Tracking helminths : from molecular diagnostics to mechanisms behind immune polarization

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Chapter 6

Dectin-1/2-induced autocrine PGE_2 signalling licenses dendritic cells to prime Th2 responses

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ABSTRACT

The molecular mechanisms through which dendritic cells (DCs) prime Th2 responses, including those elicited by parasitic helminths, remain incompletely understood. Here we report that egg antigens from *Schistosoma mansoni* (SEA), that are well known to drive potent Th2 responses, triggers DCs to produce Prostaglandin E2 (PGE_2), which subsequently in an autocrine manner induces OX40L expression to licence these DCs to drive Th2 responses. Mechanistically, SEA was found to promote PGE_2 synthesis through Dectin-1 and Dectin-2 and via a downstream signalling cascade involving Syk, ERK, $cPLA_2$, and COX-1/2. These findings were supported by *in vivo* data showing that Th2 priming by SEA was dependent on Syk expression by DCs and that Dectin-$2^{-/-}$, and to a lesser extent Dectin-$1^{-/-}$ mice, displayed impaired Th2 responses and reduced egg-driven granuloma formation following *S. mansoni* infection. In summary, we identified a novel pathway in DCs involving Dectin-1/2-Syk-PGE_2-OX40L through which Th2 immune responses are induced.

Keyword:
Dendritic cells, Soluble Egg Antigen, Omega-1, Prostaglandin E2, OX40L, Dectin-1, Dectin-2, Lipidomics

INTRODUCTION

Dendritic cells (DCs) are key players in the immune system because of their unique capacity to prime antigen-specific Th1, Th2, Th17 or regulatory T cell (Tregs) responses tailored against the pathogen they encounter [1-3]. It is well-known that allergens and parasitic helminths can evoke strong type 2 immune responses, that largely depends on DCs that prime Th2 responses [4-8]. However, the molecular mechanisms through which DCs prime Th2 responses are still not fully defined.

Soluble egg antigens (SEA) from *Schistosoma mansoni* are a widely used antigen mixture to study Th2 responses to helminths. SEA is well recognized for its ability to condition DCs for priming of Th2 responses [9-13]. Omega-1 (ω-1), a glycosylated T2 RNase [14], present in SEA was found to be a major Th2-polarizing molecule [9, 15-18]. Mechanistic studies revealed that ω-1 is bound and internalized via its glycans by the mannose receptor (MR) and that, following uptake, ω-1 impairs protein synthesis in an RNase-dependent manner that is essential for conditioning of DCs for Th2 polarization [9, 10]. However, while ω-1 by itself was sufficient to condition DCs for Th2 polarization, SEA from which ω-1 was depleted still retained most of its Th2 priming potential both *in vitro* and *in vivo*. Moreover, eggs in which ω-1 expression was silenced [19] retained most of their Th2-polarizing potential, suggesting that there are additional mechanisms through which DCs become conditioned by schistosome eggs to prime Th2 responses [20].

Lipid mediators (LMs), which arise from the enzymatic oxidation of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), docosahexaenoic acid or linoleic acid (DHA), play an important role in immunological responses. In particular, prostanoids such as tromboxanes and prostaglandins (PGs), that are derivatives of AA and that are primarily released by myeloid cells including macrophages and DCs, have been shown to have the capacity to influence immune cells by affecting their migration, differentiation, effector function and/or polarization [21-24]. Interestingly, just like their mammalian host, parasitic worms are able to synthesize PUFAs and LMs. Especially PGs and leukotrienes have been detected in many species of helminths including *S. mansoni* and are involved in different aspects of life cycle regulation and sexual maturation [25, 26]. Additionally, cercariae from *S. mansoni* have been shown to promote LM synthesis in host cells such as in keratinocytes [27]. Thus far, efforts to identify molecules responsible for Th2 polarization by helminths have primarily focused on glycans and (glyco)proteins. Whether LMs directly derived from schistosomes or derived from immune cells in response to infection by this parasite, may additionally affect immune polarization remains unknown.

To identify potential novel pathways through which Th2 responses are induced by *S. mansoni*, we set out to assess the role of PUFAs and LMs in *S. mansoni* egg-driven Th2 polarization. We here report that SEA contains various PUFAs and LMs, and that additionally SEA, independently of ω-1, induces DCs to generate a number of LMs, including Prostaglandin E2 (PGE_2). We show that this *de novo* synthesis of PGE_2 by SEA-stimulated DCs is driven by signalling through Dectin-1 and Dectin-2 and is crucial for Th2 priming. Mechanistically, we provide evidence that this PGE_2 through autocrine signalling induces OX40L expression, to licence DCs to prime Th2 responses. Finally, we show that this pathway is also crucial for Th2 priming by *S. mansoni in vivo*.

MATERIALS AND METHODS

Mice

Dectin-$1^{-/-}$ and Dectin-$2^{-/-}$ (C57BL/6 background) were housed and bred at the MIH, TUM, Germany, under SPF conditions. $Itgax^{cre}$ $syk^{fl/fl}$ mice [55] were housed and bred at the CNIC,

Madrid, Spain, under SPF conditions. All animal experiments were performed in accordance with local government regulations, and the EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes and approved by the Regiuerung von Oberbayern, animal license number 55.2-1-54-2532-28-1.

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

SEA, IPSE/α-1, ω-1 and SEAΔα-1/ω-1 from *S.mansoni* eggs were prepared and isolated as described previously [3, 20].

Human DC culture, stimulation and analysis

Peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers by density centrifugation of Ficoll as described before [10]. 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, USA) and 0.86 ng/mL of rIL-4 (R&D System, Minneapolis, MN, USA). On day two, medium including supplements was replaced. In the presence or absence (if indicated) of 100 ng/mL ultrapure LPS (*E. coli* 0111 B4 strain, InvivoGen, San Diego, CA, USA), immature moDCs were stimulated on day 6 with indicated reagents: SEA (50 μg/mL), ω-1 (500 ng/mL), IPSE (500 ng/mL), SEAΔα-1/ω-1 (50 μg/mL), PGE_2 (2.5 ng/mL, Cayman), IFN-γ (1000 U/mL) as Th1 control, 0.1 mg/mL Zymosan (Sigma-Aldrich Z4250). Alternatively, moDCs were stimulated with 2.5 ng/mL LXA_4, 2.5 ng/mL PGD_2, 12.5 μg/mL 5-HETE, 12.5 μg/mL 8-HETE, 12.5 μg/mL 11-HETE, 25 μg/mL 9-HODE or 25 μg/mL 13-HODE (all Cayman). For blocking experiments cells were pre-incubated for 60 min at 37 °C with 20 μg/mL anti-DC-SIGN (clone AZN-D1, Beckman Coulter), anti-MR (clone 15.2, Biolegend), anti-Dectin-1 (clone #259931, R&D System, Minneapolis, MN, USA), anti-Dectin-2 (clone Q7-4B5, InvivoGen), 20 μg/mL IgG1 control antibody for both anti-DC-SIGN and anti-MR, IgG2a (clone RTK2758, Biolegend) control antibody for anti-Dectin-1 and anti-Dectin-2, 1 μM R406, 4 μM UO126 (Merk-Calbiochem), 1 μM Pyrrophenone (Merck-Calbiochem), 10 μM SC-236 (Sigma-Aldrich) in combination with 10 μM Indometachine (Sigma-Aldrich), 10 μg/mL neutralizing anti-PGE_2 antibody (2B5, Cayman Chemical, Ann Arbor, USA), 10 μM EP2 (AH6809, Cayman Chemical), 10 μM EP4 (AH23848, Cayman Chemical) receptor antagonist. After 24 or 48 h of stimulation, surface expression of co-stimulatory molecules was determined by flow cytometry (FACS-Canto, BD Biosciences, Breda, The Netherlands) using the following antibodies: CD14-HV450 (clone MΦP9), CD86-FITC (clone 2331 FUN-1), CD40-APC (clone 5C3), CD80 Horizon V450 (clone L307.4) (all BD Biosciences), HLA-DR APC-eFluor 780 (clone LN3) (eBioscience, San Diego, CA, USA), CD83 PE (clone HB15e), CD1a PE (clone BL6) (both Beckman-Coulter, Fullerton, CA, USA) and CD252/OX40L FITC (clone ANC10G1, Ancell). Only live cells, which were negative for 7-AAD (eBiosciences) were included in the analysis.

Cytokine detection

1×10^4 48 h-matured moDCs were co-cultured with $1x10^4$ CD40L-expressing J558 cells for 24 h and supernatants were collected to determine IL-12p70 levels, using mouse-anti-human IL-12 (Clone 20C2) as capture antibody and biotinylated mouse-anti-human IL-12 (Clone C8.6) (both BD Biosciences) in a sandwich ELISA.

Human DC and T cell co-culture and determination of T cell polarization

For analysis of T-cell polarization, 5×10^3 48 h-pulsed moDCs were cultured with 2×10^4 allogenic naive $CD4^+$ T cells for 11 days in the presence of *Staphylococcal enterotoxin B* (10 pg/mL). On day 6 and 8, rhuIL-2 (10 U/mL, R&D System) was added to expand the T cells. Intracellular cytokine production was analysed after re-stimulation with 100 ng/mL phorbol myristate acetate plus 2 μg/mL ionomycin for 6 h, 10 μg/mL brefeldin A was added during the last 4 h. Subsequently the cells were fixed with 1.9% paraformaldehyde (all Sigma-Aldrich). The cells were permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with PE- and FITC-labelled antibodies against IL-4 and IFN-γ respectively (BD Biosciences). For blocking experiments moDC-T cell co-cultures were pre-incubated for 15 min with 10 μg/mL neutralizing PGE_2 antibody (2B5, Cayman Chemical, Ann Arbor, USA), 10 μM EP2 (AH6809, Cayman Chemical), 10 μM EP4 (AH23848, Cayman Chemical) receptor antagonist, 10 μg/mL anti-OX40L antibody (Clone 159403, R&D Systems), or IgG1 control antibody (clone P3.6.2.8.1, eBioscience).

Detection of reactive oxygen species

Detection of ROS was performed according to a published protocol (http://www.bio-protocol.org/e313) with minor modifications. In brief, after 6 h of stimulation moDCs were harvested and washed using 1% FCS RPMI and re-suspended in 50 μl of a mix containing 10 μM CM-H_2DCFDA (C6827, Invitrogen) and CD1a BV421 (clone HI149, Biolegend), followed by an incubation at 37 °C for 30 min. 7AAD was added prior to sample measurement. ROS levels were quantified by flow cytometry.

Western Blot

MoDCs were harvested after 8 h of stimulation. Then, cells were washed twice with PBS before being lysed in EBSB buffer (8% [w/v] glycerol, 3% [w/v] SDS and 100 mM Tris–HCl [pH 6.8]). Lysates were immediately boiled for 5 min and their protein content was determined using a bicinchoninic acid protein assay kit (Pierce). Proteins were separated by SDS-PAGE followed by transfer to a PVDF membrane. Membranes were blocked for 1 h at room temperature in TTBS buffer (20 mM Tris–HCl [pH 7.6], 137 mM NaCl, and 0.25% [v/v] Tween 20) containing 5% (w/v) fat free milk and incubated overnight with primary antibodies. The primary antibodies used were: COX-1 (Cell Signalling), COX-2 (Cell Signalling) and actin (Millipore). The membranes were then washed in TTBS buffer and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing, blots were developed using enhanced chemiluminescence.

Syk and ERK phosphorylation

For detection of phosphorylation of Syk (pSyk) and ERK (pERK), 2.5×10^4 immature moDCs were seeded overnight in a 96 well flat bottom plate. moDC were stimulated with SEA (50 μg/ml), SEAΔα-1/ω-1 (50 μg/mL) or ω-1 (500 ng/mL) in the presence or absence of blocking antibodies or inhibitors (R406, anti-MR, anti-Dectin-1, anti-Dectin-2, combination of anti-Dectin-1 and anti-Dectin-2 or IgG1 and IgG2 control antibodies) for indicated periods and the moDCs were fixed for 15 min with 4% ultrapure formaldehyde (Polyscience) directly in the plate. The cells were harvested and washed first with PBS and then with 0.5% of saponin for permeabilization. Cell were Intracellularly stained with anti-phospo-Try525/526 Syk PE (clone C87C1) and anti-phospo-p44/42 MAPK (Erk1/2) AF488 (clone E10) (both Cell Signalling Technology). Following 2 h incubation at

room temperature, cells were washed with 0.5% of saponin and Syk and ERK phosphorylation was determined by flow cytometry.

cPLA$_2$ activity

Cytosolic phospholipase A_2 (cPLA$_2$) activity was determined according to the manufacturer's recommendation (Cayman Chemical). Briefly, 1×10^6 moDCs stimulated with indicated reagents for 8 h were harvested. MoDCs were lysed with lysis buffer (containing 50 mM Hepes, pH 7.4, 1 mM EDTA, NP-40, protease and phosphatase inhibitors) followed by 4 round of sonication for 10 second. The cells were then concentrated using a 30 KDa filter (Millipore, Amicon). To 10 µl cell lysate, 200 µl substrate solution was added to initiate the reaction, the plate was briefly shaken and incubated for 1 h at room temperature. To stop the reaction 10 µl DTNB/EGTA was added, the plate was briefly shaken, followed by 5 min incubation at room temperature. The cPLA$_2$ activity was measured using a plate reader with absorbance of 405 nm.

Antigen binding and uptake by moDCs

SEA was fluorescently labelled with PF-647 using promofluor labelling kit (Promokine) according to the manufacturer's recommendations. Approximately 2×10^4 immature moDCs per well were seeded in a flat bottom 96-well plate. Where indicated, cells were pre-incubated with 20 µg/mL of anti-MR, anti-DC-SIGN, anti-Dectin-1, anti-Dectin-2 or control antibodies at 37 °C for 45 min. Subsequently, cells were incubated with 2 µg/mL PF-647 labelled SEA at 37 °C for 45 min for testing both binding and uptake of the antigen. After 45 min cells were washed with PBS followed by flow cytometry measurement.

Liquid chromatography tandem mass spectrometry analysis of PUFAs and LMs

20 µl of SEA or supernatants from each condition were collected 0, 6, 12 and 24 h after stimulation and stored at -80 °C until analysis. A volume of 10 µl sample was mixed with 28.4 µL methanol (MeOH) and 1.6 µL of internal standard (IS, containing: Leukotriene B4-d4, 15-HETE-d8, PGE$_2$-d4, and DHA-d5 at a concentration of 50 ng/mL in MeOH). The samples were subsequently kept at -20 °C for 10 min for completion of protein precipitation, followed by centrifugation for 10 min, 16000 ×*g* at 4 °C. Subsequently samples were diluted 1:1 with water and transferred into auto-sampler vials. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis using a QTrap 6500 (Sciex, OudekERK aan den Ijsel, The Netherlands) was carried out as described previously [56, 57].

SEA immunization and *S. mansoni* infection

Mice were injected s.c. with SEA (20 µg) in the hind footpad. Seven days later, cells from both draining and non-draining lymph nodes were isolated and analysed. For *S. mansoni* infection, mice were infected with 100 cercariae from a Brazilian strain of *S. mansoni* obtained from our in house cycle of infected *Biomphalaria glabrata* snails (also of Brazilian origin). Mice were killed after 8 weeks of infection. Liver samples were fixed in 4% buffered formalin and embedded in paraffin. Sections (4 µm) were stained with Masson blue and examined microscopically (Axioskop; Zeiss) for measuring the diameters to calculate the size of spherical granulomas. To determine the parasite burden, pieces of weighed liver and intestine samples from individual mice were digested in 4% KOH (Roth) at 37 °C for 4 h. After centrifugation, the released eggs were microscopically counted. The absolute number of eggs in the liver and intestine was then calculated in accordance to the

total organ weight. Worm burden was calculated as adult worm recovery after portal perfusion and microscopic examination of livers and intestines.

Analyses of murine T cell responses.

Antigen-specific recall responses were determined by culturing 3×10^5 LN or spleen cells per well in 96-well round bottom plates in 200 µl complete medium (RPMI containing 10% fetal calf serum, 100 U/ml penicillin/streptomycin, and 2 mM l-glutamine) in the presence of 20 µg/ml SEA or 1 µg/ml anti-CD3/CD28 antibody (eBioscience). 2.5 µg/ml IL-4R blocking antibody (M1) was added to the cultures to retain IL-4 in culture supernatants. 72 h later, culture supernatants were stored for cytokine determination. Cell culture supernatants were analysed for cytokines using the Cytokine Bead Array (BD) or the mouse Ready-Set-Go ELISA kits (eBioscience) according to the manufacturer's recommendation. Samples were analysed on a BD Canto II Flow Cytometer and Sunrise™ ELISA microplate reader (Tecan), respectively. Alternatively, assessment of cytokine production by intracellular staining of T cells from LNs was determined after polyclonal re-stimulation in 96-well round bottom plates for 5 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (1 µg/ml) in the presence of brefeldin A (10 µg/ml; all from Sigma-Aldrich) for the last 3 h. Afterwards cells were fixed with 4% PFA and subsequently stained in 0.5% saponin with the following antibodies: αCD44 (IM7), αIL-4 (11B11), IFN-γ (XMG1.2) and CD4 (RM4-5) (all BD Bioscience). Samples were analysed on a BD Canto II Flow Cytometer.

Statistical analysis

The heatmap was generated using Microsoft Excel. Data were analysed for statistical significance using two-way ANOVA test, two-sided paired Student's T-test or unpaired Student's T-test. Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA USA) for Windows.

RESULTS

LM composition of SEA and supernatants of SEA-conditioned moDCs

As a first step towards the identification of LMs that may play a role in *S. mansoni* egg-driven Th2 polarization we used a sensitive LC-MS/MS based platform to identify a total of 55 PUFAs and LMs in SEA (Table S1). We discovered that SEA contains 22 out of 55 monitored analytes, including docosahexaenoic acid, linoleic acid and AA and in relative high abundance but also PGs such as PGE_2 and PGD_2 (Figure 1A). To assess potential consumption or uptake of such lipids or synthesis by DCs in response to SEA stimulation, we tested supernatants of moDCs at 0, 6, 12, and 24 h after stimulation with LPS, LPS+SEA and LPS+ω-1. We observed that levels of the majority of the LMs present in SEA decreased over time in DC culture supernatants following SEA stimulation, indicative of consumption/uptake or degradation (Figure 1B). Interestingly, we also observed that some of these lipids (i.e. 13-HODE, LXA_4, PGD_2 and PGE_2) were accumulating over time in DC culture supernatants in response to SEA stimulation both in the presence (Figure 1B and quantitated for PGE_2 in Figure 1C) or absence of LPS (Figure S1), suggestive of active production by moDCs in response to SEA. Importantly, stimulation of moDCs with LPS alone or LPS+ω-1 did not drive accumulation of any of these compounds in the supernatants (Figure 1B). These results show that SEA contains a wide range of LMs as well as induces the release of particular LMs by DCs.

A

	Lipid mediators	ng/mg SEA
1	LA	387,09
2	AA	97,71
3	DHA	49,05
4	9-HODE	16,18
5	AdA	11,29
6	13-HODE	10,5
7	EPA	9,72
8	15-HETE	4,16
9	DPA_{n-3}	3,29
10	5-HETE	2,88
11	12-HETE	2,48
12	8-HETE	2,32
13	11-HETE	1,43
14	PGE_2	1,31
15	LXA_4	0,5
16	8*S*,15*S*-diHETE	0,14
17	PGD_2	0,13
18	8-*iso*-$PGF_2\alpha$	0,13
19	6*t*,12*epi*-LTB_4	0,09
20	6-*trans*-LTB_4	0,06
21	18-HEPE	0,04
22	PDX	0,02

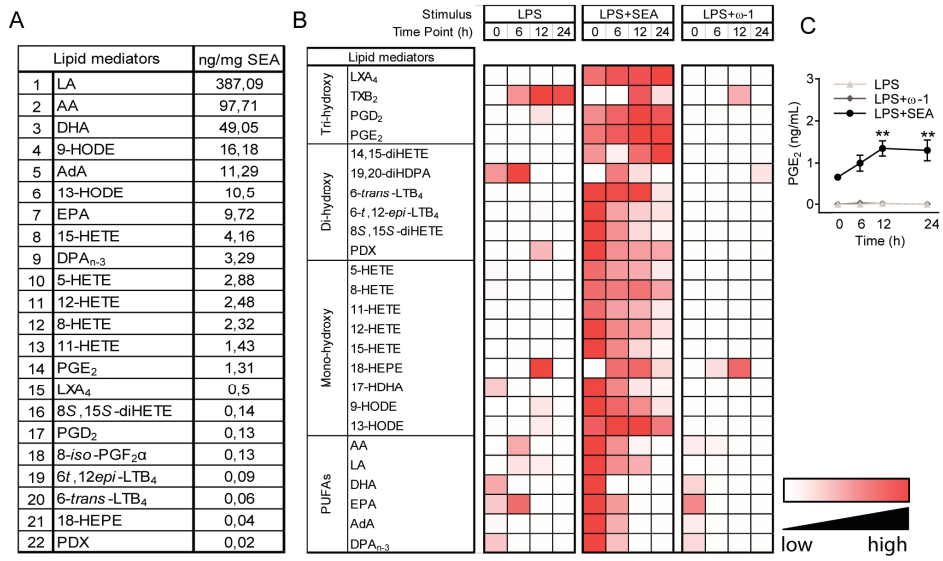

Figure 1. LM composition of SEA and in supernatants of SEA-conditioned moDCs
(A) Concentration of 22 LMs, out of 55 potentially detectable LMs, that are present in SEA from *S. mansoni* as determined by LC-MS/MS. LMs are ordered according to abundance and concentrations are determined based on internal standards. (B) MoDCs were pulsed with SEA or ω-1 in combination with LPS after which supernatants were collected at 0, 6, 12 and 24 h after stimulation. Relative amounts of PUFAs and LMs detected by LC-MS/MS in supernatants are shown in a heat-map. Data represents an average of three independent experiments. Colour coding is based on relative abundance of each lipid in comparison to other time points or stimulations. (C) PGE_2 concentration in supernatants from moDC cultures after stimulation with indicated reagents. Concentrations are determined based on an internal standard. Data represent mean ± SEM of four independent experiments. Statistical significance of different time points per condition compared to baseline (0 h) time point. *p<0.05, **p<0.01, ***p<0.001 based on two-way ANOVA test.

Th2 polarization by SEA is dependent on PGE_2 synthesis by moDCs in absence of ω-1

To test whether LMs present in SEA or generated by moDCs upon stimulation with SEA contribute to Th2 polarization by SEA, we stimulated moDCs with several of these LMs in concentrations similar to those found in SEA or in supernatants of SEA-stimulated moDCs and analysed their ability to condition DCs to induce Th2 polarization. Amongst all tested lipids, we identified PGE_2 as the only lipid capable of inducing Th2 polarization (Figure 2A). Based on this observation and given that ω-1 did not promote PGE_2 synthesis by moDCs (Figure 1B), we wondered whether PGE_2 may play a role in the previously observed ω-1-independent ability of SEA to prime Th2 responses [20]. To test this, we depleted ω-1 from SEA. Alongside ω-1, IPSE/α-1 was also depleted from this preparation, which is another glycoprotein present in SEA, but without Th2-priming capacity [20]. We found that treatment of moDCs with SEAΔα-1/ω-1 reduced expression of the Th1-polarizing cytokine IL-12 induced by LPS (Figure 2B) and promoted Th2 polarization, though less potently than total SEA (Figure 2C). In contrast to complete SEA, we could not detect PGE_2 in SEAΔα-1/ω-1 itself (time point 0 h in Figure 2D versus in Figure 1C), which suggests that during the depletion step of ω-1 and α-1 from SEA, PGE_2 was removed from SEA as well. Importantly, we observed that SEAΔα-1/ω-1 still promoted PGE_2 synthesis in moDCs (Figure 2D). This shows that SEA, in addition to containing PGE_2 itself, stimulates PGE_2 secretion by moDCs in an ω-1-independent fashion.

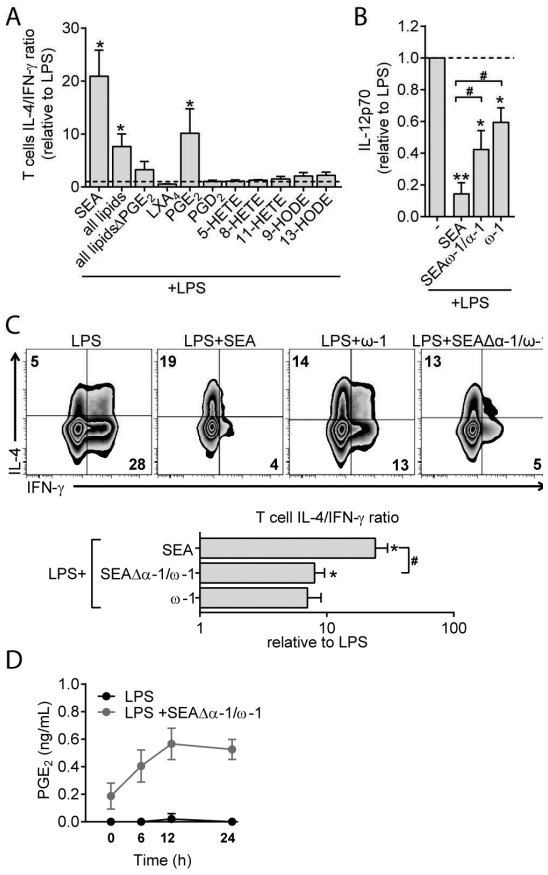

Figure 2. SEA stimulates PGE_2 secretion and primes Th2 responses independently from ω-1
(A) MoDCs stimulated with indicated lipids (concentration of 2.5 ng/mL for LXA_4, PGE_2 and PGD_2; 12,5 µg/mL for 5-HETE, 8-HETE and 11-HETE; 25 µg/mL 9-HODE and 13-HODE) were analysed for Th2 polarizing potential as described in Methods. The ratio of IL-4 over IFN-γ based on the intracellular cytokine staining was calculated relative to the control condition. (B) MoDCs were pulsed with indicated stimuli and subsequently co-cultured with a CD40L-expressing cell-line. Supernatants were collected after 24 h and IL-12p70 concentration were determined by ELISA. (C) T cell polarization was determined as in (A). Top and bottom panels show representative flow cytometry plots of intracellular staining of $CD4^+$ T cells for IL-4 and IFN-γ, and the ratio of IL-4 over IFN-γ ratio of these plots based on four experiments. Numbers in plots represent frequencies of cells in indicated quadrants. (D) PGE_2 levels as determined by LC-MS/MS in supernatants of moDCs stimulated with indicated stimuli. Data represent mean ± SEM of three independent experiments. *,#p<0.05 and **p<0.01 for significantly different with the LPS control (*) or between test condition (#) based on paired analysis (paired Student's T-test).

Next, we investigated the contribution of the synthesized PGE_2 by SEA-stimulated moDCs to ω-1-independent Th2 induction. Strikingly, when PGE_2 was neutralized using a specific anti-PGE_2 antibody during stimulation of moDCs with SEAΔα-1/ω-1, the ability of SEAΔα-1/ω-1-stimulated moDCs to drive Th2 polarization was totally lost (Figure 3A). In contrast, neutralization of PGE_2 in cultures of moDCs stimulated with ω-1 or complete SEA had no effect on the Th2-priming potential of these cells, which is consistent with our recently published study showing that ω-1, either alone or in the context of SEA can prime Th2 responses via other mechanisms [9, 10]. Moreover, we found that later neutralization of PGE_2 limited to the co-culture of SEAΔα-1/ω-1-stimulated moDCs with T cells did not impair Th2 polarization (Figure 3B), indicating that PGE_2 synthesized by moDCs does not act as a polarizing signal on T cells, but rather directly conditions moDCs in an autocrine manner to acquire a Th2-priming phenotype. In line with this observation, we found that simultaneous inhibition of the two major receptors of PGE_2, EP2 and EP4, on moDCs reduced the ability of SEAΔα-1/ω-1-stimulated moDCs to induce a Th2 response (Figure 3C). These results collectively demonstrate that SEA independently from ω-1 promotes PGE_2 synthesis by moDCs, which subsequently, in an autocrine manner, conditions these cells to acquire a Th2-polarizing phenotype.

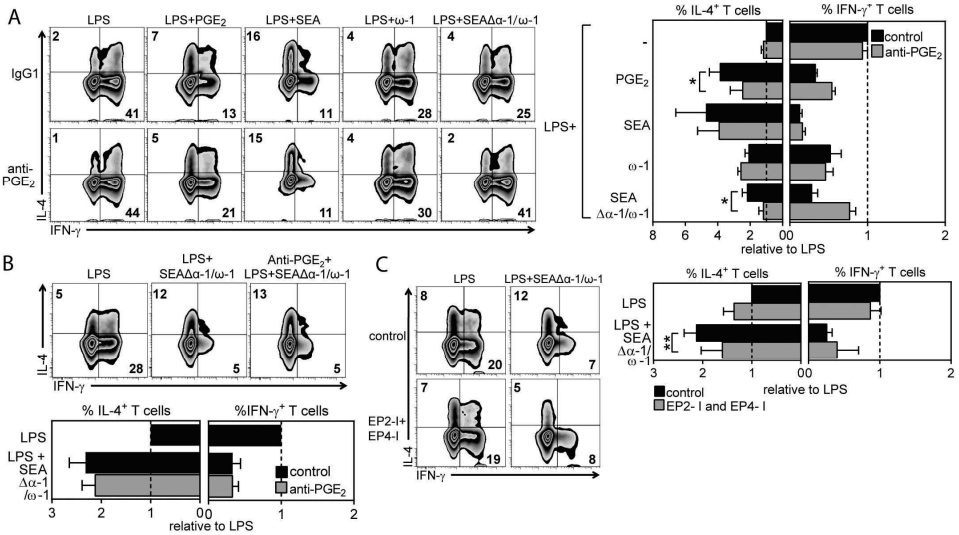

Figure 3. Omega-1-independent Th2 polarization by SEA is dependent on PGE_2 synthesis by moDCs
(A-C) T cell polarization assay as described in Figure 2A. (A) Neutralizing anti-PGE_2 antibody was added during stimulation of moDCs with indicated reagents or (B) during DC-T cell co-culture. (C) EP2 and EP4 receptor inhibitors were added during stimulation of moDCs with indicated stimuli. (A-C) Left: representative flow cytometry plots are shown of intracellular staining of $CD4^+$ T cells for IL-4 and IFN-γ. Numbers in plots represent frequencies of cells in indicated quadrants. Right: These data were used to calculate the fold change in frequency of $IL\text{-}4^+$ and $IFN\text{-}\gamma^+$ T cells polarized by moDCs stimulated with indicated stimuli relative the cytokine production by T cells polarized by LPS-stimulated moDCs, for which the values were set to 1. Bars represent mean ± SEM of at least four independent experiments. Significance was calculated based on the ratio of IL-4 over IFN-γ between conditions. *p<0.05 and **p<0.01 for significantly different from compared conditions based on paired analysis (paired Student's T-test).

OX40L is induced by SEA via PGE_2 signalling and is required for Th2 induction

MoDCs matured in the presence of PGE_2 are characterized by the expression of OX40L, a co-stimulatory molecule linked to Th2 polarization [28-30]. Moreover, an earlier study showed that moDCs stimulated with SEA express OX40L [3]. Indeed, we observed that stimulation of moDCs with PGE_2, SEA or SEAΔα-1/ω-1 induced expression of OX40L on moDCs, whereas ω-1 did not induce OX40L expression (Figure 4A). While both SEAΔα-1/ω-1 and SEA promote PGE_2 synthesis, OX40L induction by SEAΔα-1/ω-1 was lower than the levels induced by SEA. This might be explained by the fact that in contrast to SEAΔα-1/ω-1, SEA additionally contains pre-existing PGE_2 itself, resulting in higher overall concentrations of PGE_2 SEA-stimulated DCs are exposed to compared to SEAΔα-1/ω-1-primed DCs (Figure 1C versus Figure 2D). Importantly, neutralizing PGE_2 prevented the induction of OX40L expression by SEAΔα-1/ω-1 (Figure 4B). Finally, neutralizing OX40L during the co-culture with T cells significantly reduced the Th2-polarizing capacity of SEAΔα-1/ω-1-primed moDCs (Figure 4C). These data demonstrate that OX40L expression by SEAΔα-1/ω-1-conditioned-moDCs is dependent on PGE_2 and that subsequently induction of OX40L is important for Th2 polarization.

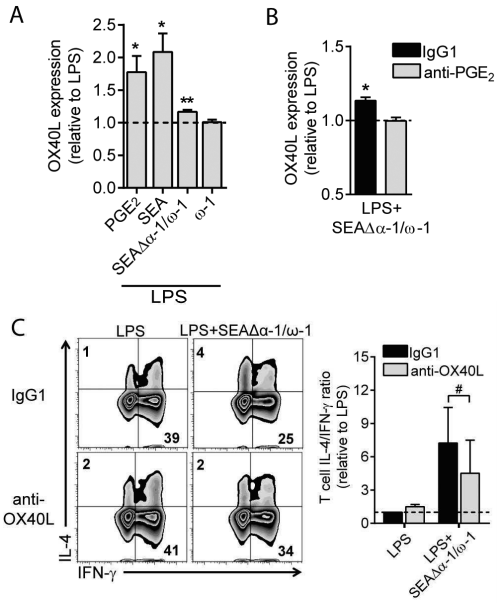

Figure 4. OX40L is induced by SEA via PGE_2 signalling and is required for Th2 induction
(A, B) MoDCs were stimulated as indicated for 48 h in the presence or absence of neutralizing anti-PGE_2 antibody after which expression of OX40L was analysed by flow cytometry. The fold change based on geometric mean fluorescence is shown relative to LPS, which is set to 1 (dashed line). (A) PGE_2 was taken along as positive control for OX40L expression. (C) T cell polarization assay as described in Figure 2C. Neutralizing OX40L antibody was added during the DC-T cell co-culture. Bar graphs represent means ± SEM of at least three independent experiments. *,#p<0.05, **,##p<0.01, ***,###p<0.001 for significant differences with the control conditions (*) or between test condition (#) based on paired analysis (paired Student's T-test).

SEA promotes PGE_2 synthesis and drives Th2 polarization via Dectin-1 and Dectin-2

Classically, central to the synthesis of PGE_2 is the release of AA from membrane phospholipids by $cPLA_2$, which can then be converted into PGs including PGE_2 through constitutively expressed COX-1 and stimulus-induced COX-2. We observed that SEA induced a small but consistent increase in $cPLA_2$ activity (Figure 5A). SEA did not change protein expression of COX-1 and was consistently expressed in all conditions. Moreover, SEA did not appear to promote COX-2 expression, nor to alter LPS-driven COX-2 expression (Figure 5B), suggesting that SEA primarily promotes PGE_2 synthesis through induction of $cPLA_2$ activation. Indeed, selective inhibition of $cPLA_2$ activity using pyrrophenone attenuated SEAΔα-1/ω-1-induced PGE_2 synthesis (Figure 5C). For these experiments in which we analysed the signalling events leading to PGE_2 synthesis we used PGE_2-free SEAΔα-1/ω-1 and not PGE_2-containing complete SEA, to be able to selectively assess *de novo* synthesis of PGE_2 by DCs. In addition, both COX-1 and COX-2 were important for PGE_2 synthesis by SEAΔα-1/ω-1, as treatment of moDCs with COX-1 and COX-2 inhibitors indomethacin and SC236 abrogated SEAΔα-1/ω-1-driven PGE_2 release (Figure 5C). Given that SEA has previously been reported to promote phosphorylation of extracellular-signal regulated kinase (ERK) [31] and that ERK can drive activation of $cPLA_2$ [32], we evaluated the role of ERK in SEA-driven PGE_2 synthesis. We found that SEA, in contrast to ω-1, induced phosphorylation of ERK (Figure 5D). and that inhibition of ERK signalling, using U0126, abrogated PGE_2 synthesis induced by SEAΔα-1/ω-1 (Figure 5E).

Next, we aimed to identify the receptors through which SEA activates this pathway leading to PGE_2 synthesis in moDCs. Previous studies have identified various CLRs through which SEA can be recognized by APCs. For human moDCs primarily fucose- and mannose-binding DC-SIGN and MR have been implicated in this process [13, 33-35]. Moreover, studies with murine APCs have also pointed to a possible role in recognition of components in SEA for Dectin-1 and Dectin-2, which are classically known for their ability to bind and respond to β-glucans and α-mannans from fungal origin, respectively [36]. Yet, whether components within SEA can be recognized and induce

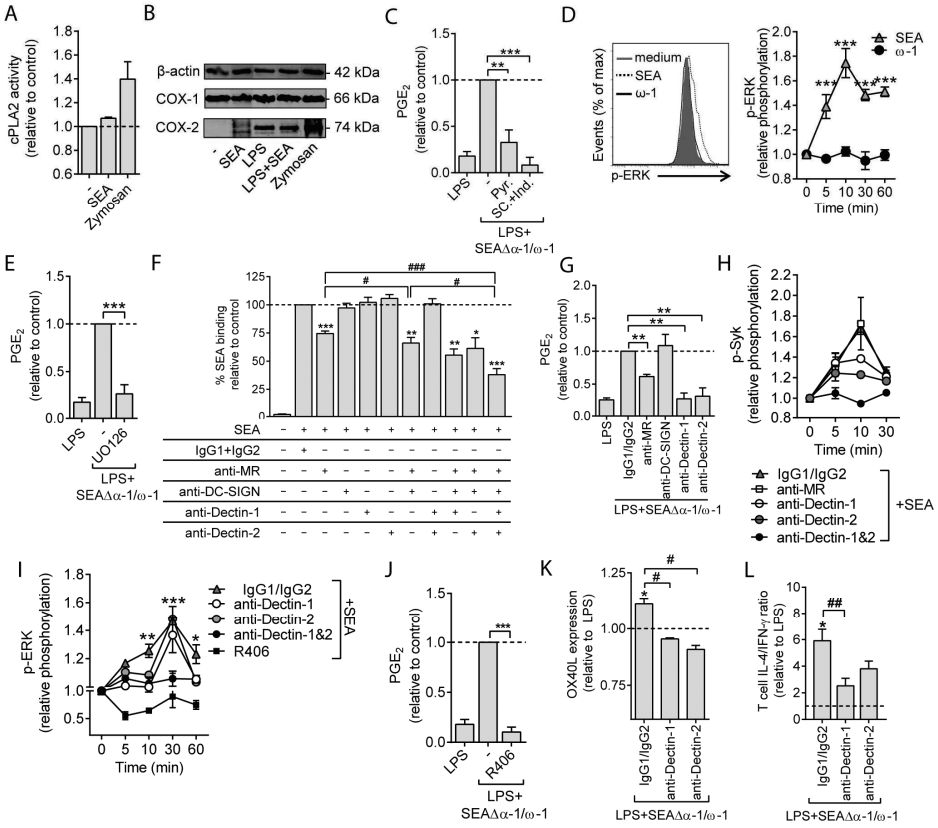

Figure 5. SEA promotes PGE_2 synthesis and drives Th2 polarization via signalling through Dectin-1 and Dectin-2

(A) $cPLA_2$ activity 8 h after stimulation. Zymosan was taken along as a positive control for $cPLA_2$ activation. (B) Protein expression of COX-1 and COX-2 were assessed by western blot. The β-actin was used as housekeeping protein. One of 3 experiments is shown. (C) Following 1 h pre-incubation with specific inhibitors for $cPLA_2$ (Pyrrophenone, [Pyr.]) or COX-1 and COX-2 (SC236 and Indometacin [SC.+ind.], respectively), moDCs were stimulated for 12 h with LPS+SEAΔα-1/ω-1 and supernatants were collected for PGE_2 determination by LC-MS/MS. (D) At the indicated time points after stimulation with depicted stimuli, phosphorylation of ERK was determined by flow cytometry. (E) PGE_2 levels were determined as in (C). U0216 was used as inhibitor of ERK. (F) MoDCs were treated 45 min with indicated blocking antibodies or isotype controls after which the cells were incubated with PF-647-labeled SEA. Antigen binding/uptake was analysed by flow cytometry and plotted as relative differences. (G) PGE_2 levels were assessed as in (C), following pre-incubation with blocking antibodies as described in (F). (H) Syk and (I) ERK phosphorylation were determined as described in (D) following pre-incubation with blocking antibodies as described in (F) or with Syk inhibitor R406. (J) PGE_2 levels were assessed as in (C). (K, L) MoDCs were pre-incubated with indicated blocking antibodies, followed by 48 h stimulation with LPS+SEAΔα-1/ω-1, after which OX40L expression was determined by flow cytometry. Data are based on geometric mean florescence. (L) Cells described in (K) were used for T cell polarization assay as described in Figure 2A. (A, C-L) data represent mean ± SEM of at least three independent experiments and are shown relative to control conditions, which are set to 1 (A, C-E, G-L) or 100% (F). *,#$p<0.05$, **,##$p<0.01$ and ***,###$p<0.001$ for significant differences with the control (*) or between test condition (#) based on paired analysis (paired Student's T-test).

signalling via human Dectin-1 and/or Dectin-2 expressed by DCs remains to be determined. As a first step towards the identification through which receptor(s) SEA induces PGE_2, we determined which of these receptors are involved in binding of SEA by moDCs. In line with earlier observations

[9, 13] blocking of MR reduced binding of fluorescently-labelled SEA, which could be further reduced when DC-SIGN binding was neutralized simultaneously. Interestingly, blocking of both Dectin-1 and Dectin-2 in conjunction with MR+DC-SIGN neutralization further reduced binding relative to blocking of just MR+DC-SIGN, suggesting that all 4 receptors contribute to recognition of glycans or glycoproteins present in SEA (Figure 5F). Next, we aimed to identify through which of these CLRs SEA promotes PGE_2 synthesis. We found that blocking of either Dectin-1 or Dectin-2, but not DC-SIGN, strongly attenuated PGE_2 synthesis induced by SEAΔα-1/ω-1, while blocking MR also resulted in reduced PGE_2 synthesis albeit to a lesser extent. This suggests a major role for Dectin-1 and Dectin-2 in SEAΔα-1/ω-1-driven PGE_2 synthesis (Figure 5G). Dectin signalling is mediated by the immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic domains of Dectins that phosphorylate and activate spleen tyrosine kinase (Syk) either directly (Dectin-1) or following FcR recruitment (Dectin-2). Syk in turn can promote ERK phosphorylation [37]. Indeed, we observed that SEA stimulation resulted in phosphorylation of Syk, which was dependent on both Dectin-1 and Dectin-2 but not MR (Figure 5H), and found that SEA-driven ERK phosphorylation was dependent on Dectin-1, Dectin-2 and Syk signalling (Figure 5I). Of note, blocking of either Dectin-1 or Dectin-2 alone only had minor effects on Syk and ERK phosphorylation, while these signals were totally blunted when both Dectin-1 and Dectin-2 signalling were blocked simultaneously, suggesting that SEA depends on both receptors to activate this pathway. In line with these findings, inhibition of Syk signalling blunted SEAΔα-1/ω-1-induced PGE_2 synthesis (Figure 5J). Finally, blocking either Dectin-1 or Dectin-2 attenuated OX40L expression (Figure 5K) as well as the Th2 response induced by SEAΔα-1/ω-1 (Figure 5L). In conclusion, these data suggest that SEA promotes PGE_2 synthesis by moDCs through MR, Dectin-1 and -2 and via a signalling cascade involving Syk, ERK, $cPLA_2$, COX-1 and 2, that is required for Th2 induction by SEA.

PGE_2 isomer generation via autoxidation contributes to Th2 induction by SEA

The observations that PGE_2 promoted Th2 induction by SEA and that this PGE_2 synthesis was dependent on COX activity, led us to hypothesize that blocking of COX activity in SEAΔα-1/ω-1-stimulated moDCs would abrogate their ability to prime Th2 responses. Surprisingly however, inhibition of COX-1 and -2 only partly reduced the Th2 response induced by these SEAΔα-1/ω-1-conditioned DCs (Figure 6A). A possible explanation for this unexpected result came from a careful reanalysis of the extracted ion chromatogram of the PGE_2 trace (*m/z* 351 -> 271) in which we noted that alongside PGE_2, moDCs stimulated with SEA produced PGE_2 isomers, also known as isoprostanes (IsoPs) (Figure 6B). Several studies have suggested that isoPs may have similar properties as PGE_2, but that in contrast to the latter they are generated by an autoxidation process directly from AA fuelled by reactive oxygen species (ROS), independently from COX activity [26]. We found isomers '1' and '2' (Figure 6B) to have a fragment ion *m/z* 189 that is characteristic for 15-series IsoPs with identical relative retention times to commercially available 15*R*-PGD_2 and 11β-PGE_2, respectively, suggesting these isomers are 15*R*-PGD_2 and 11β-PGE_2 (Figure 6C) [38]. Isomer '3' showed a somewhat different tandem MS spectrum indicating it belongs to the class of 5-series IsoPs, but this could not be confirmed due to a lack of standard material (Figure 6D) [39].

To provide a mechanistic explanation for how SEA stimulation results in IsoP generation by moDCs, we test whether SEA could induce ROS production. Consistent with earlier observations in murine DCs [33, 36], we observed that human moDCs stimulated with SEA and SEAΔα-1/ω-1, but not with ω-1, resulted in ROS production (Figure 6E) that was dependent on signalling through Syk

(Figure 6F). To determine the biological significance of the generation of these two 15-series IsoPs in response to SEA, we first determined whether 15*R*-PGD_2 and 11β-PGE_2 IsoPs could affect T cell polarization. Interestingly, these two IsoPs could condition moDCs for priming of a Th2 response, which could be blocked by treatment with anti-PGE_2 (Figure 6G). Importantly, in contrast to COX inhibition alone, pre-treatment with COX inhibitors in conjunction with ROS scavenger *N*-acetyl-*L*-cysteine (NAC), abrogated the ability of SEAΔα-1/ω-1-stimulated moDCs to prime a Th2 response (Figure 6H), suggesting that enzymatically generated PGE_2 and its isomers act in concert to condition moDCs for Th2 polarization.

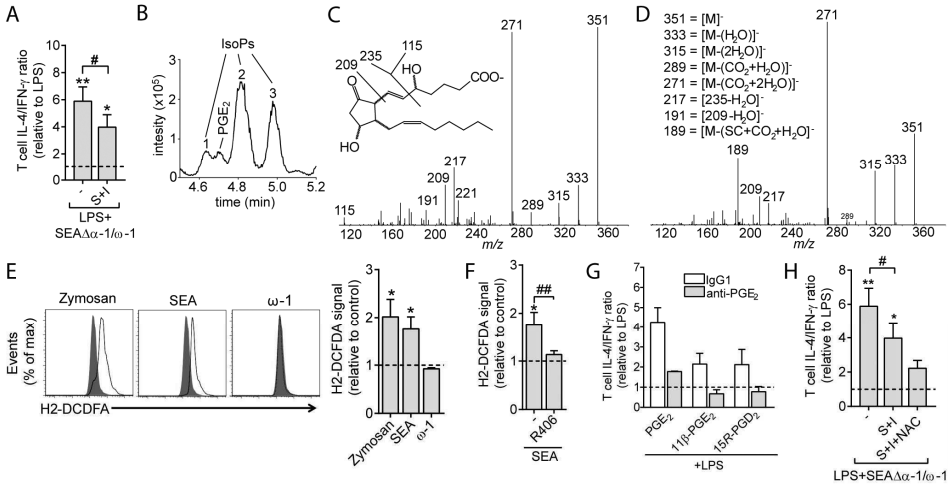

Figure 6. SEA-induced ROS production by moDCs results in PGE_2 isomers synthesis that contribute to Th2 polarization

(A) T cell polarization assay as described in Figure 2A in the presemce of COX inhibitors SC236 (S) and indomethacin (I). Bars represent mean ± SEM of at least three independent experiments. (B) LC-MS/MS trace showing the transition *m/z* 351 -> 271, the detected IsoPs are indicated by numbers. (C) Tandem MS spectrum of isomer 2, showing the fragment ion *m/z* 189, characteristic for 15-series IsoPs. (D) Showing the MS/MS spectrum of isomer 3, possibly identifying this isomer as a 5-series isoP, based on the fragment ions *m/z* 115, 217 and 191. (E) ROS generation was determined by flow cytometry (H2-DCFDA) of moDCs pulsed for 6 h with indicated reagents. On the left representative histograms for ROS induction are shown. On the right the geometric mean fluorescence of these signals is enumerated and shown as fold change relative to unstimulated moDCs (dashed line set to 1). (F) ROS production was quantified as in (E) following pre-treatment with general ROS scavenger Nac or R406 for 1 h. (G) MoDCs were stimulated with indicated PGs with or without anti-PGE_2 after which a T cell polarization assay was performed as described in Figure 2A. (H) MoDCs were stimulated with indicated reagents in the presence following 1 h pre-incubation with COX inhibitors (S+I) and NAC after which a T cell polarization assay was performed as described in Figure 2A. Bars represent mean ± SEM of at least three independent experiments. *,#$p<0.05$ and **$p<0.01$ for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (paired Student's T-test).

S. mansoni egg-driven Th2 polarization *in vivo* depends on Dectin-2 and Syk

We next aimed to assess the importance of this Dectin-Syk signalling in mediating Th2 polarization by *S. mansoni* egg antigens *in vivo*. First, to test the importance of Syk in Th2 polarization by DCs in response to egg antigen challenge *in vivo*, we made use of $Itgax^{cre}$ $syk^{fl/fl}$ mice ($CD11c^{\Delta syk}$), which selectively lack Syk expression in $CD11c^{+}$ DCs. We found that following subcutaneous immunization with SEA, $CD4^{+}$ T cells from draining LNs from $CD11c^{\Delta syk}$ mice, compared to $CD11c^{WT}$ controls, produced less Th2 cytokines *ex vivo* in response to both polyclonal (Figure 7A) and antigen-specific restimulation (Figure 7B), while IFN-γ production was not different between the

two groups of mice. These data validate our *in vitro* findings and suggest that Syk signalling in DCs plays a key role in Th2 priming by *S. mansoni* egg antigens *in vivo*.

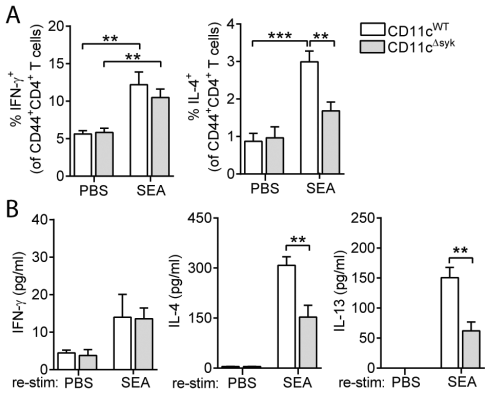

Figure 7. Th2 polarization induced by SEA mediated partly via Syk signalling *in vivo*
(A, B) WT or CD11c$^{\Delta syk}$ mice were injected with SEA in the hind footpad and draining pLNs were analysed 7 d later. (A) pLN cells were re-stimulated with PMA/Ionomycin in the presence of brefeldin A and CD4$^+$ T cells were stained for indicated intracellular cytokines. (B) pLN cells were re-stimulated with SEA for 72 h, and cytokine levels in culture supernatants were determined. Bars represent mean ± SEM of one of two independent experiments with 5 mice per group. *p<0.05, **p<0.01 and ***p<0.001 for significant differences relative to PBS treated mice based on un-paired analysis (un-paired Student's T-test).

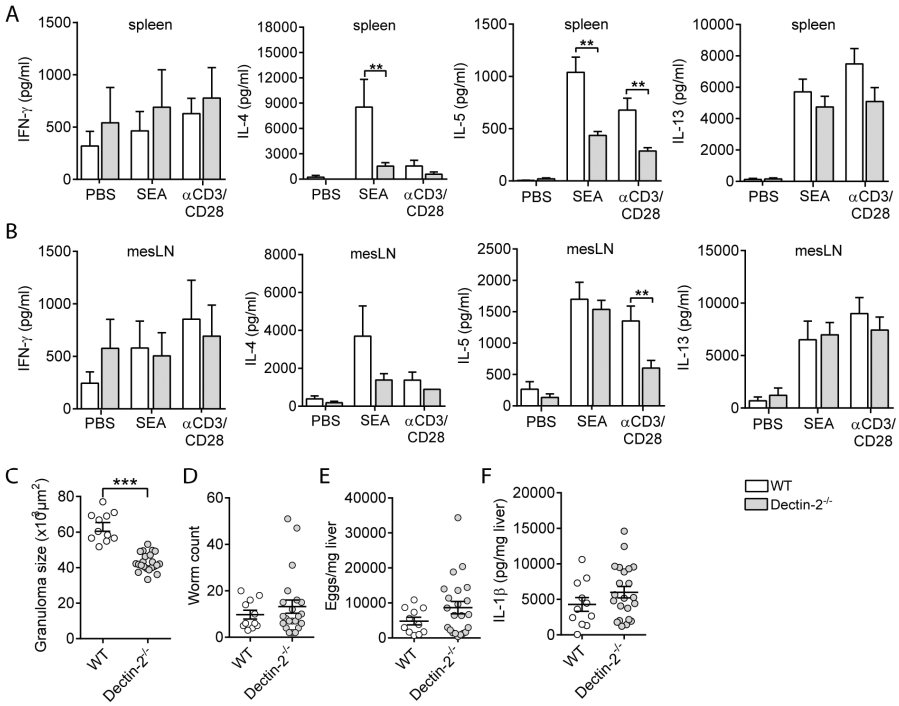

Figure 8. Dectin-2 signalling is required for induction of a Th2 response during *S. mansoni* infection
WT and Dectin-2$^{-/-}$ mice were infected with *S. mansoni*. After 8 weeks of infection cells from (A) spleens or (B) mLNs were re-stimulated with SEA or anti-CD3/CD28 for 72 h and cytokine levels were analysed in supernatants by luminex or ELISA. Bars represent mean ± SEM of combined data of at least two or three independent experiments with 5 to 10 mice per group. (C) Granuloma sizes around eggs trapped in the liver of 8 weeks infected mice were assessed in masson blue stained liver sections. Data are based on 10 mice per group. Number of worms (D) and liver and intestinal eggs (E) in 8 weeks *S. mansoni*-infected mice. (F) IL-1β protein levels in livers of 8 weeks *S. mansoni*-infected mice. **p<0.01 and ***p<0.001 for significant differences relative to the control mice based on un-paired analysis (un-paired Student's T-test).

Finally, we set out to assess the importance of this signalling axis in Th2 differentiation and Th2-driven immunopathology during a natural infection with *S. mansoni*, using Dectin-1 and Dectin-2 knock out mice (Dectin-$1^{-/-}$ and Dectin-$2^{-/-}$). During *S. mansoni* infection adult worms residing in the portal vasculature release eggs that get trapped in the liver where they induce strong Th2 responses that orchestrate the development of granulomatous lesions surrounding the eggs [8]. The intensity of the Th2 response and associated granulomatous inflammation peaks at 8 weeks after infection. To compare the Th2 response induced by this infection in WT and Dectin-$1^{-/-}$ and Dectin-$2^{-/-}$ mice, cells from mesenteric LNs and spleens from 8 weeks infected mice were re-stimulated with SEA or anti-CD3/CD28. We found that infected Dectin-$2^{-/-}$ mice displayed lower production of Th2 cytokines IL-4 and IL-5 in both lymphoid organs in comparison to their infected WT counterparts, while IFN-γ production was not different between the two groups (Figure 8A, B). In infected Dectin-$1^{-/-}$ mice only IL-5 production by splenocytes was reduced (Figure S2A, S2B). Importantly, in line with the reduced Th2 responses found in the infected Dectin-$2^{-/-}$ mice, granuloma size around the eggs trapped in the liver was smaller relative to infected WT mice (Figure 8C). Of note, this difference in Th2 response was not due to differences in infection load, as both Dectin-$2^{-/-}$ and WT mice were found to harbour similar numbers of eggs and adult worms (Figure 8D, E). Dectin-$1^{-/-}$ mice did not show an altered granulomatous response towards liver eggs (Figure S2C), nor did they show differences in numbers of adult worms and eggs (Figure S2D, S2E). We previously found that inflammasome activation, which can be triggered by SEA via Dectin-2 signalling, can alter T cell polarization and contributes to granuloma formation during *S. mansoni* infection [33]. However, levels in IL-1β protein, as readout for inflammasome activity, in liver were similar between Dectin-$2^{-/-}$ and WT mice (Figure 8F). Altogether, these findings highlight an important role for Dectin-2 in promoting Th2 differentiation and immuno-pathological outcome of this response during *S. mansoni* infection.

DISCUSSION

The molecular mechanisms through which DCs prime Th2 responses, including those elicited by helminths, are still incompletely understood. We here explored the role of PUFAs and LMs in Th2 induction by *S. mansoni eggs*, that are well known for their potent ability to elicit strong Th2 responses. This enabled us to identify a novel signalling axis in DCs involving Dectin-Syk-PGE_2-OX40L through which Th2 responses are induced *in vitro* and *in vivo*.

Some studies have been documented that different life stages of *S. mansoni* are able to produce LMs from both COX products (e.g: PGE_1, PGE_2, PGD_2 and PGA_2) and Lipoxygenase products (e.g: LTB_4, 5-HETE, 12-HETE)[26, 40]. However, the existence of LMs in eggs or SEA had not been examined before. Here we uncovered that SEA itself contains various PUFAs and LMs with potential immunomodulatory properties. In particular, the presence of the well-studied immunomodulatory eicosanoid PGE_2 caught our attention, since among the pleiotropic properties that have been attributed to this lipid, it has been associated with promoting Th2 polarization by functional modulation of DCs [3, 29]. Moreover, we found PGE_2 not only to be present in SEA, but also to be synthesized by DCs themselves in response to SEA stimulation. While, other life cycle stages of *S. mansoni* have been shown to promote PGE_2 synthesis in host cells such as cercariae in keratinocytes [27], we are the first to report and mechanistically investigate the ability of egg-derived antigens to promote PGE_2 synthesis in immune cells. Importantly, we found that this PG, in contrast to several other LMs secreted by SEA-stimulated DCs, was not only sufficient to condition moDCs for Th2 polarization, but also crucial for mediating ω-1-independent Th2 polarization by

SEA. This identifies PGE_2 as a key factor through which SEA, independently from ω-1, primes Th2 responses. A question that remains to be answered is what the relative contribution is of the pre-existing PGE_2 (present in SEA) *versus* PGE_2 synthesized by moDCs in mediating the Th2-polarizing effect. The observation that SEA from which ω-1 was depleted, was fully dependent on *de novo* synthesized PGE_2 by the moDCs for Th2 polarization, at least shows that moDC-derived PGE_2 can be sufficient for promoting a Th2 response. Moreover, the fact that SEA requires Syk signalling in DCs to prime a Th2 response *in vivo*, would argue that PGE_2 derived from SEA itself is insufficient to condition DCs for Th2 priming and that SEA instead rather depends on Syk-driven *de novo* PGE_2 synthesis for this response.

Mechanistically, we found that PGE_2 derived from moDCs acts in an autocrine manner to promote Th2 polarization by promoting the expression of OX40L in moDCs. OX40L expression has been shown to be important for Th2 polarization by DCs stimulated with various other Th2-priming stimuli, such as allergens and TSLP [30, 41, 42]. Congruent with these studies we found that OX40L expression was crucial for Th2 polarization by SEA from which ω-1 was depleted. While expression of OX40L in response to SEA has been documented before [3, 12], we now provide evidence that SEA-induced OX40L expression in moDCs is secondary to its ability to induce PGE_2 synthesis by these cells.

Moreover, we found that SEA from which ω-1 was depleted was dependent on signalling through both Dectin-1 and Dectin-2 to condition moDCs to drive Th2 polarization. Dectin-2 has been linked to Th2 polarization before in the context of allergic responses induced by house dust mite [43, 44]. In these studies Dectin-2 was found to mediate Th2 induction through the generation of cysteinyl leukotrienes by murine DCs. However, we did not observe induction of cysteinyl leukotrienes by SEA. Instead, we found that SEA interacts with Dectin-1, Dectin-2 and MR to promote PGE_2 synthesis. Downstream of these receptors we identified a pathway involving, Syk, ERK, and $cPLA_2$, that leads to the release of AA, which subsequently acts as a substrate for COX to produce PGE_2. The observation that MR also seems to play a role in SEA-driven PGE_2 synthesis, despite the fact that MR itself in contrast to Dectin-1 and Dectin-2 does not harbour an intracellular signalling motif, leads us to speculate that MR may collaborate with Dectin-1 and/or Dectin-2 to form complexes that effectively bind glycans or glycoproteins in SEA that allow for efficient activation of the signalling cascade downstream of Dectins resulting in PGE_2 synthesis. Associations of different CLRs to potentiate glycan-induced signalling have been described before for Dectin-2 and Dectin-3 [45]. Glycans derived from the cell wall of fungi such as *Candida albicans* [32] are well known to promote PGE_2 synthesis through this pathway via activation of Dectin-1, Dectin-2 and MR [46, 47]. However, in this context the production of PGE_2 seems to contribute to Th17 priming by APCs and not Th2 [46]. This difference might be explained by differences in glycan repertoire between fungi and schistosome eggs. For instance, classical β-glucans expressed by fungi are not present in SEA [48]. Therefore, the carbohydrates in SEA that mediate Dectin binding, may interact differently with, or have a lower affinity for these receptors than fungal carbohydrates do. This may induce a qualitatively and/or quantitatively different signalling cascade that could trigger sufficient PGE_2 synthesis to allow for Th2 induction to occur, without promoting the expression of high level of Th17-promoting cytokines. Secondly, fungal Dectin agonists may trigger additional PRRs to induce pro-inflammatory cytokine expression that are not activated by SEA [49]. Finally, the immunological outcome of Dectin engagement can also be cell type-dependent. For instance, Dectin-1 ligand curdlan was found to promote Th2 responses via plasmacytoid DCs while this same ligand conditioned myeloid DCs to inhibit Th2 responses [50] or

to promote Th9 responses [51]. Interestingly, similar to our observations with SEA, the conditioning of plasmacytoid DCs by curdlan to promote Th2 responses was dependent on induction of OX40L expression [50]. Currently, studies are ongoing to identify the glycoproteins or glycan moieties present in SEA that bind to Dectin-1, Dectin-2 and MR to promote this response.

We provide evidence that COX-independent generation of several PGE_2 isomers (isoPs) by SEA, independently from enzymatically synthesized PGE_2 are capable of conditioning moDCs for Th2 priming. While COX-independent generation of these isomers has been described before, as a result of auto-oxidation of AA by free radicals [26], we now here show a role for these isoPs in regulation of an immune response. In line with the free radical-dependent synthesis of isoPs, we found that SEA could drive ROS production in a Syk-dependent manner, which corroborates a recent study showing that SEA can induce ROS in murine DCs [33]. However, the latter study focused on the role of ROS in SEA-driven inflammasome activation, and did not report on other ROS-mediated effects. Our data suggest that that enzymatically synthesized PGE_2 and its ROS-induced isomers act in concert in Dectin-mediated conditioning of moDCs for Th2 priming by SEA. Moreover, we found that the widely used neutralizing anti-PGE_2 antibody [52] that we have used in this study not only neutralizes PGE_2, but also harbours cross-reactivity towards two of the main isoPs that we found to be generated by SEA-stimulated DCs. This can explain our observation that PGE_2 neutralization, in contrast to COX inhibition, did fully block Th2 polarization.

We found that mice which were deficient for Syk in their DCs, failed to mount a Th2 response following SEA immunization *in vivo*, which provides strong support for a key role of the dectin-Syk-PGE_2-OX40L axis in Th2 polarization by *Schistosoma* egg-derived antigens *in vivo*. Moreover, our studies with *S. mansoni*-infected Dectin-$2^{-/-}$ mice suggest that also during natural infection this signalling axis seems to be crucial for induction of Th2 responses. SEA has previously been reported to activate the Nlrp3-inflammasome in a Dectin-2-dependent manner and inflammasome-deficient mice were shown to have an altered T cell polarization profile and a reduction in granuloma size during *S. mansoni* infection similar to our observations reported here in infected Dectin-$2^{-/-}$ mice. However, the fact that in contrast to what was observed in inflammasome deficient mice [33], there was no reduction in total IL-1β levels in livers of Dectin-$2^{-/-}$ mice during *S. mansoni* infection, would suggest that downstream of Dectin-2 the PGE_2-OX40L axis, rather than inflammasome activation, plays a key role in Th2 priming during, and in the immuno-pathological outcome of, this infection. Nonetheless, additional studies will be needed to definitively determine the individual contribution of each pathway to the immunopathology *in vivo*. Somewhat surprising was the observation that in contrast to the *in vitro* data Dectin-1 appears to be less important for Th2 polarization *in vivo*. This may suggest that murine Dectin-1, as opposed to its human counterpart, does not play a major role in recognition of glycans present in SEA, which, although currently still speculation, might be due to differences in glycan-specificity or in expression of Dectin-1 isoforms between murine and human DCs [53, 54]. More detailed comparative studies between the SEA-binding characteristics of human and murine Dectin-1 could provide more molecular insight in the mechanisms underlying the difference in requirement for Dectin-1 in Th2 polarization by SEA between the human *in vitro* and murine *in vivo* models.

In summary, we propose a model (Figure 9) in which SEA can condition DCs for Th2 polarization independently from ω-1 by triggering Dectin-1, Dectin-2 and MR to induce in a Syk-dependent fashion the synthesis of PGE_2 and isoPs, that subsequently promote OX40L expression in an autocrine manner. OX40L then enables the SEA-stimulated moDCs to prime a Th2 response. Importantly, the fact that neutralization of PGE_2 and its isomers completely blunted the Th2-

priming ability of moDCs that had been stimulated with SEA from which ω-1 had been depleted, provides strong support for the notion that this pathway can fully account for 'residual' ability of SEA to prime Th2 polarization in the absence of ω-1. However, the fact that this same intervention had little or no effect on the Th2-priming capacity of complete SEA in our *in vitro* DC assay, suggests that the Dectin-PGE_2-OX40L signalling axis can be compensated for by the presence of ω-1, which employs distinct mechanisms to condition moDCs for Th2 polarization, and that this novel axis is only unmasked *in vitro* when ω-1 is removed from SEA. *In vivo*, however, the contribution of Dectin-PGE_2-OX40L signalling axis in egg antigen-driven Th2 polarization appears to be much more dominant, given that interference with Syk or Dectin signaling, did result in an impaired Th2 response induced by complete SEA or by a natural infection with *S. mansoni,* respectively. This would be corroborated by our previous work showing that SEA from which ω-1 was depleted was still potent in inducing a Th2 response as complete SEA *in vivo* [20] and that ω-1 knockdown in *S. mansoni* eggs by lentiviral transduction did not reduce Th2 responses induced by the eggs *in vivo* [19]. Together this leads us to speculate the Dectin-PGE_2-OX40L signalling axis plays a more important role in schistosome egg-driven Th2 polarization that *in vivo* than the one that is driven by ω-1 in which MR and suppression of protein synthesis play a central role [9].

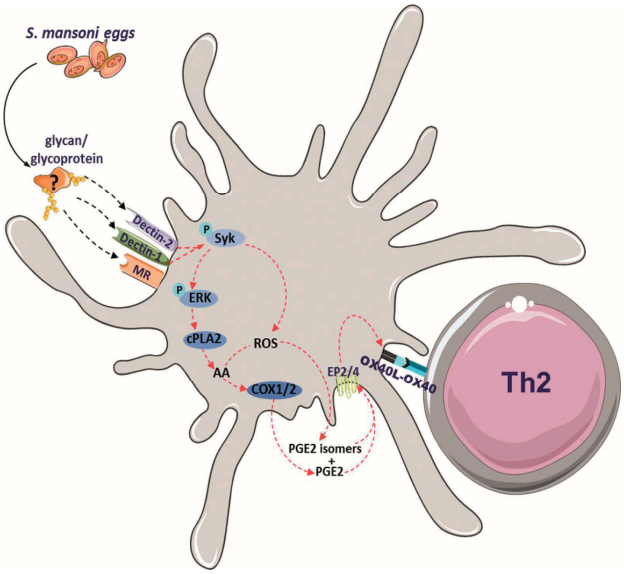

Figure 9. Proposed model of *S. mansoni* egg-driven Th2 polarization
The molecules derived from SEA recognized by Dectin-1, Dectin-2 further activate Syk and lead to two intracellular pathways in moDCs: ERK-$cPLA_2$-COX and ROS activity result in PGE_2 and PGE_2 isomers synthesis respectively. Both PGE_2 and its isomers bind to EP2 and EP4 in an autocrine loop. These series of signalling pathways give rise to a Th2-inducing phenotype of moDCs by promoting OX40L expression.

In conclusion, we have delineated a previously unrecognized pathway involving Dectin-1/2, PGE_2 and OX40L through which Th2 immunity is induced. Targeting this axis may hold promise as an approach to regulate type 2 immune responses for therapeutic purposes, not only in the context of *S. mansoni* and other helminth infections, but possibly also in major diseases of the western world, such as allergies and type 2 diabetes, that are caused by overzealous and defective type 2 immune responses, respectively.

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SUPPLEMENTARY DATA

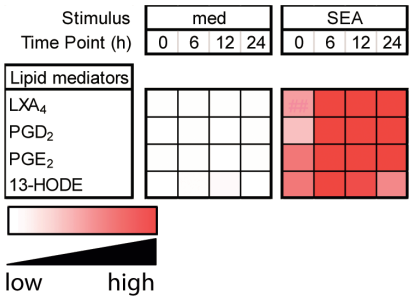

Figure S1. Heat-map of lipid changes in supernatants of SEA-stimulated moDCs

Monocyte-derived DCs were pulsed with SEA after which supernatant were collected at 0, 6, 12 and 24 h after stimulation. Relative amounts of indicated PUFAs detected by LC-MS/MS in supernatants are shown in a heat-map. Data represent one of two independent experiments.

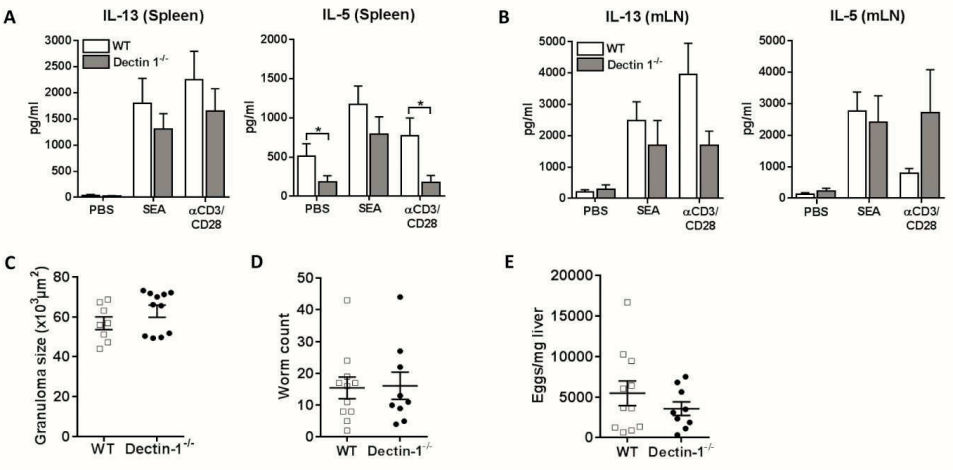

Figure S2. Dectin-1 signalling plays a minor role in Th2 priming during *S. mansoni* infection

WT and Dectin-1$^{-/-}$ mice were infected with *S. mansoni*. After 8 weeks of infection cells from (A) spleens or (B) mLNs were re-stimulated with SEA or anti-CD3/CD28 for 72 h and cytokine levels were analysed in supernatants by ELISA. Bars represent mean ± SEM of combined data of at least two or three independent experiments with 5 to 10 mice per group. (C) Granuloma sizes around eggs trapped in the liver of 8 weeks infected mice were assessed in Masson blue stained liver sections. Data are based on 10 mice per group. Number of worms (D) and liver and intestinal eggs (E) in 8 weeks *S. mansoni*-infected mice. *p<0.05 for significant differences relative to the control mice based on un-paired analysis (un-paired Student's T-test).

Table S1. Lipid Mediators (LM) including Polyunsaturated Fatty Acids (PUFAs) that were measured using LC-MS/MS.

LMs analyzed by GC-MS/MS					
1	10-HDHA	10-hydroxy Docosahexaenoic acid	29	8*S*,15*S*-diHETE	18S, 15S-dihydroxyeicosatetraenoic acid
2	11-HETE	11-hydroxyeicosatetraenoic acid	30	9-HODE	9-hydroxyoctadecadienoic acid
3	12-HETE	12-hydroxyeicosatetraenoic acid	31	9-HoTrE	9-hydroxyoctadecatrienoic acid
4	13,14dihydro-15-keto-PGE_2	13, 14dihidro-15-keto-Prostaglandin E2	32	AA	Arachidonic acid
5	13,14dihydro-15-keto-$PGF_2\alpha$	13, 14dihidro-15-keto-Prostaglandin F2α	33	AdA	Adrenic acid
6	13-HODE	13-hydroxyoctadecadienoic acid	34	ALA	α-linolenic acid
7	13-HoTrE	13-hydroxyoctadecatrienoic acid	35	AT-LXA_4	AT-Lipoxin A4
8	14,15-diHETE	14, 15-dihydroxyeicosatetraenoic acid	36	AT-RvD1	AT-Resolvin D1
9	15-HEPE	15-hydroperoxyeicosapentanoic acids	37	DHA	Docosahexaenoic
10	15-HETE	15-hydroxyeicosatetraenoic acid	38	DPA_{n-3}	Docosapentaenoic acid n-3
11	15-Keto-PGE_2	15-Keto-Prostaglandin E2	39	EPA	Eicosapentaenoic acid
12	17-HDHA	17-hydroxy Docosahexaenoic acid	40	LA	Linoleic acid
13	17-OH-DH-HETE	17-OH-DH- hydroxyeicosatetraenoic acid	41	LTB_4	Leukotriene B4
14	18-HEPE	18-hydroperoxyeicosapentanoic acids	42	LTD_4	Leukotriene D4
15	18*R*-RvE3	18R-Resolvin E3	43	LTE_4	Leukotriene E4
16	18*S*-RvE3	18S-Resolvin E3	44	LXA_4	Lipoxin A4
17	19,20-diHDPA	19, 20-dihydroxydocosapentanoic acid	45	MaR1_2	Maresin 1_2
18	20-OH-LTB_4	20-OH-Leukotrine B4	46	PDX	Protectin DX
19	5,15-diHETE	5, 15-dihydroxyeicosatetraenoic acid	47	PGD_2	Prostaglandin D2
20	5-HETE	5-hydroxyeicosatetraenoic acid	48	PGE_2	Prostaglandin E2
21	6*t*,12*epi*-LTB_4	6t, 12epi-Leukotrine B4	49	$PGF_2\alpha$	Prostaglandin F2α
22	6-*trans*-LTB_4	6-trans-Leukotrine B4	50	PGJ_2	Prostaglandin J2
23	7,17-diHDPA	7, 17-dihydroxydocosapentanoic acid	51	RvD1	Resolvin D1
24	7-HDHA	7-hydroxy Docosahexaenoic acid	52	RvD2	Resolvin D2
25	7*S*-MaR1	7S-Maresin 1	53	RvE1	Resolvin E1
26	8-HETE	8-hydroxyeicosatetraenoic acid	54	RvE2	Resolvin E2
27	8-iso-PGE2	8-iso-Prostaglandin E2	55	TxB_2	Thromboxane-B2
28	8-*iso*-$PGF_2\alpha$	8-iso-Prostaglandin F2α			