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Anti-TNF therapy in patients with rheumatoid arthritis decreases Th1 and Th17 cell populations and expands IFN-γ-producing NK cell and regulatory T cell subsets

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ABSTRACT

The aim of this work was to study the effect of anti-TNF treatment on CD4+ Th1, Th17 and regulatory T cells (Tregs), together with CD8+ T cells and NK cells from rheumatoid arthritis (RA) patients. For this purpose, 18 RA patients received adalimumab during 16 weeks and their peripheral blood lymphocytes were assessed by flow cytometry at the beginning and at the end of the study. We found that the proportion of Th17 cells was directly correlated with Th1 cells, but inversely correlated with IFN-γ-producing NK cells. A decrease was observed in Th1, Th17 cells and IFN-γ-producing CD8+ T cells by anti-TNF therapy. Conversely, the proportion of Tregs increased, as did the percentage of IFN-γ-producing NK cells. We postulate that a rise in IFN-γ production due to recovery of NK cells' function, together with expanded Tregs, contribute to decrease the Th17 response in anti-TNF-treated RA patients.

Introduction

Historically, rheumatoid arthritis (RA) was considered a Th1 disease, which implies that the immunological process is dominated by IFN-γ-secreting CD4+ T cells (Schulze-Koops and Kalden 2001). However, in the recent years this dogma has been challenged by findings describing that Th17, a T helper sub-population characterized by the secretion of IL-17 and dependent on IL-23 for its expansion, was responsible for collagen-induced arthritis (CIA) in mice (Murphy et al. 2003). Nevertheless, the participation of Th1 cells in RA pathogenesis cannot be ruled out, especially since studies in an animal model of arthritis different from CIA have demonstrated that IFN-γ, but not IL-17, is necessary for disease development (Doodes et al. 2008). In addition, Th1 cells have been found to be the predominant T cell subset in RA patients' joints (Yamada et al. 2008). Human Th17 cells have been generated *in vitro* upon activation of naïve T cells in the presence of cytokine combinations that include IL-23, IL-1β, IL-6, or IL-21, while IFN-γ induced IL-12 secretion acts repressing this differentiation (Hoeve et al. 2006; Romagnani 2008). Moreover, in CIA mice it has been demonstrated that NK cells suppress Th17 cells development by the secretion of IFN-γ (Lo et al. 2008).

On the other hand, a new focus has been posed on the balance between regulatory T cells (Tregs) and Th17 cells, guided by evidence in mice indicating that both sub-populations may share, to some extent, a common origin (Bettelli et al. 2006; Xu et al. 2007). It is thought that if this critical balance is deviated in favor of Th17 cells and against Tregs, the severity of autoimmune diseases could be significantly enhanced (Bettelli et al. 2006).

Most of the beneficial actions of anti-TNF drugs on RA patients have been attributed to their ability to antagonize the effects of TNF at late steps of the inflammatory cascade (Feldmann and Maini 2001). However, only a few studies have been carried out to elucidate how the blockade of TNF could affect earlier stages of the autoimmune process, such as T cell activation and differentiation

Abbreviations: ACR, American College of Rheumatology; CIA, Collagen-induced arthritis; PBMCs, Peripheral blood mononuclear cells; PMA, Phorbol 12-myristate 13-acetate; RA, Rheumatoid arthritis; SD, Standard deviation; TCR, T cell receptor; Tregs, Regulatory T cells.

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into different effector sub-populations, especially after the understanding of Th17 cells as relevant players in the disease. In this regard, it was demonstrated that CIA mice receiving TNF blocking therapy ameliorated arthritis without suppressing Th17 differentiation (Fujimoto et al. 2008), although this data must be validated in RA patients. Furthermore, there is a lack of reports referring to the effects of anti-TNF therapy on the function and cytokine secretion capacity of other relevant lymphoid populations, such as NK cells.

The aim of this study was to evaluate the effect of a fully human anti-TNF antibody, adalimumab, on RA patients' Th1, Th17, Tregs, CD8+ T cells and CD56+ NK cells, together with serum cytokines related to these cell lineages. We also assessed whether the percentages of these sub-populations correlate with clinical improvement. We found that TNF blockade differentially affects the studied cell populations, reducing pathogenic Th1 and Th17 cells, but increasing Tregs and IFN-γ production by CD56+ cells. These results suggest that a recovery of IFN-γ secretion ability by NK cells, together with an expansion of Tregs, could contribute to the decrease of Th17 cells observed in RA patients under anti-TNF therapy.

Materials and methods

Patients

We recruited eighteen female patients meeting the American College of Rheumatology (ACR) criteria for RA, who exhibited an active disease defined as ≥6 swollen joints, ≥9 tender joints and morning stiffness greater than 1 h, regardless of being under treatment with disease-modifying antirheumatic drugs. At study entry, patients present a mean ± standard deviation (SD) age of 50.8 ± 11.5 years and disease duration of 16.4 ± 7.5 years. All patients received 40 mg of adalimumab (kindly provided by Abbott Laboratories, Chicago, USA) subcutaneously, every other week, during 16 weeks. The ACR20 criteria were used to define response to treatment. Blood samples for cellular and serum flow cytometry analyses were drawn at study entry and 4 months after beginning adalimumab administration. The study was approved by the Ethical Committee of the Hospital Clínico Universidad de Chile, and all patients gave their written consent according to the Declaration of Helsinki.

Cell staining and flow cytometry

For IFN-γ and IL-17-producing CD4+ and IFN-γ-producing CD8+ T cells identification, fresh anticoagulated blood was mixed in a 1:1 ratio with RPMI 1640 medium (HyClone, Thermo, Utah, USA) and cultured with 15 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, St. Louis, USA), 1 μg/ml ionomycin (Sigma–Aldrich) and 3 μg/ml brefeldin A (eBioscience, San Diego, USA) for 5 h at 37 °C and 5% CO_2. Afterwards, cells were washed with 1% fetal bovine serum (HyClone) in phosphate buffered saline and incubated with anti-CD3 FITC and anti-CD8 PE-Cy5 antibodies (BD Biosciences, San Jose, USA) for 30 min at 4 °C, in darkness. Subsequently, red blood cells were lysed with ammonium chloride–potassium buffer and the remaining cells were washed and permeabilized with the Fixation and Permeabilization kit (eBioscience), incubated with anti-IFN-γ PE (BD Biosciences) or IL-17 PE (eBioscience) antibodies for 30 min at 4 °C in darkness, washed and fixed (IC Fixation Buffer, eBioscience) for flow cytometry. For NK cells and Tregs evaluation, peripheral blood mononuclear cells (PBMCs) were obtained from blood samples by density gradient with Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use. Thawed cells were allowed to recover in an overnight culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. To stimulate NK cells to express IFN-γ, PBMCs were seeded at 1×10^6 cells per ml in 24-well culture plates and incubated with PMA, ionomycin and brefeldin A as described above. After 5 h of culture, cells were harvested and stained for surface markers with anti-CD3 APC and anti-CD56 FITC antibodies (eBioscience), permeabilized and incubated with an anti-IFN-γ PE antibody (BD Biosciences). For Tregs staining, PBMCs were incubated with anti-CD4 FITC and anti-CD25 PE-Cy7 antibodies (eBioscience), permeabilized with the Foxp3 Fixation/Permeabilization Buffer Set (eBioscience) and further incubated with an anti-Foxp3 PE antibody (eBioscience) or a rat IgG2a PE antibody (eBioscience) as isotype control.

Data was acquired in a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the WinMDI 2.9 software. The lymphoid population was defined according to forward and side scatter patterns. A region was set in CD3+ CD8− cells to define the CD4+ T cell population, in CD3+ CD8+ cells to define the CD8+ T cell population and in CD3− CD56+ cells to define the NK cell population. Tregs were defined as CD4+ CD25high Foxp3+.

Serum cytokines measurement

FlowCytomix kits (Bender MedSystems, Vienna, Austria) were used to measure serum cytokines levels, according to manufacturer's instructions. Data was acquired in a FACSCalibur flow cytometer.

Statistical analyses

To compare between the percentages of cell sub-populations at baseline and after adalimumab therapy, the two-tailed Wilcoxon signed rank test was used. Differences between responder and non responder groups were analyzed using the two-tailed Mann–Whitney test. Correlations were evaluated with the two-tailed Spearman's correlation test. p values < 0.05 were considered significant. For statistical analyses and graphics, the Prism 5 software (GraphPad, La Jolla, USA) was used.

Results

Adalimumab decreases Th1 and Th17 sub-populations

First, we wanted to evaluate whether a 4-month therapy with adalimumab has the ability to influence Th1 and Th17 cells, the main effector CD4+ T cells involved in RA pathogenesis. For this purpose, peripheral blood samples from RA patients were collected at baseline and after 4 months of therapy and stimulated with PMA and ionomycin. The percentages of IFN-γ or IL-17-producing CD4+ T cells were then assessed by flow cytometry (Fig. 1A). A considerable decrease in the percentages of IFN-γ-producing CD4+ T cells was observed after 4 months of anti-TNF treatment (mean % ± SD of 17.4 ± 6.2 before therapy versus 5.8 ± 4.5 after therapy; $p = 0.0002$) (Fig. 1B). When RA patients were divided into responders and non responders according to the achievement of ACR20 criteria (11/18 and 7/18, respectively), the decrease of IFN-γ-producing CD4+ T cells was still significant for both groups ($p = 0.001$ and $p = 0.0156$, respectively) (Fig. 1B).

On the other hand, the percentages of IL-17-producing CD4+ T cells also showed a significant, but less-marked decrease after anti-TNF therapy (mean % ± SD of 1.61 ± 0.93 before therapy versus 0.97 ± 0.70 after therapy; $p = 0.0294$) (Fig. 1C). Unexpectedly, when we evaluated the responder and non responder groups independently, only the latter showed a significant reduction in this T cell sub-population ($p = 0.0156$) (Fig. 1C). No changes were detected in the percentages of CD4+ T cells among the whole lymphoid population, or in the whole leukocyte counts throughout the treatment (data not shown).