

Cytokine Secretion by Pathogen Recognition Receptor-stimulated Dendritic Cells in Rheumatoid Arthritis and Ankylosing Spondylitis

CLAUDIA PREVOSTO, JANE C. GOODALL, and J.S. HILL GASTON

ABSTRACT. Objective. Interleukin 23 (IL-23) plays a major role in differentiation and survival of IL-17-secreting CD4+ Th17 cells. Having noted a higher frequency of Th17 cells in ankylosing spondylitis (AS) and rheumatoid arthritis (RA) than in healthy donors (HD), we investigated whether IL-23 secretion is increased in these conditions.

Methods. Monocyte-derived dendritic cells (moDC) were obtained from peripheral blood of 17 HD, 16 patients with RA, and 30 patients with AS, and stimulated with ligands for several pathogen recognition receptors. Messenger RNA (mRNA) expression and cytokine secretion were analyzed by real-time polymerase chain reaction and ELISA, respectively.

Results. The combination of ligands for Toll-like receptors (TLR) 7/8 and TLR3 led to synergistic secretion of both IL-23 and IL-12p70 from all subjects; similar synergy was seen with TLR2 ligands and curdlan. However, for both combinations, moDC from patients with RA produced significantly lower amounts of IL-23 than moDC from patients with AS; in contrast, IL-12p70 secretion did not differ. Similarly, tumor necrosis factor- α , IL-6, and IL-10 were secreted at comparable levels in all subjects, whereas CXCL8 and CCL3 production was actually enhanced in moDC of patients with RA. Equivalent levels of mRNA for both IL-23p19 and IL-12p35 subunits were found in moDC from all donors, suggesting posttranscriptional regulation of IL-23 production in RA.

Conclusion. Our observations show that IL-23 production is decreased in RA and maintained in AS. Because increased numbers of CD4+IL-17+ T cells are seen in both diseases, these observations imply that there are different mechanisms underlying chronic inflammation in these 2 forms of inflammatory arthritis. (J Rheumatol First Release Aug 15 2012; doi:10.3899/jrheum.120208)

Key Indexing Terms:

DENDRITIC CELLS

INTERLEUKIN 23

RHEUMATOID ARTHRITIS

ANKYLOSING SPONDYLITIS

Th17 CELLS

Dendritic cells (DC) efficiently detect pathogens and cell damage through several pattern recognition receptors (PRR), including the Toll-like receptors (TLR). Activation of PRR results in the secretion of cytokines that affect the differentiation and function of both innate and acquired immune effector cells¹. DC have been implicated in the pathogenesis of autoimmune diseases through presentation of self-antigens, and in perpetuation of chronic inflammation through cytokine production^{2,3}; increased numbers of DC are also observed at inflammatory sites in various rheumatic diseases^{4,5,6}.

Two of the cytokines affecting T cell differentiation are interleukin 23 (IL-23) and IL-12, and activated DC are a major source of these cytokines. IL-23 and IL-12 have unique subunits (p19 and p35, respectively) covalently linked to the common p40 subunit⁷. *In vitro* stimulation of naive T cells with IL-12 induces interferon- γ (IFN- γ)-producing Th1 cells, which act against intracellular bacteria. In contrast, IL-23, together with IL-1, IL-6, and IL-21, favors differentiation and expansion of Th17 cells, which can secrete IL-17, along with IL-22, IL-21, IL-6, and tumor necrosis factor- α (TNF- α), and confer resistance to extracellular bacteria and fungi^{8,9,10,11}. The IL-23:IL-17 axis has also been implicated in murine models of autoimmunity; thus mice lacking IL-23p19 are resistant to collagen-induced arthritis, experimental autoimmune encephalomyelitis (EAE), and T-cell mediated colitis^{12,13,14}. Moreover, recent findings have shown that Th17 cells can cease to produce IL-17 (ex-Th17 cells) and instead secrete IFN- γ and granulocyte-macrophage-colony stimulating factor (GM-CSF), suggesting plasticity in T cell subsets¹⁵. Again, IL-23 is important in this respect because, together with IL-1/IL-6, it results in

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differentiation of Th17 cells, which can later proceed to GM-CSF secretion. These Th17 cells are the subset responsible for EAE, in contrast to those differentiated by IL-6 + transforming growth factor- β ^{16,17}. Certain cells of the innate immune system, including some capable of producing IL-17, also express IL23 receptor (IL-23R) and can be driven to cytokine production by IL-23; such cells are particularly active in the gut¹⁸.

IL-23 has been implicated in human diseases; lipopolysaccharide (LPS)-stimulated DC from patients with multiple sclerosis were shown to produce increased levels of IL-23¹⁹. In psoriasis, where IL-23p19 is expressed in inflamed skin, a p40-specific monoclonal antibody has shown remarkable clinical efficacy^{20,21}. Moreover, polymorphisms in the IL-23R gene are associated with susceptibility to AS²² and to psoriasis and Crohn's disease, both complicated by spondyloarthritis (SpA)^{23,24}; a hypofunctional IL-23R allele decreases susceptibility to AS²⁵. Increased numbers of Th17 cells have been reported in various forms of arthritis, including juvenile idiopathic arthritis, RA, AS, and psoriatic arthritis^{26,27,28}. In RA, while synovial cells are reported to express the IL-23p19 subunit abundantly, levels of IL-23 in synovial fluid are low^{29,30,31}.

We designed our study to determine whether monocyte-derived DC (moDC) from patients with AS and RA secrete higher levels of IL-23 than do moDC from healthy donors (HD) in response to PRR ligands, including TLR agonists and curdlan, a fungal β -glucan recognized through the C-type lectin receptor dectin-1 and the integrin CR3, together with dectin-2^{32,33}, and known to be a potent stimulator of IL-23 secretion³⁴.

MATERIALS AND METHODS

Patients. Thirty patients with AS, 16 with RA, and 17 HD were recruited for our study. Written informed consent was obtained from all patients and HD, and the work was approved by the Cambridge Cambridgeshire 2 Research Ethics Committee (Ref: 01/363; date of approval: December 13, 2001, "The role of the immune system and its component in the pathogenesis of inflammatory arthritis"). Clinical data of all donors are reported in Table 1. Patients with AS met the modified New York criteria³⁵ and those with RA fulfilled American College of Rheumatology revised criteria³⁶.

MoDC differentiation. Peripheral blood (PB) from all subjects was collected in heparinized tubes and mononuclear cells (MC) obtained by centrifugation over a Fycoll-Hypaque density-gradient. Monocytes were obtained from PBMC by positive selection using cluster of differentiation-14 (CD14) microbeads (Miltenyi Biotec) according to manufacturer's instructions. Purity of CD14+ cells, assessed by flow cytometry, was above 97%. Monocytes were cultured in 24 well plates in RPMI medium (Gibco) containing 10% heat inactivated fetal bovine serum (Biosera), L-glutamine, 50 μ g/ml gentamicin (Cydomicin; Aventis), and 10 mM HEPES buffer (Sigma-Aldrich), supplemented with recombinant GM-CSF (50 ng/ml; Biosource) and recombinant IL-4 (1000 U/ml; BD Pharmingen). At Day 6, immature moDC were harvested using cold phosphate buffered saline (PBS) and 2 mM EDTA, and counted with Trypan Blue to exclude dead cells. Four $\times 10^5$ /well viable cells were then placed in U-bottom 96-well plates. The phenotype of immature moDC (CD14-/low CD11c+ CD1c+) was checked by flow cytometry to ensure consistency of differentiation in each experiment.

MoDC stimulation with PRR ligands. Immature moDC were cultured without treatment or stimulated with various TLR ligands, and with a dectin-1 ligand, β -1,3-glucan, curdlan (125 μ g/ml; Wako Chemicals), alone or in combination. The TLR ligands used were peptidoglycan from *Bacillus subtilis* (Sigma-Aldrich) 10 μ g/ml, ultra-pure *Escherichia coli* 0111 LPS 1 μ g/ml, poly(I:C) 30 μ g/ml, Pam3CSK4, a synthetic triacylated lipopeptide 1 μ g/ml, and CL097 5 μ g/ml, all provided by Autogen Bioclear. After 24 h, supernatants were collected and stored at -20°C for cytokine measurement; poststimulation moDC were harvested to evaluate expression of the activation and maturation markers CD86, MHC class II, and CD83 using flow cytometry.

Table 1. Characteristics of patients and healthy donors (HD).

Characteristics	AS, n = 30	RA, n = 16	HD, n = 17
Age, yrs, median (range)	47.1 (23–72)	59 (32–79)	40.9 (31–61)
Sex (M:F)	23:7	6:10	10:7
Disease duration, yrs			
0–1	0	1	
1–5	6	4	
5–10	2	7	
10–20	10	2	
> 20	12	2	
BASDAI score, median (range)	4.04 (0.12–9.52)		
HLA-B27+	24, n = 27		2, n = 14
CRP, mg/l, median (range), n = 12		9.5 (0–142)	
ESR, mm/h, median (range), n = 14		10.5 (5–86)	
Swollen joint count		3 (0–24)	
Drug treatment			
None	2	0	
NSAID	17	14	
DMARD	3	14	
Current use of steroids	5	6	
TNF-blocking	7	1	

AS: ankylosing spondylitis; RA: rheumatoid arthritis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; NSAID: nonsteroidal antiinflammatory drugs; DMARD: disease-modifying antirheumatic drugs; TNF: tumor necrosis factor.

Flow cytometry. The antibodies used in the study were as follows: FITC-conjugated anti-CD14 (TüK-4; AbD Serotec), phycoerythrin (PE)-conjugated CD11c (3.9; Caltag Laboratories), allophycocyanin (APC)-conjugated CD1c (AD5-8E7; Miltenyi Biotech), and FITC-conjugated CD83 (clone HB15e) and PE-conjugated CD86 (clone 2331), both purchased from BD Bioscience. HLA-DR (L243), followed by incubation with an APC-conjugated goat anti-mouse secondary antibody, was used for staining MHC class II molecules. Stainings with isotype-matched control conjugated antibodies were performed to control for nonspecific binding for each fluorochrome. Cells were stained by incubation with antibodies at 4°C for 15 min in staining buffer [PBS, 0.1% bovine serum albumin (Sigma-Aldrich), and 0.01% sodium azide] and fixed with 2% paraformaldehyde. Cells were analyzed on a FACS Calibur flow cytometer (BD Bioscience) and data processed with WinMDI software.

Real-time quantitative polymerase chain reaction (RT-PCR). Total RNA was extracted from cells 6 hours poststimulation, using RNeasyTM columns (Qiagen) as described by the manufacturer. The expressions of IL-23p19, IL-12p35, and IL-12/IL-23p40 were quantified as ratio of fold change differences in target gene expression between treated and untreated cells relative to those in reference hypoxanthine-guanine phosphoribosyltransferase mRNA. All RT-PCR probe primer sets were obtained from Applied Biosystems (Foster City, CA, USA), and their sequence is reported in a previous study³⁷.

Measurement of cytokines by ELISA. IL-23p19/p40 heterodimer was evaluated using an eBioscience kit according to manufacturer's instructions. For measurement of biologically active IL-12p70, a sandwich ELISA was performed using purified rat anti-human IL-12p70 (0.5 mg/ml) as capture antibody, followed by a biotin-conjugated mouse anti-human IL-12 (p40/p70) antibody (0.5 mg/ml) together with horseradish peroxidase-conjugated streptavidin; all the reagents were provided by BD Pharmingen. Colorimetric data were collected using a Multiscan ELISA reader (Laboratory Systems) after the addition of the chromogen 3,3',5,5'-tetramethylbenzidine (Neogen); the concentration of cytokines in supernatants was calculated by referring to a standard curve using Ascent software (Thermolab Scientific). The sensitivity of both ELISA assays was 20 pg/ml.

Measurement of cytokines using multicytokine bead arrays. MoDC supernatants were analyzed for IL-1 β , IL-6, TNF- α , IL-10, CXCL8, and CCL3 using FlowCytomixTM simplex kits (Bender MedSystems) following the manufacturer's instructions. Briefly, a mixture of fluorescent polystyrene beads coupled with antibodies specific to each analyte was incubated with the samples. Concurrently, a biotin-conjugated antibody mixture was added that binds to the analytes captured by the first antibodies; then PE-streptavidin was added to allow fluorescent emission signals. Samples were analyzed by flow cytometry based on differential bead sizes and their distinct spectral signature; FlowCytomix Pro 2.3 software enabled calculation of the concentration of each cytokine using a standard curve generated for each analyte.

Statistical analysis. Statistical analyses were carried out using GraphPAD Prism version 4. One-way ANOVA was used to test for statistically significant differences between nonparametric datasets; the limit of significance was set at 0.05. Posthoc testing was done with Dunn's test. Spearman's rank correlation coefficient, ρ , was used to test for the correlations between nonparametric measures.

RESULTS

IL-23 and IL-12p70 production in response to PRR ligands in HD and in patients with AS and RA. IL-23 and IL-12 secretion was measured following stimulation of moDC with ligands for TLR4, TLR3, and TLR7/8, alone or in combination. The optimal concentrations of ligands and the timepoints at which maximal secretion of the cytokines occurred were determined previously (data not shown). The

levels of both cytokines were below the detection limit of ELISA assays in unstimulated (immature) moDC (data not shown), but moDC produced both IL-23 and IL-12p70 following stimulation with either TLR3 or TLR7/8 ligands, although at relatively low levels in all donors (Figure 1). Minimal amounts of IL-23 or IL-12p70 were produced upon stimulation of TLR4 with ultra-pure LPS. However, the combination of a TLR7/8 ligand with those for TLR3 or TLR4 led to marked synergistic production of both IL-23 and IL-12p70, not only in HD, but also in patients with AS and RA (Figure 1). Among the combinations we used, poly(I:C; TLR3 ligand) + CL097 (TLR7/8 ligand) was optimal. Moreover, we observed significant positive correlations between IL-23 and IL-12p70 secretion in moDC from HD and patients with AS, but not in patients with RA, using either poly(I:C) or LPS combined with CL097 (Figure 2).

When the most potent stimulus, i.e., CL097 + poly(I:C), was tested, analysis of the 3 groups by ANOVA showed a significant difference among the groups ($p = 0.028$). Posthoc testing showed that moDC from patients with AS secreted significantly more IL-23 than did moDC of patients with RA ($p < 0.05$). While patients with AS showed a 2-fold increase in the median IL-23 production compared with HD, this difference did not reach statistical significance (Figure 1A). In contrast, there were no significant differences in IL-12p70 secretion among the 3 groups when the optimal combination was used (Figure 1C). The relatively low levels of IL-12 obtained using CL097 only were, however, significantly different between patients with AS and patients with RA ($p \leq 0.05$). When stimulation with ultra-pure LPS was combined with the CL097, much lower levels of IL-23 and IL-12p70 were seen (Figure 1B and 1D), and there were no statistically significant differences among groups, although a trend was still evident toward decreased levels of IL-23 secretion by moDC from patients with RA, as compared to moDC from patients with AS.

The difference in IL-23 secretion in RA compared to patients with AS prompted us to challenge moDC with peptidoglycan (a ligand for TLR2/6) and curdlan, a dectin-1 ligand. IL-23 secretion was pronounced upon stimulation with curdlan, while peptidoglycan induced only low levels in moDC from all subjects (Figure 3). However, Pam3CSK4, a specific ligand for TLR2 paired with TLR1 rather than TLR6, enhanced IL-23 production in response to curdlan in all subjects, and ANOVA revealed a significant difference among the groups ($p = 0.033$). Although this difference again appeared to be due to the lower production by the moDC of patients with RA, this did not reach significance in posthoc testing (Figure 3). Peptidoglycan and curdlan, or curdlan in combination with Pam3CSK4, resulted in very low levels of IL-12p70 secretion in all subjects (data not shown).

We tested whether the ability to produce IL-23 and IL-12 correlated with clinical measurements in AS or RA. When using an optimal stimulus (poly I:C + CL097), we noted a

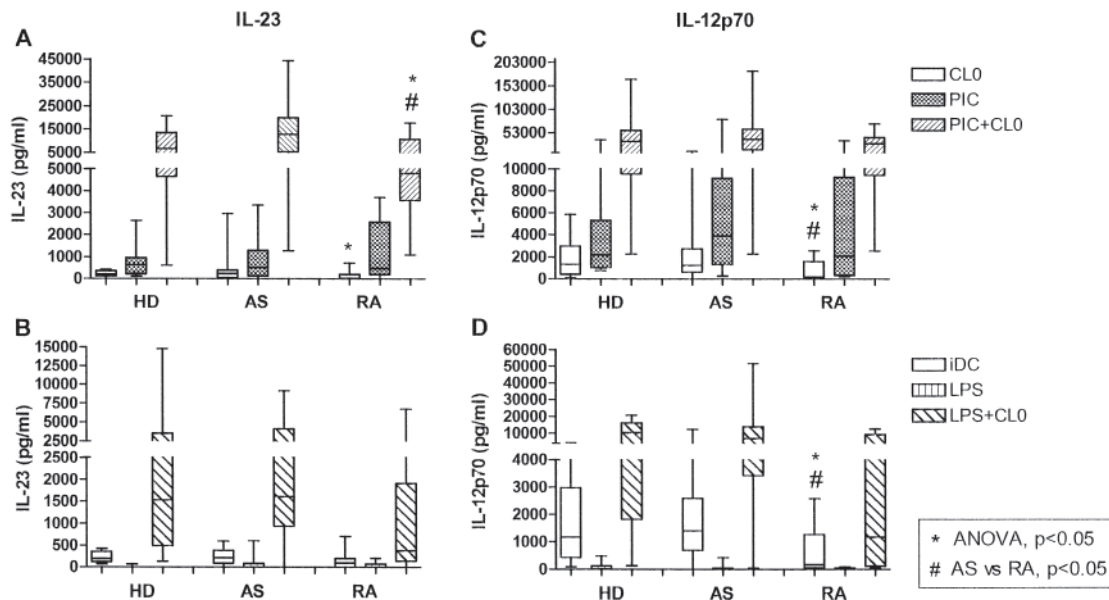


Figure 1. IL-23 and IL-12p70 secretion by monocyte-derived dendritic cells (moDC) in response to various pattern recognition receptor ligands. IL-23 (panels A, B) and IL-12p70 (panels C, D) produced by moDC from healthy donors (HD) and patients with AS and RA in response to CL097 and poly(I:C) (PIC), singly or in combination (A, C); and to CL097 and LPS, singly or in combination (B, D). Data represent results from 17 HD, 30 patients with AS and 16 with RA (CL097, PIC, PIC+CL097) or 14 HD, 24 patients with AS and 9 with RA (LPS, LPS+CL097). Dashes represent median values. * $p < 0.05$ by ANOVA, # $p < 0.05$ AS vs RA posthoc analysis.

significant correlation between the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and IL-23 production ($r = 0.39$, $p < 0.05$), but this was not found for IL-12p70 production. Similarly, when patients with AS were divided into those with low and high BASDAI, those with active disease showed higher levels of IL-23 ($p = 0.027$). No correlations with age or disease duration were seen in AS. In RA, age, disease duration, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), swollen joint counts, and presence of erosions were also not correlated with IL-23 or IL-12p70 production, and showed no differences when divided into those with high and low ESR or CRP. CRP and ESR were not measured routinely in the AS cohort because they are known to correlate poorly with disease activity as measured by clinical indices. Interestingly, treatment of AS with TNF- α -blocking drugs had no consistent effect on the ability of moDC to produce IL-23 and IL-12p70, because comparable levels were produced by patients with AS treated with nonsteroidal antiinflammatory drugs (NSAID) alone and those receiving TNF- α blockade (either etanercept or adalimumab). This may reflect our use of moDC derived from PB monocytes by *ex vivo* culture for 6 days before testing their cytokine secretion, so that effects of drug treatment or the inflammatory environment may no longer be evident.

Expression of IL-23p19, IL-12p35, and IL-12/IL-23p40 mRNA in response to PRR ligands. The findings of dissimilar secretion of IL-23 between patients with RA and those

with AS raised the question of whether this reflected altered gene transcription. Following preliminary experiments to establish the time course of expression of mRNA for IL-23 and IL-12 subunits in response to TLR agonists, cells were harvested at 6 h poststimulation. At this time any differences in mRNA expression between different donors were already established and were consistent throughout the rest of the time course (data not shown). Increases in mRNA for each subunit of IL-23 and IL-12 in response to poly(I:C) + CL097 were comparable in all groups, with a small number of outliers in each group, and no evidence of impaired transcription in RA moDC (Figure 4). Similarly, the increase in IL-23p19 RNA in response to curdlan did not differ in limited numbers of patients with RA, AS, and HD tested ($n = 2, 6, \text{ and } 4$, respectively; data not shown). These results suggest that IL-23 secretion may be regulated posttranscriptionally, and that the difference between patients with RA and those with AS occurs at this level.

MoDC activation in response to PRR ligands. To assess whether differences in IL-23 production reflected intrinsic defects in their response to PRR ligands, the effect of TLR agonists on surface expression of CD86, HLA-DR, and CD83 at 24 h poststimulation was measured by flow cytometry. Increased levels of expression for each of these markers were similar in moDC from each group (Figure 5), indicating a similar intrinsic capability of all cells to respond to activation. Similarly, the phenotype of unstimulated moDC, CD14-CD11c+CD1c+, was comparable in HD and

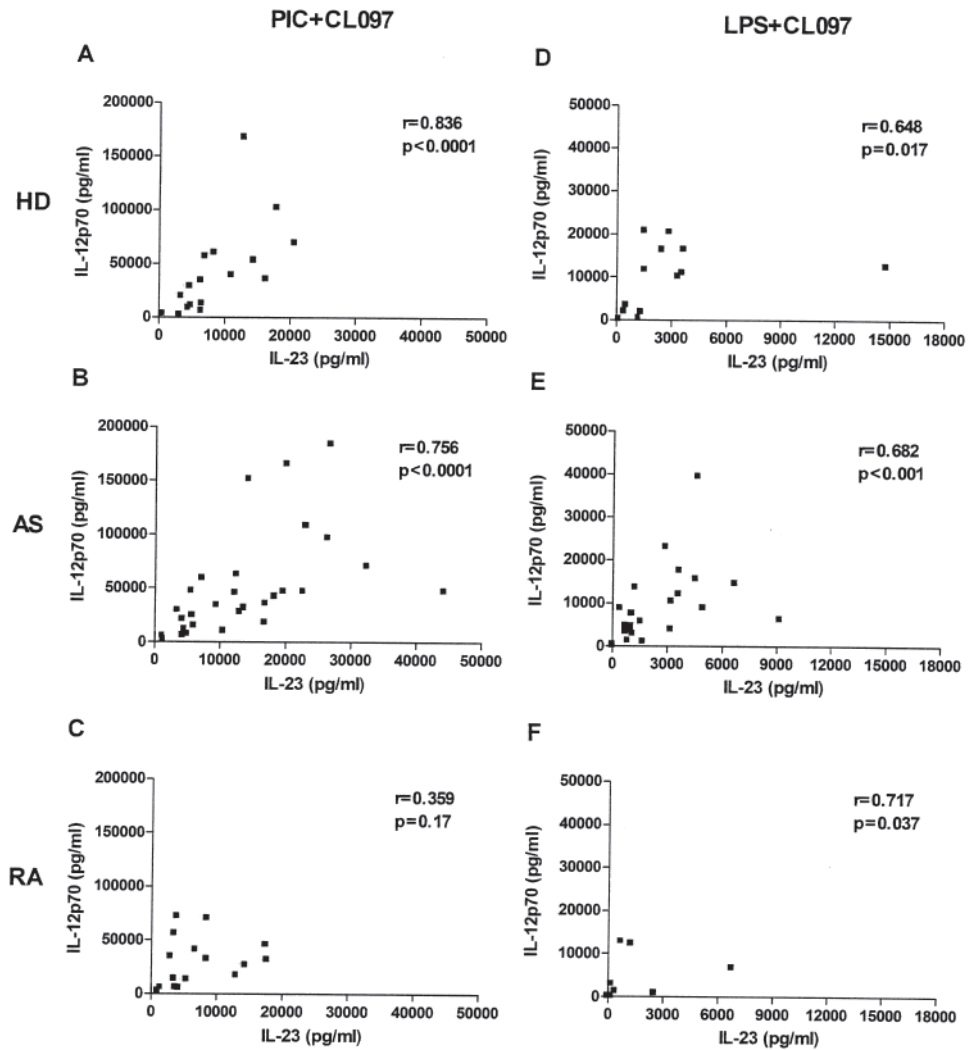


Figure 2. Correlations between IL-23 and IL-12p70 secretion in healthy donors (HD) and patients with AS and RA. Concentrations of IL-23 (X-axis) and IL-12p70 (Y-axis) obtained from monocyte-derived dendritic cells in response to a combination of CL097 and poly(I:C) (PIC+CL097, panels A, B, C), or CL097 and LPS (LPS+CL097, panels D, E, F) from HD (A, D) and patients with AS (B, E) and RA (C, F). The numbers of donors in each group are as in Figure 1. Spearman's rank correlation coefficient rho (r) and corresponding p values are shown.

patients with AS and RA (data not shown). Moreover, while different TLR ligands varied in their ability to induce expression of particular markers, all subjects showed an identical pattern of response to each of the ligands tested (Figure 6) and also to curdlan (data not shown).

Production of additional cytokines and chemokines in response to PRR ligands. Having observed differences in secretion of IL-23 in moDC from patients with RA, we widened our investigation of the response to PRR ligands by measuring a range of cytokines and chemokines using multiplex bead assays. Levels of IL-6, TNF- α , and IL-10 in response to different PRR ligands, individually or in combination, were comparable in all subjects (Figure 7). Interestingly, moDC from patients with RA produced significantly higher

levels of both CXCL8 and CCL3 than moDC from HD or from patients with AS, in marked contrast to their decreased production of IL-23.

DISCUSSION

Cytokines secreted by DC have a fundamental role in inducing and sustaining autoimmune and chronic inflammatory diseases; in particular, the balance between IL-23, which sustains the survival of Th17 cells, and IL-12, which induces Th1 cell development, may be a critical factor^{8,9,20,38}. In view of the increased numbers of IL-17-producing T cells demonstrated in both AS and RA, we hypothesized that this might be driven by IL-23, and therefore that IL-23 production might be increased in patients with AS and/or RA as

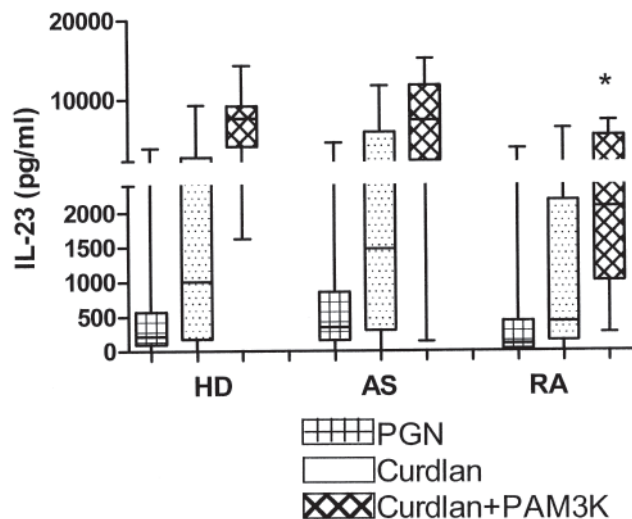


Figure 3. IL-23 secretion by monocyte-derived dendritic cells (moDC) in response to peptidoglycan and curdlan. IL-23 produced by moDC from healthy donors (HD) and patients with AS and RA in response to peptidoglycan (PGN) and curdlan singly, or curdlan and Pam3CSK4 (PAM3K) in combination. Data represent results from 17 HD and 30 patients with AS and 16 with RA (PGN and curdlan) or 12 HD and 18 AS and 10 RA (curdlan+Pam3CSK4). Dashes represent median values. * $p < 0.05$ by ANOVA.

compared to HD. However, when we analyzed IL-23 production by moDC, those from patients with RA produced significantly lower amounts of IL-23 in response to several combinations of TLR ligands than moDC of patients with AS. In contrast, secretion of the related cytokine, IL-12, was comparable in both forms of arthritis. Further, there was no global deficiency in the responsiveness of RA moDC, because they produced higher amounts of CXCL8 and CCL3 than those from HD or patients with AS, and their upregulation of surface expression of CD86, CD83, and HLA-DR was also unchanged. While expression of TLR has been shown to vary in different conditions, these data exclude levels of receptor expression as the explanation for the differences seen in IL-23 secretion, unless IL-23 production was to be much more sensitive to TLR expression levels than other TLR-mediated effects, and there is no evidence that this is the case. The differences in IL-23 secretion were not mirrored by mRNA levels, suggesting that the differences relate to posttranscriptional regulation of IL-23 synthesis. A recent report has shown that IL-23 mRNA stability is controlled posttranslationally in a way that is similar to TNF- α mRNA, involving an AU-rich region in the 3' untranslated region, and the action of tristetraprolin³⁹.

The combination of TLR7/8 and TLR3 ligands induced synergistic secretion of both IL-23 and IL-12p70 from moDC of all donors, in agreement with studies on IL-12p70 production^{40,41,42}. Synergistic effects on IL-23p19 transcription have also been reported in HD^{40,42}, but in contrast to our clear results, others did not report any synergistic effect on IL-23 secretion⁴². The reason for this difference is

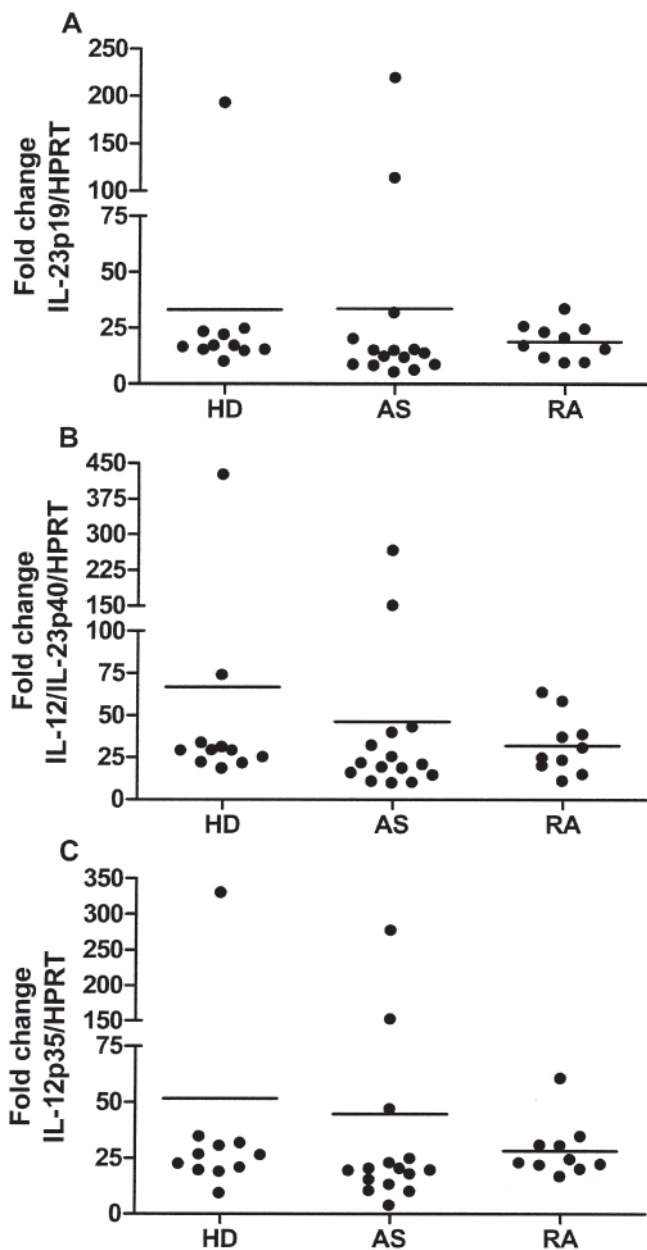


Figure 4. Expression of mRNA for IL-23 and IL-12 subunits in monocyte-derived dendritic cells (moDC) treated with TLR3 and TLR7/8 ligands. Upregulation of mRNA levels for IL-23p19 (panel A), IL-12/IL-23p40 (panel B), and IL-12p35 (panel C) in response to the combination of poly(I:C) and CL097 by moDC from healthy donors (HD; $n = 11$) and patients with AS ($n = 15$) and RA ($n = 10$). Data are reported as fold change. Dashes represent median values.

unknown, but could be due to the different imidazoquinoline derivatives used as TLR7/8 ligands, i.e., R-848 by Makela, *et al*⁴² and CL097 in our study.

Beta-glucans, including curdlan, engage the PRR, dectin-1, and have been shown to be potent inducers of IL-23 secretion and hence able to drive a robust Th17 response,

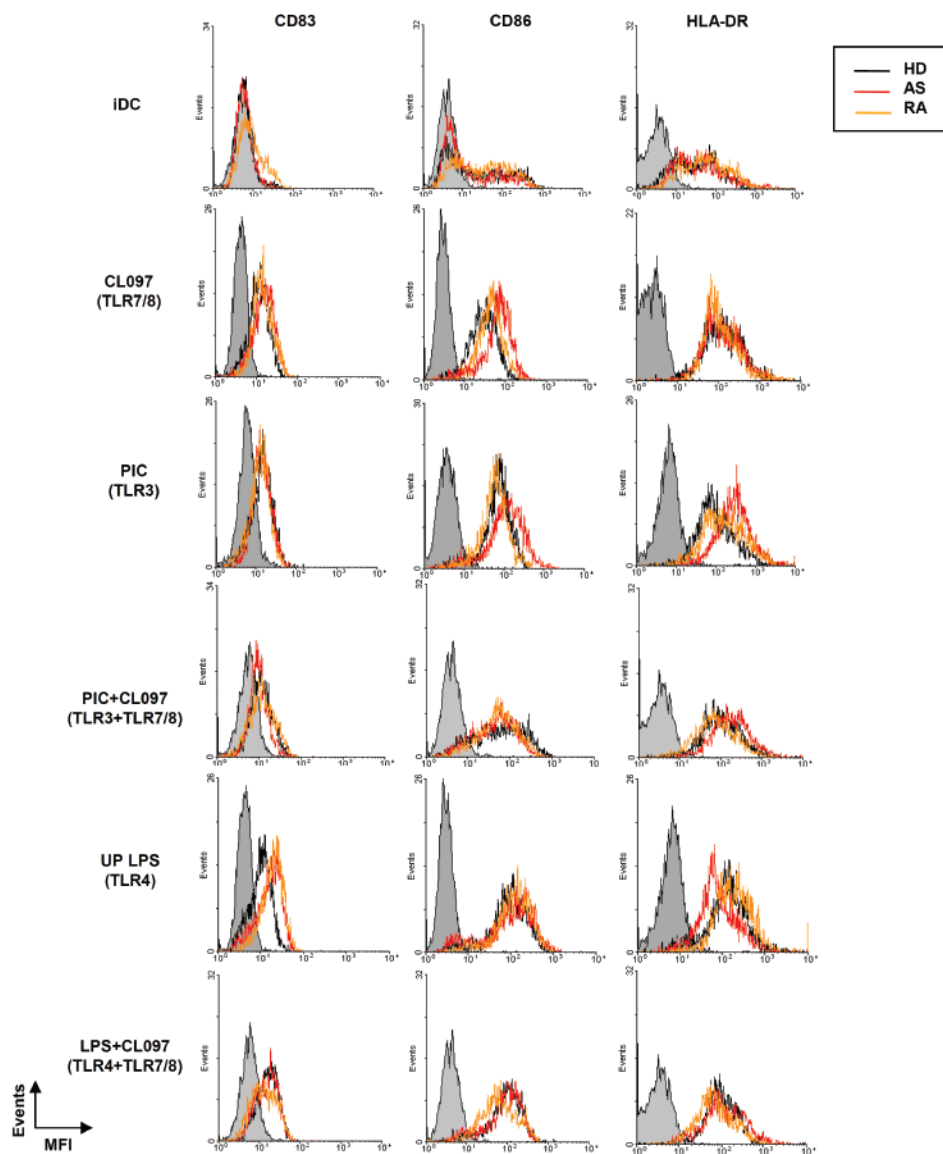


Figure 5. Expression of activation markers on monocyte-derived dendritic cells (moDC) from healthy donors (HD) and patients with AS and RA. Expression of CD83, CD86, and HLA-DR by moDC untreated (iDC) or stimulated with CL097, poly(I:C) (PIC), or LPS alone, or with CL097 in combination with either poly(I:C) (PIC+CL097) or LPS (LPS+CL097). Mean fluorescence intensity (MFI) for each marker is plotted. Data from a single representative donor from each group are shown. Gray shaded lines indicate staining with fluorochrome-conjugated isotype control antibodies.

which is needed to combat fungal infection⁴³. Recently, Plantinga and colleagues showed high levels of expression of dectin-1 in RA synovial tissues, although its contribution to synovial inflammation still remains unclear⁴⁴. Again, in our study, IL-23 secretion induced by stimulation with curdlan in combination with the TLR2 ligand, Pam3CSK4, resulted in lower levels of IL-23 from RA moDC as compared to those from patients with AS, thereby supporting our findings with the combinations of TLR3 and TLR7/8 agonists.

Although responses to single TLR ligands were much

lower than responses to combinations, production of IL-12p70 in response to the TLR7/8 ligand CL097 was significantly lower in moDC from patients with RA. This raises the possibility of a defect in TLR7/8-mediated responses in RA, but impairments in signaling by TLR7 and/or TLR8, which are located in endosomes rather than the cell surface, cannot solely account for the differential secretion of IL-23 in RA, because stimulation through TLR2 + dectin-1 also resulted in decreased secretion of IL-23 by moDC from patients with RA.

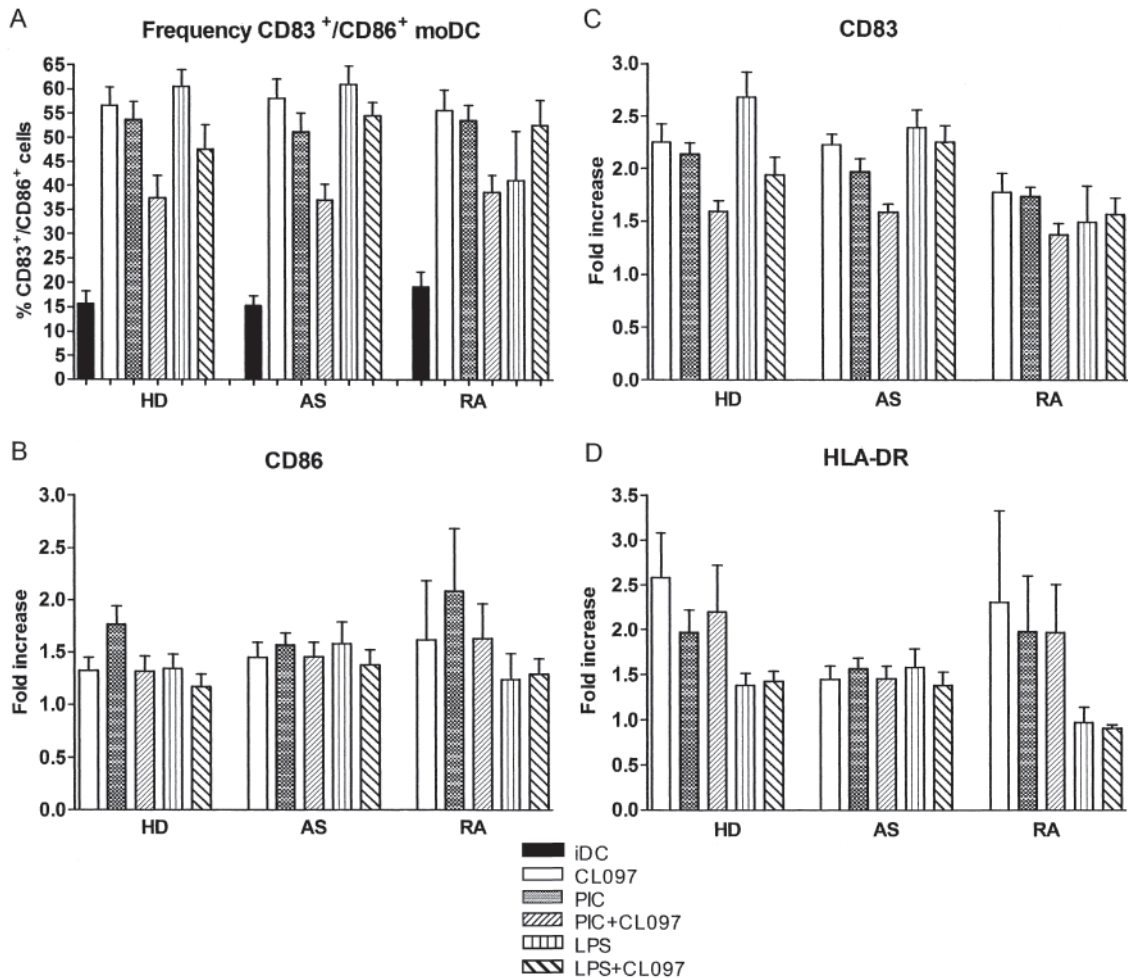


Figure 6. Analysis of activation marker expression in monocyte-derived dendritic cells (moDC) stimulated with TLR ligands. A. Percentage of moDC positive for both CD83 and CD86 either untreated (iDC) or treated with CL097, poly(I:C) (PIC), or LPS, or a combination of CL097 with poly(I:C) (PIC+CL097) or LPS (LPS+CL097) from healthy donors (HD) and patients with AS and RA, using the same conditions as in Figure 5. Panels B-D. Mean fluorescence intensity of CD86 (B), CD83 (C), and HLA-DR (D), represented as fold increase (ratio between treated and unstimulated moDC from each donor). Data represent median \pm SEM. Data were obtained from at least 11 HD, 15 patients with AS, and 10 with RA (iDC, PIC, CL097, PIC+CL097) or 7 HD, 12 with AS, and 6 with RA (LPS, LPS+CL097).

The precise roles played by IL-23 in AS and RA are still unclear. While IL-23 has been implicated in animal models of both AS and RA, there are no associations between genes involved in response to IL-23 and the generation of Th17 cells in RA. This allows the hypothesis that this cytokine pathway may be of primary importance in AS pathogenesis, as compared to acting as a downstream effector mechanism in RA. Very low levels of IL-23 have been reported in the sera of patients with SpA and RA, and these did not differ from the levels in HD sera^{29,30,45}. It is likely, therefore, that IL-23 may act locally rather than systemically⁴⁶, although a correlation between serum levels of IL-23 and disease activity measures has been reported in RA, but not SpA⁴⁵. Low amounts of biologically active IL-23 have been detected in the synovial fluid of both patients with SpA and those with

RA^{29,30,31,45}, but the main influence of IL-23 is likely to be on cell:cell interactions within synovial tissue. In this study secretion of neither IL-23 nor IL-12p70 was generally correlated with clinical measures in AS or RA, with the exception of a correlation between IL-23 secretion and BASDAI in AS. While treatment with TNF blockade could affect moDC cytokine secretion, as reported⁴⁷, this had no discernible effect in our study as far as AS was concerned, because cytokine production in 7 patients with AS treated with TNF blockade (etanercept or adalimumab) did not differ from those treated with NSAID alone (only 1 patient with RA was receiving TNF-blocking agents). Formally excluding all donors receiving anti-TNF drugs from our analysis did not affect the statistically significant differences.

Our findings suggest that moDC derived from patients

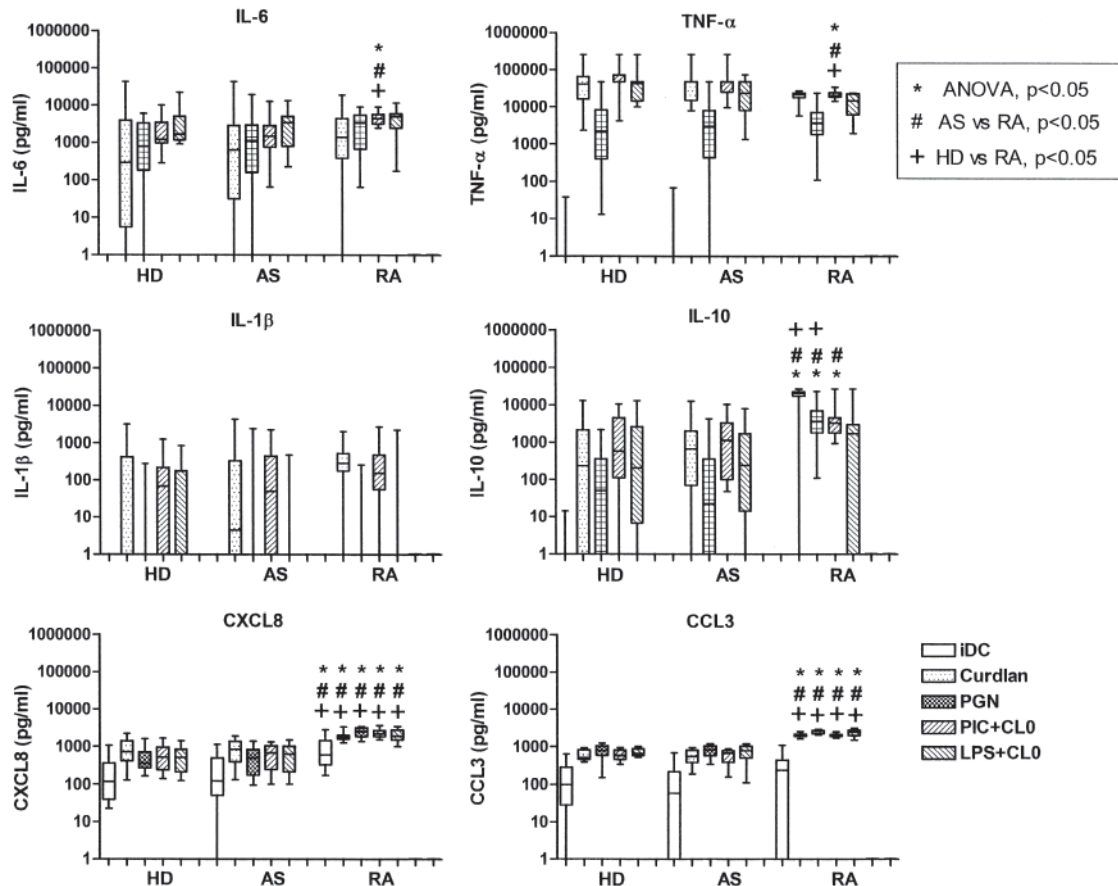


Figure 7. Secretion of cytokines and chemokines by monocyte-derived dendritic cells (moDC) from 16 healthy donors (HD), 28 patients with AS, and 16 with RA. Box and whisker plots for cytokine and chemokine secretion by moDC, untreated (iDC) or stimulated with curdlan, peptidoglycan (PGN), or combinations of CL097 with poly(I:C) (PIC+CL097) or LPS (LPS+CL097). P values are from posthoc analysis.

with RA and AS differ with regard to their ability to produce IL-23. There is a tendency in moDC from patients with RA to make lesser amounts of IL-23, while not altering their production of IL-12 and consequently altering the ratio of these 2 cytokines and their propensity to develop Th1 versus Th17 cells. In contrast, moDC from patients with AS maintain levels of secretion of both IL-23 and IL-12 comparable to those observed in HD, despite having levels of systemic inflammation similar to those with RA. These observations can be compared with the recent report from Zeng, *et al*⁴⁸ showing clearly increased IL-23 secretion from macrophages of patients with AS. While our study did not show significantly increased IL-23 production by AS DC, its maintenance in active inflammation (in contrast to RA) may point to a favoring of IL-23 secretion in DC also. Together these observations are consistent with a prominent role for IL-23 in AS pathogenesis, as recently hypothesized.

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REFERENCES

1. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783-801.
2. Lutzky V, Hannawi S, Thomas R. Cells of the synovium in rheumatoid arthritis. *Dendritic cells. Arthritis Res Ther* 2007;9:219.
3. Khan S, Greenberg JD, Bhardwaj N. Dendritic cells as targets for therapy in rheumatoid arthritis. *Nat Rev Rheumatol* 2009;5:566-71.
4. Van Krinks CH, Matyszak MK, Gaston JS. Characterization of plasmacytoid dendritic cells in inflammatory arthritis synovial fluid. *Rheumatology* 2004;43:453-60.
5. Cavanagh LL, Boyce A, Smith L, Padmanabha J, Filgueira L, Pietschmann P, et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. *Arthritis Res Ther* 2005;7:R230-40.
6. Lebre MC, Jongbloed SL, Tas SW, Smeets TJ, McInnes IB, Tak PP. Rheumatoid arthritis synovium contains two subsets of CD83-DC-LAMP- dendritic cells with distinct cytokine profiles. *Am J Pathol* 2008;172:940-50.
7. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte 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;13:715-25.
8. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003;3:133-46.
9. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1-beta and 6 but not transforming growth factor-beta

- are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007;8:942-9.
10. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;6:1123-32.
 11. Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 2010;467:967-71.
 12. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003;421:744-8.
 13. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 2003;198:1951-7.
 14. Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 2006;116:1310-6.
 15. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* 2011;12:255-63.
 16. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005;201:233-40.
 17. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 2011;12:568-75.
 18. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* 2010;464:1371-5.
 19. 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;176:7768-74.
 20. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007;8:950-7.
 21. Gottlieb AB, Cooper KD, McCormick TS, Toichi E, Everitt DE, Frederick B, et al. A phase 1, double-blind, placebo-controlled study evaluating single subcutaneous administrations of a human interleukin-12/23 monoclonal antibody in subjects with plaque psoriasis. *Curr Med Res Opin* 2007;23:1081-92.
 22. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;447:661-78.
 23. Cargill M, Schrodi SJ, Chang M, Garcia VE, Brandon R, Callis KP, et al. A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes. *Am J Hum Genet* 2007;80:273-90.
 24. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 2006;314:1461-3.
 25. Di Meglio P, Di Cesare A, Laggner U, Chu CC, Napolitano L, Villanova F, et al. The IL23R R381Q gene variant protects against immune-mediated diseases by impairing IL-23-induced Th17 effector response in humans. *PLoS One* 2011;6:e17160.
 26. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum* 2008;58:875-87.
 27. Jandus C, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P. Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis Rheum* 2008;58:2307-17.
 28. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis Rheum* 2009;60:1647-56.
 29. Brentano F, Ospelt C, Stanczyk J, Gay RE, Gay S, Kyburz D. Abundant expression of the interleukin (IL)23 subunit p19, but low levels of bioactive IL23 in the rheumatoid synovium: Differential expression and Toll-like receptor-(TLR) dependent regulation of the IL23 subunits, p19 and p40, in rheumatoid arthritis. *Ann Rheum Dis* 2009;68:143-50.
 30. Hillyer P, Larche MJ, Bowman EP, McClanahan TK, de Waal Malefyt R, Schewitz LP, et al. Investigating the role of the interleukin-23/17A axis in rheumatoid arthritis. *Rheumatology* 2009;48:1581-9.
 31. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, de Jager W, et al. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc Natl Acad Sci USA* 2010;107:14751-6.
 32. Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. *Immunol Rev* 2009;230:38-50.
 33. Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med* 2009;206:2037-51.
 34. McKenzie BS, Kastelein RA, Cua DJ. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol* 2006;27:17-23.
 35. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum* 1984;27:361-8.
 36. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
 37. Goodall JC, Wu C, Zhang Y, McNeill L, Ellis L, Saudek V, et al. Endoplasmic reticulum stress-induced transcription factor, CHOP, is crucial for dendritic cell IL-23 expression. *Proc Natl Acad Sci USA* 2010;107:17698-703.
 38. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007;204:1849-61.
 39. Qian X, Ning H, Zhang J, Hoft DF, Stumpo DJ, Blackshear PJ, et al. Posttranscriptional regulation of IL-23 expression by IFN-gamma through tristetraprolin. *J Immunol* 2011;186:6454-64.
 40. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005;6:769-76.
 41. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, et al. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum* 2005;52:2313-22.
 42. Makela SM, Strengell M, Pietila TE, Osterlund P, Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol* 2009;85:664-72.
 43. LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 2007;8:630-8.
 44. Plantinga TS, Fransen J, Takahashi N, Stienstra R, van Riel PL, van

- den Berg WB, et al. Functional consequences of DECTIN-1 early stop codon polymorphism Y238X in rheumatoid arthritis. *Arthritis Res Ther* 2010;12:R26.
45. Melis L, Vandooren B, Kruithof E, Jacques P, De Vos M, Mielants H, et al. Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis. *Ann Rheum Dis* 2010;69:618-23.
46. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 2006;25:309-18.
47. Popa C, Barrera P, Joosten LA, van Riel PL, Kullberg BJ, van der Meer JW, et al. Cytokine production from stimulated whole blood cultures in rheumatoid arthritis patients treated with various TNF blocking agents. *Eur Cytokine Netw* 2009;20:88-93.
48. Zeng L, Lindstrom MJ, Smith JA. Ankylosing spondylitis macrophage production of higher levels of interleukin-23 in response to lipopolysaccharide without induction of a significant unfolded protein response. *Arthritis Rheum* 2011;63:3807-17.