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Molecular interplay between dendritic cells and schistosomes : consequences for immune polarization

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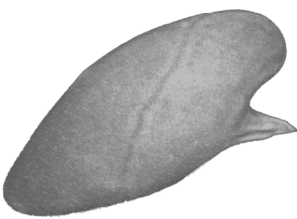
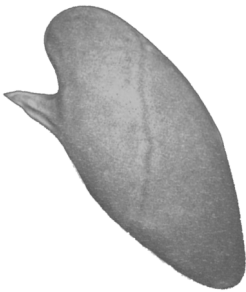
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Chapter

5



The molecular mechanisms underlying Th2 polarisation by schistosome egg-derived omega-1:

the mannose receptor and protein synthesis inhibition

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Abstract

The molecular processes and signals for Th2 priming are still not well understood. Recently, omega-1, an RNase secreted by *Schistosoma mansoni* eggs, has been identified as a single glycoprotein that drives Th2 polarization through functional modulation of dendritic cells (DCs), providing a tool to dissect the processes leading to Th2 polarization. Using omega-1 mutants we find that omega-1, through its glycans binds to both Mannose Receptor (MR) and DC-SIGN. Although, we find that omega-1 can signal via these receptors to modulate LPS-induced cytokine transcription similar to Lewis-X (Le^x)-glycoconjugates, we show that Le^x-motifs on omega-1 are not important for conditioning of DCs for induction of Th2 responses by omega-1. However, selective internalization by MR is needed for conditioning of DCs for Th2 polarization *in vitro* and accordingly mice lacking this receptor have a significantly impaired Th2 response to omega-1. Furthermore, the RNase activity is essential for Th2 polarization, shown by selective mutation of the enzymatic activity, which prevented omega-1 to impair protein synthesis, including that of IL-12, through breakdown of rRNA. Thus omega-1 conditions DCs for Th2 polarization interfering with ribosomal function following internalization by MR, providing new insights in the molecular processes that govern Th2 induction.

Introduction

Dendritic cells (DCs) play a central role in development and maintenance of immune responses during infections, as they govern both the initiation and polarization of adaptive T helper (Th) cell responses. Classically, upon recognition of invading pathogens, resting DCs undergo a process of activation, the so-called maturation, that involves stable presentation of peptides in the context of major histocompatibility complex (MHC)-II, up-regulation of co-stimulatory molecules, and production of polarizing cytokines, that all together enable the DCs to potently activate and direct CD4+ T cell differentiation [2].

This paradigm has stemmed from observations of responses towards unicellular pathogens, like bacteria, viruses and fungi. These pathogens harbour pathogen associated molecular patterns (PAMPs) that lead to classic DC activation by engaging several classes of innate pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs). Binding of PAMPs to these receptors initiates signaling cascades that generally result in the conditioning of DCs for priming of Th1- or Th17-biased responses that are instrumental in combating these classes of pathogen [4]. In contrast to this classical view of DC activation, components derived from parasitic helminths fail to induce all classic signs of DC maturation. However, although overt maturation is not observed, helminth antigens do functionally modulate DCs as they are capable of markedly inhibiting DC maturation induced by TLR ligands as well as to condition such DCs to prime Th2-polarized immune responses [5;6].

Yet despite this consistent picture, the pathways through which helminth antigens manipulate DC function and drive Th2 responses are still poorly understood [7]. Most insights in this area have come from studies with the complex mixture of soluble egg antigens (SEA) from the trematode *Schistosoma mansoni*, which is regarded as one of the most potent helminth-derived preparations that instruct DCs to drive Th2 polarization [8]. So far these studies have mainly suggested that carbohydrate structures play a role in DC modulation by SEA, given that chemical modification of glycans on proteins present in SEA is known to abolish the capacity to induce Th2 polarization [9]. SEA contains glycoproteins that carry carbohydrate structures, such as Lewis-X (Le^x), which have been shown to be recognized by DCs through binding to another class of PRR, the C-type lectins [10-13]. Since engagement of these receptors by others pathogens has been shown to suppress IL-12 production and modulate TLR-induced DC activation [14], CLR signaling has been implicated in modulation of DC by SEA. SEA has been shown to preferentially promote the phosphorylation of ERK and activation of c-fos, resulting in the suppression of IL-12 production [15;16]. Yet, ERK activation via carbohydrate-induced CLR signaling has not been reported, suggesting involvement of other signaling routes than those mediated

via CLRs [14;17]. For instance, LNFPIII, a glycan carrying Le^x, has been found to induce Th2 responses via DCs in a TLR4-dependent fashion [18]. However, the significance of this finding remains to be assessed as it has been shown that SEA does not bind to TLR4 [13;16] and is able to prime Th2 responses in the absence of TLR signaling [19].

These data illustrate that a uniform picture of the molecular mechanisms by which SEA conditions DCs for Th2 priming is still lacking. However, with the recent identification of a defined glycoprotein with RNase activity, omega-1, as the major component in schistosome eggs that is responsible for conditioning DCs for Th2 polarization [20;21], the molecular pathways through which DCs are conditioned to induce Th2 responses by schistosome eggs can now be investigated. Through site-directed mutagenesis we here show that the RNase activity as well as the glycosylation of omega-1 are essential for conditioning of DCs for Th2 induction. Furthermore, we provide evidence that Mannose receptor (MR) is critical for omega-1-driven Th2 responses and that internalization via this receptor is needed for biological activity of omega-1, as it allows omega-1 to interfere with ribosomal function and thereby to condition these cells to prime Th2 responses.

Results

RNase activity as well as glycosylation of omega-1 are essential for modulation of DC function and Th2 polarization

Earlier reports have suggested the involvement of RNase activity of omega-1 for the conditioning of DCs to prime Th2 responses [21]. However this was based on a crude chemical inactivation of the RNase activity by DEPC-treatment, which may also alter function or structures of the whole protein. Therefore, we addressed the role of RNase activity in a more stringent and specific manner by creating a mutant of recombinant omega-1 that lacked RNase activity by site-directed mutagenesis. This RNase mutant (omega-1-H58F) was created by substituting a histidine with a phenylalanine in its catalytic domain, that is known from other homologues T2 RNases to be essential for the enzymatic activity [22] (suppl Fig 1A). As glycans present in SEA have also been suggested to play a role in modulation of DCs for priming of Th2 responses [9;13;18], and potentially Th2-polarizing Le^x-motifs have been identified on glycans present on omega-1 (Meevissen *et al.*, submitted), a glycosylation mutant (omega-1-N71/176Q) was generated by one amino-acid replacement at each of the two putative N-linked glycosylation sites (suppl Fig 1A) [23]. The RNase mutant, in contrast to recombinant WT omega-1 and the glycosylation mutant failed to digest RNA (suppl Fig 1B), while a silver-stained SDS PAGE as well as an anti-omega-1 Western blot revealed only a single band of the glyco-mutant, instead of normal 3 bands due to glycosylation heterogeneity of recombinant omega-1 when expressed by HEK293 cells (suppl Fig 1C

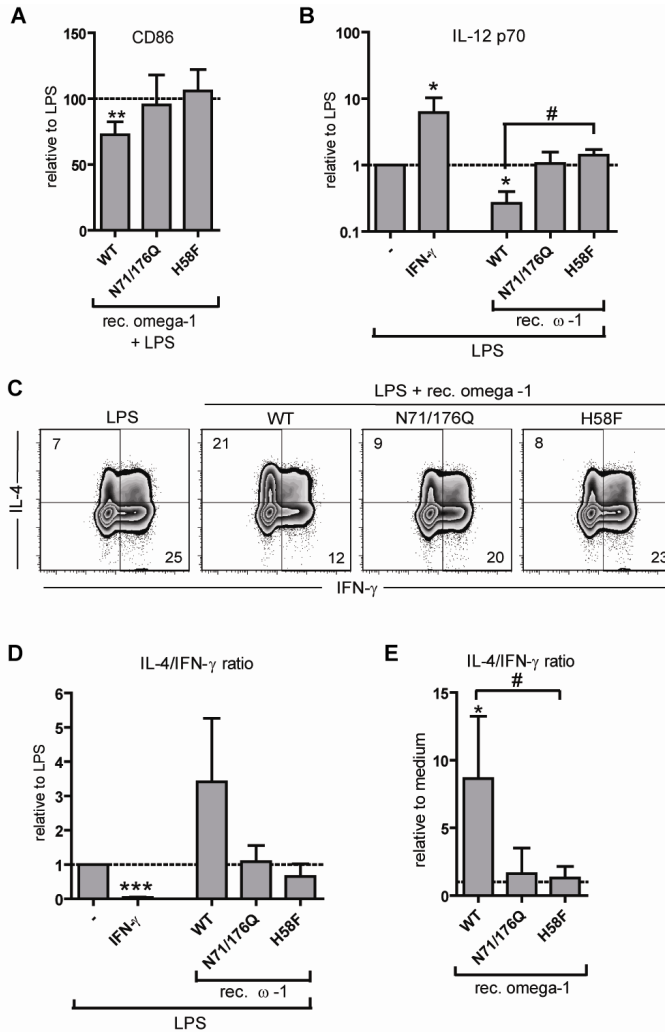


Figure 1. The RNase activity as well as glycosylation of omega-1 are essential for modulation of DC function and conditioning of these cells for priming of Th2 responses. (A) monocyte-derived DCs were pulsed for 48 h with the different variants of omega-1 (2 μ g/ml) in combination with LPS (100 ng/ml) as a maturation factor and surface expression of CD86 was determined by FACS analysis. The expression levels, based on geometric mean fluorescence, are shown relative to the DCs stimulated with LPS alone, which is set to 100% (dashed line). (B) Conditioned DCs were co-cultured for 24 h with a CD40-L expressing cell line, to mimic the interaction with T cells. IL-12p70 cytokine expression levels are shown relative to the DCs stimulated with LPS alone, which is set to 1 (dashed line). Data are representative of at least 3 independent experiments. (C-E) Conditioned DCs in the presence (C + D) or absence of LPS (E) were cultured with allogeneic naive CD4⁺ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2. Intracellular cytokine production was assayed by FACS 6 h after the stimulation of primed T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin. (C) The frequencies of each population are indicated as percentages in the plot. One representative result from 3 independent experiments is shown. Based on intracellular cytokine staining, the ratio of T cells single-positive for either IL-4 or IFN- γ was calculated relative to the control condition. (A-E) Data are representative of 3 independent experiments. Error bars represent SD. *,# $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (one-sided paired t -test). ω -1, omega-1.

and unpublished data). This confirms the successful generation of the two mutants. With regard to the glycan present on recombinant omega-1, mass spectrometric analysis of tryptic glycopeptides shows the occurrence of N-glycans on Asn₁₇₆ with the monosaccharide composition Hex₃HexNAc₆Fuc_{2/3} (suppl Fig 2), which is indicative of the presence of fucosylated LDN antennae (LDN-F), a motif previously found on another recombinant glycoprotein from HEK293 cells as well [24] and thought to have similar immunological characteristics as the Le^x-motifs present on natural omega-1 [10;25].

A well established *in vitro* culture system of human monocyte-derived DCs and naïve CD4+ T cells was used, which mimics *in vivo* DC-mediated T helper cell polarization [2], to assess the relative roles of glycosylation and RNase activity in omega-1-driven Th2 polarization. Similar to what has been found for natural omega-1 [20], recombinant WT omega-1 significantly suppressed the LPS-induced upregulation of the costimulatory molecule CD86 (Fig 1A), as well as the production of IL-12 p70 following CD40 ligation (Fig 1B), the latter which is thought to be an important characteristic of DCs that prime Th2 responses [6]. Neither the RNase mutant nor the glycosylation mutant altered LPS-induced costimulatory molecule expression or IL-12 production of DCs. Importantly, in contrast to WT-recombinant or natural omega-1-primed DCs, those conditioned with the omega-1 mutants failed to prime a Th2 response (Fig 1C). Similar results were obtained from cultures in which DCs were conditioned by the omega-1 variants in the absence of LPS (Fig 1D). The importance of glycosylation of omega-1 was further substantiated by experiments done with natural omega-1 in which the glycans were disrupted by periodate treatment (Suppl Fig 3). These data confirm and extend the earlier observations that the RNase activity of omega-1 is essential for induction of Th2 polarization via DCs. However, they also show that omega-1 requires glycosylation to exert its effects on DCs and suggest that the RNase activity and glycans as single entities are not sufficient to condition DCs to induce Th2 responses.

Omega-1 suppresses DC function by interfering with protein synthesis

While these data show that ribonucleic activity of omega-1 is essential for its modulatory effects on DCs, the molecular mechanisms through which this property enables omega-1 to alter DC function is unclear. In this respect, we observed that omega-1 not only suppresses costimulatory molecule and IL-12 expression as reported previously [20], but impairs secretion of a large set of other cytokines as well (Suppl Fig 4), indicating that the suppression is not directed to specific genes, but may be a more generalised phenomenon. Indeed, following exposure of DCs to omega-1 or SEA, a dose-dependent reduction of protein synthesis could be observed, similar to what is

found in DCs exposed to ricin, which is a well known protein synthesis inhibitor [26] (Fig 2A). As several ribonucleolytic proteins from fungal origin, so-called ribotoxins, have been described to inhibit protein synthesis through cleavage of ribosomal RNA (rRNA) [27], we tested whether omega-1 could cleave rRNA in the context of functional ribosomes in a cell free assay. As shown in figure 5 C, omega-1 was able to breakdown rRNA, while IPSE, as another *S. mansoni* egg-derived protein but lacking RNase activity, did not induce any rRNA digestion (Fig 2B), suggesting that omega-1 is able to interfere with ribosomal function by cleavage of rRNA. Colocalization experiments

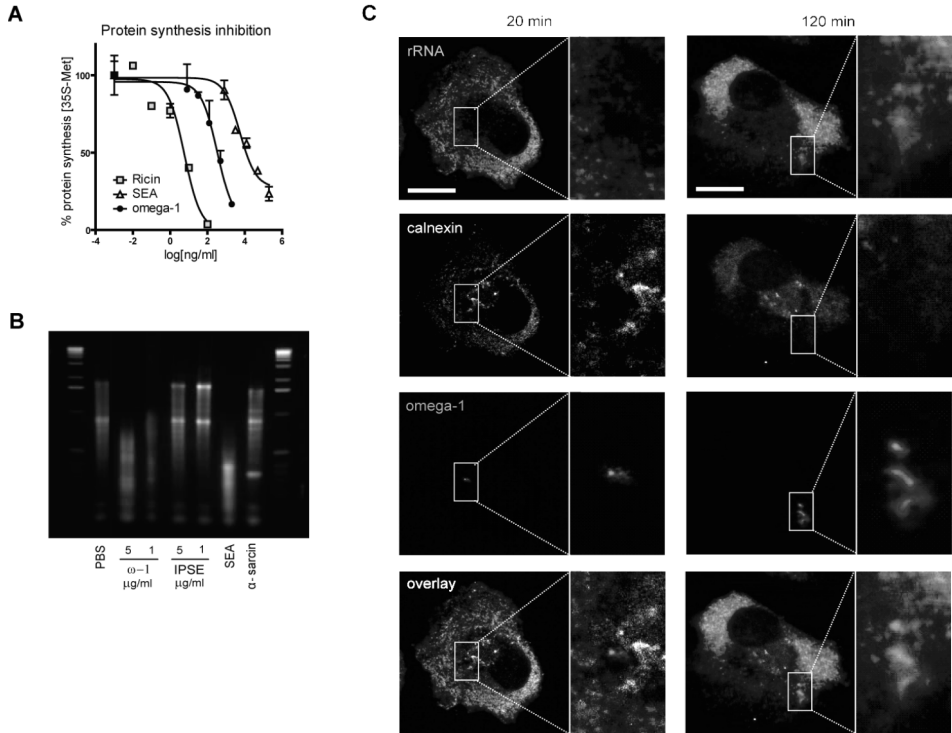


Figure 2. Omega-1 suppresses protein synthesis through interference with ribosomal function. (A) Following 16 h incubation of DCs with a concentration range of indicated reagents in the presence of LPS (100ng/ml), protein synthesis was assessed after a 2 h pulse with radioactive labelled methionine. Ricin, as potent inhibitor of protein synthesis, was taken along as positive control [1]. (B) After rabbit reticulocyte lysate containing functional ribosomes was incubated for 1 h with omega-1 or IPSE, as negative control, isolated ribosomal RNA was analysed for breakdown on a 2% agarose gel. The RNase α -Sarcin was taken along as positive control as it should give a single rRNA cleavage product when incubated with functional ribosomes [3]. (C) DCs were stimulated with PF-647-labeled omega-1 and at indicated time points cells were fixed and stained for calnexin (ER marker) and ribosomes (rRNA). Subcellular localization of the antigens was determined by confocal microscopy. The grey bars represents $2\mu\text{m}$. (A-C) Data from one representative experiment from two independent experiments is shown.

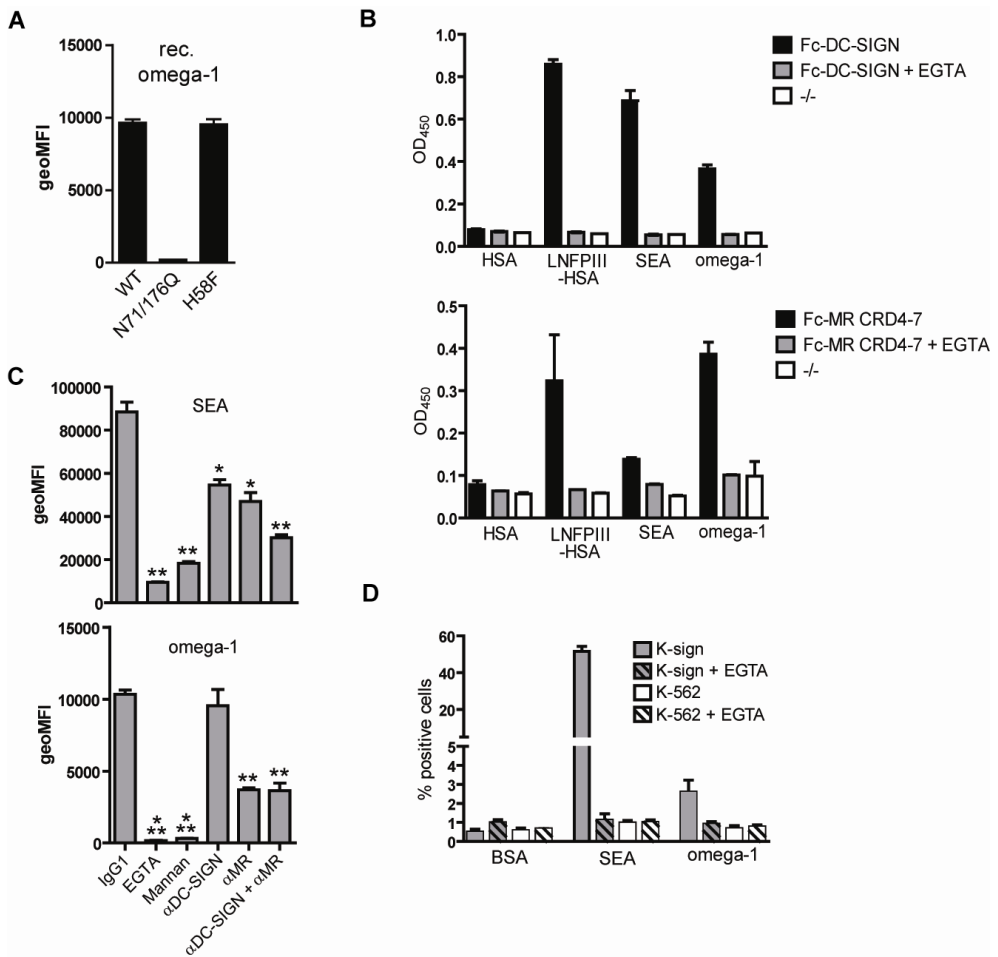


Figure 3. Omega-1 binds to DC-SIGN and Mannose Receptor (MR), but uptake of omega-1 by DCs is MR dependent. (A) DCs were incubated for 1 h with PF-647-labeled omega-1 and analysed for antigen uptake by FACS. (B) A DC-SIGN-Fc and MR-CRD4-7-Fc binding ELISA was performed on omega-1, SEA and HSA and LNFP1III-HSA as negative and positive controls respectively (C) DC-SIGN expressing cell line (K-SIGN) and a control cell line (K-562) were incubated with PF-647-labeled omega-1 and SEA in the presence or absence of EGTA to determine specificity. (D) Immature monocyte-derived DCs were pre-incubated with indicated reagents followed by incubation with PF-647-labeled SEA or omega-1 for 1h. Uptake of antigens by DCs was evaluated by FACS analysis. (A-D) Data are shown as mean \pm SD of duplicates (A, C, D) or triplicates (B), of one representative experiment out of two. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for significant differences compared to the control (one-sided t -test).

using immunofluorescence confocal microscopy by staining for calnexin as a marker for the endoplasmic reticulum (ER) and docking site for ribosomes, as well as for rRNA itself showed that 2 h after stimulation of DCs with omega-1, omega-1 co-localized with rRNA, but not with the ER (Fig 2C). Of note, apart from inhibition of protein synthesis we observed that in the absence of DC maturation induced by LPS,

omega-1 could promote apoptosis in DCs, as evidenced by an increase in DCs positive for Annexin-V and active caspase-3, 40 h after exposure to omega-1 (suppl Fig 5). Given that the onset of apoptosis was preceded by the inhibition of protein synthesis, it is likely that the omega-1-induced cytotoxicity in the absence of LPS is a consequence of the interference with ribosomal function, which would be similar to the mode of action through which ribotoxins are known to induce cytotoxicity [27]. Taken together, these data support the notion that the RNase activity enables omega-1 to modulate DC function by interfering with protein synthesis through cleavage of rRNA, which in some instances may result in apoptosis-mediated cytotoxicity.

Omega-1 binds mannose receptor (MR) and DC-SIGN, but is internalized by DCs via MR

In order to understand the role glycosylation of omega-1 plays in cellular interactions, the 3 recombinant omega-1 variants were fluorescently labelled and analysed for uptake by DCs. Recombinant WT omega-1 and the omega-1 RNase mutant, but not the mutant lacking glycosylation, were taken up by the DCs (Fig 3A). Next, potential receptors involved in recognition of omega-1 by DCs were studied. Since LNFPIII, containing the same Le^x-motifs as omega-1 (Meevissen *et al.*, submitted), is known to activate DCs via TLR4 [18], we tested whether omega-1 could activate TLR4-transfected HEK cells. However, no evidence was found for the ability of omega-1 to bind or signal via TLR4 (data not shown). As SEA has been shown to be recognized and endocytosed by human DCs via the CLRs, DC-SIGN, MR and macrophage galactose-type lectin (MGL) [13] of which the former two receptors have been described to have the capacity to bind fucose-residues such as the ones found in Le^x and LDN-F [10;25;28], we evaluated the capacity of natural omega-1 to bind to DC-SIGN or MR, by using an ELISA-based assay. Just as for SEA and HSA-Le^x, as positive controls, omega-1 was shown to bind both DC-SIGN and MR (Fig 3B). To study the role of MR and DC-SIGN in uptake of omega-1 by human monocyte-derived DCs, DCs were preincubated with EGTA (a calcium chelator that inhibits CLR function), mannan (a natural ligand that competes for binding to DC-SIGN and MR), and DC-SIGN- and MR-specific blocking antibodies, followed by a 1 h incubation with fluorescently-labelled SEA or omega-1. Uptake of SEA, as previously published [13], could be reduced by either DC-SIGN or MR blocking antibodies and even further by the combination of both antibodies. Interestingly, uptake of natural omega-1, was significantly reduced by MR but not by DC-SIGN blocking antibodies (Fig 3C). In addition, pre-incubation with the combination of both blocking antibodies did not have any additional effect on the uptake of omega-1 compared to anti-MR antibody only. Importantly, recombinant omega-1 was recognized and internalized in a similar CLR-dependent fashion by DCs, indicating that

the glycans present on recombinant omega-1 in terms of receptor binding have similar characteristics as the glycans present on natural omega-1 (suppl Fig 6). It was noted, that the blocking of uptake of omega-1 via MR was not complete. However, the fact that pre-treatment with mannan, which specifically binds with high affinity to MR and DC-SIGN, could fully block binding and uptake of omega-1, the incomplete blocking of MR by the antibody may be explained as an antibody affinity problem. Given that binding and uptake of omega-1 by DCs was not affected by DC-SIGN blocking antibodies, we tested the ability of omega-1 to bind DC-SIGN in a cell-based assay by using the K562 cell line selectively expressing DC-SIGN. A strong uptake of SEA was observed in the DC-SIGN expressing cells, but not in the ones lacking DC-SIGN expression or pre-incubated with EGTA, confirming that the uptake was DC-SIGN specific (Fig 3D). Also exposure of these cells to equivalent amounts of omega-1 resulted in binding and uptake albeit to a lower extent than SEA (Fig 3D). Thus, these observations suggest that recognition and binding of omega-1 by DCs can be mediated both DC-SIGN and MR, but that only MR is involved in uptake of omega-1 by DCs.

Omega-1 signals via DC-SIGN, but this is dispensable for Th2 polarization

The data thus far suggest that omega-1 requires its glycans to be internalized by DCs via MR, and that the RNase activity conditions DCs to prime Th2 responses. However, these observations do not exclude the possibility that glycans present on omega-1, apart from being essential in uptake of omega-1, could be involved in direct modulation of DC function via receptor-mediated signaling that, although not sufficient by itself (Fig 1), may still contribute to conditioning of DCs for induction of Th2 responses. Le^x-motifs such as present on omega-1, have recently been shown in a DC-SIGN-signaling dependent manner to enhance and suppress TLR-induced IL-10 and IL-12 production, respectively [14]. Since we found that omega-1 can bind to DC-SIGN, we asked whether omega-1 could signal via DC-SIGN, and if so, what the role of this pathway would be in the modulation of DCs to drive Th2 polarized responses. When DCs were stimulated with LPS an increase in IL-10 and IL-12 mRNA levels was observed 6 h after stimulation. Interestingly, the addition of omega-1 to these cultures, suppressed LPS-induced IL-6 and IL-12 mRNA expression, while it enhanced IL-10 mRNA levels, with similar potency as Le^x on a polyacrylamide backbone (PAA) [14] (Fig 4A). Importantly, the modulation of transcription was abolished in DCs in which LSP-1, an adaptor molecule for DC-SIGN signaling [14], was knocked down by siRNA, suggesting that modulation of cytokine expression by omega-1 is dependent on DC-SIGN signaling (Fig 4A). Since it has been shown that SEA also induces IL-10 and suppresses IL-12 expression in DCs and that this is dependent on signaling via extracellular signal-regulated kinase (ERK) [15], we determined whether omega-1

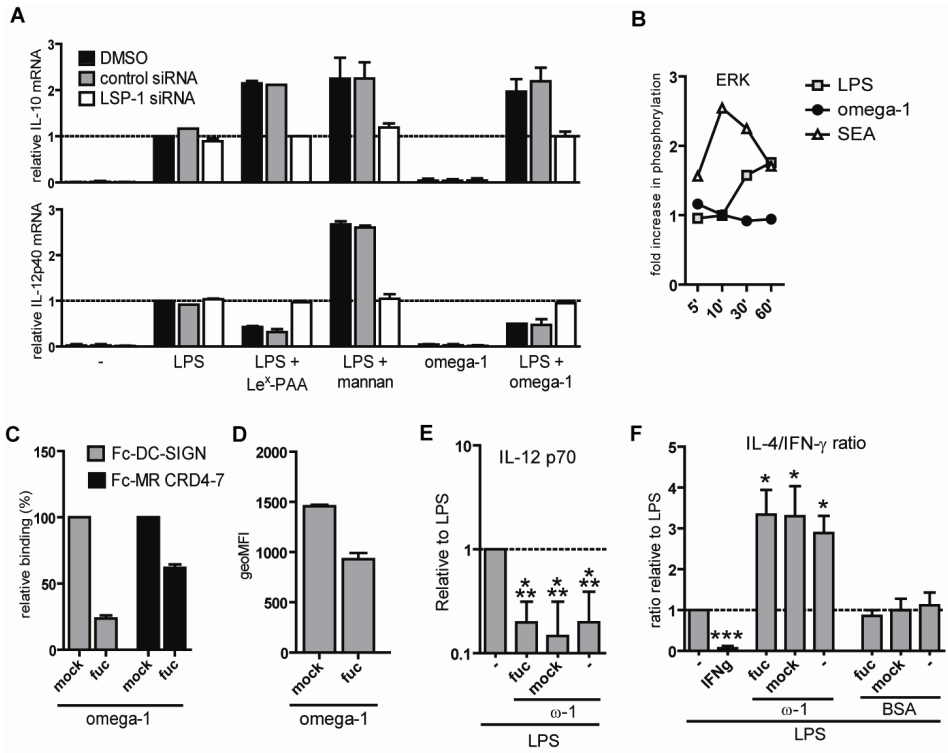


Figure 4. Omega-1 signals via DC-SIGN and LSP-1 like a Le^x-motif carrying glycoconjugate, but disruption of Le^x-motif on omega-1 does not affect its capacity to drive Th2 polarization. (A) Quantitative real-time PCR analysis of the production of cytokine mRNA in DCs treated with control or Raf-1-specific small interfering RNA (siRNA) and left unstimulated or stimulated with LPS alone or in combination with mannose-containing ManLAM, fucose-containing Le^x-PAA or omega-1. Expression is normalized to GAPDH (glyceraldehyde phosphate dehydrogenase) expression and is presented relative to expression in LPS-stimulated cells, set as 1. Data are representative of 2 independent experiments. (B) At indicated time points after stimulation phosphorylation of MAPK ERK was determined by intracellular FACS staining. Fold increase in phosphorylation is shown relative to unstimulated cells. (C) A DC-SIGN-Fc and MR-CRD4-7-Fc binding ELISA was performed on mock- and fucosidase-treated omega-1. Binding is shown relative to mock-treated omega-1. (D) Binding/uptake of omega-1 or fucosidase-treated omega-1 by DCs was determined as described in Figure. 2. (E) DCs stimulated with untreated, mock- and fucosidase-treated omega-1 (500ng/ml) were co-cultured for 24 h with a CD40-L expressing cell line, to mimic the interaction with T cells. IL-12p70 cytokine expression levels are shown relative to the DCs stimulated with LPS alone, which is set to 1 (dashed line). (F) Conditioned DCs were cultured with allogeneic naive CD4⁺ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2 and analysis as described in figure 1. IFN-γ and BSA-treated DCs (500ng/ml) were taken along as Th1 inducing control and as a control for the enzymatic treatment, respectively. Data are representative of 3 independent experiments. Error bars represent SD. * p < 0.05, ** p < 0.01, *** p < 0.001 for significant differences with the LPS control (two-sided *t*-test). ω-1, omega-1.

could phosphorylate ERK in DCs. However, in contrast to SEA, omega-1 did not induce any ERK phosphorylation either in the presence (data not shown) or absence of LPS (Fig 4B), suggesting that DC-SIGN-mediated modulation of cytokine expression by omega-1 does not involve signaling via ERK. Finally, to determine whether signaling through DC-SIGN contributes to the capacity of omega-1 to condition DCs for priming of Th2 responses, fucose-residues present in Le^x-motifs, which are known to be essential for binding to DC-SIGN [10], were specifically removed from omega-1 by fucosidase treatment (suppl Fig 7). Fucosidase-treated omega-1, which largely lost its capacity bind to DC-SIGN, was still capable to bind to MR and to be taken up by DCs, albeit to a lesser extent (Fig 4C + D). Importantly, the ability of fucosidase-treated omega-1 to modulate DC function and to drive Th2 polarization was not impaired (Fig 4E + F). Together these observations suggest that, although omega-1 is able to signal via DC-SIGN to modulate of LPS-induced cytokine expression, this pathway does not play an essential role in conditioning of DCs to prime Th2 responses.

MR, but not DC-SIGN, mediates omega-1-induced DC modulation and Th2 polarization

To further address the role of MR and DC-SIGN in the modulation of DC function by natural omega-1, DCs were stimulated with omega-1 in combination with LPS after preincubation with blocking antibodies directed against MR or DC-SIGN. Blocking of MR during stimulation of DCs with omega-1 significantly prevented inhibition of protein synthesis (Fig 5A), while blocking of DC-SIGN had no effect, suggesting that the interference with protein synthesis by omega-1 is dependent on MR-mediated internalization. Furthermore, while blocking of DC-SIGN did not alter the capacity of omega-1 to suppress LPS-induced CD86 expression (Fig 5B) and IL-12 production following CD40 ligation (Fig 5C) or to condition DCs to induce a Th2 response (Fig 5D), blocking of MR significantly reduced the modulatory effects of omega-1 on DCs and their Th2-skewing potential (Fig 5B-D). To investigate whether omega-1 is also dependent on MR to prime Th2 responses *in vivo*, omega-1 or PBS were injected subcutaneously into the footpad of WT and MR^{-/-} mice. After 7 days the draining popliteal lymph nodes (LNs) were harvested and restimulated *in vitro* with PBS, omega-1 and a polyclonal stimulus PHA and analysed for cytokine production. Antigen specific restimulation of omega-1-primed LNs from WT mice resulted in a Th2-polarized response as evidenced by elevated levels of Th2 cytokines IL-4 and IL-5 but not of Th1-associated cytokine IFN- γ (Fig 6A). Intracellular staining of LN-derived CD4⁺ T cells for IFN- γ and IL-4 revealed a significant antigen-specific Th2 polarization by omega-1 (Fig 6B). The Th2 skewing was strongly impaired in omega-1-primed LN from MR^{-/-} mice (Fig 6A + B). This was not due to a general failure of MR^{-/-} cells to produce

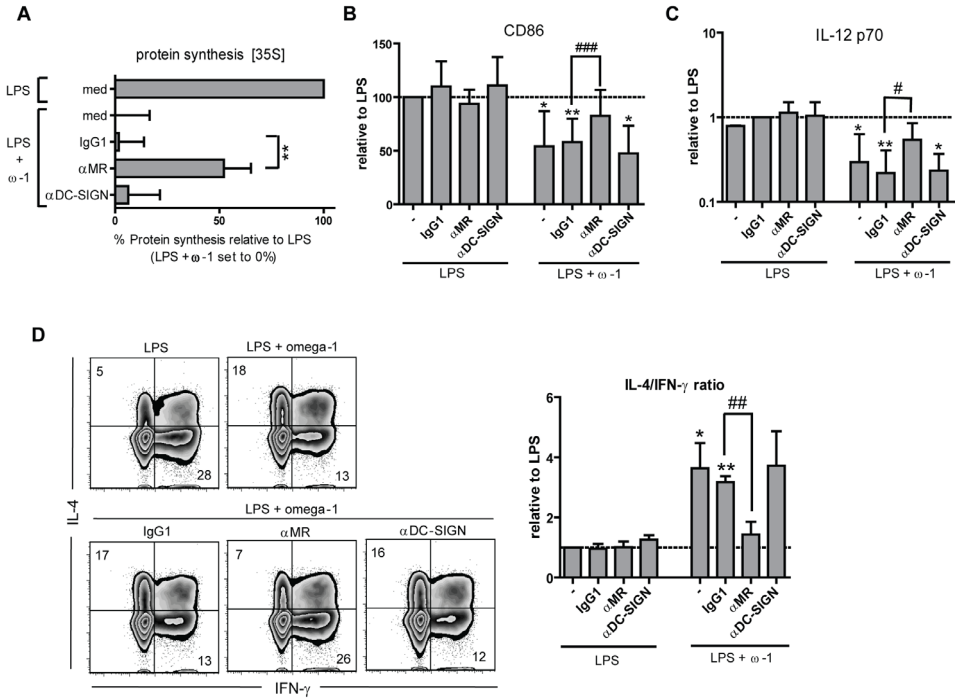


Figure 5. MR, but not DC-SIGN, mediates omega-1-induced DC modulation and Th2 polarization *in vitro*. (A) Following 1 h preincubation with blocking antibodies against DC-SIGN, MR or an isotype control (20 μ g/ml), monocyte-derived DCs were pulsed for 16 h (A) 48 h (B-D) with natural omega-1 (500ng/ml) in combination with LPS (100 ng/ml) and protein synthesis was assessed as described in FIG 2A. (B-D) DCs were stimulated as described in (A). (B) The expression levels of CD86 on DCs were assessed by FACS and are shown, based on geometric mean fluorescence, relative to the DCs stimulated with LPS alone, which is set to 100% (dashed line). (C) Conditioned DCs were co-cultured for 24 h with a CD40-L expressing cell line, to mimic the interaction with T cells. IL-12p70 cytokine expression levels are shown relative to the DCs stimulated with LPS alone, which is set to 1 (dashed line). (D) Conditioned DCs were cultured with allogeneic naive CD4⁺ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2 and analysed as described in figure 1. (A-D) Data are representative of at least 3 independent experiments. Error bars represent SD. *,# $p < 0.05$, **,### $p < 0.01$, ### $p < 0.001$ for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (one-sided paired t -test). ω -1, omega-1.

these cytokines as the responses to PHA were comparable in WT and MR^{-/-} mice (Fig 6A + B). This suggests that MR plays a crucial role in mediating Th2 polarization by omega-1 *in vivo* as well, and together these data identify MR as the principal receptor through which omega-1 primes Th2 responses.

Discussion

The molecular mechanisms through which DCs become conditioned by pathogens to prime Th1 responses have been extensively characterized. Yet, it is still poorly understood how DCs become conditioned to induce polarization towards Th2 to helminths [7]. In this respect, until now most insights into molecular processes involved in conditioning of DCs for priming of Th2 responses by helminth antigens have been based on studies with antigen preparations from schistosome eggs (SEA) as model antigen mixture with potent Th2-polarizing properties [6;16;19;29-33]. However, probably due to the complexity of the antigen preparation, a dissection of the pathways through which DCs are modulated by SEA to induce Th2 responses has been difficult. The identification of omega-1, as the major component in schistosome eggs responsible for conditioning of dendritic cells for Th2 polarization [20;21], has provided us with a tool to specifically address the molecular mechanisms through which schistosome eggs can instruct DCs to drive Th2-polarized responses.

We find that selective inactivation of the RNase activity of omega-1, through site-directed mutagenesis, renders it incapable of suppressing LPS-induced CD86 and IL-12 expression as well as conditioning human DCs for priming of a Th2 response. This confirms earlier observations that omega-1 lacking RNase activity due to chemical treatment fails to drive a Th2 response via murine DCs [21] and together establishes that both in human and mouse, omega-1-driven Th2 polarization via DCs requires RNase activity. Until now there has only been a single report that has linked an RNase, the eosinophil-derived neurotoxin (EDN), to DC-mediated Th2 polarization [34]. However, the role of the RNase activity in the modulation of DC function by EDN was not assessed. The observations that omega-1 inhibited protein synthesis and induced apoptosis in DCs, are reminiscent of the effects induced by a family of RNases secreted by fungi, mainly of the genus *Aspergillus*, known as ribotoxins, which are known to selectively cleave rRNA [27], suggested that omega-1 may modulate DCs by the same fashion. Indeed, we observed that omega-1 could cleave rRNA in the context of ribosomes, and in addition co-localized with rRNA in DCs. The finding that the integrity and yield of mRNA isolated from omega-1-stimulated DCs was not different from unpulsed control DCs (data not shown), suggests that interference with translation via selective cleavage of rRNA is the mode of action through which the RNase activity enables omega-1 to condition DCs for priming of Th2 responses. In this respect, it is interesting to note that a fungal ribotoxin aspf-1 is known as an allergen, and that its allergenicity was lost when its capacity to interfere with ribosomal function was abolished [35]. The mechanism to suppress protein synthesis in DCs by omega-1, would be in line with the well documented inhibitory effects of omega-1 as well as SEA as a whole on DC activation and TLR-induced expression of costimulatory molecules

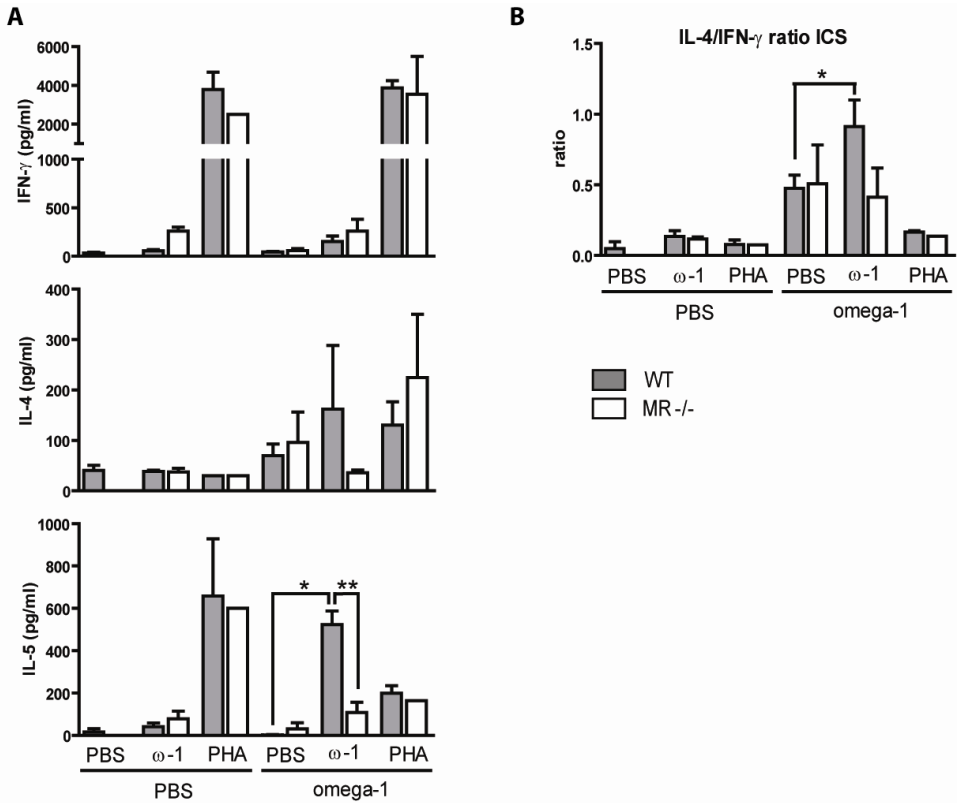


Figure 6. MR is essential for omega-1-driven Th2 polarization *in vivo*. MR^{-/-} and WT Bl/6 mice were injected s.c. with omega-1 (2 μ g in 30 μ l PBS) or PBS into the footpad. (A) After 7 days the cells from the draining lymph node (LNs) were restimulated *in vitro* for 4 d with PBS, omega-1 (2 μ g/ml) or PHA (10 μ g/ml), as polyclonal stimulus, after which cytokine production was determined by ELISA. (B) In addition, intracellular cytokine production of the CD3⁺/CD4⁺ T cells from these LNs was assayed by FACS after an additional 6 h restimulation with PMA and ionomycin. The ratio of T cells single-positive for either IL-4 or IFN- γ is shown. Data are means \pm SEM of 3 to 4 mice per group based on pooled triplicate wells for each mouse. * $p < 0.05$, ** $p < 0.01$ for significant differences based on paired analysis (one-sided paired *t*-test) ω -1, omega-1.

and cytokines [20;21]. In addition, this mode of action would also provide an explanation for the findings that omega-1 alters DC morphology as a result of cytoskeletal changes [21], since halting of translation and concomitant stress responses can affect actin rearrangements and thereby cell morphology [36]. However, it is important to note that in line with the protective effects of maturation on DC death [37], omega-1-driven cytotoxicity was not observed in LPS-matured DCs, excluding the possibility that the results obtained from our DC-T cell cultures regarding T cell polarization were influenced by potential immunomodulatory effects of apoptotic cells [38]. It remains to be established how omega-1 would be able to reach the ribosomes that are present in the cytosol. Some ribosome inactivating proteins

(RIPs) have been shown to translocate from the ER into the cytosol after retrograde transport from endosomes via the Golgi apparatus into the ER, while others are known to directly escape from endosomes into the cytosol [39]. The finding that omega-1 did not appear to colocalize with the ER marker calnexin, may argue against a role for the retrograde transport of omega-1 to the ER to translocate into the cytosol. In this respect, since omega-1 is internalized via MR, it is interesting to note that studies on cross-presentation of ovalbumin (OVA) by DCs have shown that both the internalization of OVA into endosomes as well as its translocation from these endosomes into the cytosol for cross-presentation are MR-dependent processes [40].

The cytotoxic effects of omega-1 have been documented before in a murine model in which omega-1 induced hepatotoxicity [41]. Our observations that omega-1 relies on MR to be internalized by DCs and that in the absence of uptake of omega-1, DCs are refractory to its potential cytotoxicity, may explain the selective toxicity in the liver, as apart from DCs, sinusoidal endothelial cells and Kupfer cells express MR [42;43], which would potentially render them sensitive to omega-1 induced cytotoxicity.

Apart from the enzymatic activity of omega-1, we found that the glycosylation of omega-1 is critical for its ability to modulate DCs, since omega-1 lacking native glycosylation, either by targeted mutations or by chemical disruption, failed to condition these cells for priming of Th2 responses. These observations are in agreement with previous findings that SEA is dependent on intact glycans for its Th2-polarizing capacity [11-13;44]. We found that omega-1 can bind to the CLR, DC-SIGN and MR in both ELISA- and cell-based assays, which is in line with the recognition by these receptors of fucose-residues, such as ones present in Le^x and LDN-F displayed on natural and recombinant omega-1, respectively (Meevissen *et al*, submitted) [10;25;28]. In addition we observed that omega-1 like a Le^x-carrying glycoconjugate, downregulated IL-12 and upregulated IL-10 mRNA expression induced by LPS in DCs. Interestingly, these effects were abolished in DCs silenced for LSP-1, which has recently been identified as an important adaptor molecule downstream of DC-SIGN [14]. In the same study it was also found that the signaling induced by a Le^x-carrying glycoconjugate was blocked in DCs in which DC-SIGN was silenced, showing that the signal was DC-SIGN dependent and did not involve other CLR, thereby suggesting that omega-1 can signal via DC-SIGN in a Le^x-dependent fashion to alter LPS-induced cytokine expression in DCs. Regarding signaling downstream of DC-SIGN, ERK has been shown to mediate modulation of cytokine production by SEA in DCs [15;16;31] and some studies have linked signaling via DC-SIGN [45-47] to phosphorylation of ERK. However ERK-activation was not seen in omega-1-treated DCs. Interestingly, mRNA analysis by microarray as well as real-time qPCR, did reveal the upregulation of

transcription factor c-fos a downstream target of ERK in omega-1-pulsed DCs (data not shown), similar to what has been found for SEA-treated DCs [15;16]. Given its role in suppression of IL-12 and induction of IL-10 expression in DCs [13], c-fos may be one of the transcriptional regulators that mediate cytokine modulation either downstream of CLR-signaling or following a stress response [48] induced by omega-1. However, the observation that selective defucosylation of the Le^x-motifs on omega-1, which abolishes its capacity to bind to DC-SIGN but not MR, did not affect its ability to suppress IL-12 production following CD40 ligation by LPS-stimulated DCs nor to condition these cells to induce Th2 responses, suggests that signaling via DC-SIGN by Le^x-motifs of omega-1 does not significantly contribute to its ability to condition DCs for priming of Th2 responses. This is in line with the observations that IPSE/alpha-1 with a similar Le^x-containing glycosylation profile as natural omega-1 fails to condition DCs for initiating of Th2 responses [20;49]. However, it contradicts the Th2-polarizing properties that have been assigned to LNFPIII, a glycan harboring the Le^x-motif [18]. This discrepancy might be due to the spatial dimensions of the Le^x-motif containing molecules which may affect the way it is recognized by DCs and thereby its immune polarizing properties. Indeed, it has been shown that LNFPIII is dependent on TLR4 to condition DCs for priming of Th2 responses [18], while the capacity of omega-1 to drive Th2 polarization via DCs deficient for Myd88/TRIF was unaffected [21].

Although MR and DC-SIGN have been shown to mediate binding and uptake of fucosylated-proteins by DCs [50], we observed that omega-1 was internalized by DCs in a MR-, and not DC-SIGN-dependent manner. Since fucosidase treatment strongly reduced the capacity of omega-1 to bind to DC-SIGN, but only marginally affected binding to MR, it is possible that MR may bind additional carbohydrate structures on omega-1, that results in a preferential binding to and internalization by MR. The importance of MR in recognition and uptake of omega-1 was substantiated by the finding that inhibition of protein synthesis and conditioning of DCs for Th2 polarization by omega-1 could be significantly impaired by blocking of the MR, but not by blocking of DC-SIGN. We confirmed and extended the importance of MR in Th2 polarization by omega-1 by showing *in vivo* that an antigen-specific Th2 response induced in MR-deficient mice following footpad injection of omega-1 was strongly reduced compared to the response elicited in WT mice. In this respect it is important to note that human and murine MR have a similar carbohydrate binding specificity [51]. Thus, this establishes that omega-1 relies on MR to drive Th2 polarization. Until now a role for MR in shaping of immune responses has only been documented for mannosylated antigens [52-55], while the immune modulatory effects of fucosylated epitopes from natural antigens has, as far as we are aware of, only been studied in the context of DC-

SIGN [10;52;56;57]. We now for the first time identify an essential role for MR in mediating immune polarization by a fucosylated but not mannosylated antigen.

Based in the results obtained with omega-1 a model can be proposed whereby recognition of omega-1 by MR and/or DC-SIGN triggers a signaling cascade leading to modulation of IL-10 and IL-12 transcription, but that additionally the internalisation of omega-1 by MR allows the RNase activity to interfere with ribosomal function and switch off protein synthesis that overrules the signaling-mediated DC modulation. Due to shut down of protein synthesis, the DCs will no longer be able to respond to T cell-induced CD40 ligation, resulting in a situation where T cells are primed by DCs in the absence of IL-12 and low antigen presentation and/or costimulation (suppl FIG 8), a situation that is known to favour the induction of Th2 responses [58-60]. The recent data showing that SEA-pulsed DCs, although still capable of processing antigen to present it on MHC-II, are impaired in their upregulation of surface expression of MHC-II and CD86 or IL-12 in response to CD40 ligation [20;33], as well as the fact that omega-1-primed DCs have a reduced capacity to form T cell-DC conjugates [21], would be consistent with this idea. These observations would argue that there is no active expression of Th2-polarizing signals/molecules. Nonetheless, given that SEA depleted for omega-1 still retains some Th2-polarizing capacity and that SEA, in contrast to omega-1, induces the expression of Th2-associated Notch ligand Jagged-1 and OX40-L through ERK signaling [61], and also suppresses IL-12 in an ERK-dependent fashion [15], it is still possible that SEA contains other components that in parallel with the inhibitory effects of omega-1, may actively induce the expression of Th2-polarizing factors. This may explain why SEA is such a strong Th2-polarizing agent as it would be able to exploit both positive and negative pathways to condition DCs for Th2 polarization.

Materials and Methods

Preparation and purification of *S. mansoni* egg-derived antigens

SEA, omega-1 and IPSE/alpha-1 were prepared and isolated as described previously [20;62]. The purity of the preparations was controlled by SDS-PAGE and silverstaining. Protein concentrations were tested using the Bradford or BCA procedure.

Periodate treatment of antigens

Antigens were treated with sodium periodate as previously described [63;64]. In short, sodium acetate buffer (pH 4.5) was added to the antigens to a final concentration of 0.1M prior to exposure to periodate. Antigens were incubated in 20 mM sodium periodate overnight in the dark at 4°C. The incubation was stopped by addition of an equal volume of 50 mM sodium borohydride for 30 minutes. To remove the sodium

periodate, sample buffer was exchanged to phosphate buffered saline using protein desalting spin columns according to manufacturer's recommendations (Pierce, Rockford, IL, USA).

MALDI-TOF-MS of omega-1 glycopeptides

Glycopeptides of natural or recombinant omega-1 were generated by trypsin treatment of reduced and alkylated omega-1, either in solution or in excised gel bands as described in (Meevissen *et al.*, submitted). Mass spectra were recorded using an Ultraflex II time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) as described [49].

Fucosidase treatment of antigens

Antigens were dissolved in 100 mM sodium phosphate (pH 5) and treated with α -1 \rightarrow (3,4)-fucosidase from *Xanthomonas manihotis* (0.5 mU; Sigma) overnight at 37 °C. Removal of antenna fucoses was confirmed by MALDI-TOF-MS analysis of tryptic glycopeptides.

Generation and production of WT, glycosylation mutant and RNase mutant forms of recombinant omega-1

Site directed mutagenesis was used to generate a glycosylation and RNase mutant by mutating the two putative N-linked glycosylation sites (N71/176Q) or by targeting a conserved amino-acid residue (H58F) that is known to be critical for enzymatic activity in other homologous RNases [22;23], respectively (see supplemental Figure 1). H58F and N71/176Q mutants were created by polymerase chain reaction (PCR) using mutagenic primers on a DH5 α /pProExHtb- plasmid containing the WT omega-1 sequence (Invitrogen). Introduction of the right mutations was confirmed by DNA sequencing. Subsequently, using restriction enzymes HindIII and Apal the templates for WT and omega-1 mutant were subcloned into a pSecTag2-plasmid (Invitrogen) for stable transfection into HEK cells [20]. Secreted recombinant omega-1 forms were sequentially purified from the HEK cell culture medium by immobilized metal affinity chromatography and size exclusion chromatography as described previously [20].

Human DC culture, stimulation and analysis

Monocytes were isolated from venous blood of healthy volunteers using Institutional Review Board-approved protocols by density centrifugation on ficoll followed by a Percoll gradient as described [62] and were cultured in RPMI medium supplemented with 10% FCS, human rGM-CSF (500 units/ml, a gift from Schering-Plough, Uden, The Netherlands) and human rIL-4 (250 units/ml) (R&D Systems). On day 3, culture medium

including the supplements was replaced and on day 6 immature DCs were stimulated with the indicated reagents in the presence of ultrapure LPS (100 ng/ml) (*E. coli* O111 B4 strain, InvivoGen). For CLR blocking indicated cells were pre-incubated with 20 µg/ml anti-DC-SIGN (clone AZN-D1, Beckman Coulter) or 20 µg/ml anti-MR (clone 15.2, Biolegend) for 60 min at 37 °C. As a Th1 control DCs were also pulsed with IFN-γ (1000 U/ml). After 48 h, DCs were harvested for co-culture with naïve T cells. In addition, 1×10^4 matured DCs were co-cultured with 1×10^4 CD40L-expressing J558 cells for 24 h to determine cytokine production by the DCs following activation by CD40L. IL-12p70 concentrations were determined by ELISA using mouse anti-human IL-12, clone 20C2 as capture antibody and biotinylated mouse-anti-human IL-12, clone C8.6 as detection antibody (both Becton Dickinson). Concentrations of IL-10 and TNF-α were determined by a commercially available ELISA-kit (Sanquin reagents). MIP-1β and RANTES were determined by a multiplex LUMINEX assay according to the manufacturer's instruction (InvivoGen). The expression of CD86-PE pulsed DCs was determined by FACS (FACSCanto) through staining with CD86-FITC (Becton Dickinson).

Human T cell culture and determination of T cell polarization

To determine T cell polarization, 5×10^3 48 h-pulsed DCs were co-cultured with 2×10^4 naïve T cells that were purified using a human CD4⁺/CD45RO⁻ column kit (R&D, Minneapolis, MN) in the presence of staphylococcal enterotoxin B (100 µg/ml; Sigma) in 96-well flat-bottom plates (Corning). On day 5, rhIL-2 (10 U/ml, Cetus Corp., Emeryville, CA) was added and the cultures were expanded for another 7 days. For intracellular cytokine production, the primed CD4⁺ T cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate plus 2 µg/ml ionomycin for 6 h. 10 µg/ml brefeldin A was added during the last 2 h (all Sigma). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies (BD).

DC-SIGN and MR ELISA

96-well Maxi-Sorp plates were coated overnight with antigens (1 µg/ml) in PBS at 4°C in duplicate. Plates were washed three times with TSM (20 mM Tris, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂), followed by incubation with TSM/1% BSA for 30 min. After three washes with TSM, 2 µg/ml DC-SIGN-Fc (R&D Systems) or 2 µg/ml MR-CRD4/7-Fc (a kind gift from L. Martinez-Pomares [65]) in TSM was added for 2 h, with or without a prior incubation for 15 min with EGTA (10 mM). The plates were washed five times with TSM/0.05% Tween, then bound DC-SIGN-Fc or MR-CRD4/7-Fc was detected with peroxidase-labeled goat anti-human Fc (1:5000) in TSM/0.05% Tween using standard conditions.

DC-SIGN-expressing cell line

K562 cell line stably expressing DC-SIGN or a mock transfected control (a kind gift from K. Figdor [66]) were seeded overnight in a 96 well plate at 10.000 cells/well. Where indicated, cells were pre-incubated with 10mM EGTA for 30 min at 37 °C. Subsequently, cells were incubated with 2µg/ml PF-647 labeled SEA or 500ng/ml PF-647 labeled omega-1 at 37 °C for 1 h and washed in ice cold PBS before analysis using flowcytometry.

Protein synthesis inhibition

Immature DC were seeded overnight in 96 well flatbottom plates before stimulation with indicated reagents in the presence of LPS. 16 h after stimulation protein synthesis was determined by a 2 h pulse at 37 °C with 3µCi /0,05 ml [³⁵S]-methionine (EasyTag Express Protein labeling mix, Perkin Elmer) in serum- and L-methionine free RPMI1640. After a double washing step in PBS, cells were lysed for 5 min in AV-lysis buffer (20mM Tris HCl, pH7.6, 150 mM NaCl, 0.5% DOC, 1.0% NP40, 0.1% SDS) in the presence of protease inhibitors Leupeptin and Aprotinin 200ug/ml. Lysates were transferred on a filter (Perkin Elmer) and dried. After radioactive labeled proteins were precipitated on the filter with trichloroacetic acid, filters were washed with 96% ethanol and dried. The radioactivity present on the filters was measured in a β-counter by a liquid scintillation cocktail for aqueous solution.

RNase activity assay

RNA was extracted from PBMC using the RNeasy kit (Qiagen). RNA was incubated for one hour at 37°C with with indicated antigens 0.01M Tris 0.02% Cu. Subsequently, RNA breakdown was visualized by running the samples on a 2% agarose gel containing ethidium bromide.

Ribosomal RNA breakdown

Rabbit Reticulocyte Lysate (Promega) was incubated with antigens as described by others [67]. Briefly, following 1 h incubation at 37 °C in Tris-HCl (15 mM NaCl, 50 mM KCl, 2,5 mM EDTA), the reaction was stopped with 10% SDS and RNA was extracted from the ribosomes with phenol/chlorophorm. Next, isolated ribosomal RNA was denatured at 95°C and visualized by running the samples on a 2% agarose gel containing ethidium bromide.

Analysis of dendritic cell stress and apoptosis

Immature DCs were stimulated for 40 h with indicated antigens and subsequently washed twice with PBS. For assessment of Caspase-3 activity, stimulated DCs were

fixed, permeabilized and stained according to the manufacturers' recommendations (BD). Analysis of cells undergoing apoptosis or death were stained with Annexin-V-PE and 7-AAD (both BD), respectively, according to the manufacturers' recommendations. All staining were analysed by flow cytometry using a Becton Dickinson FACSCalibur flowcytometer (BD Biosciences) and analysed using FlowJo analysis software (Tree Star).

MAPK activation analysis

Immature DC were seeded overnight in 96 well round bottom plates. At indicated times after stimulation of the cells with LPS (100ng/ml), SEA (25µg/ml) or omega-1 (500ng/ml), they were fixed for 10 minutes with 4% ultrapure formaldehyde (Polysciences) directly in the plate. Cells were harvested and washed twice in PBS/0.5% BSA. Subsequently, the DCs were permeabilized in 700 µl ice-cold 90% methanol in PBS in and left on ice for 30 minutes. Following two wash steps in PBS/0.5%BSA intracellular staining was performed for 2 hours with anti-phospho-p44/42 MAPK (ERK1/2) AF-488 (T202/Y204) (Cell Signaling Technology). After one wash in PBS/0.5%BSA MAPK activation was determined by flow cytometry using a Becton Dickinson FACSCalibur flowcytometer (BD Biosciences) and analysed using FlowJo analysis software (Tree Star)

Antigen uptake by DCs

SEA and omega-1 were fluorescently labeled with PF-647 using the Promofluor labeling kit (Promokine and according to the manufacturers recommendations. 10.000 immature DC/well were seeded in a 96 well plate. Where indicated cells were pre-incubated with 10mM EGTA, 100ug/ml Mannan (Sigma Aldrich), 20µg/ml anti-DC-sign (clone AZN-D1, Beckman Coulter) or 20µg/ml anti-MR (clone 15.2, Biolegend) for 60 min at 37 °C. Subsequently, cells were incubated with 2µg/ml PF-647 labeled SEA or 500ng/ml PF-647 labeled omega-1 at 37 °C for 1 h and washed in ice cold PBS before analysis using flowcytometry.

Confocal microscopy

Immature dendritic cells were allowed to adhere to Poly-D-Lysine coated cover slips overnight at a concentration of 80-100.000 cells/2 ml in 10% FCS/RPMI. DCs were incubated with omega-1 for 20 or 120 minutes at 37 °C (1µg/ml). Incubated cells were washed three times in 1% BSA/RPMI, fixed for 15 minutes with 4% paraformaldehyde (Sigma) in PBS, and washed twice in PBS. Next, cells were permeabilized with 0.1% Triton-X in PBS for 1 minute, washed twice in PBS and blocked for 15 minutes with 1% BSA/PBS. Cells were subsequently incubated with antibodies against rRNA (Abcam),

followed by a secondary incubation step with a G α M-AF546 antibody (Invitrogen) and Calnexin-FITC (BD) in 1% BSA/PBS. Cells were washed in PBS and cover slips were mounted on glass slides with Vectashield and analyzed by confocal microscopy. Leica AOBS SP2 confocal laser scanning microscope (CLSM) system was used, containing a DM-IRE2 microscope with glycerol objective lens (PL APO 63x/NA1.30) and images were acquired using Leica confocal software (version 2.61).

Gene silencing with siRNA

DCs were transfected with 50 nM siRNA targeted Raf-1 (M-003601-02, SMARTpool, Dharmacon) using the transfection reagent DF4 according to the manufacturer's protocol (Dharmacon). A nontargeting siRNA pool was also used (D-001206-13). This protocol resulted in a transfection efficiency of nearly 100%, as determined by flow cytometry of cells transfected with siGLO RISC-free siRNA (D-001600-01; Dharmacon). At 72 h after transfection, cells were used for experiments. Functional silencing was verified by quantitative real-time PCR and flow cytometry staining for each experiment.

Quantitative real-time PCR

Isolation of mRNA, cDNA synthesis and PCR amplification by the SYBR Green method in an ABI 7900HT sequence-detection system (Applied Biosystems) were done as described [68]. Specific primers were designed with Primer Express 2.0 (Applied Biosystems). The cycling threshold (CT) value is defined as the number of PCR cycles in which the fluorescence signal exceeds the detection threshold value. The normalized amount of target mRNA (N_t) was calculated from the CT values obtained for both target and GAPDH mRNA with the equation $N_t = 2^{Ct(GAPDH) - Ct(target)}$. Relative mRNA expression was obtained by setting N_t in LPS-stimulated samples as 1 in each experiment and for each donor.

In vivo experiments

MR^{-/-} mice on a C57BL/6 background were provided by Dr. M. C. Nussenzweig (Rockefeller University, New York, NY) and were bred and housed in the animal facility of the Institutes of Molecular Medicine and Experimental Immunology at the University Hospital, Bonn. Mice between 10 and 12 wk of age were bred under specific pathogen-free conditions and used in accordance with local animal experimentation guidelines, were immunized s.c. into one hind footpad with SEA (20 μ g), omega-1 (2 μ g) in a volume of 30 μ l in PBS and one week later the cells from the draining popliteal lymph nodes were isolated.

In vitro restimulation of lymph node cells

1.5 × 10⁶ popliteal LN cells/ml from individual animals were restimulated with 10 µg/ml SEA or 2 µg/ml omega-1. IL-5, IL-4 and IFN-γ were measured by ELISA in 4 d supernatants according to the manufacturer's recommendations (R&D). Following removal of the supernatants, cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate plus 2 µg/ml ionomycin for 6 h. 10 µg/ml brefeldin A was added during the last 2 h (all Sigma). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies (BD).

Statistical analysis

Data were analyzed for statistical significance using a one-sided paired *t*-test. All *p*-values < 0.05 were considered significant.

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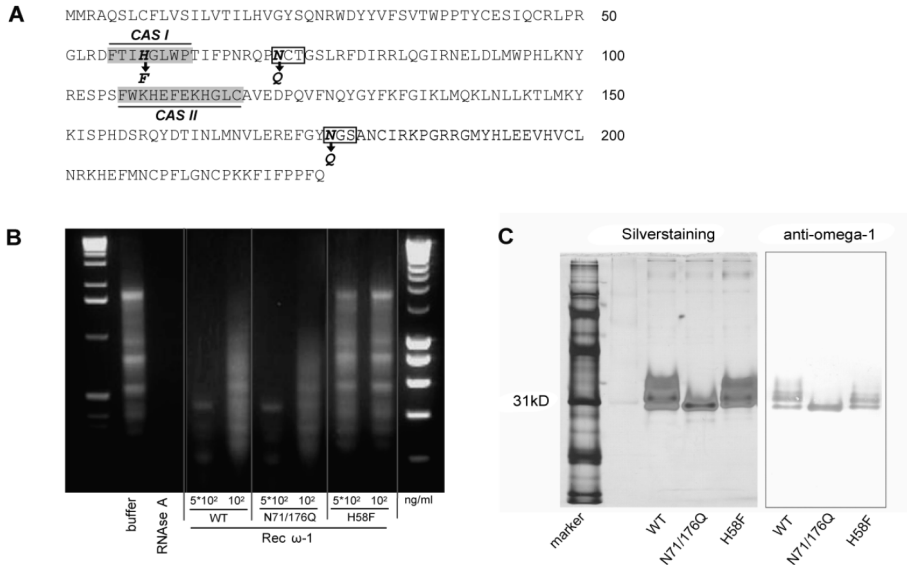
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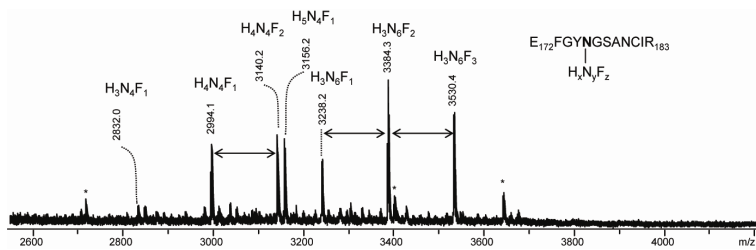
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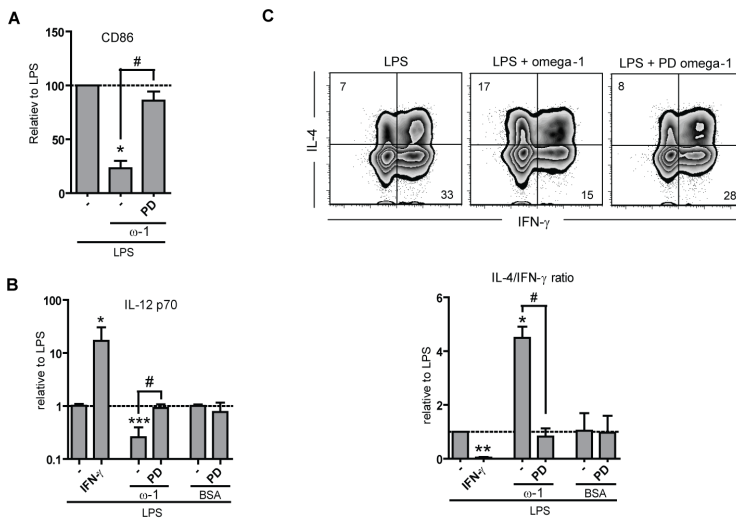
Supplemental Figures



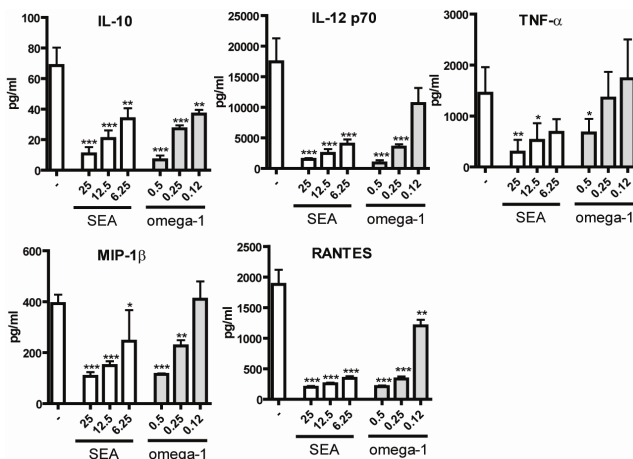
Supplemental Figure 1. Generation and evaluation of glycosylation and RNase mutants of recombinant omega-1. (A) The amino acid sequence of omega-1 is shown in which the mutation sites are depicted. The two conserved amino acid sequence (CAS)-domains essential for catalytical activity are marked in grey and the two N-linked glycosylation sites are depicted in white boxes. (B) RNA from PBMCs was incubated for 1 h with the different omega-1 variants and analysed on a 2% agarose gel for breakdown. The RNase mutant, in contrast to the glycosylation mutant and WT recombinant omega-1, fails to degrade RNA (C) The omega-1 variants were run under non-reducing conditions by SDS-PAGE and silver stained. A Western Blot by staining with a specific anti-omega-1 monoclonal antibody confirmed native conformation of the different omega-1 mutants as well as the absence of glycosylation only on the omega-1 glycosylation mutant, as evidenced by a single band instead of the 3 glycosylation forms of recombinant omega-1 normally secreted by HEK cells.



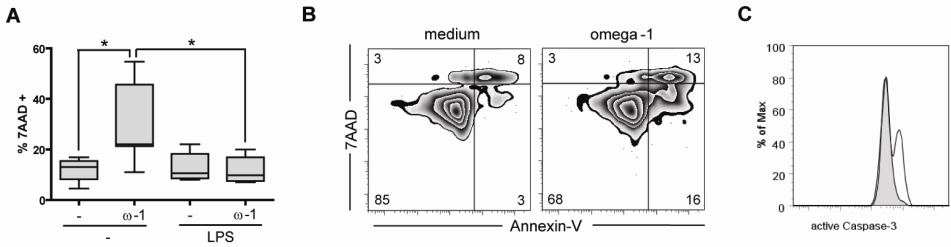
Supplemental Figure 2. Glycosylation of recombinant omega-1. MALDI-TOF mass spectrum of glycopeptides from a tryptic digest of recombinant omega-1, covering the glycosylation site N176. Recombinant omega-1 was subjected to SDS-PAGE under reducing conditions and stained with Colloidal blue. Stained bands were excised, subjected to reduction and alkylation and digested with trypsin. The MALDI-TOF-MS spectrum derived from the upper band in the SDS-PAGE pattern is depicted. Signals ($[M+H]^+$) are labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows. Signals that can not be assigned to glycopeptides are marked with asteriks (*). Based on the presence of the common N-glycan core structure (H3N2), the monosaccharide composition of the glycan species H3N6F2 and H3N6F3 indicates that these contain fucosylated antennae.



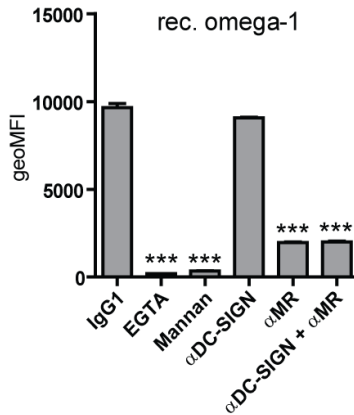
Supplemental Figure 3. Periodate treatment of natural omega-1 abolishes its capacity to modulate DC function and to induce Th2 polarization. (A) DCs were pulsed for 40 h with untreated or periodate-treated (PD) omega-1 (500ng/ml) in combination with LPS (100 ng/ml) as a maturation factor and surface expression of CD86 was determined by FACS analysis. The expression levels, based on geometric mean fluorescence, are shown relative to the DCs stimulated with LPS alone, which is set to 100% (dashed line). (B) Conditioned DCs were co-cultured for 24 h with a CD40-L expressing cell line, to mimic the interaction with T cells. IL-12p70 cytokine expression levels are shown relative to the DCs stimulated with LPS alone, which is set to 1 (dashed line). periodate- and mock-treated BSA pulsed DCs were taken along as controls (C) Conditioned DCs were cultured with allogeneic naive CD4⁺ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2 and analysed as described in figure 1. (A-C) Data are representative of 3 independent experiments. Error bars represent SD. *,# p < 0.05, ** p<0.01, *** p<0.001 for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (one-sided paired t-test). ω-1, omega-1.



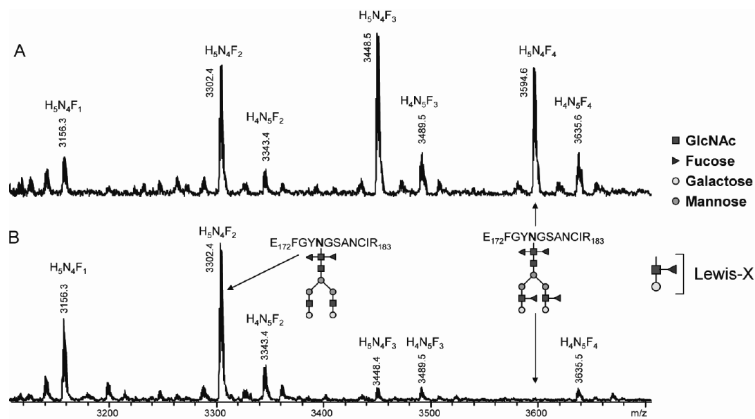
Supplemental Figure 4. Omega-1 dose dependently suppresses expression of multiple cytokines. After DCs had been pulsed for 40 h with the different antigen preparations in combination with LPS (100 ng/ml), the cells were co-cultured for 24 h with the J58 cell-line, expressing CD40-L, to mimic the interaction with T cells. Concentrations of the stimuli are given in μg/ml. Error bars represent SD. * p < 0.05, ** p<0.01, *** p<0.001 for values significantly different from the LPS control.



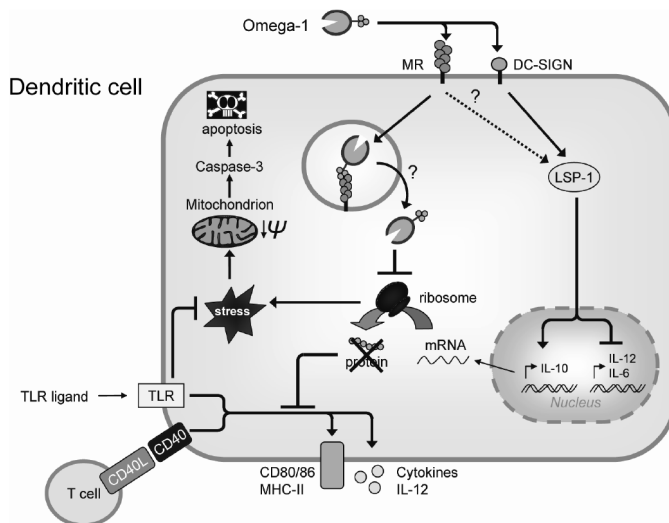
Supplemental Figure 5. Omega-1 induces apoptosis and cell death in dendritic cells through its RNase activity. (A) DC death as determined by 7-AAD staining was analysed following 40 h stimulation with omega-1 in the presence or absence of LPS (100ng/ml). Data are shown as percentage positive cells and box plots represent 25-75 percentile range with error bars showing minimum to maximum of 4 independent experiments. (B) DCs were stimulated for 40 h with omega-1 (500ng/ml) and analysed for apoptosis and cell death by staining for Annexin-V and 7-AAD, respectively. (C) 24 h stimulated DCs were stained for active effector caspase-3, as a marker for apoptosis induction. Dark line and filled diagram represent, omega-1-pulsed and unstimulated DCs, respectively. (B and C) One representative experiment out of at least 3 independent experiments is shown. * $p < 0.05$ for significant differences between conditions.



Supplemental Figure 6. Uptake of recombinant omega-1 by DCs is MR dependent. Immature DCs were pre-incubated with indicated reagents followed by incubation with PF-647-labeled recombinant omega-1 for 1h. Uptake of antigens by DCs was evaluated by FACS analysis. Data are shown as mean \pm SD of duplicates. One representative experiment out of two is shown. *** $p < 0.001$ for significant differences compared to the control (one-sided t -test).



Supplemental Figure 7. Removal of fucoses from omega-1 glycans by fucosidase treatment. MALDI-TOF mass spectra of tryptic glycopeptides covering the glycosylation site N176 of omega-1 which was either (A) mock-treated or (B) treated with α -fucosidase. Signals ($[M+H]^+$) are labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). The α -fucosidase-catalysed removal of fucose residues from omega-1 is clearly illustrated by the shift of glycopeptide signals towards species containing 2 or less fucoses (H5N4F1, H5N4F2) and the absence of species containing 3 or 4 fucoses (H5N4F3, H5N4F4) in the treated material. It has been shown that the H5N4F4 glycosylated form of E₁₇₂FGYNGSANCIR₁₈₃ contains a Le^x motif on both antennae, as well as a α 3/ α 6 difucosylated core *N*-acetylglucosamine, while the H5N4F1 and H5N4F2 species remaining after fucosidase treatment only contain the core fucoses (Meevisen *et al.*, submitted).



Supplemental figure 8. Proposed model of the molecular mechanisms through which omega-1 conditions DCs for priming of Th2 responses. Omega-1 is recognized by DCs via its glycosylation by interacting with MR and DC-SIGN, that triggers a signaling cascade dependent on LSP-1 and results in modulation of cytokine production. In addition, omega-1 is internalized by MR, that allows it via yet undefined mechanisms to translocate into the cytosol where it interferes with translation by inhibiting the function of ribosomes through cleavage of rRNA by virtue of its RNase activity. This results in potent suppression of DC activation, induced by TLR ligands or CD40 ligation, as exemplified by reduced cytokine production, including IL-12, as well as other factors such as costimulatory molecules. The inhibition of protein synthesis may also trigger stress responses that eventually result in apoptosis.