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INVESTIGATIVE REPORT

Immunosuppressive Environment in Basal Cell Carcinoma: The Role of Regulatory T Cells

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Interaction between tumour survival tactics and anti-tumour immune response is a major determinant for cancer growth. Regulatory T cells (T-regs) contribute to tumour immune escape, but their role in basal cell carcinoma (BCC) is not understood. The fraction of T-regs among T cells was analysed by immunohistochemistry followed by automated image analysis in facial BCC, peritumoural skin and normal, buttock skin. Quantitative real-time PCR (qRT-PCR) was performed for *FOXP3* and cytokines involved in T-reg attraction and T-cell activation. T-regs comprised 45% of CD4-cells surrounding BCC. *FOXP3* was highly expressed in BCC, but absent in buttock skin. Unexpectedly, expression of *FOXP3* was increased in peritumoural skin, with the *FOXP3*/CD3 fractions exceeding those of BCC ($p = 0.0065$). Transforming growth factor (TGF)-β and T-reg chemokine expression was increased in BCC and peritumoural skin, but not in buttock skin, with expression levels correlating with *FOXP3* . T-regs are abundantly present both in BCC and in peritumoural skin, mediating an immunosuppressed microenvironment permissive for skin cancer. *Key words: basal cell carcinoma; regulatory T cells (T-reg); immunosuppressive environment; chemokines.*

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Basal cell carcinoma (BCC) of the skin is the most frequent cancer worldwide and its incidence is increasing (1). BCC rarely metastasizes, but has locally invasive and destructive growth. The tumour microenvironment is characterized by dense lymphocyte infiltrates and plays a role in tumourigenesis (2), relying on continuous interaction between tumour survival tactics and host anti-tumour response. Important mediators of tumour immune escape are regulatory T cells (T-regs), which suppress conventional T cells maintaining immunological tolerance (3). In cancer, T-regs are recruited as a subpopulation of tumour-infiltrating lymphocytes, and accumulation of T-regs at tumour sites is thought to impede T-cell immunity to tumour-associated antigens (4, 5). T-regs normally comprise approximately 5–10% of peripheral $CD4^+$ T cells and are characterized by expression of the transcription factor Forkhead Box P3 (*FOXP3*) regulating the suppressive functions of T-regs (6).

The impact of T-regs is well documented in malignant melanoma, where they are markers of poor prognosis (7, 8). Increased frequencies of T-regs have been reported in squamous cell carcinoma (SCC) (9, 10). In BCC, T-regs in the cellular microenvironment surrounding tumour have been described (11), but their role in BCC is poorly understood.

This study compared T-reg density of facial BCC with peritumoural skin and non-ultraviolet (UV)-exposed skin from the buttocks. The results provide preliminary evidence indicating the role of T-regs in the formation of an immunosuppressed niche in facial skin, which may have pathogenic consequences for the development of skin cancer.

MATERIALS AND METHODS

Material for immunohistochemical staining and quantitative real-time PCR (qRT-PCR) consisted of facial BCC and peritumoural skin obtained during Mohs surgery and 4-mm buttock biopsies collected at the Department of Dermatology, Bispebjerg Hospital, Denmark ($n = 18$). The peritumoural skin was taken after complete removal of cancerous tissue. BCC material for *FOXP3*/CD4 double-staining was collected as paraffin-embedded formalin-fixed skin (2–5 mm, $n = 19$) (Department of Pathology, Rigshospitalet, Denmark).

Quantitative real-time PCR

The complete RNA and cDNA procedure is described in Appendix S1[1]. Data were analysed by the ΔΔCT method (12, 13).

Immunohistochemistry, image analysis and immunofluorescent staining

FOXP3/CD4 double-staining was performed by an indirect sequential immunoenzymatic technique (Appendix S1[1]). CD4/*FOXP3* slides were photographed with Nanozoomer 2.0 (Hamamatsu Photonics KK, Hamamatsu City, Japan) and slides were

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computed individually (Visiopharm A/S, Hørsholm, Denmark). T-regs were defined as double-positive cells (brown nuclear staining with red membrane staining); positive for both *FOXP3* and CD4, whereas non-regulatory T cells were defined as single CD4 positive (red membrane staining). The fraction between T-regs and non-regulatory T cells (double $FOXP3^+CD4^+$ cells relative to all $CD4^+$ cells) was calculated as a number index.

For immunofluorescent staining, 10-µm sections were cut using a Microm HM560 cryostat (Thermo Fisher Scientific, Waldorf, Germany). For detailed procedure and antibodies used see Appendix S1[1].

Statistical analysis

Statistical analyses were performed by unpaired Student's *t*-test (normally distributed data) or Mann-Whitney *U* test (non-normally distributed data) using GraphPad Prism 4 (GraphPad Software Inc, CA, USA). For correlation analysis linear regression was used. Statistical significance was set at $p < 0.05$.

RESULTS

High level of T-regs in the lymphocyte infiltrates surrounding basal cell carcinoma

To investigate whether T-regs were involved in the pathogenesis of BCC immunohistochemical (IHC) double staining for CD4 and *FOXP3* was performed. The fraction of double $FOXP3^+/CD4^+$ cells within all $CD4^+$ cells was quantified by the use of automated image analysis (14) (Fig. 1a, b). T-regs were found to comprise a mean of 45% (range 22–84%) of the total number of $CD4^+$ T cells. T-regs were present predominantly within the inflammatory infiltrates surrounding tumour islands consisting of both non-regulatory T cells and T-regs (Fig. 1c, d) tumour.

High level of T-regs in basal cell carcinoma and peritumoural skin, but not in normal buttock skin

To verify the IHC findings, the T-reg-related *FOXP3* mRNA levels were measured by qRT-PCR in BCC, peritumoural and buttock skin. Expression of *FOXP3* was highly increased in both BCC and peritumoural skin, whereas *FOXP3* was undetectable in the non-UV-exposed skin from the buttocks (Fig. 2a). Interestingly, the fraction of T-regs within the total number of lymphocytes (*FOXP3*/CD3) was higher in the peritumoural skin compared with BCC ($p = 0.0065$).

Increased expression of T-reg-associated chemokines in basal cell carcinoma and peritumoural skin

Since T-regs are concentrated in BCC and peritumoural skin, we sought to identify chemokines with T-reg chemotactic function. CCL17, CCL18 and CCL22 are chemokines involved in T-reg attraction in different solid cancers (15–17). In normal buttock skin we found no expression of any of the chemokines, whereas there was high expression of all 3 chemokines both in BCC and in peritumoural skin (Fig. 2b).

Immunofluorescent (IF) staining with CCL17 revealed this chemokine to be present in the stromal tissue between tumour islands, but not within the cancerous tissue, corresponding to T-regs being concentrated primarily in the vicinity of BCC and not within the tumour islands (Fig. 3).

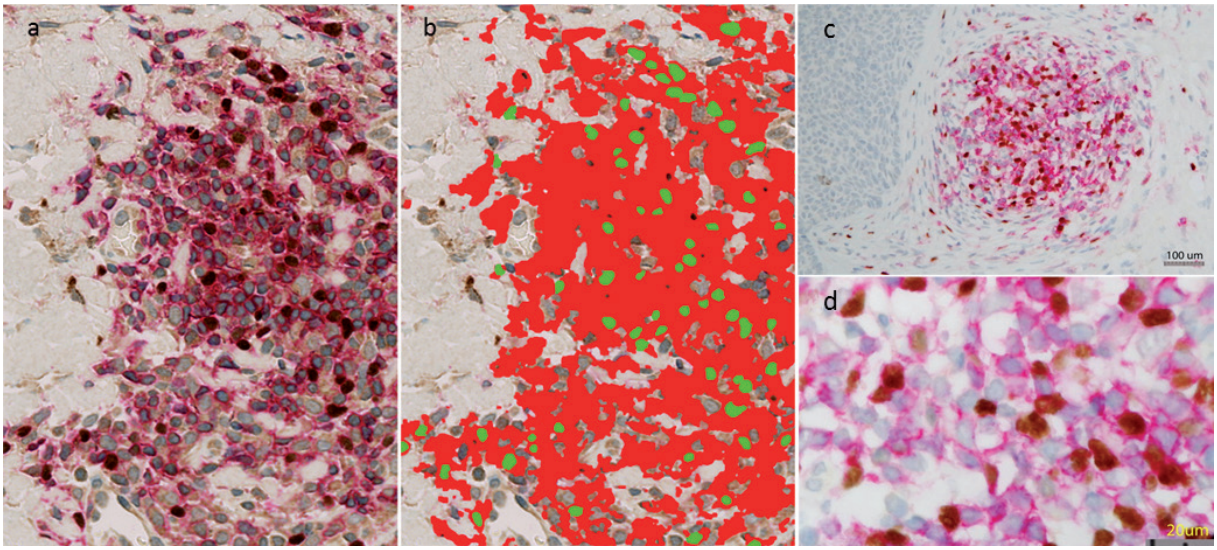

Fig. 1. Quantification of $FOXP3^+/CD4^+$ cells by image analysis. (a) *FOXP3*/CD4 double-staining. The cells with red membrane staining are the single $CD4^+$ cells and the cells with the brown nuclear staining surrounded by the red membrane staining are the double $FOXP3^+/CD4^+$ cells. (b) Image processing for the quantification of cells. *Red area*: single $CD4^+$ cells; *green area*: double $FOXP3^+/CD4^+$ cells. (c) Immunohistochemical double-staining of basal cell carcinoma (BCC) with *FOXP3*/CD4: inflammatory infiltrate near BCC with non-regulatory T cells (*red cells*) and T-regs (*brown cells with red halo*) (× 20). (d) Same photograph as (c) with a higher magnification (× 100) showing the single $CD4^+$ cells with red membrane staining and the double $FOXP3^+/CD4^+$ cells with brown nuclear staining and red membrane staining.

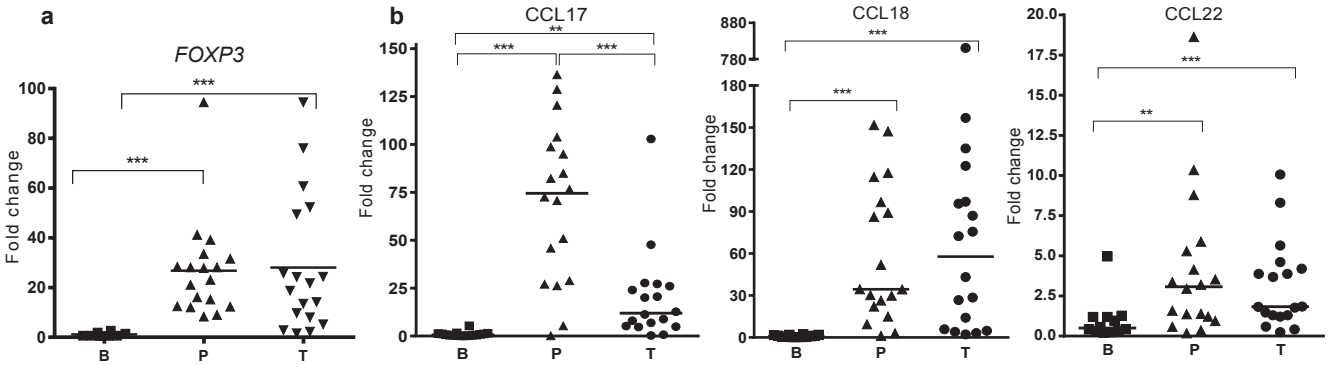

Fig. 2. Quantitative real-time PCR (qRT-PCR) analysis and correlation analysis of selected genes in basal cell carcinoma (BCC) tumours (T), peritumoural skin (P) and buttock skin (B). (a) Gene expression of Foxp3 mRNA by the use of qRT-PCR showing increased expression of *FOXP3* in BCC tumours ($n=18$) and peritumoural skin (($n=17$) compared with buttock skin (B) (($n=8$). (b) Increased expression of the chemokines CC17, CCL18, and CCL22 mRNA in BCC and peritumoural skin compared with buttock skin and higher expression of CCL17 in the peritumoural skin compared with BCC. Tumour (T), ($n=17$), peritumoural skin (P), ($n=17$), and buttock (B), ($n=15$ (14 for CCL22)).

Lack of activation of tumour-infiltrating lymphocytes in basal cell carcinoma

After finding a high T-reg concentration in BCC and peritumoural skin, we next sought to establish the phenotypes of the remaining $CD4^+$ cells. We analysed the mRNA level of T-bet (Th1), RORc (Th17), and a variety of cytokines; TNF-α (Th1), IFN-γ (Th1), IL12 (Th1), IL13 (Th2), IL17 (Th17), IL10, and TGF-β (T-reg) as well as perforin and granzyme-B (cytotoxic markers), an approach adopted previously for tumour-infiltrating lymphocytes in other solid cancers (18).

For the majority, there was no expression of any markers in the buttock skin corresponding with normal skin being in a resting state with low or absent lymphocyte infiltration. A higher, but not statistically significant, difference was found in the expression of cytokines involved in a Th1 response (T-bet, TNF-α, IFN-γ, IL12) within BCC compared with the peritumoural skin (Fig. S1[1]). When calculating the fraction of these Th1 markers out of all lymphocytes (T-bet/CD3, TNF-α/CD3, IFN-γ/CD3, IL12/CD3), there was no statistically significant increase within BCC compared with the peritumoural skin, except for TNF-α, with the highest fraction within BCC ($p=0.01$). For the cytotoxic markers granzyme-B and perforin there was no difference between BCC and peritumoural skin when assessing the fraction within CD3 cells. The expression of RORc was higher in the peritumoural skin compared with BCC and normal buttock skin, but the fraction of RORc/CD3 was highest within BCC compared with peritumoural skin ($p<0.0001$). With this increased RORc expression, we would have expected a similar increase in IL17 expression, but IL17 expression was absent in all 3 skin compartments. Th2 skewing was not predominant either, since IL13 expression was low (data not shown). Expression of TGF-β was highest in the peritumoural skin (Fig. S1[1]), whereas IL10 expression was completely absent in buttock skin, with low expression in both BCC and peritumoural skin (data not shown).

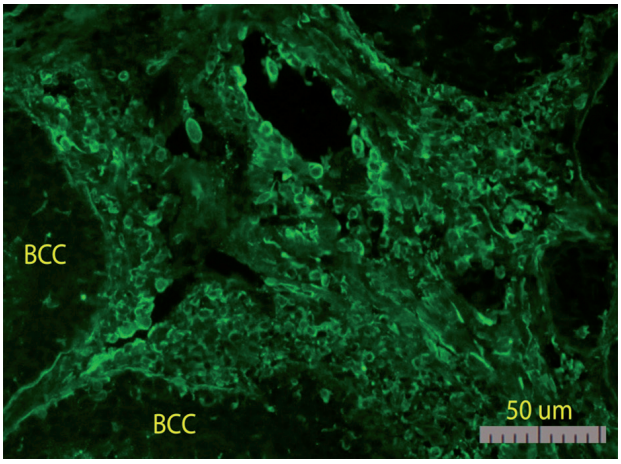

Fig. 3. Immunofluorescent staining of basal cell carcinoma (BCC) for CCL17 showing positive cells in tumour stroma surrounding BCC, but not within tumour islands (*dark area*).

DISCUSSION

This study shows that T-regs are attracted to and accumulated in BCC. Surprisingly, T-regs were found at higher concentrations in the peritumoural skin. In contrast, in the normal non-UV-exposed buttock skin, no T-reg expression was found. T-regs comprise up to 5–10% of $CD4^+$ cells in peripheral blood and 8–20% of $CD4^+$ cells in normal human adult skin (9, 19). The mean fraction of 45% T-regs of $CD4^+$ cells found in our study is therefore greatly increased.

Corresponding to a high T-reg concentration in BCC and peritumoural skin, we found a high expression of the chemokines CCL17, CCL18, and CCL22 involved in T-reg attraction in solid cancers (15–17). IF staining showed the infiltration of CCL17 being in the tumour stroma and not within BCC. This corresponds with the IHC findings of the stromal localization of T-regs. Taken together this supports the hypothesis of these

chemokines being involved in the T-reg accumulation identified within our study.

T-regs are known to cause immunosuppression partly by IL10 and TGF-β secretion (20). We found increased expression of TGF-β in the peritumoural skin compared with both BCC and the normal buttock skin. The TGF-β expression level correlated with the Foxp3 expression level both within BCC and in the peritumoural skin supporting TGF-β as a contributor of T-reg-induced immunosuppression in BCC. IL10 expression was absent in the buttock skin and the expression was surprisingly low in BCC and peritumoural skin. In mice, IL10 is involved in acute UV-induced immunosuppression (21, 22), whereas persistent IL10-related effect might primarily be caused by an immunity switch and not by continued release of the cytokine (23). This corresponds with the lack of IL10 expression in our study, since the patients have been exposed to long-term UV. Increased IL10 expression in BCC compared with normal skin and the opposite for TGF-β has been reported (11), in contrast to our results. The IL10 expression was not investigated in peritumoural skin, however, making comparison difficult.

Immunosurveillance theory describes the continuous interaction between tumour survival tactics and host anti-tumour response (24) with interferon-associated genes and cytotoxic response being part of the latter. Within BCC, we found a high expression of Th1-associated genes as well as cytotoxic mediators pointing at a partial host anti-tumour response. When calculating the fraction of Th1-associated genes and cytotoxic markers within CD3 cells the concentration was similar in BCC and peritumoural skin except for TNF-α where the expression was highest within tumour. This relative decrease in Th1-associated genes could be mediated by a maximally suppressive fraction of T-regs expressing the TNF-α receptor TNFR2 (25).

Increased RORc expression within BCC points at a skewing towards a Th17 response, but since there was no expression of IL17, the Th17 response seems to be inactivated. TGF-β is involved in the differentiation of both Th17 cells and T-regs, and consequently RORγt (encoded by RORc) and *FOXP3* are co-expressed at early stages and may antagonize each other (26). The lack of IL17 expression might accordingly be caused by T-reg expression at the expense of IL17 secretion.

The seed and soil hypothesis claims that the microenvironment hosting a tumour (the soil) is necessary for the tumour to grow (the seed) (27). Apart from facilitating mutagenesis, UV exposure dampens the immune system partly due to activation of T-regs. This has primarily been shown in animals (21, 22). With our human *in vivo* study, we show that facial peritumoural skin, chronically exposed to UV is characterized by high T-reg infiltration. We hypothesize that long-term exposure to UV induces chronic immunosuppression composing a microenvironment permissive for skin cancer growth with T-reg attraction being essential. The peritumoural skin in our study was taken after complete tumour-removal with microscopically free tumour margins. BCC is locally growing and it seems unlikely that it would impact the skin further than 0.5 cm away from the tumour periphery. Consequently, the excess of T-regs in peritumoural skin might be a sign of chronic immunosuppression induced by long-term exposure to UV. Comparing our results with a similar aged population without skin cancer and being able to show a lower T-reg population in this group compared with people with BCC, would strengthen the argument that increased T-reg leads to increased growth of BCC and is not only a consequence of chronic sun exposure. We have planned a study with this set-up.

The strength of this study is the objective quantification of T-regs among $CD4^+$ cells by image analysis. Furthermore, we provided *in vivo* data comparing BCC, peritumoural skin and normal, non-UV-exposed buttock skin from the same patients, ruling out inter-individual biological variance and different sun-exposure patterns. Unfortunately, we found it technically impossible to purify T cells from BCC samples, which precluded further confirmation of our data by flow cytometry.

In summary, we found accumulation of T-regs in BCC as well as in the peritumoural facial skin, indicating a role of T-regs in the formation of an immunosuppressed niche in BCC. Previous studies have shown T-regs being induced as a result of UV exposure in mice. Here we provide *in vivo* data of the induction of T-regs in chronic UV-exposed human skin. Based on our results, we suggest that induction of T-regs is caused by long-term immunosuppression giving rise to a permissive microenvironment susceptible to skin cancer upon mutagenesis.

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Conflicts of interest: RG reports personal fees and other from Janssen, personal fees and other from Abbvie and MSD, personal fees from Pfizer, personal fees and other from Novartis and Lilly, outside the submitted work. The other authors declare no conflicts of interest.

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