RESEARCH ARTICLE

Role of mitogen-activated protein kinase and PI3K pathways in the regulation of IL-12-family cytokines in dendritic cells and the generation of T_H-responses

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ABSTRACT. Mitogen-activated protein kinases (MAPK) are targets for the immune-modulation of dendritic cells (DC). However, our knowledge of their role in the regulation of IL-12-family cytokines is limited. This study investigated the roles of p38, JNK, p44/42 and PI3K pathways in IL-12/23/27 production by human DC, and their impact on naïve T_H-responses. We first identified TOP and UBC as robust DC housekeeping genes. Peak transcription of p35 and p40 occurred by 12h, p19 and p28 by 8h and EBI3 by 12-24h. Using selective antagonists, we showed that p38 was a positive regulator of IL-12, 23 and 27, JNK positively regulated IL-12 and IL-27, and inhibition of MEK1/2 had no marked effect. In contrast, the PI3K pathway markedly attenuated IL-23 responses and, to a lesser extent, IL-12, but not IL-27. To identify the role of these soluble factors, we co-stimulated naïve CD4+ T-cells in the presence of DC supernatant. The presence of mature DC supernatant induced not only strong IFNγ responses, but also IL-10 and IL-17A. Inhibition of p38 ablated T_{H1}, and IL-10 and IL-17A responses, whilst modestly enhancing IL-5 secretion. In contrast, inhibition of MEK1/2 abolished IL-17A production, whilst leaving other responses unaffected, whereas inhibition of JNK or PI3K had no discernable effect. In summary, we describe the expression of IL-12-family cytokines from DC and propose a modified model for their regulation. This study further clarifies the potential for therapeutic modulation through these mediators.

Keywords: dendritic cell, IL-12, IL-23, IL-27, signalling, T_H

Dendritic cells (DC) orchestrate adaptive immunity through interactions with helper T-cells (T_H), which then modulate and instruct adaptive responses. The mitogen-activated protein kinase (MAPK) and PI3K pathways play central roles in the response of DC to environmental signals. Recently, it was shown that modulation of these intracellular signalling pathways in DC differentially regulates cytokine production and the outcome of their interaction with CD4+ T-cells [1-7]. Despite the fact that these systems are now under investigation in modified vaccination and immunotherapy strategies, the full impact of modulating discrete MAPK on the secretion of T_H-modulating IL-12-family cytokines by DC and subsequent Th-priming, remains unknown.

The cytokines released from DC are essential messengers to guide T_H differentiation and the epigenetic changes imprinted on their progeny. The interleukin (IL)-12 family of cytokines produced by DC consists of IL-12-p70, IL-23 and IL-27. There has been the recent addition of a new member to this family, IL-35 [11], however, in contrast to the other IL-12-family members, IL-35 is not produced by DC and therefore serves alternative roles. IL-12 is vital to T_H-1 induction; it represses Gata-3 (the master transcription factor for T_H-2), and promotes survival and growth of T_H-1. Whilst IL-12 is important for T_H-1 induction, IL-27 can assist by inducing IL-12-receptor display by naïve T-cells and the T_H-1 master transcription factor, T-bet [8, 9]. In addition, IL-12 and IL-27 suppress the generation of the third, newly described T_H-subset, T_H-17, by inhibiting the transcription of the RORC master transcription factor [10]. However, perhaps the most important role for IL-27, in contrast to its role in T_H-1 induction, is to suppress T-cell responses by directly limiting IL-2 whilst inducing IL-10 secretion, thus producing regulatory

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T-cells [11, 12]. Initially described as supporting IFN-γ-producing memory T-cells, IL-23 also supports the establishment of T_H-17 responses. T_H-17 have specific cytokine requirements for induction (a combination of IL-1b, TGF-β, IL-6 or IL-21 depending on the system), their own cytokine profile (IL-17A, IL-17F, IL-6), and employ the RORC transcription factor. T-helper sub-set priming is highly regulated by the DC cytokine profile, and an increasingly complex picture of preferential sub-set induction is developing, focused around IL-12, IL-23 and IL-27. The IL-12-family cytokines have a unique, heterodimeric structure comprised of gene products on separate chromosomes [14]. IL-12p70 (hereafter referred to as IL-12) is composed of p35 and p40 proteins, resembling a complex of an IL-6-like protein (p35) with its soluble receptor-like counterpart (p40). Concomitant expression of each sub-unit is required for biologically active IL-12. IL-23 shares p40 with IL-12, together with the unique p19 sub-unit, whilst IL-27 is a combination of p28 (structurally similar to p35 and p19) plus another soluble receptor-like molecule, EBI3 [15, 16]. T-cell-derived IL-35 is composed of p35 and EBI3 [13].

To date, there is a sizeable body of evidence of the role for MAPK pathways in regulating the secretion of IL-12 by DC in response to commonly-studied receptors such as TLR4. IL-12 is positively regulated by MAPK p38 and Jun N-terminal kinase (JNK) [17-22]. In contrast, phosphoinositide kinase-3 (PI3K) exerts negative regulation on IL-12 transcription [23]. Furthermore several reports have also shown that the active form of the extracellular signal-related kinase (ERK), also termed p44/42, is able to counter p38 and inhibit IL-12 production by DC [24-26]. In contrast to IL-12, considerably less is known concerning upstream events involving the major MAPK pathways and PI3K for IL-23 or IL-27 expression. Much of the work to date has been limited to transcriptional investigation (assays for the more recently described IL-12-family proteins have not been widely available), and in this regard there is a paucity of data concerning appropriate housekeeping genes in human DC. Nevertheless, distinct MAPK pathways appear to control the production of the IL-12 family of cytokines in APC, and this offers opportunities for immune-modulation. Recently, Brereton and colleagues [6] examined the role of p44/42 in murine DC and showed that its inhibition suppressed IL-23, but not IL-12 production in response to TLR4 agonist. Importantly, inhibition of the up-stream kinase, MEK 1/2 in DC, suppressed their ability to induce autoantigen-specific T_H17 and T_{H1} responses *in vivo*. These data are in partial disagreement with our previous report [1] showing that inhibition of MEK 1/2 increased restored levels of IL-12 production by tumour-conditioned DC and their subsequent capacity to drive T_{H1} responses *in vitro*. The reasons for this remain unclear, but may indicate that any pronounced mis-regulation of MAPK has the potential to impede IL-12 responses.

The present study examined the contribution of the MAPK and PI3K pathways to IL-12, IL-23 and IL-27 secretion by monocyte-derived DC, and defined the effects of this for priming naïve CD4+ T-cells. As a result, we propose a revised model of IL-12-family cytokine regulation that highlights the role played by p38 as a major, positive regulator of all three cytokines. In contrast, JNK negatively regulates IL-12 and IL-27, but not IL-23; ERK inhibition has no significant effect. The PI3K pathway negatively regulates IL-12 and IL-23, but not IL-27. Importantly, manipulation of DC MAPK had pronounced effects on T_H-1 and T_H-17 priming. Such intracellular signalling pathways may be new targets for immunotherapeutic intervention against a range of pathologies, including infectious disease (*e.g.* HCV, HIV), autoimmunity, and malignancy.

METHODS AND MATERIALS

Dendritic cell generation

Monocyte-derived dendritic cells (mo-DC) were obtained from donor buffy coats by layering over Ficoll. The resultant PBMC fraction was washed in PBS before purification of monocytes by CD14-positive selection. Purity of over 95% was routinely obtained. Monocyte preparations were cultured in RPMI-1640 containing 10% (v/v) FCS, 1% (v/v) L-glutamine, GM-CSF (1,000 U/mL) and IL-4 (800U/ml). After three days, half the volume of medium containing fresh cytokines was added. Immature DC (iDC) were harvested after five or six days.

Stimulation of dendritic cells

iDC were re-plated in medium containing IFN-y (1,000 U/mL) and LPS (500 ng/mL). Cells were pre-treated with kinase inhibitors (U0126, SP203580 and SB600125, all at 10uM) or Wortmannin (0.1 uM) for one hour. Technical specifications and published research indicate that U0126, SB303580 and SP600125 are each able to inhibit their respective targets at the concentration used in human DC [20-27], and this was confirmed in our own studies (not shown). At 5-30 minutes, DC were harvested for Western blot or cultured for up to 48 hours, following which culture supernatants were collected and stored at - 80°C for ELISA and subsequent T-cell assays.

Determination of secreted cytokine

ELISA were performed for IL-12 with BD Bioscience OptEIA kits as per manufacturer's instructions, lower limits of detection were 7.8 pg/mL. Levels of secreted IL-10 were determined by ELISA (R&D System DuoSet), with a lower limit of detection corresponding to 31 pg/mL. IL-23 was assayed using eBioscience Ready-Set-Go #88-7237-22 (lower limit 15 pg/mL). In the absence of a commercially available IL-27 assay, a sandwich ELISA was established using as capture R&D anti-IL-27 and detection biotinylated anti-IL-27 R&D. Recombinant human IL-27 heterodimer (R&D Systems) was diluted for the standard curve. The sensitivity of this assay was 50 pg/mL. For all ELISA, TMB substrate reagent from BD Biosciences was used.

Western blots

Following stimulation, DCs were harvested rapidly on ice into cold PBS and cells pelleted at 180 x g for five minutes. DC cell pellets were resuspended in RIPA buffer

containing protease and phosphatase inhibitors, vortexed and incubated on ice for 30-60 minutes. Genomic DNA was sheared by passing the suspension through a fine-gauge needle, and a sample was taken for protein estimation using the BCA method. Lysed cell suspensions were stored at - 20°C in loading buffer. Fifteen to 25 µg of protein were loaded per well and electrophoresed on 10% SDS-PAGE gels, following which, proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were blocked in 5% BSA, followed by primary antibody probe overnight at 4°C. Binding of secondary antibody probe and streptavidin-HRP incubation was visualised *via* ECL (Amersham) or by using infra-red dye-labelled antibodies and LiCor Odyssey image analysis.

Quantitative PCR

DC were stimulated as described with LPS/IFN-γ and cells harvested on ice at four, eight, 12 and 24 hours after stimulation. RNA was isolated using Ambion RNAqueous 4PCR according to the manufacturer's instructions, and produced RNA with a 260/280 ratio of 1.8-2.0. cDNA was produced using Ambion Message Sensor RT kit and Oligo d(T)23 (New England Biolabs), cDNA was diluted 1/10 in Ultrapure water (Gibco) and stored at - 80°C. To the best of our knowledge, there are no published reports of housekeeping gene stability in human mo-DC. To establish an appropriate reference gene, selected cDNA samples from three donors were assayed using a Primer Design Housekeeping GeNorm kit, according to the manufacturer's instructions. Analysis of the 12 potential reference genes using the Visual Basic application GeNorm [28] ranks comparative stability and revealed TOP1 and UBC as the most stable *(table 1)*.
Relative IL-12-family sub-unit gene expression was assessed using pre-designed TaqMan Gene Expression Assays (Applied Biosciences TOP1 Hs00243257_m1, UBC Hs00824723_m1, IL-12A/IL-12p35 Hs00168405_m1, IL-12B/IL-12p40 Hs00233688_m1, IL-23A/IL-23p19 Hs00372324_m1, IL-27/IL-27p28 Hs00377366_m1 and EBI3 Hs00194957_m1). Note that m1 denotes probe exon spanning, the IL-12A assay spans exon 2/3, therefore detects only normal IL-12A mRNA transcripts not the alternative isoform missing the first part of exon 3 [29]. Gene expression assays were performed according to the manufacturer's recommendation in 96-well plates on a Stratagene Mx3000P. Cycle threshold (CT) values were obtained from MxPro (Stratagene), based on adaptive baseline readings and background-based threshold readings normalised to reference dye (rox). PCR efficiencies were calculated using the DART-PCR application, and were all approaching 100% [30]. The $2^{-}\Delta\Delta CT$ method [31] (normalising to the geometric mean of UBC and TOP1) was used to calculate the relative fold-change in mRNA levels using mDC at eight hours as the calibrator for each donor as immature DC do not express detectable levels of all sub-units.

T cell isolation and stimulation

T-cells were isolated using positive selection MACs techniques (Miltenyi). PBMC were isolated and CD4 positive cells were selected – typical purity between 92-99%. Twenty four-48 hours later, the cells were selected using CD45RA microbeads and a typical purity of 98% was obtained as determined by FACS. CD4/CD45RA double positive cells were employed as naïve T helper-cells. T-cell were cultured in T-cell medium (RPMI, 10% FCS, 1% Na pyruvate, 1xNEAA, 25mM HEPES) in a humidified atmosphere of 5% CO_2 in air.
Cell culture plates were coated with purified mouse anti human-CD3 antibody (OKT3) at 2 µg/mL in PBS overnight at 4°C, followed by aspiration of unbound antibody. T-cells (2.5 x 10^5) were plated into 48-well plates. For

Table 1
Ranking of candidate housekeeping genes by stability.
In order to determine the appropriate housekeeping genes for quantitative-PCR, DC-derived mRNA from a number of donors, under a range of relevant experimental conditions, was examined using a Primer Design Housekeeping GeNorm kit. The TOP1 and UBC genes were selected for use together as housekeeping reference genes against which changes in the genes of interest were compared

	Gene	Description (gene locus)	Median CT, SD
Least stable genes ↑	EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2 (3q28)	22.50, 1.45
	18S	18S ribosomal RNA	17.18, 1.07
	RPL13A	Ribosomal protein L13A (19q13)	22.36, 1.20
	YWHAZ	phospholipase A2 (8q23)	18.46, 0.91
	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (12q13)	26.73, 1.15
	ACTB	Beta actin (7p15)	16.84, 0.83
	SDHA	Succinate dehydrogenase complex, subunit A (5p15)	23.88, 0.77
	B2M	Beta-2-microglobulin (15q21)	15.97, 0.96
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (12p13)	18.70, 0.79
Most stable genes ↓	CYC1	Cytochrome c, isoform (8q24)	21.43, 0.70
	UBC	Ubiquitin (12q24)	18.59, 0.81
	TOP1	Topoisomerase 1 (20q12)	22.59, 0.65

experiments involving T-cell stimulation, DC were pre-treated with MAPK-inhibitor for one hour and LPS for eight hours; the supernatant was discarded. Cells were washed twice with 1ml of medium, and fresh-medium, without MAPK inhibitor, cytokines or LPS, was added. Control wells were prepared and included cell-free medium (including IL-4/GM-CSF) plus MAPK-inhibitor, which were washed in the same way as wells that contained cells. Previous experiments had shown that MAPK inhibitors, at the concentration used to treat DC (*i.e.* 10 uM), could significantly affect the cytokine profile of T-cells (data not shown). Assuming a 10% carry-over, we estimate that washing the cells as indicated and using the supernatant at 25% would result in MAPK inhibitor being present at 0.025 uM or 1:400 of the normal experimental concentration. Cells were cultured for 48hr and the cell-free supernatant harvested. The supernatant of stimulated DC was added to naïve CD4+ T-cells, giving a final concentration of 25% DC supernatant. Soluble mouse anti-human CD28 (BD Biosciences) was used to co-stimulate T-cells at 2.5 μg/mL. T-cells were incubated for four days, following which, supernatants were collected and stored for extracellular cytokine Luminex assay and cells were fixed and permeabilised for intracellular cytokine measurement.

Luminex assay

Collected supernatants were thawed on the day of analysis. Invitrogen Biosource extra-cellular protein human T_H1/T_H2 kit was multiplexed with Invitrogen Biosource human IL-17A set, and used according to the manufacturer's instructions.

Statistical analysis

Differences between two groups were assessed by performing paired, two-tailed T-tests. Resulting significance was represented as follows:
Notation P-value
* 0.01 to 0.05
** 0.001 to 0.01
*** < 0.001

RESULTS

Transcription of genes for IL-12p70, IL-23 and IL-27 by monocyte-derived DC

Previous reports have described secretion of IL-12 and IL-23 by DC [15, 32, 33], however, there is a paucity of data regarding the relative secreted levels of these cytokines and IL-27 secretion. We examined the time-course of gene transcription and protein secretion of the IL-12 family in response to TLR4 agonist plus IFNγ. There is little published information on the choice of appropriate housekeeping genes for human DC to allow accurate measurement of transcriptional changes. To establish an appropriate reference gene, cDNA samples from three donors were assayed using a Primer Design Housekeeping GeNorm kit. Appropriate analysis of the 12 reference genes [28] ranked their relative stability and revealed TOP1 and UBC as the most stable *(table 1)*. TOP1 and UBC have different functions, are not co-regulated, and are on chromosomes 20 and 12 respectively. They are therefore good candidate housekeeping genes for qPCR in human DC. Subsequent studies of IL-12-family transcription normalised mRNA levels to the geometric mean of UBC/TOP1.

As expected, none of the IL-12-family genes were constitutively transcribed by iDC, however following maturation, a pronounced increase in mRNA was observed. The five genes studied fell into three classes: p19 and p28 genes were at their transcriptional peak after eight hours, p35 and p40 after 12 hours, and EBI3 continued to increase until 24 hours *(figure 1A)*. No further increase in transcription was observed beyond 24 hours (not shown). This was consistent with the time course of protein secretion. During the first 8-12 hours, DC secreted abundant IL-12 and some IL-23, however little IL-27 was detected *(figure 1B)*. By 24 hours, mature DC were secreting IL-12, 23 and 27. IL-12 was the dominant cytokine secreted, showing a three-five-fold higher expression than IL-23 and IL-27 in the first 24 hours. In agreement with the transcription results, no further increase in secretion of IL-12, -23 or -27 was observed beyond 24 hours (not shown).

Activation of MAPK in monocyte-derived DC

Having established the profile of IL-12-family cytokine responses to TLR4-agonist, we examined the time-course of MAPK phosphorylation. In agreement with previous studies, p38, p44/42 and JNK were all phosphorylated over a period of 30 minutes *(figure 2A)*. To examine MAPK control of IL-12-family cytokine expression, we employed a panel of selective antagonists of MAPK that we had previously used successfully to investigate MAPK function in tumour-conditioned DC [1]. SB203580 [34] specifically inhibits p38 activation by binding to p38, allowing phosphorylation but not activation. However, since p38 phosphorylation, in part, relies on its own intrinsic kinase activity [35], treatment with SB203580 indirectly reduces p38 phosphorylation [36, 37]. SP600125 [38] is a reversible ATP-competitive inhibitor of JNK 1 and 2 that blocks phosphorylation. U0126 is a specific inhibitor of MEK1/2; the MAPK whose unique substrate is p44/42 [39]. The action of these inhibitors was examined by Western blotting. As shown in *figure 2B*, U0126 ablated p44/42 phosphorylation and SP600125 reduced JNK phosphorylation in accordance with their inhibitory mechanisms. Additionally, we noted that U0126 partially inhibited JNK activation. As expected and in accordance with its mode of action, SB203580 did not significantly reduce p38 phosphorylation [34].

p38 MAPK is a major regulator of IL-23 and IL-27

To examine the role of individual MAPK pathways in DC, cells were pre-treated with SB203580, SP600125 and U0126 before maturation via TLR4. In concordance with previous studies, including our own [1], p38 inhibition completely abolished IL-12 secretion (mean mDC control = 4,560 pg/mL/10^5 DC, mean SB treated = 155 pg/mL/10^5; p = 0.015). In contrast, p44/42 inhibition had little effect while JNK inhibition reduced IL-12 levels by 50% (mean mDC control = 4,560 pg/mL/10^5 DC, mean SB203580 treated = 1,848 pg/mL/10^5; p = 0.015) *(figure 3A)*.

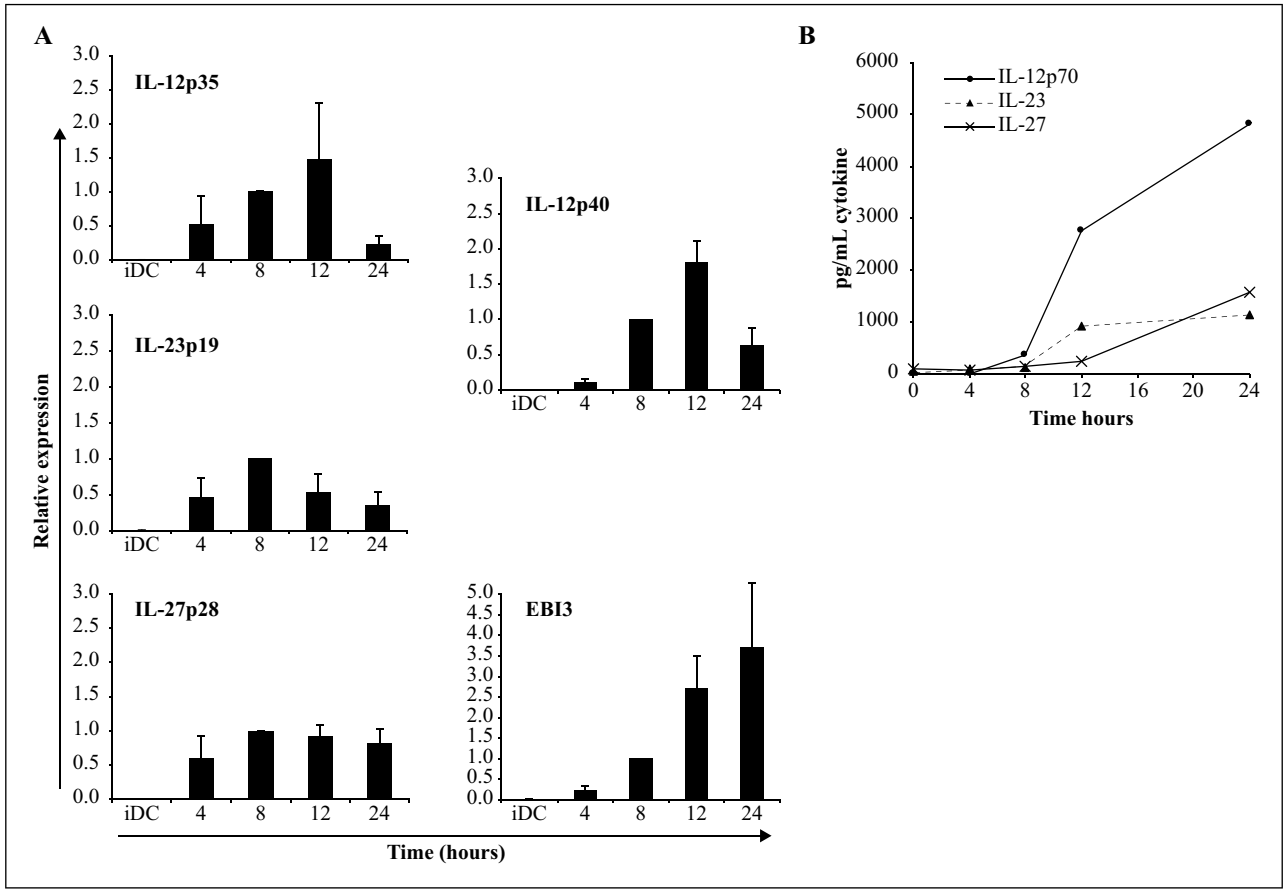

Figure 1

IL-12 family expression by DC. DC were stimulated with ultrapure LPS and IFN-γ for up to 24 hours and IL-12, IL-23 and IL-27 examined.
A) Sub-unit transcription was measured by quantitative PCR from mRNA. (Average relative expression from three donors normalised to two reference genes).
B) Secretion of each heterodimeric cytokine was measured by ELISA. Representative time-course of three donors.

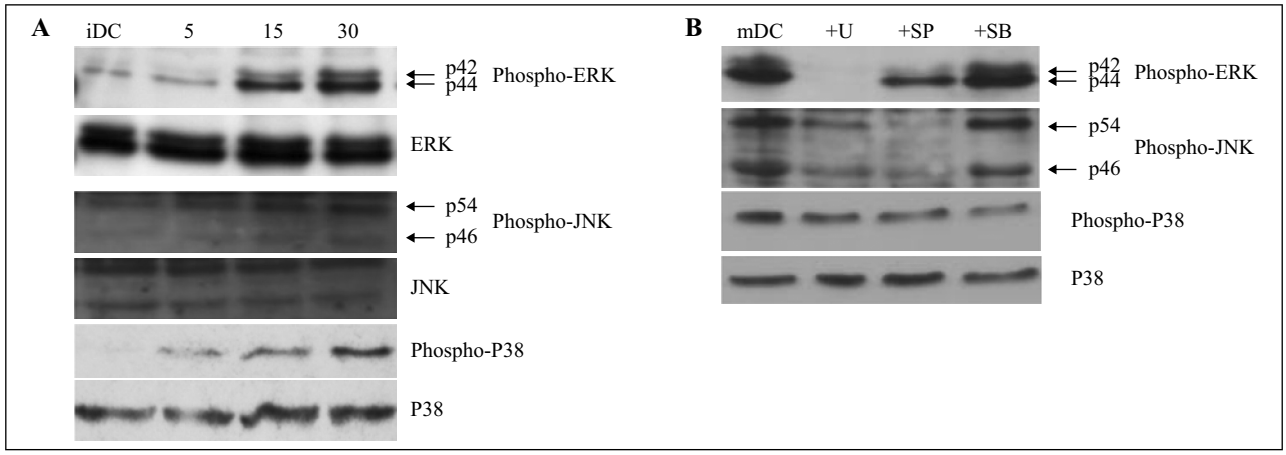

Figure 2

Phosphorylation of DC MAPK was examined by Western blotting.
A) Three major MAPK proteins are phosphorylated by LPS/IFNγ treatment of DC (representative Western blot of five donors).
B) MAPK inhibitors abolish or reduce phosphorylation of MAPK proteins at their optimal concentration (representative Western blot of six donors).

Inhibition of p38 function markedly reduced the secretion of IL-27 (mDC = 929 pg/mL/10^5, SB203580 = 131 pg/mL/10^5; p = 0.041). Secretion of IL-23 was similarly reduced following p38 inhibition (mean mDC control = 1,498 pg/mL/10^5, mean SB203580 = 224 pg/mL/10^5; p = 0.030),*(figure 3A)*. In contrast to Brereton and colleagues [6], the MEK-p44/42 pathway inhibitor U0126 had only a limited effect on all three cytokines, and this did not reach statistical significance. Inhibition of JNK reduced IL-27 secretion (mDC = 929 pg/mL/10^5, SP600125 =

498 pg/ml/10^5; p = 0.043), however, IL-23 expression was unaffected by SP600125. In summary, TLR4-dependent expression of all three cytokines required active p38, but not p44/42, whilst active JNK played a role in the secretion of IL-12 and IL-27, but was not involved in IL-23 production. The Akt/PI3K pathway is a known negative regulator of IL-12 expression. Therefore, we examined the effect of this pathway on the IL-12 family. Treatment of DC with PI3K inhibitor enhanced the secretion of IL-12 by approximately two-fold in response to TLR4-agonist. However, the secretion of IL-23 was more markedly increased (five-10-fold in some donors) following treatment of DC with Wortmannin *(figure 3B)*. In contrast, IL-27 production was unaffected. Production of IL-10 was identified in DC treated with Wortmannin (445 pg/mL/10^5 cells), but not in the supernatants of the other DC populations (not shown).

Effect of inhibition of MAPK pathways in DC on T_H-priming

To study the consequence of the downstream effect of cytokine secretion in DC, we developed a cell-free system of T_{H0} differentiation previously employed by Trinchieri and collegues [49]. Purified naive CD4+ T-cells were co-stimulated by CD3 and CD28 antibodies in the presence of cell-free supernatant from MAPK-inhibited DC. There was a pronounced induction of the IFNγ-response by CD4+ T-cells cultured in the presence of supernatant from TLR4-matured DC. This was significantly suppressed in the presence of supernatant from p38-MAPK-inhibited DC *(figure 4)* to levels that were statistically no different from those obtained with iDC. In contrast, supernatant from p44/42- and JNK-inhibited DC had only marginal effects on T_{H1} responses. Surprisingly, in light of the increase in IL-12, PI3K-inhibited DC did not increase T_{H1} responses. IL-5 levels decreased significantly with DC-maturation and were only marginally (although significantly) increased with p38-inhibition (p < 0.001). Inhibition of the p38 MAPK pathway in DC resulted in a pronounced decrease in IL-10 release from naïve T-cells. Interestingly, when naïve CD4+ T-cells were stimulated in the presence of TLR4-matured DC supernatant, there was evidence of T_{H17} responses as determined by secretion of IL-17A. T-cell secretion of IL-17A was ablated following inhibition of either the p38 or MEK-p44/42 pathways (p = 0.01). In contrast, supernatants from JNK-inhibited DC had no significantly different effect from that of mDC on IFNγ, IL-5, IL-10 or

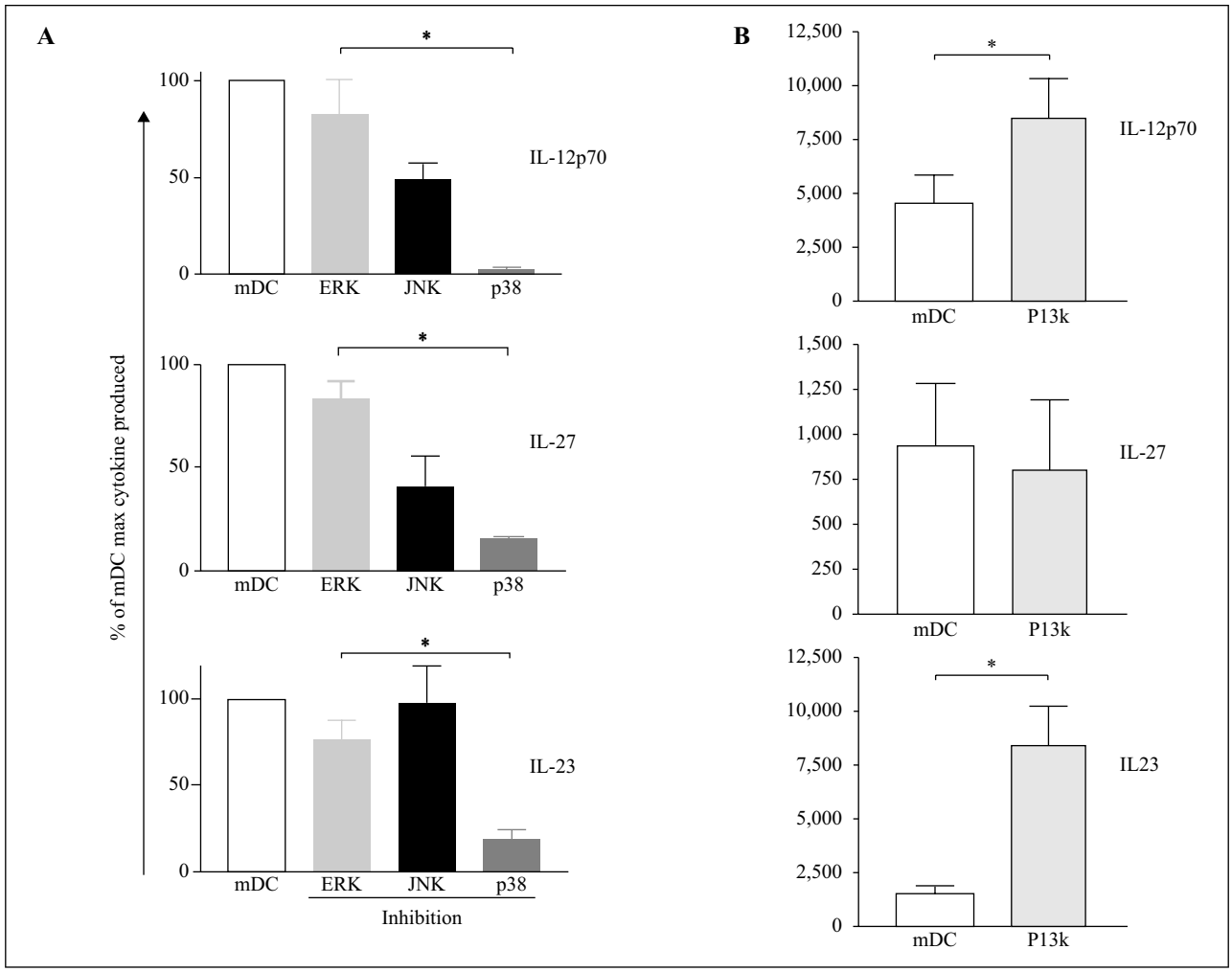

Figure 3
MAPK inhibitors differentially inhibit IL-12 family cytokines. DC were pre-treated with (**A**) MAPK and (**B**) PI3K inhibitors followed by maturation with LPS/IFNγ. Twenty four hours later, culture supernatants were collected and the level of IL-12, IL-23 and IL-27 determined by ELISA (average of six donors plus SEM). Statistical significance at p < 0.001 is indicated (*).

IL-17 secretion from naïve CD4+ T-cells. IL-4 and IL-13 were both measured in T-cell supernatants using ELISA, but values were below the detection limit for all samples (not shown). Despite producing notable alterations in IL-12 and IL-23 secretion by DC, PI3K inhibition did not increase either the T_{H1} or T_{H17} responses, and also had no significant effect on IL-4, 5, 10 or 13 secretion. Experiments were conducted to account for the effect of any carry-over of MAPK inhibitors (although they are at very low concentrations) on the cytokine profile of CD28 co-stimulated T-cells. As shown in *figure 4B*, any carry-over of cytokine or MAPK inhibitor did not significantly ($p > 0.05$) affect the cytokine output of the T-cells.

DISCUSSION

A number of recent studies have highlighted the possibility of manipulating the immune response by modulation of the intracellular signalling pathways in DC [1-7]. The present study examined the impact of the three main MAPK pathways and PI3K on the relative expression of IL-12-family cytokines. In comparison to IL-12, little is known regarding the involvement of MAPK pathways in the production of IL-23 and IL-27 by DC. By using specific inhibitors and measuring cytokine expression in mature mo-DC, we have dissected this regulation and revealed p38 and JNK as critical regulators of the APC-derived IL-12 family in the response to TLR4-agonist. Importantly, we have demonstrated that the influence of MAPK inhibition on DC-derived cytokines plays an important role in T_H-priming and differentiation, independently of the DC surface phenotype.

The transcriptional time-course for IL-12-family sub-units reflects the purported roles of these three cytokines. The "pro-inflammatory" IL-12 and IL-23 sub-units are rapidly induced, but show definite suppression of transcription at 24 hours, while the "regulatory" IL-27 sub-units continue to be transcribed after IL-12 and IL-23. In this way, IL-27 can play its key role of limiting the inflammatory responses. Our observations with ultrapure TLR4-agonist are in agreement with Schnurr *et al.*, who identified a similar time-course in *E. coli*-stimulated DC, demonstrating continued IL-27 transcription after IL-12 and IL-23 had returned to baseline [40]. However, the reported, relative levels of transcription for each sub-unit differed vastly in the Schnurr *et al.* study, showing that p28 is up-regulated 10-fold more than EBI3. Our data demonstrate a four-fold difference in favour of EBI3; this is probably due to the choice of reference gene and calibration sample. In this regard, there is a lack of any detailed information available about appropriate housekeeping genes in DC. The GeNorm application ranks reference gene stability, and of the 12 analysed, 18s RNA, as used by Schnurr *et al.*, ranks 11th most stable. This may account for the difference they observed. In contrast, we identified and employed a combination of the two most stable genes in our analysis, TOP and UBC. Future studies should consider the consequence of using sub-optimal housekeeping genes for qPCR. Furthermore, iDC did not express the genes of interest (IL-12 family), and therefore gave a result of zero, making it impossible to compare relative to untreated cells. Therefore, to ensure reliable mRNA measurement in the calibrator sample, data from the eight-hour time point were used as a reference, as all the genes of interest were expressed to some extent at this point.

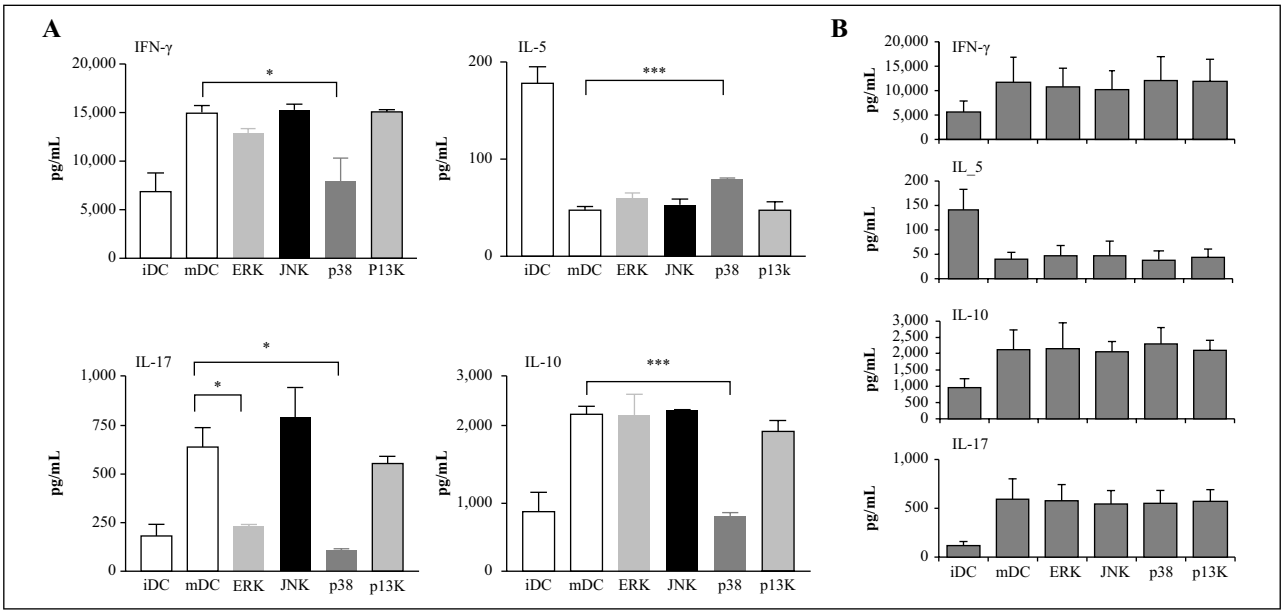

Figure 4

Consequences of DC MAPK inhibition for the generation of T_H response from naïve CD4+ T-cells.

A) Naïve CD4+ T-cells were stimulated with CD3 and CD28 antibodies for four days in the presence of conditioned medium from DC treated with the indicated MAPK inhibitors. The release of soluble T-cell-derived cytokines was determined using Luminex assays. Bars show mean plus SEM of six donors.

B) Control experiments with supernatants of DC-free cultures were conducted to account for the effect of carry-over of any MAPK inhibitors. Wells containing cytokines and MAPK inhibitors, but no cells, were treated in the same way as experimental wells, washed and harvested, and applied to naïve CD4+ T cells. Data show the mean ± SD for three donors. Statistical significance at $p < 0.01$ (*) and $p < 0.001$ (***) is indicated.

Much work to date has focused on IL-12 regulation, but considerably less is known of both IL-27 and IL-23 [41]. The present study shows that IL-12 and IL-27 are regulated in a similar fashion by MAPK pathways. This is partially supported by work in murine macrophages where TLR3/7-agonists stimulate IL-27p28 transcription in a JNK-dependant manner [43], but in the same model p38 MAPK did not play a significant role for IL-27p28 transcription. In contrast to the present study, those of Schnurr *et al.* were restricted to mRNA, and cytokine protein was not measured. IL-27p28 is remarkably similar to IL-12p35 in that it is not secreted alone, but in conjunction with its corresponding receptor-like sub-unit EBI3; if EBI3 is the target for p38 this may explain the reduction in IL-27 transcription. Alternatively, p38 has a known role in stabilising mRNA transcripts [42] *via* AU-elements in the 3'-UTR, classically described in TNF-α post-transcriptional regulation and the p35/p28 structurally-related IL-6 gene [43]. A database of AU-element-rich human mRNA (the ARED database) reveals both IL-12p35 and IL-27p28 as potential targets for such p38-based stabilisation [42]. Therefore, transcription of IL-27p28 may not always correlate with protein secretion and such data should be interpreted bearing this in mind. In contrast to IL-12 and IL-27, JNK did not play a role in IL-23 production. This, in combination with the PI3K data, indicates that each cytokine is regulated by overlapping, but distinct patterns of kinases:

– IL-12 is driven by p38 or JNK and regulated by PI3K;
– IL-27 is driven by p38 or JNK;
– IL-23 is driven by p38 and regulated by PI3K.

The work of Hawlisch describes complement protein inhibition of IL-12-family transcription, but also indicates that PI3K may negatively regulate IL-12p35, p40 and IL-23p19 sub-unit mRNA. However, this does not apply to IL-27 p28 [44]. This study also suggests that up-regulation of p44/42 signalling inhibits IL-12, and in this regard we have previously described tumour-associated regulation of IL-12p70 secretion in a p44/42-dependent manner [1]. Given we are able to modify the existing model of MAPK regulation of IL-12-family cytokines in response to TLR4 *(figure 5)*, because of the role canonically ascribed to MAPK pathways as regulators of transcription factors, it is likely that the effects we observed on IL-12-family secretion are transcriptionally mediated. In this regard, we now have evidence of a correlation between increased gene transcription and subsequent increased secretion in inhibitor-treated DC (data not shown). However, these studies have shown that the effect of MAPK and PI3K inhibitors on IL-12-family gene transcription is considerably more complex than initially assumed, and is therefore currently, the subject of further, intense investigation.

The role of MAPK inhibition in T-cell priming has been ascribed, in part, to down-regulation of MHC and co-stimulatory molecules. To assess the significance of soluble factors in T_{H1} IFN-γ induction we used an APC-free model of naïve T-cell priming. This system was recently used by Gerosa and colleagues [49], who elegantly demonstrated the differential regulation of IL-12 and IL-23 by monocyte-derived DC. Inhibition of p38 markedly reduced IL-12, 23 and 27, and was associated with reduced IFN-γ responses. However, the relative importance of each cytokine for IFN-γ in this model remains unknown, their role being currently under investigation. Despite a 50% reduction in IL-12 and IL-27 levels following JNK inhibition, this was not followed by reduced T_{H1} responses. This suggests a threshold for cytokine expression before maximal IFN-γ is reached; abrogation is below this threshold where as a 50% reduction may still provide sufficient cytokine to generate maximal T-cell responses. We are presently investigating the relative amount of IL-12-family cytokines required to generate particular T_H-responses. Alternatively, mature DC express abundant IL-12 and IL-27, and the system used here may not be sufficiently sensitive to detect functional consequences of the changes we generated. *In vivo* studies may reveal the consequence of MAPK-inhibition on T_{H1} priming, and these are presently underway in the B16 murine melanoma model.

There is considerable promiscuity in the pairing of IL-12-family subunits, as recently highlighted by the identification of IL-35 [13]. Unlike its relatives, IL-35 is not expressed by DC but derives from T-cells. The MAPK shown here to be critical for DC IL-12 family, are also known to be regulators of T-cell cytokine expression

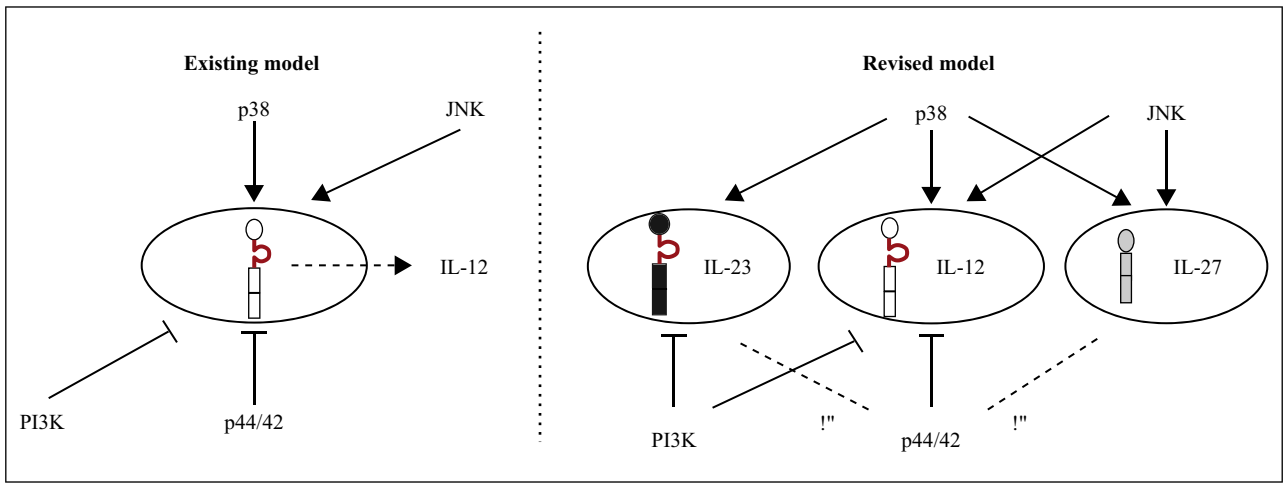

Figure 5
Model of IL-12 family regulation by MAPK and PI3K. Arrows indicate positive regulators of p38 and JNK expression, T-bars indicate negative regulators PI3K and p44/42, dotted lines remain unknown.

[45]. Convergent regulation of p35/EBI3 in these two cell types remains to be demonstrated. This study provides new evidence of the role played by DC MAPK pathways for generating IL-17 responses in CD4+ T-cells. IL-17 responses were ablated with p44/42, and p38 inhibited DC. However, IL-23 responses were not significantly reduced following U0126 treatment. Whilst T_{H17} responses were initially thought to be driven by IL-23, it is now widely accepted that a range of other cytokines including IL-1, 6, 22 and TGFβ probably orchestrate the generation of T_{H17} responses from naïve CD4+ T-cells [46-48]. In contrast, IL-23 likely plays a role in the activation of memory responses, and future studies are underway to examine the impact of the PI3K pathway on established T_{H17} responses. In summary, not only are the IL-12 family members differentially dependent on MAPK pathways, but as highlighted by recent studies [4], these pathways can be exploited to regulate immune responses.

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REFERENCES

1. Jackson AM, Mulcahy LA, Zhu XW, O'Donnell D, Patel PM. Tumour-mediated subversion of dendritic cells: Inhibiting the MEK1/2–p44/42 axis restores dendritic cell function in malignant melanoma. *Int J Cancer* 2008; 123: 623.

2. Wang S, Hong S, Yang J, *et al.* Optimizing immunotherapy in multiple myeloma: restoring the function of patients' monocyte-derived dendritic cells by inhibiting p38 or activating MEK/ERK MAPK and neutralizing interleukin-6 in progenitor cells. *Blood* 2006; 108: 4071.

3. Wang S, Yang J, Qian J, Wezeman M, Kwak LW, Yi Q. Tumor evasion of the immune system: inhibiting p38 MAPK signaling restores the function of dendritic cells in multiple myeloma. *Blood* 2006; 107: 2432.

4. Escors D, Lopes L, Lin R, *et al.* Targeting Dendritic Cells signalling to regulate the response to immunization. *Blood* 2008; 111: 3050.

5. Jarnicki A, Conroy H, Brereton C, *et al.* Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signalling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapies. *J Immunol* 2008; 180: 3797.

6. Brereton CF, Sutton CE, Lalor SJ, Lavelle EC, Mills KHG. Inhibition of ERK MAPK suppresses IL-12- and IL-1-driven IL-17 production and attenuates autoimmune diseases. *J Immunol* 2009; 183: 1715.

7. Zhao F, Falk C, Osen W, Kato M, Schadendorf D, Umansky V. Activation of p38 MAPK drives dendritic cells to become tolerogenic in *Ret* transgenic mice spontaneously driving melanoma. *Clin Cancer Res* 2009; 15: 4382.

8. Takeda A, Hamano S, Yamanaka A, *et al.* Cutting Edge: Role of IL-27/WSX-1 Signalling for Induction of T-Bet Through Activation of STAT1 During Initial Th1 Commitment. *J Immunol* 2003; 170: 4886.

9. Owaki T, Asakawa M, Fukai F, Mizuguchi J, Yoshimoto T. IL-27 Induces Th1 Differentiation via p38 MAPK/T-bet- and Intercellular Adhesion Molecule-1/LFA-1/ERK1/2-Dependent Pathways. *J Immunol* 2006; 177: 7579.

10. Colgan J, Rothman P. All in the family: IL-27 suppression of TH-17 cells. *Nat Immunol* 2006; 7: 899.

11. Awasthi A, Carrier Y, Peron JP, *et al.* A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* 2007; 8: 1380.

12. Owaki T, Asakawa M, Fukai F, Mizuguchi J, Yoshimoto T. IL-27 Suppresses CD28-Medicated IL-2 Production through Suppressor of Cytokine Signaling 3. *J Immunol* 2006; 176: 2773.

13. Collison LW, Workman CJ, Kuo TT, *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007; 450: 566.

14. Sieburth DJE, Warrington JA, Li X, *et al.* Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics* 1992; 14: 59.

15. Oppmann B, Lesley R, Blom B, *et al.* Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12. *Immunity* 2000; 13: 715.

16. Pflanz S, Timans JC, Cheung J, *et al.* IL-27, a Heterodimeric Cytokine Composed of EBI3 and p28 Protein, Induces Proliferation of Naive CD4+ T Cells. *Immunity* 2002; 16: 779.

17. Agrawal S, Agrawal A, Doughty B, *et al.* Cutting Edge: Different Toll-Like Receptor Agonists Instruct Dendritic Cells to Induce Distinct Th Responses *via* Differential Modulation of Extracellular Signal-Regulated Kinase-Mitogen-Activated Protein Kinase and c-Fos. *J Immunol* 2003; 171: 4984.

18. Yanagawa Y, Onoe K. Enhanced IL-10 Production by TLR4- and TLR2-Primed Dendritic Cells upon TLR Restimulation. *J Immunol* 2007; 178: 6173.

19. Re F, Strominger JL. Toll-like Receptor 2 (TLR2) and TLR4 Differentially Activate Human Dendritic Cells. *J Biol Chem* 2001; 276: 37692.

20. Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. A Critical Role for p38 Mitogen-Activated Protein Kinase in the Maturation of Human Blood-Derived Dendritic Cells Induced by Lipopolysaccharide, TNF-{{alpha}}, and Contact Sensitizers. *J Immunol* 2001; 166: 3837.

21. Aiba S, Manome H, Nakagawa S, *et al.* p38 Mitogen-activated Protein Kinase and Extracellular Signal-regulated Kinases Play Distinct Roles in the Activation of Dendritic Cells by Two Representative Haptens, NiCl2 and 2,4-dinitrochlorobenzene. *J Invest Dermatol* 2003; 120: 390.

22. Nakahara T, Uchi H, Urabe K, Chen Q, Furue M, Moroi Y. Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. *Int Immunol* 2004; 16: 1701.

23. Fukao T, Terauchi Y, Kadowaki T, Koyasu S. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat Immunol* 2002; 3: 875.

24. Feng GJ, Goodridge HS, Harnett MM, *et al.* Extracellular Signal-Related Kinase (ERK) and p38 Mitogen-Activated Protein (MAP) Kinases Differentially Regulate the Lipopolysaccharide-Mediated Induction of Inducible Nitric Oxide Synthase and IL-12 in Macrophages: Leishmania Phosphoglycans Subvert Macrophage IL-12 Production by Targeting ERK MAP Kinase. *J Immunol* 1999; 163: 6403.

25. Yi A-K, Yoon JG, Yeo SJ, Hong SC, English BK, Krieg AM. Role of Mitogen-Activated Protein Kinases in CpG DNA-Mediated IL-10 and IL-12 Production: Central Role of Extracellular Signal-Regulated Kinase in the Negative Feedback Loop of the CpG DNA-Mediated Th1 Response. *J Immunol* 2002; 168: 4711.

26. Wilflingseder D, Müllauer B, Schramek H, *et al.* HIV-1-Induced Migration of Monocyte-Derived Dendritic Cells Is Associated with Differential Activation of MAPK Pathways. *J Immunol* 2004; 173: 7497.

27. Riol-Blanco L, Sánchez-Sánchez N, Torres A, *et al.* The Chemokine Receptor CCR7 Activates in Dendritic Cells Two Signaling Modules That Independently Regulate Chemotaxis and Migratory Speed. *J Immunol* 2005; 174: 4070.

28. Vandesompele J, De Preter K, Pattyn F, *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3:research0034.1 - research0034.11.

29. Johansson U, Cooke SP, Londei M. Identification of a new isoform of human IL-12 p35 mRNA. *Eur J Immunogenet* 2002; 29: 439.

30. Peirson SN, Butler JN, Foster RG. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucl Acids Res* 2003; 31: e73.

31. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta] CT Method. *Methods* 2001; 25: 402.

32. Vanden Eijnden S, Goriely S, De Wit D, Goldman M, Willems F. Preferential production of the IL-12(p40)/IL-23(p19) heterodimer by dendritic cells from human newborns. *Eur J Immunol* 2006; 36: 21.

33. Vaknin-Dembinsky A, Balashov K, Weiner HL. IL-23 Is Increased in Dendritic Cells in Multiple Sclerosis and Down-Regulation of IL-23 by Antisense Oligos Increases Dendritic Cell IL-10 Production. *J Immunol* 2006; 176: 7768.

34. Cuenda A, Rouse J, Doza YN, *et al.* SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Letters* 1995; 364: 229.

35. Ge B, Gram H, Di Padova F, *et al.* MAPKK-Independent Activation of p38alpha Mediated by TAB1-Dependent Autophosphorylation of p38alpha. *Science* 2002; 295: 1291.

36. Matsuguchi T, Musikacharoen T, Ogawa T, Yoshikai Y. Gene Expressions of Toll-Like Receptor 2, But Not Toll-Like Receptor 4, Is Induced by LPS and Inflammatory Cytokines in Mouse Macrophages. *J Immunol* 2000; 165: 5767.

37. Hewison M, Freeman L, Hughes SV, *et al.* Differential Regulation of Vitamin D Receptor and Its Ligand in Human Monocyte-Derived Dendritic Cells. *J Immunol* 2003; 170: 5382.

38. Bennett BL, Sasaki DT, Murray BW, *et al.* SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001; 98: 13681.

39. Duncia JV, Santella 3rd JB, Higley CA, *et al.* MEK inhibitors: The chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorg Med Chem Lett* 1998; 8: 2839-44.

40. Schnurr M, Toy T, Shin A, Wagner M, Cebon J, Maraskovsky E. Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. *Blood* 2005; 105: 1582.

41. Hause L, Al-Salleeh FM, Petro TM. Expression of IL-27 p28 by Theiler's virus-infected macrophages depends on TLR3 and TLR7 activation of JNK-MAP-kinases. *Antiviral Res* 2007; 76: 159.

42. Frevel MAE, Bakheet T, Silva AM, Hissong JG, Khabar KS, Williams BR. p38 Mitogen-Activated Protein Kinase-Dependent and -Independent Signaling of mRNA Stability of AU-Rich Element-Containing Transcripts. *Mol Cell Biol* 2003; 23: 425.

43. Zhao W, Liu M, Kirkwood KL. p38{alpha} Stabilizes Interleukin-6 mRNA via Multiple AU-rich Elements. *J Biol Chem* 2008; 283: 1778.

44. Hawlisch H, Belkaid Y, Baelder R, Hildeman D, Gerard C, Köhl J. C5a Negatively Regulates Toll-like Receptor 4-Induced Immune Responses. *Immunity* 2005; 22: 415.

45. Nakahara T, Moroi Y, Uchi H, Furue M. Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. *J Dermatol Sci* 2006; 42: 1.

46. Kaiko GE, Horvat JC, Beagley KW, Hansbro PM. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology* 2008; 123: 326.

47. Bi Y, Liu G, Yang R. JTH17 cell induction and immune regulatory effects. *J Cell Physiol* 2007; 211: 273.

48. Korn T, Oukka M, Bettelli E. Th17 cells: Effect T-cells with inflammatory properties. *Semin Immunol* 2007; 19: 362.

49. Gerosa F, Baldani-Guerra B, Lyakh LA, *et al.* Differential regulation of IL-12 and IL-23 production in human dendritic cells. *J Exp Med* 2008; 6: 1447.