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## The functional consequences of glycosylation on dendritic cell biology

Bax, M.

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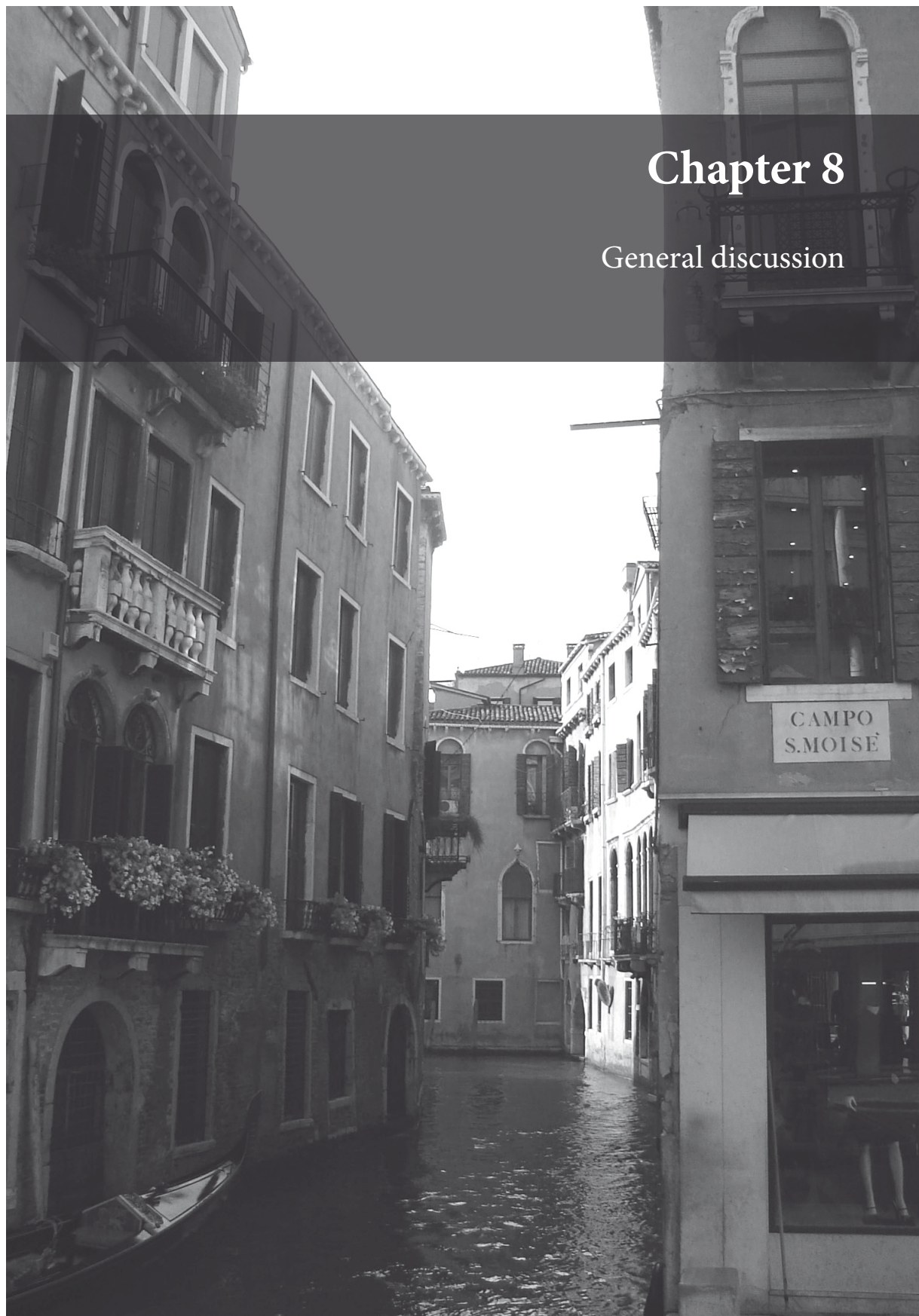
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# Chapter 8

General discussion



## ▪ Glycosylation in the immune system

In this thesis the functional consequences of glycosylation on DC biology are described. Glycosylation is the enzymatic process that mediates the addition of specific sugars to other saccharides, on proteins and on lipids. The expression of chaperons, regulatory molecules, co-factors, sugar donors and the enzymes involved in glycosylation, named glycosyltransferases and glycosidases, is reflected in the repertoire of glycans that is expressed on the cellular surface. By the process of glycosylation, glycan structures are generated, which are essential for cell viability and function<sup>1</sup>.

Glycans have important biological functions in protein maturation and turnover, receptor-ligand interactions, cell adhesion, and cell trafficking<sup>2-4</sup>. Therefore, the expression of specific glycan structures can regulate immune cell activation, differentiation, and homeostasis<sup>3</sup>. The biological information that is presented by glycans is identified and decoded by glycan-binding receptors<sup>3</sup>. Within the immune system various classes of glycan-binding receptors, or lectins, exist that recognize specific glycan structures presented by a protein or lipid<sup>5-7</sup>. Lectins recognize glycans that are expressed on self-antigens, other cells, or on pathogens. DCs express a wide range of lectins on their cell surface, including Siglecs, Galectins and C-type lectins. These lectins are involved in the detection of glycans for many functional properties including migration, adhesion, immunological activation, and the induction of tolerance<sup>8</sup>.

The first part of this discussion describes the glycosylation of immune cells and especially DCs, during activation, differentiation and homeostasis (chapter 5). In addition, the functional consequences of these changes in glycosylation during DC maturation for cellular interactions and migration are discussed (chapter 6 and 7). The second part of this discussion describes interactions of DC lectins with glycosylated (self-)antigens and pathogens (chapter 2, 3 and 4).

## ▪ Glycosylation of immune cells

The immune cell glycome is altered during cellular activation and differentiation. These changes are linked to both homeostatic as well as disease status of immune cells which can influence binding of lectins that are involved in cell trafficking, antigen-receptor activation, cytokine-receptor activation, and the induction of leukocyte apoptosis<sup>7,9</sup>.

The transition of iDCs to mDCs is essential for DCs to couple innate to adaptive immune responses. iDCs sense for pathogen in the periphery. Upon pathogen recognition, a signalling cascade initiates the DC maturation process. The phenotypic and functional changes observed during DC maturation are of critical importance for a proper induction of adaptive immunity<sup>10,11</sup>. Posttranslational protein modifications contribute to the functional switch of DCs from iDCs to mDCs. In this thesis (chapters 5, 6 and 7) we report that DC maturation is accompanied by a remodelling of cell surface glycosylation. Especially the expression of sialylated and galactosylated structures on the cell surface is changed during DC maturation.

## Dendritic cell sialylation

DC maturation is accompanied by a dramatic change of sialic acids expressed on the cell surface of DCs (Chapter 5), whereby  $\alpha$ 2,3-linked and  $\alpha$ 2,8-linked sialic acids are upregulated and  $\alpha$ 2,6-linked sialic acids are downregulated on the cell membrane<sup>12</sup>. Sialic acids have been

shown to be involved in many relevant processes in the immune system<sup>13</sup>. Sialic acids serve as ligands for sialic acid-binding lectins, including Siglecs. Sialic acid binding to Siglecs can mediate cellular interactions or cell signaling through ITIM or ITAM signalling motifs in their cytoplasmic tails, resulting in cellular activation or inhibition<sup>14</sup>.

Besides being ligands for sialic acid-specific lectins, sialic acids on the terminal ends of glycans prevent binding of these glycan structures to certain C-type lectins. By removing these terminal sialic acids from their glycan structures, immune cells can unmask ligands such as terminal galactose for C-type lectins. Hereby, new C-type lectin-ligand interactions may be induced.

#### *Sialic acids involved in cellular interactions*

In chapter 5 we describe that the upregulation of  $\alpha$ 2,3-linked sialic acids during DC maturation results in the induction of the expression of potential ligands for Sialoadhesin (Siglec-1). The functional consequences of Sialoadhesin binding to mDCs are investigated in chapter 6. The cytoplasmic region of Sialoadhesin lacks obvious signaling motifs, whereas the extracellular region is highly extended. The highly extended and evolutionary well-conserved extracellular domain of Sialoadhesin may indicate that this Siglec mediates cellular interactions<sup>14</sup>. Indeed, Sialoadhesin was observed to mediate cellular interactions between mDCs and macrophages. To accomplish this cellular contact, both mDC-expressed glycan structures terminating in  $\alpha$ 2,3-linked sialic acids and Sialoadhesin expression by macrophages are required. Sialoadhesin has been demonstrated to induce cellular interactions between macrophages and T cells as well. Wu et al reported that Sialoadhesin that is expressed by macrophages, directly binds to regulatory T cells. Binding of Sialoadhesin to regulatory T cells prohibits the expansion of regulatory T cells in an animal model of multiple sclerosis and experimental autoimmune encephalomyelitis<sup>15</sup>. The *in vitro* interaction of macrophages and DCs mediated by Sialoadhesin exists during inflammatory conditions. LPS-matured DCs and not iDCs express the ligand for Sialoadhesin. In addition, classically activated macrophages and not alternatively activated macrophages express high levels of Sialoadhesin, required for cellular interactions. The negative regulation of the expansion of regulatory T cells by Sialoadhesin also occurs during inflammatory conditions to improve an activating immune response.

#### *Sialic acids involved in migration*

We demonstrate in chapter 5 that, besides sialic acid expression, also O-glycans on the cell surface of DCs are highly modified during maturation. Sialylated core 1 O-glycans increase their abundance with respect to core 2 O-glycans due to the concomitant upregulation of the galactosyltransferase Core 1  $\beta$ 3GalT and the sialyltransferases ST3Gal I/II and the downregulation of N-acetylglucosaminyltransferase C2GnT I. Julien *et al.* investigated similar to us glycosylation changes during DC maturation as well<sup>16</sup>. Consistent with our findings, they report that maturation of DCs led to a rapid change in the expression levels of glycosyltransferases involved in O-glycosylation. Because O-glycans play a significant role in T cell trafficking, Julien et al investigated the O-glycosylation profile of human DCs in relation to DC trafficking. A down-regulation of C2GnT I mRNA and enzymatic activity was observed, coinciding with an upregulation of ST3Gal I and ST6GalNAc II mRNA, which resulted in a loss of the core 2 structures required for sialyl-Lewis<sup>x</sup> expression. Sialyl-Lewis<sup>x</sup> is needed for extravasation of DCs via selectin binding. They demonstrate that this glycan was exclusively

expressed on P-selectin glycoprotein ligand-1 in monocytes and iDCs. Sialyl-Lewis<sup>x</sup> was lost from mDCs, even though these cells retained expression of P-selectin glycoprotein ligand-1. In addition, they observed that the pattern of O-glycosylation observed in mDCs was very similar to O-glycosylation expressed by naive T cells, which home to lymph nodes as well. Lymphocyte trafficking is extensively studied. Selectin ligands that are expressed by lymphocytes bind to E- and P-selectin expressed on endothelial cells, thereby mediating the rolling step that precedes the tight attachment of lymphocyte to the endothelial lining<sup>17,18</sup>. Julien et al. conclude that O-glycans may, similar to T cell migration, have a function in DC migration<sup>16</sup>.

Related to the migration function found by Julien et al, we show that sialylation changes during DC maturation are involved in DC migration. In chapter 7 we demonstrate that the sialyltransferase ST8Sia IV, required for generating Polysialic acid, is highly expressed by mDCs. Furthermore, we show that polysialic acid expression by DCs favours their migration towards CCL21. PGE<sub>2</sub> is also known to promote human DC migration<sup>19</sup>. Our results indicate that the Polysialic acid expression that facilitates mDC migration is not mediated by PGE<sub>2</sub>, but dependent on prolonged TLR4 activation. In contrast, Julien et al. reported that the early regulation of the glycosyltransferases involved in generating sialyl-Lewis<sup>x</sup> was mediated by PGE<sub>2</sub>. We show that migration of mDCs to CCL21 is enhanced by binding of Polysialic acid to CCL21. We did not succeed in characterizing the scaffold of Polysialic acid on mDCs. In human milk, Polysialic acid was found on CD36, a member of the B class of the scavenger receptor superfamily<sup>20</sup>. Removal of Polysialic acid or O-glycans from mDCs resulted in improved anti-CD36 antibody binding, indicating that CD36 on mDCs might also be polysialylated (data not shown, chapter 7). The receptor for CCL21 is CCR7, the expression of which is upregulated during DC maturation. Polysialic acid has been found on peptides of CCR7, indicating that CCR7 might be polysialylated itself<sup>21</sup>. Accordingly to our results, glycosylation of other chemokine receptors has been shown to be important for their chemokine binding function as well. The chemokine receptor CCR5 is O-glycosylated. The O-linked glycans on CCR5, particularly the sialic acid moieties, significantly contribute to binding of the chemokine ligands. Not only sialylation, but sulfation of specific tyrosine residues in the CCR5 NH<sub>2</sub> terminus was important for efficient chemokine binding as well<sup>22</sup>. Thus, O-linked carbohydrates and tyrosine sulfates play major roles in promoting the interaction of chemokines with chemokine receptors by the negative charges of the chemokine receptor surface to allow specific, high-affinity interactions with diverse chemokine ligands. This results in enhanced migration of chemokine receptor expressing cells, such as mDCs to chemokine present in tissues, including the lymph node.

#### *Sialic acids involved in tolerance induction*

In contrast to the upregulated expression of  $\alpha$ 2,3-linked sialic acids and  $\alpha$ 2,8-linked sialic acids,  $\alpha$ 2,6-linked sialic acids are downregulated during DC maturation (chapter 5). We report that the sialyltransferase ST6Gal I, involved in coupling  $\alpha$ 2,6-linked sialic acids to underlying glycans, is highly downregulated during DC maturation. Consistent with our data, Jenner et al. reported in 2006 that tolerogenic iDCs showed high expression of  $\alpha$ 2,6-linked sialic acids, which were drastically downregulated after maturation of DCs with pro-inflammatory cytokines. Similar to us, they demonstrate that this differential expression of  $\alpha$ 2,6-linked sialic acid was reflected by transcriptional downregulation of specific glycosyltransferases during DC maturation. In addition, they report that CD4<sup>+</sup> T cells upon T cell stimulation significantly upregulate  $\alpha$ 2,6-linked sialic acid, whereas  $\alpha$ 2,3-linked sialic acid density remained largely unchanged.



A subpopulation of isolated CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells showed high expression levels of  $\alpha$ 2,6-linked sialic acids. The density of this particular carbohydrate was further increased after *in vitro* expansion of these inhibitory T cells<sup>23</sup>.

$\alpha$ 2,6-linked sialic acid can serve as ligand for inhibitory Siglecs on the surface of effector cells.  $\alpha$ 2,6-linked sialic acid is a ligand for the B-cell restricted inhibitory Siglec CD22 (Siglec-2). CD22 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) for inhibitory intracellular signaling. We showed that CD22 binds to sialylated ligands expressed by DCs (chapter 5). Recent studies have shown that DCs regulate B cell functions, whereby mouse BM-derived iDCs, but not mDCs, can inhibit B-cell receptor (BCR)-induced proliferation of B cells in a CD22-dependent manner<sup>24</sup>. This coincides with our observations that iDC, but not mDCs express high levels of ST6Gal I, required for generating the ligand for CD22.

In addition to the expression of  $\alpha$ 2,6-linked sialic acids on iDCs and regulatory T cells,  $\alpha$ 2,6-linked sialic acids are also expressed by B cells themselves. Besides binding to trans ligands, CD22 forms complexes with the BCR expressed on the same cell. Binding of CD22 to the BCR has an inhibitory role by dampening BCR signaling<sup>25</sup>.  $\alpha$ 2,6-sialylated ligand expression on neighbouring CD22 molecules promotes homotypic CD22-CD22 interactions, which decrease the frequency of CD22 binding to the BCR<sup>26</sup>. ST6Gal-1 deficiency results in an absence of ligands for CD22 expressed by other CD22 molecules, and increases the co-localisation of CD22 with the BCR. This enhanced co-localisation leads to decreased BCR activation and humoral immunity, and increased BCR endocytosis<sup>27-29</sup>.

The glycosylation of immunoglobins (Ig) secreted by B cells in addition to CD22 glycosylation, is important for B cell immune function as well. B cell activation and Ig class switching results in the secretion of Ig. Ig are glycoproteins that contain characteristic glycan structures<sup>30</sup>. Human IgG isolated from normal serum is comprised of multiple di-antennary glycoforms. The glycans of IgG are important for binding to Fc receptors and for other effector functions<sup>31</sup>. Alterations in endogenous IgG glycosylation have been reported for various immune-related diseases, including rheumatoid arthritis. In rheumatoid arthritis patients, the IgG N-glycans often lack terminal galactose and sialic acid linkages. As a result, these N-glycans bear exposed N-acetylglucosamine (GlcNAc) residues<sup>32</sup>. In patients with IgA nephropathy, O-glycans attached to IgA expose terminal GalNAc<sup>33</sup>. The terminal GlcNAc linkage is not common to mammalian species, and therefore, might promote inflammatory response through interactions with certain activating endogenous mammalian receptors and lectins<sup>34,35</sup>. The extent of IgG sialylation correlates with the level of inflammation<sup>36</sup>. Terminal  $\alpha$ 2,6-linked sialic acids, contributed by ST6Gal I, are not able to bind to pro-inflammatory lectins, and are therefore linked to anti-inflammatory effects of intravenous IgG. Consequently, IgG with  $\alpha$ 2,6-linked sialic acids could be used as a therapy for autoimmune diseases<sup>37</sup>.

## Dendritic cell galactosylation

In addition to changes in the O-glycosylation profile during DC maturation, chapter 5 describes changes in N-glycosylation during DC maturation. The N-acetyl-glucosaminyl-transferase  $\beta$ 1,3-GlcNAcT 2 and the galactosyltransferases  $\beta$ 4GalT IV and  $\beta$ 4GalT V are highly upregulated during DC maturation. These glycosyltransferases promote the substitution of N-glycans with (poly-)N-acetylglucosamine chains, or (Poly)LacNAc. In addition, by MALDI-TOF MS analysis we demonstrate that the N-glycan profile of mDC is comprised of complex bi-, tri- and tetra-

antennary glycans, which contained PolyLacNAc structures. LacNAc is the preferred ligand for most Galectins. Most lectins, like C-type lectins and Siglecs are expressed on the cell surface of antigen presenting cells, where they can modulate intercellular signaling and cell survival by glycan-dependent cell-cell interactions<sup>7</sup>. In contrast, Galectins are secreted proteins, which act in a soluble manner by binding to cell surface glycoconjugates<sup>38</sup>. Consistent with the upregulated expression of the specific N-acetylglucosaminyltransferases and galactosyltransferases during DC maturation, we demonstrate that Galectin-3 and Galectin-8 show higher binding to mDCs compared to iDCs. Galectin-3 and Galectin-8 both show enhanced binding affinity towards repeating LacNAc units (polyLacNAc)<sup>39</sup>.

In this thesis, the functional consequences of Galectin binding to DCs was not investigated. The binding of galectins to its glycan ligand can have many modulatory functions in innate and adaptive immunity<sup>40,41</sup>. Galectin-glycan interactions regulate homeostasis, activation and signaling of APCs and T cells. Because Galectins suppress cellular activation, they are studied in detail for their role in T cell and DC biology and the pathogenesis of immunological diseases<sup>42</sup>. For example, Galectins appear to be involved in attenuating graft-versus-host disease, collagen-induced arthritis, type 1 diabetes and T cell-mediated tumor rejection<sup>3</sup>. In mice, deletion of glycosyltransferases involved in synthesizing the Galectin-3 ligand results in altered APC sensitivity to cytokines. Although high expression of  $\beta$ 1,6-GlcNAcT V, encoded by *Mgat 5* was not observed in DCs, the presence of tetra-antennary N-linked glycans which contain polyLacNAc was shown by MALDI-TOF MS. Other groups have shown that this N-acetylglucosaminyltransferase initiates GlcNAc  $\beta$ 1,6-branching on N-glycans, thereby increasing PolyLacNAc ligands for galectins<sup>43</sup>. Expression of  $\beta$ 1,6-GlcNAcT V sensitized mouse leukocytes to multiple cytokines through Galectin-3-mediated cross-linking of  $\beta$ 1,6-GlcNAcT V-modified N-glycans on epidermal growth factor and transforming growth factor-beta receptors on the cell surface which delayed their removal by constitutive endocytosis<sup>44</sup>. In addition, the deletion of the PolyLacNAc synthase N-acetyl-glucosaminyltransferase  $\beta$ 1,3-GlcNAcT II markedly reduced PolyLacNAc on N-glycans in immunological tissues. The decreased PolyLacNAc levels in  $\beta$ 1,3-GlcNAcT II<sup>-/-</sup> mice resulted in reduction of the threshold for macrophage activation<sup>45</sup>.

In addition to Galectin-3 and Galectin-8, other groups have shown that Galectin-1 and Galectin-9 display binding to DCs as well. The interaction of Galectin-1 and Galectin-9 with DCs modulates DC maturation, whereby Galectin-1 promotes DC maturation accompanied with an enhanced migratory phenotype<sup>46</sup>. Galectin-9 binding to DCs favours IL-12 production by DCs and initiates DC-mediated adaptive immune responses<sup>47</sup>.

Similar to DCs, T cell-mediated processes such as activation, differentiation and homing are regulated by their glycosylation. Activated CD4<sup>+</sup> T cells can differentiate into the distinct T helper subsets, including Th1, Th2, Th17, and regulatory T cells. Each of these T cell subsets produces a unique profile of cytokines that differentially govern immune responses in various inflammatory conditions. In contrast to DCs, differential glycosylation of T helper cell subsets has been linked to their susceptibility to cell death by Galectin-induced apoptosis. By binding to glycosylated counter structures on T cells, Galectins impair T cell growth and induce T cell apoptosis. Thus, T cells can regulate their susceptibility to Galectin-induced apoptosis depending on the glycosylation status of the Galectin counter receptors<sup>38</sup>. Th1 and Th17 cells, in contrast to Th2 cells, express terminal galactosylated glycans on their cell surface. These glycan structures serve as a ligands for Galectin-1, which upon binding subsequently induces cell death. This difference in glycosylation explains the increased Th1- and Th17-cell responses

that have been observed in galectin-1-deficient mice<sup>48</sup>. In addition, Th2 cells actively secrete galectin-1, thereby promoting Th1 cell apoptosis<sup>49</sup>. Th2 cells are protected from Galectin-1 mediated cell death by virtue of their expression of terminal  $\alpha 2,6$ -linked sialic acids on their glycoproteins<sup>48</sup>. Through expression of ST6GalT I, the terminal galactose is masked with  $\alpha 2,6$ -linked sialic acid, thereby inhibiting Galectin-1 binding<sup>50</sup>.

The T cell receptor (TCR) is post-translationally modified with N- and O-linked glycan chains. The addition of glycans to TCR alters its association with other glycoproteins on the cell surface, thereby influencing signal transduction and receptor internalization by endocytosis<sup>38</sup>. Binding of Galectin-3 to  $\beta 1,6$ -branched N-glycan structures on the TCR limits TCR clustering and subsequent TCR signaling<sup>43</sup>. In  $\beta 1,6$ -GlcNAcT V knockout mice, TCR clustering is significantly increased, which coincides with a decreased threshold for T-cell activation. Accordingly, deficiency in  $\beta 1,6$ -GlcNAcT V results in T cell activation and signalling and enhanced Th1 responses<sup>43,51</sup>, leading to aggravated immune responses, including increased susceptibility to EAE and glomerulonephritis<sup>43</sup>. Treatment with high concentrations of GlcNAc increases  $\beta 1,6$ -GlcNAcT V-mediated N-glycan branching, which inhibits TCR activation and autoimmune responses in mouse models of EAE and type 1 diabetes<sup>52</sup>.

## ▪ Modulation of immune responses by lectin targeting

### Immune modulation by Siglecs

Immune cell activation and differentiation leads to a dramatic change in the expression of sialic acids. Besides the expression of sialic acids on immune cells, sialic acids are expressed on the cell surface of pathogens as well. Sialylated structures contribute to the binding of (self-)antigens and pathogens to Siglecs. Siglecs are lectins with known specificity for sialic acids expressed on the same cell (in *cis*) or on adjacent cells (in *trans*). Siglecs are predominantly expressed on myeloid and lymphoid cells. DCs express a wide range of Siglecs, including Sialoadhesin or Siglec-1, CD33 or Siglec-3, Siglec-7, Siglec-9, Siglec-10 and Siglec-15, which recognize both *cis*- and *trans*-ligands.

#### *Uptake of sialylated pathogens and antigens by DC-expressed Siglecs*

In this thesis (chapter 4) targeting of Siglec-E, the murine orthologue of human Siglec-7, in relation to antigen uptake and presentation is described. Targeting of murine Siglec-E expressed on BM-DCs with  $\alpha 2,8$ -disialylated structures resulted in trafficking to endosomes and lysosomes. Multiple mechanisms of Siglec-mediated endocytosis have been reported<sup>53</sup>. Sialoadhesin has been demonstrated to mediate endocytosis of sialylated bacterial and viral pathogens through a clathrin-mediated mechanism that does not involve DAP-12 or ITIM motifs<sup>54,55</sup>. In contrast, CD22 undergoes endocytosis through a clathrin-dependent mechanism that does require cytoplasmic ITIM motifs<sup>27,56</sup>. Endocytosis of CD33-related siglecs can be regulated by phosphorylation of their ITIM(-like) motifs<sup>56-58</sup>. In contrast to CD22, CD33 and murine Siglec-F mediate endocytosis by an clathrin-independent mechanism which facilitates trafficking to endosomes and lysosomes<sup>56,58</sup>. This mechanism is similar to endocytosis mediated by Siglec-E. We demonstrate that targeting Siglecs with glycan ligands is an efficient technique to induce siglec-dependent endocytosis. Consistent to our data, high affinity glycan ligands for CD22 (BPC-Neu5Ac $\alpha 2$ -6Gal $\beta 1$ -4GlcNAc) and Siglec-F (Neu5Ac $\alpha 2$ -3[6-SO<sub>4</sub>]



Gal $\beta$ 1-4[Fuca1-3]GlcNAc, or 6'-sulfo-sLe<sup>x</sup>) conjugated to polyacrylamide polymers were demonstrated to be rapidly endocytosed by Siglec-bearing cells as well<sup>56</sup>.

#### *Siglec targeting as immune therapy to deplete cells*

Because of the restricted expression of Siglecs on cells of the immune system, Siglec targeting is useful for immune cell therapies. To induce cell depletion in immune cell based therapies, Siglec ligands can be conjugated to toxins, to carry the therapeutic agents efficiently into Siglec-positive cells<sup>59,60</sup>. Conjugation of the endotoxin saporin to CD22 ligands was demonstrated to result in endocytosis and subsequent cell death of B cell lymphoma cell lines<sup>27</sup>. Although others and we show that synthetic glycan ligands provide an interesting opportunity to target Siglecs, the primary focus of most therapies are based on anti-siglec antibodies in order to target Siglecs<sup>59</sup>. Currently, Siglecs are used as targets in antibody-dependent therapies against lymphomas and leukaemias. CD22 and CD33 were originally identified as markers for B cell lymphoma and acute myeloid leukaemia (AML), respectively. Since CD22 is only expressed on B cells and CD33 on myeloid cells, targeting these Siglecs reduces toxicity to other cells and tissues. Moreover, antibody binding to CD22 and CD33 induces internalisation<sup>59,61</sup>, thereby carrying a toxin conjugated to the antibody directly into the target cell<sup>62-64</sup>.

The primary goal for the treatment of AML is to deplete tumour cells without killing healthy host cells. CD33 is expressed on myeloblasts of 90% of all patients suffering from AML, however CD33 is also present on many healthy myeloid cells. This indicates that killing of CD33-expressing cells may result in severe myelosuppression and neutropenia. Interestingly, CD33 is not expressed on pluripotent hemopoietic stem cells<sup>65</sup>, suggesting that these stem cells could replenish the myeloid cell compartment over time. In 2000, a calicheamicin-conjugated humanized murine anti-CD33, named Gemtuzumab Ozogamicin, was approved for cell-depletion therapy in AML patients<sup>66</sup>. Gemtuzumab Ozogamicin administered as a single agent has resulted in overall response rates of about 30% in AML patients<sup>67</sup>.

For B cell-depletion therapy in B cell lymphomas, three anti-CD22 antibodies are currently used in clinical trials; BL22, CMC-544 and Epratuzumab. BL22 is an immunotoxin, consisting of an anti-CD22 antibody conjugated to *Pseudomonas* exotoxin<sup>68</sup>. CMC-544 is an immunotoxin as well, consisting of a humanized IgG4 anti-CD22 antibody conjugated to the chemotoxin calicheamicin<sup>69-71</sup>. BL22 and CMC-544 differ from Epratuzumab, which is a humanized IgG1 anti-CD22 antibody alone<sup>72</sup>.

In addition to the treatment of lymphomas and leukaemias, Siglecs are also viewed as targets for the development of cell-directed therapies against leukocytes that mediate inflammatory, autoimmune or allergic diseases. Eosinophils and basophils express Siglec-8 in a highly cell type-restricted manner. Anti-Siglec-8 antibodies in the presence of secondary antibodies induce apoptosis of these cells by triggering a caspase-dependent signaling pathway<sup>73</sup>. This suggests that anti-Siglec-8 antibodies could be used to deplete eosinophils and basophils. Siglec-8 might therefore be an attractive target for the treatment of allergic disorders, in which hyper-eosinophilia plays an important role. In mice, Siglec-F has been assigned as the functional orthologue of human Siglec-8<sup>74</sup>. Recently eosinophilic inflammation in mice has been successfully treated with anti-Siglec-F antibodies, resulting in reduced levels of eosinophils<sup>75</sup>.

### *Siglec targeting to modify immune responses*

Chapter 3 describes that incubation of DCs with differently sialylated pathogens results in modulation of DC-mediated T cell responses. Sialoadhesin expressed on DCs recognizes *C. jejuni* lipo-oligoaccharide (LOS) expressing the GD1a/GM1a mimic with terminal  $\alpha$ 2,3-linked sialic acid, whereas Siglec-7 expressed on DCs recognizes the *C. jejuni* LOS expressing a GD1c mimic with an  $\alpha$ 2,8-linked terminal sialic acid with high affinity. We show that the linkage of sialic acids regulate DC-induced Th1/Th2 skewing. Targeting DC-expressed Sialoadhesin with LOS with terminal  $\alpha$ 2,3-linked sialic acids results in Th2 skewing, whereas Siglec-7 targeting with LOS with terminal  $\alpha$ 2,8-linked sialic acids results in DC-mediated Th1 responses. Similar to our data, Avril *et al* reported Siglec-7 binding to sialylated *C. jejuni* LOS as well<sup>76</sup>. Sialoadhesin was shown to function as receptor for the recognition of other sialylated pathogens as well, including *Neisseria meningitidis* and HIV<sup>54,55,77</sup>. The interaction of Sialoadhesin with HIV promoted trans-infection of T cells<sup>77</sup>.

Besides its role as an important pathogen uptake receptor, Sialoadhesin is also involved in autoimmune diseases. Sialoadhesin-expressing cells are believed to be crucial effector cells in inflammation associated with autoimmune diseases, such as rheumatoid arthritis. The ability of Sialoadhesin-positive cells to present antigens to T cells in secondary lymphoid tissues<sup>78</sup> and to polarize T cells towards Th2 responses, makes Sialoadhesin an attractive candidate in vaccine strategies for the treatment of autoimmune diseases<sup>59</sup>.

In chapter 4 we demonstrate that Siglec-E, the murine orthologue of human Siglec-7, acts as an endocytic receptor, as the neo-glycoconjugate  $\alpha$ 2,8-linked disialylated OVA is internalised and processed in both endosomal and lysosomal compartments. Strikingly, this uptake of  $\alpha$ 2,8-linked disialylated OVA leads to diminished MHC class II- and cross-presentation resulting in reduced CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation. Consistent with this, reduced production of the inflammatory cytokines IL-1 $\beta$ , IL-6 and IL-12p40 was observed when  $\alpha$ 2,8-linked disialylated OVA was targeted to Siglec-E compared to native OVA. Similar to our data, murine Siglec-H was shown to capture viruses, and to function as an endocytic receptor that internalizes antigen for T cell presentation as well<sup>79</sup>.

Our data provide evidence that binding and uptake of  $\alpha$ 2,8-linked disialylated OVA by Siglec-E results in dampening CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses. This can imply that  $\alpha$ 2,8-sialic acids are expressed in homeostatic conditions and interaction of  $\alpha$ 2,8-linked sialic acid binding Siglecs with their ligand can play a crucial role in maintenance of tolerance. Additionally, Miyazaki *et al.* have reported that another  $\alpha$ 2,8-linked disialylated structure, disialylated Lewis<sup>A</sup> expressed on colon epithelia, is expressed on non-malignant colon epithelial cells and its expression is decreased significantly upon malignant transformation of epithelial cells<sup>80</sup>. The physiological significance of the expression of disialylated Lewis<sup>A</sup> on epithelial cells can be proposed to protect the colon epithelia from autologous lymphocytes by binding of Siglec-7 to its ligand. Expression of  $\alpha$ 2,8-linked disialic acids is normally found on gangliosides in the nervous system. It was reported that Siglec-7 binds to ganglioside GT1b<sup>81</sup>. Furthermore, interaction of human Siglec-7 on NK cells with the ganglioside GD3 expressed on target cells leads to the suppression of NK cell-mediated cytolytic activity<sup>82</sup>.  $\alpha$ 2,8-linked disialic acids as well as Siglec-7 are also expressed by NK cells itself. Removal of *cis* ligands ( $\alpha$ 2,8-sialic acids) from NK cells by sialidase treatment results in *trans* ligand interaction leading to reduced NK-cytotoxicity<sup>82</sup>. This increase in *trans*-interaction could contribute to NK cell cytotoxicity in tissues like the brain where  $\alpha$ 2,8-linked sialic acid is abundantly expressed. The role of Siglec-E on murine DCs has not been reported yet, but can likewise be crucial for homeostatic control in the brain and the

maintenance of tolerance. Our data along with these previous studies provide evidence that human Siglec-7 or murine Siglec-E plays a novel role in homeostatic control in steady state environment.

Concluding, this thesis describes that Siglec targeting results in endocytosis of the sialylated antigens, as well as modification of immune responses through DC-mediated T cell proliferation and polarization. This, in combination with the restricted expression pattern, makes Siglecs attractive targets for cell-directed therapies.

## Immune modulation by C-type lectins

### *Glycan recognition by C-type lectins*

C-type lectins are calcium-dependent carbohydrate binding proteins<sup>7</sup> that can be divided into two categories; the mannose-type and the galactose-type C-type lectins. The mannose-type C-type lectins bind primarily to mannose- and/or fucose-terminated glycans, in contrast to galactose-type lectins, that recognize galactose- or N-acetylgalactosamine-terminated glycan structures<sup>7,83-85</sup>. The glycan structures recognized by some C-type lectins can be expressed by mammalian cells, as well as on pathogens<sup>7</sup>. This reflects the dual function of certain C-type lectins in host-pathogen recognition and homeostatic control. Such a dual function has been observed for the C-type lectins DC-SIGN, L-SIGN, mannose receptor (MR), macrophage galactose-type lectin (MGL) and Langerin. DC-SIGN and L-SIGN recognize both high-mannose and Lewis antigens, MR binds specifically to mannose, fucose, GlcNAc and sulfated glycans, whereas MGL recognizes terminal GalNAc<sup>84,86-89</sup>. Other C-type lectins, such as Dectin-1, almost exclusively interact with pathogen-specific glycan structures. Dectin-1 recognizes  $\beta$ -glucans, a fungal-specific glycan<sup>88,90,91</sup>.

C-type lectin receptors such as MR, DC-SIGN, MGL, dectin-1 and Langerin are not only involved in antigen recognition, but they also participate in the internalization and processing of antigens for loading onto MHC class I and II molecules<sup>83,92,93</sup>. By presenting antigen to T cells, the C-type lectin bearing cells instruct adaptive immunity.

### *Modulation of immune responses by targeting C-type lectins*

Modifying the glycosylation of antigens with specific glycan structures that bind with high affinity to C-type lectins can amplify immune responses against these antigens. Chapter 2 demonstrates that modifying the glycosylation of the tumour antigen gp100 with high-mannose structures enhanced the binding of gp100 to DC-expressed DC-SIGN. This improved DC uptake and antigen presentation to T cells.

Binding of specific glycan structures to C-type lectins participates in the induction of intracellular signaling cascades. Pathogens can modulate DC responses through pathogen-specific glycan structures, which affects C-type lectin receptor-induced signaling. Targeting DC-SIGN with mannose structures from the cell surface of *Mycobacterium tuberculosis* leads to high production of anti-inflammatory cytokines like IL-10<sup>93,94</sup>. Binding of mannose to the MR results in IL-10 secretion by DCs as well<sup>95</sup>. IL-10 production by DCs promotes the induction of regulatory T cells. Regulatory T cells limit immune responses directed to pathogens, which favours the survival of the pathogen<sup>96</sup>.

C-type lectin ligand binding and modulation of cytokine production can subsequently

influence the polarization of CD4<sup>+</sup> T helper cells. DC-SIGN binding to Lewis<sup>x</sup> structures on LPS of *Helicobacter pylori* or the soluble egg antigens of the parasite *Schistosoma mansoni* induces a DC-specific programming that leads to T cell differentiation towards Th2<sup>97</sup>. In contrast, binding of an *IgtB* mutant form of *Neisseria meningitidis* to DC-SIGN induces a Th1-mediated profile<sup>98</sup>. Furthermore, fucose structures promote besides Th2 also regulatory T cell responses (Gringhuis *et al.*, unpublished data). These data show that targeting DC-SIGN induces three different signaling pathways resulting in polarization of Th1, Th2 and regulatory T cells depending on the glycan ligand.

Their capability to direct antigens for antigen presentation as well as their immunomodulatory properties makes C-type lectins interesting targets for therapeutic strategies. Antigen vaccination with modified antigen glycosylation using C-type lectins as target could be a promising technique. We showed that vaccins could be developed with tumour antigens modified with high-mannose structures to induce C-type lectin-mediated T cell responses for the treatment of cancer.

## ▪ Concluding remarks

This thesis describes the functional consequences of glycosylation on DC biology. We demonstrate that the glycosylation of the DCs themselves is important for regulating immune responses. This involves upregulation of galactosylated and sialylated structures during DC maturation, thereby affecting recognition by Siglecs and Galectins. Sialoadhesin binding to sialylated structures on mDCs facilitates cellular interactions of DCs with macrophages. Furthermore, we identified polysialic acid as a marker for highly migratory mDCs. We propose that the expression of certain specific glycans by DCs should be considered as essential markers in vaccination based therapies.

In addition to the glycosylation of DCs, our work provides important knowledge on modulation of immune responses by targeting different DC-expressed lectins with glycosylated structures. Targeting of the C-type lectin DC-SIGN with high-mannose structures resulted in enhanced antigen presentation of DCs to T cells. In contrast, targeting murine Siglec-E with  $\alpha$ 2,8-linked sialylated structures resulted in reduced antigen presentation of DCs to T cells. Furthermore, we describe for the first time that the nature of DC-mediated Th responses is dependent on the linkage of the terminal sialic acid on the glycan structures and thus on the DC-expressed siglec it bound to. Targeting DC-expressed siglecs with terminal  $\alpha$ 2,3-linked sialylated structures promoted skewing of naive T cells towards Th2, whereas targeting DC-expressed siglecs with terminal  $\alpha$ 2,8-linked sialylated structures induced DC-mediated Th1 responses. These findings provide new insights in the therapeutic potential of targeting DC-expressed lectins with glycosylated structures (Figure 1).

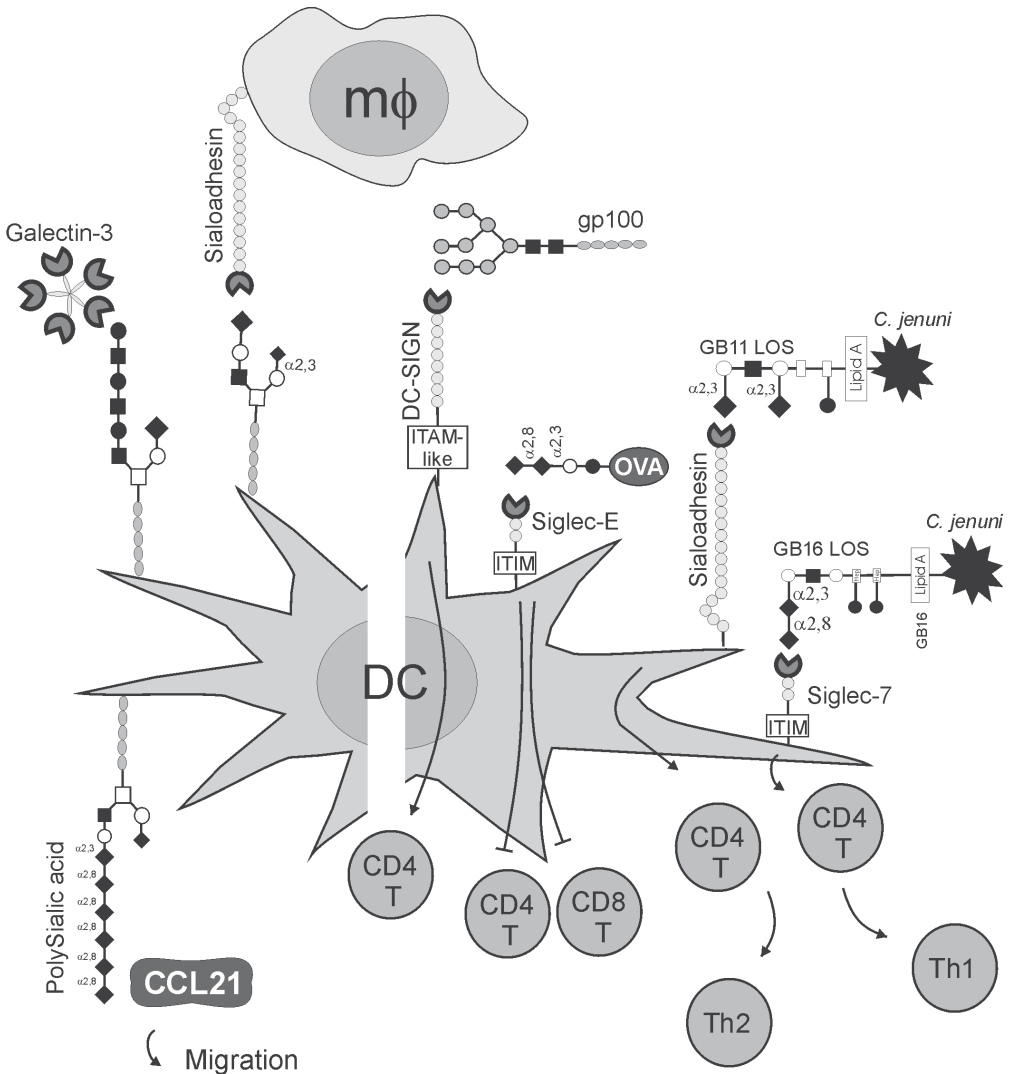


Figure 1. Functional consequences of glycosylation on DC biology.

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