Chapter 7

General discussion

Adapted from:
C-type lectin receptors for tumor-eradication: future directions
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Potency of glycan-modified antigen in DC-based immunotherapy for tumor-eradication

Many studies have shown that modulation of immune responses is possible via targeting of antigen to CLR expressed on APC. However, care must be taken when using these novel types of vaccines in the clinic. Early clinical trials on the transfer of tumor antigen-loaded DCs showed that despite the presence of detectable immune responses against the tumor antigens, clinical responses were often lacking[1]. The development of new ways of DC-targeting and advancing knowledge on the molecular mechanism of cross-presentation, DC subsets and function, as well as on avoiding tolerance induction may lead to better *in-vivo* efficacy of these DC-based therapies.

Neoglycoconjugates for the induction of antigen-specific immune-responses

In this thesis, I showed the potency of using natural ligands for CLR (glycans), instead of commonly used antibodies, to target antigen to CLR for the enhancement of antigen-specific immune responses. I examined which glycans bind with high affinity to either MGL1, MGL2 or MR expressed on murine BM-DCs (chapter 2 and 6). Subsequently, we conjugated the glycans found in these studies to the model antigen OVA, to examine their effects on antigen-specific immunity. Using these glycan-OVA conjugates, I showed that glycan-modification redirects antigens from one CLR to another, leading to rapid internalisation and modulation of immune responses. Specifically, in our studies we conjugated glycans that specifically bind the CLRs MGL1 (e.g. Le^X, chapter 3), MGL2 (e.g. GalNAc, chapter 5) or MR (e.g. sulfo-Le^A and triGlcNac, chapter 6) to OVA. The conjugation of 2 glycans per OVA molecule led to efficient targeting of the antigen to BM-DC as well ex-vivo as sp-DC, as revealed by rapid internalization of antigen via the relevant CLR.

Antigen-specific $CD4^+$ T-cell responses

When concentrating on $CD4^+$ T-cell responses, it was evident that antigen-specific $CD4^+$ T-cell proliferation was only enhanced after MGL-2 mediated uptake of OVA (OVA-GalNAc). When MGL-1 or MR were targeted, using OVA-Le^X or OVA-sulfoLeA or OVA-triGlcNAc respectively, no difference in $CD4^+$ T-cell proliferation was observed compared to native OVA induced $CD4^+$ T-cell responses. This difference in induction of $CD4^+$ T-cell responses between MGL1 and MGL2 could be due to variations in the cytoplasmic tail of these receptors, resulting

in distinguished signalling. Other studies also observed the induction of $CD4^+$ T-cells upon MGL2-targeting using GalNAc-moieties[2, 3]. However, in these studies no cross-presentation and $CD8^+$ T-cell proliferation was observed, and whether this difference in $CD4^+$ T-cell activation has consequences *in-vivo* remains to be investigated. Given that $CD4^+$ T-cell help is important for $CD8^+$ T-cell priming 4-6, it would be interesting to investigate whether MGL2-targeting in-vivo leads to even better $CD8^+$ T-cell responses via increased quantity of $CD4^+$ T-cell help. Furthermore, MGL2-targeting may be beneficial in vaccination for pathogens, where $CD4^+$ T-cell responses are essential in pathogen-clearance.

Although only MGL-2 was capable to enhance $CD4^+$ T-cell responses, all glycoconjugates induced Th1 skewing of $CD4^+$ T-cells. The differentiation of $CD4^+$ T-cells requires two signals given by the DC, namely: the expression of co-stimulatory molecules and production of cytokines. Both signals were not influenced upon incubation with glycan-modified antigen compared to native antigen. How Th1-differentiation of $CD4^+$ T-cells upon incubation with glycoconjugate loaded DCs is regulated is unclear at this moment. It is possible that co-stimulatory molecules that regulate Th1 instruction are upregulated upon initial DC-T-cell interaction.

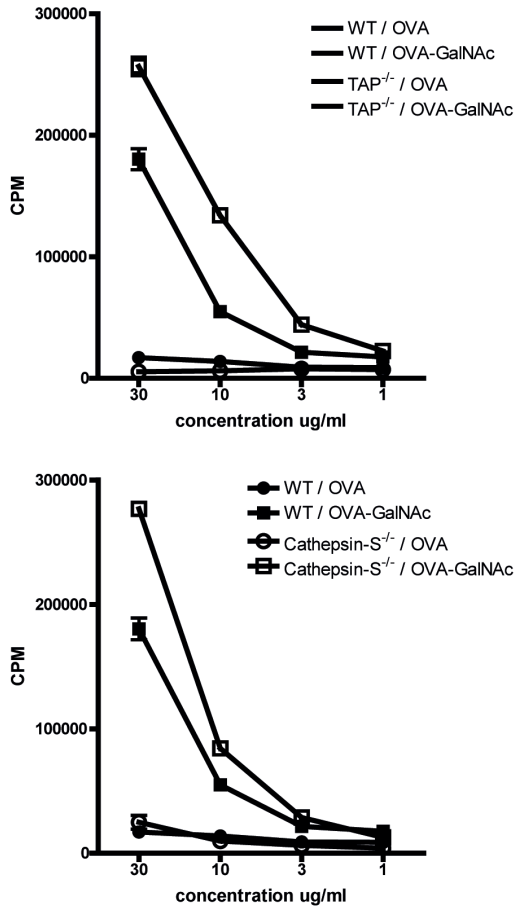
Cross-presentation and subsequent antigen-specific $CD8^+$ T-cell responses

Interestingly, all glycan-OVA conjugates investigated induced efficient cross-presentation when low concentrations (30 µg/ml) were used, in contrast to native OVA. These positive effects on cross-presentation were also observed *in-vivo*: immunization of mice with OVA-Le^X enhanced the amount of IFNγ-producing antigen-specific $CD8^+$ T-cells compared to immunization with native OVA (chapter 3). Furthermore, OVA-Le^X-immunization induced increased proliferation and IFNγ-production of adoptively transferred OT-I cells (chapter 3). Using confocal microscopy, the glycan-modified antigens were shown to preferentially locate in an early endosomal compartment. As neither expression of co-stimulatory molecules nor the production of cytokines was influenced by the glycoconjugates themselves and since the early endosomal compartment is associated with cross-presentation, the observed enhancement of cross-presentation by the glycoconjugatesis likely due to preferential localization to an intracellular 'cross-presentation' compartment.

The current proposed subcellular pathways of cross-presentation are dependent on either TAP-transport or the endosomal protease Cathepsin S[7, 8]. We have clear evidence that the molecular cross-presentation pathway used upon uptake of antigen via both MGL1 (chapter 3) and MGL2 (Box 1) is not

Box 1. cross-presentation pathway induced via MGL2-mediated uptake not TAP- or Cathepsin S-dependent.

To investigate whether MGL2 induced cross-presentation requires the same cross-presentation components as MGL1-induced cross-presentation we incubated either *TAP*$^{-/-}$ or *Cathepsin-S*$^{-/-}$ BM-DC with different concentrations of OVA-GalNAc and performed a standard proliferation assay. Consistent with cross-presentation induced by MGL1-mediated uptake of OVA via OVA-Le^{X}, CD8^{+} T-cell responses induced by MGL2-mediated uptake of OVA-GalNAc was unaffected by the absence of TAP or Cathepsin S. In conclusion, our data show that the cross-presentation route of OVA via MGL2 is similar to cross-presentation route induced by MGL1 but different from that of native OVA, which is TAP-dependent.



dependent on TAP and Cathepsin S. Antigens taken up via MGL1 and MGL2 are likely routed via the same cross-presentation pathway. However, which molecular pathway is unclear. To date, only a few TAP-independent cross-presentation routes have been described. One of these pathways involves Furin, an endoprotease which has been reported to participate in TAP-independent processing of CTL epitopes[9, 10]. The linkage of a trojan peptide sequence derived from HIV-I Tat (carrier) with a CTL epitope (cargo) was delivered directly into the endoplasmic reticulum (ER) and *trans*-Golgi network (TGN), where resident peptidases such as Furin, efficiently generated MHC class-I epitopes[9]. Subsequently, antigen-specific $CD8^+$ T-cell responses *in-vitro* as well as *in-vivo* were induced[10]. Although OVA-GalNAc and OVA-Le^X localize in the early endosome (chapter 5,[11]), confocal analysis also showed antigen which was not co-localized with either early endosomal marker EEA-1 or late endosomal / lysosomal marker LAMP-1. Whether this antigen is present in the ER, where it could be subject to Furin is currently under investigation.

In our studies we show that the glycan-modified antigens are cross-presented independently of TLR-signalling. Several groups proposed that for the induction of cross-presentation an additional TLR-signal is needed[12, 13]. In the presence of LPS, the OVA-antigen is cross-presented, but not when BM-DC of in MyD88/TRIFF$^{-/-}$ or TLR4$^{-/-}$ mice the OVA were used. Yet, also other DC-targeting strategies have been described to induce cross-presentation in the absence of a TLR-signal, such as OVA-immune-complexes targeting Fc- receptors and a TLR2 ligand-peptide conjugate that is also cross-presented in TLR2$^{-/-}$ DC[14, 15].

Whether cross-presentation is TLR-dependent or -independent could be due to differences in the nature of the antigen, efficiency of targeting to the receptor, and additional uptake-receptors like Fc-receptors, in the case of antibody-antigen conjugates.

Skewing antigen towards cross-presentation

Cross-presentation of exogenous antigens on MHC-class I molecules to $CD8^+$ T-cells is crucial for their activation. Whether an antigen is cross-presented is dependent on multiple factors. The mechanism by which antigen is internalized is important as it influences whether and via which pathway an antigen is cross-presented. Ovalbumin (OVA) which enters the DC via MR-mediated endocytosis, and in the presence of a TLR4 co-signal, is cross-presented, whereas OVA taken up through pinocytosis, enters the $CD4^+$ T-cell route[16].

Moreover, the physical form of the antigen seems to be of importance: OVA absorbed onto inert iron or polystyrene particles is cross-presented via the cy-

tosolic pathway, whereas OVA encapsulated into PLGA-particles uses the vacuolar pathway to end up as peptides on MHC class I molecules[17]. In addition, evidence exists that the size of the antigen-particles is important. DC-SIGN targeted nanoparticles of 200 nM of size were efficiently presented and enhanced T-cell responses. In contrast, DC-SIGN targeted microparticles 2 μM of size were taken up aspecifically and thereby were poorly induced T-cell responses[18]. However, $CD4^+$ and $CD8^+$ T-cell responses were not distinguished from each other, and the exact role for cross-presentation still needs to be elucidated. Moreover, the nature of the receptor involved in endocytosis of antigen seems to be crucial for the routing of antigen intracellularly. Glycan-modified OVA targeted to both mMGL1 and mMGL2 resulted in TAP- and Cathepsin S-independent processing and resulted in very efficient cross-presentation, even when low amounts of OVA were used (chapter 3 and Box 2).

Antigen can also be forced into the cross-presentation route artificially by usage of saponine-based adjuvants. Saponine-based adjuvants break down the phagosomes in which antigen is located, enabling translocation of the antigen to the cytosol and making it available for the endogenous MHC class-I presentation pathway[19, 20].

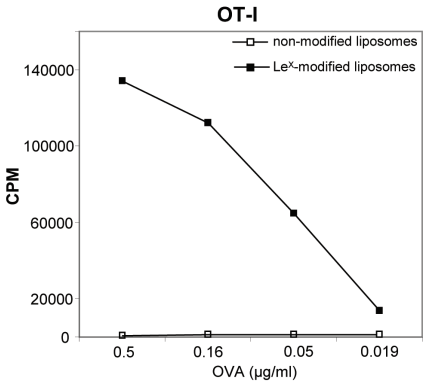
The elucidation of downstream pathways of antigen-routing and co-signalling of CLRs will provide knowledge on what intracellular signal decides the cellular localization of the antigens and whether T-helper cells, CTLs or antibody responses are induced. This information is crucial for the design of better targeted therapies.

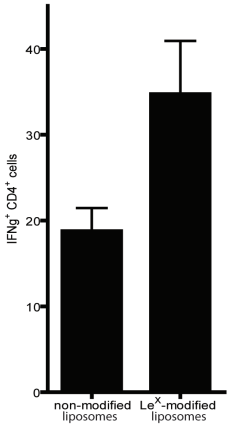
Glycan-modified antigens for tumor-eradication?

The induction of IFNγ-producing $CD4^+$ T-cells and cross-presentation of tumor associated antigens leading to an enhancement of $CD8^+$ effector T-cells, are both essential for the induction of anti-tumor responses. In this thesis we show that CLR-targeting using neoglycoconjugates, enhanced both IFNγ-producing $CD4^+$ T-cells and $CD8^+$ T-cells. However, we did not directly show the potency of MGL1/2 or MR targeting via glycoconjugates to induce effector CTL that are capable of tumor-eradication in-vivo, which is subject of current investigation. Nevertheless, we have evidence that this strategy of glycan-modified antigens can be effective for tumor-eradication. In an *in-vivo* B16-OVA tumor model, therapeutic immunization of mice with OVA-glycan conjugates (OVA-Le^B) that target DC-SIGN in combination with Treg depletion prolonged survival from B16-OVA tumors. Moreover, half of the mice showed prolonged protection against outgrowth of the tumor (unpublished observations).

Box 2 Liposomes: a delivery system for MGL1- targeting via glycans?

To investigate whether liposomes are suitable as a delivery system for MGL1 targeting via glycans, we determined their capacity to potentiate $CD8^+$ or $CD4^+$ T-cell proliferation. Liposomes incorporated with the OVA-antigen were modified with Le^X-structures, non-modified liposomes were used as a control. We show that Le^X-modified liposomes pulsed BM-DC induce significantly more cross-presentation compared to non-modified liposomes (Fig. a). Strikingly, at least 60 times lower amount of OVA-antigen is sufficient to enhance cross-presentation when using Le^X-modified liposomes compared to the glycan-antigen conjugate OVA-Le^X. Furthermore, similar as OVA-Le^X, Ie^X modified liposomes induces Th-1 skewing of naïve $CD4^+$ T-cells. Together, here we show that liposomes are a elegant delivery system suitable for MGL1-targeting via glycans, as Le^X-modified liposomes are capable to induce efficient cross-presentation and Th-1-polarization, using considerable lower amounts of antigen.





Using the model antigen OVA in mice in our studies

In our studies, we used the well-established model antigen OVA. The OVA-model is a commonly used model because of the many tools that have been developed, for example transgenic mice who bear $CD4^+$- or $CD8^+$ T-cells recognizing immunodominant epitopes of the OVA[21, 22]. Like most tumor associated antigens, OVA is poorly immunogenic. However, in the model we used, OVA is not self antigen, which is in contrast to most tumor antigens. It would be very interesting to confirm our studies using other antigens like for example TRP2, which is a tumor antigen expressed by and associated with melanoma[23]. In addition, using these models questions addressing breaking tolerance and whether and in what grade auto-immunity will be induced following our immunization strategy, can be addressed.

Adjuvants

The development of an anti-tumor vaccine stands with the induction of the right pro-inflammatory response, avoiding the induction of tolerance. CLR-mediated uptake and presentation of antigen in general does not result in maturation of the DC (with exception of Dectin-1 and -2) and this lack of maturation will generally lead to tolerance. Also CLR-mediated uptake of our glycoconjugates does not lead to maturation. Maturation of DCs is needed to induce powerful pro-inflammatory responses, through the expression of essential co-stimulatory molecules and inflammatory cytokines that allow optimal presentation of the antigen and priming of T-cells. To ensure the best anti-tumor responses in a tolerogenic tumor-micromilieu, adjuvants are often included in DC-targeting. These adjuvants include TLR-agonists, inflammatory cytokines and agonists of co-stimulatory molecules. These adjuvants will not only modify the magnitude and type, but also improve the quality of the induced response. Most TLR-agonists can induce antibody responses, but have different capacity to induce CTL and T-helper responses. TLR-2 triggering leads to the induction of T-reg rather then Th-1 which is needed for anti-tumor immunity[24, 25]. Furthermore, the activation of combinations of TLRs, like for example TLR4 with TLR7/TLR8, can induce synergistic production of cytokines inducing T-helper 1 responses[26]. Recently it was shown that the co-stimulation of TLR4 and TLR7/8 leads to the synergistic increase of the production of antigen-specific neutralizing antibodies compared to single TLR-stimulation[27]. This was due to early and increased programming of B-cell and $CD4^+$ T-cell memory and was dependent on the direct triggering of both TLRs on B-cells and DCs, as well as on T-cell help. A very interesting observation was that superior responses were induced when the an-

tigen and TLR were delivered in separate nanoparticles compared to one nanoparticle that included both the antigen and TLR stimulus. However, whether this also applies for the induction of $CD8^+$ T-cells remains to be determined. Moreover, the CLR used to target the antigen to, can often also signal, which can lead to either synergistic or inhibitory effects[28]. The addition of a TLR4-ligand to glycan-modified antigens targeting DC-SIGN or MR lead to synergistic effects on cross-presentation, especially in low antigen-concentrations ([29], and manuscript in preparation). However, when TLR4 was triggered combination with MGL1-targeting the cross-presentation was greatly diminished. This is probably due to the diversion of antigen to a different intracellular compartment unfavourable for cross-presentation in the presence of LPS (chapter 4). These observations illustrate that for every DC-targeting receptor it is important to identify which adjuvant is best to combine to obtain the desired immune response.

As an alternative to TLR-triggering, the stimulation of co-stimulatory molecules using agonistic antibodies can be used as an adjuvant. CD40 triggering on DCs leads to maturation and licences DCs to prime $CD8^+$ T-cells, and is used in multiple studies to fine-tune responses[30-33].

Multivalency of glycans on antigen and avidity for CLR: influencing immune outcome?

23% of native OVA-preparations contain high mannose structures that are able to bind the MR. For OVA to be cross-presented concentrations up to 1 mg/ml, as well as an additional TLR4-signal are required. Mass spectrometry analysis shows that about approximately 40% of our glycoconjugate preparation contains 2 additional glycan-structures (chapter 3). How is it then possible that low concentrations of our MR-targeting glycoconjugates (as little as 10 µg/ml) induce such efficient cross-presentation, in the absence of any additional TLR-signal? One possible explanation could be that different linkage leads to an altered three-dimensional structure of the antigen, herewith to improving the avidity for and binding to the CLR. In line with this, also multivalency of the glycans added to antigen will matter. A study on MR-receptor targeting showed that multi-branched mannosylated ligands were more efficient in targeting DCs compared to monomannosylated antigens[34], indicating that multivalency of glycan-CLR interaction can be beneficial for antigen uptake and processing.

Similary, differential effects on immunological outcome of a CLR due to multivalency has been reported for MGL. MGL1 and MGL2 are both described to be involved in the induction of anti-inflammatory responses, including induced

IL-10 production by APC in a DSS-colistis model[35] and altered APC adhesive function in obesity[36]. Yet, influence at the level of anti-inflammatory T-cell induction is lacking. Furthermore, mMGL is expressed on the tolerogenic alternatively activated macrophages, hinting a role for mMGL in homeostasis[37]. However, in this thesis we clearly demonstrate a pro-inflammatory role for MGL. In our studies using OVA-Le^X or OVA-GalNAc conjugates, uptake of antigen via MGL1 and MGL2, respectively lead to a beneficial and enhanced antigen presentation and induction of CTL responses. A possible explanation for this discrepancy between our data and literature may be the difference in glycan multivalency of the antigens used. The glyco-conjugates used in our studies contained only 1-2 moieties of GalNAc on each OVA-molecule. MUC1 is a heavily glycosylated tumor-antigen, consisting of elaborate repeated sequences containing multiple GalNAc-structures. It has been reported that uptake of the heavily glycosylated tumor-antigen MUC1 by human MGL, which interacts with the tumor-associated antigen MUC1 in a similar manner as murine MGL2, blocks intracellular processing and presentation and herewith the induction of CTL responses[38, 39]. Together, these data imply that fine-tuning of the glycan-density on antigen is important for each CLR to induce the most potent immune-response without tilting towards tolerance.

Nanotargeting systems

One way to increase multivalency of glycans is to encapsulate antigen a glycan-coated particulate and addition of a proper adjuvant. The use of nanotargeting systems shown to improve efficacy of targeting tremendously. Several delivery systems are being investigated, for example metallic nanoparticles, polymer microparticles and liposomes. These systems resemble pathogens in size and can amplify immune responses. Furthermore, several nanotargeting systems protect antigen from non-specific degradation and form depots and aggregates of antigen, enhancing uptake by DCs. Using this technology both the antigen, the DC-targeting component and the adjuvant can be incorporated in one particle. Also, the particle used for DC-targeting will affect internalization mechanism and consequently the pathway of presentation. Ovalbumin absorbed onto inert particles is cross-presented via the cytosolic pathway, whereas ovalbumin encapsulated into microparticles uses the vacuolar pathway[17]. Also, liposomes can be designed to force antigen into either the MHC-class I or -II presentation pathway[40]. The development of early endosomes into late endosomes / lysosomes coincides with a decreasing pH gradient, and liposomes were generated with different pH-sensitivity. This leads to breakdown of the liposome and release of antigen in either early endosomal or late endosomal / lysosomal compartment, facilitating either cross-presentation

or MHC-class II presentation, respectively. Furthermore, the MHC class I and II presentation of antigen occurred through pathways having distinct molecular and proteolytic requirements[40].

Multiple studies have shown the contribution of nanotargeting systems in amplifying T-cell- as well as B-cell responses[41-43]. In these studies the uptake of the particles is at least partly due to pinocytosis or phagocytosis. These forms of endocytosis are not APC-specific and will lead to non-specific uptake of antigen by neighbouring cells, potentially leading to unwanted side-effects. In a phase I/II study which included stage II-IV melanoma patients receiving TAA-containing virus-like nanoparticles loaded with a TLR-9 ligand, it was shown that the vaccine was well tolerated[44]. These nanoparticles were not targeted to DC and taken up by phagocytosis. Although a-specific uptake seems to induce no significant side-effects, it is preferred to add a targeting component to the nano-targeting systems. In our own studies, we used glycan-coated liposomes to specifically target several CLRs, and avoided non-APC uptake. Furthermore, CLR-targeting using these glycan-coated liposomes is at least 60 times more efficient than targeting of a soluble glycan-antigen targeting to DC (unpublished observations, box 2).

Glycan-targeting or antibody-targeting of DCs?

The use of glycans for *in-vivo* DC-targeting purposes has advantages over CLR-specific antibodies as many glycans are non-immunogenic and of self-origin, or can be produced synthetically in large scale using simple or complex chemistry depending on the complexity of the glycan structure[45]. Additionally or: alternatively glycan structures that are present on pathogens can be used when known to bind CLRs with higher affinity. In contrast, the production of humanized antibodies is expensive and can be immunogenic in patients. Moreover, antibodies themselves are glycosylated. The glycosylation of proteins is dependent on the cell line used for antibody production, and by a different glycosylation humanized antibodies can induce unfavourable immune responses[46]. A possible drawback of using glycans could be that glycans have a lower binding affinity to CLRs compared to antibodies. However, the glycan of choice can be designed to a scaffold such that it is presented in a multivalent fashion to CLRs, mimicking the glycan composition on pathogens, leading to a higher binding affinity[34].

Superior APC for targeting?

Preferentially, the antigen should be targeted to DCs only and more ideally to only those DCs that are specialized to cross-present antigen. This will reduce the amount of antigen needed to elicit immune responses, which is crucial when the amount of antigen is limiting. Especially, it has been proposed that macrophage targeting should be avoided because of rapid degradation of antigen in lysosomes, which does not favour cross-presentation. Moreover, components allowing cross-presentation are expressed at low levels by macrophages.

However, there are also beneficial roles described for macrophages that cannot be ignored. Although macrophages are not ideally equipped for cross-presentation they are described to be able to cross-present antigen *in-vivo* at least in some situations[47]. Furthermore, it has been described recently that a subset of $CD169^+$ macrophages is present in the subcapsular sinus of the lymph nodes[48]. These $CD169^+$ macrophages are capable to take up and efficiently cross-present large antigens to T-cells, inducing an immunogenic response. In addition, peptide-loaded macrophages have been reported to show similar potency as DCs to stimulate naive $CD8^+$ T-cells *in-vivo*, which then develop into effectors and memory T-cells[49]. Finally, macrophages can collaborate with DC to induce $CD8^+$ T-cell responses, as shown for the metallophilic marginal zone macrophages (MMM) and $CD8^+$ DCs in the spleen. Blood borne antigens are taken up by the MMM and may transfer antigen to splenic $CD8^+$ DCs for cross-presentation and activation of cytotoxic T lymphocytes[50]. Taken together, as the receptor that solely targets DCs is yet to be discovered, and because macrophages are a substantial APC-subset present in the skin, it would be beneficial to study whether including macrophages in a DC-targeting vaccination strategy is better or worse for anti-tumor responses. It would be interesting to see whether cross-presentation skewing saponin-based adjuvants like ISCOMATRIX will also induce efficient cross-priming of $CD8^+$ T-cells by macrophages[51].

Although macrophages can be beneficial in the induction of anti-tumor responses, the focus of targeting should lay on DCs, and preferably the DC subset that is most potent in achieving the most optimal anti-tumor response. DCs display delayed lysosomal acidification and proteolytic activity which favors cross-presentation. DCs are a heterogenous population that consists of several DC-subsets which express different sets of pattern recognition receptors, have differential capacity to take up and present antigens and produce different cytokines upon TLR-stimulation[52-54]. This results in the induction of distinct types of immune responses. Targeting antigen to a receptor expressed on 'the right

DC-subset' is required for the induction of the desired cross-presentation and induction of cellular responses. In mice, it has been shown, using antibody-targeting that not the targeting receptor but the targeted DC-subset is crucial for optimal $CD4^+$ and $CD8^+$ T-cell priming[55, 56]. Whether this applies to glycan-targeting remains to be established.

When intravenous injection is used, antigen travels through the bloodstream encountering DCs in blood or the spleen. In human blood, three different DC-subsets are identified, namely: $BDCA1^+$- , $BDCA2^+$-, and $BDCA3^+$ DCs. $BDCA1^+$ DCs express a large array of TLRs (TLR 1,2,3,4,5,6,7,8, and 10) and upon stimulation with TLR-ligands produce pro-inflammatory cytokines[57, 58]. $BDCA3^+$ DCs are recently described to be the homologue of the mouse $CD8^+$ DC which is specialized in cross-presentation. This DC-subset does express CLEC9A and TLR3 and -8[59]. They potently cross-present antigens upon co-stimulation of TLR8[52]. Although this sounds promising, if targeting of these cells *in-vivo* will induce immune responses remains to be elucidated as these $BDCA3^+$ DCs compel about 2% of the DC-population in blood. $BDCA2^+$ pDC are specialized in anti-viral responses and upon viral (TLR 7-9) stimulation produce high amounts of type-I interferons[60]. Also pDCs are described to cross-present antigens[61, 62]. Furthermore, they could add to the pro-inflammatory milieu needed for the induction of potent anti-tumor responses by the production of type-1 interferons. How all DC-subsets in blood contribute to modulating immune-responses is not yet known, and in view of *in-vivo* CLR targeting it will be most efficient to target DCs in the skin.

The most common route of vaccination is subcutaneous injection in the skin where antigen will encounter local DCs. Here, different subsets can be targeted with different outcome of immune responses. Dermal DCs (dDCs) contain a large subset which is $CD1a^+$ and a smaller $CD14^+$ subset (about 10% of dDCs). $CD14^+$ dDCs express a large array of CLRs like DC-SIGN, DEC-205, Dectin-1, DCIR, LOX-1 and Clec6. They also express TLR 2,4,5,6,8 and 10[63]. The epidermis harbours Langerhans Cells (LCs), which only express the CLRs Langerin and DCIR and the TLRs 1,2,3,6, and 10[64]. Recently, it has been shown that LCs can cross-present antigens to $CD8^+$ T-cells *in-vitro* as well as *in-vivo* in mice56. Furthermore, a direct comparison of LCs and $CD14^+$ dermal DC *in-vitro* revealed that LCs are superior in inducing $CD8^+$ responses whereas $CD14^+$ dDCs were specialized in priming $CD4^+$ T-cells into Follicular helper T-cells, inducing B-cell differentiation into antibody producing plasma cells[65]. However, if this division of labor is also operating *in-vivo* still needs to be investigated.

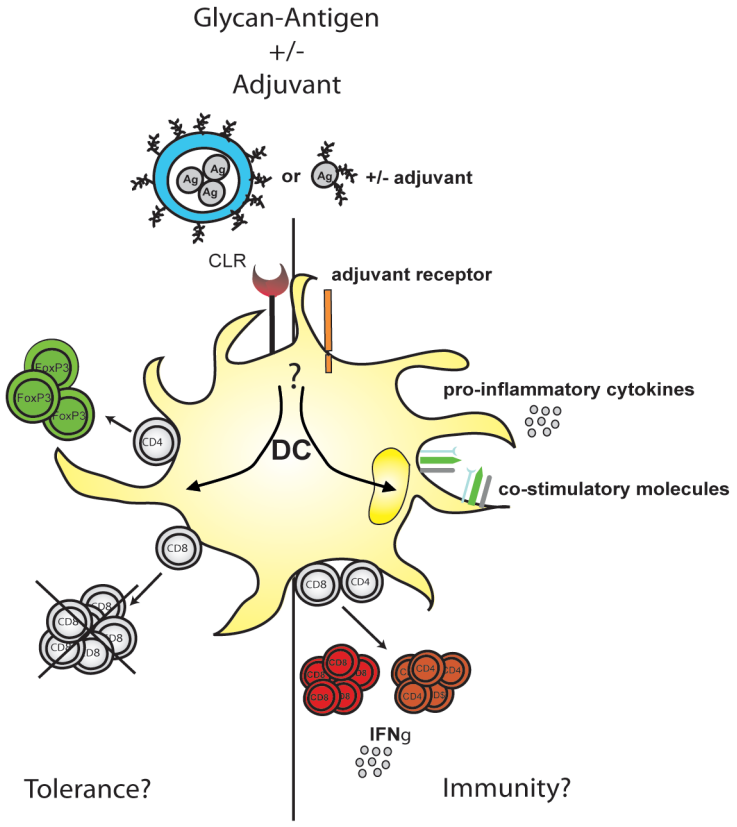


Figure 1: Shaping immune responses using DC targeting glycan-antigen conjugates.
The use of glycan-antigen conjugates can lead to the induction of CD4⁺ and CD8⁺ T-cell responses, depending on the CLR targeted. To date, no T-reg cell induction has been observed when targeting DCs with glycan-antigen conjugates without adjuvant, but this cannot be ruled out for all CLR / glycan combinations. Currently, it is insufficiently known which adjuvant can be optimally combined with the modified antigens to induce the desired immune response, this is being investigated.

Concluding Remarks

In summary, the data presented in this thesis show that antigen-targeting to DCs using glycan modified antigens is a promising tool to develop new vaccination strategies. However, the strategy can be further optimized by choosing the most optimal targeting-receptor, appropriate adjuvants and integrating all needed vaccine-components in already established delivery systems such as liposomes and nanoparticles (figure 1).

Furthermore, whether *in-vivo* DC-targeting on its own will be potent enough to induce complete tumor-eradication in patients remains to be established.

Eventually, combination therapies will probably be needed, where CLR-targeting for the induction of potent immune responses is combined with the break-down of the immunosuppressive tumor-milieu. In particular, suppressive cytokines in the tumor-environment and immune-inhibitory signals can be blocked using antibodies or soluble receptors. Furthermore, the depletion of T-regulatory cells and myeloid suppressor cells will add to the break-down of the immunosuppressive milieu.

Also combining DC-targeted therapies with targeted forms of conventional therapies like radio- and chemotherapy will be beneficial. The radio- and chemotherapy will lead to extensive cell death, which is advantageous for the induction of cross-presentation of tumor-associated antigens by DCs, inducing strong $CD8^+$ T-cell responses fighting minimal residual disease and inducing efficient memory, preventing recurrences. Combining these signals will lead to an effective anti-tumor response and long-lived memory.

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