Expression profiles of immunomodulatory genes in human solid tumors

Profiluri de expresie ale genelor imunomodulatoare în tumori solide umane

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Abstract

Due to antigen recognition, effector and regulatory function, lymphocytes have pivotal function in many disease pathways. Immune response towards foreign or altered self antigens has a considerable complexity, the fine modulation of the immune response being delivered by the interaction between costimulatory molecules, on which the final outcome of the immune response depends. Evaluation of the expression profile of immunomodulatory genes and cytokines in cancer may clear some of this complexity. Hence we used a PCR screening method in order to assess mRNA presence and the quantity of relative genes in neoplastic pathologies. We proposed to establish standard "yes or no" condition to the PCR method in order to confer a semi-quantitative attribute to our PCR. We used primers for the following genes: GITR, GITRL, IL-2, CD25, CD28, CTLA-4, TGF β1, IL-18 and GAPDH, the latter one as internal control. We collected samples from patients with mammary, kidney, rectal, ovary and colonic solid tumors, from voluntary subjects and from patients with inflammatory pathology used as controls, isolated 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. We obtained results demonstrating that there are significant differences in the expression of these genes in the above mentioned patients compared to healthy subjects and inflammatory controls.

Keywords: *gene expression, cancer, TGF β1, GITR, GITRL, CD28, CTLA-4, IL-2, IL-2Rα (CD25), IL-18.*

Rezumat

Reacţiile imune în cancer sunt de o deosebită complexitate, modularea fină a acestui răspuns fiind condiţionată de interacţiunea între moleculele costimulatoare, de care depinde de fapt finalitatea răspunsului. Evaluarea profilului de exprimare a genelor imunomodulatoare şi a citokinelor aferente în cancer poate clarifica unele aspecte ale complexităţii răspunsului imun. Astfel, am elaborat o metodă PCR semicantitativă de a evidenţia o parte din genele costimulatoare şi ne-am propus să stabilim distincţia clară de „da sau nu" pentru meto-

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da noastră PCR. Am utilizat amorse pentru următoarele gene: GITR, GITRL, IL-2, CD25, CD28, CTLA-4, TGF-β1, IL-18 şi GAPDH ca şi control intern. Am recoltat material tumoral de la bolnavi de cancer mamar, renal, rectal, ovarian şi de colon, respectiv sânge periferic de la voluntari sănătoşi şi de la pacienţi cu boli inflamatorii, şi cancer bronhopulmonar, izolând limfocitele totale din sânge şi ţesut, urmat de extragerea ARNm, şi transcrierea inversă în ADNc. S-a confirmat cantitatea şi calitatea ARNm utilizând GADPH. Rezultatele obţinute indică diferenţe semnificative în exprimarea acestor gene la pacienţii cu cancer în comparaţie cu lotul control şi cu inflamaţii.

Introduction

Effective immune responses require efficient activation of the immune stimulator system and inhibition of the immune suppressor component of the immune system (cells and cytokines). This gives rise to a dynamic equilibrium which is required in host defense. Lymphocytes have pivotal function in many disease pathways through cell surface interaction and secreted mediators.

T cell activation affects the expression of numerous cytokines, chemokines, receptors of cytokines and chemokines, signal transduction molecules and cell surface proteins.

Available data suggests functional differences between the human immune regulatory mechanism and its murine homologue.

T cell activation requires two signals, one from TCR and one from costimulatory molecules.

CD28 has a positive co-stimulatory 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 (1).

Human *GITR* (glucocorticoid-induced TNF receptor related gene) is upregulated after $CD4^+CD25^-$ T cell or lymphocyte activation (2, 3). In mouse models *GITR* is induced by glucocorticoids or by activation (4). 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 (5).

GITRL (ligand for *GITR*) was identified in humans (2, 6) and later in mice (7). Also, a soluble form of *GITRL* was identified (7). *GITRL* may be expressed in antigen presenting cells (APC): monocyte, macrophage, dendritic cells (DC) (7), 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 (1). *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 (1).

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 (8) 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 (8), $CD4^+CD25^+$ cells exert inhibitory effects by blocking the induction of the IL-2 production by the $CD4^+CD25^-$ cells at the level of RNA transcription.

Interleukin 18 (*IL-18*) is a proinflammatory cytokine, which in conjunction with some other cytokines and chemokines contribute to the tumor 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 tumor malignancy.

Jung *et al.* (9) describe increased levels of inflammation associated interleukins *IL-15*,

IL-17, *IL-18* and *IL-18*, and their binding proteins in tumor tissue. Furthermore, RT-PCR and Western blot analysis revealed that *IL-18* was upregulated in tumor tissues and contributed to tumor progression through their pro-angiogenic effect.

Park *et al.* (10) showed increased serum levels of both *IL-18* and transferrin in MCF-7 breast cancer cell line. Nicolini *et al.* (11) found that some cytokines (*IL-1*, *IL-6*, *IL-11*, *TGF β1*) stimulate whilst others (*IL-12*, *IL-18*, *IFNs*) inhibit breast cancer proliferation and/or invasion. Similarly, high circulating levels of some cytokines appear to be favorable (*soluble IL-2R*) whilst others are unfavorable (*IL-1beta*, *IL-6*, *IL-8*, *IL-10*, *IL-18*, gp130) prognostic indicators.

According to Coskun *et al.* (12) interleukin *IL-18* has potent anti-tumor effect. Both serum *VEGF* and *IL-18* levels decreased after tamoxifen therapy.

Materials and methods

Experimental model

To investigate gene expression profile in human lymphocytes we carried out a PCR based analysis in acute and chronic inflammation and controls. For this purpose we collected tissue peripheral blood mononuclear cells (PBMC) from solid tumor patients: 10 patients with breast cancer, 4 lung cancer patients, 8 rheumatoid polyarthritis patients as chronic inflammation controls and from 19 volunteers as healthy controls. Moreover, we collected tissue samples from: 10 mammary tumors, 2 patients each from kidney, rectal, ovary and colonic solid tumor and 21 tonsils resulted from acute tonsillitis extirpation. We used the latter ones as acute inflammation controls, since this is the closest tissue microenvironment which is available from human subjects, and because most of the receptors we studied are not expressed in healthy naive lymphocytes, and we aimed the profiling of lymphocytes based on different circumstances of *in vivo* stimulation.

Protocols used in this work were approved by the Ethics Committee of the Sapientia University. According to the World Medical Association Declaration of Helsinki, revised in 2000 at Edinburgh, informed consent was obtained from all subjects.

mRNA analysis

Tissue samples were obtained from untreated patients. Tumor tissue was processed under sterile conditions using Medimachine (Becton Dickinson) by mechanical mincing using Medicons (Becton Dickinson) and pressing through 70 micrometer mesh Filcons cell strainer (Becton Dickinson). From the resultant single cell suspension, lymphocytes were isolated in density gradient, using Ficoll-Paque Plus (Amersham Bioscience). Peripheral blood lymphocytes were isolated from whole blood in the same manner, using Ficoll-Paque Plus (Amersham Bioscience) according to the manufacturer's protocol.

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 double-check the presence and quality of the extracted RNA, electrophoresis of the RNA was performed in agarose gel visualized with ethidium bromide. Strictly equal amounts of RNA (based on spectrophotometric measurement) 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 a specific amplicon. Secondly, dose dependent PCR was utilized when the optimal number of cycles

Table 1. Primers used in the PCR amplification experiments

Gene	Primer pairs
TGF β1	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'

was determined so as the amplification process remains in the exponential phase. For each reaction, the numbers of cycles were interpreted to obtain yes/no results. This information was confirmed by a number of pilot experiments with positive and negative controls. Due to the fact that the starting amount of template was the same, this provided a semi-quantitative analysis of the amplicons and their comparison to the control group, and the obtained data were relative values of the intensities. The sequences of the primers used in the PCR experiments are presented in *Table 1*.

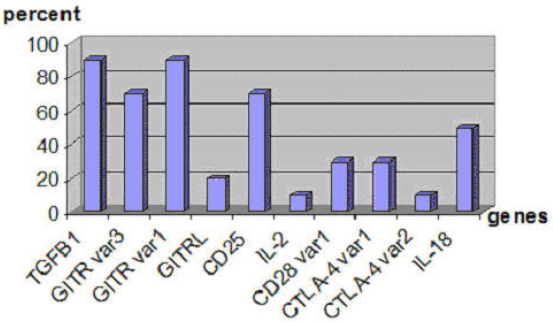

Figure 1. Percent of the positive samples in mammary tumor (n=10)

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*, for *CTLA-4 var1* and *var2*, for *CD28 var1*. 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 *Quantity-One* (BioRad), and the obtained data was processed using Excel and Matlab. Graphical representation of the data was performed in Matlab, using the box plot method. The advantage of this particular plot is that graph notch is a robust estimate of the uncertainty about the means for box-to-box comparison.

Each box has lines at the lower quartile, median, and upper quartile values. Student's *t* test was 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 mammary tumor

The mammary tumor 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) (*Figure 1*).

GITR was not expressed in PBL of the control patients. All chronic inflammation PBL samples showed elevated level of *GITR*, whilst the obtained relative intensities for the lung can-

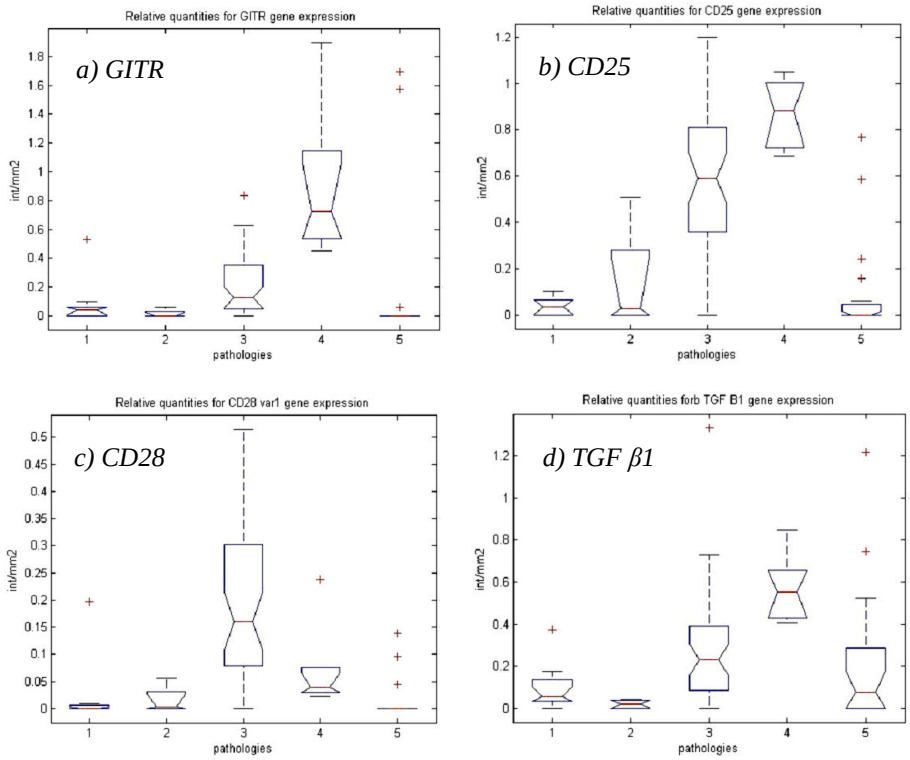

Figure 2. Relative quantities for a).*GITR mRNA* b). *CD25 mRNA* c). *CD28 mRNA* and *d). TGF β1* mRNA expression (1. breast cancer, 2. lung cancer, 3. acute inflammation, 4. chronic inflammation, 5. healthy control). Plots are illustrating the results of Quantity One volumetric analysis after background extraction and normalisation. p>0.05

cer were decreased as presented in *Figure 2a*.

In acute inflammation significant up-regulation of *GITR* was found ($p < 0.05$) compared to the breast cancer.

In other cancer samples such as kidney cancer and colonic cancer *GITR* appeared in small relative quantities in the majority of the cases.

We have found high levels of *CD25* expression in chronic inflammation in contrast to the PBL of the breast and lung cancer patients and controls, as presented in *Figure 2b*. Relative values for *CD25* expression in breast and lung cancer patients PBL were low, and statistically similar compared each other ($p < 0.05$). Moreover, in other cancer samples, in rectal and kidney tumor tissues, *CD25 mRNA* expression was similarly low.

Highest expression of *CD25* was obtained in chronic inflammation.

Following *CD28 mRNA* expression screening we have identified all eight mRNA splice variants described by Manisha Deshpande *et al.* (13). 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.

We have demonstrated upregulation of *CD28 mRNA* expression in acute tonsillitis compared to the breast cancer. Lung cancer patients presented low *CD28* expression in PBL, same as in chronic inflammation.

Control patients did not show *CD28* expression, as presented in *Figure 2c*.

Our results showed similar expression

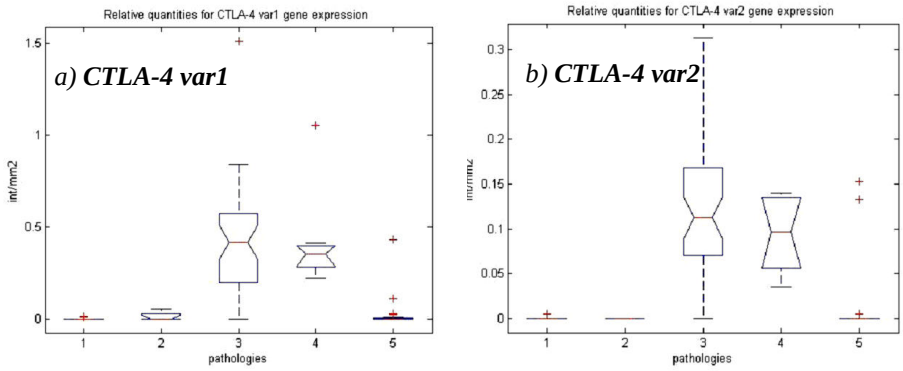

Figure 3. a). *CTLA-4 var1* and b). *CTLA-4 var2 mRNA* expression in inflammation and cancer (1. breast cancer, 2. lung cancer, 3. acute inflammation, 4. chronic inflammation, 5. control) Plots are illustrating the results of *Quantity One* volumetric analysis after background extraction and normalisation. p>0.05

of *TGF β1 mRNA* in breast cancer and in tonsil samples as presented in *Figure 2d*. We proved statistically higher ($p < 0.05$) *TGF β1* expression in PBL of the arthritis patients than in control samples or lung cancer.

CTLA-4 mRNA showed expression of both two *CLTA-4 mRNA* variants in PBL 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 *Figure 3*.

Our results showed upregulation of *CLTA-4 mRNA* expression in chronic inflammation compared to breast cancer samples where *CLTA-4 mRNA* is not expressed. *CTLA-4* variants were highly expressed in PBL of chronic inflammation patients contrary to the lung cancer patients and controls PBL. Small *CLTA-4 mRNA* expression was observed for both variants in PB of breast cancer patients.

GITRL mRNA was not detectable in the control group, but 1 from n=4 PB lung cancer samples expressed *GITRL*. *GITRL* was significantly ($p < 0.05$) upregulated in approximately half of the chronic inflammatory disease patients, as presented in *Figure 4a*. Two of n=10 solid breast tumors express *GITRL mRNA*. *GITRL* was also found to be upregulated in tonsillitis patients compared to the breast tumors.

GITRL was also found to be expressed in the rest of cancer samples expressed in the rest of cancer samples: 2 rectal tumors and 2 kidney tumor tissues.

Five from a total of 10 patients expressed IL-18 in breast cancer. Our results indicated low IL-18 expression in mammary tumor tissue compared to the tonsils as presented in Figure 4b.

Also, *IL-18* expression was determined from PB lymphocytes in rheumatoid arthritis patients (8 from n=8), mammary tumor patients (8 from n=10), similarly to the control group (8 from a total of 19 samples).

IL-18 was highly expressed in chronic inflammation, followed by control samples and mammary tumor ($p < 0.05$), as presented in *Figure 4b*.

IL-18 was highly expressed in other cancer tissue infiltrates such as ovary tumor (2 from n=2), rectal tumor (2 from n=2), kidney tumor (2 from n=2), colonic tumor (2 from n=2) and last stage uterus tumor (1 from n=1).

Our results showed elevated *IL-2 mRNA* level in acute inflammation as presented in *Figure 4c*. *IL-2 mRNA* was not expressed neither in PBL of control patients, chronic inflammation and breast cancer patients (*Figure 4c*).

Interesting *IL-2 mRNA* were found in: rectal tumors (2 from total n=2) and kidney tumors (2 from total n=2) patients.

Finally, we compared gene expression in tumor and PMBC lymphocytes in breast cancer. As we present in *Figure 5*, gene expression profile was correlated in tumor tissue and PMBC lymphocytes. Peripheral blood lymphocytes seem to have higher levels of expression than tumor tissue and, since correlation is conserved, we find it a good model of lymphocyte profiling in this case.

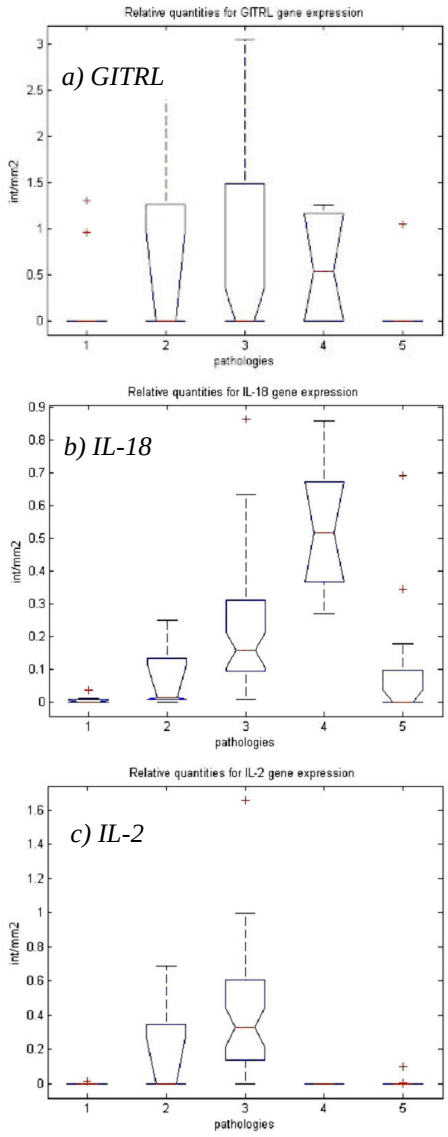

Figure 4. Relative intensities for a). *GITRL* b). IL-18 and c). IL-2 expression in cancer and inflammations (1. breast cancer, 2. lung cancer, 3. acute inflammation, 4. chronic inflammation, 5. control) Plots are illustrating the results of Quantity One volumetric analysis after background extraction and normalisation. p>0.05

Discussions

GITR is a costimulatory receptor which is reasonable to be expressed in all circumstances of immune stimulation. Treg cells constitutively express higher levels of *GITR* than conventional T cells, independently of location and activation state (14). Since GITR stimulates responder T cells and also Treg cells, the overall effect is a stimulation of the immune response, and a significant reduction of Treg suppressor activity, which is concordant with the autoimmune pathogenesis. Data already proves the role of anti-GITR antibodies in restoring this function by both removing Tregs and also stimulating responders, by inducing proliferation. Proliferating Tregs lose their suppressive capacity. Furthermore GITR seems to be at least as important, especially in CD8 T lymphocytes costimulation, as CD28 (15).

Anti-CTLA-4/anti-GITR mAb combination treatment exhibited far stronger antitumor effects compared to either antibody alone. This strong antitumor effect is explained by an increased number of CD8+ T cells infiltrating tumor sites in anti-CTLA-4 mAb-treated mice, and increased cytokine secretion and Treg resistance of tumor-specific CD8+ T cells with strongly upregulated *CD25* expression in anti-GITR mAb-treated mice (16). Cohen *et al.* demonstrated that the anti-GITR antibody enhances immune response towards melanoma by stimulating responder T cells and by inhibiting Treg cells (17). Ponte *et al.* (18) demonstrated that anti-GITR antibody is an effective vaccine adjuvant against foreign antigens, and suggested using it as a potential adjuvant in solid tumor therapy. Furthermore, this antibody was used in an adenomavirus based T cell vaccine model, eradicating permanently and

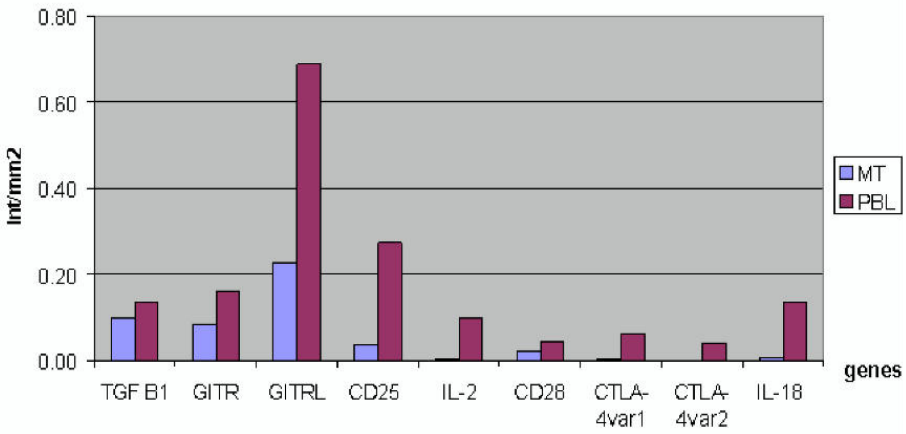

Figure 5. Comparison of tumor and PMBC lymphocyte expression profiles in breast cancer

completely the papillomavirus-induced tumors, which was not the case with other adjuvants like anti-CD4 or interferon-α (19). On the other hand, *CD25*$^+$ is a phenotypical marker for Treg cells (8).

Transforming growth factor beta, produced by macrophages, enhances tissue regeneration (20). *TGF β1* signaling 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 tumor cell's escape from an effective immune response. *TGF β1* levels are associated with tumor progression, severity, and metastatic capacity (21-25).

According to Zhang (1), *TGF β1*, *IL-2R* (*CD25*), *GITR*, *CD28* and *CTLA-4* (*CD152*) are upregulated in the tumor-specific T cells following activation versus naive T cells.

According to Baltz *et al.* (26) *GITRL* is constitutively expressed by human tumors 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. GITRL signaling alters the expression of regulatory surface molecules and stimulates production of the immunosuppressive cytokine *TGF β1* in tumor cells (26).

Tuyaerts (3) 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 (3).

Jung *et al.* (9) 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 tumor tissues. Also, RT-PCR and Western blot analysis revealed upregulation of the *IL-18* which is a pro-angiogenic factor in tumors.

IL-2 mRNA was not expressed by $CD4^+CD25^+$ cells neither anti-CD3 stimulated nor non-stimulated, but anti-CD3 stimulated $CD4^+CD25^-$ cells expressed elevated levels of IL-2 mRNA contrary to the non-stimulated $CD4^+CD25^-$ cells (8) in mouse model. Despite of the constitutive expression of CD25 on $CD4^+CD25^+$ cells, these cells did not proliferate to IL-2, anti-CD3 or anti-CD28 stimulation.

According to Thornton and Shevach (8), $CD4^+CD25^+$ cells exert inhibitory effects by blocking the induction of the IL-2 production by the $CD4^+CD25^-$ cells at the level of RNA transcription. It seems that *IL-2 mRNA* is expressed in stimulated effector cells, not in naive effector T cells or in Treg.

Conclusions

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

We have found elevated level of *CD25*, *GITR*, *GITRL*, *TGF β1* mRNA in cancer, upregulation of *IL-2* and *CD28* in acute inflammation, and upregulation of *CD25*, *GITR*, *TGF β1*, *IL-18* in chronic inflammation. We have found no *CTLA-4* expression in cancer tissue.

In control samples *IL-2*, *CD28*, *CTLA-4*, *GITR* and *GITRL mRNA* showed lack of expression or low levels of *TGF β1*, *IL-18* and *CD25*.

This data is consistent with the current theory according to which tumors obviate immune surveillance by using Treg, which protects the tumor against the host's responder T cells.

Furthermore, there is a good correlation between tissue lymphocyte expression profile and PBMC expression profile, and this data can have future clinical utility.

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

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