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Chapter 5

REGULATION OF CD8$^+$ T CELLS IN THE SKIN AND IMPLICATIONS FOR THE DEVELOPMENT OF SQUAMOUS CELL CARCINOMA

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ABSTRACT

The skin is the largest organ in the human body weighing ~3.6 kg and covering ~2m^2 in the average adult. It is the first line of defence against external aggressions and acts as a passive barrier against physical traumas and environmental insults. The fact that healthy skin contains more than twice the number of CD8$^+$ T cells found in the blood (approx. 2x10^{10} cells), underlines the key role of the skin in protection from infection and carcinogens and emphasizes the importance of this population in the maintenance of skin homeostasis. The crucial anti-tumour role played by T cells in the skin is clearly demonstrated by the high rate of skin tumour formation in organ transplant patients who receive T cell suppressive drugs to maintain the integrity of their

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transplanted organs. These patients show a 10-100 fold increased risk of cutaneous squamous cell carcinoma (SCC) development compared to healthy patients, and indeed, some renal transplant patients currently presenting to the Princess Alexandra Hospital, Brisbane, Australia, require the removal of ~120 primary skin tumours each year. However, although this relationship has been well-documented, it is still very unclear which T cell populations or mechanisms mediate cancer protection in the skin. This chapter will address $CD8^+$ T cell activation and regulation in the skin, paying special attention to what it is known about $CD8^+$ T cell activity and its link with SCC development.

Keywords: CD8, T cell, skin, squamous cell carcinoma, regulation

INTRODUCTION

The skin provides an immediate physical barrier between internal tissues and the environment. This essential organ plays a key role in protecting the body against pathogens, preventing excessive water loss, permitting sensation, and regulating body temperature. The skin is also an important site for immune system development, as recently highlighted by the finding that the skin microbiome plays a critical role in the establishment of a healthy immune system [1, 2]. In turn, a perfectly functioning immune system is essential to skin homeostasis, as inappropriate or misdirected immune activity can lead to the breakdown of skin integrity [3]. However, there is a critical knowledge gap in our understanding of how cellular mechanisms contribute to the maintenance of skin homeostasis, and particularly how immune cells, which can become potentially dangerous if dysregulated, are regulated in the skin.

T cells are white blood cells that mature in the Thymus, hence the name "T cell." There are several subsets of T cells, each with a distinct function, and the skin is a major reservoir for these cells [4]. The $CD8^+$ T cell subset plays a critical protective role by recognizing and destroying virally-infected- and malignant cells [5, 6], and influencing other cells through production of cytokines such as interferon (IFN)-γ [7, 8] and tumor necrosis factor (TNF) [9]. In the skin, $CD8^+$ T cells are responsible for immune surveillance and contribute to the prevention of infection. While most self-reactive $CD8^+$ T cells are deleted in the thymus during development through central tolerance mechanisms, this process is incomplete and peripheral tolerance is required to prevent the destruction of host tissues [10-14]. Not surprisingly, the activation of $CD8^+$ T cells is tightly regulated to avoid the unwanted destruction of

healthy, normal cells. However, the mechanisms that tightly regulate memory/effector T cell populations in the skin are not well understood, and represent a critical gap in our knowledge.

Multiple immune cell populations, including classical $CD4^+Foxp3^+$ regulatory T cells [15] (T-reg), invariant natural-killer T cells [16, 17] and $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells [18] are implicated in the control of $CD8^+$ T cells in the skin. $CD4^+Foxp3^+$ T-reg are especially abundant in the skin and modulate $CD8^+$ T cell differentiation and effector function *in vivo* by critically regulating IL-2 homeostasis [19]. Experimentally depleting $CD4^+Foxp3^+$ T-reg from the skin leads to increased $CD8^+$ T cell priming and enhanced allergic skin reactions [20]. Production of the immunosuppressive cytokine IL-10 by $CD4^+Foxp3^+$ T-regs resident in lymph nodes has been implicated in the induction of $CD8^+$ T cells that suppress contact hypersensitivity reactions in the skin [21]. Other immune populations with regulatory potential include $CD4^+CD8^+$ double-positive T cells [22, 23], regulatory macrophages, which inhibit T cell proliferation through inducible nitric oxide synthase [24] (iNOS), and plasmacytoid dendritic cells (pDC), which by expression of indoleamine 2,3 dioxygenase [25] (IDO) can reduce the T cell stimulatory potential of dendritic cells (DC). While not yet fully characterized, we have some understanding of how these cellular mechanisms work. However, the potential of other immune populations to regulate $CD8^+$ T cell responses in the skin is poorly explored.

Upon antigen stimulation, naïve T cells rapidly undergo terminal differentiation to effector and memory T cells. Compared to naive T cells, activated and memory T cells are more sensitive to antigen stimulation, exhibit faster response kinetics, reduced dependence on costimulation and can respond to lower affinity ligands. Memory T cells may be more resistant to the induction of apoptosis by virtue of increased expression of anti-apoptotic Bcl-2, although this may vary between central memory (T_{CM}) and effector memory (T_{EM}) T cells [26]. Terminal T cell differentiation transforms antigen-specific T cells from a naïve population, possessing a highly malleable differentiation potential, to committed effector and memory populations which have little or no plasticity and a reduced requirement for costimulation. This has led to the belief that they are resistant to inactivation or tolerance induction [27]. Indeed, memory $CD8^+$ T cells have been shown to provide a potent barrier to transplantation tolerance induction [28].

Recently, a new memory population, known as tissue-resident memory (T_{RM}), has been described that is phenotypically and functionally distinct from T_{CM} and T_{EM} [29, 30]. T_{RM} populations play an important role in protective

immunity to site-specific pathogens in the lung and skin [30-32], and unlike other memory populations they do not recirculate [29]. T_{RM} can be clearly distinguished from other memory T cell populations by the expression of the CD103 integrin, which binds to the $\alpha_E\beta_7$ integrin expressed by keratinocytes, and it has been hypothesized that this interaction may help T_{RM} to maintain their location within the skin [33]. Importantly, T_{RM} have now been described in multiple sites other than the skin [34] and understanding how this unique subset of $CD8^+$ T cells is regulated is crucial.

ADAPTIVE IMMUNITY AND $CD8^+$ T CELLS

The adaptive or acquired immune system is comprised of a set of highly specialized cells, T cells and B cells, which mount antigen-specific responses against foreign pathogens, and establish long-lasting immunological memory designed to trigger a rapid and robust recall response upon antigen re-exposure [35]. The capacity of T cells to recognize specific antigens rests on the tremendous diversity of their T cell receptors (TCR), which recognize cognate peptide bound to major histocompatibility complex molecules (MHC) displayed on the surface of professional antigen presenting cells (APCs) [36]. Naïve cytotoxic $CD8^+$ T cells circulate between the blood and secondary lymphoid organs where they become activated through their interaction with mature APCs. To enter into the lymph nodes naive $CD8^+$ T cells typically express on their cell surface the homing factors CC-chemokine receptor 7 (CCR7), which signaling pathway activates LFA1 (lymphocyte function-associated antigen 1) that binds ICAM1 (intercellular adhesion molecule 1) on the surface of the endothelium, and CD62L (L-selectin) that binds to the high endothelial venules, allowing the blood circulating lymphocytes to directly enter the lymph nodes [37-39]. After cognate antigen recognition, activated antigen specific $CD8^+$ T cells undergo clonal expansion and differentiate into effector T cells (T_E). This phase is known as the "expansion phase" in which a single precursor can experience more than 15 divisions over the course of 1 week [40]. Once activated, the T_E cells down-regulate the expression of CCR7 and CD62L and egress the secondary lymphoid organs to patrol the peripheral tissues and eliminate target cells by releasing cytolytic granules containing granzymes and perforin [41, 42]. This is known as the "effector phase." The production of IL-12 by activated macrophages/monocytes and dendritic cells during microbial infection stimulates the T_E cells to secrete interferon-γ, which

links the adaptive immune response with the innate immune response [43]. The release of cytokines/chemokines, such as IL-1, IL-8 and TNF, and the expression of unique combinations of selectin and integrin molecules in the infected and/or inflamed tissues set up an "area code" which favors the recall of antigen-specific T_E cells into the target organ [41]. At the same time and rapidly after priming, T_E cells express tissue-homing factors on their surface. The idea that antigen-experienced T cells have specific tissue-tropism was demonstrated by the fact that adoptively transferred lymphocytes mainly infiltrate the tissues from where they were isolated [44, 45]. For instance, T_E cells primed to migrate to the skin preferentially up-regulate CC-chemokine receptor (CCR)4 and/or CCR10, as well as cutaneous leukocyte antigen (CLA) which is a ligand of both endothelial-cell selectin (E-selectin) and platelet selectin (P-selectin) constitutively expressed by dermal micro vessels [46, 47]. Conversely, T_E cells activated in the gastrointestinal associated lymph nodes up-regulate the α4β7 integrin and CCR9, which bind the mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) and the CC-chemokine ligand 25 (CCL25) respectively; both of these ligands are expressed by epithelial cells in the small intestine [48-50].

Once the "expansion phase" is concluded, between 90 to 95% of the antigen-specific $CD8^+$ T cells undergo apoptosis. This stage is known as "contraction phase" and is believed to be important to avoid the prevalence of certain T cell clones and preserve the diversity of the T cell repertoire of the organism [51]. However, a small subset of antigen-specific T cells survive the contraction phase giving rise to long-lived "memory" T cells (T_M). T_M ($CD62L^+CCR7^+$) which circulate between blood and secondary lymphoid tissues are known as "central memory" T cells (T_{CM}), whereas those T_M ($CD62L^-CCR7^-$) circulating between blood and peripheral non-lymphoid tissues are known are "effector" T_M (T_{EM}). Nonetheless, besides their different location T_{CM} and T_{EM} cells also exhibit different phenotype and functional capacity suggesting that there is a division of labor between these two T_M populations. For instance, T_{CM} cells are self-renewing and produce high amounts of IL-2, maintaining the potential to differentiate into effector phenotypes after re-stimulation. In contrast, T_{EM} cells undergo limited homeostatic turnover, express tissue-homing factors and already exhibit an effector-like phenotype, such as the capacity to produce IFN-γ and TNF [52, 53]. Interestingly, there is a subgroup of T_{EM} that are derived from progenitors that enter the tissue during the effector phase which do not egress this compartment. These T_{EM} are also known as T_{RM} and they have lost the ability to recirculate via the blood [54-56].

TISSUE-RESIDENT MEMORY T CELLS (T_{RM})

$CD8^+$ T_{RM} cells are classically characterized by the expression of the early leukocyte activation marker CD69 and the integrin CD103 and play an important role in the control of local infections [57-60].

The down-regulation of the sphingosine-1-phosphate receptor (S1P1), which mediates cell egress from lymphoid tissues through the efferent lymphatics, is part of the transcriptional program that T_{EM} cells initiate in order to accumulate in peripheral tissues and become long-lived T_{RM} [53, 61, 62]. CD69 physically interacts with S1P1, an interaction that results in the inhibition and degradation of S1P1 preventing the departure of T cells from the infiltrating tissue [63, 64]. Indeed, $CD69^{-/-}$ $CD8^+$ T cells are unable to become T_{RM} since they fail to populate the skin and lung epitheliums [65, 66].

The expression of CD103 is thought to favor the retention of T cells in peripheral tissues by their interaction with its primary epithelial ligand E-cadherin, which is highly expressed by keratinocytes and Langerhans cells in the skin [67]. Hence, $CD103^+$ T_{RM} cells are mainly found in the epithelium of several tissues such as small intestine, salivary glands, heart, pancreas, kidney and skin [52, 53, 59]. The precise location of $CD103^+$ T_{RM} is intrinsically linked to the capacity of the tissue to secrete transforming growth factor-β (TGF-β), which is required for the expression of the integrin on the surface of $CD8^+$ T cells [65, 68-70].

Although CD69 and CD103 have been widely used to define T_{RM} cells in many compartments, recently several T_{RM} subsets have been identified that do not express these classic markers but fulfill the requirements to be considered long-lasting resident memory T cells. For example, $CD103^-$ T_{RM} are found in the secondary lymphoid tissues, small intestine, and liver [71-74], and double negatives ($CD69^-CD103^-$) represent approximately 60% of the T_{RM} in the female reproductive tract [75]. Accordingly, the expression of CD103 is tissue-dependent and the maintenance of $CD69^-$ T_{RM} could be mediated by alternative routes which in turn down-regulate S1P1, such as the zinc-finger transcription factor KLF-2 dependent-pathway [62].

Therefore, T_{RM} do not represent a homogenous population with a unique phenotypic signature, but a more complex community that shares the capacity to populate non-lymphoid tissues for long periods of time contributing to peripheral surveillance against secondary challenges.

CD8+ T CELLS IN THE SKIN

Healthy human skin contains approximately $1.1x10^6$ T cells per cm^3 which means that the body surface contains approximately twice the number of T cells than the entire circulatory system [76]. The majority of $CD8^+$ T cells in the skin are localized in the epidermis in close contact with Langerhans cells. In humans, these $CD8^+$ T cells predominantly express an αβ TCR, however in the mouse they predominantly express a γδ TCR [77, 78]. Interestingly, under resting conditions the majority of $CD8^+$ T cells in the skin are T_{EM} and co-express CLA, CCR4 and CCR6 [4]. Human skin explant and parabiotic mice studies have demonstrated that 98% of CLA^+ T_{EM} cells are in fact non-recirculating T_{RM} [4, 55]. Recently, it has been shown that the progenitor of these epithelial $CD103^+CD8^+$ T_{RM} cells lacks the expression of the effector marker killer cell lectin-like receptor subfamily G member 1 (KLRG1). The comparison of the transcriptional profiles of cutaneous T_{RM} and blood T_{CM} cells has shown that both populations share the same precursor which progressively acquires tissue-specific features to give rise to two T cell memory populations with distinct roles in protective immunity [53]. Part of this specialization program is the expression of the CD69 and CD103 surface markers in order to prevent emigration from the skin and to promote the retention of the T cells in this compartment, respectively [53, 67]. Skin long-lived memory T cells reside predominantly within the hair follicle epithelium which in turns favors the recruitment and formation of T_{RM} cells by the secretion of IL-15 and IL-17 [53, 79].

POSITIVE EFFECTS OF CD8+ T CELLS IN THE SKIN

Due to their strategic position in the epidermis, T_{RM} are relevant not only for the maintenance of skin immune homeostasis but also for the control of skin infections and malignancies.

Skin wound healing is a complex process that includes blood clotting, inflammatory cell recruitment, epithelial growth and tissue remodeling. In mice, γδ $CD8^+$ T cells, also known as dendritic epidermal T cells (DETC), contribute to several stages of this process by secreting growth factors and chemokines [80]. After the recognition of antigen released by injured keratinocytes, murine DETCs produce insulin-like growth factor 1 (IGF-1) which is required by keratinocytes during development and maintenance [81].

Similar to murine γδ $CD8^+$ T cells, in humans both γδ and αβ activated skin $CD8^+$ T cells are able to secret IGF-1 in acute wounds. However, isolated T cells from skin chronic injures failed to produce pro-healing molecules upon further stimulation, suggesting that the over-exposure to persistent antigen might led to exhaustion and malfunction of these T cell subsets [82].

The protective role of cutaneous T_{RM} cells during secondary infections has been largely documented. The fact that in humans between 50 to 80% of herpes simplex virus (HSV) reinfections are resolved within 6 to 12 hours emphasizes the relevance of a preexistent local immune control [83]. After the resolution of HSV-2 infection, a population of long-lived antigen specific $CD8^+$ T cells remains in the interphase between the epidermis and dermis in close proximity to the peripheral nerves [84]. Interestingly, the phenotypic characterization of persistent $CD8^+$ T_{RM} at the site of previous HSV-2 infection showed that they belong to the CD8αα linage and upon activation they are able to produce cytolytic granules in order limit HSV-2 shedding and morbidity [85]. Work in mice has demonstrated that sterile inflammation in the skin is enough to trigger site-specific recruitment of activated $CD8^+$ T cells, the up-regulation of the integrin CD103 on their surface and ultimately their transformation into T_{RM} in the absence of antigen stimulation. Moreover, these epidermal T_{RM} subsets exerted superior protection against HSV-1 challenge compared to their circulating T_{CM} equivalents [86]. Recently, it has been proposed that although the initial activation of T_{RM} depends on the recognition of the cognate antigen via T cell receptor, the transcriptional profile down-stream once activated results in the secretion of a wide-range of antimicrobial molecules, including IFN-γ, which allow T_{RM} to be protective against a less specific but broader pathogen array [57].

It is well established and generally accepted that numerous members of the innate and adaptive immune responses participate in the recognition and destruction of cancer cells. After transformation, malignant cells express tumor-associated antigens on their surface that can activate cytotoxic tumor-specific T cells either by direct recognition via MHC class I or via specialized antigen-presenting cells (APCs), including dendritic cells (DCs) [87]. Th1 immune responses, characterized by the expression of IFN-γ and TNF, have been shown to be necessary to eradicate cancer cells. Conversely, Th2-driven responses that led to the secretion of immune suppressive cytokines, such as IL-4, IL-5, IL-10 and IL-13, are strongly correlated with tumor persistence and poor prognosis [88]. Experiments in transgenic γδ- or αβ $CD8^+$ T cell deficient mice have demonstrated that the absence of γδ T cells in the skin increased

tumor development susceptibility upon exposure to chemical carcinogens or infusion of melanoma cell lines compared to their αβ-counterparts. In fact, the administration of a higher dose of carcinogen to αβ $CD8^+$ T cell deficient mice did not result in an increased detrimental effect, but in contrast, lead to a reduced susceptibility compared to wild-type animals, suggesting that skin αβ $CD8^+$ T cells might exert a negative effect on the generation of skin tumors [89, 90]. In subsequent studies, the efficacy of human γδ T cells in the control of skin tumor progression was assessed by using humanized mouse models in which human stem cells are adoptively transferred into severe combined immunodeficiency (SCID) mice in order to recreate the human immune system [91]. This mouse model demonstrated that human γδ $CD8^+$ T cells have significant antitumor activity against melanoma establishment and growth, however, the mechanism by which these cells favored the reduction of these tumors is still unknown [92].

PATHOGENIC EFFECTS OF $CD8^+$ T CELLS IN THE SKIN

Many cutaneous disorders arise as a direct consequence of dysregulation of the immune system in the skin. Psoriasis is a good example of a T cell mediated autoimmune disease. The main subset of T cells found in psoriatic lesions is CLA^+ and produces cytokines belonging to the Th1 repertoire. Therapies directed against Th1 products, such as monoclonal antibodies against TNF, or immunotherapies designed to skew Th1 response towards Th2, have been shown to be quite effective in the treatment of this disease [93-96]. The direct effect of tissue resident T cells on the development of this immunopathology was demonstrated by the fact that the xenotransplantation of healthy skin from psoriatic patients onto immune deficient mice resulted in the spontaneous development of psoriasis lesions. Moreover, local proliferation of resident human $CD8^+$ T cells was required for progression towards these malignant changes, since inhibiting T cell activation with monoclonal anti–human CD3 antibody muromonab-CD3 (OKT3) stopped the appearance of new lesions on the grafted skin [97]. Additionally, blocking the interaction between collagen and α1β1 integrin (VLA-1), which is specifically expressed on epidermal but not dermal T cells, prevented $CD8^+$ T cell skin retention and the subsequent psoriasis development [98].

Vitiligo is a skin disorder characterized by partial or total depigmentation of the epidermis. This disorder is triggered by the activation of skin antigen

specific $CD8^+$ T cells generated against melanocyte antigens, and results in the progressive and selective attack of melanocytes in the interfollicular epidermis and hair follicles [99]. Isolated auto-reactive T cells from vitiligo patients are able to recognize and trigger the apoptosis of healthy melanocytes *in vitro* [100, 101]. Interestingly, high numbers of cytotoxic $CD8^+$ T cells with a Th1 cytokine profile are found in vitiligo lesions, where IFN-γ production seems to drive the activation of autoimmune cytotoxic T cells and the destruction of melanocytes [102, 103].

A POTENTIAL ROLE FOR $CD8^+$ T CELLS IN THE REGRESSION OF PRECANCEROUS SCC LESIONS

From the clinical perspective, squamous cell carcinomas (SCC) comprise of cancers which originate from the squamous epithelium that covers organs such as skin, gastrointestinal tract, lungs and cervix [104]. The most frequent SCC are cutaneous, esophageal, non-small cell lung cancer and head and neck SCC, the latter of which includes the lips, oral and nasal cavities, paranasal sinuses, pharynx, larynx and parotid glands [104]. Non-melanoma skin cancers, which include cutaneous SCC and basal cell carcinoma (BCC), are the most common cancers in the Caucasian population with over 700,000 newly diagnosed cases each year in the United States. Although cutaneous SCC accounts for only 20% of the total non-melanoma skin cancer burden, it has a higher risk of metastasis compared to BCC, which makes prompt diagnosis and treatment crucial to avoid disease progression [105]. Ultra-violet (UV) radiation from the sun plays a key role in the establishment of SCC by causing DNA damage and mutations that in turn affect important anti-tumor checkpoints such as p53 [106, 107].

Precancerous SCC lesions in the skin, including Actinic Keratosis (AK), Bowen's Disease and Intraepithelial Carcinoma (IEC), undergo a high rate of spontaneous regression. Mutated p53 and the expression of cancer-testis antigens may provide $CD8^+$ T cell target epitopes [108, 109], polyclonal $CD8^+$ tumour infiltrating lymphocytes (TIL) are known to be present within AK and SCC lesions, and TILs derived from SCC lesions have been demonstrated to be cytotoxic for autologous tumour cell targets [110]. Nevertheless, precancerous lesions are frequently treated with immunomodulatory medications in order to prevent their potential progression to SCC. One such medication is imiquimod, a Toll-Like Receptor (TLR)-7/8 agonist. The

mechanism through which imiquimod induces the regression of precancerous SCC lesions is not well understood, and as a consequence the immune outcomes of imiquimod application have been studied in various contexts over many years. In mice, the topical treatment of UV-induced SCC with imiquimod has been described to induce $CD8^+$ T cell, Th17 T cell, and pDC infiltration, and the up-regulation of mRNA for IL-12/23p40, Il-12p35, IL23p19, IL17A and IFN-γ [111]. In humans, the topical application of imiquimod is thought to target TLR-7 expression on Plasmacytoid Dendritic Cells (pDC) leading to the production of IFN-α and the expression of numerous IFN-α-inducible genes, including CIG5, IRF7, OAS1, OAS2, STAT1, MxA, MxB, IFI44, IFIT1, IFI35, IDG20, and GIP2 [112, 113]. More recently, a second mechanism of action has been attributed to the inflammation induced by the topical application of imiquimod. Imiquimod itself constitutes 5% of the pharmaceutical preparation of imiquimod known as Aldara. However, the second most abundant component in Aldara after water is in fact isostearic acid, which has been shown to promote keratinocyte inflammasome activation and induce keratinocyte death by pyroptosis (Caspase-1-dependent lysis), and lead to the release of IL-1α [114]. In addition to the induction of a strong inflammatory reaction Imiquimod treatment also results in substantial immune cell, including $CD8^+$ T cell, infiltration into multiple types of neoplasia in humans, including melanoma in situ, melanoma metastasis, precancerous SCC lesions, SCC, and Basal Cell Carcinoma (BCC) [112, 115, 116]. However, not all of these neoplasia's regress as a result of imiquimod treatment, so clearly $CD8^+$ T cell infiltration alone is not evidence of regression causality.

Mouse models are enormously useful when elucidating the mechanistic role of $CD8^+$ T cells in SCC regression. Over 40 years ago, *Kripke* demonstrated that SCC are highly immunogenic tumors which typically do not establish when transplanted into immune competent mice [117]. More recently, *Girardi and colleagues* confirmed this observation, but also demonstrated that a chemically-induced transplantable SCC cell line, PDV, will establish with high reproducibility in various T cell deficient mouse lines [118]. These include $TCR\beta^{-/-}$ (lacks all αβ T cells), $CD4^{-/-}$ (lacks $CD4^+$ T cells), and $\beta_2m^{-/-}$ (lacks $CD8^+$ T cells). Furthermore, the adoptive transfer of lymphocytes from immunoprotected wild-type mice (i.e., from mice previously challenged with PDV cells) into $TCR\beta^{-/-}$ mice conferred protection against SCC establishment. However, protection was not conferred if the lymphocytes were depleted of $CD4^+$ T cells or $CD8^+$ T cells prior to transfer. Mice deficient in IFN-γ also permitted the establishment of SCC, highlighting

key roles for $CD8^+$ T cells, $CD4^+$ T cells, and IFN-γ in the prevention of (chemically-induced) SCC establishment in mice [118].

IMMUNOSUPPRESSION AND SCC DEVELOPMENT

The risk of developing SCC is greatly increased in patients rendered immunodeficient by disease or medication [119, 120]. Renal transplant recipients often receive triple therapy with combinations of Glucocorticoids (prednisolone or prednisone), calcineurin inhibitors (cyclosporine or tacrolimus) and anti-proliferative agents (azathioprine or mycophenolic acid) to prevent rejection of their allografts [121]. Worldwide, these patients suffer a 65-250 fold increased risk of non-melanoma skin cancer (NMSC) development compared with the general population [122, 123]. A single follow-up study of renal transplant recipients in Queensland, Australia, reported increased NMSC incidence with increasing duration of immunosuppression; over 50% developing at least one tumour within 10 years of immunosuppression [122]. Some renal transplant patients currently presenting to the Princess Alexandra Hospital, Brisbane, require the removal of ~120 primary skin tumours each year.

In order to try and gain insight into the connection between immune suppression and SCC incidence, numerous studies have compared the immune infiltrate of SCC in organ transplant patients (OTRs) with SCC arising in immune competent patients using established and emerging techniques [124]. *Walter and colleagues* utilised RT-PCR and immunohistochemical analysis to compare cancer-testis antigen expression, MHC class I expression, and $CD8^+$ T cell abundance in these two patient cohorts [108]. They reported that there was no difference in the proportion of patients expressing cancer-testis antigens or the number of different cancer-testis antigens expressed, and no differences in MHC class I staining, yet there was a reduction in $CD8^+$ T cell numbers in the intratumoral, peritumoral, and invasive regions in OTR SCC [108]. Their findings corroborated those of *Mühleisen and colleagues*, who, through the use of similar techniques reported a reduction in $CD8^+$ T cell proportions in the perineoplastic inflammatory infiltrate of OTR intraepithelial and invasive SCC [125]. However, in contrast to these findings, two studies from other groups have reported that the $CD8^+$ T cell density in OTR peritumoral infiltrates is not different to that in immune competent peritumoral infiltrates [126, 127]. Importantly, one of these studies, conducted by *Kosmidis*

and colleagues, confirmed their immunohistochemical observations using RNA expression analysis. While levels of CD4, T-BET, IFN-γ, and IL-17A mRNA appeared reduced in OTRs, CD8 mRNA levels did not differ significantly between groups [126]. The literature therefore stands divided as to whether there are differences in $CD8^+$ T cell numbers in SCC following immunosuppression, but seems united as a whole that $CD8^+$ T cells are present. Thus, impaired recruitment of $CD8^+$ T cells does not appear to be the underlying factor contributing to SCC abundance in OTRs. This has led us, and others, to speculate that future studies should focus on a closer examination of $CD8^+$ T cell *function* as oppose to *abundance* in order to define the contribution of $CD8^+$ T cells to SCC abundance in OTRs [120, 127, 128].

$CD8^+$ T CELL REGULATION IN SCC

As highlighted previously, imiquimod is commonly used to induce the regression of precancerous SCC lesions. SCC, by contrast, displays a low clinical response rate to Imiquimod, and Imiquimod is therefore not recommended for the treatment of SCC [129]. This would seem surprising given studies that clearly show that imiquimod causes T cell infiltration into SCC. Indeed, *Smith* et al., treated SCC with Imiquimod for 7 days prior to excision and immunohistochemical analysis, and found that $CD8^+$ T cells, which represented 10-20% of the total T cell infiltrate in untreated SCC, represented 30-50% of all mononuclear cells post imiquimod treatment [130]. Furthermore, 60% of these $CD8^+$ T cells are were granzyme B-positive, suggesting they were primed for cytotoxic activity [130]. In a separate study, $CD8^+$ T cells from excised imiquimod-treated SCC were shown to produce more IFN-γ, granzyme, and perforin, and less IL-10 and TGF-β than $CD8^+$ T cells from untreated tumours [131]. In addition, imiquimod-treated SCC showed evidence of tumour cell apoptosis and histological evidence of tumor regression [131]. However, just why excised imiquimod-treated SCC show evidence of heightened $CD8^+$ T cell activity and tumour regression, yet non-excised imiquimod-treated SCC show a poor clinical response rate, is not well understood.

It has been proposed that SCC evade immune destruction by down-regulation of vascular E-selectin and recruitment of T-regs [132]. E-selectin down-regulation may be linked to the production of Nitric Oxide by Myeloid-Derived Suppressor Cells [133], however it should be noted that while imiquimod treatment increases vascular E-selectin expression [132], this does

not appear to lead to SCC destruction *in vivo*. Another possibility is that antigen-presenting cells within SCC are somehow defective, and there is some evidence to suggest that dermal myeloid dendritic cells are deficient in their ability to stimulate T cells and that Langerhans cells are significantly decreased in number in SCC [134, 135]. However, a recent study showed that Langerhans cells isolated from human SCC are functional and may even be stronger at stimulating allogenic T cell responses than Langerhans cells from peritumoral skin [136]. Interestingly, tumour supernatant was found to enhance, rather than suppress, Langerhans cell-driven functional outcomes and Th1 bias despite the presence of immunosuppressive cytokines [136].

Recently, T-reg in SCC have been investigated to determine whether they play a role in permitting SCC growth and survival. We performed a flow-cytometry-based immunophenotypic screen of IEC and SCC lesions in immune competent patients and found that the relative abundance of $CD8^+$ T cells decreased with advancing disease stage [137]. Unexpectedly however, the relative abundance of $CD4^+FOXP3^+$ cells as a proportion of the total $CD3^+CD4^+$ T cell population did not change [137]. These results would appear to be corroborated by *Lai* et al., (2016; IHC, AK vs. SCC) and *Azzimonti* et al., (2015; IHC, well differentiated G1 SCC vs. more advanced moderately to poorly differentiated SCC G2-G3) [138, 139]. It should be noted, however, that in humans FOXP3 expression alone is not a good marker of T-regs as it can also be upregulated by activated T cells [140-142]. Subsequently, when *Azzimonti and colleagues* evaluated $FOXP3^+$ staining in concert with $CD25^+$ staining to identify $FOXP3^+CD25^+$ T-regs, they found that the abundance of T-regs was significantly increased in the peritumoral and intratumoral regions of G2-G3 SCC compared with G1 SCC [139]. T-reg analysis in humans is limited to correlative and/or observational studies, and so a functional role for $FOXP3^+CD25^+$ T-reg in the prevention of $CD8^+$ T cell-mediated SCC regression *in vivo* is difficult to define. Nevertheless, two recent and important studies have investigated the effects of T-regs on $CD8^+$ T cell *function* in the context of SCC. In a mouse model of chemically-induced SCC, *Serrels* et al., examined $FOXP3^+CD25^+$ T-reg abundance in wild-type SCC as compared with SCC lacking Focal Adhesion Kinase (FAK) [143]. FAK is a tyrosine kinase that regulates the transcription of inflammatory cytokines and chemokines leading to an immunosuppressive, pro-tumorigenic environment. SCC lacking FAK were shown to contain significantly fewer $FOXP3^+CD25^+$ T-reg and become susceptible to $CD8^+$ T cell-mediated regression. Furthermore, the treatment of palpable wild-type SCC with a FAK inhibitor, VS-4718, lead to a significant reduction in the abundance of $FOXP3^+CD25^+$ T-

reg and $CD8^+$ T cell-mediated SCC regression [143]. Using co-cultures of highly purified patient SCC-derived $CD25^{high}CD127^{low}$ T-reg and $CD3^+CD8^+$ T cells, *Lai* et al., (2016), demonstrated that T-reg suppress the proliferation of tumour-derived $CD8^+$ T cells, and the IFN-γ secretion by tumour-derived effector T cells, in response to the mitogen phytohemagglutinin (PHA) [138]. Thus, T-reg appear to play a clear role in the regulation of $CD8^+$ T cells within SCC.

CONCLUSION

The skin is the body's first barrier against external insults. Actively, $CD8^+$ T cells play a critical role in the defense against pathogens and malignancies in this compartment. Investigating this population more deeply has led to the discovery of five phenotypically and functionally distinct $CD8^+$ T cell subsets: naive, effector, effector memory, central memory and resident memory $CD8^+$ T cells. Of these five populations, $T_{RM,}$ is believed to account for more than 90% of skin $CD8^+$ T cells during the steady state. T_{RM} in the skin are defined by their non- or limited ability to recirculate via blood and their capacity to survive in the tissue for years without apparent antigen stimulation. Nonetheless, T_{RM} have been demonstrated to be highly efficient in limiting secondary infections. Interestingly, after activation with cognate antigen T_{RM} are able to express a broad range of regulatory and cytotoxic molecules that protect against antigen non-related pathogens. Ultimately, the pathogen-alert role that T_{RM} provide in the skin is antigen independent, therefore, T_{RM} are a bridge between the adaptive and the innate immune responses. Despite their important implications in pathogen control and skin maintenance, our knowledge of their individual functional roles in skin homeostasis, including how they are themselves regulated to avoid unwanted pathogenic outcomes, is still in its infancy. For example, what role if any do T_{RM} play in the emergence and/or regression of skin cancer? What are the key elements of $CD8^+$ T cell function altered by aging that explain the increased prevalence of non-melanoma skin cancer in aged individuals? How can we effectively therapeutically manipulate $CD8^+$ T cell function in the skin and which $CD8^+$ T cell subsets should we be focusing our attention on? Clearly there is a pressing need for further research into these questions, alongside many important related questions, if we are to fully elucidate the role of this key adaptive immune cell in the skin.

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