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Skin-resident antigen-presenting cells: instruction manual for vaccine development
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And

Understanding the biology of antigen cross-presentation for the design of vaccines against cancer
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Chapter 7

General Discussion
Antigen presentation by skin DC subsets

DCs are potent inducers of adaptive immune responses. To achieve this, DCs capture and internalize antigens and present the processed peptides on MHC class I and II to activate antigen-specific CD8+ and CD4+ T cells. Besides the presentation of exogenous peptides on MHC-II and endogenous antigens on MHC-I, DCs are also capable to cross-present antigens, a mechanism wherein exogenous-derived antigens are presented on MHC-I molecules to allow activation CD8+ T cells [1]. Antigen cross-presentation received a lot of attention in the field of tumor immunology, since this mechanism can potentially induce effective antigen-specific CD8+ T cell responses after internalization of tumor-derived antigens by DCs. In this thesis, antigen cross-presentation by distinct human skin APCs was studied (Chapters 3, 4 and 5). Human skin contains a dense network of APCs, which are easily accessible for vaccination through injections in the dermis or transcutaneous immunization [2;3]. However, not all skin APC populations have been described to show equal capacities to (cross-)present antigens and subsequently activate T cells. Most studies describe human LCs or CD1a+ dDCs as potent inducers of CD8+ T cell responses after internalization of protein or peptide antigens, whereas the CD14+ dDCs are referred to as stimulators of T follicular helper cells without the capacity to stimulate CD8+ T cells [4-8]. In addition, CD14+ dDCs have been linked to the induction of humoral immunity and the expansion of regulatory T cells, while the CD1a+ dDCs and LCs are described to skew CD4+ T cells to a T helper cell 1 (Th1) profile [9;10]. Nonetheless, others could not detect CD8+ T cell responses after internalization of measles virus by human LCs, suggesting that LCs were unable to cross-present antigens [11]. Additionally, recent studies also suggest that cross-presentation is not a mechanism restricted to particular DC subsets, but all DC subsets could be stimulated to cross-present antigen when specific requirements are fulfilled, such as the route of antigen internalization and the use of specific adjuvants [12;13]. This thesis adds proof for this hypothesis, by showing in chapter 3 and 4 that the route of antigen internalization is important for distinct DC subsets to be able to cross-present, whereas chapter 5 described that the formulation of the vaccine influences whether a particular DC could internalize and cross-present antigens.

Route of antigen internalization

In chapter 3, we have shown that CD14+ dDCs could be skewed to potent cross-presenting cells after internalization of antigen-encapsulated liposomes via the CLR DC-SIGN. Surprisingly, the CD14+ dDCs were even more potent stimulators compared to the CD1a+ dDCs after DC-SIGN-mediated internalization of LeX-modified liposomes (Chapter 3, Fig. 5). The enhanced cross-presentation capacity of the CD14+ dDCs was completely dependent on the antigen internalization via DC-SIGN, since uptake of untargeted 25 aa long peptides, which require processing before they can be
loaded on MHC-I, was not sufficient to induce CD8+ T cell responses by CD14+ dDCs compared to human CD1a+ dDCs or LCs (data not shown).

More evidence that the route of antigen internalization is important for antigen cross-presentation by specific DC subsets is provided in Chapter 4. Here, we have shown that human LCs were potent activators of CD8+ T cell responses after antigen internalization via the CLR langerin. However, when antigens were targeted to the CLR Dectin-1, no enhanced activation of the T cells was observed compared to isotype controls. Detailed studies in human LCs have shown that langerin-mediated internalization routes antigens to slowly-degradative EEA-1+ endosomal compartments, whereas Dectin-1-mediated internalization of antigen routes it to other EEA-1+, faster degradative compartments (Chapter 4). Previously, the acidification rates of endosomal and lysosomal compartments have been suggested to play an important role in antigen presentation [14;15]. Similar mechanisms might be involved in the langerin-mediated intracellular routing of antigen versus the Dectin-1-mediated route; langerin would facilitate routing to endosomal compartments with a relative high pH, which allows escape of antigenic peptides for loading on MHC-I. In contrast, Dectin-1 might facilitate intracellular routing to EEA-1+ endosomal and LAMP-1+ lysosomal compartments with a lower pH, where antigens are degraded too rapid for cross-presentation.

**Antigen formulation**

The formulation of antigens could have a great influence on specific receptor-mediated internalization as showed in Chapter 5. We have described an enhanced internalization of multivalent, 200 nm sized liposomes by DC-SIGN, whereas langerin was not able to internalize these relatively big nanoparticles, even though the liposomes were modified with LeY, a glycan with known binding affinity to langerin [16]. Surprisingly, human LCs were able to internalize the liposomes when modified with anti-langerin antibodies, suggesting that the binding strength to the receptor is important to allow internalization. In contrast to langerin, receptor clustering into nanodomains and tetramerization has been described for DC-SIGN and DC-SIGN molecules acquire a higher avidity for multimeric ligands when organized in these multimolecular assemblies [17]. Moreover, of all CLRs, including langerin, DC-SIGN has been shown to exhibit the highest affinity enhancement when a second or third mannose residue was added to mannose-BSA, which provides evidence that DC-SIGN binding is more affected by multivalency compared to langerin [18]. In addition, DC-SIGN-mediated internalization of gold nanoparticles (GNP) modified with α-Fucosylamide was increased when the GNP were coated with higher amounts of glycans [19]. In conclusion, DC-SIGN tetramerization and clustering into nanodomains results in a higher binding avidity to multimeric glycan-modified antigen formulations compared to langerin, which facilitate internalization of the antigens by DC-SIGN+ DCs.

Contradictory, human langerin+ LCs were successfully targeted using glycan-
modified synthetic long peptides (Chapter 5). Each peptide was conjugated to a single Le\textsuperscript{y} glycan. This modification facilitated targeting to and internalization of the antigen by langerin, but not by DC-SIGN. Although langerin and DC-SIGN show similarities; like they belong to the type II family of C-type lectins with an extracellular region consisting of a neck involved in multimerization and a C-terminal CRD and show similar specificity for high mannose, they also have differences that influences their function [20-22]. In contrast to DC-SIGN, the intracellular domain of langerin contains a proline-rich motif (WPREPPP) which might be involved in signaling and Birbeck granule formation [22;23]. Langerin is expressed at the plasma membrane, intracellularly in the Birbeck granules and at cytomembrane sandwiching structures, which form at the site where langerin accumulates on the membrane and are the initiation of a Birbeck granule [24]. Due to the presence of langerin in the Birbeck granules and the routing of langerin to Rab5\textsuperscript{+} early/sorting compartments, they are associated with antigen uptake, processing and presentation. However, the precise function and biogenesis of langerin and Birbeck granules are not yet fully elucidated. Interestingly, langerin knock-out mice lack Birbeck granules, but do not have an apparent phenotype and has been shown to respond similar as wild-type mice to tumors, bacteria, yeast and parasites [25]. Altogether, these data demonstrated that both langerin and DC-SIGN could be targeted in vivo using glycan-modified antigen formulations, but the size and multivalency of the formulation is critical to allow receptor-mediated internalization by human LCs and dermal DCs (Figure 1).

![Image](image.png)

**Figure 1.** Langerin, expressed by human epidermal LCs, facilitates the internalization of glycan-modified antigenic peptides, whereas DC-SIGN\textsuperscript{+} DCs present in the dermis can be are targeted with multivalent, glycan-modified liposomes.
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Glycans vs antibodies
The use of glycans instead of antibodies to target CLRs on skin DC subsets has advantages. Glycans are natural structures for the human body and therefore non-immunogenic and can be produced in large scale with relatively low costs[26]. Monoclonal antibodies, even when humanized, can be immunogenic in patients and are expensive to produce [27]. In addition, presenting multiple glycans in a multivalent fashion could lead to a high binding affinity by triggering receptor clustering on the plasma membrane, as described for the DC-SIGN targeting using Ley-modified liposomes in Chapter 5. Potentially, multiple CLRs on various DC subsets can be targeted using a single glycan-modified vaccine to induce a broad range of immune responses. For instance, Chapter 5 of this thesis has shown that the glycan Ley interacted with both DC-SIGN and langerin, thereby targeting human CD14+ dDCs and LCs simultaneously.

Adjuvants
CLR-mediated internalization of a DC-targeted anti-tumor vaccine in general does not result in maturation of the DC. This lack of DC maturation could potentially lead to tolerance, especially in an immunosuppressive environment. To ensure potent antigen presentation and T cell priming by the DCs, adjuvants are often included in DC-targeting anti-tumor vaccines. The combined administration of DC-SIGN targeting glycan-modified liposomes with the TLR4 ligand LPS has been shown to increase antigen cross-presentation and induction of CD8+ T cell responses by in vitro human moDCs [28]. However, human skin DC subsets seem not to mature upon addition of LPS after isolation and in vitro culture (Chapter 2) or intradermal injection (Chapter 6). Anti-tumor peptide vaccination in combination with the topical application of Aldara skin cream resulted in significantly higher migration of phenotypically mature CD1a+ dDCs with potent antigen-specific CD8+ T cell stimulatory capacities (Chapter 6). Intradermal administration of soluble TLR ligands did not induce these responses. Furthermore, adjuvants can greatly influence CRL-mediated antigen internalization and (cross-) presentation. For DC-SIGN we have shown that the intradermal vaccination of glycan-modified liposomes in the presence of GM-CSF and IL-4 as adjuvant resulted in enhanced DC mobilization and induction of antigen-specific T cell responses (Chapter 3). When we studied langerin-mediated internalization of an anti-tumor peptide vaccine by isolated human LCs, we observed a significant increase in antigen cross-presentation when the vaccine was combined with the TLR3 ligand pI:C (Chapter 4). These observations illustrate that for each human skin DC subset and the DC-targeting receptor it is important to identify which adjuvant is best to combine with DC vaccination in order to obtain most effective anti-tumor immune responses. Thus, combining these strategies with DC-targeted anti-tumor vaccination will be a promising area for further investigation to improve therapeutic outcome (Figure 2).
Figure 2. Immunotherapeutic strategies to enhance anti-tumor immunity. Generation of a large pool of effector TA-specific T cells is induced by the intradermal injection of anti-tumor vaccines. Targeting of the vaccine to a particular skin DC subset is facilitated by modification with specific glycans that bind either to DC-SIGN or langerin. Subsequent vaccine internalization induces presentation of TA-Ag and maturation of the DCs. Matured DCs migrate to draining lymph nodes to prime TA-specific CD8+ T cells and CD4+ T helper cells, leading to a large pool of cytotoxic effector T cells that are capable to infiltrate the tumor lesion and lyse tumor cells. Priming of TA-specific T cells may be enhanced by inclusion of immunostimulators such as GM-CSF and IL-21 in the DC-targeting vaccine. Systemic or intra-tumoral administered check-point inhibitors, such as anti-PD-1 and anti-CTLA-4, release the break on the anti-tumor immune response by limiting the activity of suppressive Treg and alleviate the priming and/or function of TA-specific CTLs. Similarly, anti-tumor immunity may be enhanced by manipulation of the local micro milieu via administration of DC activating antibodies (e.g., anti-CD40) or TLR ligands that act directly on the tumor cells. It is anticipated that these strategies may enhance the efficacy of DC-targeted vaccination. Tc, cytotoxic CD8+ T cell; Th, T helper cell.

**Plasticity of human DC subsets: CD14+ dDCs as targets for cancer immunotherapy**

DCs are not the only APCs present in the human skin. Besides epidermal LCs, CD1a+ dDCs and CD14+ dDCs, also a population of CD14+ macrophages is described to be present in the human dermis [8;29]. These dermal macrophages are distinct from the CD14+ dDCs, since they were autofluorescent by flow cytometry analysis, were adherent, nonmigratory and did not express CD1b and CD1c, although both APC subsets express CD14 [30;31]. Whether the CD14+ dDCs are truly DCs is currently under debate, since McGovern et al. have shown recently that human CD14+ DCs...
are not related to the human DC lineage, but are monocyte-derived macrophages that are resident in healthy skin [8]. Comparative transcriptomics analysis showed that the human CD14+ dDCs are the homolog of murine CD11b+LY6C-CD64lo-hi monocyte-derived macrophages. Despite the homology to a macrophage-like phenotype, dermal CD14+ DCs have been shown to migrate spontaneously from human skin and were able to stimulate naive T cells to a higher extent than the human dermal macrophages, albeit relatively low compared to CD1a+ dDCs [8]. In addition, no differences were observed in the capacity to stimulate recall memory T cell responses to Candida albicans between the CD14+ dDCs, CD1a+ dDCs and dermal macrophages after a pulse of the APC subsets with Candida albicans for 24 h and co-culture with autologous blood CD4+ T cells [8].

Another study also investigated the lineage relationship of the human skin DCs by hierarchical cluster analysis on gene expression profiles [32]. These authors showed that LCs clustered most closely to CD1a+ dDCs, while the CD14+ dDCs were forming a separate cluster based on gene expression profiles of PPRs, such as TLRs and CLRs [32]. Interestingly, they also described a phenotypic conversion or maturation of CD14+ dDCs after migration, suggesting that CD14-expressing cells are precursors for the CD1a+ dDCs [32]. Additionally, others also describe the capacity of cutaneous DCs and macrophages to differentiate into each other, guided by environmental cues [33-35]. These data provide evidence that human skin-resident APC subsets are not fixed subsets, but represent a rather flexible network of cells which phenotype and function are dictated by the microenvironment.

Although the CD14+ dDC are described as poor inducers of allogeneic T cells, we have shown that these cells have the capacity to stimulate CD8+ T cell clones in an antigen-dependent manner (Chapter 3). These responses were attributed to the DC-SIGN-mediated internalization and intracellular routing of the antigen, resulting in enhanced presentation of the antigen to CD8+ T cells. Furthermore, our liposomal vaccine was applied intradermally in whole skin explants in the presence of the cytokines GM-CSF and IL-4. These cytokines has been shown to enhance the migration of cutaneous DC subsets and induce an upregulation of DC-SIGN expression on CD14+ and CD1a+ dDCs (Chapter 3 and 6, [36]), thereby boosting the capacity of CD14+ DC-SIGN+ cells to capture the liposomes and migrate to the draining lymph nodes.

Combinational therapies: strategies to improve DC-based cancer immunotherapy

One of the major hurdles for the induction of efficient anti-cancer immune responses is overcoming the immune suppressive environment generated by a tumor. Many tumors employ cellular and soluble factors that suppress DC and T cell activation and function [37;38]. Therefore, strategies need to be designed that combine DC-targeted vaccination with mechanisms to boost the immunogenicity of the tumor and tumor microenvironment and enhance DC function.
Chemo- and radiotherapy

Some chemotherapies and radiotherapies has been shown to induce immunological cell death of tumor cells by revealing DAMPs that enhance DC function directly [39;40]. Chemotherapeutic drugs can also directly enhance the activation, migration and antigen presentation capacities of DCs, which resulted in a more potent anti-tumor response. Therefore, combining DC vaccination with chemo- or radiotherapy could be a successful strategy to improve clinical outcome. It already has been reported that glioblastoma multiforme (GBM) patients receiving autologous DC vaccination and subsequent chemotherapy exhibited longer recurrence times and survival compared to patients receiving only vaccination or chemotherapy [41]. In addition, administration of low-dose cyclophosphamide 1-2 days prior to immunization with a GM-CSF-secreting, whole cell vaccine (GVAX) resulted in a decrease in tumor size using a transgenic murine prostate cancer model that was not observed with the vaccine alone [42;43].

Immune checkpoint inhibitors

Tumor antigen (TA)-specific CTLs present in peripheral blood lymphocytes (PBL) or at tumor sites have been shown to up-regulate PD-1 expression, which regulates their expansion negatively [44-46]. Next to PD-1, also the inhibitory receptors Tim-3 and LAG-3 can be upregulated on tumor-infiltrating T cells and serve as markers for exhausted T cells. By contrast, anergic T cells are characterized by BTLA expression [47]. Notably, BTLA has been detected on spontaneous Mart-1- and NY-ESO1-specific CD8+ T cells in advanced melanoma patients [48;49]. The observation that PD-1 block does not alleviate the function of TA-specific CTLs on a per-cell basis argues in favor of combining this strategy with blocking other immune check-point inhibitors. Indeed, studies performed in patients and in mice with advanced melanoma showed that blockade of both PD-1 and Tim-3 acts synergistically to enhance TA-specific CD8+ T cell numbers and functions, resulting in decreased tumor growth [50-52].

It has been shown that TA-specific CD8+ T cells exhibited variable levels of dysfunction, which correlated with a specific expression pattern of markers [49]. BTLA blockade has been shown to act in concert with PD-1 and Tim-3 blockades to further enhance NY-ESO-1-specific CD8+ T cell expansion and function [49]. The specific combination of inhibitory and anergy-related molecules might indicate a hierarchical loss of T cell function in patients with advanced melanoma in context of chronic antigen stimulation. Moreover, BTLA expression is inversely correlated with CD8 T cell maturation and thus anergic BTLA+ T cells are likely to represent young TA-specific CTLs.

Alternatively, these approaches may be even more active when combined with other agents that activate or inhibit key molecular regulators of T cell function, such as, for example, the tryptophan converting enzyme indoleamine-2,3-dioxygenase (IDO) and membrane-bound CD39 and CD73 that breakdown arginase. IDO is highly
expressed in both tumor and immune cells in the tumor-environment and implicated in inhibiting anti-tumor immunity by promoting the induction of anergic and/or regulatory T cells [53;54]. Importantly, using pre-clinical animal melanoma models it was recently shown that IDO is responsible for mediating resistance to anti-CTLA-4 and anti-PD-1 therapy [55]. Two IDO inhibitors have entered clinical trials: the tryptophan analog 1-methyl-tryptophan (1-MT) and the enzymatic inhibitor of IDO termed INCB024360. Both IDO inhibitors have been effective in pre-clinical models, attenuating tumor growth in wild type but not immuno-deficient mice [56;57]. INCB024360 has now entered Phase II trials, where it will be tested as a monotherapy in ovarian cancer and as a combination therapy with ipilimumab (anti-CTLA-4) for metastatic melanoma.

Recently, combining PD-1/PD-L1 blockade with anti-cancer vaccination has shown to result in better therapeutic effects in pre-clinical murine studies[58;59]. Blockade of PD-L1 signaling during DC vaccination showed better therapeutic effects than DC vaccination alone by preventing tumor growth and prolonging survival times in a breast tumor-bearing human SCID mouse model [59]. In addition, using anti-PD-1 antibody and a multipeptide vaccine as a combination therapy regimen for the treatment of breast cancer-bearing mice prolonged the vaccine-induced progression-free survival period [58].

**Concluding remarks**

In summary, the data in this thesis show that antigen targeting to human skin DC subsets using glycan-modified antigens is a promising tool to develop new anti-tumor immunotherapies. However, the data also demonstrate that a careful selection of the DC subset, the receptor to target, formulation of the antigen and co-administration of an adjuvant is required for an optimal induction of an anti-tumor immune response. Whether DC targeting as monotherapy is sufficient enough to eradicate a tumor completely seems questionable, therefore combination therapies needs to be developed and tested. In particular, combining in vivo DC targeting with standard therapies such as chemotherapy or radiotherapy, as well as the combination with therapies influencing the suppressive tumor microenvironment would be of benefit for cancer patients.
References


