CD200 Upregulation in Vascular Endothelium Surrounding Cutaneous Squamous Cell Carcinoma

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Objective: To characterize the presence of CD200 and CD200 receptor (CD200R) in the human cutaneous squamous cell carcinoma (SCC) microenvironment and to define a possible role for the CD200 axis in immune evasion by SCC.

Main Outcome Measures: Expression of CD200 on SCC-associated blood vessels; expression of CD200 receptor on SCC-associated macrophages and T cells; and induction of CD200 on endothelial cells by SCC supernatants.

Results: CD200 gene and message were upregulated in SCC stroma. Immunostaining revealed a higher number of CD200+ cells in SCC stroma than in normal dermis (180.8 cells/mm² vs 24.6 cells/mm²) (P < .01). CD200 was further identified mainly on blood vessel endothelium in SCC. Tumor supernatant was able to induce CD200 expression on human dural blood endothelial cells in culture. CD200R was identified on macrophages and dendritic cells in SCC microenvironment.

Conclusions: CD200 expression on local blood vessels may promote tumor progression by suppressing CD200R+ myeloid cells during diapedesis. These data highlight a previously unrecognized mechanism of immune evasion by SCC and may provide guidance for the development of targeted therapy.


UTANEOUS SQUAMOUS CELL carcinoma (SCC) is responsible for most of the approximate 10 000 non-melanoma skin cancer deaths in the United States each year. While most of the 300 000 cases of SCC each year are cured by excision with clear margins, some tumors are aggressive and cause extensive local destruction or metastasize. The incidence of SCC is increased in immunosuppressed solid-organ transplant recipients, suggesting that immune surveillance plays an important role in host protection. The small number of genes required to convert nonmalignant tissue to SCC also indicates that under normal conditions, cancer events are probably not rare but rather are suppressed by immune mechanisms. How SCC evades immune surveillance in immunocompetent individuals is still not fully understood.

In this study, we aimed to further characterize the immune microenvironment of SCC, directing our attention to the stroma adjacent to SCC tumor nests. We were particularly interested in CD200, a known immunosuppressive protein. In healthy individuals, CD200 has a wide but specific tissue distribution that includes B cells, activated T cells, certain vascular endothelia, kidney glomeruli, placental cells, and neurons. Its cognate receptor, the CD200 receptor (CD200R), resides on cells of the myeloid lineage and certain subsets of B and T cells.
CD200-mediated immunosuppression seems to be exploited in various organs that require homeostatic immune privilege, such as the brain, retina, and hair follicle. CD200 has been described in several human cancers as well, presumably conferring pathologic immune privilege to tumor cells that express it. These cancers include chronic lymphocytic leukemia, multiple myeloma, acute myeloid leukemia, melanoma, ovarian cancer, and metastatic SCC. In some of these cancers, CD200 expression is correlated with progression. The role of CD200 and its receptor in primary cutaneous SCC has not been fully characterized, and their localization in the stroma adjacent to SCC has not been described.

## METHODS

### PATIENTS AND SAMPLES

Approval from the institutional review boards of Weill Cornell Medical College and The Rockefeller University and written informed consent were obtained before patients were enrolled in this study. The study was performed with strict adherence to the Declaration of Helsinki Principles. Cutaneous SCC samples were obtained during Mohs micrographic surgery and categorized as “invasive SCC” or “SCC in situ.” Normal specimens were obtained from non–sun-exposed areas of patients without skin cancer.

**LCM AND COLLECTION OF SUPERNATANTS FROM TUMOR AND NORMAL SKIN**

Normal skin samples (n=10) and SCC specimens (n=8) were subjected to laser-capture microdissection (LCM) according to the manufacturer’s protocol for the CellCut system (Molecular Machines and Industries). Freshly excised whole-tissue specimens of normal skin (n=2) and cutaneous SCC (n=6) were cut into small pieces with a scalpel and incubated for 24 hours in complete medium. Supernatants were collected, filtered, and stored at −80°C. Five supernatants were tested for endotoxin contamination using the ToxinSensor Endotoxin Detection System (GenScript).

### CELL CULTURE AND TREATMENT

Human dermal blood endothelial cells (HDBECs) isolated from adult dermis were purchased from PromoCell and maintained in Endothelial Cell Growth Media (catalogue No. C-22010) according to the manufacturer’s protocols. When confluent, cells were washed and incubated with new fresh media alone, media with 0.01-μg/mL lipopolysaccharide (LPS), or media with tumor supernatant in a 1:4 dilution for 12 hours in preparation for RNA extraction or 36 hours in preparation for flow cytometry.

### RNA EXTRACTION AND COMPLEMENTARY DNA MICROARRAY ANALYSIS

Total RNA was extracted from LCM samples using the RNeasy Micro Kit (Qiagen) and from HDBECs using the RNeasy Mini Kit (Qiagen), with on-column DNase digestion, according to the manufacturer’s protocol. Laser capture microdissection RNA was subjected to Affymetrix 2-cycle complementary DNA (cDNA) synthesis (Affymetrix), with a slight modification based on a previous report by Kube et al.** Affymetrix Human Genome U133 A2.0 arrays were used.

### QUANTITATIVE RT-PCR ANALYSIS

Laser capture microdissection total RNA was transcribed to cDNA using SuperScriptIII and Random Primers (Invitrogen), and the resulting cDNA was used for reverse transcriptase-polymerase chain reaction analysis (RT-PCR). The total RNA obtained from the HDBECs was used for RT-PCR analysis according to the manufacturer’s directions. All data were normalized to human acidic ribosomal protein (hARP). The sequences of the primers and probes for hARP are hARP-forward, GCCGTGCTAAACATGCTTCA; hARP-reverse, TGTCAAGACCCCTGCTTGAT; and hARP-probe, 6-FAM-TCCCCCCTTCTCCTTTGG GCTGG-TAMRA (GenBank accession No. NM-001002). Primers for CD200 (Hs01033303_m1) and CD31 (Hs00169777_m1) were obtained from Applied Biosystems.

### FLOW CYTOMETRY

Suspensions of cultured HDBECs were stained according to standard procedures with CD31–fluorescein isothiocyanate (FITC) (BD Biosciences, 1:100 dilution) and CD200–APC (BioLegend, 1:20 dilution). Samples were acquired using a flow cytometer (LSRII; BD Biosciences) and analyzed with Flowjo software (TreeStar Inc). Appropriate isotype controls were used to determine nonspecific staining.

### IMMUNOHISTOCHEMICAL ANALYSIS AND IMMUNOFLUORESCENCE

Frozen sections were prepared, and standard procedures were used. Immunohistochemical analysis was performed on sections of invasive SCC (n=5), SCC in situ (n=4), and normal skin (n=5) with purified mouse antihuman monoclonal antibody to CD200. Positive cells were counted in the dermis around SCC tumor nests, and cell counts per unit area (square millimeters) were determined. Immunofluorescence stainings were performed on sections of invasive SCC (n=7), SCC in situ (n=5), and normal skin (n=5), with antibodies to CD3, CD11c, CD31, CD163, CD200, CD200R, CD207, and NKp46. Detailed antibody information is provided in eTable 1 (http://www.jamaderm.com).

### STATISTICAL ANALYSIS

Microarray data was analyzed using R/Bioconductor packages (http://www.r-project.org). The Harshlight package was used to scan Affymetrix chips for spatial artifacts. Expression values were obtained using the GCRMA (guanine cytosine robust multiarray analysis) algorithm and linearly modeled in the limma package framework. For the comparison of interest, the moderated t test was used to assess differential expression. P values for each comparison were adjusted for multiple hypotheses using the Benjamini-Hochberg approach. Genes with a false discovery rate of less than 0.05 and a fold change greater than 4.0 were further analyzed with Ingenuity Pathway Analysis (Ingenuity). For surface protein expression, as quantified by manual cell counts or mean fluorescence intensity by flow cytometry as well as relative messenger RNA expression (mRNA) (in log scale), comparisons were made using the t test in Prism (GraphPad Software), with P values reported.
CD200 UPREGULATION IN SCC

Gene expression profiles of laser-captured stromal regions of SCC were compared with those of laser-captured reticular dermis of normal skin. A total of 338 upregulated and 540 downregulated probe sets were identified as differentially expressed genes (fold change >4.0; false discovery rate <0.05; eTable 2). These 878 differentially expressed genes were imported to Ingenuity Pathway Analysis to identify overrepresented biological functions (Table 1). We further explored the molecules involved in “immune responses” (Table 2). We found upregulation of CCL18, CD163, and IL7R and down-regulation of CD1c on this list. These results were consistent with previous reports, validating the accuracy of our current gene list.

CD200 EXPRESSION ADJACENT TO INVASIVE SCC

We found that CD200 was upregulated in stroma adjacent to invasive SCC (Figure 1). CD200 is a known immunosuppressive surface protein that has been identified in several human cancer cells; however, to our knowledge, no previous reports have identified CD200 in the stroma of any human cancer. We confirmed results from our gene list with quantitative RT-PCR using the same total RNA that we subjected to cDNA microarray analysis. In addition to samples of SCC stroma and normal reticular dermis, we included total RNA from laser-captured normal epidermis, actinic keratosis, SCC in situ, or invasive SCC. Figure 1A demonstrates significant upregulation of CD200 in SCC stroma compared with normal reticular dermis (P < .001). Notably, CD200 mRNA was not found in any of the keratinocyte samples (normal epidermis, actinic keratosis, SCC in situ, or invasive SCC). Representative images of CD200 immune-staining in normal skin (Figure 1B) and invasive SCC (Figure 1C) are also shown. There was a higher number of CD200+ cells in the stromal area of SCC (approximately 7-fold) than in the reticular dermis of normal skin (P < .001) (Figure 1D). Very few CD200+ cells were found directly within tumor nests, but occasionally CD200 positivity was detected at the leading edge, as previously reported (data not shown).

CD200 EXPRESSION ON ENDOTHELIAL CELLS ADJACENT TO INVASIVE SCC

We characterized the cellular distribution of CD200 in the SCC microenvironment using immunofluorescence microscopy. In invasive SCC, we found an expanse of cells and structures that were copositive for CD200 and PAL-E, a blood vessel endothelial cell marker. Nearly all PAL-E+ cells in invasive SCC coexpressed CD200. Normal skin differed strikingly; almost no PAL-E+ cells coexpressed CD200. Interestingly, in SCC in situ, some PAL-E+ structures expressed CD200, while some did not. Figure 2 shows representative images of staining in normal skin (Figure 2A), SCC in situ (Figure 2B), and invasive SCC (Figure 2C). This pattern of increasing CD200 expression with increasing progression of SCC was also apparent with CD31, a pan-endothelial cell marker. In all 3 groups, there were a few scattered LYVE-1+ lymphatic endothelial cells that expressed CD200; however, expression of CD200 on lymphatic endothelial cells did not differ across samples (Figure 2D-F). Major immune cell types including T cells (CD3+), macrophages (CD163+), and myeloid dendritic cells (CD11c+) did not express CD200 (data not shown).

CD200 EXPRESSION IN HDHECs INDUCED BY SCC TUMOR SUPERNATANT

To test whether factors in SCC could induce the expression of CD200 on HDHECs, we incubated HDHECs in culture with SCC supernatant. Treatment with LPS was used as a positive control. We cultured HDHECs with media alone, media containing LPS, and media mixed with tumor supernatant. Messenger RNA expression of CD200 in HDHECs cultured with tumor supernatant for 12 hours was significantly higher (nearly 4-fold) than HDHECs cultured with media alone (P < .01; Figure 3A). We then examined protein level expres-
sion by flow cytometry, finding increased surface protein expression of CD200 on HDBECs cultured with tumor supernatant for 36 hours compared with media alone (P < .05; Figure 3B). Representative histograms are shown in Figure 3C. The expression of CD200 on HDBECs treated with tumor supernatant was also higher than on those treated with normal skin supernatant when CD34<sup>high</sup> CD31<sup>+</sup> cells were analyzed (P < .05; Figure 4). CD34<sup>high</sup> CD31<sup>+</sup> cells likely represent a purified population of blood endothelial cells, since virtually all PAL-E<sup>+</sup> blood endothelial cells in the dermis coexpress CD34. We evaluated tumor supernatants for contamination with LPS and found that endotoxin was undetectable in all supernatants tested (<0.005 endotoxin units/mL; eTable 3). These results demonstrate that soluble factors in the tumor microenvironment are capable of inducing CD200 on dermal blood endothelial cells.

**CD200 EXPRESSION ON MACROPHAGES AND DENDRITIC CELLS IN THE SCC MICROENVIRONMENT**

To identify immune cells in the SCC microenvironment that are capable of responding to CD200, we stained for CD200R in normal skin and SCC specimens with immunofluorescence. In SCC stroma, we found high numbers of CD200R<sup>+</sup> cells. We identified these cells as a population of CD163<sup>+</sup> macrophages, of which nearly all coexpressed CD200R, and CD11c<sup>+</sup> dendritic cells, of which many coexpressed CD200R (Figure 5A and B). T cells, characterized by CD3, generally did not express CD200R in SCC (Figure 5C), though in normal skin there was occasional expression. In normal skin, nearly all macrophages, labeled with CD163, expressed CD200R, but dendritic cells, labeled with CD11c, did not. Neither CD207<sup>+</sup> Langerhans cells nor NKp46<sup>+</sup> natural killer (NK)
cells expressed CD200R in SCC (Figure 5D and E) or normal skin.

**COMMENT**

Tumor behavior is not simply a function of cancer cells themselves but also of the local “ecosystem” of a tumor, which consists of diverse stromal cells that regulate cancer progression. Endothelial cells lining local blood vessels are the first contact that most blood-borne elements, including immune cells, have with a tumor. Franses and colleagues propose that endothelial cell integrity plays a key role in tumors, promoting homeostasis when healthy but stimulating cancer growth when dysfunctional. They describe vessels that perfuse tumor as active stromal elements with “privileged access to the deepest recesses of tumors.” However, phenotypic changes in blood vessels of the SCC microenvironment have not yet been fully characterized. In the present study, we examined the expression and distribution of the immunosuppressive protein in the stroma surrounding SCC.

We found (1) that the stroma around SCC has increased gene and protein expression of CD200; (2) that CD200 is expressed by blood vessel endothelial cells in SCC to a much greater extent than in normal skin; (3) that factors released from tumor can induce expression of CD200 in HDBECs in culture; and (4) that CD200R myeloid cells are present in large numbers in the SCC microenvironment. Taken together, these results suggest that CD200 and its interaction with CD200R may be part of the mechanism of immune evasion in SCC and serve as a potential target for therapeutics for aggressive SCC.

We report herein that CD200 in the SCC microenvironment is localized mainly to microvessel endothelia. While some lymphatic vessels in SCC expressed CD200 as in normal skin, nearly all blood vessels in SCC expressed CD200, whereas very few did in normal skin. CD200 has previously been localized to blood vessel endothelium in various vascular beds including those of the lymph nodes, kidneys, liver, corpora lutea, and fallopian tubes. Its role on blood vessel endothelium is not clear. Ko and colleagues suggested that endothe-

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**Figure 2.** The number of PAL-E structures that coexpress CD200 increases with increasing invasiveness of squamous cell carcinoma (SCC). A, Immunofluorescence showed little coexpression of CD200 (green, left upper panel) and PAL-E (red, right upper panel) in normal dermis (n = 5). (The bottom panels show the merged image where double positive cells are identified in yellow.) B, Some PAL-E cells coexpress CD200 in the stroma of SCC in situ (n = 5). C, Nearly all PAL-E cells coexpress CD200 in invasive SCC (n = 7). D, There was little coexpression of CD200 (green, left upper panel) and LYVE-1 (red, right upper panel) in normal dermis (n = 5). E, There was little coexpression of CD200 and LYVE-1 in the stroma of SCC in situ (n = 5). F, There was little coexpression of CD200 (green, left upper panel) and LYVE-1 (red, right upper panel) in invasive SCC (n = 5). Scale bars = 100 µm.
lial CD200 may inhibit macrophage function, in part by downregulating macrophage adhesion molecules, to prevent aberrant diapedesis during inflammation. They found that in both mouse and human blood vessel endothelial cells, CD200 was differentially expressed and inducible depending on the type of endothelium. They reported that CD200 expression in human dermal microvascular endothelial cells in culture was not further induced with endotoxin challenge. In contrast, due perhaps to a different concentration of LPS, we found that HDBECs in culture could be induced to express higher levels of CD200, both at the gene and protein level, with both endotoxin challenge and incubation with SCC supernatant. This result suggests that factors in the tumor may be able to induce expression of CD200 on local vessels in the SCC microenvironment. Other factors on endothelial cells may also be important in the tumor microenvironment. One recent study by Clark and colleagues described reduced expression of E-selectin on vessel endothelium. They suggested that reduced E-selectin contributes to aberrant T-cell homing, increased infiltration of regulatory T cells, and ultimately reduced antitumor response.

We characterized CD200R expression in normal skin and in the SCC microenvironment. The number of CD200R+ cells was increased in cutaneous SCC compared with normal skin. We found CD200R to be localized to CD163+ macrophages and CD11c+ dendritic cells. CD200R was not found on CD207+ Langerhans cells. This is in sharp contrast to mouse data showing the expression of CD200R on mouse Langerhans cells. In human circulating monocytes have moderate levels of CD200R surface expression that is increased when they are induced in vitro to differentiate into dendritic cells. Also in line with our findings is the observation that CD11b+Gr-1+ myeloid-derived suppressor cells, but not T cells, NK cells, or mast cells, express CD200R in the stroma of chemically induced mouse cutaneous SCC. Myeloid-derived suppressor cells are thought to represent a mixture of immature myeloid cells that expand systemically in
malignant neoplasms, differentiate predominantly into dendritic cells and macrophages in tissues, and suppress T-cell responses.31 CD200R+ macrophages and dendritic cells found in the stroma of SCC may be stimulated by cognate ligand CD200 during diapedesis. It has been reported that CD200 binding to CD200R on macrophages and dendritic cells represses proinflammatory activation.8,32-35 It inhibits MAP kinases p38, ERK, and JNK, the common signaling pathways involved in classic activation of macrophages, theoretically maintaining M2 cells in their polarized state.36 The immunosuppressive role of CD200 on dendritic cells remains controversial. Jenmalm and colleagues37 did not observe any effects of CD200R on human monocyte-derived dendritic cells in vitro. In contrast, in mice, a soluble CD200-Ig protein did induce tolerogenic functions in dendritic cells.38 Our findings may suggest a novel mechanism responsible in part for the protumoral change in SCC-associated macrophages and dendritic cells that we have previously reported. Macrophages in the SCC microenvironment produce protumoral products like MMP9, MMP11, and VEGF-C and fail to eradicate SCC.24,39 Myeloid dendritic cells from SCC are poor stimulators of allogeneic T-cell proliferation compared with those from normal and peritumoral skin, despite similar levels of phenotypic maturity.23 Further investigation exploring the nature and function of CD200R-myeloid cells in human cancer would be of great interest.

Blockade of CD200-CD200R interaction by an antibody against CD200 has been shown to enhance antitumor activity against CD200-expressing human tumors in a mouse model.14,40 A phase 1/2 trial studying this anti-CD200 monoclonal antibody (ALXN6000) in patients with CD200-expressing B-cell chronic lymphocytic leukemia or multiple myeloma has recently been completed (Alexion Pharmaceuticals, NCT00648739). In addition to potentially modulating immune response in human CD200-expressing tumors, our results suggest that a CD200 antibody might also be able to modulate immune response in non-CD200–expressing tumors that are associated with CD200-rich stroma. In support of this idea, Rygiel and

Figure 4. CD200 expression is induced to a greater degree on CD34+ human dermal blood endothelial cells (HDBECs) treated with tumor supernatant compared with CD34+ HDBECs treated with normal skin supernatant. A, CD31+ HDBECs were further gated on CD34+ populations and analyzed for the expression of CD200. The value 25.3 reflects 25.3% of all cells. B, Superimposed histograms of fluorescence intensity of CD34+ HDBECs from selected samples of media plus lipopolysaccharide (LPS) (red), normal skin supernatant (blue), and squamous cell carcinoma (SCC) tumor supernatant (green) illustrate a stepwise shift toward greater surface expression of CD200. The orange line indicates background fluorescence intensity (FMO). C, When cultured for 36 hours in media containing tumor supernatant, HDBECs were found to have greater CD200 surface expression than when cultured with normal skin supernatant, as measured by mean fluorescence intensity (MFI). Error bars indicate mean (SEM).
colleagues recently demonstrated that CD200-knockout mice showed markedly less outgrowth of a chemically induced endogenous skin tumor compared with wild-type mice irrespective of tumor cell CD200 expression.

In conclusion, the ability of SCC to progress relies on evasion of the skin’s normal immune surveillance. We have shown that factors in the tumor can induce expression of the immunosuppressive surface protein CD200 on SCC-associated vascular endothelium and that this may affect the function of tumor-associated myeloid cells. It should be noted that angiogenesis and phenotypic changes in blood vessels are common elements affecting cancer behavior. Therefore, the induction of CD200 on vascular endothelium in other cancers may be a promising avenue of further study. Our findings suggest that anti-CD200 therapies may be promising treatments for aggressive SCC and other cancers that may be found to share these features.

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Figure 5. CD200 receptor (CD200R) is located on most CD163+ macrophages and occasional CD11c+ dendritic cells in the squamous cell carcinoma (SCC) microenvironment. A, Immunofluorescence in invasive SCC shows that the majority of CD163+ cells (green, left upper panel) coexpress CD200R (red, right upper panel). (The bottom panels show the merged image where double positive cells are identified in yellow.) B, Some CD11c+ cells (red, left upper panel) coexpress CD200R (green, right upper panel). C, There was little coexpression of CD3 and CD200R in invasive SCC. D, There was little coexpression of CD207/Langerin and CD200R in invasive SCC. E, There was little coexpression of NKp46 and CD200R in invasive SCC. Scale bars = 100 µm.
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