Chapter 7

General Discussion

Soluble egg glycoproteins of *Schistosoma mansoni* exhibit potent immunomodulatory properties including those that lead to T-helper 2 (Th2) polarization and granuloma formation [1]. The glycans carried by these proteins are thought to be responsible for a large part of these properties by directing interactions with host lectins. Until now, most experiments supporting this line of thought have been performed with soluble egg antigen (SEA) preparations and/or synthetic, schistosome-related glycoconjugates [2-6]. However, SEA comprises a very complex mixture of soluble glycoproteins [7] and therefore it is difficult to determine which individual glycoproteins or specific structural characteristics of SEA are involved. Moreover, when using synthetic glycoconjugates as a basis for immunological studies, certain aspects of native glycoproteins are not addressed, such as the spatial presentation of glycans and the influence of the underlying protein structure. Comprehensive analysis of single, native *S. mansoni* glycoproteins in terms of glycan and protein structure, lectin binding behavior as well as functional capacities is essential to achieve a more profound understanding of immunological properties of schistosome eggs. In this thesis, the structural features of the glycans expressed on three major egg glycoproteins and the role that these glycans play in various aspects of host-parasite interplay have been studied.

One of these egg glycoproteins is the major ES component omega-1 [8]. Recently, omega-1 has been shown to harbor potent Th2 polarizing activities via modulation of dendritic cells (DCs), *in vitro* using human blood-derived cells and *in vivo* in mouse models [9;10]. We show in **chapter 2** that omega-1 carries core-difucosylated diantennary N-glycans with one or more Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX) motifs in the antennae. These structural observations are particularly interesting because a) the same glycans are present on IPSE/α1, a second major ES glycoprotein with immunomodulatory properties [11;12]; b) LeX as part of model glycoconjugates can modulate DC activation via DC-SIGN signaling, resulting in a immunological phenotype that is thought to be capable of inducing Th2 polarization [3;4;13-15]. It is reasonable to speculate that omega-1 uses this mechanism to condition DCs for Th2 priming. However, in **chapter 5** we show that DC-SIGN is dispensable for omega-1-induced Th2 polarization. Moreover, IPSE/α1, despite having similar glycans and C-type lectin receptor (CLR)-binding characteristics as omega-1 ([12] and **chapter 4**), does not exhibit the same Th2-polarizing properties observed for omega-1 [10]. Interestingly, the reported immunomodulatory effects of IPSE/α1, i.e. induction of IL-4 release in human and murine basophils [16;17] and reduction of cellular infiltration into granulomas [18], do not require intact glycosylation [16-18]. While literature suggests that respectively IgE binding- and chemotactic activities of IPSE/α1 are responsible for the immunological properties of this glycoprotein [11;16-18], we show in **chapter 5** that the mechanism through which

native omega-1 induces DC-mediated Th2 polarization is based on internalization of omega-1 via LeX-MR interactions, allowing its RNase activity to functionally modulate DCs.

In accordance with a major role for MR in the immunomodulatory effects of omega-1, we demonstrate that MR is the dominant receptor on monocyte-derived immature DCs (iDCs) that recognizes the LeX-dominated glycosylation on soluble IPSE/α1 and omega-1, while DC-SIGN interactions seem to be of only minor importance (**chapter 4 and 5**). In contrast to a dominant role for MR over DC-SIGN for these *soluble* LeX-expressing glycoproteins, MR binds only marginally to *plate-coated* LeX glycoconjugates (**chapter 4** and [19]), while DC-SIGN displays a strong affinity for these coated glycoconjugates as well as *plate- and particle-coated* SEA (**chapter 4** and [5;20;21]). These findings imply that the specificity of these CLR is at least partially dependent on the molecular presentation of the ligand.

Several studies describing the CLR-dependent molecular mechanisms of immunomodulatory molecules from other pathogens in addition imply that the multivalency characteristics of a ligand can influence the mechanism of action. Ligation of MR on DC by specific mannosylated molecules, including the mannose-capped lipoarabinomannans (ManLAM) from *Mycobacterium species*, has been demonstrated to promote anti-inflammatory responses via the induction of anti-inflammatory cytokines and interference with pro-inflammatory TLR signalling [15;22;23]. Other mannosylated ligands for MR, including mannan and thyroglobulin, are not able to induce these signalling pathways [22]. Cross-linking of MR by anti-MR antibodies leads to similar effects as ManLAM, indicating that the polyvalent nature of ManLAM is involved in its MR-mediated effects [22;23]. For DC-SIGN, it has been shown that pathogens expressing mannosylated (e.g. *Mycobacterium tuberculosis* and human immunodeficiency virus type 1; HIV-1) as well as fucosylated (*Helicobacter pylori*) glycan structures on a pathogenic surface can interfere with TLR-induced signaling via modulation of a signaling complex associated with DC-SIGN [15;24]. The type of glycan moiety recognized by DC-SIGN is critically important for the recruitment or dissociation of signaling molecules to the signaling complex. More importantly, the glycan moiety thereby affects the consequential modulation of cytokine responses. Mannose-mediated DC-SIGN signaling leads to upregulation of IL-10, IL-12 and IL-6 transcript while fucose-mediated signaling induces a more anti-inflammatory profile with enhanced IL-10 levels and decreased IL-12 and IL-6 levels [15].

These findings, combined with ours, indicate the presence of at least two separate mechanisms via which interplay of pathogen-associated or -derived glycoconjugates with CLRs can condition DCs for immune modulation of T cells: a) CLR-mediated internalization of bioactive molecules; and b) CLR-induced interference with TLR signalling. Which mechanism is exploited by pathogen-derived molecules may depend on the glycan motifs expressed, their

spatial presentation, and co-stimulatory factors provided by the targeted molecule itself and/or surrounding pathogenic molecules. Interestingly, the fucosylated and mannosylated pathogeni-derived molecules that are thought to induce functional DC signaling, are expressed on the surface of pathogens. Such a multivalent presentation of glycan ligands might be a prerequisite for functional signalling in DCs leading to Th polarization, and could explain why omega-1 and IPSE/α1, being secreted glycoproteins, are unable to use this specific type of mechanism (**chapter 5**).

For kappa-5, the third major egg glycoprotein studied, no immunomodulatory function has been described in literature. We show in **chapter 3** that kappa-5 mainly carries core-difucosylated and -xylosylated triantennary N-glycans that are substituted with GalNAcβ1-4GlcNAc (LacdiNAc, LDN) antennae, a minority of which carry an additional α3-linked fucose on the GlcNAc residue (LDN-F). These glycans mediate interaction of kappa-5 with DC-SIGN, MGL and MR (**chapter 4**), and at the same time are a target for IgE-binding (**chapter 3**), suggesting multiple roles for these glycans in the host-pathogen interaction. In **chapter 6**, we demonstrate in a pulmonary mouse model that native kappa-5 coated to Sepharose beads can induce type 2-polarized granulomatous responses, and that the LDN motifs on kappa-5 are involved in this process. These results indicate a dual role for kappa-5 as a granulomagenic as well as a Th2-modulating agent.

The mechanism by which LDN induces granulomatous responses remains to be elucidated, but its recognition by CLRs on DCs suggests that CLR-expressing antigen-presenting cells (APCs) are likely to be involved. Also, preliminary data indicate a role for the chemokines CCL17 and CCL22, as mRNA expression of these glycoproteins could be induced in DCs upon kappa-5 stimulation (data not shown). CCL17 and CCL22 have been previously shown to be upregulated in presensitized mice upon tail vein injections of *S. mansoni* eggs [25], Moreover, these chemokines can functionally act via CCR4 which has been shown to be expressed on a wide array of immune cells including Th2 cells, eosinophils and macrophages, cells that are major constituents of the *S. mansoni* egg-induced granuloma [26-29]. Neutralization of either of these chemokines in mice resulted in reduced egg granuloma size and eosinophil numbers as well as increased cytokine levels [26]. Given these and our findings, we hypothesize that recognition of LDN motifs by CLRs on APCs leads to the release of CCL17 and CCL22 and therefore the attraction of CCR4-expressing immune cells. This may provide one mechanism of action via which eggs can induce and/or modulate type 2 granuloma formation.

Unpublished findings indicate that kappa-5 is not the only granulomagenic component in SEA or ES, as depletion of kappa-5 does not completely abrogate the capacity of these mixtures to

induce granuloma formation in a pulmonary mouse model (data not shown). Likewise, SEA depleted of omega-1 is still able to induce Th2 polarization in a mouse model [10], indicating that other soluble egg (glyco)proteins are in addition involved in these immunomodulatory processes. Considerable data is now available on the proteome and glycome of *S. mansoni* eggs [7;30;31]. However, apart from the glycoproteins described in this thesis, it is generally unknown which proteins express which glycans, complicating the search for new immunogenic molecules. Still, the structural information of SEA and ES glycans provides interesting clues. Omega-1 and IPSE/α1 have been shown to be the main carriers of LeX motifs within the ES glycome [31], while kappa-5 is the major LDN-expressing glycoprotein within SEA (**chapter 3**). Therefore, we suggest that the remaining immunoactive soluble egg glycoproteins can modulate immune responses via other, yet undetermined, glycans and/or protein motifs. Major glycan motifs on these glycoproteins include α3-fucose and β2-xylose core modifications, fucosylated LDN motifs, and Fucα1-2Fucα1-3-HexNAc elements [30;31].

For the fucosylated LDN motifs present on *S. mansoni* egg glycoproteins (see Table 2 in **chapter 1**), we and others have shown that DC-SIGN recognizes the majority of these structures (**chapter 4** and [20,21]). Interestingly, LDN-based structures are expressed by many other helminth parasites that induce Th2-polarized responses, including *Haemonchus contortus* [32], *Fasciola hepatica* [33] and *Trichinella spiralis* [34;35]. This is in contrast to LN/LeX motifs, that have only been found on schistosomes and the nematode *Dictyocaulus viviparus* [31;33]. Thus, the fucosylated LDN motifs form common helminth ligands for DC-SIGN, although the significance of these motifs remains unknown.

Fucα1-2Fucα1-3 (DF) elements are frequently occurring on LDN structures of egg O-glycans, while only a minority of N-glycans seem to display this element [31;36]. DF has so far not been reported in any species other than schistosomes, and therefore could potentially be very interesting in terms of immune recognition. Indeed, DF elements elicit high antibody responses during *S. mansoni* infection in humans and chimpanzees [37-39]. Moreover, LDN-DF glycoconjugates induce a pronounced production of pro- and anti-inflammatory cytokines in peripheral-blood mononuclear cells (PBMCs) from non-exposed humans, to a level far higher than the production induced by other glycoconjugates including those terminating in LeX and LDN-F [40]. The latter observation may indicate that DF elements may be involved in innate recognition of eggs by APCs. Our glycoconjugate array data show that DC-SIGN, MGL and MR do not or only slightly bind the DF-element on a single GlcNAc residue (**chapter 4**), and it was previously demonstrated that DC-SIGN is unable to bind LDN-DF [20], indicating that DC-SIGN, MGL and MR do not play a role in the recognition of DF elements by the immune system. To date, no other receptors have been found that display affinity for this element.

In addition to the described properties of soluble egg glycoproteins, insoluble components of the *S. mansoni* egg shell as well as glycolipids may also contain (glycosylated) molecules with immunomodulatory properties. Egg glycolipids have been shown to express LDN, LDN-F and LeX, as well as the DF-element in the form of repetitive Fucα1-2Fucα1-3-HexNAc stretches [36,41], and have been linked to innate immunity [40]. Recently, it has been shown that the egg shell also appears to be glycosylated [42]. Moreover, we found that egg shell N-glycans are dominated by Galβ1-4GlcNAc (LacNAc, LN) motifs (unpublished finding), which as a part of asialofetuin on Sepharose beads have been shown to harbor granulomagenic properties [43].

Concluding remarks

Pathogens are recognized by the innate immune system via a multitude of conserved molecules that are bound by pattern recognition receptors (PRR) such as CLRs, leading to activation of the adaptive immune system. However, pathogens have evolved to modulate immune responses via the same group of molecules to ensure survival within the host. Schistosomes are particularly persistent, as clearance of these parasites usually does not occur without treatment. Immunogenic glycan motifs of egg molecules are suggested to play a major role in the immunomodulation leading to long term survival.

While affecting CLR-induced signaling in DCs seems to be a common mechanism for pathogens to polarize Th-cell responses, we have shown that the *S. mansoni* egg glycoprotein omega-1 uses LeX-CLR interplay for its internalization into DCs, which is an essential step for the Th2 priming effect of this glycoprotein (**chapter 2 and 5**). In the case of IPSE/α1, intact glycosylation is dispensable for its anti-inflammatory and Th2 regulatory effects, although its glycans are efficiently recognized by CLRs (**chapter 4**). For kappa-5, we have shown that LDN glycosylation is involved in its granulomagenic properties (**chapter 3 and 6**). The exact mechanisms via which the immune system mediates this effect remain unclear, although CLR recognition of kappa-5 glycans might play a role (**chapter 4**). The approach used in this thesis, in which studies on primary structural features, binding properties, as well as functional effects of a single glycoprotein are combined, has helped to gain insight in the mechanisms of action of three *S. mansoni* egg glycoproteins and could also be useful for the identification of other glycosylated molecules involved in helminth-induced immune responses.

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