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Nitric oxide-producing myeloid-derived suppressor cells inhibit vascular E-selectin expression in human squamous cell carcinomas

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Abstract

Squamous cell carcinomas (SCC) are sun-induced skin cancers that are particularly numerous and aggressive in immunosuppressed individuals. SCC evade immune detection at least in part by down-regulating E-selectin on tumor vessels, thereby restricting entry of skin homing T cells into tumors. We find that nitric oxide potently suppresses E-selectin expression on human endothelial cells and that SCC are infiltrated by nitric oxide-producing iNOS⁺ CD11b⁺ CD33⁺ CD11c⁻ HLA-DR⁻ myeloid-derived suppressor cells (MDSC). MDSC from SCC produced NO, TGFβ and arginase and inhibited endothelial E-selectin expression *in vitro*. MDSC from SCC expressed the chemokine receptor CCR2 and tumors expressed the CCR2 ligand HBD3, suggesting CCR2-HBD3 interactions may contribute to MDSC recruitment to SCC. Treatment of SCC *in vitro* with the iNOS inhibitor L-NNA induced E-selectin expression at levels comparable to imiquimod-treated SCC undergoing immunologic destruction. Our results suggest that local production of NO in SCC may impair vascular E-selectin expression. We show that MDSC are critical producers of NO in SCC and that NO inhibition restores vascular E-selectin expression, potentially enhancing T cell recruitment. iNOS inhibitors and other therapies that reduce NO production may therefore be effective in the treatment of SCC and their premalignant precursor lesions actinic keratoses.

Introduction

Over 700,000 SCC are diagnosed each year in the United States (Rogers et al.). The treatment of non-melanoma skin cancers, of which SCC is the second most frequent type, account for 4.5% of all Medicare cancer costs (Berg and Otley, 2002; Housman et al., 2003).

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Conflicts of Interest

None to report.

Although most are curable by surgical excision, 4% metastasize to the lymph nodes and 1.5% of SCC patients die from metastatic or locally aggressive disease (Brantsch et al., 2008). SCC are a leading cause of death among organ transplant recipients. These patients have a 65- to 250-fold increased risk of developing SCC; nearly 10% of these cancers metastasize and the majority of patients die as a result (Berg and Otley, 2002; Euvrard et al., 2003). Currently, wide surgical excision is the only treatment for invasive SCC. In addition to the burden of invasive cancers, actinic keratoses, the premalignant precursor lesion of SCC, are the third most frequent reason in the U.S. for consulting a dermatologist (Feldman et al., 1998). Over 5.2 million physician visits are made each year for the treatment of actinic keratoses at a cost of over \$900 million (Warino et al., 2006).

Immune evasion in human SCC appears to primarily result from aberrant T cell homing. Vessels in SCC lack expression of E-selectin, a skin addressin that is expressed at baseline by cutaneous postcapillary venules, is up-regulated with inflammation, and by binding to cutaneous lymphocyte antigen (CLA) on skin homing T cells, mediates the first step of T cell recruitment into skin (Chong et al., 2004; Clark et al., 2008; Kupper and Fuhlbrigge, 2004). As a result, these tumors exclude CLA⁺ skin homing T cells, the cell type that provides cutaneous immune surveillance and would be expected to contain tumor specific T cells (Clark, 2010).

Topical treatment of SCC with the TLR7 agonist imiquimod induces endothelial activation, massive infiltration of tumors by CLA⁺ T cells producing IFN γ , perforin, and granzyme, tumor cell death and histologic evidence of tumor regression (Clark et al., 2008; Huang et al., 2009). This rapid and effective immune response suggests that primed SCC-specific T cells exist in the circulation but these cells cannot gain access to the tumor. Imiquimod can be used to treat SCC in poor surgical candidates (Peris et al., 2006). In solid organ transplant recipients, a course of imiquimod was effective and did not engender graft rejection in the 6 or 12 months that patients were followed (Brown *et al.*, 2005; Ulrich *et al.*, 2007) but concerns linger that repeated use of imiquimod could engender graft rejection or shorten the life of an allograft. There is therefore a need to understand why blood vessels in SCC fail to express T cell homing addressins such as E-selectin and to identify novel agents that can induce endothelial activation and restore appropriate T cell homing without broad, nonspecific activation of the immune system. Below, we present our findings that nitric oxide (NO) production in SCC contributes to suppression of E-selectin expression by tumor vessels and that agents that inhibit NO production may be effective therapeutic strategies for the treatment of SCC.

Results

Vascular E-selectin expression correlates with tumor infiltration by CLA⁺ T cells and histologic evidence of tumor regression

We previously observed qualitative increases in vascular E-selectin expression and T cell infiltration after TLR7 agonist treatment of SCC (Clark et al., 2008; Huang et al., 2009). To quantitatively study the relationship of vascular E-selectin expression and tumor infiltration by skin homing CLA⁺ T cells, we counted the percentage of tumor vessels expressing E-selectin and the number of infiltrating CLA⁺ T cells in untreated SCC and SCC treated with

TLR7 agonist prior to excision. In agreement with earlier observations, vascular E-selectin expression was absent or low in untreated tumors and markedly up-regulated in SCC treated with the TLR7 agonist imiquimod (Fig. 1, a and d). In untreated tumors, 6.7% of blood vessels in tumors expressed E-selectin (n=5, SEM 1.53) whereas 34.2% of blood vessels expressed E-selectin in tumors treated with TLR7 agonist prior to excision (n=3, SEM 3.04). The difference between treated and untreated SCC was statistically significant ($p < 0.0001$). Likewise, recruitment of CLA⁺ T cells was low in untreated SCC but greatly enhanced in treated tumors (Fig. 1, b, c, and d). Untreated tumors were infiltrated by a mean 21.3 CLA⁺ T cells per high power field (HPF, n=6, SEM 5.91) whereas tumors treated with TLR7 agonist prior to excision contained a mean 256.6 CLA⁺ T cells per HPF (n=3, SEM 5.91, $p = 0.0002$). There was in fact a strong linear correlation between vascular E-selectin expression and infiltration by CLA⁺ T cells (correlation coefficient $R = 0.94$) and only tumors with both features had histologic evidence of tumor regression (Fig. 1d).

SCC are infiltrated by NO producing CD11c⁻CD11b⁺HLA-DR⁻ myeloid-derived suppressor cells

Cells expressing iNOS were prominent in untreated SCC (Fig. 2, a and b)(Clark et al., 2008). We previously observed faint staining of iNOS⁺ cells for CD11c, suggesting a possible dendritic cell lineage. However, follow up staining with multiple anti-CD11c antibodies demonstrated that these cells were in fact CD11c⁻, CD11b⁺, and HLA-DR⁻, a phenotype shared by myeloid derived suppressor cells identified in several human cancers (MDSCs) (Fig. 2c,d,h)(Corzo *et al.*, 2010; Diaz-Montero *et al.*, 2009; Filipazzi *et al.*, 2007; Gabitass *et al.*, 2011). iNOS⁺ cells lacked expression of the macrophage marker CD163, the T cell marker CD3 and the endothelial cell marker CD34 (Fig. 2e-g) and were CD14⁻ (data not shown). Further characterization by flow cytometry analysis of dispersed cells isolated from collagenase-treated SCC demonstrated that CD11b⁺ HLA-DR⁻ cells expressed CD33, a subset expressed iNOS and the majority expressed TGFβ, a phenotype consistent with human MDSC (Fig. 3a). RT-PCR analysis of CD11b⁺ cells isolated by magnetic bead separation from collagenase dispersed tumors demonstrated that CD11b⁺ cells expressed arginase I (Fig 3b). iNOS⁺ CD11b⁺CD11c⁻HLA-DR⁻ cells were present in 16/16 untreated SCC (10 SCC were studied by immunostaining of cryosections and 6 by flow cytometry analysis of collagenase treated tumors). As we previously reported, iNOS⁺ cells were not present in imiquimod treated SCC undergoing regression (Clark *et al.*, 2008). CD11b⁺ HLA-DR⁻ cells made up a mean 6% of total tumor cells in collagenase digested SCC but were rare (0.8%) in normal human skin (Fig. 3c). Selective gating on iNOS-expressing cells demonstrated that MDSC were the prominent cell type expressing iNOS in most tumors, although it was also expressed by other cell types within the tumor microenvironment (Fig. 3d). A mean 51% of total iNOS⁺ cells in four collagenase digested SCC were CD11b⁺ CD11c⁻ HLA-DR⁻ CD33⁺ MDSC (SEM 10.3).

To confirm that NO was produced within the SCC tumor microenvironment, we measured nitrate and nitrite levels from tumor supernatants using the Griess method (Fig. 3e). Although levels varied, significant NO production was observed in all tumors analyzed (significance of difference medium vs. SCC supernatants, $p < 0.05$). To confirm that MDSC in SCC produced NO, we isolated CD11b⁺ cells using magnetic bead separation of

collagenase dispersed tumors. CD11b enrichment produced a fairly uniform population of CD11b⁺ HLA-DR^{low} cells (Fig. 3f) and analysis of the culture supernatants of these cells demonstrated that they produced NO (Fig. 3g).

NO inhibits E-selectin expression by human dermal microvascular endothelial cells IN VITRO

NO inhibits the expression of endothelial adhesion receptors on human umbilical vein endothelial cells (HUVEC) and reduces adhesion of dendritic cells to endothelial monolayers *in vitro* (De Caterina et al., 1995; De Palma et al., 2006). Gene expression analyses have found significant differences between HUVEC and the microvascular endothelial cells found in tissues such as the skin (Chi et al., 2003). We studied the effects of NO on human dermal microvascular endothelial cells (DMEC). To mimic the physiologic stimulation likely to occur within the tumor microenvironment, DMEC were co-cultured with TLR7 agonist-stimulated T-cell depleted peripheral blood mononuclear cells (APC). Under these conditions, NO potently inhibited endothelial E-selectin expression (Fig. 4a and b). However, NO only partially inhibited E-selectin expression when endothelial cells were stimulated with 10 ng/ml of TNF β . A similar biology was observed in umbilical vein endothelial cells (HUVEC); NO completely inhibited E-selectin expression after physiologic stimulation with APC but only partially inhibited expression after intense endothelial stimulation with TNF β (Fig. 4b).

MDSC from SCC suppress endothelial E-selectin expression in vitro

To determine if cell types in SCC are capable of suppressing endothelial E-selectin expression, we cultured human HUVEC in the presence of unfractionated cells from collagenase dispersed SCC tumors. We observed a modest suppression of E-selectin under these conditions (Fig. 4c). We then separated collagenase dispersed SCC into CD11b⁺ and CD11b⁻ fractions using magnetic bead separation (Fig. 3f) and tested the ability of these cells to suppress endothelial E-selectin expression (Fig. 4d and e). CD11b⁺ cell fractions reproducibly inhibited endothelial E-selectin expression whereas CD11b⁻ fractions had no effect or modestly increased E-selectin expression.

MDSC from SCC express CCR2 and SCC produce the CCR2 ligand HBD3

MDSC from both mice and humans express the chemokine receptor CCR2 and CCL2 production by human tumors has been implicated in the migration of MDSC into tumors (Huang *et al.*, 2007). MDSC from collagenase dispersed SCC expressed CCR2 by flow cytometry analysis whereas T cells from the same tumors did not (Fig. 5a). Co-immunostaining for iNOS and CCR2 in tumor cryosections confirmed that iNOS⁺ cells expressed CCR2 (Fig. 5b). Quantitative RT-PCR analysis of SCC tumors showed that the CCL2 ligands CCL2, CCL7, CCL13 and HBD2 were expressed at comparable levels in normal skin and SCC, whereas only the CCL2 ligand HBD3 was expressed at significantly higher levels in SCC (Fig. 5c). Immunostaining of SCC cryosections confirmed production of HBD3 in SCC tumors (Fig. 5d).

iNOS inhibition induces SCC vascular E-selectin expression *IN VITRO*

To determine if NO production in tumors locally inhibits vascular E-selectin expression, we cultured portions of human SCC for 24 hours in the iNOS inhibitor L-NNA in the presence or absence of TNF β and then assayed for E-selectin expression by immunostaining of cryosections. We observed a marked up-regulation of E-selectin expression in tumors treated with iNOS inhibitor (Fig. 6, a and b). E-selectin was expressed on a mean 1.9% of blood vessels in SCC treated with control medium (n=4 tumors, SEM 0.58). After treatment with iNOS inhibitor, E-selectin was expressed by a mean 27.1% of tumor vessels (n=4, SEM 1.06, control medium vs. iNOS inhibitor p<0.0001). As expected, treatment of SCC with TNF β also increased expression of E-selectin (mean 19.6% positive vessels, SEM 2.17, n=4, control medium vs. TNF β treated p= 0.0002). Combining iNOS inhibition and treatment with TNF β provided no additional increase in E-selectin expression above the use of iNOS inhibitors alone (iNOS inhibitor vs. TNF β /iNOS inhibitor p=0.45).

Discussion

Impairment of T cell homing is a major mechanism by which cutaneous SCC evade immune responses. Vessels in SCC tumors lack expression of E-selectin and exclude the population of CLA⁺ T cells thought to provide immune surveillance in the skin (Clark et al., 2008). Induction of E-selectin expression on blood vessels by TLR7 agonist therapy leads to infiltration of the tumors by CLA⁺ T cells producing IFN γ , perforin and granzyme, and is associated with histologic evidence of tumor regression (Clark et al., 2008; Huang et al., 2009). These clinical responses suggest that tumor specific T cells exist within the circulation but cannot gain access to the tumor. Thus, the induction of appropriate T cell homing addressins on tumor vessels has the potential to restore homing and potentially induce tumor destruction.

We report here that human SCC are infiltrated by a population of NO producing cells that express CD11b, CD33 and lack CD11c and HLA-DR, a phenotype suggestive of MDSC (Diaz-Montero *et al.*, 2009; Gabrilovich and Nagaraj, 2009). MDSC are a heterogeneous population of myeloid cells that are enriched in the circulation of cancer patients as well as in many animal tumor models. MDSC play a major role in cancer related immunosuppression and can potently suppress T cell responses (Nagaraj *et al.*, 2010). The majority of CD11b⁺CD11c⁻HLA-DR⁻ cells isolated from SCC expressed i-NOS, TGF β , and arginase I, three critical effector mechanisms used by MDSC to suppress T cell responses (Gabrilovich, 2004; Jia *et al.*, 2010; Li *et al.*, 2009). CD11b⁺HLA-DR⁻ cells comprised approximately 6% of total cells in SCC, but were rare in normal skin (Fig. 3c).

iNOS, a key enzyme that catalyzes NO production, is expressed in a variety of human cancers including malignant melanoma, breast, lung, prostate, and colorectal cancers (Lechner et al., 2005). iNOS expression correlated with progression in human astrocytoma and prostate cancer, and patients with iNOS⁺ melanomas had decreased survival (Ekmekcioglu *et al.*, 2006; Lechner *et al.*, 2005; Tanese *et al.*, 2011). NO has a variety of effects on immune cells including inhibition of T cell activation, proliferation, and cytokine production (Bogdan, 2001) and animal studies suggest it may also reduce the adhesion of leukocytes to blood vessels. Inhibition of NO increased leukocyte rolling and adhesion in

mesenteric venules in cats and rats and P-selectin expression was increased in rats after perfusion with NO inhibitors (Davenpeck et al., 1994; Kubes et al., 1991). In a mouse cancer model in which mouse mammary adenocarcinoma and human colon carcinoma cell lines were injected into the skin, treatment of animals with the iNOS inhibitor L-NAME led to increased rolling and stable adhesion of leukocytes to tumor vessels (Fukumura et al., 1997).

We find that NO is produced by SCC tumors and that CD11b⁺ MDSC expressed iNOS and are significant sources of NO production within the SCC microenvironment. Small numbers of CD11b⁺ HLA-DR^{-low} cells isolated from SCC potently suppressed endothelial E-selectin expression, suggesting that even though MDSC comprise a relatively small percentage of total tumor cells, they may play a critical role in down regulating vascular E-selectin and impairing T-cell trafficking into tumors. In a mouse model of B16 melanoma, MDSC inhibited the migration of activated CD8 T cells into tumors but the mechanism of impaired T-cell homing was not identified (Lesokhin *et al.*, 2011). Our results suggest that NO produced by MDSC inhibits vascular E-selectin, likely impairing T cell migration in to tumors, and that this may be another mechanism by which MDSC impair anti-tumor immunity.

Human studies have been limited but *in vitro* treatment of HUVEC with NO has been shown to reduced addressin expression and dendritic cell adhesion (De Caterina et al., 1995; De Palma et al., 2006). We found that NO completely abrogated the expression of E-selectin when DMEC were physiologically stimulated with activated APC, but could only partially inhibit E-selectin expression when endothelial cells were strongly and directly stimulated with 10 ng/ml of TNF β (Fig. 4a, b). It may therefore be possible to overcome impaired E-selectin expression by either inhibiting iNOS activity or by potently and directly stimulating endothelial cells. We found that HUVEC and DMEC derived from human skin responded very similarly to NO, suggesting that HUVEC, which are easier to obtain and grow, may be useful in drug screens used to identify novel agents that activate endothelial cells.

MDSC in both humans and animal models express the chemokine receptor CCR2 (Huang *et al.*, 2007; Lesokhin *et al.*, 2011). CCL2 is produced by human breast, gastric and ovarian cancers and inhibition of CCR2-CCL2 signaling in mouse cancer models reduced MDSC migration and MDSC-induced tumor cell growth (Huang *et al.*, 2007). In addition to their antimicrobial function, HBD-2 and HBD-3 can induce cell chemotaxis via CCR2 (Rohrl *et al.*, 2010). HBD3/CCR2 interactions promoted migration of macrophages into tumors in a mouse model of oral carcinoma (Jin *et al.*, 2010). We found that iNOS⁺ MDSC in SCC tumors expressed CCR2 and SCC tumors expressed the CCR2 ligand HBD3, suggesting that CCR2-HBD3 interactions may play a role in the recruitment of MDSC to tumors (Fig. 5).

Lastly, we tested the ability of iNOS inhibitors and TNF β to restore E-selectin expression in freshly excised human SCC tumors. iNOS inhibition alone markedly enhanced E-selectin expression on tumor vessels (Fig. 6). Remarkably, iNOS inhibition alone induced E-selectin expression at similar or greater levels than TNF β . Moreover, E-selectin was expressed at levels comparable to those observed in SCC undergoing immunologic destruction after topical treatment with TLR7 agonist (Fig. 1d and 6b). SCC undergoing immunologic

destruction after TLR7 agonist therapy expressed E-selectin on a mean 34.2% of blood vessels as compared to 27.2% of blood vessels following *in vitro* treatment with iNOS inhibitor ($p>0.05$). Although the limitations of working with humans preclude us from directly demonstrating that T cell recruitment into SCC is enhanced as a result of iNOS inhibition, these studies strongly suggest that iNOS inhibition induces vascular E-selectin at levels capable of enhancing T cell entry into tumors. To our knowledge, it is previously unreported in a human cancer that iNOS activity impairs the expression of vascular addressins critical for T cell recruitment.

Our results suggest that treatment with iNOS inhibitors or potent stimulators of endothelial activation both have the potential to restore addressin expression in SCC without the attendant widespread immune activation observed with TLR agonists such as imiquimod. A topically applied inhibitor of iNOS is currently in clinical trials for the treatment of neuropathic pain (LaBuda et al., 2006). Because immune evasion in SCC is primarily an issue of impaired T cell homing, our studies suggest that topically applied iNOS inhibitors or potent endothelial activating agents may be effective, either alone or in combination with other therapies, in the treatment of SCC and their premalignant precursor lesions, actinic keratoses.

It is becoming increasingly appreciated that 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). Impaired T cell homing as a result of decreased vascular addressin expression has been reported in a number of human cancers, including malignant melanoma, breast, gastric and lung cancers (Madhavan et al., 2002; Piali et al., 1995; Weishaupt et al., 2007). 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 (Weishaupt et al., 2007). Our work suggests that local production of NO within tumors could be a common mechanism for impaired T cell homing. If this proves to be the case, iNOS inhibition used in concert with agents that enhance the presentation of tumor antigens have the potential to enhance immune responses to many human cancers.

Materials and methods

SCC samples

Tumor samples consisted of tumor removed prior to taking the first Moh's section during Moh's micrographic excision of biopsy-proven squamous cell carcinomas removed from immunocompetent individuals. Acquisition of tumor samples and all studies were approved by the Partners Institutional Review Board and were performed in accordance with the Declaration of Helsinki. Because this work utilized discarded tissues with no identifiable personal information, the Partners IRB ruled that no informed written patient consent was required.

Immunofluorescence studies

Five μm sections were cut from SCC blocks frozen in OCT. Sections were fixed in acetone, air dried, rehydrated in PBS and blocked with human IgG (Jackson Immunoresearch). Sections were incubated with a biotinylated anti E-selectin (clone 68-5H11, BD) at 5 $\mu\text{g}/\text{ml}$, rinsed in PBS/1% BSA, and co-stained with streptavidin-PE (1 $\mu\text{g}/\text{ml}$, R&D Systems) and CD31 FITC (1:40 clone WM59, BD). Sections were rinsed and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). E-selectin⁺ blood vessels were enumerated in ten 100X high power fields (HPF). To quantify CLA⁺ T cells, sections were co-stained with PE anti-CD3 (1:40, BD) and FITC anti-CLA (1:25, BD). The number of CLA⁺ infiltrating T cells was determined by counting CLA⁺ cells directly in 10 HPF or by counting total T cells in 10 HPF and multiplying by the % CLA⁺ T cells of total T cells obtained from 5 HPF. For examination of iNOS-expressing cells, sections were co-stained with directly conjugated monoclonal antibodies (BD) and FITC anti-iNOS (1:20, BD). For HBD3 staining, sections were stained with rabbit anti human HBD-3 mAb (1:100, FL-67 Santa Cruz Biotech) followed by Alexa Fluor 488 goat anti rabbit polyclonal antibody (1:100, Invitrogen). In all studies, DAPI nuclear stain was used to confirm the presence of invasive tumor as determined by the presence of large atypical keratinocyte nuclei. Sections were photographed using a Nikon Eclipse 6600 microscope equipped with Nikon Plan Fluor objective lenses. Images were captured with a SPOT RT model 2.3.1 camera (Diagnostic Instruments) and were acquired with SPOT 4.0.9 software (Diagnostic Instruments).

Isolation and flow cytometry analysis of T cells and MDSC from SCC tumors

For isolation of MDSC from SCC, tumors were minced and dissociated with 0.2% type I collagenase (Invitrogen) and 30 Kunitz Units/ml of DNase (Sigma) for two hours at 37° with vigorous shaking. CD11b⁺ cells were isolated from collagenase treated tumors by staining with anti-CD11b-PE mAb (R&D systems) and anti-PE micro-beads (Miltenyi Biotech) followed by AutoMACS separation (Miltenyi Biotech). For TGF β staining, cells were stimulated overnight with 1 $\mu\text{g}/\text{ml}$ LPS from *E. coli* (Sigma) and 100 IU/ml IFN γ (R&D systems) to activate MDSC (Greifenberg *et al.*, 2009). Flow cytometry analysis was performed using directly conjugated monoclonal antibodies from BD. Analysis of flow cytometry samples was performed on a Becton Dickinson FACSCanto instrument and data were analyzed using FACSDiva software (BD).

Assay of SCC supernatants and CD11b⁺ cells for NO production

For analysis of SCC supernatants, 3 mm³ tumor fragments were cultured for 24 hours in Iscoves medium supplemented with 10% human AB serum, fungizone, gentamicin, penicillin/streptomycin, L-glutamine and 0.6 mM L-arginine. Supernatants were concentrated using Microcon centrifugal filter devices (Millipore Corp). For analysis of NO production from SCC CD11b⁺ cells, CD11b⁺ cells were isolated from collagenase treated SCC by magnetic bead separation as described and 10,000 CD11b⁺ cells were incubated for 12 hours with 1 $\mu\text{g}/\text{ml}$ *E. coli* LPS and 100 IU/ml IFN γ . Supernatants were assayed for nitric oxide using the QuantiChrom Nitric Oxide assay kit or the Parameter Total NO/Nitrite/Nitrate Assay Kit (R&D Systems), as per the manufacturer's instructions.

Endothelial cell studies

Human dermal microvascular and umbilical vein endothelial cells (Lonza) were cultured in EGM-2 bulletkit growth medium (Lonza). Endothelial cells at 90% confluency were stimulated for 12 hours with activated APC or 10 ng/ml TNF- α (R&D systems) with or without 0.5 mM nitric oxide donor spermine NONOate (Sigma-Aldrich). Human peripheral blood APC were isolated from products discarded following plasmapheresis of healthy donors by density centrifugation using histopaque-1077 (Sigma) and depletion of T cells using the Pan-T isolation kit and AutoMACS instrument (Miltenyi Biotech). APC (2.5×10^6) and 3 μ M imiquimod (added to stimulate APC) were added to wells containing cultured endothelial cells for 12 hr. Endothelial cells were then stained with directly conjugated antibodies to CD31 and E-selectin (BD) and acquired on a BD FACSCanto flow cytometer. Data were analyzed using FACS Diva software (V6.1). For experiments involving co-cultures of endothelial cells with cells from SCC tumors, cells obtained from collagenase treated SCC were rested overnight at in complete Iscove's medium in the presence of 10 ng/ml GM-CSF (R&D systems) to maintain myeloid cell viability (Ko *et al.*, 2009). Cells from SCC were added to endothelial cells in a 1:1 ratio and stimulated with 1 ng/ml TNF- α (R&D systems). In a second group of experiments, 5,000 AutoMACS-enriched CD11b⁺ cells from collagenase treated SCC were added to wells containing 5,000 endothelial cells stimulated with TNF α 1 ng/ml; immunostaining of endothelial cells was performed 12 hours later as described.

Quantitative real-time PCR for CCR2 ligands and arginase I

Total RNA was isolated from cryosections of SCC and normal skin or from cells isolated from SCC tumors using the RNeasy Lipid Tissue kit (Qiagen), according to the manufacturer's instructions. cDNA was generated using the QuantiTect reverse transcription kit (Qiagen) and quantitative real-time PCR was performed using the ABI StepONE plus instrument and the Fast SYBR green master mix (Applied Biosystems). Expression of each ligand transcript was determined relative to the reference gene transcript, GAPDH, and calculated as $2^{-(C_{t, \text{ligand}} - C_{t, \text{GAPDH}})}$. The primers used to detect the ligands and the reference transcripts were purchased from Origene Technologies and were as follows: hBD2 (F-GGCGTAGAAGTTCTCTGTCTCC; R-GAAGCAGGAGAAAAGGATGGAG), hBD3 (F-GGTGAAGCCTAGCAGCTATGAG; R-GCCGCCTCTGACTCTGCAATA), CCL2 (F-AGAATCACCAGCAGCAAGTGTCC, R-TCCTGAACCCACTTCTGCTTGG), CCL7 (F-ACAGAAGGACCACCAGTAGCCA; R-GGTGCTTCATAAAGTCTGGACC), CCL13 (F-GATCTCCTTGAGAGGCTGAAG; R-TCTGGACCCACTTCTCCTTTGG), GAPDH (F-GAGTCAACGGATTTGGTCGT; R-CATGGGTGGAATCATATTGGA).

IN VITRO treatment of SCC with iNOS inhibitor and TNF β

SCC were cultured for 24 hours in control medium (Iscoves, 10% human AB serum, fungizone, gentamicin, penicillin/streptomycin, L-glutamine, 0.6 mM L-arginine) in the presence or absence of 0.6 mM iNOS inhibitor *N*_ω-Nitro-L-arginine, (L-NNA, Sigma) and/or 10 ng/ml TNF β (R&D), then embedded in OCT, cryosectioned and stained for CD31 and E-selectin as described.

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Abbreviations used

CLA	cutaneous lymphocyte antigen
SCC	squamous cell carcinoma
NO	nitric oxide
MDSC	myeloid-derived suppressor cell
iNOS	inducible nitric oxide synthase
HBD	human beta defensin

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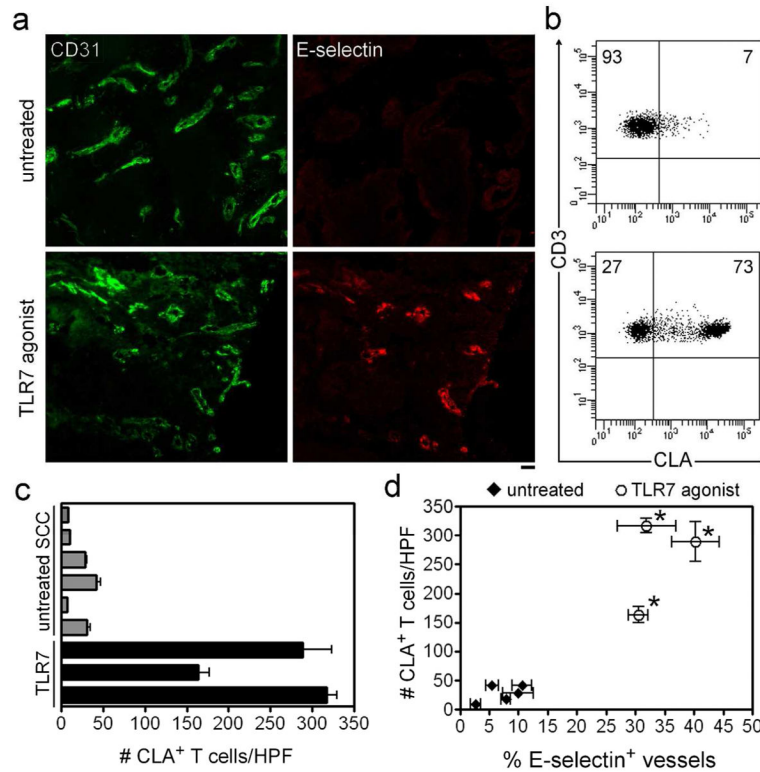


Figure 1. E-selectin expression on tumor vasculature correlates quantitatively with infiltration by CLA⁺ T cells

(a) Untreated human SCC lacked vascular expression of the skin T cell homing addressin E-selectin. Cryosections of tumor were co-stained for CD31 (a blood vessel marker, left panels) and E-selectin (right panels). SCC treated with the TLR7 agonist imiquimod showed up-regulation of E-selectin expression on tumor vessels (lower panels). Similar findings have been demonstrated in a total of 12 untreated tumors and six imiquimod treated tumors. Scale bar = 100 μ M. (b) CLA⁺ T cells are excluded from untreated human SCC but are present in imiquimod treated tumors. Shown are T cells isolated from untreated (upper panel) and imiquimod treated (lower panel) SCC tumors. (c) Exclusion of CLA⁺ T cells from SCC was reversed by topical treatment with TLR7 agonist prior to excision. The absolute numbers of CLA⁺ T cells per high power field (HPF) infiltrating untreated SCC (gray bars) and TLR7 agonist treated tumors (TLR7, black bars) are shown. (d) Vascular E-selectin expression correlated with the number of CLA-expressing T cells infiltrating SCC and histologic evidence of tumor regression. The mean and SEM for both the number of CLA-expressing T cells and the % E-selectin positive vessels are shown. These studies demonstrate a strong correlation of vascular E-selectin expression with the ability of tumors to recruit CLA⁺ skin homing T cells. Tumors designated with an asterisk (*) had histologic evidence of tumor regression.

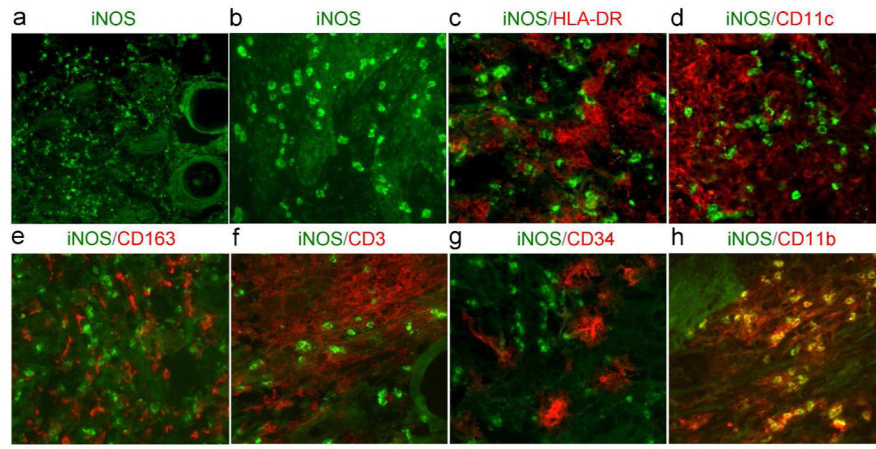


Figure 2. iNOS⁺ cells infiltrating SCC tumors lack HLA-DR and express CD11b, a phenotype consistent with MSDC

(a,b) Co-staining of SCC cryosections demonstrated that iNOS⁺ cells were evident in SCC tumors. (c,d) iNOS⁺ cells lacked expression of the dendritic cell markers HLA-DR and CD11c, (e) the macrophage marker CD163, (f) the T cell marker CD3, and (g) the endothelial marker CD34. (h) iNOS⁺ cells did express the myeloid marker CD11b. CD11b expression together with a lack of HLA-DR expression is a phenotype suggestive of MSDC. Similar findings were observed in a total of eight SCC tumors. Scale bar = 100 μ M.

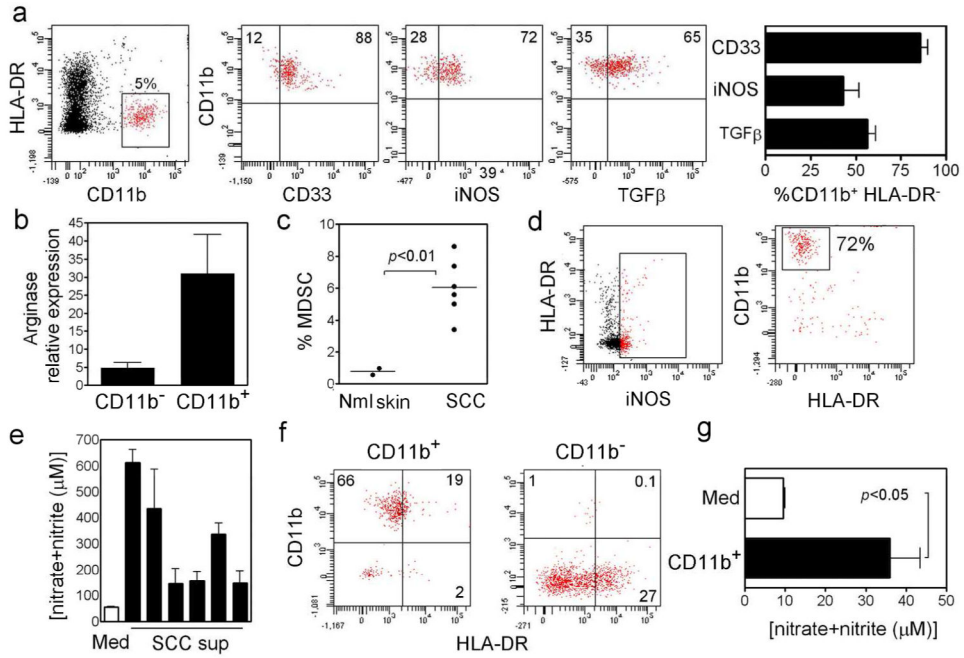


Figure 3. MDSC are a major source of nitric oxide production in SCC tumors

(a) SCC tumors were dispersed by treatment with collagenase and the population of CD11b⁺HLA-DR⁻ cells were studied by flow cytometry. Most CD11b⁺HLA-DR⁻ cells expressed CD33, approximately half expressed iNOS and over half produced TGFβ. Representative histograms are shown and the mean and SEM of multiple donors are shown on the right (n=5 for CD33 and iNOS, n=3 for TGFβ) (b) Arginase I was increased in CD11b⁺ cells isolated by magnetic bead separation from collagenase dispersed tumors as assayed by qRT-PCR. The mean and SEM of 3 donors are shown. (c) In collagenase dispersed tumors, a mean 6% of total tumor cells were CD11b⁺ HLA-DR⁻CD33⁺ MDSC but these cells were rare in normal skin (Nml skin). (d) In many tumors, MDSC represented the majority of iNOS-expressing cells but non-MDSC cell types also expressed iNOS. (e) Analysis of SCC tumor supernatants using the Griess method demonstrated that NO was produced in SCC tumors. For each tumor, the mean and SEM of duplicate measurements of nitrate+nitrite are shown. (f,g) CD11b⁺ MDSC from SCC produce NO. CD11b⁺ MDSC were enriched by magnetic bead separation from collagenase treated tumors and cultured in vitro. Culture supernatants were analyzed for the presence of NO by the Griess method. The mean and SEM of measurements from three SCC are shown.

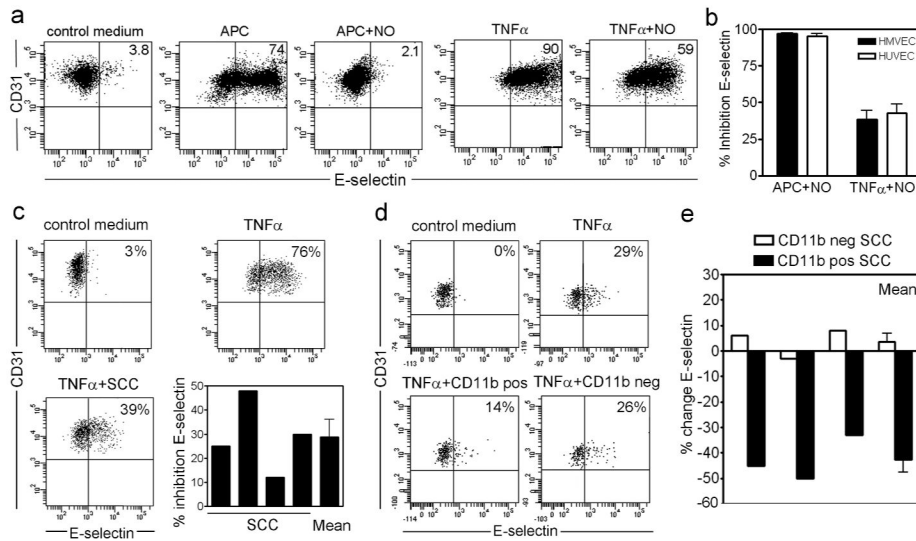


Figure 4. NO and MDSC from SCC inhibit human endothelial cell E-selectin expression
 (a) Human skin dermal microvascular cells (DMEC) were stimulated with either TLR7 agonist activated APC (APC), mimicking physiologic stimulation, or the potent endothelial activator $\text{TNF}\beta$ in the presence or absence of the NO donor spermine NONOate (NO). Treated cells were immunostained for E-selectin and analyzed by flow cytometry. NO potently suppressed E-selectin upregulation in response to stimulated APC but only partially inhibited expression when endothelial cells were directly stimulated with $\text{TNF}\beta$. (b) Percent inhibition of E-selectin expression by NO after stimulation with APC or $\text{TNF}\beta$. The mean and SEM of three different endothelial donors are shown. Human umbilical vein endothelial cells (HUVEC) responded similarly in that NO completely abrogated E-selectin expression in response to stimulated APC (APC+NO) but only partially inhibited expression after stimulation with $\text{TNF}\beta$. (c-e) MDSC from SCC suppress endothelial E-selectin expression in vitro. (c) Human HUVEC were stimulated with $\text{TNF}\beta$ in the presence or absence of collagenase dispersed cells from SCC tumors. A modest but reproducible inhibition in E-selectin expression was observed. Representative histograms and individual results from four SCC tumors are shown, along with the mean and SEM of these measurements. (d,e) CD11b^+ MDSC from SCC tumors suppress endothelial E-selectin expression. CD11b^+ and CD11b^- cell populations were obtained by magnetic bead separation from collagenase dispersed SCC tumors. HUVEC were stimulated with $\text{TNF}\beta$ in the presence or absence of CD11b^+ and CD11b^- cells. (d) Representative histograms and (e) individual results from three SCC tumors are shown, along with the mean and SEM of these measurements. CD11b^+ MDSC reproducibly inhibited expression of endothelial E-selectin.

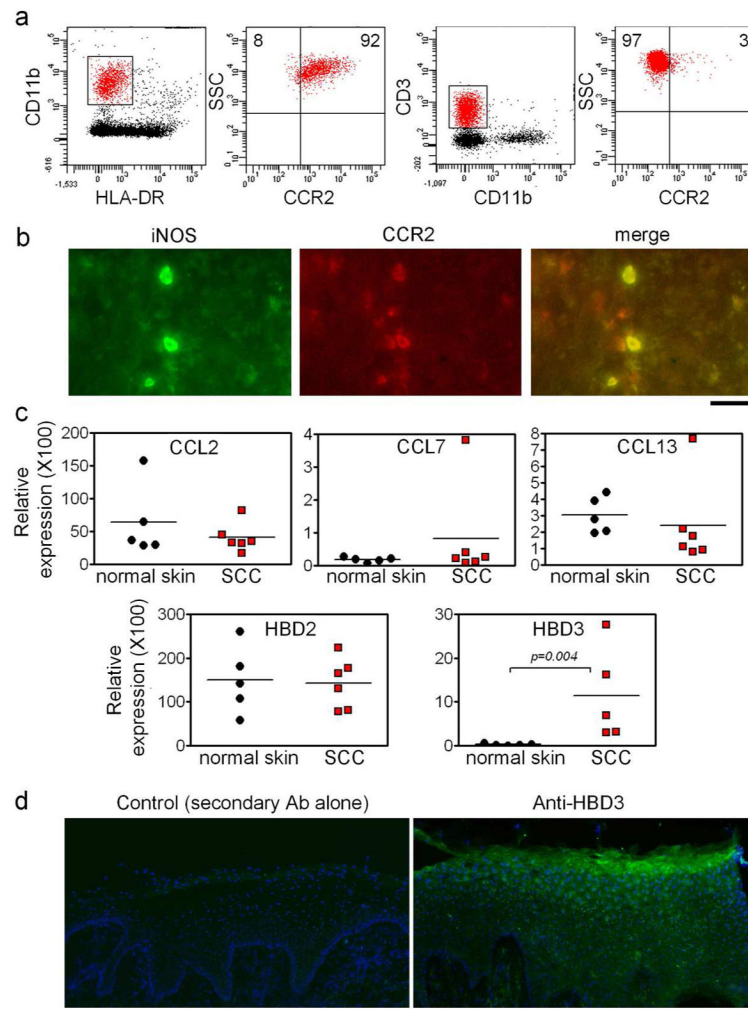


Figure 5. MDSC from SCC express CCR2 and SCC produce the CCR2 ligand HBD3
 (a) CD11b⁺HLA-DR⁻ MDSC from collagenase dispersed SCC uniformly expressed CCR2 whereas CD3⁺ T cells lacked CCR2 expression. (b) Immunostaining of SCC cryosections confirmed that iNOS⁺ cells in SCC tumors co-express CCR2. (c) RT-PCR analysis of SCC tumors and normal human skin demonstrated selective production of the CCR2 ligand HBD3 in SCC. (d) Immunostaining of SCC cryosections confirmed production of HBD3 by SCC tumor cells. Scale bar = 100 μ M.

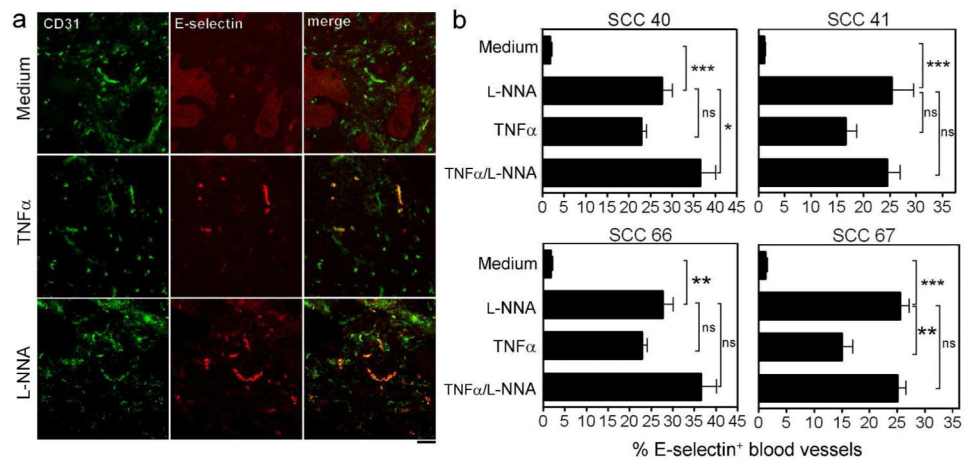


Figure 6. Inhibition of iNOS activity restores vascular E-selectin expression in SCC tumors
 (a) *In vitro* treatment of human SCC induced vascular E-selectin expression. SCC tumors were cultured for 24 hours in medium alone, TNF β or with the iNOS inhibitor L-NNA. Tumors were then cryosectioned and stained for vascular E-selectin expression. SCC treated with TNF β or iNOS inhibitor showed induction of vascular E-selectin expression on a subset of vessels. (b) iNOS inhibition potently induced vascular E-selectin expression. The mean and SEM of the % E-selectin expressing vessels in 10 high-power fields are shown. *In vitro* treatment of tumors with iNOS inhibitor induced vascular E-selectin at levels comparable to or greater than that observed after treatment with TNF β . Results from four SCC tumors are shown; comparable results were observed in seven additional SCC. *** p <0.0005, ** p <0.01, * p <0.05. Scale bar = 500 μ M.