Chapter 4
Molecular and Cellular Interplay in SCC Including Immunomodulation and Clinical Implications

Valerie Yanofsky, John A. Carucci, and Günther F.L. Hofbauer

Introduction

CSCC is the second most common form of cancer found in the United States, and accounts for approximately 20% of all non-melanoma skin cancers. Several important risk factors for the development of SCC include skin type, with Fitzpatrick types I and II skin being the highest risk, cumulative exposure to UV radiation, age, and immune status [1, 2]. These are covered in detail in Chaps. 1 and 2. In particular, the incidence of SCC is thought to be over 100 times greater in immunosuppressed solid-organ transplant recipients (OTR) as compared to the general population [3]. The epidemiology and clinical management of SCC in the context of immunosuppression is covered fully in Chap. 10 but is briefly reviewed by way of introduction here.

SCC lesions found in OTRs may display more aggressive clinical behavior [4] than that seen in the immunocompetent. Transplant-associated SCC (TSCC) often occurs in patients at a younger age, and may demonstrate increased rates of local recurrence, which may reach as high as 13.4 %, as well as elevated metastatic rates approximating 8 % within the first 24 months post-excision [5, 6]. In some cases, OTRs may develop hundreds of rapidly growing SCC, a phenomenon known as

V. Yanofsky, BS
Ronald O. Perelman Department of Dermatology, NYU-Langone Medical Center,
550 First Avenue, New York, NY 10016, USA
e-mail: valerie.yanofsky@med.einstein.yu.edu

J.A. Carucci, MD, PhD (✉)
Ronald O. Perelman Department of Dermatology, New York University Langone Medical Center, 240 East 38th Street, 12th Floor, New York, NY 10016, USA
e-mail: john.carucci@nyumc.org

G.F.L. Hofbauer, MD
Dermatologische Klinik, Universitätsspital Zürich,
Gloriastrasse 31, Zürich, ZH 8091, Switzerland

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catastrophic carcinomatosis, which can result in extensive local tissue damage and widespread disease [4, 7]. Metastatic SCC in OTRs is often fatal, with some studies showing a 3-year mortality as high as 46 % [8, 9]. SCC lesions in immune suppressed patients may therefore be considered to be "high-risk" tumors. See Chaps. 1, 3, and 10 for a thorough discussion of clinical data related to SCC and immunosuppression, and Chap. 2 for definitions of high-risk SCC.

Although high-risk SCC lesions found in immunocompromised hosts may present as a single solitary tumor, the far more common clinical picture involves multiple lesions, which arise consistently on chronically photo-damaged skin [5, 10]. This gives rise to the notion of "field cancerization," which refers to a broad area of skin that may appear clinically and histopathologically normal, but has in fact been transformed through exposure to multiple carcinogens, such as UV rays and immunosuppressive agents, to become tumorigenic. These areas typically contain distinct molecular and immune profiles, and may be affected by multiple subclinical and clinical actinic keratosis (AK) and in situ (epidermal) SCC lesions [11, 12]. Extensive skin damage associated with field cancerization provides a rationale for the initiation of field therapy, which represents a multi-pronged approach aimed at targeting an entire field rather than individual lesions in an effort to eradicate both clinically apparent and subclinical AK, in situ, and dermally invasive SCC lesions [13, 14]. Field therapy (covered in Chap. 5) may lead to markedly improved long term outcomes for patients with field cancerization.

Comparing and contrasting SCC development in immune competent patients with high-risk SCC in OTRs provides us a unique opportunity to explore the tumor microenvironment, and the precise molecular changes which may underlie the disparate clinical behaviors seen between these two populations. Although high-risk SCC can develop in patients without known immune dysfunction (see Chap. 2), the OTR model allows us to gain a greater understanding of the generation of antitumor immunity, and the various factors which may contribute to field cancerization and catastrophic carcinogenesis. This chapter will therefore highlight the important molecular pathways and key immune features which may give rise to SCC lesions. In particular, we will focus on the novel molecular interactions and distinct immune phenotypes seen in immunosuppressed OTRs as a proposed mechanism underlying the development of high-risk SCC tumors. As this chapter focuses on molecular pathways and cellular interactions, see Chap. 3 for a discussion of genetic and epigenetic changes impacting SCC production and prognosis.

Cellular Pathways of SCC Formation

All SCC lesions are believed to begin via the repeated, uncontrolled division of transformed keratinocytes [15]. Ultraviolet (UV) exposure is accepted as the main pathogenic factor inducing a primary mutation in keratinocytes, which may ultimately lead to the development of SCC. This process is believed to occur in a classic, stepwise fashion, in which a single transformed clone gains a growth advantage

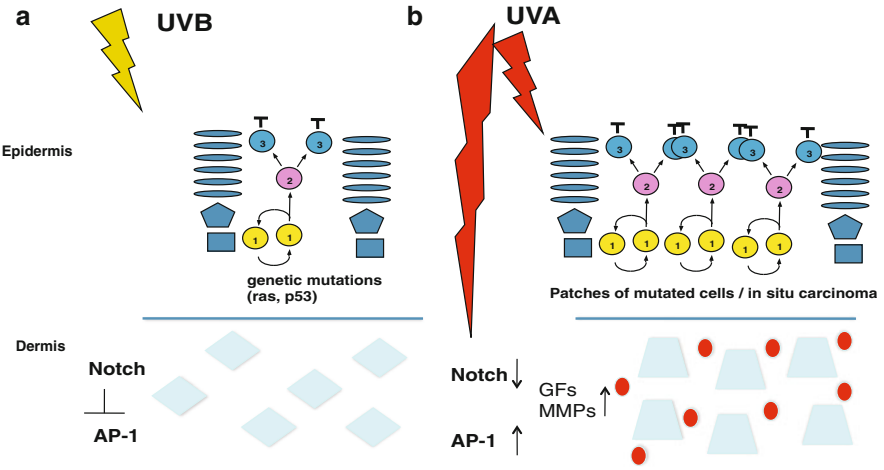

Fig. 4.1 *The cellular pathways of SCC formation and field cancerization*. (**a**) UV exposure results in a primary genetic mutation in keratinocytes, which promotes cellular proliferation and the acquisition of further mutations. This ultimately results in the formation of precursor SCC lesions. (**b**) Normal appearing skin adjacent to tumor tissue may contain unique molecular and genetic profiles, resulting in a tumorigenic focus. For example, Notch1, a regulator of keratinocyte differentiation, has been shown to be down-regulated in the dermis underlying actinic keratosis lesions. This results in increased expression of AP-1 family transcription factors, leading to elevated levels of pro-tumor cytokines. Kindly provided by Prof. Gian-Paolo Dotto, MD PhD

which allows it to acquire more genetic alterations [16]. Accumulation of transformed clones results in a microscopic focus of abnormal cells within the skin, which form precursor SCC lesions known as actinic keratosis (AK) or Bowen's disease (SCC in situ, Fig. 4.1a). These lesions are confined locally by the skin's basement membrane, and are therefore prevented from invading adjacent tissues [15–17]. Ultimately, further mutations enable the tumor to progress and breach the basement membrane, which results in the infiltration of nearby structures and subsequent metastasis.

This model is likely incomplete since some SCC, particularly very high-risk, poorly differentiated and sarcomatoid tumors do not appear to arise from a prior AK or in situ SCC lesion. Other mechanisms probably exist which can initiate SCC carcinogenesis, including viral HPV tumor promotion which plays a role in anogenital and nail fold SCC, but likely has a minimal role in UV-induced SCC. Still, since most SCCs do arise within sun-exposed regions and many occur in clinically actinically damaged skin, the UV model is likely relevant to most SCCs as further explained below.

UV rays most commonly induce a mutation or deletion of the p53 gene, resulting in an inactivation of its tumor-suppressor protein product [18]. This protein is thought to play a prominent role in protecting the integrity of the genome by triggering apoptosis of mutated cells during the cell cycle. Deletion of this gene therefore promotes unchecked progression through the cell cycle and resistance to cellular death [19]. Accordingly, $p53^{-/-}$ mice will have an increased propensity for developing AK-like lesions and SCC secondary to UV exposure [20]. The presence of p53 mutations

have been found in a significant percentage of CSCC lesions, as well as in AKs, demonstrating that dysplastic lesions may acquire genetic mutations prior to becoming SCC [21]. In fact, the prevalence of p53 mutations is over 15-fold higher in clinically unremarkable sun-exposed skin as compared to non-exposed skin, which further supports the notion of field cancerization which may be subclinical and sets the stage for the acquisition of new mutations, which may drive tumor development and progression [22]. UV-induced p53 mutations are occurring constantly in our skin and are also constantly being repaired via DNA repair mechanisms. The devastating effects of failure of this natural repair system are seen in xeroderma pigmentosum. This disease is due to defects in DNA repair and results in aggressive SCCs beginning in childhood which invariably lead to death.

While keratinocytes in chronically sun-exposed areas of the skin may show multiple changes detectable before cancer formation occurs, other cells may similarly be involved in a close interplay in the process of field cancerization. For instance, the Notch1 gene is known to be a master regulator of differentiation in many tissues, and has been found to contribute to keratinocyte homeostasis in the epidermis [23]. Notch1 has also been shown, however, to play a key role in the differentiation of dermal fibroblast cells. Accordingly, a site-specific deletion of CSL/RBP-Jκ in the mouse dermis led to the formation of multiple SCC lesions, reenacting the clinically observed phenomenon of field cancerization [24]. Furthermore, human skin may demonstrate similar patterns of Notch1 downregulation in dermal tissue underlying AK lesions, with a corresponding increase in the AP-1 family transcription factors such as c-Jun and Fos (Fig. 4.1b).

Another factor which may play an important role in the promotion of field cancerization is the presence of chronic inflammation. Notch 1 is a regulatory gene which contributes to keratinocyte homestasis [23]. In a mouse model of field cancerization with impaired dermal Notch signaling, widespread low-grade inflammation could be observed long before the presence of multiple tumors though the mechanism by which this occurs is not known.

This link between chronic inflammation and SCC seen in the laboratory is consistent with the clinical occurrence of SCC within chronically inflamed or damaged skin such as is seen in Marjolin's ulcers and the devastating condition of recessive dystrophic epidermolysis bullosa in which aggressive SCC forms within the chronically scarred tissues and is the leading cause of death in this disease. SCC formation could be reduced in the Notch 1 mouse model above via the use of broad anti-inflammatory agents such as cyclooxygenase-2 inhibitors prior to tumor development which resulted in a dramatic reduction if not total suppression of tumor formation [24].

Inflammation therefore appears to be a critical precursor for the induction of field cancerization in this model. Similarly, inflammation was shown to be a key player in the chemical carcinogenesis model of SCC formation through the presence of the RAGE receptor, and its ligands S100A8/A9 [25]. S100A8/A9 belong to the calcium-binding family of S100 proteins which are commonly expressed in inflammatory diseases at large, e.g. S100A7 (psoriasin) in psoriasis. Among other ligands

they may bind to the receptor for advanced glycation end products (RAGE), thus mediating a pro-inflammatory effect. Both the RAGE receptor and its ligands appear to be upregulated in SCC lesions, and expression levels may vary following the use of immunosuppressive drugs in organ transplant recipients. This suggests a possible role for RAGE and S100A8/A9 in the formation of high-risk SCC lesions associated with OTRs [26].

Carcinogenic Impact of Medication on Keratinocytes

While light skin type and cumulative UV exposure are thought to be the principal risk factors for the development of SCC, the administration of certain medications is also known to play a pivotal role in SCC formation. In particular, the risk of developing SCC in OTRs on chronic immunosuppressive therapy is thought to be 100-fold greater than that of the general population [3]. The effect of immunosuppression itself and the subsequent decreased immune surveillance it entails may facilitate SCC formation.

In addition to the impact of immunosuppressive drugs on the immune system, direct drug-induced effects extending beyond immunosuppression have recently been identified in keratinocytes, and may serve as potential contributors to the formation of high-risk SCCs in OTRs. These mechanisms are discussed below.

Calcineurin Inhibition Drives SCC Formation through the Promotion of ATF3 in Keratinocytes

Calcineurin inhibitors (cyclosporine A and tacrolimus) were amongst the first immunosuppressive agents used to prevent transplant rejection in OTRs, and they remain at the cornerstone of modern transplant medicine. They are thought to exert their immunosuppressive effect largely through the inhibition of calcineurin, which prevents the activation of lymphocytes and thereby suppresses the initiation of an adaptive immune response. The expression of calcineurin is not unique to lymphocytes of the immune system, however, and has been found in multiple different cells of the body. Cyclosporine A may therefore exert widespread systemic effects in addition to its role in immune suppression.

In keratinocytes, calcineurin has recently been shown to inhibit the expression of Activating Transcription Factor 3 (ATF3), a member of the enlarged AP-1 family of transcription factors. ATF3 expression has been shown to play a role in the pathogenesis of several different epithelial cancers, and therefore may be of potential interest in keratinocytes as well. Subsequent experimentation has shown calcineurin inhibition to directly result in increased ATF3 expression, which in turn, results in increased binding of ATF3 to various sites in the promoter region of p53. This results in the

effective inhibition of p53 mRNA expression [27]. As mentioned previously, p53 is a key tumor suppressor gene in charge of cell cycle regulation, and loss of p53 function is known to catalyze tumor formation in epithelial cells [19]. Furthermore, in vivo experiments conducted in mice have shown that the use of cyclosporine A resulted in a sharp increase in both ATF3 mRNA and protein levels, with a corresponding downregulation of p53 protein expression. This led to a subsequent increase in epithelial tumor formation [27]. This newly described mechanism for calcineurin inhibition in keratinocytes may help explain the disproportionate increase in keratinocyte-derived cancers relative to overall malignancies seen in OTRs.

In contrast to systemic calcineurin inhibition with cyclosporine A, topical calcineurin inhibitors such as tacrolimus and pimecrolimus, widely used in the treatment of atopic dermatitis and other inflammatory skin disease, have yet to be associated with any increased incidence of SCC [28, 29]. While the exact mechanism underlying this observed discrepancy is not fully understood, it is thought that the inhibition of calcineurin in epidermal keratinocytes alone may be insufficient to incite the development of squamous cell carcinoma itself. Rather, SCC formation may rely on the synergistic effect of the local dermal and inflammatory microenvironment in order to overcome host tumor defenses mounted by the body's immune system. These systemic cellular host immune defenses are impaired by systemic but not topical calcineurin inhibition which may explain why systemic inhibition with oral drugs (notably cyclosporine A) is associated with SCC formation while topical inhibition has not emerged in association with increased cutaneous carcinogenesis to date.

Azathioprine Photosensitizes Skin to UVA and Facilitates Direct DNA Damage of Keratinocytes in OTR

Another medication commonly used to prevent transplant rejection in OTRs is the anti-metabolite compound azathioprine (AZA). AZA works by incorporating the metabolite 6-thioguanine (6-TG) into cells during de novo DNA synthesis, thereby blocking effective synthesis and impairing cellular function. More recently, the newer purine analogues mycophenolic acid and mycophenolate mofetil have become the first-line anti-metabolite agents used in transplantation medicine. AZA continues to be used, however, in older patients with long-standing immunosuppressive regimens, as well as those with chronic inflammatory conditions such as inflammatory bowel disease [30].

While patients on azathioprine do not typically complain of photosensitivity, experimental data have previously identified AZA as a potent photosensitizer in the ultraviolet A (UVA) range [31]. More importantly, keratinocytes under the influence of AZA were shown to be directly susceptible to DNA damage induced by UVA. This is particularly significant since it was previously believed that only ultraviolet B (UVB) rays were powerful enough to induce direct DNA damage, as evidenced by UVB signature mutations such as the cyclobutane pyrimidine dimer and 6-4 photoproduct formation commonly found in the skin. Further experiments effectively

demonstrated, however, that AZA treatment may result in clinically demonstrable UVA-induced photosensitivity [32]. Accordingly, clinical studies have shown that switching renal transplant recipients from AZA to alternative anti-metabolites such as mycophenolate mofetil can improve UVA-related photosensitivity in as little as 3 months [33]. Furthermore, UVA exposure in these patients, once switched to mycophenolate, did not inflict the same degree of molecular damage, as measured by p53 induction in photo-exposed skin as they had suffered while on azathioprine.

Interestingly, a long-term follow up conducted on a small group of renal transplant recipients was able to detect the persistence of the AZA metabolite 6-TG in circulating lymphocytes as long as 2 years after discontinuation of the drug, with a continued further improvement of photosensitivity in two out of four patients. These latter observations suggest that azathioprine may continue to exert a carcinogenic effect long beyond its period of use. While no formal data incriminate azathioprine as an isolated culprit for the increased incidence of SCC seen in OTRs, it may still be prudent to limit its use, especially in light-skinned patients, and to consider switching away from azathioprine if SCC occurs, based on the current evidence highlighted above [34].

The Tumor Microenvironment in High-Risk SCC

The body's immune system is equipped with all the necessary components to effectively identify, target, and eradicate malignant SCC cells. The generation of such anti-tumor immunity, however, is a complex process which is dependent on the precise and dynamic interplay of several critical immune mediators. Furthermore, the nature of an immune response may be molded by a variety of chemical signals present in the surrounding microenvironment [35].

Typically, the initiation of anti-tumor immunity begins with dendritic cells, which are considered professional antigen-presenting cells and serve as an important link between the innate and adaptive immune systems [36]. These cells will recognize and process specific tumor-associated antigens, with subsequent presentation to naïve CD4+ and CD8+ T-cells. This results in the generation and activation of antigen-specific effector T cells, including T-helper (Th) and cytotoxic T cells (Tc), which may directly attack invading cancer cells [37]. Accordingly, any defect or suppression of function in the above-mentioned immune cells may result in defective or impaired anti-tumor immunity. In the remainder of this chapter, we will systematically describe the presence and function of key immune cells, including dendritic cells, macrophages, and effector T cells, which are found in the SCC tumor microenvironment. We will further highlight the distinct immune features observed in transplant-associated SCC (TSCC) as compared to SCC, which may be contributing to the impaired immunity and accelerated tumor growth seen clinically. Additionally, we will characterize the unique soluble factors and signaling molecules present in the SCC microenvironment, which may be interacting with immune cell function to promote neoplastic transformation and protect the tumor from host immunity.

Dendritic Cells in the Tumor Microenvironment

Dendritic cells (DC) are considered to be professional antigen-presenters based on their ability to sample the surrounding environment for foreign invaders, and present associated antigens in the context of MHC class II and co-stimulatory molecules [36]. Their prevalence in the peripheral tissues such as the skin supports their role as gatekeepers of immunity. Cutaneous DCs may be loosely subdivided into three main subsets, which can be distinguished based on their location and differential expression of surface molecules in the steady state. These include: (1) epidermal Langerhans cells (LCs) which patrol the epidermis; (2) dermal myeloid dendritic cells (mDCs) which patrol the dermis; and (3) plasmacytoid dendritic cells (pDCs) which are antigen presenting cells that circulate in blood and lymphatics [38, 39].

DCs are thought to be the first immune cells to encounter local tumor antigen in SCC, and are therefore critical for the initiation of tumor immunity. Indeed, we have previously studied the presence of DCs in SCC lesions, and found significantly reduced quantities of both LCs and mDCs as compared to normal skin (Fig. 4.2). This suggests a disruption in DC-generated immunity in SCC, which may in part account for a more tumor permissive environment [40, 41].

In addition to the decreased amount of mDCs, we have also evaluated both the phenotype and function of mDCs extracted from SCC lesions compared to those taken from peritumoral or healthy skin. We found that tumor-associated mDCs were, in fact, poor stimulators of T cell proliferation and activation when compared to their peritumoral or healthy skin counterparts. Furthermore, this discrepancy was directly the result of defective mDC function and not due to impaired DC maturation. This was evidenced by comparable levels of the DC maturation markers CD83 and CD86 in tumoral, peritumoral, and healthy-skin mDCs [40]. Consistent with our findings, tumor-associated mDCs extracted from BCC lesions have also been shown to be deficient activators of the T cell response when compared to normal cutaneous mDCs [42]. Although the precise mechanism underlying this observed effect remains unclear, we have shown the SCC immune microenvironment to be rich in soluble immunosuppressive factors such as IL-10, TGF-β, and VEGF-A [40]. These molecules were found to be elevated in both lesional tissue and adjacent peritumoral skin, which supports the notion that normal-appearing skin adjacent to tumor sites may in fact contain a unique pro-tumoral composition. Additionally, IL-10, TGF-β and VEGF-A have been linked to the direct inhibition of mature mDC stimulatory function [43]. This suggests tumor cells may secrete immunosuppressive molecules which effectively suppress proper mDC function, resulting in the impaired generation of anti-tumor immunity.

In contrast to the impaired mDC function seen in SCC, LCs harvested from SCC lesions have actually been shown to have an enhanced ability to stimulate CD4+ and CD8+ T cell proliferation in vitro when compared to cells taken from matched, non-tumor bearing skin [44]. Furthermore, SCC-derived LCs may efficiently polarize the T cell population towards a predominantly Th1 response, which results in the increased secretion of IFN-γ. This is thought to be a critical mediator in the successful

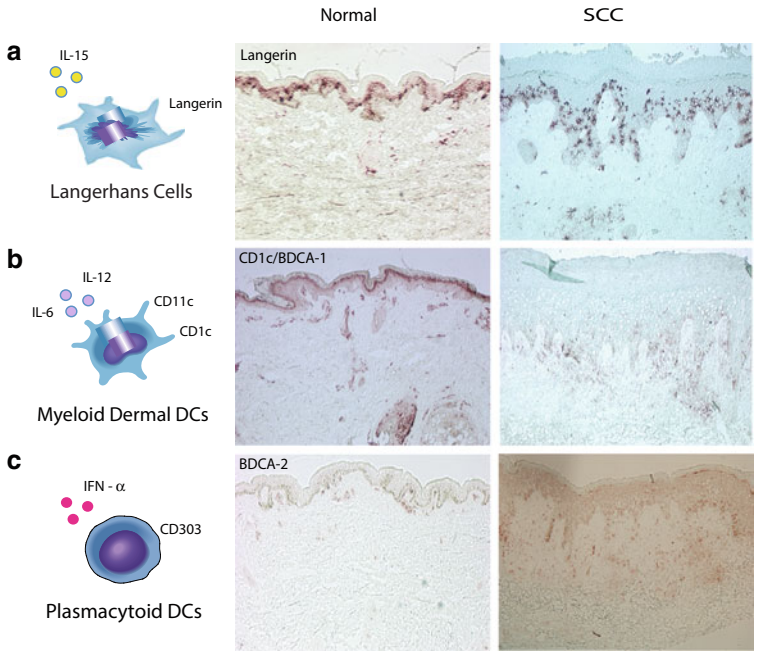

Fig. 4.2 *The distribution of cutaneous dendritic cell subsets in normal skin versus human squamous cell carcinoma* (*SCC*). The human skin contains three main subsets of DCs which can be distinguished based on location and the differential expression of surface molecules in the steady state (*left*). Representative immunohistochemistry (*right*) demonstrating the relative distribution of DC subsets in normal skin versus SCC, including (**a**) Langerhans cells (**b**) dermal myeloid DCs and (**c**) plasmacytoid DCs

generation of anti-tumor immunity [45]. It would therefore appear that the SCC microenvironment may actually serve to promote, rather than inhibit, LC activation and the initiation of an anti-tumor response. Accordingly, subsequent study has revealed that non-tumor LCs cultured in the presence of tumor supernatant (TSN) will lead to the increased proliferation of both CD8+ and CD4+ T cells, with a shift towards a Th1 cell response [44].

Despite the stimulatory effect of the SCC microenvironment on LC function, and the enhanced type 1 anti-tumor response generated in vitro, LCs remain incapable of preventing SCC tumor growth in vivo. This effect may be due to a variety of different reasons, including the dramatically reduced number of LCs found in both lesional and peritumoral skin [40, 41, 46]. Additionally, these cells may have impaired patterns of migration, and defective mechanisms of T cell priming in the draining lymph nodes. This would effectively prevent the activation of a T cell response and subsequently inhibit the launch of a full-scale immune attack [47, 48]. In fact, current research has shown that the application of TSN directly to SCC lesions in mice resulted in a markedly diminished migration of LCs to draining lymph nodes in vivo [41, 49]. Finally, much of our knowledge concerning LC

function in the SCC microenvironment is derived from the study of migrating cells taken from pre-existing tumors. The role of LCs in the tumor initiation stage thus remains largely unknown. Several recent studies have suggested LCs may actually accelerate SCC development in mutated keratinocytes, resulting in a pro-tumor effect in cutaneous carcinomas [50, 51].

While the SCC tumor microenvironment is distinctive in that it contains markedly low levels of mDCs and LCs, it is also notable for containing relatively large quantities of pDCs [40]. These cells are thought to be the primary foot soldier of the innate immune system due to their tremendous ability to produce interferon-α (IFNα) in response to foreign invasion [52]. This cytokine has been shown to have both antiviral and antitumor effects, and may therefore be beneficial in tumor eradication [53]. Additionally, it has recently been shown that pDCs are capable of recognizing, processing, and cross-presenting foreign antigen to CD8+ T cells [54, 55]. Although this process is found to be less efficient in pDCs when compared to their mDC counterparts, these findings support the notion that pDCs may, in fact, be effective activators of the anti-tumor immune response [56]. Accordingly, it has been shown that the elevated amounts of pDCs are indeed associated with increased clearance of BCC lesions following treatment with imiquimod [57]. Further research is currently needed in order to more accurately define the role of pDCs in human cutaneous carcinomas.

Recently, a novel subtype of mDCs has been identified which is associated mainly with an inflammatory response. These highly specialized DCs are characterized by the secretion of inflammatory mediators such as TNF, iNOS, IL-20 and IL-23, and are hence labeled TIP-DCs [39, 40]. The presence of TIP-DCs has been previously described in psoriasis, where it was shown that treatment of psoriatic lesions with the anti-CD11a agent efalizumab strongly reduced their influx into the affected areas. This suggests that these cells may be playing an active role in driving keratinocyte hyperproliferation [58]. Alternatively, TIP-DCs have been shown to exert a direct immunosuppressive effect by catalyzing the metabolism of L-arginine, which results in the subsequent production of nitric oxide and reactive oxygen species. In particular, reduced concentration of L-arginine has been shown to prevent the development of antigen-specific T cells, and nitric oxide may inhibit activated T cell proliferation [59]. We have previously evaluated the SCC microenvironment for the presence of TIP-DCs, and found a significant influx of these cells in the dermal regions surrounding SCC tumor nests. While their precise role in SCC remains somewhat unclear, TIP-DCs may be contributing to immune dysfunction through the secretion of immunosuppressive cytokines which inhibit effector T cell production.

Effector T Cells in the Tumor Microenvironment

Following antigen uptake and processing, peripheral DCs will migrate through the afferent lymphatics to nearby lymph nodes for presentation to naïve T cells. Binding of T cells to the MHC-antigen complex and co-stimulatory molecules on the DC

surface results in the activation and subsequent differentiation of T cells into highly specific effector cells [60]. Typically, these cells can be subdivided into one of three categories based on the distinct molecules they produce. These include: (1) cytotoxic CD8+ T cells (Tc), which bind to MHC class I molecules on the DC surface, and release specialized cytotoxins including perforin, granzymes, and IFN-γ in response to cytosolic pathogens; (2) CD4+ Th1 cells, which bind to MHC class II molecules and release IL-2, IFN-γ and other activating molecules, thereby enhancing the anti-tumor function of macrophages, natural killer cells (NK) and Tc cells; and (3) CD4+ Th2 cells, which also bind to MHC class II molecules, and drive B-cell dependent humoral immunity through the release of B cell growth factors IL-4 and IL-5 [61–64]. Tc and Th1 cells are generally thought to be the key mediators of anti-tumor immunity.

Since T-cell mediated immunity is critical in controlling tumor growth, we have previously characterized the presence of tumor-associated CD4+ and CD8+ T cells in SCC as compared to those found in transplant-associated SCC (TSCC) and normal skin. Not surprisingly, we found a significantly greater number of CD8+ T cells associated with both SCC and TSCC as compared to normal skin [65]. Previous research has shown that CSCC is commonly associated with increased T cell infiltrates, however the clinical persistence of cancer suggests that these T cells are unable to destroy the tumor [66, 67]. Interestingly, however, our results show fewer numbers of CD8+ T cells in TSCC as compared to SCC lesions. This decrease in anti-tumor Tc cells may be one of several factors contributing to the increased proliferative behaviors seen in TSCC. Accordingly, recent studies have indeed shown reduced amounts of tumor-infiltrating CD8+ T cells to be associated with more aggressive tumor phenotypes and increased risk of lymph node metastasis [68].

In addition to increased CD8+ T cells in the SCC and TSCC microenvironment, we have also found a significantly increased number of Foxp3+ regulatory T cells (Tregs) in tumor tissue as compared to normal skin [65]. These cells are known to promote immune tolerance, and are thought to be critical for the prevention of autoimmunity [69, 70]. Recent evidence has shown, however, that Tregs may also play a role in the suppression of an appropriate anti-tumor response through the direct inhibition of effector T cell proliferation and cytokine production [71–73]. Additionally, DCs co-cultured with Tregs have been shown to down-regulate the expression of co-stimulatory molecules, which may impair their ability to stimulate T cell proliferation and contribute to immune system dysfunction [74–76]. In fact, recent studies have shown that increased levels of Tregs are correlated with a poor prognosis and decreased survival rates in a variety of cancers, including gastric, breast and ovarian carcinoma [71, 77, 78]. Of note, when evaluating the presence of Tregs in SCC and TSCC, we actually found an increased ratio of Tregs to CD8+ Tc cells in TSCC as compared to SCC [65]. This disruption of the Treg to Tc cell balance may further exacerbate the immune impairment seen in SCC, which may result in a severely compromised ability to launch an immune attack. This may again be contributing to the more aggressive tumor phenotypes seen in TSCC.

Another significant disparity that is found between the immune microenvironment in TSCC as compared to SCC is a decreased percentage of IFN-γ secreting

CD4+ T cells (Th1) in TSCC [65]. This is somewhat consistent with our previous research, which has shown the TSCC microenvironment to contain a predominantly Th2 phenotype [74]. As mentioned previously, Th1 cells are thought to be critical for the generation of anti-tumor immunity, thus decreased levels may contribute to the more tumor permissive environment seen in TSCC [65, 79]. More interestingly, however, we found the TSCC microenvironment to contain a significant increase in the percentage of CD8+ interleukin-22 (IL-22) producing cells as compared to non-transplant associated SCC (Fig. 4.3a) [65]. IL-22 is a member of the IL-10 family of cytokines, and has been implicated in a number of benign keratinocyte hyperproliferative conditions such as psoriasis and atopic dermatitis [80–84]. Recent evidence, however, suggests that IL-22 may also play a role in driving the growth of a number of malignant processes, such as mantle cell lymphoma, hepatocellular carcinoma, colon carcinoma and pancreatic cancer [85–89]. While the precise nature of IL-22 in SCC remains to be defined, we have provided evidence that IL-22 administration may in fact enhance the proliferation of CSCC cells in vitro (Fig. 4.3b). More specifically, we found treatment with IL-22 resulted in an eight-fold increase in cell numbers as compared to those cells grown without IL-22 supplementation (Fig. 4.3c) [65]. Furthermore, the SCC microenvironment was associated with increased gene expression of IL-22, and increased protein expression of IL-22, the IL-22 receptor, and associated downstream modulator pSTAT-3 [65]. These findings serve to reinforce the importance of the IL-22 pathway in SCC and TSCC, and support the notion that increased IL-22 may be driving accelerated SCC growth in organ transplant recipients.

Macrophages, Immune Suppressive Molecules, and Other Competing Forces in the SCC Microenvironment

Tumor behavior is not simply a function of cancer or immune cells themselves, but rather is dependent on the composition of the local surroundings. The tumor microenvironment can thus be thought of as the collection of cells, soluble factors, signaling molecules and extracellular matrix components that together may serve to regulate neoplastic transformation and tumor progression [90]. We have previously described the unique DC and effector T cell components which make up the SCC microenvironment. We will now focus on the presence of macrophages, associated soluble factors, and immunosuppressive molecules, which may also be contributing to the complex immune environment seen in SCC.

Macrophages are considered to be the body's principal phagocytic cells, and like DCs, they are thought to play a key role in the initiation of both the innate and the adaptive immune response. They are often one of the major populations of leukocytes found in solid tumors, where they have been shown to alternatively promote or inhibit tumor growth [91–93]. In particular, tumor-associated macrophages (TAMs) have been shown to promote early eradication of tumor cells in vitro [94]. They are also, however, associated with a negative prognosis in several human cancers, and

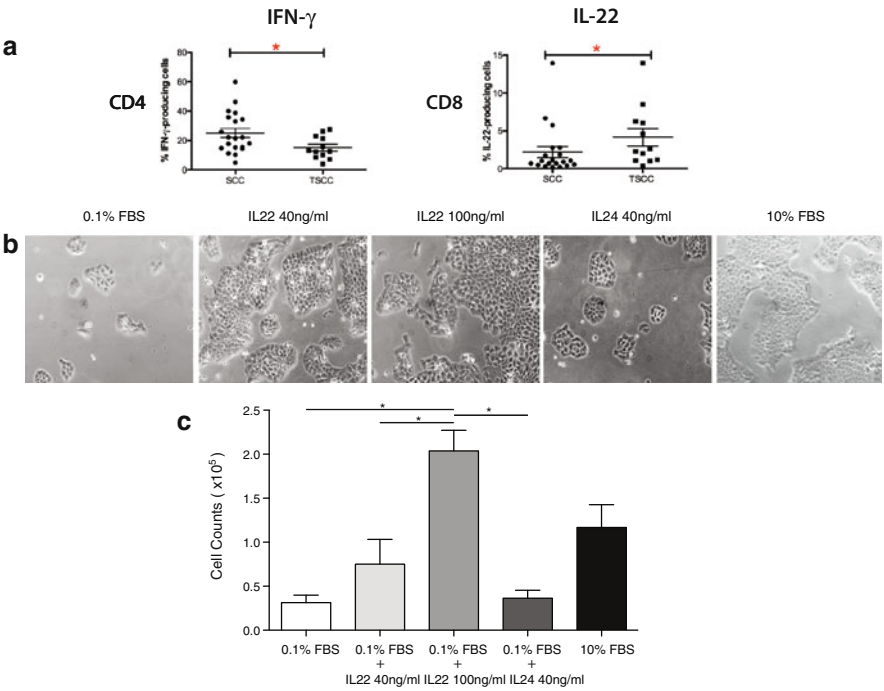

Fig. 4.3 *Transplant-associated SCC (TSCC) contains a unique immune phenotype which may be driving accelerated SCC growth.* (**a**) We stimulated T cell "crawl outs" taken from SCC and TSCC and determined T cell phenotype through intracellular cytokine staining. We found TSCC lesions to contain a significant decrease in CD4+ IFN-γ producing cells, and a significant increase in CD8+ IL-22 producing cells ($p < 0.05$). (**b**) IL-22 was found to drive accelerated SCC growth in vitro. A431 cells were cultured in full media (10 % FBS) or in serum starvation media (0.1 % FBS) with or without the addition of the indicated cytokines for 72 h. Cells cultured in full media, and in starvation media supplemented with IL-22 (40 and 100 ng/ml) show considerably greater proliferative behavior with increased colony formation when compared to those grown in starvation media alone or supplemented with IL-24 (40 ng/ml) (**c**) Cell counts were performed after 72 h of cultivation in the indicated conditions. The addition of 100 ng/ml IL-22 to the starvation media resulted in a hyperproliferation of tumor cells and eightfold increase in cell numbers when compared to those grown in serum starvation alone, or serum starvation supplemented with IL-22 (40 ng/ml) or IL-24 (40 ng/ml) (one-way ANOVA, $p < 0.001$)

have been shown to release angiogenic factors which may directly contribute to tumor growth [93, 95]. We have previously shown that TAMs are significantly upregulated in the SCC microenvironment as compared to normal skin. Additionally, the phenotype and function of TAMs in SCC was found to be heterogeneous, with evidence for both anti- and pro-tumor function [35]. For instance, TAMs were found to have upregulated expression of the IFN-γ receptor, which reflects an enhanced ability to respond to IFN-γ with antigen presentation and the initiation of a Th1 immune response. This would be expected to prevent tumor progression [96]. Conversely, TAMs were also found to produce factors which encouraged tumorigenesis.

These include the pro-lymphangiogenic vascular endothelial growth factor-C (VEGF-C), as well as matrix metalloproteinases (MMPs) 9 and 11, which promote local invasion and metastatic spread through the degradation of the extracellular matrix [35, 97]. MMPs may also release matrix-sequestered angiogenic factors which encourage tumor growth [98]. The abundant influx of TAMs in the SCC microenvironment may therefore be serving to promote tumor growth and carcinogenesis.

As mentioned above, the SCC microenvironment contains a large amount of TAMs which may secrete pro-tumoral factors such as VEGF-C. VEGF-C is a critical mediator of lymphangiogenesis, and is thought to stimulate the proliferation and increased survival of lymphatic endothelial cells [99]. Additionally, increased lymphatic vessel density (LVD) and VEGF-C expression have been shown to be effective predictors of lymph node metastasis in several different cancers, including oral and supraglottic SCC, as well as melanoma [100–102]. Accordingly, we have shown the SCC tumor microenvironment to contain increased LVD in the juxtatumoral dermis, as well as an upregulation of several different lymphangiogenesis genes. SCC lesions also contained increased levels of VEGF-C as compared to normal skin, likely the result of secretion by associated TAMs. Moreover, these findings were not only present in SCC lesions, but also apparent in matched adjacent non-tumor bearing skin. This supports the concept of field cancerization, and may facilitate the recurrence and metastasis of SCC tumors despite treatment with excision and seemingly clear margins [97].

Finally, endothelial cells lining local blood vessels are often the first contact that many blood-borne elements, including certain immune cells, have with a tumor. Endothelial cell integrity may therefore play a key role in tumor development by allowing various immune components to gain entry to the tumor microenvironment [103]. Recent research has suggested that reduced expression of endothelial E-selectin in the local SCC tumor vasculature may result in aberrant T cell homing and increased infiltration of Tregs into the tumor [66]. Additionally, we have shown that the SCC tumor microenvironment contains significantly greater expression of the endothelial protein CD200, a known immunosuppressive protein which often regulates immune cell entry into areas of immune privilege, such as the brain, retina and hair follicle [104–106]. This effect can be manipulated in human cancers to confer pathologic immune privilege to tumor cells, thereby helping the tumor evade immune detection. Furthermore, we found several factors released from SCC lesions were actually capable of inducing the expression of CD200 on endothelial cells in vitro. This may help explain our finding that nearly all vessels in SCC expressed CD200, whereas very few did in normal skin [104]. We also saw a significant increase in the expression of CD200 receptor (CD200R) on myeloid cells in the SCC microenvironment, which may, in part, help account for the mDC dysfunction seen in SCC [40, 104]. Taken together, these results suggest that CD200, and its interaction with CD200R on myeloid cells, may be a critical mechanism of immune evasion in SCC.

Summary and Conclusions

Squamous cell carcinoma (SCC) remains a significant cause of morbidity and mortality in organ transplant recipients (OTRs). Lesions in this patient population tend to be more numerous, rapidly growing, and more aggressive when compared to SCC from immune competent patients, with elevated rates of recurrence and metastases [4, 7]. Additionally, extensive body surface area involvement often renders surgery, the primary treatment modality, difficult or disfiguring. There thus exists a real need for the development of effective medical therapies for the treatment of filed cancerization and aggressive SCC in OTRs. This process is dependent, however, on a thorough and comprehensive understanding of SCC pathogenesis and the various factors which may allow tumor cells to evade immune detection. Furthermore, targeting specific lesions themselves may be inadequate in the context of field cancerization, which suggests that normal appearing skin adjacent to tumor tissue may actually contain unique genetic mutations which render the area tumorigenic.

Comparing low-risk SCC tumor development in immune competent patients versus high-risk transplant-associated SCC (TSCC) provides us with a novel opportunity to explore the intricacies of the tumor immune microenvironment, and allows us to gain a greater understanding of the various factors which may drive SCC proliferation. SCC lesions are thought to occur as a direct result of chronic exposure to carcinogenic stimuli such as UV light. This eventually results in a genomic mutation in keratinocytes, which confers a growth advantage such that the transformed cell may continue to acquire new mutations. These rapidly-proliferating mutated cells will ultimately form a microscopic tumor focus within the skin. While this process of tumor development is thought to be consistent for all SCC lesions, there are various tumorigenic factors which may catalyze cellular damage and increase carcinogenesis. For instance, the use of immunosuppressive agents such as Cyclosporine A and Azathioprine may be directly driving SCC formation through the respective inhibition of the p53 tumor suppressor protein and photosensitization of the skin to UVA. These medications are commonly used in OTRs, and may, in part, explain the high prevalence of SCC seen in this population.

Additionally, the immune microenvironment associated with SCC is thought to be a dynamic milieu, comprised of opposing forces driving tumor promotion and tumor suppression (Fig. 4.4). We have found SCC lesions to contain a significantly reduced number of mDCs and LCs when compared to normal skin, as well as an increased number of pDCs. Furthermore, mDC function appears to be impaired in SCC lesions, with an inability to stimulate an appropriate T cell response. Other unique features of the tumor microenvironment include increased amounts of Tregs, which is further highlighted by an increased ratio of Tregs to CD8+ Tc cells in transplant-associated SCC (TSCC). TSCC lesions also contain a significantly decreased amount of CD4+ IFN-γ producing cells, as well as a significantly increased amount of CD8+ IL-22 producing cells. The former is thought to be a key mediator of anti-tumor immunity, and may help explain the more tumor permissive environment seen in TSCC. The latter has been shown to directly drive SCC proliferation in vitro, and may therefore contribute to the more aggressive tumor phenotypes seen in TSCC.

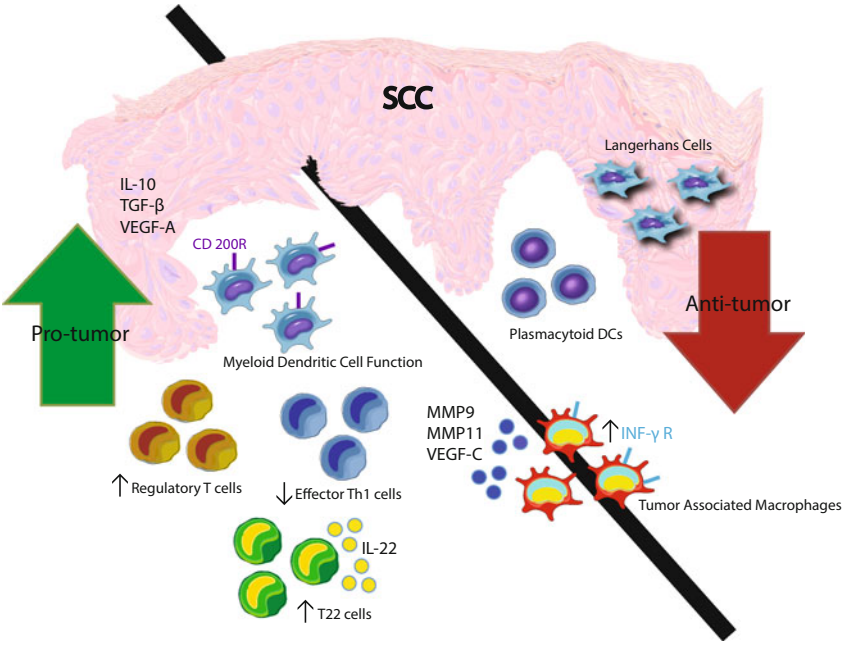

Fig. 4.4 *The SCC immune microenvironment is a dynamic milieu comprised of competing forces driving tumor progression and tumor suppression*. The SCC microenvironment is associated with an increased influx of pDCs, which secrete the anti-tumoral cytokine IFNα. The tumor microenvironment also works to promote LC function and enhance their ability to stimulate effector T cells. Conversely, mDC function is suppressed in SCC lesions. SCC is also associated with an influx of Tregs, which may create a more tumor permissive environment. Additionally, TSCC will contain a significant decrease in Th1 cells as well as a significant increase in T22 cells, which serve to further promote tumor proliferation. Tumor associated macrophages display heterogeneous behavior. On the one hand they demonstrate an enhanced ability to respond to IFN-γ and stimulate Th1. They also, however, secrete pro-angiogenic immunosuppressive factors such as MMP 9, MMP 11 and VEGF-C

High-risk SCC lesions may therefore be attributed to a number of different factors, which taken together serve to promote both a decrease in immune surveillance, as well as an increase in mutagenic and proliferative signals. Furthermore, aberrant gene patterns in the surrounding tissue increase the likelihood of tumor recurrence and new tumor formation. Treatment of these lesions may therefore require a comprehensive, multi-pronged approach aimed at targeting individual lesions themselves, the surrounding tumor microenvironment, and the affected field in order to effectively eradicate lesions and ensure a more long term tumor-free survival.

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