Dendritic-cell immunotherapy: from *ex vivo* loading to *in vivo* targeting

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Abstract | The realization that dendritic cells (DCs) orchestrate innate and adaptive immune responses has stimulated research on harnessing DCs to create more effective vaccines. Early clinical trials exploring autologous DCs that were loaded with antigens *ex vivo* to induce T-cell responses have provided proof of principle. Here, we discuss how direct targeting of antigens to DC surface receptors *in vivo* might replace laborious and expensive *ex vivo* culturing, and facilitate large-scale application of DC-based vaccination therapies.

Adjuvant
An agent that does not have any specific antigenic effect in itself, but stimulates the immune system to increase the response to antigens.

Cross-presentation
The mechanism by which certain antigen-presenting cells take up, process and present extracellular antigens on MHC class I molecules to stimulate $CD8^+$ T cells.

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doi:10.1038/nri2173
Published online
14 September 2007;
corrected online
17 September 2007

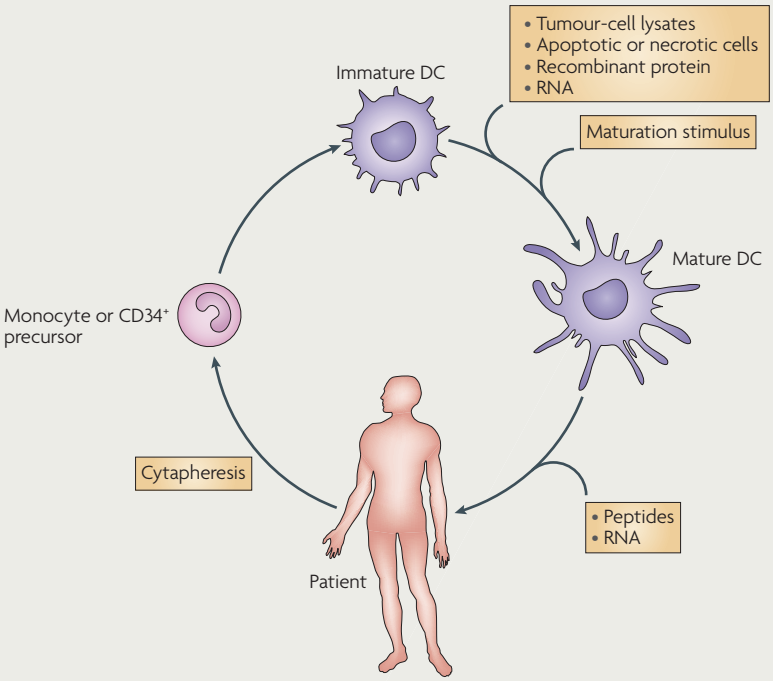
Dendritic cells (DCs) are key regulators of T- and B-cell immunity, owing to their superior ability to take up, process and present antigens compared with other antigen-presenting cells (APCs)[1]. They were introduced as adjuvants in vaccination strategies that aimed to induce antigen-specific effector and memory cells (BOXES 1,2). A more direct strategy involves the loading of DCs with antigens through their surface receptors *in vivo* (BOX 3; FIG. 1). In the mid-1980s it became evident that antibodies enhance specific T-cell responses by promoting Fc receptor (FcR)-mediated recognition of opsonized antigens by APCs[2,3]. This led to the hypothesis that targeted delivery of antigen to cell-surface molecules expressed by APCs might increase T-cell-mediated immune responses. Exploiting bispecific antibodies, Snider and Segal targeted antigen specifically to FcRs for IgG (FcγRs) or MHC molecules on APCs *in vitro*, resulting in enhanced antigen presentation to T cells[4]. These findings were corroborated by *in vivo* studies that showed strong humoral responses to antigens targeted to these cell-surface molecules in the absence of adjuvants[5,6]. Later, the identification of receptors that are more or less specifically expressed by DCs resulted in the development of vaccination strategies that target these professional APCs (TABLE 1). So far, these targeting studies have revealed that the efficacy of *in vivo* DC vaccination depends on numerous factors, including the expression pattern and biological properties of the specific receptor and the maturation or activation status of the DC. In addition, several recent publications have challenged the ideas on how antigens are handled by DCs. These reports cover the areas of cross-presentation, antigen processing by the immunoproteasome and the effect of DC maturation factors on antigen capture, processing and presentation. In this Review, we discuss the implications of these studies for the rational design of novel DC vaccination strategies, mainly focusing on strategies that induce immunity.

Targeting DC receptors

Many receptors used in targeting studies belong to the C-type lectin receptor (CLR) family (TABLE 1). The CLRs are a family of calcium-dependent lectins that share primary structural homology in their carbohydrate-recognition domain (CRD). Through their CRD, the CLRs bind to specific self or non-self sugar residues. Many endocytic transmembrane receptors of the CLR family are expressed by DCs and are implicated in antigen capture[7]. Most CLRs are type II CLRs, which have their amino-termini located within the cytoplasm. CD205 (also known as LY75 and DEC205) and the mannose receptor belong to the type I CLR group, which have their N-termini located extracellularly. Approaches to targeting CLRs fall into two categories: first, strategies based on the binding of natural receptor ligands; and second, strategies that exploit antibodies that are directed against the receptor. In this section, we discuss several targeting studies that focus on the well-characterized CLRs — the mannose receptor, CD205 and DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin (DC-SIGN).

Targeting the mannose receptor. The mannose receptor is expressed by various cell types, including immature DCs (iDCs) and macrophages (TABLE 1). The sugars mannose and mannan have been widely applied in preclinical mouse studies for targeted delivery of antigens to the mannose receptor, resulting in enhanced antigen presentation by MHC class I and II molecules[8]. In a Phase I clinical trial, patients with advanced carcinoma of the

Box 1 | **DC-based vaccines using *ex vivo* loaded DCs to induce immunity**



The recognition that dendritic cells (DCs) are important regulators of immune responses, together with the development of techniques to obtain large numbers of DCs *in vitro* from isolated monocytes, has stimulated research on DC-based vaccination strategies, with the first clinical study being published in 1996 (REF. 109). Most 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 or $CD34^+$ precursors that are isolated from patient blood by cytapheresis, as illustrated in the figure. These cells are cultured in the presence of various cytokine mixtures to produce immature DCs, and loaded with antigen either before or following DC maturation.

These first clinical trials have provided valuable information on DC-based therapy. First, the therapy is safe and well tolerated, side effects are constrained to induration of the skin at the injection site and a mild fever. Second, they emphasize the importance of the quality of DCs, especially their migratory capacity and ability to induce potent T-cell responses. Notably, only a small percentage of the DCs injected in current trials actually migrate from the injection site into the draining lymph node to present the antigen to T cells. This might be due to suboptimal maturation protocols and could be enhanced by preconditioning DCs with pro-inflammatory cytokines or Toll-like receptor agonists[110]. Third, they have resulted in the development of various immunomonitoring tools to study the mechanisms underlying successful vaccination that will help to shape future vaccine design. Finally, these studies have unequivocally demonstrated that DC vaccination can induce immunological responses in many of the patients. So far, studies have generally been pursued in patients with late-stage cancer with a poor prognosis. These patients probably suffer from immunosuppression as a result of a large tumour burden and prior radiation therapy or chemotherapy. This might be one of the reasons why, to date, clinical responses have only been observed in a minority of patients.

breast, colon, stomach and rectum were treated with mannan conjugated to part of the tumour-associated antigen mucin 1 (MUC1). This resulted in antigen-specific humoral responses in half of the patients and cytotoxic T lymphocyte (CTL) responses in a minority of patients, but no apparent clinical responses[9]. A pilot Phase III clinical study on oxidized mannan conjugated to MUC1 in patients with early disease showed promising results. Patients with stage II breast cancer that were treated in this trial, and evaluated 5 years after the last individual started treatment, revealed that all patients receiving immunotherapy were free of tumour recurrences, whereas the recurrence rate in patients receiving placebo was 27%[10]. A large multicentre trial is now in progress to confirm these findings. Although the sugar residues that were used in these vaccination studies bind to the mannose receptor, they lack receptor specificity and probably target the antigens to multiple lectins with overlapping binding specificities[8]. The use of mannose-receptor-specific antibodies has confirmed that antigens which are targeted to the mannose receptor on human DCs enhance uptake and presentation of the antigen by both the MHC class I and class II pathways[11–13]. Notably, the antibody in these studies was generated in mice carrying human immunoglobulin transgenes, resulting in a highly specific, low immunogenic targeting antibody suitable for use in humans.

Injection of the human-mannose-receptor-targeting antibody in human-mannose-receptor-transgenic mice induces humoral responses that can be qualitatively and quantitatively enhanced by the co-administration of an adjuvant[13]. Although B cells can acquire antigen directly from DCs, it remains unclear whether the targeted DCs directly induce B-cell immunity in this way[14]. The humoral responses could have also been induced by a combination of T-cell help induced by the targeted DCs and B cells directly capturing antigen, in this case the targeting antibody. Therefore, DC-targeting therapies that aim to induce humoral responses might benefit from the co-administration of adjuvants and, perhaps, non-targeted antigen.

Targeting CD205. CD205 is a second member of the type I CLR family that is a suitable target for *in vivo* antigen-targeting studies. CD205 recycles through late endosomal or lysosomal compartments and mediates antigen presentation[15]. In mice, CD205 expression is relatively DC restricted: it is highly expressed by mature DCs (mDCs), thymic epithelium, at low levels by B cells and at very low levels by T cells and granulocytes[16]. Therefore, CD205 is an excellent target to study *in vivo* DC targeting. Instead of inducing immunity, delivery of the model antigen ovalbumin (OVA) to CD205 without additional maturation stimuli results in the induction of regulatory T (T_{Reg}) cells and T-cell tolerance[17–19]. By contrast, co-administration of DC maturation stimuli with targeted OVA leads to a strong induction of OVA-specific $CD4^+$ and $CD8^+$ T-cell responses[18,20,21]. In addition to CTL responses, the $CD4^+$ T cells that are induced by this targeting strategy provide long-lived T-cell help for humoral responses[22]. Mahnke and co-workers used a B16 melanoma model to explore CD205 targeting in tumour therapy. Targeting of two melanoma antigens to CD205 together with a DC maturation stimulus cured 70% of the mice from pre-existing tumours[23]. In addition to mouse CD205, human CD205 mediates cross-presentation of the targeted antigen. Antibody-mediated delivery of HIV protein antigen to CD205 in human monocyte-derived DC and T-cell co-cultures induces the presentation of

Box 2 | **Progress in the development of DC-based vaccines**

The development of techniques to generate large numbers of dendritic cells (DCs) *ex vivo* resulted in a number of studies exploring DC-based vaccination strategies. The first clinical study involved B-cell lymphoma patients and used immature DCs (iDCs) that were administered intravenously. The iDCs were loaded with the immunoglobulin protein produced by each tumour (idiotype protein), which is tumour specific as the malignant cells are monoclonal and will produce identical immunoglobulin receptors with unique antigenic variable regions. Subsequent studies varied in the way DCs were generated, loaded with antigen or administered. Some of the key studies are shown in the TIMELINE.

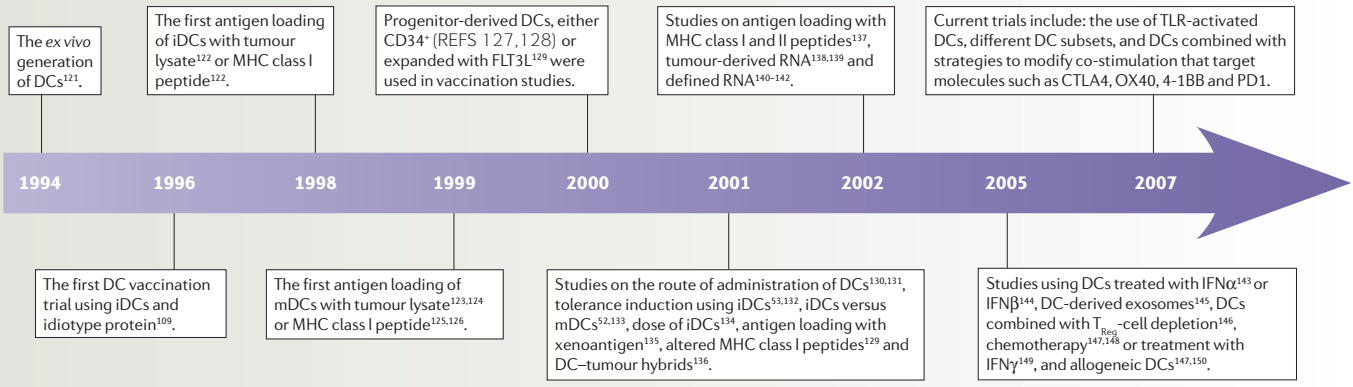
Initial studies used iDCs until it was found that they induced tolerance instead of immunity, resulting in a switch to the use of mature DCs (mDCs). The types of DC used in vaccination studies included FLT3L-expanded DCs, $CD34^+$ DCs, DCs generated with IFNα or IFNβ and allogeneic DCs. DCs were pulsed with peptides, loaded with proteins or transfected with RNA encoding specific antigens (defined RNA). To increase the range of tumour-specific antigens that were presented, DCs were transfected with tumour-derived RNA or fused with tumour cells to generate DC–tumour hybrids. Furthermore, DC-derived exosomes were used for vaccination purposes. Exosomes are membrane vesicles of endocytic origin that are secreted by many cell types. DC-derived exosomes pulsed with peptides are capable of inducing peptide-specific T-cell responses[111]. Several clinical trials evaluated combinations of DC-based therapy with other therapies, such as depletion of regulatory T cells, chemotherapy or administration of IFNγ. Novel strategies that are currently in clinical trials include the use of TLR-ligand-activated DCs, use of various DC subsets and DC-based therapy in combination with strategies that target co-stimulation molecules, such as CTLA4, OX40, 4-1BB or PD1. 4-1BB, tumour-necrosis factor receptor superfamily, member 9; CTLA4, cytotoxic T-lymphocyte antigen 4; FLT3L, FMS-related tyrosine kinase 3 ligand; IFN, interferon; OX40, tumour-necrosis factor receptor superfamily, member 4; PD1, programmed cell death 1; TLR, Toll-like receptor.

many different MHC-class-I-restricted peptides on various HLA subtypes[24]. However, CD205 expression in humans is less DC restricted than in mice, and targeting constructs might therefore be endocytosed by several other cell types as well. Although human CD205 expression levels are highest in mDCs, CD205 is also expressed by B cells, T cells, monocytes, macrophages and natural killer (NK) cells[25].

Targeting DC-SIGN. DC-SIGN is predominantly expressed on iDCs, and at lower levels on mDCs and macrophages[24,26,27]. Unfortunately, the mouse is not suitable as a preclinical model to study DC-SIGN targeting, as multiple forms of DC-SIGN are expressed in mice, which seem functionally unrelated to the human receptor[28,29]. Therefore, the feasibility of targeting DC-SIGN *in vivo* was assessed in a monkey model, using a mouse antibody specific for human DC-SIGN that crossreacts with the cynomolgus monkey homologue. Following injection, the antibody efficiently targets DCs in the draining and mesenteric lymph nodes, as hardly any antibody-free DC-SIGN molecules could be detected (C. Pereira, R.T., K. Hebeda, A. Kretz-Rommel, S. Faas, C.G.F. and G.J. Adema, unpublished observations). Subsequently, the mouse hypervariable domains were cloned into human framework regions, resulting in a humanized antibody with a hybrid IgG2–IgG4 constant domain that prevents binding to FcRs. Targeted delivery to human monocyte-derived DCs of a model antigen conjugated to the humanized DC-SIGN-specific antibody leads to presentation of the antigen by MHC class I and II molecules, and elicits both naive and memory T-cell responses *in vitro*[30].

The CLR targeting approaches that are most likely to enter the clinic in the near future target DC-SIGN, CD205 and the mannose receptor. DC-SIGN is the most DC-specific receptor (TABLE 1), which might be advantageous because the targeting vector will not be scavenged by other cell types that could result in lower targeting efficiencies and possible undesired side effects. CD205, however, seems more potent in mediating cross-presentation *in vitro* than the other two receptors[24]. The fact that CD205 is expressed by different DC subsets when compared with the mannose receptor and DC-SIGN[27,31] makes it difficult to predict which targeting strategy is most likely to be successful.

Timeline | **Progress in the development of DC-based vaccines**



4-1BB, tumour-necrosis factor receptor superfamily, member 9; CTLA4, cytotoxic T-lymphocyte antigen 4; DCs, dendritic cells; FLT3L, FMS-related tyrosine kinase 3 ligand; iDCs, immature DCs; IFN, interferon; mDCs, mature DCs; OX40, tumour-necrosis factor receptor superfamily, member 4; PD1, programmed cell death 1; TLR, Toll-like receptor; T_{Reg} cell, regulatory T cell.

Box 3 | **Pros and cons of *in vivo* targeting versus *ex vivo* loading**

A major advantage of vaccines based on strategies targeting antigens to dendritic cells (DCs) *in vivo* is that they can be produced in bulk quantities, whereas vaccines based on DCs loaded with antigen *ex vivo* require the vaccine to be tailor-made for each individual. In addition, the opportunity to target natural DC subsets *in vivo* might have advantages over loading more artificial *ex vivo* cultured DCs (see table). However, *ex vivo* culture conditions can be carefully controlled, and DC quality can be checked before the cells are administered to the patient. Furthermore, most of the receptors used for *in vivo* DC-targeting strategies are expressed by other cells as well, although it remains to be established whether this significantly affects targeting efficacy. Additional differences between *in vivo* and *ex vivo* strategies might include the duration of antigen presentation and the stability of the vaccine following administration, but these factors will vary considerably depending on the nature of the targeting vector and the *ex vivo* antigen-loading strategy.

	***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

Immunoproteasome
The standard proteasome is composed of 14 α and β subunits, of which three, β1, β2 and β5, are involved in peptide-bond cleavage. Interferon-γ induces the expression of the immunosubunits β1i, β2i and β5i that can replace the catalytic subunits of the standard proteasome to generate the immunoproteasome, which has distinct cleavage-site preferences.

C-type lectin receptor
A receptor belonging to the family of Ca^{+}-dependent lectins that share primary structural homology in their carbohydrate-recognition domains.

Regulatory T cell
(T_{Reg}cell). A specialized subpopulation of $CD4^{+}$ T cells that suppresses immune responses to maintain tolerance to (self) antigens.

B16 melanoma model
A well-characterized model to study tumour growth in C57BL/6 mice. There are many sublines of the B16 mouse melanoma cell line, each with its own characteristics.

Hypervariable domains
Three regions within the immunoglobulin variable region that are highly divergent. Together they form a surface that is complementary to the antigen.

Framework regions
Regions adjoining the hypervariable domains, located at the N terminus of the immunoglobulin.

Humanized antibody
Genetically engineered antibody in which the hypervariable domains of a non-human antibody have been transplanted onto a human antibody.

Receptor usage and quality of response

Owing to differences in intracellular receptor routing, signalling pathways and expression patterns, one can predict that the type of immune response that is induced depends mainly on the receptor that is targeted. Several studies compared immune responses induced by targeting different receptors expressed by distinct DC subtypes in mice: the $CD8^{+}$ and $CD8^{-}$ DCs. Unfortunately, it remains difficult to directly translate the results from these studies to the human situation as the human equivalents of various mouse DC subsets are yet to be identified. For instance, human DCs lack CD8 expression and the human equivalent of $CD8^{+}$ DCs remains elusive[32].

Mouse splenic $CD8^{+}$ DCs were shown to cross-present antigen to T cells *in vitro*, in contrast to $CD8^{-}$ DCs[33]. $CD8^{+}$ DCs express relatively high levels of proteins that are involved in MHC class I presentation, whereas the $CD8^{-}$ DCs express relatively high levels of proteins that are implicated in the MHC class II presentation pathway, indicating that these subsets are specialized in presenting antigens to $CD8^{+}$ and $CD4^{+}$ T cells, respectively[34]. Corbett and co-workers immunized mice, without additional maturation stimulus, with four rat IgG2a antibodies: CD205-specific antibody, F4/80-like-receptor (FIRE)-specific antibody, CD209a (also known as CIRE)-specific antibody and a non-targeting control antibody. FIRE is a member of the epidermal growth factor–transmembrane-7 protein family[35], and CIRE was proposed to be the mouse homologue of DC-SIGN[29,36]; both are expressed on $CD8^{-}$ DCs. CD205 is predominantly, but not exclusively, expressed on $CD8^{+}$ DCs[37,38]. Immunization of mice elicited rat-IgG-specific responses after FIRE and CIRE targeting, but not after targeting of CD205 (REF. 39). These findings seem consistent with a targeting study in which OVA antigen complexed to antibodies directed against dectin-1 (also known as CLEC7A) or CD205 was used in combination with a maturation stimulus. Like CIRE, dectin-1 is a CLR that

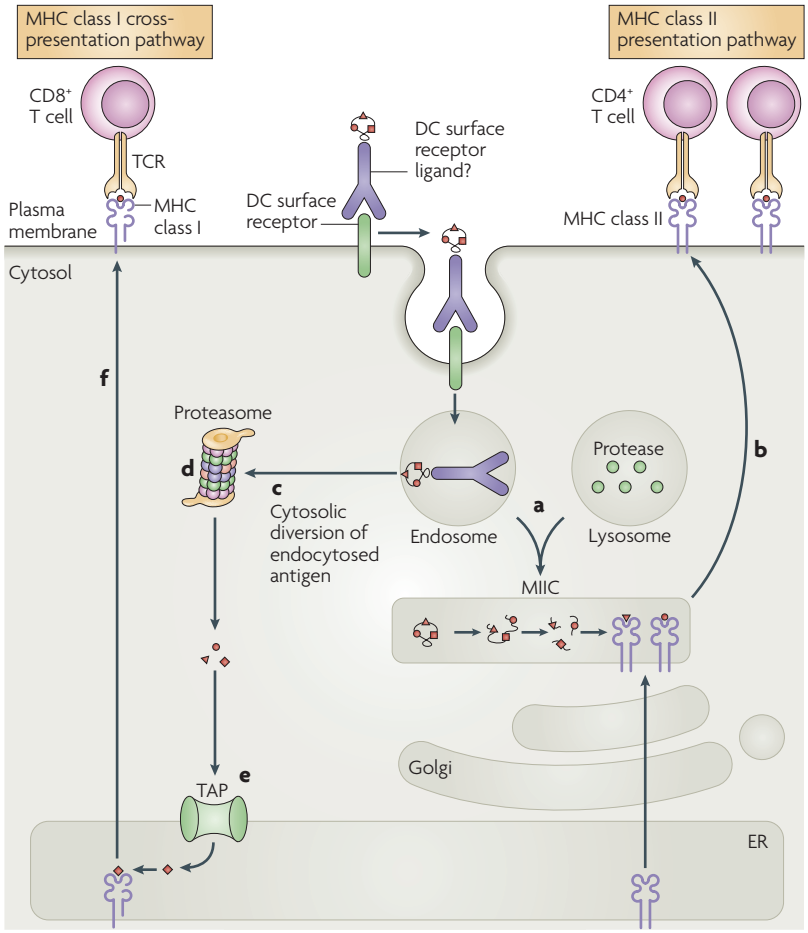


Figure 1 | **Intracellular fate of antigens targeted to DC surface receptors.** Targeting vectors bind to dendritic cell (DC) surface receptors and are internalized. Most targeting vectors enter the DCs by the endocytic pathway. Most of the targeted protein or peptide antigen remains in the endosome, which fuses with protease-containing lysosomes, resulting in degradation of the antigen into smaller peptides (**a**). These peptides are loaded onto MHC class II molecules that reside in the MHC class II compartment (MIIC), and are presented at the cell surface to $CD4^+$ T cells (**b**). However, some DCs are capable of cross-presenting endocytosed antigens on MHC class I molecules to $CD8^+$ T cells. Small quantities of antigen escape from the endosome to the cytosol (**c**) and gain access to the MHC class I processing pathway, although the mechanism of access of exogenous antigen to the MHC class I pathway remains poorly understood. Antigens are broken down into peptides by the immunoproteasome (**d**) and transported to the endoplasmic reticulum (ER) (**e**), where they are further trimmed and loaded onto MHC class I molecules. Subsequently, the loaded MHC class I molecules are transported to the cell surface, where the peptides are presented to $CD8^+$ T cells (**f**). Various strategies have been used to actively direct the endocytosed antigens to the MHC class I cross-presentation pathway. These include methods to enhance endosomal escape, processing by the proteasome and transport into the ER. In contrast to protein and peptide antigens, most viral targeting vectors have an inherent capacity to escape from the endosome, and drive expression of antigens directly into the cytosol, resulting in effective MHC class I loading. TAP, transporter associated with antigen processing; TCR, T-cell receptor.

is expressed on $CD8^-$ DCs. Whereas CD205-specific antibody conjugates mediated strong $CD8^+$ T-cell responses, $CD4^+$ T-cell responses were relatively weak and no antibody response was elicited. By contrast, dectin-1 targeting mediated strong $CD4^+$ T-cell responses, yet $CD8^+$ T-cell responses were relatively weak[40]. A third study shows that targeting OVA to CD205 results in strong $CD8^+$ and modest $CD4^+$ responses, whereas targeting DC inhibitory receptor 2 (DCIR2), a type II CLR that is expressed by $CD8^-$ DCs, has the opposite effect[34]. Taken together, these studies show that antigens delivered to receptors on mouse $CD8^+$ DCs are presented preferentially to $CD8^+$ T cells, but $CD8^-$ DCs are specialized in presenting to $CD4^+$ T cells. However, it should be emphasized that the outcome of targeting different receptors is not merely dictated by the DC subtype that is targeted. Expression of most, if not all, of the receptors used for targeting is not restricted to DCs, and other cell types might modulate the responses observed. Furthermore, the choice of targeting antibody will affect both the efficiency of antigen internalization and the quality of the immune response. Internalization studies with a panel of antibodies directed against the CLR liver- or lymph-node-specific ICAM3-grabbing non-integrin (L-SIGN) reveal that the antibodies are internalized with various efficiencies, which do not necessarily correlate to their binding affinities[41], suggesting that internalization efficiency also depends on the receptor epitopes that are recognized by the various antibodies. In addition, receptors such as DC-SIGN can be successfully targeted through monovalent receptor ligands or single-chain antibodies[41], whereas others such as FcRs require crosslinking to induce internalization. Moreover, continuous triggering of the FcαR with single-chain antibodies inhibits the activating responses of heterologous FcRs[42].

Following the engagement of a specific receptor, antigen presentation will also be affected by intracellular routing of the targeted receptor. In contrast to the mannose receptor, CD205 recycles through late endosomal compartments, which seems to be crucial for efficient presentation of antigens on MHC class II molecules[15]. In addition, the routing of a receptor might be influenced by the targeting moiety. For example, dectin-1 recycles to the cell surface after binding laminarin, but not after binding glucan phosphate[43].

Apart from being differentially routed, specific cell-surface receptors trigger distinct intracellular signalling pathways on ligand binding, thereby modulating immune responses. This is shown by various studies on FcRs. DCs express receptors for IgG (FcγR), IgA (FcαR) and IgE (FcεR). The human FcγR family consists of activating receptors FcγRI (CD64), FcγRIIa/c (CD32a/c) and FcγRIIIa/b (CD16a/b), and the inhibitory receptor FcγRIIb (CD32b)[44]. FcγRI and FcγRIIIa, signal through the FcR γ-chain, which has an immunoreceptor tyrosine-based activation motif (ITAM) and is shared with FcεRI and FcαRI. FcγRIIa signals through an ITAM in its cytoplasmic tail. In contrast to the activating FcγRs, FcγRIIb mediates its inhibitory effect through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail. FcR-mediated internalization of immune complexes of IgG and antigen can result in DC maturation and priming of antigen-specific $CD8^+$ T cells *in vivo*. Whether these IgG-containing immune complexes induce protective immunity depends on the balance between activating and inhibitory signalling by the various FcγRs that are expressed on the DC, as reflected by the potent responses induced by immune

Table 1 | **Receptors used for targeting studies***

Targeted receptor	Receptor family	Expression by human cells	Co-stimulation required for induction of CTL response	Refs
Mannose receptor	CLR	iDCs (low on mDCs), monocytes, macrophages, subsets of endothelial cells, retinal pigment epithelium, kidney mesangial cells, tracheal smooth muscle cells	Yes	11–13, 67
CD205	CLR	mDCs (low on iDCs), thymic epithelial cells, monocytes, B cells, NK cells, T cells	Yes	17–20, 23,34
DC-SIGN	CLR	iDCs (low on mDCs), macrophages, megakaryocytes	Unknown‡	30,112
LOX1	CLR	iDCs, macrophages, fibroblasts, smooth muscle cells, endothelial cells	No	61
Dectin-1	CLR	iDCs (low on mDCs), monocytes, macrophages, neutrophils, eosinophils, B cells, subpopulation of T cells	Unknown§	40
FcγRI	FcR	DCs, monocytes, macrophages, activated neutrophils	Unknown‡	113
FcγRIIa	FcR	DCs, monocytes, macrophages, neutrophils, eosinophils, platelets	Unknown‡	114
FcγRIII	FcR	DCs, NK cells, macrophages, neutrophils, stimulated eosinophils	Unknown‡	115
FcγR	FcR	mDCs (low on iDCs), monocytes, macrophages, neutrophils, eosinophils	Unknown‡	116
CD11c–CD18	Integrin	DCs, monocytes, macrophages, granulocytes, NK cells, activated B cells, certain CTLs	Yes	21
MACI	Integrin	DCs, monocytes, macrophages, granulocytes, NK cells, subsets of T and B cells	No‖	117–120
CD40	TNF-receptor superfamily	DCs, B cells, macrophages, endothelial cells, keratinocytes, fibroblasts, $CD34^+$ haematopoietic cell progenitors, thymic epithelial cells	No	60,90
Siglec-H	Siglec	No human orthologue identified	Yes	108

*Summary of dendritic cell (DC) surface receptors that have been used for the targeting of antigens to DCs. Unfortunately, the expression of most receptors is not restricted to DCs. The table shows the expression pattern of the receptors in human cells. In addition, it indicates whether antigen targeting to the receptor required co-stimulation for induction of CTL responses in mouse studies. ‡No specific *in vivo* targeting studies have been performed, or only humoral responses were assessed. §Conditions without maturation stimuli were not studied. ‖MAC1 was targeted with antigen conjugated to the N-terminal catalytic domain of adenylate cyclase toxin from *Bordetella pertussis*. CLR, C-type lectin receptor; CTL, cytotoxic T lymphocyte; DC-SIGN, DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin; FCR, FC receptor; iDC, immature DC; LOX1, lectin-type oxidized low-density lipoprotein receptor 1; MAC1, macrophage receptor 1; mDC, mature DC; NK, natural killer; Siglec, sialic-acid-binding immunoglobulin-like lectin, TNF, tumour-necrosis factor.

complexes in FcγRIIb-deficient mice[45]. Thus, specifically targeting antigen to ITAM-containing FcRs seems to be a promising strategy to induce immunity. Additionally, reagents to selectively block or activate the activating or inhibitory FcRs now provide tools to skew the outcome of antibody-based therapies towards immunity or tolerance[45]. It has become evident that CLRs, similar to FcRs, have a role in mediating intracellular signalling events. DCIR and myeloid inhibitory C-type lectin-like receptor (MICL) contain ITIMs in their cytoplasmic domains, whereas DC immuno-activating receptor (DCAR) associates with the ITAM-containing FcR γ-chain, and dectin-1 has an ITAM in its cytoplasmic domain[46]. Therefore, triggering of these CLRs is likely to have functional consequences, as was shown for dectin-1, which was reported to signal through the tyrosine kinase SYK (spleen tyrosine kinase), resulting in interleukin-2 (IL-2) and IL-10 production by DCs[47,48]. Furthermore, triggering of dectin-1 (REF. 49) and other CLRs, such as DC-SIGN[50] and blood DC antigen 2 (BDCA2; also known as CLEC4C)[51], modulates cytokine production following Toll-like receptor (TLR) stimulation.

In conclusion, the receptors exploited for targeted delivery of antigens are not inert portals shuttling antigen into the DC. Differences in their expression by distinct DC subsets, the intracellular signalling cascades they induce and their intracellular routing have consequences for the immunological outcome of *in vivo* DC-based therapy.

DC maturation and activation

Maturation and activation of DCs is required for upregulation of co-stimulatory molecules, enhancement of their APC function and expression of chemokine receptors that promote migration to nodal T-cell areas. As discussed earlier, mere targeting of antigen to DC receptors without providing proper activation and maturation stimuli can result in tolerance in mice. These findings are consistent with DC-based therapy studies in humans showing that DC maturation is a prerequisite for the induction of immunity[52,53]. Targeting antigen to iDCs to silence the immune system seems to be an attractive strategy for the treatment of allergy, transplant rejection, autoimmunity and perhaps also for chronic inflammatory diseases. By contrast, vaccination strategies aimed at inducing immunity to fight cancer or infectious diseases need to include means to mature the targeted DC. Two factors are of crucial importance when applying maturation stimuli: timing and route of administration.

ITAM
(Immunoreceptor tyrosine-based activation motif). A structural motif containing a tyrosine residue that is found in the cytoplasmic tails of several signalling molecules. The consensus sequence consists of Tyr-Xaa-Xaa-Leu/Ile, and the tyrosine is a target for phosphorylation by Src tyrosine kinases and subsequent binding of proteins containing SRC homologue 2 domains.

ITIM
(Immunoreceptor tyrosine-based inhibitory motif). A structural motif found in the cytoplasmic domains of many receptors that negatively regulates intracellular signalling complexes. The consensus sequence consists of Ile/Val-Xaa-Tyr-Xaa-Xaa-Leu/Val.

Maturation and activation stimuli can be applied systemically, separate from the targeting vector. Systemic application of adjuvants such as α-galactosylceramide[54], CD40-specific antibody[20] and TLR ligands[55] enhance $CD8^+$ T-cell responses against co-administered antigens. However, applying stimuli too long before or too long after the antigen can impair antigen cross-presentation[56,57]. Furthermore, certain DC receptors, such as CD205, seem to lose their endocytic capacity on full DC maturation[58], abolishing uptake of targeted antigens. In addition to separate administration of antigen and maturation stimuli, both can be combined within a single targeting vector. Several targeting vectors directed against certain receptors, such as TLR2 (REF. 59), CD40 (REF. 60) and LOX1 (lectin-type oxidized low-density lipoprotein receptor 1; also known as OLR1)[61] have an inherent capacity to mature DCs. Alternatively, antigens and maturation stimuli can be physically linked, for example, by packaging them into targeting liposomes[21] (FIG. 2). Linkage of protein antigens to TLR ligands, such as R848 (REF. 62) (which signals through TLR7 and TLR8), CpG[63] (which signals through TLR9) and profilin[64] (which signals through TLR11), was reported to enhance antigen presentation to T cells. Moreover, an elegant study by Blander and Medzhitov shows that both the antigen and TLR ligand need to co-localize within the same phagosome for efficient MHC class II antigen presentation to occur[65]. Instead of using a single maturation stimulus to activate DCs, it could be advantageous to use selected combinations of maturation stimuli. The TLR ligands polyI:C (which signals through TLR3) and R848 act synergistically, resulting in mDCs that are specialized in orchestrating cellular responses[66]. Targeting antigen to the mannose receptor on human DCs in combination with R848 and polyI:C seems to be optimal in inducing T helper cell and CTL responses in cell-culture assays[67].

Antigen processing and presentation

Antigens targeted to DC receptors are concentrated in compartments that are competent for processing by proteases, resulting in the partial degradation of the antigen into peptides that are presented on MHC class I and II molecules. FIGURE 1 shows the intracellular routing of endocytosed targeted protein antigen, which is eventually presented to $CD4^+$ and $CD8^+$ T cells. Advancing knowledge on the intracellular routing of antigen allows for rational design of effective vaccines, as many of the steps shown in FIG. 1 can be manipulated to induce optimal T-cell priming.

Cross-presentation. Whereas exogenous antigens are presented on MHC class II molecules and will readily induce $CD4^+$ T-cell responses, endogenous antigens are generally presented on MHC class I molecules. However, DCs have the capacity to cross-present exogenous antigens on MHC class I molecules. The ability of DCs to cross-present antigen is a major opportunity for *in vivo* targeting strategies aimed at generating potent cellular responses directed against tumours or pathogens that are inefficiently cleared by the humoral immune system. This is especially true because many of the vaccines on the market today induce immunity based on antibody-mediated immune responses. A recent report by Burgdorf *et al.* suggests that whether APCs cross-present an antigen depends on the way it enters the cell. Specifically, OVA antigen that enters APCs by pinocytosis is transported to lysosomes and presented to $CD4^+$ T cells, whereas OVA entering the cell by binding to the mannose receptor is retained for at least 6 hours within early endosomes and presented to $CD8^+$ T cells[68]. Cross-presentation is not an efficient process, and endocytosed soluble antigens seem less efficiently cross-presented when compared with phagocytosed particulate antigens[69,70]. Similar to the conventional MHC class I processing pathway, cross-presentation requires antigen to be processed by the cytosolic-based proteasome. Subsequent transport of the derived peptide antigens into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) results in loading onto MHC class I molecules[71]. Although still under debate, the relative efficiency of cross-presentation by the phagosome route has been attributed to phagosome–ER fusion during or soon after phagosome formation, whereby the phagosome acquires ER-resident proteins, including the protein-translocation SEC61 complex that mediates transport of antigen into the cytosol[72]. Endosomal proteins that escape proteolysis gain access to the ER, from where they may be transported into the cytosol[73]. Much effort is being spent to improve vaccine efficacy by enhancing cross-presentation efficiency.

Facilitating endosomal escape. One possible way to stimulate cross-presentation is to increase cytosolic delivery of the exogenous protein or peptide antigen[74,75]. There are a number of studies on substances that facilitate endosomal escape for the cytoplasmic delivery of proteins or DNA, including various polymeric particles, cell-penetrating peptides (CPPs) and fusogenic peptides. However, most reports fail to unequivocally show that endosomal escape is substantially facilitated, as they rely on sensitive techniques to demonstrate protein (enzymatic activity) or DNA (reporter gene) in the cytoplasm.

Many studies have addressed the ability of CPPs to deliver cargo into the cytoplasm of cells. CPPs are positively charged peptides that are internalized after binding to negatively charged surface proteoglycans. There is much debate over whether CPPs actively facilitate endosomal escape of conjugated cargo[76]. Several studies have reported increased MHC class I presentation of antigens after fusion to CPPs[77–79]. However, the results did not unequivocally demonstrate whether this was due to a CPP-mediated increase in receptor-mediated endocytosis, enhanced endosomal escape, or both. Direct comparison of targeting antigen to DCs by CPPs or a DC-SIGN-specific antibody reveals similar levels of cross-presentation, suggesting that CPPs do not substantially facilitate endosomal escape (P.J.T., B. Joosten, A. Reddy, D. Wu, A. Kretz-Rommel, G.J. Adema, R.T. and C.G.F., unpublished observations).

More promising candidates for cytoplasmic delivery of conjugated cargo are fusogenic peptides, which are based on peptide sequences found in viral transmembrane

Toll-like receptors
(TLRs). A family of membrane-spanning proteins that recognize structurally conserved molecules that are shared by various microorganisms. Signalling through TLRs generally results in immune activation.

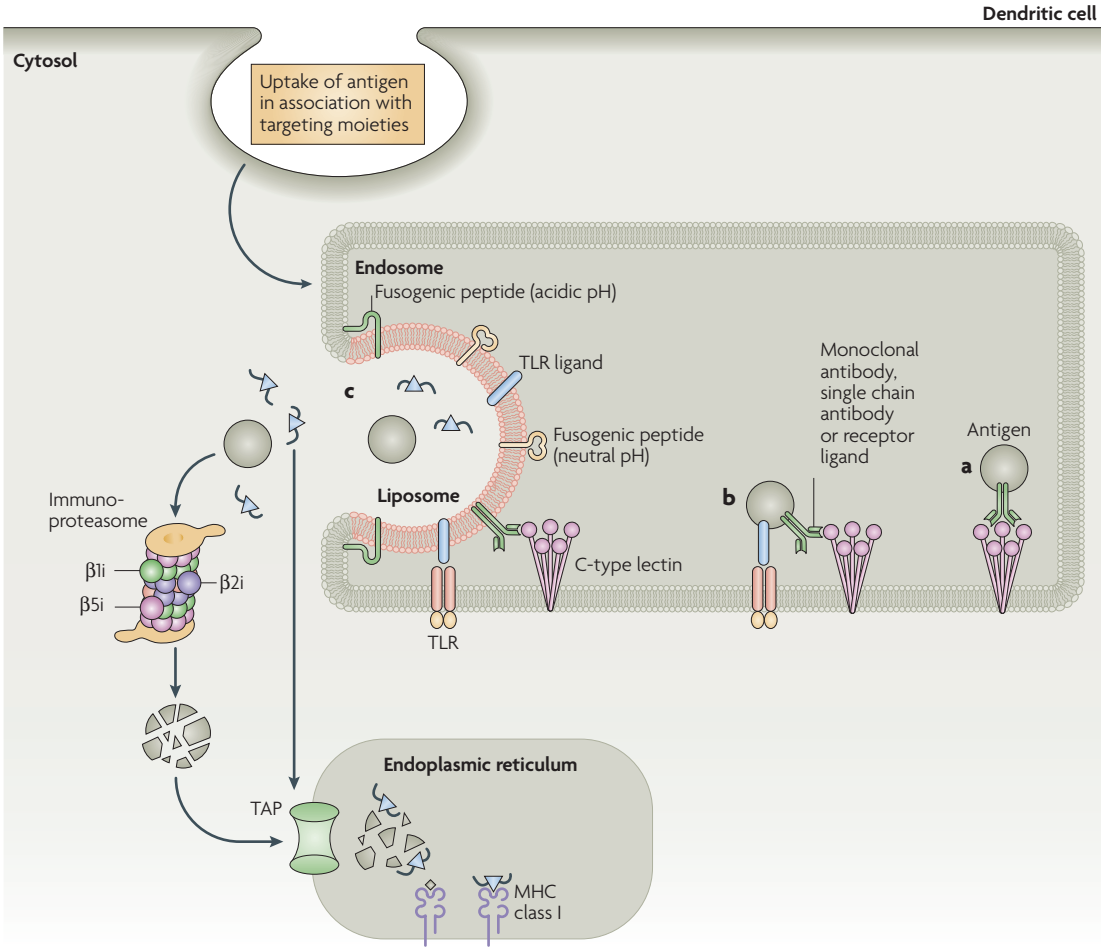


Figure 2 | **Vaccines delivering MHC class I antigens.** Protein or peptide antigen can be targeted to dendritic cell (DC) surface receptors by targeting moieties, such as antibodies or sugars. The antigen can be directly linked to the targeting moiety and be administered in combination with maturation stimuli (**a**). Alternatively, the maturation stimuli, for example, Toll-like receptor (TLR) ligands, can be introduced in the targeting construct itself (**b**). Instead of directly linking the antigen to the targeting moiety, it might be packaged within a microparticle, for example, a liposome, carrying targeting moieties on its surface (**c**). Microparticles have the advantage that they can be readily loaded with multiple protein and peptide antigens, DC maturation stimuli and other factors that enhance antigen presentation, such as fusogenic peptides. Owing to the acidic pH of late endosomes, these fusogenic peptides undergo a conformational change, resulting in leakage and possibly fusion of the liposomal and endosomal membranes, promoting cytosolic delivery of liposomal content. Subsequent to delivery of protein and peptides into the cytoplasm, the immunoproteasome digests the proteins into peptides. Protein degradation by the immunoproteasome generates a peptide pool that differs from that of the standard proteasome, as the immunoproteasome contains the unique β1i, β2i and β5i catalytic immunosubunits. Epitopes exclusively generated by the standard proteasome need to be incorporated into the vaccine as peptide antigens. These peptides, together with those generated by the immunoproteasome, enter the endoplasmic reticulum through TAP (transporter associated with antigen processing), are loaded onto MHC class I molecules and presented to $CD8^+$ T cells.

proteins. Viruses are obligatory intracellular pathogens that have to deliver their genome into the cytoplasm without being degraded in endosomes. Some enveloped viruses circumvent endosomal degradation by entering cells by direct fusion of the viral membrane with the plasma membrane, whereas others, such as influenza viruses, require exposure to the mildly acidic pH within endosomal vesicles to induce membrane fusion. This fusion process of viral and host membranes depends on transmembrane proteins that are anchored on the viral surface[80]. Fusogenic peptides that are based on the N-terminal sequences of the influenza virus haemagglutinin protein destabilize membranes in a pH sensitive manner and have been applied to enhance cytoplasmic delivery of DNA and proteins[81–83]. Laus and co-workers showed that a combination of a fusogenic peptide and a CPP significantly enhances cross-presentation of protein antigen *in vitro*, whereas the fusogenic peptide or CPP alone had no effect[84]. It is likely that both increased endocytosis induced by the CPP and the endosomal escape mediated by the fusogenic peptide are required. In addition, DCs loaded *ex vivo* with protein antigen conjugated to a fusogenic peptide and a CPP resulted in priming of significant CTL responses in mice[84]. For *in vivo* DC-based vaccination strategies, CPPs are not very attractive, as they enter virtually every cell type[85]. By contrast, a

vaccine consisting of protein or peptide antigen coupled to a fusogenic peptide and a DC-specific targeting antibody might be extremely powerful in priming CTL responses (FIG. 2).

DNA vaccination. The use of DNA vaccines circumvents the need for cross-presentation because antigens that are encoded by the DNA are endogenously expressed and access the classical MHC class I pathway. DNA can be administered by various delivery systems, such as live attenuated viruses, bacteria, liposomes, polymer microparticles, bacterial ghosts or virosomes. One example of targeted delivery of DNA complexed to polymer microparticles that has already entered clinical trials is DermaVir, which is composed of a mixture of polyethylenimine-mannose and plasmid DNA that encodes HIV proteins. Although the mechanism of action has not been completely unravelled, it implies transduction of Langerhans cells in the skin that subsequently mature, migrate to the lymph node and present the antigen to $CD4^+$ and $CD8^+$ T cells[86]. Vaccination led to a reduction in viral load in SIV-infected non-human primates with AIDS, induction of antiviral immune responses and increased survival times[87].

Adenovirus-based vectors represent another promising strategy for targeted gene delivery. Adenoviral DNA does not integrate into host chromosomal DNA and is transiently expressed[88]. Despite some safety concerns due to reports of incidental vector-related deaths, many clinical trials involving adenoviral vectors are now under way. Moreover, Gendicine, an adenovirus carrying the human tumour-suppressor gene tumour protein p53 (TP53) has already been marketed in China for the treatment of head and neck squamous-cell carcinoma[89]. Although DCs are relatively resistant to adenoviral infection, this can be overcome by retargeting the virus to specific surface receptors, such as CD40 and DC-SIGN[90,91]. Excitingly, recent reports show that the adenoviral fibre protein, which mediates binding of the virus to its receptor, can be modified to incorporate single chain antibodies[92]. This will allow the generation of adenoviral vectors with an inherent capacity to deliver genes to DCs through a receptor of choice.

Preventing rapid degradation of antigen. Antigens must be degraded for loading onto MHC molecules. This has led to the notion that protease-sensitive antigens might be presented more efficiently than proteins that are relatively resistant to proteases. However, several recent studies show that the endosomal, phagosomal and lysosomal milieu of DCs is much less destructive than was initially thought. Protease activity in phagosomes and lysosomes in DCs seems relatively modest when compared with macrophages[93,94]. Furthermore, delivery of proteases to the phagosome is significantly reduced following maturation of the DC[93]. This relatively mild proteolytic environment seems to be crucial for optimal antigen presentation by MHC class II molecules[94]. Exacerbated antigen degradation might destroy many potential peptides for T-cell recognition and possibly prevent formation of an antigen depot inside the DC, abolishing dissemination of the antigen throughout the lymphatic system. These observations have major implications for DC vaccine design and the choice of antigen in particular, as is elegantly demonstrated in a study by Delamarre and colleagues, who determined the immunogenicity of proteins with the same amino-acid sequence and structure, but with different susceptibility to lysosomal proteolysis[95]. Indeed, only those proteins that were relatively resistant to proteolysis elicited strong antibody and $CD4^+$ T-cell responses[95]. So, vaccines might be significantly improved by modifying the antigen to improve lysosomal protease resistance. Alternatively, antigens can be protected from rapid degradation by incorporation into slow-release systems. Incorporation of antigen into poly(D,L-lactide-co-glycolide) microspheres was shown to result in prolonged and more efficient antigen presentation by MHC class I and II molecules[96].

Apart from the recruitment of proteases, early DC phagosomes acquire the NADPH oxidase 2 (NOX2), resulting in a relatively high phagosomal pH. This limits the hydrolytic capacity of the phagosome, which seems to be crucial for efficient cross-presentation[97]. Strikingly, cross-presentation can be actively enhanced by inhibiting endosomal acidification with lysosomotropic agents. Accapezzato and co-workers showed that vaccine-induced $CD8^+$ T-cell responses are boosted by oral administration of the lysosomotropic malaria drug chloroquine[98]. They treated individuals, who had responded to hepatitis B virus (HBV) vaccination several years before, with or without chloroquine, followed by a booster dose of HBV vaccine. Approximately half of the individuals treated with chloroquine developed $CD8^+$ T-cell responses to the HBV antigen, whereas none of the controls did[98].

The immunoproteasome. For MHC class I presentation, antigens are degraded into peptides in the cytosol by the proteasome, a large multicatalytic protease complex[99]. The standard proteasome is constitutively expressed by most cell types. Interferon-γ (IFNγ) induces the expression of the immunosubunits β1i (low molecular mass protein 2 (LMP2; also known as PSMB9)), β2i (multicatalytic endopeptidase complex subunit 1 (MECL1; also known as PSMB10)) and β5i (LMP7; also known as PSMB8) that can replace the catalytic subunits β1, β2 and β5 of the standard proteasome, resulting in the formation of the immunoproteasome (FIG. 2). However, in APCs such as DCs the immunosubunits are constitutively expressed, and mDCs contain almost exclusively immunoproteasomes.

Although the rate of protein proteolysis by both the standard proteasome and the immunoproteasome are comparable, they target distinct cleavage motifs, thus generating peptide pools that only partially overlap[100,101]. Accordingly, mice deficient in one of the immunosubunits exhibit a T-cell repertoire that differs from wild-type mice[100,102,103]. This might be exploited by vaccines directed against proteins that have been seen by the immune system before, and to which tolerance

Single chain antibody
An antibody consisting of only one heavy and one light chain.

Poly (D,L-lactide-co-glycolide) microspheres
Biodegradable microparticles suitable for drug or antigen delivery, consisting of a polymeric ester of lactic and glycolic acid that is approved for application in humans.

Lysosomotropic
Having affinity for, and thus accumulating in, lysosomes. Lysosomotropic weak bases that are capable of crossing biological membranes selectively accumulate in acidic compartments by protonation, thereby affecting organelle pH and function.

might have been induced, including tumour antigens. Such proteins might contain peptide epitopes that are readily presented by tumour cells, but are poorly generated by DCs because these predominantly express the immunoproteasome. For example, DCs are inefficient in generating the Melan-$A_{26–35}$ peptide from whole protein, in contrast to tumour cells. Therefore, DCs transduced with lentivirus harbouring the coding sequences for Melan-A protein are incapable of inducing Melan-$A_{26–35}$-specific CTLs in mice, whereas DCs transduced with virus containing only the Melan-$A_{26–35}$ peptide sequence do. These studies elegantly demonstrate that precursor T cells recognizing the epitope are available, and can expand properly[104]. Potent DC-based vaccines should therefore contain peptides that are readily generated by the standard proteasome, but not by the immunoproteasome, thus priming T-cell subsets that have not previously encountered this epitope, and limiting the chance of pre-existing tolerance.

Opportunities and challenges

In the previous sections we have distilled ideas from the current literature that describe opportunities to improve *in vivo* targeting strategies. The discovery of TLRs and our advancing knowledge on how DCs discriminate self from non-self provides the immunological community with tools to boost antigen presentation and T-cell triggering. Extensive knowledge of tumour-associated antigens and differences between the immunoproteasome and the standard proteasome provides many novel and effective means to improve vaccines. Studies on antigen routing for MHC class I and II presentation allow for the design of vaccines that effectively deliver the antigen for processing into the desired intracellular pathway.

So far, many targeting studies have been performed using relatively simple antigen delivery systems consisting of antigen conjugated to antibodies or receptor ligands. Sugars targeting CLRs are relatively easy to produce, but lack specificity and might interact with numerous soluble and cell-surface-bound lectins. By contrast, antibodies recognize their targets with high specificity and affinity. Although antibodies can be modified to include an antigen, it might prove difficult to combine multiple antigens, DC maturation stimuli and other features to improve antigen presentation within a single antibody. This will require more complex delivery systems, such as antibodies complexed to liposomes or polymer microparticles. An example of a hypothetical delivery system with many of the features discussed in this Review is shown in FIG. 2: a liposomal vaccine carrying targeting moieties, protein and peptide antigens, a TLR ligand and fusogenic peptides allowing endosomal escape. As discussed above, targeted viral vectors seem to be an attractive alternative for protein and peptide vaccines, but are still in the initial stage of development. It will take considerable effort to show proof of principle, that they are safe and do not confer their altered tropism to other wild-type viruses.

The balance between immunity and tolerance is often shifted towards tolerance, especially in cancer. Although outside the scope of this article, we must not forget that T_{Reg} cells suppress the actions of tumour-specific helper and effector T cells. In cancer therapy, the challenge remains to tilt the balance from tolerance towards immunity. Although potent DC-based vaccines might induce antitumour responses that are strong enough to induce immunity, the possibility remains that the DCs simultaneously expand the T_{Reg}-cell population, diminishing the effect of vaccination[105]. Therefore, DC therapy in combination with T_{Reg}-cell depletion poses an attractive prospect.

Although the current literature describes many opportunities to improve DC-based vaccines, some obstacles remain. First, we are only beginning to understand the signalling pathways of the receptors that have been used for targeting purposes, and we need to discover how signalling of the various receptors affects immunological outcome on targeting. Second, the observation that targeting antigen to receptors expressed on distinct DC subtypes dictates the quality of the T-cell response in mice raises the question of what DC subtype should be targeted in humans. One could target DCs residing in the T-cell areas of lymph nodes directly, using receptors such as CD205 (REF. 31). However, targeting DCs in peripheral tissue might provide the opportunity to instruct T cells to home to specific sites. Peripheral DC subsets include Langerhans cells in the skin, which display good cross-presentation capabilities[106] and could be targeted using the CLR Langerin, and dermal DCs, which can be targeted through DC-SIGN or the mannose receptor. Plasmacytoid DCs (pDCs) are another DC subset that captures antigen through specific receptors and presents it to $CD4^+$ T cells, and could be targeted by blood DC antigen 2 (BDCA2)[51,107]. Although it remains to be determined whether human pDCs cross-present, targeting antigen to sialic-acid-binding immunoglobulin-like lectin H (Siglec-H) on pDC precursors induces antigen-specific $CD8^+$ T cells in mice[108]. Despite basic similarities between mouse and human DCs, it remains difficult to directly compare human DC populations with mouse subtypes owing to differences in cell-surface-marker expression and to the fact that few studies have analysed human DC populations that are freshly isolated from tissues[32]. Studies on human DC subsets are also hampered by the fact that DCs are difficult to isolate in large quantities. Protocols are available to culture DCs with specific subtype characteristics from monocytes or $CD34^+$ precursors, but the question remains how closely they resemble the actual *in vivo* situation[32]. Although these *in vitro* studies have shown that all human DC subsets present exogenous antigen, determining how presentation by the various subsets translates into immunological outcome remains a major challenge. Despite these obstacles, the preclinical studies carried out in mice hold great promise for *in vivo* DC-based vaccination strategies. Moreover, results from clinical trials involving *ex vivo* loaded DCs have now provided proof of principle, giving impetus to the development of novel DC-based vaccination strategies. These vaccines are likely to be safe, relatively inexpensive and provide long lasting, protective immunity or tolerance.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
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CD4 | CD40 | CD205 | CLEC4C | CLEC7A | IFNγ | IL-2 | IL-10 | MUC1 | OLR1 | PSMB8 | PSMB9 | PSMB10 | TLR2 | TP53

FURTHER INFORMATION
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