Expansion of $CD4^+CD25^-GITR^+$ regulatory T-cell subset in the peripheral blood of patients with primary Sjögren's syndrome: correlation with disease activity

A. Alunno[1], G. Nocentini[2], O. Bistoni[1], M.G. Petrillo[2], E. Bartoloni Bocci[1], S. Ronchetti[2], E. Lo Vaglio[1], C. Riccardi[2], R. Gerli[1]

[1]SSD di Reumatologia, Dipartimento di Medicina Clinica e Sperimentale, Università degli Studi di Perugia, Italy;

[2]Sezione di Farmacologia, Tossicologia e Chemioterapia, Dipartimento di Medicina Clinica e Sperimentale, Università degli Studi di Perugia, Italy

SUMMARY

Objectives: $CD4^+CD25^{high}$ regulatory T cells (T_{REG}) represent a suppressive T-cell subset that plays a key role in the modulation of immune responses and eventually in the prevention of autoimmunity. There is growing evidence that patients with autoimmune and inflammatory chronic diseases show an impairment of T_{REG} cells or activated effector T cells unresponsive to T_{REG}. Glucocorticoid-induced TNFR-related protein (GITR) is a widely accepted marker of murine T_{REG} cells, but little is known of its role in humans. The aim of the present study was to investigate the characteristics of different subsets of T_{REG} cells in Sjögren's syndrome and the potential role of GITR as a marker of human T_{REG} cells.

Methods: Fifteen patients with primary Sjogren's syndrome (SS) and 10 sex- and age-matched normal controls (NC) were enrolled in the study. $CD4^+$ T cells were separated from peripheral blood by magnetic cell sorting (negative selection). Cell phenotype was analyzed by flow-cytometry using primary and secondary antibodies. Disease activity was assessed using the EULAR Sjögren's syndrome disease activity index (ESSDAI).

Results: Although the proportion of circulating $CD25^{high}GITR^{high}$ subset was similar in SS patients and normal controls, an expansion of the $CD25^-GITR^{high}$ cell population was observed in the peripheral blood of SS patients. Interestingly, this expansion was greater in patients with inactive rather than active disease.

Conclusions: The number of $CD4^+CD25^-GITR^{high}$ cells increases in SS as compared to normal controls. Furthermore, the fact that the expansion of this cell subset is mainly observed in patients with inactive disease suggests that these cells may play a role in counteracting inflammatory response.

Key words: *Regulatory T cells, Sjögren's syndrome, GITR, FoxP3.*

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INTRODUCTION

Regulatory T cells (T_{REG}) represent a T-cell subtype with functional suppressive activity that is a key factor in modulating immune response and, therefore, in the development of autoimmunity. There is much evidence to confirm a central role for this cell *subset* in the pathogenesis of systemic autoimmune or chronic inflammatory diseases given the anomalies in their number or alterations in their function in patients with these pathologies. There is further evidence to suggest that effector T cells activated in these pathologies are resistant, at least in part, to the action of T_{REG} cells. The particular phenotypic characteristic of T_{REG} cells that has allowed them to be isolated, first in mouse and subsequently in humans, is the high expression of a receptor molecule on their surface, known as CD25 (IL2Rα). This is associated with the intracellular expression of the transcription factor *forkhead winged helix* (Fox)P3 (1, 2). FoxP3, previously known as JM2, was initially identified as the gene the mutations of which were associated with a murine autoimmune syndrome, the

Corresponding author:
Prof. Roberto Gerli
SSD di Reumatologia
Via Enrico dal Pozzo, Padiglione X
06122 Perugia, Italy
E-mail: gerlir@unipg.it

X-linked autoimmunity-allergic disregulation (XLAAD) syndrome. This was later identified in humans and became known as the *immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome* (3, 4). Subsequent studies showed that human murine T_{REG} cells constitutively express FoxP3. It was seen that this gene is needed until, during the initial stages of development of the immune system, a progenitor T cell differentiates itself into a T_{REG} cell. This observation has reinforced its phenotypic and functional importance. In fact, FoxP3 is essential for the induction and maintainance of the functional characteristics of T_{REG} cells, such as maintaining the anergy to the receptor stimuli and the reduced production of IL-2 following their intereaction with the thymic stromal cells (5-6).

Subsequently, other surface molecules were identified as T_{REG} *markers* such as the *cytotoxic T lymphocyte antigen-4* (CTLA-4), CD127 and the *glucocorticoid-induced TNF receptor-related* (GITR) (7, 8). However, studies of some of these are still ongoing given that their specificity as T_{REG} cell markers requires further confirmation. For example, recently activated effector T cells can increase their CD25 surface expression by appearing, even though transiently, as $CD25^{high}$ cells, just as GITR can be differently expressed according to the stage of cell activation. Finally, some molecules are only recognized as T_{REG} *markers* in mouse, but their role in humans is still a subject of controversy. Given this, for the moment, co-expression of these molecules and of FoxP3, the most specific T_{REG} *marker*, provides the best definition of this cell population. However, the methodology for determining FoxP3 involves cell permeabilization. This could identify other surface molecules and is not, therefore, a suitable method to use to identify normal surface markers. Also, for the same reason, FoxP3 cannot be used to separate T_{REG} cells when they are to be used *in vivo* and reinfused in patients.

For the moment, it is possible to subdivide the T_{REG} $CD4^+$ cells into two distinct subpopulations. Natural T_{REG} cells are generated in the thymus in the first stages of life following specific interactions with the resident stromal cells. On the other hand, adaptive and induced T_{REG} cells are produced in peripheral blood throughout life and are the result of the conversion of naïve $CD4^+CD25^-$ T cells in the presence of a particular cytokine *milieu* and of appropriate antigenic stimulation (9).

As reported above, T_{REG} have a suppressive action that is directed towards circulating autoreactive lymphocytic clones. In spite of the fact that the immune system mitigates the mechanisms such as to prohibit most of the auto-reactive T cells entering the blood circulation, as for example, the negative thymic selection, some manage to elude them making continuous immune surveillance essential throughout life. The suppressive activity of the T_{REG} expresses itself mainly through cell-to-cell contact and, therefore, the consequent trigger of pro-apoptotic *pathways*, but it has also been suggested that this suppression could be mediated by soluble factors produced by the T_{REG} themselves, such as IL-10 and TGF-β (10).

Many attempts have been made over the years to understand the exact role of the T_{REG} in the pathogenesis of systemic autoimmune pathologies. In the beginning, animal models of chronic inflammatory pathologies were investigated such as, for example, collagen-induced arthritis (CIA) (11-14). Subsequent studies focused on evaluating the possible involvement of T_{REG} in the human manifestations of these pathologies in order to identify phenotypic and/or functional alterations of these cells during the disease course.

Data published on Sjögren's syndrome (SS), are all substantially in agreement that the percentages of peripheral blood of T_{REG} cells, identified by high CD25 expression and/or expression of FoxP3, of affected patients are identical to or slightly lower than those of healthy controls (15-18). Furthermore, extremely interesting results are emerging from immunohistochemical studies of the salivary glands in SS. These show a correlation between the lymphocytic infiltrate of the salivary glan-

ds (evaluated both by the Chisholm score and by the *focus score*) and the number of T_{REG} cells present in the saliva (16-18). In other words, even though the presence of a T_{REG} cell population has been observed at the site of inflammation, these cells do not seem to be able to reduce the lymphocytic infiltrate or, consequently, the parenchymal damage caused by it. Given this, it is even more important to standardize the information we have concerning the phenotypic and functional mechanisms of this cell population in the pathogenesis of systemic auto-imune diseases. The aim of this study was, therefore, to make a qualitative and quantitative evaluation of the T-cell subpopulations in patients with SS and to investigate the possible role of GITR as a *marker* of human T_{REG} cells.

MATERIALS AND METHODS

A total of 15 SS patients of the Rheumatology Department of the University of Perugia were enrolled in the study. All patients were female (mean age 52.7±13.7 years). Ten healthy age-matched women were enrolled as control.

All patients underwent clinical evaluation and blood tests: erythrocyte sedimentation rate (ESR), C-reactive protein test (CRP), complete blood count, sieroprotein electrophoresis, immunoglobulin (IgG, IgA, IgM) and complement fraction (C3, C4). Disease activity was calculated from study enrolment according to the EULAR *Sjögren's syndrome disease activity index* (ESSDAI) (19). No patient received either *disease modifying anti-rheumatic drugs* (DMARDs) or corticosteroids; patients only received topical treatment with artificial tears for Sjogren's syndrome. Blood samples were placed in heparin test tubes and centrifuged with a Hystopaque 1077 (SIGMA) at 1800 rpm at room temperature for 30 min. The peripheral blood mononuclear cells (PBMCs) obtained were subjected to magnetic sorting (negative selection) to obtain the $CD4^+$ T cells (CD4 human isolation kit II and large depeletion column, Miltenyi Biotech). Briefly, the non-$CD4^+$ T cells, e.g. $CD8^+$ T cells, T γδ cells, granulocytes, B cells, NK cells, monocytes, dendritic cells and erythroid cells were removed after incubation with a selection of biotin-conjugated antibodies towards CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γδ and glycophorin A. Cells were separated using anti-biotin microbeads with a *large depletion* (LD) column (Miltenyi Biotech). Cytometric analysis showed that $CD4^+$ T-cell purity was over 98%.

Phenotypic characterization was carried out by cytometric methods. The identified $CD4^+$ cells were colored with anti-GITR-PE (Biolegend), anti-CD25 biotinilato (Miltenyi Biotech) and respective isotypes. Cytometric analysis was carried out using a Beckman Coulter EPICS XL-MCL and EXPO32 ADC software. Statistical analysis was performed using Student's *t*-test or by calculating Spearman's correlation coefficient. $P<0.05$ was considered significant.

RESULTS

There was a statistically significant increase in the $CD25^-GITR^{high}$ subpopulation in the peripheral blood of SS patients compa-

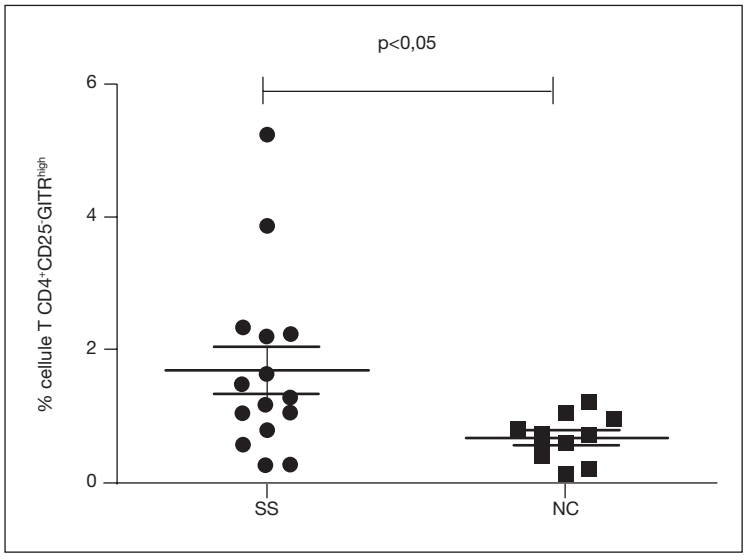

Figure 1 - Expression (%) of the CD4+CD25-GITRhigh T-cell subpopulation in SS patients (n=15) and healthy controls (n=10); Student's t-test.

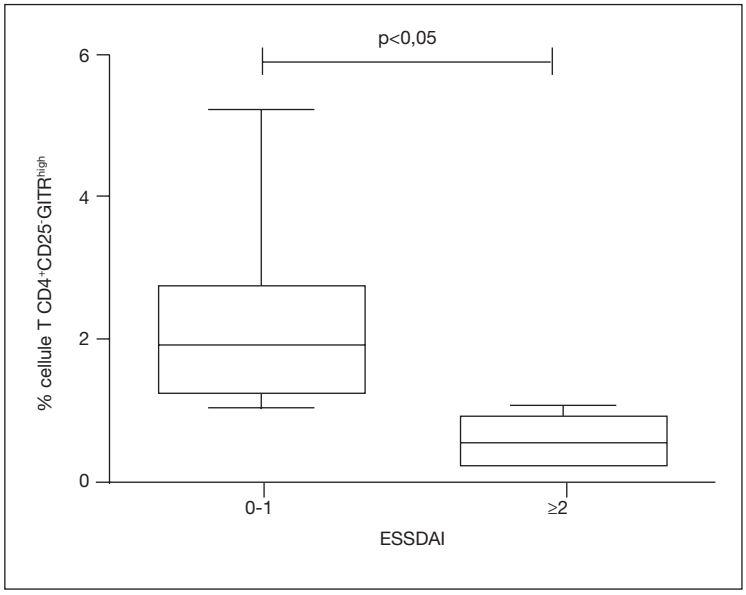

Figure 2 - Expression (%) of the CD4+CD25-GITRhigh T-cell subpopulation in SS patients according to disease activity (ESSDAI≥2 n=5; ESSDAI 0-1 n=10); Student's t-test.

red with controls (2.2±1.3% *vs* 0.7±0.3%; P<0.05) (Fig. 1).

Phenotypic and functional characteristics of $CD25^{-}GITR^{high}$ cells in SS patients were analyzed by dividing the patients according to their disease activity and clinico-serological parameters. Patients were, therefore, divided into two groups according to disease activity using the ESSDAI.

Patients with inactive disease showed a statistically significant greater expansion in the percentage of $CD25^{-}GITR^{high}$ cells compared to those patients with active disease (Fig. 2).

Finally, a statistical analysis of the clinico-serological characteristics did not show any significant correlation between these parameters and the percentage values of the two cell *subsets* analyzed (*data not shown*).

DISCUSSION

The present study has shown that patients with SS experience an expansion of T cells in peripheral blood with low or no levels of expression of CD25 and high levels of GITR on their surface. Our previous phenotypic study in healthy subjects and patients with systemic lupus erythematosus (SLE) showed that this cell population expands also in lupic patients and presents T_{REG} cell characteristics expressing the FoxP3 transcription factor at an intracellular level (20).

As demonstrated in the murine model (21), there is growing evidence that GITR could be a T_{REG} *marker* that is co-expressed with CD25. However, the observed correlation between FoxP3 and GITR expression and the lack of any correlations between FoxP3 and CD25 in $CD25^{-}GITR^{high}$ cells seem to suggest a role for GITR as a T_{REG} *marker* also in humans, as already demonstrated in mouse (3). Finally, the sub-analyzsis performed by subdividing patients according to disease activity showed expansion of $CD25^{-}GITR^{high}$ cells was significantly higher in patients with inactive disease. This could be explained, at least in part, by the possible trigger of compensatory mechanisms aimed at counterbalancing the excessive activity of the autoreactive effector T cells in patients with autoimmune diseases such SS. In particular, in patients in whom the disease has been quiescent or relatively inactive, these compensatory regulatory mechanisms, such as the expansion of suppressive subpopulations, seem to be more efficacious.

However, the crucial point of this observation is the fact that apparently $CD25^{-}GITR^{high}$ cells function normally in healthy subjects. It is, therefore, possible that in SS patients effector T cells are, at least in part, resistent to suppression by T_{REG} cells. In spite of the expansion of these cells in patients, this does not necessarily mean there is an equilibrium between pro- and anti-inflammatory stimuli. Consideration should also be given to the fact that, from an *in vivo* systemic phlogistic point of view, there may be a reduction in the functional activity of T_{REG} cells as hypothesized in studies carried out on synovial fluid of patients with active rheumatoid arthritis (22, 23). Therefore, in spite of the high number of $CD25^{-}GITR^{high}$ T cells, their suppressive action could be challenged by the pro-inflammatory cytokine *milieu* in the target

organs. Studies are ongoing to evaluate the $CD25^{-}GITR^{high}$ cells infiltrating the salivary gland tissue of SS patients during the disease course. In fact, characterization of conventional CD25hiGITRhi T_{REG} cells showed a discrepancy between the results obtained in peripheral blood and those obtained in the target organs in patients with rheumatoid arthritis or SS (16-18, 21, 22). The presence of $CD25^{-}GITR^{high}$ T cells in the T cell infiltrate characteristic of chronic autoimmune sialadenitis would confirm their pathogenetic involvement, supported by their expansion in peripheral blood, even though this does not appear to be able to contrast the inflammatory process that damages the glandular parenchyma. On the other hand, ongoing *in vitro* studies aimed at testing $CD25^{-}GITR^{high}$ function in patients with SS, will help provide greater clarification.

In conclusion, our results show that T_{REG} cells are very likely involved in the pathogenesis of human auto-immune diseases. However, the mechanisms are still not completely clear. Animal models of the disease, although important for our understanding of the multiple mechanisms involved, must be integrated with more in depth studies into the human pathology. This would throw light on the etiopathogenetic aspects that require further explanation and would provide further elements to promote the therapeutic use of T_{REG} cells in the treatment of systemic auto-immune pathologies (24).

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