Ph.D. THESIS

INVESTIGATIONS OF REGULATORY T CELLS IN POLISYSTEMIC AUTOIMMUNE DISEASES AND HODGKIN'S LYMPHOMA

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Introduction

The ability of the immune system to discriminate between self and non-self results in immunological tolerance, which can be defined as the lack of responsiveness towards certain molecules, and can be acquired by central or peripheral mechanisms. Central tolerance occurs during the ontogeny of T cells and leads to elimination of self-reactive T cells by clonal deletion in the thymus. Peripheral tolerance takes place throughout life, and is usually designed to control responses towards foreign antigens that are not harmful or antigens recognized with low affinity. However, sometimes the immune system can develop tolerance towards tumoral antigens or viral antigens, with chronic exposure, and in these letter cases, tolerance would rather be abrogated. The mechanisms that control peripheral tolerance include anergy and suppression, are mainly realized by specific cell subsets of regulatory T (Tr) cells. The $CD4^+CD25^+$ and the IL-10 producing $CD4^+$ T cells (induced Tr1 cells) are the best characterized subsets. TGF-β producing, Th3 type regulatory cells are also frequently investigated. These three types of regulatory T cells are indeed separate subsets with distinct function differentiation patterns and specialised functions.

Naturally occuring (or thymically derived) $CD4^+CD25^+$ T regulatory cells comprise approximately 5-10% of the peripheral $CD4^+$ T cells in human and mice. These cells were defined in 1995 by Sakaguchi and colleagues, who showed that the passive transfer of T cells lacking in the $CD4^+CD25^+$ subset into athymic nude mice resulted in the spontaneous development of various T-cell-mediated autoimmune diseases. These cells appear to be capable of suppressing a wide variety of immune cells, consisting of those from both the innate and adaptive immune system.

Various efforts have been made at identifying unique cell surface markers expressed by naturally occuring regulatory T cells, but no definitive marker has so far been found. The most widely recognised and used marker for $CD4^+CD25^+$ is the high leveled expression of the IL-2 receptor alpha chain molecule. CD25 is constituvely expressed at a higher mean density even on resting regulatory T cells. Other cell surface receptor molecules include the glucocorticoid –induced tumornecrosis factor receptor (GITR), CTLA-4, galectin-1, CD38, CD62L, OX-40L, CD103, TNFR2 and TGF-βR1. Naturally regulatory T cells also express high levels of CD5, L-selectin and CD45RO.

The relatively new promising functional markers of naturally regulatory T cells are the forkhead box transcription factor (FoxP3) and lymphocyte activation gene (LAG-3). Both FoxP3 and LAG-3 are crucial for regulatory T cells function and generation. In the thymus, regulatory thymocytes gather in the fibrous septe and m,edullary areas, and require higher avidity interactions between the T cell receptor (TCR) and thymic stroma expressing class II MHC-self-peptide complexes for development. Studies involving knock out mice demonstrate that CD28, CD40 and IL-2 are critical for regulatory T cell development and survival. FoxP3 is probably the most critical molecule int he generation as well as function of naturally regulatory T cells. Disruption of the FoxP3 equavalent in mice results in autoimmune disease in multiple organs as well as uncontrolled lymphoproliferation due to the lack of regulatory cells. FoxP3 is a negative regulator of T cell activation perhaps via repression of cytokines like IL-2. Further studies are needed to elucidate not only the biochemistry of the FoxP3 protein, but the precise molecular mechanisms by which this molecule mediates its regulatory function and its role in regulatory T cell development.

Regulatory T cells are capable of suppressing the proliferation and cytokine production of convetional $CD4^+$ and $CD8^+$ lymphocytes, dendritic cells and monocytes. Once stimulated, regulation of by Tr cells is independent of antigen specificity of the target cells. In order to achieve Tr suppressive function, TCR and IL-2 stimulation is necessary.

Naturally regulatory T cells suppressive function requires direct cell-cell interaction without the need of cytokines. One line of data suggests that the TGF-β in Tr cells may exert its regulatory function by its expression in the cell surface via membrane-proximal mechanisms and not necessarily act as a soluble factor. Another form by which Tr cells have been recently shown to mediate their regulatory function is by a cell-cell mediated mechanism involving cytotoxic effector functions associated with the synthesis of perforin, CD18 and granzyme-A in a Fas independent manner. It is probable that Tr cells suppress immunological responses in multiple ways, which may involve negative signals produced by inhibitory Tr surface molecules, cytotoxic killing, APC function down-regulation, induction of other regulatory cells, as well as a number of other cell-cell interactions.

The production of IL-10 is a distinguishing feature of Tr1 cells. These cells can be induced in vitro by differentation of resting or naïv $CD4^+$ T cells in the presence of IL-10. Tr1 cells do not express high levels of CD25 and FoxP3, and do not mediate suppression via cell-cell contact. Some studies revealed that human Tr1 cells suppress the production of immunoglobulin by B cells and by modulating the antigen-presenting capacity of monocytes and dendritic cells.

Hodgkin's lymphoma

Hodgkin lymphomas (HL) are characterized by the presence of a small proportion - usually less than 1% - of neoplastic cells, the multinucleated, so-called Reed–Sternberg cells and their mononuclear variants (R–S cells), and a majority of reactive cells, mostly lymphocytes admixed with histiocytes, plasma cells and eosinophils. There are two main types of HL, the nodular lymphocyte predominance subtype (NLPHL) and the classical type, that includes mixed cellularity (MC), nodular sclerosis (NS), lymphocyte-rich and lymphocyte-depleted cases.

Mixed Connective Tissue Disease

Mixed connective tissue disease (MCTD) is a chronic systemic inflammatory disorder affecting many organs. The autoantibodies to U1RNP are specific to MCTD. However, the mechanism involved in the pronounced autoantibody production is largely unknown. Hassan et al. reported increased serum TNF-α and IL10 levels in MCTD patients, which suggests an immunoregulatory disturbance in MCTD. In a previous study our group described an increased intracytoplasmatic IL10 production by $CD4^+$ T cells in patients with MCTD.

Systemic Lupus Erythematosus

Systemic lupus erythematousus (SLE) is an autoimmune disease of unknown etiology that manifests as a pleomorphic systemic disease mainly affecting females (female to male ratio 9:1). The variety of autoantibodies found in the serum of patients indicate that SLE is an autoimmune disease, but the mechanisms leading to the aberrant responses are not clearly understood although it is thought that a number of genetic and environmental factors may be involved. Environmental (or non-genetic) exposures could include infectious agents, chemicals or other compounds capable of modulating immune responses such as occupational/environmental pollutants or drugs, and behavioural factors such as smoking and diet. Environmental exposures may lead to the production of autoreactive T cells and autoantibodies, the stimulation of pro- and antiinflammatory cytokines, and target end-organ damage, but are not so convincing as agents causing SLE. Exposure to viruses increases

antibody titres, but these may be the result of polyclonal B cell activation. Other data suggest that there may be a link between infections early in life and an increased prevalence of antinuclear antibodies and SLE in adults. Early exposure to microbial antigens or vaccines may predispose to lupus-like autoimmune disease. The amount and timing of exposure to different environmental factors may therefore play a significant and complex role in the pathogenesis of SLE and other autoimmune diseases.

Aims

In our study, we investigated the ratio and the absolute number of naturally regulatory CD4+/CD25high T cells, CD4+/IL-10+ Tr1 type T cells in polysystemic autoimmune diseases (systemic lupus erythematosus, mixed connective tissue disease) and Hodgkin's lymphoma. At the start of our studies, there was no data about the absolute number and percentage of these regulatory cells in SLE, MCTD and Hodgkin's lymphoma. Our aim was to provide basic data about the modifications of regulatory T cells in the diseases mentioned above:

To determaine the alterations in the percentage and absolute number of regulatory T cells in SLE. Is there any correlation between the disease activity index (SLE-DAI) and the number of regulatory T cells? Is there effect of plasmaphereis treatment on regulatory T cells in patients with severe SLE?

To determine the changes in the percentage and absolute number of regulatory cells in MCTD and Hodgkin's lymhoma. Is there any correlation between the disease activity and the number of regulatory T cells? Does the type of treatment influence the number of regulatory T cells in MCTD or Hodgkin's lymphoma?

Patients and methods

Hodgkin's lymphoma

We randomly chose patients with HL (n = 94: 52 women, 42 men) treated at our department, being in a long-lasting complete remission, not taking any cytostatic and immunosuppressive drugs and who had not been treated with either radiotherapy (RT) or stem cell transplantation in the previous 3 years. The average duration of HL was 8.2 years (range 3–33). The average age was 44 years (range 20–77). 54 patients were Epstein-Barr virus positive and 42 were negative. 39 patients were in stages I–II and 55 in the stages III–IV. The histological typing of disease showed 60 patients with mixed cellularity, 28 with nodular sclerosis, 2 with lymphocyte predominance, 2 with nodular lymphocyte predominance and 2 with lymphocyte depletion according to WHO lymphoma classification.
The peripheral blood of 41 healthy Caucasian subjects (matched for age and sex) were studied as 'negative' controls. In addition, in a group of 'positive controls', 47 women with breast cancer, also being in a long-lasting remission, were investigated. Patients with breast cancer had been treated by RT and chemotherapy (CT) earlier, but in the previous 2 years they had only received hormone therapy.
After all subjects gave their informed consent to participate, approval was obtained from the Institutional Review Board.

Mixed connective tissue disease (MCTD)

Forty eight patients with MCTD (mean age 53 ± 9 yrs), 47 women and one man participated in this study. The mean disease duration was 13 ± 7 yrs. The diagnosis of MCTD was established according to the criteria by Alarcon-Segovia and Villareal. Disease activity was determined by SLAM score ranges 3-27. All patients fulfilled the criteria for MCTD. Twenty healthy women served as controls.

Systemic lupus erythematosus (SLE)

Seventy-two Hungarian lupus patients (63 female and 9 male) were enrolled in the study. All patients fulfilled 4 or more of the ACR revised diagnostic criteria. Their age at the time of the study was 34.4±13.9 (mean±SD) years, disease duration was 9.4±8.12 years. Fifty-three patients had inactive and 19 patients had active disease. Activity was defined by having at least one clinical and one laboratory manifestation, which indicated the necessity to change therapy. Disease activity index (SLE-DAI) was < 5 in those with inactive disease, while SLE-DAI was ≥5 in those with active disease. There was more striking female dominance in active patients. Disease duration was shorter in active cases. Active patients were younger, had significantly higher SLE-DAI and anti-dsDNS autoantibody concentration, and also required higher daily methylprednisolon dose. More patients received other immunsuppressants (12/53 vs. 12/19).Control group consisted of 41 age- and sex matched healthy volunteers.

We also investigeted the changes of regulatory T cells in 5 SLE patients who were treated with repeated plasmapheresis. The average of their SLEDAI was 11.6 before the

treatment. There was no change in their taking of drugs during the series of plasmapheresis. Therefore, all the changes found in the distribution of peripheral cell could be related to the effect of plasmapheresis. Negative side-effects of immunosuppressive treatments and different disease manifestations were the reasons of the repeated plasmapheresis treatments.

Determination of Lymphocytes Subpopulations

Cells in 100 µl of heparinized whole blood were stained with the adequate amount of lymphocyte subset-specific monoclonal antibodies. After a 25-min incubation in the dark and at room temperature, the red blood cells were lysed. The resting lymphocytes were washed and suspended in 800 µl of 1% paraformaldehyde. We determined the proportion of the $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ cells. Anti-CD69 and anti-HLA-DR monoclonal antibodies were used as activation markers. The $CD4^+CD25^{high}$ suppressor T-cell population, $CD4^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ cells were also determined. The major part of the $CD4^+CD25^{high}$ regulatory T cells was proven to be Foxp3-positive. Intracellular Foxp3 staining was done according to the manufacturer's instructions. The following reagents were used: human anti-CD8-FITC, anti-CD3-FITC (Sigma, St. Louis, Mo., USA), anti-CD56-PE (Becton Dickinson, Franklin Lakes, N.J., USA), anti-CD4-PE, anti-CD19-PerCP (Immunotech, Marseille, France), anti-CD3-FITC/HLA-DR-PE (Serotec Ltd, Oxford, UK), anti-CD-69-Cy5 (Pharmingen, San Diego, Calif., USA), and anti-Foxp3-PE (eBioscience, San Diego, Calif., USA).The equipment (FACSCalibur/Becton Dickinson/flow cytometer) counted 5,000 lymphocytes (40000 for testing $CD4^+CD25^{high}$ cell population) in each sample

Determination of Intracytoplasmic Cytokines

The intracytoplasmic content of IL-10 was measured by flow cytometry in the peripheral $CD4^+$ and $CD8^+$ T cells. Lymphocytes were stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin at 37°C in 5% CO_2 milieu for 4 h. In order to retain the de novo synthesized cytokines in the cells, the Golgi apparatus was blocked by Brefeldin-A. Afterwards, the cell surface CD4 and CD8 molecules were stained with Quantum Red conjugated monoclonal antibodies. After the staining, erythrocytes were lysed and the membrane of lymphocytes was permeabilized. Cytokines in the Golgi apparatus were also stained with monoclonal antibodies. Cells were fixed with paraformaldehyde. The following reagents were used: human anti-CD4-QR, anti-CD8-QR, PMA, ionomycin, Brefeldin-A (Sigma), lysing solution, permeabilizing solution, anti-intracytoplasmic IL-10 (Caltag Laboratories, San Francisco, Calif., USA).

Statistical Analysis

Statistical analysis was performed using SPSS.11.0 software. We used Student's t-test when the distributions were normal, otherwise we used the Mann-Whitney non-parametric test. Data are presented as median with the first and third quartiles. The non-parametric Kruskal-Wallis test was used to compare the various subgroups of HL patients arranged according to state and therapy, and the data were presented as median and range (min-max). The p values <0.05 were considered statistically significant.

Results

Systemic Lupus Erythematosus

The ratio of $CD4^+CD25^{high}Foxp3^+$ T cells was significantly lower in SLE patients (3.06±1.45%, 3.07% (2.08, 4.22) p<0.001) than in normal controls (4.26±1.0% 4.31% (3.40, 4.91)). The absolute number of natural Treg cells also decreased in lupus (0.019±0.012x10^9/l 0.016x10^9/l (0.009,0.026)) compared to controls (0.039±0.017x10^9/l 0.038x10^9/l (0.0257, 0.048)) in significant manner (p<0.001). However, we did not found significant difference in the ratio and number of $CD4^+CD25^{high}Foxp3^+$ cells between patients with active and those with inactive disease.

The ratio of $CD4^+IL\text{-}10^+$ cells were significantly elevated in lupus patients as compared to those of measured in healthy individuals (20.92±14.01% v.s.15.5±11.65%, 18.67% (11.05, 28.3) vs. 12.95% (7.8, 16.2)) p<0.03), but their absolute number were not significantly different. Patients in the active or inactive phase of SLE did not differ as regards the ratio and the number of $CD4^+IL\text{-}10^+$ regulatory T cells.

Significant positive correlation was found between the number and ratio of $CD4^+CD25^{high}Foxp3^+$ cells (r: 0.437, p<0.001), as well as between the number and ratio of $CD4^+IL\text{-}10^+$ cells (r: 0.768, p<0.001). Neither the number, nor the ratio of the two examined regulatory cell types was found to correlate significantly with SLE-DAI, anti-dsDNS level or daily corticosteroid dose. Although, disease activity and anti-dsDNA concentration changed inversely with $CD4^+CD25^{high}$ cell number. Not surprisingly, a significant positive correlation was shown between SLE-DAI and anti-dsDNA level (r: 0.505, p<0.001), as well as between SLE-DAI and daily corticosteroid dose (r: 0.499, p<0.001), and between anti-dsDNA concentration and daily steroid dose (r: 0.367, p=0.002).

Five patients with sever acute form of SLE resistant to other types of therapy were treated with repeated plasmapheresis and followed up from the aspect of changes in the number of peripheral $CD4^+CD25^{high}$ T cells and the activity of disease (SLEDAI).The number of $CD4^+CD25^{high}$ T cells was found to be elevated and SLEDAI decreased in all the five patients one day after the last treatment of repeated plasmapheresis compared to the starting values (in patient 1.= 0.11 versus 0.042; in patient 2.= 0.072 versus 0.012; in patient 3.= 0.11 versus 0.031; in patient 4.= 0.103 versus 0.037; in patient 5.= 0.073 versus 0.012 G/L). ANOVA analysis showed that these changes in the SLEDAI and number of $CD4^+CD25^{high}$ T cells are statistically significant (p=0.0013). Linear regression pointed to the decrease of SLEDAI parallel with the increase of the number of $CD4^+CD25^{high}$ T cells (r= -0.96, r^2=0.92, p=0.008). When we summarised these elevations statistically in all patients, we found a highly significant increase at the end of the treatments compared to the starting values in the number of $CD4^+CD25^{high}$ T cells (0.093 ±0.026 versus 0.016 ± 0.006; p<0.01).

It was crucial to know whether the increase in the $CD4^+CD25^{high}$ T cells observed during repeated plasmapheresis could derive from the relatively increased concentration of the cellular part of blood during the plasmaexchange. Therefore, we analysed simultaneously the changes in the number of $CD4^+CD25^{high}$, all $CD4^+$ T cells and all lymphocytes in the perpheral blood during the series of plasmapheresis. It could be clearly seen from the data of Figure 4. that the changes in the number of all lymphocytes and $CD4^+$ cells took place in parallel. The increase in the number of $CD4^+CD25^{high}$ cells, however, was gradual, it grew treatment by treatment. Thus, this phenomenon can be regarded to be a plasmapheresis specific alteration and it can be independent of the actual presence of other subsets of lymphocytes in the peripheral blood.

Mixed Connective Tissue Disease

The percentage and the absolute number of $CD4^+CD25^{high}$ cells were significantly lower in the peripheral blood of patients with MCTD compared to healthy controls (3.5 ± 1.6 % vs 4.26 ± 1.0 %; $p<0.01$; 0.03 ± 0.017 G/L vs 0.04 ± 0.016 G/L; $p<0.04$). The percentage and the absolute number of $CD4^+CD25^+$ Treg cells were also lower in patients with active MCTD than in those with inactive disease (2.67 ± 1.2 % vs 4.0 ±1.5 %; $p<0.001$; 0.022 ± 0.011 G/L vs 0.035 ± 0.018 G/L; $p<0.01$).

There was an elevation in the percentage and absolute cell number of $CD4^+IL10^+$ cells in patients with MCTD compared to controls (6.1 ± 4.4% vs 2.2 ± 0.63 %; $p<0.002$, absolute cell number: 0.119 ± 0.09 G/L vs 0.062 ± 0.02 G/L; $p<0.02$). The percentage of $CD4^+IL10^+$ Treg cells was higher in the active stage of MCTD than in the inactive disease (10.0 ± 5.9 %, vs 4.9 ± 5.9; $p<0.005$). However, we did not find any significant difference in the absolute number of $CD4^+IL10^+$ Treg cells between the patients with active or inactive disease (0.116 ± 0.09 vs 0.118 ±0.1; ns).

Hodgkin's Lymphoma

We found a significant elevation in the absolute number of the $CD4^+IL\text{-}10^+$ (Tr1), the $CD4^+CD25^{high}$ suppressor and $CD8^+IL\text{-}10^+$ suppressor T cells in the patients with HL and patients with breast cancer compared to the healthy controls ($CD4^+IL\text{-}10^+$ in HL patients: median = 0.072 G/L(first quartile = 0.055 G/L; third quartile = 0.1 G/L cells/l); in BC patients (positive control): 0.2 G/L(0.12 ; 0.28); in the healthy control group: 0.008 G/L(0.001 ; 0.03), $p < 0.001$; $CD4^+CD25^{high}$ in HL patients: 0.084 G/L(0.037; 0.180); in BC patients: 0.053 G/L(0.038; 0.065); in healthy controls: 0.034 G/L(0.024; 0.049); $CD8^+IL\text{-}10^+$ in HL patients 0.081 G/L(0.056; 0.13); in BC patients: 0.330 G/L(0.12; 0.550) in healthy controls: 0.072 G/L(0.012; 0.095); $p < 0.001$). However, whereas the elevation in the number of $CD4^+CD25^{high}$ T cells was significantly higher and characteristic of the patients with HL, both the $CD4^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ cells were more significantly elevated in the cancer patients than in those with lymphoma. Comparing the changes in the number of circulating $CD4^+CD25^{high}$, $CD4^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ regulatory T cells, we did not find any significant change in their distribution in the various groups. Their peripheral appearance seemed to be independent of the duration of time since therapy (3–5, 6–10, >10 years), the stages of disease (stages I–II or III–IV) and the types of therapy (CT, RT or combined modality treatment).

Discussion

Systemic Lupus Erythematosus

Autoimmune disorders, such as SLE are characterised by the loss of self-tolerance. It can partly be attributed to alterations of regulatory cell populations. Sakaguchi described at first, that a certain group of $CD4^+$ T-cells has special role in controlling autoreactive T cells. Later these cells have been identified as $CD4^+CD25^{high}$ natural regulatory T-cells. Different experiments confirmed, that the loss or the reduction of nTreg cells lead to the development of autoimmune disorders, while administration of these cells may give reversal to autoimmune phenomena. Based on these observations it could be hypotesized that $CD4^+CD25^{high}$ cells may have important role in the development of human SLE. However, only few papers concerning with the role of nTreg cells in human lupus have been published in the literature. In the present study we measured the ratio and the number of natural regulatory T cells in SLE patients and found both to be reduced. This may indicate that the hampered nTreg cell ratio can be attributed to the reduction in the number of these cells. Unequivocally, Miyara *et al.* evidenced that the decrease in the proportion of nTreg cell can not be attributed to their accumulation in lymph nodes or other affected target organs, nor to be killed by soluble factors, but they demonstrated *in vitro* a heightened sensitivity to Fas-induced apoptosis.

Data are conflicting as regards the association between these cells and the activity of the disease. Liu *et al.* examined 94 lupus patients and they also found reduced $CD4^+CD25^{high}$ cell number in patients, but similar with our results there was not statistical significant difference between patients with active and inactive disease. Analysing their publication in more details it can be realised that they had data about the activity of lupus only in 21 patients, while in our present study the disease activity of all patients were characterised by clinical and laboratory parameters. Crispin *et al*. analysed the data of 30 SLE patients and revealed lower $CD4^+CD25^{high}$ cell number in those with active lupus as compared with inactive disease. It is important that they entered only untreated patients in the study. It calls the attention for the possible modifying role of immune suppressive therapy. Karagiannidis *et al*. described that in asthma patients steroid treatment up-regulated the expression of Foxp-3 and therefore the number of nTreg cells. Suarez *et al*. published enhanced percentage of $CD4^+CD25^{high}$ cells in 110 unselected lupus patients. This increment was unrelated to clinical manifestations but correlated with glucocorticoid therapy. We speculate that the number and ratio of $CD4^+CD25^{high}$ regulatory cells in our study did not decreased further in patients with active SLE, as they received higher daily corticosteroid doses that could up-regulate the Foxp-3 expression. The fact that there were no significant differences in the number and ratio of both natural and inducible Treg cells between active and inactive patients may also reflect that immune regulatory abnormalities exist even in the inactive phase of the disease and in treated patients as well. Natural regulatory T cells can well be candidates for therapeutic manipulations. La Cava *et al.* used the strategy of tolerizing lupus-prone mice with an arteficial peptide based on sequences commone to several anti-dsDNA antibodies, and $CD4^+CD25^{high}$ cells were raised under such circumstances. The same team used another tolerizing strategy, a minigen vaccination with DNA encoding T cell epitopes presented by MHC class I molecules, which produced similar effect on nTreg cells as described it Hahn.

$CD4^+CD25^{high}$ cells are able to inhibit anti-dsDNA production in B cells, induced by T-helper cells, as it was reported by Fields et al. They suggest that nTreg cells suppress the activity of SLE through the inhibition of anti-dsDNA production. Similarly, Lee *et al.*found inverse correlation between CD4+ regulatory T cell population and anti-dsDNA antibody

levels in pediatric patients with SLE. In our study, there was no statistically significant correlation between $CD4^+CD25^{high}$ cells and anti-dsDNA concentration, however we found a significant weak negative relation between these parameters.

Natural regulatory T-cells are supposed to suppress immune reactivity through two pathogenic pathways, i.e. cell-cell contact and immunosuppressive cytokines, such as IL-10. In cell-to-cell contact CTLA-4 molecule and GITR, expressed on $CD4^+CD25^{high}$ cells, seems to have special importance. Anti-CTLA-4 therapy inhibited immunosuppressive effect of $CD4^+CD25^{high}$ cells in an experimental colitis model and increased autoantibody production. On the other rout cytokines, among the others such as IL-12 and especially anti-inflammatory IL-10 play important role in performing suppressore activity nTreg cells. Crispin *et al.* measured reduced IL-12 and high IL-10 concentartions in the supernatants of PBMC obtained from active SLE patients. $CD4^+$ cells expressing intracytoplasmic IL-10 also belong to regulatory cells and called as Treg1. These cells develop from peripheral $CD4^+$ cells in consequence of *in vivo* or *in vitro* stimulation/induction by antigens. Vieira has shown that Treg1 cells (inducible regulatory T cells) are present in the lack of natural regulatory cells and their activity does not require Foxp-3, however $CD4^+IL\text{-}10^+$ cells have similar suppressive influence as $CD4^+CD25^+$ cells. It is known that serum IL-10 concentration is elevated in SLE patients. Based on that, efforts were performed in few pilot studies with anti-IL-10 therapy, however results were not so promising. Previously we and others found that not also serum IL-10 concentration but the ratio and/or number of $CD4^+IL\text{-}10^+$ cells increased mainly in patients with active lupus. We described the modulating effect of plasmapheresis on $CD4^+$ T cell subgroups. According to our present findings the ratio, but not the absolute number of Treg1 cells were significantly elevated in SLE patients as compared to controls, but there was not significant difference as regards disease activity. This probably indicates that $CD4^+IL\text{-}10^+$ cells are under permanent antigenic stimuli *in vivo* even in inactive phase of SLE, and that the increase of Treg1 cell ratio within the $CD4^+$ cell population can be a compensatory mechanism.

According to our present results and previous literature data we can conclude that the number and the ratio of $CD4^+CD25^{high}$ cells decreased and those of $CD4^+IL\text{-}10^+$ cells increased irrespective to or in association with clinical activity of lupus, and this probably has important role in the pathogenesis of SLE. Alterations shown in inactive phase of lupus may indicate the persistance of distrubed immune regulation even in clinically asymptomatic patients, however corticosteroid therapy and other immunosuppressants may prevent the development of more profound alterations, expected in the active phase of SLE. To confirm this hypothesis further studies are required.

Plasmapheresis is known to decrease the levels of circulating immune complexes and concentration of pathologic autoantibodies. Consequently, it may control immune complex and autoantibody mediated diseases, such as myasthenia gravis or different types of rapidly progressive glomerulonephritis. It was Jones *et al.*, who first indicated plasmapheresis in SLE. We are careful about recommending plasmapheresis as a general treatment for SLE, but this procedure may be useful in some severe cases of disease, mainly in those which are accompanied with kidney and central nervous system involvements or with pulmonary haemorrhage, or secondary anti-phospholipid syndrome. According to previous data, plasmapheresis has some immunomodulating effects. It can induce the activation of complement system, increases the complement receptor 1 (CD35) expression on lupus erythrocytes. Both effects can improve the defective immune complex clearing in SLE patients.

In our previous work, we described the effect of plasmapheresis on Th1/Th2 balance. In this study we found that as the number of $CD4^+CD25^{high}$ T cells increased after plasmapheresis, also the activity of SLE (the values of SLEDAI) decreased. This inverse

relation can be an indirect provement for the activity of this type of suppressor cells appearing in elevated percent and number in the peripheral blood of SLE patients after repeated plasmapheresis treatments.

In addition, we could observed the elevating effect of plasmapheresis on the number of the peripheral CD4+CD25high T cells not only in SLE but also in patients with myasthenia gravis and Guillain-Barré syndrome, but following a little bit other kinetics than was found in SLE. (data are not published).

It also has to be considered, however, that Liu and coworkers describing the decrease in the level of $CD4^+CD25^+$ T cells in SLE patients did not find a correlation between the percent of these suppressor cells and the individual values of SLEDAI. But without knowing how their patients took their drugs, how they were treated pharmacologically during the measurements, it is hardly possible to accept the final conclusion of their measurements. (In their paper there is no information about the drug treatments for example.) We suppose that in their patients with high percent of $CD4^+CD25^+$ T cells and with low SLEDAI, the elevated percent of suppressor cells just derives from the high dose of glucocorticoids used for the treatment, increasing the frequency and the FOXP3 expression of this type of suppressor cells in the periphery. In our measurements, it had to be stressed that there was no change in the dose and the type of drugs taken by the patients during the series of plasmapheresis. That is why we can regard the changes found on the suppressor cells and SLEDAI to be plasmapheresis related alterations. We do think that a strong correlation can be between the significant elevetion of $CD4^+CD25^+$ T cells and the decrease in SLEDAI induced by repeated plasmapheresis. Furthermore, as we carried out measurements of self-control type, we did not need other groups of controls for the plasmapheresis study. In our system the relation between the elevated number of suppressor cells induced by plasmapheresis and the decreased values of SLEDAI can be clearly observed. The determination of the suppressor activity of $CD4^+CD25^{high}$ T cells before and after plasmapheresis seemed to be technically as difficult that although we tried it, we had to give it up from ethical and theorethical points of view.

At the same time, we are aware of the limitation of the use of plasmapheresis in the treatment of SLE. Especially the increased chance for infections may have a current danger.

In summary, we present here the first data on the significant elevation in the number of peripheral $CD4^+CD25^{high}$ suppressor T cells in SLE patients treated by repeated plasmapheresis resulting in clinical improvements, reflected in the remarkable decreases in the values of SLEDAI. Thus, this beneficial effect can be one of the reasons to use this additional method for the treatment of SLE. The application of plasmapheresis, however, may have some limitations and needs serious considerations from the aspect of infections during the treatment of autoimmune patients.

Mixed Connective Tissue Disease

The purpose of this study was to determine the number of $CD4^+CD25^{+high}$ and $CD4^+$IL-10 Treg cells in the peripheral blood of patients with MCTD. Decreased $CD4^+CD25^{+high}$ T cell number have been reported to be associated with various autoimmune disorders. Liu et al. described low levels of $CD4^+CD25^{+high}$ suppressor T cells in the peripheral blood of patients with SLE. Monk et al. also found that the decrease of $CD4^+CD25^{+high}$ regulatory T cells may contribute to the pathogenesis of SLE, furthermore functional abnormalities of this cell type may also exist. The number of $CD4^+CD25^{+high}$ cells

has been described to be reduced in patients with myasthenia gravis, while in the inflamed synovium of patients with rheumatoid arthritis (RA) this cell type has been shown to be elevated. Pop et al. reporteded a decreased Foxp3 and TGF-beta1 coexpressing $CD4^+CD25^+$ regulatory T cell number during autoimmune diabetes. On the other hand, Putnam et al. did not find significant differences in the number or in the in vitro regulatory function of $CD4^+CD25^{+high}$ T cells in chronic human type 1 diabetes subjects.

In accordance with previous findings, we found a decreased percentage and absolute number of $CD4^+CD25^{+high}$ T suppressor cells in the peripheral blood of patients with MCTD as compared to healthy controls. Interestingly, in the active stage of the disease the number of $CD4^+CD25^{+high}$ Treg cells showed further decrease.

Type 1 regulatory T cells are induced mainly in the presence of IL-10, produce high levels of IL-10 and TGF-beta and suppress activation and cytokine production of effector cells in a cell contact-independent but IL-10 dependent manner. Level of IL-10 and TGF-beta secreting T cells were shown to be elevated in the synovial membranes of patients with reactive arthritis compared to RA. In addition, $CD4^+$IL10 Treg regulatory cells inhibit T cell expansion in vivo and in certain murine disease models such as experimental autoimmune encephalomyelitis (EAE) via IL-10 dependent mechanisms. In humans, Tr1 cells limit immune response to Mycobacterium tuberculosis antigens and regulate HIV replication . The role of $CD4^+$IL10 Treg cells in different infections is discussed by O'Garra et al.. Tr1 cells are able to regulate both Th1 and Th2 responses while Th1 and Th2 cells also reciprocally regulate the development and function of each other.

The biological basis for the reduction and dysfunction of Treg cells in MCTD remains unclear. However, in a murine model of asthma, it has been demonstrated that IL-6 and soluble IL-6 receptor (sIL-6R) together decrease Treg cell number and function in the lungs. In sera of patients with MCTD high levels of IL-6 have been described. Elevated serum levels of both IL-6 and sIL-6 may play an important role in the Treg cell development and function. Binding of IL-6 to the membrane bound IL-6 receptor (mbIL-6R) can suppress the proliferation of $CD4^+CD25^{+high}$ T cells. TGF-beta has also been shown to inhibit the IL-2 dependent T cell proliferation. In addition, high level of IL-10 in serum of patients with MCTD has been reported.

Similarly to previous data we found an increase in the percentage of IL-10 expressing CD4+ T cells in MCTD patients in comparison to controls, as well as in patients with active and inactive disease.

The above-mentioned data suggest that the chronic inflammation in patients with MCTD leads to the elevation of IL-6 and IL-10, therefore the high level of these cytokines modulate the number of regulatory cells. One of the effects of elevated IL-10 can be that it induces Tr1 proliferation while high level of IL-6 can suppress the proliferation of naturally occurring $CD4^+CD25^{+high}$ T cells. However, the exact mechanism of regulation and the interaction between Tr1 and $CD4^+CD25^{+high}$ T cells still remains unclear.

Hodkin's Lymphoma

Our new observations are that the number of all the three types of immunoregulatory T cells, $CD4^+/CD25^{high}$, $CD4^+/IL\text{-}10^+$ and $CD8^+/IL\text{-}10^+$ lymphocytes are elevated in the peripheral blood of patients with HL being in a long-lasting complete remission (not taking any drugs) compared to the healthy controls. The increase in the number of $CD4^+/CD25^{high}$ cells seems to be characteristic of HL because it is elevated only in this disease compared to the carcinoma patients and healthy controls. In the patients with breast carcinoma, however, the number of

$CD4^+/IL\text{-}10^+$ and $CD8^+/IL\text{-}10^+$ T cells is increased compared to the Hodgkin's patients and healthy controls.

The elevation in the number of $CD4^+/CD25^{high}$ cells in the peripheral blood reflects the same tendency found earlier in the lymph nodes of Hodgkin's patients. In addition, Sasada et al. demonstrated that the percentages of $CD4^+/CD25^{high}$ T cells were elevated in gastrointestinal malignances. In their experiments, patients with higher percentages of $CD4^+/CD25^{high}$ T cells had a poorer prognosis compared to the patients with lower percentages. In addition, the fludarabine therapy was able to significantly decrease the elevated frequencies and suppressive function of $CD4^+/CD25^{high}$ T cells in patients with chronic lymphocytic leukemia. Our results show that the distribution of these cells seems to be independent of the duration and stage of disease and the type of therapy applied in HL patients. It has to be stressed that these elevations in the number of immunosuppressive regulatory T cells were found to be independent of therapy. Therefore, we do suggest here the existence of a specific correlation between the number of $CD4^+/CD25^{high}$ T cells and HL itself. Our additional finding is that beside $CD4^+/CD25^{high}$ cells, the numbers of the two other forms of immunoregulatory T cells, $CD4^+/IL\text{-}10^+$ (Tr1) and the newly recognized $CD8^+/IL\text{-}10^+$ T cells are also increased in Hodgkin's patients compared to the controls, sustaining an immunosuppressive state in the peripheral blood what is also independent of the duration and stage of the disease and the types of therapy. In the peripheral immunosuppression found in patients with breast carcinoma, however, the dominating role of $CD4^+/IL\text{-}10^+$ and $CD8^+/IL\text{-}10^+$ T cells seems to be more significant than that of $CD4^+/CD25^{high}$ cells.

This study has been part of a more complex work on peripheral lymphocyte subsets in patients with HL. During these experiments we could confirm these earlier, well-known observations that the decreased CD4/CD8 ratio, the increased expression of $CD3^+/HLA\text{-}DR^+$ and $CD3^+/CD69^+$ cells were really characteristic of the lymphocytes of patients with HL. In addition, we could not find any correlation between the elevation in the $CD4^+/CD25^{high}$ T cells and Epstein-Barr virus infection, or the circulating level of IL-10 in HL patients. None of these data have been published to date.

We conclude that the measurement of the number of $CD4^+/CD25^{high}$ T cells in the peripheral blood of Hodgkin's patients can demonstrate some disease-specific change in the immunoregulation in these patients. However, further investigation is needed on how to answer the following two questions: (a) Is the increased number of $CD4^+/CD25^{high}$ cells (immunosuppression in periphery and in the lymph nodes) a predisposing factor for the disease? (b) Is the increased number of $CD4^+/CD25^{high}$ cells (immunosuppression in the periphery and in the lymph nodes) a consequence of the disease manifested already?

Summary

One of the most important features of the immune system is the ability to discriminate between self and non-self, and based on this discrimination, to tolerate self antigens. This immunological tolerance can be acquired by central or peripheral mechanisms. Although immunological tolerance means lack of responsiveness towards self antigens, it also includes active processes, like suppression, which is controlled by specific T cells subsets. These regulatory T lymphocytes (Tr) include CD4+/CD25+^{bright} and interleukin (IL)-10 producing CD4+ cells.

To gain a better understanding on the underlying immunological mechanisms contributing to the development of systemic autoimmune diseases and malignant conditions, we aimed at investigating the distribution of Tr cells in patients with systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Hodgkin's lymphoma and breast cancer. Absolute and relative numbers of CD4+/CD25+^{bright} and CD4+/IL-10+ T cells were determined by flow cytometry in the peripheral blood of 72 patients with SLE, 48 patients with MCTD, as well as in 94 Hodgkin's disease and 47 breast cancer sufferer. Results were compared to those from …healthy volunteers. We also compared the distribution of Tr cells between active and inactive stage of SLE and MCTD. Five SLE patients underwent repeated plasmapheresis treatment. The Tr subsets in these patients were closely monitored and the results were compared to other laboratory and clinical data during the course of plasmapheresis treatment.

Our data show that the percentage and absolute number of CD4+/CD25+^{bright} cells was decreased in SLE and MCTD patients compared to healthy controls. There was no significant difference between SLE and MCTD subjects. However, in MCTD patients with active disease, the absolute number of CD4+/CD25+^{bright} Tr cells was significantly lower than in those with inactive disease. No difference in the absolute number of CD4+/CD25+^{bright} Tr cells between active and inactive SLE patient was observed, though. In SLE patients undergoing plasmapheresis, the number of CD4+/CD25+^{bright} Tr cells increased gradually. Remarkably, the increase in the number of CD4+/CD25+^{bright} Tr cells was coupled with a decrease in disease activity index (SLEDAI). Regarding CD4+/IL-10+ T cells, their frequency was significantly elevated in both autoimmune diseases. Moreover, the absolute number of CD4+/IL-10+ T cells was significantly higher in MCTD, but not in SLE patients compared with healthy controls. We could not find significant differences between active and inactive MCTD and SLE patients regarding the percentage or absolute number of CD4+/IL-10+ T cells. Our results reflect impaired immunoregulatory mechanisms in SLE and MCTD, characterized by decreased number or percentage of CD4+/CD25+^{bright} cells. The ratio of these cells seems to fluctuate during the course of the disease, and lower numbers are associated with active disease. The higher frequency of CD4+/IL-10+ T cells may reflect counterbalancing process.

Hodgkin's lymphoma patients, as well as breast cancer patients were characterized by elevated numbers of CD4+/CD25+^{bright} and CD4+/IL-10+ T cells compared to controls. Moreover, Hodgkin's lymphoma patients had significantly increased number of CD4+/CD25+^{bright} T cells than breast cancer patients, while the latter had significantly higher number of CD4+/IL-10+ T cells compared to those with Hodgkin's lymphoma. Duration and stage of the disease, as well as the type of therapy did not significantly affect these findings, suggesting profound immunoregulatory abnormalities. Increased numbers of regulatory cells may suppress immune response against altered self-antigens that can occur in malignant diseases, and thereby contribute to the development and propagation of the tumor.

Our data suggest that impaired immunoregulatory mechanisms may play an important role in the development and maintenance of autoimmune and malignant diseases.

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