Immunosuppression affects CD4+ mRNA expression and induces Th2 dominance in the inflammatory microenvironment of cutaneous squamous cell carcinoma in organ transplant recipients

Maria Kosmidis[1,6], Piotr Dziunycz[1,6], Mayte Suárez-Fariñas[2], Beda Mühleisen[1], Leo Schärer[3], Severin Läuchli[1], Jürg Hafner[1], Lars E. French[1], Carsten Schmidt-Weber[4], John A. Carucci[5], Günther F.L. Hofbauer[1]

[1] Department of Dermatology, University Hospital Zurich, Zurich, Switzerland

[2] Laboratory for Investigative Dermatology, The Rockefeller University, 1230 York Ave, New York, NY, 10065

[3] Dermatopathologische Gemeinschaftspraxis, Friedrichshafen, Germany

[4] Allergy and Clinical Immunology, Imperial College London, UK

[5] Department of Dermatology, Weill Medical College of Cornell University, 1305 York Ave, New York, NY, 10021

[6] These authors contributed equally

Key words

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Corresponding author

Günther Hofbauer, MD, Department of Dermatology, University Hospital Zurich, Gloriastrasse 31, 8091 Zürich, Switzerland, Phone +41 44 255 1111, Fax +41 44 255 45 49, hofbauer@usz.ch

Abstract

Squamous cell carcinoma (SCC) is the most frequent cancer in organ transplant recipients (OTRs):. Compared to the general population, OTRs are at a 60- up to 100-fold risk. The immune system plays a major role in the fight against SCC, however little is known about the local inflammatory response in SCC at all. We analyzed quantity and quality of the perineoplastic inflammatory SCC microenvironment in immunocompetent patients and immmunosuppressed OTRs.

SCC RNA was analyzed in 8 groups relating to Th1 vs. Th2 response: GATA3, IL-12, TH1TH2, ST IL13, ST IL-4, IFN-γ up/ down; IL-4 using Gene Set Enrichment Analysis (GSEA). SCC from 15 immunocompetent patients and 13 OTRs was analyzed by real-time RT-PCR for CD4, CD8, TBET, GATA-3, FOXP3, RORC, IFN-gamma, IL-4, TGF-beta, IL-10 and IL-17A mRNA expression characterizing Th1, Th2, T-reg and Th17 populations. Immunohistochemistry was performed in SCC for FOXP3 expression.

RNA expression profile of SCC patients was analyzed for 8 different set of genes relating to Th1 vs. Th2 response: GATA3, IL-12, TH1TH2, ST IL13, ST IL-4, IFN-γ up/ down; IL-4 using Gene Set Enrichment Analysis (GSEA). SCC from 15 immunocompetent patients and 13 OTRs was analyzed by real-time RT-PCR for CD4, CD8, TBET, GATA-3, FOXP3, RORC, IFN-gamma, IL-4, TGF-beta, IL-10 and IL-17A mRNA expression characterizing Th1, Th2, Treg and Th17 populations. Immunohistochemistry was performed in SCC for CD3, CD4, CD8 and FOXP3 expression.

Considerable inflammation was seen in both patient groups. SCC in immunocompetent patients and OTRs was associated with a mixed Th1 and Th2 gene expression signature. CD4+ mRNA was diminished in immunosuppression, while CD8+ did not vary. Skin adjacent to SCC in OTRs showed Th2 expression pattern as compared to immunocompetent patients. T-BET as well as INF-gamma mRNA expression was decreased in the OTR group. On the other hand GATA-3 and IL-4 mRNA expression did not differ. Although Th17 weighted inflammation, characterized by expression of RORC mRNA was unchanged, IL-17A mRNA level was markedly decreased with immunosuppression. Regulatory T cells, characterized by FOX-P3 and TGF-beta mRNA level, were decreased in OTRs. IL-10 mRNA expression did not vary between the groups.

The inflammatory microenvironment of cutaneous squamous cell carcinoma in organ transplant recipients is characterized by an attenuated state of local immune response comprising a lesser quantity of helper T cell response and a Th2 polarized quality. Our findings support the hypothesis that non -tumor-bearing

skin adjacent to SCC in OTRs is not necessarily normal and that the local microenvironment may contribute to a field effect contributing to higher recurrence rates and more aggressive behavior observed in these patients.

Introduction

Squamous cell carcinoma of the skin (SCC) is a common tumor occurring on sun-damaged skin. Metastasis from these SCCs is a rare event in the general population. The immune system plays a major role in the fight against SCC. An inflammatory response eliminates most intraepithelial lesions before invasive SCC develops [1, 2]. Recognition and elimination of tumor antigen-bearing cells may be one mechanism of tumor development [3]. The magnitude of the inflammatory response overall seems clinically important for clearance of intraepithelial lesions [4]. Likewise, lack of tumor surveillance by the immune system is of importance [2]. Drug-induced immunosuppression in organ transplant recipients (OTRs) dramatically alters the course of SCC. Skin cancers are the most frequent malignancies following organ transplantation [5, 6]. The incidence of NMSC increases steadily with time after transplantation and varies in the United States and Western Europe from 5% to 10-27% at 2, 10 and 20 years, respectively [7-9]. In OTR the ratio of SCC to BCC (4/1) is reversed compared with the population at large, and this reversal increases with decreasing latitude, sun exposure and length of follow-up [5, 6].. Compared to the general population, OTRs are at a 60- up to 100-fold risk of SCC development [5, 10]. SCC in OTRs is characterized by a higher risk for metastasis in up to 20% and shows a more aggressive course than SCC in the immunocompetent, general population.

The relationship between immunosuppression and the development of skin malignances is well recognized, however, the mechanism underlying this relationship is not well understood. The quantity and quality of the inflammatory infiltrate have not been studied extensively [11]. Differences in the perineoplastic inflammatory microenvironment on cellular and cytokine levels may explain the differing characteristics of SCC in immunocompetence and drug-induced immunosuppression. We therefore analyzed the perineoplastic inflammatory microenvironment in SCC regarding quantity and quality in immunocompetent patients and OTRs.

Materials and methods

Ethical considerations

Ethical approval was obtained from the institutional review board for processing specimens from clinically indicated excisions.

SCC Th1/Th2 pathway analysis

RNA Isolation: Total RNA isolation and reactions were performed as previously described [12]. Briefly, SCC tumor samples from transplant recipients and non transplant recipients, removed at Mohs surgery (n=10-12), and patient-matched site-matched perineoplastic non-lesional (PTNL) skin (n=10-12), were obtained at the time of repair after clear margins were achieved. Normal skin was obtained from abdominoplastic reductive surgery (n=4). All samples were snap-frozen and stored in liquid nitrogen. Individual frozen samples were placed in 1ml of room temperature RLT buffer with 1% β-mercaptoethanol (Qiagen, Valencia, CA) and immediately homogenized at full power for 30 seconds using a PowerGen 1000 homogenizer (Fisher Scientific, Pittsburgh, PA). Homogenates were sonicated on ice for 20 seconds at full power. DNA was removed with on-column DNAse digestion using an RNAse-free DNAse Set (Qiagen, Valencia, CA). RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. Total RNA concentration and purity were evaluated using an Ultraspec 2100 pro-spectrophotometer (Amersham Biosciences, Piscataway, NJ).

Statistical analysis: SCC microarray data has been previously published by Haider, et al [12]. We wanted to evaluate differential expression of sets of genes or pathways relating to Th1 vs. Th2 response. We included GATA3, IL-12, TH1TH2, [13]; ST IL-13 and ST IL-4 [14]; IFN-γ up/ down [15]; IL-4 [16] which were either known pathways or gene sets established by our group. We first estimated mean fold change between SCC, TSCC, PTNL, TPTNL and normal skin for each pathway. We then tested the significance of those findings using Gene Set Enrichment Analysis (GSEA) [17]. GSEA is a method that determines whether a previously defined set of genes shows statistically significant differences between two biological states, for example SCC vs. normal skin, using rank statistics. If the genes in the gene set or pathway in question are ranked highly in "SCCs vs. normal fold change", the ES score will be near 1, if the opposite effect occurs, then the value approaches -1. Because we were working with several lists, a normalized enrichment score (NES) allowed us to compare the enrichment score between lists [17].

Real time RT-PCR

Tissue samples: Measurements were performed with fresh material from clinically indicated excisions. 3mm punch specimens from the SCC border area were collected at the time of surgery, immediately homogenized in TRIzol reagent (Invitrogen AG, Basel, Switzerland), and then stored at -80°C. All specimens' diagnoses were confirmed by a board-certified dermatohistopathologist.

mRNA extraction and reverse transcription: For mRNA extraction, the samples were thawed, and total mRNA was isolated using TRIzol reagent following the instructions provided by manufacturer. Complementary DNA (cDNA) was synthesized by reverse transcription using First strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics AG, Rotkreuz, Switzerland), following the protocol provided, but with an incubation time of 90 minutes. After a 1:4 dilution with sterile water, cDNA was stored at -20°C and used as template for subsequent quantitative real-time polymerase chain reactions (RT-PCR).

PCR primers: cDNA amplifications were performed using Ready-to-use amplification primer mix for RT-PCR (Search LC GmbH, Heidelberg, Germany), with primers corresponding to CD8, CD4 and to specific transcription factors and cytokines defining CD4 subpopulations. Those are T-box expressed in T cells (TBET) and INF-gamma characterizing Th1; GATA-binding protein 3 (GATA-3) and IL-4 for Th2 cells; Forkhead Box P3 (FOX-P3), TGF-beta, and IL-10 characterizing Tregs. We also measured the expression of RAR-related orphan receptor C (RORC) and IL-17 characterizing Th17 cells (RORC primer forward: AGTCGGAAGGCAAGATCAGA; RORC primer reversed: CAAGAGAGGTTCTGGGCAAG; IL-17A primer forward: CCCCAGTTGATTGGAAGAAA; IL-17A primer reversed GAGGACCTTTTGGGATTGGT). Amplification of Glyceralaldehyde-3-phosphate dehydrogenase (GAPDH; primers purchased from Search LC GmbH, Heidelberg, Germany) as housekeeping gene was performed for relative quantification.

RealTime - PCR: All PCR amplifications were carried out on a Light Cycler instrument (LightCycler 2.0, Roche Diagnostics, AG, Switzerland) applying the recommended running conditions. Each sample was processed in a total volume of 20ul reaction mixture, including 15 ul H2O, 2 ul primer mix, 2 ul polymerase (Light Cycler Fast Start DNA Master SYBR Green I, Roche Diagnostics AG, Mannheim,

Germany) and 1 ul of diluted cDNA. With each set of reactions and for each primer, negative and positive controls (H_2O and leukocyte cDNA respectively) were included. All samples and controls were processed in triplicates. Amplification of the requested sequence was verified by melting temperature analysis.

Statistical analysis: Statistical analyses were performed using GraphPad Prism 5 and SPSS 15.0. For relative quantification, we applied the comparative delta-Ct method using GAPDH mRNA expression as internal reference [18]. CD4 and CD8 were normalized to GAPDH, and then compared between immunocompetent patients and OTRs in order to quantify inflammation. Expression of Th1-, Th2-, Treg- and Th17-specific transcription factors and cytokines was normalized to GAPDH for the quality of the inflammatory microenvironment. Expression of transcription factors and cytokines was analyzed with Mann-Whitney test or Kruskal-Wallis-test followed by the post-hoc Dunn's test. Expression patterns of transcription factors and cytokines were compared between immunocompetent patients and OTR groups by Greenhouse-Geisser test. For box plot presentation, results are displayed in logarithmic scale.

Immunohistochemistry

3- to 5-um adjacent sections from formalin-fixed paraffin-embedded tissue were used for immunohistochemistry as described previously [19]. The deparaffinized sections were heated in a 100-W household microwave oven at maximum power for three times 5 minutes each in 10 mmol/L citric acid for antigen retrieval. Primary antibody was then applied for 60 minutes at room temperature. Immunohistochemical stainings were performed with monoclonal IgG mouse antibodies specifically binding human CD3 (A 0452; Dako, Glostrup, Denmark), CD4 (NCL-1F6; Novocastra, Newcastle upon Tyne, UK) and CD8 (M 7103; Dako). FOX-P3 was stained using rabbit polyclonal ab10563 (Abcam Limited, Cambridge, United Kingdom). Secondary staining was performed using the alkaline phosphatase anti-alkaline phosphatase method. Normal epidermal cells, lymphocytes, fibroblasts as well as other cells of the subcutaneous tissue served as internal negative controls, sections of known positive tissue such as tonsils for lymphocyte antigens as external positive controls. Immunoreactivity was assessed over the perineoplastic inflammatory microenvironment and rated as 0-5%, 6-25%, 25-50%, 51-75%, 76-100% of all inflammatory cells. These results will be published in a larger context [11]. Statistical analysis was performed using Microsoft Excel 2000 and SPSS for Windows 11.5.

Results

Patient characteristics

RT-PCR: SCC specimen from 13 organ transplant recipients (12 male, 1 female) with mean transplant duration of 13.2 years were collected. In nine patients, immunosuppressive treatment consisted of Cyclosporine A in combination with other immunosuppressive drugs. One patient was receiving Sirolimus. Immunocompetent patients comprised 14 individuals (10 male, 4 female), mean age 77.4 years, without immunosuppressive medication or other immunodeficiencies. RT-PCR patients' and tissue sample characteristics are summarized in Supplemental Table S1.

Immunohistochemistry: Immunohistochemical staining was performed with tissue material from 42 immunosuppressed and 43 immunocompetent patients. Of the 42 organ transplant recipients, 32 patients had received a kidney, 4 had received a lung and 6 a heart graft, respectively. Gender of the immunocompetent patients was adjusted to 74% male, as this is the proportion of male organ transplant recipients at Zurich University Hospital. Age was closely matched. Patient characteristics for immunohistochemistry are summarized in Supplemental Table S2.

SCC in immunocompetent patients is associated with a mixed Th1 and Th2 gene expression signature.

The genomic signature for Th1 and Th2 was analyzed in our samples from SCC and non-tumor bearing skin from immunocompetent patients. The mean fold changes for GATA3, IL-2, IL-4, ST IL-13, ST IL-4, Th1Th2, "IFN-γ up" and "IFN-γ down" were evaluated in SCC, adjacent non-tumor bearing skin, and normal skin as per methods. IFN-γ up/down refers to genes up or down regulated with addition of IFN-γ to cultured keratinocytes [15]. In SCC vs. normal skin, and non-tumor bearing skin vs. normal skin, we found significantly increased fold changes for GATA3, IL-12, ST IL-4, and "IFN-γ up" (p-values all <0.05) (Figure 1). The significance of those findings was also tested using Gene Set Enrichment Analysis (GSEA). In GSEA, statistically significant enrichment was observed for "IFN-γ up", ST IL-4, and Il-12 in both SCC and adjacent, non–tumor bearing perilesional skin with "IFN-γ down" enriched in normal skin (Supplemental Table S3).

SCC in organ transplant recipients is associated with a mixed Th1 and Th2 gene expression pattern.

The genomic signature for Th1 and Th2 was analyzed in our samples from SCC and non-tumor bearing skin from immunocompromised transplant recipients. The fold changes of GATA3, IL-2, IL-4, ST IL-13, ST IL-4, Th1Th2, "IFN-γ up" and "IFN-γ down" were evaluated TSCC vs., normal skin. In TSCC, we observed significantly increased fold changes for IL-12, ST-IL-4 and "IFN-γ up" (p-values all <0.05) (Figure 1), GSEA shows significant enrichment for IFN-γ "up" and ST-IL4 in the TSCC group (Supplemental Table S4).

CD4+ mRNA expression is less pronounced in SCC of OTRs, while CD8+ does not differ significantly

Real-time PCR analysis showed a significant reduction in CD4 mRNA expression (p=0.009; Fig. 2b; Table 3) in the inflammatory microenvironment of OTRs (OTR mean $8.29*10^{-4}$, SD $\pm 1.12*10^{-3}$, interquartile range $7.25*10^{-5}$ – $1.16*10^{-3}$; immunocompetent mean $3.49*10^{-3}$, SD $\pm 3.44*10^{-3}$, interquartile range $8.68*10^{-4}$ – $4.94*10^{-3}$).

By contrast, cytotoxic CD8+ mRNA is at about the same expression level in both groups (p=0.1; Fig. 2a; OTR mean $8.64*10^{-4}$, SD $\pm 1.16*10^{-3}$, interquartile range $5.32*10^{-5}$ – $15.52*10^{-4}$; immunocompetent mean $22.35*10^{-4}$, SD $\pm 33.47*10^{-4}$, interquartile range $2.71*10^{-4}$ – $30.56*10^{-4}$). Assessing CD3, CD4 and CD8 immunoreactivity by immunohistochemistry showed no significant changes (Fig. 4).

Local inflammation is Th2 polarized in SCC from OTRs

We analyzed expression of genes corresponding to GATA3, IL-2, IL-4, ST IL-13, ST IL-4, Th1Th2, "IFN-γ up" and "IFN-γ down" in SCC of immunocompetent patients vs. OTRs. In this "head to head" comparison, SCC from immunompetent patients showed increased mean fold change for GATA3 pathway and decreased mean fold change for IFN-γ up pathway indicative of an enhanced Th2 response. Non tumor bearing skin adjacent to excised SCC in immunocompetent patients showed increased mean fold change for Gata3, IL-4, ST-IL13, and ST-IL4, also indicative of a Th2 response (Figure 1). That these were increased compared to SCC in OTRs is consistent with the overall diminished inflammatory response in these patients, As microarray is a rudimentary method for gene expression analysis and needs to be further validated, we explored the nature of the infiltrate in OTRs vs. immunocompetent patients by real-

time PCR on higher number of samples and found differences in Th2 vs. Th1 composition. The analysis showed significant decrease in Th1 weighted inflammation, measured by TBET mRNA expression, in OTR SCC (p=0.0056; Fig.2c; OTR mean $4.17*10^{-5}$, SD ±5.05*10-5, interquartile range 1.28*10-5 – 5.68*10-5; immunocompetent mean 1.65*10-4, SD ±1.66*10-4, interquartile range 4.4*10-5 – 2.38*10-4). Similarly, expression of the Th1 specific cytokine INF-gamma was significantly decreased with immunosuppression (p=0.02; Fig. 2d; OTR mean $1.56*10^{-4}$, SD $\pm 2.87*10^{-4}$, interquartile range $2.1*10^{-5}$ – $1.52*10^{-4}$; immunocompetent mean $5.65*10^{-4}$, SD $\pm 8.3*10^{-4}$, interquartile range $8.63*10^{-5}$ – $8.2*10^{-4}$). On the other hand, GATA-3 mRNA expression, measured by real-time PCR, did not differ with immunosuppression (p=0.08; Fig. 2e; OTR mean $1.89*10^{-2}$ SD $\pm 1.01*10^{-2}$, interquartile range $1.03*10^{-2}$ – $2.75*10^{-2}$; immunocompetent patients mean $1.15*10^{-2}$ SD $\pm 1.02*10^{-2}$, interquartile range $3.08*10^{-3}$ – $1.74*10^{-2}$), but the increased expression of IL-4 in OTRs could not be confirmed with real-time PCR (p=0.14; Fig. 2f; OTR mean $3.12*10^{-6}$, SD $\pm 4.32*10^{-6}$, interquartile range $7.71*10^{-10}$ – $6*10^{-6}$; immunocompetent mean $5.84*10^{-6}$, SD $\pm 6.46*10^{-6}$, interquartile range $8.52*10^{-7}$ – $1.1*10^{-5}$). Moreover, real-time PCR analysis showed that in the perineoplastic microenvironment of both immunocompetent patients and OTRs, GATA-3 mRNA expression markedly exceeds T-BET mRNA expression ($p<0.001$ in both groups; Fig. 3a, 3b).

FOXP3 mRNA and protein expression is diminished in OTRs

Perineoplastic mRNA levels of FOX-P3, a transcription factor associated with Tregs, are diminished in perineoplastic inflammatory infiltrate of OTRs as measured by both real-time PCR (p=0.045; Fig. 2g; mean 6.89*10-4 SD ±6.64*10-4, interquartile range 1.35*10-4 – 1.2*10-3; immunocompetent patients: mean 2.44*10-3, SD ±4.96*10-3, interquartile range 4.6*10-4 – 1.74*10-3), and immunohistochemical staining (p=0.04; Fig. 5). A similar difference was also observed for mRNA levels of TGF-beta, an immunosuppressive cytokine associated with Tregs. Its mRNA expression was significantly decreased (p=0.036; Fig. 2h) with immunosuppressive treatment (OTR mean $8.93*10^{-3}$ SD $\pm 4.8*10^{-3}$, interquartile range $3.96*10^{-3}$ – $13.2*10^{-3}$; immunocompetent mean $13.91*10^{-3}$ SD $\pm 5.9*10^{-3}$, interquartile range $9.97*10^{-3}$ – $17.53*10^{-3}$). Detailed analysis of OTR SCCs sub-divided according to immunosuppressive treatment showed a significant decrease in TGF-beta mRNA expression with prednisone treatment compared to immunocompetent SCC ($p<0.05$, Fig. 3b). On the other hand, mRNA expression of IL-10, another key immunosuppressive cytokine produced by Tregs, did not vary with immunosuppression (p=0.10; Fig. 2i;

OTR mean $1.19*10^{-4}$ SD $\pm 1.82*10^{-4}$, interquartile range $4.18*10^{-5}$ – $1.46*10^{-4}$; immunocompetent mean $2.27*10^{-4}$ SD $\pm 2.43*10^{-4}$, interquartile range $6.35*10^{-5}$ – $3.43*10^{-4}$).

RORC mRNA expression is unchanged with immunosuppression, while IL-17A mRNA expression is decreased in OTRs

Expression of Th17 specific transcription factor RORC was similar in both patients groups (p=0.34; Fig. 2j; OTR mean 9.72*10-4, SD ±1.33*10-3, interquartile range 2.15*10-4 – 1.25*10-3; immunocompetent patients: mean 4.53*10-4, SD ±4.56*10-4, interquartile range 9.79*10-5 – 8.74*10-4). However expression of the Th17 cytokine, IL-17A was significantly decreased in OTRs (p=0.016; Fig. 2k; OTR mean $5.03*10^{-5}$, SD $\pm 5.18*10^{-5}$, interquartile range $1.33*10^{-5}$ – $7.43*10^{-5}$; immunocompetent mean $6.78*10^{-4}$, SD $\pm 9.4*10^{-4}$, interquartile range $5.19*10^{-5}$ – $1.19*10^{-3}$). Detailed analysis of OTR SCC sub-divided according to immunosuppressive treatment showed a significant decrease in IL-17A mRNA expression with prednisone treatment compared to immunocompetent SCC ($p<0.05$, Fig. 3a).

Transcription factor but not cytokine expression differs between patient groups

Overall analysis with the Greenhouse-Geisser test shows that expression patterns of transcription factor mRNA' (p=0.003) but not cytokines mRNA (p=0.241) differ with immunosuppression (Fig. 4). Detailed analysis showed that the RORC/TBET mRNA relationship differs with immunosuppression. In OTRs, but not in immunocompetent patients, RORC was found at a markedly higher expression level than T-BET ($p<0.05$), but there is no difference in the relationship between IL-17A and IFN-gamma expression levels in both groups. Moreover, in OTRs the IL17A/IL4 and IL17A/TGF-beta mRNA expression relationship decreases when compared to immunocompetent patients (Fig. 4).

Discussion

Multiple clinical phenomena highlight the close relationship between cutaneous SCC risk and immunity. For example, actinic keratoses, benign lesions that can progress to SCC, are known to regress spontaneously in immunocompetent patients with prolonged photoprotection, presumably via immunological mechanisms [20]. On the other hand, dendritic cells from human SCC show diminished capacity to present antigens to T cells in a microenvironment rich in IL-10, TGF-β, and VEGF-A [21]. Skin cancers result both from a decrease in immunosurveillance and from the direct oncogenic effects linked to some immunosuppressants [22], although it is difficult to know what mechanisms predominate. The magnitude of the inflammatory response overall seems clinically important for the clearance of lesions [4].

In our study, considerable inflammatory activity was present in neoplasms from both immunocompetent and immunocompromised patients. CD4 mRNA expression was reduced in OTRs, whereas CD8 mRNA expression remained at similar level. An association with reduced CD4+ cells in the peripheral blood of renal transplant recipients and skin cancer has been reported [23], and may be reflected in our study on the level of the inflammatory microenvironment of squamous cell carcinoma of the skin. Interestingly, the incidence of NMSC seems proportional to the level of immunosuppression: lower CD4 counts were found in OTR with NMSC versus patients without NMSC [23]. Recent findings suggest that depletion in CD4+ T cells may result in a significant increase in UVB-induced cutaneous inflammation [24]. In humans, CD4+ T cells make up the predominant lymphocytic infiltrate in the skin in response to UV exposure [25], and these cells were found to be important in inducing regression of both actinic keratotic lesions (SCC precursors) as well as invasive SCC [26-29].

In rejection and transplantation tolerance a predominantly Th2-weighted inflammatory response correlates with tolerance [30]. Similar to our findings, a reduced anti-donor-specific reactivity, including a state of immunologic tolerance, has been associated with a decrease in expression of the Th1-associated cytokines IL-2 and IFN-γ in PBMC. Conversely, IL-4, IL-5, and IL-13 production typical of Th2 cells correlates with long-term allograft survival. Th1 cells can regulate the function of Th2 cells, while in turn Th2 cells can down-regulate an aggressive Th1 response [31]. Our data show that the perineoplastic infiltrate in SCC of OTRs appears Th2-weighted, recapitulating previous systemic observations on the level of the microenvironment. Non–tumor bearing skin adjacent to SCC in OTRs differs from

immunocompetent normal skin. The local microenvironment significantly inhibiting Th1 response, thus favoring Th2, may result in a field effect permitting the higher recurrence rates and more aggressive course of SCC in OTRs.

Lately, regulatory T cells have gained attention for their function in the suppression of immune responses. Many cancers display higher proportions of CD4+CD25+ regulatory cells, which are thought to down-regulate the anti-tumor response [32, 33]. Our results, however, show that OTRs display a significantly lower proportion of FOXP3+Tregs as well as a lower expression of TGF-beta, a key cytokine for regulatory T cells, within the perineoplastic inflammatory infiltrate. Generally, a reduction in Tregs should favor the anti-tumor response [33]. How may SCC in OTRs be different? The calcineurin inhibitor cyclosporine A negatively impacts regulatory T cell generation and function [34, 35] and is a mainstay of the immunosuppressive regiment in our OTRs. This may well be the reason for the lower proportion of FOXP3+ Tregs in OTRs. However, the decrease of TGF-beta expression in OTRs was unexpected, as cyclosporine A administration was shown to increase production of this cytokine in the transplanted organ [36, 37]. Detailed analysis of OTR SCC according to immunosuppressive drugs showed that decreased TGF-beta expression correlates with prednisone treatment. This finding is consistent with previous reports, in which prednisone was shown to decrease TGF-beta expression in experimental models [38]. Another explanation for the decreased mRNA expression of regulatory T cells is the impact of Th1 cells on the Treg subpopulation [39]. Wang *et. al*. showed the requirement of IFN-gamma for the induction of FoxP3 and the conversion of CD4+ CD25+ T cells to CD4+ regulatory T cells. Along these lines, the decreased IFN gamma expression found in our SCC of OTRs may explain the reduced expression of FoxP3 in OTRs.

T-helper 17 (Th17) cells are a newly described lineage of CD4+ T cells, characterized by their production of interleukin-17A (IL17A) and other cytokines. Physiologically, Th17 cells are responsible for the protection against certain pathogens and are involved in inflammation and recruitment of innate immunity. Th17 cells are also linked to autoimmune tissue destruction previously attributed to Th1 activity [40, 41]. Naturally occurring Th17 cells have been found in the tumor microenvironment in animal models and in patients, but their specificity and function remain unclear [42, 43]. In our measurements, we found that although Th17-associated transcription factor expression RORC was at the same level in immunocompetent patients and OTRs, the Th17 specific cytokine, IL-17A was decreased in SCC of

OTRs. Furthermore, detailed analysis of OTR group shows decreased IL-17A expression in perineoplastic tissue correlated mostly to prednisone. Recent reports show that IL-17 may be involved in allograft rejection to the same degree as IFN-gamma, and several independent studies showed that immunosuppression inhibits expression of both cytokines [44-47], favoring graft taking. Reduced IL17A expression may weaken the anti-tumor response against SCC in OTRs.

In summary, we find the perineoplastic infiltrate of SCC in OTRs to be distinct from the one in immunocompetent patients. Reduced CD4+ cells, Th1 cells as well as IL17A-expressing cells probably compromise the local inflammatory response in SCC of OTRs. Reduced numbers of perineoplastic Tregs in OTRs probably result from the immunosuppressive medication and may not permit an increase in inflammation sufficient to stem the dramatically increased SCC carcinogenesis in OTRs. Local alteration of the tumor microenvironment by targeting the distorted perineoplastic infiltrate of SCC in OTRs could prove beneficial against SCC formation.

Acknowledgements

Laboratory team, Department of Dermatology, University Hospital Zurich, Switzerland

Department of Biostatistics, Institute for Social and Preventive Medicine, University of Zurich, Switzerland

References

Figure legends

Figure 1

Microarray analysis of fold change for Th1 and Th2 genes in SCC and non-tumor bearing skin vs. normal skin and transplant SCC (TSCC) and transplant non-tumor bearing skin (TNT) vs. normal skin. SCC and surrounding tissue form immunocompetent patients as well as transplant patients is associated with mixed Th1 and Th2 gene expression pattern. Non-tumor-bearing skin adjacent to SCC in immunocompetent patients compared to non-tumor-bearing skin of transplant patients, shows a Th2 expression pattern. (*p<0.05, **p<0.01, ***p<0.0001).

Figure 2

(A) CD8 mRNA expression normalized to GAPDH mRNA was expressed at about the same level in both, immunocompetent patients (IC) and organ transplant recipients (OTR) (p=0.37). The mRNA level was normalized to GAPDH mRNA expression in each sample individually.

(B) CD4 helper T cell mRNA expression normalized to GAPDH mRNA is diminished in OTRs (p=0.009). The mRNA level was normalized to GAPDH mRNA expression in each sample individually.

(C) Th1 weighted inflammation, characterized by expression of T-BET mRNA normalized to GAPDH mRNA expression is decreased in organ transplant recipients (p=0.0053), similarly (D) mRNA expression of INF-gamma normalized to GAPDH, is significantly decreased in OTR group (p=0.02).

(E) GATA-3 mRNA as a marker for Th2 weighted inflammation is not significantly changed, however shows tendency to increase with immunosuppression (p=0.08). (F) IL-4 mRNA expression does not differ significantly (p=0.14).

(G) Regulatory T cell response characterized by FOX-P3 mRNA is decreased in organ transplant recipients (p=0.045). (H) TGF-beta is decreased with immunosuppression approaching significance (p=0.036), while (I) IL-10 mRNA expression is unchanged (p=0.1).

(J) Th17 weighted inflammation, characterized by expression of RORC mRNA expression is similar in both groups of patients (p=0.34), however (K) IL-17A is markedly decreased in immunosuppressed patients (p=0.016).

Figure 3

IL-17A (A) and TGF-beta (B) mRNA expression was significantly decreased in patients from OTR group after prednisone treatment, when compared to immunocompetent group. The effect was not observed after CsA, or combined prednisone and CsA treatment. *p<0.05

Figure 4

mRNA expression pattern of transcription factors differs within and between patient groups (*p<0.0001* in both immunocompetent (IC) and organ transplant recipients (OTR) groups). Out of the measured transcription factors, the most abundant in both groups was GATA-3, which expression was significantly increased as compared to T-BET, FOX-P3 and RORC (A, B). In contrast to immunocompetent patients, in OTR group T-BET was at lower expression than RORC (B).

Overall, cytokine mRNA expression levels differ within the patient groups (p< 0.0001 in both immunocompetent and OTR groups). INF-gamma mRNA level is lower than TGF-beta but higher than IL-4 expression in both tested groups. INF-gamma, IL-10 and IL-17A are expressed at similar level in the tumor microenvironment of both immunocompetent patients and OTRs. All three cytokines IL-4, TGF-beta and IL-10 are expressed at different levels in both immunocompetent patients and OTRs, however the pattern remains the same. Among them, the most abundant cytokine is TGF-beta, while the lowest expression level shows IL-4. Difference between IL-4 and IL-17A mRNA expression can be observed only in immunocompetent patients, where IL-17A is more abundant than IL-4. On the other hand, OTRs show significantly lower expression of IL-17A compared to TGF-beta mRNA level, and the difference is not detected within immunocompetent group. The mRNA level was normalized to GAPDH mRNA expression in each sample individually. *p<0.05, **p<0.01, ***p<0.001

Figure 5

Immunohistochemical staining: CD3, CD4, and CD8 immunoreactivities do not differ in the perineoplastic infiltrate of OTRs (open bars) and immunocompetent patients (closed bars). FOXP3+ staining shows reduced immunoreactivity in OTRs (*p=0.04*).

Figure 1

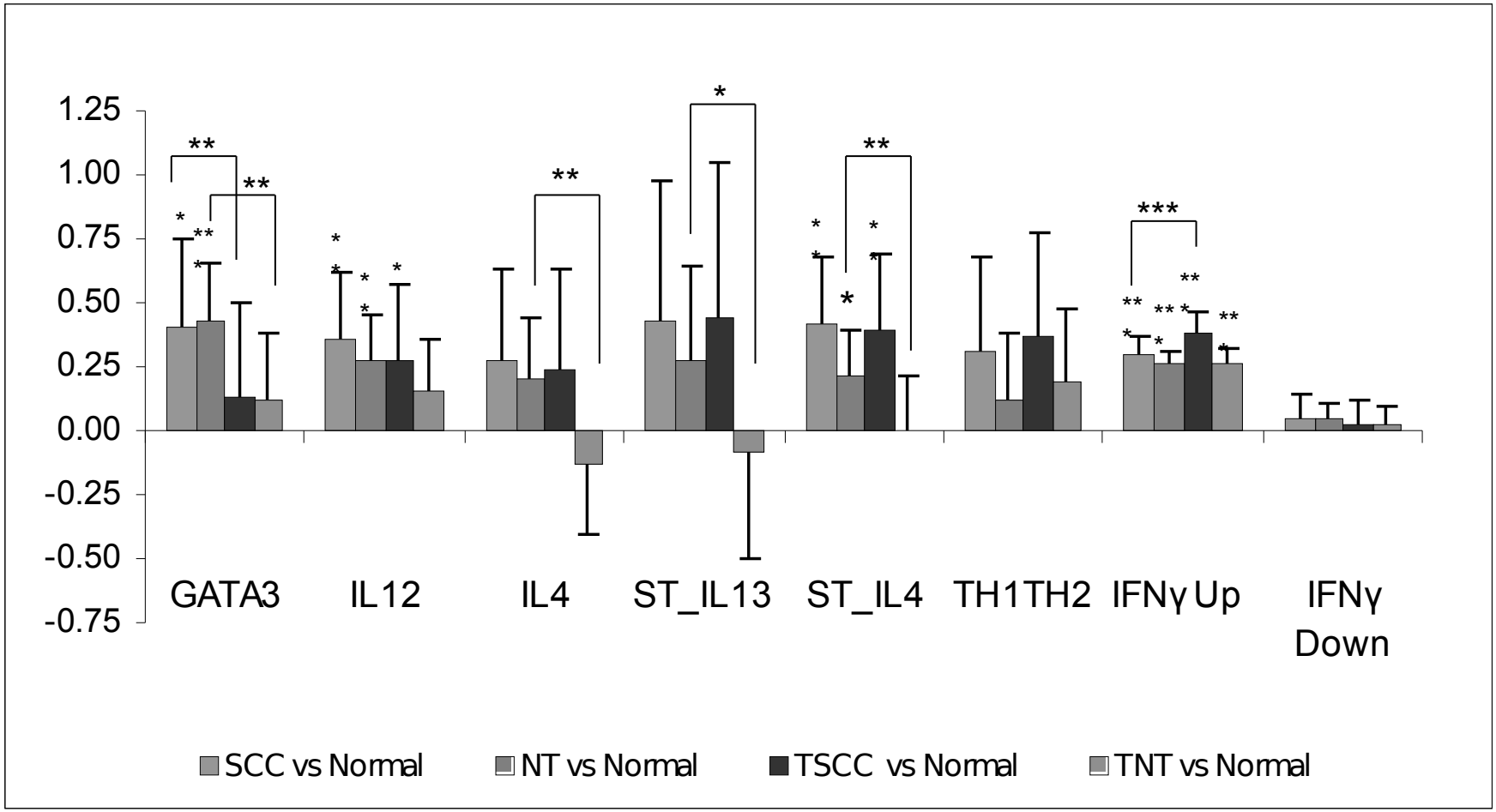

1.25
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
GATA3
IL12
IL4
ST_IL13
ST_IL4
TH1TH2
IFNγ Up
IFNγ Down
SCC vs Normal
NT vs Normal
TSCC vs Normal
TNT vs Normal

Figure 2

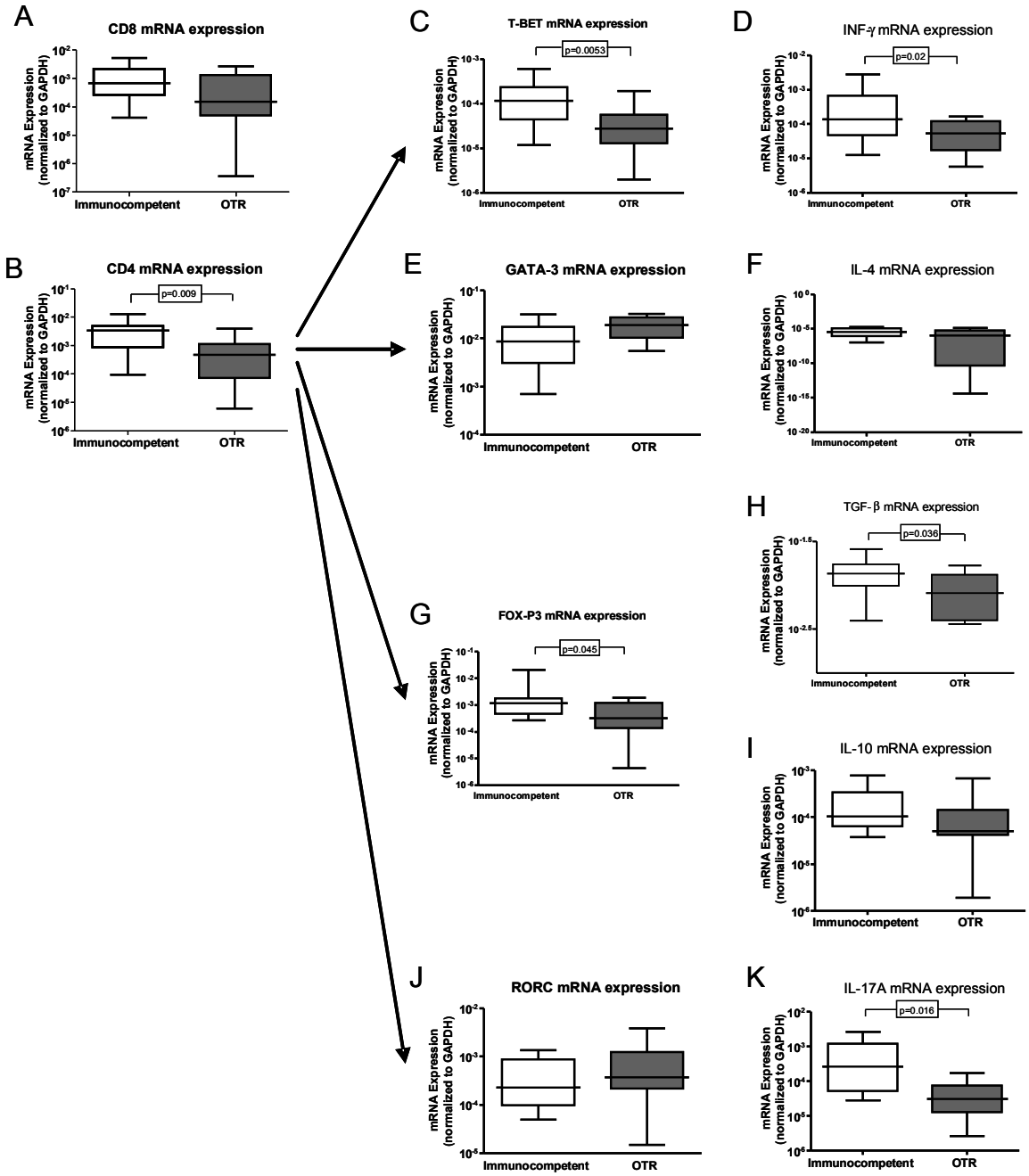

A
CD8 mRNA expression
mRNA Expression (normalized to GAPDH)
Immunocompetent
OTR
B
CD4 mRNA expression
p=0.009
C
T-BET mRNA expression
p=0.0053
D
INF-γ mRNA expression
p=0.02
E
GATA-3 mRNA expression
F
IL-4 mRNA expression
G
FOX-P3 mRNA expression
p=0.045
H
TGF-β mRNA expression
p=0.036
I
IL-10 mRNA expression
J
RORC mRNA expression
K
IL-17A mRNA expression
p=0.016

Figure 3

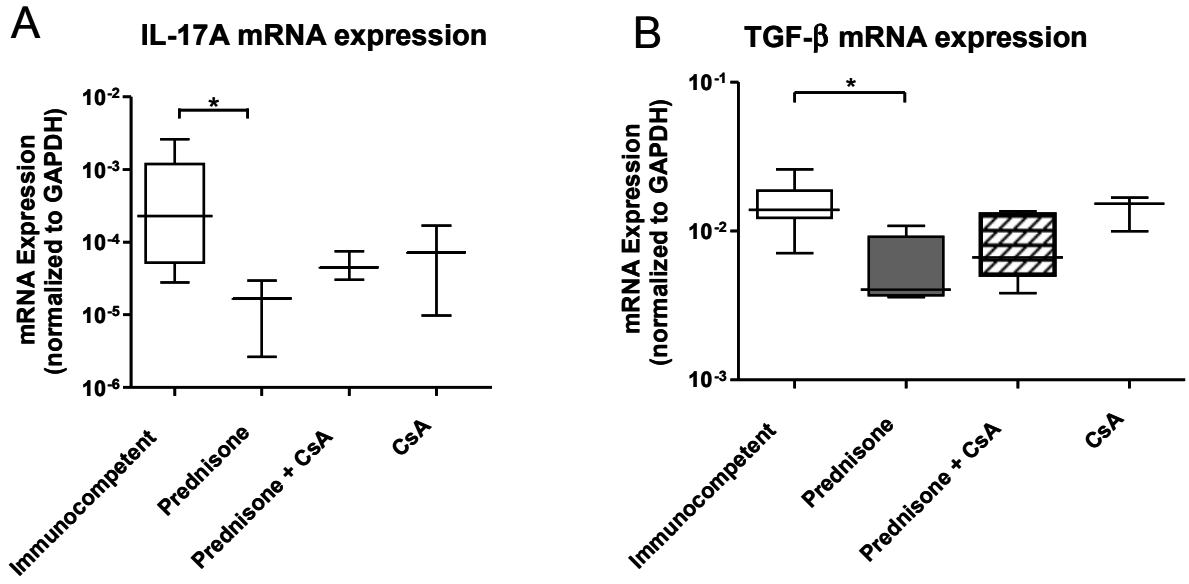

A
IL-17A mRNA expression
mRNA Expression (normalized to GAPDH)
10-2
10-3
10-4
10-5
10-6
*
Immunocompetent
Prednisone
Prednisone + CsA
CsA
B
TGF-β mRNA expression
mRNA Expression (normalized to GAPDH)
10-1
10-2
10-3
*
Immunocompetent
Prednisone
Prednisone + CsA
CsA

Figure 4

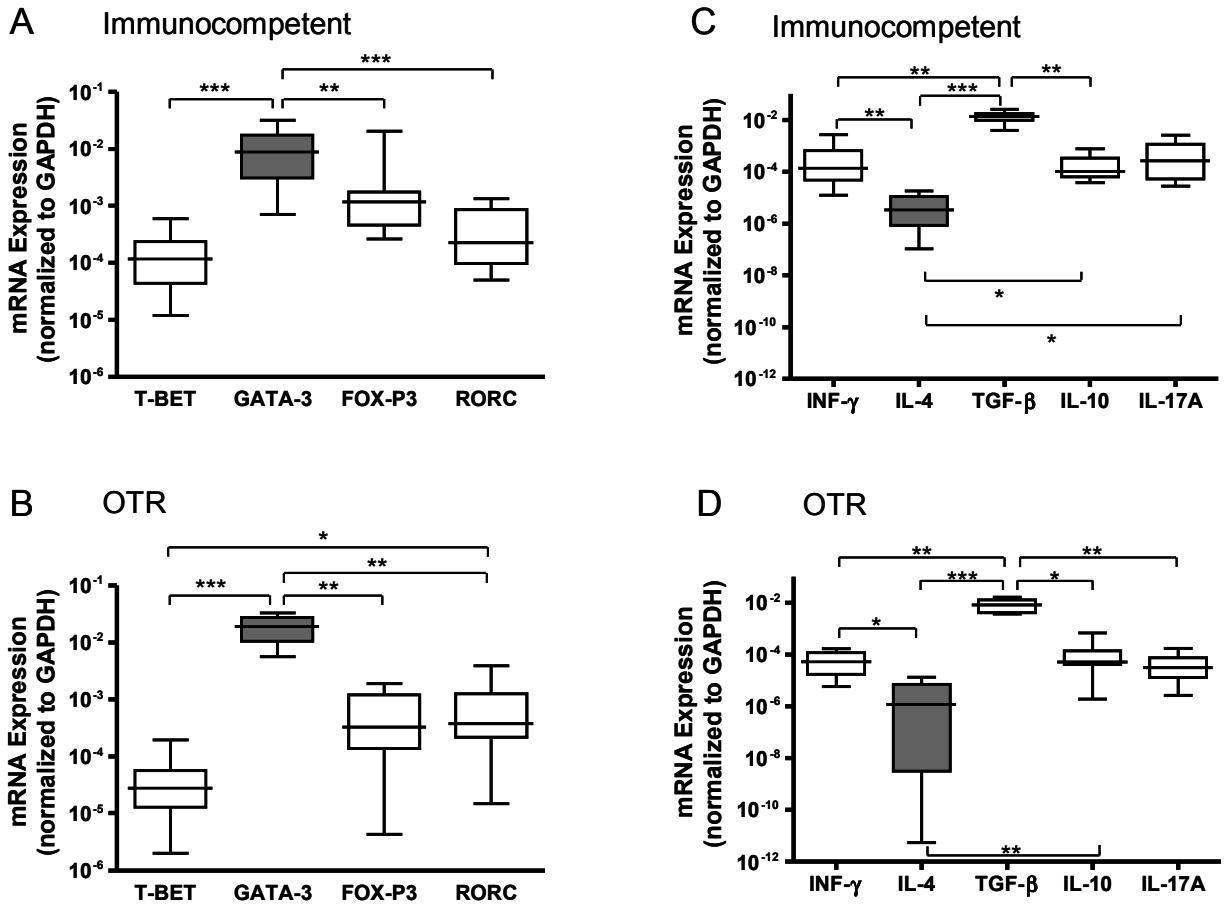
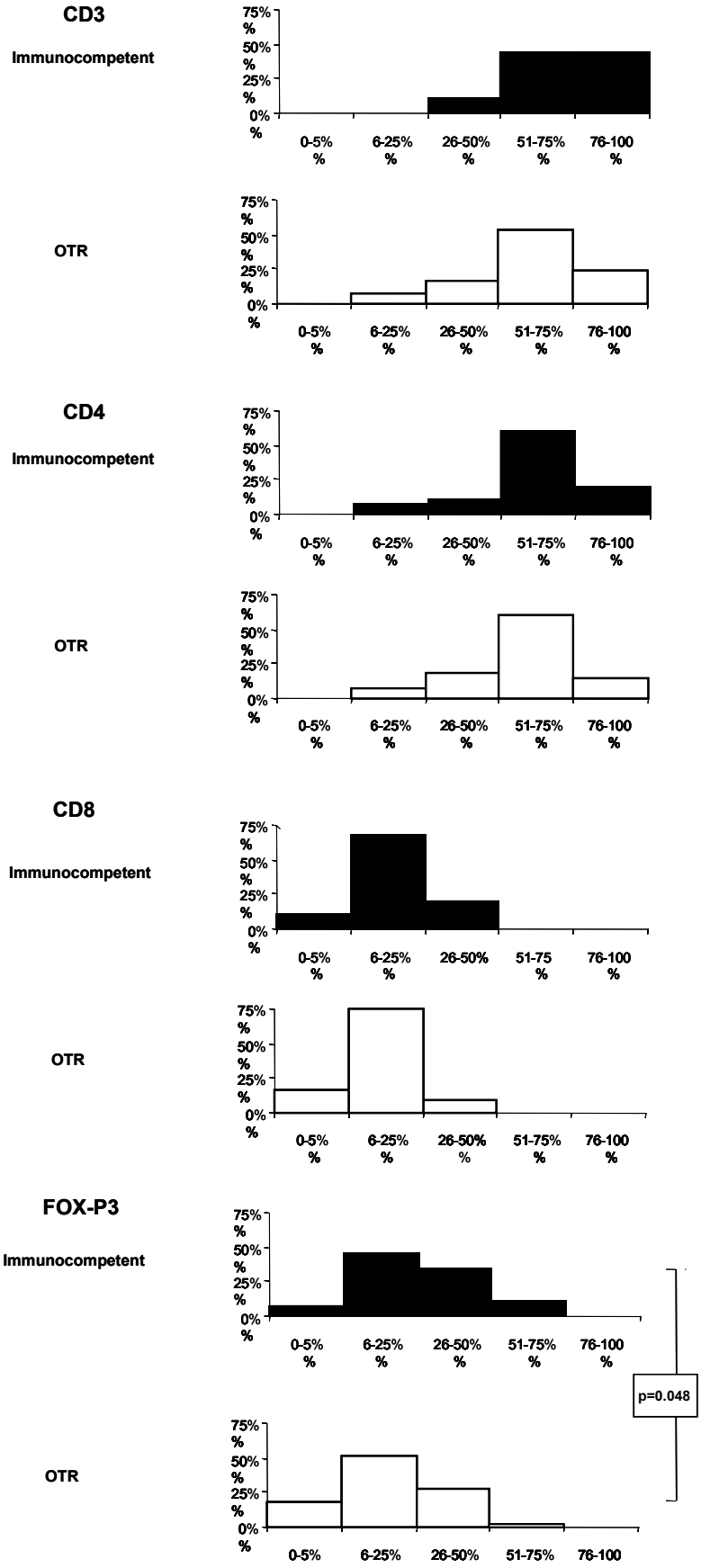

A
Immunocompetent
mRNA Expression (normalized to GAPDH)
T-BET
GATA-3
FOX-P3
RORC
B
OTR
C
Immunocompetent
INF-γ
IL-4
TGF-β
IL-10
IL-17A
D
OTR

Figure 5


CD3
Immunocompetent
OTR
CD4
Immunocompetent
OTR
CD8
Immunocompetent
OTR
FOX-P3
Immunocompetent
OTR
75%
50%
25%
0%
0-5%
6-25%
26-50%
51-75%
76-100
51-75
p=0.048

Supplemental Table legends

Table S1

Baseline characteristics of patients (real time RT-PCR).

IC: immunocompetent patients; OTR: organ transplant recipients; CyA: Cyclosporine A; MMF: Mycophenolate Mofetil; AZA: Azathioprine; y: years; m: months; mg/d: milligram per day

Table S2

Baseline characteristics of patients (immunohistochemistry)

n: number; y: years

Table S3

(a) GSEA results for SCC vs. Normal Comparisons show mixed Th1 and Th2 genes expression. SCC of immunocompetent patients is characterized by increased expression of genes corresponding to IFNgamma-up, ST IL-4 and IL-12. (b) GSEA results for NT vs. Normal Comparisons show increased expression of genes corresponding to IFNgamma-up, ST IL-4 and decreased expression of genes corresponding to IFNgamma-down in non-tumor-bearing skin adjacent to SCC of immunocompetent patients.

SIZE – number genes in the gene set, ES – Enrichment Score, NES – Normalized Enrichment Score, NOM p-value – Nominal p-value, FDR q-value – False Discovery Rate, RANK AT MAX - the position in the ranked list at which the maximum enrichment score occurred.

Table S4

(a) GSEA results for TSCC vs. Normal Comparisons show increased expression of genes related to IFNgamma-up and ST IL-4 and decreased expression of IFNgamma-down genes in SCC of transplant patients. (b) GSEA results for TNT vs. Normal Comparisons show increased expression of IFNgamma-up related genes in non-tumor-bearing skin adjacent to SCC of transplant patients.

SIZE – number of analyzed genes, ES – Enrichment Score, NES – Normalized Enrichment Score, NOM p-value – Nominal p-value, FDR q-value – False Discovery Rate, RANK AT MAX - the position in the ranked list at which the maximum enrichment score occurred.

Table 1

Baseline Characteristics of Subjects (RT-PCR)		IC	OTR	Total
Gender	Male	11	12	22
	Female	4	1	6
	Total	15	13	28
Age (y)	Mean (± SD)	77.4 (±10.0)	63.7 (±10.2)	71(±12.1)
	Range	56-93	39-81	56-93
Organ transplant	Kidney		7	
	Lung		3	
	Heart		2	
	Liver		1	
Duration of transplant (y)	Mean		13.2	
	Range		2m -34	
Immunosuppressive regimen	CyA, MMF, Prednisone		4	
	CyA, AZA, Prednisone		2	
	Rapamycin, AZA, Prednisone		1	
	CyA, AZA		3	
	AZA, Prednisone		1	
	MMF, Prednisone		1	
	Prednisone		1	
Drug dosage (mg/d)	CyA mean		168.1	
	CyA range		70-300	
	Rapamycin		2	
	AZA mean		90	
	AZA range		50-150	
	MMF mean		1362.9	
	MMF range		500-2500	
	Prednisone mean		6.8	
	Prednisone range		5-10	
SCC differentiation	Good	5	7	12
	Moderate	2	2	4
	Poor	3	0	3
	Not classified	5	4	9
SCC cutaneous location	Head	13	9	22
	Body	2	4	6

Table 2

Baseline Characteristics of Subjects (Immunohistochemistry)		IC	OTR	Total
Patients		43	42	85
Gender	Male [n (%)]	32 (74)	36 (86)	68 (80)
Age (y)	Age [mean (± SD)]	66.0 (±0.74)	61.3 (±1.07)	63.7 (±0.67)
	Range	56-93	39-81	56-93
Organ transplant	Kidney		32	
	Heart		6	
	Lung		4	

Table S3

a)

Enriched In SCC	Gene Set	SIZE	ES	NES	NOM p-value	FDR q-value	RANK AT MAX
	IFNG Up	270	0.45	2.07	0	0	1174
	ST_IL_4_	23	0.63	1.86	0	0.002	545
	IL12	19	0.58	1.62	0.029	0.04	2449
	ST_IL_13_	7	0.68	1.46	0.058	0.098	1360
	IL4	11	0.59	1.41	0.097	0.124	545
	TH1TH2	16	0.48	1.27	0.18	0.221	2630
	GATA3	14	0.42	1.11	0.343	0.411	1585
Enriched In Normal							
	IFNG Down	199	-0.29	-1.25	0.069	0.288	1337

b)

Enriched In NT	Gene Set	SIZE	ES	NES	NOM p-value	FDR q-value	RANK AT MAX
	IFNG Up	270	0.54	2.21	0	0	808
	ST_IL_4_	23	0.57	1.53	0.033	0.088	398
	IL12	19	0.56	1.43	0.06	0.148	2138
	ST_IL_13_	7	0.64	1.34	0.143	0.193	1299
	IL4	11	0.58	1.29	0.181	0.228	907
	TH1TH2	16	0.51	1.27	0.181	0.226	3189
	GATA3	14	0.44	1.06	0.398	0.471	1649
Enriched In Normal							
	IFNG Down	199	-0.29	-1.23	0.046	0.406	1154

Table S4

a)

Enriched in TSCC	Gene Set	SIZE	ES	NES	NOM p-value	FDR q-value	RANK AT MAX
	IFNG Up	270	0.44	2.11	0	0	1137
	ST_IL_4_	23	0.52	1.57	0.029	0.11	731
	TH1TH2	16	0.51	1.44	0.071	0.141	3436
	IL12	19	0.49	1.43	0.08	0.133	2815
	ST_IL_13_	7	0.55	1.19	0.258	0.354	1641
	IL4	11	0.45	1.08	0.38	0.498	731
	GATA3	14	0.23	0.63	0.915	0.98	2220
Enriched in Normal							
	IFNG Down	199	-0.28	-1.28	0.047	0.171	1080

b)

Enriched in TNT	Gene Set	SIZE	ES	NES	NOM p-value	FDR q-value	RANK AT MAX
	IFNG Up	270	0.48	2.27	0	0	844
	GATA3	14	0.5	1.35	0.145	0.305	675
	TH1TH2	16	0.38	1.05	0.398	0.638	3588
	IL12	19	0.36	1.05	0.41	0.582	2873
	ST_IL_4_	23	0.34	1.03	0.417	0.559	590
	ST_IL_13_	7	0.37	0.8	0.706	0.801	675
Enriched in Normal							
	IL4	11	-0.44	-1.13	0.316	0.578	87
	IFNG Down	199	-0.2	-0.96	0.578	0.518	1870