Local immune response in cutaneous basal cell carcinoma

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This review has been accepted as a thesis together with three original papers by the University of Copenhagen 7th of September 2016 and defended on 16th November 2016.

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Dan Med J 2017;64(10):B5412

The three original papers are:

I. Immunosuppressive environment in basal cell carcinoma: the role of regulatory T-cells (T-regs). Omland SH, Nielsen PS, Gjerdrum LMR, Gniadecki R. Acta Derm Venereol. 2016 Apr 27. doi: 10.2340/00015555-2440.

II. Cancer associated fibroblasts (CAFs) are activated in cutaneous basal cell carcinoma and in the peritumoural skin. Omland SH, Wettergren EE, Mourier T, Hansen AJ, Asplund M, Mollerup S, Gniadecki R. Accepted Aug 2017 BMC Cancer

III. High diversity of the T-cell receptor repertoire of tumor-infiltrating lymphocytes in basal cell carcinoma. Omland SH, Hamrouni A, Gniadecki R. Exp Dermatol. 2017 May;26(5):454-456. doi: 10.1111/exd.13240. Epub 2017 Jan 19.

BACKGROUND

The understanding of cancer treatment and tumor biology has shifted from primarily focusing on cancer as a cell-intrinsic phenomenon to inclusion of the tumor surroundings and immune response upon tumor development. Tumor immunogenicity is increasingly recognized as an essential contributor to tumor progression, and immunotherapy is evolving as an important therapeutic actor for several cancer types. In malignant melanoma in particular, immunotherapy shows promising potential (1,2). In keratinocyte carcinomas such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin, immunotherapy is so far of limited use with approval of a few topical immunostimulators for restricted indications for superficial BCC (3) and actinic keratosis (4). The overall purpose of this thesis is to investigate and characterize the microenvironment in BCC with focus on the tumor-immune interactions.

BASAL CELL CARCINOMA (BCC)

BCC of the skin is the most frequent cancer worldwide and the incidence is increasing (5). In Denmark, 12.000 new cases of BCC were registered in 2014 (6), and the incidence rate is among the highest in Europe (7). BCC is a slowly growing tumor that rarely metastasizes but has a locally invasive and destructive growth. Multiple histological subtypes exist in which broader classification can divide BCC into six major categories: 1) nodular 2) superficial 3) infiltrative 4) morphoeic 5) micronodular and 6) a group of more rare subtypes including tumors of mixed types (8). The most common subtypes of BCC are the nodular with a predilection for the face followed by the superficial BCC that is more often seen on the trunk (9) (Figure 1). The infiltrative and morphoeic cases of BCC are more aggressive, characterized by a diffuse and destructive growth pattern (8,10).

BCC often develops on sun-exposed skin such as the face and neck as ultraviolet radiation (UVR) is a major etiological factor. In particular recreational sun exposure and sunburns seem to be causative (11). While age is another significant risk factor, a concerning trend is arising with more cases of BCC now appearing in the younger population (7). Other risk factors are fair skin type and ionizing radiation (12). Immunosuppression also plays an essential role as solid organ transplant recipients undergoing lifelong immunosuppressive therapy possess a well-described increased risk of BCC (13). Similarly, patients undergoing allogeneic hematopoietic stem cell transplantation are at increased risk of developing BCC (14). Stimulation of antitumor immunity in BCC, e.g. by the topical immunostimulator imiquimod often results in tumor resolution further highlighting the immunogenicity of BCC (15).

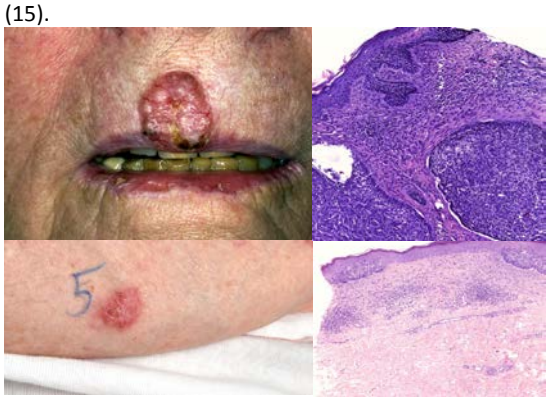

Figure 1. Clinical and histological photos of BCC. Top left shows a nodular BCC and top right shows the histology of a nodular BCC with isolated tumor islands in the dermis. Lower photo left is a superficial BCC on the abdomen and corresponding histology with tumor islands attached to the epidermis at the lower right photo. Own photos.

A broad panel of treatment options exists for BCC. They are divided into surgical and non-surgical procedures. The method of choice depends on whether the BCC is a high-risk or a low-risk tumor. High risk tumors are defined by fulfilling one or more of the following criteria: 1) facial tumors in the H-zone, 2) tumors with a diameter >2 cm, 3) aggressive histological subtype, 4) poor clinical definition of tumor margins, and 5) recurrent lesions (3). In general, surgery or radiotherapy is the treatment of choice for high-risk tumors. Surgical procedures include standard excision with excision of clinically normal surrounding skin or Mohs micrographic surgery. Mohs surgery is a technique where skin tumors are excised at stages guided by per-operative histological examination of the entire deep and lateral margins. By this per-operative histological examination, free tumor margins are ensured with achievement of high cure-rates (>95%) (16). Non-surgical treatments for low-risk tumors include curettage combined with electrodessication, cryotherapy, topical immunostimulaters such as imiquimod or 5 flourouracil and photodynamic therapy (PDT) (3).

BCC and the tumor microenvironment

Tumor immunogenicity is increasingly recognized as an essential contributor to tumor progression and increased understanding of the ability to escape host anti-tumor response is necessary for improvement of treatment modalities. The cancer immunosurveillance theory describes the continuous interaction between tumor survival tactics and host anti-tumor response (17). This theory comprises three phases; the elimination phase, the equilibrium phase, and the escape phase. In the elimination phase, innate and adaptive immunity collaborates to inhibit and destroy the tumor before it becomes clinically apparent. If escape from the elimination phase occurs, the tumor cells enter the equilibrium phase with ongoing crosstalk between tumor cells and immune regulatory cells. Tumor outgrowth is thus equilibrated and limited due to efficient antitumor response. This phase can last for several years. Progressing to the escape phase, the restriction of the tumor cells is no longer adequate and tumor growth is increasing partly through subversion of the immune system (18).

Tumor-infiltrating lymphocytes (TILs)

The crosstalk between tumor cells and these immune regulatory cells, called tumor infiltrating lymphocytes (TILs), is essential for tumor growth in a variety of cancers and presence of TILs is generally considered a characteristic of activation of the host's immune response (19). For many cancers, infiltration of TILs at tumor sites is a prognostic marker correlated to improved disease outcome (20,21). However, phenotype and activity state of the TILs is pivotal. While there is a balance between the tumor-promoting and tumor-inhibiting effects during the equilibrium phase of cancer immunosurveillance, this balance is skewed towards domination of tumor-promotion when entering the escape phase. In this phase the antitumor activities of TILs seem to be inhibited. The knowledge of TILs in BCC is sparse but probably essential given the frequent observation of dense lymphocyte infiltrates surrounding BCC (Figure 2), the increased risk of BCC upon inhibition of the immune system and the regression of tumor following topical immunostimulators.

While abundance of TILs is correlated to improved disease outcome owing to their anti-tumor response, a subpopulation of TILs is in general known to cause local immunosuppression supporting tumor growth. This subgroup of TILs inhibiting adequate anti-tumor response is known as regulatory T-cells (T-regs) (22).

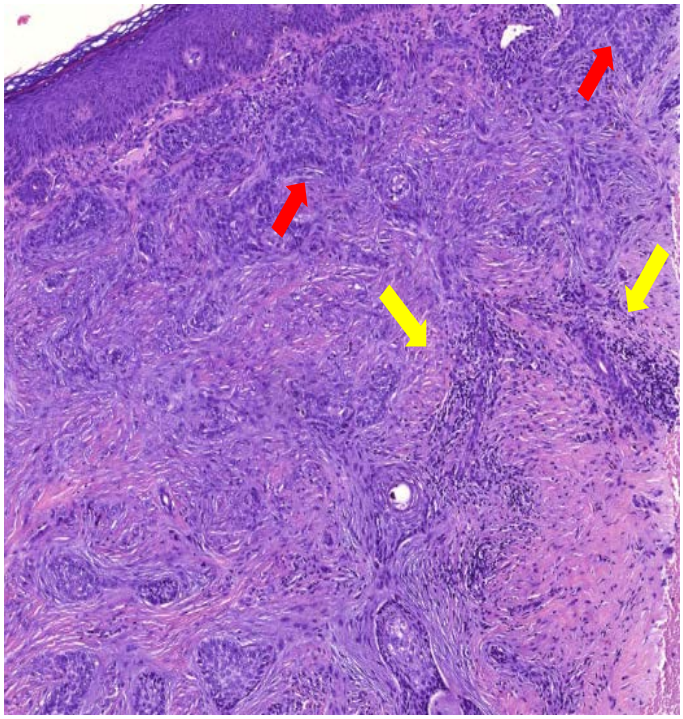

Figure 2 showing an infiltrative BCC with irregular tumor islands diffusely infiltrating the dermis (red arrows). Many of the tumor islands are surrounded by peritumoral infiltration (yellow arrows).

Regulatory T-cells (T-regs)

T-regs comprise a subpopulation of CD4 positive T-cells being indispensable in maintaining immunologic tolerance in healthy individuals by suppressing conventional T-cells (23). Upon neoplasia, T-regs are recruited as a subpopulation of TILs where accumulation at tumor sites leads to suppressed activation and suppressed expansion of tumor-antigen specific T-cells (24). T-regs are characterized by the expression of the transcription factor Forkhead boxp3 (Foxp3) involved in the regulation of the suppressive functions provided by T-regs (25). Their tumor-suppressive activities are mediated in a variety of ways, amongst others by secretion of different immune regulatory cytokines such as TGF-β and IL10. In the healthy individual, T-regs represent approximately 5-10% of the peripheral CD4 positive T-cells. It is well-established that T-regs are overrepresented in and correlated to worse disease outcome for different solid cancers such as pancreas (26), breast (27), and head-and neck cancer (28). For skin malignancies, the impact of T-regs is well documented in malignant melanoma where they are markers of poor prognosis (29,30) and in SCC they have been reported at increased frequencies compared with normal skin (31). Concerning BCC, presence of T-regs in the tumor microenvironment has been shown in association with a Th2

dominant immune response (32) but their role in BCC is poorly understood.

Source of TILs

An immune response dominated by clonal expansion of T-cells directed towards tumor antigens could provide efficient tumor inhibition. In malignant melanoma, restriction of the T-cell receptor (TCR) repertoire has been found in support of antigen-driven clone selection (33,34).The TCR is a dimeric transmembrane receptor of the T-cell, consisting of an α- and a β-chain. T-cells develop in the thymus, and since the possible antigen pool that a T-cell can meet throughout life is enormous, the T-cells are designed to be able to interact with a wide range of proteins by possible generation of very broad receptor diversity. This diversity of the TCR is produced by the recombination, for both the α- and the β-chain, of one of multiple of the gene segments V (variable), D (diversity) (for the β-chain only) and J (joining) to a C (constant) chain gene (35). Additional nucleotide insertion and deletion creates the junctional diversity of the highly variable complementarity-determining region 3 (CDR3) (Figure 3). This nucleotide insertion and deletion leads to variations in both the length and amino acid sequence of the CDR3 region.

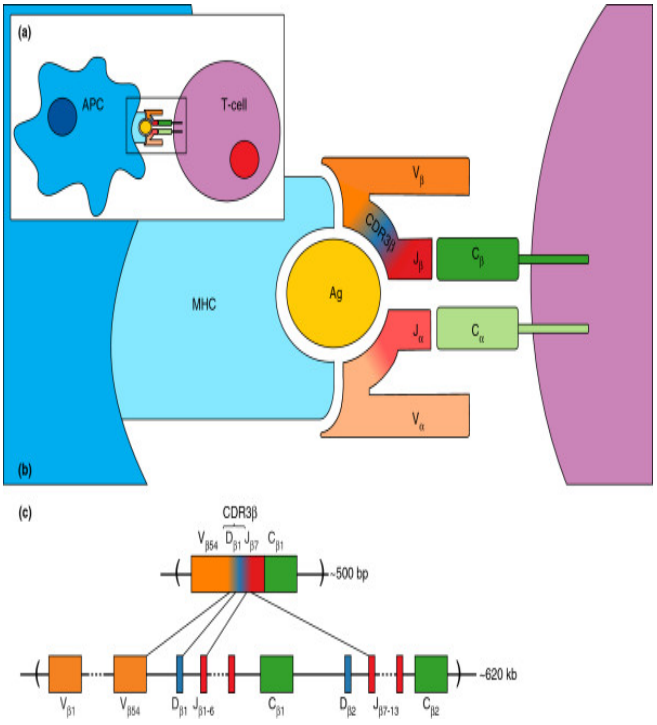

Figure 3. The figure illustrates the interaction between a T-cell by the T-cell receptor binding to an antigen-presenting cell (APC) (a) shown in more detail in figure b with illustration of the TCR composition with the α and the β-chain each being composed of a constant region (C) and a variable region (V) and junctional region (J) making up the highly specific CRD3 region. Figure c illustrates the somatic recombination of the different V, J and D segments. The major histocompatibility complex (MHC) molecule is responsible for antigen-presentation and TCRs are only capable of recognizing antigens presented by this antigen-presenting protein. The figure is shown with permission from Woodsworth DJ et al. (35).

Every T-cell clone has a unique CDR3 region and the frequency of this specific sequence is hence a measure of the occurrence of its corresponding T-cell clone. It is debated whether restricted TCR repertoire found at tumor sites could lead to oligoclonal expansion of specific T-cells in tumor compartments and thereby improve anti-tumor response (36). Oligoclonal expansion or clonal selection is the principle of individual antigen response where antigen-specific lymphocytes proliferate upon recognition of antigen and differentiates into antigen-specific effector cells (37). Studies of the TCR repertoire in various cancer types have primarily focused on the β-chain (36,38) due to previous technical challenges in sequencing both the α and the β-chain. This is also the case for BCC, where analysis of the TCR β repertoire suggested restricted diversity (39). With newer sequencing tools such as next-generation sequencing (NGS), sequencing of both the TCR-α and β-chain is now possible (40). By analyzing the TCR repertoire of TILs in BCC it is possible to establish whether antigen-specific T-cell recruitment is present. Furthermore, knowledge of the source of TILs could be provided. The human skin is protected by T-cells homing to the skin (resident skin T-cells) as well as by a pool of circulating T-cells. The skin resident T-cells provide rapid immune protection against previously recognized common skin pathogens (41). These skin resident T-cells also contribute to skin diseases such as psoriasis and the cutaneous T-cell malignancy mycosis fungoides (42). Whether TILs involved in BCC are recruited from this pool or from the quickly recirculating group of peripheral T-cells is not known.

Tumor stroma and cancer associated fibroblasts (CAFs)

Apart from tumor cells and cells of the immune system, also supportive cells of the connective tissue are involved in tumor development. Evidence is emerging that crosstalk between neoplastic and stromal cells contributes to tumor growth (43). Cells of the tumor stroma include macrophages, fibroblasts, endothelial cells, and adipocytes. The two former are considered essential in tumor progression. Macrophages that infiltrate in tumor surroundings are named tumor associated macrophages (TAMs) and for some cancer types increased number of TAMs is associated with worsened disease outcome (44,45). Additionally, fibroblasts within tumors and in the tumor surroundings are essential in cancer growth, being involved in progression, growth and invasiveness (46). These fibroblasts, termed cancer associated fibroblasts (CAFs), are characterized by the expression of a variety of markers, such as fibroblast activated protein-α (FAP-α), platelet-derived growth factor receptor β (PDGRF-β) and prolyl-4-hydroxylase (P4H) (46,47,48). Some of the pro-carcinogenic activities induced by CAFs involve secretion of matrix metalloproteinases, recruitment of T-regs by secretion of chemokines (43,49) and induction of epithelial-to mesenchymal transition (EMT). EMT is a process where epithelial cells lose their cell-to-cell contact and gain mesenchymal properties. For cancer cells undergoing EMT, invasive and migratory abilities are obtained (46). Furthermore, CAFs are capable of generating a desmoplastic stroma contributing to improved growth conditions for tumor cells (50). The tumor-promoting abilities of CAFs and correlation to worsened prognosis has been shown for different cancers such as head and neck cancer (43), pancreatic cancer (51), colorectal carcinoma (52) and lung cancer (53). In breast cancer, the central role of CAFs in promoting tumor growth is well described (49,54). Additionally, fibroblasts from cancer-free tissue in the tumor vicinity have been found to exhibit tumor-promotion (55,56). In BCC, cross-talk between CAFs and related matrix-remodeling has been suggested (57) but the role of CAFs in BCC is widely unknown.

1.2.5 Ultraviolet radiation (UVR) and local immunosuppression of the skin

Besides the well-known DNA damages following UV-exposure, in particular UVB (middle wave length range 290-320 nm) (58), UVR also mediates both systemic and local immunosuppression of the skin. Upon UVR, structural changes in the chromophores lead to secretion of a variety of mediators followed by release of different immunosuppressive cytokines such as IL4, IL10 and tumor necrosis factor α (TNF-α) (59,60). This affects the function and migration of different immune cells including the important epidermal antigen presenting cells; the Langerhans cells, which migrate to the lymph nodes that drain the UV-irradiated skin. IL10-producing monocytes are recruited to the skin and subsequently drained to the lymph nodes upon antigen stimulation. This leads to abnormal antigen presentation both in the skin and draining lymph nodes which results in dampened secretion of IL12 and IL23, cytokines which are involved in T-cell activation and repair of UV-induced DNA damage (61). Additionally, the balance between Th1 helper cells and T-regs are skewed with increased fraction of T-regs. This local immunosuppression is to a large extent based on generation of T-regs at tumor sites where T-reg mediated IL10 secretion is a major contributor to the immunosuppressive activities (62). This local UV-immunosuppressive effect has primarily been shown in murine studies, where UVB-induced skin tumors were rejected upon transplantation onto syngeneic healthy mice but accepted when transplanted onto immunosuppressed mice or to mice pre-exposed to UVB (63). In humans, local and systemic immunosuppression upon UV-exposure has been shown in delayed-type hypersensitivity (DTH) reactions. Antigen-specific responses are measured after vaccination and UV-exposure, where suppression of elicitation reactions is seen, both at local UVR sites and at more distant non-UVR areas (64). The relation between T-reg development and skin cancer in chronic UV-exposed human skin is largely unknown. In vivo human studies of psoriasis have shown a correlation between suppression of inflammation (decrease in non T-reg infiltration) and increase in T-reg infiltration upon UV-exposure (65).

AIM OF THE THESIS

BCC is the most frequent cancer with increasing incidence worldwide. The slowly growing behavior of BCC and the fact that BCC barely ever metastasizes indicates this neoplasm to possess solid survival mechanisms other than rapid cell turnover and metastasizing abilities. Inhibition of adequate anti-tumor response could be an essential contributor to the survival tactics provided by BCC given the knowledge on BCC being an immunogenic tumor.

The overall aim of the thesis is to characterize the local immune response in the tumor microenvironment in BCC.

Specific study objectives are to:

1. Investigate T-regs in facial BCC and compare their density with the corresponding density in peritumoral skin and non UV-exposed skin (study I).

2. Characterize the contribution of the mesenchymal component to the immune response in BCC with focus on CAFs (study II).

3. Analyze the TCR repertoire in the lymphocytes of BCC in order to investigate whether a restricted TCR repertoire is present within BCC in support of specific tumor-antigen induced immune response (study III).

MATERIALS AND METHODS

PATIENTS AND BIOLOGICAL MATERIAL

For all three studies (I, II, III) we used facial BCC and corresponding peritumoral skin obtained during Mohs surgery at the Department of Dermatology, Bispebjerg Hospital in the period from January 2014 through 2015. All tumors were prior to surgical examination histologically verified as being BCC and per-operative histological examination verified the diagnosis. The peritumoral skin was taken during closure of the wound after microscopic verification of cancer free tissue. In study I and II, skin from the buttocks was also included in order to compare with normal skin that had not been exposed to UVR within the same patients. The sample material from the buttocks was collected as 4 mm punch biopsies. All material was immediately snap frozen and subsequently stored at -80°C until analysis.

We prospectively collected the material for the three studies during Mohs surgery. In study I and II, we collected skin from 18 patients (BCC n=18, peritumoral skin n=18 and buttock skin n=18), and the material was split in two with one half being used for study I and the other half for study II. In study II we additionally included skin from 4 patients (BCC n=4 and peritumoral skin n=4) for the next-generation sequencing analysis. In study III, we included further 38 BCCs and 28 samples of adjacent control skin, hence 10 BCCs did not have corresponding control skin. One patient had two tumors and corresponding two control skin samples. We included peripheral blood from five healthy volunteers for study III which was used as control material. Formalin-fixed paraffin embedded samples of BCC (n=19) were collected retrospectively for immunohistochemical Foxp3/CD4 double staining in study I.

The studies were approved by the Danish Regional Ethics Committee, protocol number: H-4-2013-197 (and two amendment protocols) and the Danish Data Protection Agency, journal number: BBH-2014-008, I-Suite: 02675. All participants gave signed informed consent.

Methods

Immunohistochemistry, automated image analysis and immunofluorescence

Identification of T-regs in BCC in study I was done by immunhistochemical double staining of BCC with antibodies towards Foxp3 and CD4 followed by quantification with automated image analysis. Immunohistochemistry is a technique used to detect antigens in tissue by adding antibodies directed towards the antigen of interest. Subsequent antibody-antigen interaction with conjugation of the antibody to an enzyme leads to a color-producing reaction. The density and distribution of the stain can then be visualized by microscopy (66). We performed double Foxp3/CD4 staining and T-regs were recognized as double positive (brown nuclear staining with red membrane staining) while the non-regulatory T-cells only stained positive for CD4 (red membrane staining). The quantification was done by the use of automated image analysis as illustrated in Figure 4.

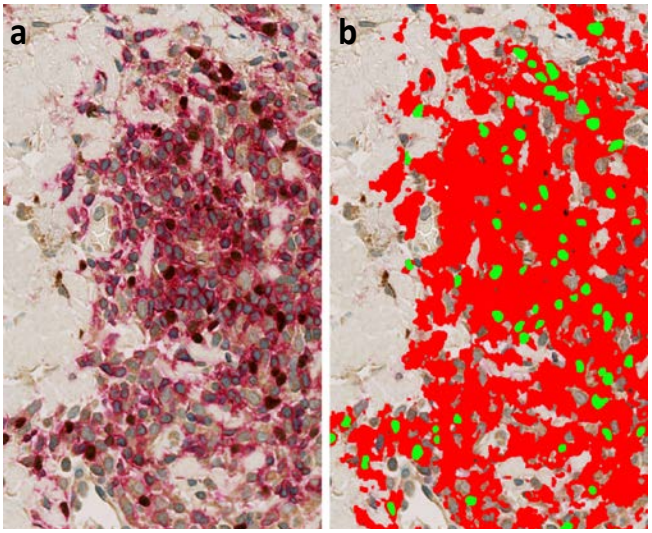

Figure 4. Illustration of the immunohistochemical Foxp3/CD4 double staining of BCC and automated image analysis. T-regs are double positive for Foxp3/CD4 visualized as cells with brown nucleus and red membrane while the non-regulatory T-cells only are single positive with red membrane staining but without staining of the nucleus (a). Automated image analysis showing how the T-regs were quantified with the green area representing the T-regs and the red area the non-regulatory T-cells (b). Photo modified from study I (67).

The principle of immunofluorescence is in general the same as that of immunohistochemistry. Instead of visualization by the use of a peroxidase reaction, the antibody that binds to antigen is detected by a fluorophore; a fluorescent chemical compound, which can re-emit light upon light excitation. We used Alexa Flour as fluorophores for our studies (I, II).

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used in study I and II to investigate and compare the messenger RNA (mRNA) level of a variety of genes expressed in BCC, peritumoral skin and buttocks skin. After RNA extraction from the obtained skin samples, complementary DNA (cDNA) synthesis was performed and subsequently qRT-PCR analysis. The qRT-PCR technique is based on the polymerase chain reaction, a technology that allows for amplification of a single copy or a few copies of pieces of DNA. This technique generates thousands to millions of copies of a particular DNA sequence of interest. The overall method is based on thermal cycling; initial melting leading to separation of the double DNA strand is followed by repeated healing and cooling cycles, in which cooling lets the primers anneal and increased temperature lets the polymerase extend the primers. Primers are strands of short nucleic acid sequences that serve as the starting point for DNA synthesis. A fluorescent dye binds to the product performed. Quantification of the specific gene of interest is analyzed by evaluation of the number of amplification cycles required to reach a particular threshold fluorescence level, termed the cycle threshold (CT) value (68). Data were analyzed by the delta-delta CT-method with GAPDH used as reference gene (69).

Next-generation sequencing (NGS)

Sequencing of BCC and peritumoral skin transcriptome in study II was performed by the use of NGS on the Illumina HiSeq 2000 platform (study II) and on the Illumina MiSeq platform (study III).

NGS refers to a collection of sequencing technologies capable of generating large volumes of sequence data at a high speed and relatively low cost (70). The NGS technique comprises four steps; 1) sample collection, 2) template generation, 3) sequencing, and 4) data analysis. After collection of sample material, a library of sequencing material must be prepared. This involves cDNA fragmentation (100 bp in study I and 250 bp in study III) and adapter ligation. The adapter sequence is annealed to the DNA fragment and is complementary to the parts of the PCR index primers. The index sequence is unique for each fragment enabling mixing of several samples (multiplex sequencing) since the index can identify the sample origin of a sequence. Following library construction, the libraries are clonally amplified for sequencing by bridge amplification. Since we were interested in quantitative information on relative abundance, that is the expression level of certain genes within BCC compared with peritumoral skin, we performed mRNA sequencing, also known as transcriptome sequencing where the transcriptome refers to the set of all mRNA molecules transcribed from DNA in a given cell population. The sequencing principle of NGS is the same as Sanger sequencing referred to as first generation sequencing (71): both techniques are based on incorporation of fluorescently labelled bases to the single stranded DNA template that should be sequenced. Millions of single stranded DNA fragments (templates) bind to a minute glass slide where they are fixed upon the entire sequencing reaction. The four nucleotides (A, G, C and T) used for elongating the sequence are added at the same time together with DNA polymerase; an enzyme allowing for nucleotide incorporation. All four nucleotides are labelled with a unique fluorescent label and blocked, and one base pr. cycle is incorporated into each template molecule. Each cycle of nucleotide incorporation is followed by image capture of the fluorescence signal acquiring four different images, one for each nucleotide. After the capture of the newly incorporated terminating base, a restoration step converts the base to a non-fluorescent, non-terminating base, after which a new round of single-base extension can start over, followed by image capture. After *n* imaging cycles, the fluorescence color in each image from each template position is linked to yield a DNA sequence. With this technique each fragment on the glass slide can be sequenced in parallel yielding a very high throughput (72). After sequencing, mapping of the sequenced reads to the human genome is performed.

Statistics

Statistical analyses in study I and II 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 $P<0.05$.
The NGS data in study II were analyzed following estimation of dispersions, and genes differentially expressed between BCC and peritumoral skin were tested using the ExactTest function in EdgeR.
In study III, two different diversity indices were used to estimate the diversity in the TCR repertoire: The Shannon index and the Simpson index. A diversity index is a quantitative measure that reflects how many different types of species there are in a given community/dataset. At the same time a diversity index takes into account how evenly the entities are distributed. While the Shannon index gives more weight to the richness of sequences (the

number of read sequences), the Simpson index is more a reflection of homogeneity or the distribution of the sequences. By using both, we get a better description of both diversity components; that is the richness as well as the distribution.

RESULTS AND DISCUSSION

Characterization of the microenvironment in BCC and peritumoral skin (Study I and II)

Quantification of T-regs in BCC, peritumoral-and buttock skin by the use of IHC and qRT-PCR (study I)

Previous studies

Increased numbers of T-regs have been described as markers of poor prognosis in a variety of cancers (73,74). In cutaneous SCC, increased T-reg infiltration upon tumor development from pre-invasive to invasive carcinoma has been found (75). Previous literature on T-regs in BCC is sparse with one study describing an attenuated immune state mediated by T-regs (32).

Own results and discussion

By quantification of the T-reg fraction within all CD4 positive cells (double Foxp3/CD4 positive cells) in BCC by the use of immunohistochemistry and automated image analysis, we found that T-regs comprise 45% in mean of the total CD4 positive cells (range 22-84%). This fraction is highly increased compared with healthy blood where T-regs represent 5-10% of T-cells and in healthy skin up to 20% (76). The T-regs were primarily localized within the inflammatory infiltrates in the tumor surroundings and rarely within the tumor islands (Figure 5). In order to understand whether the T-reg accumulation was specific to BCC and very near tumor surroundings or if the more distant tumor periphery was also infiltrated by T-regs, we compared the Foxp3 mRNA levels of BCC with that of adjacent tumor-free skin. Additionally, to understand if T-regs were generally found in high levels in the normal skin, we also included normal non-UV exposed skin from the buttocks for comparison. We found an increased expression of Foxp3 in both BCC and peritumoral skin but absence of Foxp3 expression in the buttock skin ($p<0.001$) (Figure 6). The findings suggest that T-regs may play a role not only in the local immune environment of BCC but also in the peritumoral surroundings.

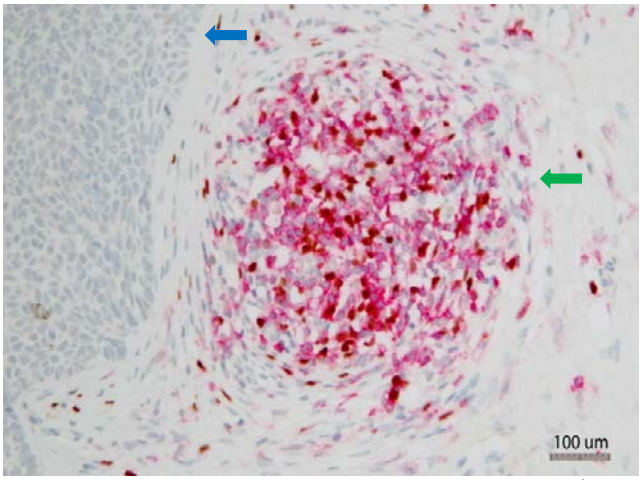

Figure 5. Immunohistochemical double staining of BCC with Foxp3/CD4: The blue arrow points at a BCC tumor island and the green arrow shows the inflammatory infiltrate with non-regulatory T-cells (red cells) and T-regs (brown cells with red halo). The inflammatory infiltrate is seen adjacent to but not within BCC (x4 magnification).

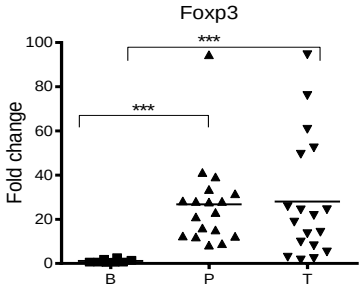

Figure 6. mRNA expression levels of Foxp3 within BCC (T), peritumoral skin (P) and normal buttock skin (B) showing highly increased Foxp3 expression in BCC and peritumoral skin compared with normal non-UV exposed buttock skin where no expression was seen . ***p-value <0.001. Figure modified from study I.

Expression of CAF markers in BCC, peritumoral-and buttock skin analyzed by the use of NGS, qRT-PCR, immunohistochemistry and immunofluorescence (study II)

Previous studies

In breast cancer, CAFs have been thoroughly described as promoters of tumor growth by interaction with cancer cells (49,77). Furthermore, the surrounding stroma in the tumor free tissue also harbors CAFs capable of mediating tumor promotion (55). A few studies have described the presence of CAFs within BCC (57,78) but the knowledge of the impact of CAFs in BCC is sparse.

Own results and discussion

The idea to focus on CAFs came from the data of the transcriptome sequencing in BCC where we found a consistent footprint of abnormal matrix metabolism. There were 65 genes out of 542 which expression was upregulated in BCC and which were coding for extracellular matrix components or enzymes involved in matrix metabolism including collagen XI, a collagen not normally present in healthy skin. PDGFR-β and P4H were among the overexpressed genes indicating increased CAF levels within BCC. We confirmed the NGS results by qRT-PCR and included FAP-α as an additional CAF marker. The mRNA levels of FAP-α, PDGFR-β and P4H as well as collagen XIA revealed high expression of the three CAF markers as well as collagen XI within BCC compared with normal buttock skin ($p<0.01$). PDGFR-β, P4H and collagen XIA were also increased in the peritumoral skin, but almost completely lacking in the normal buttock skin ($p<0.01$-0.001) (Figure 7). FAP-α expression was restricted to BCC (BCC vs peritumoral and BCC vs buttock skin $p<0.01$).

Even though FAP-α, PDGFR-β and P4H are well-recognized markers of CAFs, none of them is fully specific or sensitive to all CAFs (46). A concerted increased expression of all three CAF-markers in BCC and absence in the normal skin, however, supports that there is an actual activation of fibroblasts in BCC. IHC staining with FAP-α, PDGFR-β, and P4H visualized CAFs in BCC and independently confirmed the qRT-PCR data.

Additionally, the increased level of collagen XI in particular within BCC but also in the peritumoral skin points at definite changes in the stroma and the peritumoral skin. Increased levels of this collagen has been found in a variety of cancers (79,80), and assigned a metastatic potential (81,82). BCC rarely metastasizes and we

speculate that collagen XI may be an essential component of the niche allowing the tumor to grow invasively.

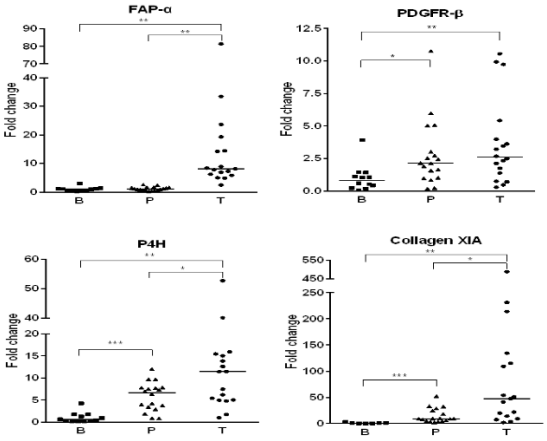

Figure 7. mRNA expression levels of the CAF-markers FAP-α, PDGFR-β and P4H as well as collagen XI illustrating increased expression of the genes both within BCC (T) but also in the peritumoral (P) skin with no or minimal expression in the normal buttock skin (B). *p-value<0.05, **p-value<0.01, ***p-value <0.001. Figure reproduced from study II.

Recruitment of lymphocytes to BCC (study I, II and III)

Attraction of T-regs to BCC by CAF-induced chemokine secretion (study I and II)

Previous studies

Migration of T-regs to tumor sites is partly induced by chemokine-mediated selective recruitment, and elevated chemokine expression has been found correlated to increased T-reg expression in a variety of cancers. Among the chemokines involved in T-reg accumulation are CCL17, CCL18, CCL22 and CXCL12 (83,84,85). The direct effect of chemokines on T-reg attraction has been demonstrated in vitro upon neutralization of CCL22 leading to decreased ability of malignant cells to recruit T-regs (86). Furthermore, T-reg proportions in healthy blood have been shown to increase upon co-culture with CAFs (43). Additionally, secretion of chemokines and cytokines are among the mechanisms whereby CAFs can affect the anti-tumor response (55).

Own results and discussion

Based on the knowledge of certain chemokines being essential for T-regs to move to and accumulate in tumor sites we detected, by the use of qRT-PCR, the mRNA levels of CCL17, CCL18, CCL22 and CXCL12 (study I and II). We found high expression of all four chemokines in BCC and peritumoral skin, whereas there was no detectable expression in the normal buttock skin (Figure 8). In order to confirm the qRT-PCR findings and to search for a correlation between T-regs and related chemokines, we performed immunofluorescent staining with CCL17 revealing presence of CCL17 in the BCC stroma close to but not within the tumor cells. This corresponds to CCL17 expression being elevated primarily in the peritumoral skin. To verify a correlation between chemokines involved in T-reg accumulation and CAFs, we performed immunofluorescent double staining of the CAF markers FAP-α and PDGRF-β and the chemokines CXCL12, CCL17, and CCL22. These stainings revealed double positive FAP-α/CXCL12, FAP-α/CCL17 and PDGFR-β/CCL17 in BCC (Figure 9) pointing at a correlation between CAFs and these chemokines.

Together these findings support that T-regs are attracted to BCC by the chemokines CCL17, CCL18, CCL22 and CXCL12 and that the secretion of CCL17 and CXCL12 is partly CAF-mediated. Hence, CAFs seem to be involved in indirect anti-tumor response by aiding in T-reg recruitment.

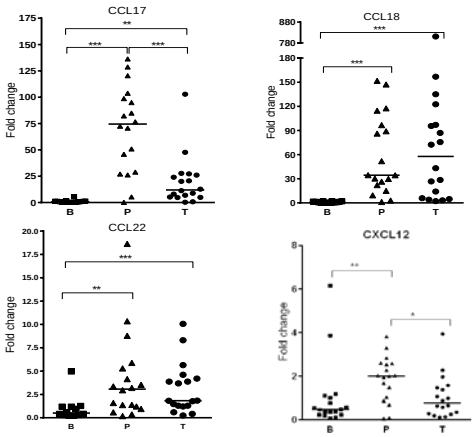

Figure 8. mRNA expression levels of the chemokines CCL17, CCL18, CCL22 and CXCL12. Corresponding to the immunofluorescent staining with CCL17 with abundance in tumor stroma and not within BCC, this cytokine is expressed at the highest level in the peritumoral skin. The figure shows high expression levels of all four chemokines in BCC and peritumoral skin with almost no expression in the normal buttock skin. *p-value<0.05, **p-value<0.01, ***p-value <0.001. Figure modified from study I and II.

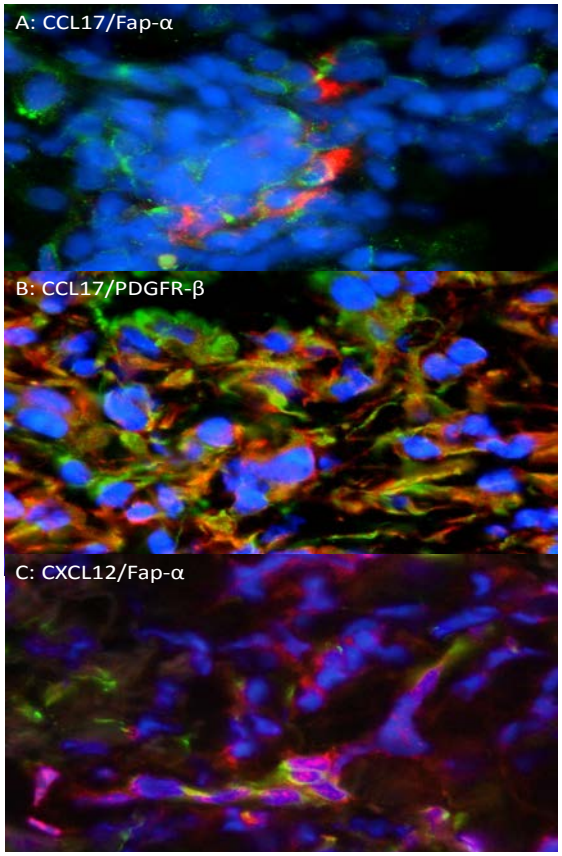

Figure 9. Immunofluorescent double staining of BCC showing correlation between the CAF markers FAP-α and PDGFR-β and the chemokines CCL17 and CXCL12. A: CCL17 (green)/FAP-α (red), B: CCL17 (green)/PDGRF-β (red) C: CXCL12 (green)/ FAP-α (red). Figure reproduced from study II.

TCR-repertoire in BCC and peritumoral skin and source of T-cell recruitment in BCC analyzed by the use of NGS (study III)

Previous studies

While T-reg attraction to tumor sites leads to inhibition of adequate anti-tumor response, overall recruitment of TILs is in contrast associated with improved disease outcome (87). Sufficient T-cell response is mediated by recognition of antigens by corresponding TCRs expressed on T-cells (88). It is debated whether restricted TCR repertoire in tumors could cause oligoclonal expansion of T-cells specifically directed towards tumor-antigens and thereby improve the anti-tumor response (36). The human skin is protected by blood/lymph circulating T-cells and by a pool of skin resident T-cells where the latter provides fast immune response towards previously recognized common skin pathogens (41,89). While skin-resident T-cells are involved in skin inflammatory diseases such as psoriasis, the source of TILs involved in BCC is yet unknown.

Own results and discussion

By the use of NGS, the mRNA coding for the TCR α-and β-chains, as a measure of the TIL variability in BCC, was sequenced and we found a very high diversity of the TCR repertoire in the blood from the healthy individuals. This was anticipated since the blood derived from healthy donors, where minor clonal expansion would be expected. The TCR diversity in BCC and peritumoral skin was only slightly lower, both for the α-and the β-chain.

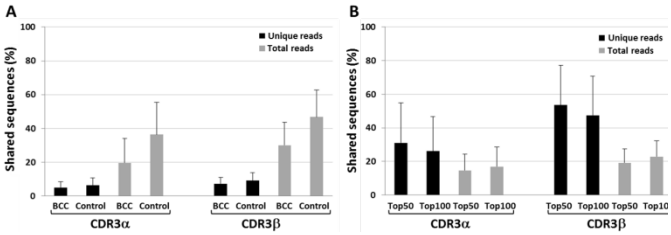

Figure 10**.** Illustration of the shared sequences between BCC and corresponding control skin. (A) shows the proportions of the shared sequences in terms of unique reads and in terms of total reads for all the clonotypes in BCC and control skin. (B) shows the top-50 and top-100 most frequent BCC clonotypes for the α-chain and the β-chain in mean ± SD. Figure reproduced from study III.

Attempting to identify if a restricted TCR repertoire was present pointing at an antigen-specific immune response, the V-J pairing for the 30 most frequent V-Jα and V(D)J-β combinations was compared between BCC and surrounding control skin revealing no preferential VJ pairing and no preferential V or J gene usage. This is opposite to a previous work where preferential Vβ families in TILs of BCC were found compared with peripheral blood monocytes and in comparison with control skin. Comparison between BCC and control skin was only performed for five samples, though and the predominant Vβ differed from individual to individual (39). Therefore, no solid evidence for selection of specific T-cells seem clear. Additionally, this study was performed by the use of PCR and southern blot making these data much less precise than those obtained by NGS.

Finally, by comparing the CDR3 length distribution of the more frequent clonotypes, we found similar CDR3 length Gaussian distribution between BCC, peritumoral skin and healthy blood.

Together, this broad TCR repertoire and random VJ usage and pairing as well as similar CDR3 lengths do not support antigen-driven clone selection.

In order to seek for antigen-specific clonotypes characteristic for BCC and establish the origin of T-cells (skin versus blood); the CDR3 clonotypes in BCC were compared with the CDR3 clonotypes in the corresponding control skin. We found, that around 10% of the CDR3 clonotypes were shared between BCC and the adjacent control skin (both for the α-chain and the β-chain). In terms of total reads, however, these shared clonotypes comprised around 20-40% (α-chain) and 30-50% (β-chain) (Figure 10A), meaning that the shared clonotypes were among the high-frequency clonotypes representing about 20% of the total reads. Since an antigen-specific clone would be expected to be frequent, we considered the top-50 or top-100 most frequent BCC clonotypes and by this we found that only 30 (α-chain)-50% (β-chain) of these clonotypes were also detected in the corresponding control skin (Figure 10B). Hence, a large proportion (50-70%) of the clonotypes in BCC was not detected within the corresponding control skin. Additionally, when searching for clonotypes repeatedly detected in more than two samples, we could only detect very few.

As mentioned, the possible T-cell source in BCC is the blood from where T-cells quickly recirculate after termination of response, or the skin resident T-cells. With non-specific T-cell recruitment from the pool of skin-resident T-cells, we would expect to see broad overlap in terms of TCR repertoire between BCC and adjacent skin. Partial overlap would be expected upon specific recruitment from the skin resident T-cell pool, whereas little overlap in terms of TCR repertoire would be assumed with T-cell recruitment from the blood. Since there was low overlap of the TCR repertoire between BCC and adjacent control skin, we hypothesize that the T-cells are primarily recruited from the blood. Furthermore, given that no restricted TCR repertoires were found, we argue that the T-cell attraction to BCC is probably more a reflection of random T-cell accumulation than a specific antigen-driven anti-tumor immune response.

Local immunosuppression mediated by T-regs and CAFs within BCC and peritumoral skin (study I and II)

T-reg and CAF-mediated cytokine secretion and correlation to chronic UV-exposure (study I and II)

Previous studies

The tumor seed and soil hypothesis highlights the importance of the microenvironment for malignant cells to grow; for a tumor (the seed) to grow, the surrounding milieu (the soil) must be prepared for it (90). In the human skin, where UVR is a major risk factor for BCC by causing DNA signature mutations such as C to T or CC to TT, UV-exposure also affects the skin causing local immunosuppression. This could cause the skin (the soil) to be receptive of the mutated keratinocytes (the seed) and/or dampen the repair of the UV-induced mutations. Increased T-reg expression has been shown to be pivotal in this UV-induced local immunosuppression of the skin (63). In psoriasis, where enhanced inflammation is causing disease, decline in inflammation and corresponding

clinical improvement upon UV-exposure has been shown related to increased T-reg expression (65). Also UV-mediated secretion of certain cytokines is affecting the skin with significance on skin tumors; Increased IL6 expression upon UV-exposure has been shown in cutaneous SCC, where UV-induced CAFs can mediate tumor-promoting IL6 secretion (91). Additionally, the impact of UVR on fibroblasts has been shown to cause enhanced expression of matrix metalloproteinases (92).

Own results and discussion

By the use of qRT-PCR and confirmed with immunohistochemistry, we compared the IL6 expression levels within BCC, the peritumoral skin and the normal buttock skin and found highly increased IL-6 expression primarily in the peritumoral skin. There was no expression in the normal buttock skin and hardly any within BCC (Figure 11).

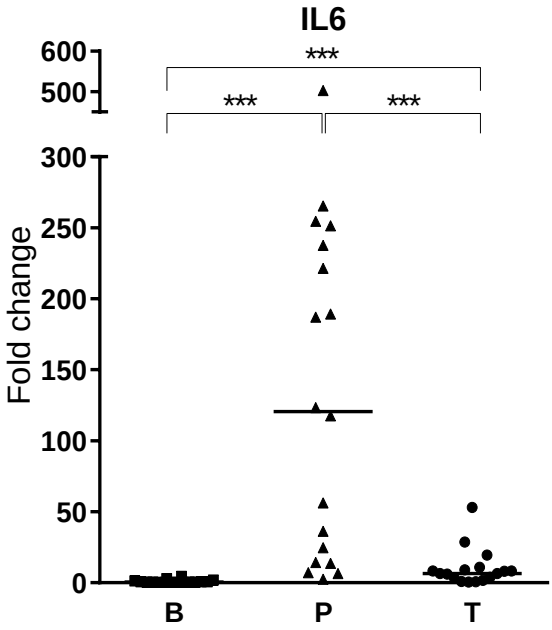

Figure 11. mRNA expression levels of IL6 in BCC (T), peritumoral skin (P) and normal non UV-exposed buttock skin (B). The figure illustrates the highly increased expression levels of IL6 primarily in the peritumoral skin. ***p-value<0.001. Figure modified from study II.

In breast cancer, fibroblasts with a phenotype intermediate between those of CAFs in cancer tissue and fibroblasts in healthy breast tissue has been detected in the tumor free surgical margins (55). Based on our results showing different expression of CAF markers within BCC and peritumoral skin with FAP-α expressing cells being restricted to BCC, we suggest that the same phenomenon applies to BCC where activation and functioning of these different fibroblast phenotypes may vary. Since IL6-expression was rather specific to the peritumoral compartment, we hypothesize that this cytokine is selectively secreted from the intermediate fibroblasts in the peritumoral stroma. The ability of IL6 in mediating EMT (93) supports that the primary functions of this cytokine is mediated in stromal compartments. All BCC cases and corresponding peritumoral skin included in our analyses were facial; hence exposed to long-term UVR. Based on this we hypothesize, that fibroblasts in the peritumoral surroundings of BCC are induced upon chronic UV-exposure mediating increased IL6 secretion thereby causing a soil permissive of skin cancer growth.

Given that UV-exposure is pivotal in generating local immunosuppression where T-regs, at least in mice, are essential contributors, our findings of increased T-reg concentration not only within BCC but also in the peritumoral surroundings is not that surprising. In murine studies, the immunosuppressive effects of acute UV-induced T-regs are highly caused by IL10 expression (94). In our study, when investigating the mRNA levels of IL10 by the use of qRT-PCR, we found very low expression levels both in BCC and peritumoral skin and no expression in the normal buttock skin. The mRNA expression levels of TGF-β, another important T-reg related cytokine, was enhanced both in BCC and peritumoral skin, highest in the peritumoral skin but absent in normal non-UV exposed buttock skin. The TGF-β expression level correlated with the Foxp3 expression level both within BCC and in the peritumoral skin supporting TGF-β as a contributor of T-reg induced immunosuppression in BCC (Figure 12). Lack of IL10 secretion in our study could be caused by multiple factors; Firstly, difference in mouse and man is a reasonable assumption. Secondly, IL10-secretion has been described to increase upon acute UV-exposure causing an immunity switch after which continued secretion is stopped (95). Given the chronic UV-exposure of the patients in our studies, IL10 secretion could have been increased at an earlier point. A previous study on BCC and T-regs showed increased IL-10 expression in BCC compared with normal skin and the opposite for TGF-β (32) opposing our results. The comparison was between BCC and normal skin and not peritumoral skin, making comparison difficult.

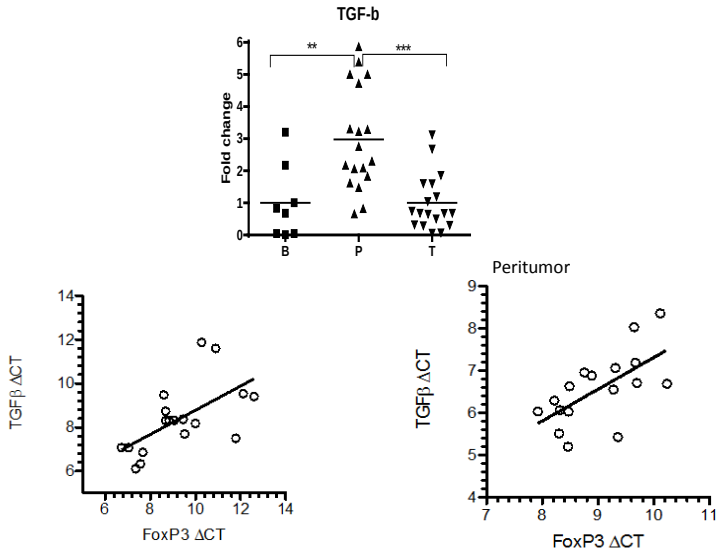

Figure 12. The top figure shows the mRNA expression level of TGF-β in BCC (T), peritumoral skin (P) and normal buttock skin (B) revealing increased levels of TGF-β in the peritumoral skin. The lower figures illustrate the correlation between Foxp3 and TGF-β in tumor and in the peritumoral skin. **p-value<0.01, ***p-value<0.001. Figure modified from study I.

TILs in BCC by analysis of mRNA expression levels of T-cell markers (study I)

Previous studies

Th1 orientation of an immune response is in general considered favorable in terms of inhibiting cancer growth, and increased mRNA expression levels of Th1 associated genes is associated to

improved prognosis for some cancer types (96,97). In spontaneously regressing BCC, increased CD4 infiltration and IFN-γ secretion has been found compared with non-regressing tumors (98,99). Furthermore, imiquimod, a Toll-like receptor 7 agonist induces the secretion of different cytokines such as IL1, TNF-α (100), IFN-α (101), IFN-γ and activation of Th1 mediated cell response (102). In contrast, Th2 polarization has been shown in BCC in combination with increased T-reg density suggesting immune skewing induced by T-regs (32). Hence, skewing of the immune response might affect the progression of BCC growth.

Own results and discussion

Attempting to search for a specific immune response directed towards BCC, we sought to establish the phenotypes of the non-regulatory T-cells to investigate if polarization towards a specific inflammatory response was present. Based on previous work on TILs by Han et al (103), the mRNA level of T-bet (Th1), ROR-c (Th17), and a variety of cytokines; TNF-α (Th1), IFN-γ (Th1), IL12 (Th1), IL13 (Th2), IL17 (Th17), as well as perforin and granzyme-B (cytotoxic markers) was investigated. For the majority of markers there was no expression in the normal buttock skin which was expected since normal healthy skin is in a resting state without activation of an immune response. Although detecting a higher expression of Th1 markers in BCC compared with normal buttock skin, when comparing the expression levels in BCC and peritumoral skin, we could not detect any statistically significant difference in expression of these cytokines (TNF-α, IFN-γ, IL12 and T-bet) (Figure 13).

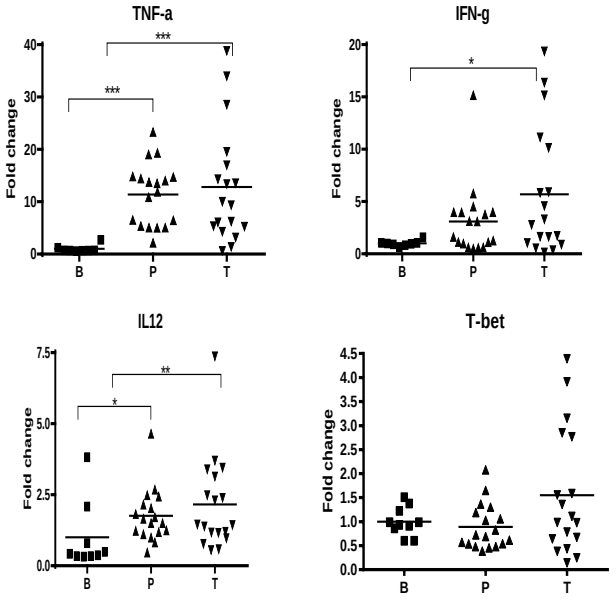

Figure 13 showing the mRNA expression levels of the TH1 markers TNF-α, IFN-γ, IL12 and T-bet. There is no expression of any markers in the normal buttock skin (B) except for a low level of T-bet. Increased expression of IL12 and TNF-α is seen in BCC (T) and peritumoral skin (P) compared with normal buttock skin and increased levels of IFN-γ in BCC compared with buttock skin. There was no statistically significant difference in expression levels of any of the markers between BCC and peritumoral skin. *p-value<0.05, **p-value<0.01, ***p-value<0.001. Figure modified from study I.

Since the majority of lymphocytes observed microscopically in relation to BCC were localized in the surroundings and not within BCC, the fraction of these specific markers within the total number of lymphocytes in BCC and corresponding peritumoral skin was a more representative measure than analyzing the markers isolated. When calculating this fraction of Th1 markers out of all lymphocytes (T-bet/CD3, TNF-α/CD3, IFN-γ/CD3, IL12/CD3), there was still no statistically significant difference in expression levels between BCC and peritumoral skin, except for TNF-α where a higher fraction was found within BCC (p=0.01). For the cytotoxic markers perforin and granzyme-B, we did not detect any difference between BCC and peritumoral skin when comparing the fraction of these markers within CD3 cells. Overall ROR-c expression was higher in the peritumoral skin compared with BCC and normal buttock skin, but the fraction of ROR-c/CD3 was increased in BCC compared with the fraction in the peritumoral skin (p<0.0001). Since ROR-c is a marker of a Th17-response, we expected a corresponding increase in IL17 expression but there was no expression of IL17 in any of the three skin areas. Inverse correlation of T-reg and Th17 has been reported for breast and ovarian cancer (86,104), and in ovarian cancer T-regs have been shown to inhibit Th17 cell development (86). Moreover, TGF-β induced Foxp3 has been shown to inhibit Th17 cell differentiation by inhibition of RORγt (encoded by ROR-c) (105) and the lack of IL17 expression in our study might accordingly be caused by T-reg expression at the expense of IL17 secretion. Finally, given that there was no IL13 expression, Th2 skewing did not seem predominant either (data not shown). In conclusion, no characteristic immune skewing could be detected in support of a tumor-directed antigen response. This lack of specific anti-tumor response corresponds with the findings of a broad TCR-repertoire without characteristics of antigen-specific anti-tumor response as found in paper III.

CONCLUSION

- T-regs are present at high concentration within BCC and peritumoral skin. They are attracted to the tumor environment by a variety of chemokines such as CCL17, CCL18 and CCL22. T-regs seem to mediate an immunosuppressive niche and inhibit adequate anti-tumor response (study I)

- CAFs are detected within BCC where there is a high expression of the CAF-markers FAP-α, PDGFR-β and P4H. CAFs are involved in T-reg attraction since correlation between the chemokines CCL17 and CXCL12 and CAFs were shown. Fibroblasts in the peritumoral surroundings seem to be of intermediate type between those in BCC and normal fibroblasts in the normal buttock skin and these intermediate type fibroblasts might be induced upon chronic UV-exposure (study II).

- There is a broad TCR repertoire in BCC and peritumoral skin without characteristics of a specific anti-tumor response. There is low overlap in terms of TCR repertoire between BCC and peritumoral skin. Based on this, the TILs in BCC are most likely recruited primarily from the pool of circulating T-cells in the blood and not the skin resident T-cells (study III).

GENERAL DISCUSSION, PERSPECTIVES AND

FUTURE RESEARCH

In this thesis, the microenvironment in BCC was investigated with focus on the tumor-immune interactions. The overall conclusion from our studies was that BCC resides in an immunosuppressed niche and is therefore shielded from eradication by the immune system. Further, an unexpected finding was that features of immunosuppression were also detected in normal facial skin even in the absence of tumor cells. The results of the studies showed that this immunosuppressive niche was partly mediated by T-regs and CAFs; high levels of T-regs and CAFs were found both within BCC but also in the peritumoral skin whereas the non UV-exposed buttock skin contained neither T-regs nor CAFs. Correspondingly, increased expression of the chemokines CCL17, CCL18, CCL22 and CXCL12 involved in T-reg attraction were detected in BCC and peritumoral skin but not in buttock skin. Furthermore, correlation between CCL17 and CXCL12 and CAFs was shown pointing at a role for CAFs in attracting T-regs to BCC thereby indirectly contributing to local immunosuppression. This interaction between T-regs and CAFs in mediating dampened anti-tumor response has previously been shown by induction of T-regs upon co-culture of peripheral blood cells with CAFs (43). Moreover, induction of CAFs by TGF-β, an essential T-reg secreted cytokine, has been shown in wound healing (46) supporting the possible interaction between CAFs and T-regs and suggesting the interactions being bidirectional. Figure 14 summarizes schematically some of the mediators involved in and activated upon BCC growth.

Specific T-cell mobilization upon tumor-antigen recognition would lead to efficient anti-tumor response where skewing of the T-cell response could be characteristic for such a response. A characterization of the composition of the inflammatory infiltrates in BCC was done by analyzing the mRNA expression of various T-cell markers. There was no statistically significant difference in the expression levels of any T-cell markers in BCC compared with peritumoral skin except for TNF-α and ROR-c. For both TNF-α and ROR-c, a higher fraction within all CD3 cells was found in BCC compared with peritumoral skin. However, lack of corresponding IL17 expression points at this Th17 response being inactivated. This inactivation is likely caused by T-regs, since inverse correlation of T-reg and Th17 has been related to a variety of cancers (86,104). Hence, it seems that adequate anti-tumor response is inhibited in BCC, even though the findings of increased TNF-α suggests a certain level of anti-tumor response. In the normal buttock skin we did not detect expression of any T-cell markers corresponding to healthy skin being in a resting state.

This lack of specific immune response was confirmed by investigating the TCR repertoire. If a specific anti-tumor response was present, we would have expected a rather narrow diversity in the TCR repertoire within BCC. The opposite was found, however, with broad diversity of the TCR repertoire both in BCC and in the surrounding control skin. Lack of oligoclonal response in many tumors has been explained by ongoing mutations and thereby shifting of the dominant tumor antigens. This would lead to constant new clones leading to a mixture of different clones. This is probably not the case for BCC, however, since BCC is a very monogenic tumor. Lack of clonality is therefore more likely caused by other suppressive mechanisms such as those mediated by T-regs and CAFs.

The findings of T-regs and fibroblasts with cancer associated phenotype in the surroundings of BCC in our studies supports previous research describing the importance of the stromal compartment in order for BCC to grow. This local tumor milieu could be generated not only upon BCC development but also by long term exposure to UVR. Given the lack of functional experiments in this thesis, it cannot be conclude whether T-reg attraction and CAF-induction was initiated by mutated epithelial cells or prior to BCC development generating an immunosuppressed environment where malignant cells are more prone to grow. It seems likely that this is a combinatory event where both chronic UV-exposure and neoplastic cells interact, underscoring the seed and soil hypothesis.

With the design of our studies, we compared BCC, peritumoral skin and normal buttock skin samples derived from the same patients. By this we are able to rule out interindividual differences, thus increasing the reliability of our findings. Furthermore, this is the first comprehensive characterization of the local immunosuppressive environment in BCC in vivo analyzing both immune cells, cells of the connective tissue and the TCRs of the TILs.

The lack of functional experiments in the thesis weakens some of the conclusions of the studies. Isolation of T-cells from BCC was of great interest and we attempted to do this by the use of two different experimental set-ups (106). By isolating T-cells from BCC, we would have been able to perform flow cytometry resulting in more accurate analysis of the T-cell infiltrate. Unfortunately, we failed with these experiments and we have not been able to identify the reason. Limited amount of sample material could be one of the reasons and relative sparse amount of T-cells within BCC could also be part of the explanation. Knowing that fibroblasts are easy to culture, a future experiment with functional studies on CAFs is currently planned. Isolation of CAFs/fibroblasts from BCC, peritumoral skin and normal buttock skin will be performed and upon UVR exposure the impact on cytokine and chemokine secretion as well as T-reg induction, will be analyzed. Inclusion of facial samples from individuals without skin cancer will provide more solid scientific basis to the impact of UVR in the induction of T-regs and CAFs.

In conclusion, the studies on the local immune response upon BCC development have shown that an immunologic response is generated in line with BCC being an immunogenic tumor, but this response is not specific. Additionally BCC is capable of generating a protective niche in the microenvironment composed of both T-regs and CAFs causing local immunosuppression and hindering of adequate anti-tumor response. This lack of adequate anti-tumor response is more pronounced in the immunosuppressive population who possess an increased risk of developing skin cancer, a finding we have previously described in recipients of allogeneic stem cell transplantation (14). Aiming at gaining further knowledge on the general role of immunosuppression and skin cancers, we are currently analyzing epidemiological data of the risk of skin cancer in HIV-infected individuals compared with immunocompetent persons. Additionally, we are interested in further understanding which factors contribute to the increased risk of keratinocyte carcinoma in the immunosuppressed population. Therefore, we are investigating whether changes in the microbiome can be detected in pre-invasive keratinocyte carcinoma in immunosuppressive patients compared with immunocompetent to understand if microbial changes contribute to the increased risk of keratinocyte carcinoma in the immunosuppressed population.

In a clinical context, further development of immunotherapy as a treatment modality for BCC, as supported by the findings of the present studies, holds great promise. A few topical immunostimulators are already approved for superficial BCC. Overcoming the inhibitory mechanisms described in this thesis by the use of immunotherapy could introduce a new path in clinical treatment of BCC.

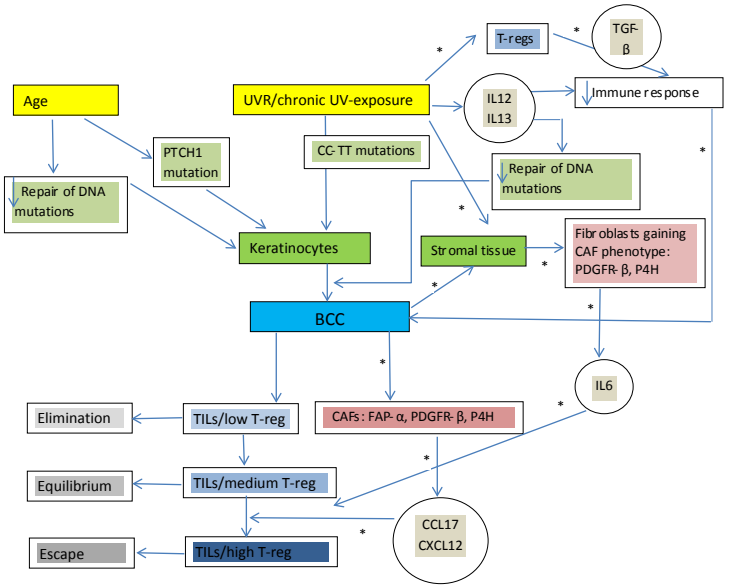

Figure 14. Overview of the mediators involved in BCC growth. The areas marked with an asterisk are those investigated in this thesis. The scheme, despite being far from covering, illustrates the complex interactions of environmental effectors and following mediators where each contributor is often involved in multiple steps.

LIST OF ABBREVIATIONS

BCC: basal cell carcinoma
UVR: ultraviolet radiation
SCC: squamous cell carcinoma
PDT: photodynamic therapy
TILs: tumor-infiltrating lymphocytes
T-regs: regulatory T-cells
Foxp3: Forkhead Box P3
TCR: T-cell receptor
NGS: next-generation sequencing
CAFs: cancer associated fibroblasts
TAMs: tumor associated macrophages
FAP-α: fibroblast activated protein
PDGRF-β: platelet-derived growth factor receptor β
P4H: prolyl-4-hydroxylase
EMT: epithelial-to-mesenchymal transition
UVR: ultraviolet radiation
UVB: medium wavelength ultraviolet radiation, 290-320 nm
TNF-α: tumor necrosis factor α
qRT-PCR: quantitative real time polymerase chain reaction
mRNA: messenger RNA
cDNA: complementary DNA

SUMMARY

BCC is an immunogenic tumor highlighted by the increased risk in immunosuppressed individuals and the frequent occurrence of tumor infiltrating lymphocytes (TILs) in the tumor surroundings. Immunotherapy is evolving as a promising treatment strategy for several cancer types where topical immunostimulators are among the possibilities for superficial BCC.
The overall aim of this thesis is to characterize the immunologic response upon BCC as well as characterizing the surrounding tumor stroma.
The aim was achieved by the use of a variety of laboratory techniques; immunohistochemistry, immunoflourescence, qRT-PCR and NGS.

Tumor microenvironment

T-regs are a subpopulation of the CD4 positive T-cells normally comprising around 5-10% of the peripheral T-cells and up to 20% of the skin resident T-cells. In the healthy individual they are crucial in hindering autoimmune diseases whereas the role in cancer is less advantageous with association to tumor progression for a variety of cancer types. By investigating the presence of T-regs in BCC by immunohistochemistry in study I, it was found that T-regs comprised 45% in mean of the total CD4 positive cells in BCC. The increased T-reg concentration was confirmed with qRT-PCR showing increased Foxp3 expression levels in BCC as well as in the peritumoral skin. In the normal non-UV exposed buttock skin, no Foxp3 expression was found. Hence, T-regs seem to play a role both in BCC but also in the tumor surroundings.

Tumor surroundings are essential in terms of the ability for a tumor to grow. Apart from interaction between immune and cancer cells, also crosstalk with cells of the connective tissue such as CAFs is essential. In study II, NGS revealed increased expression of the CAF-markers P4H and PDGFR-β in BCC. Subsequent qRT-PCR confirmed this and also showed increased expression in the peritumoral skin whereas no expression was found in the normal buttock skin. FAP-α expression was seen only within BCC. CAFs are thus highly present within BCC and we further hypothesize that fibroblasts in the peritumoral skin acquire a phenotype intermediate between normal fibroblasts and CAFs in BCC. This intermediate phenotype might be induced by chronic UV-exposure mediated by increased IL6 expression. This corresponds to our findings of highly increased IL6 expression primarily in the peritumoral skin and to previous literature describing CAF-induced tumor-promoting IL6 expression upon UV-exposure in cutaneous SCC.

Recruitment of TILs to BCC

mRNA expression levels of the chemokines CCL17, CCL18, CCL22 and CXCL12, involved in T-reg attraction to tumor sites were increased both in tumor and peritumoral skin with lack of expression in the normal skin. Correlation between the chemokines CCL17 and CXCL12 and CAF markers was found by IF establishing a role for CAFs in attracting T-regs to tumor sites.

Efficient immunologic anti-tumor response could be provided by clonal expansion of T-cells directed against tumor-antigens. If this was the case, then restricted TCR-repertoire in BCC compared with surrounding skin would be seen. Analysis of the α and β-chain of the TCR was performed showing a high diversity of TCR repertoire in BCC and lack of predominant V(D)J-gene usage, no preferential VJ pairing or specific CDR3 length distribution. Therefore, no support of antigen-driven clone selection was found. This corresponds with lack of obvious anti-tumor skewing towards a Th1, Th2 or Th17 polarization.

Conclusion

To summarize it has been shown, with these studies on the local immune response upon BCC development, that an immunologic response is generated in line with BCC being an immunogenic tumor. This response is not specific, though. Additionally, BCC is capable of generating a protective niche in the microenvironment composed of both T-regs and CAFs breeding local immunosuppression and hindering of adequate anti-tumor response. In a clinical perspective, further research in improving immunotherapy for BCC is promising since an immunological response is present but needs to be reactivated.

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