



Suppressed Activity of Tumor-Specific T Cells in Human Merkel Cell Carcinomas

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Abstract

Merkel cell carcinomas (MCC) are rare but highly malignant skin cancers associated with a novel polyomavirus. We studied T cells in MCC to determine how these virally mediated tumors evade immune responses. MCC tumors were infiltrated by T cells, including effector, central memory and increased numbers of CD4 and CD8 FOXP3⁺ regulatory T cells. Infiltrating T cells showed markedly reduced activation as evidenced by reduced expression of CD69 and CD25. Treatment of MCC tumors in vitro with IL-2 and IL-15 led to T cell activation, proliferation, enhanced cytokine production and loss of viable tumor cells from cultures. Expanded tumor-infiltrating lymphocytes showed TCR repertoire skewing and upregulation of CD137. MCC tumors implanted into immunodeficient NOD/SCID/IL2 receptor γ chain^{null} mice failed to grow unless human T cells in the tumor grafts were depleted with denileukin diftitox, suggesting tumor-specific T cells capable of controlling tumor growth were present in MCC. 50% of non-activated T cells in MCC expressed PD-1, a marker of T cell exhaustion, and PD-L1 and PD-L2 were expressed by a subset of tumor dendritic cells and macrophages. In summary, we observed tumor-specific T cells with suppressed activity in MCC tumors. Agents that stimulate T cell activity, block Treg function or inhibit PD-1 signaling may be effective in the treatment of this highly malignant skin cancer.

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CITATION TO PUBLISHED WORK

Portions of this thesis were adapted from the following publication:

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GLOSSARY

MCC	Merkel cell carcinoma
Merkel cell polyomavirus	MCV
CLA	Cutaneous lymphocyte antigen
SCC	Squamous cell carcinoma
NO	Nitric oxide
TILs	Tumor infiltrating lymphocytes

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare and highly malignant neuroendocrine cancer that arises in the skin. The tumor typically presents as a rapidly growing, painless, flesh-colored or bluish-red intracutaneous nodule, with a predilection for sun-exposed areas. MCC is more frequent in immunosuppressed individuals, and the Merkel cell polyomavirus (MCV) has been implicated in its etiology (Becker *et al.*, 2008; Feng *et al.*, 2008; Rodig *et al.*, 2012). Chronic immune suppression, such as occurs in AIDS, CLL, or solid organ transplantation substantially increases the relative risk of developing MCC, even compared to other cancers; however, only 8% of patients fall in this category of severe and chronic immune suppression. Most patients are over 70 years of age (71.6%) (Albores-Saavedra *et al.*, 2010), and likely have some degree of immune response impairment and dysregulation due to immunosenescence.

The incidence rate of first primary MCC in the United States has tripled in 14 years, from 0.2 per 10⁵ persons in 1992 - when the specific immunologic profile for MCC was first established (Agelli *et al.*, 2010) - to 0.6 per 10⁵ persons in 2006 (Albores-Saavedra *et al.*, 2010). This trend is likely due to several factors, such as higher UV exposure, a larger number of elderly and immune suppressed individuals, as well as increased awareness, diagnosis, and reporting of the disease.

The initial treatment in most cases is wide local excision with negative margins. This may be followed by observation, lymph node dissection, radiation therapy, and chemotherapy. Despite these efforts, MCC has a mortality rate of 30%, making it a more deadly cancer than malignant melanoma (Agelli and Clegg, 2003).

In 2008, a novel virus designated the Merkel cell polyomavirus (MCV) was found to be integrated into the genome of 80% of Merkel cell tumors (Feng *et al.*, 2008), a finding that has since been confirmed by multiple studies worldwide (Paolini *et al.*, 2011; Foulongne *et al.*, 2008; Becker *et al.*, 2009). Several lines of reasoning suggest a direct oncogenic role for the virus in cases of MCV+ tumors. First, the clonal integration of the MCV DNA in the primary tumor and any metastases indicate that MCV infection and genome integration preceded tumorigenesis and clonal expansion (Feng *et al.*, 2008). In addition, these integrated MCVs all contain a signature mutation or deletion of the

large T (LT) antigen helicase that results in a truncated LT antigen and renders the virus non-transmissible (Shuda *et al.*, 2008). The truncated LT antigens, which retain intact retinoblastoma (Rb) interacting domains, are expressed in MCV+ tumors (Shuda *et al.*, 2009) and provide a possible mechanism for oncogenesis. In fact, MCC cell lines derived from MCV+ tumors are dependant on T antigen expression for their survival, both in vitro (Houben *et al.*, 2010) and in vivo, an effect which can be largely ascribed to LT antigen and its interaction with Rb (Houben *et al.*, 2012). Despite the oncogenic potential of MCV, later epidemiological studies show MCV to be a very common infection of both children and healthy adults. MCV DNA is detected in up to 80% of normal skin samples, with viral loads increasing sharply with age (Hashida *et al.*, 2016).

T cells specific for MCV oncoproteins are present in the blood and tumors of patients with MCC and these patients have levels of circulating antibodies specific for MCV oncoproteins that fluctuate with disease activity (Iyer *et al.*, 2011; Paulson *et al.*, 2010). However, these immune responses are insufficient in most cases to control growth of the cancer, suggesting that MCC tumors have potent immune evasion strategies.

Even with the malignant nature of this tumor, there have been 50 reported cases of complete regression of both primary and metastatic Merkel cell tumors following a diagnostic procedure such as biopsy or fine needle aspiration (Ahmadi Moghaddam *et al.*, 2016). Histopathology of the specimens generally shows extensive infiltration by many CD4 and CD8 T lymphocytes, accumulation of foamy macrophages, and widespread fibrosis, suggesting that the biopsy may have triggered tumor regression by stimulating the immune system.

This cellular nature of complete spontaneous regressions, combined with the increased incidence of MCC in immune suppressed individuals, particularly those with T cell defects such as AIDS, CLL, and organ recipients on T cell immunosuppressant medication, strongly suggests that the immune system and especially T cell mediated immunity play important roles in controlling the growth of this tumor. We hypothesized that MCC-specific T cells are present in the tumors, but are held in an inactive state by immunosuppressive elements within the tumor microenvironment. This report details our studies of the local tumor microenvironment in MCC tumors. We find these tumors

contain T cells capable of restraining tumor growth but that their activation is suppressed. We present findings that tumor resident regulatory T cells and T cell exhaustion may be two strategies used by MCC to evade immune destruction.

MATERIALS AND METHODS

Patient samples.

MCC tumors and squamous cell carcinomas (SCC) were obtained from the Department of Dermatology at Brigham and Women's Hospital, the Cutaneous Oncology Program at the Dana Farber/Brigham and Women's Cancer Center, and the Fred Hutchinson Cancer Research Center, University of Washington. Acquisition of tumor samples and all studies were approved by the Institutional Review Board of the Dana Farber Cancer Institute/Harvard Cancer Center and were performed in accordance with the Declaration of Helsinki. Written informed consent was received from participants prior to inclusion in the study. Twelve primary tumors and three metastases were studied. Six out of six tumors studied tested positive for the presence of the Merkel cell polyomavirus, four by RT-PCR and two by immunohistochemical staining for large T antigen as described (Rodig *et al.*, 2012). Normal human skin was obtained as discarded tissues following plastic surgery procedures.

Isolation of T cells from MCC tumors and normal skin.

T cells were isolated from normal skin, biopsy-proven MCC tumors, and SCC tumors as previously described (Clark *et al.*, 2006b).

Flow cytometry studies.

Flow cytometry analysis of T cells was performed using directly conjugated monoclonal antibodies from: BD Biosciences (CD3, CD4, CD8, CD25, CD69, CD45RO, CD45RA, CD56 and CD137), BD PharMingen (CLA, CD73), Abcam (CD39), Beckman Coulter (L-selectin), R&D Systems (CCR7), and eBioscience (FOXP3, clone PCH101).

IOtest Beta Mark kit (Beckman Coulter) was used for TCR repertoire analysis. For cytokine production analysis, T cells from MCC tumors were stimulated with either control medium or 50 ng/ml PMA and 750 ng/ml ionomycin for 6 h, with 10 µg/ml Brefeldin-A (Calbiochem) added after 1 h. Cells were stained for surface markers, fixed, permeabilized, and stained with directly conjugated anti-cytokine antibodies. Analysis of flow cytometry samples was performed on Becton Dickinson FACScan or FACSCanto instruments, and data were analyzed using FACSDiva software (V5.1).

Immunofluorescence studies.

MCC tumors were embedded in OCT, frozen, and stored at -80°C. 5 µm cryosections were cut, air dried, fixed in acetone, rehydrated in PBS, and blocked with 20 µg/ml of human IgG (15 min, Jackson ImmunoResearch Laboratories). Sections were incubated with primary antibody (30 min), followed by three rinses in PBS/1% BSA. If necessary, secondary antibody was added (1:100 dilution, 30 min), followed by three rinses. Sections were mounted using Prolong Gold anti-fade mounting medium with DAPI (Invitrogen) and examined by a microscope (Eclipse 6600; Nikon) equipped with a [40×/0.75] objective lens (Plan Fluor; Nikon). Images were captured with a camera (SPOT RT model 2.3.1; Diagnostic Instruments) and acquired with SPOT [4.0.9] software (Diagnostic Instruments). Antibodies from: BD Biosciences (CD3, PD-1, PD-L1, PD-L2, HLA-DR), Abcam (CD163), and R&D Systems (CD11c), Biolegend (CD56, clone HCD56).

Cell viability and cytotoxicity studies.

Autologous tumor cells present in explant cultures were co-cultured with nonexpanded or expanded T cells from three week explant cultures and tumor cell viability was assessed by flow cytometry after 18 hours by cell scatter and/or exclusion of 7-AAD viability stain (BD Biosciences). A second, shorter term cytotoxicity assay was carried out by incubating autologous T cells at the indicated T cell to tumor cell ratio for two hours in the presence of a fluorogenic caspase substrate; cytotoxic tumor cell death was assayed by flow cytometry as per manufacturer's instructions (CyToxiLux Kit, Oncolmmunin).

Mice xenografted with MCC tumors.

Freshly excised MCC tumors were divided into four equal portions at least 3 mm in each dimension and implanted subcutaneously on the dorsal flank of NOD/SCID/IL2-receptor γ -chain^{null} mice (Jackson Laboratories). Mice received 50 μ L intraperitoneal injections on days 0, 2, 6, 7 and 9 of either saline or denileukin diftitox (7.2 mcg/mL). Tumor size was monitored by palpation for 8 weeks, at which point they were harvested and measured.

RESULTS

MCCs are infiltrated by a mixed population of skin-homing effector memory, central memory and regulatory T cells

Immunostaining of MCC cryosections demonstrated the presence of tumor infiltrating lymphocytes (TILs) in MCCs (Figure 1a), which in some cases infiltrated into tumor nests (Figure 1b). In other tumors, T cells surrounded the tumor but did not penetrate into it, similar to the peritumoral pattern previously described to correlate with better survival (Figure 1c) (Andea, 2008; Paulson *et al.*, 2011). Cells isolated from primary MCC tumors using short-term explant cultures (Figure 1d) included both CD4⁺ and CD8⁺ CD45RO⁺ memory T cells (Figure 1e).

Human squamous cell carcinomas of the skin evade immune responses by downregulating vascular E-selectin expression excluding cutaneous lymphocyte antigen (CLA) expressing skin-homing effector memory T cells (T_{EM}) and by recruiting FOXP3⁺ regulatory T cells (Tregs) with a L-selectin/CCR7⁺ central memory T cell (T_{CM}) phenotype (Clark *et al.*, 2008). A subset of MCC lacked CLA⁺ T cells, suggesting skin-homing T cells were excluded (Figure 1f). Immunostaining of MCC tumors demonstrated that CLA expression correlated with the pattern of T cell infiltration into tumors. 3/3 MCC with higher levels of T cell CLA expression were infiltrated by increased number of T cells and T cells were present within the present within the tumor nests themselves (Figure 1a,b) whereas in 4/4 tumors with decreased T cell CLA expression, T cells surrounded but did not penetrate into the tumor, the peritumoral

pattern that has been correlated previously with poorer survival (Figure 1c) (Paulson *et al.*, 2011). However, the presence of CLA⁺ skin-homing TILs in tumors was not entirely protective. Of the three patients with >50% CLA⁺ TILs, only one remained free of disease following primary excision and local radiotherapy; one had recurrent disease that responded to combination chemotherapy and brachytherapy, and one died from disease. L-selectin/CCR7⁺ T_{CM} were significantly enhanced in MCC compared to normal skin, suggesting the preferential recruitment of T_{CM} to these tumors (Figure 1g). FOXP3⁺ Treg were also increased in MCC tumors as compared to normal skin (Figure 1h) but this did not correlate with decreased survival. Of the three patients with >20% tumor Tregs, only one died; one had recurrence that responded to chemotherapy and brachytherapy, and one remained free of disease following primary excision and radiotherapy.

T cells infiltrating MCC show decreased activation

CD69 is a marker of early T cell activation that is expressed by roughly half of the T cells isolated from non-inflamed human tissues including skin and gut (Bos *et al.*, 1987; Clark *et al.*, 2006a; Kunkel *et al.*, 2002). The CD69 expression of MCC TILs was much lower than T cells isolated from normal, non-inflamed human skin (Figure 2a, c), suggesting decreased activation. CD25 is a marker for later stages of T cell activation that is expressed by both activated T cells and FOXP3⁺ Tregs. Normal human skin contains a significant population of CD25⁺ FOXP3⁻ activated T cells and a smaller population of CD25⁺ FOXP3⁺ Tregs (Figure 2b)(Clark *et al.*, 2006a). Although normal numbers of MCC TILs expressed CD25, the vast majority of these were FOXP3⁺ Tregs (Figure 2a-c); the CD25⁺ FOXP3⁻ activated effector T cells present in normal skin were absent. Taken together, these results demonstrate a marked inhibition of activation in the MCC TILs.

Culture of MCC tumors in IL-2 and IL-15 leads to T cell proliferation, activation, and expansion of CD8 T cells

Immunostimulatory cytokines including IL-2 and IL-15 have been used *in vitro* to expand TILs with antitumor activity from malignant melanoma (Mueller *et al.*, 2008;

Rosenberg *et al.*, 2011). We expanded T cells from MCC tumors with IL-2 and IL-15 for one week and observed marked increases in cell size and granularity, enhanced activation as evidenced by increased expression of both CD69 and CD25, increased numbers of CD25⁺ FOXP3⁻ activated effector T cells, and reduced percentages of CCR7⁺/L-selectin⁺ T_{CM} (Figure 3a, b). After three weeks of stimulation, we observed T cell proliferation, particularly CD8 T cells, as indicated by the proliferation marker Ki-67 (Figure 3d). In contrast to these population shifts, we found no changes in the percentages of CLA⁺ skin-homing or FOXP3⁺ Tregs (Figure 3c). Further studies demonstrated that IL-15 was critical for enhancing T cell activation and proliferation, as these were mostly unchanged by IL-2 treatment alone (Figure 4).

In vitro treatment of MCC tumors with IL-2 and IL-15 enhances T cell cytokine production

We next studied T cell cytokine production in MCC tumors in the presence or absence of treatment with IL-2 and IL-15. In one patient, T cells showed markedly enhanced production of IFN γ and TNF α by both CD4 and CD8 T cells and enhanced CD4 IL-17 production after IL-2/IL-15 treatment (Figure 5a,b). The patient was 90 years-old at diagnosis with a high-risk, 3-cm primary lesion and evidence of micrometastases. She was treated with wide excision and local radiation therapy and remains well despite the high-risk nature of her primary lesion. A second subset of patients demonstrated increased production of IFN γ and/or TNF α but little IL-17 production (Figure 5c). A third group of patients showed significant production of Th2 cytokines (IL-4, IL-13) and IL-10 and no enhancement of IFN γ , TNF α , and IL-17 production after treatment with IL-2/IL-15 (Figure 5d).

T cells isolated from patient MCC6 at the time of initial biopsy showed low levels of IFN γ and TNF α production even after IL-2/IL-15 treatment. However, T cells isolated from the same primary tumor 19 days later at the time of full excision showed enhanced production of IFN γ and TNF α , suggesting that the initial biopsy event triggered either migration of Th1 T cells into the tumor or allowed enhanced cytokine production (Figure 5e).

MCC tumors contain CD8⁺ FOXP3⁺ Treg and plasmacytoid dendritic cells.

Classical FOXP3⁺ Tregs are CD4⁺ but suppressive CD8⁺FOXP3⁺ Tregs have been described in human transplant recipients, mouse models of autoimmune diseases, and the microenvironments of many tumors (Chang *et al.*, 2002; Rifa'i *et al.*, 2004; Xystrakis *et al.*, 2004; Yang *et al.*, 2010). We observed a discrete population of CD8⁺FOXP3⁺ T cells in MCC that expanded in concert with effector T cells during IL-2/IL-15 treatment (Figure 6a). These CD8 Tregs expressed the skin-homing addressin CLA, but lacked CCR4 and had a CCR7⁻ non-T_{CM} phenotype (Figure 6b). The majority expressed intracellular CTLA-4 and GITR, two immunomodulatory molecules also expressed by CD4⁺ Tregs (Sakaguchi, 2000), and nearly all expressed high levels of HLA-DR, a marker for high suppressive capacity in humans (Baecher-Allan *et al.*, 2006). Expression of HLA-DR, GITR and intracellular CTLA-4 by T cells from normal human skin is also included (Figure 6c). Immunostained tumor cryosections revealed frequent CD123⁺ plasmacytoid dendritic cells (PDC) (Figure 6d,e), which have been shown to induce formation of CD8⁺FOXP3⁺ Tregs in the tumor microenvironment of ovarian carcinoma (Wei *et al.*, 2005).

TILs in MCC show evidence of T cell exhaustion

PD-1 expression by TILs in tumors in the presence of PD-1 ligands can indicate T cell exhaustion (Ahmadzadeh *et al.*, 2009; Blank *et al.*, 2006; Chapon *et al.*, 2011; Jin *et al.*). Despite the low levels of activation observed in MCC TILs, a significant percentage of both CD4⁺ and CD8⁺ TILs expressed PD-1 (Figure 7a, b, c). PD-1 expression by TILs was reduced after treatment of tumors with IL2/IL-15, particularly among CD8⁺ T cells (Figure 7 b,c). TIL expression of PD-1 by tumor T cells was significantly higher than that of T cells from normal skin and blood (Figure 7c). Treatment of tumors with IL-2 and IL-15 reduced TIL expression of PD-1 to levels observed in normal skin T cells (Figure 7c). Two PD-1 ligands, PD-L1 and PD-L2, were present in the tumor microenvironment but not expressed by the tumor cells themselves (Figure 8d,e). Instead, they were expressed by a population of CD11c⁺ dendritic cells (Figure 7f). In addition, a small subpopulation of CD163⁺ macrophages within the tumor also expressed PD-L1 and PD-L2 (data not shown).

MCC tumors contain tumor-specific T cells capable of controlling tumor growth

T cells expanded from MCC treated in vitro with IL-2 and IL-15 showed skewing of the TCR repertoire among both CD4⁺ and CD8⁺ T cells, suggesting expansion may be antigen-specific (Figure 8a-c). The expansion of T cells and simultaneous loss of viable tumor cells suggested that viable tumor cells may be killed by expanding T cells (Figure 8d). Cytokine treated CD8⁺ TILs showed upregulation of CD137, a marker of antigen-specific T cell activation used to identify melanoma or virus-specific TIL after in vitro expansion (Figure 8e) (Hernandez-Chacon *et al.*, 2011; Wehler *et al.*, 2008). Significant killing of tumor cells was observed after culture of autologous tumor cells with IL-2/IL15 expanded tumor T cells as compared to that induced by non-expanded TILs (Figure 8f). A shorter term cytotoxicity assay utilizing a fluorogenic caspase substrate also demonstrated tumor cell killing by expanded TILs that was superior to that observed using non-expanded TILs from the same tumor (Figure 8g). 2 mm MCC tumor fragments implanted subcutaneously into NOD/SCID/IL2-receptor γ -chain^{null} (NSG) mice failed to grow in size (Figure 8i,j). Hypothesizing that T cells transferred with the tumor may be preventing tumor growth, we isolated T cells from tumors five days after implantation into mice. T cells from implanted tumors showed enhanced activation, as shown by increased expression of CD69 and CD25 (Figure 8h). T cell expression of PD-1 declined, as did the percentage of FOXP3⁺ Treg. We treated implanted mice systemically with denileukin diftitox, a recombinant fusion protein that selectively depletes human CD25-expressing T cells, including activated effector and regulatory cells (Ho *et al.*, 2004; Morse *et al.*, 2008). MCC implanted into denileukin diftitox treated mice grew and could be transferred to additional animals (Figure 8i,j).

Metastatic lesions of MCC also contain T cells that can be activated and expanded by treatment with IL-2 and IL-15.

Similar to our findings in primary tumors, T cells from IL-2/IL-15 treated MCC metastases expanded, upregulated expression of activation markers CD69 and CD25, and CD8 expression of CD137 was upregulated (Figure 9a,b). There was enhanced IFN γ production and skewing of the T cell repertoire and increased percentages of

effector T cells (Figure 9c,d). In brief, metastatic lesions also contain T cells, and their activation and cytokine production was enhanced by IL-2/IL-15 treatment.

DISCUSSION

As with other virally associated cancers, T cell immunity plays a critical role in the susceptibility and immune responses to MCC. Incidence is markedly increased in immunosuppressed individuals and withdrawal of iatrogenic immunosuppression or biopsy itself has induced regression of MCC (Friedlaender *et al.*, 2002; Heath *et al.*, 2008; Muirhead and Ritchie, 2007; Val-Bernal *et al.*, 2011; Wooff *et al.*, 2010). Although 80% of MCCs have genomic integration of MCV and produce viral proteins including small and large T antigens, these tumors are still highly malignant in immunocompetent individuals (Becker *et al.*, 2009; Feng *et al.*, 2008; Foulongne *et al.*, 2008; Nakamura *et al.*, 2010; Paolini *et al.*, 2011; Shuda *et al.*, 2009; Shuda *et al.*, 2008). 92% of MCC occur in immunocompetent individuals, and mortality is 30%, making MCC a more fatal cancer than malignant melanoma (Heath *et al.*, 2008). The highly malignant nature of this virally mediated cancer suggests that MCC has potent strategies for evading immune response and that neutralization of these strategies may enhance anti-tumor immunity.

Cancer destruction requires not only the generation of tumor-specific T cells but also the ability of these T cells to access the tumor once they are generated (Gajewski, 2007). T cells are imprinted with expression of tissue specific addressins and preferentially migrate to the peripheral tissue in which they first encountered antigen (Campbell and Butcher, 2002; Kupper and Fuhlbrigge, 2004). MCC-specific T cells should express the skin-homing addressin CLA because MCC are cutaneous tumors and T cells will have first encountered antigen within the skin-draining lymph nodes. We observed decreased numbers of skin-homing CLA⁺ T cells in a subset of MCC, suggesting the presence of a T cell homing defect in at least some tumors. Impaired T cell homing as a result of decreased vascular addressin expression has been reported in a number of human cancers, including malignant melanoma, cutaneous SCC, breast,

gastric, cervical and lung cancers (Clark *et al.*, 2008; Madhavan *et al.*, 2002; Piali *et al.*, 1995; Trimble *et al.*; Weishaupt *et al.*, 2007). Indeed, prior studies have shown that the presence of CD8 T cells within the MCC tumor itself is correlated with better patient outcomes (Paulson *et al.*, 2011). However, patients with tumors that showed infiltration with CLA⁺ skin-homing T cells did not have a markedly improved survival, suggesting that other immunosuppressive mechanisms were also at work.

Our results suggested that at least some MCC tumors contained tumor-specific T cells but that activation of these T cells was markedly suppressed. Expression of the activation markers CD69 and CD25 was severely decreased in MCC TIL, lower even than T cells from normal, non-inflamed human skin (Figure 2). In vitro culture of MCC with the T cell activating cytokines IL-2 and IL-15 led to marked activation and expansion of tumor T cells, loss of viable tumor cells from cultures and upregulation of CD137 on CD8 T cells, a marker of antigen-specific T cell activation that has been used to identify melanoma or virus-specific TIL after in vitro expansion (Figure 8) (Hernandez-Chacon *et al.*, 2011; Wehler *et al.*, 2008). T cells expanded from MCC tumors killed autologous tumor cells in vitro (Figure 8f,g). Significantly, MCC tumors implanted subcutaneously into NSG mice, an immunodeficient mouse strain that lacks T cells, B cells and NK cells, failed to grow unless their activated T cells were depleted from the transplanted tumor by treatment with denileukin diftitox (Figure 8h-j) (Ho *et al.*, 2004; Morse *et al.*, 2008). These experiments suggest that at least some MCC tumors contain T cells capable of controlling tumor growth.

These findings suggest that potent mechanisms for the suppression of T cell activation exist within the MCC tumor microenvironment and that intratumoral administration of T cell activating agents may be capable of stimulating TILs, thereby enhancing immune responses. Indeed, intratumoral injection of interferon-beta was reported to induce MCC regression in four patients (Paulson, 2011). We found that the combination of IL-2 and IL-15 was critical for the expansion and activation of MCC TILs. Surprisingly, IL-2 alone had little effect on proliferation and cytokine production of MCC TILs (Figure 4) whereas IL-15 alone enhanced cytokine production but resulted in less proliferation (data not shown). Both IL-2 and IL-15 induce antigen-independent proliferation of T cells and participate in bystander T cell proliferation during immune

responses (Lodolce *et al.*, 2001). Under normal conditions, IL-2 plays a crucial role in sustaining Tregs and maintaining peripheral tolerance, whereas IL-15 controls the survival and turnover of memory T cells (Sprent *et al.*, 2008). In a mouse model of adoptive immunotherapy for melanoma, T cells expanded *in vitro* with IL-15 were longer-lived and more effective *in vivo* than those generated with IL-2 (Mueller *et al.*, 2008). Our results suggest intratumoral therapy with potent activators of T cell function, including the combination of IL-2 and IL-15, may be effective in enhancing local antitumor immunity.

Our studies have identified Treg inhibition and T cell exhaustion as two possible mechanisms for the inhibition of T cell activity we observe in MCC. MCC contained increased numbers of both CD4 and CD8 Tregs (Figure 1, 4). CD8 Tregs contribute to disease progression in prostate and colorectal cancer and have also been described in ovarian cancer and malignant melanoma (Chaput *et al.*, 2009; Kaufman *et al.*; Kuniwa *et al.*, 2007; Wei *et al.*, 2005). CD8 Tregs were more efficient than CD4 Tregs in reducing CD4 T cell proliferation and Th1 cytokine production (Filaci *et al.*). MCC are also heavily infiltrated by PDC, a cell type known to induce the formation of CD8 Tregs (Wei *et al.*, 2005). Our results suggested that therapies that inhibit Treg activity, such as ipilimumab, may be useful in MCC. In fact, a clinical trial started in 2014 is examining this very treatment (clinical trial NCT02196961) and currently recruiting participants.

Our most interesting finding, however, was that PD-1 was expressed on approximately half of MCC T cells, despite their markedly suppressed activation status. PD-1, a CD28/CTLA-4 family member, is expressed by activated T cells but when expressed in tumors in the presence of its ligands PD-L1 or PD-L2, it can be a sign of T cell exhaustion (Jin *et al.*). PD-1 is upregulated on T cells in malignant melanoma (Blank *et al.*, 2006; Chapon *et al.*, 2011), and increased PD-1 expression correlates with an exhausted phenotype and impaired effector function (Ahmadzadeh *et al.*, 2009). MCC TILs showed markedly suppressed activation and PD-1 expression was therefore unlikely to be the result of T cell activation and more likely reflected T cell exhaustion. Activation and expansion of TILs with IL-2 and IL-15 led to marked decreases in TIL PD-1 expression along with potent enhancement of cytokine production and anti-tumor cytotoxicity, consistent with recovery from the exhausted phenotype (Figures 5, 6 and

Figure 5). We observed a population of dendritic cells and macrophages within MCC that expressed PD-L1 and PD-L2 and may be responsible for the induction of PD-1 on tumor T cells. These findings suggested that medications that target the PD-1 pathway may have a role in the treatment of patients with MCC (Brahmer *et al.*, 2010). Research performed since the publication of this paper has confirmed our findings (Afanasiev *et al.*, 2013; Lipson *et al.*, 2013;) and laid the groundwork for clinical trials with very encouraging results.

An early phase II trial for pembrolizumab - a humanized monoclonal IgG4 antibody - as a first-line treatment in 25 patients with stage IIIb or stage IV MCC, showed a 56% objective response rate with four (16%) complete responders compared with median 23% response rate of standard chemotherapy regimens. Most impressively, there was a 67% rate of progression-free survival at 6 months compared with 6.7% rate for standard chemotherapy. 86% of the responders continued to respond to treatment after 6 months (Nghiem, *et al.*, 2016). Avelumab, an anti-PD-L1 monoclonal antibody, has been trialed in 84 patients with stage IV chemotherapy refractory MCC and has shown a 31.8% response rate, with 82% of those ongoing at time of publication after a median of 10.4 months followup (Kaufman *et al.*, 2016). Interestingly, in contrast to other cancer types, neither pembrolizumab nor avelumab response correlated with PD-1/PD-L1 expression or MCV status, which might be attributed to small sample size and high response rates.

In addition to these two phase II trials, there have been several case reports of off-label application of immunotherapies targeting the PD-1/PD-L1 axis. A case report of off-label pembrolizumab in metastatic etoposide treated MCC showed effective treatment response (Winkler *et al.*, 2016). Another case report using nivolumab, a humanized IgG4 monoclonal anti-PD-1 antibody, reported 11 months of partial response with no new sites of disease (Walocko *et al.*, 2016). Nivolumab is currently in a clinical trial that is recruiting participants (NCT02488759) while another PD-L1 antibody durvalumab is in the pre-recruitment phase (NCT02643303).

MCC are virally associated cancers that should be eminently recognizable by the immune system. The fact that MCC are highly malignant in immunocompetent individuals suggests they must have formidable strategies for evading immune responses. We found that MCC tumors utilize a spectrum of immune evasion strategies,

including impairment of T cell homing and local suppression of T cell activation. Impaired T cell activation likely results in part from locally high concentrations of both CD4 and CD8 Tregs and expression of PD-L1 and PD-L2 within the tumor microenvironment, leading to T cell exhaustion. Our work suggested that adjuvant therapy of high risk patients with agents that stimulate T cell activity, block Treg function, and inhibit PD-1 signaling may enhance antitumor immunity in patients with MCC.

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Figures

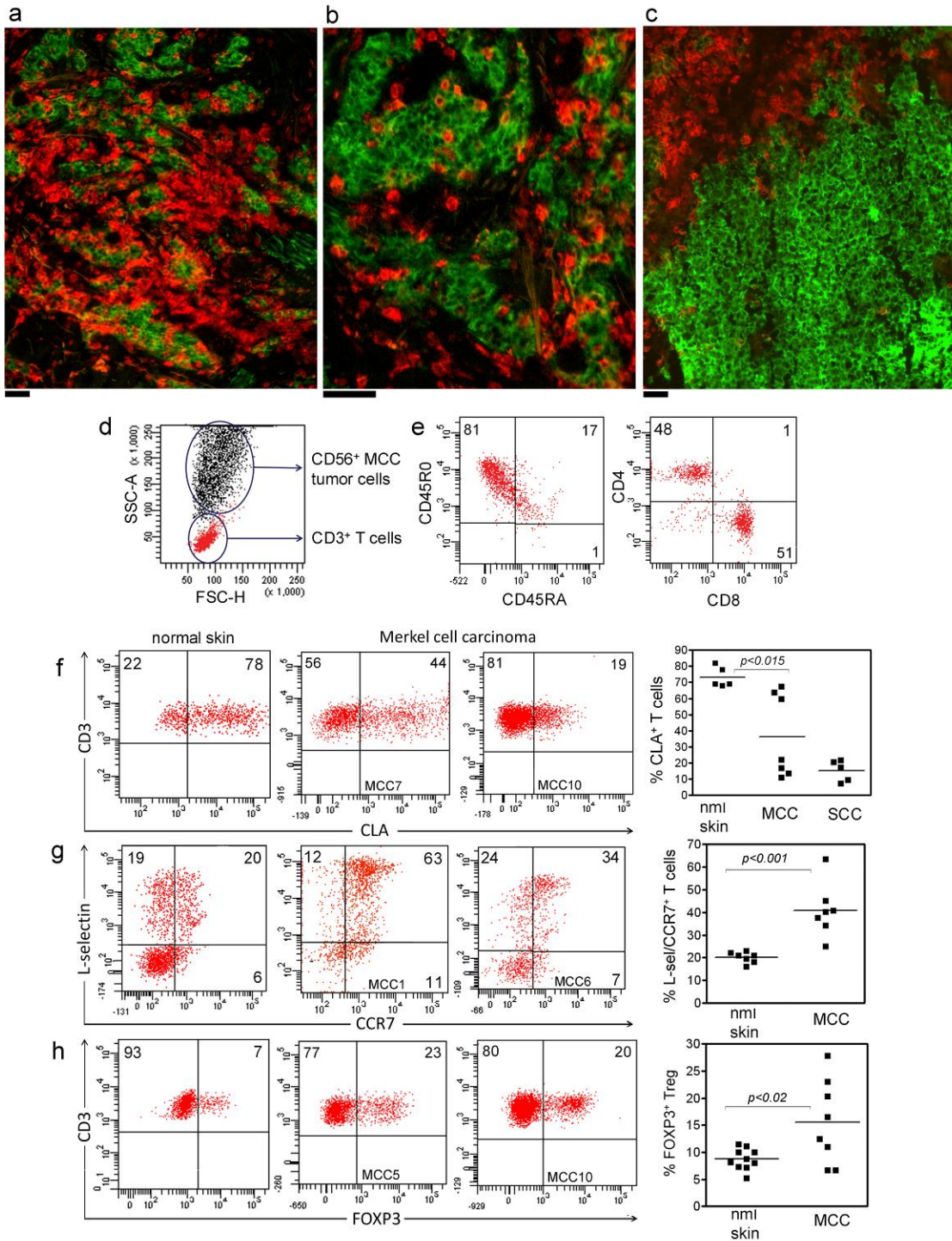


Figure 1

Figure 1. Merkel cell carcinomas are infiltrated by a mixed population of effector memory, central memory, and regulatory T cells. (a) MCC cryosection immunostained for T cells (α CD3, red) and MCC tumor cells (α CD56, green) demonstrates numerous infiltrating T cells. (b) Higher power view shows T cells infiltrating within tumor nests. (c) In some tumors, T cells were located in a peritumoral distribution. (d) Short term cultures of MCC tumors allowed isolation of both T cells and tumor cells. The presence of T cells was studied by direct T cell isolation or immunostaining in 12 primary MCC tumors and three metastatic lesions. (e) In 8/8 MCC, TILs were CD45RO⁺ memory T cells with both CD4⁺ and CD8⁺ T cells. (f) MCC tumors had varied recruitment of CLA⁺ skin-homing T cells. The percentage of CLA⁺ T cells in normal skin (nml skin), MCC, and SCC are shown. A subset of MCC was infiltrated by CLA⁺ T cells whereas a second group of tumors excluded these T cells. The homing defect in this second subset was as pronounced as that observed in SCC, a tumor known to evade immune responses at least in part by excluding CLA⁺ T cells. (g) MCC were infiltrated by higher percentages of L-selectin/CCR7⁺ T_{CM} and (h) FOXP3⁺ Tregs compared to normal skin. All histograms are gated to show CD3⁺ T cells.

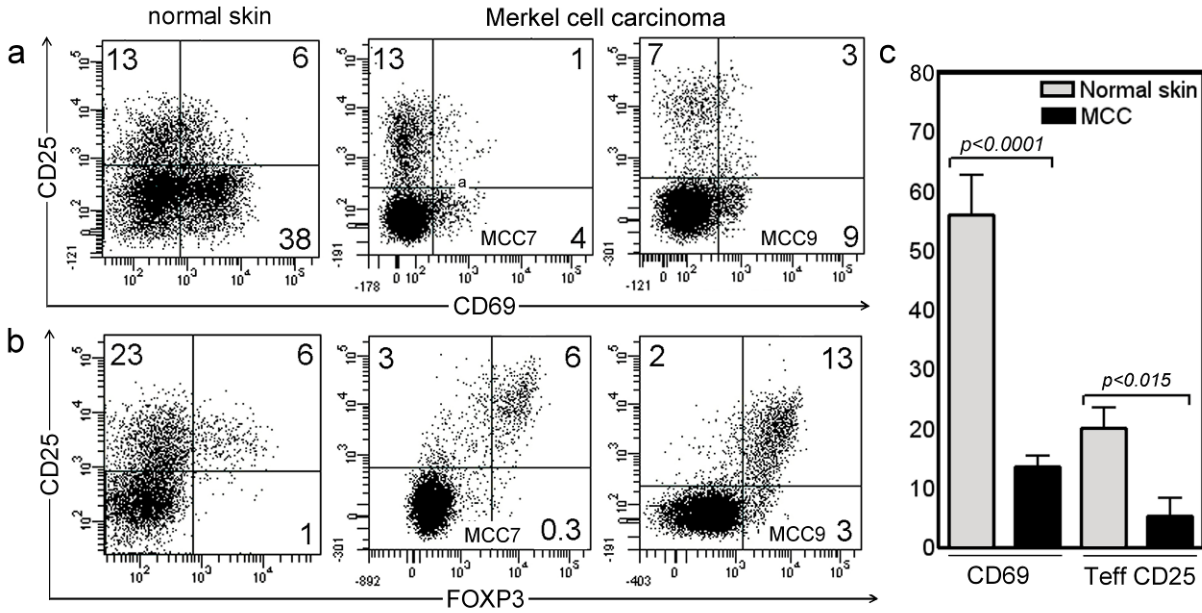


Figure 2. T cells infiltrating MCC show decreased activation. (a) MCC TILs showed markedly reduced expression of the early activation antigen CD69. (b) Expression of the activation marker CD25 was largely restricted to FOXP3⁺ Treg. CD69 and CD25 expression from two tumors are shown; similar findings were observed in six additional tumors. The CD25⁺FOXP3⁻ activated T cell population observed in normal skin was absent from MCC tumors. (c) The mean and SEM of CD69⁺ and CD25⁺FOXP3⁻ T cells from 8 MCC tumors are shown, compared to 4 samples of normal skin. All histograms are gated to show CD3⁺ T cells.

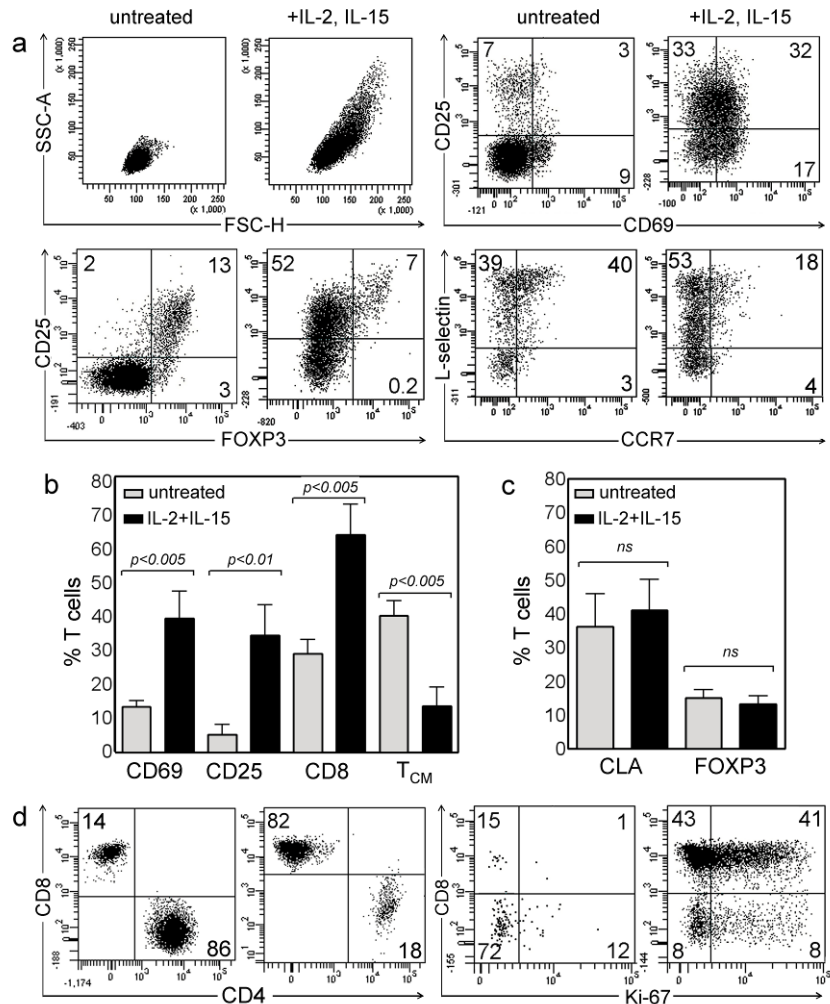


Figure 3. Culture of MCC tumors in IL-2 and IL-15 leads to T cell activation, proliferation, and expansion of CD8 T cells. (a) T cells from tumors treated for one week with IL-2 and IL-15 showed marked upregulation of CD69 and CD25, increased percentages of CD25⁺FOXP3⁻ activated effector T cells and reduced percentages of CCR7⁺/L-selectin⁺ T_{CM}. The mean and SEM from seven tumors of parameters that **(b)** changed or **(c)** remain unchanged after one week of IL-2 and IL-15 treatment are shown. The percentages of CLA⁺ skin-homing and FOXP3⁺ Tregs were not altered by cytokine treatment of tumors. **(d)** By three weeks of culture, CD8⁺ T cells predominated and marked T cell proliferation was evident, as indicated by the proliferation marker Ki-67. All histograms are gated to show CD3⁺ T cells.

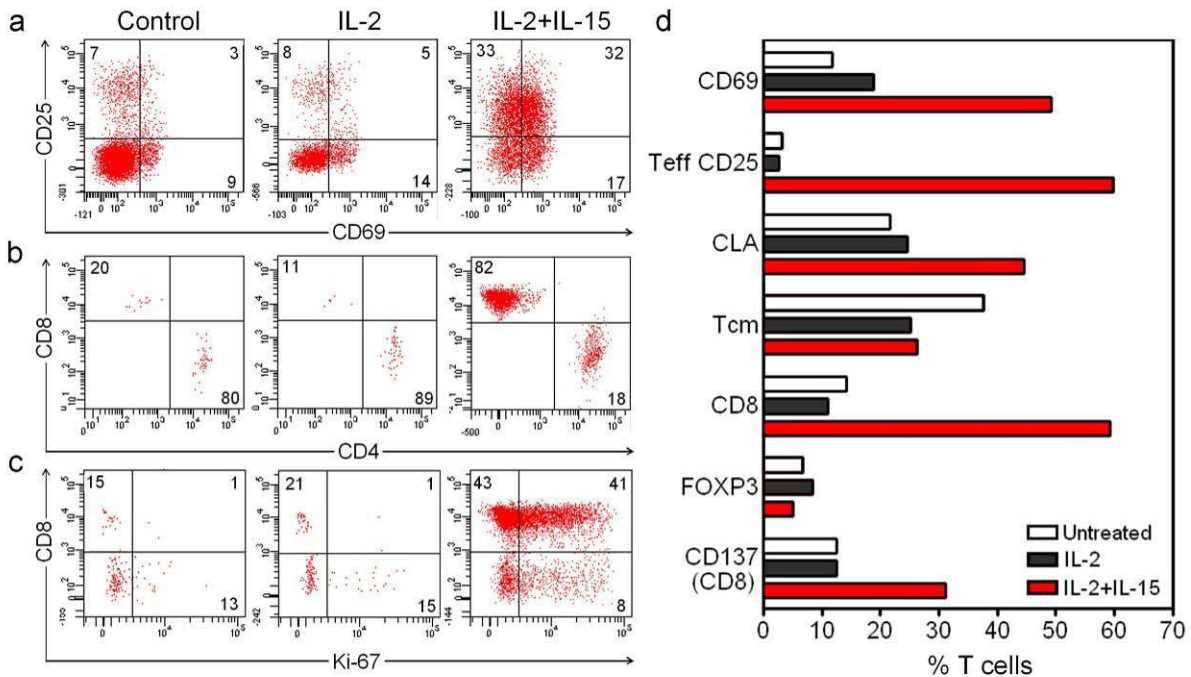


Figure 4. A combination of IL-2 and IL-15 was optimal for the activation and expansion of T cells from MCC. T cells isolated from the same MCC tumor in the presence of no exogenous cytokines (control), IL-2 alone (IL-2) or a combination of IL-2 and IL-15 (IL-2+IL-15) are shown. Inclusion of both IL-2 and IL-15 induced (a) maximal T cell activation, (b) preferential expansion of CD8 T cells and (c) maximal T cell proliferation, whereas IL-2 alone was minimally effective. IL-15 alone induced T cell activation but less overall T cell proliferation (data not shown). (d) Effects of IL-2 alone vs. IL-2+IL-15. CD137 expression of CD8 T cells was enhanced only in tumors treated with both IL-2 and IL-15. A representative tumor is shown; similar results were obtained in two additional tumors. All histograms are gated to show CD3⁺ T cells.

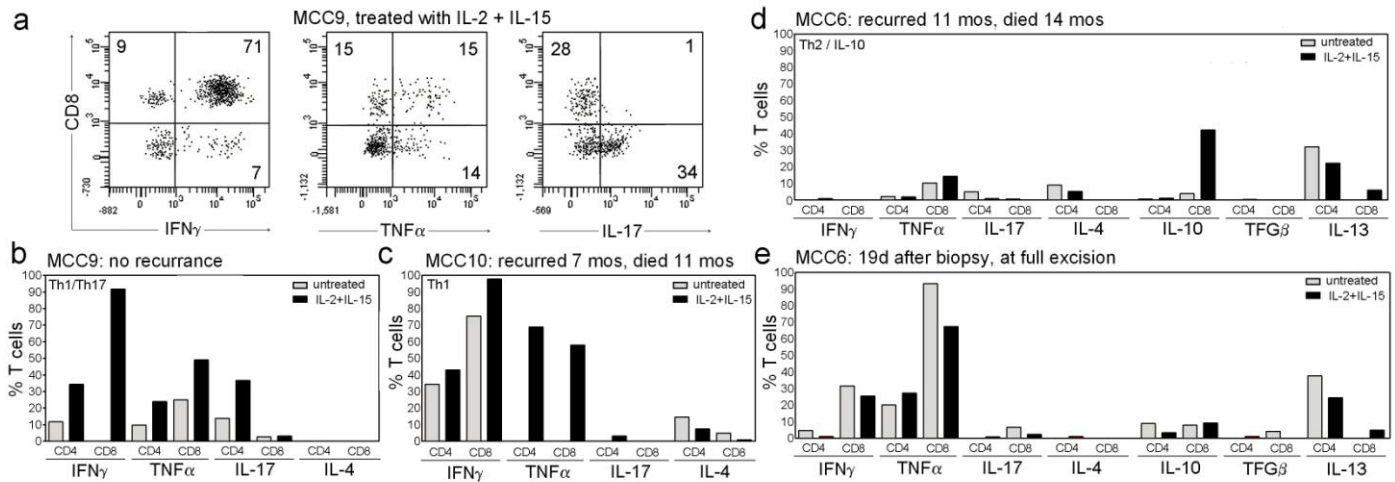
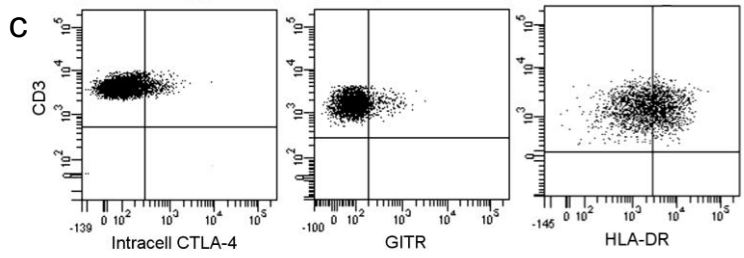
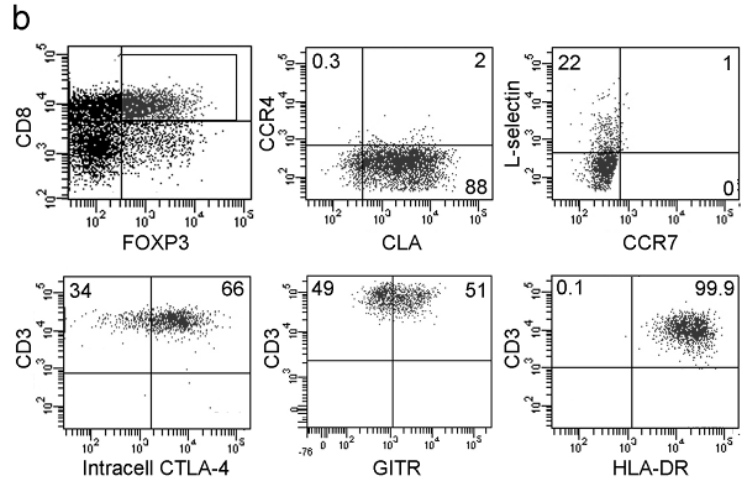
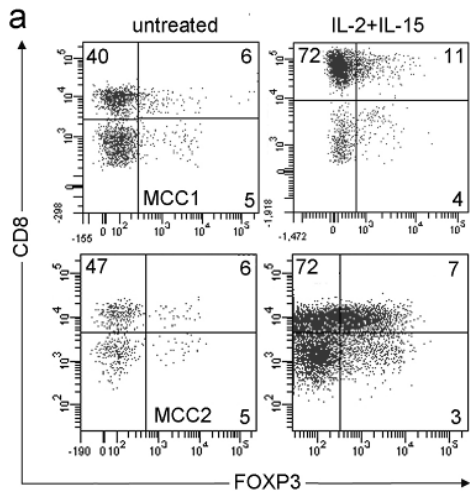
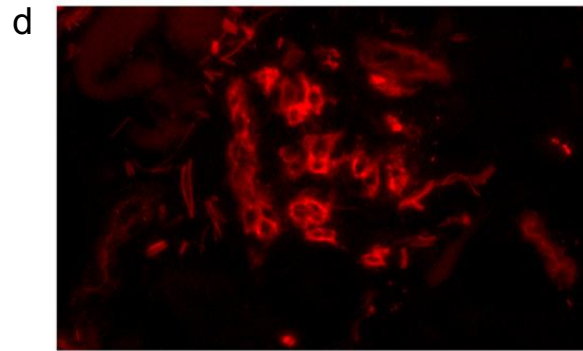


Figure 5. In vitro treatment of MCC tumors with IL-2 and IL-15 enhances T cell cytokine production. T cells were isolated from 5 MCC tumors cultured for two weeks in control medium (untreated) or IL-2 and IL-15 (IL-2+IL-15); cytokine production was assayed by intracellular cytokine staining after stimulation with PMA and ionomycin. (a, b) One patient showed enhanced production of IFN γ , TNF α and IL-17 production in treated tumors. (c) A second subset of patients (2/5) demonstrated increased production of IFN γ and TNF α but little IL-17. (d) Expanded TIL from a third group of patients (2/5) showed significant production of Th2 cytokines (IL-4, IL-13) and IL-10 but no enhancement of IFN γ , TNF α and IL-17 production. (e) T cells isolated from the same tumor shown in panel D 19 days after the initial biopsy showed enhanced production of IFN γ and TNF α , suggesting migration of Th1 T cells into the tumor occurred following the biopsy procedure. All histograms are gated to show CD3⁺ T cells.



CD123 (red)



CD123 (red) DAPI (blue)

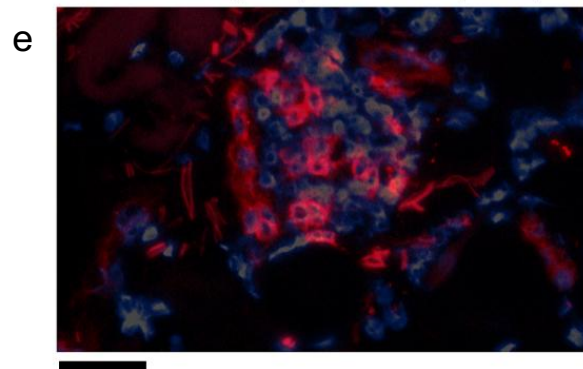


Figure 6. MCC tumors contain CD8⁺ FOXP3⁺ Treg and plasmacytoid dendritic cells (PDC). (a) MCC tumors contained CD8⁺ FOXP3⁺ Tregs and similar or increased numbers of these cells were evident after two weeks of TIL expansion with IL-2/IL-15. Two representative tumors are shown. (b) Most CD8⁺ Treg expressed CLA and lacked the T_{CM} markers L-selectin/CCR7, consistent with a skin-tropic effector T cell phenotype. The majority of CD8⁺ Tregs expressed intracellular CTLA-4 and GITR, and all expressed high levels of HLA-DR, a marker of highly suppressive Tregs. All histograms are gated to show CD3⁺ T cells. (c) Expression of HLA-DR, GITR and intracellular CTLA-4 on T cells from normal human skin. (d) MCC cryosections immunostained for CD123 demonstrated the presence of PDC in tumors, a cell type known to induce formation of CD8⁺ Tregs. (e) PDC were present within tumor nests. A superimposed nuclear stain demonstrates the presence of PDC in tumor nests. A representative tumor is shown; similar results were obtained in seven additional tumors.

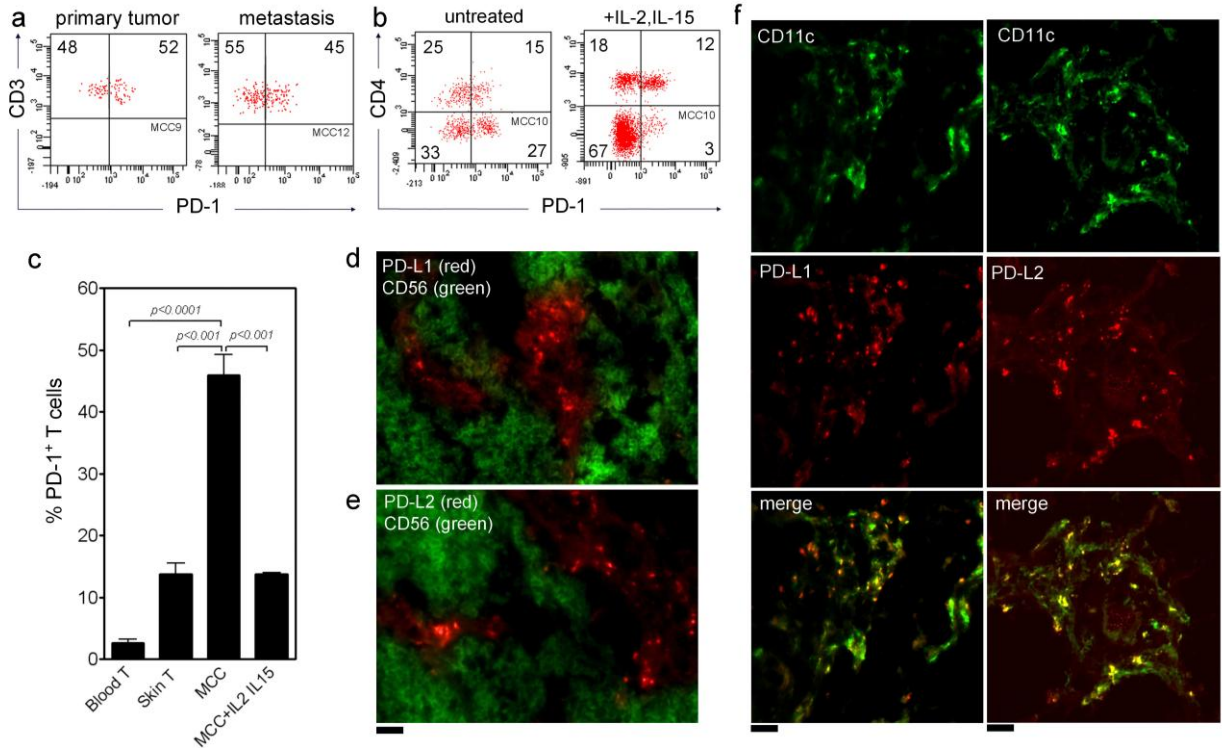


Figure 7. PD-1 is expressed by T cells and PD-1 ligands are expressed within the tumor microenvironment in MCC. (a) A significant proportion of MCC TILs expressed PD-1. T cells from two representative tumors are shown; similar results were observed in four additional tumors. **(b)** PD-1 was expressed by both CD4⁺ and CD8⁺ T cells in untreated MCC and PD-1 expression was reduced, especially on CD8⁺ T cells, after treatment of tumors with IL-2 and IL-15. **(c)** T cells from untreated MCC expressed PD-1 at markedly higher levels than those from normal human skin and blood. TIL expression of PD-1 was significantly reduced after treatment of tumors with IL-2 and IL-15. **(d,e)** MCC tumor cryosections immunostained for PD-L1 or PD-L2 (red) and CD56 (green, delineating tumor cells) show that although PD-L1 and PD-L2 expressing cells are frequent in tumors, they are not tumor cells. Representative cryosections are shown; comparable results were observed in 6 additional tumors. All histograms are gated to show CD3⁺ T cells. **(f)** PD-L1 and PD-L2 are expressed by dendritic cells in MCC. Cryosections immunostained for the dendritic cell marker CD11c and PD-L1 or PD-L2 are shown. PD-L1⁺ or PD-L2⁺ dendritic cells appear yellow in merged images. In addition to CD11c⁺ dendritic cells, PD-L1 and PD-L2 were also expressed by a small subset of CD163⁺ macrophages (data not shown). Representative cryosections are shown; similar results were obtained in 6 additional tumors.

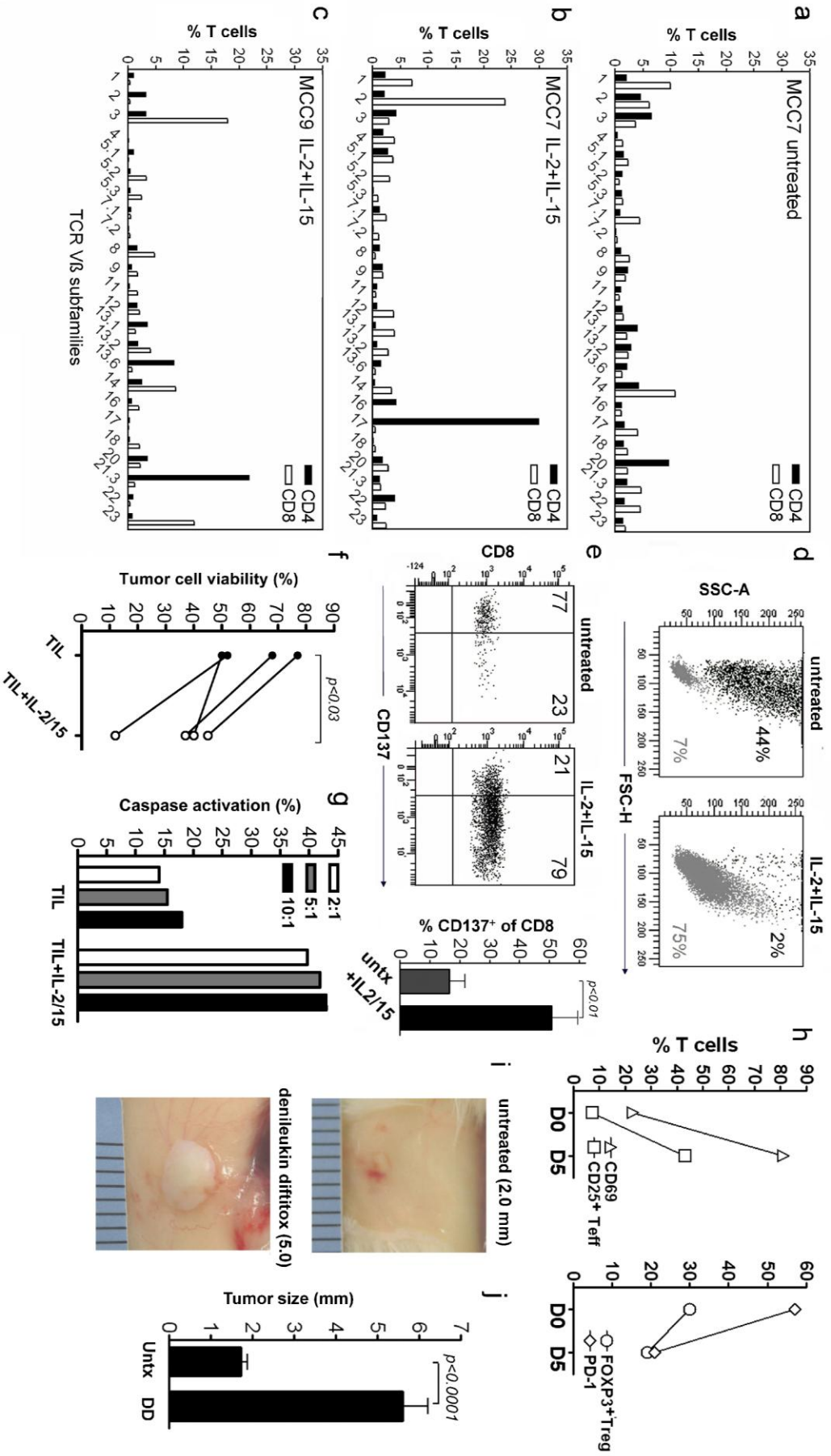


Figure 8. MCC tumors contain tumor-specific T cells capable of controlling tumor growth. (a-c) Expansion of TIL by treatment of tumors with IL-2 and IL-15 led to marked skewing of the T cell repertoire. Two representative tumors are shown; similar results were observed in three additional tumors. Tumors were cultured for three weeks in IL-2 and IL-15 or control medium. (d) Tumors treated for three weeks with IL-2 and IL-15 showed marked expansion of CD3⁺ T cells (gray) and loss of viable CD56⁺ tumor cells (black) from the cultures. (e) CD8⁺ T cells tumors treated for three weeks showed upregulation of CD137, a marker of antigen-specific T cell activation. Representative histograms and the mean and SEM of CD137 expression by non-expanded and expanded CD8 T cells from five MCC are shown. Histograms are gated to show CD8⁺T cells. (f) Death of autologous tumor cells was observed after co-culture with autologous expanded T cells from IL-2 and IL-15 treated tumors and was greater than that observed after co-culture with non-expanded T cells from the same tumors. (g) A short term cytotoxicity assay measuring caspase activation demonstrated cytotoxic killing of autologous tumor cells by T cells isolated from IL-2 and IL-15 treated tumors. The ratios of T cells:tumor cells are shown. (h) T cells isolated from MCC before (D0) and five days after (D5) subcutaneous implantation into immunodeficient mice showed increased expression of activation antigens CD69 and CD25, reduced percentages of FOXP3⁺ Treg and reduced expression of PD-1. (i) MCC tumors implanted subcutaneously into immunodeficient mice did not grow unless activated T cells transferred with the graft were depleted with IP administration of denileukin diftitox. Grafts from the same original MCC tumor are shown eight weeks after implantation; the mean and SEM of tumor sizes are shown (n=28).

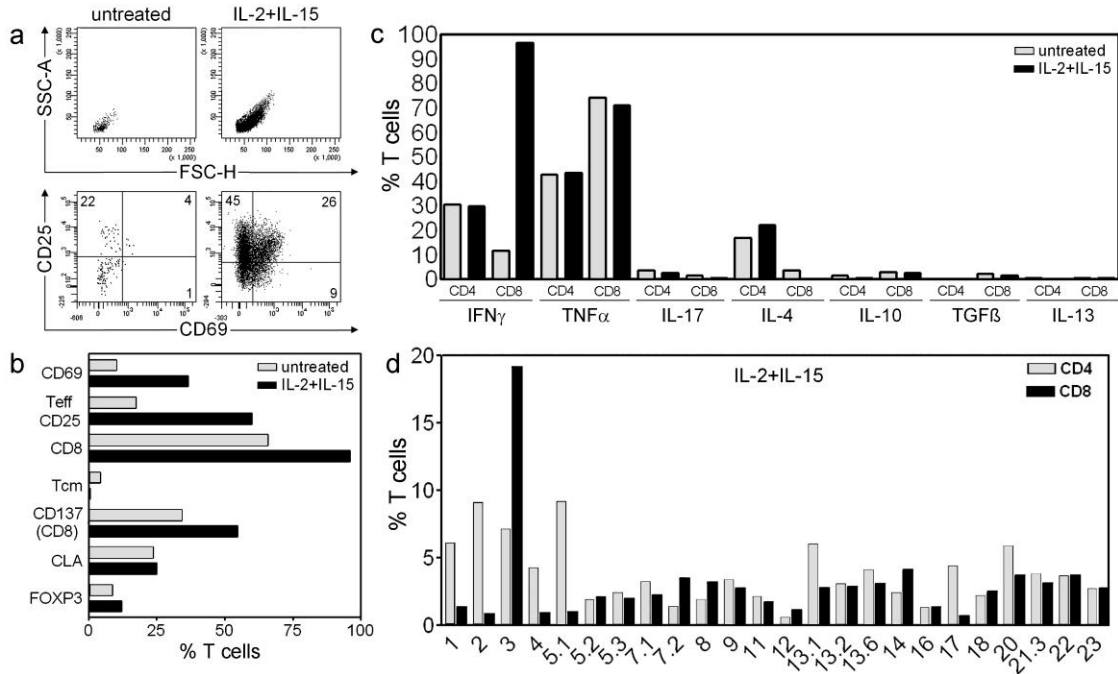


Figure 9. MCC metastases also contain T cells that can be activated and expanded by treatment with IL-2 and IL-15. (a) T cells from IL-2/IL-15 treated MCC metastases showed expansion and increased expression of the activation markers CD69 and CD25. **(b)** CD8⁺ effector cells preferentially proliferated and CD137 expression was enhanced on CD8 T cells. **(c)** IFN γ production was markedly enhanced and there was **(d)** significant skewing of the T cell repertoire. All histograms are gated to show CD3⁺ T cells.