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Metabolism and lipid mediators as regulators of innate immune cell function: implications for inflammation and immune responses

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High-Mannose Glycans From *Schistosoma mansoni* Eggs Are Important for Priming of Th2 Responses via Dectin-2 and Prostaglandin E_2

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Abstract

The parasitic helminth *Schistosoma mansoni* is a potent inducer of type 2 immune responses by stimulating dendritic cells (DCs) to prime T helper 2 (Th2) responses. We previously found that *S. mansoni* soluble egg antigens (SEA) promote the synthesis of Prostaglandin E_2 (PGE2) by DCs through ERK-dependent signalling via Dectin-1 and -2, that subsequently induces OX40L expression, licensing them for Th2 priming. Yet, the ligands present in SEA involved in driving this response, and whether specific targeting of PGE2 synthesis by DCs could affect Th2 polarization are unknown. We here show that the ability of SEA to bind Dectin-2, drive ERK phosphorylation, PGE2 synthesis, OX40L expression, and Th2 polarization is impaired upon cleavage of high mannose glycans by Endoglycosidase H treatment. This identifies high mannose glycans present on glycoproteins in SEA as important drivers of this signalling axis. Moreover, we find that OX40L expression and Th2 induction are abrogated when microsomal prostaglandin E synthase-1 (mPGES) is selectively inhibited, but not when a general COX-1/2 inhibitor is used. This shows that *de novo* synthesis of PGE2 is vital for the Th2-priming function of SEA-stimulated DCs, as well as pointing to the potential existence of other COX-dependent lipid mediators that antagonize PGE2-driven Th2 polarization. Lastly, specific PGE2 inhibition following immunization with *S. mansoni* eggs dampened the egg-specific Th cell response. In summary, our findings provide new insights in the molecular mechanisms underpinning Th2 induction by *S. mansoni* and identifies druggable targets for potential control of helminth driven-Th2 responses.

Introduction

Helminth parasites are known to provoke strong T helper 2 (Th2) cell–polarized immune responses, which can contribute to protective immunity, but may also lead to immunopathology. Yet, the underlying molecular mechanisms through which helminths activate this type of immune response are still incompletely understood. A better understanding of how Th2 responses are initiated by helminths may help to identify pathways that could be targeted to shape Th2 responses in therapeutic settings, not only in the context of helminth infections, but also inflammatory disorders such as allergies that are characterized by aberrant Th2 responses.

Dendritic cells (DCs) play a highly important role in the immune system by functioning as a bridge between the innate and adaptive immune system. Their specialized role as antigen presenting cells (APCs) allows them to prime responses depending on the pathogen or stimulus encountered (1,2). Upon helminth infection, DCs are key players in inducing the differentiation and activation of Th2 cells (3,4). As such, elucidating the mechanisms by which parasite antigens license DCs to induce Th2 responses will be key to unravel how helminths activate this type of immune response.

Schistosoma mansoni soluble egg antigens (SEA) are one of the most commonly used antigen preparations to study the immune response against helminths (5–7). SEA is complex mixture of highly immunogenic antigens that are capable of activating DCs, driving robust Th2 polarized immune responses (7,8). Within SEA, omega-1 (ω1), a glycoprotein with T2 Ribonuclease activity, has been identified as a key driver of Th2 responses (9), both *in vitro* and *in vivo*, by conditioning DCs for Th2 priming in a mannose receptor-dependent fashion

Importantly however, SEA depleted of ω1 (Δω1-SEA), can still promote a Th2 response, highlighting the existence of additional omega-1-independent mechanisms through which *S. mansoni* eggs can condition DCs for Th2 priming (5). More recently, we identified that components in SEA, independently from omega-1, can trigger a Dectin-1 and -2-dependent signalling pathway, involving Syk-dependent ERK phosphorylation, to increase COX1/2 activity, resulting in elevated oxidation of arachidonic acid and *de novo* PGE2 synthesis. This PGE2 in turn acts in an autocrine manner on DCs to induce OX40L expression, thereby endowing DCs with the ability to prime a Th2 response (10). However, the molecular determinants in SEA that interact with Dectin-1 and -2 to induce this signalling cascade remained unidentified.

Many proteins in SEA are heavily glycosylated and their reported immunomodulatory effects are in many cases glycan dependent (11,12). Correspondingly, on DCs, SEA

has been shown to interact with and signal through several glycan-binding C-type lectin receptors, such as the macrophage galactose-type lectin (MGL), CD209 (DC-SIGN) and CD206 (Mannose Receptor) to modulate TLR-induced cytokine production and T cell-priming capacity (13–17). The main glycan moieties present in SEA that are thought to mediate interaction with these receptors are Galβ1,4(Fucα1,3)GlcNAc (LeX), GalNAcβ1,4(Fucα1,3)GlcNAc (LDNF), and GalNAcβ1,4GlcNAc (LDN) (13,18). However, it is currently unknown which component(s) in SEA act as ligands for Dectin-1 and Dectin-2. Both transmembrane C-type lectins are well known to bind β-Glucan and high mannose glycans, respectively (19,20). As SEA has been shown to contain high mannose glycans (21), and since Δω1-SEA requires Dectin signalling (10), we here explored whether SEA components were capable of binding to, and subsequently activating signalling downstream of Dectin-1- and Dectin-2.

Additionally, it remains to be determined whether pharmacological targeting of the PGE2/OX40L signalling pathway in DCs, in particular PGE2 synthesis, could be used to manipulate the egg-induced Th2 response. We previously found that while antibody-mediated PGE2 neutralization was able to fully block omega-1 independent Th2 priming by DCs, COX1/2 inhibition only had a partial effect (10). This prompted us to explore the possibility that a more targeted pharmacological intervention is needed, i.e. by selectively inhibiting PGE2 synthesis to effectively block Th2 priming.

Materials and Methods

Preparation and purification of S. mansoni egg–derived antigens

SEA and Δω1-SEA from *S. mansoni* eggs were prepared and isolated as described previously (22)

EndoH treatment of SEA and Δω1-SEA

SEA and Δω1-SEA were treated with Endo-H (NEB #P0702S) according to their non-denaturing protocol conditions. Succinctly, for both SEA and Δω1-SEA, 500 µg was treated with Endo-H and 500 µg was mock-treated. Samples were not denatured. Buffer was directly added along with 12.500 units of Endo-H. Samples were then incubated at 37 °C for 24h. Removal of oligomannose N-glycans was confirmed by mass spectrometry (FigS1).

Purification of Mannose-9 from human serum

High mannose glycans were cleaved from human serum proteins, using Endo-H (NEB #P0702S) and incubating at 37°C for 48h. Released glycans were purified by

application of C18 SPE-(J.T.Baker, #7020-03) and Carbon SPE columns (Supelco, #57088) and labelled with anthranilic acid (AA) by reductive amination. To remove labelling reagent excess, Acetonitrile (ACN) was added to a final concentration of 75%, and the sample loaded onto Bio-Gel P10 Gel resin (catalog no.: 1504144; Bio-Rad) previously conditioned with 80% ACN. Glycans were eluted with MQ and dried using a Speedvac. Glycans were then further fractionated using reverse phase HPLC (RP-HPLC) and analysed by MALDI-TOF MS, yielding pure Man9 glycan. Above methodology is described in more detail by Petralia *et al* (23)

AA to AEAB label conversion

2-amino-N-(2-aminoethyl)-benzamide (AEAB) labelled Man9 glycans generated from AA labelled Man9 as previously described (24). In short, 20 µg of both Maltopentose-AA (generated from maltopentose (Sigma #SMB01321) as described for Man9 above) and Man9-AA were mixed with 50 µL EDC (10 mg/mL in DMSO) and 50 µL HOBt (10mg/mL in DMSO). Subsequently, we added 10 µL of 5% (v/v) EDA and 0.5 M MES buffer (pH = 6.5). Samples were then vortexed for 1 min and incubated at room temperature for 3h and then quenched with 1.1 mL of cold ACN. Samples were then vortexed and stored at -20 °C for 30 minutes. The cloudy reaction mixture was centrifuged for 10 min at 10 000 x g. Supernatant was discarded and the precipitate was dried under vacuum. Once dry, it was dissolved in 100 µL of Mili-Q and applied to a RP-HPLC C18 column for purification.

Generation of Man9-labelled NHS gold nanoparticles

Both Man9-AEAB and Maltopentose-AEAB were conjugated to 100nm NHS-Activated Gold Nanoparticles using a kit (Cytodiagnostics #CGN10K-100-1) and following the manufacturers protocol. As a deviation to the protocol, glycans were diluted to 0.1 µg/µL using protein re-suspension buffer and 1x PBS was used instead of conjugate storage buffer. Reaction efficiency was estimated to be ≈58% via RP-HPLC with fluorescence detection to quantify the percentage of recovered uncoupled material.

Human DC culture, stimulation, and analysis

Peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers by density centrifugation in Ficoll as described before (25). Monocytes were isolated by positive magnetic cell sorting using CD14-microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured in 10% FCS RPMI medium supplemented with 20 ng/mL rGM-CSF (BioSource/Invitrogen, Carlsbad, CA) and 0.86 ng/mL of rIL-4 (R&D Systems, Minneapolis, MN). On days 2-3, medium, including supplements was replaced.

Immature moDCs were stimulated on day 5-6 in the presence or absence (if indicated) of 25 ng/mL ultrapure LPS (Escherichia coli 0111 B4 strain; InvivoGen, San Diego, CA), along with indicated reagents: SEA (20 µg/mL), Δω1-SEA (20 µg/mL), 50 µg/mL Zymosan (Z4250; Sigma-Aldrich, St. Louis, MO), CIII (10 µM), Indomethacin (Sigma-Aldrich #I7378, 50 µM), 5 µg/mL, 2 µg/mL or 0.2 µg/mL of either Man9- or Maltopentose-bound nanoparticles, or inactivated nanoparticles.

After 48h of stimulation, surface expression of co-stimulatory molecules was determined by flow cytometry (FACS-Canto; BD Biosciences, Breda, the Netherlands or Aurora; Cytek, Amsterdam, the Netherlands) using the following antibodies: CD1a (clone HI149), CD14 (clone MΦP9), CD86 (clone 2331 FUN-1), CD40 (clone 5C3), and CD80 (clone L307.4) (all BD Biosciences); HLA-DR (clone LN3) CD83 (clone HB15e) (both eBioscience, San Diego, CA); and CD252/OX40L (clone ANC10G1; Ancell, Bayport, MN). Only live cells that were negative for Zombie NIR (BioLegend Europe BV, Amsterdam, the Netherlands) were included in the analysis. Acquired samples were unmixed using SpectroFlo version 3 (if measured with Aurora), and analyzed with FlowJo.

Human DC and T cell coculture and determination of T-cell polarization

For analysis of T-cell polarization, 5×10^3 moDCs pulsed for 48 h were cultured with 2×10^4 allogenic naïve CD4+ T cells for 7 days in the presence of Staphylococcal enterotoxin B (10 pg/mL). On day 7, T cells were replated and rhuIL-2 (10 U/mL; R&D Systems) was added to expand the T cells. On day 9-10 T cells were split with medium containing the same concentration of rhuIL-2. Intracellular cytokine production was analyzed on day 12 after restimulation with 100 ng/mL phorbol myristate acetate, 2 µg/mL ionomycin and 10 µg/mL brefeldin A for 4 h. Subsequently, the cells were fixed with 2% paraformaldehyde (all Sigma-Aldrich). The cells were permeabilized with permeabilization buffer (eBioscience #00-5523-00) and stained with antibodies against IL-4 and IFN-γ, respectively (BD Biosciences). Acquired samples were unmixed using SpectroFlo version 3 (if measured with Aurora), and analyzed with FlowJo.

Measurements of PGE2 levels in culture supernatants

Lipid mediators (LM) and polyunsaturated fatty acids (PUFA) were measured using reverse-phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS/MS) as previously described (26), with some modifications. Briefly, 2 µL internal standard (IS) mix of deuterated lipid standards consisting of PGE2-d_4, 15-HETE-d_8, Leukotriene B_4-d_4, DHA-d_5, 8-iso-PGF2a-d_4 and 14(15)-EET-d_{11} (50 ng/mL in MeOH) were added to 400 µL culture supernatants. Lipids were extracted and purified by solid phase extraction (SPE) after protein precipitation with 1.2 mL MeOH. The dried

extracts were reconstituted in 100 µL 40% MeOH and transferred into a micro vial glass insert. 40 µL sample was injected and analyzed using a Shimadzu Nexera LC40-system with an autosampler coupled to a QTrap 6500 mass spectrometer (Sciex). Kinetex C18 50 × 2.1 mm, 1.7 µm column and C8 precolumn (Phenomenex) were used for LC separation. LC-MS/MS chromatograms were integrated manually using Sciex OS (Sciex). The results were reported as relative peak area of lipids to the internal standards. PGE2-d_4 IS was used for reporting area ratios of PGE2, TxB2 and PGF2a.

ERK phosphorylation

For detection of ERK phosphorylation (pERK), 2.5×10^4 immature moDCs were seeded overnight in a 96-well flat-bottom plate. moDCs were stimulated with SEA (25 µg/mL), Δω1-SEA (25 µg/mL), for indicated periods, and the moDCs were fixed for 15 min with 4% ultrapure formaldehyde (Polysciences, Warrington, PA) directly in the plate. The cells were harvested and washed first with PBS and then with 0.5% of saponin for permeabilization. Cells were intracellularly stained with anti–phospo-p44/42 MAPK (Erk1/2) (clone E10) (both Cell Signalling Technology). Following 2 h incubation at room temperature, cells were washed with 0.5% of saponin, and ERK phosphorylation was determined by flow cytometry.

Dectin ELISA

96-well high-binding half-area microplates (Corning #10052511) were used for the Dectin-1/2-hFc binding ELISAs. The appropriate antigens were coated in 50 µL TSM (20mM Tris-HCl, 150mM NaCl, 2mM $CaCl_2$ and 2mM $MgCl_2$ at pH 7.4) overnight at 4°C; SEA of *Schistosoma mansoni* (50 µg/mL), Mock or Endo-H treated SEA of *Schistosoma mansoni* (50 µg/mL), Zymosan (20 µg/mL), NHS gold nanoparticles (5 µg/mL), or 1% BSA in TSM. After overnight coating, the plate was washed 3 times with an excessive amount of TSM/0.005% Tween. After washing, the plate was blocked for 1 h with 100 µL TSM/1% BSA at room temperature. After blocking, the plate was washed 3 times, again with an excessive amount of TSM/0.005% Tween. Following the washing, 50 µL Dectin-1-hFc (Sino Biological #10215-H01H) or Dectin-2-hFc (Sino Biological #10250-H01H) at a concentration of 10 µg/mL in TSM/0.005% Tween was incubated for 2h at room temperature. After incubation, the plate was washed 5 times again with an excessive amount of TSM/0.005% Tween. Next, 50 µL of Monoclonal Biotin Mouse anti-Human IgG1-Fc (Invitrogen #05-3340) (1:500 in TSM/0.005% Tween) was added to the plates, along with HRP-Strep (BD 51-9002813) and incubated for 1 h. The plate was then washed again for 6 times. We used 50 µL of TMB ELISA substrate solution (ThermoFisher #34021) for 30 min, followed by 25 µL of H_2SO_4 1.8 M to stop the coloring reaction. ELISA readout was performed at 450 nm, with absorbance correction at 570 nm, by MultiskanTM FC Microplate Photometer

(ThermoFisher, type 357).

Mice

Wild type (WT) mice, both male and female and all on a C57BL/6J background, were bred under SPF conditions at the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Mice were culled through cervical dislocation. Animal experiments were performed when the mice were between 8 and 16 weeks old. Animal experiments were performed in accordance with local government regulations, EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, as well as approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD). Animal license number AVD116002015253.

SEA immunization

Mice were injected subcutaneously with 5000 *S. mansoni* eggs, in the hind footpad, and either injected i.p. with vehicle, or 1 mg CIII/20 g of body weight. Injections with either CIII or vehicle were then repeated on day 2 and day 4 post-immunization. Seven days later, mice were sacrificed and cells from both draining and nondraining lymph nodes were isolated and analysed as described below.

Analysis of murine T-cell responses

Antigen-specific responses were determined by culturing 5×10^5 LN cells per well in high binding 96-well flat-bottom plates (Corning #3590) in 200 µL complete medium (RPMI containing 10% FCS, 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine) in the presence of 20 µg/mL SEA along with 2.5 µg/mL IL-4R blocking antibody to retain IL-4 in culture supernatants. After 48 h, culture supernatants were stored for cytokine determination. Cell culture supernatants were analyzed for cytokines using the Cytokine Bead Array (BD) according to the manufacturer's recommendation. Samples were analyzed on a BD Canto II Flow Cytometer. Alternatively, cytokine production was assessed by intracellular staining of T cells from LNs after polyclonal restimulation in 96-well flat-bottom plates for 4 h with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL), ionomycin (1 µg/mL), and brefeldin A (10 µg/mL; all from Sigma-Aldrich). Afterwards, cells were fixed with 2% PFA and subsequently stained in eBioscience permeabilization buffer: IL-4 (11B11), IFN-γ (XMG1.2), IL-13 (eBio13A), IL-17A (TC11-18H10.1), and CD4 (RM4-5), IL-10 (JES5-16E3) (all BD Bioscience or BioLegend). Samples were analyzed on a BD Canto II Flow Cytometer or Cytek Aurora. Acquired samples were unmixed using SpectroFlo version 3 (if measured with Aurora), and analyzed with FlowJo.

Statistical analysis

Data were tested for normality using the Shapiro–Wilk test. Statistical tests used are indicated in the figure legends. Generally, data were compared using one-way ANOVA for more than two groups, or two-way ANOVA for comparing multiple parameters across two or more groups, with Tukey's post-hoc test for multiple comparison. If comparing parameters within the same sample, a paired or repeated-measures test with Geisser-Greenhouse correction was used. p values < 0.05 were considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). All statistical analyses were performed using GraphPad Prism v.9.0.

Results

SEA Binds to Dectin-2 in a High-Mannose-Dependent Manner

We previously reported that blocking antibodies against Dectin-1 and -2 in DCs were able to impair omega-1-independent Th2 priming by SEA (10). However, it was not assessed whether components in SEA were directly interacting to Dectin-1 and/or -2 to promote this response. To determine this directly we performed a binding ELISA with a construct consisting of the carbohydrate binding domain of Dectin-1 or Dectin-2 coupled to a human IgG1 Fc domain. Zymosan, a known ligand for Dectin-1 and -2 was taken along as positive control. We observed that both Dectin-1 and Dectin-2 were directly able to bind to components present in SEA (Fig1. A, B).

These receptors are C-type lectins that preferentially bind to sugar residues. It was shown in previous studies (8) that high mannose glycans (e.g. Man9), known to be Dectin-2 ligands (20,27), are present in high frequency in the mixture. To assess if the presence of these high mannose moieties was required for SEA to interact with Dectin-1 and -2, we treated SEA with endoglycosidase-H (EndoH), to specifically remove N-linked oligomannose glycans, including high mannose, without affecting complex N-linked glycans or O-linked glycans (FigS1). EndoH hydrolyses the glycosidic linkage of high mannose glycans between GlcNAc1 and GlcNAc2, resulting in free glycans with only one GlcNAc residue present in the core, while PNGase-A cleaves off glycans between GlcNAc 1 and asparagine, resulting in free glycans with two GlcNAc residues present in the core. The success of EndoH treatment can thus be confirmed in the top spectrum due to the absence of high mannose glycans with two GlcNAc residues., indicating that all high mannose glycans were released by Endo-H before the PNGase-A treatment.

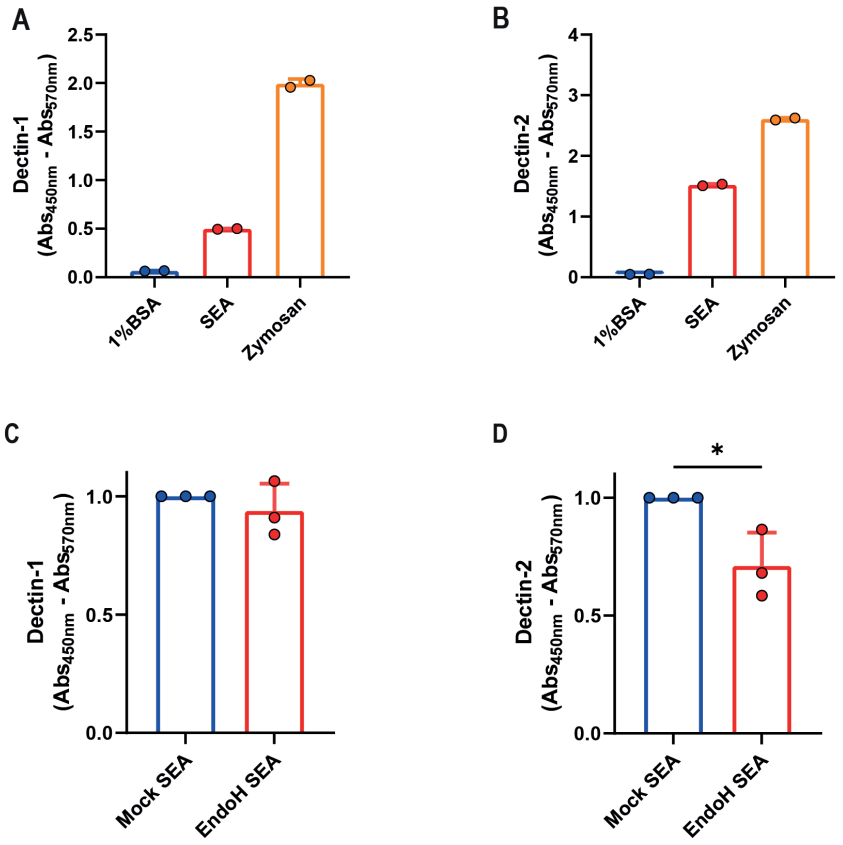

Figure 1. Dectin-1 and Dectin-2 directly bind to SEA
Binding ELISA of indicated molecules was performed as described in Materials and Methods for (A) Dectin-1, and (B) Dectin-2. Binding ELISA of indicated molecules for (C) Dectin-1 and (D) Dectin-2. (A), (B), are representative plots of 3 experiments (n = 2 per experiment, mean ± SD). (C) and (D) represent pooled data of 3 independent experiments shown with data normalized by the Mock SEA condition and compared using a paired one-tailed t-test (n = 2 -3 per experiment, mean ± SD). *p < 0.05.

As assessed in a Dectin-1 and -2 binding ELISA, the hydrolysis of high mannose glycans in SEA did not affect binding by Dectin-1. However, this treatment did significantly decrease binding of SEA by Dectin-2, suggesting that SEA directly interacts with the latter in a partly high mannose-dependent manner (Fig1. C, D).

High-Mannose Glycans in SEA Are Required, but Not Sufficient, to Induce PGE2 and OX40L Expression and Th2 Priming by moDCs

Considering SEA requires the presence of oligomannose glycans, including Man9, to bind to Dectin-2, we wondered if the presence of these glycans was important for induction of the signalling cascade leading to OX40L expression and Th2 polarization

by moDCs. EndoH-treated SEA lost its ability to induce ERK phosphorylation (Fig2. A), OX40L expression (Fig2. B), and subsequent Th2 polarization by moDCs (Fig2. C). The latter two readouts were performed in the presence of LPS as neutral DC maturation factor (5). Correspondingly, the synthesis of PGE2 was also reduced in moDCs stimulated with EndoH-treated SEA (Fig2. D). These effects were not confounded by the presence of omega-1 (ω1) as moDCs stimulated with SEA depleted of ω1 (Δω1-SEA), which had undergone EndoH treatment, were also compromised in their ability to induce a Th2 response compared to DCs stimulated with mock treated control Δω1-SEA (Fig2. E). These data suggest that high mannose residues are important for ω1-independent Th2 polarization by SEA.

To investigate if high mannose glycans themselves are sufficient to recapitulate the effects of SEA, we coupled AEAB-labelled mannose-9 (Man9) oligosaccharides isolated from human serum to N-Hydroxysuccinimide (NHS)-activated gold nanoparticles. Interestingly, while these loaded nanoparticles were efficiently covered with high mannose glycans (FigS2. A-D) and were able to directly bind to Dectin-1 and -2 as determined by ELISA (FigS3. A, B), they were not able to replicate the effects of SEA on moDCs in terms of inducing OX40L on moDCs or condition them for Th2 polarization in a concentration of glycans labelled to NPs ranging from 0.2-5 μg/mL (Fig2. F, G). This indicates that, while high mannose glycans are required for the activation of the dectin-OX40L axis by SEA, they are not sufficient to activate this pathway.

Selective Inhibition of PGE2 Synthesis Impairs OX40L Expression, and Th2-Priming by moDCs

We previously found that SEA-driven Th2 polarization via Dectin-1/2 is critically dependent on PGE2, one of the downstream products of COX. However, in contrast to PGE2 neutralization experiments, COX inhibition was only able to modestly decrease Th2 priming (10). We wondered whether this could be explained by the fact that COX inhibition does not only inhibit PGE2 synthesis, but also affects the synthesis of other COX products that may affect the Th2 priming capacity of DCs. To test this and avoid this potentially confounding issue, we used CIII, an inhibitor that specifically targets microsomal Prostaglandin E synthase-1 (mPGES) without affecting other COX-derived products (28) (FigS4).
Corresponding with inhibition of PGE2 synthesis by both drugs, their incubation reduced the Δω1-SEA-driven expression of OX40L by moDCs (Fig3. A). While no statistical difference was found when comparing CIII to Indomethacin ($p = 0.442$) (Fig3. B), CIII treatment significantly lowered the Th2-priming ability of Δω1-SEA-treated moDCs. In contrast, this was not the case for treatment with Indomethacin, suggesting that targeting PGE2 synthesis itself is superior in modulating Th2 priming in this setting to targeting COX further upstream.

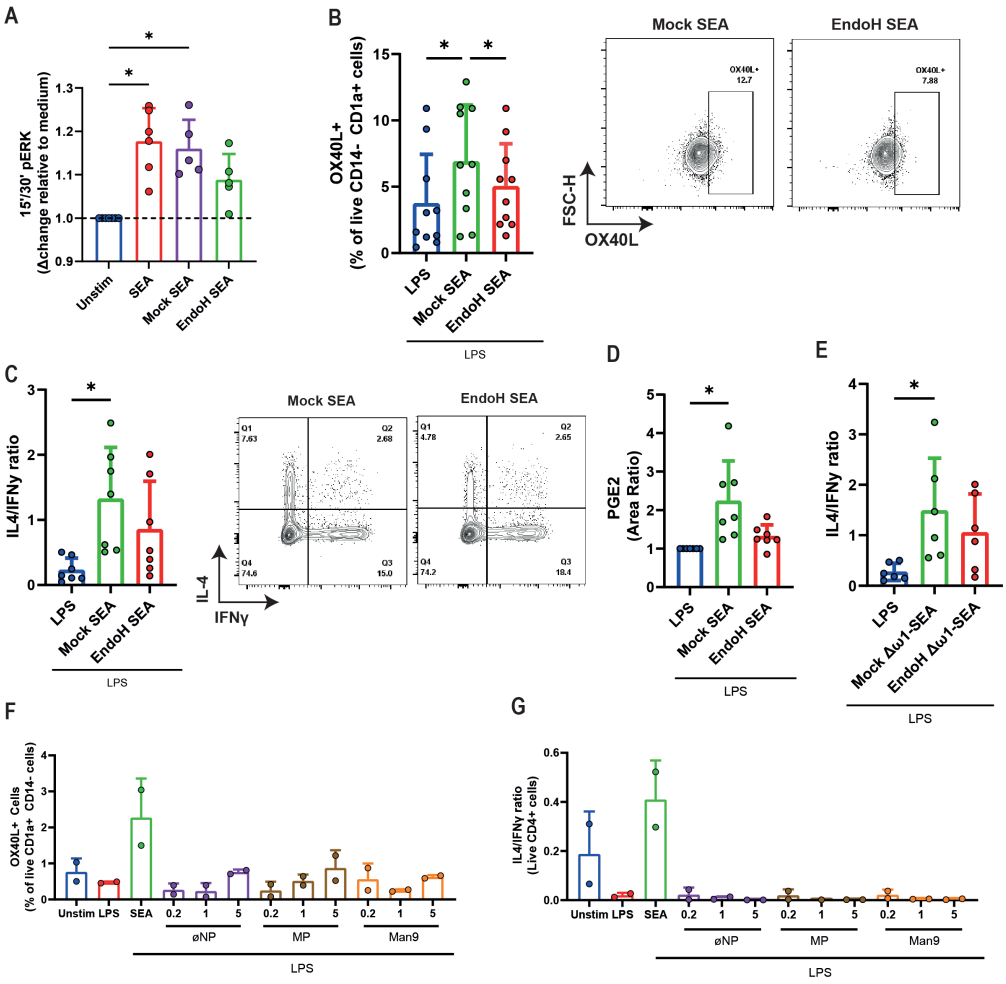

Figure 2. Man9 is required, but not sufficient, to induce OX40L in moDCs and subsequent Th2-priming

(A) ERK phosphorylation was measured with FACS after stimulating moDCs for 15 and 30 minutes with the indicated stimuli. (B) OX40L expression by moDCs was measured via FACS in m after 48h of stimulation with mock-treated SEA or with EndoH-treated SEA, all in the presence of LPS. (C) Th1- and Th2-priming abilities of moDCs treated with indicated stimuli were analysed as described in Materials and Methods. The ratios of IL4+IFNy- percentage over IL4-IFNy+ percentages are based on intracellular staining following PMA/Iono/BrefA stimulation. (D) PGE2 levels (measured in area ratio) in supernatants from moDC cultures after stimulation with indicated stimuli. (E) IL4/IFNy ratio of CD4+ T cells primed by moDCs treated for 48h with indicated stimuli. (F) moDCs were stimulated with inactivated nanoparticles (øNP) Maltopentose-coated nanoparticles (MP) or Man9-coated nanoparticles and OX40L expression was measured via FACS. (G) Th1- and Th2-priming abilities of moDCs treated with indicated nanoparticles were also measured, as mentioned in (C) and (E). (A)-(D) data points represent individual donors pooled from 3-5 experiments with data compared using a paired one-way ANOVA (n = 5-10, mean ± SD). (F) and (G) data points represent data from 2 individual donors. *p-value <0.05.

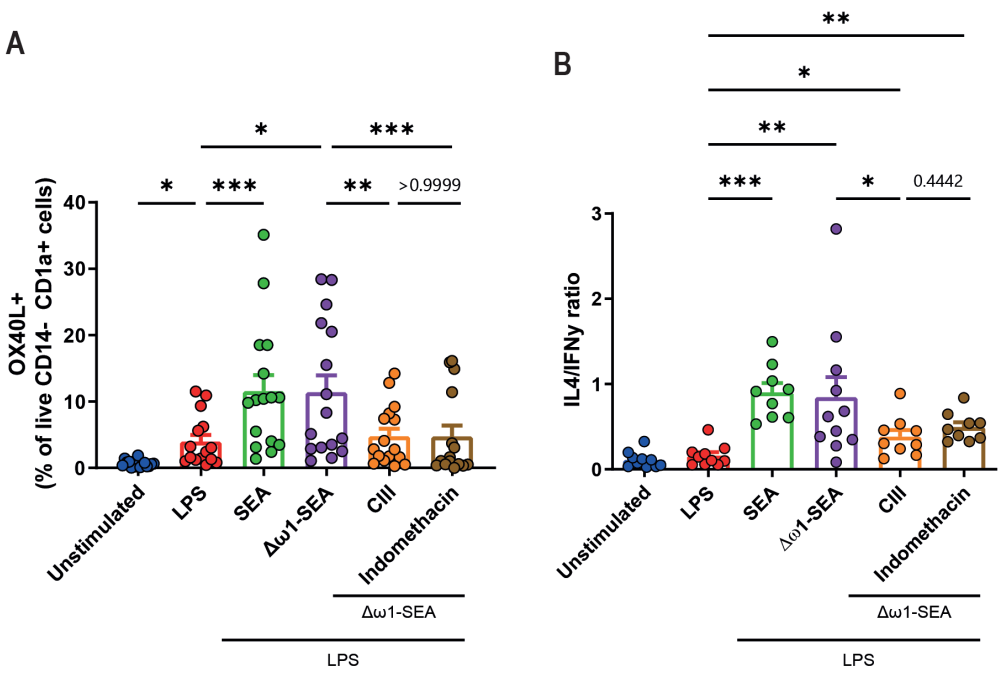

Figure 3. Selective inhibition of mPGES, but not general COX inhibition, in moDCs reduces Th2 priming following SEA stimulation
(A) OX40L expression in moDCs following 48h stimulation with indicated stimuli and (B) Th1- and Th2-priming abilities as described in Fig2. Data points in (A)-(B) represent data 7 to 16 individual donors. Data were compared using a paired one-way ANOVA (median ± SD). *p <0.05, **p < 0.01 ***p < 0.001.

Inhibition of PGE2 Synthesis Impairs the T Cell Response to S. mansoni Eggs In Vivo

In view of these results we obtained *in vitro*, we wondered if PGE2 synthesis inhibition would also reduce Th2 polarization *in vivo*. To test this, we injected *S. mansoni* eggs in the footpad of wild type mice, followed by i.p. injections of CIII day 0, 2 and day 4 post-challenge. On day 7 mice were sacrificed and the $CD4^+$ T cell response in the draining and non-draining lymph nodes was characterized.

Mice challenged with *S. mansoni* eggs and treated with CIII displayed an overall lower number of $CD4^+$ T cells in draining LNs (Fig4. A), resulting in a lower number of IL4-, IL13-producing Th2-polarized T cells upon polyclonal restimulation, when compared to egg immunized mice injected with the vehicle control. However, this was also true for the number of IFNγ- and IL-17-producing Th cells. As a consequence, CIII treatment did not alter the ratio between IL4- and IFNγ-producing $CD4^+$ T cells (Fig4. B-F) No difference was seen in IL10-producing $CD4^+$ T cells (Fig4. G). To assess antigen-specific cytokine responses, cells that had been isolated from the LNs of

immunized mice were restimulated with SEA. While no difference was seen in the IL-4 response (Fig4. H), cell cultures from egg immunized mice receiving CIII displayed a near significant lower IL-13 (Fig4. I) and a decreased IFNγ (Fig4. J) response to the antigens, while the IL-17 (Fig4. K) and the IL-10 (Fig4. L) levels remained unaffected. This indicates that optimal priming of Th2 cell responses upon *S. mansoni* egg challenge *in vivo*, but also that of other concomitant egg-induced Th cell responses, rely on *de novo* PGE2 synthesis.

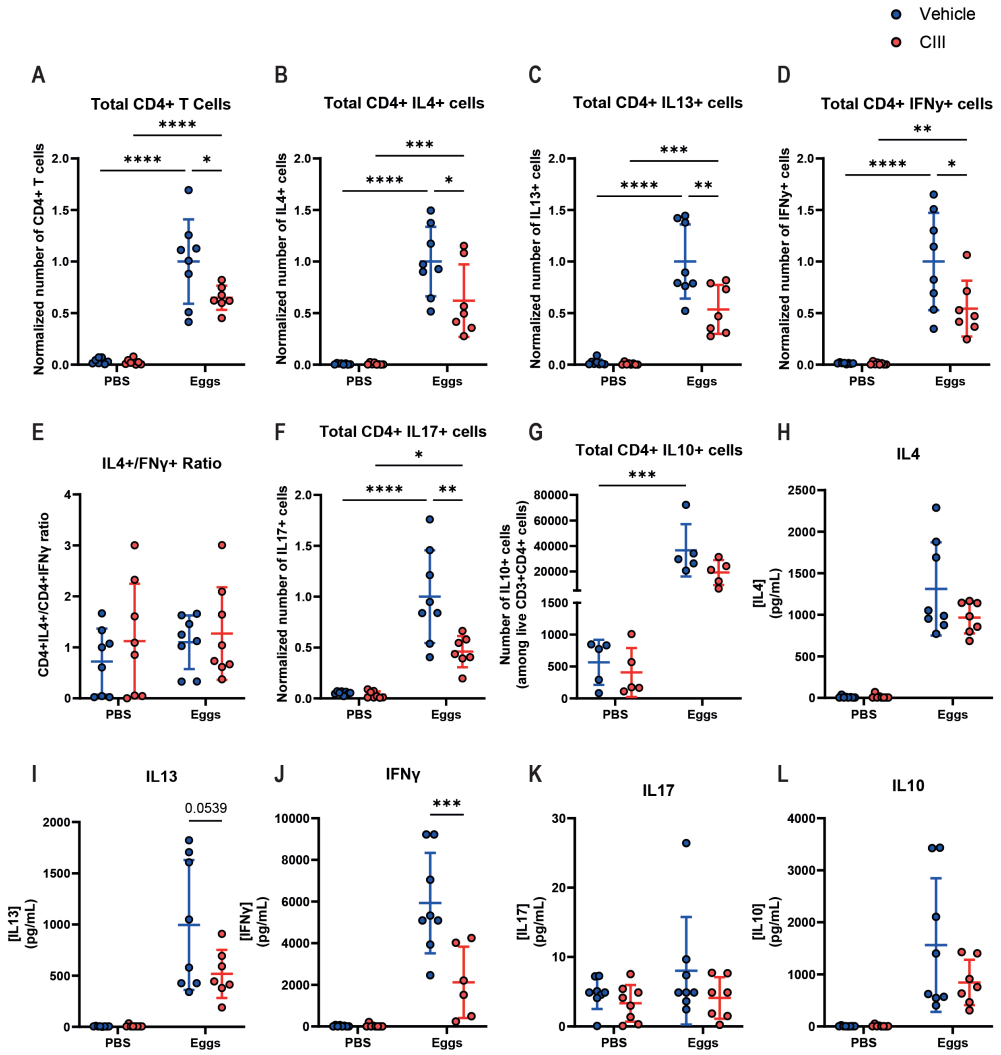

Figure 4. *De novo* PGE2 synthesis is required for optimal induction of CD4⁺ T cell responses by *S. mansoni* eggs *in vivo*

(A) Total number of live CD4⁺ T cells in non-draining and draining lymph nodes in mice injected with CIII or Vehicle following injection with *S. mansoni* eggs, and total number of live CD4⁺ T cells producing IL4 (B),

IL13 (C), and IFNy (D), along with the ratio of IL4+/IFNy+ (E) are shown. CD4+ T cells producing IL17 (F), and IL10 (G) were also measured. (H)-(L) Antigen-specific cytokine production of indicated ex vivo T cells was measured using a CBA assay in supernatant collected after 24h stimulation with SEA. Data points represent individual mice from 2 experiments. Data were compared using a 2-way ANOVA (n = 7-8, mean ± SD). Number of cells was normalized by using the average of vehicle + eggs condition from each respective experiment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

It has previously been shown that SEA drives type 2 immune responses, even if depleted of ω-1, one of its main Th2-inducing molecules (5,10). While it was demonstrated that this ω-1-independent Th2-priming was reliant on dectin signalling and PGE2 synthesis in DCs, the ligands in SEA that trigger this signalling cascade had not been identified, nor had it been determined if selective chemical inhibition of PGE2 synthesis in DCs would impair their Th2-priming abilities following SEA stimulation.
Here we have shown that Dectin-1 and -2 are able to directly bind components in SEA, and that for Dectin-2 this is in part dependent on the presence of high mannose N-glycans. Dectins are traditionally associated with anti-fungal responses (29–31), however we show that both Dectin-1 and Dectin-2 can also bind to helminth-derived glycoproteins. This aligns with previous work showing that both Dectin-1 and Dectin-2 play a role in driving the immune response against other helminths (32,33).

While Dectin-2 is known to bind to mannose residues (20,27), we have demonstrated that Dectin-1, which typically binds to β-Glucans (34), can also bind to SEA, albeit in a high mannose-independent manner. The motifs in SEA behind Dectin-1 binding remain elusive, as SEA does not contain β-glucans typically found in fungi (21). However, there is data showing that Dectin-1 can bind to N-glycans present on tumour cells (35), and to an unknown ligand present in T cells that was resistant to tunicamycin/N-glycosidase treatment, but susceptible to trypsin treatment (36), suggesting Dectin-1 may bind to other carbohydrates than classically thought, and perhaps even non-glycan components.

The importance of high mannose glycans present in SEA in mediating binding to Dectin-2, was further extended in functional studies in which treatment of SEA with the enzyme EndoH, to hydrolyse high mannose glycans from its glycoproteins, not only reduced Dectin-2 binding but also translated into lower pERK levels, PGE2 synthesis, OX40L expression, and, subsequently, impaired Th2-priming ability by moDCs. However, while we here provide evidence for a requirement of high mannose glycans in promoting this Dectin-2/PGE2/OX40L signalling axis by SEA, these glycan moieties alone do not appear to be sufficient to drive this response, as loading gold nanoparticles with Man9 residues did not mimic the effects seen with SEA. This might

be due to possible differences in coating density of Man9 glycans between the native proteins and the nanoparticles (37), as glycan density may influence the extent to which multimers of Dectin-2 (or other glycan-binding receptors) can be formed, which can affect the signalling strength downstream of those receptors (38). Alternatively, there might be a contribution of an unknown co-receptor engaged by other glycans or proteins in SEA to the signalling cascade. For instance, we previously reported that CD206 on moDCs is needed for optimal expression of PGE2 following SEA stimulation (10), which may suggest that Dectin-2 may act in concert with other glycan-binding receptors to drive this response. Finally, a not mutually exclusive possibility is that Man9 facilitates Dectin-2-dependent endocytosis of a carrier protein that subsequently modulates DC function for Th2 priming, analogous to what previously had been described for ω-1, that requires its glycans to be internalized after which its ribonuclease activity modulates DC function (9).

In addition, we demonstrate that selective chemical inhibition of PGE2 synthesis is superior in inhibiting Th2 polarization by DCs stimulated with helminth antigens, compared to targeting COX. Some conflicting results have been published regarding the effects of COX2 inhibition on Th1 and Th2 responses. While some studies indicate COX2 activity/PGE2 synthesis induce a Th2 response (39–42), some point to the opposite, showing instead an inhibition of the Th2 response and an induction of Th1 activity (43–46). We postulate that these diverse outcomes in part stem from effects on synthesis of lipid mediators downstream of COX2 other than PGE2, such as PGD_2 and PGI_2, which have been shown to exert diverse immunomodulatory effects , that includes modulation of Th1 and Th2 differentiation (47–54).

These *in vitro* findings were largely recapitulated *in vivo*, as targeted PGE2 inhibition reduced the Th2 response following immunization with *S. mansoni* eggs. We found there was lower Th2 cell expansion in immunized mice treated with the inhibitor, as well as reduced Th2 cell cytokine production following antigen-specific restimulation. Of note, also egg-induced Th1- and Th17-associated cytokine production was reduced, suggesting a more general dampening effect of mPGES inhibition on *S. mansoni* egg-driven Th cell priming, that is not limited to the Th2 response specifically. It is known that PGE2 signalling can contribute to DC maturation, antigen uptake, and migration to lymph nodes (55–57), and, as such, additional studies would be required to evaluate to what extent the effects of PGE2 inhibition on T cell priming, in the context of *S. mansoni* egg challenge, are secondary to changes in DC biology as a whole.

In conclusion, we show here that SEA is able to directly bind to Dectin-1 and -2 on moDCs, the latter in a partially high mannose-dependent manner. We also reported on the importance of high mannose residues present in SEA in inducing the

previously identified PGE2/OX40L signalling axis (10), that licenses DCs to promote a Th2 immune response. Additionally, we found that the specific targeting of PGE2 synthesis, by chemical inhibition of mPGES, is able to impair Th2 priming by DCs both *in vitro* and *in vivo*. As the expression of high mannose glycans is shared with several other parasitic helminths, such as *Fasciola hepatica* (58) and *Brugia malayi* (23), it will be interesting in future studies to explore whether high mannose glycan-driven Dectin-2/PGE2/OX40L signalling axis is a more common pathway through which helminths elicit Th2 responses. Finally, our data provide first proof of principle that targeting PGE2 synthesis with specific chemical inhibitors could be a strategy to dampen pathological type 2 immunity in the context of schistosomiasis, but possibly also in other type-2 immunity driven diseases, such as allergies.

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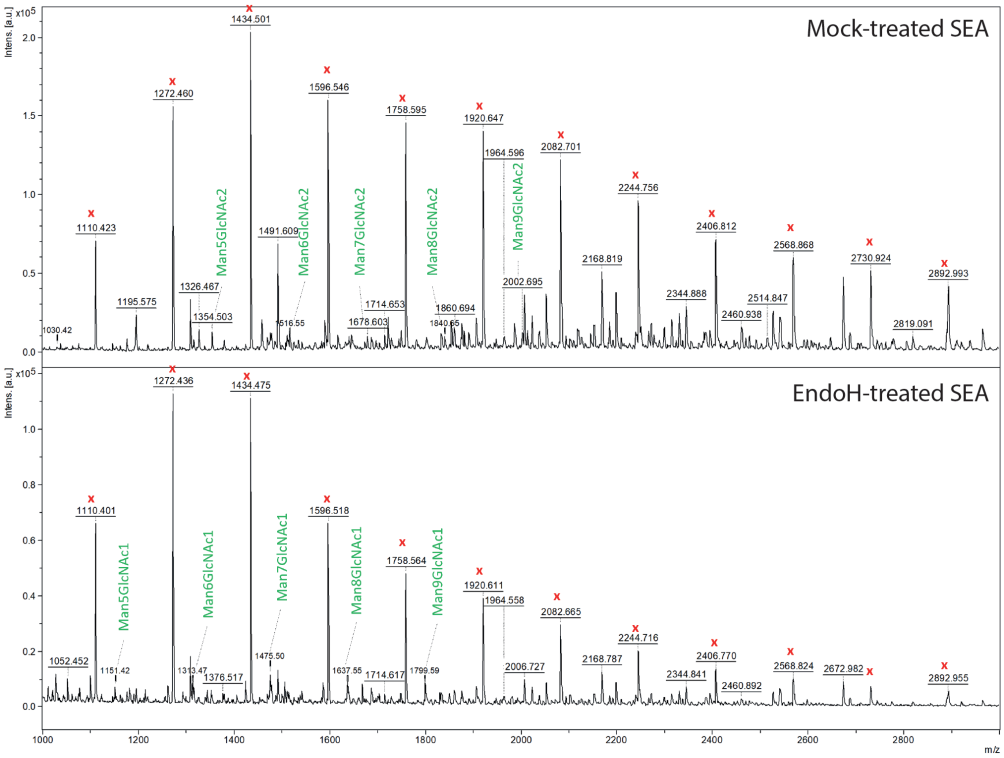
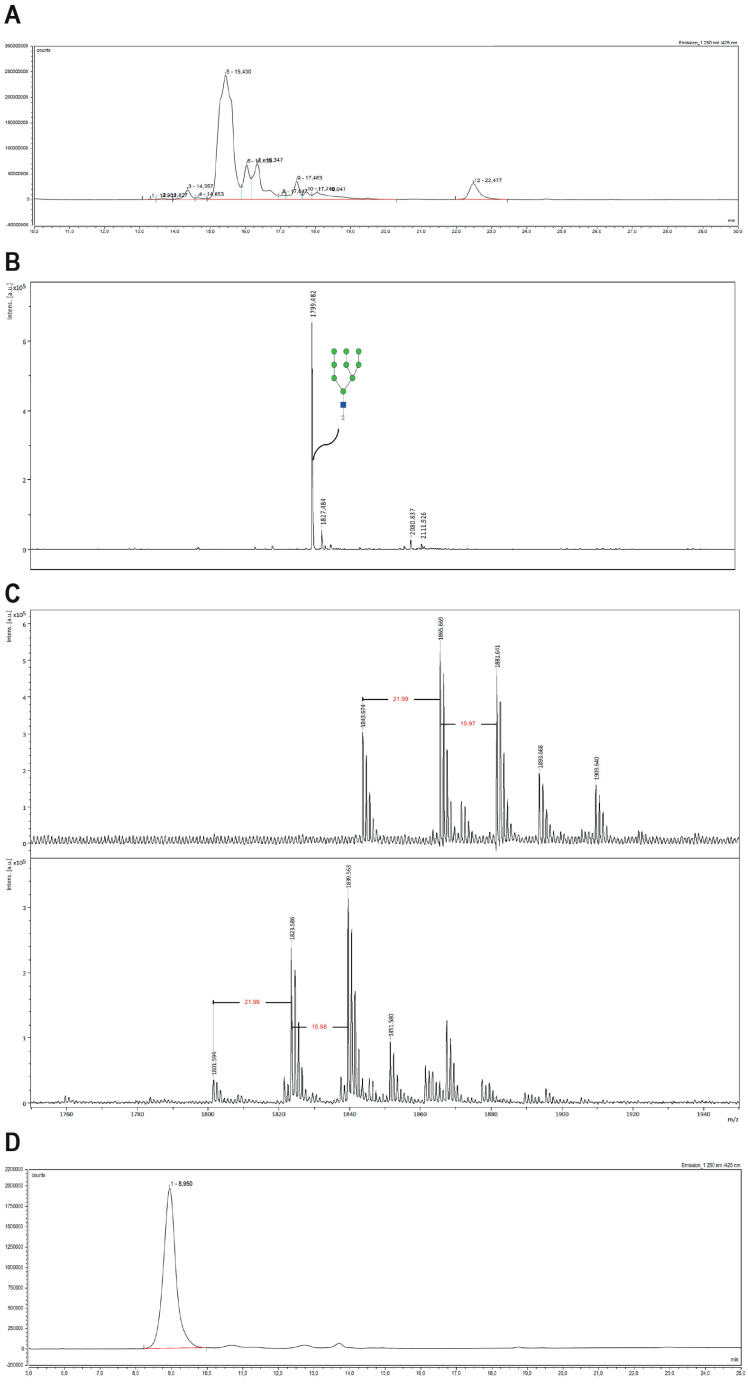
Supplementary Material

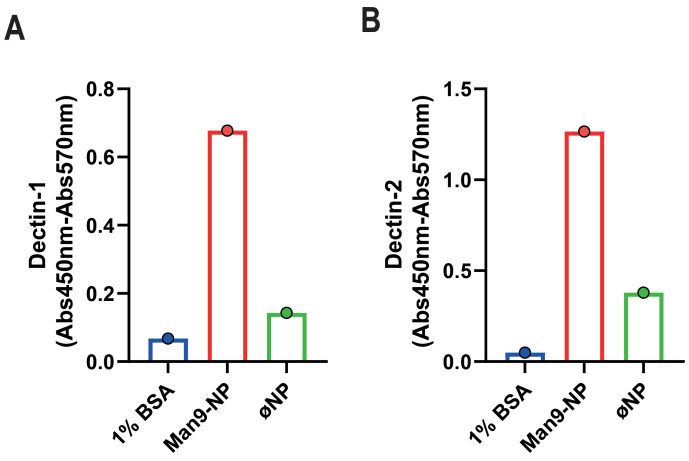


Supplemental Figure 1. MALDI-TOF MS of Mock- and EndoH-treated SEA N-glycans
MALDI-TOF mass spectra of PNGase-A released AA-labelled N-glycans of Mock (top) or EndoH (bottom) treated SEA.

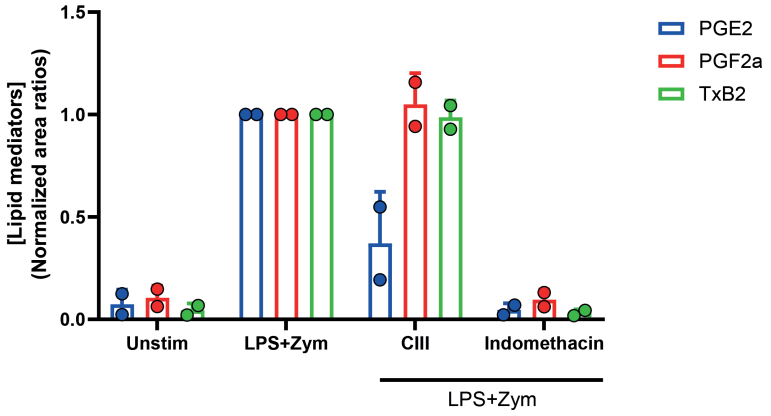


(legend on next page)

Supplemental Figure 2. Purification of Man9 from human serum and AEAB labelling
(A) RP-HPLC profile of high mannose glycans cleaved from human serum with EndoH, and (B) MALDI-TOF mass spectrum of Man9 after purification, as described in materials and methods. (C) MALDI-TOF mass spectra of AEAB-labelled Man9 (top) and AA-labelled Man9 (bottom). Successful AEAB labelling, as described in materials and methods, can be identified by an increase of 42 m/z ratio of the peaks, with Man9-AEAB having a m/z ratio of 1843.674 (top) and Man9-AA a m/z ratio of 1801.594 (bottom). (D) RP-HPLC profile of Man9-AEAB generated and purified as described in materials and methods.



Supplemental Figure 3. Nanoparticle Dectin binding ELISA
Dectin-1 (A) and Dectin-2 (B) binding ELISA to Man9-coated nanoparticles (Man9-NP) or inactivated nanoparticles (øNP). Data are from one experiment.



Supplemental Figure 4. Effects of COX and mPGES inhibitor on lipid species synthesis
(A) PGF2a, PGE2 and TxB2 concentrations in supernatants from moDC cultures after stimulation with indicated reagents. Cells were stimulated with Zymosan and LPS to induce high levels of COX-dependent lipid species, along with either CIII to inhibit mPGES or Indomethacin to inhibit COX. To confirm the specificity of CIII for inhibition of PGE2 synthesis, we measured PGE2 levels, but also mPGES-independent, COX-dependent lipids, such as PGF2a and TxB2 and compared the effect of CIII to Indomethacin. Data points represent data from 2 individual donors.

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