EXTENDED REPORT

High production of proinflammatory and Th1 cytokines by dendritic cells from patients with rheumatoid arthritis, and down regulation upon FcγR triggering


Objective: To assess whether DC from RA produce altered cytokine levels and whether this is regulated by triggering of Fc gamma receptors (FcγR).

Methods: The production of proinflammatory (TNFα, IL1, IL6), Th1 (IL12, IFNγ), and Th2 (IL10) cytokines profiles of immature DC (iDC) from patients with RA and healthy subjects upon triggering of FcγR dependent and independent pathways was investigated. iDC, derived from blood monocytes by standardised protocols, were stimulated with immune complexes (IC) at day 6 for 48 hours and, subsequently, for 2 days with LPS in the presence or absence of IC or IFNγ, resulting in fully matured DC (mDC). IL1, IL6, TNFα, IFNγ, IL12, and IL10 levels in supernatants were measured by ELISA and RIA.

Results: mDC from patients with RA showed a markedly increased production of IL1, IL6, TNFα, and IL10 compared with DC from healthy donors. Triggering of FcγR decreased the production of proinflammatory cytokines IL1, IL12, and IFNγ by iDC and mDC in RA and controls. The production of IL6 and TNFα decreased in patients with RA, whereas it was increased in controls. Triggering of FcγR independent mechanisms using IFNγ increased the production of proinflammatory and Th1 cytokines, which was more pronounced in RA.

Conclusion: FcγR dependent pathways influence cytokine production by DC. A skewed balance towards proinflammatory and Th1 cytokines in RA can, at least partly, be restored by triggering FcγR on DC in RA. Insight into the mechanism which determines the FcγR balance might lead to new strategies to abrogate Th1 driven inflammatory processes in RA.

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown cause characterised by synovial inflammation and subsequent damage of cartilage and underlying bone. RA synovial tissue is infiltrated by activated macrophages, dendritic cells, and T cells and shows evidence of synovial hyperplasia and neoangiogenesis. Despite extensive research on the immunological mechanisms involved, the exact pathophysiological pathways remain unclear. It is currently well accepted that a complex system, in which numerous inflammatory cells, chemokines, and cytokines are implicated, dominates the inflammatory cascade of RA. Involvement of the cytokines tumour necrosis factor α (TNFα), interleukin (IL1), and IL6 has been studied thoroughly. The proinflammatory cytokines TNFα and IL1 are crucial in RA and their neutralisation results in clinical and radiological improvement. Other cytokines regulate the balance between Th1 (IL12) and Th2 (IL10) immune reactions. Both clinical and experimental evidence strongly suggests a cytokine balance skewed towards Th1 responses in arthritis. 1

Dendritic cells (DC) are professional antigen presenting cells which orchestrate innate and adaptive immune responses and have a critical role in the regulation of both peripheral and central tolerance. In addition, DC are producers of large amounts of pro- and anti-inflammatory cytokines and chemokines which attract numerous effector cells to the site of inflammation. Nowadays, a large body of evidence points towards an important role for DC in both experimental arthritis and RA. Immature DC (iDC) are scattered throughout the body and are highly specialised for antigen uptake, which is mediated by several scavenger receptors including Fc gamma receptors (FcγR). After antigen uptake or stimulation by proinflammatory cytokines, DC are triggered to undergo full maturation. This process is characterised by down regulation of the receptors involved in antigen uptake. We recently showed that DC obtained from patients with RA lack the normal down regulation of FcγR during maturation. Moreover, activated mature DC are abundantly present in synovial tissue from patients with RA and express high levels of FcγRII in comparison with those of healthy subjects. These data underline the potential role of DC during inflammatory processes in RA, both systemically and locally.

FcγR in humans can be divided into activation (FcγRI, FcγRIIa, and FcγRIII) and inhibitory (FcγRIIB) subtypes. FcγRII and FcγRIII are mainly involved in the binding of IgG-antigen complexes (immune complexes (IC)), which are abundantly present in RA. The relevance of IC and the coordinated expression of activation and inhibitory FcγR in the activation of effector cells and experimental arthritis has been demonstrated in several studies. Of high interest are the results obtained recently from a study which examined the potential role for FcγR balance in the orchestration and polarisation of immune responses. These studies clearly show that, depending on the balance between the opposing

Abbreviations: DC, dendritic cells; EUSA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorter; FcγR, Fc gamma receptor; HAGGS, heat aggregated gamma immunoglobulins; IC, immune complexes; IDC, immature dendritic cells; IFNγ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; mDC, mature dendritic cells; RA, rheumatoid arthritis; RIA, radioimmunoassay; TNFα, tumour necrosis factor α
FcyR subtypes, triggering by IC can lead to either maintenance of tolerance or initiation of immunity. Measurement of IL12 and IL10, two cytokines diametrically opposed in their action to instruct Th1/Th2 T cell profiles, suggested the importance of FcyR activation in the balance of these cytokines. The importance of the inhibitory FcyR subtype was further substantiated by the findings that only DC without an intact FcyRIIb signalling pathway were able to show full maturation, activation, and T cell activation upon engagement of IC.

Taken together, these data are highly suggestive for a potential role for DC and FcyR expression in the initiation and modulation of the inflammatory cascade during RA. Elucidation of the mechanisms responsible for the pro-inflammatory response and Th1 cytokine balance in RA is a prerequisite to the development of effective immunomodulatory treatments for RA as has been shown with genetically modified DC during experimental arthritis. 31 32 In this study we therefore investigated the production of proinflammatory cytokines (IL1, TNFα, and IL6), Th1 (interferon (IFN)γ and IL12) cytokines, and Th2 (IL10) cytokines by immature and fully mature DC under basal conditions and after stimulation via FcyR dependent and independent pathways in RA. This study provides firm evidence that triggering of DC via FcyR dependent pathways leads to a diminished production of IL6 and TNFα, which is specific for RA. Furthermore, triggering of FcyR down regulates production of IL12 whereas IL10 is clearly up regulated. These results may provide new clues for the therapeutic application of ex vivo “trained” DC to abrogate or diminish the Th1 driven inflammatory process in RA.

PATIENTS AND METHODS

Patients

A total of 26 patients with RA and 20 healthy controls were enrolled in the study. All patients fulfilled the American College of Rheumatology criteria 33 for RA and gave informed consent for the study. All patients were treated with disease modifying antirheumatic drugs alone or in combination with non-steroidal anti-inflammatory drugs. Excluded from the study were patients who received treatment with systemic steroids or biological agents. The medical ethics committee of the University Medical Centre, Nijmegen approved the study protocol.

Generation of monocyte derived DC

Venous blood was collected in 10 ml lithium heparin Vacutainer tubes (Becton & Dickinson, Mountain View, CA) and the peripheral blood mononuclear cell fraction was obtained by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Roosendaal, The Netherlands). After several stringent washing procedures the cells were allowed to adhere for 1 hour at 37°C in RPMI-1640, Dutch modification (Life Technologies, Breda, The Netherlands), enriched with 2% heat inactivated human serum in six well culture plates (Costar, Badhoevedorp, The Netherlands). Adherent monocytes were cultured in RPMI-1640, Dutch modification, supplemented with 10% heat inactivated fetal calf serum and antibiotic-antimycotic agent (Life Technologies) in the presence of 500 U/ml IL4 (Schering-Plough, Amstelveen, The Netherlands) and 800 U/ml granulocyte-monocyte-colony stimulating factor (Schering-Plough) for 6 days. Fresh complete culture medium was added after 3 days.

At day 6 the iDC were harvested. To generate mDC, iDC were transferred to new six well culture plates and cultured for two more days in the presence of complete culture medium supplemented with 2 μg/ml lipopolysaccharide (LPS) (E coli, Sigma Chemicals, St Louis, MO). The expression of cell surface markers characterising both the iDC and mDC characteristics was checked by fluorescence activated cell sorter (FACS) techniques, as described previously. 34 Briefly, the first layer was attached after incubation of 1 x 10⁷ monocyte derived DC for 30 minutes at 4°C with the monoclonal antihuman antibodies against CD14, CD16, CD32, CD64 (all DAKO, Glostrup, Denmark); DC-SIGN, 35 CD80 (Becton Dickinson); CD83 (Beckman Coulter, Mijdrecht, The Netherlands); CD86 (Pharmingen, San Diego, CA, USA); MHC-I (clone W6/32), MHC-II DQ (clone SPV-L3), and MHC-II DP/DR (clone Q1513). After a washing step, the cells were incubated with fluorescein isothiocyanate conjugated goat antihorse IgG for 30 minutes at 4°C in complete darkness. Subsequently, the cells were then analysed by FACS techniques (Calibur, Becton & Dickinson). Cells were gated according to their forward and side scattering patterns and the expression of DC-SIGN and CD83 for iDC and mDC, respectively. For each marker 10⁴ cells were counted in the gate. During all FACS procedures, mouse IgG2b was used as an isotype control and the same instrumental settings were used. In all FACS analyses, the amount and position of viable cells were controlled using propidium iodide staining.

FcγR dependent and independent stimulation of DC

To investigate the effect of FcγR dependent or independent stimulation on cytokine production by iDC, fresh medium with or without heat aggregated gamma immunoglobulins (HAGGS, final concentration 100 μg/ml) was added at day 6 and incubated for 48 hours. To study the effect of FcγR triggering during DC maturation, iDC were stimulated with LPS on day 6 in the presence or absence of HAGGS for two more consecutive days. FcγR independent triggering of iDC and mDC was achieved by adding interferon gamma (IFNγ), final concentration 1000 U/ml, Boehringer Ingelheim, Ingelheim am Rhein, Germany) in the same manner as with HAGGS. HAGGS were obtained by heating human IgG (Sigma Chemicals) at 63°C for 30 minutes. The solution was centrifuged at 12 000 g for 10 minutes thereafter, and the concentration of HAGGS was determined by reading the absorbance at 280 nm. Supernatants were collected at 48 hours depending on the assessment of iDC or DC during maturation, respectively. During the whole experiment, the same batch of heat inactivated fetal calf serum was used.

Radioimmunoassay (RIA) for IL1

Polyclonal antibodies for IL1β were kindly provided by Sclavo (Siena, Italy). Human recombinant IL1β was radiolabelled by the chloramine-T method. 35 The RIA was performed as previously described. 36 Briefly, all samples and standards were prepared and mixed with a standard buffer which contained 13 mM Na2 EDTA, 0.02% sodium azide, 0.25% bovine serum albumin (Boehringer, Marburg, Germany), and inactivating units aprotinin, pH 7.4 (Bayer, Leverkusen, Germany). For measurement of IL1β in supernatants, 10 μl of sample or standard was added to the buffer. The mixture was incubated for 1 day at room temperature. After the addition of tracer (about 7000 dpm/100 μl) the incubation was continued for two more days. Separation of bound and free tracer was achieved by the addition of 100 μl of a separation agent containing sheep antirabbit IgG and 0.01% rabbit IgG (Sigma, St Louis, MO). After incubation for 30 minutes, the antibody complex was completely precipitated by the addition of 1 ml 7.5% polyethylene glycol 6000 (Merck, Darmstadt, Germany). The range of the standard curve was 20–3000 pg/ml with a sensitivity of 40 pg/ml. To minimise interassay variations, all samples from the same experiments were analysed in the same run in duplicate. The

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interassay variation of our RIA is estimated at ≤15%, whereas the intra-assay variation is ≤10%.

**Enzyme linked immunosorbent assay (ELISA) for IL6, IL10, IL12, and TNFα**

IL6 was measured by using a commercially available ELISA (Pelikine Compact human IL6 ELISA kit, CLB, Amsterdam, The Netherlands) according to the manufacturer’s instructions. The lower limit of detection with this assay was 8 pg/ml. IL10 (detection limit 3.9 pg/ml) concentration was assessed by an ELISA, as described recently. In this system, a horseradish peroxidase system was used employing 3,3'-5,5'-tetramethylbenzidine as substrate (DAKO, Glostrup, Denmark). As primary and secondary antibody 9D7 and 12G8, obtained from Pharmingen, were used respectively, at a concentration of 1 μg/ml. The measurement of IL12p70 (detection limit 0.2 ng/ml) was performed by a specific sandwich ELISA. As primary and secondary antibodies antihuman IL12p70 and biotin labelled antihuman IL12p40/70 were used, respectively (Endogen, Woburn, MA). A streptavidin-horseradish peroxidase conjugate was used to specifically stain the biotin labelled monoclonal antibodies and was visualised by incubation with 3,3'-5,5'-tetramethyl-benzidine substrate. Absorption was measured at 450 nm. TNFα production was measured by an ELISA (detection limit 2 pg/ml) using an experimental arrangement previously described for components of the plasminogen activator system.

**Statistical analysis**

Differences between the DC from RA or healthy controls were calculated by the Mann-Whitney U test and Student’s t test, as appropriate. Values of p were two sided and the level of significance was set at p<0.05.

**RESULTS**

Flow cytometric characterisation of immature and mature DC

Figure 1 shows the results of the FACS analysis of a panel of membrane markers on iDC and mDC. As expected, iDC express high levels of FcγR II and DC-SIGN, intermediate levels of FcγRI and III, costimulatory (CD80 and CD86) and MHC molecules, and low levels of CD14 (the monocyte marker) and CD83 (mDC marker). Upon full maturation, DC clearly reflect an up regulation of costimulatory molecules CD83 and MHC, while the expression of FcγRI, II, and IIIa and CD14 clearly decreased. As demonstrated before, for the majority of markers, no differences were seen between the
Production of proinflammatory and Th1 cytokines in RA patients with RA and healthy donors.

Th2 (IL10) cytokine production by iDC and mDC from the same group of 26 patients with RA and 20 healthy subjects. (B) Th1 (IFNγ, IL12) and Th2 (IL10) cytokine production by iDC and mDC from the same group of patients with RA and healthy donors.

Figure 2  Production of pro- and anti-inflammatory cytokines by iDC and mDC from patients with RA and healthy controls. (A) Production of proinflammatory cytokines (pg/ml) IL1, IL6, TNFα by iDC and mDC from 26 patients with RA and 20 healthy subjects. (B) Th1 (IFNγ, IL12) and Th2 (IL10) cytokine production by iDC and mDC from the same group of patients with RA and healthy donors.

Figure 3  FcγR dependent stimulation with anti-IgG complexes (IC) and effects on the production of proinflammatory, Th1, and Th2 cytokines by mature DC. The figure shows the percentage change in cytokine production of iDC cocultured with LPS and HAGGS compared with that of iDC cocultured with LPS alone from 15 patients with RA and 15 healthy donors.

**Cytokine production by iDC and mDC from patients with RA and healthy controls**

We first investigated the production of proinflammatory (IL1, IL6, and TNFα), Th1 (IFNγ, IL12), and Th2 (IL10) cytokines by iDC at day 6 and after full maturation with LPS (mDC, day 8) from 21 patients with RA and 18 healthy donors. Figures 2A and B show that iDC from patients with RA and from healthy subjects produced similar levels of proinflammatory, Th1 and Th2 cytokines. As expected, stimulation of iDC with LPS significantly increased the production of IL1, IL6, and TNFα in both groups. However, comparison of patients and controls showed clearly higher levels of IL1 (mean (SD); 341 (47) v 116 (7) pg/ml; p = 0.005), IL6 (6027 (1185) v 661 (220) pg/ml; p < 0.005), and TNFα (3494 (762) v 587 (128); p < 0.005) in RA. Fully matured DC produced higher levels of IL12 and similar levels of IFNγ than iDC, and there were no differences between patients with RA and healthy controls (fig 2B). In contrast, the production of IL10 was significantly higher in mDC from patients with RA (225 (53) v 38 (15) pg/ml; p = 0.003) than in healthy subjects.

**Triggering of FcγR results in distinct cytokine responses between patients with RA and healthy controls**

Because previous findings from our group suggested that DC from patients with RA have an increased expression of inhibitory FcγRIIB,15 we decided to test its repercussions on the production of cytokines. Therefore FcγR were triggered with heat aggregated IgG immunoglobulins (HAGGS).

As expected, the addition of HAGGS to DC from healthy controls cocultured with LPS before maturation significantly increased production of TNFα (110%, p = 0.006) and IL6 (160%, p = 0.002) in comparison with DC cocultured with LPS alone (fig 3). In contrast, additional FcγR triggering of DC from patients with RA reduced the production of these cytokines with TNFα (−38%, p = 0.03) and IL6 (−40%, p = 0.03). After FcγR stimulation, the production of IL1 decreased both in DC from patients with RA (−63%, p = 0.008) and from healthy donors (−38%, p = 0.005). The secretion of IFNγ (−8% v −14%) and IL12 (−67% v −65%) decreased equally in patients with RA and healthy donors, respectively, but was significant for only the latter.

The production of IL10 increased significantly upon FcγR dependent stimulation by DC from patients with RA (57%, p = 0.01) and healthy controls (92%, p < 0.01). Although the level of IL10 was already high in mDC of patients with RA (fig 3) it further increased to 354 (44) pg/ml v 73 (16) pg/ml (p < 0.005) in controls.

Because the expression of FcγR changes during DC maturation we also investigated the effect of FcγR engagement by IC on cytokine production by iDC. The production of IL1, IL6, TNFα, and IFNγ by iDC was similar to that by mDC after triggering of FcγR in both patients with RA and healthy controls, whereas opposite reactions were observed for IL12 and IL10 (table 1).

**Cytokine production by DC stimulated via FcγR independent pathways (IFNγ)**

Table 2 shows that the addition of IFNγ to iDC dramatically increased production of IL6 (48-fold), TNFα (84-fold), and IL12 (82-fold) by DC from patients with RA and led to much higher levels of these cytokines in RA than in control DC.
Table 1  IL12/IL10 production by iDC and mDC upon FcγR triggering

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<th>Immature DC</th>
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<td></td>
<td>RA</td>
<td>Control</td>
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<tr>
<td>IL12</td>
<td>Basal</td>
<td>HAGGS % p</td>
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<tr>
<td>RA</td>
<td>56 (12)</td>
<td>78 (10)</td>
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<td>RA</td>
<td>31 (10)</td>
<td>9 (4)</td>
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Table 2  Cytokine production by iDC and mDC upon stimulation with IFNγ

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<th>Immature DC</th>
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<tr>
<td></td>
<td>RA</td>
<td>Control</td>
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<tr>
<td>IL6</td>
<td>Basal</td>
<td>IFNγ % p</td>
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<tr>
<td>RA</td>
<td>178 (39)</td>
<td>6587 (1800)</td>
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<tr>
<td>RA</td>
<td>62 (40)</td>
<td>5202 (1500)</td>
</tr>
<tr>
<td>IL12</td>
<td>56 (12)</td>
<td>4270 (180)</td>
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<tr>
<td>IL10</td>
<td>31 (10)</td>
<td>5 (2)</td>
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Results are shown as mean (SD).
During maturation with LPS, the addition of IFNγ further increased the secretion of IL6, TNFα, and IL12 by DC both from patients with RA and controls.

Although the percentage increase of cytokine production by mDC was less than that of iDC, mDC from patients with RA secreted enormous amounts of these cytokines, which were significantly higher than the levels secreted by DC from controls.

In contrast, stimulation with IFNγ of DC clearly decreased production of IL10 by iDC and mDC from both patients with RA and healthy controls.

**DISCUSSION**

We have previously observed an increased expression of FcγRII, probably subtype IIb, on DC from patients with RA and suggested that this might function as a counter mechanism to suppress inflammation. Therefore, we investigated whether this phenomenon has biological consequences. To this aim we studied the production of various proinflammatory cytokines, Th1, and Th2 cytokines by DC after triggering of FcγR with IC and after FcγR independent stimuli such as IFNγ. Our results clearly show that fully matured DC from patients with RA produce significantly higher levels of proinflammatory (IL1, IL6, and TNFα) and Th2 (IL10) cytokines than those of healthy donors. These results are in line with previous reports, which suggest a balance towards proinflammatory cytokines and Th1 responses by inflammatory cells in RA.

Intriguingly, triggering of FcγR, abundantly expressed on DC, resulted in clear differences in IL6 and TNFα production between DC from patients with RA and healthy controls. Whereas stimulation by IC in healthy subjects led to an increased production of IL6 and TNFα, the opposite effect was observed by DC from patients with RA. That FcγR triggering leads to an increased production of TNFα by monocytes and macrophages from healthy donors has been previously reported. However, we are the first to describe a reversed effect in RA and suggest a different balance of activation and inhibitory FcγR on DC from patients with RA in comparison with controls. As only IL6 and TNFα and not IL1, IL10, IFNγ, and IL12 are differentially influenced by the balance in FcγR expression, it is suggested that this is not a generalised phenomenon and might provide a new tool enabling the production of several cytokines to be tuned specifically. In agreement with recent observations, the balance of IL12 and IL10, two cytokines diametrically opposed in function, altered dramatically upon FcγR stimulation on DC. Activation of FcγR led to a skewed IL12/IL10 balance towards the latter in both groups but was more pronounced in RA.

The much higher production of cytokines after stimulation with LPS or IFNγ by DC from patients with RA is intriguing and might suggest the presence of imprinting phenomena in DC. It is well known that DC development is influenced by a plethora of environmental factors which favour distinct DC phenotypes. DC obtained from patients with RA are probably primed by a variety of stimuli such as cytokines, chemokines which are abundantly present in the serum and joints of patients with RA. Accumulating evidence suggests that the DC phenotype can be defined by its Th1/Th2 cytokine profile which, in turn, determines the polarisation of the T cell response. However, it remains a matter of debate how DC influence this decision and whether the ability to respond differently is acquired by environmental factors or is an intrinsic property. A large body of evidence points towards DC having a key role in the orchestration of the innate and adaptive immune responses. Furthermore, involvement of DC in the modulation and control of both central and peripheral tolerance is generally well established. DC perform these pivotal functions and the expression of both inhibitory and activation FcγR is of paramount importance; this has been elegantly addressed by several studies. Anderson et al. showed that only macrophages stimulated with antigen directed to FcγR result in the production of antigen-specific T cells, which preferentially produce Th2 cytokines. Moreover, the development of immunity or tolerance against tumour antigens was largely dependent on the balance between activation or inhibitory FcγR. Additionally, the involvement of FcγR in synovial inflammation was clearly demonstrated in several models of experimental arthritis and RA. Our data might support the hypothesis that DC in RA represent a protective cell type but fail to orchestrate this protective effect to the full extent owing to an as yet unknown cause. This hypothesis is further underlined by preliminary data, which show that DC from patients with RA cannot increase the expression of FcγRII to levels achieved by DC of healthy controls after certain stimuli (manuscript in preparation). Recently, genetic alterations in the regulatory regions in the FcγRIIb gene were found. However, both environmental and intrinsic factors may be implicated in determining the balance between inhibitory and activation FcγR. Possibly, one or more of these potential factors is affected in RA. The profiles of cytokine production observed after FcγR triggering and the differences between patients with RA and healthy controls strongly favour this hypothesis. However, an intrinsic defect cannot be excluded and is potentially reflected by the inability to fully employ their protective phenotype in vivo.

DC are preferentially located in the perivascular regions of the synovial tissue, where they fulfil an important role in the chemoattraction and instruction of other inflammatory cells. Therefore, from a therapeutic point of view, manipulation of cytokine profiles and behaviour of DC represents a very attractive strategy to orchestrate and control the autoimmunity seen in RA. The fact that our data show that triggering of FcγR, at least partly, overrules cytokine production in response to activation of Toll-like receptors (LPS stimulation) is highly interesting. It was suggested recently that high levels of IL6 induced by activation of Toll-like receptors block the activity of regulatory T cells in vivo. This phenomenon might explain the beneficial effects of intravenous immunoglobulin seen in many diseases (reviewed by Mackay and Rosen). Strong evidence for a clinical effect in RA, however, is lacking and might be due to our suggested FcγR regulatory defect. Further elucidation of the mechanisms involved in the regulation of FcγRIIb might provide new clues for the modulation of DC function in autoimmune diseases such as RA.

In summary, the present study provides strong evidence that DC may play a part in the pathogenesis of RA owing to their high production of proinflammatory and Th1 cytokines, which is differentially regulated by FcγR on DC from patients with RA and healthy controls. More insights into these mechanisms might provide new clues about the regulation of (auto)immunity and tolerance in DC in RA and might expand our therapeutic armamentarium to control this disease.

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