Helminth infections induce immunomodulation : consequences and mechanisms

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TLR ligation in the context of bacterial or helminth extracts in human monocyte derived dendritic cells: molecular correlates for Th1/Th2 polarization

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Summary

Recognition of pathogens by dendritic cells (DCs) through interaction with pattern recognition receptors (PRRs), including Toll like receptors (TLRs), is crucial for the initiation of appropriate adaptive immune responses. Yet, the characteristics and differences in molecular profiles of DCs with different T cell polarizing capacities are still poorly defined. To address this issue, the molecular profile of human monocyte derived DCs was characterized after exposure to Th1 activating bacterial extracts from *Listeria monocytogenes* (HKLM) and *Escherichia coli* and the Th2 activating helminth derived phospholipids (PS) from *Schistosoma mansoni* and *Ascaris lumbricoides*, all with TLR2 activating capacity. We find that the ratio of activated MAP kinase *p*-ERK / *p*-p38 is lower in the DCs stimulated with the bacterial products compared to the DCs stimulated with the helminth products, which correlates with the Th1 and Th2 polarizing capacity of these compounds. Furthermore, the mRNA expression profiles induced by the bacterial and helminth derived products differ widely. Notch ligand delta-4 and transcription factor c-fos are differentially regulated and show a strong correlation with Th1 and Th2 polarization, respectively. The molecular profile induced by the two bacteria share a comparable molecular profile, while DCs exposed to the Th2 promoting lipid extracts have a profile that is similar to that induced by *S. mansoni* derived glycoproteins (SEA). These data show that TLR2 activating compounds embedded within different antigen sources can induce very distinct DC programming and suggest that the polarizing capacity of compounds can be predicted with the molecular signature they induce in DCs.

Introduction

Dendritic cells (DCs) are antigen presenting cells that play a pivotal role in the initiation of adaptive immune responses. These cells function as sentinels in the periphery where they are able to recognize and respond to stimuli from the environment they reside in, some of which could be products from invading micro-organisms or helminths. Upon such exposures DCs undergo phenotypic changes that allow them to effectively migrate to lymph nodes and prime appropriate T cell responses [88, 190].
The type of compounds encountered by DCs will determine to a large extent the nature of the T cell polarization promoted by these DCs. For this, DCs have to be able to distinguish these different classes of molecules. To this end, DCs express several classes of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors, Nod-like receptors and RIG-I like receptors that are able to recognize specific pathogen derived components, the so-called pathogen associated

molecular patterns (PAMPs). Upon engagement of these receptors, signalling cascades are initiated that involve activation of the mitogen activated protein kinases (MAPKs) and NF-κB, and induction of expression of genes involved in DC maturation and the ability to prime and skew T cell responses. It is known that intracellular organisms are primarily capable of instructing DCs to induce T helper (Th) 1 responses [191], whereas extracts of parasitic helminths have been demonstrated to drive Th2 skewed responses [40, 96, 191].

Relatively much is known about the signalling pathways in DCs induced after triggering of PRRs [103, 192, 193], however, the molecular characteristics that are different for DCs that have been activated by Th1 or Th2 promoting PAMPs are much less understood [194, 195]. We set out to address this issue by characterizing human monocyte derived DCs after exposure to bacterial and helminth derived products. The characterization of the DCs comprised gene expression analysis of 25 genes that have been linked to activation and T cell polarizing properties of DCs. These molecular profiles of the DCs were correlated to their T cell polarizing capacity. In this study we used Gram-positive heat killed *Listeria monocytogenes* (HKLM) and Gram-negative *Escherichia coli*, both of which stimulate TLR2 activity and induce Th1 polarization. In addition, *Schistosoma mansoni* and *Ascaris lumbricoides* derived phosphatidylserine containing preparations (PS) were used, that also activate TLR2, but drive Th2 responses. We show that the signalling routes and the resulting mRNA expression profiles following stimulation by the bacterial and helminth derived products are very distinct. This indicates that not all extracts that contain TLR2 activating components lead to similar DC programming and suggests that there is a general molecular DC1 and DC2 signature that can be used to predict Th1 and Th2 skewing potential of DCs.

Materials and methods

Antigen preparation

Phosphatidylserine containing preparations (PS) were extracted from 4 gram of *A. lumbricoides* worms (expelled from infected humans) or from schistosomal worms, collected from golden hamsters 45-48 days after infection with *S. mansoni*, as described before [96]. Mass spectrometry was used to confirm the presence and composition of PS species in both lipid preparations, as described before [196]. Schistosomal egg antigen (SEA) was prepared from schistosomal eggs, collected from trypsin treated liver homogenate of the *S. mansoni* infected hamsters. *E. coli* (ATCC 11775) and *L. monocytogenes* (kind gift of J. van Dissel, LUMC, Leiden, The Netherlands) were grown at 37°C for 18 h in Brain Heart Infusion (BHI)

bouillon (Biomerieux). Cultures were washed with PBS, quantified, and frozen in aliquots. In addition *L. monocytogenes* was heat inactivated for 2 hours and 45 minutes at 80°C before storage.

Dendritic cell culture and naïve T cell polarization

Monocytes were isolated and immature DCs were cultured as described before [96]. At day 6 or 7 immature DCs were matured with LPS (ultrapure, *E. coli* 0111 B4 strain, invivogen) (100ng/ml) in the presence of IFN-γ (1000 U/ml), heat killed *L. monocytogenes* (HKLM; 10^8/ml), *E. coli* (10^7/ml), SEA (50 μg/ml), PS lipid extract derived from *Ascaris* worms (an equivalent of 120 mg of worm per ml) or PS lipid extract derived from schistosomal worms (an equivalent of 20 worm-pairs per ml). For RNA isolation, DCs were harvested 16 hours after stimulation, as pilot experiments in our lab indicated that the expression levels of most genes had changed at this time point. DCs were snap-frozen in liquid nitrogen and kept at -80 °C until RNA isolation. For measuring cytokine production by DCs and for co-culture with naïve T cells, DCs were matured for 48 hours after stimulation, after which produced cytokines were measured in the harvested supernatant. Levels of IL12p70 were determined by ELISA using monoclonal antibodies 20C2 and biotinylated mouse-anti-human IL-12 C8.6 (both Becton Dickinson) as coating and detection antibodies, respectively. Levels of IL-23 were determined by ELISA using monoclonal antibodies ebio473p19 and biotinylated mouse-anti-human IL-12 C8.6 (both Becton Dickinson) as coating and detection antibodies, respectively. To determine T cell polarization, 5 x 10^3 mature DCs were cocultured with 2 x 10^4 naïve T cells that were purified using a human CD4+/CD54RO- column kit (R&D, Minneapolis, MN) in the presence of SEB (100 pg/ml; Sigma) in 96-well flat-bottom plates (Costar). On day 5, rhuIL-2 (10 U/ml, Cetus Corp., Emeryville, CA) was added and the cultures were expanded for another 5-9 days. To measure the frequency of IL-4- and IFN-γ- producing T cells, Th cells were restimulated with PMA and ionomycin in the presence of brefeldinA (all Sigma) during 5 or 6 hours and stained with anti-hu-IL-4-PE and anti-hu-IFN-γ-FITC (both BD Biosciences).

RNA isolation, DNase treatment and cDNA synthesis

RNA isolation was performed using Trizol reagent (Invitrogen, Breda, The Netherlands) according the manufacturers' instructions, with a minor modification: 3 μl of glycogen (Invitrogen) was added to all samples after they were homogenized in Trizol for a few minutes at room temperature (RT). DNAse treatment and cDNA synthesis were performed following standard procedures.

Analysis of gene expression levels
Primers and Taqman probes were provided as a Taqman gene expression kit (Applied Biosystems, Foster City, California) or designed using Primer Express (Applied Biosystems) and synthesized by Biolegio (Malden, The Netherlands) and Eurogentec (Seraing, Belgium), respectively (sequences available upon request). Real time qPCR was performed using Eurogentec PCR reagents, in a volume of 25 μl on an ABI PRISM 7700 Sequence Detection System (SDS, Applied Biosystems), using the following program: 10 minutes at 95°C, 40 cycles of 15 seconds denaturation at 95°C and 60 seconds annealing and amplification at 60°C. Results were monitored and analysed with SDS software (Applied Biosystems).
Gene expression was normalized to the housekeeping gene TAF-1 and calculations were performed as described using the $2^{-\Delta\Delta CT}$ method [197]. Analysis of the expression of 6 different housekeeping genes in a subset of the samples indicated that TAF-1 was a stable housekeeping gene in our samples upon stimulation. Spotfire software (spotfire.tibco.com) was used to generate a heatmap and perform hierarchical clustering of the genes.

MAP Kinases
20 or 60 minutes after stimulation of immature DCs (day 6), cells were fixed for 10 minutes with 4% ultrapure formaldehyde (Polysciences) directly in the plate. Cells were harvested and washed twice in PBS/0.5% BSA. Subsequently, the DCs were permeabilized in 700 μl ice-cold 90% methanol in PBS in and left on ice for 30 minutes. Following two wash steps in PBS/0.5%BSA intracellular staining was performed for 2 hours at room temperature in the dark with anti-phospho-p44/42 MAPK AF-488 (T202/Y204), anti-phospho-p38 MAPK AF-647 (T180/Y182), anti-phospho-SAPK/JNK AF 647 (T183/Y185), anti-Phospho-Akt (Ser473) AF-488 (all Cell Signalling Technology), and anti-human c-fos-PE (BD). After one wash in PBS/0.5%BSA MAPK activation was determined by flow cytometry using a Becton Dickinson FACSCalibur flowcytometer (BD Biosciences) and analysed using FlowJo analysis software (Tree Star).

Statistical analysis
Data were analysed using SPSS (v14.0) and GraphPad Prism4. Differences amongst stimuli were analysed by a Mann-Whitney test. Differences relative to LPS stimulation were determined using a one sample t-test. Correlations between expression of genes and/or T-cell responses were calculated by a two-tailed Spearman's-rho test. Differences were considered significant when P-values were below 0.05.

Results

TLR2 activating components that induce Th1 or Th2 polarization via dendritic cells

To study the molecular characteristics of DCs exposed to compounds that engage TLRs, yet lead to differential skewing of immune responses in terms of Th1 and Th2 induction, different pathogen derived products from bacterial or helminth origin were chosen with a known Th1 and Th2 inducing capacity. For this study Gram-negative *E. coli* and Gram-positive heat killed *L. monocytogenes* (HKLM) were used as bacterial stimuli that induce Th1 responses. A schistosome (a trematode) derived phosphatidylserine containing lipid preparation (schPS) and a similar preparation from the nematode worm *A. lumbricoides* (ascPS), both containing mainly phosphatidylserine species with two attached acyl chains and some lysophosphatidylserine species (with only a single attached acyl chain) (figure 5.1A and B, respectively), were chosen as Th2 inducing compounds. Stimulation of HEK cells transfected with TLRs showed that all stimuli could activate TLR2, with additional potent TLR4 stimulation by *E. coli* (figure 5.1C). IFN-γ and schistosome derived soluble egg antigen (SEA), stimuli that do not show strong TLR activating capacity in our experiments (figure 5.1C), and induce Th1 and Th2 responses, respectively, were used as controls.

To assess the T cell polarizing capacity of DCs exposed to these compounds, stimulated human monocyte derived DCs were cocultured for two weeks with allogeneic naïve $CD4^+$ T cells and IL-4 as well as IFN-γ production was determined by intracellular staining upon T cell restimulation (figure 5.1D). DCs were stimulated with the different compounds in the presence of LPS, to ensure equal maturation and to rule out potential effects on polarization due to differences in maturation status of the DCs. We found that in all conditions expression of maturation markers was significantly higher than levels measured on immature DCs and overall similar to the levels induced by LPS alone (data not shown). As expected, *E. coli* induced a strong Th1 response comparable to DCs stimulated with IFN-γ, while HKLM induced a moderately polarized Th1 response. Conversely, the helminth derived compounds, as shown before for schPS [96], and SEA [96, 191], but also the *A. lumbricoides* derived phospholipids instructed DCs to drive Th2 skewed responses with the strongest polarization induced by SEA (figure 5.1D).

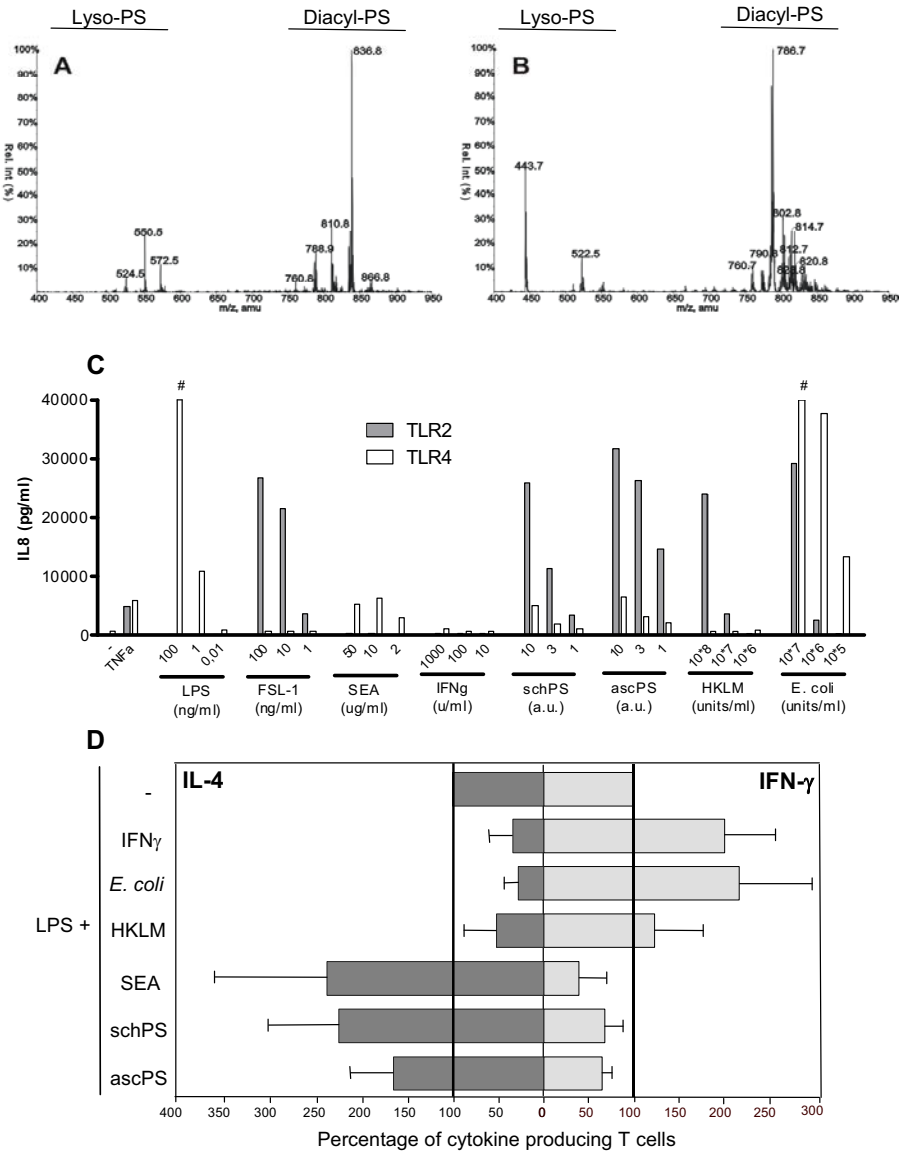

Figure 5.1. TLR activation and T-cell polarization by the different compounds. Mass spectrometry analysis of schPS (A) and ascPS (B). Samples were analysed by LC/MSMS in the negative mode. Neutral loss scans of 87 amu, corresponding to the loss of serine from the phospholipid were obtained. The relative intensity is shown of the detected phosphatidylserine species (indicated by their distinct m/z ratios). C. Activation of TLR2 and TLR4 transfected HEK293 cells. HEK cells were stimulated and IL-8 production in response to activation is shown. CD14 transfected HEK cells were used as negative controls (not shown). # out-positive, a.u. arbitrary units. D. T cell polarization was determined by measuring the percentages of cells with intracellular IFN-γ and IL-4 production by FACS analysis. T-cell polarization after LPS stimulation alone was set to 100% (indicated by the bold lines). Relative amounts of IFN-γ and IL-4 positive T cells induced by the stimuli in the presence of LPS are given. Dark gray (left); IL-4, Light grey (right); IFN-γ. Polarization profiles (IL-4/IFN-γ ratio; n ≥ 4) for all stimuli were significantly different from LPS stimulation alone; $p < 0.05$.

MAPK activation

To obtain a better understanding of the molecular processes in DCs that could underlie the observed differences in T cell polarizing capacity of these helminth- and bacteria-derived compounds, we set out to investigate in more detail the molecular characteristics of the DCs exposed to the different stimuli. To study the intracellular signalling routes activated upon exposure to the helminth and bacterial derived products, we analysed the activation of the MAPKs . ERK (ERK1/2) and p38 are two effector kinases of the MAPK family and are known to play an important role in shaping immune responses [198]. p38 has been shown to regulate DC maturation and proinflammatory responses, while activation of ERK has been related to anti-inflammatory and Th2 responses [199]. As has been described before [200], exposure of DCs to LPS alone led to preferential phosphorylation of p38 (figure 5.2A, B). The Th1 promoting stimuli IFN-γ and *E. coli* even further increased the activation of this MAPK resulting in a reduced *p*-ERK / *p*-p38 ratio, 20 minutes after stimulation (figure 5.2B,C), whereas for HKLM this ratio did not change. In contrast, the Th2 inducing compounds PS and SEA increased this ratio. Interestingly, the high *p*-ERK / *p*-p38 ratio induced by these Th2 polarizing stimuli was the result of different activation profiles for SEA versus the lipid preparations: SEA significantly induced phosphorylation of ERK, whereas the helminth derived lipids impaired p38 activation, but showed no effect on ERK activation (figure 5.2D and 2E). The *p*-ERK / *p*-p38 ratio showed a positive correlation with Th2, and negative correlation with Th1 polarization (R^2 = 0.36 and -0.47, respectively, figure 5.2F and G). In conclusion, for all components tested, the *p*-ERK/*p*-p38 ratio only 20 minutes after DC stimulation can be used to predict the outcome of the T cell response in terms of Th1 and Th2. This shows that very early events in DC activation already determine the fate of the DCs in terms of its T cell polarizing capacity.

Gene expression analysis

To further characterize the molecular profile of the differentially stimulated DCs we performed mRNA expression analysis, using real-time PCR, on a selected set of genes involved in TLR signalling and T cell polarization (table 5.1, figure 5.3A). Upon maturation with LPS, the expression of most genes was increased (data not shown). All data shown are relative to what is seen in mature DCs without any polarizing agents added, i.e. DCs stimulated with LPS. Stimulation of DCs from different individuals with the same stimulus showed very consistent profiles (data not shown). Clustering analysis revealed that the gene expression profiles of Th1 and Th2 polarizing agents clustered in separate groups (top of figure 5.3A). Within the Th1 stimuli, DCs exposed to bacterial products derived of *L. monocytogenes* and *E. coli* had a remarkably similar profile that was different from the profile induced by IFN-γ. For the Th2 stimuli,

both helminth derived lipid preparations showed a very comparable profile, which resembled the expression profile induced by SEA for most of the genes (figure 5.3A). However, expression levels in PS pulsed DCs were generally lower than in SEA stimulated DCs which is in accordance with the less pronounced effects on activation of the MAPKs by the PS preparations. Next, we related expression levels of individual genes to the T cell polarizing capacities of the DCs, to identify potential mechanisms through which different pathogen derived compounds induce differential T cell polarization. Members of the IL-12 cytokine family are well known for driving Th1 polarization [88]. Indeed the expression of both IL-12 p40 and p35, but also IL-23 p19 were shown to be upregulated in DCs stimulated with Th1 inducing stimuli and reduced in DCs stimulated with helminth derived compounds (figure 5.3A). This was confirmed at the protein level when IL-12 and IL-23 production by DCs were measured by ELISA (figure 5.3B,C).

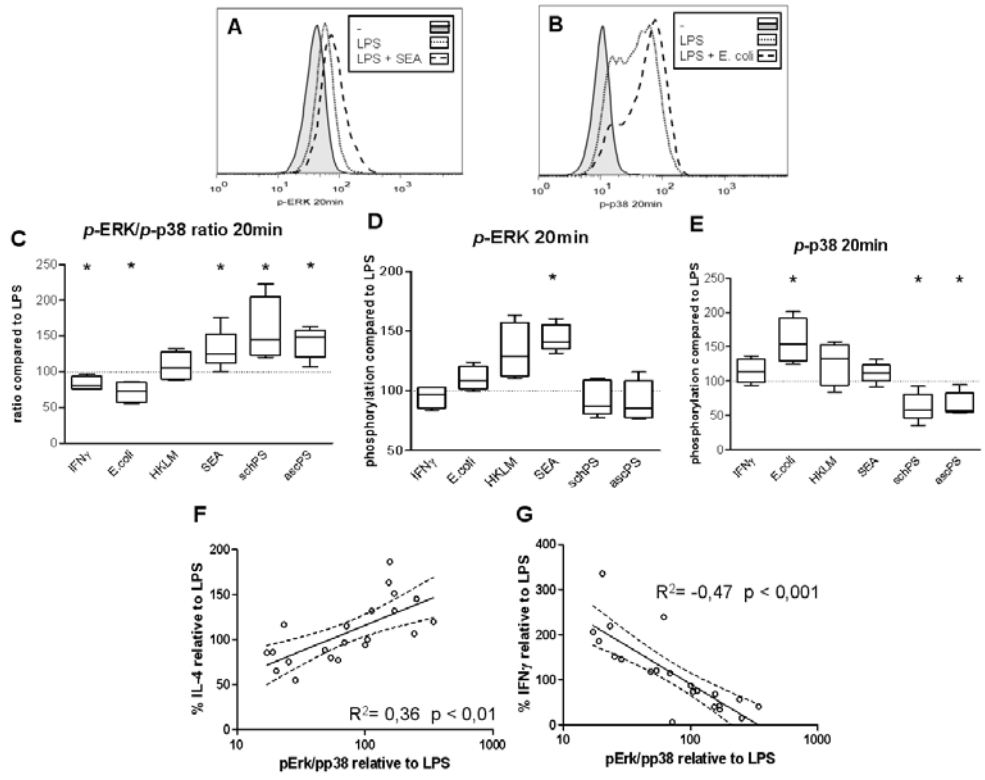

Figure 5.2. MAP kinase activation in dendritic cells. Representative histograms of (A) ERK and (B) p38 phosphorylation in DCs 20 minutes after stimulation. C-E. Ratios of p-ERK / p-p38 (C), phosphorylation of ERK (D) and p38 (E) 20 minutes after stimulation in the presence of LPS. Expression induced by LPS was set to 100% (dashed line), relative expression levels or ratios are shown. * P<0.05 compared to LPS stimulation. F and G. Correlation of p-ERK / p-p38 ratio and IL-4 (F) or IFN-γ (G) production by T-cells. All data are relative to stimulation with LPS only.

With respect to T cell polarization, other genes of interest are the notch ligand family members delta-1, delta-4 and jagged-2, since expression of delta and jagged on DCs has been associated with induction of Th1 and Th2 responses, respectively [230]. For jagged-2 and delta-1 no significant differences were found between the stimuli (figure 5.3A). However, delta-4 was upregulated by the bacterial Th1 inducing stimuli, and downregulated by the Th2 inducing lipids. Moreover, expression levels of delta-4 correlated with the IL-4/IFN-γ cytokine ratio produced by the T cells of the stimuli that activate TLR2 (R^2=-0.87, figure 5.3D). Yet, in SEA and IFN-γ stimulated DCs delta-4 expression was not altered. Therefore, Delta-4 seems to associate with T helper cell polarization only when TLR2 is also engaged.

Conversely, we found higher c-fos mRNA levels in PS and SEA pulsed DCs compared to HKLM and *E. coli* stimulated DCs. c-fos has been shown before to mediate SEA induced repression of IL-12 secretion by DCs [199]. Indeed, correlation analysis revealed that in DCs stimulated with bacterial products or helminth-derived lipids, mRNA levels of c-fos were positively correlated with Th2 induction (R^2 = 0.667, figure 5.3E).

Discussion

DCs express a range of PRRs that allow them to recognize different pathogens and initiate appropriate adaptive immune responses. Pivotal to this process is the proper integration of PRR derived signals into a molecular activation profile of DCs that leads to a particular T cell polarizing capacity. This study demonstrates that TLR activation in the context of different bacterial and helminth derived extracts can lead to very distinct molecular activation profiles of human DCs which correlate with their T cell polarizing capacity in terms of Th1 and Th2 skewing.

One of the major signalling cascades triggered upon engagement of TLRs is the MAPK pathway. Differential activation of MAPK p38 and ERK in DCs has been associated with specific T cell polarization; p38 is thought to be important in mediating DC maturation and pro-inflammatory / Th1 responses, whereas ERK activation has more often been associated with anti-inflammatory / Th2 responses [232]. At 20 minutes after stimulation, we observed decreased *p*-ERK/*p*-p38 ratios in the Th1 promoting DCs.

Of the two Th1 polarzing agents, *E. coli* induced stronger p38 activation, compared to HKLM. The fact that *E.coli*, unlike HKLM, strongly activates TLR4 (figure 5.1C), which is known to lead to preferential p38 activation and induction of pro-inflammatory responses, may explain this [200].

Table 5.1. Description of genes

		Gene	Function	Ref.
Cytokines & chemokines		IL-12 p35	Together with p40 forms the cytokine IL-12 involved in Th1 polarization	[191, 201]
		IL-12 p40	Together with p35 forms the cytokine IL-12 involved in Th1 polarization	[191, 201]
		IL-23 p19	Together with p40 forms cytokine IL-23 in DCs which augments Th1 and Th17 responses	[202-204]
		TNF-α	General pro-inflammatory cytokine mediating local inflammation. Its expression is dependent on NF-kB signalling	[205]
		CXCL-10 (IP-10)	Chemotactic factor for T cells. Its expression is dependent on the IFN-responsive gene pathway	[206, 207]
		CCL22	Chemokine involved in recruitment of Th2 effector cells	[208]
		TGF-β	Cytokine with anti-inflammatory properties, by inhibiting activity and function of both T cells and DCs	[209, 210]
		IL-10	Cytokine that potently suppresses immune responses and in particular DC and T cell responses, by downregulating pro-inflammatory effectors	[210, 211]
Toll like receptor signalling		TLR2	Receptor of innate immunity for recognition of mainly lipid containing compounds	[212, 213]
		TLR4	Receptor of innate immunity for recognition of LPS	[212, 213]
		MyD88	Proximal, most common adaptor of TLR signalling, shared by all TLRs except TLR3	[212, 214]
		NOD2	Intracellular peptidoglycan receptor implicated in activation of NF-kB but also in inhibition of TLR2 signalling	[215-217]
		TRIF (TICAM1)	TLR3 and TLR4 specific adaptor which mediates the MyD88-independent pathway preferentially leading to induction of IFN-responsive genes	[218, 219]
		Tollip	Inhibitor of IRAK activity and thereby TLR signalling	[220]
		SOCS-1	Inhibitor of LPS-TLR4 signalling pathway as well as TLR induced JAK/STAT signalling. Potential negative regulator of Th1 responses	[221, 222]
		SOCS-3	Inhibitor of JAK/STAT signalling but also positive regulator of APC function by suppression of STAT3, which normally inhibits TLR signalling.	[221, 222]
Modulators of T cell activation & polarization	Intracellular	DUSP2	Phosphatase modulating MAP kinase signalling balance	[223]
		IDO	Enzyme that catabolizes tryptophan to kynurenines, which are able to induce T cell apoptosis and inhibition of proliferation. Expression induced by IFN-γ	[224]
		c-Fos	Transcription factor activated by MAP kinases which induces IL-10 production and is involved in DC mediated Th2/anti-inflammatory responses	[108, 199]
	Membrane bound	PD-L1 (B7-H1)	Costimulatory molecule and ligand for PD-1 on T-cells. It has inhibitory function in T cell proliferation and cytokine production. Might be stimulatory for Th2 response	[225-227]
		PD-L2 (B7-DC)	Costimulatory molecule and ligand for PD-1 on T-cells. Reported to have synergic activity with other costimulatory molecules as well as inhibitory activity on T cell activation	[225-227]
		LIR-7 (ILT-1)	Receptor with unknown ligand(s) with possible immune suppressive properties, but also implicated in immune activation	[228, 229]
		Jagged-2	Ligand for notch-receptor on T cells; influences T cell skewing	[230]
		Delta-4	Ligand for notch-receptor on T cells; influences T cell skewing	[230, 231]
		Delta -1	Ligand for notch-receptor on T cells; influences T cell skewing	[230]

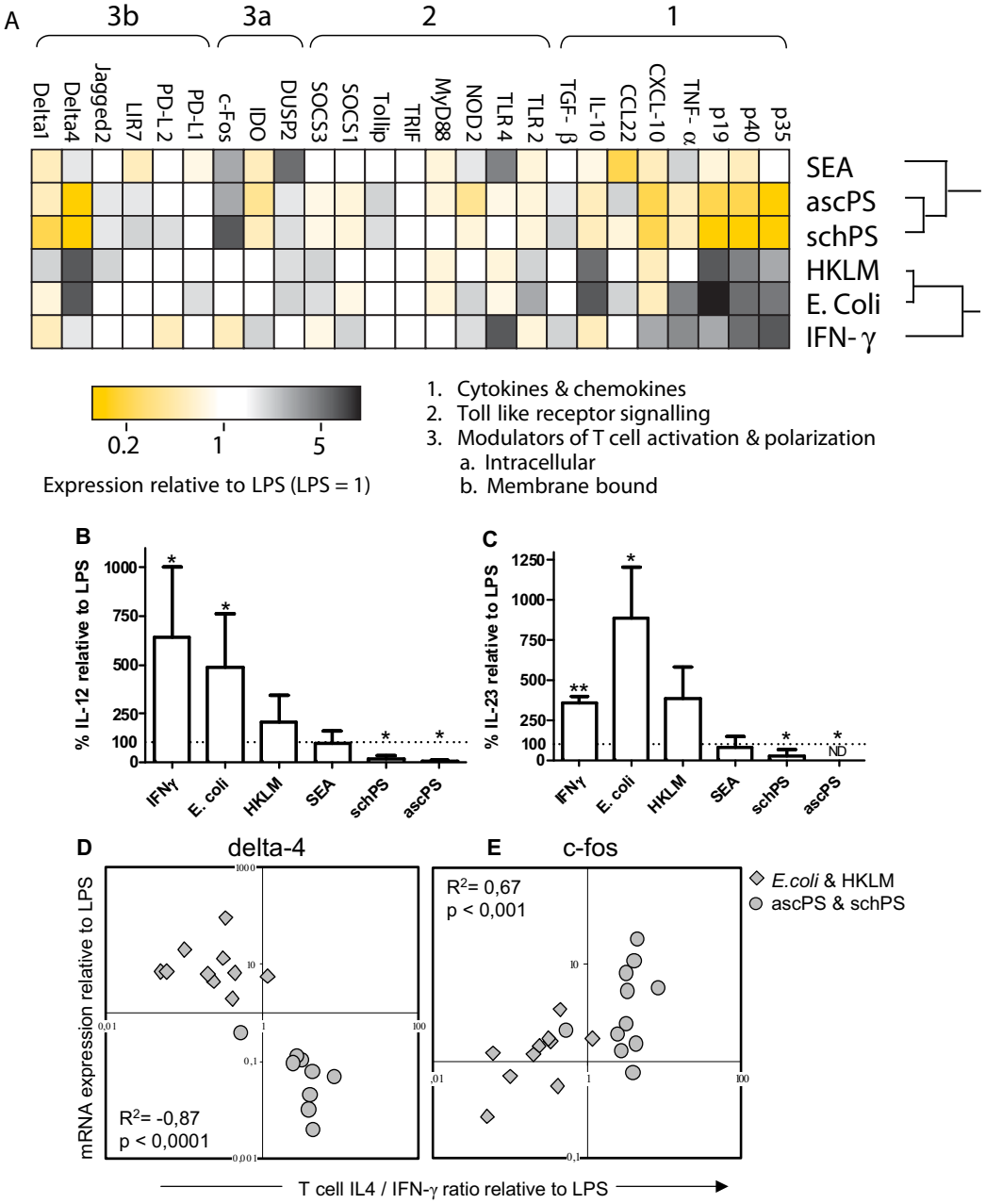

Figure 5.3. Distinct mRNA expression levels after stimulation of immature DCs with Th1 and Th2 inducing compounds. A. mRNA expression levels of the different genes compared to LPS (representing a value of 1). Black and yellow colors represent expression levels higher and lower, respectively, relative to LPS pulsed DCs. Expression was determined by real-time PCR with TAF-1 as housekeeping gene. Stimuli were clustered hierarchically according to expression profiles (top of figure). B and C. Amount of IL-12 and IL-23 present in the supernatant of DCs 48 hours after stimulation, relative to the amount produced in the presence of LPS only. ND: not detectable, * $p < 0.05$, ** $p < 0.01$ compared to LPS stimulation only. D and E. Association of delta-4 (D) and c-fos (E) mRNA expression levels with T cell polarization for TLR2 activating stimuli. Diamonds represent HKLM or *E. coli*, circles the helminth derived lipids. Relative expression levels to LPS control condition (set to 1) are shown.

Comparison of the MAPK activation profile of IFN-γ with the Th1 inducing bacterial products revealed a similar reduction in the *p*-ERK/*p*-p38 ratio. This could be explained by the fact that IFN-γ induced STAT signalling is known to potentiate TLR4 mediated effects resulting in selective enhancement of p38 phosphorylation [233, 234]. These data are in agreement with the increased mRNA expression levels of pro-inflammatory mediators such as IL-12, IL-23 and TNF-α in all the Th1 promoting DCs. In contrast, all helminth derived stimuli increased the *p*-ERK/*p*-p38 ratio in the DCs. This is consistent with what others have found for SEA [108] and also for other helminth derived Th2 polarizing antigens such as LNFPIII [93] and ES-62 [235] in murine DC models. Taken together, an increased *p*-ERK/*p*-p38 ratio appears to be an important characteristic of antigen presenting cells exposed to helminth derived antigens that skew responses towards Th2.
Comparison of the MAPK activation profile induced by the helminth-derived lipids compared to SEA, revealed that SEA, like other helminth derived antigens such as LNFPIII and ES-62, induces a higher *p*-ERK/*p*-p38 ratio by increasing activation of ERK, whereas the lipids influenced the *p*-ERK/*p*-p38 ratio by specifically impairing p38 phosphorylation. Thus, although the lipids share the properties of other Th2 inducing helminth antigens described so far, they appear to achieve this differently which is exemplified by a different modulation of the MAP kinase signalling pathway.
This difference in MAPK activation between SEA and the lipid preparations is further substantiated by the finding that c-Fos protein levels were elevated and sustained in DCs stimulated with SEA, which is in line with a previous study showing that SEA stabilizes c-Fos through selective activation of ERK [108], whereas in PS pulsed DCs this increase was lower and more transient (Everts *et al*, unpublished data).

Comparison of the mRNA expression profiles of TLR activating bacterial and helminth derived compounds revealed that, unlike the Th2 inducing phospholipids, exposure of DCs to Th1 promoting stimuli preferentially led to the induction of the Th1 promoting IL-12 and IL-23, both at the mRNA and protein level. The level of expression of these cytokines reflected the degree of p38 activation that drives pro-inflammatory gene expression. The fact that these cytokines were expressed to a higher level in the *E. coli* and IFN-γ stimulated DCs, probably contributes to the stronger Th1 induction seen with these stimuli compared to HKLM pulsed DCs. Although the expression profile of the pro-inflammatory cytokines was shared by the IFN-γ stimulated DCs, expression of other genes like IL-10, delta-4 and TLR4 was clearly different from the bacteria conditioned DCs. These differences are likely to be caused by the fact that *E. coli* and HKLM

harbor PAMPs that activate DCs through multiple PRRs, whereas IFN-γ signals via PRR independent pathways that involve STAT signalling.
Far less is known about the mechanisms leading to induction of Th2 responses, compared to Th1 responses. Both lipid preparations induced a nearly identical molecular profile in DCs. This is in agreement with the similar molecular species composition present in the lipid preparations: both PS preparations contain predominantly diacyl phosphatidylserine species (molecules with m/z ratios above 700), and a few lysophosphatidylserine species (molecules with a single attached acyl chain and m/z ratios between 400 and 600) (figure 5.1A, B). Combining the mRNA expression levels and MAPK activation for the Th2 inducing stimuli we found that both lipid preparations as well as SEA induce Th2 polarization and a *p*-ERK / *p*-p38 ratio dominated by ERK. However, SEA induces higher levels of activated MAPKs and a stronger Th2 response. It appears therefore, that the ratio between *p*-ERK and *p*-p38 is important for the polarization of the immune response, whereas the strength of this response is dependent on the absolute amounts of the activated MAPKs.

Recent work highlighted a role for certain Notch ligands, including jagged-2, in skewing towards Th2 responses [230]. Our findings suggest that jagged-2 mRNA is not important in the Th2 skewing for the conditions tested in this study. However, high levels of delta-4 were found upon stimulation of human DCs with bacterial compounds and helminth derived compounds show a decreased delta-4 expression and induce a Th2 response, which is in agreement with the finding that the Notch ligand delta-4 is involved in Th1 skewing [230]. Moreover, recently it was found that delta-4 expressing mouse DCs not only drive Th1 by inducing IFN-γ production by T-cells, but also by actively inhibiting Th2 development via counteraction of IL-4 [236]. This is in agreement with our findings in human DCs that expression of delta-4 is not only positively associated with Th1 induction, but that delta-4 is also negatively associated with the development of a Th2 response. Moreover, we found that this up- and downregulation of delta-4 expression can both exist in the context of TLR2 signalling.

One of the genes that were found to be positively associated with Th2 inducing DCs was the transcription factor c-fos. c-Fos has been shown to mediate IL-12 suppression in SEA pulsed DCs, which is generally thought to be a prerequisite for Th2 induction [108, 199]. In addition, the observation that c-fos mRNA expression was strongly positively correlated with Th2 induction not only for SEA, but also for PS, further supports the notion that this transcription factor plays a role in the promotion of helminth antigen dependent Th2 skewing. However, as described above, the kinetics of c-fos translation seems different between the lipids and SEA. Therefore, it

remains to be established whether c-fos in PS pulsed DCs plays a similar role compared to the role in SEA induced Th2 polarization.

Several studies have shown that TLR2 activation may lead to many different outcomes; Th2 [108, 237], Treg [238] as well as Th1 [239]. It has been suggested that these different outcomes may be the result of heterodimerization of TLR2 with different receptors, such as TLR1 or TLR6 [240, 241], or liaisons with other receptors including Nod-like receptors and C-type lectins [194, 242]. In our study, the compounds used from helminths or bacteria are mixtures that would be expected to signal via additional receptors besides TLR2. *E. coli* has been shown to activate TLR4 and NOD1 [243, 244], whereas resistance to *Listeria* infection was related to the presence of functional NOD2 [245]. Relatively little is known about Th2 skewing by the helminth derived compounds, but in a previous study for schistosomal lipids it was shown that TLR2 activation was not needed for Th2, but rather for regulatory responses [96]. Therefore, these results suggest that the engagement of additional PRRs along with TLR2 and TLR4 are likely to play a role in the Th2 polarizing capacity of DCs [245, 246].

In conclusion, the study presented here indicates that TLR ligation on monocyte derived DCs in the context of bacterial or helminth derived extracts leads to profound differences in the activation or expression of various markers at the level of MAPK phosphorylation, mRNA expression levels and protein products thereof. Interestingly, several of these molecular markers are strongly correlated with the T cell polarizing capacity of these DCs. This not only gives new insights about the processes involved in Th1 and Th2 polarization but it also suggests that there is a common molecular Th1 and Th2 signature in human DCs that can be used to differentiate between Th1 and Th2 inducing DCs as well as to predict the strength of skewing in terms of Th1/Th2 balance. Thereby, this study provides new insights in molecular pathways involved in the capacity of human DCs to distinguish bacterial and helminth derived antigens via PRRs including TLRs and the proper integration of these signals leading to the induction of appropriate immune responses.

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