

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

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Omega-1, a glycosylated T2 ribonuclease (RNase) secreted by *Schistosoma mansoni* eggs and abundantly present in soluble egg antigen, has recently been shown to condition dendritic cells (DCs) to prime Th2 responses. However, the molecular mechanisms underlying this effect remain unknown. We show in this study 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 both ribosomal and messenger RNA. These experiments reveal an unrecognized pathway involving MR and interference with protein synthesis that conditions DCs for Th2 priming.

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Abbreviations used: CLR, C-type lectin receptor; Le^x, Lewis-x; moDC, monocyte-derived DC; MR, mannose receptor; mRNA, messenger RNA; rRNA, ribosomal RNA; SEA, soluble egg antigen; TLR, Toll-like receptor.

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 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 MHC-I and -II, up-regulation of co-stimulatory molecules, and production of polarizing cytokines, which collectively enable DCs to potently activate and direct CD4⁺ T cell responses (Kapsenberg, 2003).

This paradigm is largely based on observations of responses toward pathogens, like bacteria, viruses, and fungi. These pathogens harbor pathogen-associated molecular patterns that lead to classical DC activation by engaging several classes of innate pattern recognition receptors, including the Toll-like receptors (TLRs).

Binding of pathogen-associated molecular patterns to these receptors initiates signaling 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 (Akira et al., 2006). In contrast to this classical view of DC activation, components derived from parasitic helminths, when co-cultured with DCs, fail to induce the 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 (Carvalho et al., 2009).

Despite this consistent picture, the pathways through which helminth antigens manipulate

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DC function and drive Th2 responses are still poorly understood (MacDonald and Maizels, 2008). The majority of the studies have been conducted with a complex mixture of soluble egg antigens (SEAs) 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 (Carvalho et al., 2009; Phythian-Adams et al., 2010). 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 (Okano et al., 1999). In this respect, another class of pattern recognition receptors expressed by DCs, the carbohydrate-binding C-type lectin receptors (CLRs), has been suggested to play a role in modulation of DC function by SEA (van Liempt et al., 2007). For instance, SEA contains carbohydrate structures such as Lewis-x (Le^x) that can be recognized by DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin; van Die et al., 2003; van Liempt et al., 2006; Gringhuis et al., 2009). 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 (Bergman et al., 2004; Gringhuis

et al., 2009). In addition, more recently it has been shown that SEA can modulate cytokine responses through another CLR, dectin-2 (Ritter et al., 2010). Finally, multiple studies have raised the possibility that TLRs are involved in SEA-mediated Th2 induction (Thomas et al., 2003; Correale and Farez, 2009). However, direct evidence for involvement of specific receptors or downstream pathways in SEA-driven Th2 polarization has been missing.

The recent identification of omega-1, a glycosylated T2 RNase, as the major component in schistosome eggs that is responsible for conditioning DCs for Th2 polarization (Fitzsimmons et al., 2005; Everts et al., 2009; Steinfelder et al., 2009) has allowed us to dissect the molecular pathways involved in a precise manner. Through site-directed mutagenesis, we show that both the RNase activity and 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 translation by degrading ribosomal RNA (rRNA) and messenger RNA (mRNA) and thereby to condition these cells to prime Th2 responses.

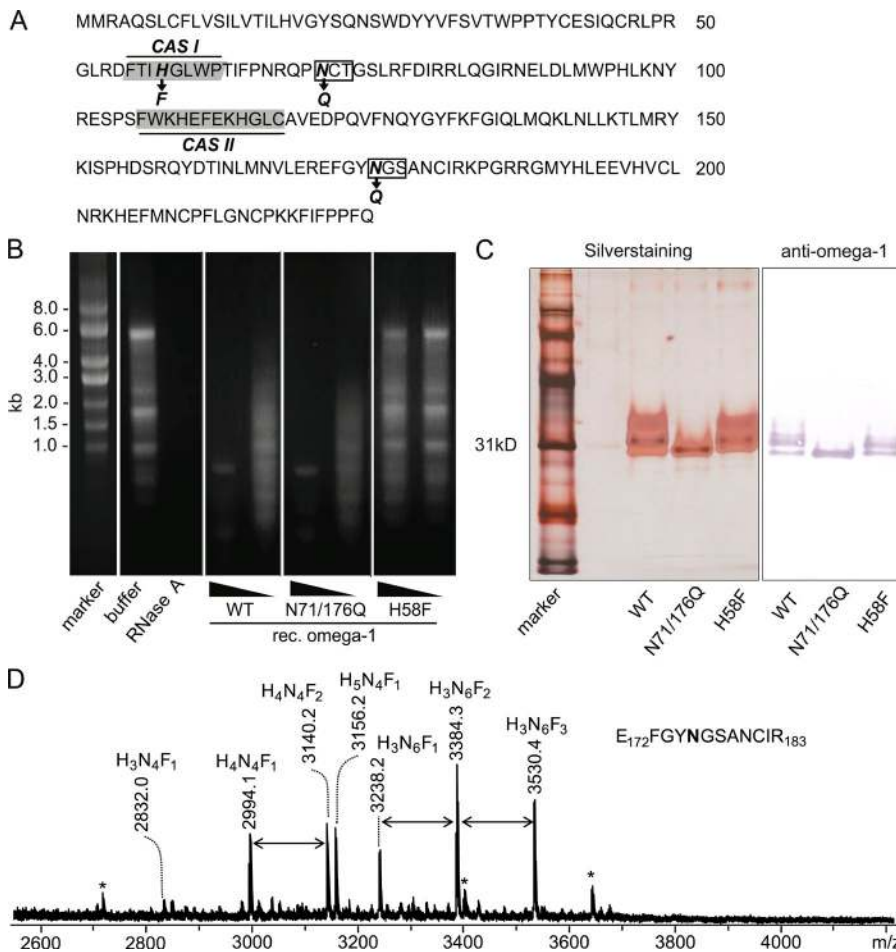


Figure 1. Generation and evaluation of glycosylation and RNase mutants of recombinant omega-1. (A) The amino acid sequence of omega-1 (NCBI Protein database accession no. ABB73003.1) is shown in which the mutation sites are depicted. The two conserved amino acid sequence (CAS) domains essential for catalytic activity are marked in gray, and the two N-linked glycosylation sites are boxed. (B) RNA from PBMCs was incubated for 1 h with the different omega-1 variants (500 and 100ng/ml) and analyzed on a 2% agarose gel for breakdown. RNase A was used as a positive control. One of two experiments is shown. (C) The omega-1 mutants were run under nonreducing conditions by SDS-PAGE and silver stained. A Western blot by staining with a specific anti-omega-1 monoclonal antibody was in line with the absence of glycosylation only on the omega-1 glycosylation mutant. (D) MALDI-TOF mass spectrum of glycopeptides from a tryptic digest of recombinant WT 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 asterisks.

RESULTS

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

The RNase activity of omega-1 has been proposed to play a role in the conditioning of DCs to prime Th2 responses (Steinfeldt et al., 2009). However, this was based on a chemical inactivation of the RNase activity by diethylpyrocarbonate treatment, which can result in off-target modification of histidines as well as other amino acids that could alter the function or structure of the protein (Wolf et al., 1970). Therefore, we addressed the role of RNase activity in a more stringent and specific manner by creating a mutant of recombinant 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 (Irie and Ohgi, 2001), was replaced by phenylalanine (omega-1 H58F; Fig. 1 A). Apart from RNase activity, glycosylation of omega-1 may also be important for its Th2-priming capacity because chemical modification of glycans on proteins present in SEA is known to abolish the ability of SEA to induce Th2 polarization (Okano et al., 1999). Moreover, potentially Th2-polarizing Le^x glycan motifs have recently been described to be present in glycans on omega-1 (Meevissen et al., 2010). 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 N-linked glycosylation sites (omega-1-N71/176Q; Fig. 1 A; Fitzsimmons et al., 2005; Meevissen et al., 2010).

An RNase assay showed that the RNase mutant did not have any RNase activity, whereas the RNase activity of the glycosylation mutant was unaffected (Fig. 1 B). In addition, the banding patterns of recombinant WT omega-1 and the mutants on silver-stained SDS-PAGE and anti-omega-1 Western blots were in line with the absence of carbohydrates on the glycosylation mutant as indicated by a single band instead of the three glycoforms of the recombinant WT omega-1 and the RNase mutant (Fig. 1 C). With regard to 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 Hex₃HexNAc₆Fuc_{2/3} (Fig. 1 D). This composition is indicative of the presence of GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) antennae, a glycan element previously found on a protein from HEK293 cells (Yan et al., 1993), the cell type in which recombinant omega-1 is expressed. LDN-F motifs frequently occur on helminth glycoproteins, and the characteristics of LDN-F with respect to binding to CLRs are similar to those of the Le^x element (van Die et al., 2003; van Liempt et al., 2006; Meevissen et al., 2010; Meevissen et al., 2011).

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 (moDCs) and naive CD4⁺ T cells was used, which mimics in vivo DC-mediated Th cell polarization (Kapsenberg, 2003). Similar to natural omega-1 (Everts et al., 2009), recombinant

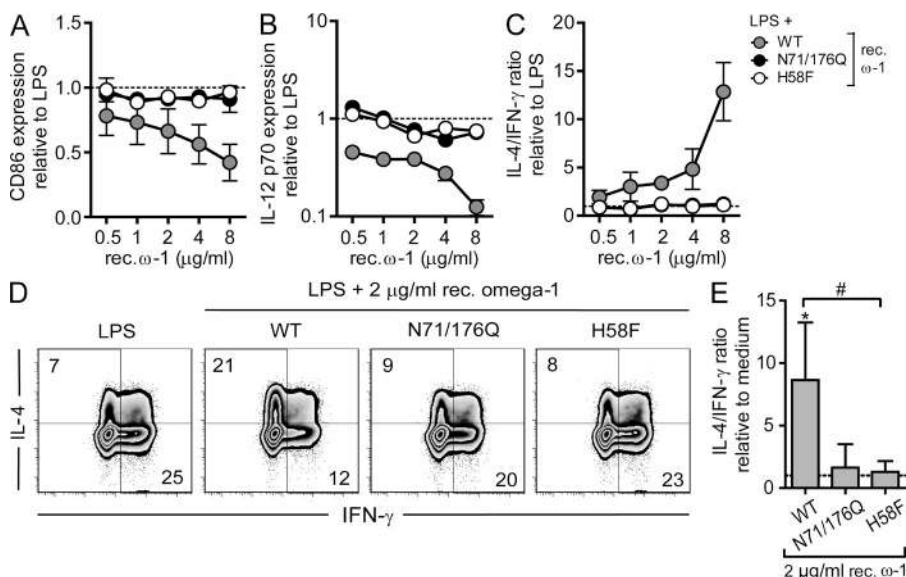


Figure 2. The glycosylation and RNase activity of omega-1 are essential for conditioning human DCs to prime Th2 responses.

(A) Human moDCs were pulsed for 48 h with increasing concentrations of the mutant variants of recombinant omega-1 (ω -1) in combination with 100 ng/ml LPS 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 1. Data are based on two independent experiments and shown as mean \pm SD. (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. Data are representative of triplicate wells from one of two independent experiments and shown as mean \pm SD. (C) DCs conditioned as described in A were cultured with

allogeneic naive CD4⁺ T cells for 12 d 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 PMA and ionomycin. 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. Data are based on two independent experiments and shown as mean \pm SD. (D) An example of T cell polarization assay as described in C induced by the different recombinant omega-1 variants. The frequencies of each population are indicated as percentages in the plot. One representative result from five independent experiments is shown. (E) T cell polarization assay as described in C but in the absence of LPS. Data are representative of three independent experiments. Bars represent mean \pm SD. * and #, $P < 0.05$, for significant differences compared with control conditions (*) or between test conditions (#) based on paired analysis (two-sided paired Student's t test). H58F, RNase mutant; N71/176Q, glycosylation mutant.

WT omega-1 consistently and significantly suppressed in a concentration-dependent manner the LPS-induced up-regulation of the co-stimulatory molecule CD86 (Fig. 2 A), as well as the production of IL-12 p70 after CD40 ligation (Fig. 2 B), which is an important characteristic of Th2-priming DCs (Carvalho et al., 2009). However, both the glycosylation mutant and the RNase mutant failed to alter LPS-induced CD86 expression or IL-12 production of DCs at any of the concentrations tested. Importantly, in contrast to DCs primed with recombinant WT omega-1, those conditioned with either mutant did not prime a Th2 response (Fig. 2, C and D). Similar results were obtained with cultures in which DCs were conditioned by the omega-1 mutants in the absence of LPS (Fig. 2 E). 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 (Mohrs et al., 2001). 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. After the s.c. injection of the antigens into the footpad, the draining popliteal 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 IL-4 production in vivo (Fig. 3). Importantly, although recombinant WT omega-1 induced a marked Th2 response and the production of IL-4, both mutants were significantly impaired to prime this response as evident from lower frequencies (Fig. 3, A and B) as well as total numbers of huCD2⁺ T cells (Fig. 3 C) in the draining LN. Collectively, 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 the 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 human DCs was dependent on the glycans present on omega-1. Although human DCs were capable of binding fluorescently labeled recombinant WT omega-1 or the RNase mutant as determined by FACS analysis, DCs failed to bind the glycosylation mutant, demonstrating that glycans present on omega-1 are essential for recognition by DCs (Fig. 4 A). Given the importance of glycosylation of omega-1 for binding to DCs, we explored the involvement of carbohydrate-binding CLRs in the recognition and uptake of omega-1. Although DCs readily bound fluorescently labeled omega-1, binding of natural

omega-1 was totally prevented when DCs were preincubated with the calcium-chelator EGTA, which abolishes CLR binding to carbohydrate ligands (Fig. 4 B). In contrast, treatment of DCs with EGTA after 1-h incubation with omega-1 could not reduce the fluorescent signal of omega-1, indicating that by then all bound omega-1 had been internalized. This suggests that DCs recognize and rapidly internalize omega-1 in a CLR-dependent manner. SEA has been reported to be recognized and endocytosed by human moDCs via the CLRs DC-SIGN and MR (van Liempt et al., 2007), which have the capacity to bind fucose residues such as those found in Le^x (Taylor et al., 1992; van Die et al., 2003; van Liempt et al., 2006), a glycan motif present on natural omega-1 (Meevissen et al., 2010). To determine whether MR and DC-SIGN are involved in recognition and internalization of natural omega-1, DCs were preincubated with

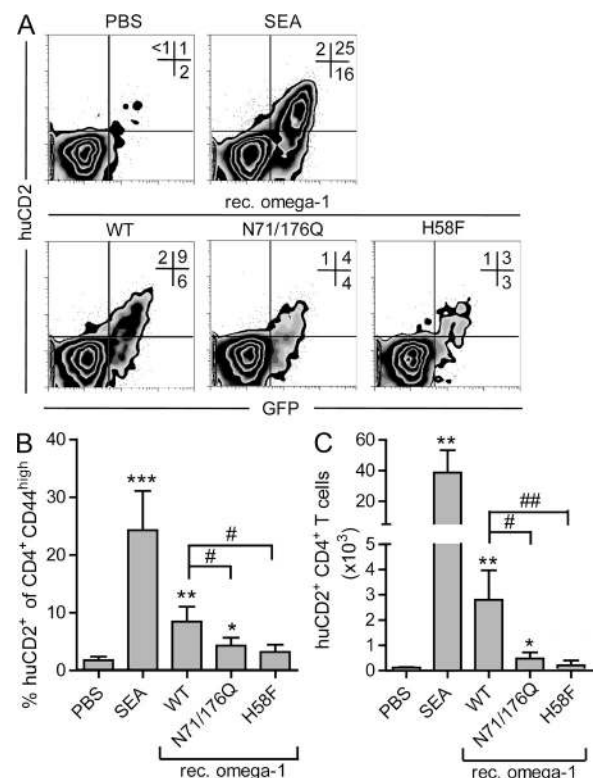


Figure 3. Glycosylation and RNase activity are essential for omega-1 to prime Th2 responses in vivo. 4get/KN2 IL-4 dual reporter mice were injected s.c. with 20 μ g SEA or 3 μ g WT or mutant recombinant omega-1 into the footpad. After 7 d, the frequency of GFP⁺ and huCD2⁺ within the CD4⁺CD44^{high} effector T cell population was determined by flow cytometry in the draining popliteal LNs. (A–C) Depicted are concatenated FACS plots (A), frequencies of huCD2⁺ within the CD4⁺CD44^{high} population (B), and total huCD2⁺ T cell numbers in draining LNs (C) of combined data of four mice per group. (A) The frequencies of each population are indicated as percentages in the plots. One of three independent experiments is shown. Bars represent mean \pm SD. * and #, $P < 0.05$; ** and ##, $P < 0.01$; ***, $P < 0.001$ for values significantly different from the PBS control (*) or between test conditions (#) based on two-sided t test. H58F, RNase mutant; N71/176Q, glycosylation mutant.

mannan (a natural ligand which competes for binding to DC-SIGN and MR) or DC-SIGN- and MR-specific blocking antibodies, followed by a 1-h incubation with fluorescently labeled SEA or natural omega-1. As reported previously (van Liempt et al., 2007), uptake of SEA by human moDCs could be reduced by mannan and either DC-SIGN or MR blocking antibodies in an additive manner (Fig. 4 C). With regard to omega-1, pretreatment with mannan could fully block binding and uptake of omega-1 by DCs. Interestingly, binding and uptake of natural omega-1 were significantly reduced by MR but not by DC-SIGN blocking antibodies (Fig. 4 C). Preincubation with the combination of both blocking antibodies did not have any additional effect on the uptake of omega-1 as compared with preincubation with anti-MR antibody alone. In addition, we found that recombinant omega-1 was recognized and internalized by DCs in a similar

MR-dependent fashion as natural omega-1 (Fig. 4 D). 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 labeled SEA was readily bound by both the DC-SIGN- and MR-expressing cells, which was not observed upon preincubation with EGTA or in parental 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 (Fig. 4 E) but not in the cell line expressing DC-SIGN (Fig. 4 F). It should be noted that in these uptake experiments (Fig. 4, C and D), blocking with anti-MR antibody was not complete ($\pm 40\%$ reduction). However, given that blocking the binding of omega-1 to the cell line selectively expressing MR with the anti-MR antibody was not complete either (not depicted),

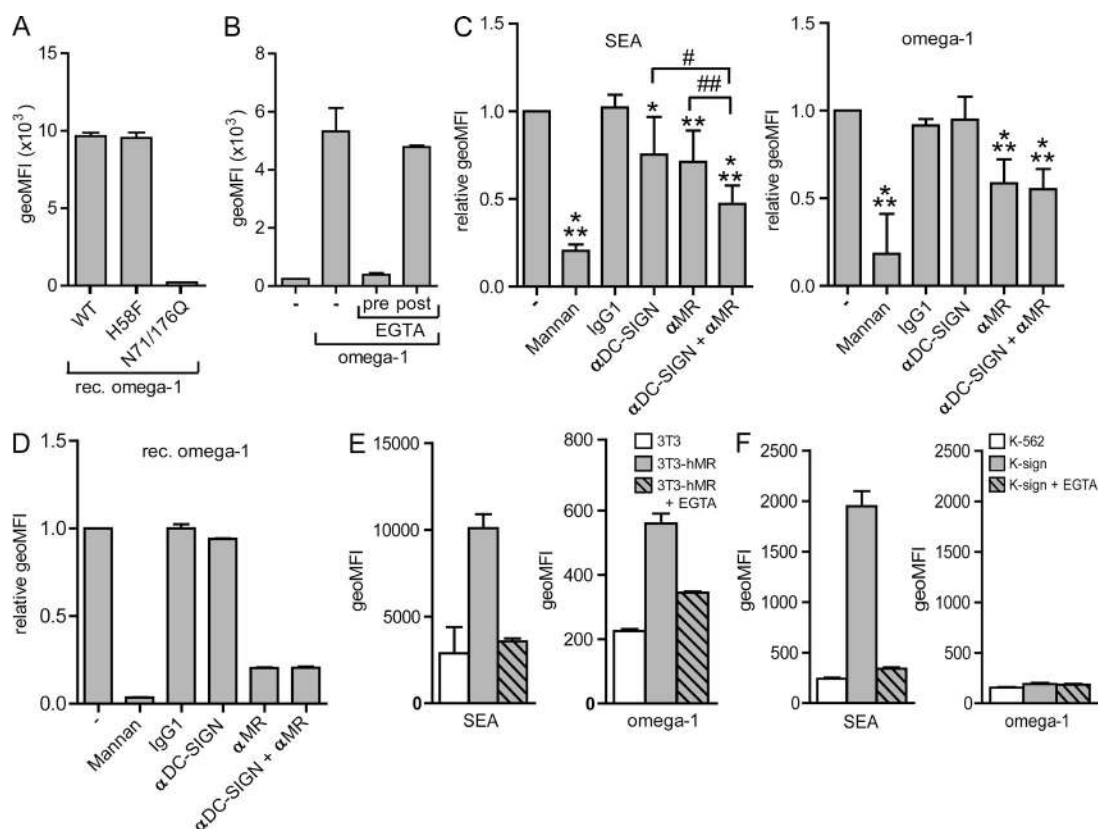


Figure 4. MR mediates recognition and internalization of omega-1 by human DCs. (A) Human moDCs were incubated for 1 h with PF-647-labeled recombinant WT omega-1, the glycosylation mutant, or the RNase mutant and analyzed for uptake of antigens by FACS analysis. One representative experiment with duplicate samples out of two experiments is shown. Bars represent mean \pm SD. (B) Human moDCs were incubated for 1 h with PF-647-labeled omega-1 and, where indicated, either preincubated (pre) with EGTA to prevent omega-1 binding to CLRs a priori or treated afterward with EGTA (post) to remove any CLR-bound omega-1 from the cell surface. One of two independent experiments is shown, and data represent mean \pm SD of duplicates. (C and D) A binding/internalization assay of natural (C) and recombinant omega-1 (D) by immature moDCs was performed analogous to B after preincubation with the indicated reagents. Binding and internalization are shown relative to control pretreatment. (C) Data are based on five experiments and are shown as mean \pm SD. (D) One of two independent experiments is shown, and data represent mean \pm SD of duplicates. (E and F) 3T3 cell line-expressing MR (E) and K-SIGN-expressing DC-SIGN (F) 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 two is shown. Bars represent mean \pm SD. * and #, $P < 0.05$; ** and ##, $P < 0.01$; ***, $P < 0.001$ for significant differences compared with control conditions (*) or between test conditions (#) based on two-sided Student's *t* test. H58F, RNase mutant; MFI, mean fluorescence intensity; N71/176Q, glycosylation mutant.

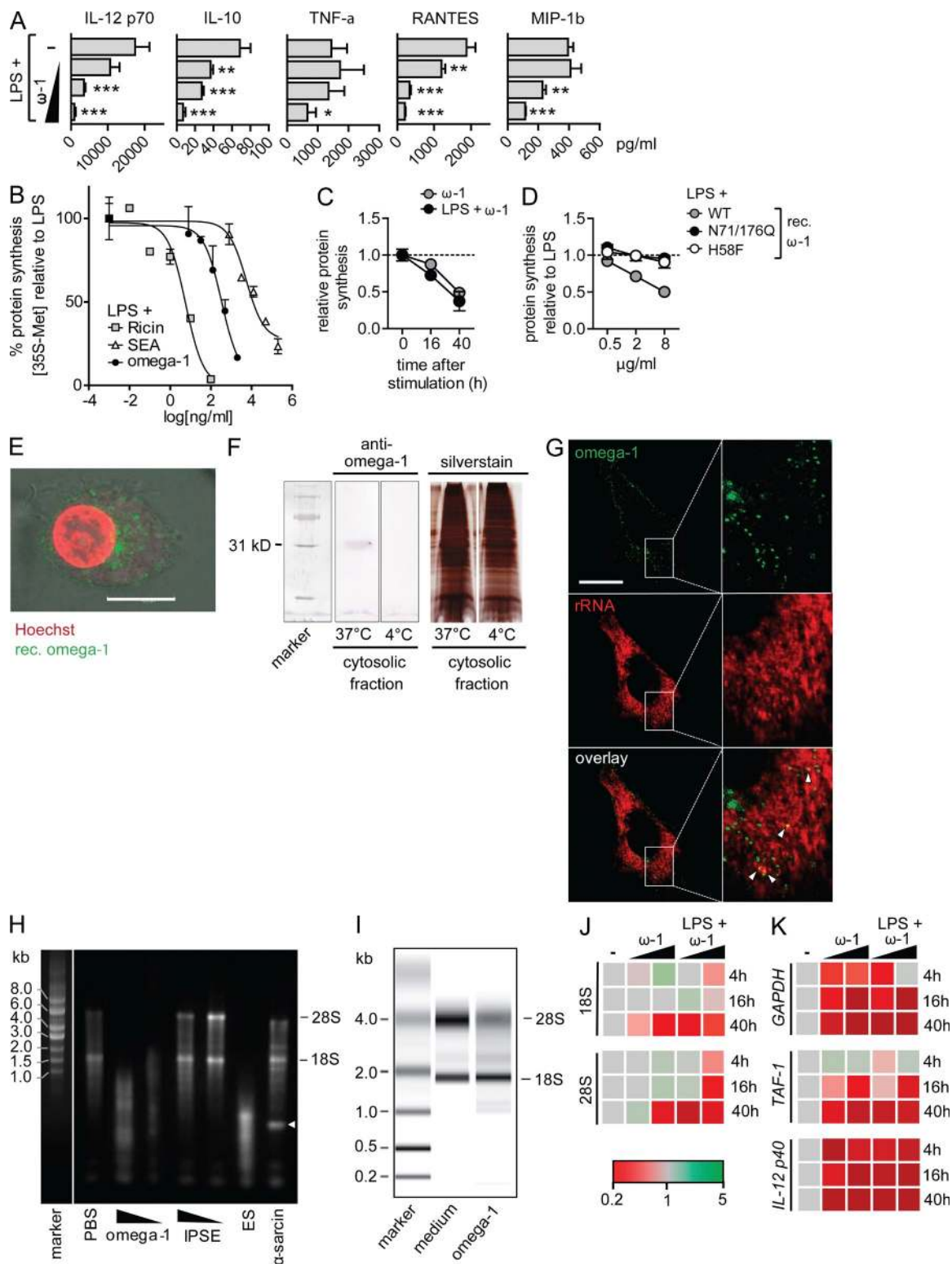


Figure 5. Omega-1 suppresses protein synthesis through breakdown of rRNA and mRNA. (A) After human moDCs had been pulsed for 40 h with 125, 250, and 500 ng/ml omega-1 (ω -1) in combination with 100 ng/ml LPS, the cells were co-cultured for 24 h with the J558 cell line, expressing CD40-L, to mimic the interaction with T cells. Bars represent mean \pm SD of triplicate wells of one of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for values significantly different from the LPS control. (B) After 16-h incubation of human DCs with a concentration range of indicated reagents in the presence of 100 ng/ml LPS, protein synthesis was assessed after a 2-h pulse with radioactively labeled methionine. Ricin, as potent inhibitor of protein synthesis, was taken along as positive control (Montanaro et al., 1973). One of two experiments is shown. Data points represent mean \pm SD of duplicates.

it is likely that a low affinity of the anti-MR antibody accounts for this finding rather than that other receptors are involved. Collectively, our data show that recognition and internalization of omega-1 by human DCs are dependent on its glycosylation and that MR is a major CLR involved in this process.

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 human 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 (Everts et al., 2009), but also to secrete other cytokines and chemokines (Fig. 5 A). This suggested that the suppression may not be gene specific but could be the result of general inhibition of protein synthesis. Indeed, after 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 (Fig. 5 B; Montanaro et al., 1973). In addition, this inhibition by omega-1 was time dependent and observed in both the presence and absence of LPS (Fig. 5 C). The capacity to inhibit protein synthesis was dependent on its RNase activity and uptake via its glycans because the RNase as well as the glycosylation mutant failed to interfere with protein synthesis (Fig. 5 D). As several fungal ribonucleolytic proteins, so-called ribotoxins, have been described to inhibit protein synthesis through cleavage of rRNA after translocation into the cytosol (Lacadena et al., 2007), we first evaluated the localization of omega-1 in human DCs after uptake. We found that omega-1 was efficiently internalized by DCs and present throughout the cell after 1 h (Fig. 5 E and Video 1). In addition, Western blots revealed the presence of omega-1 in the cytosolic fraction of omega-1-stimulated

DCs after 3 h (Fig. 5 F). In line with this, colocalization experiments using immunofluorescence confocal microscopy by staining for rRNA showed that 2 h after stimulation of DCs with omega-1, omega-1 partially colocalized with rRNA (Fig. 5 G). We next 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, whereas IPSE, another *S. mansoni* egg-derived protein which lacks RNase activity but has identical glycans as omega-1 (Wuhrer et al., 2006), did not induce any rRNA degradation (Fig. 5 H), indicating that omega-1 is able to interfere with ribosomal function by cleavage of rRNA. Analysis of the integrity of rRNA isolated from omega-1-exposed DCs by gel electrophoresis showed a preferential breakdown of 28S rRNA (Fig. 5 I). This was confirmed by real-time PCR, which additionally revealed breakdown of 18S at later time points (Fig. 5 J). Because the known T2 RNases have no sequence-specific RNase activity (Irie and Ohgi, 2001), we evaluated whether omega-1, which belongs to the T2 RNase family, may additionally impair protein synthesis by degrading not only rRNA but also mRNA transcripts in a generic manner. When DCs were stimulated with omega-1, both in the presence or absence of LPS, a concentration- and time-dependent loss in mRNA transcripts of housekeeping genes *TAF-1* and *GAPDH* as well as *IL-12* p40 could be observed (Fig. 5 K), suggesting that omega-1 targets mRNA transcripts in a general manner in DCs as well. Collectively, these data support the notion that the RNase activity enables omega-1 to modulate human DC function by interfering with protein synthesis through cleavage of rRNA and mRNA after translocation into the cytosol.

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

(C) As described in B, protein synthesis by human DCs was followed over time after exposure to 500 ng/ml omega-1 either in the presence or absence of 100 ng/ml LPS. Data are shown relative to unstimulated or LPS-stimulated controls as depicted by the dotted line. Data are representative of two independent experiments and are depicted as mean \pm SD. (D) Protein synthesis by human moDCs after exposure to increasing concentrations of the recombinant omega-1 variants was assessed as described in B. Data are shown relative to LPS-stimulated DCs. Data are representative of two independent experiments and are depicted as mean \pm SD. (E) DCs were stimulated with FITC-labeled recombinant omega-1 for 1 h, and uptake was visualized by confocal laser-scanning microscopy. Nuclei were stained with Hoechst. One of three experiments is shown. See Video 1 for z-stacked images. (F) Cytosolic fractions of human DCs stimulated for 3 h with omega-1 were run under nonreducing conditions by SDS-PAGE and analyzed by Western blot for the presence of omega-1 or silver stained to control for input. DCs incubated at 4°C were taken along as controls as these cells have surface-bound but not internalized omega-1. One of two experiments is shown. (G) Human DCs were stimulated with 1 μ g/ml FITC-labeled omega-1 and after 2 h fixed and stained for rRNA. Subcellular localization of omega-1 was determined by confocal microscopy. One representative cell from three independent experiments is shown. Arrowheads depict areas where rRNA and omega-1 colocalize (yellow). Bars, 10 μ m. (H) After rabbit reticulocyte lysate containing functional ribosomes was incubated for 1 h with 1 and 5 μ g/ml omega-1, 1 and 5 μ g/ml IPSE as negative control, or 25 μ g/ml ES (schistosome egg excretory/secretory products), containing omega-1, isolated rRNA 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 (arrowhead; Kao et al., 2001). One of three independent experiments is shown. (I) rRNA was isolated from 24-h omega-1-stimulated human DCs and was visualized by running a laboratory-on-a-chip picogel. One of three experiments is shown. (J and K) rRNA or mRNA expression of the indicated genes in DCs was assessed by real-time quantitative PCR at different time points after stimulation with 500 ng/ml and 2 μ g/ml omega-1 in the presence or absence of 100 ng/ml LPS. Data are shown relative to unstimulated or LPS-stimulated controls, which were set to 1. RNA expression was normalized based on a genomic real-time quantitative PCR for *ccr5*. Data represent the mean of three independent experiments. H58F, RNase mutant; N71/176Q, glycosylation mutant.

omega-1, we used blocking antibodies directed against MR or DC-SIGN. Blocking of MR during the stimulation of human DCs with omega-1 significantly prevented the inhibition of protein synthesis (Fig. 6 A), whereas 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 (Fig. 6 B) and IL-12 production after CD40 ligation (Fig. 6 C) or to condition DCs to induce a Th2 response (Fig. 6 D). The importance of MR was further substantiated by the observations that in contrast to their WT counterparts, MR^{-/-} murine DCs, when conditioned with omega-1, failed to prime a Th2-skewed allogeneic T cell response in vitro (Fig. 6 E). 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 was injected s.c. into the footpad of WT and MR^{-/-} mice. After 7 d, 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 indicated 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^{-/-} (Fig. 7 A). Furthermore, intracellular staining for IFN-γ and IL-4 after antigen-specific restimulation of CD4⁺ T cells from omega-1-primed LNs showed a significant increase in IL-4-producing T cells from WT but not MR^{-/-}

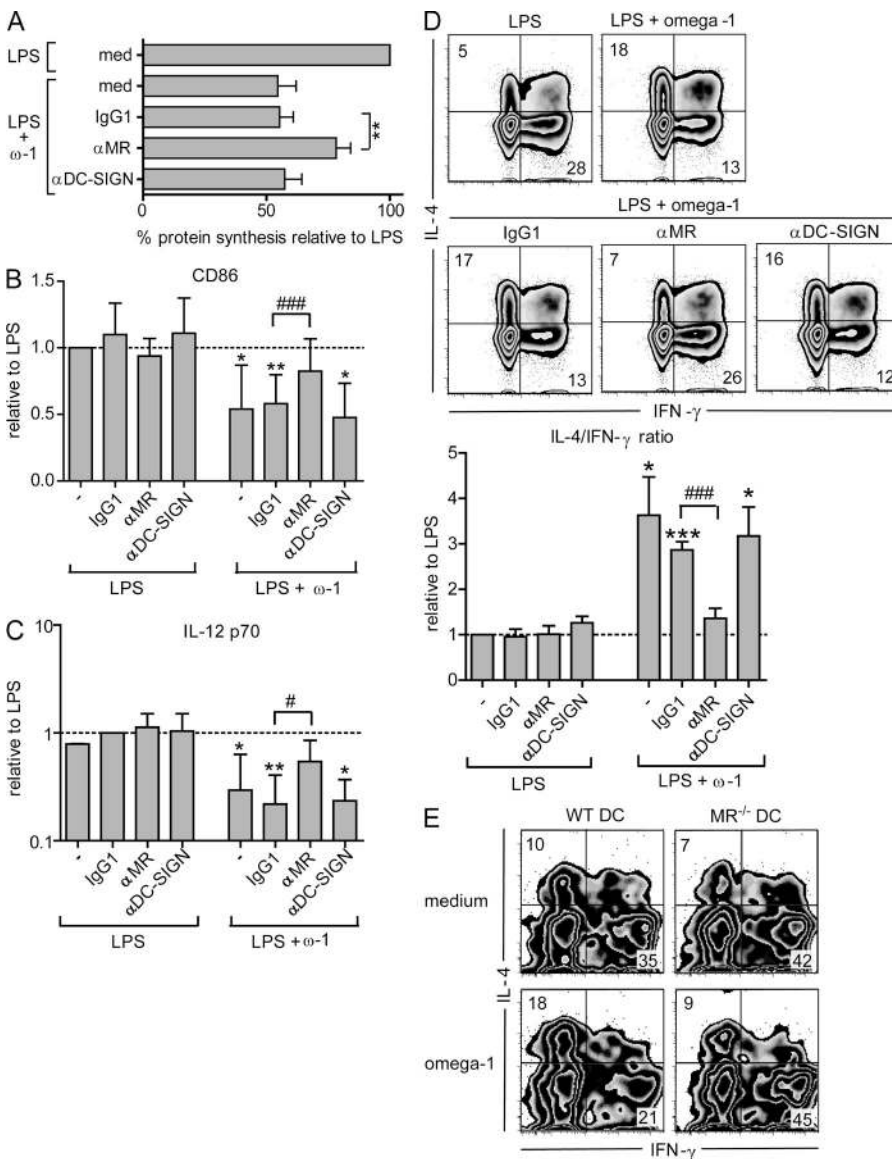


Figure 6. MR mediates omega-1-induced DC modulation and Th2 polarization in vitro. After 1-h preincubation with blocking antibodies against MR, DC-SIGN, or an isotype control (20 μg/ml), human moDCs were pulsed for 16 (A) or 48 h (B–D) with 500 ng/ml natural omega-1 (ω-1) in combination with 100 ng/ml LPS. (A) Protein synthesis was assessed as described in Fig. 5 B. One representative experiment based on duplicate samples out of three experiments is shown. Data are shown as mean ± SD. (B) The expression levels of CD86 on human DCs assessed by FACS are based on geometric mean fluorescence, relative to the DCs stimulated with LPS alone, which is set to 1 (dashed line). Data are based on three independent experiments and shown as mean ± SD. (C) Conditioned human 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 three independent experiments and shown as mean ± SD. (D) Conditioned human moDCs were cultured with allogeneic naive CD4⁺ T cells for 12 d in the presence of staphylococcal enterotoxin B and IL-2, and T cell polarization was analyzed as described in Fig. 1. FACS plots of one representative experiment out of six are shown. Bar graphs are based on six independent experiments and represent mean ± SD. (E) Murine splenic WT or MR^{-/-} DCs from a C57BL/6 background were co-cultured with naive BALB/c CD4⁺ T cells in the presence 2 μg/ml omega-1. After an expansion with rIL-2 at day 3, T cells were restimulated on day 6 with PMA and ionomycin and analyzed for intracellular cytokines. One representative experiment out of three is shown. * and #, P < 0.05; **, P < 0.01; *** and ###, P < 0.001 for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (two-sided paired Student's t test).

mice (Fig. 7 B). The failure of $MR^{-/-}$ mice to prime a Th2-polarized response to omega-1 was not caused by a general failure of $MR^{-/-}$ cells to produce these cytokines as the responses to PHA were comparable in WT and $MR^{-/-}$ mice (Fig. 7, A and B). Collectively, these data show that MR is essential for priming of Th2 responses by omega-1 in vivo.

DISCUSSION

Using omega-1, a single glycosylated T2 RNase secreted by *S. mansoni* eggs, we studied the molecular mechanisms involved in conditioning DCs to induce Th2 responses. By generating mutants of omega-1, we could show that both the glycosylation and the 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

(Frison et al., 2003) and omega-1 harbors fucose-containing Le^x -glycan moieties (Meevisen et al., 2010), we observed that omega-1 significantly bound only to an MR- but not to a DC-SIGN-expressing cell line and that internalization by DCs was mainly MR dependent and did not involve DC-SIGN. Lack of strong binding and uptake of omega-1 by DC-SIGN might be explained by the fact that in most DC-SIGN binding studies polyvalent Le^x -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 would present Le^x at a low valency (Meevisen et al., 2011). In line with this observation, DC-SIGN blocking experiments suggest that interaction with DC-SIGN does not play a major role in omega-1-driven Th2 polarization via DCs. In contrast, the importance of MR in recognition and uptake of omega-1 was substantiated by the finding that conditioning of both human and murine DCs for Th2 polarization by omega-1 were significantly impaired when MR was blocked or when the DCs were deficient

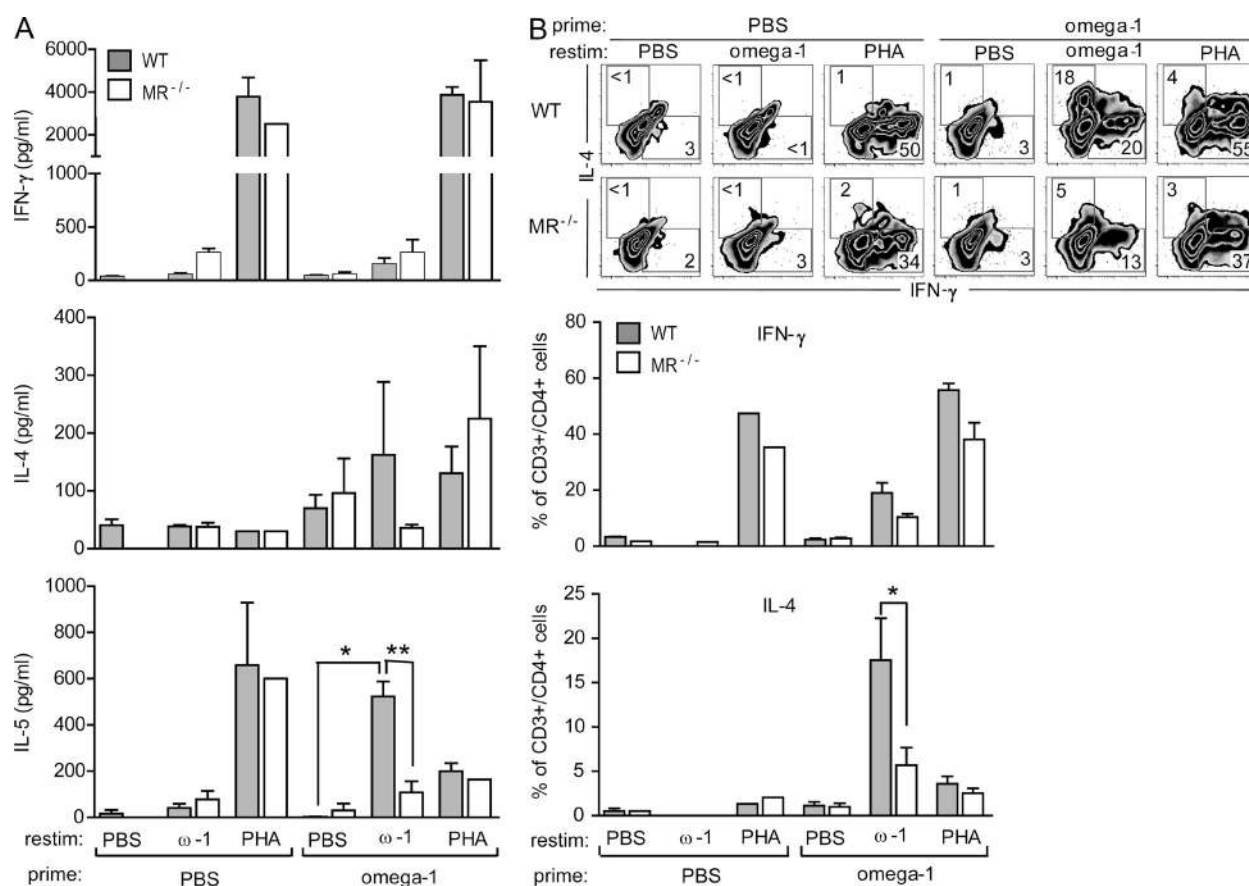


Figure 7. MR is essential for omega-1-driven Th2 polarization in vivo. $MR^{-/-}$ and WT Bl/6 mice were injected s.c. with omega-1 (ω -1; 2 μ g in 30 μ l PBS) or PBS into the footpad. (A) After 7 d, the cells from the draining LNs were restimulated in vitro for 4 d with PBS, 2 μ g/ml omega-1, or 10 μ g/ml PHA, 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. FACS plots show concatenated data from four mice. The bar graphs represent the percentage of T cells single-positive for either IL-4 or IFN- γ . One of two independent experiments is shown. Data are means \pm SEM of four 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 Student's *t* test).

for MR, respectively. Furthermore, we confirmed and extended the importance of MR in Th2 polarization by omega-1 in vivo by showing that an antigen-specific Th2 response induced in MR^{-/-} mice after footpad injection of omega-1 was strongly reduced compared with the response elicited in WT mice. In this respect, it is important to note that human and murine MRs have a similar carbohydrate-binding specificity (East and Isacke, 2002). Thus, this establishes that omega-1 relies on MR to drive Th2 polarization. Apart from schistosome egg-derived antigens, it was recently shown that MR can also recognize glycosylated antigens derived from schistosome larvae in the skin and that MR^{-/-} mice display a Th1-biased antigen-specific T cell response in the skin-draining LNs after infection with cercaria (Paveley et al., 2011). This study along with our data indicates that MR may play a role in shaping of Th2-polarized immune responses during different stages of schistosome infection.

In vitro studies with DCs have shown that MR cross-linking with antibodies (Chieppa et al., 2003) or by mannosylated antigens (Yamamoto et al., 1997; Op den Brouw et al., 2009) can drive an antiinflammatory cytokine program in DCs away from a Th1-promoting profile (Chieppa et al., 2003) and that allergen-driven Th2 polarization by DCs is in part dependent on MR (Li et al., 2010; Royer et al., 2010). These studies suggest that engagement of MR may be sufficient to promote Th2 polarization, potentially via signaling events. However, our data demonstrate that MR binding alone is not sufficient for Th2 induction by omega-1 because glycans present on omega-1, in the absence of RNase activity, fail to program DCs to induce Th2 responses. This is in line with the observation that IPSE/α-1, another major glycoprotein secreted by *S. mansoni* eggs with identical glycosylation as omega-1 (Wuhrer et al., 2006), which can bind the cell line expressing MR (unpublished data) but lacks RNase activity, is unable to prime Th2 responses (Everts et al., 2009).

Apart from its glycosylation, omega-1 requires its RNase activity to induce a Th2 response via modulation of human DCs. It was observed that omega-1 in an RNase-dependent manner impaired protein synthesis and that DCs exposed to omega-1 displayed a progressive reduction in mRNA content of several unrelated genes as well as in rRNA levels. The drop in mRNA transcripts from both housekeeping genes (*taf1* and *gapdh*) and inducible genes (*il12b* p40), as well as rRNA (28S and 18S), suggests that omega-1 does not degrade specific transcripts but targets the global RNA pool in DCs. Although it currently remains to be determined what the relative contribution of each of these processes and their relative timing is to the impairment of protein synthesis, it is most likely that the observed inhibition in protein synthesis is a combined effect of degradation of mRNA transcripts and interference with ribosomal integrity caused by rRNA cleavage. These data support the view that as a consequence of RNA breakdown, reduced protein synthesis 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. The major birch pollen allergen, Bet v 1 (Bufe et al., 1996), was identified as an RNase. Furthermore, some fungal RNases that appear to selectively cleave rRNA, such as mitogillin and Asp f 1, are known to be allergens (Kao et al., 2001). Interestingly, for Asp f 1 it was found that its allergenicity was lost when its capacity to interfere with ribosomal function was abolished (García-Ortega et al., 2005). In addition, a study has linked an endogenous RNase, the eosinophil-derived neurotoxin, to DC-mediated Th2 polarization (Yang et al., 2008). 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. In this respect, it is interesting to note that RNase T2 homologues can be identified in the genomes of *Schistosoma japonicum* and *Schistosoma haematobium*, as well as of the nematodes *Brugia malayi*, *Loa loa*, and *Ascaris suum* (Hillwig et al., 2009; Zhou et al., 2009; Young et al., 2012). However, it is currently unknown whether these parasites actually express these T2 RNases and if they do, whether they play any role in Th2 polarization in their host. Collectively, our data suggest that for an RNase to harbor a Th2-priming capacity, it needs to be recognized by DCs and routed to reach the cytosol where in turn its enzymatic activity would result in suppression of protein synthesis, yet without shutting down DC function altogether or inducing cell death before T cell priming has occurred.

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 (Sandvig and van Deurs, 2005). In this respect, because omega-1 is internalized via MR, it is interesting to note that cross-presentation of OVA by DCs, a process which requires translocation of the antigen from endosomes into the cytosol, has been shown to be dependent on MR (Burgdorf et al., 2007, 2008). Mechanistically, it was demonstrated that binding of the MR to OVA leads to polyubiquitination of MR, resulting in the recruitment of the ATPase p97, a member of the ER-associated degradation machinery, toward the endosomal membrane. p97 in turn was found to provide the energy to pull out the MR ligand into the cytoplasm (Zehner et al., 2011). 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.

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 (Everts et al., 2009; Steinfeldt et al., 2009). 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 (Steinfeldt et al., 2009) because halting of translation and concomitant stress responses can affect actin rearrangements and thereby cell morphology (Pelling et al., 2009). Importantly, during interactions with naive 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, a situation which is known to favor the induction of Th2 responses (Oswald et al., 1994; Constant et al., 1995). This mechanism would be different from a default hypothesis for Th2 induction (Wang et al., 1994; Yates et al., 2000) 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 up-regulation of surface MHC-II and CD86 or expression of IL-12 in response to CD40 ligation (Marshall and Pearce, 2008; Everts et al., 2009), as well as with the observation that omega-1-primed DCs have a reduced capacity to form T cell-DC conjugates (Steinfeldt et al., 2009).

Collectively, 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 the MR. Subsequently, after 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 vaccines against parasitic worm infections or approaches to counterbalance unwanted Th1 responses in hyperinflammatory diseases (Jäger and Kuchroo, 2010; Ricardo-Gonzalez et al., 2010).

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 (de Jong et al., 2002; Everts et al., 2009). The purity of the preparations was controlled by SDS-PAGE and silver staining. Protein concentrations were tested using the Bradford or BCA procedure (Woodward et al., 1985; Hamilton et al., 1999).

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 homologous T2 RNases (Irie and Ohgi, 2001; Fitzsimmons et al., 2005), respectively. H58F and N71/176Q mutants were created by PCR using mutagenic primers on a DH5alpha/pProExHtb plasmid (Invitrogen) containing the WT omega-1 sequence (NCBI Protein database accession no. ABB73003.1). Successful mutation was confirmed by DNA sequencing. Subsequently, using restriction enzymes SfiI and ApaI, the templates for WT and mutant omega-1 were subcloned into a pSecTag2 plasmid (Invitrogen) for stable transfection into HEK cells (Everts et al., 2009). 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 (Everts et al., 2009).

Human DC culture, stimulation, and analysis. Monocytes were isolated from venous blood from healthy volunteers according to protocols approved by the Institutional Review Board of Leiden University Medical Center by density centrifugation on Ficoll followed by CD14⁺ MACS isolation (Miltenyi Biotec) or a Percoll gradient as described previously (Sallusto and Lanzavecchia, 1994) and were cultured in RPMI medium supplemented with 10% FCS, 50 ng/ml human rGM-CSF (Invitrogen), and 25 U/ml human rIL-4 (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 100 ng/ml of ultrapure LPS (*Escherichia coli* 0111 B4 strain; InvivoGen). For CLR blocking, the indicated cells were preincubated 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 1,000 U/ml IFN-γ. After 48 h, DCs were harvested for co-culture with naive T cells. In addition, 10⁴ matured DCs were co-cultured with 10⁴ CD40L-expressing J558 cells for 24 h to determine cytokine production by the DCs after 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 BD). Concentrations of IL-10, TNF, MIP-1β, and RANTES were determined by a multiplex LUMINEX assay according to the manufacturer's instruction (InvivoGen). The expression of CD86 on pulsed DCs was determined by FACS (FACSCanto) through staining with CD86-FITC (BD).

Murine T cell polarization assay. Splenic CD11c⁺MHCII⁺ DCs and CD62L⁺CD4⁺ T cells were isolated by sorting from naive splenocytes derived from C57BL/6 and BALB/c mice, respectively. 2.5 × 10⁵ CD4⁺ T cells were co-cultured with 1.25 × 10⁵ splenic DCs and stimulated with 2 µg/ml omega-1. At day 3, T cells were expanded with 30 U/ml rIL-2 (R&D Systems) and at day 6 restimulated with 50 ng/ml PMA plus 2 µg/ml ionomycin for 5 h. 10 µg/ml brefeldin A was added during the last 2 h (all Sigma-Aldrich). The cells were stained with a combination of IL-4-PE and IFN-γ-APC antibodies (BD).

Human T cell culture and determination of T cell polarization. To determine T cell polarization, 5 × 10³ 48-h-pulsed DCs were co-cultured with 2 × 10⁴ naive T cells that were purified using a human CD4⁺/CD45RO⁻ column kit (R&D Systems) in the presence of 10 pg/ml staphylococcal enterotoxin B (Sigma-Aldrich) in 96-well flat-bottom plates (Corning). On day 5, 10 U/ml rhuIL-2 (R&D Systems) was added, and the cultures were expanded for another 7 d. For intracellular cytokine production, the primed CD4⁺ T cells were restimulated with 50 ng/ml PMA plus 2 µg/ml ionomycin for 6 h. 10 µg/ml brefeldin A was added during the last 2 h (all Sigma-Aldrich). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies.

DC-SIGN- and MR-expressing cell line. K562 cell line stably expressing DC-SIGN (a gift from S.I. Buschow and K. Figdor, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands; Geijtenbeek et al., 2000) or 3T3 cell line stably expressing human MR (a gift from J.L. Miller [University of Oxford, Oxford, England, UK] and G. Brown [University of Aberdeen,

Aberdeen, Scotland, UK]; Miller et al., 2008) and their respective parental control cell lines were seeded overnight in a 96-well plate at 10,000 cells/well. Where indicated, cells were preincubated with 10 mM EGTA for 30 min at 37°C. Subsequently, cells were incubated with 2 µg/ml PF-647-labeled SEA or 500 ng/ml PF-647-labeled omega-1 at 37°C for 1 h and washed in ice-cold PBS before analysis using flow cytometry.

Protein synthesis inhibition. Immature DCs were seeded overnight in 96-well flat-bottom plates before stimulation with the indicated reagents in the presence of LPS. At the indicated time points after stimulation, protein synthesis was determined by a 2-h pulse at 37°C with 3 µCi/0.05 ml [³⁵S]methionine (EasyTag Express Protein labeling mix; PerkinElmer) in serum-, cysteine-, and L-methionine-free RPMI 1640. After a double washing step in PBS, cells were lysed for 5 min in AV lysis buffer (20 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.5% DOC, 1.0% NP-40, and 0.1% SDS) in the presence of 200 µg/ml of protease inhibitors leupeptin and aprotinin. Lysates were transferred on a filter (PerkinElmer) and dried. After radioactive-labeled proteins were precipitated on the filter with trichloroacetic acid, filters were washed with 96% ethanol and dried. Using a liquid scintillation cocktail for aqueous solution, the radioactivity present on the filters was measured in a β-counter.

RNase activity assay. RNA was extracted from PBMCs using the RNeasy kit (QIAGEN). RNA was incubated for 1 h at 37°C with the indicated antigens in 0.01 M Tris 0.02% Cu. Subsequently, RNA breakdown was visualized by running the samples on a 2% agarose gel containing ethidium bromide.

rRNA breakdown. Rabbit reticulocyte lysate (Promega) was incubated with antigens as described previously (Kao et al., 2001). In brief, after 1-h incubation at 37°C in Tris-HCl (15 mM NaCl, 50 mM KCl, and 2.5 mM EDTA), the reaction was stopped with 10% SDS, and RNA was extracted from the ribosomes with phenol/chloroform. Next, isolated rRNA was denatured at 95°C and visualized by running the samples on a 2% agarose gel containing ethidium bromide.

Analysis of rRNA integrity in human DCs. mRNA was isolated from DCs conditioned by omega-1 for the indicated time points using an RNeasy mini kit (QIAGEN) according to the manufacturer's recommendations. Integrity of rRNA was visualized using an RNA 6000 Pico kit in a 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's recommendations.

RNA and DNA isolation, DNase treatment, and cDNA synthesis. Total DNA and RNA were simultaneously extracted using a DNeasy blood and tissue kit (QIAGEN) as per the manufacturer's instruction, except for that no RNase reaction was performed. DNase treatment was performed using RQ1 RNase-Free DNase (Promega) as per the manufacturer's instruction. cDNA synthesis was performed according to standard procedures.

Analysis of gene expression levels. Primers and TaqMan probes were provided as a TaqMan gene expression kit (Applied Biosystems) or designed using Primer Express (Applied Biosystems) and synthesized by Biogio. Primers for DNA PCR for CCR5 were a gift from E. Boon (Leiden University Medical Center, Leiden, Netherlands; Boon et al., 2007). Real-time quantitative PCR was performed using the ABI PRISM 7700 Sequence Detection System (SDS; Applied Biosystems). mRNA expression levels were normalized based on DNA input as determined by CCR5 PCR. Data were visualized as heat maps using Tableau software.

Antigen uptake by human DCs. SEA and omega-1 were fluorescently labeled with PF-647 using the Promofluor labeling kit (Promokine) according to the manufacturer's recommendations. 10,000 immature DCs/well were seeded in a 96-well plate. Where indicated, cells were preincubated with 10 mM EGTA, 100 µg/ml Mannan (Sigma-Aldrich), 20 µg/ml anti-DC-SIGN (clone AZN-D1), or 20 µg/ml anti-MR (clone 15.2) for 60 min

at 37°C. Subsequently, cells were incubated with 2 µg/ml PF-647-labeled SEA or 500 ng/ml PF-647-labeled omega-1 at 37°C for 1 h and washed in ice-cold PBS before analysis using flow cytometry.

Confocal microscopy. For live cell imaging, purified HEK-omega-1 was fluorescently labeled with NHS (*N*-hydroxysuccinimide)-fluorescein (Thermo Fisher Scientific) according to the manufacturer's instructions. After protein labeling, nonreacted NHS-fluorescein was removed using Zeba desalt spin columns (Thermo Fisher Scientific). Live cell imaging was performed with a TCS Sp5 inverse confocal laser-scanning microscope (Leica) and analyzed with LAS AF software. In detail, 2.5×10^4 DCs were added to a channel of an IV0.4 µ-slide (Ibidi) and incubated for 2 h at 37°C and 6% CO₂ with 10 µl fluorescein-labeled HEK-omega-1 (0.3 mg/ml). Nuclei of the cells were then counterstained with Hoechst 33342 (1:10,000) for 30 min. For confocal microscopy of fixed DCs, cells were allowed to adhere to poly-D-lysine-coated coverslips overnight at a concentration of 80,000–100,000 cells/2 ml in 10% FCS/RPMI. DCs were incubated with 1 µg/ml omega-1 for 2 h at 37°C. Incubated cells were washed three times in 1% BSA/RPMI, fixed for 15 min with 4% paraformaldehyde (Sigma-Aldrich) in PBS, and washed twice in PBS. Next, cells were permeabilized with 0.1% Triton X-100 in PBS for 1 min, washed twice in PBS, and blocked for 15 min with 1% BSA/PBS. Cells were subsequently incubated with antibodies against rRNA (Abcam), followed by a secondary incubation step with a GαM-AF546 antibody (Invitrogen) in 1% BSA/PBS. Cells were washed in PBS, and coverslips were mounted on glass slides with Vectashield and analyzed by confocal microscopy. The AOBSP2 confocal laser-scanning microscope (Leica) system was used, containing a DM-IRE2 microscope with glycerol objective lens (Plan APOchromat 63×/NA 1.30), and images were acquired using Leica confocal software (version 2.61).

Cytoplasmic omega-1 Western blotting. Cytoplasmic extracts of omega-1-incubated DCs were prepared using Nuclear Extraction kit (Active Motif) as per the manufacturer's instructions. Cytoplasmic extracts were concentrated 10-fold and subjected to 12% SDS-PAGE followed by silver staining or blotting onto nitrocellulose membrane. For silver staining, 30 µg/cm was applied, and for Western blotting, 100 µg/cm was applied. Omega-1 was then detected by the monoclonal anti-omega-1 antibody 140-3E11 and an alkaline phosphatase-labeled goat anti-mouse IgG (1:10,000) detection antibody (Dianova). Visualization was performed by the substrate/chromogen mixture of 0.033% (wt/vol) nitro blue tetrazolium and 0.017% (wt/vol) 5-bromo-4-chloro-indolyl phosphate (Serva) in 0.1 M Tris-buffered saline, pH 9.5.

In vivo experiments. 4get/KN2 (Mohrs et al., 2005) mice were bred and housed in the animal facility of the Trudeau Institute and used at 8–12 wk of age. MR^{-/-} mice on a C57BL/6 background were provided by M.C. Nussenzweig (The 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 of the Trudeau Institute and Molecular Medicine and Experimental Immunology at the University Hospital of Bonn and by the Animal Studies Committee of Washington University in St. Louis School of Medicine. Mice were immunized s.c. into one hind footpad with 20 µg SEA and 3 µg omega-1 in a volume of 50 µl, and the draining popliteal LNs were analyzed 1 wk later.

In vitro restimulation of LN 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 Systems). After removal of the supernatants, cells were restimulated with 50 ng/ml PMA plus 2 µg/ml ionomycin for 6 h. 10 µg/ml brefeldin A was added during the last 2 h (all Sigma-Aldrich). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies.

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.

Online supplemental material. Video 1 shows z-stacked images of live DCs internalizing omega-1. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20111381/DC1>.

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Author contributions: B. Everts designed, performed, and interpreted most experiments and prepared the manuscript. M.H.J. Meevisen performed the biochemical treatments and glycosylation analysis of omega-1. C.H. Hokke supervised the omega-1 glycosylation treatments and analysis and part of the antigen binding experiments. H. Haas, G. Schramm, and B. van der Hoeven isolated natural omega-1 and IPSE and generated the recombinant WT omega-1 and mutants. T. Scholzen performed life cell imaging. S. Burgdorf, M. Mohrs, and E.J. Pearce provided the MR^{-/-} mice and 4get/KN2 mice. L. Hussaarts and N.N. Driessen participated in many DC experiments and antigen uptake experiments. A.J. van der Ham executed many DC experiments and performed most cytokine ELISAs and protein synthesis inhibition experiments. H.H. Smits performed many of the murine experiments and together with M. Yazdanbakhsh supervised all aspects of this study.

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