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Human squamous cell carcinomas evade the immune response by down-regulation of vascular E-selectin and recruitment of regulatory T cells

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Squamous cell carcinomas (SCCs) of the skin are sun-induced skin cancers that are particularly numerous in patients on T cell immunosuppression. We found that blood vessels in SCCs did not express E-selectin, and tumors contained few cutaneous lymphocyte antigen (CLA)$^+$ T cells, the cell type thought to provide cutaneous immunosurveillance. Tumors treated with the Toll-like receptor (TLR)7 agonist imiquimod before excision showed induction of E-selectin on tumor vessels, recruitment of CLA$^+$ CD8$^+$ T cells, and histological evidence of tumor regression. SCCs treated in vitro with imiquimod also expressed vascular E-selectin. Approximately 50% of the T cells infiltrating untreated SCCs were FOXP3$^+$ regulatory T (T reg) cells. Imiquimod-treated tumors contained a decreased percentage of T reg cells, and these cells produced less FOXP3, interleukin (IL)-10, and transforming growth factor (TGF)-β. Treatment of T reg cells in vitro with imiquimod inhibited their suppressive activity and reduced FOXP3, CD39, CD73, IL-10, and TGF-β by indirect mechanisms. In vivo and in vitro treatment with imiquimod also induced IL-6 production by effector T cells. In summary, we find that SCCs evade the immune response at least in part by down-regulating vascular E-selectin and recruiting T reg cells. TLR7 agonists neutralized both of these strategies, supporting their use in SCCs and other tumors with similar immune defects.

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Abbreviations used: CCL, CC chemokine ligand; CLA, cutaneous lymphocyte antigen; hpf, high power field; iNOS, inducible nitric oxide synthase; PDC, plasmacytoid DC; SCC, squamous cell carcinoma; TLR, Toll-like receptor; T reg, regulatory T.

More than 100,000 squamous cell carcinomas (SCCs) of the skin are diagnosed each year in the United States (1). Nonmelanoma skin cancer, of which SCC is the second most frequent type, is the fifth most costly cancer, accounting for 4.5% of all Medicare cancer costs (2). The premalignant precursors to SCC, actinic keratoses, are the third most frequent reason in the United States for consulting a dermatologist (3). More than 5.2 million physician visits are made each year for the treatment of actinic keratoses at a cost of more than 900 million dollars annually (4).

Solid organ transplant recipients on immunosuppressive medications frequently develop multiple and aggressive SCCs (5). These individuals have a 65–250-fold increased risk of SCCs, nearly 10% of these tumors metastasize, and the majority of these patients die as a result (5, 6). The development of SCCs in transplant recipients is linked to the use of medications that suppress T cell activity (6). T cell function therefore appears critical to the immunological control of SCCs. We present our findings that SCCs from

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both healthy and immunocompromised individuals evade the immune response, at least in part, by down-regulation of vascular E-selectin, exclusion of skin-homing memory T cells, and recruitment of regulatory T (T reg) cells.

RESULTS

SCCs are infiltrated by diverse noncutaneous central memory T cells

SCCs of the skin commonly have associated T cell infiltrates, but the clinical persistence of the cancer suggests that these T cells are unable to destroy the tumor (7). We isolated T cells from human invasive SCCs and compared them with T cells from normal human skin, the population thought to provide immunosurveillance (Fig. 1) (8, 9). Peripheral tissue effector T cells express tissue-specific homing receptors and preferentially recirculate through the tissue in which they first encountered their antigens (10, 11). Skin resident T cells express the skin addressins cutaneous lymphocyte antigen (CLA) and CCR4, which bind to E-selectin and CC chemokine ligand (CCL) 22 on skin endothelium (9, 11, 12). T cells from SCCs did not express CLA and CCR4 (Fig. 1 A) and instead expressed L-selectin and CCR7, markers of central memory T cells that are normally found only in the blood or lymph nodes (13). Studies of cryosections confirmed that T cells from SCCs lack the skin addressin CLA (Fig. 1 B). T cells from SCCs developing in transplant recipients also lacked CLA and CCR4 expression (not depicted). Few SCC T cells were Th2 biased as demonstrated by the lack of two independent markers for Th2 bias, ST2L and the γ-IFN receptor β chain (Fig. 1 A) (14–16). Analysis of cytokine production by intracellular flow cytometry confirmed that most T cells from SCCs were Th1 biased (Fig. 1 C).

T cells resident in human skin have a diverse T cell repertoire, consistent with their role in immunosurveillance against a variety of pathogens and tumors (9). T cells in cervical cancers have a more biased repertoire, reflecting local expansion of tumor-specific T cell clones (17). We analyzed the TCR repertoire of T cells infiltrating SCCs by flow cytometry for Vβ TCR subfamilies and found that these cells were diverse, without detectable Vβ bias (Fig. 1 D).

Blood vessels of invasive SCCs in skin do not express E-selectin

E-selectin is a ligand for CLA that is expressed on postcapillary venules in the skin. Vascular E-selectin is up-regulated with inflammation and supports the entry of CLA$^+$ T cells into the skin under both normal and inflamed conditions (11, 12, 18). We examined tumor vessels for E-selectin expression by immunohistochemistry and found that blood vessels in areas surrounding the tumor (peritumoral areas) expressed E-selectin, but that blood vessels within the tumor parenchyma did not. (Fig. 2, A–C). Staining for CD31 was included to

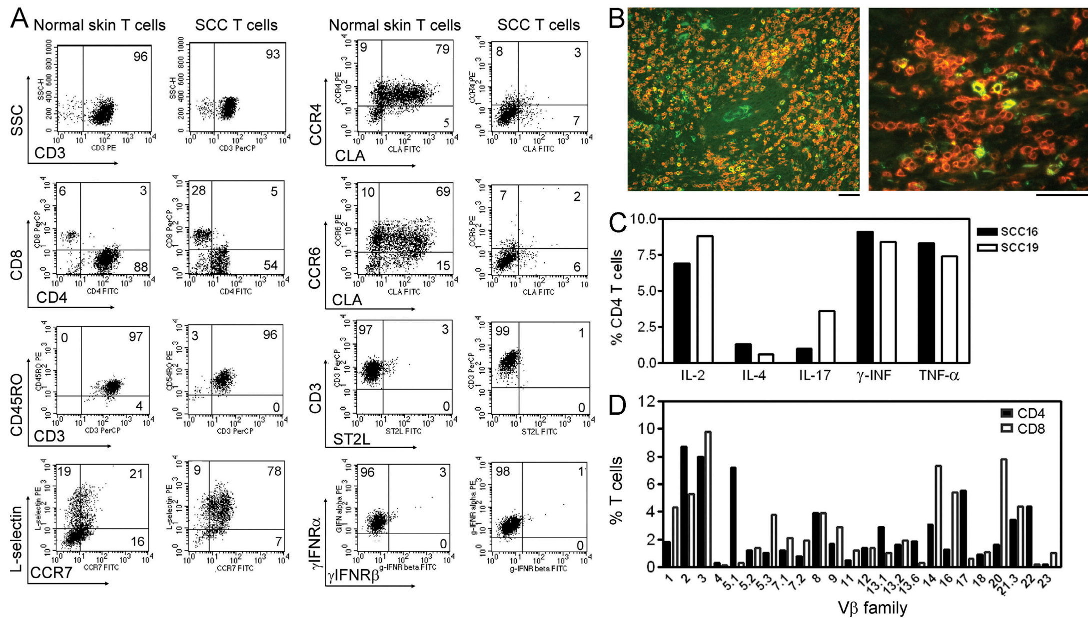

Figure 1. T cells infiltrating SCCs are noncutaneous central memory T cells. (A) T cells isolated from SCCs were memory (CD45RO$^+$) Th1-biased T cells that lacked expression of skin-homing addressins (CLA, CCR4) and instead expressed markers characteristic of central memory T cells (L-selectin/CCR7), a cell type that is usually restricted to blood or lymph nodes. In contrast, T cells from normal human skin expressed high levels of the skin-homing addressins CLA and CCR4 and most lacked CCR7/L-selectin coexpression. Similar results were observed in three additional SCC samples. (B) Confirmation that T cells infiltrating SCCs lack CLA expression. Frozen sections of invasive SCCs were stained for CD3 (red) and CLA (green). Only a small number of T cells infiltrating SCCs were skin-homing T cells (CD3$^+$CLA$^+$, yellow). A higher magnification of the same field is also shown. (C) Intracellular cytokine analysis of CD4$^+$ T cells from two SCC tumors demonstrated a Th1 bias with few Th2 or Th17 T cells present. Unstimulated cells produced no detectable cytokines (not depicted). (D) Analysis of T cells from SCCs by flow cytometry for TCR Vβ expression demonstrated significant TCR diversity. Analysis of two additional tumors showed comparable diversity. Bars, 100 µm.

identify blood vessels. This finding was observed in all SCCs studied; we performed immunohistochemical stains on tumors from four patients and conducted similar studies using three-color immunofluorescence analysis on tumors from five additional patients. Hoechst nuclear stain was used to confirm the presence of invasive tumor in immunofluorescence studies. Two additional SCCs excised from transplant recipients on immunosuppressive medications also lacked vascular E-selectin within the tumors (not depicted).

Imiquimod-treated SCCs express vascular E-selectin and are infiltrated by CLA+ skin-homing T cells

Imiquimod is a topical Toll-like receptor (TLR)7/8 agonist that is effective in the treatment of a wide range of skin

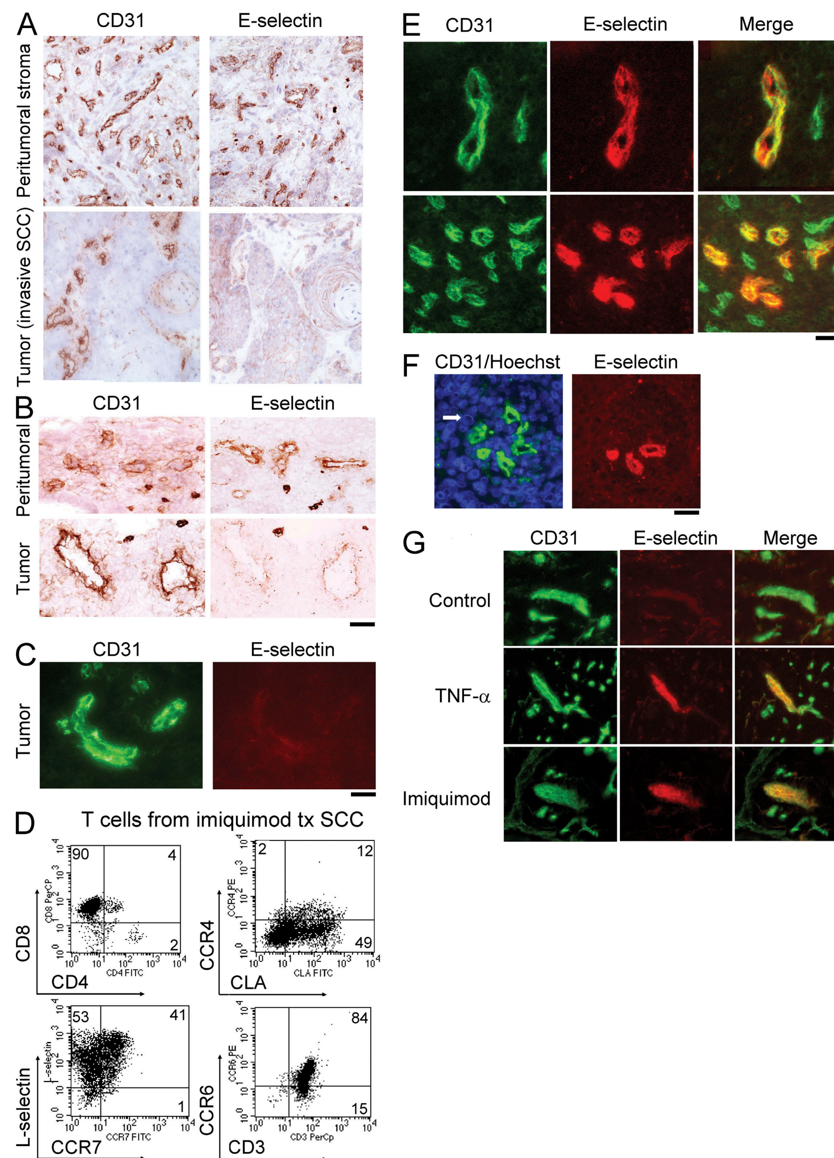

Figure 2. Blood vessels in areas of invasive SCCs do not express E-selectin, but imiquimod treatment induces vascular E-selectin expression and normalization of T cell homing. (A) Serial sections of SCCs demonstrated the presence of CD31+ blood vessels in areas of invasive SCCs, but these vessels lack E-selectin expression. (B) A second example is shown, in which tumor and peritumoral areas were present in a single stained section. (C) Immunofluorescence studies on a third sample stained for CD31 (green) and E-selectin (red), showing no E-selectin expression on tumor vessels. (D) SCCs treated with imiquimod before excision were heavily infiltrated with CLA+ (skin-homing) cytotoxic T cells. (E) A subset of tumor vessels in imiquimod-treated SCCs expressed E-selectin. (F) A second sample showing E-selectin expression on a subset of vessels embedded within a tumor nodule after imiquimod treatment. Large atypical keratinocytes forming a tumor nodule are demonstrated by Hoechst stain (blue). (G) In vitro treatment of SCC tumor tissue with imiquimod up-regulated E-selectin on a subset of tumor vessels. 2-mm bread loaf sections of untreated SCC tumor were incubated for 24 h in control medium, TNF-α, or imiquimod. Samples were then frozen, sectioned, and stained for CD31 and E-selectin. In all studies, Hoechst nuclear stain was used to identify areas of invasive tumor. Bars: (B, C, E, and F) 100 µm.

malignancies, including basal cell carcinomas and SCCs (19). We studied invasive SCCs treated with imiquimod from 10 to 14 d before tumor excision (mean 12.7 d). All treated tumors exhibited areas of fibrosis surrounding the tumors, consistent with stromal changes of tumor regression (not depicted). Imiquimod-treated SCCs contained >80% CD8^{+} cytotoxic T cells and, in contrast to untreated tumors, the majority of these T cells expressed CLA (Fig. 2 D). The majority also lacked L-selectin and CCR7 coexpression, suggesting a shift toward effector memory cells, the type normally found within the skin (9). T cells also expressed CCR6, but most lacked CCR4.

In contrast to untreated tumors, vessels of imiquimod-treated SCCs expressed E-selectin on a subset of tumor vessels (Fig. 2, E and F). Human endothelial cells have not previously been shown to respond to imiquimod. To study the ability of imiquimod to induce E-selectin expression ex vivo, we cultured portions of an untreated SCC tumor with imiquimod or TNF-α in vitro. Imiquimod and TNF-α had similar effects; both induced E-selectin expression on a subset of tumor vessels, likely representing postcapillary venules (Fig. 2 G).

Imiquimod indirectly up-regulates E-selectin on human endothelial cells

To determine if endothelial cells were the direct targets of imiquimod, we measured the expression of TLR7 and TLR8 in dermal microvascular endothelial cells by immunostaining and real-time PCR. Blood vessels in SCCs expressed TLR7 and TLR8 by immunostaining (Fig. 3 A). However, TLR antibodies can be cross-reactive, so expression was confirmed by real-time PCR analysis of purified cultures of dermal microvascular endothelial cells (Fig. 3 B). Although purified endothelial cells expressed TLR7 and TLR8, they did not up-regulate E-selectin when treated in vitro with imiquimod (Fig. 3 C). In contrast, inclusion of activated APCs in the co-cultures or overnight treatment with TNF-α resulted in a robust induction of E-selectin.

To determine if SCC tumor cells could suppress endothelial expression of E-selectin, we co-cultured endothelial cells with the SCC cell line SCC13 in the presence or absence of imiquimod and TNF-α (Fig. 3 D). We found no effect on the baseline or induced levels of endothelial E-selectin, suggesting that SCC tumor cells themselves do not directly suppress vascular E-selectin expression.

SCCs are infiltrated by FOXP3^{+} T reg cells

We further characterized the central memory T cells infiltrating SCCs and found they contained a large population of CD25^{hi}CD69^{lo} T cells, a phenotype similar to that of natural T reg cells (Fig. 4 A) (20). Natural T reg cells, which develop as a separate lineage within the thymus, can be distinguished from other T cells by their constant and high expression of the transcription factor FOXP3 (21–23). We and others have found excellent correlation of high FOXP3 expression with suppressive ability (20). T cells isolated from SCCs of both normal and

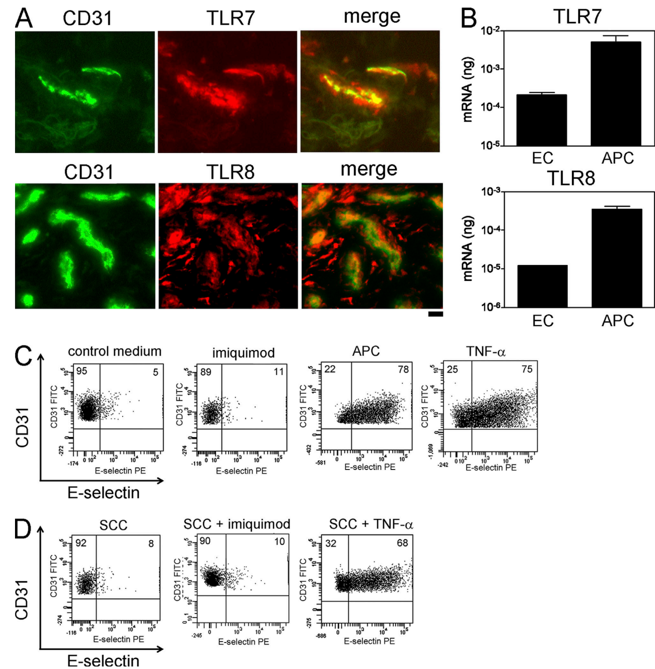

Figure 3. Imiquimod up-regulates E-selectin on endothelial cells by an indirect mechanism. (A) Immunofluorescence studies on frozen sections of SCC tumor stained for the vascular marker CD31 (green) and TLR7 or TLR8 (red). (B) Confirmation of TLR7 and TLR8 expression by real-time quantitative PCR. Cultured dermal microvascular endothelial cells were analyzed for TLR7 and TLR8 expression and compared with T cell–depleted peripheral blood mononuclear cells (APCs). (C) Imiquimod does not directly induce E-selectin on endothelial cells. Purified human endothelial cells were cultured with imiquimod or activated APC for 3 d or TNF-α for 12 h, and then harvested and assayed by flow cytometry for the expression of CD31 and E-selectin. Experiments using two additional endothelial cell donors produced similar results. (D) SCC tumor cells do not suppress baseline or induced E-selectin expression on endothelial cells. SCC13 tumor cells were co-cultured with endothelial cells in control medium (SCC), with imiquimod (SCC+imiquimod) or with TNF-α (SCC+TNF-α). No changes in basal or induced levels of endothelial E-selectin were observed. Experiments using two additional endothelial donors produced similar results. Bar, 100 µm.

immunocompromised patients had greatly increased numbers of FOXP3^{+} T reg cells when compared with the population found in normal skin (Fig. 4 A). SCC FOXP3^{+} T reg cells were CD4^{+}, lacked expression of addressins found on skin resident T reg cells (CLA, CCR4, and CCR6) (20), and instead expressed markers of central memory T cells (Fig. 4 B, L-selectin/CCR7^{+}). FOXP3 is expressed at high and constant levels by T reg cells, but expression of FOXP3 is also transiently increased in activated non–T reg cells (20, 21, 24). Our earlier work has shown that although activated T cells increase expression of FOXP3, this transient increase was small and did not obscure the identification of true T reg cells, which expressed FOXP3 at levels a log higher than activated non–T reg cells (20). CD127 negativity has recently been reported to discriminate between T reg cells and effector

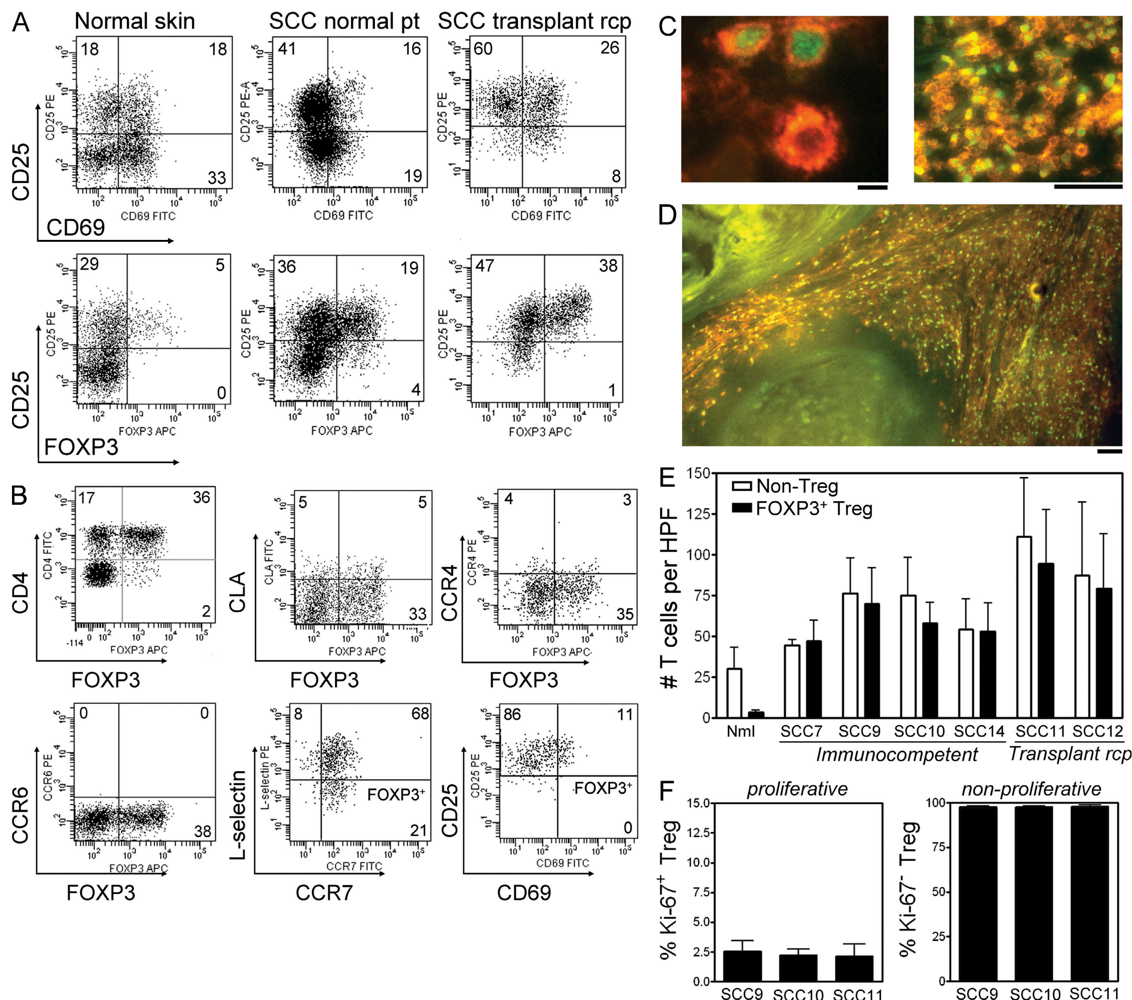

Figure 4. SCCs are heavily infiltrated by FOXP3+ T reg cells recruited from blood. (A) T cells isolated from SCCs developing in normal individuals and transplant recipients contained many $CD25^{hi}FOXP3^+$ T reg cells. (B) T reg cells isolated from SCCs were $CD4^+$ central memory T cells (L-selectin/$CCR7^+$) and were distinct from cutaneous T reg cells found in normal skin as shown by their lack of expression of key skin-homing addressins (CLA, CCR4). The last two graphs are gated to show only $CD3^+FOXP3^+$ T cells. (C) Direct study of $FOXP3^+$ T reg cells in areas of invasive SCCs using immunofluorescence staining of frozen sections. SCCs were stained for CD3 (red) and FOXP3 (green). Two $FOXP3^+$ T reg cells are shown at the top of the left image, and a $FOXP3^-$ nonregulatory T cell is shown on the bottom. A larger field is shown in the right image. (D) A lower magnification image of another SCC, demonstrating that large numbers of $FOXP3^+$ T reg cells (red cells with green nuclei) surround nodules of invasive tumor, which appear as pools of green secondary to nonspecific staining of tumor keratin. (E) Enumeration of T reg cells in frozen sections of SCCs. The number of T reg cells and nonregulatory T cells were counted in 10 high power (40X) fields in SCCs from normal patients (Immunocompetent) and transplant recipients (Transplant rcp) and the results were compared with that of normal skin. Shown are the mean and SD of counts from 10 fields. (F) $FOXP3^+$ T reg cells are not locally expanded within SCCs. SCC sections were costained for FOXP3 and Ki-67, a marker of cell proliferation. Proliferative and nonproliferative $FOXP3^+$ T reg cells were counted in 5 hpf for each donor; the mean and SD for each tumor are shown. SCC9 and 10 are from immunocompetent individuals; SCC11 is from a transplant recipient. Bars: (C, left) 10 µm; (C, right, and D) 100 µm.

cell populations in humans (25–27); we found that SCC $FOXP3^+$ T cells lacked expression of CD127, suggesting that they do not represent recently activated non–T reg cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071190/DC1).

Our method of T cell isolation from skin depends upon the ability of T cells to migrate out of the skin in response to chemokines produced by dermal fibroblasts (28). T cells with central memory markers may not migrate efficiently to skin cell chemokines because they express different chemokine receptors (Fig. 1). We therefore confirmed our findings using primary SCC tumor tissue. We used three-color immunofluorescence staining of frozen sections of SCC tumors to enumerate non–T reg ($FOXP3^-CD3^+$) and T reg ($FOXP3^+CD3^+$) cells in areas of invasive SCCs. Nearly 50% of the T cells infiltrating SCCs from both normal and immunosuppressed patients were $FOXP3^+$ T reg cells (Figs. 4 E and 5 C). Approximately 50–60% of the total T cells in SCCs were $CD4^+$ (Fig. 1 A), suggesting that the vast majority of $CD4^+$ T cells in SCCs are actually $FOXP3^+$ T reg cells.

To determine if T reg cells are recruited from the blood or locally expanded within the tumor, we stained tumors for expression of Ki-67, an antigen expressed by dividing cells. We observed very few proliferating T reg cells in SCCs, suggesting that recruitment of these cells from the blood is the predominant mechanism for their accumulation within SCCs (Fig. 4 F).

Imiquimod treatment is associated with decreased percentages of FOXP3+ T reg cells

T cells isolated from SCC tumors treated with imiquimod contained decreased percentages of FOXP3+ T reg cells (Fig. 5 A). We counted the absolute number of T reg cells in the cryosections of imiquimod-treated tumors and found that this drop in the percentage of T reg cells resulted from a marked influx of non–T reg cells into the tumor, most of which were cytotoxic CD8+ T cells (Figs. 5 B and 2 D). FOXP3+ T reg cells were still present in treated tumors, but the percentage of these cells was reduced to roughly 10%, similar to that found in normal human skin (Fig. 5, B and C).

Imiquimod inhibits T reg cell function

TLR8 agonists can block the ability of T reg cells to suppress T cell responses, suggesting that imiquimod may have an effect on tumor-associated T reg cells (29). We isolated T reg cells from normal human skin and studied the effect of imiquimod on these cells. In vitro treatment of purified skin T cells with imiquimod for 1 wk had no direct effect on the viability of FOXP3+ T reg cells, suggesting that T reg cells are not depleted by imiquimod (Fig. 5 D). Our previous studies demonstrate that skin-resident natural T reg cells proliferate in response to culture with dermal fibroblasts and IL-15 (20). We found that imiquimod only slightly reduced proliferation of FOXP3+ T reg cells under these conditions (Fig. 5 E). To study T reg cell function, we cultured skin explants with

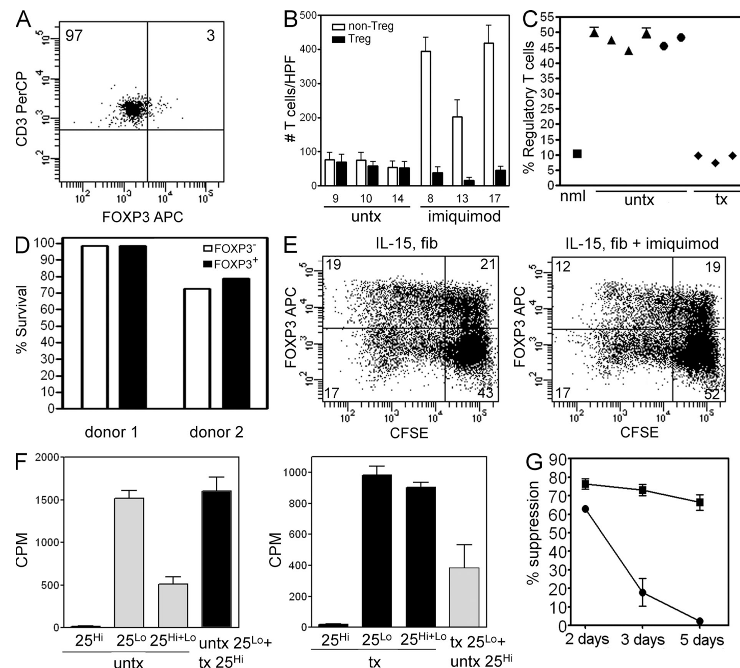

Figure 5. Imiquimod-treated SCCs contain decreased percentages of FOXP3+ T reg cells and imiquimod treatment in vitro blocks the ability of T reg cells to suppress. (A) T cells isolated from imiquimod-treated SCCs contain few detectable FOXP3+ T reg cells. (B) Direct enumeration of FOXP3+ T reg cells in sections of imiquimod-treated SCCs (tx). Counts from untreated SCCs (untx) are shown for comparison. Mean and SD are shown. (C) Percentage of FOXP3 T reg cells infiltrating normal human skin (nml), untreated SCC tumors (untx, triangles; SCCs from healthy individuals, circles; SCCs from transplant recipients) and imiquimod-treated SCCs (tx). Bars indicate the SD of the percentage of T reg cells from 10 hpf. (D) Imiquimod does not affect the viability of nonregulatory T cells (FOXP3−) and FOXP3+ T reg cells (FOXP3+) isolated from human skin. Viability was assessed after 1 wk of incubation in either control medium or imiquimod. (E) Imiquimod only slightly inhibits the proliferation of skin-derived T reg cells. T cells from human skin were labeled with CFSE and cultured with dermal fibroblasts and IL-15 for 1 wk in the presence or absence of imiquimod. Cells were then stained for FOXP3 expression. FOXP3+ T reg cells that have proliferated are shown in the top left quadrant of each histogram. (F) Imiquimod treatment paralyzes regulatory T cell function. T cells isolated from human skin were cultured for three days in control medium (untx, gray bars) or imiquimod (tx, black bars), and then separated into T reg cells, enriched CD25hi T cells (CD25hi), and responder CD25lo T cells (CD25lo). Cells were stimulated with soluble anti-CD3 and –CD28 and proliferation was assayed by incorporation of [3H]thymidine. Untreated CD25hi suppressed CD25lo T cell proliferation, but pretreatment of CD25hi cells with imiquimod blocked suppression. Suppression of imiquimod-treated CD25lo was restored by adding untreated CD25hi T cells, demonstrating that the suppressive defect was is the CD25hi subset. (G) At least three days of imiquimod pretreatment is required for loss of suppressive function. Skin T cells were cultured in control medium (squares) or imiquimod (circles) for the indicated length of time, and then cells were sorted and analyzed for suppressive ability. Bars indicate the SD of experiments from two different skin donors.

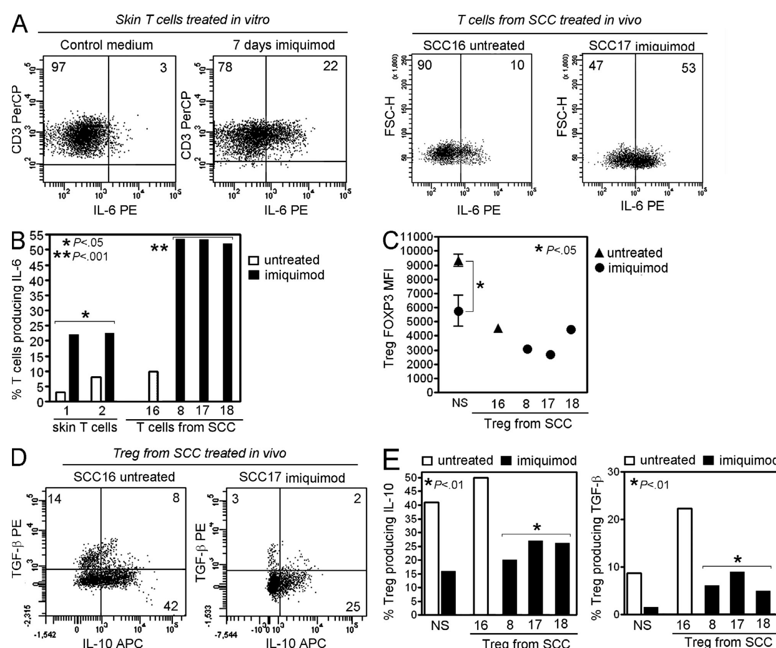

Figure 6. Imiquimod induces IL-6 production by effector T cells and reduces FOXP3 and production of IL-10 and TGF-β by T reg cells. (A) Imiquimod treatment induces IL-6 production by T cells in vitro and in vivo. Imiquimod or control medium was added to explant cultures of normal human skin for 1 wk. T cells were then isolated from these cultures and examined for IL-6 production. Imiquimod treatment of purified skin T cells alone had no effect. T cells were isolated from SCCs that were either untreated (SCC16) or treated in vivo with topical imiquimod (SCC8, 17, 18) and analyzed for IL-6 production. Histograms of SCC T cells are gated to show only $CD3^+$ T cells. (B) IL-6 production from multiple donors after in vitro (skin T cells) or in vivo (SCC; 8, 17, 18) treatment with imiquimod. (C) FOXP3 expression as assayed by mean fluorescence intensity (MFI) under identical staining conditions in T cells from normal skin explant cultures (NS) treated for 1 wk with control medium (triangle) or imiquimod (circle, mean and SD of three determinations are shown) or from untreated SCCs (16; triangle) or SCCs treated in vivo with imiquimod (circles; 8, 16, 18). (D) IL-10 and TGF-β production as determined by intracellular cytokine analysis of $FOXP3^+$ T reg cells isolated from untreated (SCC16) or imiquimod treated SCCs (SCC17). (E) IL-10 and TGF-β production by $FOXP3^+$ T reg cells isolated from normal skin explant cultures (NS) treated for 1 wk with control medium (white bars) or imiquimod (black bars) and by $FOXP3^+$ T reg cells isolated from untreated SCCs (16) or SCCs treated in vivo with imiquimod (8, 17, 18).

either imiquimod or control medium for 3–5 d. We then collected T cells from explant cultures, isolated enriched populations of $FOXP3^+$ T reg cells by high speed flow cytometry sorting for $CD3^+CD4^+CD25^{hi}CD69^{lo}$ T cells, and tested the ability of these cells to suppress the proliferation of T cells isolated from the same skin sample, as described previously (20). When suppression assays, which lasted 6 d, were performed in the absence of imiquimod, we found that $CD25^{hi}$ T reg cells pretreated for 3 d with imiquimod failed to suppress T cell proliferation, whereas untreated $CD25^{hi}$ T reg cells from the same sample of skin did suppress T cell responses (Fig. 5 F). The reciprocal experiment showed that pretreatment of both $CD25^{lo}$ responder cells and $CD25^{hi}$ T reg cells with imiquimod showed no suppression, but the addition of untreated $CD25^{hi}$ T cells restored suppression. This effect depended on the pretreatment of T reg cells with imiquimod; 5 d of pretreatment with imiquimod produced similar results, but 2 d of treatment induced only a partial loss of suppressive ability, suggesting that inactivation of T reg cells required at least 3 d of imiquimod treatment (Fig. 5 G). When imiquimod was additionally added to suppression assays, we found that effector T cells were less susceptible to suppression by untreated T reg cells (not depicted). These findings suggested that imiquimod may affect both T reg and non–T reg cells.

Imiquimod indirectly up-regulates IL-6 production by effector T cells

In our suppression assays, imiquimod was added to skin explant cultures during the final period before T cell collection. We could not determine from these experiments if the effects of imiquimod on T cells were direct or were mediated by factors produced by other cells within the skin.

Imiquimod induces the production of IL-6 from human monocytes, plasmacytoid DCs (PDCs), and keratinocytes, and the production of IL-6 by T cells or nearby DCs has been shown in mice to render T cells resistant to suppression by T reg cells (30–34). T cell expression of IL-6 in response to imiquimod has not been previously reported. We found that the addition of imiquimod to skin explant cultures for 1 wk induced IL-6 expression in 22% (SD of 0.2) of skin T cells compared with a baseline expression of 5.5% (SD of 2.5; Fig. 6, A and B). We then examined T cells isolated from SCCs treated in vivo with imiquimod and found that 53% (SD of 0.64) of T cells from treated tumors produced IL-6,

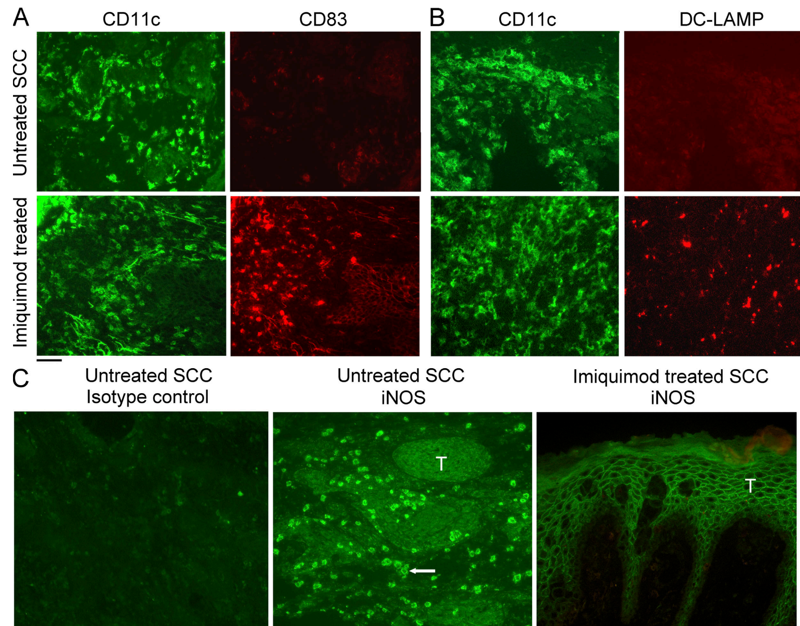

Figure 7. SCCs contain immature and iNOS⁺ DCs. Cryosections of untreated and imiquimod-treated SCCs were immunostained for the DC marker CD11c and markers of DC maturation CD83 (A) and DC-LAMP (B). Numerous immature DCs were present in untreated SCCs, whereas treated SCCs contained significant numbers of mature DC. Untreated SCCs also contained a population of cells that expressed high levels of iNOS (C, indicated by arrow), but these cells were not detected in imiquimod-treated SCCs. There was also some staining of tumor cells (T) in both untreated and treated tumors. A concentration-matched isotype control for iNOS staining is shown in the first image. Bars, 100 µm.

compared with 10% from an untreated SCC (Fig. 6, A and B). In contrast, culture of purified skin T cells with imiquimod did not induce IL-6 production (not depicted), suggesting that IL-6 induction occurs by an indirect mechanism.

Imiquimod indirectly decreases expression of FOXP3, IL-10, TGF-β, CD39, and CD73

Our suppression assays demonstrate that imiquimod inhibits the ability of T reg cells to suppress. To study this further, we examined the effect of imiquimod on T cells isolated from human skin. When imiquimod was added to explant cultures for 1 wk before T cell isolation, expression of the FOXP3 protein by T reg cells was significantly decreased (Fig. 6 C). FOXP3 protein expression by T reg cells correlates with their ability to suppress T cell responses (21). We then examined FOXP3 expression in T reg cells isolated from SCCs and found that although levels of FOXP3 in imiquimod-treated tumors tended to be lower than in untreated SCCs, this difference was not significant (Fig. 6 C).

T reg cells can suppress T cell responses by the production of cytokines such as IL-10 and TFG-β, and by cell-contact mechanisms shown recently to involve, in part, the action of CD39 and CD73 on T reg cells (35). Imiquimod added to skin explant cultures decreased the production of both IL-10 and TFG-β by FOXP3⁺ T reg cells (Fig. 6 E). Moreover, in addition to being present at fivefold lower numbers, T reg cells isolated from SCCs treated in vivo with imiquimod produced less IL-10 and TFG-β (Fig. 6, D and E). Lastly, we found that CD39 and CD73 were preferentially expressed by FOXP3⁺ T reg cells versus FOXP3⁻ effector T cells, and that treatment in vitro with imiquimod reduced the expression of both CD39 and CD73 on T reg cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071190/DC1). Treatment of purified skin T cells with imiquimod did not affect T reg cell surface marker expression or cytokine production, arguing for an indirect mechanism of effect. In summary, we find that imiquimod decreases T reg cell FOXP3, CD39, and cytokine production and that this effect is dependent on the presence of other cells resident in skin.

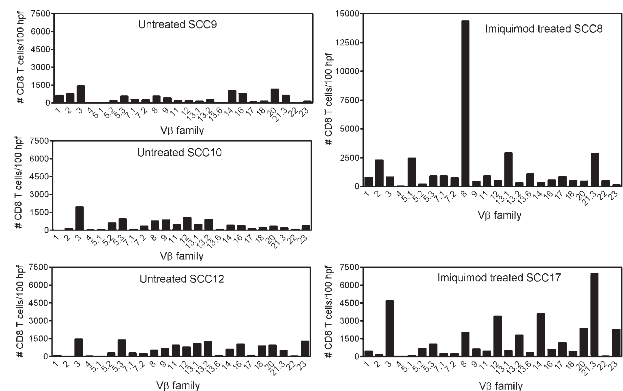

Figure 8. Imiquimod treated tumors contain expanded populations of TCR-biased cytotoxic T cells. T cells were isolated from untreated and imiquimod-treated SCCs and analyzed for Vβ expression by flow cytometry. Data are presented as the absolute number of CD8⁺ T cells of each Vβ family in 100 hpf. Imiquimod-treated tumors contained larger numbers of cytotoxic T cells, and these T cells had clearly biased Vβ repertoires, consistent with local expansion of tumor-specific T cells.

SCCs are infiltrated by immature and inducible nitric oxide synthase (iNOS)+ DCs

We have found that two important effects of imiquimod, the induction of vascular E-selectin and immunomodulation of T reg and effector T cells, appear to occur via indirect mechanisms. Human dermis contains macrophages and dermal DCs, and SCCs also contain PDCs (36, 37); these cell types have been reported to respond directly to imiquimod. We analyzed SCCs by immunostaining and found that these tumors contain large numbers of $CD11c^+$ DCs (Fig. 7, A and B, DC). However, most DCs were immature, as demonstrated by their lack of DC-LAMP and CD83 expression. In contrast, imiquimod-treated SCCs contained mature DCs expressing both CD83 and DC-LAMP, consistent with reports that imiquimod induces maturation of DCs (31, 38). Additionally, a population of cells expressing high levels of iNOS was present in untreated but not imiquimod-treated SCCs (Fig. 7 C). These cells co-stained weakly for CD11c, but were negative for CD34 (not depicted), suggesting that they represent DCs as opposed to myeloid-derived suppressor cells (39, 40).

Imiquimod-treated tumors contain expanded clonal populations of cytotoxic T cells

Our findings suggest that imiquimod treatment allows the entry of tumor-specific T cells into SCCs and may also block the ability of T reg cells to suppress the activity and proliferation of these cells once they enter the tumor. Expansion of tumor-specific T cells would be expected to produce a skewing of the T cell repertoire in imiquimod-treated tumors. To evaluate this, we analyzed the TCR repertoire of $CD8^+$ T cells isolated from untreated and imiquimod-treated tumors using flow cytometry for TCR Vβ families (Fig. 8). Untreated tumors were infiltrated by small numbers of diverse CD8 T cells, but imiquimod-treated tumors contained larger numbers of $CD8^+$ T cells with a markedly skewed Vβ repertoire. This skewed repertoire, together with the histological changes of tumor regression seen in treated tumors, suggests successful proliferation and infiltration of tumor-specific $CD8^+$ T cells in treated but not in untreated tumors.

DISCUSSION

Cutaneous immunosurveillance is an invisible process when it is functioning properly. Normal human skin contains 1 million memory T cells/cm^2, and there are nearly twice as many T cells resident in normal skin than are present in the entire circulation (9). This suggests that immune surveillance of the skin is a high priority for the immune system. The susceptibility of individuals on T cell–immunosuppressant medications to SCCs suggests that T cells may play a role in controlling these tumors. We were intrigued by the fact that SCCs are heavily infiltrated by T cells that nonetheless fail to control tumor growth. We find that SCCs from both healthy and immunocompromised individuals exclude skin-homing memory T cells, and instead recruit a population of T reg cells normally restricted to the blood and lymph nodes.

Effector memory T cells preferentially migrate through the peripheral tissue in which they first encountered antigen (10, 11). This tissue-specific migration ensures that tissues are populated by T cells specific for pathogens likely to be encountered again in that tissue. Such T cells can migrate preferentially because they express addressins that bind to specific counterreceptors on the endothelium of a particular tissue. For example, CLA on skin-homing T cells binds to E-selectin expressed on cutaneous postcapillary venules, supporting the entry of T cells into the skin under both normal and inflamed conditions (11, 12, 18). SCC-specific T cells should express CLA because they first encounter antigen within the skin-draining lymph nodes. We have found that SCCs do not express E-selectin on tumor vessels and are not infiltrated by CLA^+ skin-homing T cells. The tumor therefore excludes the population of skin-homing memory T cells expected to contain tumor-specific T cells. We observed a lack of vascular E-selectin and exclusion of CLA^+ T cells from SCCs arising in both normal and immunosuppressed individuals, suggesting that aberrant T cell homing occurs in tumors from both clinical groups.

Impaired T cell homing also occurs in other types of human cancer. Reduced expression of adhesion molecules on blood vessels has been described in human breast, gastric, and lung cancer (41, 42). Melanoma metastases express low levels of the addressins E-selectin, P-selectin, and ICAM-1, and this is associated with low numbers of T cells within the metastatic tumor nodules (43). Because of its readily accessible location, cutaneous SCC is a model cancer in which to study the defective T cell homing that may underlie poor immune responses to several human cancers. Additional studies of the nature of endothelial cells within SCCs will be critical to understanding how these tumors regulate the expression of vascular addressins.

T reg cells can suppress the activation, cytokine production, and proliferation of other T cells, and they are crucial to the development and maintenance of self-tolerance (21, 44, 45). We have found that up to 50% of the T cells infiltrating cutaneous SCCs from both normal and immunosuppressed individuals are $FOXP3^+$ T reg cells. These cells form a dense infiltrate surrounding tumor nests and are well positioned to impair the responses of effector T cells that gain access to the tumor. T reg cells from SCCs lack CLA and CCR4 and are therefore distinct from the T reg cells that populate normal skin (20). Instead, T reg cells from SCCs coexpress L-selectin and CCR7. This phenotype is similar to that of central memory T cells, a type of cell found normally only in the blood or lymph nodes. We observed no local proliferation of T reg cells in tumors (Fig. 4 F), and thus recruitment from the blood may be the primary mechanism for enrichment of these cells in tumors. T reg cells are recruited to ovarian carcinoma by the interaction of tumor CCL22 with CCR4 on T reg cells, and recruitment of T reg cells to Hodgkin lymphomas also involves CCR4 (46). However, CCR4 is highly expressed by T cells that provide immunosurveillance of the skin (9), and recruitment of this subset would likely be detrimental to tumor

survival. In fact, we see no expression of CCR4 on the T reg cells infiltrating SCCs, suggesting another addressin must be responsible for recruiting these and other central memory T cells into tumors. Preliminary studies in our laboratory have shown that SCC vessels do not express peripheral node addressin, a group of L-selectin ligands that recruit memory and naive L-selectin–expressing T cells into lymph nodes, nor do they express the CCR7 ligands CCL19 and CCL21 (unpublished data) (47). This is consistent with the lack of CD45RA$^+$ naive T cells in tumors. Ongoing studies in our laboratory are focused on identifying the vascular ligands expressed by SCC tumor vessels that support the recruitment of central memory T cells.

Imiquimod is a topical immune response modifier that is effective in the treatment of basal cell carcinomas, SCCs, and SCC precursor lesions actinic keratoses (19). Imiquimod induces tumor regression via both immunological and nonimmunological mechanisms by activating TLR7 and TLR8 and binding to adenosine receptors (48–51). Imiquimod stimulates blood mononuclear cells to produce a variety of inflammatory cytokines, including IFN-α, TNF-α, IL-1, IL-12, IL-6, IL-8, and IL-10 (32, 52–56). Clinical response to topical imiquimod has been associated with the migration of PDCs into the skin and subsequent cytokine production (57, 58). It has been suggested that SCCs may be infiltrated by Th2-biased T cells, and that clearance of these tumors after imiquimod therapy might be a result of a shift from Th2- to Th1-biased immunity (59). However, we found very few Th2-biased T cells in SCCs (Fig. 1 A). Thus, lack of tumor destruction is not likely a result of Th-2 bias among tumor-infiltrating T cells.

Our results show that treatment of SCC with imiquimod is associated with induction of E-selectin on tumor vessels, infiltration by CLA$^+$ skin-homing CD8$^+$ cytotoxic T cells, and histological evidence of tumor regression. In vitro treatment of SCCs with imiquimod induced E-selectin on tumor vessels, suggesting that this medication may act to restore normal T cell homing, allowing CLA$^+$ skin-homing T cells access to the tumor, where they can initiate tumor destruction. We have found that SCC blood vessels and dermal microvascular endothelial cells express TLR7 and TLR8, but do not respond directly to imiquimod. Vascular responses to imiquimod therefore require the presence of APCs or other imiquimod-responsive cell types. To respond directly to TLR7/8 agonists, a cell must take up and deliver agonists to the endosomal compartment where TLR7/8 are located, and endosomes must subsequently undergo acidification and maturation (60). Thus, human endothelial cells may not respond directly to TLR7/8 agonists because they are nonphagocytic or lack endosomal maturation.

In addition to its effect on E-selectin expression, imiquimod treatment of SCC results in a fivefold reduction in the percentage of tumor-infiltrating FOXP3$^+$ T reg cells. Treated tumors contain vastly increased numbers of CD8$^+$ T cells, in essence diluting out tumor T reg cells. In a mouse model of sarcoma, it was the relative percentage of FOXP3$^+$ T reg cells that distinguished progressively growing tumors from those that spontaneously regressed (61). Thus, the dilution of tumor T reg cells by recruitment or local expansion of CD8$^+$ T cells may tip the balance toward immunological destruction.

We hypothesized that imiquimod may inhibit tumor-associated T reg cells, given that TLR8 ligation was recently shown to block the suppressive ability of T reg cells (29). Indeed, we found that treatment of T reg cells with imiquimod in vitro blocked their ability to suppress T cell proliferation without reducing viability. Imiquimod treatment decreased the expression of FOXP3 and production of the cytokines IL-10 and TGF-β in T reg cells isolated from human skin. CD39 and CD73 have been recently implicated in contact-dependent suppression by T reg cells in mice; we found that both CD39 and CD73 were preferentially expressed by T reg cells and that expression was down-regulated after treatment with imiquimod (35). These effects were observed if imiquimod was added to the skin explant cultures before T cell isolation, but direct treatment of purified skin T cells had no effect, suggesting an indirect mechanism mediated by another cell type present in skin. Candidate responsive cells in skin include PDCs, macrophages, and keratinocytes, each of which has been shown to respond directly to imiquimod (30–32). T reg cells isolated from SCCs treated in vivo with imiquimod also had decreased IL-10 and TGF-β production, confirming that the effects we see in vitro are also present in vivo.

In addition to the effect on T reg cells, imiquimod induced the production of IL-6 by effector T cells, albeit by an indirect mechanism. To our knowledge, imiquimod-stimulated IL-6 production by T cells has not been reported previously. IL-6 production by murine T cells renders them resistant to suppression by T reg cells, and early reports suggest a similar effect occurs in human psoriatic T cells (34, 62, 63). Thus, TLR agonists such as imiquimod both decrease the suppressive activity of T reg cells and increase the resistance of effector cells to suppression. Again, this effect was indirect and required the presence of other cells in skin. Over 50% of effector T cells isolated from imiquimod-treated SCCs produced IL-6, compared with 10% in an untreated tumor, confirming the in vivo relevance of this finding. Studies to identify the imiquimod-responsive cells in skin and the signals that mediated endothelial and T cell responses to imiquimod are ongoing in our laboratory.

The indirect nature of imiquimod's effects on endothelial and T cells highlights the critical role of innate immune cells such as DCs in tumor responses. We found large numbers of immature DCs in untreated SCCs, whereas mature DCs were evident only after imiquimod treatment. Immature DCs within tumors can prevent proper antigen presentation and can induce the formation of T reg cells (64). We also observed a population of iNOS-expressing DCs in untreated, but not treated, SCCs. Nitric oxide (NO) impairs the ability of human endothelial cells to express E-selectin in vitro (65, 66). Exogenously produced NO down-regulates the expression of MAdCAM-1 on gut vessels, decreases lymphocyte rolling, and has been proposed as a possible therapy for inflammatory

bowel disease (67). We are currently investigating the possibility that NO production suppresses E-selectin expression on tumor vessels.

We have evidence for three novel mechanisms of action for imiquimod that may initiate and sustain the immunological destruction of SCC. First, imiquimod up-regulates E-selectin on tumor vessels. This E-selectin expression is associated with an influx of CLA$^+$ cytotoxic T cells into the tumor, thereby delivering potentially tumor-reactive T cells to the cancer while at the same time diluting out tumor T reg cells. Second, imiquimod inhibits the suppressive activity of T reg cells, decreasing the levels of FOXP3 protein and surface molecules associated with contact inhibition, as well as reducing the production of immunosuppressive cytokines. Lastly, imiquimod induces the production of IL-6 by effector T cells that may render them resistant to suppression by T reg cells.

Impaired T cell homing and recruitment of regulatory T cells are features of many human cancers. We find that the TLR7 agonist imiquimod neutralizes both of these defenses, supporting the use of this medication in SCCs and in other tumors that use similar strategies to evade the immune response.

MATERIALS AND METHODS

SCC samples. Tumor samples consisted of curetted tumor removed before taking the first Mohs section during Mohs micrographic excision of biopsy-proven SCCs. Acquisition of tumor samples and all studies were approved by the Institutional Review Board of the Dana Farber Cancer Institute and were performed in accordance with the Declaration of Helsinki. Tumors were divided into bread loaf sections. Adjacent sections were used for (a) immunohistochemical or immunofluorescence studies to confirm the presence of invasive tumor cells and (b) T cell isolation, as described in the following section. The SCC13 SCC cell line was provided by J. Rheinwald (Brigham and Women's Hospital, Boston, MA).

Isolation of T cells from SCC tumors and normal skin. The clinically evident portions of biopsy-proven invasive SCC tumors were obtained. The tumors were divided into bread loaf sections. One section was histologically examined to confirm the presence or absence of invasive SCC tumor. Sections without evidence of tumor were designated peritumoral. Tumor-infiltrating T cells were isolated from sections adjacent to those studied by histology. T cells were isolated from SCC tumors in the absence of exogenous cytokines, as previously described (28). For normal skin studies, T cells were isolated from skin discarded after plastic surgery procedures from 3 wk explant cultures (28). Skin was provided by T. Cochran (Boston Center for Plastic Surgery, Boston, MA) and E. Eriksson (Brigham and Women's Hospital, Boston, MA).

Flow cytometry studies. Flow cytometry analysis of T cells was performed using directly conjugated monoclonal antibodies obtained from: BD Biosciences (CD3, CD4, CD8, CD25, CD69, CD45RO, and IL-10), BD PharMingen (CLA, CCR4 [1G], CD73, and CCR6), Abcam (CD39), Beckman Coulter (L-selectin), R&D Systems (TGF-β, CCR7, and CD127), and eBioscience (FOXP3, clone PCH101). 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. SCC tumors were embedded in OCT, frozen, and stored at −80°C until use. 5-μm cryosections were cut, air dried, fixed for 5 min in acetone, rehydrated in PBS, and blocked with 20 μg/ml of human IgG (Jackson ImmunoResearch Laboratories) for 15 min at room temperature. Sections were incubated with primary antibody for 30 min, and then rinsed three times in PBS/1% BSA for 5 min. If necessary, secondary antibody was added (1:100 dilution) for 30 min, followed by three rinses. Sections were stained with 0.5 μg/ml Hoechst stain for 2 min, rinsed briefly in PBS/1% BSA, and then mounted using Prolong anti-fade mounting medium (Invitrogen) and examined immediately by immunofluorescence microscopy. Antibodies were obtained from the following: BD Biosciences (CLA, CD3, CD8, CD31, E-selectin, and Ki-67), Imgenex (TLR8 clone 44C143, TLR7, CD83, and DC-LAMP), and R&D Systems (CD11c). In all studies, Hoechst nuclear stain was used to confirm the presence of invasive tumor. Sections were photographed using 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 were acquired with SPOT 4.0.9 software (Diagnostic Instruments).

Cytokine analysis. T cells from SCC tumors were stimulated with either control medium or 50 ng/ml PMA and 750 ng/ml ionomycin for 6 h; 10 μg/ml Brefeldin A (Calbiochem) was added after 1 h. Cells were stained for surface markers, fixed, permeabilized, stained with anticytokine antibodies, and examined by flow cytometry.

TCR diversity analysis of tumor-infiltrating T cells. T cells were isolated from SCC tumors via 1 wk explant cultures and examined by flow cytometry for Vβ expression, CD3, CD4, and CD8. Vβ staining was performed using the IOtest Beta Mark TCR V β Repertoire kit (Beckman Coulter) as per manufacturer's instructions. For Fig. 6, the number of CD8 T cells in 100 high power fields (hpf) was calculated for each Vβ family by the following formula: (percentage of Vβ expression) × (mean number of T cells in 1 hpf from Fig. 4 E and Fig. 5 B) × (100).

Immunohistochemical studies. For detection of CD31 and E-selectin on SCC blood vessels, 5-μm sections were cut and stored as described in Immunofluorescence studies. Sections were fixed in −20°C acetone for 5 min, air dried, and incubated with 4 μg/ml primary antibody or 4 μg/ml of mouse IgG as a negative control for 1 h at room temperature. Sections were washed in PBS three times for 5 min, and then incubated with a 1:200 dilution of secondary antibody at room temperature for 30 min. Sections were washed three times in PBS, incubated with ABC-peroxidase at room temperature for 30 min, and then washed three times in PBS. The substrate reaction was performed for 30 s (CD31) or 2.5 min (E-selectin). Sections were then counterstained with hematoxylin (Gill's No. 1; Thermo Fisher Scientific). Primary anti-CD31 was obtained from Dako, anti–E-selectin was purchased from R&D Systems, and secondary antibody was biotinylated horse anti–mouse IgG (Vector Laboratories). The Vectastain Elite standard ABC-peroxidase kit and NovaRED substrate were obtained from Vector Laboratories.

Real-time quantitative PCR for endothelial cell expression of TLR7 and TLR8. Human adult dermal microvascular endothelial cells were purchased from Cambrex Corporation and cultured in endothelial basal media (Clonetics Corp.) supplemented with 25 μg/ml dibutyryl cyclic AMP (Sigma-Aldrich), 1 μg/ml hydrocortisone acetate (Sigma-Aldrich), 20% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. RNA was isolated from second passage cultures. For comparison, identical studies were performed on T cell–depleted PBMCs, isolated from peripheral blood by Ficoll density centrifugation followed by T cell depletion using the pan–T cells isolation kit (Miltenyi Biotech) and AutoMACS instrument (Miltenyi Biotech). For RNA extraction and cDNA synthesis, cells were placed in RNALater RNA-stabilizing reagent (QIAGEN) and frozen at −80°C for later use. Frozen cells were then lysed and homogenized, and total RNA was extracted using QIAGEN RNeasy mini kits as specified by the manufacturer. Avian RT first-strand kits (Sigma-Aldrich) were used to synthesize cDNA from total RNA. The concentration of total RNA was determined at the optical density at 260 nm (OD_{260}), and discrepancies in the amount of total RNA extracted were corrected by loading the same amount and concentration of RNA for cDNA synthesis. The purity of cDNA was

determined by the OD_{260}/OD_{280} ratio. For primer design, nucleotide sequences were determined from PubMed (National Center for Biomedical Information), and the primers were custom designed using primer3 software. Primer pairs were as follows: TLR7, 5′-TGGAAATTGCCCTCGTTGTT-3′ and 5′-GTCAGCGCATCAAAAGCATT-3′; TLR8, 5′-CTTCGATACCTAAACCTCTCTAGCAC-3′ and 5′-AAGATCCAGCACCTTCAGATGA-3′. Real-time RT-PCR analysis was performed using the iCycler (Bio-Rad Laboratories) with SYBR Green kits (Bio-Rad Laboratories) and mRNA quantification by the standard curve method, as previously described (68, 69). In brief, for each transcript analyzed, a standard curve with predetermined concentrations and serial diluted respective PCR amplification products from 0.1 to 0.00001 ng was constructed. This approach allows the standards to be amplified in the same way as the template cDNA in the unknown samples because the product sequence and size are identical. Levels of Cyclophilin A mRNA served as an internal control to normalize samples for variations in sample volume loading, presence of inhibitors, and nucleic acid recovery during extraction and cDNA synthesis procedures. The normalized initial concentration of each transcript in every sample was converted to the initial copy number by using the following formula: Amount (copies/μl) = 6 × 1,023 (copies/mole) × concentration (grams/microliter)/molecular mass (grams/mole), where the mean molecular weight of double-stranded DNA equals the number of base pairs × 660 Daltons/base pair. All analyses were performed in triplicate.

In vitro treatment of endothelial cells with imiquimod. Human endothelial cells (Lonza Group) from two different donors were expanded with EGM-2 BulletKit growth media (Lonza Group). Cells were cultured on RepCell temperature-responsive plates (CellSeed). 3 μM imiquimod or 10 ng/ml TNF-α was added for indicated lengths of time, either alone or in combination with APC. APCs were isolated from human blood by ficoll density centrifugation and depletion of T cells using the Pan-T isolation kit, followed by MACS separation (Miltenyi Biotech). 4.5 million APCs were added to each well of a 6-well plate (CellSeed), and the combined culture was maintained in EGM-2 medium. APCs cultured in EGM-2 endothelial media became activated, produced inflammatory cytokines, and induced endothelial cell E-selectin in the presence or absence of imiquimod. On the day of FACS analysis, plates were cooled to room temperature to promote spontaneous release of endothelial cells. Released cells were stained with directly conjugated antibodies to CD31 and E-selectin (BD Biosciences) and analyzed by flow cytometry. For experiments with SCC13, SCC13 cells (provided by J. Rheinwald) were cultured with endotheilial cells for 3 d, and TNF-α (if present) was added for the last 12 h.

In vitro treatment of SCC tumor with TNF-α or imiquimod. Freshly excised SCC tumor was divided into 2-mm-thick slices. Slices were incubated for 24 h in control medium (Iscove's modified medium [Mediatech] with 20% heat-inactivated FBS [Sigma-Aldrich], penicillin and streptomycin, and 3.5 μl/liter β-mercaptoethanol) alone or with the addition of 1 ng/ml TNF-α or 3 μM concentration of imiquimod. 10,000× (30 mM) imiquimod stocks were made by solubilizing imiquimod cream in DMSO. Stocks were then diluted 1:10 in culture medium, and 1 μl of this 1,000× stock was added to each milliliter of culture medium. For control medium samples, an equivalent amount of DMSO was added to the control culture medium (a 1:10,000 dilution). After 24 h, the SCC slices were embedded in OCT, frozen in liquid nitrogen, and stored at −80°C until sectioning. Sections were then cut, stained, and photographed as described in Immunofluorescence studies.

Imiquimod treatment of skin T cells. To study the effect of imiquimod on skin T cell viability, T cells were isolated from normal skin as described in Isolation of T cells, and cultured for 1 wk on monolayers of feeder human dermal fibroblasts in either control medium (Iscove's modified medium with 20% heat inactivated FBS, penicillin and streptomycin, and 3.5 μl/liter β-mercaptoethanol, with 1:10,000 DMSO) or medium containing 3 μM imiquimod. T cells were then harvested, counted, stained for CD3 and FOXP3, and analyzed by flow cytometry. Percentage of survival was calculated as the number of T cells (imiquimod-treated)/(control medium-treated) × 100 for both $CD3^+FOXP3^-$ (non-T reg cell) and $CD3^+FOXP3^+$ (T reg cell) T cells. To assess the effect on proliferation, T cells from normal human skin were labeled with 0.5 μM CFSE (Invitrogen) per manufacturer's directions and cultured for 1 wk on dermal fibroblast monolayers and IL-15 (20 ng/ml; R&D Systems) either with or without 3 μM imiquimod. T cells were stained for CD3 and FOXP3 and analyzed by flow cytometry. For regulatory cell functional assays, 3 μM imiquimod was added to explant cultures during the last 2–5 d before T cell collection, and cells were then harvested and assayed for regulatory activity.

Functional assays of regulatory T cell activity. T cells were isolated from untreated or imiquimod-treated explant cultures and assayed for regulatory T cell functional, as previously described (20). If included, imiquimod was present at 3 μM concentration.

Online supplemental material. In Fig. S1. $FOXP3^+$ T reg cells were isolated from SCC via explant cultures and stained for surface CD127 and nuclear FOXP3 as described in Materials and methods. In Fig. S2, for expression of CD39 and CD73, imiquimod or control medium was added to explant cultures of normal human skin for the last 7 d before T cell isolation. T cells were then collected from explant cultures, stained for surface CD39 and CD73 and for nuclear FOXP3, and examined by flow cytometry. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071190/DC1.

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