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Expression of immunomodulatory genes in inflammatory and autoimmune diseases

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Abstract

Immune response in inflammation has a wide complexity, involving APC-lymphocyte and lymphocyte-lymphocyte communication. Fine modulation of the immune response is delivered by the interaction between co-stimulatory molecules, on which the final outcome of the immune response depends. Evaluation of the expression profile of certain co-stimulatory genes and cytokines in inflammation may clear some of this complexity. Hence a PCR method is proposed in order to asses mRNA presence and quantity in some pathologies. We proposed to establish standard "yes or no" condition to the PCR in order to confer a semi-quantitative attribute to our PCR. We constructed primers for the following genes: GITR, GITRL, IL-2, CD25, CD28, CTLA-4, TGF-β1 and IL-18. The GAPDH gene was used as internal control. Samples were collected from patients with tonsillitis, rheumatoid polyarthritis, systemic lupus erythematosus and voluntary subjects as controls, isolating lymphocytes from blood and tissue, and extracted the total mRNA, followed by reverse transcription to cDNA. We confirmed quality and quantity of the total mRNA using GAPDH. Afterwards we performed PCR of the above mentioned genes.

The obtained results demonstrate that there are significant differences in the expression of these genes in response to inflammation.

Keywords: gene expression, inflammation, costimulatory genes, immune modulation, lymphocytes

Introduction

Inflammation is the natural response of the organism against external or internal pathogen agents or altered internal factors. The innate and adaptive immune responses are complex processes during which a lot of cells are involved with their specific receptors and corresponding ligands by which the inter- and intracellular communication is realized.
Through this "cross-talk" between receptors from the same or different cells many signals arise, and these signals determine if the immune defense is triggered, and the type, localization, duration, and intensity of the immune response, or if the cells remain indifferent to the antigen and become anergic [1]. The classical immunology explains lymphocyte activation with the two signal model in which the antigen specificity depends on the first signal, and the second signal, called co-stimulatory signal, and defines the type, intensity, duration and localization of the immune defense.

Hence, the first signal resulted through antigen presentation by the MHC on APC towards the TCR system of the T lymphocyte, system which involved the TCR and the CD4, CD8, CD3 co-receptors. The second signal results from the interaction between the B7.1

(CD80) and B7.2 (CD86) co-stimulatory receptors from APC and the CD28 receptor of the T lymphocyte, finalized in positive co-stimulation [2]. CD28 expression was followed by the expression of CTLA-4 (CD152), which is the alternative receptor for B7.1 (CD80) and B7.2 (CD86), and resulted in negative co-stimulation. Other genes such as IL-2Rα, IL-2 [3] in their promoter regions contain CD28 inducible elements and are considered immunomodulatory genes. As some other co-stimulatory genes were identified as TNFs (ex. GITR, OX40, 4-1BB), the high complexity of the immune modulation is obvious.
Recent studies emphasized that CD4+CD25+ and CD8+CD25+ Tregs (regulatory T lymphocytes) are important players in immune regulation, alongside the APC and responder T lymphocytes (CD4+CD25-) [3, 4].

A specific microenvironment given by cytokines (IL-2, TGF B, IL-6, IL-10 etc.) is required for lymphocyte (APC, T responder, Treg) "cross-talk", as reported by Stephens et al. [4].

As a consequence of the aforementioned mechanisms, the immune cells enter into a costimulatory dialog, signals are transmitted in both directions, and it depends on these signals if the lymphocytes are activated, differentiated, transformed in effectors or memory cells, or if the lymphocytes enter in anergy and apoptosis [5, 6].
Accumulated data indicate a functional hierarchy of the costimulatory receptors and ligands, involved in the "fine–tuning" [7] of the immune response.
In this study we aimed to assess mARN expression of certain immunomodulatory genes in human ex-vivo cellular systems in order to evaluate gene expression profile in inflammatory pathology. To obtain gene expression profile we determined the percent of positive samples for the studied genes, and the expressed relative quantities. These relative quantities were compared to the relative quantities obtained for the control group. To obtain the mRNA expression profile, semi-quantitative valuation of the mRNA transcripts was performed for the following genes: *TGFβ1 (Transforming growth factor beta 1) (NM_00066019), GITR (Glucocorticoid-induced tumor necrosis factor receptor) (NM_148902), GITRL (Glucocorticoid-induced tumor necrosis factor receptor ligand) (NM_005092), CD28 (NM_006139), CTLA-4 (Cytotoxic T-Lymphocyte–associated antigen 4) (NM_005214), IL-2Rα (CD25) (Interleukin 2 receptor, alpha) (NM_000417), and the genes of various cytokines such as: IL-2 (Interleukin 2) (NM_000586), IL-18 (Interleukin 18, interferon-gamma-inducing factor) NM_001562)(NCBI accession numbers -www.genenames.org).*

Materials and methods:

Experimental model

Previous studies showed modified immunmodulatory gene expression in the rodent inflammation model [8, 9, 10].

To investigate gene expression profile in humans we carried out a PCR based analysis in acute and chronic inflammation and controls, respectively. For this purpose peripheral blood samples were collected, from 19 volunteer controls and also from 42 samples of tonsils used as acute inflammation models. Also, peripheral blood samples were collected from 8 rheumatoid polyarthritis patients and from 4 systemic lupus erythematosus patients, and used as chronic inflammation model.

mRNA analysis

Total lymphocytes were isolated from integral peripheral blood, and the infiltrate derived from tissues was processed and stored frozen at -85 °C until use. Total RNA was extracted in all cases starting from same number of viable cells, from $10x10^6$ cells. The total RNA was extracted using *Trizol ® LS Reagent* (Invitrogen), according to the manufacturer's

instruction. Concentration of RNA was determined at 260 nm using UV/VIS (*VarianCarry*) spectrophotometer, and to determine the purity of the obtained RNA, the A_{260}/A_{280} ratio was calculated. To confirm the presence and quality of the extracted RNA, electrophoresis of the RNA was performed in agarose gel visualized with ethidium bromide. Equal amounts of RNA from different samples were used for reverse transcription in cDNA, using *iScript ™ cDNA Synthesis Kit* (BioRad). The cDNA obtained was used for gene specific PCR. Primers were designed excluding the possibility of genomic DNA amplification and according to specific amplification of possible splice variants of the mRNA. PCR optimization was performed for each primer pair to obtain specific amplification products and to interpret the results semi-quantitatively. First the optimal hybridization temperature of the primer pairs was established in order to obtain specific amplicon. Secondly, dose dependent PCR was utilized when the optimal number of cycles was determined such that the amplification process to be in logarithmic phase. This provided semi-quantitative analysis of the amplicons and their comparison to the control group, and the obtained data were relative values of the intensities. We used the following primers: TGFβ_1 5'- GCC CTG GAC ACC AAC TAT TGC T -3' and 5'- AGG CTC CAA ATG TAG GGG CAG G-3'; IL-2 5'- GCT ACA ACT GGA GCA TTT ACT GCT G -3' and 5'- CTA CAA TGG TTG CTG TCT CAT CAG C-3'; IL-2Rα 5'- GAT GGA TTC ATA CCT GCT GAT GTG G -3' and 5'- TCC ACT GGC TGC ATT GGA CTT TGC A -3'; GITR: 5'- TTG GAA CAA GAC CCA CAA CG -3' and 5'- GGC ACC TCC AGC AGC AGC T -3'; GITRL 5'- CTT TAA GCC ATT CAA GAA CTC A -3' and 5'- CCC AAC ATG CAA TTC ATA AGT CC-3'; 5'- ATG CTC AGG CTG CTC TTG GCT -3' and 5'- TCA GGA GCG ATA GGC TGC GA -3'; CTLA-4: 5'- CTT CTC TTC ATC CCT GTC TTC TGC -3' and 5'-ATT GCT TTT CAC ATT CTG GCT CTG-3'; IL-18: 5'- GCT TGA ATC TAA ATT ATC AGT C -3' and 5'-GAA GAT TCA AAT TGC ATC TTA -3'; Amplification products were separated in 1,5% agarose gel electrophoresis and visualized in UV in the presence of ethidium bromide. As far as the identified variants of the genes are concerned, we identified more variants, but we used for this study as follows: for GITR var1; CTLA-4: var1 and2; for CD28 var1 (NCBI nucleotide database). The internal control used for PCR was GAPDH (NM_002046) housekeeping gene. Also, GAPDH expression was used for data normalization.

Statistical analysis

Total amount of the amplification products was calculated using the volumetric analysis method of *QuantityOne* (BioRad), and the obtained data was processed using Excel and Matlab. Briefly, background was extracted using the software, compared always to the relative gel, afterwards GADPH extraction was performed manually using Excel. Graphical presentation of the data was performed in Matlab, using the box plot method. Each box has lines at the lower quartile, median, and upper quartile values. Student's t tests were used where two groups of the intensity values were compared. P value of less than 0.05 was considered statistically significant.

Results

Gene expression profile in the control group

First, the gene expression on the control group was evaluated. *TGF B1* was expressed on more than 60% of the controls as presented in Figure 1. *GITR* was expressed in a few cases (3 from total number of 19). *GITR ligand* appears only in one case (n=19). The presence of *GITR* and *GITRL* in relatively high quantities in the same control can be explained with the uncertain immunological history of the control.

Obtained data shows weak expression of the *CD25*, *IL-2*, *CD28*, *IL-18* immunostimulatory genes in a few controls (5 cases for *CD25*, 3 cases for *IL-2*, 3 cases for *CD28* and 8 cases for

IL-18, from the total of 19 controls) presented in Figure 2 . We obtained similar data *CTLA-4 var1* and *var2* (3 cases).

These results suggested that the control group's expression pattern may be described by high expression of the *TGFB1* genes, absence of *GITRL* and moderate expression of the *GITR*, *CD25*, *IL-2*, *CD28*, *CTLA-4 var1, CTLA-4 var2* and *IL-18* genes.

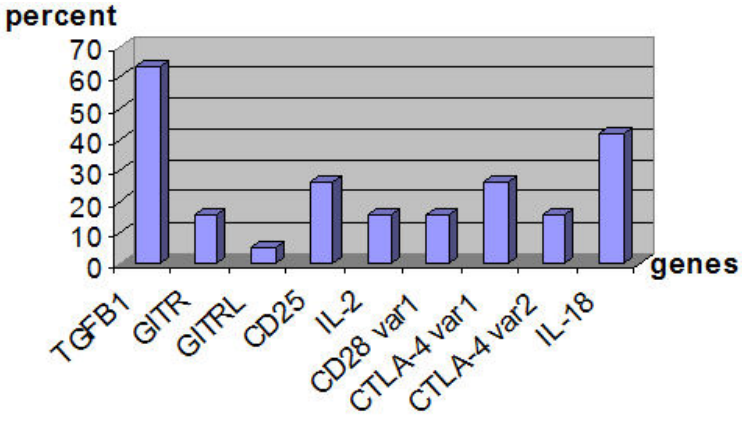

Figure 1. Percent of the positive controls for certain immunomodulatory genes

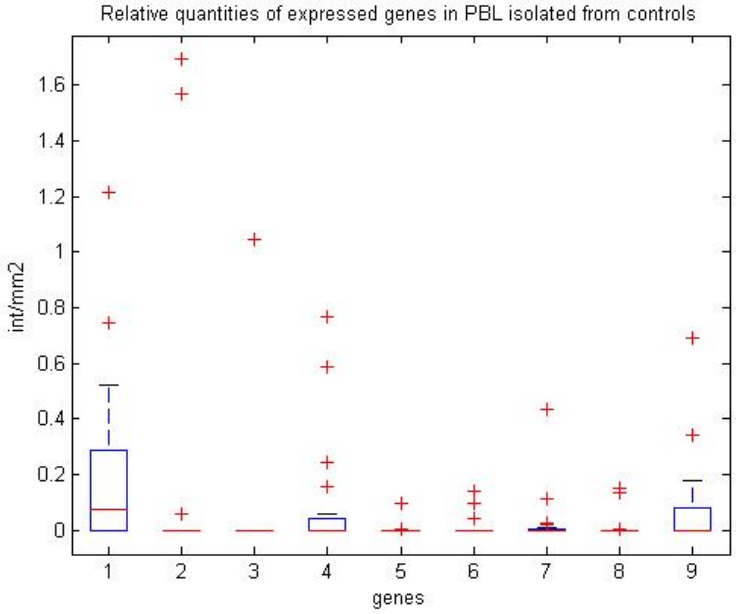

Figure 2. Relative quantity expressed in PBL isolated from controls (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

Gene expression profile in acute tonsillitis

Immunomodulatory gene expression screening in acute tonsillitis shows more than 80% positive patients for the studied genes excluding *GITRL*. *GITRL* was expressed in half of the tonsillitis patients, all in high quantities, as presented in Fig 3. Compared to the control group, the relative *TGFB1* expression was upregulated. *GITR var1* was upregulated in tonsillitis patients (95%) in contrary to the control group where *GITR var1* was expressed in 15% of the studied controls. The *GITRL, CD25*, *IL-2*, *CD28*, *CTLA-4 var1, CTLA-4 var2* and *IL-18* genes were significantly upregulated ($p<0.05$) in acute tonsillitis patients, compared to the controls as presented in Figure 4. The real expression profiles in controls as opposed to the tonsillitis patients were shown in the image of the obtained gels, as presented in Figure 4.

Expressed relative quantity from tissue infiltrate in acute tonsillitis (1. TGF B1, 2.

GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

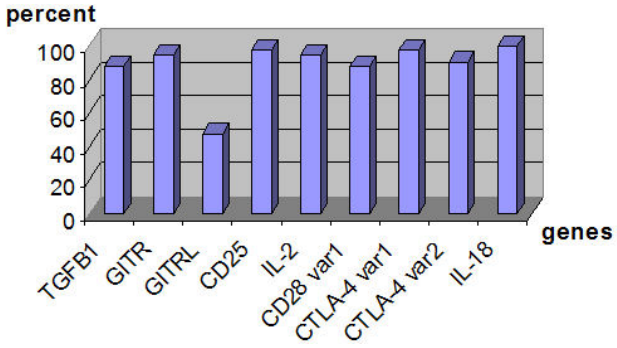

Fig 3: Percent of the positive samples in acute tonsillitis

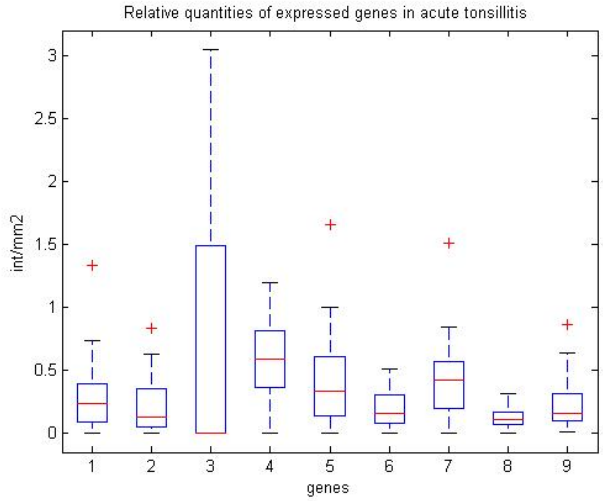

Figure 4. Expressed relative quantity from tissue infiltrate in acute tonsillitis (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

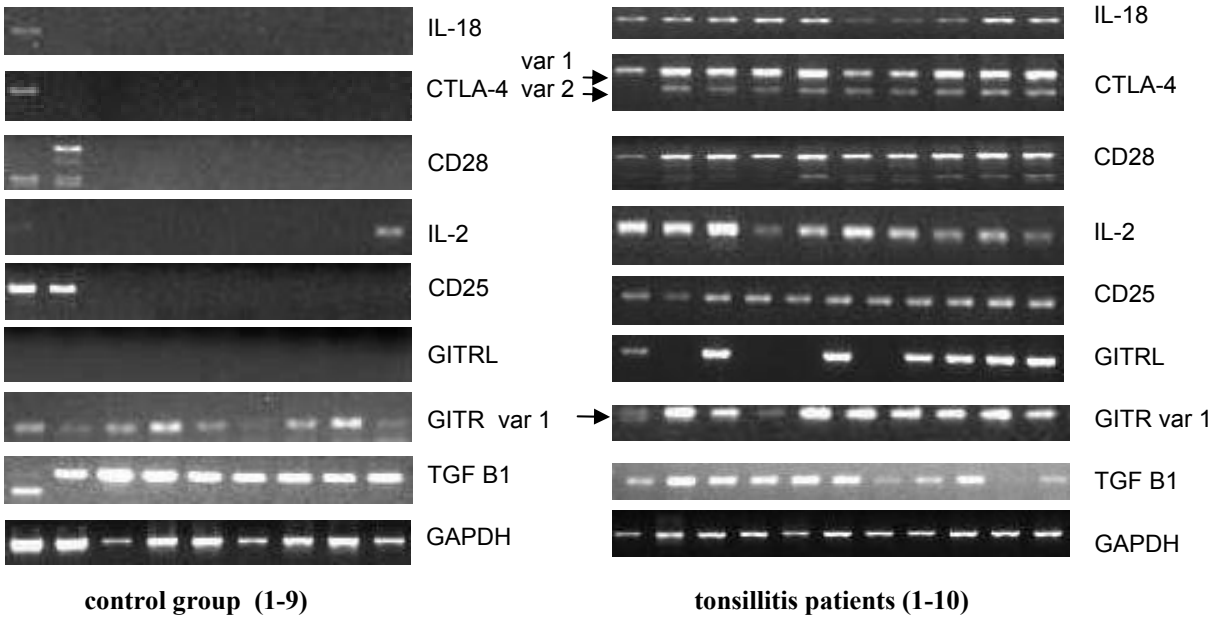

Figure 5. PCR analysis of the mRNA expression in the control group and the acute tonsillitis patients. The PCR products were analyzed by 1,5% agarose gel electrophoresis

Gene expression profile in rheumatoid polyarthritis

Obtained data shows that all genes taken into study are expressed in 100% of rheumatoid polyarthritis samples. Exceptions were GITRL which was expressed in 50% of the cases (4 from a total of n=8) and IL-2, which lacks completely (Figure 6). Enhanced relative quantities of gene expression were demonstrated in the majority of the studied genes, as presented in Figure 7. The comparison to the control group indicated significant *TGFB1, GITR, GITRL*, *CD25, CD28 var1*, *CTLA-4 var1*, *CTLA-4 var2* and *IL-18* gene upregulation ($p<0.05$), probably as a result of inflammation. *IL-2* lacks, accompanied by consecutive *CD25* upregulation.

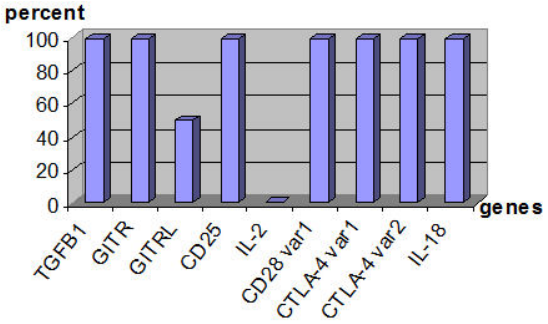

Figure 6. Percent of the positive samples in rheumatoid polyarthritis

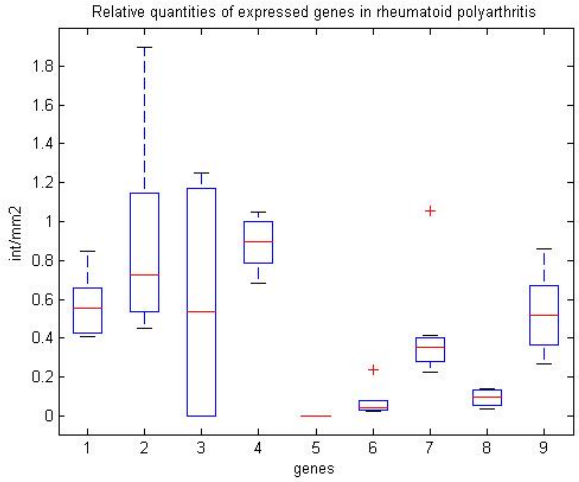

Figure 7. Relative quantities of gene expression from PBL in rheumatoid polyarthritis (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

Gene expression profile in systemic lupus erythematosus

Our study indicated highly similar gene expression profile in systemic lupus erythematosus and in rheumatoid polyarthritis. The percent of positive samples for the studied genes was the same as in rheumatoid polyarthritis, but *GITRL* was expressed in 50% of the cases (2 from the total of n=4) and *IL-2* was expressed only in a single case (25%, total n=4), as presented in Figure 8. Similarly, the relative quantity expressed of these genes was the same as in rheumatoid polyarthritis (Figure 9).

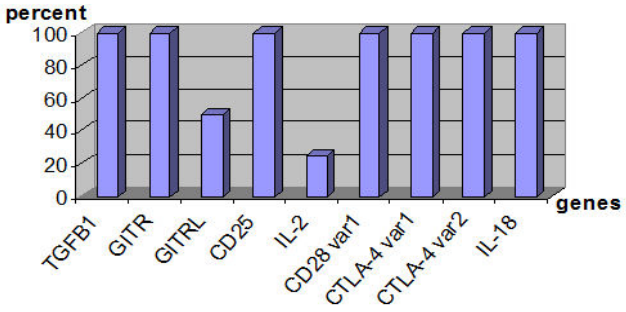

Figure 8. Percent of the positive samples in systemic lupus erythematosus

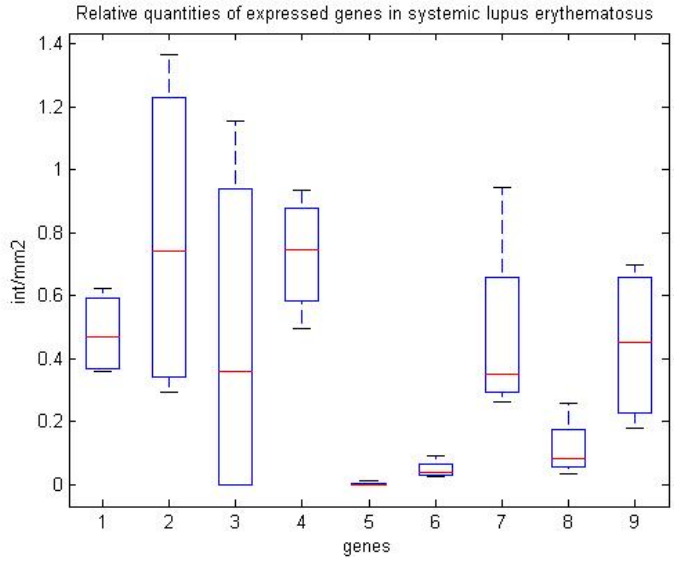

Figure 9. Relative quantities of gene expression from PBL in systemic lupus erythematosus (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

Discussion

GITR is expressed in activated macrophages [12] and in mononuclear cells, and *GITR* is upregulated upon activation of the $CD4^+CD25^-$ responder T lymphocytes. *GITR* is used as phenotypic marker for $CD4^+CD25^+$ T suppressor lymphocytes. Murine GITR is a promoter of effector T response in CD4 and CD8 T subpopulations, and following physiological triggering at also renders Treg cells ineffective by inducing their proliferation. In a murine model there is an important interaction between GITR and CD28, upregulating each other [13].

GITRL is constitutively expressed on: macrophages, dendritic cells (DC), B lymphocytes, endothelial cells, thymocytes and activated T lymphocytes. As Tuyaers reports, hGITRL expression is not detectable in different PBMC subsets, but is detected on the HUVEC line EA.Hy92 and on the EBV-transformed B cell lines 888-EBV, 1087-EBV and 1088-EBV [14]. Recent studies show elevated *GITR* and *GITRL* expression in macrophages from human atherosclerotic plaques [11], in staphylococcal enterotoxin B stimulated human PB monocytes [12] and synovial fluid macrophages and PB from RA patients [15].

According to our screening GITRL was not expressed on the PB lymphocytes control group.

There is also evidence, that murine GITRL autostimulates GITR on CD8 lymhocytes, and has a key role in fine-tuning, which in this subpopulation GITR seems to exceed in importance CD28 [13]. Stimulation of the synovial fluid macrophages with *anti-GITR mAb* resulted in dose dependent induction of the inflammatory cytokines [6, 15].

In concordance with this data we obtained high levels of *GITR* and *GITRL* mRNA from RA patients compared to the control group. So in our study, GITR-GITRL system seems to have a proinflammatory behavior in both acute and chronic inflammation.

According to these data our gene screening showed *IL-18 mRNA* upregulation in PB lymphocytes of RA patients. We could not detect any variation in the *IL-2 mRNA* levels in comparison to the healthy controls but the *CD25 mRNA* was found to be upregulated in arthritis and lupus samples. *IL-2* is required for $CD4^+CD25^-$ responder T lymphocytes proliferation in the presence of the $CD4^+CD25^+$ Tregs. Similarly, *IL-2* secreted by $CD4^+CD25^-$ responder T lymphocytes is needed for $CD4^+CD25^+$ Treg function [4,16]. According to Thornton (1998) *IL-2 mRNA* can not be expressed by stimulated $CD4^+CD25^+$ T lymphocytes or by unstimulated $CD4^+CD25^-$ and $CD4^+CD25^+$ lymphocytes [16].

In concordance with Mottonen et al. we demonstrated *CTLA-4 mRNA var 1* and *var 3* overexpression in PB lymphocytes of RA patients [17] as well as *CD28 mRNA*. CD28 triggering is pro-, CTLA-4 triggering is anti-costimulatory effect. These receptors are extensively studied, and our data is in concordance with most results obtained by other authors.

In PB lymphocytes of RA patients transforming growth factor (TGF) B1 was upregulated in comparison to controls, inflammation. TGFB1, a cytokine produced by efferocytotic macrophages, suppresses the inflammation and enhances the regeneration of tissue and regulates proliferation, differentiation, adhesion, migration, and other functions in many cell types. Many cells have TGF B receptors, and the protein positively and negatively regulates many other growth factors. TGF-beta, which is usually elevated in tumor patients, has its role as promoter of inflammation. Baltz [18] determines increased levels of the suppressor cytokines TGFB1 in the supernatant of Treg.

IL-18 is a proinflammatory cytokine. In several animal models antibodies that neutralize endogenous IL-18 reduce the severity of disease. Endotoxin lethality is prevented by anti-IL-18. AntiIL-18 antibody protects liver cells from activated T cell injury as well [19].

Obtained mRNA expression profile in the PB of the RA and systemic lupus erythematosus patients presented statistically significant similarity ($p<0.05$) for the studied genes.

In lymphocyte infiltrate from acute tonsillitis the majority of the studied genes were upregulated compared to the controls. Compared to the chronic inflammations *TGF B1 mRNA, IL-18, GITR* and *CD25* were downregulated, and *IL-2* and *CD28* was upregulated in acute tonsillitis. This possibly suggests a large number or a high activity of the T responder $CD4^+CD25^-$ as consequence of acute inflammation [16]. *GITR, GITRL*, *CTLA-4 var1* and *CTLA-4 var2* were highly expressed in both acute and chronic inflammation. This is reasonable, because in acute inflammation immune stimulation prevails. In chronic inflammation GITR, GITRL and CTLA are probably of Treg origin [17]. CTLA-4 on T cells inhibits activation and regulates peripheral tolerance [20].

To summarize, our data contributes to the understanding of biological functioning of lymphocytes in the pathology of inflammatory disease, and endorse further studies of the different subpopulations in order to establish each receptor's specific role in the fine-tuning.

Acknowledgments

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