

# Tumor Necrosis Factor- $\alpha$ Blockade Leads to Decreased Peripheral T Cell Reactivity and Increased Dendritic Cell Number in Peripheral Blood of Patients with Ankylosing Spondylitis

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**ABSTRACT. Objective.** To study the effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) antagonist (etanercept) treatment on the peripheral T cell reactivity of patients with ankylosing spondylitis (AS).

**Methods.** Peripheral blood mononuclear cells were collected from 40 patients with AS at baseline, after 2 and 6 weeks of etanercept treatment or placebo treatment, and from healthy controls. The number of cells secreting various cytokines was detected by enzyme linked immunospot. Serum soluble interleukin 2 (IL-2) receptor level was measured by ELISA. T cell proliferation was assayed with the WST-1 live cell-staining method. The myeloid dendritic cell (mDC) and regulatory T cell (Treg) levels were analyzed by fluorescence activated cell sorting.

**Results.** After 2 and 6 weeks of etanercept treatment, the number of TNF- $\alpha$ -secreting monocytes decreased. Although the T cell proliferation rate remained stable, the number of T cells secreting IL-2 and interferon- $\gamma$  under anti-CD3/anti-CD28 stimulation was significantly decreased. The level of serum soluble IL-2R (sIL-2R), a T cell activation marker, also declined. The changes in T cell reactivity were correlated with a significant increase in MHC Class II-positive mDC cells in circulation. An increase in Treg cell numbers was also observed.

**Conclusion.** The anti-TNF- $\alpha$  therapy blockaded MHC Class II-positive mDC maturation, enhanced regulatory T cell levels, and suppressed the functions of effector T cells. The reduced T cell reactivity could contribute to the efficacy of the TNF- $\alpha$  antagonist therapy in patients with AS. (First Release Nov 1 2008; J Rheumatol 2008;35:2220-8; doi:10.3899/jrheum.080219)

## Key Indexing Terms:

ANKYLOSING SPONDYLITIS                      ETANERCEPT                      TUMOR NECROSIS FACTOR- $\alpha$   
PERIPHERAL BLOOD MONONUCLEAR CELLS                      ENZYME LINKED IMMUNOSPOT

Ankylosing spondylitis (AS) is a chronic inflammatory joint disease that predominantly affects young adults. It can lead to stiffness and deformity of the vertebral column, with invalidating deformities. Until recently, treatment for AS was mainly based on nonsteroidal antiinflammatory drugs (NSAID) and regular physiotherapy<sup>1</sup>. However, these approaches fail to halt the progression of disease. Disease

modifying antirheumatic drugs, such as sulfasalazine, methotrexate, and leflunomide, appear less beneficial in AS than in other rheumatic diseases such as rheumatoid arthritis (RA)<sup>2,3</sup>. Therefore, focus has switched to anti-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) therapies for patients with AS in recent years.

It has been shown that TNF- $\alpha$  is one of the major regulators in the inflammation process<sup>4,5</sup>. Various TNF- $\alpha$  antagonists, such as infliximab (an anti-TNF- $\alpha$  antibody) and etanercept (a recombinant TNF- $\alpha$  receptor/Fc fusion protein) have been shown to be efficacious in a large proportion of patients with AS<sup>6-8</sup>. However, the underlying mechanism by which TNF- $\alpha$  antagonists improve the symptoms of patients with AS is still unclear. Given the clear linkage of HLA-B27 allele to AS, it has been hypothesized that antigen-specific T cell activation plays an important role in AS, and TNF- $\alpha$  blockade may suppress T cell reactivity<sup>9-11</sup>. In spite of reports by 2 independent groups, it remains controversial whether TNF- $\alpha$  antagonist treatment enhances or suppresses the function of T cells in patients with AS<sup>12-14</sup>.

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We investigated changes in T cell reactivity in terms of cell proliferation and cytokine secretion, as well as the levels of antigen-presenting cells and regulatory T cells after etanercept treatment. A possible linkage between TNF- $\alpha$  blockade and change in T cell reactivity was proposed.

## MATERIALS AND METHODS

**Patients and study design.** Our study was approved by the local independent ethics committee. Forty patients  $\geq 18$  years of age, who met the 1984 modified New York criteria for definite AS<sup>15</sup> and had evidence of active inflammatory spondylitis despite treatment [defined as a Bath AS Disease Activity Index (BASDAI) score of at least 4/10], were enrolled. All patients were HLA-B27-positive. Exclusion criteria included spondylitis other than AS and clinical or radiological evidence of complete spinal ankylosis.

In our placebo-controlled double-blind study, patients were randomly assigned to receive etanercept (Shanghai Celgen Bio-pharmaceutical Co., Ltd., China) or placebo treatment. In the etanercept group patients were treated with 50 mg etanercept subcutaneously once weekly for 6 weeks, while patients of the placebo group were treated with placebo for 6 weeks. Patients were allowed to take drugs previously prescribed for AS if doses had not been changed for  $\geq 4$  weeks before the study. Clinical evaluations were performed by 2 blinded investigators. Blood specimens were obtained from patients at baseline, 2 weeks, and 6 weeks after treatment. The healthy subjects were blood donors and staff from the Department of Rheumatology, Chinese PLA General Hospital.

**Evaluation of clinical response.** The primary efficacy endpoint was achievement of Assessment in Ankylosing Spondylitis (ASAS) 20% at Week 6; ASAS 50% and 70% response rates were also evaluated. We also used the mean improvements in several scores: the Bath AS Functional Index (BASFI), a 50% improvement or more of the initial BASDAI (BASDAI 50%), as well as improvement in the serum C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) during the 6 weeks of treatment.

**Preparation of peripheral blood mononuclear cells (PBMC).** Peripheral blood was collected in heparinized tubes and diluted 1:1 with phosphate buffered saline (PBS). Mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque and suspended in RPMI-1640 with 10% fetal calf serum (FCS).

**Elispot assays.** Ninety-six PVDF bottom-well plates (Millipore, Bedford, MA, USA) were coated with 100  $\mu$ l/well primary antibody (Diacclone, Stockholm, Sweden) overnight at 4°C;  $3 \times 10^5$  cells/well PBMC were added for interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin 2 (IL-2). For TNF- $\alpha$ , PBMC were seeded first in 35 mm dishes at 37°C for 3 h, the adhesive cells were collected and suspended in RPMI-1640, and then added to 96 PVDF bottom-well plates at  $2 \times 10^4$  cells/well and incubated overnight. Wells were washed with PBS and biotinylated detection antibody was added for 2 h at 37°C. Streptavidin-alkaline phosphatase was added at a dilution of 1:5000 for 1.5 h at 37°C and then BCIP/NBT was distributed in each well. Cytokine-secreting cells represented by individual spots on the plates were counted with the ImmunoSpot<sup>®</sup> system (CTL, Cleveland, OH, USA). IL-2 and IFN- $\gamma$  assays were performed simultaneously with and without 5  $\mu$ g/ml of anti-human CD3 and 1  $\mu$ g/ml of anti-human CD28 (Biosciences, San Diego, CA, USA). As the number of cells spontaneously secreting IL-2 and IFN- $\gamma$  was negligible, only stimulated cell count was presented for these 2 cytokines. All assays were performed in double wells. Percentage change of the number of cytokine-secreting cells = (number of cytokine-secreting cells at wk 2 or wk 6 with etanercept or placebo treatment minus number of cytokine-secreting cells before treatment)/number of cytokine-secreting cells before treatment  $\times 100\%$ .

**ELISA detection of serum soluble IL-2 receptor.** Blood samples were collected into vacutainer tubes (Becton-Dickinson) and centrifuged at 2500 rpm for 15 min. Sera were collected and frozen at -80°C. The frozen sera were thawed and their sIL-2R levels were assayed with the sIL-2R ELISA

kit (Bender MedSystems, Vienna, Austria). Optical density readings were performed at 450 nm with a Victor3 1420 multilabel counter (Perkin-Elmer, Wellesley, MA, USA).

**Proliferation assay.** CD4+ and CD8+ T cells were first enriched using RosetteSep<sup>®</sup> Human CD4+/CD8+ T cell enrichment cocktail (Stemcell Technologies, Vancouver, BC, Canada) from whole blood and suspended in RPMI-1640<sup>16</sup>. The cells were added into 96-well plates at a density of  $3 \times 10^5$  cells/well and the assays were performed simultaneously with and without 5  $\mu$ g/ml of anti-human CD3 and 1  $\mu$ g/ml anti-human CD28. Each sample was carried out in triplicate wells. The plates were incubated for 96 h and then WST-1 reagent (Roche, Mannheim, Germany) was added, 10  $\mu$ l/well, for 3.5 h at 37°C. The values were read with a Victor3 1420 multilabel counter at 450 nm. Cell proliferation rate and percentage change of the cell proliferation rate were calculated as follows: cell proliferation rate = (value of stimulated cells minus value of unstimulated cells)/value of unstimulated cells  $\times 100\%$ .

Percentage change of the cell proliferation rate = (cell proliferation rate at wk 2 or wk 6 with etanercept or placebo treatment minus cell proliferation rate before treatment)/cell proliferation rate before treatment  $\times 100\%$ .

**Flow cytometry.** The red blood cells in whole blood were lysed with FACS Lysing Solution (Becton-Dickinson, San Jose, CA, USA) and stained with anti-CD25 (PE), anti-CD4 (fluorescein isothiocyanate, FITC), and anti-CD3 (Percp), and analyzed with a FACScan cytometer for CD25<sup>high</sup> T cells. Cells were stained with anti-HLA-DR (Percp)/anti-HLA-abc (Percp), Lin1 (FITC), and CD11C (PE) and analyzed for dendritic cell subsets.

**Statistical analysis.** Data were analyzed using SPSS for Windows, release 13.0. ASAS20, ASAS50, ASAS70, and BASDAI were calculated by chi-squared test, and CRP, ESR, and BASFI were calculated by paired t test. All comparisons were 2-sided. A significance level of 5% was used;  $p < 0.05$  was considered significant.

Nonparametric methods were used for Elispot and FACS experimental statistics throughout the report. Differences between etanercept and placebo group at baseline were analyzed with the Mann-Whitney U-test, and analyses for matched pairs were performed with Wilcoxon's signed-rank test. Paired t test was used for ELISA and proliferation experimental statistics. Bonferroni adjustment was used to correct a value.

## RESULTS

**Clinical efficacy.** A total of 40 patients were enrolled in our study; 21 were assigned to receive etanercept and 19 were assigned to the placebo group. The average age of patients was  $25.5 \pm 6.8$  years. Most participants were male (M/F 39/1). The treatment groups had similar baseline disease scores and concomitant use of NSAID.

Significantly more etanercept patients than placebo patients [15 (71.4%) vs 1 (5.2%);  $p < 0.01$ ] were ASAS20 responders at Week 6, the primary efficacy endpoint. Significant improvements in the etanercept group were evident by Week 2, the earliest assessment point, and were sustained thereafter. There were also significantly more responders in the etanercept group at the ASAS50 level ( $p < 0.01$ ) and at the ASAS70 level at Weeks 4 and 6 ( $p < 0.05$ ).

After 6 weeks, a BASDAI 50% response was achieved by 10/21 (47.6%) patients in the etanercept group and 2/19 (10.5%) placebo patients ( $p = 0.0106$ ). Between baseline and Week 6, the BASFI scores improved significantly ( $p = 0.0201$ ) from  $35.33 \pm 23.66$  to  $19.10 \pm 19.62$  in the etanercept group. In addition, the acute-phase reactants CRP and ESR were also significantly decreased in etanercept treated

patients ( $p < 0.0001$ ) at Week 6, with changes of 75.8% and 72.6%, respectively.

*Frequency of TNF- $\alpha$ -secreting cells was decreased after etanercept treatment.* The number of TNF- $\alpha$ -producing cells was determined by Elispot. The number of TNF- $\alpha$ -secreting cells in AS patients at baseline was significantly higher than in healthy controls ( $p < 0.025$  was considered significant after Bonferroni adjustment). After 6 weeks of etanercept treatment, a clear reduction in the frequency of TNF- $\alpha$ -secreting cells was observed (not statistically significant;  $p < 0.017$  was considered significant after Bonferroni adjustment). In contrast, no obvious change was detected in the placebo group (Figure 1A). Figure 1B shows the correlation between change of TNF- $\alpha$ -producing cell number and BASDAI after 6 weeks of etanercept/placebo treatment. Although there was no significant correlation between change of cell numbers secreting TNF- $\alpha$  and BASDAI, most of the patients who showed reduced BASDAI scores also had reduced TNF- $\alpha$ -secreting cells after 6 weeks of etanercept treatment.

*Frequency of IL-2 and IFN- $\gamma$ -secreting T cells was decreased after etanercept treatment.* Without stimulation, the number of cells spontaneously secreting IL-2 or IFN- $\gamma$  was extremely low (about 1 in 100,000; data not shown). When PBMC were stimulated with anti-CD3 plus anti-CD28 antibodies, T cells were activated and cells started to secrete IL-2 or IFN- $\gamma$ . The number of IL-2 and IFN- $\gamma$ -producing cells after T cell stimulation was significantly higher in patients with AS compared to controls ( $p < 0.025$  was considered significant after Bonferroni adjustment). After 2 and 6 weeks of etanercept treatment, the frequencies of T cells that secreted either cytokine clearly decreased ( $p < 0.017$  was considered significant after Bonferroni adjustment). No change was observed in the placebo group (Figures 2A, 2C). Figures 2B and 2D show the correlation between changes of IL-2 or IFN- $\gamma$ -producing cell numbers and BASDAI after 6 weeks of etanercept/placebo treatment. There was no significant correlation; however, most of the patients who showed reduced BASDAI scores also had reduced TNF- $\alpha$ -secreting cells after 6 weeks of etanercept treatment, whereas this correlation was not observed in the placebo group.

*Serum soluble IL-2 receptor levels were decreased after etanercept treatment.* The soluble IL-2 receptor level in serum is a biomarker for T cell activation. sIL-2R levels in patients with AS were significantly higher than in controls ( $p < 0.025$  was considered significant after Bonferroni adjustment; Figure 3). After 2 weeks of etanercept treatment, sIL-2R levels clearly decreased ( $p < 0.017$  was considered significant after Bonferroni adjustment).

*No changes in T cell proliferation after etanercept treatment.* CD4+ or CD8+ T cells were isolated from whole blood cells and stimulated with anti-CD3/CD28 antibodies.

T cell proliferation was measured as the difference in the number of living cells between stimulated and nonstimulated cells. There was an increase in the proliferation rate of CD4+ T cells after 2 weeks of etanercept treatment, but the proliferation rate dropped back to baseline at Week 6 (Figure 4A). There was no significant change in the proliferation rate of CD8+ T cells (Figure 4B).

*MHC Class II-positive myeloid dendritic cells (mDC) were increased in circulation after etanercept treatment.* The percentage of MHC Class II-positive mDC (Lin-/CD11c+/HLA-DR+) in PBMC was measured by flow cytometry. Patients with AS had lower levels of MHC Class II mDC cells than controls (Figure 5;  $p < 0.025$  was considered significant after Bonferroni adjustment). A significant increase was observed after 6 weeks of etanercept treatment ( $p < 0.017$  was considered significant after Bonferroni adjustment). Meanwhile, the level of MHC Class I-positive mDC (Lin-/CD11c+/HLA-ABC+) in patients with AS was slightly lower than in controls (not statistically significant) as measured by flow cytometry. No significant change in MHC Class I mDC level was observed after drug treatment (data not shown).

*Regulatory T cell (Treg) level was increased after etanercept treatment.* CD4+CD25<sup>high</sup> Treg in patients at baseline were fewer than in controls (Figure 6), but the difference was not statistically significant ( $p < 0.025$  was considered significant after Bonferroni adjustment). After 6 weeks of etanercept treatment, there was an increase in Treg levels compared with Week 0, which was also statistically insignificant ( $p < 0.017$  was considered significant after Bonferroni adjustment).

## DISCUSSION

We showed that Chinese patients with active AS had similar efficacy profile to 6 weeks of etanercept treatment compared to the other reports. Further, we studied changes caused by etanercept treatment at the molecular and cellular levels.

Using the Elispot assay, we first demonstrated that TNF- $\alpha$  secretion by peripheral monocytes was reduced after etanercept treatment. Since TNF- $\alpha$  blockade kept monocytes at a less activated stage, it was not surprising that the number of monocytes spontaneously secreting proinflammatory cytokines was reduced. In Elispot, the cytokine is captured directly around the secreting cell, before it is diluted in the supernatant. Not only is this assay much more sensitive than conventional ELISA measurements, but it also allows us to directly count how many cells actually secrete the cytokine.

We further demonstrated a decrease in the numbers of IL-2 and IFN- $\gamma$ -secreting T cells under *in vitro* stimulation conditions. This decrease was only observed in T cells from the treatment group but not in those from the placebo group. T cell cytokine secretion has been studied by 2 independent groups in patients with AS treated with infliximab, an anti-TNF- $\alpha$  antibody. One group showed that infliximab down-

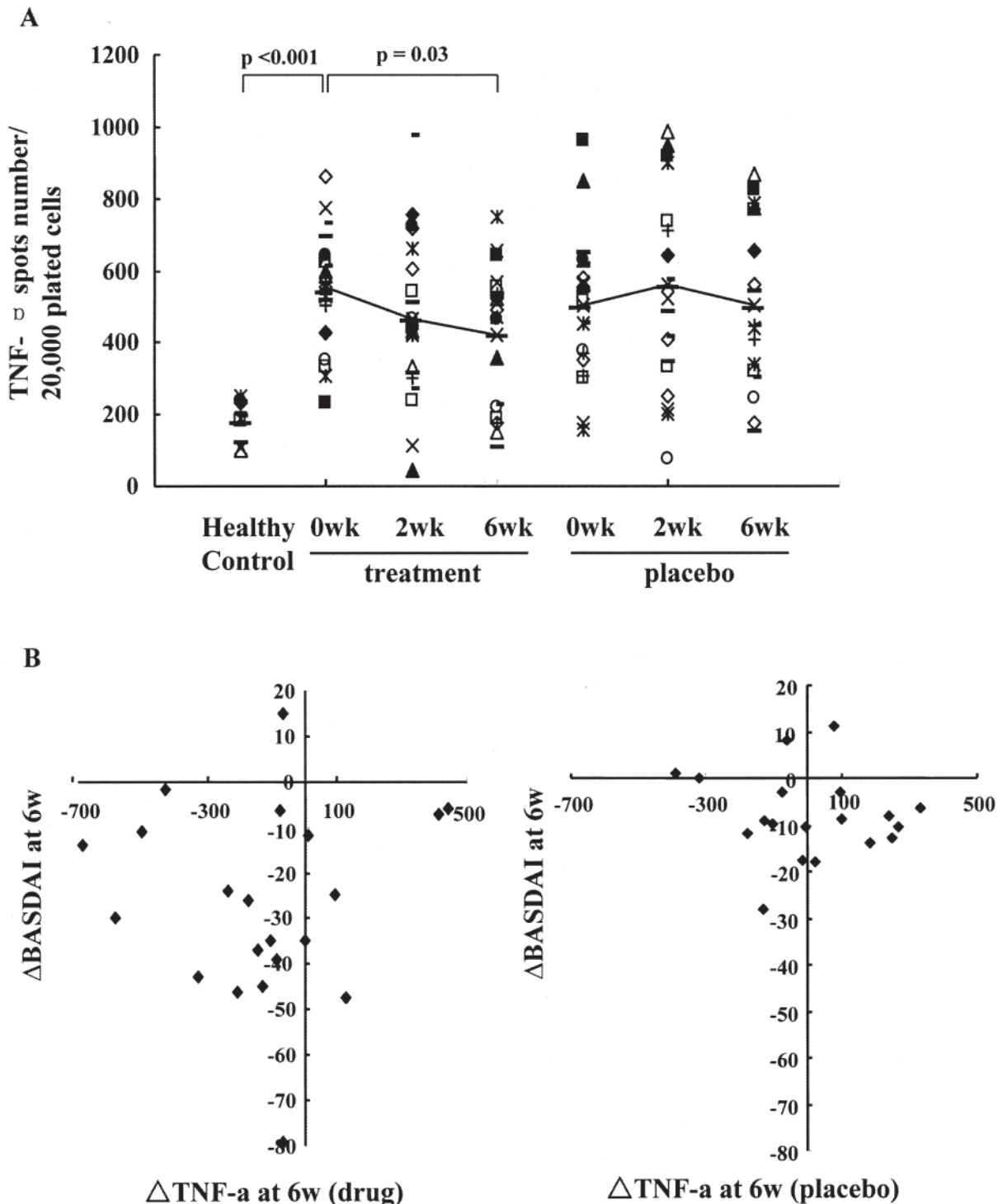


Figure 1. Number of TNF- $\alpha$ -secreting monocytes was decreased after etanercept treatment. A. Number of TNF- $\alpha$ -secreting cells compared between controls and patients with AS at baseline (Mann-Whitney U-test;  $p < 0.025$  considered significant after Bonferroni adjustment). After 2 and 6 weeks of etanercept treatment, number of TNF- $\alpha$ -secreting cells in treatment and placebo groups was compared (Wilcoxon's signed-rank test;  $p < 0.017$  considered significant after Bonferroni adjustment). B. Correlation between change of TNF- $\alpha$ -secreting cells and BASDAI after 6 weeks' etanercept/placebo treatment.

regulates the number of both IFN- $\gamma$  and TNF- $\alpha$ -producing T cells<sup>12</sup>, and another group reported that infliximab induced a significant and persistent increase in the level of IFN- $\gamma$  and

IL-2-producing T cells in patients with spondyloarthropathy<sup>14</sup>. The first group also reported that etanercept treatment upregulated the level of TNF- $\alpha$  and IFN- $\gamma$ -producing T cells

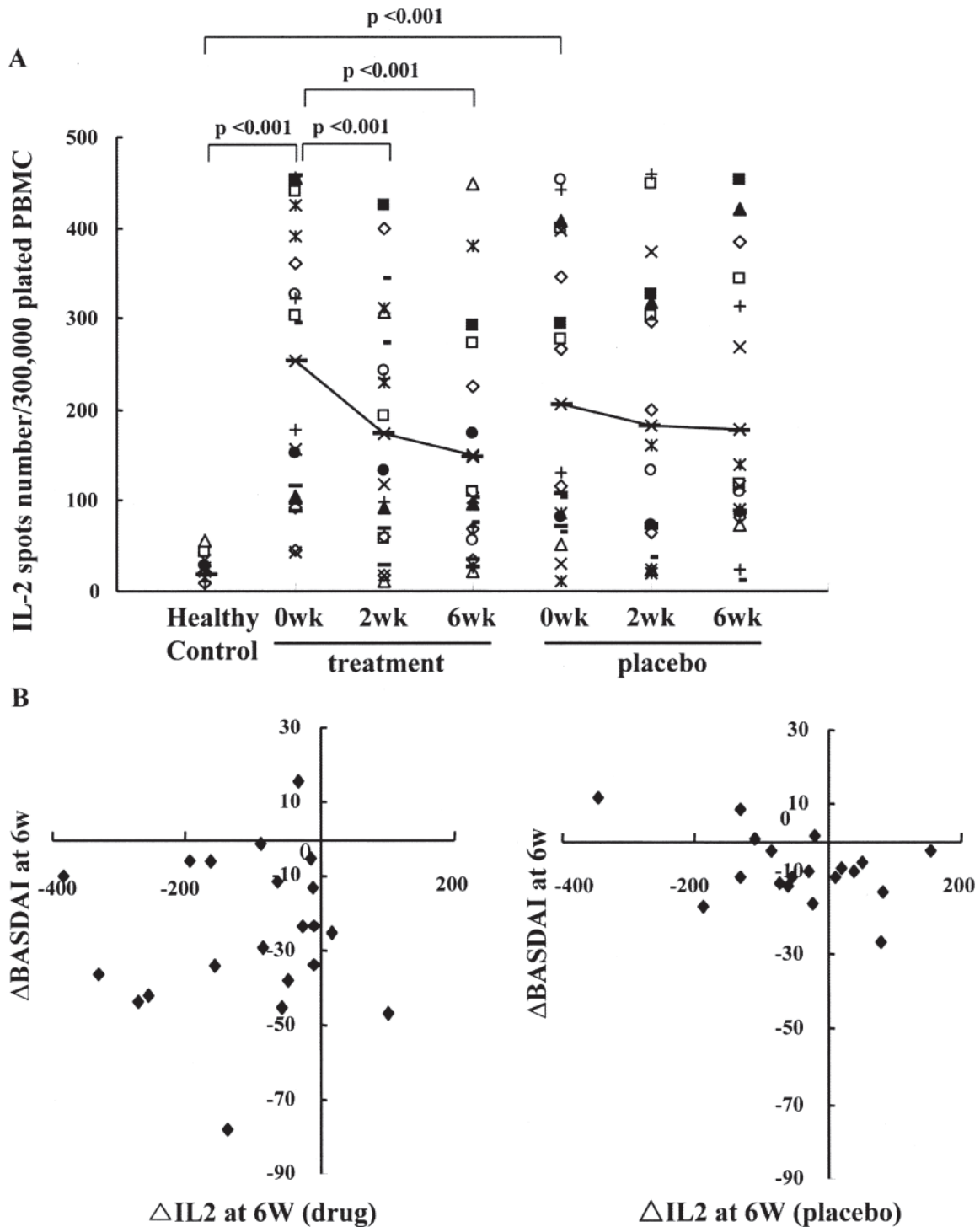


Figure 2. Number of T cells that secrete IL-2 and IFN- $\gamma$  in response to T cell receptor activation. A. Number of cells secreting IL-2 in response to anti-CD3/CD28 antibody stimulation compared at baseline between controls and patients with AS (Mann-Whitney U-test;  $p < 0.025$  considered significant after Bonferroni adjustment). After 2 or 6 weeks of treatment, number of IL-secreting cells under stimulation was compared with baseline in etanercept and placebo groups, respectively (Wilcoxon's signed-rank test;  $p < 0.017$  considered significant after Bonferroni adjustment). Lines across the graphs represent mean values. B. Correlation between change of IL-2-secreting cells and BASDAI after 6 weeks' etanercept/placebo treatment.

in patients with AS<sup>13</sup>. We noted that both groups were using intracytoplasmic cytokine staining followed by flow cytometry as the main method to assay T cell cytokine production.

Due to the sensitivity limitation of flow cytometry, phorbol myristate acetate (PMA)/ionomycin stimulation was used to achieve the maximum T cell activation. PMA is a strong

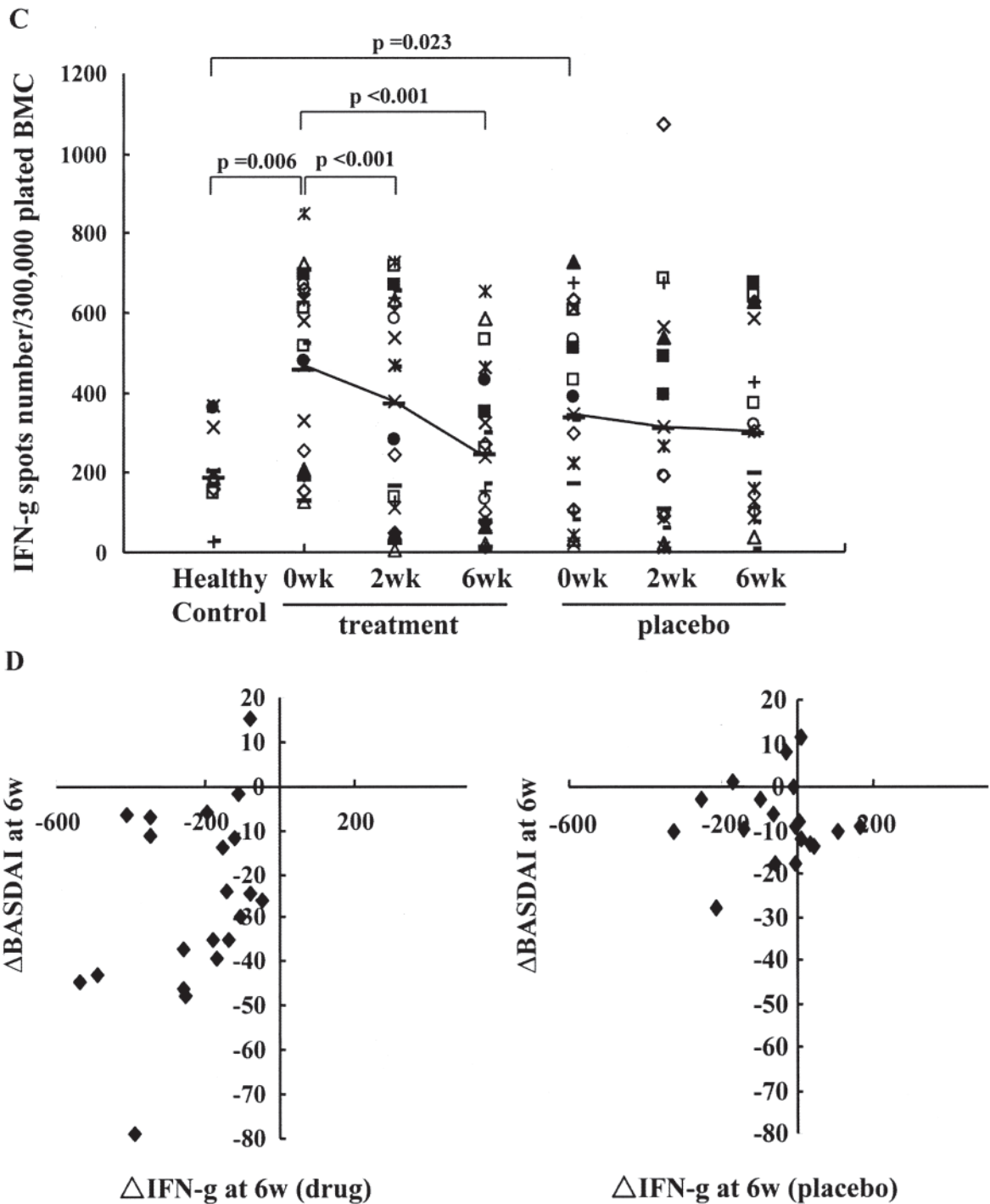


Figure 2. C. Numbers of cells secreting IFN- $\gamma$  in response to anti-CD3/CD28 antibody stimulation were compared at baseline between controls and patients with AS. After 2 or 6 weeks of treatment, number of IFN- $\gamma$ -secreting cells under stimulation was compared with baseline in etanercept and placebo groups, respectively. Statistical analysis was the same as that for IL-2. Lines across the graphs represent mean values. D. Correlation between change of IFN- $\gamma$ -secreting cells and BASDAI after 6 weeks etanercept/placebo treatment.

protein kinase C activator and ionomycin is known to carry calcium into cells<sup>17</sup>. Since those 2 reagents bypassed the T cell receptor and its early signaling events, activating T cells this way may not reflect the T cell reactivity under physiological conditions. The Elispot assays we used are orders of

magnitude more sensitive than flow cytometry, and allowed us to use anti-CD3/CD28 antibody, a more physiologically relevant method to activate T cells<sup>18,19</sup>.

Consistent with the above results, we also demonstrated that T cell activation was elevated in patients with AS before

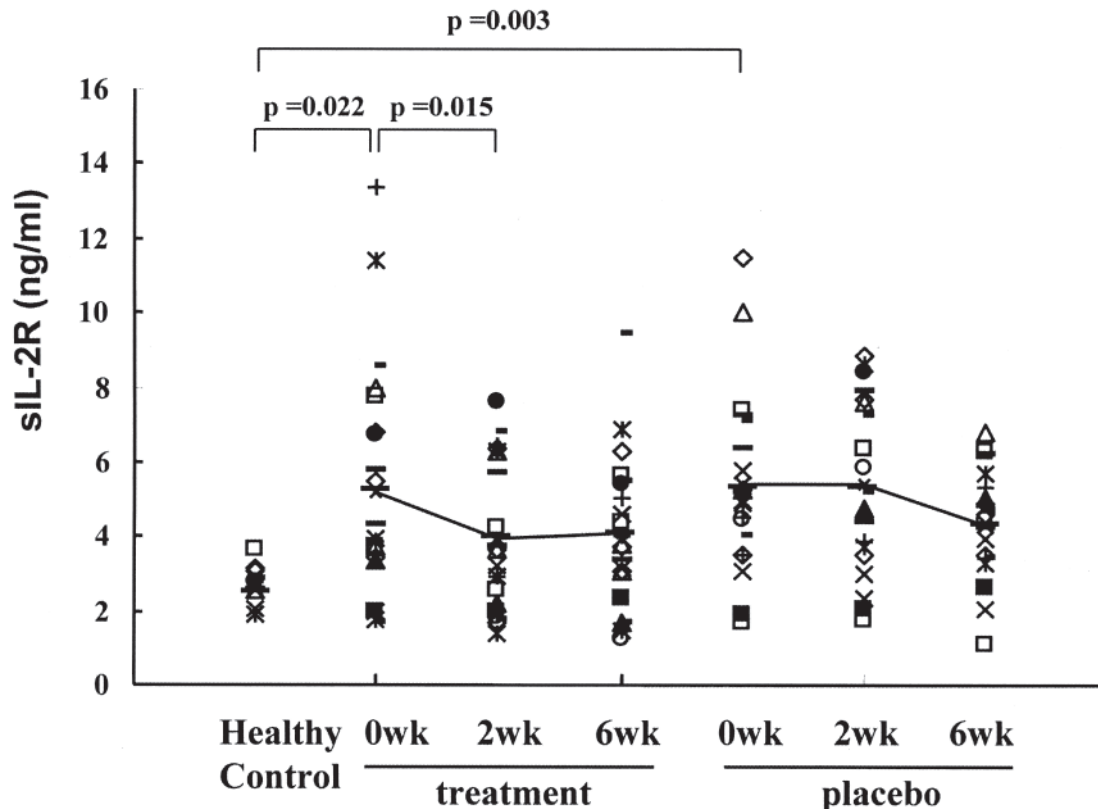


Figure 3. sIL-2R in sera were decreased after etanercept treatment, as measured by ELISA. sIL-2R level in patients with AS was significantly higher than in controls (Mann-Whitney U-test;  $p < 0.025$  considered significant after Bonferroni adjustment). sIL-2R level decreased after 2 or 6 weeks of etanercept treatment, and sIL-2R in placebo group also declined after 6 weeks (Wilcoxon's signed-rank test;  $p < 0.017$  considered significant after Bonferroni adjustment).

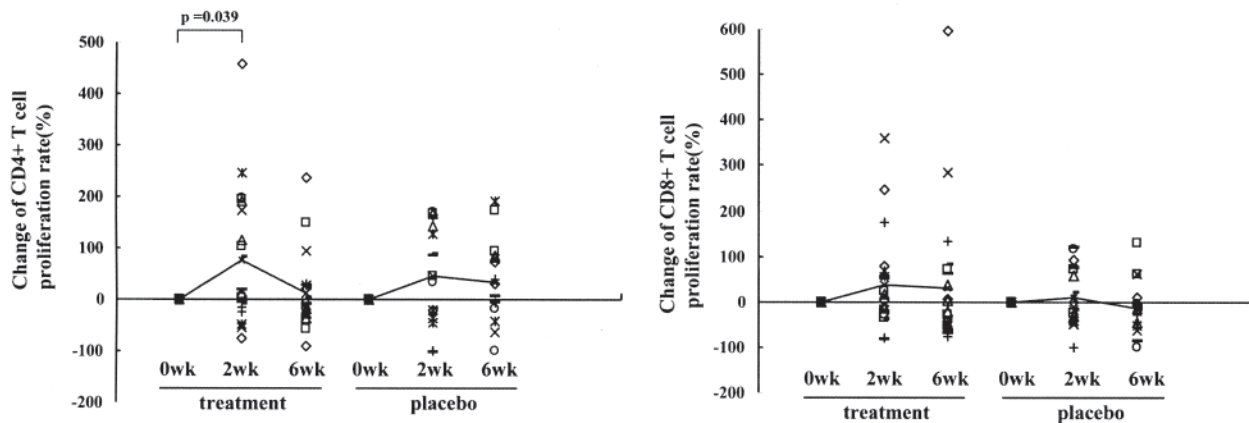


Figure 4. Etanercept treatment did not lead to a reduction in CD4+ or CD8+ T cell proliferation rate. CD4+ or CD8+ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies, and living cell numbers were measured by WST-1 assay. Cell proliferation rate was calculated by comparing cell number difference between stimulated and nonstimulated cells. Data represent percentage of changes between the proliferation rate at Week 2 (or 6) and Week 0. A. CD4+ T cell proliferation rate. B. CD8+ T cell proliferation rate. Lines across the graphs represent mean values.

treatment and reduced after etanercept treatment by measuring serum sIL-2R level as a T cell activation biomarker.

Knowing that T cell reactivity was reduced after TNF- $\alpha$  blockade, we tried to find a link between TNF- $\alpha$  function and T cell reactivity. One obvious approach is to assess dendritic cell (DC) levels before and after etanercept treatment.

DC are "professional" antigen-presenting cells. Because of their capacity to stimulate naive T cells, DC play a central role in the initiation of primary immune responses<sup>20</sup>. Upon exposure to antigens, the peripheral DC upregulate their costimulatory molecules, migrate to draining lymph nodes, and interact with T cells to stimulate or tolerate them. DC

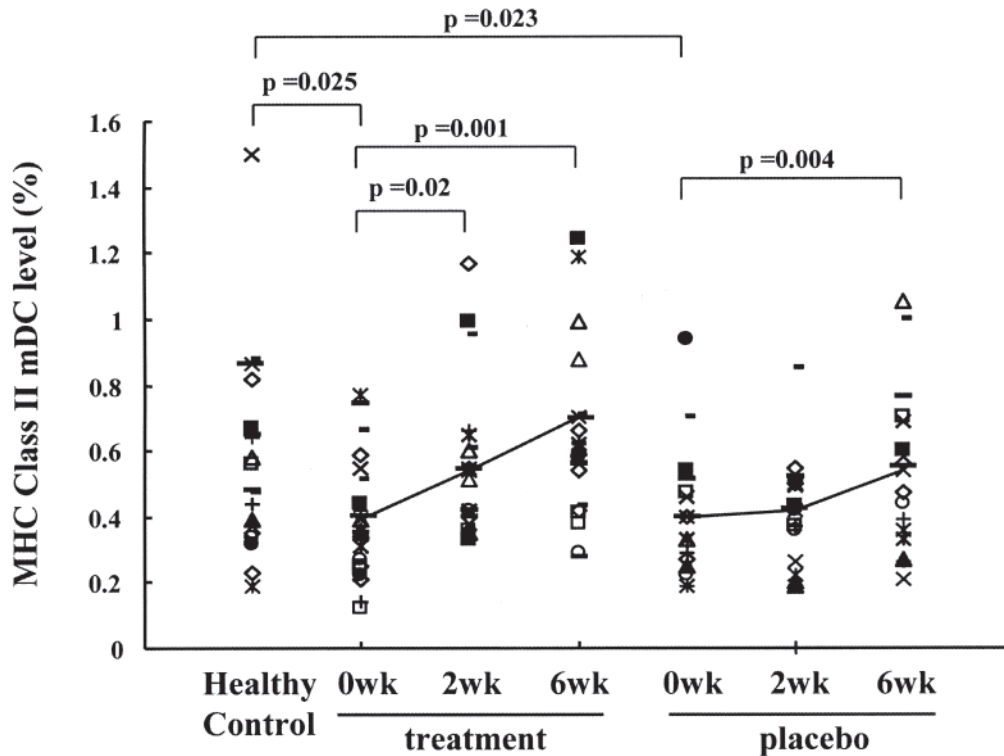


Figure 5. Circulating MHC class II-positive myeloid dendritic cell (mDC) level was increased after etanercept treatment. Three-color labeling and flow cytometry assay were used to determine mDC levels in PBMC. Levels of MHC class II-positive (Lin-/CD11c+/HLA-DR+) from patients with AS and controls were compared (Mann-Whitney U-test;  $p < 0.025$  considered significant after Bonferroni adjustment). MHC class II mDC level changes after 2 or 6 weeks of treatment are shown (Wilcoxon's signed-rank test;  $p < 0.017$  considered significant after Bonferroni adjustment). Lines across the graphs represent mean values.

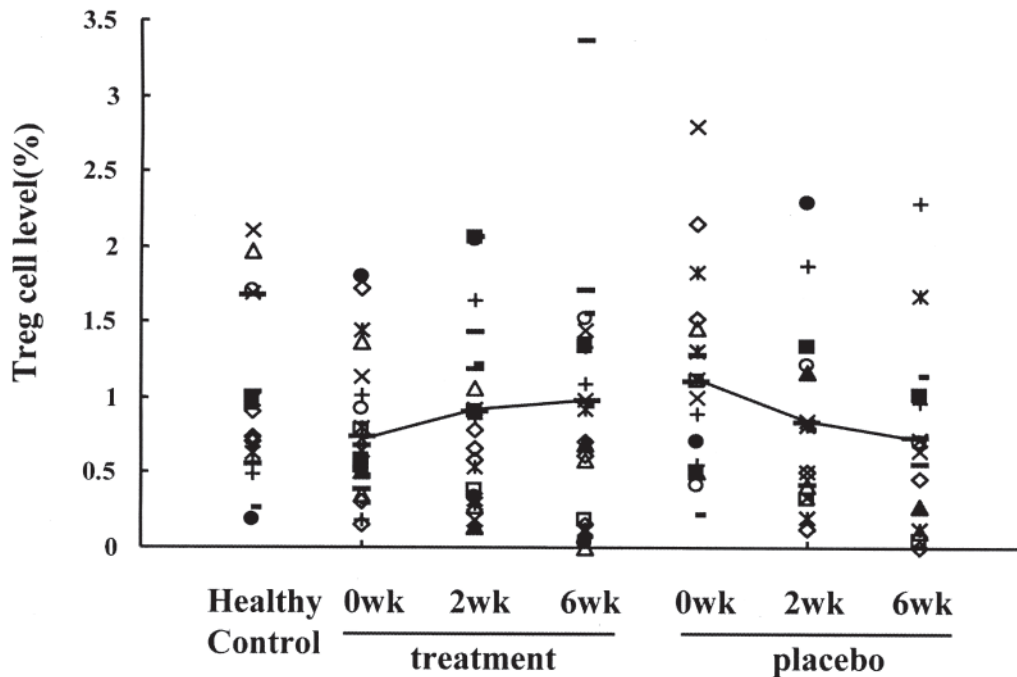


Figure 6. The CD4+CD25<sup>high</sup> regulatory T cell (Treg) level in PBMC was increased after etanercept treatment, as analyzed by FACS. Treg level in patients with AS at baseline was compared with that in controls (Mann-Whitney U-test;  $p < 0.025$  considered significant after Bonferroni adjustment). Treg level changes after treatment are shown (Wilcoxon's signed-rank test;  $p < 0.017$  considered significant after Bonferroni adjustment). Lines across the graphs represent mean values.



have been found in synovium and joint fluid in RA, often at the center of a cluster of T cells<sup>21</sup>. We observed that patients with AS have fewer MHC class II-bearing myeloid DC (mDC-DR) in circulation compared to healthy controls. After etanercept treatment, the circulating mDC-DR level was significantly increased. According to previous reports, those circulating DC are immature DC that do not express activation markers such as CD83<sup>22</sup>. The increase in circulating mDC level suggested a blockade of mDC maturation after neutralizing TNF- $\alpha$  activity. The blockade of mDC maturation can at least partially explain the reduction of T cell reactivity.

Although not statistically significant, we observed that patients with AS had lower CD4+CD25<sup>high</sup> Treg levels compared with healthy controls, and this reduction was reversed by etanercept treatment. This observation is in agreement with studies in patients with RA, showing that infliximab treatment increased Treg counts of patients compared to those of normal controls, and reduced spontaneous apoptosis of CD4+CD25+ T cells<sup>23</sup>. Treg are instrumental in the maintenance of peripheral immune tolerance and in the control of adaptive immune responses. The interplay between Treg and antigen-responsive T cells is modulated by DC, and immature mDC can support Treg development<sup>24</sup>. Thus, an alternative explanation to the reduction of T cell reactivity is through the enhanced activity of Treg. Further work is under way to test this hypothesis.

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