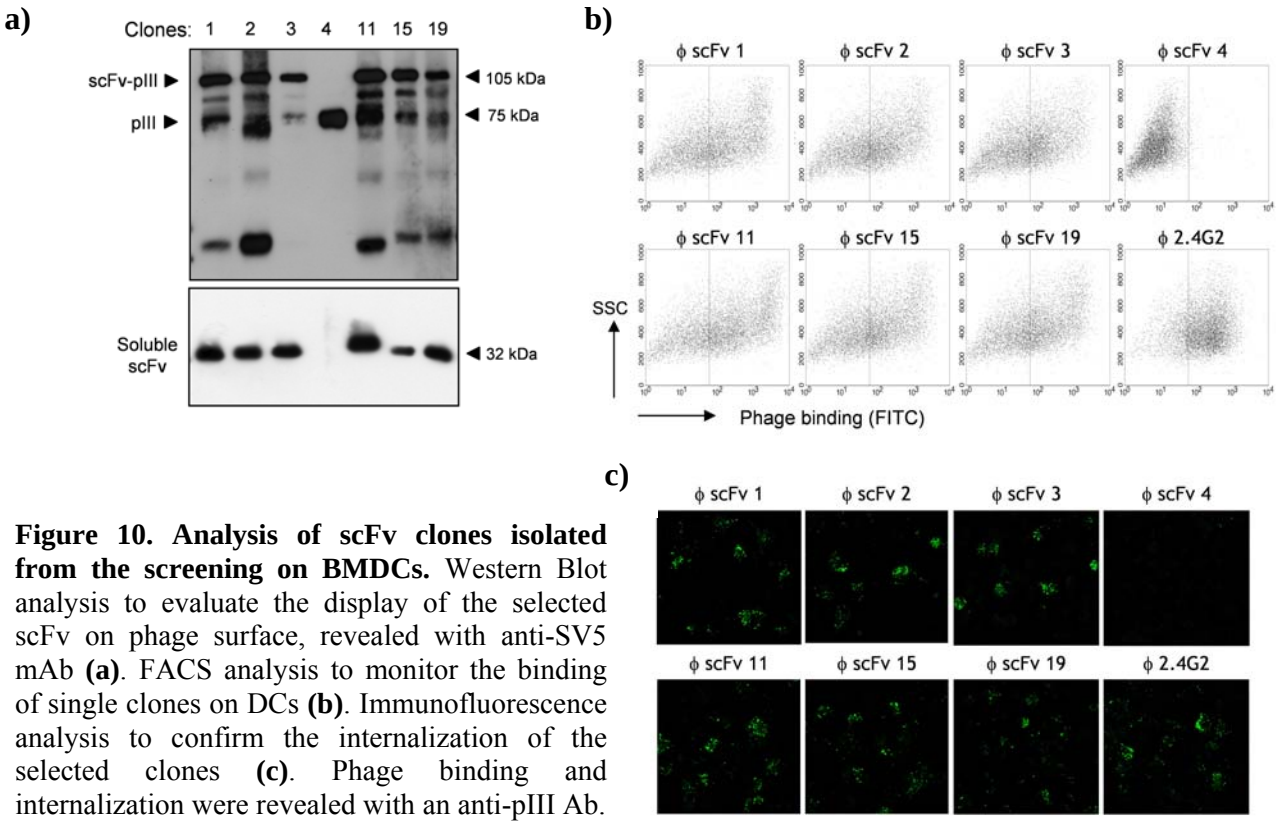


Figure 10. Analysis of scFv clones isolated from the screening on BMDCs. Western Blot analysis to evaluate the display of the selected scFv on phage surface, revealed with anti-SV5 mAb **(a)**. FACS analysis to monitor the binding of single clones on DCs **(b)**. Immunofluorescence analysis to confirm the internalization of the selected clones **(c)**. Phage binding and internalization were revealed with an anti-pIII Ab.

3.1.4 Selection of internalizing phages by panning on splenic DCs

DCs are a phenotypically heterogeneous population of cells both in mice and in humans. DCs generated in vitro by supplementing GM-CSF resemble monocyte-derived DCs. However, it is unclear whether they correspond to any of the lymphoid-organ-resident DCs subsets found in steady state in vivo (7). Therefore, we decided to perform a new screening on freshly isolated splenic DCs. DCs were isolated from the spleen of C57Bl/6 mice and purified by magnetic sorting. The panning was carried out as previously described. Upon binding and internalization, extracellular phages were inactivated by subtilisin treatment. Cell lysate were used to infect TG1 cells to amplify phages for the next round of selection. A total of three rounds were performed.

We evaluated the enrichment of binders by FACS analysis and found that only a percentage of $CD11c^+$ DCs stained positive for the pool of phages recovered from the third round of selection, suggesting that we may have isolated Abs directed against a subset of splenic DCs (Fig. 11).

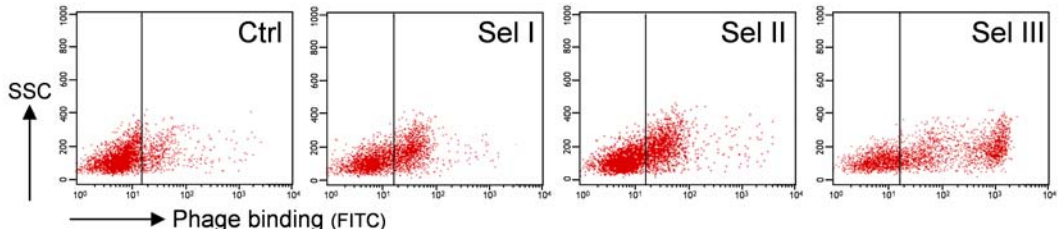


Figure 11. Evaluation of the phage panning procedure. Flow cytometry analysis on splenic DCs to monitor the enrichment of binders in the polyclonal phage preparations from each round of selection.

Fingerprinting analysis of 36 randomly picked clones revealed that 97% of them contained a full length scFv. We were able to identify 4 different fingerprinting patterns with three of them being more represented: clone1, 11 and 3 with 33%, 24% and 21% of frequency respectively (Fig. 12). Sequence analysis revealed a similarity between the clones. By searching against VBASE database we found that all the VH sequences belonged to the VH 1 family and the VL sequences to the Vλ 1 family. The CDRs H3 region length ranged from 9 to 11 aa and was identical in the last 5 aa in the most represented clones (scFv-1, scFv-3 and scFv-11) Only in one clone (scFv-9) that recurred only once, the CDR H3 region differed completely in sequence, but not in length, respect to the others (Table V).

Each clone was phenotypically functional and well displayed on the phagemid (Fig. 13a). FACS analysis showed that all the scFv-phage selected were able to bind to a percentage of splenic purified $CD11c^+$ DCs (Fig. 13b), thus validating our screening.

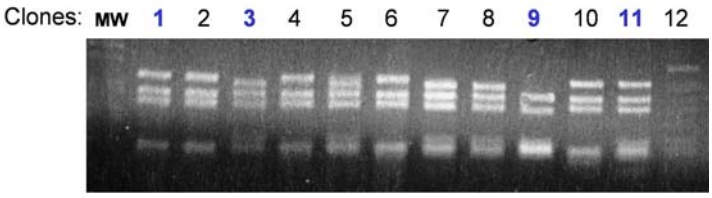


Figure 12. Diversity of the pool of scFv clones isolated after three rounds of selection. BstN1 fingerprinting of randomly picked single clones from the third round of selection. ScFv clones with a different fingerprinting profile are indicated in blu.

Table V. Analysis of the families and CDR3 regions of clones isolated from the phage selection.

VH segments				
	VH family	VH CDR3	CDR 3 length	JH
Clone 1	VH 1	MEQQHDAFDI	10	JH3a
Clone 3	VH 1	GAFEDDAFDI	10	JH3a
Clone 9	VH 1	ENYGSGAGMD	10	JH6a
Clone 11	VH 1	EVGASPDAFDI	11	JH3a

VL segments				
	VL family	VL CDR3	CDR 3 length	JL
Clone 1	Vλ 1	SWDDSLSGY	9	Jλ1
Clone 3	Vλ 1	AWDDSLSGY	9	Jλ1
Clone 9	Vλ 1	TWDDSVNSY	9	Jλ1
Clone 11	Vλ 1	TWDDRLNAW	9	Jλ1

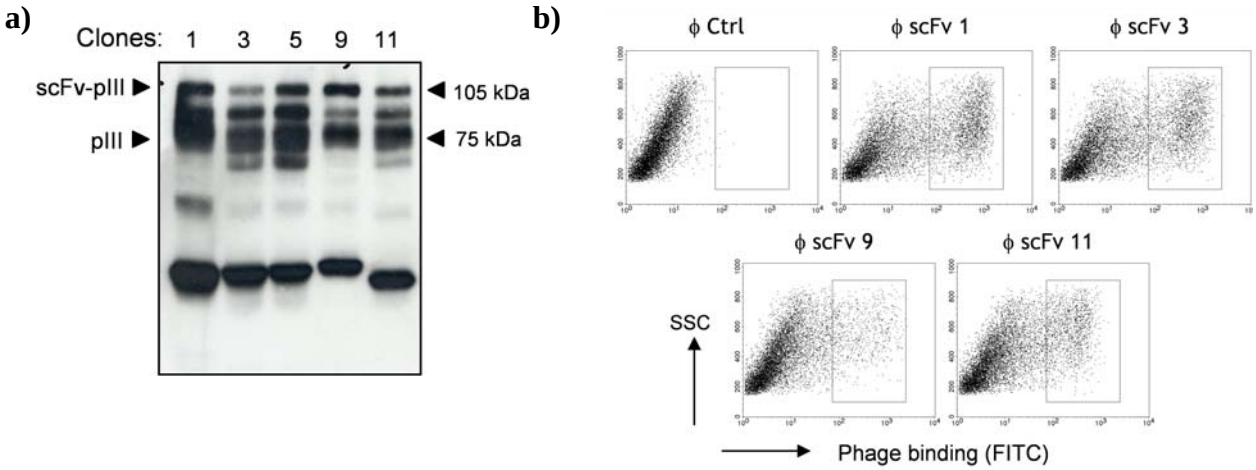


Figure 13. Analysis of scFv clones isolated from the screening on splenic DCs. Western Blot analysis to evaluate the display of the selected scFv on phage surface, revealed with anti-SV5 mAb **(a)**. FACS analysis to monitor the binding of single clones on DCs using an anti-pIII Ab **(b)**.

3.2 Receptor identification

The analysis of the scFvs recovered from the panning on splenic DCs and BMDCs revealed a sequence identity between the two most represented clones of each screening (scFv-1 and scFv-2 respectively). This strong selective pressure may derive from the high level of expression of the Ag, either a receptor or a specific structure/domain shared by different surface molecules, in both BMDCs and splenic DCs. Alternatively, a higher stability of the dominant scFv-phagemid may have conferred a growth advantage and thus favoured its amplification in each round of selection. Indeed, the sequence similarity and the constant CDR H3 length of the scFvs isolated from both screenings may denote a peculiar conformation of the Ag binding site and suggest an identity in the type of structure recognized by each clone. In light of these findings, it was of primary interest to characterize the Ag recognized by the dominant scFv, designated scFv-2E5 hereafter.

To this purpose, we pursued two complementary approaches. In a first set of experiments, we set out to precisely define the pattern of expression of the Ag recognized by scFv-2E5 on different DCs subsets and on other cells of hematopoietic origin. In parallel, we carried out immunoprecipitation experiments and mass spectrometry analysis to unequivocally identify the nature of the Ag.

To obtain a large amount of properly folded protein and avoid the purification step from *E.coli* periplasmic extract, we expressed both scFv-2E5 and a scFv-Ctrl in mammalian cells by stable transfection.

By genetically linking the variable regions of the scFv to the third constant domain of human been shown to be well secreted from mammalian cells by means of its human IgG1 secretion signal sequence(175).

Cell supernatants containing either scFv-2E5-CH3 or a scFv-Ctrl-CH3 proteins were thus used to assess the expression of the putative receptor on different cells type. Notably, the γ1-CH3 is a suitable tag for binding detection and due to its lack of binding to FcRγ it does not require a pre-blocking step when used in flow cytometry assays.

3.2.1 Binding of scFv-2E5 on DCs subsets

We initially evaluated the binding of scFv-2E5 on the different DCs subsets found in various lymphoid organs: spleen, lymph nodes (either peripheral and mesenteric)and thymus DCs subsets were identified by the differential expression level of specific cell markers such as: CD11c, CD8, CD11b and B220. Upon treatment of the organs with collagenase/DNaseI, the DCs population was enriched by low-density fractionation and recovered cells were incubated with either scFv-2E5 or scFv-ctrl. After extensive washing, scFv-2E5 binding was revealed using an anti-human IgG Ab and evaluated by gating on the different DCs subtypes. As shown in figure 14a, analysis of the conventional DCs, derived from both spleen and lymph nodes and defined as $CD11c^{hi}$ cells, revealed a tight correlation between CD8α expression and binding of scFv-2E5, resulting in two predominant populations of DCs: $CD8\alpha^+$ scFv-$2E5^+$ and $CD8\alpha^-$scFv-$2E5^-$. Plasmacytoid DCs, defined as $B220^+$ and $CD11c^{int}$, did not shown any specific staining, indicating the lack of expression of the receptor recognized by scFv-2E5 on this specific subset. Analysis of the so called migratory DCs: Langherhans and dermal DCs from peripheral LNs and DCs from lamina propria (mesenteric LNs), was evaluated by gating on a population of $CD11c^+$ cells that expresses high levels of MHC class II. We could not detect any staining on these migratory DCs suggesting the lack of expression of the Ag recognized by scFv-2E5. We confirmed this finding, by repeating the analysis on Langerhans and dermal DCs isolated from epidermis. Altogether, these results indicate that the antigen recognized by scFv-2E5 is uniquely expressed by the $CD8\alpha^+$ subset of lymphoid organ resident DCs.

3.2.2 Binding of scFv-2E5 on hematopoietic cells

We next investigated scFv-2E5 binding specificity for other hematopoietic cell types present in the spleen. We evaluated the binding on B cells, T cells, monocytes and macrophages (Fig. 14b) using a panel of different Abs: B220, CD3, Ly6c, CD11b, and F4/80. Whereas both T lymphocytes ($CD3^+$ cells) and monocytes (defined by gating on $CD11b^+$ and $Ly6c^+$ cells) were negative, we could detect scFv-2E5 binding on both macrophages ($F4/80^{hi}$ and $Ly6c^+$) and on a percentage (30%) of B cells. Altogether, these data indicate that scFv-2E5 recognizes a cell surface marker specifically expressed by antigen presenting cells.

We next analyzed scFv-2E5 binding on two different B cells lines: A20 (IgG expressing mature B cells) and WEHI (immature IgM expressing). As shown in figure 14c, scFv-2E5 clearly stained WEHI, but not A20 cells. Although this analysis is not sufficient to unequivocally link the

receptor expression to the cell maturation state, it indicates that at least in B cells, the expression of the Ag recognized by scFv-2E5 is regulated.

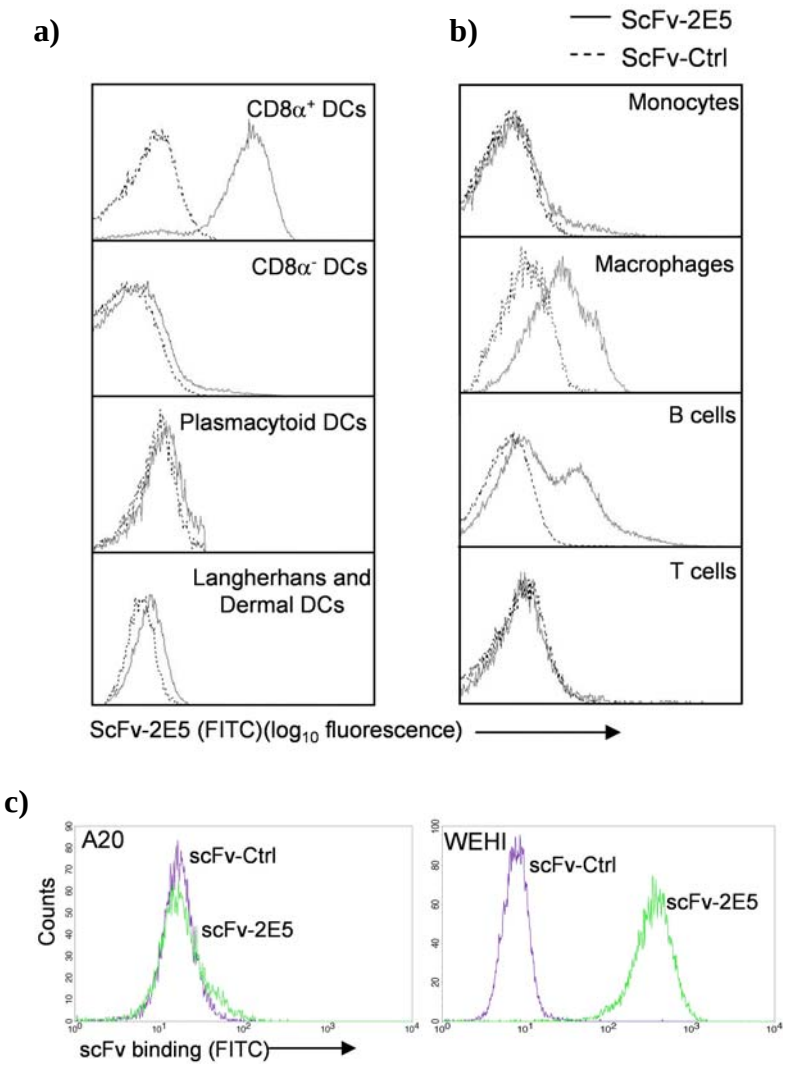


Figure 14. Binding profile of scFv-2E5 on splenic DCs and hematopoietic cells. DCs from the spleen of C57BL/6 mice were labelled with scFv-2E5 and a panel of different markers gating on: CD11c^{hi} and CD8α$^{+/-}$ for conventional DCs, and CD11c^{int} and B220^{+} for plasmacytoid DCs. Langherhans and dermal DCs were isolated from the epidermis, and identified by gating on CD11c^{+} cells **(a)**. Monocyte were defined by gating on Ly-6C^{hi}, CD11b^{hi} and CD11c^{-} cells, macrophages as F4/80^{+} and Ly-6C^{int}, B lymphocytes by expression of B220 and T cell by expression of CD3 **(b)**. Filled line, scFv-2E5. Binding profile on the B cells line A20 and WEHI **(c)**.

3.2.3 Antigen identification

To define the nature of the antigen targeted by scFv-2E5, we performed immunoprecipitation (IP) experiments using extracts of BM-DCs and WEHI B cell line. In order to visualize the IP antigens, cell surface proteins were biotynilated before preparing cell lysate. Cell extracts were incubated with scFv-2E5 or an irrelevant scFv (scFv-control). Upon IP, presence of biotinylated surface proteins was revealed in a western blot analysis by streptavidin. These experiments identified a DCs specific protein with an apparent molecular masses of 90kDa. The same experiment performed on WEHI cells revealed a band of approximately 100kDa, that may indicate a cell type dependent post-translational modification. As further control, we repeated the IP on biotinylated extracts from a T cells line (BO9) that was negative for scFv2E5 binding. As expected, Western Blot analysis did not reveal any biotinylated protein (Fig. 15a).

For precise receptor identification, nonbiotinylated cell extracts were IP and separated by SDS-PAGE. MALDI Mass Spectrometry was used to analyze the IP proteins with a molecular mass corresponding to the band previously revealed by Western Blot. Protein gel pieces were excised and tryptically digested. Extracted peptides were mixed with α-cyano-4- hydroxycinnamic acid matrix and subjected to MALDI analysis. Mass spectrometry data were acquired with a MALDI TOF/TOF and subsequently subjected to database searching using Mascot program against Swissprot. Protein data base searching revealed that the primary sequence of the peptides obtained from trypsin digestion matched portions of mouse class B scavenger receptor CD36 (Fig. 15b). To validate the results from MS we carried out a series of Western Blot analysis.

CD36 is a highly N-glycosilated protein and indeed treatment with the amidase PNGaseF, to specifically remove N-linked glycans, revealed a band of approximately 55kDa, which correspond to the molecular mass of CD36 (Fig. 15c). We next repeated the IP experiment on biotynilated or not biotinylated cells extracts and compared the IP proteins using either streptavidin or an anti-CD36 antibody. The commercial Ab detected a specific band that likely coincided with the one revealed by streptavidin (Fig. 15d). To further confirm the identity of the antigen recognized by scFv-2E5, full-length cDNA sequence of CD36 was amplified and transfected in 293T cells. FACS analysis showed that scFv-2E5 specifically stained CD36-transfected but not mock transfected 293T cells (Fig. 15e). These results thus demonstrate that scFv-2E5 indeed recognizes an epitope in the extracellular portion of CD36.

To prove our initial hypothesis that the scFvs similarity in the CDR H3 region was correlated to the specificity of the Ag recognized, we repeated the FACS assay on CD36-transfected 293T

cells. We tested two scFvs, isolated from the screening on splenic DCs, whose CDR H3 (last 5 aa) were either similar (scFv-3) or different (scFv-9) from scFv-2E5. Whereas scFv-3 clearly stained CD36-transfected 293T cells proving the specificity of this scFv for the receptor, in case of scFv-9 we did not observe any binding (Fig. 15f). This result indicates that the CDR H3 region do play a critical role in defining the Ag specificity and suggests that the other scFvs, isolated from both the screenings, may likely recognize CD36.

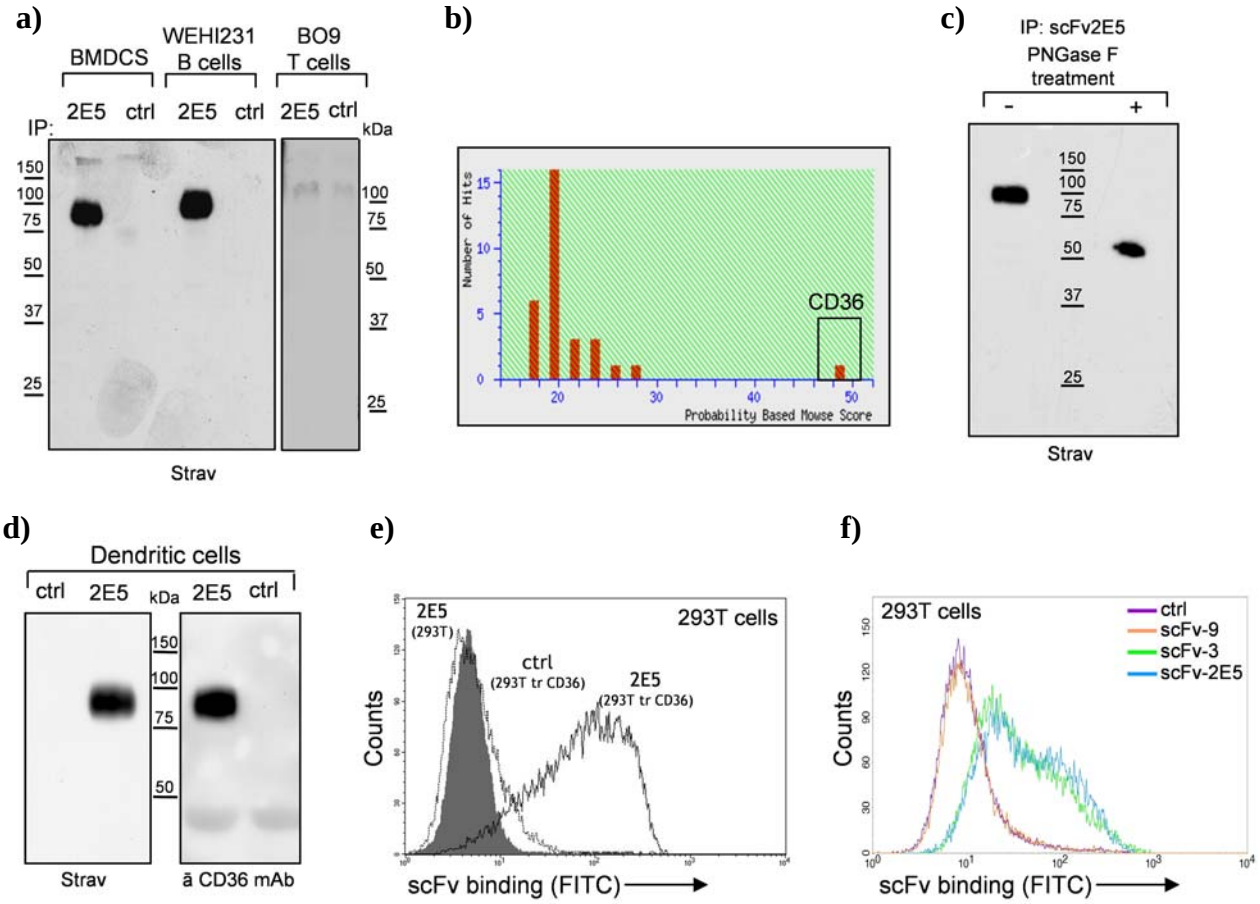


Figure 15. ScFv-2E5 recognizes an epitope in the extracellular portion of the scavenger receptor CD36. Western blot of extracts from cell-surface biotinylated DCs, WEHI or BO9 T cells, immunoprecipitated with scFv-2E5 (2E5) or an irrelevant scFv (ctrl) and revealed with streptavidin-HRP **(a)** Graphic representation of the data obtained from Mass Spectrometry analysis of the proteins immunoprecipitated by scFv-2E5 **(b)**. Western blot of extracts from biotinylated WEHI cells upon immunoprecipitation with scFv-2E5 or scFv-ctrl and PNGaseF treatment. IP proteins were revealed with streptavidin-HRP **(c)**. Western blot of extracts from biotinylated DCs or BO9 T cells, immunoprecipitated with scFv-2E5 or scFv-ctrl and revealed with streptavidin-HRP or an anti-CD36 Ab, as indicated **(d)**. Binding of scFv-2E5 or scFv-ctrl to 293T cells transiently transfected with the cDNA encoding mouse CD36 **(e)**. Binding of different scFvs isolated from the screening to 293T cells transfected with CD36 cDNA **(f)**.

3.3 Antigen presentation efficiency of the antibody/receptor pairing

Results described in the previous sections provide the proof of principle that antibodies directed against DCs endocytic receptors can be isolated by phage-display and subsequently used to identify the cognate receptor.
Having isolated a high affinity antibody against CD36, a multiligand scavenger receptor with a role in the uptake of apoptotic bodies and in innate immunity, we decided to characterize in detail the immune responses elicited by targeting this receptor on DCs.

To evaluate the capacity of the antibody/receptor pairing to deliver antigens to the cellular compartments involved in Ag processing and presentation, we designed a DNA cassette that allows us to readily produce recombinant scFv proteins that include the antigenic epitopes. We chose as a model antigen ovalbumin and evaluated the immunogenicity of different scFv-OVA fusion proteins containing either the single MHC class I OVA epitope (scFv-$OVA_{257-264}$) or the whole OVA protein. In the latter case, we produced both a monomeric (scFv-OVA) and a dimeric form (SIP-OVA) of the recombinant OVA proteins to allow receptor cross-linking. Finally, we tested all these molecules for their antigen presenting properties in different set of in vitro and in vivo assays.

3.3.1 Production of scFv-$OVA_{257-264}$ epitope fusion protein

To produce our recombinant OVA fusion proteins, we initially modified the phagemid vector *pDAN3* by cloning between the two tag sequences (SV5 and His6) a short linker containing two restriction enzyme sites: AgeI and NsiI. We chose these sites because of their low frequency of recurrence in the DNA coding for the variable regions of antibodies. This DNA cassette thus allows to insert in frame with the scFv any desired antigenic epitope flanked by the sequence of the two restriction enzyme sites.
Ovalbumin was chosen as model antigen due to the availability of well characterized systems to evaluate Ag processing and presentation on both MHC class I and MHC class II molecules.
To test the efficiency of different scFvs to target antigens to the cross-presentation pathway, we cloned in the DNA cassette the sequence coding for the ovalbumin class I epitope SIINFEKL. To allow the correct processing of the OVA class I peptide, inserted in an exogenous context, both natural N-terminal and C-terminal flanking sequences (5aa each) were included (176) (Fig. 16a).
ScFv-$OVA_{257-264}$ recombinant molecules were produced in bacteria either as soluble proteins or displayed on phagemid and in eukaryotic cells (HEK 293T) by transient transfection. In this case,

the scFv-OVA DNA cassette was subcloned in the mammalian expression vector *pcDNA3* (Fig. 16b). Expression of the recombinant scFv-OVA$_{257-264}$ (scFv-DEC205, scFv-2.4G2, scFv-CD36 and scFv-ctrl) proteins was evaluated by Western Blot analysis and functionality assessed by citofluorimetry (Fig. 16c-d).

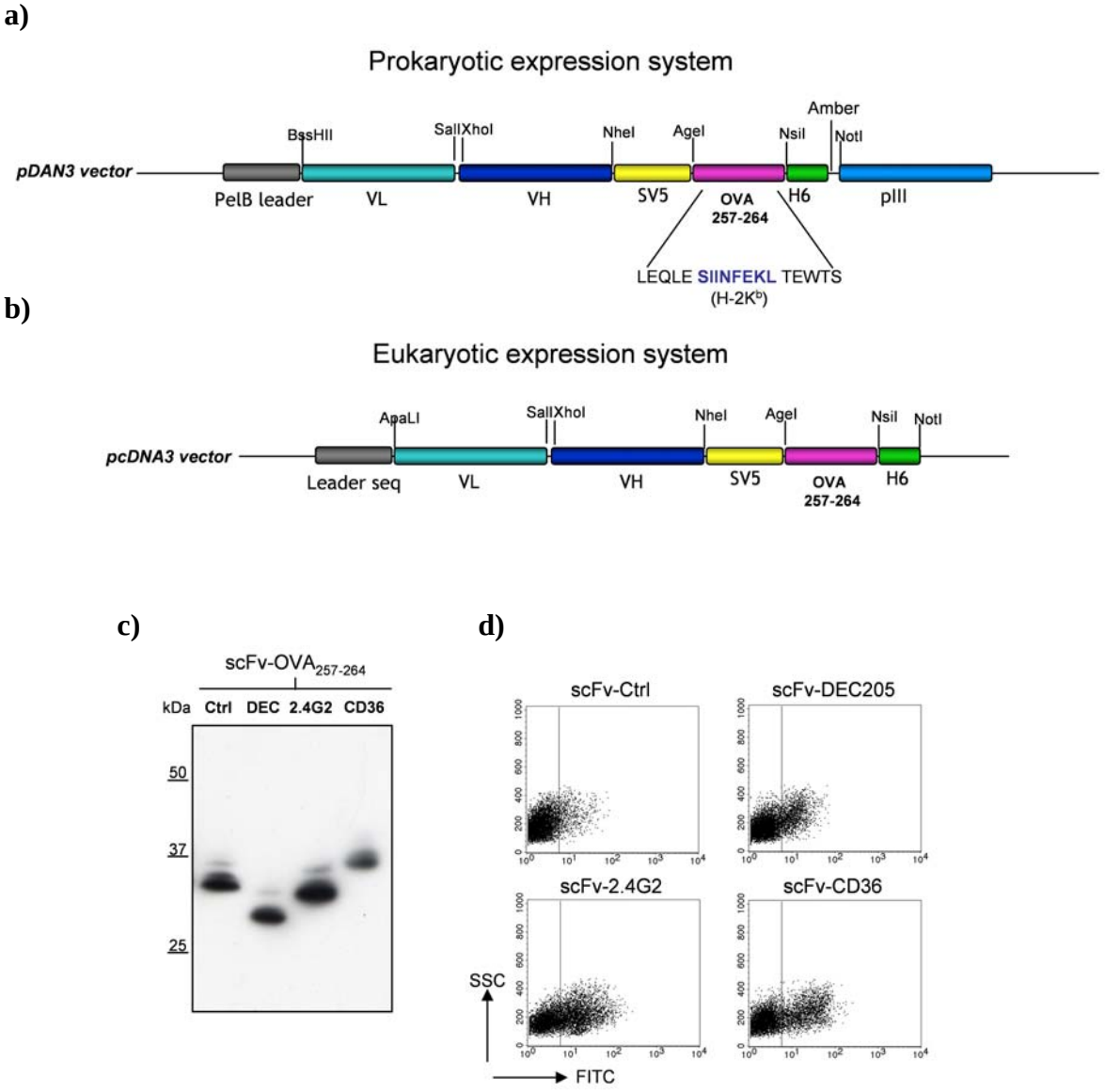


Figure 16. Production of scFv-OVA$_{257-264}$ recombinant proteins. Schematic representation of the DNA constructs that allow the production of scFv-OVA$_{257-264}$ fusion proteins in bacteria **(a)** and eukaryotic cells **(b)**. Western blot of scFv-OVA$_{257-264}$ proteins produced by transient transfection in 293T cells, revealed with an anti-SV5 mAb **(c)**. Binding of scFv-OVA$_{257-264}$ proteins to splenic DCs, detected with a SIPanti-SV5 **(d).**

3.3.2 In vitro antigen cross-presentation assay using scFv-OVA$_{257-264}$ proteins

To compare the ability of the different scFv-OVA$_{257-264}$ fusion proteins to enter the cross-presentation pathway, we initially performed in vitro cross-prentation assays using as antigen specific T cells the hybridoma B3Z. This cell line carries a β-galactosidase construct driven by the NF-AT element from the IL-2 promoter (177) and allows to measure the OVA-specific response either by evaluating IL-2 secretion or using chlorophenol red β-galactosidase (CPRG) as a substrate to detect LacZ activity in B3Z lysates. In these experiments, DCs were pulsed with different concentration of Ag before adding B3Z cells and co-cultured for 18 h before measuring LacZ activity. The assay was repeated several times, varying both the BM-DCs maturation state, the pulsing conditions (1-3h to o/n) and the form of the Ag used (soluble protein or recombinant phage). Despite the many conditions tested, we did not observed B3Z cells activation with any of the targeting scFvs (anti-DEC, anti-CD36 and anti-FcγR). Only the incubation of DCs with the peptide SIINFEKL or with a high concentration of soluble OVA (100 μg) resulted in B3Z activation (Fig. 17a).

We reasoned that the lack of T cell proliferation may derive from the high threshold of activation of the of B3Z cells. We thus repeated the cross-presentation assay using CD8$^+$ T cells isolated from OT-I mice, which carries a transgenic TCR (Vα2/Vβ5) specific for the OVA SIINFEKL peptide restricted by H-2K^b (178).

In this set of experiments, scFv-OVA$_{257-264}$ proteins were produced by transient transfection in 293T cells. Supernatants containing the secreted recombinant proteins were dialyzed to get rid of peptides deriving from protein degradation and that could have been presented by DCs without direct processing. DCs were incubated with graded amount of scFv-OVA$_{257-264}$ for 4 h and then washed. OT-I cells were co-cultured with DCs for 18 h before evaluating the expression of the T cell early activation marker CD69. Although in some experiments we detected OT-I activation by pulsing DCs with the scFvs targeting DEC-205, the FcγRs and CD36, but not with scFv-Ctrl, these data were not always reproducible (Fig. 17b). Collectively, these experiments were not sufficient to clearly evaluate whether Ags delivered via CD36 would access the cross-presentation pathway, yet they suggested that the OVA class I epitope could be correctly processed and presented when fused to the scFv.

The variability in the outcome and in the extent of the response may both derive from an imprecise quantification of the protein concentration or by the presence of products of degradation or contaminants in the cells supernatants used as Ag source. Notably, not all the recombinant scFv-OVA$_{257-264}$ proteins were produced with the same efficiency upon 293T transfection and indeed

protein quantification by Western Blot could not ensure that exactly the same amount of Ag was used in the assays. Several attempts were carried out to purify scFv- $OVA_{257-264}$ proteins, either from bacteria periplasmic extracts or from cells supernatant by His6 tag affinity chromatography, but the protein was not pure and the efficiency always very low.

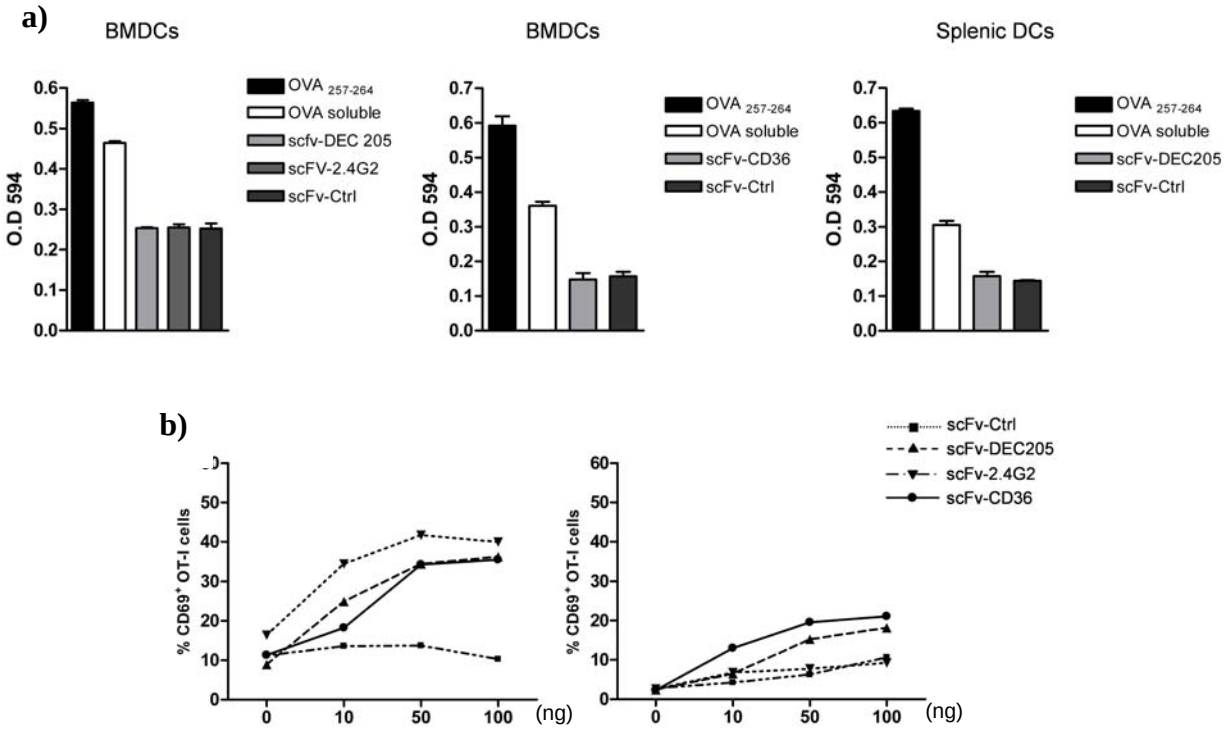


Figure 17. *In vitro* targeting of DCs endocytic receptors by scFv-$OVA_{257-264}$ recombinant proteins. Activation of B3Z cells co-cultured for 18 h with DCs pulsed with an equal amount (100ng) of scFv-$OVA_{257-264}$, soluble OVA (100µg) or $OVA_{257-264}$ peptide (1µM). CPRG was used as substrate to detect LacZ activity in B3Z lysates **(a)**. Activation of OT-I cells co-cultured for 18 h with DCs pulsed with graded amount of scFv-$OVA_{257-264}$ proteins. Expression of CD69 marker is taken as an index of OT-I cell activation **(b)**.

3.3.3 In vivo antigen cross-presentation assay using scFv-OVA$_{257-264}$ proteins

In parallel to the in vitro assays, we tested whether scFv-OVA$_{257-264}$ proteins were able to target DCs in vivo and induce the activation of adoptively transferred OVA specific T cells (OT-I). scFv-OVA$_{257-264}$ proteins (anti-CD36 or Control) were produced by 293T transfection and Western Blot was performed to estimate protein concentration by comparison to a previously quantified standard.

OT-I T cells activation was assessed by flow cytometry either by evaluating CD69 expression at day1 post injection or by looking at the cell proliferation profile at day3. To track OT-I cells in vivo, we labeled them with the fluorescent dye CFSE, which allows each cell division to be visualized by flow cytometry as a sequential 2-fold reduction in fluorescent intensity. Labeled OT-I were adoptively transferred into congenic C57Bl/6 mice, followed by subcutaneous injection with comparable amount of scFv-OVA$_{257-264}$ proteins.

Although in some experiments the percentage of OT-I cells that underwent proliferation was higher in mice primed with scFv-antiCD36 respect to mice immunized with scFv-ctrl (Fig. 18a), these results were not reproducible. Moreover, the number of OT-I cells we tracked was too low to clearly evaluate the extent of T cell proliferation.

In a second set of experiments, scFv-OVA$_{257-264}$ proteins were injected intravenously and OT-I cells evaluated for CD69 expression. Despite several attempts, we were never able to detect a clear pattern of T cell activation (Fig. 18b). Collectively, these results are not sufficient to assess the ability of scFv-antiCD36 to target DCs in vivo and to deliver the antigen to the cross-presentation pathway.

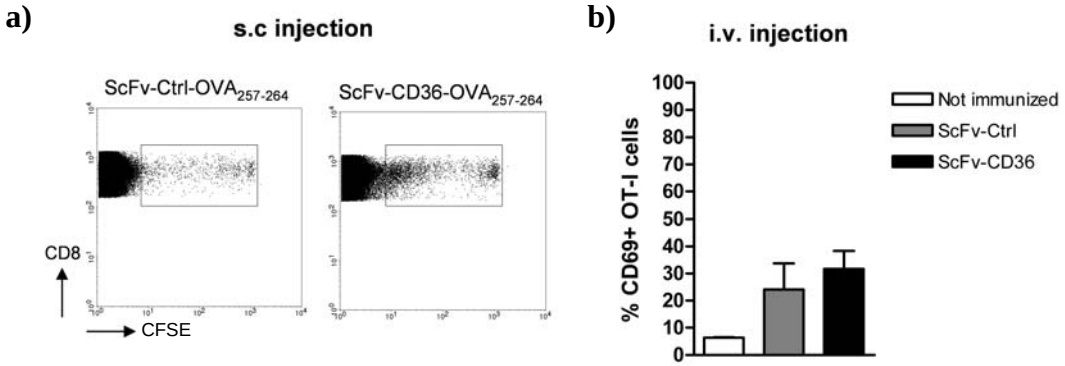
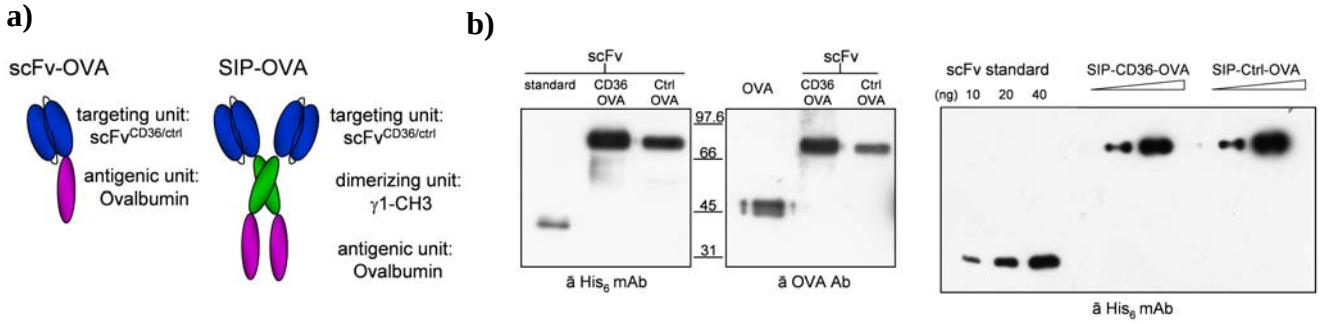


Figure 18. *In vivo* targeting of DCs via CD36 by injection of scFv-OVA$_{257-264}$ recombinant proteins. C57BL/6 mice were injected i.v. with 1x10^6 CFSE labeled OT-I cells and subsequently injected s.c. with an equal amount (200ng) of scFv-CD36-OVA or scFv-Ctrl-OVA, as indicated. T cell proliferation was measured at day 3 in draining LNs. Dot plot represent the CFSE dilution profile of OT-I cells at day 3 **(a)**. Mice were adoptively transferred with 1x10^6 OT-I cells and then i.v. injected with scFv-OVA$_{257-264}$, as described above. T cells activation is detected by evaluating CD69 expression at day 1 post injection **(b)**.

3.3.4 Production of scFv-OVA fusion proteins

To improve the immunogenicity of our recombinant fusion proteins, we replaced the MHC class I OVA epitope with the full-length sequence of the antigen, which allows to evaluate in parallel the processing and presentation on both MHC class I and MHC class II molecules. We reasoned that the lack of a clear T cells activation profile upon immunization with scFv-OVA$_{257-264}$ proteins may derive from the poor processing efficiency of the OVA class I peptide when directly fused to the scFv. This hypothesis was supported by the findings that even immunization with our positive control: scFvDEC-OVA$_{257-264,}$ did not always elicit efficient T cell priming. Secondly, lack of T cells activation may derive from the inefficient internalization of the scFv-CD36 molecule upon receptor binding. Indeed, some receptors such as FcRs require cross-linking to induce internalization. We thus engineered a dimeric variant of scFv-OVA protein which contained downstream of the scFv, the third constant domain of human IgG (SIP-OVA proteins) to induce CD36 cross-linking (Fig. 19a).

The scFvs-OVA and SIPs-OVA were produced in 293T cells by transient transfection. Protein production and functionality were assessed by Western Blot and FACS analysis respectively (Fig. 19b and c). Notably, the expression of the different scFvs molecules by transfected cells was similar and protein concentration was estimated around 2-4 mg/litre. We next set up a protein purification protocol taking advantage of the His6 tag present at the C-terminus of the different forms of scFv/SIP-OVA molecules. Anti-His6 affinity chromatography was performed and protein concentration and purity was evaluated by SDS-PAGE and Comassie staining. We obtained pure protein preparations with a concentration of 20-30 mg/litre (Fig. 19d).



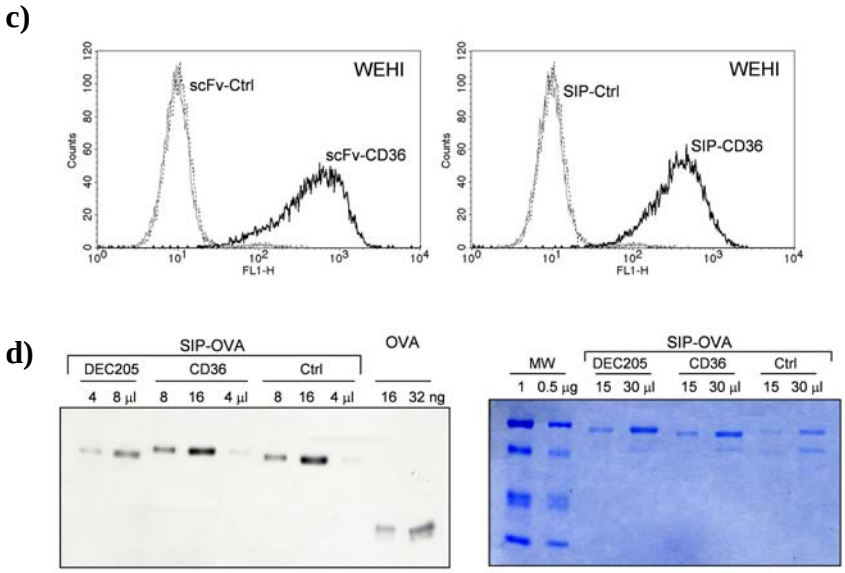


Figure 19. Eukaryotic expression of scFv-OVA and SIP-OVA recombinant proteins. Schematic representation of the monomeric scFv-OVA and dimeric SIP-OVA recombinant molecules **(a)**. Western blot of recombinant Ag molecules detected with either anti-H_6tag or anti-OVA Abs **(b)**. FACS analysis to evaluate binding of recombinant Ag proteins on WEHI cells, using an anti-OVA Ab **(c)**. Western Blot and Comassie staining of purified SIP-OVA proteins **(d)**.

3.3.5 Internalization of SIP-CD36-OVA fusion protein

To initially study whether the SIP-CD36-OVA protein was endocytosed upon receptor binding, we performed a flow cytometry based internalization assay. DCs were incubated with SIP-CD36-OVA or SIP-Ctrl-OVA at 4°C to allow receptor binding. Cells were then washed and incubated either at 4°C or 37°C for different time points (15 to 60 min). The amount of SIP-OVA remaining at the cell surface was evaluated by FACS using an anti-OVA Ab. As shown in Fig.25a, SIP-CD36-OVA was rapidly internalized with ~70% of the protein removed from cell surface within 30 min at 37°C (Fig. 20a).

We further confirmed this result by immunofluorescence analysis. Upon CD36 binding, cells were incubated at 4°C or 37°C for 30 min and SIP-CD36-OVA internalization was detected by anti-hIgG Ab. Data indicate that SIP-CD36-OVA was efficiently internalized at 37°C while it remained associated to the plasma membrane at 4°C (Fig.20b).

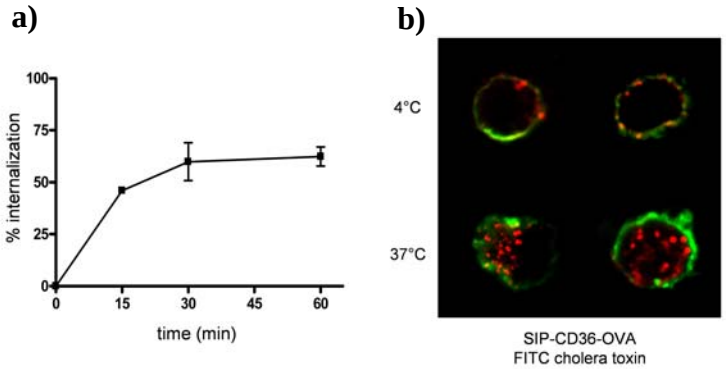


Figure 20. Internalization of the recombinant anti-CD36-antigen fusion. Time course of SIP-CD36-OVA internalization. After binding at 4°C, DCs were incubated either at 37°C or at 4°C for the indicated time points. The plot shows the ratio of remaining surface-bound $scFv^{CD36}$-OVA at the two temperatures, measured with an anti-OVA Ab. Data show means of duplicate measurements **(a)**.

Confocal microscopy of SIP-CD36-OVA internalization by DCs. After binding at 4°C, cells were washed and incubated for 30 min at 4 °C or 37°C, as indicated. Fixed and permeabilized DCs were stained with $Alexa^{594}$-conjugated anti human Ab and FITC-conjugated cholera toxin subunit B, to counter stain plasma membranes. Images from the central Z-section of representative cells are shown **(b)**.

3.3.6 Role of CD36 in antigen presentation by targeting DCs in vitro

To investigate the capacity of SIPCD36-OVA to deliver OVA-derived peptide antigens to the MHC class I and MHC class II pathways of antigen presentation, we performed *in vitro* proliferation assays using as reporter cells OT-I or OT-II T lymphocytes. Whereas OT-I TCR is specific for $OVA_{257-264}$ peptide, OT-II are transgenic for an MHC class II restricted αβTCR specific for the $OVA_{323-339}$ peptide determinant presented in the context of MHC class II I-A^b (179). DCs isolated from the spleen of C57Bl/6 mice were incubated with graded concentration of the recombinant SIP-CD36-OVA or SIP-Ctrl-OVA proteins or soluble OVA and then co-cultured with OT-I or OT-II cells. T cell proliferation was evaluated three days later by measuring [^{3}H]thymidine incorporation. SIP-CD36-OVA induced OT-I and OT-II proliferation at a concentration as low as 20 ng/ml, whereas no proliferation was detected even at the highest concentration (1600 ng/ml) of SIP-Ctrl-OVA. Relative to soluble OVA, uptake via CD36 increased the efficiency of presentation by at least 400 fold for MHC class I epitope (Fig. 21a) and by 300-fold for MHC class II epitope (Fig. 21b). These results clearly demonstrate that targeting CD36 on splenic DCs increases the efficiency of presentation of protein antigens on MHC class II and, more importantly, is able to deliver OVA to the MHC class I pathway of Ag cross-presentation.

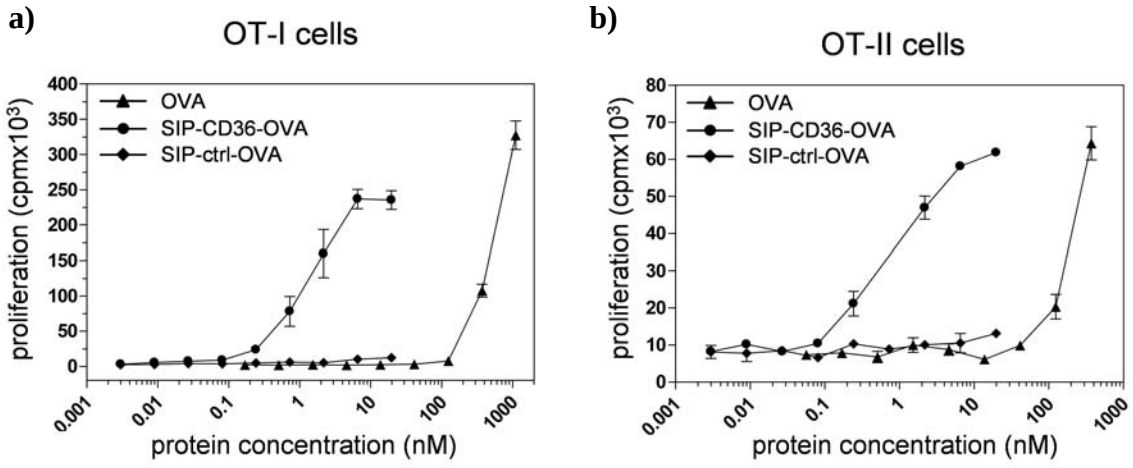


Figure 21. *In vitro* targeting of CD36 on splenic DCs. Proliferation, measured as [^{3}H]-thymidine uptake, of OT-I cells **(a)** or OT-II cells **(b)** co-cultured for 72 h with spleen derived DCs pulsed with different concentrations of SIP-CD36-OVA (●), SIP-Ctrl[l]-OVA (♦) or OVA alone (▲).

Previous data have shown that also B cells express high levels of CD36. We thus asked whether CD36 targeting on B cells leads to cross-presentation of $OVA_{257-264}$ derived peptide. To compare the efficiency of cross presentation mediated by DCs versus B cells we isolated, from either spleen or lymph nodes, $CD11c^+$ DCs, $CD19^+$ B cells and $CD11c^-$ lymph nodes cells and pulsed them with different amount of SIP-CD36-OVA, SIP-DEC205-OVA or SIP-Ctrl-OVA. After 4 h, Ag was washed and pulsed APC were co-cultured with CFSE labelled OT-I cells. T cells activation and proliferation were assessed after 3 days by measuring secretion of IL-2 in the supernatants and by evaluating the CFSE profile by cytofluorimetry. Results show that only DCs are able to efficiently take up and present OVA to OT-I cell. Neither B cells nor DCs depleted lymph nodes cells stimulated T cell proliferation above the back-ground levels, even at Ag and cell doses 10-fold higher than those required to observe proliferation by DCs. SIP-Ctrl-OVA did not cause T cell activation even at the highest Ag dose (1μg). Splenic DCs pulsed with SIP-DEC205-OVA were twice as efficient than those pulsed with CD36 to induce T cells proliferation, whereas the same percentage of proliferated OT-I was observed in the case of DCs isolated from LNs and pulsed either with SIP-CD36-OVA or SIP-DEC205-OVA (Fig. 22a). These results were confirmed by measuring IL-2 production by ELISA. Only T cells primed by DCs secreted IL-2 (Fig. 22b) indicating that CD36 targeting induces efficient cross-presentation exclusively in DCs.

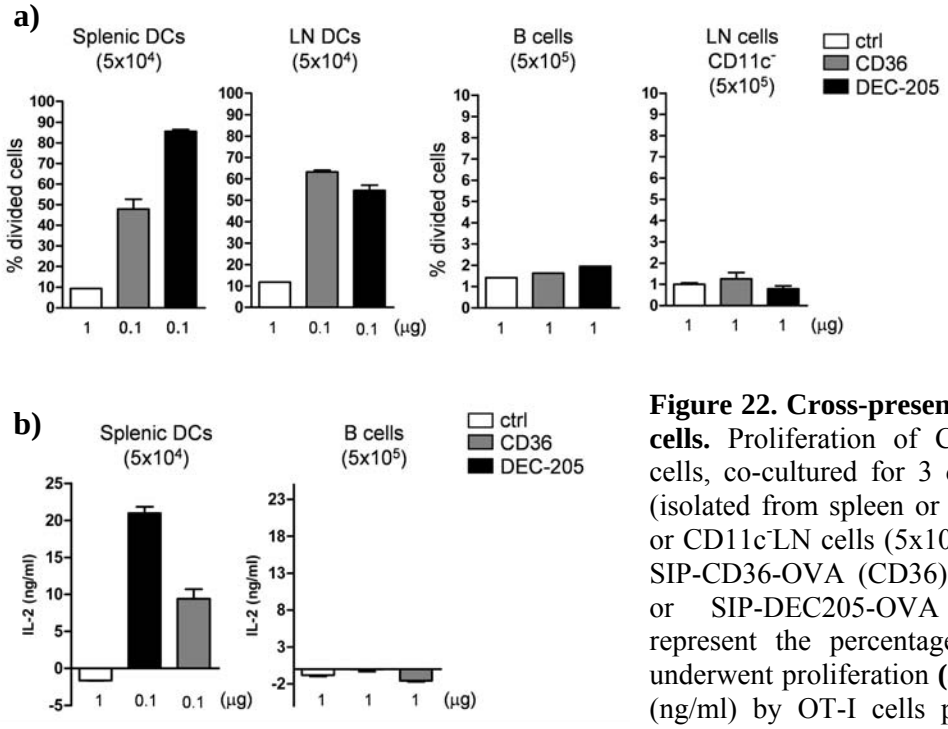


Figure 22. Cross-presentation by DCs and B cells. Proliferation of CFSE-labeled OT-I T cells, co-cultured for 3 days with $5x10^4$ DCs (isolated from spleen or LNs), B cells ($5x10^5$) or $CD11c^-$LN cells ($5x10^5$), pre-incubated with SIP-CD36-OVA (CD36), SIP-Ctrl-OVA (ctrl) or SIP-DEC205-OVA (DEC-205). Plots represent the percentage of OT-I cells that underwent proliferation **(a)**. Production of IL-2 (ng/ml) by OT-I cells primed by DCs or B cells, as previously described **(b)**.

3.3.7 In vivo targeting of DCs using SIP-OVA proteins

We next asked whether targeting antigen to $CD11c^+$ $CD8\alpha^+$ DCs via CD36 is able to induce potent immune responses in vivo.

At first, we investigated the ability of SIP-CD36-OVA to induce proliferation of OVA specific $CD4^+$ T cells. Mice were adoptively transferred with CFSE labelled OT-II cells followed by immunization with graded doses of SIP-CD36-OVA or SIP-Ctrl-OVA. T cell proliferation was evaluated as CFSE dilution in the draining lymph node at day 3 upon injection. We detected OT-II proliferation only in lymph nodes of mice immunized with of SIP-CD36-OVA (200 ng) whereas priming with a higher amount of SIP-Ctrl -OVA (500 ng) did not elicit OT-II activation (Fig. 23). These data indicate that the recombinant antiCD36-OVA protein is able to target DCs in vivo and more importantly, it enhances the efficiency of MHC-class II peptide antigen presentation.

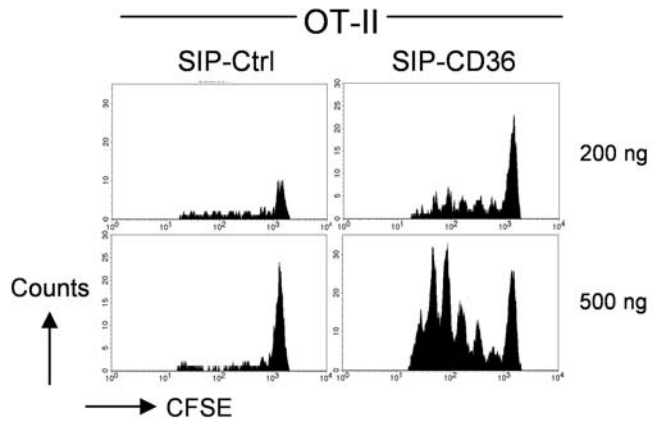


Figure 23. *In vivo* targeting of DCs via CD36 improves the efficiency of antigen presentation on MHC class II molecules. C57BL/6 mice were injected i.v. with $1x10^6$ CFSE labeled OT-II T cells and subsequently injected s.c. with graded doses of SIP-CD36-OVA or SIP-Ctlr-OVA T cell proliferation was measured at day 3 in draining LNs. Histograms show one representative CFSE dilution profile of OT-II cells at day 3.

We then focused on the ability to trigger cytotoxic T lymphocytes since this is particularly challenging for not replicating antigens and would be valuable for the design of effective vaccines.

We compared the relative efficiency of scFv-CD36-OVA versus SIP-CD36-OVA to elicit OT-I activation in vivo. Mice, adoptively transferred with CFSE labelled OT-I cells, were immunized with equal amount of scFv-CD36-OVA, SIP-CD36-OVA or scFv-Ctrl-OVA. T cells proliferation was evaluated 3 days upon transfer by flow cytometry. Results show that immunization of mice with either the scFv-CD36 or SIP-CD36 OVA recombinant proteins, induced a similar extent of OT-I proliferation and accumulation at both doses tested (100-300 ng), whereas scFv-Ctrl-OVA completely failed to activate the $CD8^+$ T cells response even at the highest Ag dose (Fig. 24a). These data indicate that targeting CD36 on DCs is a valuable means to enhance T cells priming in vivo and

suggest that scFv-CD36 is efficiently internalized by DCs even without triggering the cross-linking of the receptor.

We next compared the OT-I response elicited by targeting DCs in vivo either via CD36 or DEC205. Mice were adoptively transferred with CFSE labelled OT-I cells and s.c. injected with increasing amounts of SIP-CD36-OVA, SIP-DEC205-OVA or SIP-Ctrl-OVA. Antigen specific T cell proliferation was determined in the draining lymph nodes at day 3 upon transfer. Practically all the OT-I cells in lymph node of SIP-CD36-OVA immunized mice entered cell cycle and underwent up to 6 divisions after a dose of just 100 ng of OVA protein, whereas immunization with a highest dose of SIP-Ctrl-OVA (300 ng) did not induce naïve OT-I cells to divide (Fig. 24b). We next compared the response elicited by targeting CD36 versus DEC205 and found that immunization with SIP-DEC205-OVA was more efficient in inducing entry in cycle since as little as10ng of protein induced extensive T cell proliferation (Fig. 24b). Collectively, these data indicate that although less efficient than DEC205, delivery of antigens through CD36 *in vivo* enhances the uptake and processing for peptides presentation on MHC class I molecules.

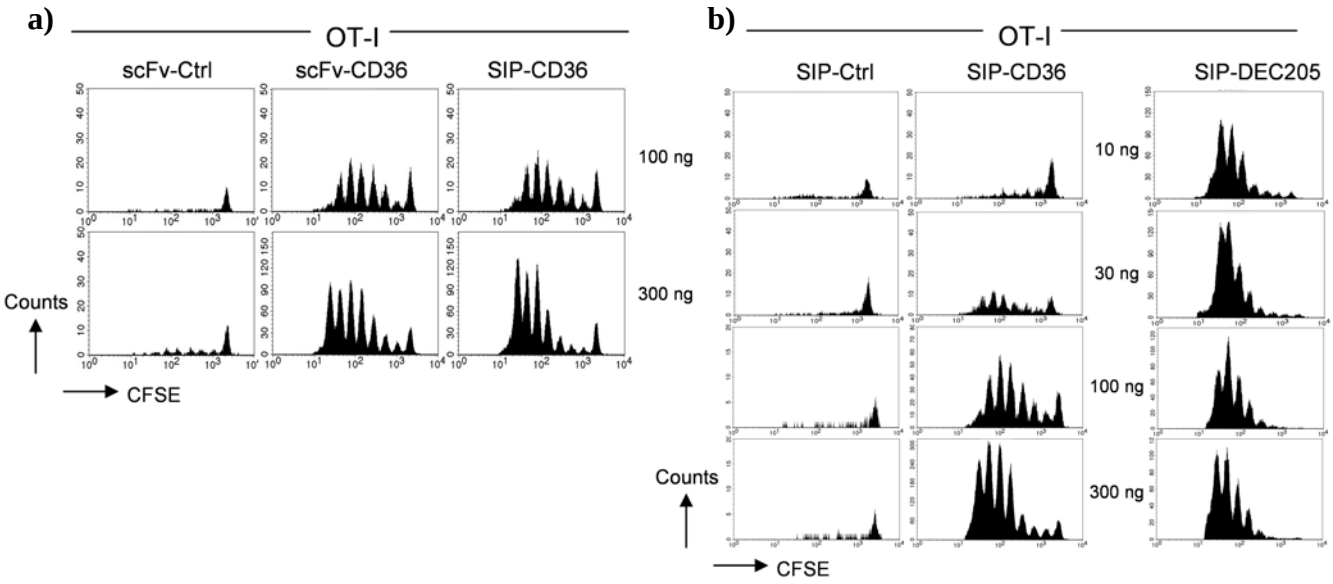


Figure 24. CD36-targeted antigen accesses the cross-presentation pathway *in vivo.* C57BL/6 mice were injected i.v. with $1x10^6$ CFSE labeled OT-I T cells and subsequently injected s.c. with graded doses of scFv-Ctrl-OVA, scFv-CD36-OVA or SIP-CD36-OVA **(a)**, or with SIP-Ctrl-OVA, SIP-CD36-OVA or SIP-DEC205-OVA **(b)**, as indicated. T cell proliferation was measured at day 3 in draining LNs. Histograms show one representative CFSE dilution profile of OT-I cells at day 3.

3.3.8 Characterization of the $CD8^+$ T cells response elicited by targeting CD36 on DCs

We have shown that targeting $CD8\alpha^+$ conventional DCs through CD36 induces the active proliferation of adoptively transferred OVA specific $CD8^+$ T cells. However, it is now clear that the effective presentation of the antigen and the subsequent T cell proliferation do not necessarily imply the induction of an immune response. Previous studies have in fact demonstrated that targeting immature, steady state DCs via DEC-205 results in the induction of peripheral tolerance rather than immunity. Although Ag specific $CD8^+$ T cells initially expand upon DCs priming, they fail to differentiate in effector cytotoxic T cells and undergo a deletional mechanism (86).
Therefore, procedures aimed at the induction of protective immune responses by targeting DCs in vivo, should in addition induce the activation of DCs in order to provide the T cells with the co-stimulatory signals required for their differentiation into effector cells and the development of memory.

To evaluate the long-term effects of targeting Ags to CD36 receptor on DCs, we followed the fate of the OT-I cells that proliferated upon immunization with SIP-CD36-OVA and SIP-DEC205-OVA, for comparison. To track the T cells in vivo, we isolated OT-I $CD8^+$ cells from OT-I mice expressing a specific isoform of the CD45 marker (CD45.1) and adoptively transferred into C57Bl/6 mice expressing the CD45.2 isoform. We next evaluated the number of OVA-specific T cells by means of an Ab that specifically recognizes the CD45.1 isoform.
Mice were adoptively transferred with 2×10^6 OT-I cells and s.c injected with 300 ng of SIP-CD36-OVA, SIP-DEC205-OVA or SIP-Ctrl-OVA either in the presence or absence of adjuvant (anti-CD40 mAb) to induce DCs activation. At day 12 upon transfer, we evaluated the expansion of the antigen specific effector T cells in the spleen. In mice immunized with SIP-CD36-OVA in the absence of adjuvant we found a 2 to 3-fold expansion of OT-I cells, relative to mice injected with SIP-Ctrl-OVA, or PBS. Notably, the co-administration of anti-CD40 mAb with SIP-CD36-OVA did not significantly increase T cell expansion. In contrast, immunization with SIP-DEC205-OVA, despite initial T cell proliferation, did not induce detectable expansion of antigen specific effector cells unless co-delivered with the anti-CD40 mAb (Fig. 25a). Still, T cell expansion was higher in mice primed with SIP-CD36-OVA than in mice immunized with SIP-DEC205-OVA plus adjuvant. Altogether these results indicate that, as already shown by previous works, antigen targeting to $CD8^+$ DCs via the DEC205 receptor requires a maturation stimulus to induce immunity. Instead, targeting the same DCs subset via CD36 induces T cell expansion *per se*.

To evaluate the effector functions of the OT-I cells present in the spleen at day 12 post injection, we monitored their capacity to produce IFN-γ upon in vitro restimulation with the $OVA_{257-264}$ peptide. In the absence of adjuvant, we detected a high proportion of IFN-γ secreting OT-I cells only in mice immunized with SIP-CD36-OVA. Moreover, the percentage of IFN-γ producing OT-I cells did not significantly increase when the Ag was co-administered with anti-CD40 mAb. In contrast, the few OT-I cells that persisted in mice primed with SIP-DEC205-OVA without adjuvant, were unable to secrete IFN-γ and were rescued only upon co-administration of anti-CD40 mAb (Fig.25b).

To confirm the induction of effector OT-I cells upon targeting Ags to CD36, we performed an *in vivo* cytotoxicity assay. Mice were adoptively transferred with $1{,}5\times10^6$ OT-I cells and then s.c injected with 200 ng of SIP-CD36-OVA or SIP-Ctrl-OVA either with or without anti-CD40 antibody. At day 12 after immunization, we injected a mixture of OVA peptide-pulsed and non-pulsed syngeneic splenocytes to assess the cytolytic activity of *in vivo* primed OT-I cells. Effective cytotoxicity was observed only in lymph nodes and spleen of mice immunized with SIP-CD36-OVA regardless of the co-administration of anti-CD40. In mice primed with SIP-CD36-OVA ~90% of the peptide-pulsed targets were eradicated from lymph nodes, while a small proportion of target cells could still be detected in the spleen (Fig. 25c). Together, these data demonstrate that a single immunization with SIP-CD36-OVA is sufficient to induce a durable formation of effector memory T cells even in the absence of any additional maturation stimuli.

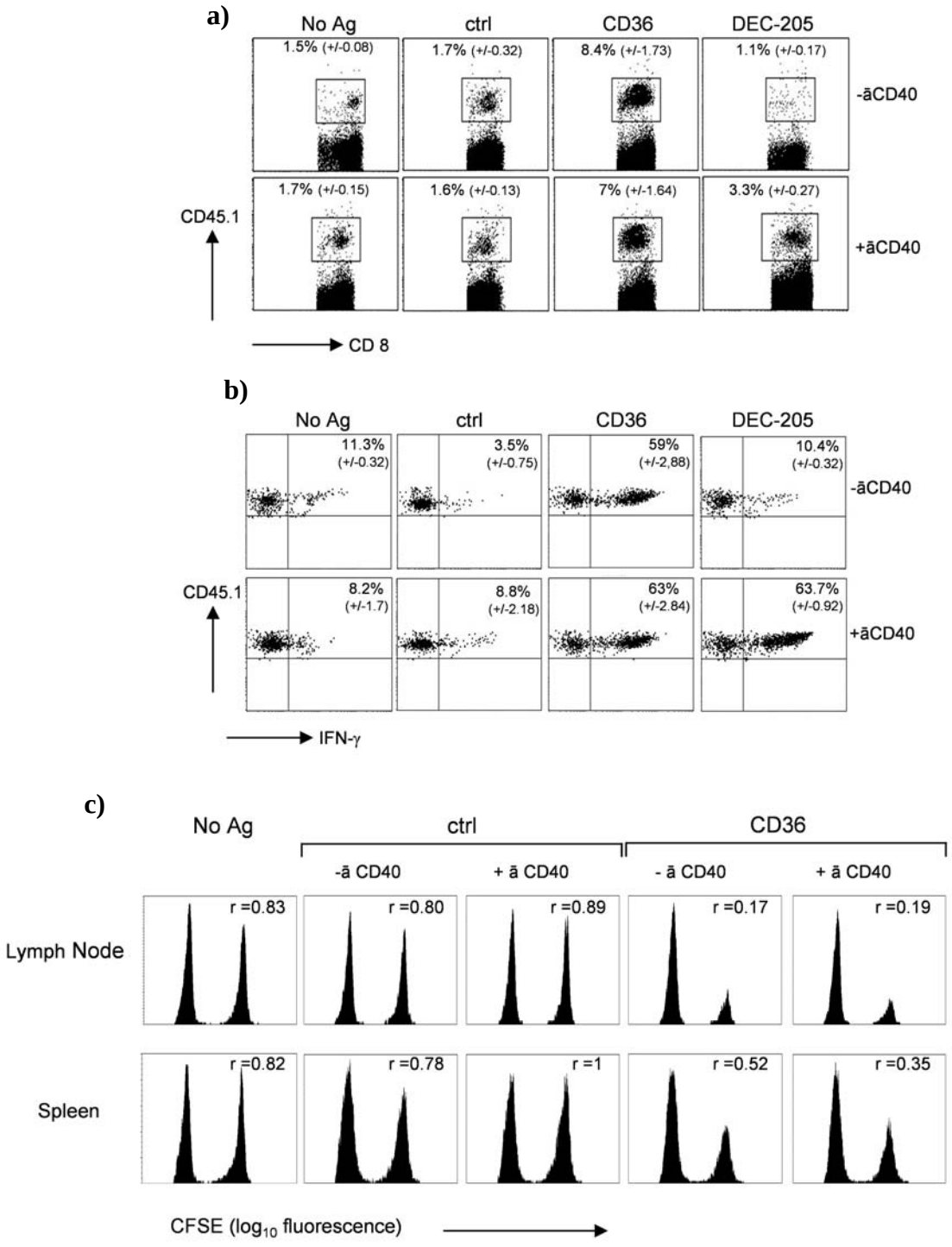


Figure 25. CD36-targeting to steady state DCs induces long lasting cytotoxic T cells. 1.5 x10^6 OT-I (CD45.1^+) T cells were adoptively transferred into CD45.2^+ C57BL/6 mice followed by immunization with 0,3μg of SIP-CD36-OVA, SIP- DEC-205-OVA or SIP-Ctrl-OVA, with or without the addition of anti-CD40 Ab (25μg). Spleens were harvested at day 12 to determine by flow cytometry: **(a)** the percentage of persistent CD45.1^+ /CD8^+ cells (OT-I) and **(b)** their ability to produce IFN-γ, following re-stimulation *in vitro*. **(c)** In vivo cytotoxicity assay. Mice were treated as above. The ability of primed OT-I cells to kill an Ag specific target cell was evaluated at day 12 by injecting mice with a mix of CFSE labelled syngeneic splenocytes pulsed (CFSE^{high}) or not pulsed (CFSE^{low}) with the $OVA_{257-264}$ class I peptide. 15h post-injection, the ratio between CFSE^{high} and CFSE^{low} (r) was evaluated as a measure of specific cytotoxic T lymphocyte activity.

3.3.9 Characterization of the endogenous T cells response elicited by targeting CD36

Having demonstrated the ability of SIP-CD36-OVA to induce priming and differentiation of adoptively transferred OT-I cells into effector memory T cells, we next sought to determine whether targeting OVA to CD36 could activate, as well, the endogenous naïve repertoire that contains low frequency of antigen-specific T cells.

To this purpose, naïve C57Bl/6 mice were immunized twice in 14 days with 500 ng of SIP-CD36-OVA or SIP-Ctrl-OVA. 7 days after the second immunization, lymphocytes isolated from blood were tested for the ability to produce IFN-γ upon *in vitro* restimulation with $OVA_{257-264}$ peptide. As shown in figure 26a, mice injected with SIP-CD36-OVA, but not with SIP-Ctrl-OVA, developed an Ag specific CTL response as demonstrated by the percentage (4%) of IFN-γ secreting $CD8^+$ T cells.

In parallel, we examined the humoral immune response by measuring the titres of OVA-specific IgG antibodies by ELISA. High titres of anti-OVA specific antibodies were found in mice primed with SIP-CD36-OVA, but not with SIP-Ctrl-OVA indicating that targeting Ag to DCs through CD36 is effective in eliciting T cell help for humoral responses (Fig. 26b).

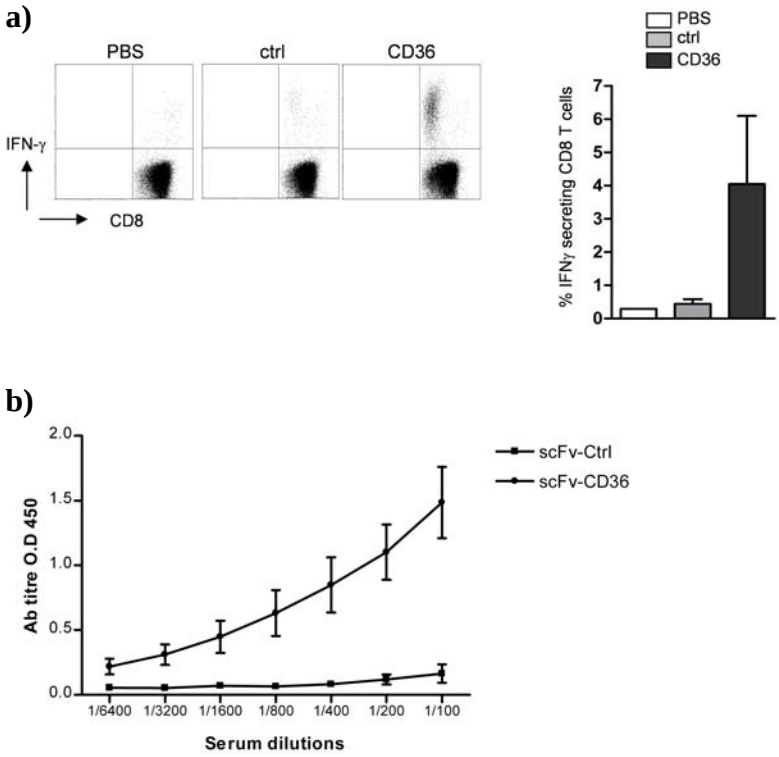


Figure 26. Evaluation of the endogenous OVA specific T cell response. (a) Induction of OVA specific $CD8^+$ T cells. Mice were immunized twice at 1 week interval with an equal amount (0,5μg) of SIP-CD36-OVA (CD36), SIP-Ctrl-OVA (ctrl) or PBS. After 7 days, lymphocytes isolated from blood were re-stimulated in vitro with the $OVA_{257-264}$ peptide. Dot plots show one representative IFN-γ secretion profile in the different groups (left panel). Data expressed as means (n=3) of IFN-γ secreting $CD8^+$T cells (right panel). **(b)** OVA specific IgG Ab titers determined by ELISA from sera of animals immunized as in (a). Ab titre is expressed as means of 5 mice per group.

3.3.10 Tumor protection experiments

We have demonstrated that targeting DCs via CD36 results in the effective induction of OVA specific cellular and humoral immunity. We next sought to determine whether the activation of the endogenous Ag-specific T cell response could confer protection against the graft of a OVA expressing tumour cells. As tumour model we chose E.G7 cells, a C57Bl/6 thymoma transfected with OVA cDNA which is insensitive to NK cell-mediated lysis and to anti-OVA mediated complement-dependent lysis (180) (181).

C57BL/6 mice were immunized at day 0 and 7 with 500 ng of SIP-CD36-OVA or SIP-Ctrl-OVA and 7 days after the second immunization, mice were challenged by s.c injection of $2x10^5$ E.G7-OVA cells into the right flank. Tumour size was measured 2, 3 and 4 weeks after challenge. Results showed that immunization with SIP-CD36-OVA, increased the efficiency of tumour rejection and induced a significant delay in tumour growth compared to mice injected with SIP-Ctrl-OVA ($p=0.0005$). In contrast, immunization with SIP-Ctrl-OVA did not affect tumour rejection and up to 100% of mice developed tumours comparable to not immunized mice (Fig. 27). 50% of mice primed with SIP-CD36-OVA remained tumour free after 40 days. Altogether these results demonstrate that *in vivo* targeting of a tumour Ag to CD36 triggers a strong and protective anti-tumour immune response.

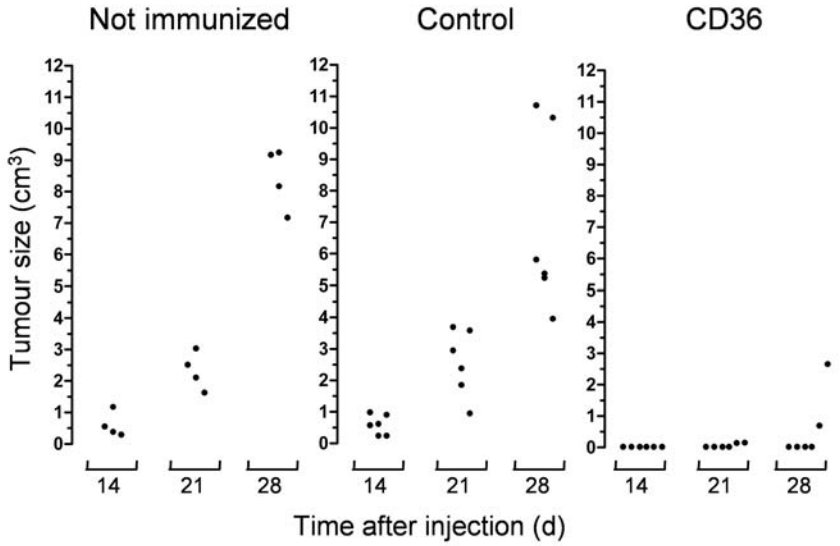


Figure 27. Anti-tumor activity of endogenous T cells primed by CD36 targeted DCs. Growth of EG7-OVA tumor cells s.c injected in C57BL/6 mice not immunized (n=4) or s.c immunized (n=6) on day 0 and 7 with 0,5µg of SIP-CD36-OVA or SIP-Ctrl-OVA. Tumor size was measured 14, 21 and 28 days after challenge. The average tumor size was expressed in cubic centimetres. Data shows one representative out of three experiments.

4 DISCUSSION

Dendritic cells play a prominent role in the orchestration of a broad repertoire of immune responses. This led in the recent years, to the development of novel potential therapies aimed at harnessing DCs immunostimulatory properties for the treatment of chronic infections and cancer, or at silencing/reverting some of their functions during chronic inflammatory conditions, i.e autoimmunity or organ transplantation (182).

The new concept of mobilizing the DCs system directly in situ, within their natural context, has proved feasible in animal models, upon the identification of receptors that are primarily expressed by DCs and the isolation of antibodies directed against these surface molecules. Recent studies have shown that immunization with antigens coupled to antibody that are directed against DCs endocytic receptors greatly increases the efficiency of antigen presentation on both MHC class I and MHC class II molecules and results in the induction of antigen specific T cell mediated and humoral responses (27, 129, 145, 150). So far, these targeting studies have revealed that the efficacy of in vivo DCs vaccination depends on numerous factors that are related not only to the nature and biological properties of the receptor targeted, but also to the specific DCs subset that expresses the receptor (127-129) and on the maturation or activation status of the DCs (85, 86). Besides the intrinsic nature of the receptor and the DCs subset targeted, a second factor that likely affect the outcome of the immune response is related to the selection of an appropriate antibody/receptor pairing. For instance, antibodies directed against the same receptor, but recognizing different epitopes or domains, may differ in their internalization potential and as a consequence, in the amount of antigen delivered into the cell compartments involved in antigen processing (183).

Based on these observations, it is difficult to predict which strategy among the proposed ones is the most efficient on a comparative basis. Thus, there is the necessity to identify new receptors, characterize their mode of action and develop technologies that facilitate comparative studies.

With the aim to improve current existing strategies we set up a screening of phage-displayed libraries of Ab fragments (scFv) on DCs. Whole-cell panning screening is a powerful technique in that it provides new Ab candidates that recognize antigens in their native conformation, an essential requirement for their in vivo application, and it allows the isolation of Abs that trigger a specific biological function (i.e. internalization). In addition, once the phage carrying the scFv with the desired specificity is identified, its sequence can be isolated and modified to create fusion proteins that include antigenic epitopes. This provides us with an easy and rapid method to test new endocytic receptors for antigen presentation. Finally, recombinant scFv proteins can be produced in large amount and subsequently used to identify their cognate receptors.

In this study we provide the proof of principle of such methodology. This is the first report that describes the successful isolation of internalizing antibodies by phage display on mouse dendritic cells and it thus provides the rationale for the further development of such a promising technology (i.e. screening performed on specific dendritic cells subsets or on dendritic cells of human origin).

The initial part of this work has focused on the setting up and optimization of the phage screening procedure. We identified some parameters that are important for the enrichment of phages that specifically bind to DCs (i.e. blocking solution and washing conditions) and are crucial for the preferential recover of endocytosed phage particles (i.e. internalization time and treatment of the cells with subtilisin). Our results show that we successfully applied a procedure to select, from a filamentous phage display library, scFvs Ab fragments internalized by DCs. The fact that after three rounds of panning we recover a pool of phages characterized by similar hypervariable regions, suggests that the conditions under which the screening has been performed may be too stringent, leading to the isolation of the scFv-phage particles with the highest affinity and internalization potential. Therefore, a reduction in the number/stringency of the washings may lead to the isolation of a greater number of different clones. Further analysis of the scFv sequences recovered after two rounds of panning may indeed support this hypothesis. Alternatively, the limited set of scFv-clones may be the result of a bias in the original scFv-phage library. A higher frequency in the original library or a greater stability of these scFv-phagemid may have conferred a growth advantage and thus favored their amplification in each round of selection.

The selected Ab fragments showed a peculiar binding profile on DCs, with only a percentage of the cells staining positively (Fig. 11). We reasoned that this feature was particularly relevant, considering the heterogeneity of the DCs population isolated from mouse lymphoid organs (4) and the specific roles that these DCs subsets play in the initiation of different type of immune responses (3).

We thus focused on the characterization of a dominant clone, scFv-2E5. We demonstrated that it recognizes a putative receptor primarily expressed by the $CD8\alpha^+$ subset of lymphoid organ DCs (Fig. 14a). Thanks to the high affinity of scFv-2E5 for its ligand and taking advantage of standard biochemical techniques coupled to mass spectrometry analysis, we were able to identify the molecule recognized by scFv-2E5 as the mouse scavenger receptor CD36 (Fig. 15).

The finding that other scFvs sharing a sequence similarity in the CDR H3 region did recognize the same receptor, whereas scFv 9 which carry a different sequence did not, suggests that the last 5 aa in the third hypervariable region of the heavy chain may indeed be critical for the binding to CD36.

CD36 belongs to the class B scavenger receptor family. It has been implicated in multiple biological processes that define it as a multiligand scavenger receptor (34). CD36 was shown to be expressed on DCs and cells of hematopoietic origin, including platelets and macrophages, but also on microvascular endothelium, adypocytes and muscle cells. Our results confirm the unique distribution pattern of CD36 among DCs, its preferential expression on the $CD8\alpha^+$ splenic DCs (25, 26), and further demonstrate its lack of expression on Langherhans, interstitial and plasmacytoid DCs (Fig.14). We also detect CD36 on macrophages and on a percentage of B cells, where its expression was shown to correlate with the expression level of the B-cell differentiation factor Oct-2 (184).

CD36, until recently best known as a receptor for thrombospondin-1 (TSP-1), also bind long-chain fatty acids (185), modified LDL (186), anionic phospholipids (38), uric acid (187) and collagen I and IV. It is reported to play a role in diverse cellular processes including foam cell formation (188), fatty acid transport (189) and suppression of angiogenesis (190).

CD36 has also been implicated in the innate immune response to $GRAM^+$ bacteria such *S. aureus* . *Hoebe et al* first demonstrated the selective and nonredundant role of CD36 as sensor for microbial diacylglycerides. Upon binding to bacterial lipoteichoic acid (LTA), CD36 forms a cluster with the heterodimer TLR2/TLR6 within lipid raft, followed by the internalization of the complex. These data collectively indicated that CD36 acts as a facilitator or co-receptor for diacylglyceride recognition through the TLR2/6 complex (40). Due to these multiple functions, it would be interesting to map the epitopes recognized by the various scFvs directed against CD36 and test whether they are able to prevent the binding of the natural ligands or alter some of the biological functions of the receptor.

Few studies have addressed the role of CD36 in APCs. At first, CD36 was shown to be involved in the engulfment and removal of apoptotic cells in human DCs (24) implicating its possible role in the cross-presentation of antigens derived from apoptotic bodies. However, subsequent studies indicated that CD36 is not essential for either cross-priming nor cross-tolerance since $CD36^{-/-}$ APC were fully competent to carry out cross-presentation of Ags derived form apoptotic cells in vivo (25, 26). Most recent data suggest that CD36 is selectively implicated in the presentation of Ags derived from apoptotic bodies on MHC class-II molecules. *Janssen et al.* demonstrated that in Oblivious mice, which carry a nonsense allele of *Cd36* and are equivalent to $CD36^{-/-}$ mice, the $CD4^+$ T cell response to apoptotic cells was abrogated (191).

In the present work we show a new function for CD36 receptor in adaptive immunity.

We produced an antibody based vaccine, by rescuing the sequence that code for the variable regions of the scFv directed against CD36 and fused them to an antigenic unit. As model antigen we chose ovalbumin due to the availability of systems that easily allow to evaluate the presentation of MHC class I and MHC class II epitopes to antigen specific T cytotoxic (OT-I) and T helper (OT-II) lymphocytes. We show that targeting an Ag to DCs via CD36 results in its rapid internalization (Fig. 20) and mediates its processing and presentation on both MHC class I and MHC class II molecules. In vitro, CD36-mediated uptake allows the efficient presentation of Ags found at nM concentration (Fig. 21). In addition, we show that this property is unique of DCs since B cells, that express the receptor and bind the recombinant antibody, were unable to cross-present the OVA epitope to T cells and thus trigger T cells proliferation (Fig. 22).

Most importantly, we showed that our recombinant scFv is able to target DCs in vivo. Immunization of mice with a low amount (0,1μg) of SIP-CD36-OVA elicits extensive proliferation of adoptively transferred antigen specific CTL and TH lymphocytes (Fig. 23-24).

Remarkably, CD36 can be successfully targeted through a monovalent single-chain antibody. We show that targeting with the dimeric form of the recombinant antibody (SIP) did not apparently increased the efficiency of antigen presentation and the extent of OVA specific $CD8^+$ T cells proliferation, indicating that the cross-linking of the receptor is not required to induce its internalization.

These data clearly indicate that targeting antigens to CD36 elicits both CTL and T_H responses. This means that upon internalization, the antibody/receptor complex follows an intracellular route that allows the antigen to gain access to both MHC class I and MHC class II loading compartments.

CD36-TLR2/6 heterocomplex has been shown to target the Golgi compartment upon binding and internalization of bacterial derived lipoteichoic acids (41). Whether CD36 follows a similar route upon targeting with the scFv-OVA protein, remains to be established.

It has been described that the mechanisms of Ag uptake can dictate the intracellular destination compartment and thus determine the presentation of Ag to $CD4^+$ and/or $CD8^+$ T cells. For instance, pinocytosis conveyed OVA to lysosomes for MHC class II-restricted presentation, whereas mannose receptor mediated endocytosis results in the delivery of ovalbumin into stable early endosomal compartment that eventually lead to cross-presentation (126). Moreover, the fate of an antigen captured by receptor mediated endocytosis will depend on the specific intracellular route followed by the receptor. For example, the mannose receptor is predominantly found in early endosomes, whereas DEC205 recycle through late endosomal compartments, property that seem to be crucial for

the presentation of antigens on MHC class II molecules (192). To date, there are no reports describing the specific recycling route followed by CD36. We are therefore performing co-localization studies using early endosomes and lysosomes specific markers to map the internalization pathway of the scFv-OVA/CD36 complex. However, it has to be kept in mind that the intracellular routing of a receptor may also be influenced by the targeting moiety and so by the specific epitope engaged by the antibody or ligand. For example, dectin-1 recycles to the cell surface after binding laminarin, but not after binding glucan phosphate (193). Therefore, the route followed by scFv/CD36 complex will not necessarily be the same of that followed by the receptor upon binding with other ligands.

The type of immune response elicited by targeting different endocytic receptors on DCs may depend on the specific DCs subset that expresses the receptor. For instance, antigens delivered to receptors on mouse $CD8^+$ DCs are presented preferentially to $CD8^+$T cells, whereas antigens targeted to $CD8^-$ DCs are able to mediate strong $CD4^+$ T cell responses.

Ags captured via the C-type lectin DEC205 and the mannose receptors that are both $CD8\alpha^+$ specific are cross-presented. We provide evidences of a further $CD8\alpha^+$ specific receptor, CD36, that induces cross-presentation of endocytosed Ags.

Among lymphoid-organ resident DCs, those expressing the $CD8\alpha$. marker are clearly the most efficient at cross-presenting cellular (119), soluble (121, 122) and pathogens associated Ags (59, 124). The ability of $CD8\alpha^+$ DCs to process exogenous Ags for presentation on MHC class-I molecules may thus depend on the expression of a specialized pathway to promote the generation of MHC class-I peptides from internalized Ags. Thus it is tempting to speculate that targeting any endocytic receptors on $CD8\alpha^+$ would deliver the antigen to this specialized machinery and so lead to cross-presentation.

Although the dichotomy between $CD8^+$ and $CD8^-$ DCs well describe the effects elicited by targeting Ags to receptors such as DCIR-2 (127), dectin-1 (128), CIRE and FIRE (129) on $CD8^-$ DCs, this paradigm may be an over-simplification and may lead to the erroneous view that $CD8^+$ DCs are not so efficient in priming T_H lymphocytes.

In the present work we demonstrate that targeting CD36 on $CD8^+$ DCs results in the formation of both MHC-I and MHC-II/peptide complexes and thus in the active proliferation of both $CD8^+$ and $CD4^+$ T lymphocytes. Most importantly, we show that immunization of mice with the recombinant scFvCD36-OVA protein induces a strong anti-OVA antibody response (Fig. 26b), indicating that $CD8^+$CD36 targeted DCs can trigger both arms of the immune system.

Several studies have shown that targeting DEC205 on $CD8^+$ DCs elicits weaker CD4 T cell responses respect to DCIR-2 or dectin-1 on $CD8^-$ DCs (127, 128). However, this effect may be a peculiar property of DEC205 and be related to the intracellular pathway followed by the antigen upon receptor internalization. DEC205 is indeed very efficient in delivering antigen to the MHC class I presentation pathway, and we also show, in a comparative study, that targeting this C-type lectin receptor is more efficient in inducing OT-I activation than CD36 (Fig. 24b). It is tempting to speculate that upon internalization via DEC205, the majority of the antigen is delivered to the MHC class I loading compartment, leaving only a minor amount available for MHC class II presentation. The situation may be different in case of antigen captured via CD36, where probably a higher amount of endocytosed protein remains available for MHC class II loading. Therefore, a direct comparison of the efficiency of DEC205 and CD36 to activate antigen specific $CD4^+$ T cells it is of crucial importance to better elucidate the properties of the lymphoid organ resident $CD8^+$ DCs.

The capacity of CD36 to deliver antigens to the MHC class I loading compartment is particularly valuable, since it makes this scavenger receptor a good candidate for vaccination strategies aimed at generating potent cellular responses directed against tumours or pathogens that are inefficiently cleared by the humoral immune system.

Our results show that targeting low amount of antigen to DCs via CD36 not only results in the activation of large numbers of TCR-transgenic T cell is vivo, but it is also able to prime the endogenous naïve repertoire that contains a low frequency of antigen/specific T cells (Fig. 26a). A second major concern when designing a DCs based vaccination strategy, derives from the necessity to deliver the antigen coupled to a maturation signal, since mere targeting of antigens to DCs receptors without providing proper activation may result in tolerance in mice (85, 86). These findings are consistent with DC-based therapy studies in humans showing that DC maturation is a pre-requisite for the induction of immunity (194).

The C-type lectin DEC205 has been the prototype target receptor to induce immune responses against various Ags (144, 146, 159). Induction of protective cellular immunity via DEC205 however, required the systemic administration of a second maturation signal in the form of CD40-specific antibody to mimic T cell help. Some receptors such as TLR2, TLR4 and CD40 or FcγRs have an inherent capacity to mature DCs, so that targeting vectors directed against these molecules do not necessitate the addition of adjuvants to induce immunity (143, 149, 150, 195). Moreover, targeting antigens to DCs receptors like CIRE and FIRE results in the induction of humoral immunity in the absence of additional "danger" signals, even though receptor engagement does not seem to directly activate DCs.

In the present study, we demonstrate that when antigens are targeted to DCs through CD36 such additional maturation signal is dispensable to generate long lasting protective immune responses. Specifically, immunization of mice with SIPCD36-OVA, in the absence of CD40 adjuvant effect, induces the differentiation of adoptively transferred $CD8^+$ T cells into long term effector cells (Fig. 25) and more importantly elicits an immune response that protect mice in tumor graft experiments (Fig. 27). Thus, it is tempting to speculate that CD36 provides *per se* a DCs maturation signal. A recent work by *Urban et al*. demonstrated that CD36 ligation on mouse DCs increases the production of the immunomodulatory cytokine TGFβ, but do not directly up-regulates the expression of co-stimulatory molecules (196). It would be interesting to investigate whether triggering of CD36 with our scFv molecule results in the activation of a similar signaling pathway, or whether the engagement of CD36 in our model may activate pro-inflammatory responses by co-engaging TLRs at the cell surface, as shown to occur during the uptake *S. aureus* (42).

Alternatively, the immune response observed in mice vaccinated with SIP-CD36-OVA may be modulated by other cell types that express the receptor. Although we demonstrate that only DCs are able to cross-present OVA captured via CD36 (Fig. 22), this does not rule out an involvement of other APCs, especially B cells, in the presentation of OVA epitopes to $CD4^+$ T cells. As a last possibility, processing of Ags internalized via CD36 may be less selective in consuming Ags for the MHC class I pathway thus leaving it available for the class-II pathway. This would lead to more efficient $CD4^+$ T cell activation bypassing the need of adding exogenously the help provided by the anti CD40 Ab. In favor of these last two hypothesis is the fact that immunization via CD36 induces also high levels of Ag specific Abs (Fig. 26b). Collectively, these data indicate that targeting an Ag to CD36 *in vivo* is a novel approach to initiate protective immune responses and may represent a valuable means to induce $CD8^+$ T cell-mediated immunity.

5 CONCLUSIONS

In this work we provide the proof of concept that screening of antibody libraries on DCs is a powerful tool to identify new antibody/receptor pairings of potential clinical interest. This is the first report that describes the successful application of the phage display technology to isolate internalizing antibodies on mouse dendritic cells and it thus provides the rationale for the design of novel phage screening directed against specific subsets of murine DCs or DCs population of human origin.

In the present study, we focus on the properties of a selected high affinity antibody directed against CD36, a $CD8\alpha^+$ DCs specific receptor whose role in the induction of immune responses is still unclear. Using a recombinant anti-CD36 Ab fused to ovalbumin we demonstrate that *in vitro* targeting of DCs via CD36 results in the processing and presentation of the antigen on both MHC class I and MHC class II molecules. Moreover, we show that *in vivo* delivery of OVA to DCs through CD36 elicits the active proliferation of adoptively transferred $CD4^+$ and $CD8^+$ antigen specific T cells and the differentiation of CTLs into long-term effector cells. Remarkably, such a response is induced in the absence of any added DCs maturation stimulus and thus it differs from that triggered by the targeting of DEC205 receptor, the golden standard to target $CD8\alpha^+$ DCs.

Finally, we demonstrate that immunization of mice with the recombinant anti-CD36 Ab fused to OVA is able to prime the endogenous pool of antigen specific $CD8^+$ T cells, induces the production of anti-OVA antibodies and confers protection against an OVA expressing tumor, in the absence of any additional adjuvancy.

Collectively, these data are of interest for the fundamental understanding of $CD8\alpha^+$ DCs biology and for the potential relevance of the method presented to identify and test new candidate therapeutic molecules.

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