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Polarization of host immune responses by helminth-expressed glycans

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Helminth parasites bias host CD4$^+$ T helper (Th) cells toward Th2 responses, drive alternative activation of macrophages, and expand T regulatory cells. Helminth-expressed carbohydrates play critical roles in driving much of this immune cell biasing. Studies on helminth glycans have focused on Lewis X, LDN, LDN-DF, other fucosylated structures, chitin, tyvelose, and trehalose, which interact with host antigen presenting cells (APCs) minimally via C-type lectins and/or Toll-like receptors (TLR). Here, we review recent findings on helminth glycan activation of APCs via C-type lectin/TLRs and introduce the concept that glycosylated helminth molecules require endocytosis to function as immune modulators. Second, we describe unpublished data showing that *in vivo* glycoconjugates comprising multiple copies of glycans on carriers are directly immune modulatory. Lastly, we discuss the observation that CD14 negatively regulates alternative activation of APCs during helminth infection. We close with a discussion on the use of immune modulatory glycans as vaccine adjuvants and as antiinflammatory therapeutics.

Keywords: helminth parasites; glycans; LNFPIII; Lewis X; alternative activation; macrophages; dendritic cells

Introduction

Initial studies on parasite modulation of host immune responses focused on why host immune responses were unable to control parasite infections, rather than on how parasite glycans could modulate immune responses.[1] During infection by African trypanosomes the parasites switch expression of variant surface glycoproteins, coincident with the rise of the host antibody response to the existing variant surface glycoproteins.[2,3] In *Leishmania*-infected cells, parasite-expressed lipophosphoglycans and proteophosphoglycans inhibit maturation of host endosome/lysosomes, which allows parasites to evade lysosomal killing and degradation.[4,5] The ability of parasites to actually bias host immune responses was initially shown in *Leishmania*-infected mice,[6–8] and subsequently during helminth infection.[9–13,14]

Importantly for the discussion here, the potent ability of helminth infection, or administration of helminth extracts, to bias host CD4$^+$ T cells toward Th2 responses is largely due to helminth-expressed glycans.[15–17] The importance of glycans in biasing CD4$^+$ T cells was initially demonstrated by using low concentrations of sodium metaperiodate to alter glycan structures on saline soluble schistosome egg antigens (SEA). Comparing metaperiodate-treated SEA to intact or mock-treated SEA, the study demonstrated that as expected, intact or mock-treated SEA drove CD4$^+$ Th2 responses *in vivo*, while metaperiodate-treated SEA did not.[18] Subsequently, Tawill *et al.*[15] showed that enzymatic removal of glycans from extracts of filarial worms rendered them unable to drive Th2 respnoses.[15] Thus, two independent studies, employing two different methods to remove or alter glycan structures, demonstrated that helminth-expressed glycans were essential for driving host CD4$^+$ Th2 responses. It is worthwhile to note that neither of these studies demonstrated that particular helminth glycans were *directly* biasing host immune responses. Rather, these studies showed that worm glycans need to be present in order for host immune Th2 responses to occur.

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One of the immune modulatory glycans expressed by schistosomes and other helminth parasites is Lewis X. Our initial identification of this schistosome carbohydrate was done using monoclonal antibodies produced from mice immunized with schistosome eggs or with soluble egg antigens. In a collaborative effort, the resulting anti-egg monoclonal antibodies (mabs) were screened for binding to day 9–10 fetal mouse brain, a source rich in variant glycoforms. Interestingly, one mab, E.5, bound to fetal brain in a pattern that was identical to the pattern previously reported for stage-specific embryonic antigen-1 (SSEA-1). The glycan structure for SSEA-1 is lacto-*N*-fucopentaose III (LNFPIII), which we used to inhibit mab E.5 from binding to schistosome soluble egg antigens. Interestingly, in addition to being a developmental antigen, LNFPIII is also abundant in human breast milk,[19] and LNFPIII contains the Lewis X trisaccharide. Based on these studies, we initially reported LNFPIII as a schistosome sugar. However, subsequent studies demonstrated that schistosomes actually express the Lewis X trisaccharide, which is contained within the LNFPIII pentasaccharide[a]. In addition to Lewis X, schistosomes have been reported to express molecules that have poly-Lewis X on them, as well as other fucosylated sugars, such multiply fucosylated, LacdiNac, multiply fucosylated, and DF-LDB-DF.[20–27] For studies performed with LNFPIII, we utilized neoglycoconjugates displaying multiple copies of LNFPIII linked to different carrier molecules. The neoglycoconjugates comprised 8–12 LNFPIII molecules conjugated to carriers such as the 42 kDa dextran or human serum albumin via linker-spacer methods. In addition, other groups have examined responses to Lewis X conjugated to bovine serum albumin or to polyacrylamide. Of note, to our knowledge, while schistosomes or other helminth parasites may express poly-Lewis X, the presentation of helminth Lewis X is different from the LNFPIII neoglycoconjugates.

A new concept presented here is *how* helminth-expressed glycans induce immune modulation of host immune responses. For example, for the majority of glycosylated helminth molecules,[28,29] it is not known if the glycans on these molecules directly activate host immune cells. Perhaps for many of the glycans present on helminth molecules their roles may be to facilitate binding to host cells via one or more C-type lectin receptors, leading to endocytosis of the helminth molecules?

Below, we present evidence indicating that for schistosome soluble egg antigens endocytosis indeed appears to be a key aspect in the maturation of antigen-presenting cells that drive $CD4^+$ Th2 responses. Clearly, some glycans expressed by helminths are able to directly bind to and activate host antigen presenting cells. However, as noted earlier, for all studies demonstrating direct activation of host cells by helminth glycans, the glycans have been presented as multivalent arrays on carrier molecules. Yet, it is critical to note that multivalent, conjugate arrays are not how these glycans are expressed *in situ* on helminth molecules.

One mechanism that helminth parasites utilize to drive anti-inflammatory responses *in vivo* is via the induction of alternatively activated APCs.[9,10,16,30] In the remainder of this paper we present data demonstrating that CD14 negatively regulates the ability of helminth-expressed glycans to drive alternative activation of macrophages. We then discuss recent studies demonstrating that helminth-expressed glycans, administered as multivalent glycoconjugates, have potent, therapeutic activity *in vivo* against inflammation-based diseases. We conclude with a discussion of where research on parasite glycans is headed, and how these studies will provide insight into the control of inflammation-based diseases. We include an overview of the use of parasite glycans as immuno-potentiators (or adjuvants) for vaccines, and the feasibility of using defined parasite glycans as therapies to suppress or prevent development of proinflammatory diseases.

Immune modulation during helminth infection

Infection with helminth parasites is associated with increased production of the anti-inflammatory cytokine interleukin (IL)-10, and $CD4^+$ T cell-mediated Th2 immune responses (IL-4, IL-13, and IL-5).[31,32] In an attempt to define the class of helminth molecules contributing to immune biasing, Okano *et al.* showed that glycans in *S. mansoni* eggs were largely responsible for inducing Th2 responses.[18] This study compared immune

[a] Therefore, throughout this article we will use "LNFPIII" and "Lewis X" interchangeably.

responses in mice injected with schistosome SEA (a known, potent inducer of Th2 responses) treated with sodium metaperiodate, which alters glycan structures, compared to mice injected with mock-treated SEA. The results showed that unlike intact or mock-treated SEA, metaperiodate-treated SEA could not drive Th2 responses *in vivo*.[18] A similar finding was presented in a subsequent study by Tawill *et al.*, which demonstrated that enzymatic removal of glycans on extracts of nematode parasites largely abrogated Th2 responses.[15] Additional studies examining the immune modulatory or biasing roles of cestode and nematode glycans report similar findings.[33–35] Taken together, these studies strongly suggest that some helminth carbohydrates play critical roles in the overall process of generating $CD4^+$ Th2 responses seen during helminth infection, or following treatment with helminth extracts.

Sugar structures on helminth parasites were initially defined using antiparasite monoclonal antibodies. The first of these studies screened a series of murine mabs produced from mice immunized with schistosome eggs or with soluble egg antigens. As mentioned above, the anti-egg mabs were screened against day 9–10 fetal mouse brain as a rich source of variant glycoforms and the mab E.5 bound to fetal mouse brain in a pattern that was identical to that described for stage SSEA-1,[19] which was known to be LNFPIII. The fact that LNFPIII could block mab E.5 from binding to SEA suggested that this was the sugar found on schistosomes. However, this conclusion was incorrect, as shown by Levery *et al.*, who demonstrated that a major constituent of schistosome egg antigens was actually Lewis X, the terminal trisaccharide of LNFPIII.[36] Additional structurally related glycans in schistosome eggs have been identified using mabs of sera from infected animals or patients.[37,38] Lewis X motifs are found in schistosomes and in nematodes such as *Dictyocaulus viviparous*,[39–41] while other helminthes express core alpha 1-3 fucose. Nematodes also express chitin,[42–44] trehalose, and tyvelose motifs.[45] Table 1 lists some of the glycan structures expressed on different helminth parasites and, where known, the host receptors that bind/interact with the glycans. Table 1 also lists the types of immune responses induced by these different glycan structures.

Regarding helminth glycan induction of immune modulation or polarization, more studies have been performed with molecules that contain Lewis X or LNFPIII. For studies on LNFPIII, the pentasaccharide is presented as a neo-glycoconjugate comprising multiple copies of LNFPIII (8–12) conjugated to carriers such as the 42 kDa dextran or to human serum albumin. As multivalent conjugates, LNFPIII induced *in vivo* expansion of B-1 B cells and production of IL-10 and PGE_2.[46] Further, Vellupillai *et al.* demonstrated a role for LNFPIII/Lewis X in driving IL-10 production in anergic peripheral blood mononuclear cells from schistosome-infected patients in Brazil.[46] LNFPIII/Le^x glycoconjugates were also shown to induce proliferation of suppressor macrophages and to drive Th2 responses under *in vivo* and *in vitro* conditions.[47,48] Taken together, these studies led to the concept that the $\alpha1\rightarrow3$-fucose group was required to induce IL-10 and PGE_2. Further evidence in support of $\alpha1\rightarrow3$-fucosylated glycans driving Th2 and anti-inflammatory responses during helminth infection comes from studies on *Haemonchus contortus* infection of sheep. This study showed, for example, that parasite-specific IgE inserum of *H. contortus*-infected sheep binds to Lewis X. IgE is indicative of Th2 responses and thus it was concluded that Lewis X motifs play a role in driving Th2 responses in *H. contortus*-infected sheep;[49] in fact, it may be that these responses were due to expression of core a $\alpha1\rightarrow3$-fucose. Subsequent studies showed that schistosome adult worms and eggs express *N*-glycans containing $\alpha1\rightarrow3$-fucose or $\beta2$-xylose, and that both were able to induce Th2 responses in mice.[50]

It has been known for some time that helminth glycans are expressed on both proteins and lipids; yet, whether helminth glycans expressed on proteins and lipids behave differently has only recently been investigated. Evidence suggests that both helminth glycoproteins and glycolipids can modulate immune responses. For example, Lewis X, LDNF, or fucosylated LDN [$\beta1\rightarrow4$(Fuc$\alpha1\rightarrow2$ Fuc$\alpha1\rightarrow3$) GlcNAc] motifs are found in the glycosphingolipid fraction of schistosome eggs, cercariae, and adult worms.[25,26,51] When these fractions were tested on human PBMCs, the egg glycosphingolipids-induced production of IL-10, IL-6, and TNF-α, whereas the adult worm glycosphingolipids induced only proinflammatory responses.[25,26,51] Furthermore, the ability of the adult worm glycosphingolipids to drive proinflammatory responses required intact fucose residues on the glycolipids, via a mechanism that

Table 1. List of helminth glycans that have been reported to bind to host innate receptors and modulate/polarize immune responses

Glycans	Helminth species	Receptors/immune cells involved		Biological function/s	References
		Human	Mouse		
Lewis X	Schistosomes Nematodes	DC-SIGN MGL	SIGNR-1; SIGNR-3; MGL1	Th2 immune modulation	75, 93–94
LDN	Schistosomes Nematodes	MGL	MGL2 Galectin3	B cell response	17, 20, 66
LDNF	Schistosomes Nematodes	MGL DC-SIGN TLR4	MGL	Humoral immune response (IgG, IgM); Th1 response	67, 95
α1,3 core fucose	Schistosomes *H. contortus* *C. elegans*	–	–	Humoral immune response (IgE); Th2	50
Core beta 2-xylose	Schistosomes	DC-SIGN MR?		Th2	50
High mannose *N*-glycans	Schistosomes	DC-SIGN L-SIGN MR	SIGNR1/3 MR	Humoral response? Immune suppression?	64, 95–97
Tn antigen	Schistosomes	MGL	MGL2	B cell response; Th2; immune suppression?	93, 98
	Tape worms	Galectin-3			
Chitin	Nematodes	–	–	Macrophage polarization	43

required the dendritic cell receptors TLR4 and DC-SIGN.[25,52] The reports of adult worm glycosphingolipids driving proinflammatory responses is unique and suggests that glycosylated helminth molecules are not solely purposed for driving $CD4^+$ Th2 responses or for inducing alternatively activated macrophages.[25,26,51,52]

Immune modulatory schistosome IPSE/alpha-1 and omega-1 glycoproteins

To search for immune modulatory proteins, schistosome soluble egg antigens were fractionated. The first described hepatotoxic egg antigen was omega-1, which was later shown to be a ribonuclease.[28,53] Much later, IPSE/alpha-1 was described as a major secretory glycoprotein from schistosome eggs, which also express Lewis X motifs on core-difucosylated *N*-glycans.[28] Subsequently, IPSE/alpha-1 was shown *in vivo* to drive IgE-dependent but antigen-independent IL-4 production by murine basophils.[54] Further, recombinant IPSE/alpha-1 induces IL-4 in a dose-dependent manner, suggesting that the Lewis X motifs are not required. In back to back publications, omega-1 was shown *in vitro* to drive maturation of human DCs that could stimulate naive $CD4^+$ T cells to Th2 cells, or, in the second study, murine DCs were activated with omega-1 *in vitro* and then used to drive maturation of naive OVA-Tg $CD4^+$ T cells to Th2 cells;[29,55] activity of omega-1 was abrogated when it was treated with DEPC (diethylpyrocarbonate) or protease. Similar to SEA, omega-1 downregulated LPS-induced dendritic cell maturation and production of proinflammatory cytokines.[55] A subsequent study by Meevisse *et al.* showed the presence of Lewis X motifs on native omega-1 protein by Western blot analysis and by mass spectrometry.[29] Native omega-1 also has at least two N-glycosylation sites, each of core-difucosylated dientennary glycans with one or more Lewis X motifs in the antenna. Native omega-1

displays other sugars, including tandem repeats of Lewis X and difucosylated LN and LDN. An interesting question to solve regarding omega-1 will be to determine if omega-1-expressed glycans are required for optimal Th2-inducing activity.

Unlike native omega-1, recombinant omega-1 expressed in human HEK293 cells had a decreased ability to drive Th2 responses. The reduced potency of the recombinant molecule may be due to differences in glycosylation pattern or, simply, to the fact that the recombinant form is not glycosylated. Further studies need to be performed to determine whether Th2 responses induced by omega-1 require the presence of glycans for optimal activity.[55] It may be that the glycans on omega-1 bind to C-type lectin receptors on APCs, facilitating endocytosis.[29] Endocytosis of omega-1 may be critical for maturation of APCs that drive Th2 responses.

Recently, IPSE/alpha-1 and omega-1 were tested for their ability to upregulate Foxp3-expressing T cells. This study demonstrated that injection of omega-1 into NOD mice was sufficient to induce Foxp3 and IL-4 expression, whereas IPSE/alpha-1 injection did not induce Foxp3 expression.[56] Following the initial observations of omega-1 as a hepatotoxin, Abdulla *et al.*, demonstrated that IPSE/alpha-1 is also hepatotoxic, and that omega-1 and IPSE/alpha-1 together account for the majority of hepatotoxic activity secreted by schistosome eggs (see Table 1).[57]

Helminth glycans drive alternative activation of antigen-presenting cells

In addition to dendritic cells, macrophages take up pathogen antigens; for macrophages this process can lead them to become either classically activated (CA) or alternatively activated (AA).[58] In general, AA macrophage induction is thought to require IL-4 and/or IL-13.[59] Helminth infection generally drives AA, and during schistosome infection of mice this process has been demonstrated to be IL-4 and/or IL-13 dependent.[60]

Several studies have investigated whether glycans expressed by helminths drive AA maturation independent of IL-4 and/or IL-13. Atochina *et al.* asked if schistosome glycans could induce alternative activation of macrophages unable to respond to IL-4/IL-13 using IL-4Rα$^{-/-}$ mice.[59] Peritoneal macrophages harvested 18–20 hours postintraperitoneal injection of LNFPIII conjugates from wild-type or IL-4Rα$^{-/-}$ mice showed equal expression of the alternative activation markers *ym1, fizz1,* and *arg1.* These results showed for the first time that helminth-expressed glycans can drive IL-4/IL-13–independent alternative activation (Fig. 1).[59] In addition to studies on LNFPIII/LeX conjugates, several groups have recently begun investigating nematode-expressed chitin as an immune modulator. Chitin is ubiquitous, both nematode parasites and the free-living nematode *Caenorhabditis elegans* express it.[42–44] With regard to nematode-expressed chitin having immune modulatory activity, Reese *et al.* found that it induced recruitment of IL-4–producing innate cells in both lungs and the peritoneal cavity of mice.[61] Although injection of chitin led to the recruitment of AA orArg1-expressing macrophages, the study by Reese *et al.* did not determine if alternative activation in response to chitin was an IL-4–dependent or –independent event.[61] However, a recent study by Satoh *et al.*

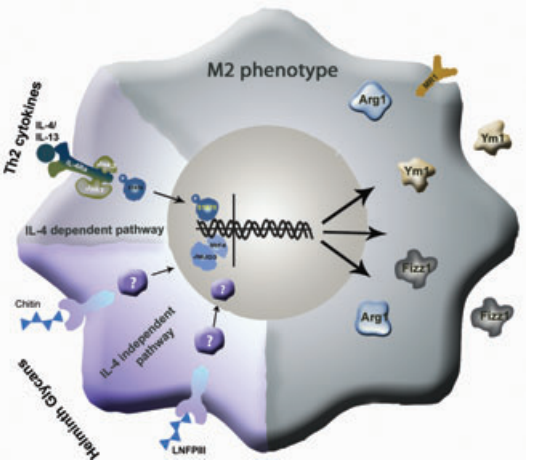

Figure 1. Helminth glycans are able to drive IL-4/IL-13–independent alternative activation of macrophages in a process that does not utilize IL-4Rα. IL-4 or IL-13 can bind to the IL-4Rα on macrophage surfaces and initiate alternative activation. IL-4Rα ligation activates JAK1 and JAK3, which in turn help in activation of STAT6 transcription factor. Activated STAT6 translocates to the nucleus and regulates the expression of genes involved in alternative activation (AA) of macrophages. Ym1, Fizz1, Arg1, and MR1 are AA markers expressed in response to IL-4 stimulation. The helminth glycan chitin induces alternative activation of macrophages independent of the IL-4Rα pathway through an unknown signaling cascade that involves activation of JMJD3, which in turn activates IRF4. Similarly, the helminth glycan LNFPIII/Lewis X, through an unknown signaling cascade, also induces expression of AA markers independent of IL-4Rα.

showed that intraperitoneal injection of chitin into mice-induced recruitment of AA macrophages and eosinophils in a JMJD3-dependent fashion.[43] In addition, Satoh *et al.* showed that expression of AA markers on macrophages was severely impaired in *jmjd3*$^{-/-}$ and *irf4*$^{-/-}$ mice, compared with WT controls. Interestingly, *jmjd3*$^{-/-}$ macrophages were alternatively activated when stimulated with IL-4, suggesting that chitin-induced alternative activation is independent of IL-4–mediated signaling but requires the JMJD3 and IRF4 pathways.[43] Lastly, Gundra *et al.* reported that TLR2$^{-/-}$ mice infected with *Taenia solium,* the tapeworm that causes neurocystocercosis, showed reduced expression of AA macrophage markers (*Arg1*, *Fizz1*, and *Ym1*) in brain, compared with infected WT controls.[62] Currently, it is not known if *T. solium* glycans are involved in TLR2-mediated alternative activation of macrophages.

In addition to AA macrophages, studies have shown that DCs stimulated with helminth glycans can drive maturation of CD4$^+$ T cells to a Th2 phenotype in both human and mouse models.[63,64] Thomas *et al.* showed that murine bone marrow-derived dendritic cells (BMDCs), pretreated with LNFPIII and then cocultured with naive CD4$^+$ T cells, induced Th2 cells that produced IL-4, coincident with downregulation of IFN-γ.[30] Van Liempt *et al.* made a similar observation with human iDCs, showing that soluble egg antigens are internalized by iDCs via C-type lectin receptors. This process inhibited LPS-induced IL-12 production, as well as expression of the maturation markers CD86/CD80.[64] Chitin has also been shown to suppress proliferation of CD4$^+$ naive T cells and to inhibit the expression of PDL-1 on macrophages independent of TLRs or STAT6-mediated signaling.[34] Taken together, helminth-expressed glycans drive alternative activation of macrophages and DCs. To date, the cellular receptors and signaling cascade(s) involved in glycan-mediated alternative activation are incompletely described.

The role of C-type lectins in APC binding and uptake of helminth glycans

The apparent requirement of glycans for helminth parasites to drive maximal immune biasing suggests that APC expressed C-type lectin receptors (CLRs) play a role in facilitating this immune modulation. As discussed briefly in the introduction, whether this "facilitation" is simply due to binding and subsequent endocytosis of glycosylated helminth molecules, or to direct CLR-induced signaling, is not well understood at this time. The majority of studies on APC C-type lectin binding to helminth glycans have been performed with schistosome soluble egg and adult worm molecules. Glycans expressed in/on *S. mansoni* eggs, larvae, or adult worms have been shown to bind to DC-SIGN, mannose receptor (MR1), MGl1, the scavenger receptor C-type lectin, and galectin.[52,64–68] C-type lectins have generally been considered to play roles in phagocytosis and/or antigen uptake.[69] As listed in Table 1, CLRs expressed on innate cells recognize pathogens/parasites through glycan-mediated interactions. Van Die *et al.* reported that human dendritic cells interact with fucosylated glycans of schistosome SEA via DC-SIGN.[67] Further, DC-mediated internalization of SEA glycans was dependent on one or more CLR, DC-SIGN, MGL1, and MR1.[64] It will be interesting to determine if CLRs are involved in development of Th2 responses; the new observations presented here suggest that this is the case, at least for schistosome egg antigens.

The observations that helminth-expressed glycans may bind to multiple C-type lectins makes it difficult to investigate the function(s) of a single receptor. For a defined class of CLRs, ascribing specific immune modulatory roles is difficult—for example, in the mouse genome harbors several homologues of DC-SIGN; among these SIGNR-1, -3, and -5 are closely related to human DC-SIGN.[70] In further complicating matters, Koppel *et al.* found that unlike human DC-SIGN murine SIGNR-1 not only binds to Le$^{x/y}$, Le$^{a/b}$ antigens, and mannose-containing glycans, but also has specificity for sialylated-Le^x.[71] Murine SIGNR (mSIGNR)-3 is most closely related to human DC-SIGN, and shows binding specificity toward both mannose and fucosylated sugars (Le^x) and the ability to endocytose glycan-expressing molecules.[70] In addition to the variability in mSIGNRs compared to human DC-SIGN, analysis of the mouse genome shows that there are also two copies of the macrophage C-type lectins (MGL) 1 and 2, both of which have been shown to bind Le^X.[72] MGL1 and MGL2 are expressed on alternatively activated macrophages during parasitic infection and allergic airway inflammation.[73] Although a defined signaling cascade downstream of the majority of these CLRs is lack-

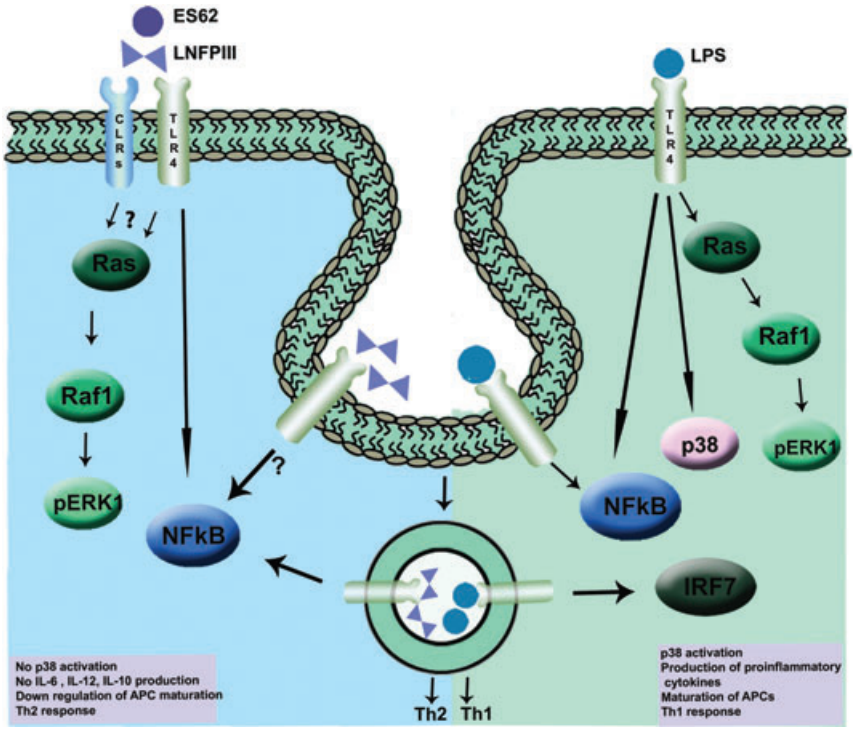

Figure 2. Helminth glycans induce altered signaling downstream of Toll-like receptors in antigen-presenting cells. LNFPIII glycan or ES62 glycoprotein ligation of APC cell surface receptors leads to activation (phosphorylation) of ERK and the p50 subunit of NF-κB, without activating or inducing p38 activation and production of IL-6, TNF-α or IL-12 cytokines. Whether or not helminth glycans need to be endocytosed by APCs to drive cell signaling cross-talk with TLR-dependent signaling, leading to APC activation that drives $CD4^+$ Th2 responses is not known. The signaling patterns induced by helminth glycans are distinct from those induced by LPS, activating ERK, NF-κB, and p38, resulting in the production of proinflammatory cytokines and generation of Th1 responses.

ing, DC-SIGN, mannose receptor (MRR), blood DC-SIGN antigen 2 (BDCA2), DC immunoreceptor (DCIR), and myeloid C-type lectin-like receptor (MICL) are CLRs reported to induce signaling that modulates TLR-mediated immune responses.[74]

Helminth glycan binding to, and signaling through, C-type lectins can influence signaling through Toll-like receptors (TLRs). For example, van Liempt *et al.* reported that glycans in SEA, via binding to DC-SIGN, MGL1, or MR1, modulate TLR-induced immune responses of iDCs.[64] Later Gringhius *et al.* elegantly showed that ligation of mannose-containing glycans to DC-SIGN leads to Raf-1 phosphorylation and acetylation of p65, which results in augmentation of LPS-induced IL-10, IL-12, and IL-6 production.[75] Subsequently, fucose-containing glycans, such as Lewis X, were shown to downregulate LPS-induced IL-12 and IL-6 and to upregulate IL-10 in a Raf-1-independent manner.[75] Van Stijn *et al.* demonstrated that fucose-containing glycosphingolipids derived from adult worms use both TLR4 and DC-SIGN to induce maturation of human DCs-producing IL-6, IL-12, IL-8, and TNF-α. This was not seen with glycosphingolipids derived from eggs (Fig. 3).[52] Similarly, Jenkins *et al.* reported that TLR4 was sufficient to induce glycan-dependent induction of proinflammatory responses from peritoneal macrophages in response to secretory soluble glycans from *S. mansoni* larvae. The ability of soluble larval antigens to induce IL-12 was intact after treatment with proteases, suggesting that glycans in the larval secretions induced these responses.[76] Taken together, these studies suggest that C-type lectins, by binding to helminth-expressed glycans, generate divergent signaling pathways capable of modulating TLR signaling.[64,67,75,77] These studies also suggest that in addition to protein antigens and ODNs, helminth glycans can generate Th1 or Th2 responses, depending on whether they are linked to lipids or proteins;

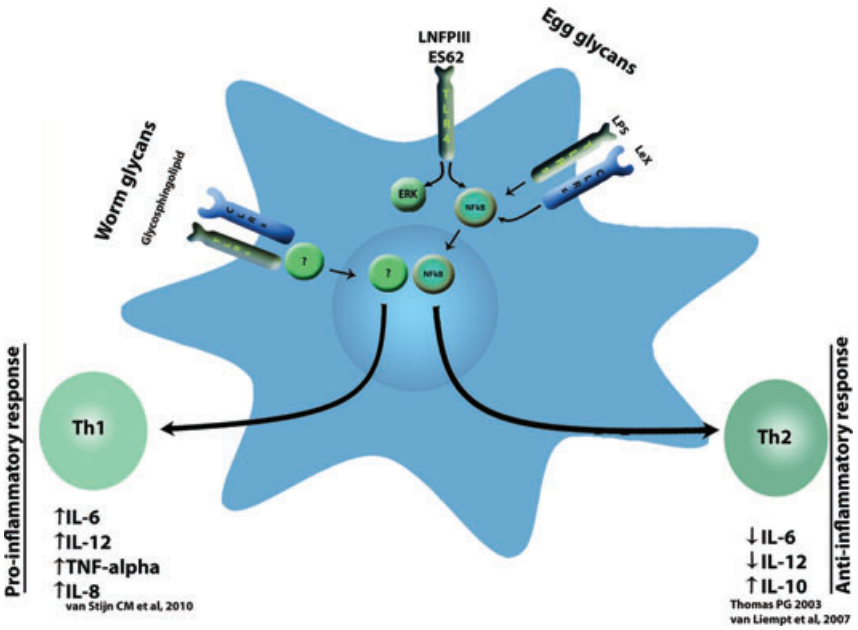

Figure 3. Helminth glycans may drive Th1 or Th2-type responses, depending on the expression of molecules (protein, lipid) they express. Egg glycans or Lewis X-containing glycoconjugates bind to CLRs such as DC-SIGN on human APCs, and downregulates LPS-induced IL-6 and IL-12 and upregulates IL-10 production. Lewis X-containing glycans activates ERK and NF-κB signaling, eventually leading to APC activation, which induces $CD4^+$ T cell Th2 responses in a TLR4-dependent fashion. In contrast, fucose-containing glycosphingolipids from adult worms ligate DC-SIGN and TLR4 to drive APC maturation and $CD4^+$ T cells to produce proinflammatory cytokines IL-6, IL-8, IL-12, and TNF-α.

and in the case of glycosphingolipids, the nature and/or length of the lipid portions may be important. Also clear is the idea that CLRs are major PRRs with important roles in glycan-induced immune suppression during helminth infection, and they can signal with or antagonize TLRs (Fig. 3). Future studies dissecting how different glycans interact with different PRRs that regulate immune homeostasis will be exciting. Defining the signaling pathways that lead to pro- versus antiinflammatory responses will be difficult in systems where multiple PRRs are ligated.

CD14 and APC endocytosis in Th2 biasing and alternative activation of APCs

Little is known about the signaling cascade induced in APCs following helminth glycan stimulation. Studies with chitin suggest that alternative activation of macrophages involves activation of the demethylase protein JMJD3, which epigenetically regulates expression of IRF4. This pathway regulates expression of alternative activation markers *Ym1*, *Fizz1*, and *MR1*, suggesting an IL-4/IL-13–independent pathway for chitin induction of the alternative activation pathway, similar to the earlier finding by Atochina *et al.*, that helminth-expressed LNFPIII/LewisX drives an IL-4/IL-13–independent alternative activation of macrophages *in vivo*.[59] The murine macrophage receptors that drive LNFPIII/LewisX-mediated alternative activation have not been identified. However, several studies suggest that DC-SIGN and the scavenger receptor C-type lectin (SRCL) are the relevant receptors for LNFPIII on human DCs and macrophages, respectively.[75,78] To date, the signaling cascade induced by LNFPIII has been shown to involve activation of ERK, but not JNK or p38, coincident with transient translocation of NF-κB, independent of IκB degradation (Fig. 2).[79]

Recently, we asked if molecules normally involved in the host proinflammatory response to microbial ligands might also play a regulatory role in antiinflammatory responses, such as alternative activation. CD14 is a PRR known to associate with signaling mediated by TLR2, 4, 7, 8 and 9, and yet CD14 has also been shown to

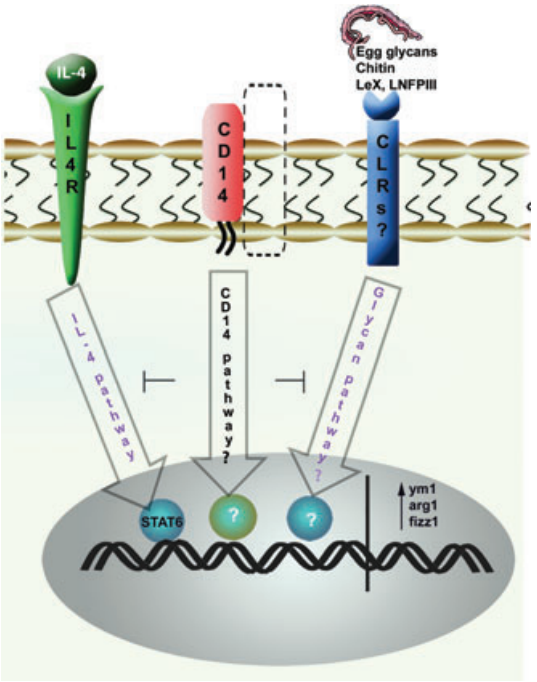

Figure 4. CD14 negatively regulates alternative activation of macrophages. IL-4/IL-13 or helminth glycans are able to induce alternative activation of macrophages using different APC cell surface receptors and subsequent signaling cascades. CD14, an innate receptor, can negatively regulate both IL-4Rα -dependent and -independent (helminth glycan induced) AA macrophage activation through an unknown mechanism. (Tundup S, Harn DA, unpublished). Dotted lines represent unknown receptor or downstream signaling molecules that may help CD14 in mediating negative regulation of AA macrophages.

induce signaling independent of TLRs.[80–83] For these experiments, we analyzed alternative activation in schistosome infected, schistosome egg injected, or LNFPIII-conjugate injected $CD14^{-/-}$ or WT mice. We found that schistosome-infected $CD14^{-/-}$ mice had significantly increased recruitment of AA macrophages to hepatic egg granulomas, compared to infected WT mice (Tundup *et al.,* unpublished observations). Similarly, examination of lung granulomas demonstrated a significant increase in AA macrophages in schistosome egg-injected $CD14^{-/-}$ mice, compared to WT mice. Importantly, we also found that injection of LNFPIII conjugates into $CD14^{-/-}$ mice resulted in increased expression of alternative activation markers *Ym1*, *Fizz1*, and *Arg1* on peritoneal macrophages, compared to macrophages from LNFPIII-injected wild-type mice (Tundup *et al.,* unpublished observations). Taken together, these observations suggest that macrophage alternative activation via helminth glycans is negatively regulated by CD14, possibly via signaling crosstalk, though this concept has not yet been validated (Fig. 4). We are currently attempting to determine if CD14 plays a similar negative regulation role in chitin-induced alternative activation.

In an attempt to understand how helminth-expressed glycans "program" APCs to drive $CD4^+$ Th2 responses and alternative activation, we asked whether APC endocytosis of helminth antigens or helminth glycans is a required process for APC-mediated Th2 biasing; we utilized inhibitors of endocytosis (e.g., dynasore) to test this. We observed that bone marrow-derived APCs stimulated with schistosome soluble egg antigens, or with LNFPIII conjugates, were unable to drive naive $CD4^+$ T cells to produce Th2 mediators (IL-13) if inhibitors of dynasore was added to the APC cultures (Table 2) (Srivastava *et al.,* unpublished observations). This is an important observation, as it suggests that helminth glycan-induced Th2 responses by APCs requires that the APCs endocytose the helminth molecules. The results using schistosome soluble egg antigens suggest that the majority of schistosome antigens require APC endocytosis to drive maturation in a way that will drive $CD4^+$ Th2 responses.

Helminth-expressed glycan induction of antiinflammatory responses *in vivo*

A long-term observation of developing country populations is the relative lack of inflammation-based disease. One tenet of the *hygiene hypothesis* is that this lack of inflammatory disease may be due to the high prevalence of helminth infection in these countries. Experimentally, helminth infection, or administration of helminth extracts such as schistosome saline soluble egg antigens, have been used as therapies to prevent, or to reverse, proinflammatory autoimmune diseases, including type 1 diabetes, inflammatory bowel disease, and experimental autoimmune encephalomyelitis.[84–88]

In addition to experimental studies in animals, the importance of helminthes as anti-inflammatory agents was demonstrated in studies showing that 65–70% of therapy-refractive inflammatory bowel disease patients improved after ingesting a drink containing *Trichuris suis* eggs.[89] This is consistent

Table 2. Antigen presenting cells must endocytose SEA or LeX/LNFPIII to mature into Th2-driving cells

LeX/LNFPIII	SEA	Dynasore	Endocytosis	Th2 (IL-13)
+	−	−	Yes	Enhanced
+	−	+	No	Reduced
−	+	−	Yes	Enhanced
−	+	+	No	Reduced

Dendritic cells were treated with LNFPIII with or without pretreatment with endocytosis inhibitor dynasore or carrier molecule DMSO. Supernatant was used to detect IL-13 levels by ELISA. Endocytosis was quantified using anti-LeX antibody on a confocal microscope.

with the conclusion that helminthes express and/or secrete immune modulatory molecules.

We have demonstrated that administration of LNFPIII conjugates was sufficient to treat/prevent psoriatic lesions in *fsn/fsn* mice, and recently, with our collaborators, to extend cardiac allograft survival following transplantation.[90,91] Most recently, LNFPIII conjugates have been used therapeutically to reduce clinical symptoms in experimental autoimmune encephalomyelitis.[92] In both the cardiac allograft transplantation study and the EAE study, administration of LNFPIII conjugates induced a number of antiinflammatory mechanisms *in vivo*, including upregulation of alternatively activated macrophages and increases in $Foxp3^+$ T regulatory cells. However, despite the dramatic impact of LNFPIII conjugate therapy on reducing inflammation-based diseases *in vivo*, the LNFPIII multivalent conjugates employed in these studies are not identical to the way these glycans are expressed on helminth molecules. These studies support the concept that helminth infection, or administration of helminth glycans, represents a new paradigm for the treatment of inflammation-based diseases.

Conclusion

Helminth glycans play important roles biasing $CD4^+$ T cell responses to Th2 type, as well as driving an anti-inflammatory state via alternative activation of APCs. Further, a new paradigm has recently emerged showing that depending on the chemical nature of and perhaps the length, the lipid portion of glycosphingolipids can also drive proinflammatory T helper cell immune responses. The immune modulatory properties of helminth glycans suggest that these molecules may be used as vaccine adjuvants, for both Th1 and Th2, or, potentially, as immunotherapeutics to treat inflammation-based diseases. In this regard, several animal model studies have been performed to show the potential of LNFPIII conjugates as anti-inflammatories *in vivo*.

Using defined helminth glycans to induce anti-inflammatory mediators may lead to the discovery of new classes of molecules that can be used to treat/prevent inflammation-based diseases, such as type 1 diabetes, autoimmune diseases, cardiovascular diseases, and obesity/metabolic diseases. Further study of helminth glycosphingolipids may aid in the development of new proinflammatory adjuvants for vaccines. Future studies should focus on innate receptors for these glycans, comparing murine and human APCs, as well as studies to define the downstream signaling pathway that drives IL-4/IL-13-independent alternative activation and production of anti-inflammatory mediators.

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Conflicts of interest

The authors declare no conflicts of interest.

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