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Analysis of T cell receptors in rheumatoid arthritis: the increased expression of HLA-DR antigen on circulating $\gamma\delta^+$ T cells is correlated with disease activity

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SUMMARY

The phenotypic characteristics of peripheral blood T cells, isolated from 37 rheumatoid arthritis (RA) patients and 17 healthy controls were determined with special emphasis on $\gamma\delta^+$ T cells and $CD4^-CD8^-$ $\alpha\beta^+$ T cells. Two- and three-colour automated flow cytometry analyses were performed using a panel of MoAbs directed against differentiation antigens and T cell receptor molecules. The results demonstrated: (i) no significant difference between the percentages of $CD4^-CD8^-$ $\alpha\beta^+$ T cells in patients and controls; (ii) a significant decrease of the $\gamma\delta^+$ T cell level in the peripheral blood of RA patients relative to controls; (iii) phenotypic abnormalities of circulating $\gamma\delta^+$ T cells in RA patients suggestive of an activation status *in vivo*. These abnormalities included a significant reduction in the density of the T cell differentiation antigen CD3 and an increase in the expression of HLA-DR antigen. The level of circulating $HLA\text{-}DR^+/\gamma\delta^+$ T cells was significantly higher in patients with active disease. $HLA\text{-}DR^+/\gamma\delta^+$ T cells were also present in the synovial fluid obtained from three patients with an active disease. In addition, preliminary experiments showed that the activated $\gamma\delta^+$ T cells were predominantly Vδ1. Taken together, these data support the involvement of $\gamma\delta^+$ T cells in the pathogenesis of RA.

Keywords T cell receptors $\gamma\delta$ T cell rheumatoid arthritis

INTRODUCTION

Human T lymphocytes express two distinct types of antigen receptor (TCR) on their cell surface in association with the CD3 molecular complex [1]. The great majority of peripheral blood T lymphocytes express a disulphide-linked $\alpha\beta$ receptor and include the helper-inducer $CD4^+$ T cell subset, the cytotoxic-suppressor $CD8^+$ T cell subset and a recently identified small population of double-negative $CD4^-CD8^-$ $\alpha\beta^+$ T cells whose function remains unclear [2]. A minor set of peripheral blood T lymphocytes (about 5%) possesses a second form of the TCR composed of a $\gamma\delta$ heterodimer [3]. Most peripheral $\gamma\delta^+$ T cells lack CD4 and CD8 antigens, and approximately 30% express the CD8 antigen. The functions of $\gamma\delta^+$ T cells are still obscure, although they have been shown to respond to mitogenic stimuli [4], alloantigens [5], recall antigens [6], mycobacterial antigens [7] and to display cytotoxic [8] and natural killer (NK)-like activities [9]. MoAbs specific to differentiation antigens and T cell receptors enable an analysis of the different $\alpha\beta^+$ and $\gamma\delta^+$ T cell subsets in the peripheral blood of healthy and diseased individuals, and in T cell infiltrates of tissues affected by inflammatory processes.

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology in which different T cell populations might play a central role [10]. The participation of the helper-inducer subset is strongly suggested by the presence of activated $CD4^+$ T cells in the mononuclear cell infiltrate of the synovia of RA patients [11]. Conflicting results have been obtained concerning the representation of $\gamma\delta^+$ T cells in RA peripheral blood [12–16], but the constant elevation of this population in the RA synovial fluid (SF) [13,15,16] supports its pathogenic role. Although no study has focused on the $CD4^-CD8^-$ $\alpha\beta^+$ T cells in RA patients, the marked expansion of this population in the MRL/lpr-lpr lupus-prone mice that develop histopathologic manifestations of the joints identical to those observed in RA [17] raises the question of its participation in the pathogenetic mechanisms of RA.

The objective of this study was to analyse the phenotypic characteristics of peripheral blood T cells in a large series of patients with RA with special emphasis on $CD4^-CD8^-$ $\alpha\beta^+$ and $\gamma\delta^+$ T cells.

PATIENTS AND METHODS

Patients

Peripheral blood samples were obtained from 37 RA patients (10 males and 27 females) who met the criteria of the American

Rheumatism Association [18]. The mean age of the patients was 59 ± 13 years (range 29–75 years) and the mean disease duration was 10 ± 7 years. Fourteen patients were taking non-steroidal anti-inflammatory drugs, 16 corticosteroids, 19 methotrexate, three gold derivatives, and two D-penicillamine. Disease activity was assessed according to the Ritchie index (≥10), the patient's perception of morning stiffness (lasting at least 45 min) and erythrocyte sedimentation rate (ESR > 28 mm). The patients were classified as active when they met three of these criteria. Thirteen patients had active RA and 24 were in complete or incomplete remission. Blood samples were obtained from 22 healthy individuals and used as controls.

Cell preparations

Mononuclear cells from heparinized peripheral blood were isolated by density gradient centrifugation on Ficoll–Hypaque (Pharmacia, Les Ulis, France). Cells at the interface were collected and washed three times and adjusted to 10^7/ml in PBS supplemented with bovine serum albumin (Sigma Chemical Co., St Louis, MO), and 0·1% sodium azide (PBS-BSA-azide). Synovial fluid was obtained from the knees of RA patients by joint aspiration and the mononuclear cells were prepared as described above.

Monoclonal antibodies

Biotin-conjugated, FITC-conjugated and PE-conjugated MoAbs were used in this study. Biotinylated antibodies were revealed by streptavidin-allophycocyanin (APC) (Molecular Probes, Clinisciences, France). In the three-colour automated flow cytometry analysis, the following combinations were used: anti-αβTCR (WT31, Becton Dickinson, Montain View, CA)/anti-CD4 (T4, Coulter Immunology, Hialeah, FL)/anti-CD8 (T8, Coulter); anti-TCR-γ/δ-1 (Becton Dickinson)/a mixture of T4/T8 and anti-CD3 (Dako, Glostrup, Denmark). In the two-colour automated flow cytometry analysis, the cells were labelled with the anti-TCR-γ/δ-1 and one of the following MoAbs: anti-CD3, anti-CD45RA (2H4, Coulter), anti-CD29 (4B4, Coulter), or anti-HLA-DR (I3, Coulter) which reacts with a monomorphic determinant of HLA-DR molecules. γδ T cell subsets were analysed using the MoAb Vδ2 (a kind gift from Dr T. Hercend) specific to Vδ2 and δ-TcS-1 (T Cell Sciences, Cambridge, MA) specific to Vδ1Jδ1 or Vδ1Jδ2. Isotype-matched MoAbs that did not react with human cells were used as controls to exclude non-specific binding.

Labelling procedures

One hundred microlitres of the cell suspension were mixed with the MoAb conjugates. Five microlitres of each antibody were used in each test except for the WT31 MoAb which was used at a lower concentration (2·5 μl/test) to specifically stain the αβTCR [19]. When two or three antibodies were used simultaneously, they were added together and incubated at 4°C in the dark for 30 min. Then, the cells were washed twice and the biotinylated antibodies were revealed by the addition of 7·5 μl of streptavidin-allophycocyanin and 92·5 μl of PBS-BSA-azide. The cells were incubated at 4°C for 30 min and washed twice again. The cells were immediately analysed or fixed with 1% paraformaldehyde in PBS. These fixed samples could be kept for several days before being washed with PBS and analysed.

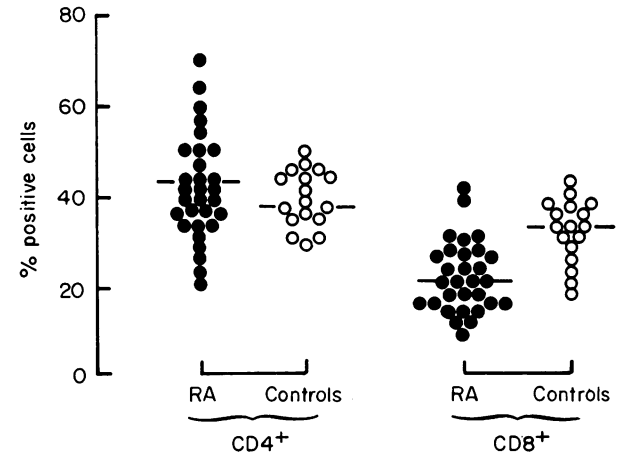

Fig. 1. Percentages of lymphocytes expressing the CD4 or the CD8 antigens in the peripheral blood of patients with rheumatoid arthritis (RA) and controls evaluated by three-colour cytofluorometry using FITC-WT31, PE-anti-CD4 and biotinylated-anti-CD8. The data are expressed as the percentage of $CD4^+$ or $CD8^+$ cells relative to the whole lymphocyte population. Arithmetic means are represented by the horizontal lines.

Flow cytometry analysis

An EPICS-ELITE flow cytometer (Coulter Electronics Inc, Hialeah, FL), equipped with a 500 mW argon laser and a helium-neon laser, was used. Appropriate settings of forward and 90° light scatter gates were used to analyse 10 000 lymphoid cells/test. Cell labelling was analysed on a log-fluorescence scale. FITC was measured using a 530/30 nm band-pass filter (530 nm centred), PE with a 555/95 nm band pass filter and APC with a 675 nm long pass filter. The FITC, PE and APC gains were optimized using a mixture of PE-non-specific IgG2a and FITC-non-specific IgG1. Positive cells were determined by setting a threshold with reference to non-specific MoAb. The fluorescent compensations were then adjusted using a mixture of PE-anti-CD4, FITC-anti-CD8 and biotinylated anti-CD3. Antigen density was indirectly ascertained by the mean fluorescent intensity (MFI) of the cells analysed.

Statistical analysis

Results are expressed as the arithmetic means ± s.d. The means were compared using the Mann–Whitney *U*-test because the values were not normally distributed.

RESULTS

Phenotypic characteristics of $\alpha\beta^+$ T cells

The phenotypic characteristics of circulating $\alpha\beta^+$ T cells were evaluated in RA patients and controls by three-colour cytofluorometry using FITC-WT31, PE-anti-CD4 and biotinylated anti-CD8. The levels of $CD4^+$ T cells were not different in patients and controls (42 ± 11% *versus* 39 ± 6%) (Fig. 1). In contrast, the percentage of $CD8^+\alpha\beta^+$ T cells was significantly decreased in RA patients (12 ± 7% *versus* 20 ± 6% in controls, $P < 0.001$) and accounted for the decreased level of $CD8^+$ lymphocytes in RA patients (21 ± 7% *versus* 32 ± 6%, $P < 0.001$). No double-positive ($CD4^+/CD8^+$)$\alpha\beta^+$ T cells could be detected. To determine the percentage of the $CD4^-CD8^-$ $\alpha\beta^+$ T cells, we first checked that, for each sample, the addition

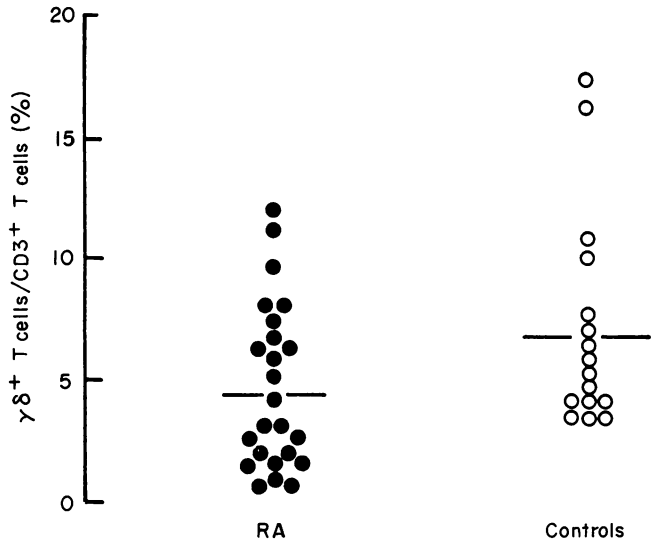

Fig. 2. Levels of $\gamma\delta^+$ T lymphocytes in the peripheral blood of rheumatoid arthritis (RA) patients and controls evaluated by two-colour cytofluorometry using FITC-anti-TCR-γ/δ-1 and PE-anti-CD3. The data are expressed as the percentage of $\gamma\delta^+$ T cells relative to the percentage of total CD3$^+$ T cells. The horizontal lines represent the arithmetic means.

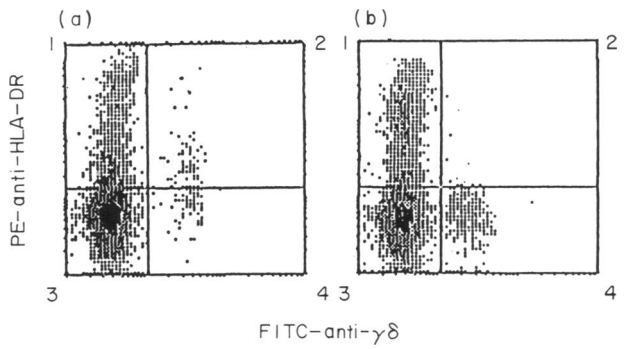

Fig. 3. The flow cytometry profiles using two-colour immunofluorescence of mononuclear cells isolated from the peripheral blood of a patient with active rheumatoid arthritis (RA) (a) and that of a healthy control (b). Staining with FITC-anti-TCR-γ/δ-1 is plotted along the abscissa (green fluorescence) while that with PE-anti-HLA-DR is plotted along the ordinate (red fluorescence). The cells stained with both fluorescent molecules are in the upper right quadrant.

of the number of double-negative $\alpha\beta^+$ T cells and double-negative $\gamma\delta^+$ T cells corresponded to the number of double-negative CD3$^+$ T cells. This evaluation was considered satisfactory because the ratio of double-negative $\alpha\beta^+$ plus double-negative $\gamma\delta$ T cells on CD3$^+$ double-negative T cells in the RA population was 99·6±11% (range 82–120%). The level of circulating CD4$^-$CD8$^-$$\alpha\beta^+$ T cells (expressed as percentage of total lymphocytes) observed in controls (1·2±0·6%) was the same as that reported previously [2] and was not different from that observed in patients (1·2±0·7%). Thus, no expansion of CD4$^-$CD8$^-$ $\alpha\beta^+$ T cells was observed in peripheral blood of patients with RA.

Fig. 4. Levels of HLA-DR$^+$ $\gamma\delta^+$ T cells in the peripheral blood of rheumatoid arthritis (RA) patients and controls evaluated by two-colour cytofluorometry. The data are expressed as the percentages of HLA-DR$^+$ $\gamma\delta^+$ T cells relative to the percentage of total $\gamma\delta^+$ T cells. ▧, RA patients with inactive disease; ■, RA patients with active disease; □, healthy controls.

Table 1. CD4$^-$CD8$^-$ $\alpha\beta^+$ and $\gamma\delta^+$ T cells in paired synovial fluid (SF) and peripheral blood from three rheumatoid arthritis patients

	Patient					
	1		2		3	
Phenotype	SF	PB	SF	PB	SF	PB
CD4$^-$CD8$^-$ $\alpha\beta^+$ (%)*	1·2	1·4	1·5	1·2	1·2	1·0
$\gamma\delta^+$ (%)†	6·6	6·3	6·1	6·7	8	7·9
DR$^+\gamma\delta^+$ (%)‡	20	4·5	18·6	4·5	20	13

* Expressed as the percentage of total lymphocytes.
† Expressed as the percentage of CD3$^+$ T cells.
‡ Expressed as the percentage of $\gamma\delta^+$ T cells.
PB, Peripheral blood.

Phenotypic characteristics of γδ⁺ T cells

The levels of circulating T lymphocytes expressing the $\gamma\delta^+$ TCR were analysed by two-colour cytofluorometry using FITC-conjugated anti-TCR-γ/δ-1 and PE-conjugated anti-CD3. Figure 2 shows that the percentage of circulating $\gamma\delta^+$ T lymphocytes among CD3$^+$ T lymphocytes in RA patients (4·4±3%) was significantly lower than that observed in controls (6·8±4%, $P<0·025$). During this procedure, we noticed that, in the healthy control group, CD3 density on $\gamma\delta^+$ T cells was higher than that on $\gamma\delta^-$ T cells, whereas in RA patients CD3 density was frequently lower on $\gamma\delta^+$ T cells than on CD3$^+\gamma\delta^-$ T cells. Thus, we compared the CD3 MFI on $\gamma\delta^+$ T cells from RA patients and controls. The CD3 MFI on $\gamma\delta^+$ T cells was significantly lower in patients (6·45±2·1 *versus* 8·67±2·3, $P=0·05$), while CD3 MFI on $\alpha\beta^+$ T cells was the same in patients and controls (5·53±1 *versus* 5·48±1·2). Since a low CD3 antigen density on T cells has been reported to be associated with an activation status [20], this observation prompted us to analyse HLA-DR expression on circulating $\gamma\delta^+$ T cells by two-colour cytofluorometry. The percentage of $\gamma\delta^+$ HLA-DR$^+$ T cells among $\gamma\delta^+$ T cells was significantly higher in RA patients (22·7±18%) than in controls (2·2±1·3%,

Table 2. $\gamma\delta^+$ T cell subsets in the synovial fluid (SF) and peripheral blood from seven rheumatoid arthritis patients

	Patient								
	4		5		6	7	8	9	10
Phenotype	SF	PB	SF	PB	PB	PB	PB	PB	PB
$\gamma\delta^+$ (%)*	2·2	2·2	2·2	5·8	0·9	1·8	6	5·5	6·3
$DR^+, \gamma\delta^+$ (%)†	42	3·2	42	1·6	34	32	15	34	26
$V\delta2^+/\delta$-TCS-1^+‡	0·6	18	0·5	26	0·4	0·1	0·16	0·2	0·15
$V\delta2^+, DR^+$ (%)§	BD	2·6	12	2·6	21	BD	11	12	6
δ-TcS-$1^+, DR^+$ (%)	78	BD	90	BD	29	59	19	33	23

* Expressed as the percentage of $CD3^+$ T cells.

† Expressed as the percentage of $\gamma\delta^+$ T cells.

‡ The relative proportions of $V\delta2^+$ and δ-TcS-1^+ were calculated as a ratio of $V\delta2^+$ to δ-TcS-1^+ T cells.

§ Expressed as the percentage of $V\delta2^+$ or δ-TcS-1^+ T cells.

BD, Below detectable levels; PB, peripheral blood.

$P<0{\cdot}001$) in whom, virtually, no HLA-DR^+ $\gamma\delta^+$ T cells could be detected. Figure 3 shows the flow cytometry profiles of circulating lymphocytes isolated from a healthy control and from a patient in whom the percentage of HLA-DR^+, $\gamma\delta^+$ T cells reached 80%.

The phenotypic characteristics of $\gamma\delta^+$ T cells were further studied. The percentages of circulating $CD8^+$ $\gamma\delta^+$ T cells were determined by two-colour cytofluorometry; patients and controls were found to have identical levels (approximately 25%). Anti-CD29 and anti-CD45RA MoAbs allow separation of the $\alpha\beta^+$ T cell population into two subgroups at different stages of maturation, referred to as naive ($CD45RA^+$) and memory ($CD29^+$) T cells. So far, little information has been obtained concerning the expression of these markers on $\gamma\delta^+$ T cells. Similar percentages of $CD45^+\gamma\delta^+$ (52 ± 10 and 46 ± 16) and $CD29^+\gamma\delta^+$ T cells (55 ± 9 and 52 ± 10) were observed in patients and controls, respectively.

Correlation between the percentage of circulating $\gamma\delta^+$ T cells and the disease activity

RA patients were classified into two groups according to their disease activity evaluated by the criteria defined in the Patients and Methods section. The level of circulating $\gamma\delta^+$ T cells was not different in patients with active and inactive disease. However, in the former group, the percentage of circulating HLA-$DR^+\gamma\delta^+$ cells ($41\pm25\%$) was significantly higher than in the latter ($7{\cdot}15\pm3{\cdot}15\%$; $P<0{\cdot}005$) (Fig. 4).

The HLA-$DR^+\gamma\delta^+$ T cell population is present in the synovial fluid

Synovial fluid was obtained from three RA patients with active disease. Table 1 shows the levels of $\alpha\beta^+$ and $\gamma\delta^+$ T cell populations and the percentage of HLA-$DR^+\gamma\delta^+$ T cells in these three samples. Similar levels of double-negative $\alpha\beta^+$ and $\gamma\delta^+$ T cells were observed in paired SF and peripheral blood. In contrast, the percentages of HLA-$DR^+\gamma\delta^+$ T cells in SF were sharply increased compared with those in the paired peripheral blood sample.

$DR^+, \gamma\delta^+$ T cells are predominantly $V\delta1$

The use of MoAbs enables one to divide the $\gamma\delta^+$ T cells into two different subsets. The $V\delta2$ and the δ-TcS-1 define populations characterized respectively by the $V\delta9V\delta2$ or the $V\delta1$ usage. Seven additional RA patients were studied to determine whether the HLA-DR antigen was expressed predominantly by a $\gamma\delta^+$T cell subset. The results observed in seven blood samples and two paired SF are presented in Table 2. In most patients the $V\delta1^+$ T cell subset predominated in the blood and was associated with an increased percentage of $DR^+, \gamma\delta^+$ T cells. Interestingly, in the two patients (5 and 6) who had high proportions of $V\delta2^+$ T cells and virtually no circulating $DR^+\gamma\delta^+$ T cells, SF had high percentages of $DR^+\gamma\delta^+$ T cells and δ-TcS-1^+ T cells. In most patients the $DR^+\gamma\delta^+$ T cells were predominantly $V\delta1$.

DISCUSSION

The major observation derived from this study concerns the level and the phenotypic characteristics of circulating $\gamma\delta^+$ T cells in patients with RA. The percentage of $\gamma\delta^+$ T cells was significantly reduced in RA patients. This result is similar to that reported by Smith *et al.* [16] but differs from those reported by other groups who found increased [13,15] or normal levels [13,21]. The reason for these discrepancies remains unclear but might be due, at least partly, to the marked variation in $\gamma\delta^+$ T cell values observed in both patients and controls. In this study, patients with active and inactive disease had comparable $\gamma\delta^+$ T cell levels. At variance with our results, Meliconi *et al.* [14] reported a reduced percentage of circulating $\gamma\delta^+$ T cells only in patients with joint effusion.

In RA patients we found that $\gamma\delta^+$ T cells had the phenotype of an *in vivo* activation status. First, a decreased density of the CD3 antigen was observed in circulating $\gamma\delta^+$ T cells, a phenomenon reported to be associated with an activation status in models of *in vitro* T cell activation [20]. Second, a large number of peripheral blood $\gamma\delta^+$ T cells expressed the HLA-DR molecule in RA patients, whereas this activation marker could not be detected in controls. Interestingly, higher HLA-$DR^+/\gamma\delta^+$ T cell values were observed in patients with active disease. These observations strongly suggest the participation of $\gamma\delta^+$ T

cells in the pathogenesis of RA, in as much as the HLA-DR$^+\gamma\delta^+$ T cell population was also present in the SF obtained from three patients with active disease. So far, no study has determined the expression of activation markers on $\gamma\delta^+$ T cells in the peripheral blood or SF of patients with adult RA. However, Kjeldsen-Kragh *et al.* [21] reported the presence of $\gamma\delta^+$ T cells expressing HLA-DR and IL-2 receptors in the SF of patients with juvenile rheumatoid arthritis (JRA), in contrast to their peripheral blood in which no $\gamma\delta^+$ T cells expressing these antigens could be detected. One should also mention that, in JRA, T cell clones expressing the CD3$^+$CD4$^-$CD8$^-$WT31$^-$ phenotype could be derived from synovial T lymphocytes cloned directly in IL-2 (without phytohaemagglutinin), a procedure which allows selective growth of T cells expressing functional receptors for IL-2 [22]. Interestingly, in our five patients from whom the SF was obtained, the level of HLA-DR$^+\gamma\delta^+$ T cells was higher in the SF than in the blood. This observation suggests that, in RA, either the synovium is the site of activation of $\gamma\delta^+$ T cells, which would subsequently migrate into the general circulation, or circulating activated $\gamma\delta^+$ T cells migrate preferentially to the synovium. Analysis of $\gamma\delta^+$ T cell subsets demonstrated that in both peripheral blood and SF δ-TcS-1$^+$ T cells were predominant. These data are in accordance with those published previously [14,16,23] and, in addition, showed that the δ-TcS-1$^+$ T cell population preferentially expressed the HLA-DR antigens.

The physiological role of $\gamma\delta^+$ T cells in the immune response remains unknown. Although some authors have reported that these cells reacted with a number of infectious agents including bacteria, viruses and parasites, their repertoire remains to be characterized. Functional studies showed that $\gamma\delta^+$ T cells exhibited cytotoxic activities and produced lymphokines [24]. Their relevance to autoimmune processes comes from studies performed in RA and systemic lupus erythematosus (SLE). For instance, $\gamma\delta^+$ T cells of RA patients showed enhanced reactivity to a fraction of mycobacteria cross-reactive with cartilage [25]; IL-2-dependent $\gamma\delta^+$ T cell lines derived from SLE peripheral blood had the ability to selectively augment the production of pathogenic autoantibodies [26]. In this study, the presence of $\gamma\delta^+$ T cells expressing predominantly the Vδ-1 gene segment may reflect a non-specific migration. However, the activation status of $\gamma\delta^+$ T cells which preferentially involves a $\gamma\delta^+$ T cell subset suggests a potentially antigen-driven process.

A very limited number of studies have evaluated the levels of double-negative $\alpha\beta^+$ T cells in healthy individuals and in patients with autoimmune diseases [2,27]. In our study, double-negative $\alpha\beta^+$ T cells represented approximately 1% of circulating lymphocytes in controls and were not expanded in the peripheral blood and SF of patients with RA. This observation does not necessarily support the involvement of double-negative $\alpha\beta^+$ T cells in the pathogenetic mechanisms of the disease; however, one should mention that this normal level does not preclude the participation of this T cell population. For example, in mice with type II collagen autoimmune arthritis (a model for human arthritis), double-negative T cells, although present at normal levels, have been shown to display aberrant expression of a voltage-gated K$^+$ channel called type 1 [28]; this abnormality is considered to be restricted to autoimmune diseases. A similar study in humans would provide information about the role of double-negative $\alpha\beta^+$ T cells in RA.

A decreased level of CD8$^+$ peripheral blood lymphocytes was observed in RA patients and was demonstrated to be due to a reduced representation of CD8$^+\alpha\beta^+$ T cells. Previous studies have shown that patients with active disease are deficient in circulating CD8$^+$ T cells [15,29], although these results have been disputed [11]. The phenotypic characterization of the CD8$^+\alpha\beta^+$ T cells will be analysed by three-colour cytofluorometry to determine which subpopulation is reduced numerically.

Our findings suggest a role for the $\gamma\delta^+$ T cell subset in the development and progression of RA. Further investigation of activation markers, the repertoire and functions of HLA-DR$^+\gamma\delta^+$ T cells through automated cytofluorometry and T cell clone technology may provide important insights into the pathogenesis of the disease.

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