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Frequency and functional activity of Th17, Tc17 and other T-cell subsets in Systemic Lupus Erythematosus

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ABSTRACT

To compare frequency and functional activity of peripheral blood (PB) Th(c)17, Th(c)1 and Treg cells and the amount of type 2 cytokines mRNA we recruited SLE patients in active ($n = 15$) and inactive disease ($n = 19$) and healthy age- and gender-matched controls ($n = 15$). The study of Th(c)17, Th(c)1 and Treg cells was done by flow cytometry and cytokine mRNA by real-time PCR. Compared to NC, SLE patients present an increased proportion of Th(c)17 cells, but with lower amounts of IL-17 *per* cell and also a decreased frequency of Treg, but with increased production of TGF-β and FoxP3 mRNA. In active compared to inactive SLE, there is a marked decreased in frequency of Th(c)1 cells, an increased production of type 2 cytokines mRNA and a distinct functional profile of Th(c)17 cells. Our findings suggest a functional disequilibrium of T-cell subsets in SLE which may contribute to the inflammatory process and disease pathogenesis.

1. Introduction

Until very recently, the dominant paradigm for understanding the pathogenesis of autoimmune diseases, including Systemic Lupus Erythematosus (SLE) stated that Th1 and Th2 cells orchestrate autoimmune responses [1]. Some studies suggested that Th2 cells (producers of IL-4 and IL-10) were the key mediators [2–4] while others emphasized the role of Th1 cells (producers of IFN-γ, IL-2, TNF-α) in SLE [5]. The discovery of a new subset of $CD4^+$ T cells, named Th17 after their signature cytokine, IL-17 is originating a major revision in this paradigm. Many recent studies suggest that Th17 cells are the primary drivers of inflammatory responses in a variety of autoimmune diseases, including Crohn's disease, rheumatoid arthritis and multiple sclerosis [6,7]. The role of IL-17 producing cells in SLE pathogenesis is far less clear [8]. Some studies in mouse models and in human lupus provide evidence for a role of IL-17 [9–11], while others argue against this [12,13]. Studies on the influence of IL-17 in SLE disease activity are scarce and results are contradictory [8]. In the $CD8^+$ T cell compartment, Tc1 and Tc2 subsets exist, characterized by the same hallmark cytokines as Th1 and Th2, respectively. Recently, a subpopulation of $CD8^+$ T cells producing IL-17 (Tc17) was also discovered [14–16], but its role in SLE has not been examined. It has been suggested that regulatory T (Treg) cells (TGF-β producers another T-cell subset with an important role in suppressing autoimmunity may be defective in SLE [17,18]. A reciprocal relationship exists between these different T-cell subsets. Development of Th1 and Th2 subsets is mutually antagonistic and both cell types suppress Th17 [19,20]. Treg cells suppress other effector T cells but, surprisingly, Treg-derived TGF-β enhances Th17 differentiation [21,22].

Because SLE immune response is highly heterogeneous and in view of the reciprocal interrelationships of the T cell subpopulations, the role of Th(c)17 cells in SLE might be better elucidated with integrated evaluation of their cytokine production.

The aim of this study was to evaluate and compare the frequency and functional activity of Th17 cells and other peripheral blood T-cell subsets, namely Th(c)1, Tc17 and Treg cells, and the production of type 2 cytokines mRNA in active and inactive SLE and in healthy control subjects.

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2. Materials and methods

2.1. Patients

We recruited patients with SLE fulfilling the 1997 ACR classification criteria for the disease [23,24]. Disease activity at the time of evaluation was scored according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI, 2000) [25,26]. Patients were divided into two groups, according to their SLEDAI score (active disease = SLEDAI $\geqslant$ 5) [27]. Medication at time of evaluation was recorded.

2.2. Controls

As controls, we recruited healthy age- and gender-matched bone marrow donors at the Histocompatibility Center of Coimbra. Pregnancy and age under 18 were exclusion criteria for participation. The study protocol was approved by the local ethics committee. All participants gave and signed informed consent.

2.3. Blood samples

One 20 mL PB sample was collected from each participant on the day of inclusion and clinical evaluation. Samples were sent to the laboratory identified with a code number and analysis was performed without knowledge of the participant disease status.

2.4. Immunofluorescence staining of peripheral Th(c)1 and Th(c)17 subsets after in vitro stimulation with PMA/ionomycin, in the presence of Brefeldin A

500 microliters of each PB sample were diluted L/L (vol/vol), in RPMI-1640 medium (Gibco; Painactive SLEy, Scotland, UK), supplemented with 2 mM L-glutamine. About 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma, Saint Louis, MO, USA), 1 μg/mL of ionomycin (Boehringer Mannheim, Germany) and 10 μg/mL of Brefeldin A (Golgi plug-Sigma, Saint Louis, MO, USA) were added and the sample was incubated for 4 h at 37 °C in a humidified incubator with 5% CO_2 concentration.

Each cultured PB sample was aliquoted and stained in three different tubes (200 mL/tube) following an intracytoplasmatic permeabilization and staining protocol in order to analyse separately the intracellular expression of IL-2, TNF-α and IFN-γ in IL-17-positive and IL-17-negative T cell subpopulations, within CD4 T cell and CD8 T-cell subsets. All cell aliquots were stained with IL-17 PE (clone 41802; R&D Systems, Europe) and separately with IL-2 (clone MQ1-17H12; BD Pharmingen, San Diego, CA, USA), TNF-α (clone MAb11; BD Pharmingen, San Diego, CA, USA) and IFN-γ (clone 4S.B3; BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions for fixation and permeabilization. These mAb were added to each tube after staining cells for surface expression of mAb directed against T lymphocytes subsets – anti-CD3-PerCP (clone SK7; BD, San Jose, CA, USA) and anti-CD8 APC (clone SK1; BD, San Jose, CA, USA). Among positive CD3 cells, CD4 positive T cells were identified after exclusion of γδ-T-cell subset according to their higher reactivity with anti-CD3 monoclonal antibody and typical light scatter.

2.5. Immunofluorescent staining of peripheral regulatory T cells (Treg)

In aliquots corresponding to Treg evaluation, anti-CD25-FITC (clone M-A251; Pharmingen BD, San Diego, CA, USA), anti-CD127 PE (clone hIL-7R-M21; BD, San Jose, CA, USA), and anti-CD4 PerCP-cy5.5 (clone SK3; BD, San Jose, CA, USA), were added and the samples were incubated for 15 min at room temperature in darkness. After this incubation period, 2 mL of FACS Lysing Solution (BDB, San Jose, CA) diluted 1:10 (vol/vol) in distilled water was added and after 10 min of incubation the cells were washed with 2 mL of PBS and resuspended in 0.5 mL of PBS before analysis in flow cytometry.

2.6. Flow cytometric analysis

Single-cell suspensions were prepared and stained for 15 min at room temperature in darkness with optimal dilutions of each mAb. Results illustrate the percentage of positive cells within each cell subset or/and their mean fluorescence intensity (MFI). T lymphocytes were identified according to their positivity for CD3 and typical light scatter. The cytokine production was evaluated within the different T-cell subsets on an electronic $CD3^+$ gating, after a first acquisition of 20,000 of total events. The identification and quantification of Treg were done based on the following phenotype: $CD4^+$, $CD25^{bright}$ and $CD127^{-/low}$ expression. Finally, expression of cell surface markers and production of cytokines were assessed using the flow cytometer (FACSCalibur; BD, San Jose, CA, USA), and data were analysed using Infinicyt software (Cytognos, Spain).

2.7. Treg cell sorting

As other researchers, we used the expression of CD127 as a viable surface biomarker to differentiate effector ($CD127^+$) cells from Treg ($CD127^{-/low}$) cells in humans [12]. Thus, $CD25^{bright}/CD127^{-low}$ CD4 T cells, defined as Treg cells in our experiments, were purified using a FACSAria cell sorter (BD, San Jose, CA, USA). The purity of the isolated cell populations was evaluated and it was consistently greater than 90%.

2.8. Gene expression analysis

Study of mRNA expression of IL-4 and IL-10 from whole blood was performed in blood collected collection in PAXgene Blood RNA Tube (PreAnalytiX GmbH, Switzerland) with automated RNA purification in QIAcube (Qiagen, Hilden, Germany). Total RNA isolation from sorted Treg cells was performed using RNeasy Micro Kit (Qiagen, Hilden, Germany).

RNA integrity and quantification were analyzed using 6000 Nano Chip® Kit, in Agilent 2100 bioanalyzer (Agilent, Walbronn, Germany). One microgram of RNA was reverse transcribed with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) using oligo(dT) plus random hexamers according to manufacturer's instructions.

Relative quantification of gene expression by real-time PCR was performed using thermocycler LightCycler 480 II (Roche, Basel, Switzerland). Normalization for gene expression quantification was performed with geNorm Housekeeping Gene Selection Human Kit (Primer Design, Southampton, UK) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) to select optimal housekeeping genes to this study [28].

Real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for IL-4 (QT00012565), IL-10 (QT00041685), FOXP3 (QT00048286), TGFB1 (QT00000728) and endogenous controls ATP5B (QT00055622), B2M (QT00088935), RRN18S (QT00199367) and SDHA (QT01668919) together with QuantiTect SYBR Green PCR Kit Gene expression (Qiagen, Hilden, Germany) according to manufacturer's instructions. Reactions were performed with the following thermal profile: 10 min at 95 °C plus 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Quantitative real-time PCR results were analyzed with Lightcycler 480 software (Roche, Basel, Germany) and quantification was performed in qBase*Plus* software package (Biogazelle, Zulte, Belgium).