Relative increase of Th17 phenotype in senescent CD4+CD28^{null} T cells from peripheral blood of patients with rheumatoid arthritis

Sirs,

As people age, the immune system loses protective abilities while assuming inflammatory functions, enabling the development of chronic inflammation and immune-mediated tissue damage (1). A hallmark of immunosenescence is the accumulation of CD4$^+$ cells that lack expression of the CD28 molecule (2). Recently, Wahlin and colleagues described a high number of CD4$^+$CD28^{null} cells in patients with rheumatoid arthritis (RA), in association with elevated levels of IgG against cytomegalovirus (CMV) and an increased burden of atherosclerosis (3). Although this study is enlightening, since it supports that chronic inflammation is probably responsible for the excess of cardiovascular mortality observed in RA patients, it does not evaluate the T-helper phenotype displayed by these senescent cells (4). Acknowledging that helper phenotypes are key to the effector functions of T cells, we investigated the proportion of Th1, Th2 and Th17 phenotypes by evaluating the transcription factor that is committed to each lymphocyte phenotype (T-bet, GATA-3, and ROR-γt, respectively) in CD4$^+$CD28^{null} cells from RA patients. Thirty-nine consecutive patients with RA meeting the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria, naïve to biologic disease-modifying antirheumatic drugs, were recruited (5). Five healthy blood donors were included as controls. Disease activity was assessed by the DAS-28-CRP score. As a surrogate for atherosclerotic burden, the person's risk of developing myocardial infarction or stroke over the next 10 years was calculated with the QRISK3 web calculator (6). This study was approved by the local ethics committee (no. 13-816) and performed according to the Declaration of Helsinki. All individuals consented to participate.

From all participants, 6 mL fasting venous blood were collected in EDTA-treated tubes, and peripheral blood mononuclear cells (PBMC) were separated by ficoll density gradient. Colour flow-cytometry was performed in a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) using monoclonal antibodies against CD4-PercP and CD28-PE or CD28-FITC (Biolegend, San Diego, CA, USA) to define the CD4$^+$CD28^{null} subpopulation. After that, cells were fixed, permeated, and incubated with antibodies against T-bet-FITC (Biolegend), GATA3-PE, and RORγt-APC (eBioscience, San Diego, CA, USA). Gating strategy has already been described (2).

Table I. Main clinical and laboratory data of participants.

	Rheumatoid arthritis (n=39)	Healthy controls (n=5)	*p*-value
Age, years	43 ± 10	34 ± 6	0.138
Female, n (%)	36 (92)	3 (60)	0.090
Disease duration, years	5 ± 7	-	-
Diabetes, n (%)	6 (15)	0	1.000
Hypertension, n (%)	9 (23)	0	0.566
Sjögren's syndrome, n (%)	6 (15)	0	1.000
RF +, n (%)	34 (87)	-	-
Anti-CCP +, n (%)	33 (84)	-	-
CRP, mg/L	15 ± 31	-	-
DAS28-CRP score	3.2 ± 2.0	-	-
Glucocorticoid use, n (%)	10 (25)	-	-
Synthetic DMARDs, n (%)	39 (100)	-	-
Total CD4$^+$/10,000 PBMC	4,929 ± 1,120	6,120 ± 1,721	0.080
% CD4$^+$ lacking CD28 expression	2.2 ± 1.7	0.8 ± 0.8	**0.040**
% T-bet +	37.3 ± 25.9	58.1 ± 34.4	
% RORγt +	53.3 ± 26.8	39.5 ± 36.5	**0.007**
% GATA-3 +	9.4 ± 10.3	2.4 ± 3.7	

Data are presented as mean ± standard deviation unless otherwise specified. Significant *p*-values are in bold.
RA: rheumatoid arthritis; RF: rheumatoid factor; Anti-CCP: anti-cyclic citrullinated protein antibodies; CRP: C-reactive protein; DAS28-CRP: Disease Activity Score-28 with CRP; DMARD: disease-modifying anti-rheumatic drug; PBMC: peripheral blood mononuclear cells.
Synthetic DMARDs encompass the use of methotrexate, leflunomide, sulfasalazine, and hydroxychloroquine, either alone or in combination.

Table I summarises the main clinical characteristics of participants. Despite the fact that patients were young, 15% already had diabetes and 23% had hypertension, so the relative risk of suffering a major cardiovascular event (QRISK3 score) was increased up to 2.4±3.33. The total number of CD4+/10,000 PBMC was similar between patients and controls (4,929±1,120 *vs*. 6,120±1,721; p=0.080); however, the percentage of CD4$^+$CD28^{null}/CD4$^+$ cells was significantly higher in patients (2.2±1.7% *vs*. 0.8±0.8%; p=0.040). When evaluating the proportion of CD4$^+$CD28^{null} cells that express one or other of the transcription factors, a significantly higher percentage of cells that express RORγt (53.3% *vs*. 39.5%) and GATA-3 (9.4% *vs*. 2.4%), but lower of the cells expressing T-bet (37.3% *vs*. 58.1%) were found in patients as compared to healthy controls, respectively. Among CD4$^+$CD28^{null} cells, a RORγt/T-bet ratio ≥1 was found in 56% of patients, but only in 20% of control subjects, although a RORγt/T-bet ratio ≥1 was unable to differentiate disease activity (DAS28-CRP, 3.1±1.5 *vs*. 3.3±1.4; p=0.880) or the frequency of anti-cyclic citrullinated peptide antibodies (84% *vs*. 78%; p=0.681) and rheumatoid factor (89% *vs*. 85%; p=1.0).

Additional studies showed no differences between T-helper phenotype distribution and comorbidities, disease duration, body mass index, anti-cyclic citrullinated peptide antibodies, rheumatoid factor or C-reactive protein levels (data not shown). There was also no correlation between disease activity and CD4$^+$CD28^{null} cells (Spearman's rho -0.003) or the percentage of cells expressing RORγt (rho 0.03), GATA-3 (rho 0.07) and T-bet (rho 0.03). Similarly, no correlation was found between the QRISK3 score and CD4$^+$CD28^{null} cells (rho -0.13), RORγt (rho 0.07), GATA-3 (rho 0.01) or T-bet (rho -0.08) expressing cells.

The novelty of this study is the characterisation of senescent T cells in RA as preferentially polarised towards a Th17 phenotype, rather than a Th1 phenotype. This is in line with the increased number of circulating Th17 cells observed in RA, which in turn causes a decrease in the function of regulatory T cells (7). Indeed, Th17 cells induce inflammation by secreting IL-17, which activate fibroblast-like synoviocytes and promote osteoclast maturation and function. IL-17 may also recruit and activate neutrophils, macrophages, and B cells, increasing inflammation in RA (8). In contrast, regulatory T cells attenuate inflammation by secreting inhibitory cytokines to suppress the function of effector T cells, mainly Th17 cells (8). Increases in certain subpopulations of CD4$^+$CD28^{null} cells may also indicate which stimuli trigger T cells to become Th17 effector cells in epithelial tissues or elsewhere. In addition to the aforementioned association with CMV infection, the expansion of senescent T cells could also be driven by chronic exposure to bacterial and fungal antigens, including those of the microbiota. In this sense, there is a consistent association between the relative abundance of *Porphyromonas gingivalis*, a bacterium belonging to the gastrointestinal microbiota, with excessive citrullination of peptides (through a pathway that uses peptidyl arginine deiminase) and the induction or exacerbation of arthritis (9). Furthermore, transcriptomes of rheumatoid synovial tissue show gene-patterns similar to those induced by bacterial and fungal triggers (10).

In conclusion, having highly active lymphocytes including senescent CD4$^+$CD28^{null}

cells polarised towards a Th17 effector phenotype may facilitate an imbalance in pro-inflammatory Th17 and anti-inflammatory regulatory T responses, favouring immune-mediated tissue damage in RA.

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