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EXPRESSION OF SOME IMMUNOMODULATORY GENES INVOLVED IN HUMAN INFLAMMATORY PATHOLOGY

SUMMARY OF THE DOCTORAL THESIS

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CONTENT

keywords: immune modulation, costimulatory genes, mRNA expression, inflammation, cancer, *TGF β1*, *GITR*, *GITRL*, *CD28*, *CTLA-4*, *IL-2*, *IL-2Rα* (*CD25*), *IL-18*.

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 intracellulary 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 (Clark R., 2005). 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 costimulatory signal, 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 coreceptors. The second signal results from the interaction between the B7.1 (CD80) and B7.2 (CD86) costimulatory receptors from APC and the CD28 receptor of the T lymphocyte, finalized in positive costimulation. 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 costimulation. Other genes such as IL-2Rα, IL-2 (Ermann J., Fathmann C.G., 2003) in their promoter regions contain CD28 inducible elements and are considered immunomodulatory genes. As some other costimulatory 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-) (Beissert, S. 2006, Stephens G. L., 2004).

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 (Stephens G. L., 2004).

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 (Rudiger A, 2006, Bae E.M, 2008).

Accumulated data indicate a functional hierarchy of the costimulatory receptors and ligands, involved in the "fine–tuning" of the immune response.

In this study we aimed to asses mRNA 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 tumour necrosis factor receptor) (NM_148902), GITRL (Glucocorticoid-induced tumour 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).*

CD28 has a positive costimulatory function on the T cell, whilst *CTLA-4* (cytotoxic T-lymphocyte associated antigen 4, also known as *CD152*) has a negative regulatory effect. Regulation of the *CD28* and *CTLA-4* costimulatory molecules expression plays a basic role in the homeostasis of the immune system. *CTLA-4* is expressed at low levels on resting T cells and it is upregulated upon T cell activation. *IL-2* (interleukin 2) *mRNA* is not expressed by $CD4^+CD25^+$ cells neither anti-CD3 stimulated nor unstimulated, but anti-CD3 stimulated $CD4^+CD25^-$ cells express elevated levels of

IL-2 mRNA contrary to the unstimulated $CD4^+CD25^-$ cells (Thornton A.M., 1998). in mouse model. Despite of the constitutive expression of CD25 on $CD4^+CD25^+$ cells, these cells do not proliferate to *IL-2*, anti-CD3 or anti-CD28 stimulation.

According to Thornton and Shevach (Thornton A.M., 1998), $CD4^+CD25^+$ cells exert inhibitory effects by blocking the induction of the IL-2 production by the $CD4^+CD25^-$ cells at the level of RNA transcription.

Human *GITR* (glucocorticoid-induced TNF receptor related gene) is upregulated after $CD4^+CD25^-$ T cell or lymphocyte activation (Kwon B.S., et al., 1999, Tuyaerts S. et al., 2007). In mouse models *GITR* is induced by glucocorticoids or by activation (Nocentini G., et al., 1997). Accumulated data indicates that *GITR* is particularly critical in protection from apoptosis in murine models.

GITR is constitutive and highly expressed on $CD4^+CD25^+$ regulatory T cells (Treg), and the removal of GITR or the antibody blockade of *GITR* results in autoimmune disease (Nocentini G., et al., 1997).

GITRL (ligand for *GITR*) was identified in humans and later in mouse. Also, soluble form of *GITRL* was identified. *GITRL* may be expressed in antigen presenting cells (APC): monocyte, macrophage, dendritic cells (DC) endothelial cells, but not in resting and activated T cells.

TGF β1 (transforming growth factor, beta 1) can have immunostimulatory or immunosuppressive effects on T cells in a context-specific fashion (Zhang X., 2002). *TGF β1* secreted by Th3 lymphocytes may either costimulate $CD8^+$ or down-regulate the expression of *IL-2Rα* (interleukin 2 receptor alpha, also known as *CD25*), which inhibits T cell proliferation .

Interleukin 18 (*IL-18*) is a proinflammatory cytokine, which in conjunction with some other cytokines and chemokines contribute to the tumour specific microenvironment. There are some controversial data regarding *IL-18* in cancer. In this context some authors describe pro-neoplastic effects of the interleukin-18 in various cancers. Increased serum levels of *IL-18* in cancer patients are correlated with tumoral malignancy.

Jung *et al.* (Jung M.Y. et al., 2009) describe increased levels of inflammation associated interleukins *IL-15, IL-17, IL-18* and *IL-18,* and their binding proteins in

tumour tissue. Furthermore, RT-PCR and Western blot analysis revealed that *IL-18* was upregulated in tumour tissues and contributed to tumour progression through their pro-angiogenic effect.

Park *et al.* (Park S. et al., 2009) show increased serum levels of both *IL-18* and transferrin in MCF-7 breast cancer cell line.

II. MATERIALS AND METHODS

Experimental model

Previous studies showed modified immunomodulatory gene expression in the rodent inflammation model.

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.

II.1. 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. The internal control used for PCR was GAPDH (NM_002046) housekeeping gene. Also, GAPDH expression was used for data normalization.

II.2. Processing of the gels, 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.

III. PERSONAL CONTRIBUTIONS, RESULTS AND DISCUSSION

III.1. *Gene expression profile in the control group, inflammations and neoplastic disease*

III.1.1. 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 Fig 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 Fig 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.

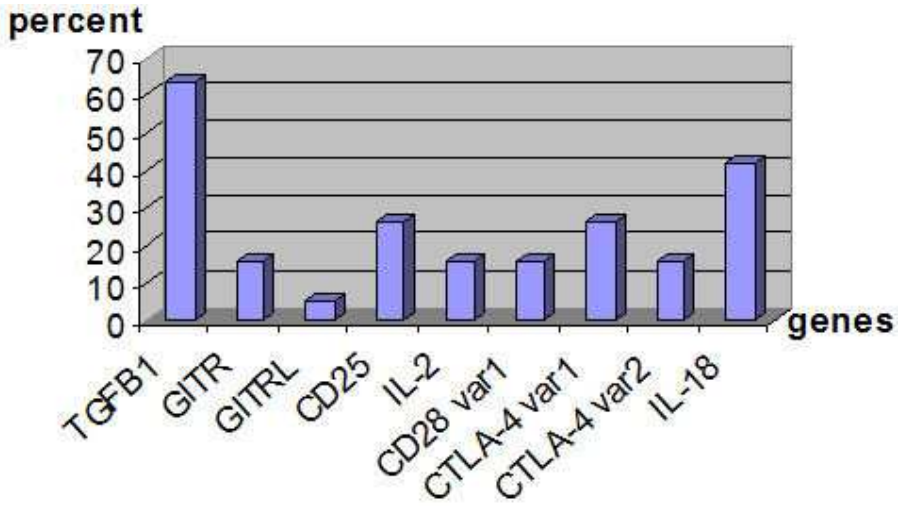


Fig 1: Percent of the positive controls for certain immunomodulatory genes

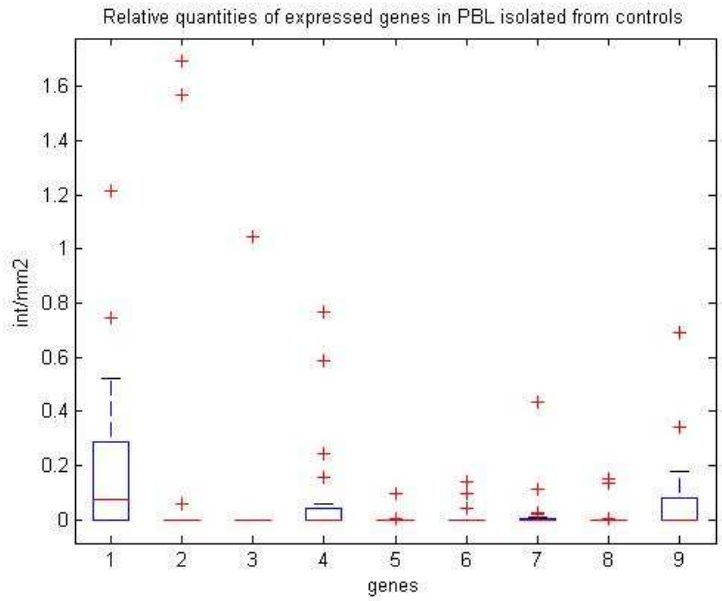


Fig 2: Relative quantity expressed in PBL isolated from controls (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

Thus, we demonstrated weak expression of *GITR*, *GITRL*, *CTLA-4 var1* and *CTLA-4 var2* costimulatory genes, which characterize Treg and weak expression of the *IL-2*, *CD28*, which are activation markers. The expression profile of the costimulatory genes in controls shows de baseline of the immune system.

III.1.2. 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 *TGFB 1* expression was upregulated. *GITR* was upregulated in tonsillitis patients (95%) in contrary to the control group where *GITR* 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 Fig 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 Fig 5.

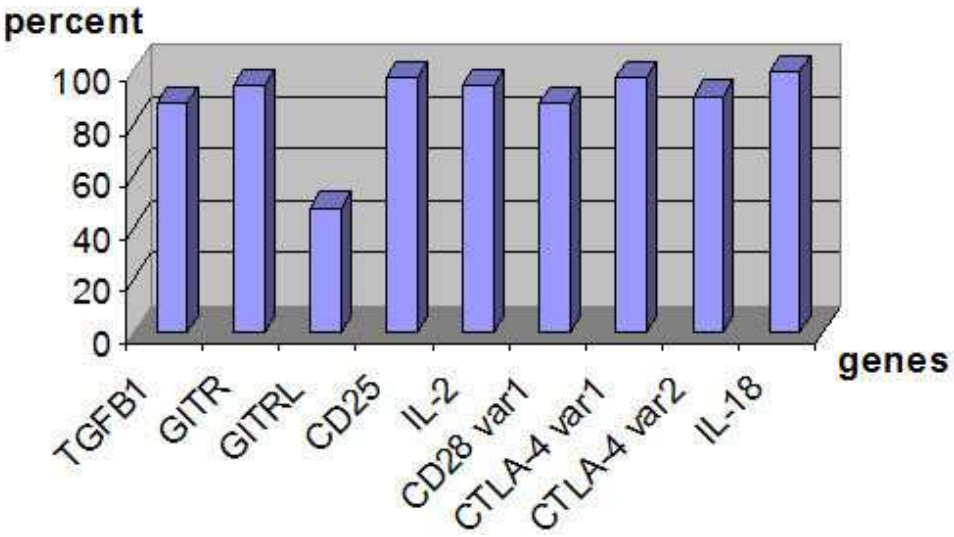


Fig 3: Percent of the positive samples in acute tonsillitis

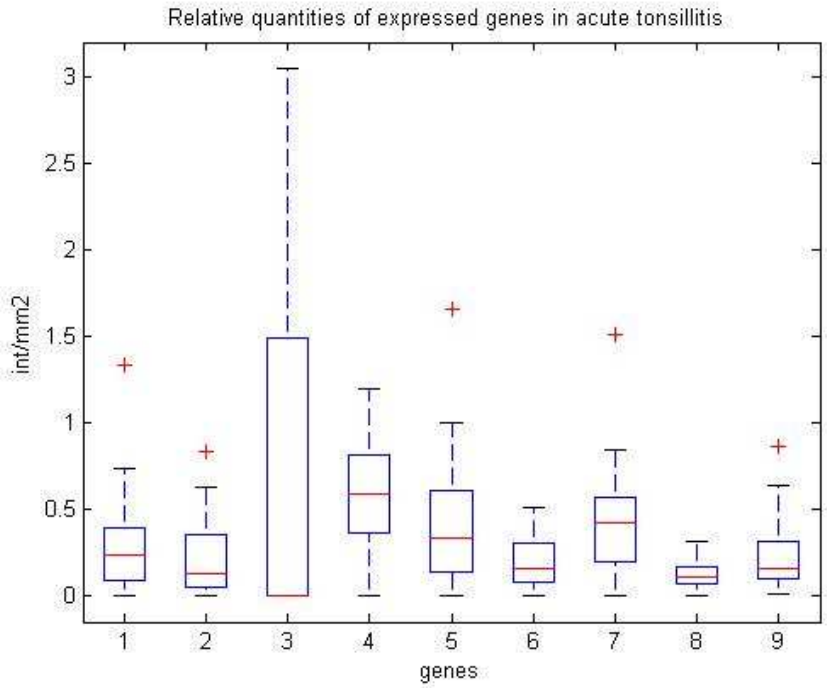
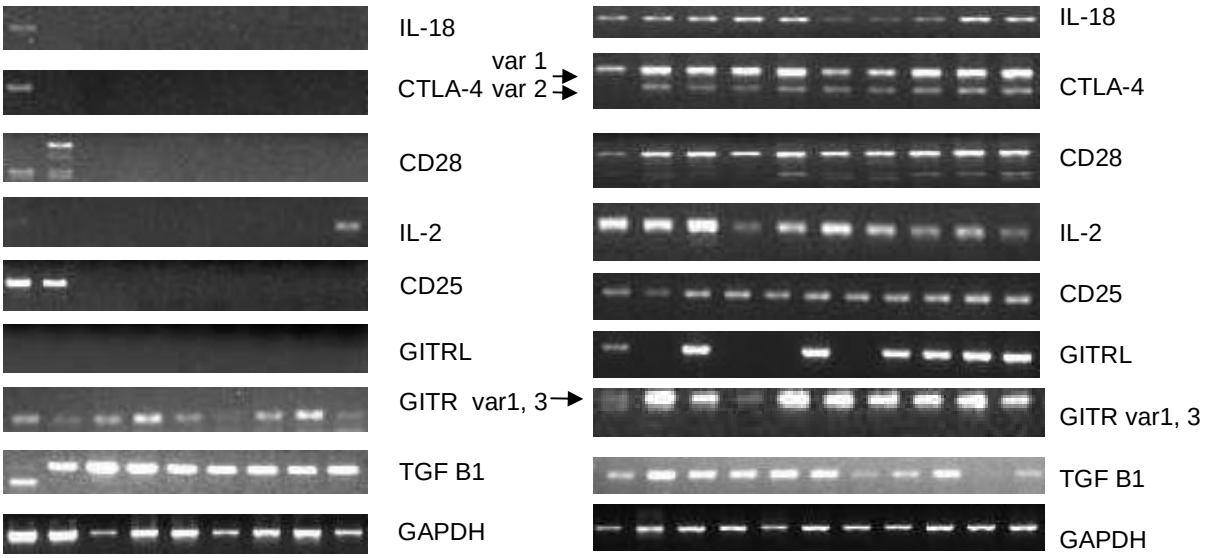


Fig 4: Expressed relative quantity from tissue infiltrate in acute tonsillitis (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).



control group (1-9) **tonsillitis patients (1-10)**

Fig 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

In acute tonsillitis *IL-2* and *CD28* were upregulated, *CD25*, *TGF β1*, *GITR* and *IL-18* were downregulated, and *CD25*, *TGF β1*, *GITR* and *IL-18* were similar compared to the chronic inflammations.

IL-2 and *CD28* are activation markers of the T lymphocytes, and upregulation of these genes in acute tonsillitis indicated responder lymphocytes presence in inflamed tonsils. We assume that high *CD25*, *GITR* and *CTLA-4* expression is of Treg origin.

III.1.3. 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 (Fig 6). Enhanced relative quantities of gene expression were demonstrated in the majority of the studied genes, as presented in Fig 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.

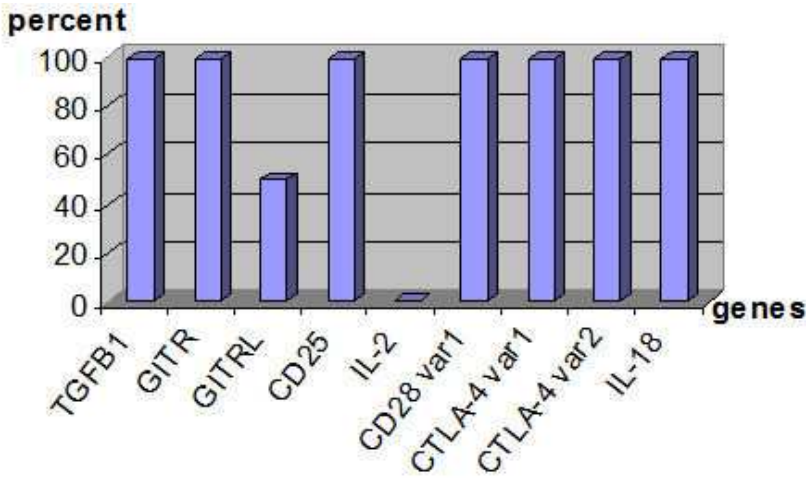


Fig 6: Percent of the positive samples in rheumatoid polyarthritis

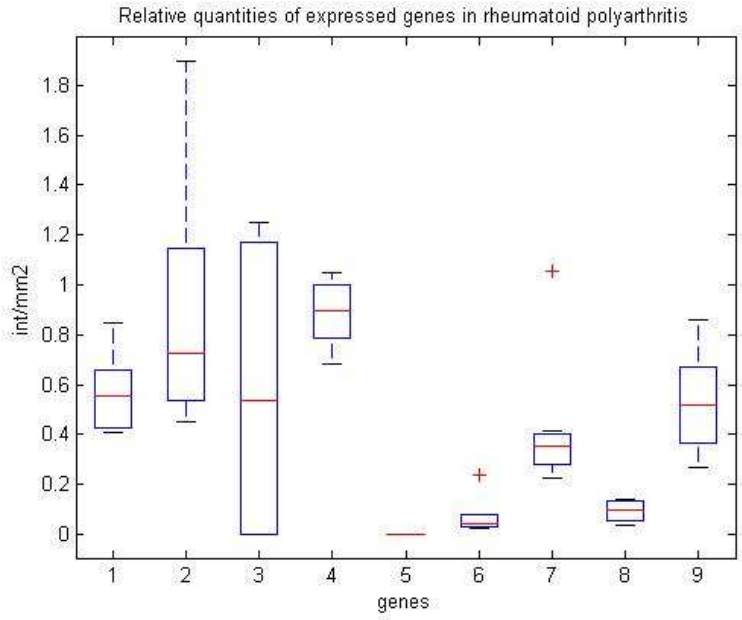


Fig 7: Relative quantities of gene expression from PBL in rheumatoid polyarthritis (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

III.1.4. 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 Fig 8. Similarly, the relative quantity expressed of these genes was the same as in rheumatoid polyarthritis (Fig 9).

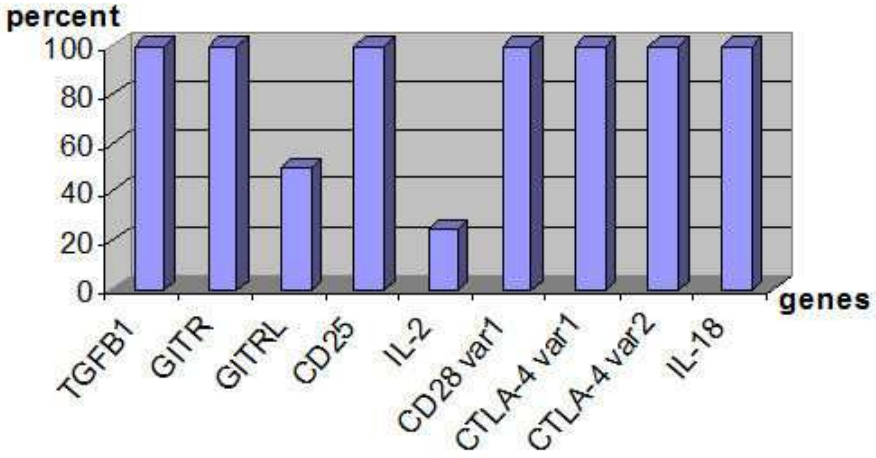


Fig 8: Percent of the positive samples in systemic lupus erythematosus

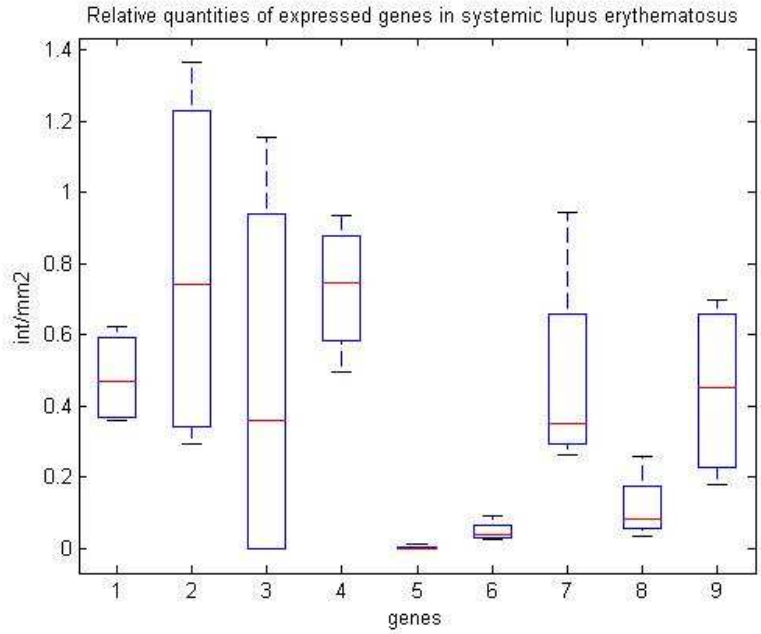


Fig 9: Relative quantities of gene expression from PBL in systemic lupus erythematosus (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

Rheumatoid polyarthritis is a systemic autoimmune inflammation caused in inefficient control of the autoreactive T lymphocytes or of the antibody secreted B lymphocytes by the Treg (Xue H. et al., 2010). Some authors identified altered functions of the Treg in peripheral blood and in synovium of rheumatoid arthritis patients.

Systemic lupus erythematosus is a systemic autoimmune inflammation caused by hyperactive B lymphocytes in nuclear antigen directed autoantibody production. Also, hyperactive effectoric T lymphocytes were identified in systemic lupus erythematosus.

We proved *GITR, GITRL, CD25, TFG β1, CTLA-4* mRNA upregulation in PBL of the RA and LES patients. CD28 and IL-2 were downregulated in these samples.

GITR, CD25, TFG β1, CTLA-4 are Treg markers, hence, we assume that altered or anergic Tregs are involved in evolution of chronic inflammation.

We found similar ($p>0.05$) and specific gene expression profile in RA and LES.

III.1.5. Gene expression profile in mammary tumour

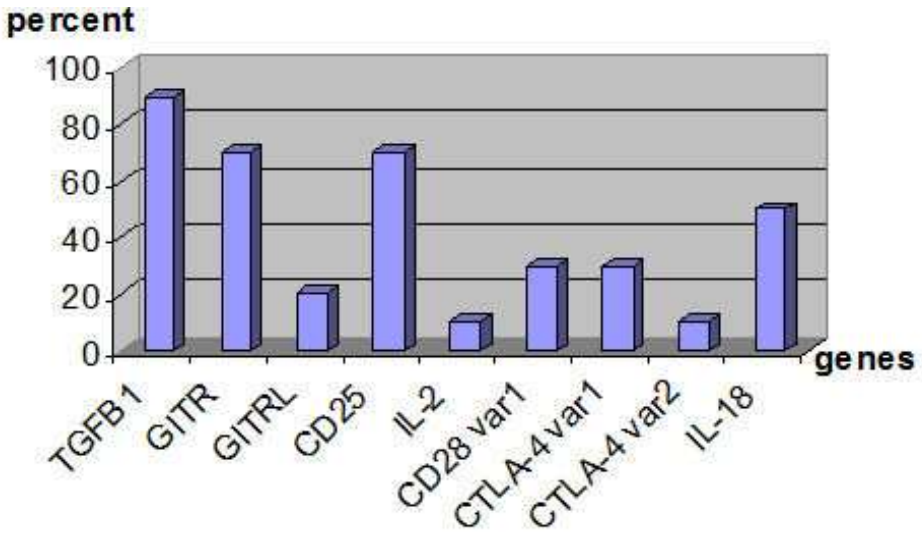


Fig 10: Percent of the positive samples in mammary tumour

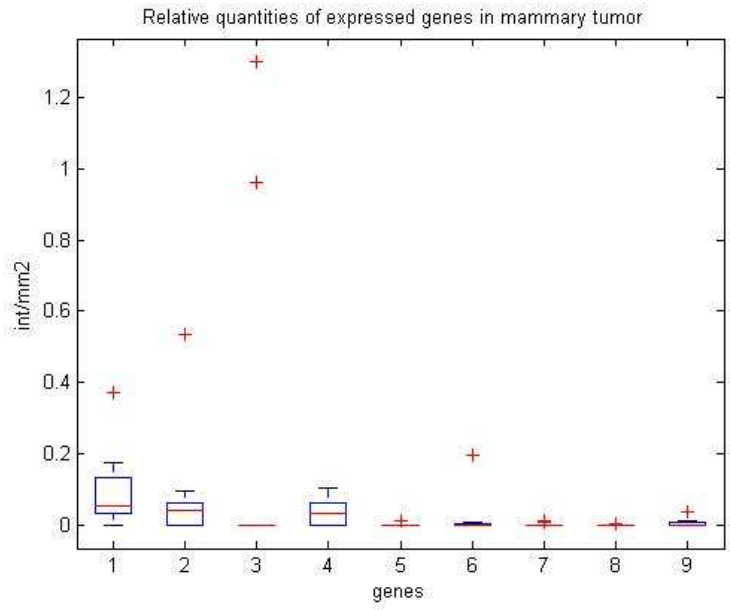


Fig 11: Expressed relative quantity from tissue infiltrate in breast cancer (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18).

The mammary tumour gene expression profile was characterized by the expression of the *TGF β1*, *GITR* and *CD25* genes in high percentages, whilst the rest of the genes were expressed only in a few cases: *GITRL* (in 2 from total n=10), *IL-2* (1 from n=10), *CD 28 var1* (3 from n=10), and *CTLA-4 variants 1 and 2* (1 from n=10) (Fig 10).

Considering the quantitative aspect of gene expression, our results indicated the downregulation of the studied genes compared both to the controls and to the rest of the pathologies. *GITR* was significantly downregulated in comparison to studied inflammations, similar to the *GITRL, CD25, CD28 var1, CTLA-4 var1, CTLA-4 var2, IL-18 and IL-2* genes as presented on Fig 11.

III.1.6. Gene expression profile in lung cancer

GITRL, *IL-2* and *CTLA-4* are expressed only in few lung cancer samples as presented in Fig 12. Low relative intensities was fined for the majority of the genes (Fig 13), and higher relative intensities for *GITRL*, *CD25*, *IL-2* and *IL-18*. Relative quantities for the studied genes were low compared to the controls and the rest of the pathologies.

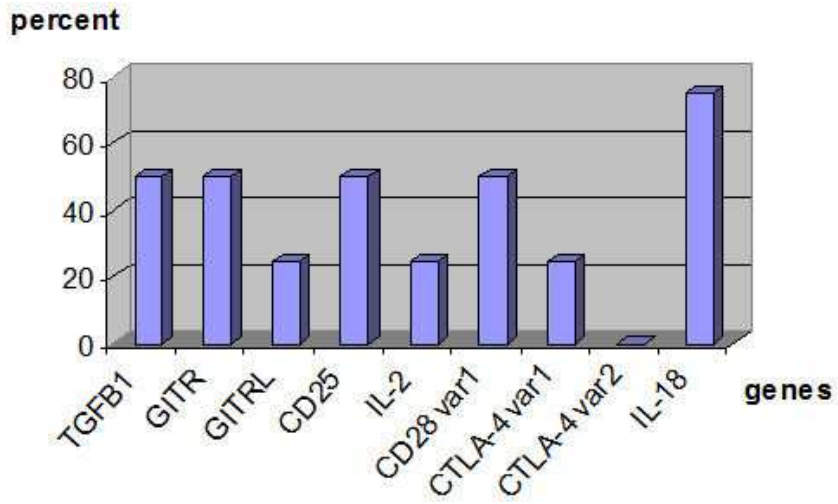


Fig 12. Percent of the positive samples in lung cancer

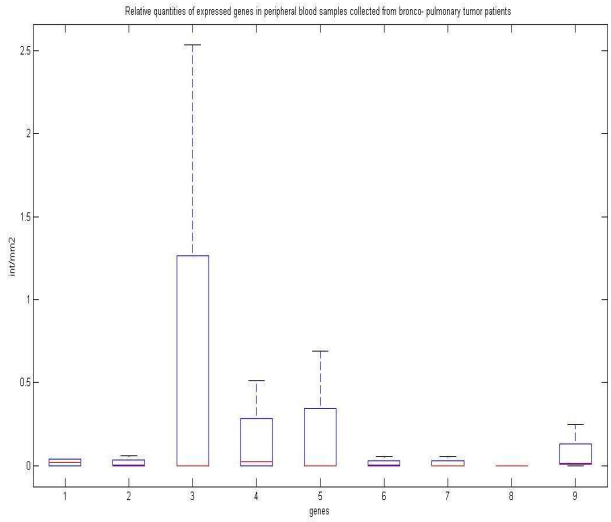


Fig 13. Relative quantity of RANm in lung cancer (1. TGF B1, 2. GITR, 3. GITRL, 4. CD25, 5. IL-2, 6. CD28 var1, 7. CTLA-4 var1, 8. CTLA-4 var2, 9. IL-18)

Zhang X. *et al.* using DNA microarray demonstrated expression of more than 100 genes (Zhang X. et al., 2002) in tumour specific, active, murine T lymphocytes, where *TGF β*, *CD25*, *4-1BB*, *GITR*, *CD28*, *CTLA-4*, *OX40* were upregulated. On the contrary, naïve T lymphocytes express only 37 genes.

CD25, *CTLA-4* and *GITR* are constitutively expressed on Treg and there are phenotypical markers for it.

Our results show downregulation of the studied costimulatory genes compared to the acute and chronic inflammations. Compared to the controls we demonstrated lack of *IL-2* and *CTLA-4*, similar intensities for *TGF β1* and *IL-18*, and upregulation of *CD25*, *CD28*, *GITR* and *GITRL*.

CD25, *CD28*, and *GITR* upregulation is due to increased number or activity of the Treg in tumour tissue compared to controls. These Tregs contribute to the intratumoral immune suppression. Similarly, *GITRL* and *TGF β1* contribute to the intratumoural immune tolerance.

III.2. Expression of the costimulatory genes in the control group, inflammations and neoplastic disease

III.2.1. CD25 mRNA expression

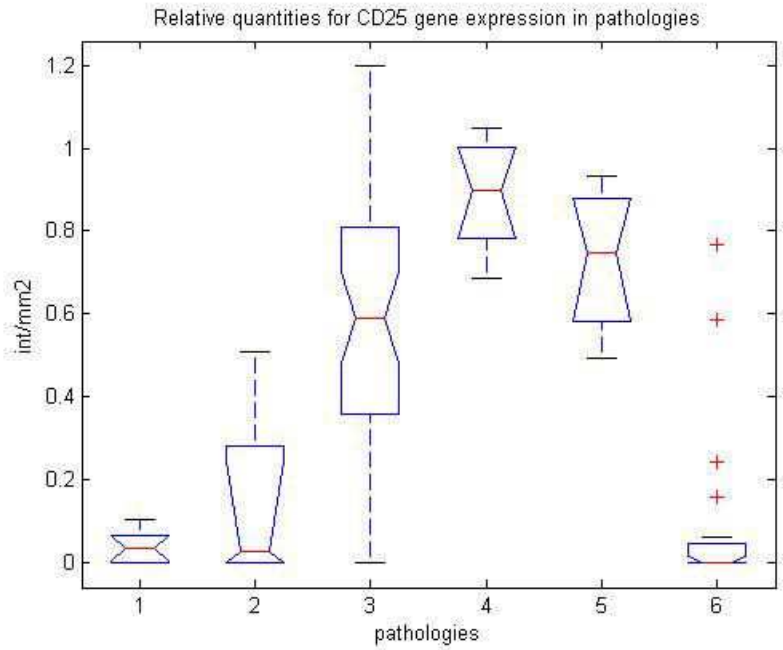


Fig 14. Relative intensities of *CD25 mRNA* expression (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

We have found upregulation of *CD25* expression in chronic and acute inflammation in contrast to the neoplastic samples and controls, as presented in Fig 14. Highest

expression of *CD25* was obtained in chronic inflammation. Relative values for *CD25* expression in breast and lung cancer patients were low, and statistically similar compared each other and to the controls ($p < 0.05$). Moreover, in other cancer samples, in rectal and kidney tumour tissues, *CD25 mRNA* expression was similarly low, not very different compared to the controls.

On the other hand, $CD25^+$ is phenotypical marker for Treg cells.

III.2.2. IL-2 mRNA expression

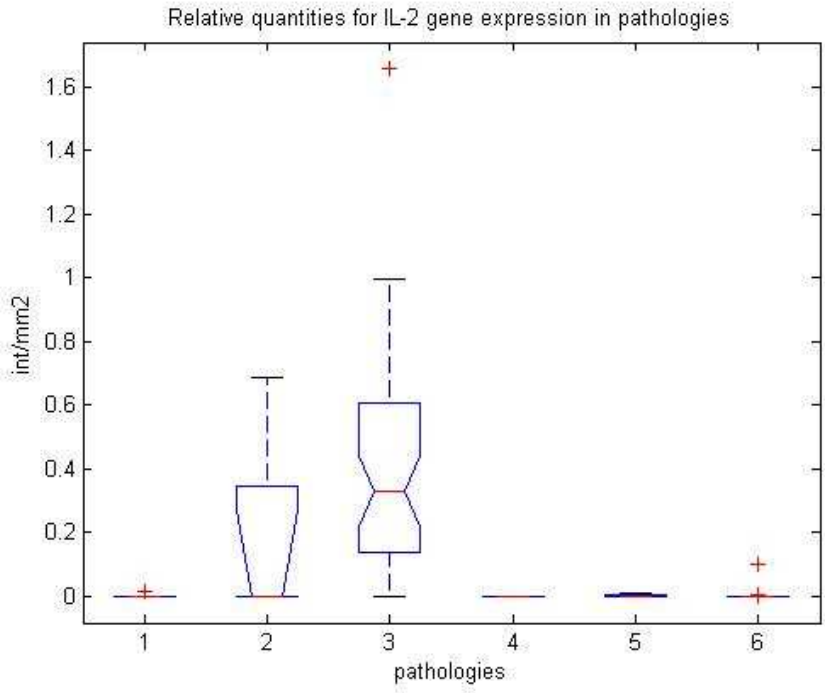


Fig 15. Relative intensities for *IL-2* expression in cancer and (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

Our results showed elevated *IL-2 mRNA* level in acute inflammation as presented in Fig 15. *IL-2 mRNA* was not expressed neither in control patients, chronic inflammation and breast cancer patients (Fig 15). Exceptions were found to be: rectal tumours (2 from total n=2), kidney tumours (2 from total n=2) and 1 PB sample from 4 cancer patients.

III.2.3. CD28 mRNA expression

Following *CD28 mRNA* expression screening we have identified all eight mRNA splice variants described by Manisha Deshpande *et al.* (Manisha Deshpande et al. 2002.) as presented on Fig 16. These eight mRNA splice variants appeared in peripheral blood samples as well as in tissue infiltrate. Statistical analysis has been done using only (the largest) variant 1, NM_006139.

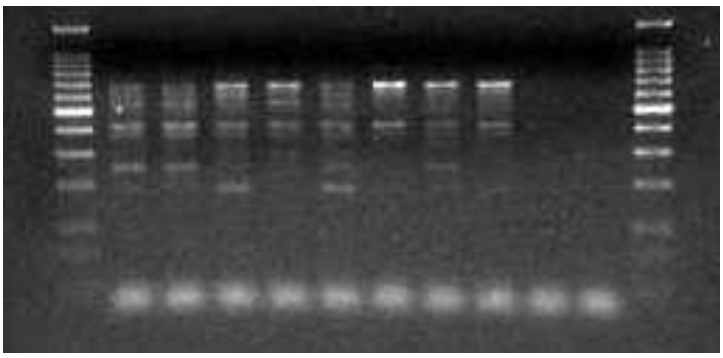


Fig 16. PCR for *CD28*

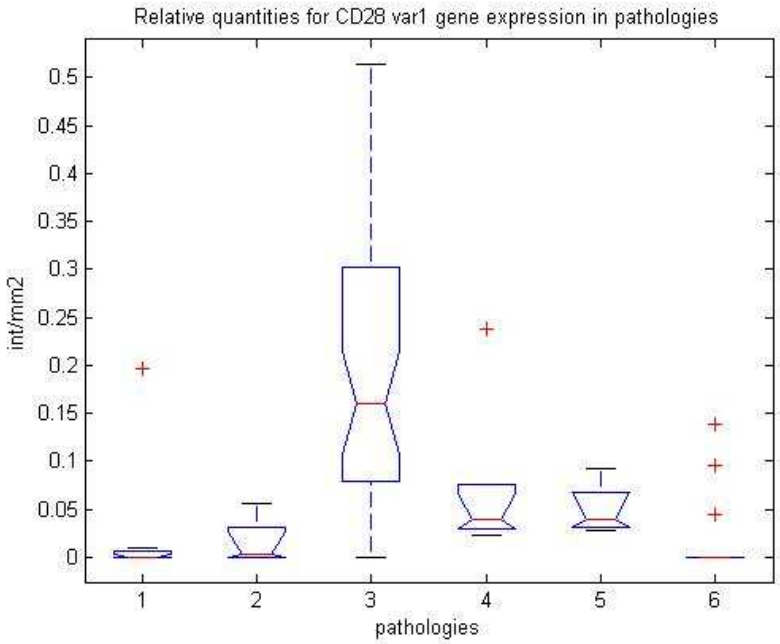


Fig 17. *CD28 mRNA* expression in cancer and inflammations (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

We have demonstrated upregulation of *CD28 mRNA* expression in acute inflammation compared to the chronic inflammation, cancer and control group ($p < 0.05$). Breast and lung cancer patients presented low *CD28* expression, same as in kidney and rectal tumours.

Control patients did not show *CD28* expression, as presented in Fig 17.

III.2.4. TGF β1 mRNA expression

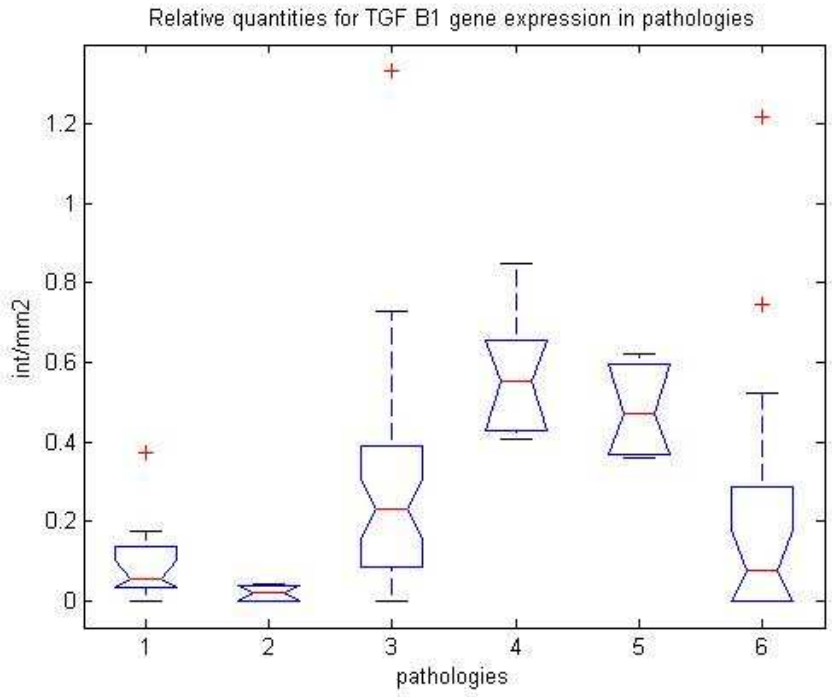


Fig 18. Relative intensities for *TGF β1* mRNA expression (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

Our results showed less expression of *mRNA* in cancer than in acute and chronic inflammation samples as presented in Fig 18. We proved statistically similar ($p < 0.05$) *TGF β1* expression in cancer and control samples.

TGF β1 signalling was shown to be implicated in suppressive effects of the Treg in the murine and human models. Elevated level of soluble *TGF β1* was observed by some authors in the supernatant of Treg cell cultures, where this gene contributes to the

tumour cell's escape from an effective immune response. *TGF β1* levels are associated with tumour progression, severity, and metastatic capacity (Łuczyński W. et al., 2010), (Sato Y. et al., 2010), (Zhao X.P. et al., 2010), (Domschke C. et al., 2004), (Chod J. et al., 2008).

III.2.5. CTLA-4 mRNA expression

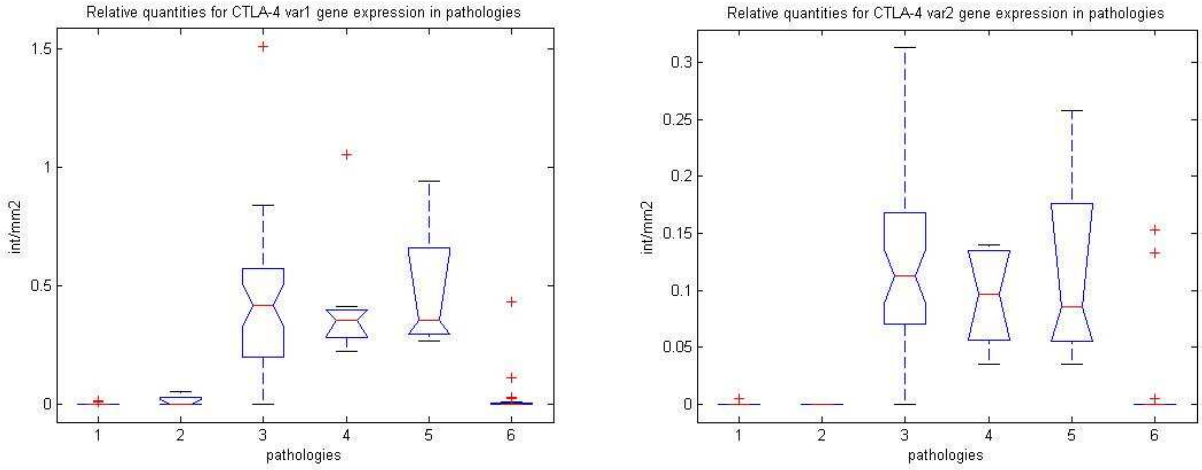


Fig 19. Relative intensities for CTLA-4 var1 and CTLA-4 var2 mRNA expression (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

CTLA-4 mRNA showed expression of both two *CLTA-4 mRNA* variants in PB and tissue: mRNA variant 1 (NM_005214) and mRNA variant 2 (NM_001037631). The expression profiles found for *CTLA-4* variants were highly similar as presented in Fig 19.

Our results showed upregulation of *CLTA-4 mRNA* expression in chronic and acute inflammation compared to controls and cancer samples. *CTLA-4* variants were expressed neither in controls nor in solid breast tumour tissue.

Small *CLTA-4 mRNA* expression was observed for both variants in PBL of breast cancer patients and in solid kidney tumour samples.

According to Zhang, *TGF β1*, *IL-2R* (*CD25*), *GITR*, *CD28* and *CTLA-4* (*CD152*) are upregulated in the tumour-specific T cells following activation versus naive T cells (Zhang X. et al., 2002).

III.2.6. GITRL mRNA expression

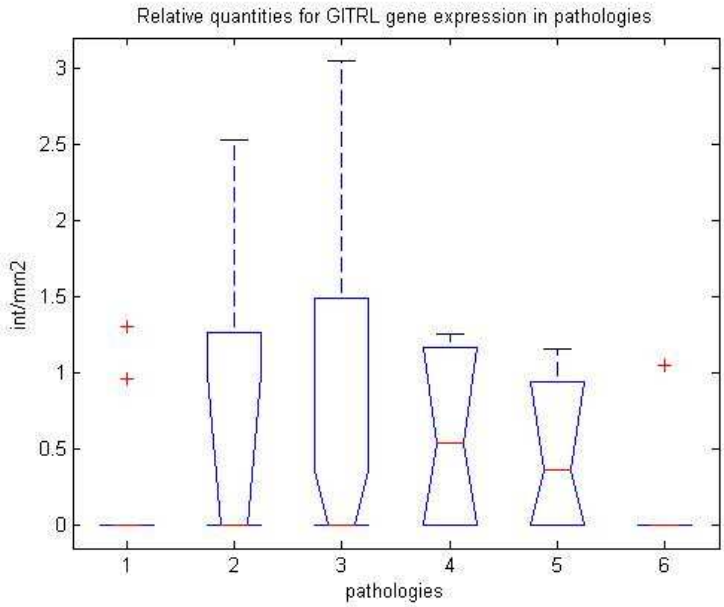


Fig 20. Relative intensities for *GITRL* expression in cancer and inflammations (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

GITRL mRNA was not detectable in the control group, but 2 of n=10 solid breast tumours and 1 from n=4 PB lung cancer samples expressed *GITRL*. *GITRL* was significantly ($p < 0.05$) upregulated in approximately half of the acute and chronic inflammatory disease patients, as presented in Fig 20. *GITRL* was also found to be expressed in the rest of cancer samples: 2 rectal tumours, 2 kidney tumour tissues, and the 2 PB samples of breast cancer patients.

According to Baltz *et al.* (Baltz K.M. et al., 2007) *GITRL* is constitutively expressed by human tumours and directly modulates their immunogenicity, cytokine release and interaction with NK cells found to express *GITR*. GITRL stimulation markedly reduces expression of immunostimulatory molecules CD40 and CD45 (Baltz

K.M.et al., 2007). GITRL signaling alters the expression of regulatory surface molecules and stimulates production of the immunosuppressive cytokine *TGF β1* in tumour cells (Baltz K.M.et al., 2007). Tuyaerts could not detect *hGITRL* expression in different PBMC subsets but they detected *GITRL* on different transformed cell lines such as HUVEC line EA.Hy926, and on the EBV-transformed B cell lines 888-EBV, 1087-EBV and 1088-EBV (Tuyaerts S.et al., 2007).

III.2.7. GITR mRNA expression

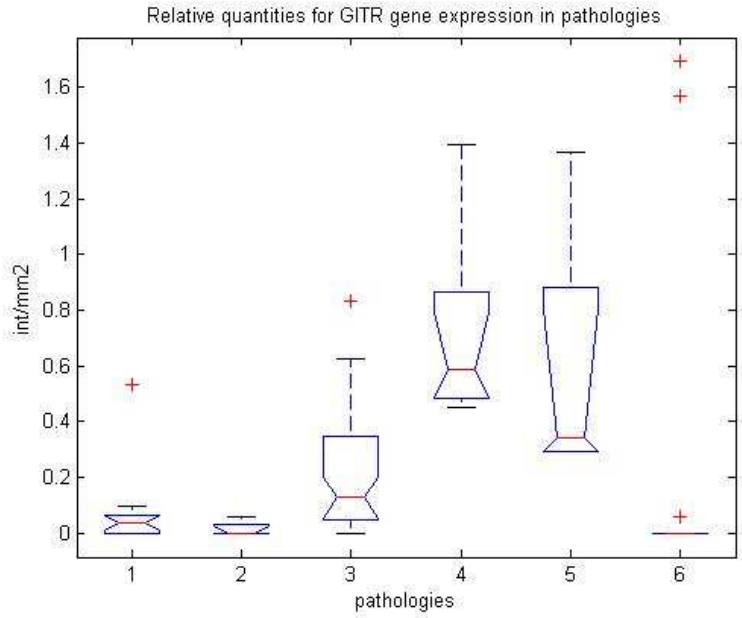


Fig 21. Relative quantities for *GITR mRNA* expression (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

GITR was not expressed in PBL of the control patients. In chronic and acute inflammation significant upregulation of *GITR* was found ($p < 0.05$) compared to the controls. All chronic inflammation samples showed elevated level of *GITR*, whilst the obtained relative intensities for the acute inflammation were decreased as presented in Fig 21.

In lung and breast cancer *GITR* was expressed, but it showed significant downregulation compared to the inflammations. In other cancer samples such as kidney cancer, colonic cancer and PB of the breast cancer *GITR* appeared in small relative quantities in the majority of the cases.

Treg cells constitutively express higher levels of *GITR* than conventional T cells, independently of location and activation state (Coe D. et al., 2010).

Anti-CTLA-4/anti-GITR mAb combination treatment exhibited far stronger antitumour effects compared to either antibody alone. This strong antitumour effect is explained by an increased number of CD8+ T cells infiltrating tumour sites in anti-CTLA-4 mAb-treated mice, and increased cytokine secretion and Treg resistance of tumour-specific CD8+ T cells with strongly upregulated *CD25* expression in anti-GITR mAb-treated mice. Cohen *et al.* demonstrated that anti-GITR antibody enhances immune response towards melanoma by stimulating responder T cells and by inhibiting Treg cells (Cohen A.D. et al., 2010). Ponte *et al.* (Ponte J.F. et al., 2010) demonstrated that anti-GITR antibody is an effective vaccine adjuvant against foreign antigens, and suggested using it as a potential adjuvant in solid tumour therapy. Furthermore, this antibody was used in an adenomavirus based T cell vaccine model, eradicating permanently and completely the papillomavirus-induced tumours, something that was not the case with other adjuvant like anti-CD4 or interferon-α (Hoffmann C. et al., 2010).

III.2.8. IL-18 mRNA expression

Our results indicated low *IL-18* expression in mammary tumour tissue. 5 from total 10 patients expressed *IL-18*, similarly to the control group (8 from a total of 19 samples).

IL-18 was highly expressed in other cancer tissue infiltrates such as ovary tumour (2 from n=2), rectal tumour (2 from n=2), kidney tumour (2 from n=2), colonic tumour (2 from n=2) and last stage uterus tumour (1 from n=1). In ovary tumour, rectal tumour, kidney tumour and colonic tumour cases *IL-18* were upregulated compared to the mammary tumour samples and the controls ($p < 0.05$).

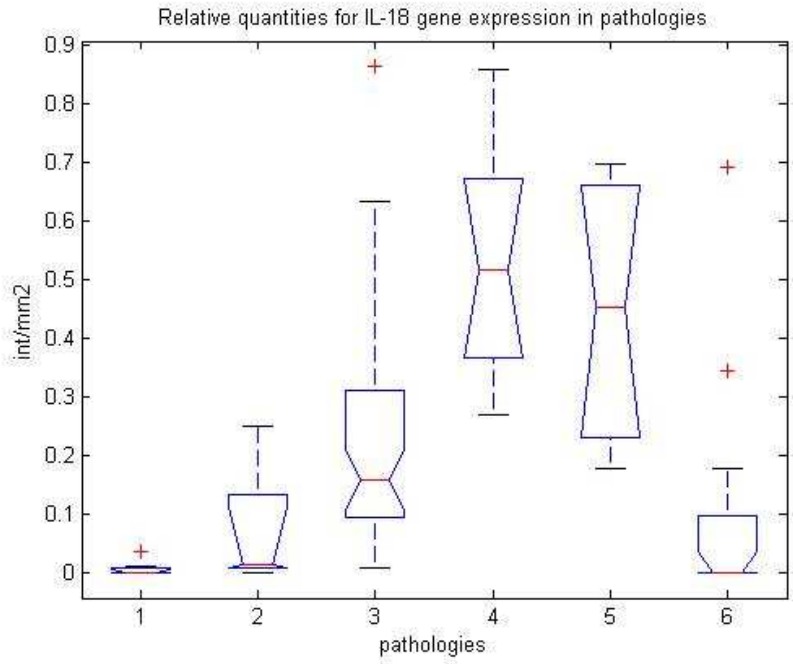


Fig 22. *IL-18 mRNA* expression in inflammation and cancer (1. breast cancer, 2. lung cancer, 3. acute tonsillitis, 4. rheumatoid polyarthritis, 5. systemic lupus erythematosus 6. control)

Also, *IL-18* expression was determined from PB lymphocytes in mammary tumour patients (2 from n=2), ovary (1 from n=1), rectal (1 from n=1) and lung tumour (n=4).

Relative quantity of *IL-18* from peripheral blood was the same as in tumour infiltrate, apart from mammary tumour patients ($P < 0.05$) where the expression was higher in PB lymphocytes. The highest expression of *IL-18* was found in chronic inflammation, followed by acute inflammation, as presented in Fig 22.

Jung *et al.* found increased expression of some inflammation associated cytokines such as IL-15, IL-17, IL-18 and IL-18 binding protein (IL-18bp), and elevated level of chemokines in tumour tissues. Also, RT-PCR and Western blot analysis revealed upregulation of the *IL-18* which is a pro-angiogenic factor in tumours.

IV CONCLUSIONS

In this study our purpose was to evaluate the mRNA expression for some costimulatory genes.

The problem is rather complex, due to the complexity of the immune regulation, but also due to the fact that the immune suppressor or immune stimulator function of the same gene depends on physiological conditions, cytokine microenvironment, implicated cell line or intensity of the stimulatory signal. Moreover, differences appear depending on the experimental model, human or murine model, or in vitro, in vivo or ex-vivo conditions.

To obtain physiologically veritable data, we chose to use ex-vivo human model, based on preliminary murine experimental data.

To demonstrate mRNA expression differences, samples from acute and chronic inflammation patients, neoplastic patients and controls were used. As chronic inflammations we chose rheumatoid polyarthritis and systemic lupus erythematosus, considered the most common chronic autoimmune diseases, and acute tonsillitis, which is a common inflammation but no preliminary data are available.

In this study we used total lymphocytes isolated from peripheral blood or tissue, and the obtained values represent the sum of the gene expressions in various cell lines where T responders and Tregs are most important.

We evaluated mRNA expression semi-quantitatively, preceded by exigent optimization of the reactions, data analysis, and the obtained data are relative values of the intensities.

In consequence, we obtained the percentage of the positive samples and the relative expression for each gene, and based on this we could build expression profiles in inflammations and cancer. These profiles allowed comparisons to the control group.

Graphical presentation of the data was performed in the Matlab program, using the notched box plot method, a method which exhibits minimum, median, and maximum values, distributions and statistical significance indicators of the data.

In conclusion, we proved upregulation of mRNA expression for some immunomodulatory genes, and that some other genes are not expressed:

1). These results suggested that the control group's expression pattern may be described by moderate expression of the *TGFB1*, *CD25* and *IL-18,* lack of the *GITR, GITRL, IL-2, CD28, CTLA-4 var1* and *CTLA4 var2* genes.

2). 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.

3). Our study indicated highly similar ($p<0.05$) gene expression profile in PBL of the systemic lupus erythematosus and in rheumatoid polyarthritis patients.

4). In PB lymphocytes of chronic inflammations patients transforming growth factor (TGF) β1 was upregulated in comparison to controls and acute inflammation, similarly *GITR, CD25* and *IL-18*. Relative quantities of the *GITRL, IL-2, CTLA var1, 2* were similar (P<0,05), and *IL-2* and *CD28* were downregulated.

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 presumably of Treg origin. CTLA-4 on T cells inhibits activation and regulates peripheral tolerance.

5). We have found elevated level of *CD25, CD28, GITR, GITRL, TGF β1* mRNA in cancer. We have found no *IL-2* and *CTLA-4* expression in cancer tissue.

High intensities for *CD25, GITR, GITRL, TGF* β1 are caused probably by the Treg. This data is consistent with the current theory according to which tumours obviate immune surveillance by using a tumour-specific Treg clone, which protects the tumour against the host's responder T cells.

6). *GITR* is expressed in activated macrophages and in mononuclear cells, and *GITR* is upregulated upon activation of the $CD4^+CD25^-$ responder T lymphocytes. *GITR* is phenotypical marker for Treg $CD4^+CD25^+$. 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.

GITRL is constitutively expressed on antigen presenting cells (macrophages, dendritic cells (DC), B lymphocytes) and endothelial cells. As Tuyaerts 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. Recent studies show elevated *GITR* and *GITRL* expression in macrophages from human atherosclerotic plaques, in synovial fluid macrophages and PB from RA patients. Stimulation of the synovial fluid macrophages with *anti-GITR mAb* resulted in dose dependent induction of the inflammatory cytokines

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

There is also evidence that murine GITRL autostimulates GITR on CD8 lymphocytes, and has a key role in fine-tuning, and that in the CD8 subpopulation GITR seems to exceed in importance CD28.

7). Interleukin 18 (*IL-18*) is a proinflammatory cytokine. In several animal models antibodies that neutralize endogenous IL-18 reduce the severity of disease.

In concordance with this data we obtained high levels of *IL-18 mRNA* in peripheral blood lymphocytes of chronic inflammation patients and moderate upregulation in acute inflammation.

8). *IL-2* and *CD28* are activation markers for T lymphocytes.

Accordingly, our results show significant upregulation of *IL-2* and *CD28* mRNA in acute tonsillitis, since in acute inflammation effectoric T cells prevail. *IL-2 mRNA* was not expressed neither in control patients, chronic inflammation and breast cancer patients. According to Thornton *IL-2* is expressed in stimulated effector T cells and 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 (Thornton S. et al., 1998).

9). Despite the fact that we could not demonstrate *IL-2 mRNA* expression neither in chronic inflammation nor in breast cancer patients, the high affinity IL-2 receptor, *CD25 mRNA* was found to be upregulated in arthritis and lupus samples.

10). In concordance with Mottonen *et al*. we demonstrated *CTLA-4 mRNA var 1* and *var 2* overexpression in chronic inflammations. In PB lymphocytes of RA and LES patients *CD28 mRNA* were upregulated compared to the controls. CD28 triggering has pro-, CTLA-4 triggering has anti-costimulatory effect. These receptors are extensively studied, and our data is in concordance with most results obtained by other authors.

11). Our results showed higher expression of *TGF β1 mRNA* in chronic inflammation samples than in acute inflammation samples and controls. We proved statistically similar ($p < 0.05$) *TGF β1* expression in cancer and control samples.

Transforming growth factor beta, produced by macrophages, enhances tissue regeneration. Many cells have TGF B receptors, and the protein positively and negatively regulates many other growth factors. TGF-beta, which is usually elevated in tumour patients, has its role as promoter of inflammation. Baltz determines increased levels of the suppressor cytokines TGFB1 in the supernatant of Treg.

TGF β1 levels are associated with tumour progression, severity, and metastatic capacity.

Our determinations show similar TGF *β1* expression in cancer, acute tonsillitis and control patients.

Our findings are the first step in evaluating human cells, and endorse further studies in this direction. Although structural and functional changes exists between human and murine fine-tuning, elucidating these mechanisms seems a valid direction in order to find new approaches in autoimmune disorders management and cancer immunotherapy.

Taken together, our data contributes to the understanding of biological functioning of lymphocytes in inflammation and neoplastic disease.

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VI. LIST OF THE AUTHOR'S SCIENTIFIC PAPERS

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2. **I. Mészáros,** T. L. Krausz, E. Fischer-Fodor, Sz. Lányi, *Expression profiles of immunomodulatory genes in human solid tumors*, Revista Română de Medicină de Laborator, in press.