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Myeloid dendritic cells correlate with clinical response whereas plasmacytoid dendritic cells impact autoantibody development in rheumatoid arthritis patients treated with infliximabChristophe Richez^{1,2}, Thierry Schaefferbeke¹, Chantal Dumoulin¹, Joël Dehais¹, Jean-François Moreau^{2,3} and Patrick Blanco^{2,3,4}¹Département de Rhumatologie, CHU Bordeaux, place Amélie Raba-Léon, 33076 Bordeaux, France²UMR-CNRS 5164, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux, France³Département de Virologie et d'Immunologie biologique, CHU Bordeaux, place Amélie Raba-Léon, 33076 Bordeaux, France⁴Service de Médecine Interne, CHU Bordeaux, 1 Avenue de Magellan, 33600 Pessac, FranceCorresponding author: Christophe Richez, christophe.richez@mac.com Patrick Blanco, patrick.blanco@chu-bordeaux.fr

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Arthritis Research & Therapy 2009, **11**:R100 (doi:10.1186/ar2746)This article is online at: <http://arthritis-research.com/content/11/3/R100>© 2009 Richez *et al.*; licensee BioMed Central Ltd.This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.**Abstract**

Introduction The objective of our study was to identify the significance of the subtypes of dendritic cell (DC), specifically myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), in rheumatoid arthritis (RA) pathogenesis through their longitudinal follow-up in patients receiving infliximab.

Methods Circulating mDC and pDC levels were evaluated by flow cytometry in RA patients (n = 61) and healthy volunteers (n = 30). In RA patients, these levels were measured before and during infliximab therapy. Their counts were correlated to RA disease activity markers and anti-nuclear antibody occurrence. IFN α production was measured by ELISA in serum of RA patients and, *in vitro*, in supernatant of peripheral blood mononuclear cells stimulated by influenza virus in the presence or absence of infliximab. Statistical evaluations were based on Mann-Whitney tests or Wilcoxon's signed-rank tests.

Results RA patients with active disease were characterized by a baseline decrease in both circulating pDCs and mDCs. Disease activity markers inversely correlated only with mDC level. This level increased in RA patients responsive to infliximab therapy, to reach the level observed in controls. Conversely, anti-nuclear antibody appearance during infliximab therapy correlated inversely with pDC level and was associated with increased serum IFN α level and circulating plasma cells number. *In vitro* studies revealed that infliximab kept pDCs in an IFN α secreting state upon viral stimulation allowing differentiation of B cells into anti-nuclear antibody-secreting plasma cells.

Conclusions This study reveals two distinct roles for pDC and mDC in RA. Circulating mDCs mainly contribute to RA activity, whereas pDCs seem to be involved in appearance of anti-nuclear antibodies under infliximab therapy through the ability of this drug to keep pDCs in an IFN α secreting state.

Introduction

Dendritic cells (DCs) represent a critical link between innate and adaptive immune systems. Two DC subsets, myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), have been identified in humans. These DC subsets recognize different microbial pathogens through specific receptors, which in turn induce different types of innate and adaptive immune responses [1]. Abnormalities of DC homeostasis have been involved in the pathophysiology of various human dis-

eases, including autoimmune diseases [2]. In systemic lupus erythematosus (SLE), an autoimmune disease characterized by the presence of an autoimmune reaction against nuclear components, pDCs secrete large amounts of IFN α . This secretion promotes the differentiation of monocytes into mDCs. These mDCs capture circulating nucleic acid-containing bodies and activate autoreactive T cells and B cells, leading to the increased production of autoantibodies by plasma cells [3,4].

ANA: anti-nuclear antibody; DAS28: Disease Activity Score in 28 joints; DC: dendritic cell; ELISA: enzyme-linked immunosorbent assay; EULAR: European league against rheumatism; FCS: fetal calf serum; IFN: interferon; IL: interleukin; mDC: myeloid dendritic cell; PBMC: peripheral blood mononuclear cell; PBS: phosphate-buffered saline; pDC: plasmacytoid dendritic cell; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; TNF: tumor necrosis factor.

Rheumatoid arthritis (RA) is a common inflammatory disease, yet its pathogenesis remains incompletely understood. It is probable that DCs could play a key role in its pathogenesis as they have been reported to infiltrate the synovium in RA patients [5,6]. These synovial DCs are more mature than DCs from peripheral blood: they express various activation markers, secrete large amounts of various cytokines (IL-12, TNF α , IL-6), and are able to activate autologous T lymphocytes as well as B lymphocytes [7-9]. Trying to dissect and decipher the exact roles of mDC and pDC subsets in this disease, however, remains difficult because both subsets are present in RA synovial fluid and infiltrate synovial tissues [10,11].

Anti-TNF α therapies have improved the prognosis of RA, although these agents may induce a number of adverse effects including autoimmunity. Anti-nuclear antibodies (ANAs) develop in 30 to 60% of the patients given anti-TNF α regimens [12-14] and, occasionally, clinical lupus develops during the course of therapy [15,16]. The mechanism responsible is still unclear. The TNF/TNF-receptor system appears to play an important role in SLE pathogenesis, as is exemplified by TNF α -induced amelioration of murine lupus nephritis [17] and an increased soluble TNF-receptor correlation with disease activity [18]. These data suggest a role of anti-TNF α in exacerbation or induction of lupus-type autoimmunity and, therefore, could explain some events occurring in patients treated by TNF α blockers. Despite these observations, a recent study has suggested that SLE can be treated with infliximab, although autoantibodies to double-stranded DNA and cardiolipin were increased [19].

To understand the implication of DC subsets in RA immunopathology, we examined peripheral pDC and mDC numbers in patients suffering from active RA and the evolution of these numbers during the course of infliximab treatment. Our study demonstrates that RA activity correlates with fluctuations in mDC numbers and reveals a possible role for the pDCs, through their sustained IFN α production, in the ANA production induced by infliximab.

Materials and methods

Study population

Sixty-one patients with active RA (Disease Activity Score in 28 joints (DAS28) >5.1), who fulfilled the revised classification criteria of the American College of Rheumatology for RA [20], were evaluated before and after infliximab therapy. Table 1 summarizes the characteristics of these patients.

Infliximab (Shering-Plough, Levallois-Perret, France) was given at a dose of 3 mg/kg intravenously at weeks 0, 2 and 6 and then every 8 weeks in combination with stable doses of methotrexate 7.5 to 15 mg/week orally or intramuscularly. Only patients on stable prednisone doses \leq 10 mg/day and nonsteroidal anti-inflammatory drug treatment were included. According to EULAR response criteria [21], a positive clinical

Table 1

Baseline characteristics of the study patients

Characteristic	Baseline value
Age (years)	55 (24 to 82)
Sex ratio (female:male)	7:3
Disease status	
Disease duration (years)	14 (2 to 34)
Rheumatoid factor positivity (%)	76
Anti-cyclic citrullinated peptide positivity (%)	59
Disease Activity Score in 28 joints score	6.14 \pm 1.38
C-reactive protein (mg/l)	36.2 \pm 30.5

Data presented as mean (range) or mean \pm standard deviation unless otherwise indicated.

response to infliximab therapy was defined as a drop in the DAS28 from baseline by >1.2 or as a DAS28 <3.2 at week 14.

In addition, 30 healthy blood donors were included in the study. These donors were matched with patients for sex and age. Synovial fluid was obtained from 11 patients suffering from osteoarthritis.

The study was approved by the local Ethics Committee, and all patients gave informed consent.

Enumeration of blood dendritic cell precursors and plasma cells by flow cytometry

Whole blood samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Pont-de-Claix, France) with 10⁶ white blood cells acquired per analysis. DC subsets were measured using a DC kit from BD Biosciences. Peripheral blood mDC and pDC subsets were defined by the concomitant lack of lineage markers, HLA-DR expression, and mutually exclusive membrane expression of CD11c or CD123, respectively. Absolute numbers of blood DC precursors were calculated as the percentage of white blood cells expressed per milliliter of peripheral blood. Enumeration of blood DC was evaluated as published elsewhere [22]. Plasma cells were analyzed by gating on CD19⁺ cells and by calculating the percentage of CD20^{neg}/CD38^{high} cells.

Synovial fluid was obtained at the initial time point from patients with RA (n = 9) and from patients with osteoarthritis (n = 11), with knee effusions. This synovial fluid was diluted appropriately with PBS in order to avoid clot formation. Synovial mDC and pDC subsets were defined by the concomitant lack of lineage markers (CD3⁻, CD14⁻, CD16⁻, CD56⁻, CD8⁻ and CD19⁻), HLA-DR expression, and mutually exclusive membrane expression of CD11c or CD123, respectively. Results were expressed as the percentage of mDCs or pDCs among

cells without the following lineage markers: CD3, CD14, CD16, CD56, CD8 and CD19.

IFN α quantification

Serum samples were collected and were stored at -80°C . IFN α levels were quantified with a human IFN α ELISA kit (BioSource International, Camarillo, CA, USA), according to the manufacturer's instructions. The detection limit of this IFN α ELISA is 25 pg/ml. This assay has been used previously by others groups for measurement of IFN α in the serum [3,23].

Preparation of cell culture

Peripheral blood mononuclear cells (PBMCs) of adult donors were isolated using Ficoll-Paque Plus (Amersham Biosciences, Saclay, France) gradient centrifugation. PBMCs (1×10^6 cells/well) were cultured in RPMI supplemented with 10% FCS, and were stimulated *in vitro* with live influenza virus (10^4 particles; Charles River Laboratories, Wilmington, MA, USA) with or without TNF α (10 $\mu\text{g/ml}$; R&D Systems, Lille, France) or TNF α blockers (Infliximab 20 $\mu\text{g/ml}$; Schering-Plough) in 96-well U-bottom plates. The infliximab dose used *in vitro* is comparable with the infliximab serum concentration found *in vivo* during the first weeks after the infusion [24]. After 24 hours incubation, supernatants were collected. Depending on the conditions, cells were further incubated in fresh RPMI with live influenza virus (10^4 particles; Charles River Laboratories). After 24 hours, the supernatants were again collected for IFN α quantification by ELISA.

Plasma cell generation and antibody production

PBMCs were isolated by Ficoll-Paque Plus (Amersham Biosciences, Saclay, France) gradient centrifugation – from RA patients treated by infliximab who had developed significant ANA titers, from healthy donors and from SLE patients. PBMCs (1×10^6 /well) were then cultured with 10^4 influenza virus particles (Charles Rivers, Wilmington, MA, USA) with or without TNF α (10 $\mu\text{g/ml}$; R&D Systems) or TNF α blockers (Infliximab 20 $\mu\text{g/ml}$; Schering-Plough, Levallois-Perret, France) in a 48-well plate in 10% FCS RPMI supplemented with rhIL-2 (50 U/ml; R&D Systems, Lille, France). At day 15, supernatants were collected and tested for ANAs. The resulting B cells were analyzed using flow cytometry after gating on CD19 $^+$ cells and by calculating the percentage of CD20 $^{\text{low}}$ /CD38 $^{\text{high}}$ cells.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat software (version 3.0a for Macintosh; GraphPad Software, San Diego, CA, USA). Mann-Whitney tests were used for mean comparisons between groups. Wilcoxon's signed-rank test was used for the analyses of matched pairs. Correlation between DCs and activity markers were assessed using linear regression, given with the r^2 correlation coefficient. $P < 0.05$ was considered statistically significant.

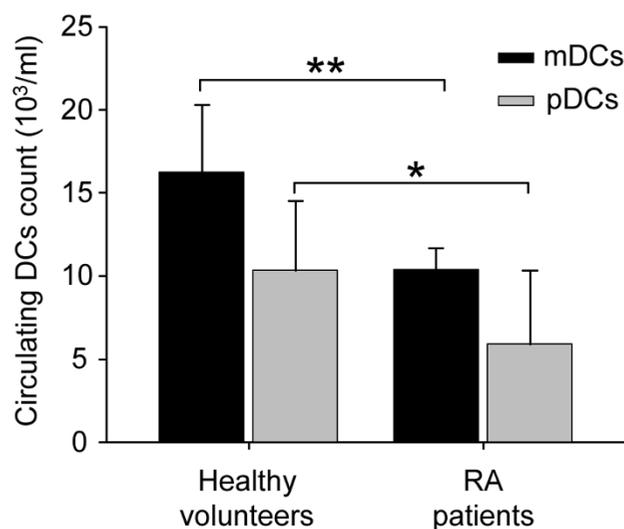
Results

Blood dendritic cell subsets in RA and their correlation with disease activity

To better delineate the involvement of known DC subsets in RA pathogenesis, we compared the number of circulating CD11c $^+$ HLA-DR $^+$ CD123 $^-$ mDCs and CD11c $^+$ HLA-DR $^+$ CD123 $^+$ pDCs in peripheral blood from 61 active RA patients (free of TNF α -blocker treatment) and from 30 healthy volunteers. Interestingly, RA peripheral blood was characterized by a decreased number of both pDC and mDC subsets (mean \pm standard deviation): mDC count = $10,214 \pm 7,576$ cells/ml in the RA group versus $16,228 \pm 4,057$ cells/ml in the healthy control group ($P = 0.0002$), and pDC count = $6,098 \pm 4,710$ cells/ml in the RA group versus $10,313 \pm 4,201$ cells/ml in the healthy control group ($P < 0.0001$) (Figure 1). We concluded that RA patients are characterized by a quantitative deficit in their peripheral circulating DCs.

We then looked for a correlation between absolute counts of blood DCs and the clinical status or laboratory tests known to reflect disease activity (DAS28, Health Assessment Questionnaire score, and C-reactive protein level). In RA patients, mDC counts were inversely correlated with each of these markers ($P < 0.05$, $r^2 = 0.07$, $P < 0.02$, $r^2 = 0.11$ and $P < 0.05$, $r^2 = 0.11$, respectively, for DAS28, Health Assessment Questionnaire score and C-reactive protein level). We did not find any statistical correlation between the pDC counts and DAS28, Health

Figure 1



Circulating dendritic cell subset levels in patients with active rheumatoid arthritis and in healthy volunteers. Dendritic cell (DC) subsets were measured in the peripheral blood of patients with rheumatoid arthritis (RA) ($n = 61$) and in healthy subjects ($n = 30$). The mean numbers per milliliter of blood of CD11c $^+$ CD123 $^-$ Lin $^-$ HLA-DR $^+$ myeloid dendritic cells (mDCs) and CD11c $^+$ CD123 $^+$ Lin $^-$ HLA-DR $^+$ plasmacytoid dendritic cells (pDCs) are shown (mean \pm standard deviation). * $P < 0.0001$ and ** $P < 0.001$, Mann-Whitney U test.

Assessment Questionnaire score or C-reactive protein level (Figure 2a,b,c).

The levels of both DC subsets are therefore decreased in the blood of RA patients with active disease, but only mDCs correlate inversely with disease activity – suggesting that this mDC decrease could reflect a migration to inflamed tissues. Accordingly, we found a higher percentage of mDCs in synovial fluid from active RA patients compared with that from patients with osteoarthritis (percentage \pm standard deviation: mDC = $52.5 \pm 13.7\%$ in the RA group vs. $17.4 \pm 18.3\%$ in the osteoarthritis control group; $P = 0.0005$). In contrast, the percentage of pDCs in synovial fluid was not different between the RA and the osteoarthritis groups (percentage \pm standard deviation: pDC = $8.4 \pm 10.9\%$ in the RA group vs. $2 \pm 3.9\%$ in the osteoarthritis control group, $P = 0.1119$) (Figure 2d). The preferential migration of mDCs to inflamed joints was also suggested by the increase of the mDC:pDC ratio in synovial fluid compared with that found in peripheral blood (median, 3.8:1; $P < 0.01$, Wilcoxon matched-pairs test) (Figure 2e).

Evolution of dendritic cell subset counts in infliximab-treated RA patients and correlation with the treatment response

Our initial results suggest that mDCs migrate from the blood to the inflamed synovial compartment. If this is the case, it seemed likely that effective therapy might block this migration and increase the blood mDC level.

Responders to the infliximab regimen ($n = 46$) were defined by a DAS28 decrease >1.2 after 14 weeks of infliximab therapy, whereas nonresponders ($n = 13$) were patients defined by a DAS28 variation <1.2 at week 14. Responders showed a substantial increase in their numbers of circulating mDCs (mean \pm standard deviation = $11,915 \pm 8,630$ cells/ml at day 0 vs. $15,868 \pm 11,467$ cells/ml at week 14, $P < 0.05$ using Wilcoxon matched-pairs test) (Figure 3a), whereas the blood pDC level did not change significantly ($5,632 \pm 3,035$ cells/ml at day 0 vs. $6,555 \pm 4,656$ cells/ml at week 14, $P = 0.23$) (Figure 3b). In contrast, nonresponders did not show statistically significant changes in mDC and pDC counts, and some patients even showing a decrease in both DC subsets during the course of treatment (mean \pm standard deviation: mDCs = $7,991 \pm 4,275$ cells/ml at day 0 vs. $8,386 \pm 3,689$ cells/ml at week 14, $P = 0.41$; and pDCs = $5,542 \pm 3,525$ cells/ml at day 0 vs. $4,649 \pm 2,032$ cells/ml at week 14, $P = 0.27$) (Figure 3a,b). These data suggest the existence of a relationship between the fluctuations of the mDCs present in the blood and the variations of disease activity.

Plasmacytoid dendritic cell number and blood IFN α levels correlate with anti-nuclear antibody positivity in infliximab-treated RA patients

The development of ANA is one of the most common side effects of TNF α -blocker therapies [25,26]. We therefore looked for a correlation between ANA appearance and DC count evolution in RA patients treated with infliximab.

The ANA levels were determined at the same time as the peripheral DC levels. After 14 weeks of treatment, we separated infliximab-treated patients into two groups: patients with positive ANA ($n = 30$) and patients with negative ANA ($n = 16$). The ANA level was considered positive when the serum dilution giving a positive signal in the indirect immunofluorescence on Hep-2 cells was above 1:250 and negative at the beginning of the treatment, or if the dilution increment reached at least three times the dilution observed at treatment onset. All of the data were obtained on day 0 of treatment onset and at week 14.

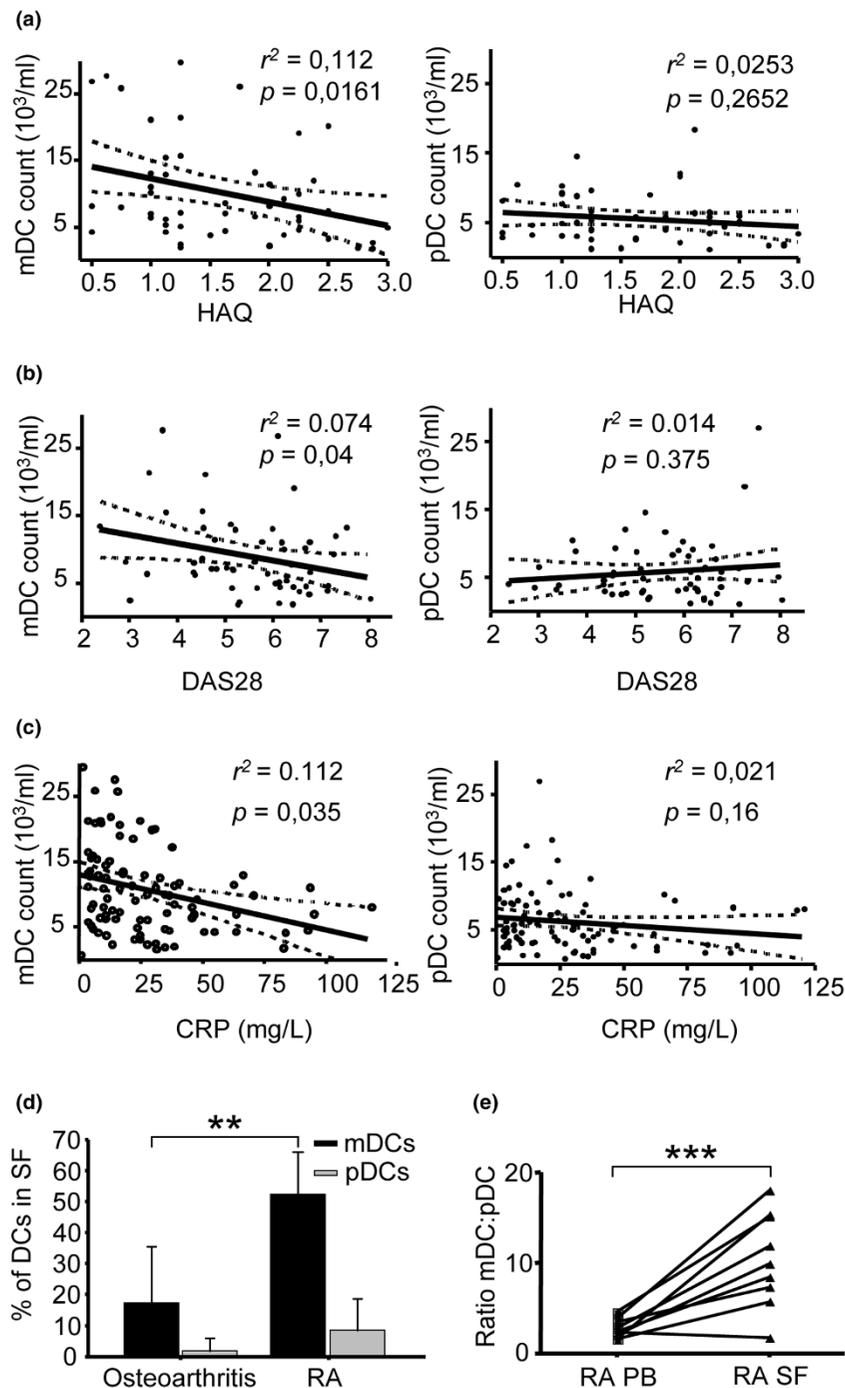
At week 14, the pDC levels were statistically lower in the ANA-positive group when compared with the ANA-negative group (mean \pm standard deviation: circulating pDCs = $5,509 \pm 3,161$ cells/ml vs. $9,324 \pm 5,834$ cells/ml, $P < 0.01$) (Figure 4a). Although no statistically significant difference was found in the mDC subset between the two groups (data not shown), the decrease of peripheral pDC counts correlated with the increase of ANA titers ($P = 0.02$, $r^2 = 0.15$) (Figure 4b).

Because IFN α and pDCs have been implicated in autoantibody production in SLE pathogenesis [3], we measured the IFN α level in the blood of both ANA-positive and ANA-negative RA patients treated by infliximab. We found that RA patients developing ANA were characterized by higher levels of IFN α (310 pg/ml vs. 47 pg/ml, $P < 0.01$), suggesting that infliximab influences pDC homeostasis and promotes the production of ANAs through the secretion of IFN α (Figure 4c).

Anti-TNF α antibody infliximab keeps plasmacytoid dendritic cells in an IFN α secreting state

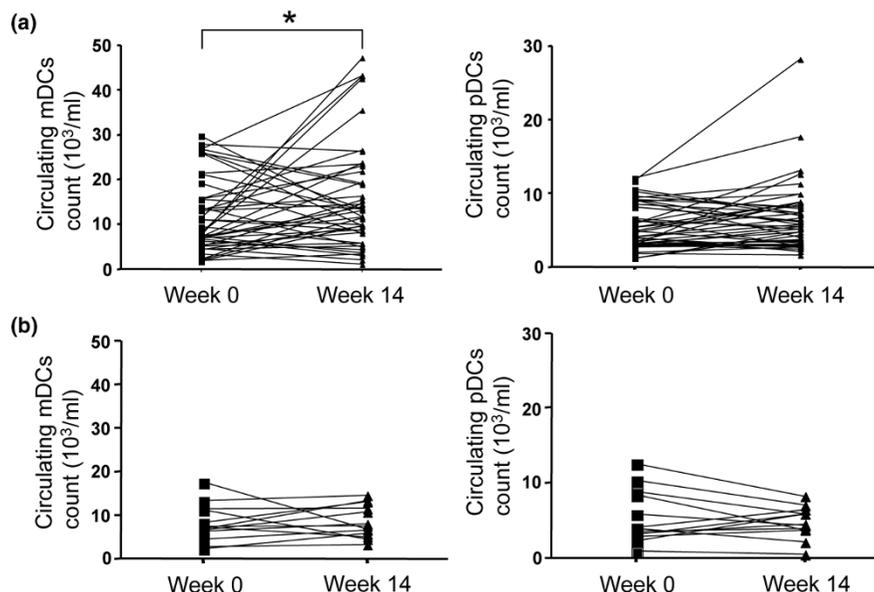
The presence of higher amounts of IFN α in RA ANA-positive patients prompted us to analyze the effects of infliximab on pDCs' ability to secrete IFN α *in vitro*. PBMCs from control donors were exposed to influenza virus alone or in the presence of infliximab. Influenza virus was used as a well-known strong pDC-IFN α inducer. We did not find any increase in cellular apoptosis of the cells in any of the conditions tested (data not shown). In both conditions (virus alone or virus + infliximab), we detected high levels of IFN α in the supernatant collected after 24 hours culture, without any differences between the two conditions (Figure 5). Repeat exposure of PMBCs to influenza virus, however, was able to induce large IFN α production only in the presence of infliximab. Furthermore, PBMCs pretreated with TNF α were unable to secrete significant amounts of IFN α . Although these studies were performed

Figure 2



Correlation between circulating dendritic cell subsets and disease activity markers. Circulating plasmacytoid dendritic cell (pDC) and myeloid dendritic cell (mDC) counts (mean numbers/ml blood) from rheumatoid arthritis (RA) patients ($n = 60$) plotted against **(a)** Health Assessment Questionnaire (HAQ) score, **(b)** Disease Activity Score in 28 joints (DAS28), and **(c)** C-reactive protein (CRP) level. **(d)** The mDC level in synovial fluid (SF) of patients with active RA is significantly increased compared with that in osteoarthritis patients. Dendritic cell (DC) subsets were measured in the SF of patients with RA ($n = 9$) and in osteoarthritis patients ($n = 11$). The percentage of $\text{CD11c}^+\text{CD123}^+\text{HLADR}^+$ mDCs and $\text{CD11c}^-\text{CD123}^+\text{HLADR}^+$ pDCs in Lin^- cells (CD3^+ , CD14^+ , CD16^+ , CD56^+ , CD8^+ , CD19^+) are shown (mean \pm standard deviation). ** $P < 0.001$, Mann–Whitney U test. **(e)** The mDC:pDC ratio in SF from RA subjects is significantly increased compared with the ratio in matched peripheral blood (PB) samples. Squares and triangles indicate individual matched samples ($n = 9$). The ratio is calculated from the percentage of mDCs and pDCs in Lin^- cells. *** $P < 0.01$, Mann–Whitney U test.

Figure 3



Evolution of circulating myeloid and plasmacytoid dendritic cell counts over 14-week treatment with infliximab. Evolution of circulating myeloid dendritic cell (mDC) and plasmacytoid dendritic cell (pDC) counts over a 14-week period of treatment with infliximab in (a) responder patients ($n = 46$) and (b) nonresponder patients ($n = 13$). Squares indicate matched samples. * $P < 0.05$, using Wilcoxon matched-pairs test.

with PBMCs, it is probable that pDCs were the major source of $\text{IFN}\alpha$ given that they are the major $\text{IFN}\alpha$ -producing cells in peripheral blood. These data strongly suggest that infliximab maintains pDCs in an $\text{IFN}\alpha$ secreting state by quenching $\text{TNF}\alpha$.

Infliximab increases plasma cell generation and promotes *in vitro* anti-nuclear antibody secretion

Jego and colleagues showed that pDCs exposed to viral infection were able to activate the B-lymphocyte compartment and to promote the generation of plasma cells and/or plasmablasts in an $\text{IFN}\alpha$ -dependent and IL-6-dependent fashion [4]. To delineate the consequences of the sustained $\text{IFN}\alpha$ secretion induced by infliximab, we compared the proportion of circulating $\text{CD}19^+\text{CD}20^+\text{CD}38^+$ plasma cells in RA ANA-positive patients ($n = 10$) and RA ANA-negative patients ($n = 10$). RA ANA-positive patients exhibited a significant increase ($P < 0.001$) of the proportion of circulating plasma cells compared with RA ANA-negative patients (Figure 6a). The percentage of plasma cells in RA ANA-positive patients was similar to that observed in SLE patients.

We then tested, *in vitro*, whether infliximab effects plasma cell generation from B lymphocytes. PBMCs were cultured with influenza virus with or without infliximab. After 15 days, we measured the proportion of $\text{CD}19^+\text{CD}20^+\text{CD}38^{\text{high}+}$ plasma cells. PBMCs cultured with influenza virus + infliximab were characterized by a higher proportion of plasma cells. Interestingly, concomitant addition of $\text{TNF}\alpha$ with virus stimulation inhibited plasma cell generation (Figure 6b). To analyze the *in*

vitro effects of infliximab on ANA secretion, we repeated the experiment with PBMCs from RA ANA-positive patients, SLE patients and healthy control individuals. These PBMCs were cultured for 15 days in the presence of influenza virus with or without infliximab. After 15 days the secretion of ANA was found only in supernatants from cells from RA ANA-positive patients or SLE patients, and was further increased in the presence of infliximab (Figure 6c).

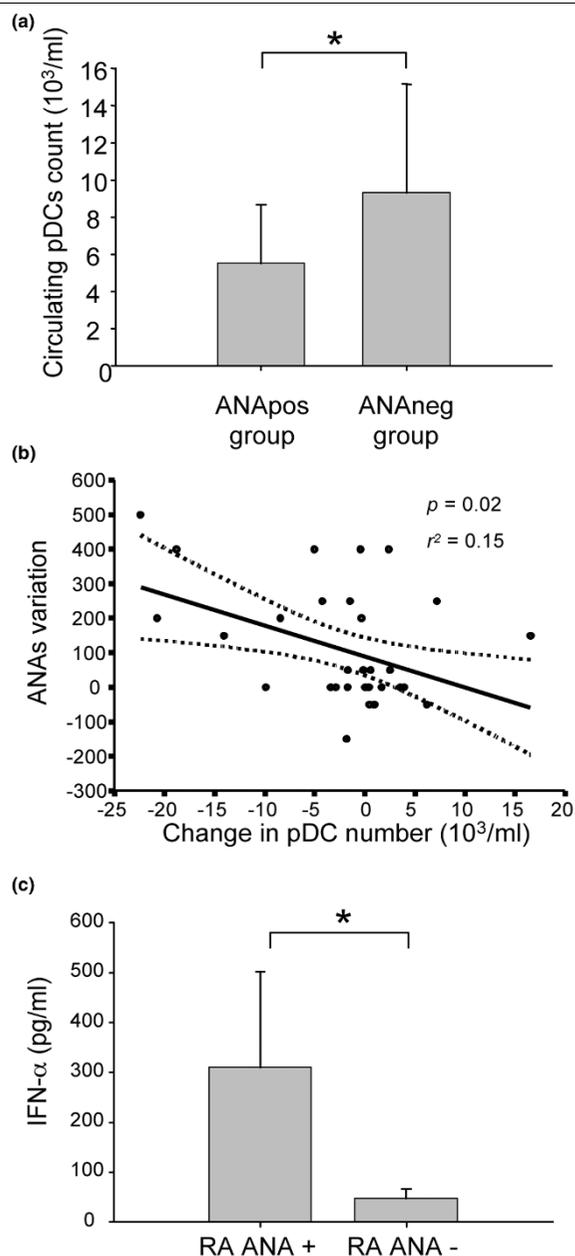
Taken together, those results suggest that infliximab promotes pDCs in an $\text{IFN}\alpha$ secreting state and allows for the differentiation of B lymphocytes into ANA-secreting plasma cells.

Discussion

DCs are thought to play a key role in driving the immunopathogenic response underlying chronic inflammatory arthritis. Various studies [9,27-29] have shown that both mDCs and pDCs accumulate in synovial tissue and synovial fluid of RA patients. The evolution of circulating peripheral blood DC counts under $\text{TNF}\alpha$ blocker therapy has never been studied, however, but it may provide important information on the implication of both subsets in RA pathogenesis.

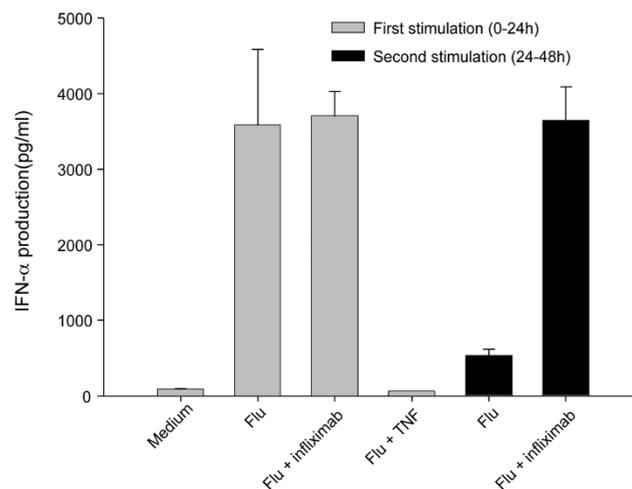
In the present study we show that RA patients are characterized by a significant decrease in circulating mDCs and pDCs, consistent with previous results from Jongbloed and colleagues [11]. We, however, found that only mDC counts correlated inversely with RA activity as assessed by the Health Assessment Questionnaire score, DAS28, and C-reactive protein level, and that the percentage of mDC was increased

Figure 4



Plasmacytoid dendritic cell number, blood IFN α and anti-nuclear antibody positivity in infliximab-treated rheumatoid arthritis patients. **(a)** Circulating plasmacytoid dendritic cell (pDC) levels in rheumatoid arthritis (RA) patients after 14 weeks of infliximab therapy. pDC subsets were measured in the peripheral blood of patients, and two groups were individualized: patients with positive anti-nuclear antibody (ANA) (ANAPos, $n = 30$) and patients with negative ANA (ANAneg, $n = 16$). Mean \pm standard deviation shown. * $P < 0.01$, Mann-Whitney U test. **(b)** Correlation between ANA levels and pDC variations under infliximab therapy. ANA levels (1/dilution) and the pDC amount were measured on the same blood draw, before each infliximab infusion. **(c)** Detecting IFN α in serum of RA patients treated by infliximab and developing or not ANA. IFN α levels (pg/ml) were measured in peripheral blood of RA patients under infliximab therapy with ANA ($n = 30$) or without ANA ($n = 16$). * $P < 0.001$, Mann-Whitney U test.

Figure 5

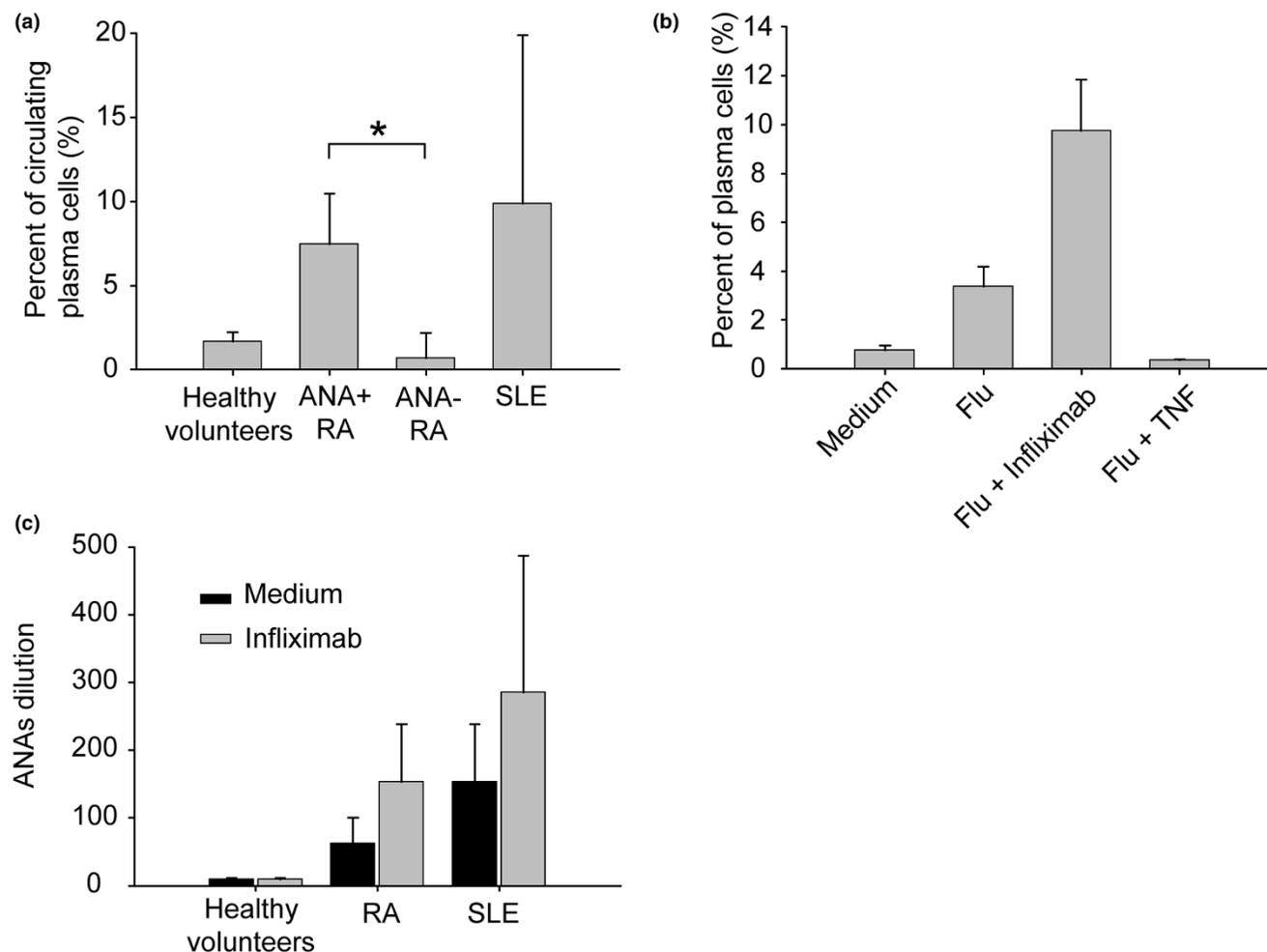


Infliximab maintains plasmacytoid dendritic cells exposed to influenza virus in an IFN α secreting state. Peripheral blood mononuclear cells from control donors were exposed to influenza virus alone (Flu) or to influenza virus in the presence of infliximab. After 24 hours of incubation, the supernatant was collected and IFN α levels were measured by ELISA. The cell pellets were then washed, resuspended in fresh medium, and exposed for an additional 24 hours to influenza virus. Supernatants were analyzed by ELISA. Data are expressed as the mean \pm standard error of the mean of three independent experiments.

in the inflamed synovial tissue. Moreover, in the presence of effective infliximab therapy, circulating mDC counts increased to reach levels observed in healthy volunteers. Our results suggest that, among DCs, mDCs have a prominent role in clinical disease manifestations in RA patients since their circulating numbers correlate directly with disease activity, and treatment with infliximab corrects mDC count abnormalities in infliximab-responsive patients.

The lack of correlation between pDC counts and RA clinical evolution was unexpected because pDCs are known to play a central role in various inflammatory diseases, including psoriasis [30], Sjogren's syndrome [31] and SLE [3]. It is probable that other unknown parameters may alter pDC homeostasis in RA patients. Psoriasis [32-34] and SLE (or more frequently the appearance of ANA) [12-16] have been described as an adverse effect of TNF α -blocker therapy. In both diseases, pDCs are implicated in pathogenesis through their ability to produce high amounts of IFN α [3,30]. In the case of SLE, this occurs through uptake of the immune complex on the pDC cell surface and the subsequent internalization and delivery of the self-DNA or self-RNA within the complex to intracellular TLR9 or TLR7, respectively [35-37]. In the case of psoriasis, the endogenous antimicrobial peptide LL37 forms a complex with self-DNA that is delivered to and retained within early endocytic compartments of pDCs to trigger TLR9 and to induce IFN α production [38]. Interestingly, a recent study has reported an increased IFN α expression and more severe psoriasis

Figure 6



Infliximab enhances plasma cell differentiation. **(a)** Circulating plasma cell levels in rheumatoid arthritis (RA) patients treated by infliximab with anti-nuclear antibody (ANA) ($n = 10$) or without ANA production ($n = 10$), in patients with active systemic lupus erythematosus (SLE) ($n = 10$), and in healthy volunteers ($n = 10$). Plasma cell levels were measured in peripheral blood. The mean number/milliliter of $CD38^+CD19^+CD20^-$ is shown (mean \pm standard deviation). * $P < 0.001$, Mann-Whitney U test. **(b)** Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured out in the presence of influenza virus (Flu) with or without infliximab or $TNF\alpha$. After 10 days, we analyzed by flow cytometry the proportion of $CD19^+CD20^-CD38^{high+}$ plasma cells. Data expressed as the mean \pm standard error of the mean of three independent experiments. **(c)** PBMCs from healthy donors, from RA patients treated by infliximab and developing ANAs, and from SLE patients were cultured in the presence of influenza virus with or without the $TNF\alpha$ blocker, infliximab. After 15 days, ANA titers were measured in the supernatants. Data expressed as mean \pm standard error of the mean of three independent experiments.

riatic skin lesions in patients treated with TNF blockers [34], implicating $IFN\alpha$ in the pathogenesis of psoriasis.

As previously proposed by Palucka and colleagues in systemic-onset juvenile idiopathic arthritis [39], we thought pDCs may be preferentially involved in the ANA response frequently found in RA patients – which increases under treatment. Indeed, we found a direct correlation between ANA levels and decreased pDC variation. Furthermore, serum $IFN\alpha$ was significantly increased in patients developing ANAs. Several studies [39-41] evaluating $IFN\alpha$ production in autoimmune diseases have measured $IFN\alpha$ gene expression and $IFN\alpha$

inducible gene expression instead of measuring serum $IFN\alpha$ protein levels because of the limited sensitivity of the ELISA assay. In our study, however, the serum level of $IFN\alpha$ induced by influenza was high enough to be detected at the protein level, allowing the same ELISA assay to be used for both *in vivo* and *in vitro* measurement of $IFN\alpha$. Our results suggest that migration of pDCs – which are known to enter lymph nodes when they produce $IFN\alpha$ [42] – occurs, leading to their decreased numbers at the periphery in the ANA-positive group. Moreover, this $IFN\alpha$ secretion from pDCs has been previously described to induce plasma cell differentiation and, therefore, autoantibody production [4]. Accordingly, *in vivo*,

we found increased plasma cell generation in RA patients developing ANAs during infliximab therapy.

IFN α -secreting pDCs have been described as being immature or precursor DCs [43]. TNF α is known to differentiate immature DCs into a more mature stage [44] and to inhibit IFN α induced by viruses [45]. TNF α -mediated maturation of pDCs could block the IFN α -producing ability of pDCs. Conversely, pDCs stimulated by viruses secrete high amount of IFN α and TNF α that could act in an autocrine loop to control IFN α secretion through pDC maturation.

We confirm that TNF α blocks the ability of pDCs to secrete IFN α upon viral stimulation, and that the TNF α antagonist, infliximab, keeps pDCs in an IFN α secreting state. This result and our *in vivo* data described above are consistent with a previous report showing that, *in vitro*, TNF α blockers inhibit virus-induced maturation of pDCs and increase IFN α secretion [39]. The authors suggested that this inhibition may explain the increase of ANA production in patients treated with TNF α blockers. We confirmed their findings by showing, *in vitro* and *in vivo*, the ability of infliximab to increase IFN α secretion, plasma cell differentiation and ANA generation. de Rycke and colleagues, however, have previously described differences in ANA induction between infliximab and etanercept in patients suffering from spondylarthropathy [13]. It will therefore be important to determine in future work whether other TNF blockers (adalimumab and etanercept) have the same ability as infliximab to maintain pDCs in an IFN α secreting state.

Conclusions

Although both subtypes of circulating DCs are reduced in active RA patients' peripheral blood, only mDC levels correlated with disease activity, suggesting a possible link to RA pathogenesis. The exact role of pDCs in RA remains unclear, but these cells seem likely to play an important role in lupus-like complications of infliximab therapy as they do in lupus.

We confirmed that infliximab acts on the regulation of IFN α system *in vivo* and *in vitro*, by enhancing plasma cell differentiation, which is ultimately responsible for autoantibody secretion. Our results emphasize the balance between IFN α and TNF α in RA, and provide mechanistic insights into the possible roles of DC subsets in mediating the shift in autoimmune disease manifestations by therapeutics that inhibit TNF α . These findings may also be relevant in other autoimmune diseases where the role of IFN α and TNF α has been suggested, such as psoriasis [30,46].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CR, TS, J-FM and PB designed the study. CR and CD collected clinical patient data. CR, TS and PB performed all

experiments and analyzed the data. CR and PB drafted the manuscript. JD followed up the patients. All authors read and approved the final document.

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