EXTENDED REPORT

Dendritic cells from patients with rheumatoid arthritis lack the interleukin 13 mediated increase of Fc γ RII expression, which has clear functional consequences

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Background: Dendritic cell (DC) function is largely tailored by Fc gamma receptors (Fc γ R) and is critical for every immune response.

Objective: To compare interleukin (IL) 13 mediated regulation of Fc γ RII and its related DC function between healthy controls and patients with rheumatoid arthritis (RA).

Methods: DC were derived from peripheral blood mononuclear cells according to standardised protocols. Fc γ RI, II, and III expression and DC phenotype were assessed by FACS analysis. The level of cytokine production and chemokine expression was measured by Luminex and real time quantitative polymerase chain reaction techniques. Antigen uptake capacity was studied by DC fluorescent heat aggregated immunoglobulins and FACS analysis.

Results: Replacement of IL4 by IL13 clearly increased the expression of Fc γ RII on DC from healthy controls (CDC), but had no effect on DC from patients with RA (RADC). The lower production of inflammatory mediators by IL13 CDC upon Fc γ R mediated triggering suggests that IL13 induces up regulation of specifically Fc γ RII. RADC co-cultured with IL4 already displayed an inhibitory DC phenotype, but this inhibitory phenotype was not augmented by the addition of IL13. The defective Fc γ RII regulation was further substantiated by the finding that IL13 CDC increased antigen uptake capacity, whereas IL13 RADC did not.

Conclusion: IL13 regulates the expression of inhibitory Fc γ RII in normal subjects but not in RA, potentially resulting in a chronic proinflammatory immune reaction in RA. Unravelling the underlying mechanisms of Fc γ RII regulation might lead to new therapeutic targets in RA.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterised by inflammation and destruction of the synovial joints. Although the precise mechanisms involved in its pathogenesis remain to be elucidated, it is clear that a massive influx of inflammatory cells, including macrophages and dendritic cells (DC), in the joint is one of its key elements.¹ DC are the professional antigen presenting cells that control the innate and adaptive immune response, and orchestrate the balance between tolerance and immunity.² Nowadays, evidence points towards the potential role for DC in both the initiation and perpetuation of synovial inflammation in RA. At first, DC are strategically located in the perivascular regions and ectopic lymphoid structures in RA synovium.^{3–5} Secondly, in RA synovium DC-specific chemokines, including CCL17, CCL18, and CCL19, are significantly more highly expressed than in normal subjects.⁶ Finally, it has been shown that genetically modified DC can abrogate collagen induced arthritis,⁷ whereas collagen pulsed DC drive inflammatory arthritis.⁸

For the recognition and processing of antigens, DC are equipped with several receptors, including Fc gamma receptors (Fc γ R). Fc γ R constitute a group of receptors designed to recognise IgG containing immune complexes (IC), which are abundantly present in serum and synovial fluid of patients with RA.⁹ In humans three classes can be distinguished, Fc γ RI, Fc γ RII, and Fc γ RIII.^{10–11} Fc γ RII is further divided in two subtypes: Fc γ RIIa and Fc γ RIIb. Fc γ RIIa, together with Fc γ RI and III, activates cellular responses upon triggering. In contrast, Fc γ RIIb is a unique inhibitory Fc γ R.^{12–14}

Although the mechanism is not fully elucidated, it is generally accepted that the balance between activating and inhibitory Fc γ R is intimately controlled by pro- and anti-inflammatory cytokines such as interleukin (IL) 4 and interferon γ (IFN γ). Pricop *et al* demonstrated that IL4 was responsible for the increased expression of inhibitory Fc γ R, whereas IFN γ reversed this balance in favour of the activating Fc γ R subtypes.¹⁵ Interestingly, we have shown that monocyte derived DC from patients with RA with active disease expressed higher levels of the inhibitory Fc γ RII than healthy donors, which was translated into an altered DC behaviour.^{5–6} Perhaps, this increased expression of Fc γ RIIb is explained by the presence of local factors during early DC development. IL4 is a plausible candidate, but is not found to be increased in RA. On the contrary, IL13, a cytokine which resembles many functions of IL4, is abundantly expressed in RA synovial fluid and highly secreted by synovial fluid macrophages during synovitis.^{17–19} The potential role for IL13 in regulating the balance of Fc γ R during RA is therefore more likely.

Accumulating evidence that points toward the critical situation for Fc γ R in arthritis originates from experimental

Abbreviations: CDC, DC from healthy controls; DC, dendritic cell(s); FACS, fluorescence activated cell sorter; Fc γ R, Fc gamma receptor; HAGGs, heat aggregated gammaglobulins; IC, immune complex(es); iDC, immature monocyte derived DC; IFN γ , interferon γ ; IL, interleukin; LPS, lipopolysaccharide; mDC, fully matured DC; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RA, rheumatoid arthritis; RADC, DC from patients with RA; TNF α , tumour necrosis factor α

Table 1 Phenotypic characteristics of iDC and mDC from healthy donors and patients with RA

CD Marker	Immature DC controls		Immature DC RA		Mature DC controls		Mature DC RA	
	% Cells	MFI	% Cells	MFI	% Cells	MFI	% Cells	MFI
CD14	10 (7)	26 (11)	13 (9)	28 (10)	6 (4)	22 (7)	8 (5)	18 (11)
CD80	51 (24)	26 (13)	50 (17)	33 (15)	99 (9)	110 (13)	98 (7)	99 (19)
CD83	11 (8)	20 (11)	12 (8)	17 (10)	97 (8)	53 (16)	91 (8)	61 (11)
CD86	76 (15)	105 (25)	69 (12)	116 (21)	98 (6)	260 (21)	99 (11)	272 (23)
MHC-I	90 (14)	133 (11)	91 (8)	141 (16)	99 (9)	435 (41)	98 (4)	429 (34)
MHC-II DP/DR	92 (11)	264 (18)	85 (13)	278 (21)	99 (9)	888 (32)	99 (5)	845 (41)

Results are shown as mean (SD).

arthritis models. In antigen induced arthritis and IC arthritis, for example, chondrocyte death and cartilage erosion are heavily dependent on the presence of activating FcγR.^{20–22} Likewise, absence of the inhibitory FcγRIIb renders mice susceptible to collagen induced arthritis, and it has been suggested that FcγRIIb reduces both joint inflammation and destruction by inhibiting the activation of FcγR and clearance of IC.^{14–23} The finding that DC obtained from patients with RA express higher levels of the inhibitory FcγRIIb led us to speculate that inhibitory FcγRII is up regulated during inflammation as a potent counteracting mechanism mediated by IC aimed at dampening the proinflammatory response. Here we postulate that IL13 has a critical role in the up regulation of FcγRIIb in healthy controls but that this response is absent in patients with RA, leading to an inadequate inhibition of the proinflammatory response which is normally mediated by IC.

Here we demonstrate that the up regulation of FcγRII upon stimulation with IL13 in healthy donors is absent in RA, which results in an altered production of proinflammatory mediators. These findings provide evidence that the regulation of FcγR expression and related DC function might contribute to dampening of the immune response during normal conditions but fails in RA.

PATIENTS AND METHODS

Patients

Fifteen patients with RA and 16 healthy controls were enrolled in the present study. All patients fulfilled the American College of Rheumatology criteria for RA²⁴ and gave informed consent for the study. Patients who received treatment with systemic steroids or biological agents (anti-tumour necrosis factor α (TNFα) and IL1 receptor antagonist) were excluded from the study. The medical ethics committee

Figure 1 Expression of FcγRII upon replacement of IL4 by IL13 on DC of healthy controls and patients with RA. (A) Percentage of FcγRII positive cells (left panel) and MFI of FcγRII expression (right panel) after co-culture of DC from healthy donors (n=16) with various concentrations of IL13 and compared with IL4 (500 U/ml). (B) Comparison of the percentage of FcγRII positive cells (left panel) and mean fluorescence intensity (MFI) of FcγRII expression between DC from healthy donors (n=16) and patients with RA (n=15) co-cultured with IL4 (500 U/ml) and IL13 (5 ng/ml). *p<0.05.

Figure 2 Effect of Fc γ R triggering on cytokine production by DC from healthy controls and patients with RA after co-culture with IL4 or IL13. The production of TNF α , IL6, IL10, and IL12 by DC co-cultured with IL4 or IL13 from healthy controls and patients with RA was measured. The graphs display the percentage of cytokine production upon combined stimulation with LPS and HAGGs compared with that after stimulation with LPS alone.

of the University Medical Centre Nijmegen approved the study protocol.

Generation of monocyte derived DC

Monocyte derived DC cultures were obtained using essentially the same protocol as described previously.⁶ Briefly, a peripheral blood mononuclear cell fraction was obtained by density gradient centrifugation from full blood. Thereafter, the cells were allowed to adhere for 1 hour at 37°C in RPMI-1640, enriched with 2% heat inactivated human serum. Adherent monocytes were cultured in RPMI-1640, supplemented with 10% heat inactivated fetal calf serum and antibiotic-antimycotic agent (Life Technologies) in the presence of either IL4 (500 U/ml; Schering-Plough, Amstelveen, The Netherlands) or IL13 (R&D systems Inc, USA) alone or in combination and granulocyte monocyte-colony stimulating factor (800 U/ml; Schering-Plough) for 6 days. To generate fully mature DC (mDC), immature DC (iDC) were transferred to new six well culture plates and cultured for 2 more days in the presence of complete culture medium supplemented with 2 μ g/ml lipopolysaccharide (LPS; *E coli*, Sigma Chemicals, St Louis, MO).

Phenotypic characterisation of iDC and mDC

The expression of cell surface markers characterising both iDC and mDC characteristics was checked by fluorescence activated cell sorter (FACS) techniques as described previously.⁵ The following monoclonal antibodies were used as the first layer: CD14, CD16, CD32, CD64 (all DAKO, Glostrup, Denmark), CD80 (Becton Dickinson), CD83 (Beckman Coulter, Mijdrecht, The Netherlands), CD86 (Pharmingen, San Diego, CA, USA), major histocompatibility complex (MHC)-I (clone W6/32), MHC-II DQ (clone SPV-L3), and MHC-II DP/DR (clone Q1513). Cells were gated according to their forward and side scattering patterns. During all FACS procedures, mouse IgG2b was used as an isotype control and the same instrumental settings were used.

Fc γ R dependent and independent stimulation of DC during maturation

To study the effect of Fc γ R mediated triggering, DC stimulated with LPS and heat aggregated gammaglobulins (HAGGs; final concentration 100 μ g/ml) were compared with DC stimulated with LPS alone. To compare IL4 and IL13, DC were costimulated with one of these cytokines. HAGGs were obtained as previously described.¹⁶ Supernatants were then collected after 48 hours of incubation.

Cytokine and chemokine measurement

The secretion of the inflammatory mediators IL1, IL6, TNF α , IL10, and IL12 by iDC, mDC, and iDC stimulated with HAGGs during maturation was measured using Luminex technology as described in detail previously.²⁵

Chemokine expression was studied using real time polymerase chain reaction (PCR) techniques using essentially the same protocol as recently described.⁶ Briefly, the sequence of primers and probes for the chemokines CCL17, CCL18, CCL19, and CCL3 was used as described previously.⁶ The probes specific for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and porphobilinogen deaminase (PBGD) were labelled with a VIC fluorescent group at the 5' end.

PCR reactions were performed in accordance with the Taqman assay instructions, using an end concentration of 175 nmol/l probe and 600 nmol/l primers.²⁶⁻²⁷ The amplifications were performed on a ABI/PRISM 7700 sequence detector system (PE-Applied Biosystems).

Fc γ R mediates antigen uptake capacity by DC

To obtain data about the functionality and level of Fc γ RII subtypes on IL13 and IL4 DC, we studied the uptake of antigens by these cells, as described previously.²⁸ Therefore, human gammaglobulins (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 10 mg/ml were labelled with fluorescein isothiocyanate (FITC) using the FluoReporter FITC protein

Figure 3 Expression of chemokines CCL18, CCL17, CCL19, and CCL3 by DC co-cultured with IL4 or IL13. mRNA expression of CCL18, CCL17, CCL19, and CCL3 by DC from healthy controls (n = 16) and patients with RA (n = 15) was measured by real time quantitative PCR techniques. The chemokine expression level by DC co-cultured with IL4 (500 U/ml) was compared with that co-cultured with IL13 (5 ng/ml).

Figure 4 Effect of Fc γ R triggering on the mRNA expression of chemokines by DC from healthy controls and patients with RA after co-culture with IL4 or IL13. The mRNA expression of CCL18, CCL19, CCL17, and CCL3 by DC co-cultured with IL4 or IL13 from healthy controls and patients with RA was studied. The graphs display the percentage of chemokine expression upon combined stimulation with LPS and HAGGs compared with that after stimulation with LPS alone.

labelling kit (Molecular Probes, Eugene, OR, USA) and thereafter heated to 63°C for 30 minutes. iDC were resuspended in phosphate buffered saline containing 0.1% bovine serum albumin at a concentration of 4×10^6 cells/ml. DC from six patients and six healthy subjects were used in six different experiments. Every experiment was performed using simultaneous culture of DC from healthy donors and patients with RA.

RESULTS

DC phenotype is not altered by stimulation with IL13

iDC expressed intermediate levels of CD14, costimulatory molecules (CD80 and CD86), and MHC molecules and lacked CD83, the mDC marker (table 1). Upon triggering with LPS, the DC mature which coincides with a decreased expression of CD14, up regulation of CD80, CD86, and MHC and clear expression of CD83. No differences were seen between DC from the 16 healthy controls and the 15 patients with RA.

To study the effect of IL13, we next investigated the phenotype of DC cultured in the presence of IL13. With this aim, we added different concentrations of IL13 (1, 5, and 20 ng/ml) to the DC cultures instead of 500 U/ml IL4. No phenotypic differences were seen between DC cultured in the presence of IL4 or IL13, either in healthy controls or in those from patients with RA (data not shown).

IL13 mediated up regulation of FcγRII does not occur on DC from patients with RA

Because we hypothesised that FcγRII expression by DC might be mediated by IL13, we first studied the effect of IL13 on the expression of FcγR on DC from healthy donors (CDC, $n = 16$). Figure 1A shows that the number of FcγRII expressing (mean (SD) 66 (3) v 56 (5), $p = 0.08$) cells and the mean (SD) fluorescence intensity (MFI) (77 (6) v 49 (7), $p = 0.007$) increased upon stimulation with low amounts of IL13 (1 ng/ml) compared with that of IL4 (500 U/ml). The addition of higher concentrations of IL13 (5 ng/ml) further up regulated FcγRII positive cells (82 (4), $p = 0.001$) and MFI (123 (5), $p = 0.001$) but levelled by 20 ng/ml of IL13. Therefore, 5 ng/ml IL13 was considered to be the optimal concentration for DC in this study.

To investigate whether IL13 would bring about the same effect in RA, DC from patients with RA (RADC, $n = 15$) were studied. In corroboration with previous findings from our group, RADC co-cultured with IL4 displayed a higher expression of FcγRII than CDC (fig 1B).⁵ In contrast, stimulation with IL13 of DC from patients with RA did not significantly alter the number of FcγRII expressing cells (66

(7) v 72 (3), $p = 0.10$) or the MFI (91 (15) v 77 (12), $p = 0.09$) (fig 1B).

Of note, the percentage IL4 DC that expressed FcγRI and FcγRIII was similar to that of IL13 DC both from healthy controls (FcγRI: 15 (14) v 23 (16), FcγRIII: 15 (13) v 22 (19)) and patients with RA (FcγRI: 15 (6) v 16 (9), FcγRIII: 15 (8) v 20 (10)). The same trend was seen for the MFI (data not shown).

Altered regulation of FcγRII has functional consequences for the production of cytokines upon FcγR mediated triggering

We hypothesised that IL13 mediates selective up regulation of FcγRIIb on CDC but not on RADC and envisaged that this would affect the secretion of cytokines upon FcγR mediated triggering.

Figure 2 shows that FcγR mediated triggering of IL4 CDC significantly increased secretion of TNFα (56%) and IL6 (34%), IL10 (100%), whereas IL12 secretion was inhibited (40%), which is in line with previously published reports.¹⁶ In contrast, IC mediated triggering of IL13 DC led to a clearly decreased production of TNFα (68%, $p < 0.001$) and IL6 (73%, $p < 0.001$), a further decrease of IL12 (89%, $p = 0.02$) and further increased production of IL10 (162%, $p < 0.01$). These results demonstrate that IL13 mediated up regulation of FcγRII on CDC clearly results in inhibitory DC phenotype upon FcγR mediated triggering in comparison with IL4 DC.

We next analysed whether the altered IL13 mediated regulation of FcγRII expression as seen in DC from patients with RA affects the cytokine production upon FcγR mediated stimulation. IL4 DC from patients with RA decreased the secretion of TNFα ($p = 0.001$), IL6 ($p = 0.002$), and IL12 ($p = 0.001$) upon stimulation with IC whereas the production of IL10 ($p = 0.002$) increased (fig 2), indicating that RADC already display an inhibitory subtype, which is in line with previous research from our group.¹⁶ Intriguingly, IL13 RADC failed to inhibit the production of TNFα, IL6, and IL12 production as potentially as IL4. Similarly, the up regulation of IL10 production upon IC mediated triggering was less with IL13 DC than with IL4 DC (fig 2). These results thus parallel our findings seen with FACS analysis and suggest that the IL13 mediated regulation of FcγRIIb on DC is severely altered in RA. The administration of non-heated IgG did not have any effect on DC for cytokine production. Of note, we tested whether a potentially different expression of the IL13 receptor might explain the altered response to IL13 between DCs from healthy controls and patients with RA. However,

Figure 5 IL13 mediated regulation of FcγR dependent uptake of fluorescent HAGGs. The percentage of fluorescent cells (A) and mean fluorescence intensity (B) as a marker for uptake of fluorescent HAGGs over a period of time is shown (x axis). DC from patients with RA ($n = 6$) co-cultured with IL13 or IL4 are compared with CDC ($n = 6$) co-cultured with IL13 and IL4 respectively. * $p < 0.05$.

the expression of IL13 receptor was similar between the two groups (data not shown).

FcγR mediated inhibition of chemokine expression by CDC is absent in RADC

DC are involved in the attraction of T cells, which is mainly orchestrated by the excretion of chemokines. We investigated the effects of IL13 on expression of the chemokines CCL17, CCL18, CCL19, and CCL3 both by FcγR independent and dependent pathways.

IL13 CDC expressed significantly less of the chemokines CCL17 ($p = 0.003$ and $p = 0.01$), CCL18 ($p = 0.006$ and $p = 0.01$), and CCL19 ($p = 0.04$ and $p = 0.004$) than IL4 DC in healthy donors and patients with RA, respectively (fig 3). The same trend was seen for CCL3.

IC mediated triggering of IL13 of CDC resulted in a significantly decreased expression of CCL17 ($p = 0.004$), CCL18 ($p = 0.008$), CCL19 ($p = 0.006$), and CCL3 ($p = 0.04$) in comparison with that of IL4 CDC, suggesting that the anti-inflammatory effect of IL13 is, at least partly, mediated by FcγR modulation (fig 4). In contrast with CDC, the expression of CCL17, CCL18, CCL19, and CCL3 was less efficiently inhibited by IL13 DC from patients with RA after stimulation with IC. This further substantiates the finding that DC from healthy controls respond differently to IL13 than DC from patients with RA, which might have profound biological consequences for the response on IC.

IL13 increases IC uptake capacity of CDC but not of RADC

FcγRII is important in the uptake and processing of antigen-immune complexes. Therefore, we studied whether different expression of FcγRII on stimulation with IL13 between CDC and RADC has consequences for the antigen uptake capacity by performing six consecutive cultures, each taking one patient with RA and one healthy control simultaneously. Figure 5 shows that stimulation of CDC with IL13 increased the uptake of immunoglobulins compared with IL4 DC both for the number of positive cells ($p = 0.03$) and mean fluorescence intensity ($p = 0.02$). In sharp contrast with CDC, the antigen uptake capacity by RADC was not increased by IL13 and was similar to that of CDC cultured with IL4. Because FcγRI and FcγRIII are also involved in the uptake of IC, we measured the expression of these FcγR subtypes as well. Both FcγRI and FcγRIII were expressed at similar levels on IL4 and IL13 DC from both patients with RA and healthy controls.

DISCUSSION

Here we demonstrate that replacement of IL4 by IL13 during culture clearly increases expression of FcγRII on CDC, whereas this effect is absent on RADC. This phenomenon could not be explained by a potential relative resistance for IL13 of RADC, because IL13 itself strongly inhibits the production of inflammatory mediators equally for RADC and CDC, or by IL13 receptor expression because this was found to be similar for CDC and RADC (data not shown). In addition, we demonstrated that the altered response to IL13 seen by RADC has major consequences for the production of inflammatory mediators upon stimulation of FcγR dependent pathways. The functionality of this IL13 mediated up regulation of FcγRII was further substantiated by the finding that IL13 increased the antigen uptake capacity by DC from healthy donors but failed to orchestrate the same effect on DC from patients with RA. Intriguingly, RADC co-cultured with IL4 already displayed a diminished capacity for IC uptake in comparison with those from healthy controls. Because the cell surface expression of FcγRII was found to be higher on RADC, there might also be a defect in the

functionality of the FcγRII in RA which needs to be examined in further studies.

DC express high levels of FcγR and are at least partly controlled by the triggering of these receptors. The expression of FcγR expression is tightly regulated by many pro- and anti-inflammatory cytokines, of which IL4 and IFNγ have been the most thoroughly investigated. Although, the effects of IL4 on monocytes and macrophages on phenotype and function are largely known, the effect on DC has not been similarly scrutinised. IL4 is not present in large quantities during synovial pathology, which makes an important role less likely. In contrast, IL13, a cytokine generally accepted as having potent anti-inflammatory effects, shares many biological functions with IL4 and is present in large amounts during synovial inflammation.^{18 19 29 30} The presence of IL13 in RA serum and synovial fluid was further substantiated by the finding that the blockade of IL13 in RA serum and synovial fluid, at least partly, diminished the up regulation of FcγRIIb (manuscript in preparation). To our knowledge, the precise effects of IL13 on FcγR expression have not yet been determined. Here we present new data which suggests that IL13 mediates the up regulation of FcγRIIb more potently than IL4, which is translated into a FcγR mediated inhibitory DC phenotype in healthy subjects. In addition, we provide firm evidence that in RA such FcγRII regulatory mechanism seems to be unresponsive to IL13, which might potentially result in a failure to mount an adequate inhibitory response to IC.

Every signal that ignites the immune response has to be terminated to prevent the establishment of chronic inflammation. Envisaged in the light of DC function, the main task of which is to prime T cells and thus control the balance between immunity or tolerance, it is tempting to speculate that the balance between activating and inhibiting FcγR should act as a counteractive mechanism to dampen the proinflammatory response after elimination of the provocative factor. Such a counteractive mechanism is perhaps set in motion by the production of IL13 during the early phases of inflammation. Later on in the immune response, when IL13 production subsides, the balance of the expression of FcγR is altered towards the inhibitory subtype. Subsequent triggering by IC, produced in large quantities during later stages of an immune response, activate inhibitory pathways through FcγR mediated mechanisms aimed at dampening the proinflammatory response and restoring an immunological steady state. In RA, however, this counteractive mechanism fails for yet unidentified reasons, potentially leading to a vicious circle of uncontrolled proinflammatory responses, which ultimately result in irreversible joint destruction as seen in RA.

The critical role for FcγR in the orchestration and polarisation of immune responses has recently been studied by Anderson *et al*, who demonstrated that macrophages can direct the production of antigen-specific T cells, which preferentially produce Th2 cytokines, exclusively when triggered with antigen directed against FcγR.^{31 32} In addition, the development of immunity or tolerance against tumour antigens was largely dependent on the balance between activating or inhibitory FcγR.³³ Further evidence for a role of FcγR in the modulation of autoimmunity is provided by the fact that intravenous immunoglobulins are beneficial in several inflammatory conditions.^{34–36} The fact that intravenous immunoglobulins are ineffective in the treatment of RA underlines our finding that such counteractive mechanism, mediated by FcγR, is defective in this condition.³⁷

In conclusion, we provide evidence for an aberrant expression and regulation of the inhibitory FcγRIIb by IL13 on DC from patients with RA, which has profound functional consequences. Because inhibitory FcγR are pivotal for the regulation of immune responses a potentially defective

FcγRII regulation might explain why RA is a chronic, Th1 driven disease. Because DC treatment in autoimmune diseases holds great promise, further research into the responsible pathways involved in FcγR mediated DC function might lead to new therapeutic targets.

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