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Antibody-Mediated Delivery of Antigen to Dendritic Cells

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Abstract

Dendritic cells (DCs) are specialized antigen-presenting cells that control T cell responses. DCs play a dual role in inducing and orchestrating adaptive immune responses upon infection but also maintaining T cell tolerance. The superior capacity of DCs to control immunogenicity has initiated the development of DC-targeted vaccines that aim at inducing potent, durable and adjustable immune responses that could be clinically favourable in various human disorders. Specific delivery of antigen to DCs has been assayed in a research setting for a couple of decades and these efforts have now enabled implementation of DC-targeted vaccines in a clinical setting. The present review discusses targeting of DCs with special focus on antibody-mediated delivery of antigen.

Keywords: Dendritic cells; C-type lectins; Targeting; Immune responses

Abbreviations

mAb: Monoclonal antibody; Ag: Antigen; APC: Antigen-presenting cell; CFA: Complete Freund´s adjuvant; CLIP: Class II-associated invariant chain peptide; CLR: C-type lectin receptor; CRD: Carbohydrate recognition domain; DAMPs: Damage associated molecular patterns; DCs: Dendritic cells; EE: Early endosomes; ER: Endoplamic reticulum; HCMV: human cytomegalovirus; HER2: Human epidermal growth factor receptor 2; HEV: High endothelial venule; HIV: Human immunodeficiency virus: Ii: Class II invariant chain; ITAM: Immunoreceptor tyrosine-based activation motif; LE: Late endosomes; MHC: Major histocompatibility complex; OVA: Ovalbumin; PAMPs: Pathogen-associated molecular patterns; PRRs: Pattern recognition receptors; scFv: Single chain fragment variable; TLR: Toll-like receptor; Tregs: Regulatory T cells

Dendritic cells

DC functions

Dendritic cells (DCs) are a heterogeneous group of antigen-presenting cells (APCs) specialized in picking up, processing and presenting antigens (Ags) to T cells [1-3]. Although these cells share many common features, multiple different subsets with distinct phenotype, localization and role in controlling the type of immune response have been identified [3]. DCs are found in most tissues including skin and mucosal surfaces, which are the most common sites of entry for microbial pathogens, but also in secondary lymphoid organs, in which adaptive immune responses to such pathogens are initiated [4]. The different DC subsets express distinct pattern recognition receptors (PRRs), e.g. C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid inducible gene-I (RIG-I)-like receptors [5,6]. Ligands for these receptors include pathogenic structures, also known as pathogen-associated molecular patterns (PAMPs) that enable detection of pathogenic (non-self) molecules. Furthermore, PRRs recognize non-infectious structures of endogenous origin that derive from tissue destruction and cell death - the damage-associated molecular patterns (DAMPs) [5,7]. Receptor activation initiate intracellular signalling that triggers different cellular responses including internalization of Ags, production of cytokines and chemokines followed by induction of adaptive immunity [5,8].

The interaction of DCs with T cells can lead to either different forms of immune responses or to T cell tolerance, depending on both the maturation state and the environmental signals received by the DCs [6,8,9]. In the steady state, immature, migratory DCs act as sentinels in peripheral tissues, continuously sampling the environment for Ags. Upon encounter with antigenic structures, these cells may undergo maturation transforming the immature DCs, having weak T cell stimulatory capacity, into potent T cell stimulating mature DCs [6,10,11]. The maturation process involves redistribution of major histocompatibility complex (MHC) molecules from intracellular endocytic compartments to the cell surface, decrease in Ag internalization and upregulation of the surface expression of co-stimulatory molecules required for T cell activation [6,8,12]. In addition, DC maturation results in a switch in chemokine receptor expression with down-regulation of receptors for inflammatory chemokines and up-regulation of receptors for chemokines produced in secondary lymphoid tissues, which facilitate the migration of DCs from peripheral tissues to these tissues. Immature DCs express a unique repertoire of inflammatory chemokine receptors, e.g CCR1, CCR2, CCR5 and CCR6, that bind to chemokines such as CCL2, CCL5 and CCL20 allowing their access into peripheral tissues [13–15]. When DC mature, many of these chemokine receptors are down-regulated while other receptors are up-regulated, e.g. CCR7 [12,16]. CCR7 recognizes two chemokines, CCL19 and CCL21, which mediates entry of DCs into secondary lymphoid tissues. CCL21 is expressed by endothelial cells of high endothelial venules (HEV) and of lymphatic vessels but also by stromal cells present in the T cell zone of secondary lymphoid tissues [16]. CCL19 is produced by stromal cells and mature DCs in the T cell zone [17]. Besides attracting maturing DCs to

lymphoid tissues, CCL19 and CCL21 also promote extravasation of $CCR7^+$ naïve and memory T cells through HEV thereby orchestrating the encounter of DCs and T cells in the T cell zone of lymph nodes [18]. Arriving in the secondary lymphoid tissues, Ag-bearing DCs efficiently trigger an immune response by T cells that recognize one of the antigenic peptides presented in complex with a MHC molecule on the DC surface membrane [17]. Migration to the lymph nodes also occurs in the steady state, although at lower rates [16]. In this case, antigenic peptides are presented on immature DCs and engagement with a T cell receptor, recognizing the peptide-MHC complex, will in most cases lead to T cell tolerance by induction of unresponsiveness in the T cells, induction of T cell apoptosis, or by generation of regulatory T cells (Tregs) [6,9]. Interestingly, studies have shown that in order for steady state DCs to remain in the immature and tolerogenic state, the suppressive activity of Tregs is required. The ability of DCs to present Ags in a tolerogenic manner constitute a mechanism by which peripheral tolerance complements central tolerance as a means of controlling autoreactive T cells [6].

Antigen presentation by DCs

APCs usually present exogenous Ags on MHC II molecules and activate $CD4^+$ T helper cells, while Ags that are generated intracellularly, like cytosolic Ags of viral origin, usually are presented on MHC I molecules and activate $CD8^+$ cytotoxic T cells [19,20]. However, DCs have the capacity to present exogenous Ags on MHC I as well as on MHC II molecules [21].

Upon internalization of Ags from the plasma membrane, Ag is located in vesicular compartments named early endosomes (EE), characterized by a neutral ph. Endosomal maturation causes fusion of EE with late compartments resulting in late endosomes (LE) displaying lower pH than EE. Further acidification of LE is mediated by fusion with lysosomes containing proteases and hydrolases with low pH optima which can degrade the luminal contents into small peptides for presentation on the MHC II molecules [22,23]. Newly synthesized MHC II molecules assemble (α and β chains) in the endoplasmic reticulum (ER) and associates with the accessory molecule class II invariant chain (Ii) that targets this complex to acidic endosomal compartments, e.g. LE [24]. Here, Ii is proteolysed until only a small piece of Ii, known as CLIP, remains bound in the peptide-binding groove. Upon a conformational change in the MHC II molecule, mediated by chaperones, CLIP is released facilitating loading of antigenic peptides [23,24]. Notably, DCs harbour mechanisms that prevent the rapid acidification of endosomal compartments allowing endocytosed contents to remain intact for a prolonged time which has been associated with enhanced ability to present exogenous Ags on MHC I [25,26].

For presentation of endogenous Ags on MHC I molecules, cytosolic proteins are degraded into small peptides by proteasomal proteolysis, translocated via TAP transporters into the endoplasmic reticulum (ER) for further trimming by aminopeptidases. The optimized peptides are subsequently loaded onto newly synthesized MHC I molecules and transported toward the Golgi stacks and the plasma membrane. The molecular mechanism involved in this exit still remains unknown [27].

The presentation of exogenous Ags on MHC I molecules is termed cross-presentation. Two main routes have been proposed for this mechanism: the cytosolic and vacuolar pathway [22,27]. The cytosolic pathway involves transport of endocytosed Ag into the cytosol for proteasomal degradation followed by translocation of peptides into ER via the TAP transporter and thereby into the classical MHC I pathway [27,28]. Alternatively, several studies have reported that the source of MHC I molecules in this pathway may be recycled MHC I that gain access to retro-transported cytosolic Ags in cellular compartments like the phagosomes and specialized endosomes [27,29,30]. The vacuolar pathway does not require translocation of Ag from endosomal compartments to the cytosol, but utilizes acidic lysosomal proteases for generation of antigenic peptides in the endocytic pathway [27,31,32]. For efficient endosomal peptide/MHC I loading, MHC I molecules must be delivered into the peptide loading compartment, which is achieved by constitutively internalization of cell surface MHC class I molecules [33,34]. A conserved tyrosine residue in the cytosolic tail of MHC I is required for MHC I endocytosis and its targeting to lysosomal vesicles in which internalized peptide-MHC I complexes disassociate in the acidic environment (pH ~ 5) facilitating peptide exchange [31,35]. Endosomal peptide loading may contribute to rapid cross-presentation of endocytosed antigenic peptides decreasing the risk for competition with endogenous peptides present in the ER [22]. Following assembly of peptide-MHC I complexes, these complexes must be translocated to the cell surface. The exact route by which this happens remains unknown but it likely depends on the location for peptide loading. Peptides loaded in the ER are probably transported via the biosynthetic pathway to the cell surface, whereas endosomal loading may rely on the endosomal recycling pathway for surface presentation of peptide-class I complexes [31]. During endocytosis, membrane proteins and lipids are continuously taken up into endosomal compartments and to ensure steady surface display, most of these proteins and lipids must rapidly be returned to the plasma membrane through endosomal recycling. Studies on inhibition of endosomal recycling demonstrated impaired cross-presentation of exogenous Ags, supporting the hypothesis that endosomal recycling pathways are involved in cross-presentation [35-38].

Regardless of the particular pathways used, the ability of DCs to cross-present Ags is an important feature for vaccination strategies aiming at generating potent cellular responses directed against tumours or pathogens that are inefficiently cleared by the humoral immune response [39].

Targeting Dendritic Cells

Since the 1990s, the immunotherapeutic potential of DCs has been explored in clinical settings [39,40]. The idea of harnessing the potential of DCs to induce immune responses, coupled with the capacity to generate large numbers of DCs *ex vivo*, gave rise to *ex vivo* antigen-loaded DC-based vaccines [39,40]. Such vaccines consist of *ex vivo* generated Ag-loaded autologous DCs that are administered to patients with the intention of inducing Ag-specific immune responses [39]. Although these vaccines proved safe, clinical results have been limited. Furthermore, the production of these cell-based vaccines is very laborious and expensive [41]. Most likely, one of the major reasons for the limited clinical effects of vaccination with Ag-loaded DCs is that the DCs, after being manipulated *in vitro*, have a poor immuno-stimulatory potential, when reintroduced into the patient's body.

An alternative strategy to the *ex vivo*-generated DC-based vaccines, is to directly deliver Ags to specific surface receptors on DCs *in vivo*. In this case, manipulation of the cells *ex vivo* is avoided. Instead, the cells are targeted *in situ*, which might benefit from reaching multiple and rare DC populations in their natural environments. Furthermore, such vaccines could be standardized off-the-shelf products bringing this treatment to many more patients than possible with *ex vivo* method.

During the past decades many studies have explored the effect of targeted delivery of Ag to different receptors on DCs and several of these reported augmented immune responses. Nonetheless, it is still important to seek to identify new candidate targets that might induce more effective and longer lasting immune responses. The majority of studies have only investigated one or few target structures and it is therefore not known whether targeting the same Ag to another receptor would elicit markedly different immune responses. In order to evaluate the relative efficacy of different targets, it is important to study the effects of targeting these molecules in parallel. Inspired by this, our group conducted an *in vivo* targeting study that compared the effect of targeting to eight different targets; CD11c, CD36, CD205, Clec6A (Dectin-2), Clec7A (Dectin-1), Clec9A (DNGR-1), Siglec H and PDC-TREM in the absence of adjuvant [42]. The results demonstrated enhanced humoral responses upon targeting to six of the selected targets [42]. As a follow-up, an *in vitro* screening assay was developed [43]. In both of these studies, unconjugated rat mAbs directed against target structures on murine DCs were used as both targeting devices and Ag. The results demonstrated that this very simple approach can be useful in screening of candidate targets for Ag-delivery prior to more complicated studies with relevant targeted Ags. The maturation stage of the DC is of great importance for the ability to activate antigen-specific T cells [6,8]. Maturation may be induced by stimulation of the cell's PRRs. Many of the molecules investigated for their potential as targets for Ag-delivery are indeed PRRs, so that the interaction between DCs and the vaccine induce both internalization and maturation in the targeted cells. Another possibility is to target the Ag to a suitable endocytic receptor on the DC but then include a PRR ligand in the vaccine. DCs carry numerous PRRs and it has been described that the type of PRR targeted influence the character of immune response initiated by the DC [41,44]. Furthermore, Ag-delivery to specific subpopulations of DCs may allow for the design of DC-targeted vaccines that take advantage of the distinct functional characteristics of the different DC subpopulations [41]. It is therefore hoped, that DC-targeting vaccines may be created to induce different types of immune responses appropriate for different clinical situations. In particular, it is hoped that the DC-targeting approach may lead to the development of vaccines able to induce effective immune responses against cancer. Figure 1 illustrates how a DC-targeting anti-cancer vaccine could function. DC-targeting vaccines may likewise be designed for induction of potent immune responses against infectious diseases and possibly also for induction of tolerance as desired in different autoimmune diseases.

Targeting of antigens to specific receptors

Several strategies have been developed to deliver Ag to DC surface receptors. One approach for specific delivery of Ags to DCs is by the use of monoclonal antibodies (mAbs). Antibody-based targeting is performed either by conjugation of the Ag of choice to a mAb specific for selected surface molecules or by genetic engineering in which the Ag is fused to a single-chain fragment variable (scFv) specific for the target receptor [45-47]. Another approach is ligand-based targeting in which Ags are conjugated to pattern recognition receptor ligands, such as Toll-like receptor (TLR) ligands (e.g. CpG) and CLR ligands (e.g. complex oligosaccharides), or to other ligands, such as the chemokine XCL1 [48,49]. A third approach is to use more complex structures, like liposomes or viruses, that carry a receptor ligand or receptor-specific antibody on their surface and in which the Ag or DNA encoding the Ag is incorporated [46,50]. his review

focuses on targeting via mAbs specific for surface structures present on DCs.

CD205/DEC-205: CD205 is a type I CLR containing 10 carbohydrate recognition domains (CRDs) and a specific triad of acidic amino acids in its carboxyl terminus [51]. The human and mouse CD205 are structurally similar, and the only difference is the number of CRDs of which human CD205 contains 10 CRDs while mouse CD205 contains 9. Unlike mouse CD205, which is predominantly expressed on DCs [52], human CD205 is expressed on several cell types, including DCs, monocytes and B, T and NK cells [51]. CD205 is an endocytic receptor with a still unknown ligand specificity [53,54]. CD205 has been described to recognize ligands expressed during apoptosis and necrosis through a pH-dependent mechanism indicating a function as a recognition receptor for dying cells [53,54]. Furthermore, the high-level expression of CD205 on DCs in the T cell area of lymphoid tissues suggests a function in the regulation of T cell responses [55,56]. CD205 is internalized by means of coated pits and vesicles, and recycles through late endosomes or lysosomes rich in MHC II molecules rather than via early endosomes. This distinct intracellular trafficking of CD205 is mediated by the specific acidic amino acids at its cytoplasmic tail [55,57].

Initial studies on targeting of Ag to CD205 using rabbit antibodies specific for mouse CD205 demonstrated a 100-fold more efficient T cell response compared to a non-targeting rabbit IgG [55]. Later, Bonifaz et al. demonstrated that *in vivo* targeting of ovalbumin (OVA) to CD205 induced tolerance in the steady state whereas strong immunity required co-administration of DC maturation signals, like anti-CD40 or CFA [58]. The necessity for DC maturation was supported in a study by Corbett et al. in which targeting of CD205 only triggered antibody responses in the presence of CpG adjuvant [59]. In addition, two separate groups demonstrated that, in the presence of adjuvant, targeting of the human immunodeficiency virus (HIV) Gag p24 to CD205 using a fusion construct induced both high levels of Ag-specific Th1 responses as well as improved cross-presentation and priming of Ag-specific $CD8^+$ T cells [60,61]. Interestingly, a recently published study described potent HIV-specific $CD4^+$ and $CD8^+$ T cell responses in the GI tract upon nasal delivery of HIV Ags to DCs via CD205 [62]. Targeting of Ag to CD205 has also been reported by our group as well as others to induce strong humoral responses, both in the presence or absence of adjuvant [42,60,62]. Several studies have explored the effect of targeting tumour-specific Ags to CD205 as a means of inducing protective and therapeutic immune responses. Mahnke et al. described that targeting of melanoma Ags (tyrosine-related protein-2 and gp100) to CD205 *in vivo* induced potent melanoma-specific CD4 and CD8 responses in mice when a Toll-like receptor ligand (CpG) was co-injected. CD205-targeted vaccination protected the mice against challenge with tumour cells and slowed the growth of an established B16 melanoma tumour [63]. In addition, Bonifaz et al. demonstrated that injection of an OVA-linked mAb specific for CD205 in combination with a DC maturation stimulus (agonistic anti-CD40) primed naïve $CD4^+$ and $CD8^+$ T cells and mediated both protective and therapeutic effects towards an OVA-expressing B16 melanoma [64]. A study by Wang et al. determined the immunogenicity of a CD205-HER2 (human epidermal growth factor receptor 2) fusion protein in a mouse breast cancer model and described that this construct elicited strong $CD4^+$ and $CD8^+$ T cell responses as well as humoral immunity specific for HER2 Ags. In addition, CD205-HER2 vaccinated mice were protected from tumour challenge, a protection mediated by both $CD4^+$ and $CD8^+$ T cells [65]. The first phase 1 clinical trial using a vaccine, CDX-1401, composed of a human mAb specific for CD205 fused to the full-length tumour antigen NY-ESO-1 was published in 2014 (Figure 2). The vaccine proved safe and induced humoral and cellular responses against NY-ESO-1 in patients with tumours that expressed NY-ESO-1 [66].

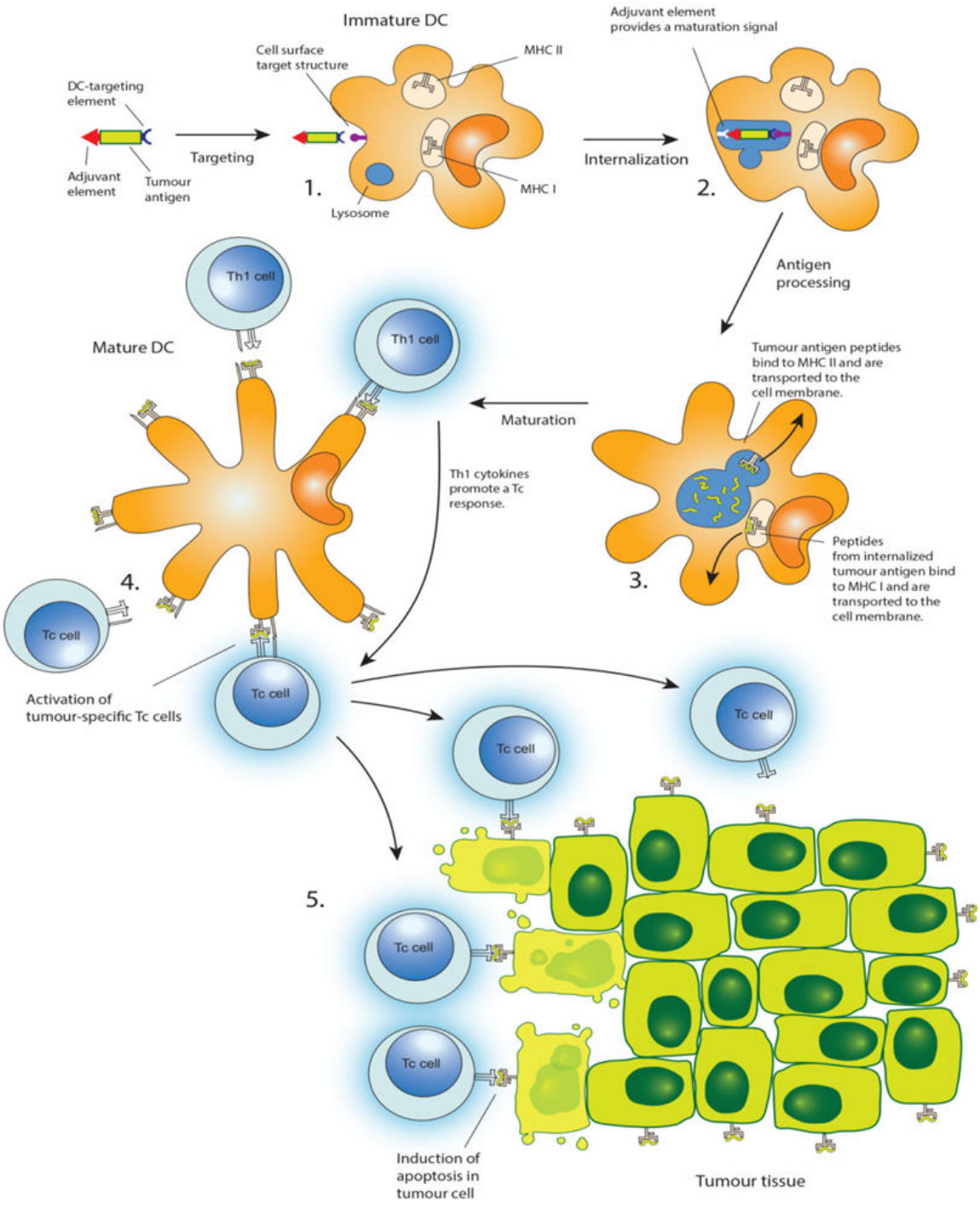

Figure 1: Principle of DC-targeted vaccination against cancer. 1. An anti-cancer vaccine could be composed of three elements; i) the DC-targeting element, which can be an antibody or a scFv of an antibody against the target molecule, or it can be a natural ligand for the target molecule ii) the tumour antigen, and iii) an adjuvant element that initiates activation and maturation of the DCs. 2. Upon interaction with the targeted DC, the vaccine is internalized and the integrated adjuvant element (e.g. a PRR ligand) conveys a maturation signal to the DC. 3. The vaccine is degraded and tumour-specific antigenic peptides are transferred both to MHC II and MHC I molecules. The DC migrates to T-cell-dominated areas of secondary lymphoid tissue while undergoing maturation. 4. The fully mature DC with a high expression of MHC and co-stimulatory molecules activates antigen-specific T helper cells (Th) and cytotoxic T cells (Tc). Tumour-specific Tc proliferates and differentiates into active Tc effector cells supported by cytokines secreted by the activated Th1 cells. 5. The Tc effector cells attack the tumour cells inducing apoptosis in cells exhibiting the cognate peptide/MHC I complexes on their cell membrane.

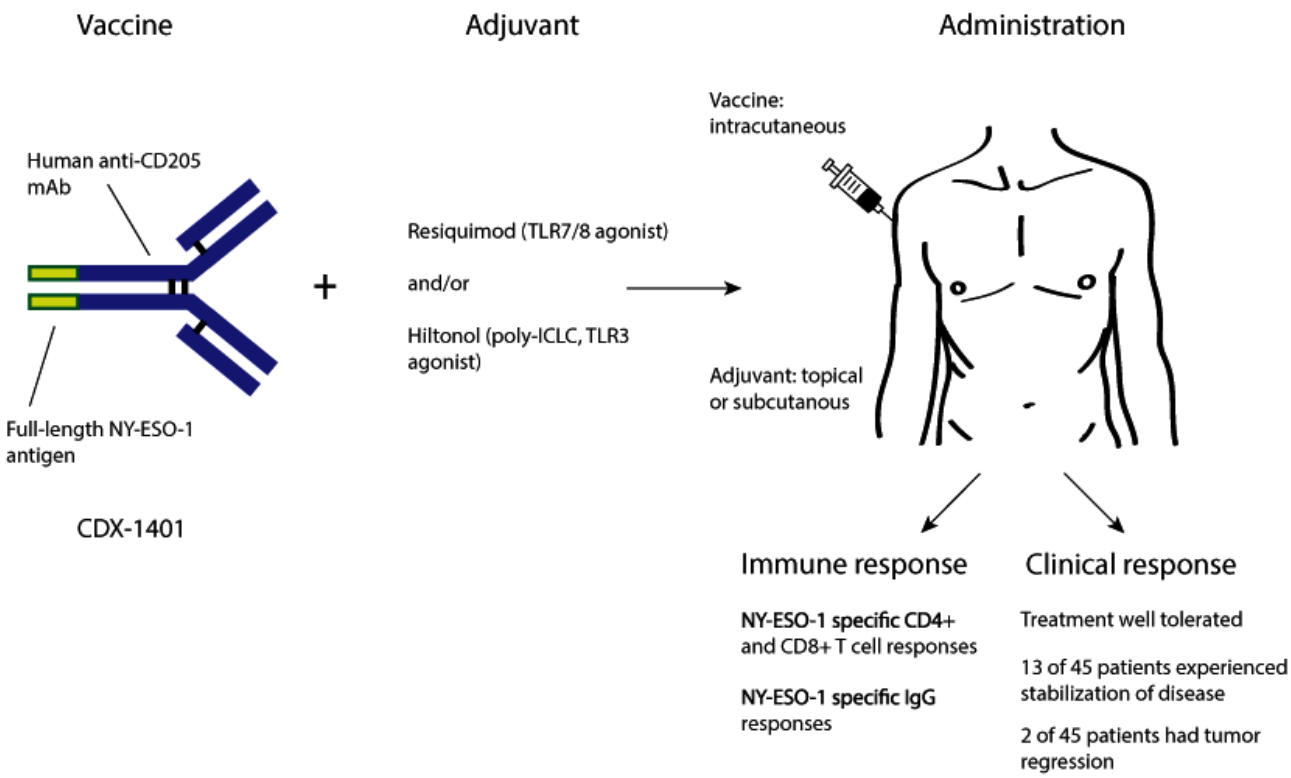

Figure 2: CD205-targeting NY-ESO-1 anti-cancer vaccine CDX-1401. The CDX-1401 vaccine (Celldex Therapeutics) is a fusion protein comprising the tumour Ag NY-ESO-1 in full length fused to a human anti-CD205 antibody. This vaccine was used in the first phase 1 study of a protein vaccine targeting DCs *in vivo* in man. A total of 45 patients with advanced malignancies were enrolled. The vaccine was given in combination with TLR ligands. The figure summarises the results of this phase 1 study [66].

CD209/DC-SIGN: CD209/DC-SIGN (DC-specific ICAM 3-grabbing non-integrin) is a member of the type II CLRs with a single CRD displaying specificity for mannose residues [67]. The structure of the mouse and human homologues of CD209 is very different, but the expression pattern is similar with both receptors predominantly expressed on DCs and some macrophages [68,69]. Human CD209 can interact with several pathogens including viruses, e.g. HIV, human cytomegalovirus (HCMV) and Ebolavirus, bacteria, e.g Mycobacterium, and parasites, e.g. *Leishmania mexicana* [70,71]. In contrast, studies on mouse CD209 could not demonstrate interaction with HIV, HCMV, the Ebolavirus glycoprotein or *Leishmania mexicana*, which may be due to the structural differences of the two receptors [68,72]. CD209 has also been reported to be a cell adhesion receptor that upon binding to ICAM-3 mediates transient interaction between DCs and resting T cells [69]. Furthermore, CD209 functions as an Ag receptor that induces endocytosis of soluble ligands into late endosomal/lysosomal compartments, resulting in processing and subsequent presentation of antigenic peptides in complex with MHC II to $CD4^+$ T cells [73]. The dual role of CD209 in adhesion and Ag uptake provides DCs with a functional receptor that in peripheral tissues takes up Ag and upon arrival in secondary lymphoid tissues mediates interaction with T cells [73].

Based on the great functional differences among human and mouse CD209, mouse CD209 is inadequate as a model for human CD209 explaining the lack of *in vivo* targeting studies on mouse CD209 [46]. Instead, several targeting studies have been performed using the human CD209 [73-77]. A study from 2008 compared the efficiency of *in vitro* targeting of Ag to DCs using either a humanized antibody specific for human CD209 or cell-penetrating peptides (CPPs) [75]. CPPs are positively charged peptides that deliver macromolecules such as proteins, oligo-nucleotides, and plasmid DNA into living cells [78]. The exact mechanism by which CPPs mediate intracellular delivery of their cargo is still not clear, but it has been demonstrated that CPPs enter cells via endocytosis [79]. CPPs have been proposed to favour cross-presentation as they may facilitate uptake and endosomal escape of conjugated Ags [75]. The study demonstrated that CPPs and anti-CD209 were equally potent in mediating cross-presentation of conjugated Ags when targeted to human monocyte-derived DCs *in vitro* [75]. Another study by Tacken et al. demonstrated that cross-linking of a model Ag, keyhole limpet hemocyanin (KLH), to an anti-human CD209 antibody effectively induced both Ag-specific naïve and recall T cell responses *in vitro* [74]. Later on, Tacken et al. evaluated the effect of targeting CD209 through antibodies specific for the neck region of the receptor, as previous studies all used antibodies directed at the CRD of CD209 [76]. They found that anti-neck and anti-CRD antibodies were differentially internalized. Anti-CRD induced a clathrin-dependent internalization and mainly channelled Ags into late endosomal compartments, whereas the uptake of anti-neck was clathrin-independent and shuttled Ags into early endosomal compartments rich in MHC I molecules [76]. This study demonstrated

that intracellular routing of targeted Ags depends on the particular epitope recognized by the targeting antibodies. In addition, Tacken et al. investigated the effect of targeting anti-neck/OVA-conjugates to bone marrow-derived DCs from mice carrying the human CD209 transgene under the promoter of CD11c. The targeted DCs were able to induce proliferation of both $CD4^+$ (OT-II) and $CD8^+$ (OT-I) OVA-specific T cells [76]. Later on, a similar setup with anti-CD209 conjugated to OVA induced strong and persistent OT-II and OT-I T cell responses, which efficiently protected the mice from infection with an OVA-expressing *Listeria monocytogenes* [80].

Clec9A/DNGR-1/CD370: Clec9A is a type II CLR with one CRD and a cytoplasmic immunoreceptor tyrosine-based activation-like motif [81]. Clec9A has been shown to be expressed by the mouse $CD8\alpha^+$ subset of cDCs as well as by mouse pDCs [82,83]. Clec9A is expressed at the cell surface as a glycosylated dimer and can mediate endocytosis but not phagocytosis [81]. Clec9A is a DAMP recognition molecule that senses the presence of necrosis and is specialized for the uptake and processing of dead-cell-associated Ags [11,84]. The dead-cell ligand for Clec9A has been identified as a filamentous form of actin complexed with molecules containing the calponin homology-based actin binding domain (ABD) motif of cytoskeletal molecules [11].

Several studies have investigated the potential of targeting Ags to Clec9A in the mouse [82–86]. Targeted delivery of Ags to Clec9A using mAbs induced a marked enhancement of humoral responses, even in the absence of adjuvants [83,85,87]. Clec9A was reported to elicit long-lived, affinity-matured Ab responses with extensive $CD4^+$ T cell expansion and a high degree of transformation into follicular helper T cells, which are crucial for antibody production [85,86]. Enhanced CD4 and CD8 T cell proliferative responses was also reported upon delivery of different Ags (e.g. OVA, MUC1) to Clec9A either in the presence or absence of adjuvants, which slowed the tumour growth, mediated eradication of established tumours and/or prevented tumour implantation [82,83,88]. Notably, our group observed only weak antibody responses following targeting to Clec9A in the absence of adjuvants [42] which has also earlier been reported by Joffre et al. [42,84]. In addition, Joffre et al. showed that in the absence of adjuvants, targeting of Ag to Clec9A lead to proliferation of Ag-specific naïve $CD4^+$ T cell that differentiated into $Foxp3^+$ regulatory T cells. In contrast, when anti-Clec9A was administered in combination with an adjuvant, tolerance was prevented and targeting promoted development of potent antibody and Th1 or Th17 responses [84]. A study by Lahoud et al. compared Ag-delivery to three different receptors predominantly expressed by CD8α+ DCs, namely Clec9A, CD205 and Clec12A. They found that induction of cytotoxic T cells required co-administration of adjuvants and were mediated by Ag-delivery to Clec9A and CD205 but not to Clec12A [85].

Clec7A/Dectin-1/CD369: Clec7A is a type II CLR with a single CRD and an activating ITAM in its cytoplasmic tail [89]. Clec7A is atypical compared to other CLRs in that carbohydrate recognition is $Ca2^+$-ion independent [90]. In the mouse, Clec7A was originally identified as a DC-specific marker [89], however, later studies demonstrated that both in mice and humans, Clec7A is expressed on several other cell types, including macrophages, monocytes and neutrophils [91,92]. On mouse DCs, the expression of Clec7A has been reported to be restrained to the CD8α- subset of DCs [93]. Clec7A is a major receptor for β-glycans with the ability to recognize and endocytose a number of fungal species including *Candida albicans*, *Pneumocystis jiroveci* and *Saccharomyces cerevisiae* [94]. Upon interaction with such organisms *in vitro*, Clec7A mediates uptake and killing of live fungal particles through induction of the respiratory burst and production of protective inflammatory cytokines and chemokines [94]. Signalling from Clec7A is sufficient for some of these responses, but induction of respiratory burst and proinflammatory cytokines requires cooperative signalling from MyD88-coupled TLRs, like TLR2 and TLR6 [95,96]. Clec7A display other functions beside the anti-fungal activity and can recognize yet undefined endogenous ligands on T cells leading to improved proliferation of CD3-activated T cells suggesting that Clec7A may function as a co-stimulatory molecule [89]; a function supported by its expression on APCs in the T cell areas of secondary lymphoid tissues. Furthermore, human Clec7A can facilitate the uptake of apoptotic cells by DCs mediating cross-presentation of cellular Ags [97].

Carter et al. investigated the effect of targeting of Ags to Clec7A *in vivo* in mice and demonstrated that injection of OVA-anti-Clec7A conjugates, in combination with poly I:C, induced both $CD4^+$ and $CD8^+$ T cell responses as well as OVA-specific antibody responses [93]. A recently published study by our group also reported strong humoral responses following targeting of Ag to DCs through murine Clec7A, however, in the absence of adjuvant [42]. Furthermore, a study using human Clec7A showed that targeted delivery of Ag to human Clec7A also elicited enhanced $CD8^+$ T cell responses [98].

CD11c: CD11c, also known as αX, forms with the β chain CD18 a heterodimeric receptor designated complement receptor 4. The receptor is a member of the family of β2-integrins that also includes LFA-1 and Mac-1. CD11c/CD18 has been reported to interact with complement protein (e.g. iC3b and iC3b-opsonized particles), cell adhesion molecules (e.g. ICAM-1, ICAM-2, and VCAM-1), matrix proteins (e.g. fibrinogen and collagen) and bacterial cell wall components (e.g. LPS) [99–101]. The exact function of CD11c is still not fully elucidated, but CD11c has been described to be involved in phagocytosis, cell migration, cellular adhesion to endothelium as well as in cytokine production by monocytes and macrophages [99,101–103]. The human CD11c/CD18 complex is expressed on several subsets of DCs but also on macrophages, monocytes, granulocytes, activated B cells and some T cell populations [101,104]. The mouse CD11c complex has a more restricted expression pattern and CD11c are widely used as a DC-specific surface marker [105].

We and others have shown strong antibody responses following Ag-delivery to CD11c [42,106]. Interestingly, White et al. described that, apart from the ability to target Ag, anti-CD11c antibodies can deliver a powerful adjuvant effect even if the Ag was targeted to other molecules on the DCs [107]. Castro et al. showed that *in vivo* targeting of Ag to DCs via CD11c resulted in efficient Ag processing and presentation of peptides on both MHC I and MHC II molecules inducing robust $CD4^+$ and $CD8^+$ T cell responses [102]. Furthermore, studies using fusion proteins, consisting of the extracellular domain of human HER2 fused to the single-chain fragment variable specific for CD11c, have shown that vaccination with such fusion proteins in mice induced strong HER2-specific CD4+ and CD8+ T cell responses as well as HER2 antibody responses. Additionally, vaccination protected mice from subsequent challenge with HER2-positive murine breast tumour cells. Therapeutic effects were also seen as vaccination elicited rejection of established HER2-positive tumours [105]. Similar results were obtained in a study using a DNA vaccine coding for the extracellular domain of human HER2 fused to the scFv of a mAb specific for CD11c. In this study, mice immunized with the DNA vaccine in combination with low-dose cyclophosphamide demonstrated effective eradication of existing HER2-positive tumours [108].

Important aspects of targeting antigens to DCs

When using mAbs as Ag targeting devices, the outcome of the immune response induced by DCs depends on several parameters of which some are related to the DC subset that is targeted, whereas others are related to the choice of target receptor or to the particular mAb used [41]. Regarding the influence of the particular mAb, Tacken et al. described that the intracellular routing of Ag targeted to CD209 depends on the particular epitope recognized by the targeting mAb [76]. Similarly, other authors have reported very different outcomes after targeting Clec9A; some anti-Clec9A mAbs induced strong humoral responses in the absence of adjuvant, whereas other anti-Clec9A mAbs were unable to mediate humoral responses in the absence of adjuvant [42,82,83,85,87]. Similar results have been published for targeting of Clec7A and CD205 [42]. Differences in affinity or epitope specificity may explain the very different outcomes [45]. Another prerequisite for an effective immune response is the presence of suitable T and B cell epitopes in the Ag or the vaccine. In support of this, several studies have shown that, when mAbs are used as both targeting device and as Ag, the isotype of the mAb may affect the outcome of targeting [83-85], which possibly arise from a lack of appropriate T cell epitopes in some subclasses, while present in other subclasses [45]. For instance, cancer vaccines should contain both Th and Tc epitopes in order to obtain an effective anti-tumour response.

Other important features of targeting are related to the targeted DC and the targeted receptor. The DC subset presenting the Ag may determine the type of immune response obtained. Consequently, targeting specific subsets can be utilized as a means of inducing specific responses. Thus, *in vivo* studies have demonstrated that the $CD8\alpha^+$ DCs possess a superior ability to present exogenous Ags on MHC I molecules [109,44], whereas CD8α- DCs are more efficient at presenting Ag to $CD4^+$ T cells [59,44,110]. The effect of targeting also depends on how the different target receptors handle Ags for presentation. Several studies have shown that different receptors shuttle Ags into distinct endosomal pathways leading to dissimilar processing and presentation of the Ag [41,57,44,111]. For instance, CD206 and CD40 mediate Ag localization to early endosomal compartments rich in recycling MHC I molecules, which favour cross-presentation of antigenic peptides, however, the Ag presenting efficacy is different for the two targets [111,112]. In contrast, targeting of Ag to CD205 channels Ag directly to late endosomal/lysosomal compartments, which are rich in MHC II molecules. However, several studies have reported superior MHC I cross-presentation upon targeting to CD205 [57,61]. Thus, trafficking to specific endosomal compartments is not the only determinant of the efficiency of targeting. Another parameter that has received attention is the speed of internalization. Initial studies described that slow Ag internalization was correlated to improved Ag presentation by providing a depot of Ag that was continuously released over extended periods providing sustained presentation of peptide-MHC complexes [113]. On the contrary, a recently published study targeting OVA to different DC surface receptors described no correlation between the speed of internalization and Ag presentation on MHC I and MHC II [44].

The route of administration may also affect the outcome of targeting. The choice of injection site could influence the access to different subsets of DCs and affect the pattern of DC migration, ultimately affecting the level of T cell activation induced by vaccination [12]. A study, in which patients with advanced melanoma were vaccinated with Ag-loaded DCs, investigated the effect of intradermal versus intranodal administration and showed superior T cell activation upon intradermal vaccination [114]. Another study reported that intradermal delivery of DCs induced stronger effector T cell activation than intravenous administration [115]. In contrast, a murine *in vivo* targeting study investigated the effect of intravenous, intraperitoneal, or subcutaneous administration and described no significant differences in the humoral responses elicited by Ag-delivery [42]. It is very likely that the optimal administration route depends on the type of vaccine (*ex vivo* Ag-loaded DC vaccines versus *in vivo* targeted DC vaccines), however, the optimal route for such vaccines is still a matter of debate. Interestingly, a recent study shows that the immunological balance within the tissue at the injection sites can direct the elicited immune response either in a stimulatory or a suppressive direction, why the cytokine micromilieu may be manipulated in order to improve the effect of Ag-delivery [12].

Conclusion

Antibody-targeted delivery of Ags is a new way of improving the efficacy of vaccination. So far, a large number of studies in the mouse have reported potent and improved immune responses upon targeted delivery of Ags to DC, substantiating the potential of this approach. However, the translation of these results into a clinical setting is still in its very beginning. Results from the first clinical trial investigating the effect of an anti-CD205/NY-ESO-1 fusion protein vaccine given to melanoma patients have just recently been published [66]. Results from these and future clinical trials will reveal whether the potential of DC-targeted vaccines envisioned based on *in vivo* animal studies will be fulfilled. It is possible that DC-targeting anti-cancer vaccines could become a valuable complement to checkpoint blockade therapies [116] or other forms of immunotherapy. Clinical trials that combine targeted Ag-delivery with different immune-modulating agents (e.g. flt3 ligand, anti-IDO1) are already being carried out (clinicaltrials.gov).

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