Influence of anti-tumor necrosis factor therapy (Adalimumab) on regulatory T cells and dendritic cells in rheumatoid arthritis

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Abstract

Objective

To investigate whether anti-TNF therapy could have an effect on dendritic cells (DCs) and regulatory T cells in rheumatoid arthritis (RA) patients.

Methods

A four-colour flow cytometric technique was used to measure $CD4^+CD25^+$ T cells i.e. $CD4^+CD25^{high+}$ (regulatory T cells) and $CD4^+CD25^{low+}$ (activated T cells)), DCs as well as the in vitro, intracellular, lipopolysaccharide-stimulated cytokine production of DCs.

Results

Clinical and laboratory parameters of disease activity decreased after anti-TNF treatment. Before anti-TNF therapy, RA patients demonstrated a decreased count of Th2-promoting lymphoid DCs as compared to controls and after anti-TNF therapy this decrease was sustained. Intracellular cytokine production was only found in the myeloid DCs population and there was a higher production of TNF-α and IL1- β as compared to healthy controls. Treatment did not alter this cytokine production. Before anti-TNF therapy, the percentage $CD4^+CD25^{low+}$ T cells was significantly elevated in RA patients than in healthy controls.

Conclusion

These results demonstrate anti-TNF to be a potent anti-inflammatory drug, as mirrored by the decrease in clinical and biological parameters as well as the decrease in activated $CD4^+$ T cells. However, in this study no demonstrable effect on DCs and regulatory T cells was found.

Key words

Dendritic cells, regulatory T cells, rheumatoid arthritis, anti-TNF therapy.

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Introduction

Several data indicate that DCs are likely to have a role in the pathogenesis of autoimmune diseases like rheumatoid arthritis (RA) (1). DCs are the most potent, professional antigen presenting cells (APC). Myeloid DCs (DC1) and lymphoid DCs (DC2) can polarize naive T cells into Th1 and Th2 effector cell development, respectively (2, 3). It is known that RA is a Th1-mediated disease (4) and is characterized by infiltration of different immunocompetent cells into the synovium where a sustained overproduction of pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) is observed.

Besides Th1-type $CD4^+$ T cells, a different T-cell subpopulation, i.e. the regulatory T cells, might play a role in auto-immune diseases like RA. The term "regulatory T cells" describes a variety of T cells that display primarily suppressive functions. Much interest is paid to the $CD4^+$ $CD25^+$ T cells, whereas it has been demonstrated that only those expressing high levels of CD25 display regulatory activity, indicating that the whole $CD4^+$ $CD25^+$ T-cell population contains a relatively high proportion of previously activated T cells (5). These spontaneously occurring regulatory T cells can suppress the activation and the function of autoreactive T cells that escape other mechanisms of tolerance (6, 7).

In the treatment of RA, anti-TNF therapy has proven to be effective in improving both clinical disease activity and laboratory parameters of inflammation (8-12). We have shown that anti-TNF therapy down-regulates the capacity of monocytes to produce pro-inflammatory cytokines and induces a shift to a more pronounced anti-inflammatory Th2 cytokine balance (13). Furthermore, it has been shown that anti-TNF therapy downregulates the CD23 expression on T-cell activated B cells and this is related with the presence of circulant immune complexes containing TNF-α (14).

The aim of this study was to investigate if anti-TNF therapy has modulating effects on DCs and regulatory T cells.

Materials and methods

Study population

Ten patients (6 females, 4 males; age 39-77, median 56) with active RA despite stable treatment with methotrexate (7.5-15 mg/week) and non-steroidal anti-inflammatory drugs (NSAIDs) or low-dose corticosteroids (4-12.5 mg/day) fulfilling the diagnostic criteria of the American College of Rheumatology for the classification of RA (15) were enrolled in this study. They were evaluated before and after 12 weeks of treatment with the fully human, anti-TNF monoclonal antibody adalimumab (40 mg, sub-cutaneously, every other week). During this study period the dose of methotrexate, NSAIDs and corticosteroids remained unchanged.

Disease activity was scored clinically (number of painful and number of swollen joints) and biologically (erythrocyte sedimentation rate and C-reactive protein). DAS28 scores (16) were also calculated. Responders to anti-TNF therapy were defined as having a decrease in DAS28 of > 1.2 (17).

The ethics committee of the University Hospital of Antwerp approved this study. All patients gave informed consent for the study. Ten healthy age- and sex-matched individuals served as a control group.

Blood sampling

Heparinized whole blood (9 mL; Greiner Bio-one, Frickenhausen, Germany) was processed within 1 hour after collection for the analysis of T-cell subsets, dendritic-cell phenotype and cytokine production.

Flowcytometrical identification of DCs

DCs were identified using a combination of fluorochrome-conjugated monoclonal antibodies to different membrane markers (18, 19). All reagents were obtained from BD Biosciences (Erembodegem, Belgium): 20 μL lineage cocktail 1-FITC (Lin1-FITC), containing a mixture of CD3, CD14, CD16, CD56, CD19 and CD20 antibodies; 5 μL anti-CD34-FITC; 5 μL anti-CD123-PE; 5 μL anti-CD11c-APC; and 10 μL anti-HLA-DR-PerCP were incubated with 100 μL whole blood for 15 minutes at 4°C in the dark. Cells

were fixed and lysed with Facslysis (BD Biosciences) for 20 minutes at room temperature. Cells were spun at 400 g for 10 minutes, and 0.5 mL phosphate buffered saline (PBS) containing 0.1% NaN_3 was added.

Data acquisition was performed on a 4-colour FACScalibur (BD Biosciences) using a well-defined gating strategy in Cell-Quest Pro software (19) and a representative example is showed in Figure 1. At least 5 x 10^5 cells were measured to obtain a minimum of 1000 HLA-DR positive DCs. Different cell populations could then be identified: myeloid DCs (DC1, $CD11c^+$ $CD123^{dim+}$); lymphoid DCs (DC2, $CD11c^-$ $CD123^{high+}$); and ldDCs (less-differentiated DCs, $CD11c^-$ $CD123^{dim+}$), a DC subpopulation that was previously described by Hagendorens *et al.* (19). Results were expressed as absolute number of DCs (10^6/L) in white blood cells.

Intracellular cytokine analysis in circulating DCs

Intracellular cytokine analyses were preformed as described previously (20). Briefly, 1 mL of heparinized whole blood was stimulated for 6 hours with 1 μg/mL lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich Corp, St. Louis, MO, USA), in the presence of brefeldin A (Sigma-Aldrich), added during the last 5 hours of stimulation. After incubation at 37°C in a 5% CO_2 humid atmosphere, DCs were identified using a combination of fluorochrome-conjugated monoclonal antibodies and different membrane markers as described (20). Thereafter, red blood cells were lysed and white blood cells were fixed with Facslysis during 20 minutes at room temperature. After centrifugation, cell membranes were made permeable with 0.3% saponin (Sigma-Aldrich) in PBS. Cells were stained during 30 minutes at 4°C, with monoclonal anti-cytokine antibodies labelled with phycoerythrin (PE) (anti-human IL-1β-PE, anti-human TNF-α -PE, anti-human IL-12-PE, and anti-human IL-10-PE (BD)). Irrelevant isotype-matched monoclonal antibodies conjugated with PE were used as negative controls. After staining, cells were washed with PBS, spun for 5 minutes at 400 g and re-suspended in 0.5 mL PBS containing 0.1% NaN_3.

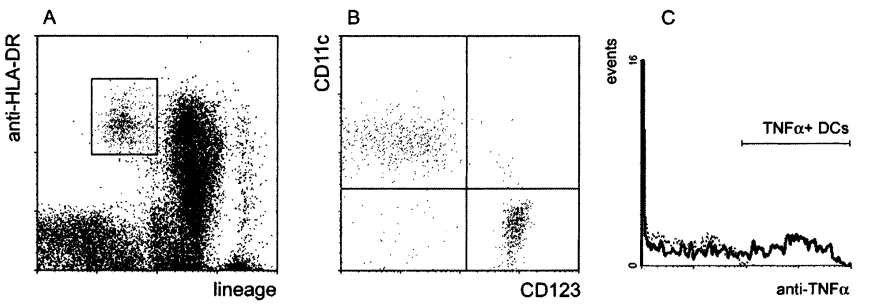

Fig. 1. Gating strategy for identification of dendritic subtypes and TNF-α production. (**A**) Dendritic cells were characterised as cells negative for lineage markers (DClineage-FITC) and positive for HLA-DR-PerCP. (**B**) After gating DCs, a CD-123-PE/CD11c-APC plot showed 3 distinct cell populations: myeloid DCs were $CD11c^+$ $CD123^{dim+}$, lymphoid DCs are $CD11c^-$ $CD123^{high+}$ and $CD11c^-$ $CD123^{dim+}$ cells were considered as less differentiated DCs (ldDC). (**C**) Within the gate of $CD11c^+$ DCs, intracellular TNF-α producing DCs were determined in a histogram of the cytokine measured. The dotted line represented the isotype match irrelevant antibody and the marker was set on the 99th percentile.

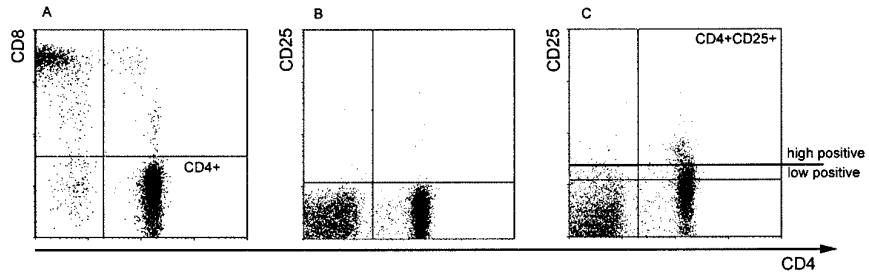

Fig.2. Gating strategy for identification of T-cell subtypes.
(**A**) The different T-cells were characterised as $CD3^+CD4^+CD8^-$ (T-helper cells) and $CD3^+CD4^-CD8^+$ (T-cytotoxic cells) T-cells. (**B**) For detecting the $CD4^+CD25^+$ T-cell population, an irrelevant antibody was used as negative control and the marker was set on the 99th percentile. (**C**) $CD4^+CD25^{+high}$ T-cells were defined as having a fluorescence intensity of CD25 expression exceeding channel 400.

Data acquisition was performed as described above for the DCs phenotype. Gating procedure was performed as shown in Figure 1 and described before (20). Results were expressed as the percentage and absolute number of cytokine-producing DCs. Because preliminary data showed no detectable intracellular IL-10 and IL-12 production by circulating DCs after LPS stimulation, IL-10 and IL-12 measurements were not further performed in this study.

Flowcytometrical analysis of T cell subsets

Fifty μL whole blood was incubated for 15 minutes in the dark at 4°C with 10 μL of three-colour monoclonal antibody panels (CD3-PerCP + CD4-FITC + CD8-PE, BD Biosciences) and 5 μL of CD25-APC (BD Biosciences).

Subsequently, the red blood cells were lysed with Facslysis for 20 minutes at room temperature. The cells were pelleted at 400 g for 10 minutes and 0.5 mL PBS, containing 0.1% NaN_3, was added and analyzed on a 4-colour FACScalibur within 24 hours using CellQuest Pro software. Through gating on sideward scatter and $CD3^+$ cells, the different T-cell subsets were determined (13). Results are expressed as median (range) percentage (%) and as absolute numbers (10^6/L).

For detecting the $CD4^+CD25^+$ T cell population, an irrelevant, isotype-matched monoclonal antibody conjugated with APC (BD Biosciences) was used as a negative control.

$CD4^+CD25^{high+}$ T cells were defined as having a fluorescence intensity of CD-25 expression exceeding channel 400 (21). Results are expressed as a percentage of the $CD4^+$ T cells. Figure 2 shows a representative example of the gating strategy for the T-cell subtypes.

Statistics

Data analysis was performed using SPSS 11.0 for Windows statistical package. Results are expressed as me-

dians and corresponding ranges. The Mann-Whitney U-test and Wilcoxon signed-rank test were used as appropriate. A p-value < 0.05 was considered statistically significant.

Results

Disease activity

Most clinical and biological parameters of disease activity decreased significantly after anti-TNF treatment (Table I). There were 7 responders to the anti-TNF therapy.

DCs subsets

Before treatment, a decreased number of lymphoid DCs was found in the peripheral blood of RA patients as compared to controls ($p < 0.05$) (Table II). No significant differences in absolute numbers of circulating DCs ($p = 0.15$) and no shift in the ratio of myeloid DCs to lymphoid DCs ($p = 0.1$) were found between RA patients and controls. As depicted in Table II, anti-TNF did not alter numbers of DCs and DCs subsets.

Intracellular cytokine production by circulating DCs

The percentage of TNF-α producing DCs was significantly higher in RA patients compared with controls ($p = 0.007$) (Fig. 3). Furthermore, the percentage of IL1-β producing DCs tended to be higher in the RA patients ($p = 0.05$). After anti-TNF therapy no alterations was found. When only evaluating the responders, the percentage TNF-α producing DCs remained increased ($p = 0.03$). When expressed as absolute numbers no differences were found.

T-cell subsets

Before treatment, the absolute numbers of peripheral blood lymphocytes, $CD3^+$, $CD4^+$ and $CD8^+$ T cells were significantly lower in RA compared with controls (Table III). Twelve weeks of therapy with anti-TNF resulted in a normalization of the lymphocyte counts.

Before anti-TNF therapy, the percentage $CD4^+CD25^+$ T cells was significantly higher in RA patients compared with controls ($p < 0.03$) and there was normalization after treatment ($p = 0.26$). As shown in Figure 4 this was mainly due to the differences in the subpopulation $CD4^+CD25^{low+}$ T cells. These normalizations were also found when only evaluating the responders ($p = 0.17$, $p = 0.49$ for $CD4^+CD25^+$ T cells and $CD4^+$ $CD25^{low+}$ T cells, respectively).

No difference was found in the subpopulation $CD4^+CD25^{high+}$ T cells between patients at baseline and controls or between patients before and after treatment (Fig. 4).

Table I. Different parameters in RA patients before (d0) and after 12 weeks of therapy with anti-TNF. Given values are expressed as median (min-max).

	Before therapy d0	After therapy w12	p-value
ESR (mm/h)	37.5 (16-120)	24 (2-108)	0.02
CRP (mg/dL)	1.9 (<0.35-6.5)	0.6 (< 0.35-5.9)	0.05
Ritchie index	19 (8-25)	5 (0-27)	0.04
Number of swollen joints	6 (4-20)	4 (0-13)	0.26
DAS28	6.2 (5.4-7.6)	4.1 (2.4-7.6)	0.01

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS: Disease Activity Score.

Table II. Absolute numbers of DCs, myeloid DC ($CD11c^+$ $CD123^{dim+}$), lymphoid DC ($CD11c^-$ $CD123^{high+}$) and less-differentiated DCs (ldDC, $CD11c^-$ $CD123^{dim+}$) detected in peripheral blood of controls and RA patients before and after therapy. Given values are expressed as median (min-max) x 10^6cells/L.

	Controls	RA before therapy (n = 10)	RA after therapy (n = 10)
DC	24 (18-53)	19 (6-45)	19 (6-66)
$CD11c^+$ $CD123^{dim+}$	12 (8-23)	8 (4-21)	10 (3-35)
$CD11c^-$ $CD123^{high+}$	5 (2-19)	2 (0-12)§	2 (0-11)
$CD11c^-$ $CD123^{dim+}$	8 (2-12)	5 (2-11)	7 (2-22)

§$p < 0.05$ vs. controls.

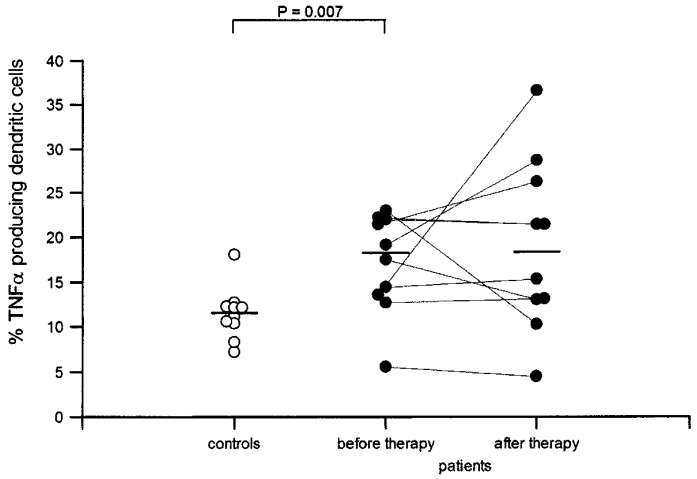

Fig. 3. Percentage of TNF-α producing DCs after 6 hours stimulation with 1 μg/ mL lipopolysaccharide from *E. coli* of controls (n =10) and RA patients before and after anti-TNF therapy (n = 10). Bars represent the median.

Discussion

Although the pathogenesis of RA is complex and not completely understood, it is generally accepted that the disease process involves presentation of a putative antigen by APC and activation of autoreactive T cells. The observation that anti-TNF rapidly suppresses clinical and biological disease activity stimulated us to investigate whether this therapy could affect DCs or regulatory T cells. In our study, before anti-TNF therapy, there were de-

Table III. Lymphocyte subsets in healthy controls and RA patients before and after 12 weeks of anti-TNF therapy adalimumab. Results are expressed as median (range) percentage (%) and as absolute numbers (10^6/L).

	Controls (n = 10)	RA before therapy (n = 10)	RA after therapy (n = 10)
$CD3^+$ (%)	69 (57-75)	63 (11-78)	61 (34-84)
$CD4^+$ (%)	40 (31-46)	45 (5-57)	42 (19-71)
$CD8^+$ (%)	23 (15-35)	12 (5-30)*	16 (8-28)
Lymphocytes (x 10^6/L)	2100 (1600-3500)	1100 (300-1900)*	1700 (400-2900)§
$CD3^+$ (x 10^6/L)	1469 (928-2436)	676 (89-1490)*	958 (212-1670)§
$CD4^+$ (x 10^6/L)	783 (527-1558)	529 (43-830)*	617 (108-1052)§
$CD8^+$ (x 10^6/L)	549 (256-760)	109 (14-562)*	187 (65-596)§

*$p < 0.04$ vs. controls.
§$p < 0.03$ vs. RA patients before treatment.

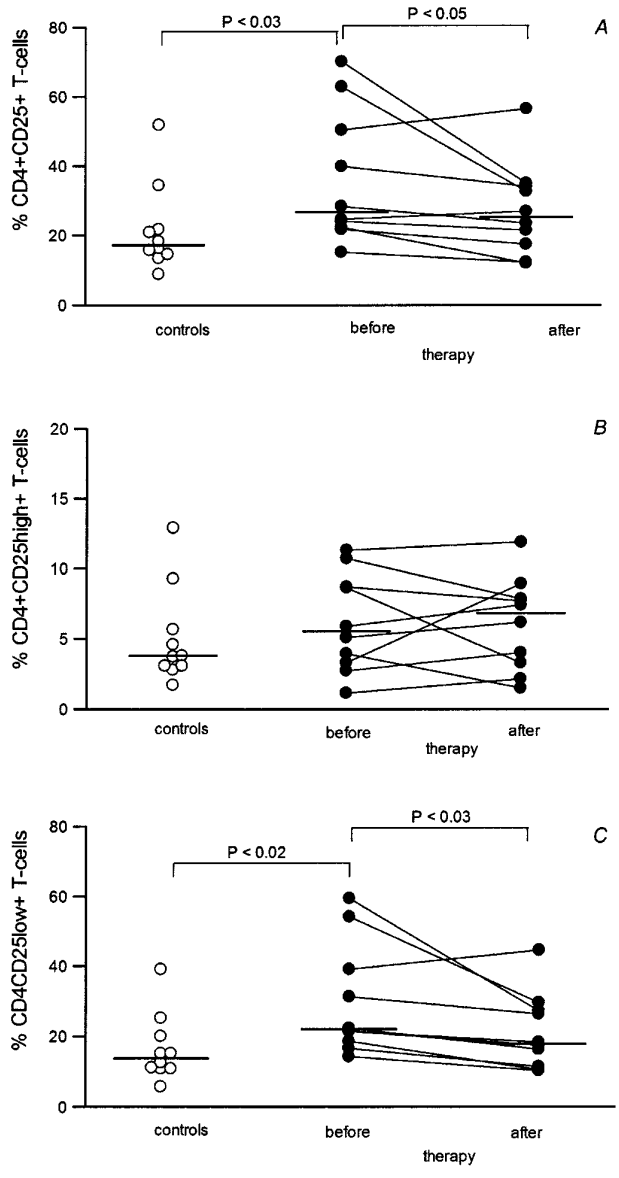

Fig. 4. Percentage of $CD4^+CD25^+$ T cells (**A**), $CD4^+CD25^{high+}$ T cells (**B**) and $CD4^+CD25^{low+}$ T cells (**C**) in peripheral blood from healthy controls (n =10) and RA patients before and after anti-TNF therapy (n = 10).
Results are expressed as a percentage of total $CD4^+$ T cells. Bars represent the median.

creased counts of peripheral blood lymphoid DCs in RA patients as compared to healthy controls. This decrease in lymphoid DCs might result in a down-regulation of peripheral blood Th2 cells and a relative predominance of myeloid DCs inducing a Th1 cytokine production in RA. Although recruitment of lymphoid DCs to the inflamed joints, a phenomenon that has been demonstrated recently, can not be excluded (22-24). It also has to be mentioned that treatment with prednisolone, although in our study low doses were used, might have an effect on the number lymphoid DCs (25). When anti-TNF therapy was given we could not observe alterations of these lymphoid DCs counts. Concerning *in vitro* stimulated cytokine production an increase was observed in the percentage TNF-α and IL-1β producing myeloid DCs, which could impair the Th1-Th2 cytokine balance with a further skewing to the Th1 cytokine responses. Recently, Radstake *et al.* (26) also reported elevated cytokine production in cultured DCs of RA patients. In our study, twelve weeks of anti-TNF therapy did not result in an altered cytokine production. Perhaps longer treatment with anti-TNF therapy could change the percentage TNF-α producing DCs.

Apart from Th1 and Th2 induction, it has been suggested that DCs might promote the development of regulatory T cells (27, 28). In animal models, evidence has emerged that regulatory T cells play an important role in the maintenance of immunological self-tolerance (29). However, the role of these cells in the pathogenesis of human autoimmune diseases such as RA remains to be delineated.

In this study, no differences were found in regulatory T cells between healthy controls and the RA patients. This is in line with a previous report (30).

On the other hand there was an increased percentage of peripheral blood $CD4^+CD25^+$ T cells in RA patients compared with the blood of healthy controls and this was due to an increase in the subpopulation of activated $CD4^+$ T cells. The presence of these activated $CD4^+$ T cells have been demonstrated in the peripheral blood of RA patients earlier (31).

We found that anti-TNF therapy significantly decreased activated $CD4^+$ T cells, reflecting suppression of disease activity, but did not alter regulatory T cells. Obviously, our data do neither

exclude functional changes nor changes of regulatory T cells at the target organ. This data diverge from the recently described increase in both T cell subpopulations, respectively $CD4^+$ $CD25^{high+}$ and $CD4^+CD25^{low+}$, in RA patients that actually did respond to anti-TNF therapy (32). In this study of Ehrenstein *et al.*, patients were classified as responders and non-responders to anti-TNF. When we reanalysed our results and only included responders our results of decreased activated $CD4^+$ T cells after therapy remained. This discrepancy might, to some extent, be attributed to some important differences between these two studies. First, we phenotyped these cells in whole blood samples and not after cell isolation. Second, there were differences between the RA populations enrolled (type of anti-TNF therapy, concomitant treatment). They also showed that the $CD4^+CD25^{high+}$ subset has a functional defect that is restored in RA patients treated and responding to a TNF blocking agent.

The observed increase in peripheral blood T lymphocytes and $CD4^+$ and $CD8^+$ cells after anti-TNF therapy is inline with previous reports (13, 33, 34) and might reflect a diminished migration of T cells into the inflamed target tissues possibly due to a treatment-induced down-regulated expression of adhesion molecules by synovial endothelia (35).

In conclusion our data demonstrate that anti-TNF is a potent anti-inflammatory drug, as mirrored by the decrease in clinical and biological parameters of disease activity and the decrease in activated $CD4^+$ T cells. On the other hand we did not observe phenotypical changes in DCs and regulatory T cells and further functional studies are necessary.

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