Chapter 5

Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by mannose receptor

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Abstract

Omega-1, a glycosylated ribonuclease secreted by *Schistosoma mansoni* eggs and abundantly present in soluble egg antigen (SEA), has recently been shown to condition dendritic cells (DCs) to prime Th2 responses. However, the molecular mechanisms underlying this effect remain unknown. We show here by site-directed mutagenesis of omega-1 that both the glycosylation and the RNase activity are essential to condition DCs for Th2 polarization. Mechanistically, we demonstrate that omega-1 is bound and internalized via its glycans by the mannose receptor (MR) and subsequently impairs protein synthesis by degrading ribosomal RNA. These studies reveal an unrecognized pathway, involving MR and interference with protein synthesis that conditions DCs for Th2 priming.

Introduction

Dendritic cells (DCs) play a central role in the development and maintenance of immune responses during infection, as they govern both the activation and polarization of adaptive T helper (Th) cells. Classically, upon recognition of invading pathogens, resting DCs undergo a process of activation, 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 collectively enable DCs to potently activate and direct $CD4^+$ T cell responses [1].

This paradigm is largely based on observations of responses towards 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 signalling cascades that generally result in the conditioning of DCs for priming of Th1- or Th17-biased responses which are instrumental in combating prokaryotic and single cell eukaryotic pathogens [2]. In contrast to this classical view of DC activation, components derived from parasitic helminths fail to induce all traditional signs of DC maturation. However, although overt maturation is not observed, unlike immature DCs, helminth antigen-treated DCs are altered such that they prime Th2-polarized immune responses [3].

Despite this consistent picture, the pathways through which helminth antigens manipulate DC function and drive Th2 responses are still poorly understood [4]. The majority of the studies have been conducted with a complex mixture of soluble egg antigens (SEA) from the trematode *Schistosoma mansoni*. SEA is regarded as one of the most potent helminth-derived antigenic extracts that instruct DCs to drive Th2 polarization [3;5]. 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 their capacity to induce Th2 polarization [6]. In this respect, another class of PRRs expressed by DCs, the carbohydrate-binding C-type lectin receptors (CLRs), have been suggested to play a role in modulation of DC function by SEA [7]. For instance, SEA contains carbohydrate structures, such as Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX), that can be recognized by DC-SIGN [8-10]. Engagement of this receptor by components from pathogens such as *Helicobacter pylori* has been shown to suppress IL-12 production and modulate TLR-induced DC activation and T cell polarization [8;11]. In addition, more recently it has been shown that SEA can modulate cytokine responses through another CLR, Dectin-2 [12]. Finally, a number of studies have raised the possibility that TLRs are involved in SEA-mediated Th2 induction [13;14]. However, direct evidence

for involvement of specific receptors or downstream pathways in SEA-driven Th2 polarization has been missing.

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 [15;16], has allowed us to dissect the involved molecular pathways in a precise manner. Through site-directed mutagenesis we show that the RNase activity as well as the glycosylation of omega-1 are essential for programming 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

Omega-1 requires both its glycosylation and RNase activity to condition DCs for priming of Th2 responses

A role for the RNase activity of omega-1 has been proposed in the conditioning of DCs to prime Th2 responses [16]. However, this was based on a chemical inactivation of the RNase activity by DEPC-treatment, which may also alter the function or structure 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 wildtype (WT) omega-1 lacking RNase activity by site-directed mutagenesis. Specifically, a histidine residue in its catalytic domain, known from other T2 RNases to be essential for the enzymatic activity, was replaced by phenylalanine (omega-1 H58F) [17] (Supplementary figure 1A). Apart from RNase activity, glycosylation of omega-1 may also be important for its Th2-priming capacity, since chemical modification of glycans on proteins present in SEA is known to abolish the ability of SEA to induce Th2 polarization [6]. Moreover, potentially Th2-polarizing LeX glycan motifs have recently been described to be present in glycans on omega-1 [18]. To address the role of glycosylation in Th2 priming by omega-1, a glycosylation mutant was generated by a single amino-acid replacement at each of the two putative N-linked glycosylation sites (omega-1-N71/176Q) (Supplementary figure 1A) [19]. An RNase assay showed that RNase mutant did not have any RNase activity, while that the RNase activity of the glycosylation mutant was unaffected (Supplementary figure 1B). In addition, using a silver-stained SDS PAGE as well as an anti-omega-1 Western blot, we confirmed the absence of glycosylation on the glycosylation mutant. On the other hand, the mass shift of the RNase mutant on a silver-stained SDS PAGE was identical to recombinant WT omega-1, suggesting the glycosylation on the RNase mutant was still intact (Supplementary figure 1C). With regard to

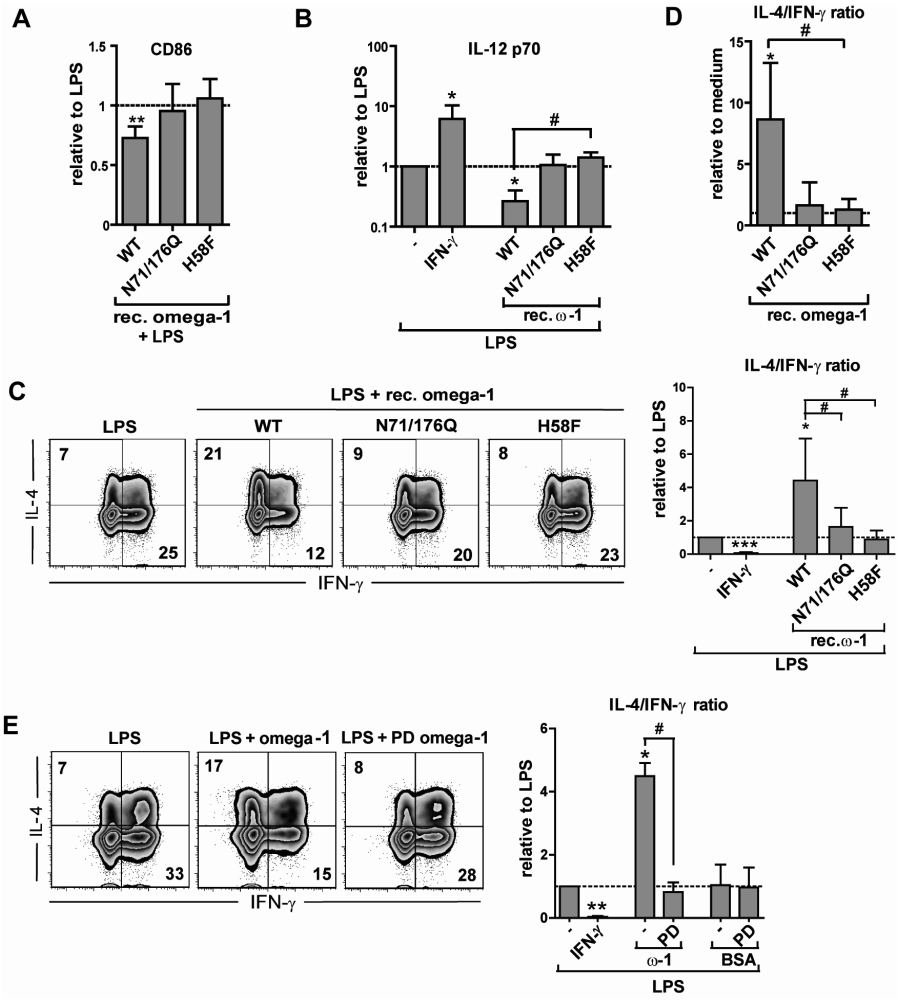

Figure 1. The glycosylation and RNase activity of omega-1 are essential for conditioning DCs to prime Th2 responses (A) Monocyte-derived DCs were pulsed for 48 h with the different variants of recombinant omega-1 (2 μg/ml) in combination with LPS (100 ng/ml) as a maturation factor and surface expression 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%. (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. (C+D) Conditioned DCs 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. 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. (E) DCs were pulsed for 40 h with mock- or periodate-treated (PD) natural omega-1 (500 ng/ml) in combination with LPS (100 ng/ml). Conditioned DCs were cultured with allogeneic naive $CD4^+$ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2 and analyzed as described in (D). Data are representative of 3 independent experiments. Bars represent mean ± 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 (two-sided paired *t*-test). ω-1, omega-1.

the glycans present on recombinant WT omega-1 and the RNase mutant, mass spectrometric analysis of tryptic glycopeptides showed the presence of N-glycans on Asn176 with the monosaccharide composition $Hex_3HexNAc_6Fuc_{2/3}$ (Supplementary figure 2). This is indicative of the presence of fucosylated LDN antennae (LDN-F), a glycan element previously found on a protein from HEK293 cells, the cell type in which recombinant omega-1 is expressed [20]. LDN-F is thought to have similar immunological characteristics as the LeX motifs present on natural omega-1 [9;10;18].

To assess the role of glycosylation and RNase activity in omega-1-driven Th2 polarization, 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 Th cell polarization [1]. Similar to natural omega-1 [15], recombinant WT omega-1 consistently and significantly suppressed the LPS-induced upregulation of the costimulatory molecule CD86 (Figure 1A), as well as the production of IL-12 (Figure 1B), which is an important characteristic of Th2-priming DCs [3]. However, both the glycosylation as well as the RNase mutant failed to alter LPS-induced CD86 expression or IL-12 production of DCs. Importantly, in contrast to DCs primed with recombinant WT omega-1, those conditioned with the glycosylation mutant or the RNase

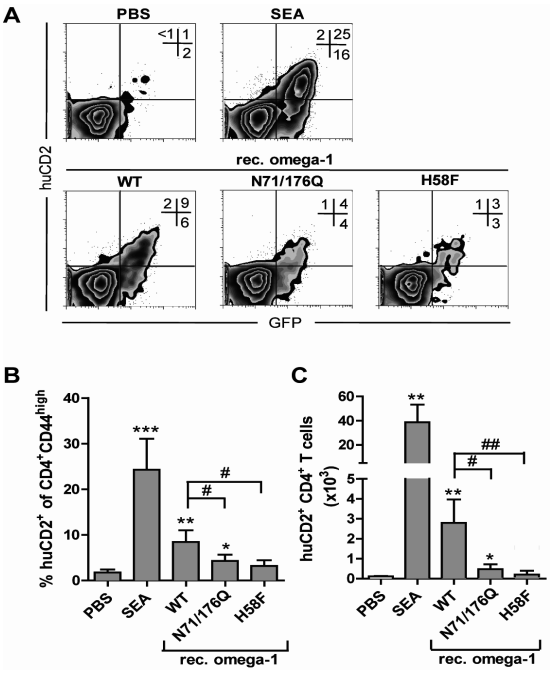

Figure 2. Glycosylation and RNase activity are essential for omega-1 to prime Th2 responses *in vivo* 4get/KN2 IL-4 dual reporter mice were injected s.c. with SEA (20 µg) or WT recombinant omega-1 and the two mutants (3 µg) into the footpad. After 7 days the frequency of GFP^+ and $huCD2^+$ within the $CD4^+CD44^{high}$ effector T cell population was determined by flow cytometry in the draining popliteal lymph nodes. Depicted are (A) concatenated FACS plots, (B) frequencies of $huCD2^+$ within the $CD4^+CD44^{high}$ population and (C) total $huCD2^+$ T cell numbers in draining lymph nodes of combined data of 4 mice per group. (A) The frequencies of each population are indicated as percentages in the plots. One of 3 independent experiments is shown. Bars represent mean ± SD. *,# $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for values significantly different from the PBS control (*) or between test conditions (#) based on two-sided *t*-test.

mutant did not prime a Th2 response (Figure 1C). Similar results were obtained with cultures in which DCs were conditioned by the omega-1 variants in the absence of LPS (Figure 1D). The importance of omega-1 glycosylation for polarizing towards Th2 was further substantiated by experiments performed with natural omega-1 on which the glycans were disrupted by periodate treatment (Figure 1E). These data show that the RNase activity and the glycosylation of omega-1 are both essential, but as single property not sufficient, for the induction of Th2 responses via DCs.

Omega-1 requires both its glycosylation and RNase activity to prime Th2 responses in vivo

To test whether the *in vivo* Th2-priming capacity of omega-1 is dependent on glycosylation and RNase activity, recombinant WT omega-1 or its mutants were administered to 4get/KN2 IL-4 dual-reporter mice [21]. In these mice IL-4-competent cells are GFP^+ and IL-4-producing cells additionally express huCD2, allowing the direct visualization of Th2 differentiation and IL-4 production. Following the s.c. injection of the antigens into the footpad, the draining popliteal lymph nodes (LNs) were harvested on day 7 and $CD4^+CD44^{high}$ effector T cells were analyzed for the expression of GFP and huCD2 directly ex vivo. Injection of SEA resulted in a significant increase of GFP^+ and $huCD2^+$ cells, reflecting the induction of Th2 differentiation and acute IL-4 production *in vivo* (Figure 2). Importantly, while recombinant WT omega-1 alone also induced a marked Th2 response and the production of IL-4, both mutants were significantly impaired to prime this response as evidenced by lower frequencies (Figures 2A and 2B) as well as total numbers of $huCD2^+$ T cells (Figure 2C) in the draining LN. Taken together, these data show that the glycosylation and the RNase activity of omega-1 play a crucial role in Th2 polarization induced by omega-1 *in vivo*.

Omega-1 is internalized by DCs via mannose receptor (MR)

To get a better understanding of how glycosylation is involved in omega-1-driven Th2 polarization, we tested whether recognition of omega-1 by DCs was dependent on glycans from omega-1. To this end an assay was used that quantifies binding and uptake of fluorescently-labelled antigens by DCs (Supplementary figure 3). While DCs were capable of binding and internalizing recombinant WT omega-1 or the RNase mutant, DCs failed to bind and internalize the glycosylation mutant, demonstrating that glycans present on omega-1 are essential for recognition by DCs (Figure 3A). Given the importance of glycosylation of omega-1 for binding to DCs, we explored the involvement of carbohydrate-binding CLRs in the recognition of omega-1. When DCs were pre-incubated with calcium-chelator EGTA, which blocks CLR function, binding and internalization of natural omega-1 was totally prevented (Figure 3B),

suggesting that DCs recognize omega-1 via CLRs. SEA has been reported to be recognized and endocytosed by human DCs via the CLRs DC-SIGN and MR [7], that have the capacity to bind fucose-residues such as ones found in LeX [9;10;22], a glycan motif present on natural omega-1 [18]. To determine whether MR and DC-SIGN are involved in recognition and internalization of natural omega-1, DCs were pre-incubated with mannan (a natural ligand that competes for binding to DC-SIGN and MR), or DC-SIGN- and MR-specific blocking antibodies, followed by an 1h incubation with fluorescently-labelled SEA or natural omega-1. Uptake of SEA, as previously published [7], could be reduced by mannan and either DC-SIGN or MR blocking antibodies in an additive manner. With regard to omega-1, pre-treatment with mannan could almost completely block binding and uptake of the molecule. Interestingly, binding and uptake of natural omega-1 were significantly reduced by MR but not by DC-SIGN blocking antibodies (Figure 3C).

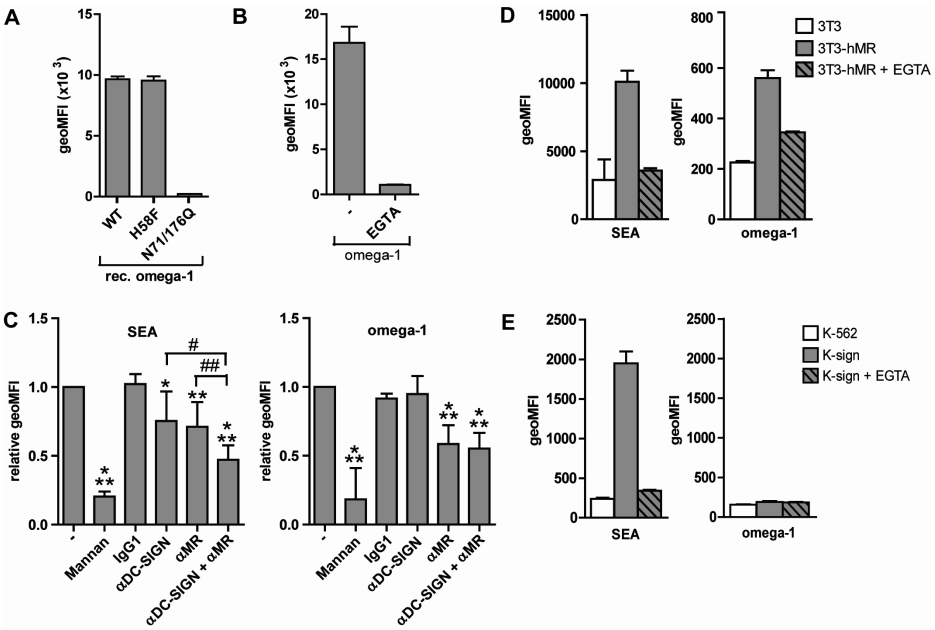

Figure 3. Mannose Receptor (MR) is the principal receptor through which omega-1 is recognized and internalized by DCs (A) Human monocyte-derived DCs were incubated for 1 h with PF-647-labeled recombinant WT omega-1, the glycosylation mutant or the RNase mutant and analyzed for uptake of antigens by FACS analysis. One representative experiment with duplicate samples out of 2 experiments is shown. (B) A binding and internalization assay on immature monocyte-derived DCs was performed as described in (A) following preincubation with EGTA. One representative experiment based on duplicate samples out of 5 is shown. Data are shown as mean ± S.D. (C) A binding and internalization assay on immature monocyte-derived DCs was performed as described in (A) following preincubation with indicated reagents. Binding and internalization are shown relative to mock pre-treatment. Data are representative of 5 experiments. (D) 3T3 cell-line expressing MR and (E) K-SIGN expressing DC-SIGN or parental control cell lines (3T3 and K-562) were incubated with PF-647-labeled omega-1 and SEA in the presence or absence of EGTA to determine specificity. One representative experiment based on duplicate samples out of 2 is shown. Bars represent mean ± SD. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for significant differences compared to the control (two-sided *t*-test).

Pre-incubation with the combination of both blocking antibodies did not have any additional effect on the uptake of omega-1 as compared to pre-incubation with anti-MR antibody alone. Of note, we found that recombinant omega-1 was recognized and internalized by DCs in similar MR-dependent fashion as natural omega-1 (data not shown). To further investigate the observations of selective recognition and uptake of omega-1 by MR, we made use of the K562 and 3T3 cell lines selectively expressing human DC-SIGN and MR, respectively. Fluorescently-labelled SEA was readily bound by both the DC-SIGN- and MR-expressing cells, which was not observed upon pre-incubation with EGTA or in parent control cell lines lacking CLR expression. In line with the DC-binding and uptake data, omega-1 binding could be observed in the cell line expressing MR (Figure 3D), but not in the cell line expressing DC-SIGN (Figure 3E). It should be noted, that not only in DC uptake experiments but also in the omega-1 binding experiments with the cell line selectively expressing MR, blocking with anti-MR antibody was not complete (Figure 3C and data not shown), suggesting that a relatively low affinity of the anti-MR antibody accounts for this finding rather than that other receptors are involved. Taken together, our data suggest that recognition and internalization of omega-1 by human DCs is dependent on its glycosylation and that MR is the primary CLR involved in this process.

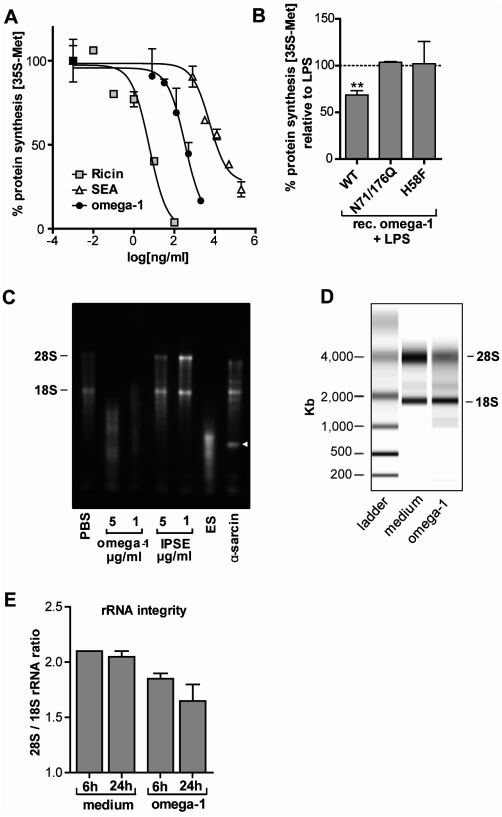

Figure 4. Omega-1 suppresses protein synthesis through interference with ribosomal function (A) Following 16 h incubation of DCs with a concentration range of indicated reagents in the presence of LPS (100 ng/ml), protein synthesis was assessed after a 2 h pulse with radioactive labelled methionine. Ricin, as potent inhibitor of protein synthesis, was taken along as positive control [23]. (B) Protein synthesis by DCs following exposure to the recombinant omega-1 variants (2 µg/ml) was assessed as described in (A). (C) After rabbit reticulocyte lysate containing functional ribosomes was incubated for 1 h with omega-1 or IPSE/α1, as a negative control, isolated ribosomal RNA was analyzed 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 (white arrowhead) [35]. (D) rRNA isolated from 24h omega-1-stimulated DCs and was visualized by running a lab-on-a-chip picogel and (E) 28S/18S rRNA ratio was determined from these samples as a measure for rRNA integrity. (A-E) One representative experiment from 3 independent experiments is shown. Bars represent mean ± SD.

Omega-1 suppresses DC function by interfering with protein synthesis

Next we examined the molecular mechanism through which the RNase activity of omega-1 exerts its modulatory effects on DCs. We noted that omega-1-stimulated DCs in response to CD40 ligation were not only impaired in their capacity to produce IL-12 p70, as reported previously [15], but also fail to express a large panel of other cytokines (Supplementary figure 4). This indicates that the suppression may not be gene specific, but could be the result of inhibition of protein synthesis globally. 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, a well known protein synthesis inhibitor [23] (Figure 4A). The capacity to inhibit protein synthesis was dependent on its RNase activity and uptake via its glycans, since the RNase as well as the glycosylation mutant failed to interfere with protein synthesis (Figure 4B). As several fungal ribonucleolytic proteins, so-called ribotoxins, have been described to inhibit protein synthesis through cleavage of ribosomal RNA (rRNA) [24], we tested whether omega-1 could cleave rRNA in the context of functional ribosomes in a cell free assay. Omega-1 was able to break down rRNA, while IPSE/α1, another *S. mansoni* egg-derived protein that lacks RNase activity but has identical glycans as omega-1 [25], did not induce any rRNA digestion (Figure 4C), indicating that omega-1 is able to interfere with ribosomal function by cleavage of rRNA. Finally analysis of the integrity of rRNA isolated from omega-1-exposed DCs revealed a selective breakdown of 28S rRNA (Figure 4D), resulting in time dependent decrease in 28S/18S ratio (Figure 4E). 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.

MR mediates omega-1-induced protein synthesis inhibition, DC modulation and Th2 polarization

To address the role of omega-1 binding by MR in mediating RNase-dependent DC modulation and Th2 priming by omega-1, we used blocking antibodies directed against MR or DC-SIGN. Blocking of MR during the stimulation of DCs with omega-1 significantly prevented the inhibition of protein synthesis (Figure 5A), while blocking of DC-SIGN had no effect, showing that the interference with protein synthesis by omega-1 is dependent on MR. In line with these observations, blocking of MR significantly reduced the capacity of omega-1 to suppress LPS-induced CD86 expression (Figure 5B) and IL-12 production following CD40 ligation (Figure 5C) or to condition DCs to induce a Th2 response (Figure 5D). As a last step, murine MR-/- splenic DCs were tested for their Th2-priming capacity in response to omega-1. While OVA peptide-pulsed WT splenic DCs, when conditioned with omega-1, primed a Th2-skewed OVA peptide-specific T cell response *in vitro*, MR-/- splenic DCs failed to do so (Figure 5E).

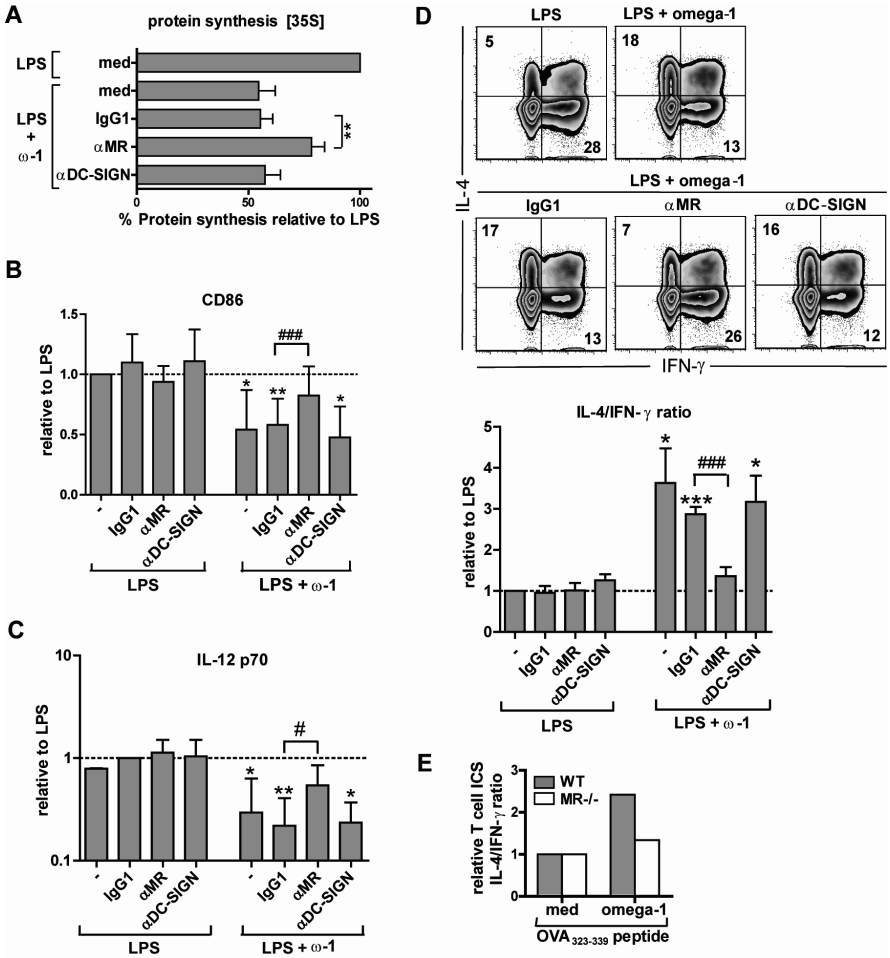

Figure 5. MR mediates omega-1-induced DC modulation and Th2 polarization *in vitro*. Following 1 h pre-incubation with blocking antibodies against MR, DC-SIGN or an isotype control (20 μg/ml), monocyte-derived DCs were pulsed for 16 h (A) or 48 h (B-D) with natural omega-1 (500 ng/ml) in combination with LPS (100 ng/ml). (A) Protein synthesis was assessed as described in Fig. 4 A. One representative experiment based on duplicate samples out of 3 experiments is shown. (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). Data are based on 3 independent experiments (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). Data are based on 3 independent experiments. (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 T cell polarization was analyzed as described in figure 1. Data are based on 6 independent experiments. (E) Splenic WT or MR-/- DCs were co-cultured with OVA-specific OT-II cells for 4 days in the presence of indicated antigens. At day 4 T cells were restimulated with PMA and ionomycin and analyzed for intracellular IL-4/IFN-γ cytokine ratio as described in Fig. 1 D. 1 representative experiment out of 2 experiments is shown. Bars represent mean ± 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 (two-sided paired *t*-test). ω-1, omega-1.

These data establish that MR is essential for the omega-1-driven Th2 polarization via DCs *in vitro*.

Omega-1 requires MR to prime Th2 responses in vivo

Finally, to investigate the role of MR in Th2-priming by omega-1 *in vivo,* natural omega-1 or PBS were injected subcutaneously into the footpad of WT and MR-/- mice. After 7 days the draining popliteal LNs were harvested and restimulated *in vitro* with PBS, omega-1 or a polyclonal stimulus PHA and analyzed 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-associated cytokine IL-5 but not of Th1-associated cytokine IFN-γ, which was absent in LN cells derived from MR-/- (Figure 6A). Furthermore, intracellular staining for IFN-γ and IL-4 following antigen-specific restimulation of $CD4^+$ T cells from omega-1-primed LNs, showed a significant increase in the ratio between IL-4- and IFN-γ-producing T cells from WT but not MR-/- mice (Figure 6B). The failure of MR-/- mice to prime a Th2-polarized response in response to omega-1 was not due to a general failure of MR-/- cells to produce these cytokines as the responses to PHA were comparable in WT and MR-/- mice (Figures 6A and 6B). Taken together, these data show that MR is essential for priming of Th2 responses by omega-1 *in vivo*.

Discussion

Utilizing omega-1, a single molecule derived from *Schistosoma mansoni*, we studied the molecular mechanisms involved in conditioning DCs to induce Th2 responses. By generating mutant proteins we could show that both the glycosylation and RNase activity of omega-1 are essential for its potent Th2-inducing activity both *in vitro* and *in vivo*. The glycan structures on omega-1 suggested that CLRs might play a role in its interaction with DCs. Although both MR and DC-SIGN have been shown to mediate binding and uptake of fucosylated antigens by DCs and omega-1 harbours fucose-containing LeX moieties [18], we observed that omega-1 only significantly bound to a MR-, and not DC-SIGN-expressing cell-line and was internalized by DCs in a MR-, and not DC-SIGN-dependent manner. Lack of potent binding and uptake of omega-1 by DC-SIGN might be explained by the fact that in most DC-SIGN binding studies polyvalent LeX-containing beads or conjugates have been used, which may be bound by DC-SIGN with a higher affinity than soluble glycoproteins, such as omega-1 that presents LeX at a low valency [27]. In line with this observation, DC-SIGN blocking experiments suggest that interactions with DC-SIGN do not play a major role in omega-1-driven Th2 polarization via

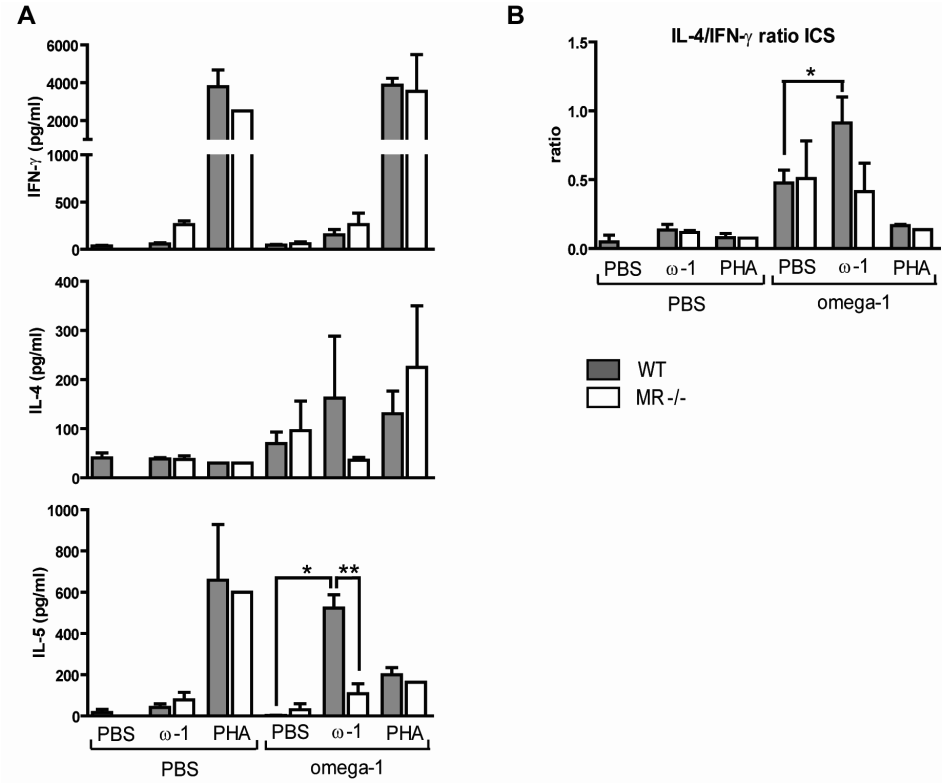

Figure 6. MR is essential for omega-1-driven Th2 polarization *in vivo* MR-/- and WT Bl/6 mice were injected s.c. with omega-1 (2 µg in 30 µl PBS) or PBS into the footpad. (A) After 7 days the cells from the draining lymph node (LNs) were restimulated *in vitro* for 4 days with PBS, omega-1 (2 µg/ml) or PHA (10 µg/ml), as polyclonal stimulus, after which cytokine production was determined by ELISA. (B) 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 percentage of T cells single-positive for either IL-4 or IFN-γ is shown. One experiment of 2 independent experiments is shown. Data are means ± s.e.m. of 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 (two-sided paired *t*-test). ω-1, omega-1.

DCs. On the other hand, 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 was significantly impaired by blocking of MR, but not 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 [28]. Thus, this establishes that omega-1 relies on MR to drive Th2 polarization. This is consistent with earlier observations that crosslinking of MR with antibodies [29] or engagement by mannosylated antigens [30;31] can drive an anti-inflammatory cytokine program in DCs away from

a Th1-promoting profile [29] and that allergen-driven Th2 polarization by DCs is in part dependent on MR [32;33]. These studies point towards a direct role for MR in Th2 induction, potentially via signalling events. However, our data demonstrate that MR binding alone is not sufficient for Th2 polarization by omega-1, since glycans present on omega-1, in absence of RNase activity, are not sufficient to program DCs to induce Th2 responses. This is in line with the observation that IPSE/α1, another major glycoprotein secreted by *S. mansoni* eggs with identical glycosylation as omega-1 [25], which can bind a cell line expressing MR (chapter 4 of this thesis) but lacking RNase activity, fails to prime Th2 responses [15].

Furthermore, we specifically confirmed that apart from glycosylation, omega-1 requires its RNase activity to induce a Th2 response via modulation of DCs by using an RNase mutant. It was observed that omega-1 could cleave rRNA in the context of ribosomes in a cell free assay, and could induce 28S rRNA breakdown 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. Some RNases have been linked to Th2 polarization before. For instance, the major birch pollen allergen, Bet v 1 [34], was identified as an RNase. Furthermore, some fungal RNAses that selectively cleave rRNA, such as mitogillin and Aspf-1, are known to be allergens [35]. Interestingly, for Aspf-1 it was found that its allergenicity was lost when its capacity to interfere with ribosomal function was abolished [36]. In addition, a report has linked an endogenous RNase, the eosinophil-derived neurotoxin, to DC-mediated Th2 polarization [37]. Although these studies have not specifically addressed the role of RNase activity in direct priming of Th2 responses, they do highlight the possibility that Th2 priming through interference with ribosomal function may not be a unique feature of *S. mansoni*-derived omega-1, but may be shared by other RNases as well. However, such an RNase needs to be recognized by DCs and routed in a specific manner to reach the ribosomes and exert its enzymatic activity which in turn would result in suppression of protein synthesis, yet without shutting down DC function altogether or inducing cell death before T cell priming has occurred. One would predict that only certain RNases or ribosome-inactivating proteins will meet those requirements, and as such would be able to drive Th2 responses.

It remains to be established how omega-1 would be able to reach the ribosomes present in the cytosol. Some ribosome-inactivating proteins have been shown to translocate from the ER into the cytosol after retrograde transport or by direct escape from endosomes into the cytosol [38]. In this respect, since omega-1 is internalized via MR, it is interesting to note that cross-presentation of OVA by DCs, a process that requires translocation of the antigen from

endosomes into the cytosol, has been shown to be dependent on MR [39;40]. Mechanistically, it was demonstrated that binding of the MR to OVA leads to poly-ubiquitination of MR, resulting in the recruitment of the ATPase p97, a member of the ER-associated degradation machinery, towards the endosomal membrane. p97 in turn was found to provide the energy to pull out the MR ligand into the cytoplasm [41]. This suggests that the MR itself can regulate the transport of its ligand, into the cytoplasm and provides a mechanism through which omega-1 could be translocated into the cytosol of DCs.

Apart from inhibition of protein synthesis, we observed that omega-1 could promote apoptosis in DCs in a RNase dependent manner (unpublished data), reminiscent of ribotoxins that are known to induce cytotoxicity by interfering with ribosomal function [24]. It is important to note that in line with the protective effects of maturation on DC death [42], 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. Furthermore, our observations that omega-1 relies on MR to be internalized by DCs may explain the documented hepatotoxic effects of *S. mansoni* egg-derived protein fractions containing omega-1 [43;44], given that apart from DCs, sinusoidal endothelial cells and Kupfer cells express MR [45;46], which would render them sensitive to omega-1-induced cytotoxicity.

The suppression of protein synthesis in DCs by omega-1, would be in line with the documented inhibitory effects of omega-1 as well as SEA on DC activation and TLR-induced expression of co-stimulatory molecules and cytokines [15;16]. In addition, this mode of action would also provide an explanation for the finding that omega-1 alters DC morphology as a result of cytoskeletal changes [16], since halting of translation and concomitant stress responses can affect actin rearrangements and thereby cell morphology [47]. Importantly, during interactions with naïve T cells, omega-1-conditioned DCs will, in contrast to unconditioned DCs, be largely refractory to respond to CD40 ligation by T cells, as their protein synthesis machinery is impaired. As a consequence, T cells are primed in the absence of IL-12 and in the context of low antigen presentation and/or co-stimulation, a situation that is known to favour the induction of Th2 responses [48;49]. This mechanism would be different from the initially proposed 'default hypothesis' for Th2 induction [50], as it represents a dominant and active suppression of signals during DC-T cell interactions. Such a model of active suppression of DC signals for Th2 polarization would be in line with 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 [15;51], as well as the observations that omega-1-primed DCs have a reduced capacity to form T cell-DC conjugates [16].

Taken together, based on our data we propose a model in which the glycans present on omega-1 do not play a dominant role in functional modulation of DC function for induction of Th2 responses, but instead are essential for efficient recognition and internalization by DCs via MR. Subsequently, following translocation into the cytosol omega-1 programs DCs to drive Th2 polarization in an RNase dependent manner by interfering with ribosomal function and protein synthesis. These studies have uncovered a novel mechanism through which DCs can be programmed to drive Th2 responses. It will be of great interest to study whether targeting of MR and the protein synthesis machinery to condition DCs for priming Th2 responses is unique to schistosome-driven Th2 polarization, or a mechanism that is also involved in the initiation of other Th2-polarized immune responses, found during other helminth infections or allergies. In addition, the insight may help the design of Th2-polarizing molecules, that could be used in the development of immune-driven manipulation of metabolic disorders such as insulin resistance, vaccines against parasitic worm infections or approaches to counterbalance unwanted Th1 responses [52;53].

Materials and Methods

Preparation and purification of S. mansoni egg-derived antigens

SEA, omega-1 and IPSE/α1 were prepared and isolated as described previously [15;54]. 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 [55;56]. 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 4ºC. 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 recombinant omega-1 were generated by trypsin treatment of reduced and alkylated omega-1, either in solution or in excised gel bands [18]. Mass spectra were recorded using

an Ultraflex II time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) as described [25].

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 [20;85], respectively (see supplemental Figure 1). H58F and N71/176Q mutants were created by polymerase chain reaction (PCR) using mutagenic primers on a DH5α/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 ApaI the templates for WT and omega-1 mutant were subcloned into a pSecTag2-plasmid (Invitrogen) for stable transfection into HEK cells [15]. 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 [15].

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 [15] and were cultured in RPMI medium supplemented with 10% FCS, human rGM-CSF (500 units/ml, Invitrogen) 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 0111 B4 strain, InvivoGen). For CLR blocking indicated cells were pre-incubated with 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. 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, $1x10^4$ matured DCs were co-cultured with $1x10^4$ 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, TNF-α, 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).

Murine DC and T cell polarization assay

Splenic $CD11c^+MHCII^+$ DCs and OT-II $CD4^+$ T cells were isolated by sorting from naive splenocytes. 5×10^5 Tg $CD4^+$ lymphocytes/ml were co-cultured with 2.5×10^5 syngeneic splenic DCs/ml and stimulated interchangeably with 100 μg/ml OVA. At d4, $CD3^+/CD4^+$ 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). and analyzed for intracellular IL-4/IFN-γ cytokine ratio.

Human T cell culture and determination of T cell polarization

To determine T cell polarization, 5 x 10^3 48 h-pulsed DCs were co-cultured with 2 x 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 (10 pg/ml; Sigma) in 96-well flat-bottom plates (Corning). On day 5, rhuIL-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).

TLR-transfected HEK cell activation

HEK-293-CD14, HEK-293-CD14/TLR2 and HEK-293-CD14/TLR4 cells were maintained in DMEM culture medium, supplemented with 10% FCS, 10 μg/ml ciprofloxacin and 5 μg/ml puromycin. For stimulation experiments, cells were seeded at 3.5 x 10^4 cells/well in 96-well flatbottom plates and were stimulated the next day. For stimulation of HEK-293-CD14/TLR4 cells, 12.5% supernatant of MD-2 transfected cells was added. IL-8 production was measured in supernatants after 22 hours using a commercial kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations.

DC-SIGN- and MR-expressing cell line

K562 cell line stably expressing DC-SIGN (a kind gift from K. Figdor [57]) or 3T3 cellline stably expressing human MR (a kind gift from G. Brown [58]) and their respective parental control cell lines 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 [^{35}S]-methionine (EasyTag Express Protein labeling mix, Perkin Elmer) in serum- and 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 1 h at 37 ˚C 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 [35]. 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 ribosomal RNA integrity in human DCs

mRNA was isolated from DCs conditioned by omega-1 for indicated time points using RNeasy mini Kit (Qiagen) according to the manufacturers recommendations. Integrity of rRNA was visualized and quantified using Agilent RNA 6000 Pico Kit in a 2100 Bioanalyzer (Agilent) according to the manufacturers recommendations.

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.

In vivo experiments

4get/KN2 [59] mice were bred and housed in the animal facility of the Trudeau Institute and used at 8-12 weeks of age. 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. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Mice were immunized s.c. into one hind footpad with SEA (20μg), omega-1 (3μg), in a volume of 50 ul and the draining popliteal lymph nodes were analyzed one week later.

In vitro restimulation of lymph node cells

1.5×10^6 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 day 4 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 two-sided paired Student's *t*-test or where indicated a two-sided unpaired Student's *t*-test. All p-values < 0.05 were considered significant.

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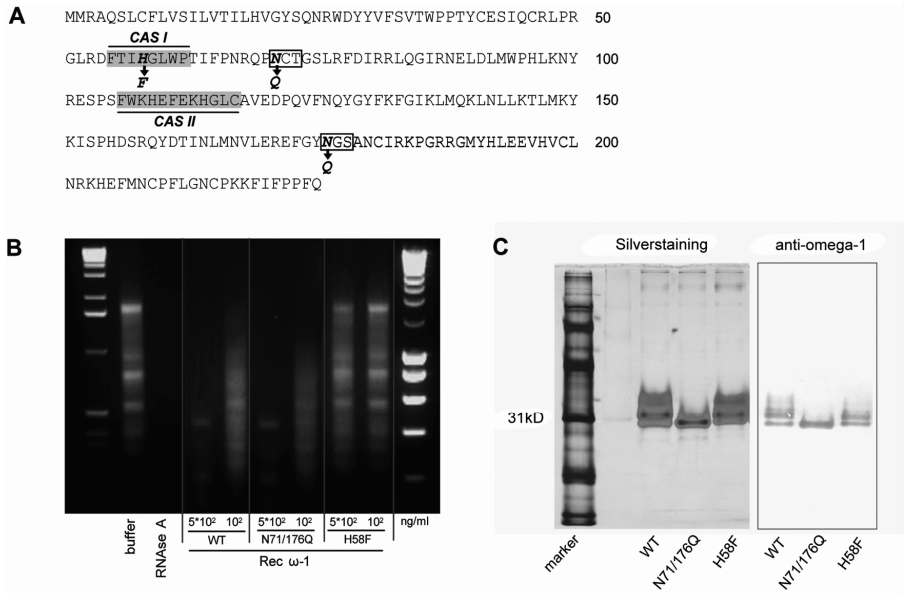
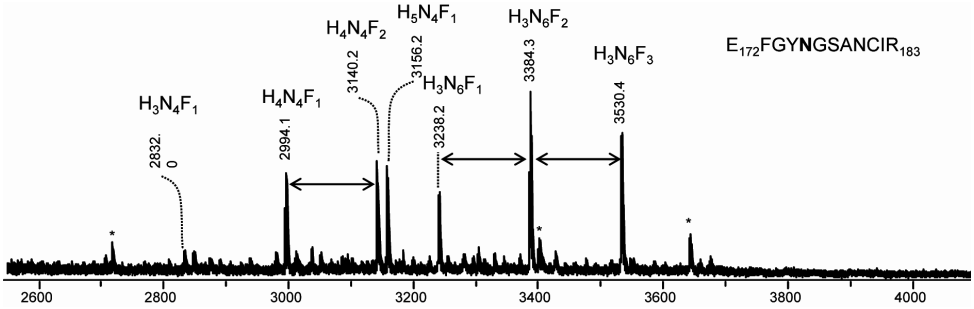
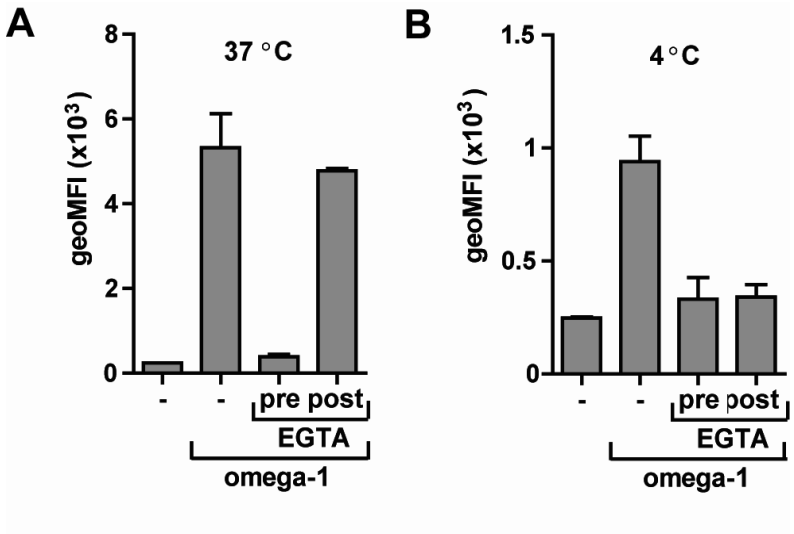
Supplementary figures



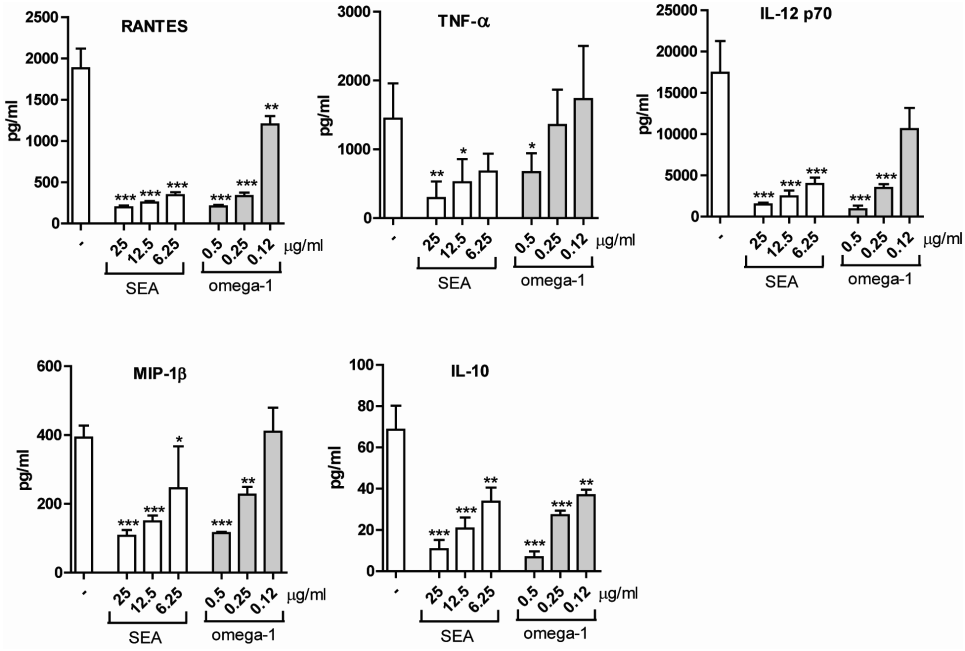
Supplementary figure 1. Generation and evaluation of glycosylation and RNase mutants of recombinant omega-1 (A) The amino acid sequence of omega-1 is shown in which the mutation sites are depicted. The two conserved amino acid sequence (CAS)-domains essential for catalytical activity are marked in grey 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 analyzed on a 2% agarosegel 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 HEK293 cells.



Supplementatry 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 cannot be assigned to glycopeptides are marked with asteriks (*). Based on the presence of the common N-glycan core structure (H_3N_2), the monosaccharide composition of the glycan species $H_3N_6F_2$ and $H_3N_6F_3$ indicates that these contain fucosylated antennae.



Supplementary figure 3. Validation of an assay for binding and uptake of antigens by DCs (A) DCs were incubated for 1 h with PF-647-labeled omega-1 at 37 °C and, where indicated, either preincubated with EGTA to prevent binding to CLRs or treated afterwards with EGTA to remove any potential antigen bound to CLRs on the cell surface. After 1h incubation at 37 °C, EGTA treatment afterwards did not remove any fluorescently-labeled omega-1 from the surface, given that the fluorescent intensity of the DCs was not affected, suggesting that all omega-1 is internalized by DCs, (B) As a control, the same assay was performed at 4 °C, a temperature that precludes receptor-mediated uptake, to show that EGTA treatment afterwards, can abolish surface binding of omega-1.



Supplementary 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 J558 cell-line, expressing CD40-L, to mimic the interaction with T cells. Concentrations of the stimuli are given in μg/ml. Bars represent mean ± SD of triplicate wells. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for values significantly different from the LPS control.