The Immunosuppressive Surface Ligand CD200 Augments the Metastatic Capacity of Squamous Cell Carcinoma

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Abstract

CD200 (OX-2) is a cell surface glycoprotein that imparts immune privileges by suppressing alloimmune and autoimmune responses through its receptor, CD200R, expressed primarily on myeloid cells. The ability of CD200 to suppress myeloid cell activation is critical for maintaining normal tissue homeostasis but may also enhance the survival of migratory neoplastic cells. We show that CD200 expression is largely absent in well-differentiated primary squamous cell carcinoma (SCC) of the skin, but is highly induced in SCC metastases to the lymph node and other solid tissues. CD200 does not influence the proliferative or invasive capacity of SCC cells or their ability to reconstitute primary skin tumors. However, loss of CD200 impairs the ability of SCC cells to metastasize and seed secondary tumors, indicating that the survival of CD200⁺ SCC cells may depend on their ability to interact with CD200R⁺ immune cells. The predominant population of CD200R⁺ stromal cells was CD11b⁺Gr-1⁺ myeloid-derived suppressor cells, which release elevated levels of granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor when in the presence of SCC cells in a CD200-dependent manner. Collectively, our findings implicate CD200 as a hallmark of SCC metastasis and suggest that the ability of CD200⁺ SCC keratinocytes to directly engage and modulate CD200R⁺ myeloid-derived suppressor cells is essential to metastatic survival. Cancer Res; 70(7): 2962–72. ©2010 AACR.

Introduction

Solid tumor metastasis is a complex multistep process, during which neoplastic cells must invade tissue, enter and navigate via the circulatory system, and extravasate to colonize a distant foreign tissue (1, 2), and the success of this process is highly dependent on modulating the immune system (3, 4). Some well-known examples of tumor-associated immune modulation include loss of tumor antigens, alterations in HLA class I antigens (5, 6) and proinflammatory (Th1) and anti-inflammatory (Th2) T helper cell–derived cytokine production (7–9), decreased antigen-presenting cell activity, secretion of proapoptotic factors, and recruitment of immunosuppressive cells, all of which may disable antitumor effector T cells (10).

CD200 is a widely expressed cell surface glycoprotein that acts as a potent suppressor of CD200R-expressing immune cells (11–13). The immunosuppressive capacity of CD200 is crucial for maintaining homeostasis in a number of tissues, including the central and peripheral nervous system (14), vascular endothelia (15), skin (16), and lymphoid cells (12). Mice lacking CD200 are normal in appearance but exhibit elevated numbers of activated CD11b⁺ macrophages and granulocytes (11). As a result, CD200-null animals display chronic central nervous system inflammation, early onset of experimental autoimmune encephalomyelitis, and increased susceptibility to autoimmunity in experimental autoimmune uveoretinitis due to a failure to adequately inactivate CD200R¹ macrophages in response to injury (11, 17). Therefore, during homeostasis, CD200 maintains an appropriate level of activated macrophages to preserve tissue integrity; whereas during injury-induced heightened inflammatory states, CD200 also provides immune privilege to CD200⁺ tissues to escape macrophage-mediated tissue damage (16, 18). CD200R, a transmembrane glycoprotein, is expressed mostly in myeloid cells (13, 19) and smaller subsets of lymphoid-derived cells (13, 20). There are five described CD200R isoforms, CD200R1–R5, of which CD200R1 is reported to be the major mediator of CD200 immunosuppressive signaling (13, 21).

Several lines of evidence suggest that the immunosuppressive capacity of CD200 may also stimulate cancer development. CD200 is upregulated in chronic lymphocytic leukemia (22), multiple myeloma (23), and acute myeloid leukemia (24). Furthermore, CD200-expressing melanoma (25) and ovarian cancer cells downregulate Th1 cytokine production in coculture with mixed leukocytes (26), indicating that CD200-mediated immune suppression may also be featured in tumor progression and/or metastasis. This idea is supported in a model of B cell lymphocytic leukemia, in which ectopic CD200 expression inhibited the ability of lymphocytes to clear tumor cells, whereas CD200-negative tumor cells were rejected by CD200R⁺...
peripheral mononuclear blood cells (25). Finally, CD200 expression has also been observed in a tumorigenic keratinocyte line isolated from carcinogen-treated mouse skin (16); however, the significance of CD200 in epithelial tumor metastasis remains to be determined.

We have identified CD200 induction as a hallmark of metastatic squamous cell carcinoma (SCC). CD200 does not influence primary tumor development but promotes the survival of metastatic SCC cells and their ability to seed secondary tumors. Finally, CD200R1+ myeloid-derived suppressor cells (MDSC) were implicated as the primary myeloid immune cell type potentially responsible for supporting the survival of CD200+ metastatic SCC cells.

Materials and Methods

**Antibodies.** Antibodies were used against human CD200, mouse CD200, CD3e, CD86, α6 integrin-FITC (BD Biosciences); NK1.1-488, e-kit, CD123, HMC class II, CD11b-FITC, CD11b-PE, Gr-1-FITC, Gr-1-PE, CD11c (BioLegend); Langerin, Foxp3 (eBioscience); Keratin 14 (Covance); CD200R1 (R&D Systems); CD4-APC (Abcam); granulocyte colony-stimulating factor (G-CSF; Santa Cruz Biotechnology); anti-rabbit horseradish peroxidase (Jackson ImmunoResearch); species-specific AlexaFluor-488, -594, -647, and -680 (Invitrogen); and human α6 integrin (kindly provided by Fiona Watt, Cambridge Research Institute, Cambridge, United Kingdom).

**Two-stage skin carcinogenesis.** Murine skin tumors were induced by a standard two-stage chemical carcinogenesis protocol in FVB mice as previously described (ref. 27; see Supplementary Materials and Methods). All mice were housed and sacrificed according to Institutional Animal Care and Use Committee and NIH guidelines for experimental endpoints including tumor size and morbidity.

**Tissue harvesting.** Skin tumors were harvested from 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate–treated mice upon sacrifice and residual normal skin on the periphery of the tumors was removed using surgical scissors. Mice were also examined internally, and enlarged lymph nodes and lung tumor specimens were extracted with forceps and cleaned from connective tissue. De-identified human cutaneous SCC specimens were obtained from the Dermatology Department and human metastatic SCC specimens from the Herbert Irving Comprehensive Cancer Center Tissue Bank at Columbia University under institutional review board approval. Normal skin and tumor specimens were embedded in optimal cutting temperature compound and cryopreserved or fixed in 10% neutral buffered formalin and paraffin-embedded.

**Cell culture.** Primary keratinocyte cultures were established from normal skin, benign papillomas, primary cutaneous SCC, or metastatic SCC to the lymph node or lung as previously described (28, 29). All cells were plated on collagen and fibronectin-coated dishes in the presence of a mitotically arrested fibroblast feeder layer (28) at 32°C in Williams’ Medium E (Invitrogen) supplemented with 8 μg/mL of transferrin (Invitrogen), 409 mg/L of glutamine (Invitrogen), 20% fetal bovine serum (Hyclone), 10 ng/mL of epidermal growth factor (Invitrogen), 5 μg/mL of insulin (Sigma), 0.5 μg/mL of hydrocortisone (MP Biomedicals), 10−10 mol/L of cholera enterotoxin (ICN), and 1x penicillin-streptomycin (Invitrogen). All cell lines used are listed in Supplementary Table S1. Cell passages 1 to 20 were used in experiments.

**Cytokine array.** pRS-NS or pRS-CD200 Lung Met cells (8×10^6) were cocultured with fluorescence-activated cell sorted (FACS) CD200R1+ immune cells (1.6×10^6) freshly isolated by FACS CD200R1+ immune cells (1.6×10^6) freshly isolated by FACS and visualized by indirect immunofluorescence on glass coverslips and quantified by flow cytometry analysis.

**Flow cytometry.** All flow cytometry experiments were performed on freshly isolated tumor cell suspensions or pRS-CD200-transduced, pRS-NS-transduced, or untransduced Lung Met cells (8×10^6). Lungs were analyzed at 7 or 35 d postinjection for the presence of metastatic SCC (n = 3–6 mice per time point; see Supplementary Materials and Methods).

**In vitro studies.** pRS-NS or pRS-CD200 Lung Met cells (8×10^6) were cocultured with fluorescence-activated cell sorted (FACS) CD200R1+ immune cells (1.6×10^6) freshly isolated by FACS and visualized by indirect immunofluorescence on glass coverslips and quantified by flow cytometry analysis. Flow cytometry. All flow cytometry experiments were performed on freshly isolated tumor cell suspensions or pRS-CD200-transduced, pRS-NS-transduced, or untransduced Lung Met 1, 2, and 4 cultured keratinocytes (see Supplementary Materials and Methods). In vitro studies. To measure proliferation, pRS-NS or pRS-CD200 Lung Met 1, 2, or 4 cells were plated into 12-well plates at 50,000 cells/well and cell counts were performed using a hemacytometer in 48-h increments for a period of up to 2 wk. Each time point was counted in triplicate for each group and average cell numbers were statistically compared using a Student’s t test (P < 0.05) between pRS-NS and pRS-CD200 groups for each Lung Met cell line.

For invasion assays, pRS-NS or pRS-CD200 Lung Met 1, 2, or 4 cells were plated at 2×10^6 cells per well in Matrigel invasion chambers according to the protocols of the manufacturer (BD Biosciences). After 48 h, membranes were stained with 1% toluidine blue, mounted and microscopically evaluated for the number of invasive cells on five randomly chosen fields of view per insert. Normal mouse epidermal keratinocytes were analyzed as a control. The average number of cells per membrane was statistically compared using the Student’s t test (P < 0.05) between pRS-NS and pRS-CD200 groups for each Lung Met cell line.

For skin tumor reconstitution, pRS-NS or pRS-CD200 Lung Met 1, 2, or 4 cells were resuspended in HBSS solution and 1.0×10^5 (n = 3 mice per cell group) or 5.0×10^5 (n = 3 mice per cell group) cells were implanted onto the dorsal fascia of recipient mice in silicon grafting chambers as previously described (ref. 28; see Supplementary Materials and Methods). To measure metastasis, pRS-NS or pRS-CD200 Lung Met 1, 2, or 4 cells were resuspended in HBSS and 2×10^5 cells were injected into the tail vein of fully immunocompetent female FVB mice (n = 9 mice per cell group). Lungs were analyzed at 7 or 35 d postinjection for the presence of metastatic SCC (n = 3–6 mice per time point; see Supplementary Materials and Methods).
from DMBA/12-O-tetradecanoylphorbol-13-acetate–induced skin tumors. Lung Met/immune cell cocultures were incubated at 32°C for 24 h in complete Williams’ Medium E, after which supernatants were collected, filtered, and hybridized to the Mouse Inflammation Antibody Array 1 according to the protocols of the manufacturer (RayBiotech). Each experimental group was run in duplicate and controls included supernatants harvested from Lung Met or CD200R1+ immune cells cultured alone. For densitometry analysis, individual cytokine values were corrected by subtraction from the average background reading for each membrane followed by normalization to the average positive control signal (n = 6 positive control readings per membrane). Average-corrected cytokine levels (n = 2 cytokine Readings per membrane) were statistically compared between all of the following groups: CD200R1+ cells alone, pRS-NS Lung Met cells alone, pRS-CD200 Lung Met cells alone, CD200R1+/pRS-NS Lung Met cultures, and CD200R1+/pRS-CD200 Lung Met cultures (Student’s t test; P < 0.05).

Results

**CD200 is induced in metastatic SCC.** To determine a role for CD200 in skin carcinogenesis, we analyzed CD200 expression in primary and metastatic SCC induced by classic two-stage chemical carcinogenesis in FVB mouse skin (27, 31). In response to topical DMBA/12-O-tetradecanoylphorbol-13-acetate treatment, FVB mice develop cutaneous SCC that metastasize to the lymph node and lung (ref. 27; Fig. 1A), a model that is analogous to human SCC pathogenesis (32, 33). CD200 was present in the hair follicle bulge but not in the epidermis of normal murine skin (ref. 16; Fig. 1A) and was also observed in the dermal papilla of telogen and anagen hair follicles (Fig. 1A; Supplementary Fig. S1). No CD200 expression was detected in benign papillomas (0 of 9) or well-differentiated (WD) SCC of the skin (0 of 105) and pRS-CD200 Lung Met cells alone, CD200R1+/pRS-NS Lung Met cultures, and CD200R1+/pRS-CD200 Lung Met cultures (Student’s t test; P < 0.05).

Next, we quantified the number of CD200+ cells from freshly isolated primary and metastatic SCC that coexpress the keratinocyte surface receptor α6 integrin by flow cytometry analysis. Little to no α6+/CD200+ cells were observed in WD SCC (average 3.9%, n = 5; Fig. 1B); however, a substantial population of α6+/CD200+ cells was detected in PD SCC (average 68%, n = 8; Fig. 1B). Interestingly, expression of CD200 within PD SCC was consistently localized in tumor keratinocytes invading the underlying muscle fascia (Fig. 1B). Importantly, virtually all (LN Met: average 88%, n = 7; Lung Met: average 93%, n = 3) keratinocytes isolated from metastatic SCC specimens expressed CD200 (Fig. 1B).

A similar expression pattern for CD200 was observed in human cutaneous and metastatic SCC. CD200 expression was rarely observed in human WD SCC (1 of 20, 5%) but was more frequent in human PD SCC (2 of 7, 29%; Fig. 2; Table 1). Notably, 85% (11 of 13) of human metastatic SCC to the lymph node and 100% (7 of 7) of metastatic SCC seeding a variety of other secondary organs exhibited CD200 induction (Fig. 2; Table 1). No CD200 expression was detected in normal human epidermis (Fig. 2). These observations confirm that CD200 induction is a hallmark for murine and human metastatic SCC and indicate that the CD200+ pool of SCC keratinocytes rapidly expands during late stage carcinogenesis.

**CD200 does not influence SCC keratinocyte proliferation or invasion.** Established cultures of LN and Lung Met SCC keratinocytes maintained their CD200 expression status, whereas no CD200 expression was observed in K14+ keratinocytes isolated from skin, papilloma, or SCC (Fig. 3A and B). The molecular fingerprint of DMBA-induced cutaneous SCC is the transversion mutation in the 61st codon of H-ras (34, 35). We detected the H-ras 61st codon mutation in two of the three (67%) Lung Met specimens (Lung Met 2 and 4), confirming that these tumors were derived from DMBA-induced skin tumors (Supplementary Fig. S2).

To measure the effects of CD200 on proliferation, short hairpin RNA–transduced (pRS-NS or pRS-CD200) Lung Met 4 cells (Fig. 3B) were plated at equal densities and counted every 48 hours for a period of 2 weeks, by which time, all cells had reached confluency. No significant difference in log phase growth was observed between pRS-NS (doubling rate = 1.6 × 10^6) and pRS-CD200 (doubling rate = 1.6 × 10^6) Lung Met 4 cells (Fig. 3C). Similar results were obtained for Lung Met 1 and 2 cultures (data not shown), pRS-NS and pRS-CD200 Lung Met 4 cells were also evaluated for their capacity to invade through Matrigel. Although Lung Met cells were 6-fold to 7-fold more invasive than normal mouse epidermal keratinocytes (data not shown), there was no significant difference in the invasive capacity between pRS-NS and pRS-CD200 Lung Met 4 cells (Fig. 3D). Similar results were obtained for Lung Met 1 and 2 cultures (data not shown). Collectively, these results indicate that CD200 does not influence the proliferation or invasion of metastatic SCC cells.

**CD200 does not influence primary cutaneous SCC formation.** To assess the role of CD200 in primary cutaneous...
SCC formation, pRS-NS or pRS-CD200 Lung Met 4 cells were surgically implanted onto the dorsal fascia of fully immunocompetent FVB mice and evaluated for their ability to reconstitute cutaneous SCC (Fig. 4A). FVB mice are an inbred strain and highly syngeneic, thereby circumventing problems related to host-graft compatibility. Within 5 to 7 weeks postgrafting, both pRS-NS and pRS-CD200 Lung Met 4 cells (1 x 10^5 or 5 x 10^5 cells/graft) were capable of regenerating cutaneous SCC (Fig. 4A and B). There was no difference in SCC size as determined by tumor diameter (data not shown) or weight between pRS-NS and pRS-CD200 Lung Met 4 grafts (Fig. 4B). Similar results were obtained in SCC grafts obtained from Lung Met 1 and 2 cells (data not shown). These data indicate that CD200 may not play a role in primary SCC formation, an idea consistent with the onset of CD200 induction in late stage PD SCC.

**Loss of CD200 diminishes SCC metastatic potential.** To determine whether CD200 may be essential for SCC metastasis, pRS-NS or pRS-CD200 Lung Met 4 keratinocytes were assessed for their ability to seed secondary tumors in the lung following tail vein injections in FVB mice. The tail vein assay is a well-established model of experimental metastasis that tests the ability of tumor cells to cross the alveolar-capillary barrier and form tumors in the lung. Under these conditions, a 5-week incubation was the latest allowable time point for harvesting tissues as the morbidity related to lung metastasis was typically too severe by 6 weeks postinjection (data not shown). Upon sacrifice, animals were subjected to gross inspection to detect the presence of secondary tumors in internal organs; however, SCC were only observed in the lung. Harvested lung tissues were analyzed by FACS analysis for CD200 and K14 (K14 is not normally expressed in lung tissue) expressing metastatic SCC keratinocytes. At 7 days postinjection, low levels of both pRS-NS (average, 0.76%) and pRS-CD200 (average, 0.48%) Lung Met cells were detected in total lung cell suspensions but these levels were not statistically different (P = 0.11; n = 3 mice per group; Fig. 4C). However, the percentage of K14+ Lung Met cells increased 4-fold in pRS-NS–injected mice (n = 3 mice), whereas the percentage of K14+ cells actually decreased ~2-fold in pRS-CD200–injected mice (P = 0.0001; n = 6 mice) by 35 days (Fig. 4C). Consequently, the metastatic capacity of pRS-NS Lung Met cells was 8-fold to 9-fold higher compared with pRS-CD200 Lung Met cells (P = 0.0001; Fig. 4C). Similar results were obtained for all three Lung Met cell lines (data not shown). These data indicate that CD200 promotes SCC metastasis by supporting the survival of metastatic cells seeded in secondary tissues.

**CD200R-expressing immune cells are present in cutaneous and metastatic SCC.** To determine whether CD200+ SCC cells may interact with CD200R+ immune cells during skin carcinogenesis, DMBA-induced skin and metastatic tumors were analyzed for the presence of CD200R1-expressing cells. CD200R1+ cells were present in the stroma of benign papilomas (data not shown), WD SCC, LN Met, and Lung Met (Fig. 5A and Supplementary Fig. S3B) and were tightly associated with SCC keratinocytes suggestive of direct cell-cell contact (Fig. 5A). Little to no CD200R1+ cells were found to coexpress NK1.1, CD3, or c-kit, thereby excluding a natural killer cell, lymphoid, or mast cell phenotype (Supplementary Fig. S3C). In addition, no significant labeling for CD123, Langerin, CD86, or CD11c was observed in the CD200R1+ population by FACS analysis (data not shown). However, a predominant population of CD200R1+ cells was found to coexpress the myeloid lineage marker CD11b (average, 87.9% of CD200R1+ cells coexpress CD11b; P = 0.0001; Fig. 5B). Furthermore, most of the CD200R1+/CD11b+ cell population also expressed Gr-1 (average, 74.5% of CD200R1+/CD11b+ cells coexpress CD11b; n = 5; Fig. 5B). Finally, the remaining population of CD200R1+ cells coexpressed the MHC class II surface molecule (average, 8.4% of CD200R1+ cells coexpressed MHC class II; Fig. 5B), potentially marking a population of Langerhans cells (36). The coexpression of CD200R1 and CD11b in stromal SCC cells identified by FACS analysis was confirmed by immunofluorescence labeling in tissue sections of WD SCC (Fig. 5B and Supplementary Fig. S3B) and LN Met (Supplementary Fig. S3B).

Immunosuppressive regulatory T-cells are also implicated in cutaneous SCC progression (37); however, we could not correlate any distribution of Foxp3+ cells with the presence or absence of CD200 (Supplementary Fig. S4A). The identification of Foxp3+ regulatory T cells was confirmed by CD4 colabeling (data not shown). Collectively, these studies identify the major CD200R1+ stromal cell present in primary skin SCC as CD11b+/Gr-1+ MDSCs.

**Metastatic SCC cells modulate MDSC activity via CD200–CD200R interaction.** To gain insight into a potential mechanistic link between the CD200–CD200R interaction and SCC metastasis, pRS-NS or pRS-CD200 Lung Met keratinocytes were cocultured with equal amounts of CD200R1+ cells.

### Table 1. CD200 expression in human and murine SCC

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Tumor number (n)</th>
<th>CD200+ (%)</th>
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</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cutaneous tumors (n = 27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD SCC</td>
<td>20</td>
<td>1 (5)</td>
</tr>
<tr>
<td>PD SCC</td>
<td>7</td>
<td>2 (29)</td>
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<tr>
<td>Metastatic SCC (n = 20)</td>
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<td></td>
</tr>
<tr>
<td>Lymph node (head-neck)</td>
<td>13</td>
<td>11 (85)</td>
</tr>
<tr>
<td>Other secondary site*</td>
<td>7</td>
<td>7 (100)</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cutaneous tumors (n = 47)</td>
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<td></td>
</tr>
<tr>
<td>Papilloma</td>
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<td>0</td>
</tr>
<tr>
<td>WD SCC</td>
<td>24</td>
<td>0</td>
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<tr>
<td>PD SCC</td>
<td>14</td>
<td>14 (100)</td>
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<tr>
<td>Metastatic SCC (n = 17)</td>
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</tr>
<tr>
<td>Lymph node</td>
<td>14</td>
<td>14 (100)</td>
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<tr>
<td>Other secondary site*</td>
<td>3</td>
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*Other secondary sites include liver, bone, abdomen, chest wall, larynx, and oral mucosa in human (one specimen per site) and lung in mouse (all three).
MDSCs extracted from DMBA-induced skin tumors, after which the levels of 40 secreted proinflammatory cytokines were analyzed on Inflammation Antibody Arrays. We observed a substantial increase in the release of 2 out of 40 cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), and G-CSF, in cocultures consisting of pRS-NS Lung Met (CD200⁺) cells and CD200R1⁺ MDSCs but not in cocultures of pRS-CD200 Lung Met (CD200 knockdown) cells and CD200R1⁺ MDSCs or any of the single Lung Met or MDSC cultures (Supplementary Fig. S4B). In addition, the net production values of GM-CSF and G-CSF in pRS-NS Lung Met/CD200⁺ and CD200R1⁺ MDSC cocultures compared with pRS-CD200 Lung Met/CD200R1⁺ MDSC cocultures were 4-fold (P = 0.049) and 20-fold (P = 0.002) higher, respectively (Fig. 5C). These results show a functional role for the interaction between CD200 ligand in metastatic SCC cells and CD200R1⁺ immune cells to promote metastasis. A similar model has been reported in leukemia cells that exhibit increased surface levels of CD47, an immunoglobulin-like ligand that binds its receptor, SIRPα, expressed on macrophages. The CD47–SIRPα interaction blocks macrophage phagocytosis of leukemia cells, thereby stimulating metastasis (38).

CD200 has recently been established as a marker of a stem cell population residing in the bulge region of the hair follicle (39) and is thought to be one of a number of immunosuppressive molecules expressed by hair follicle stem cells that protect against immune destruction, thereby preserving the regenerative capacity of hair appendages (40). Interestingly, the hair follicle bulge has recently been shown to be a site of origin for cutaneous SCC stem cells (41) and some normal phenotypic markers of bulge keratinocyte stem cells, such as CD34 expression, are preserved in cutaneous SCC (41). Along these lines, it is feasible that CD200 expression in SCC cells may mark a population of cancer stem cells. However, we were unable to detect CD200⁺ keratinocytes in papillomas, the benign precursor to SCC. Although we did detect a small population of CD200⁺ keratinocytes in WD SCC by FACS analysis, we cannot rule out the possibility of contaminating normal cells contributing to this population. The results of our tumor reconstitution studies would suggest that, if CD200 does label SCC stem cells, it is not required for their development in the skin. Moreover, the uniform expression of CD200 in metastatic SCC is not entirely consistent with the notion that cancer stem cells are rare cells that maintain differentiated and phenotypically distinct progeny in tumors.

Using a mouse multistage model of SCC metastasis, we identified the predominant CD200R1⁺ immune cell species infiltrating the stroma of cutaneous and metastatic SCC as CD11b/Gr-1⁺ MDSCs. In the mouse, MDSCs have been well described as highly immunosuppressive CD11b/Gr-1⁺ cells that commonly infiltrate solid tumors or other sites of inflammation (42, 43). To our knowledge, this is the first report of CD200R expression in MDSCs infiltrating any tumor type, and therefore, provides a new dynamic to the behavior of tumor-promoting MDSCs. In a normal tissue microenvironment, ligation to CD200 would suppress the activity of CD11b/Gr-1⁺ mature myeloid cells. However, in a tumor microenvironment, activation of CD200R in MDSCs may stimulate...
Figure 3. CD200 does not influence SCC keratinocyte proliferation or invasion. A, CD200, DAPI, and K14 immunofluorescence in normal mouse epidermal keratinocytes (NEK), papilloma (Pap), WD SCC, and LN Met–cultured keratinocytes. Arrowhead points to positive detection of CD200 (LN Met). All three images for each column represent the same field of view. B, top: CD200 and K14 immunofluorescence in untransduced and pRS-CD200-transduced LN Met cells. Arrows point to the presence (untransduced) or loss (pRS-CD200) of CD200 expression. Dotted line delineates the boundary of a pRS-CD200 keratinocyte colony. Both images for untransduced and pRS-CD200 cells represent the same field of view. FACS histograms showing CD200 surface levels in Lung Met 1, 2, and 4 keratinocytes (red line). IgG control for each plot is outlined in blue, or Lung Met 4 cells transduced with pRS-NS and pRS-CD200 short hairpin RNA. Bars designate gate set for FACS sorting into α6+/CD200+ (pRS-NS) and α6+/CD200− (pRS-CD200) cell populations. C, average counts of Lung Met 4 cells transduced with pRS-NS (△) or pRS-CD200 (●) in 48-h increments (n = 3 per time point). Log phase of the growth (days 6–12) was used to determine the slope of each growth curve. D, bar graph showing the average number of cells per insert as a measure of the invasive capacity of Lung Met 4 cells transduced with pRS-NS or pRS-CD200 (n = 3 inserts per group).
the ability of these cells to promote tumorigenesis by immune suppression or other means. This concept is supported in part by our in vitro data demonstrating that the general effects of CD200+ tumor cells engaging CD200R1+ MDSCs are stimulatory rather than suppressive. Interestingly, MDSCs are present in the stroma of benign papillomas and WD SCC well before the onset of CD200 induction. This would also suggest multiple roles for MDSCs in carcinogenesis in that the previously reported immunosuppressive mechanisms of MDSCs (42, 43), and perhaps CD200R-independent, may support the early development and malignant conversion of neoplastic cells in the primary tumor site. Whereas in later stages of carcinogenesis, CD200+ SCC cells use MDSCs in a CD200R-dependent manner to gain immune privilege and seed secondary tumors. We observed that MDSCs are stimulated to release elevated levels of G-CSF and GM-CSF upon interaction with metastatic SCC cells in a CD200-dependent manner. A number of studies have reported that G-CSF administration could enhance tumor angiogenesis, growth, and malignant progression in vivo (44), whereas GM-CSF has been shown primarily to stimulate antitumor immune function in mice and humans (45). Although it is conceivable that the modulation of MDSC behavior via the CD200–CD200R interaction may establish a highly immunosuppressive, prometastatic milieu through G-CSF and GM-CSF production, the mechanistic significance of these proinflammatory cytokines remains to be determined. Along these lines, a more comprehensive delineation of MDSC behavior resulting from CD200R activation will be the focus of future studies.

The pattern of CD200 induction was preferentially localized to the invading front of tumor cells in PD SCC, indicating that CD200 may play roles in local tumor invasion as well as metastasis and that the proximity of neoplastic cells to tumor stromal-derived factors might be a key factor to CD200 induction. In fact, both IFNγ and tumor necrosis factor-α could induce CD200 expression (46). Therefore, it is reasonable to assume that CD200 may be predominantly important for primary and metastatic tumor cells as they invade certain microenvironments and, in as much, might not be fundamental for the metastatic process. However, we found CD200 expression present in human metastatic SCC specimens isolated from at
Figure 5. CD200R1+ stromal cells are CD11b−/Gr-1− MDSCs. A, CD200R1 immunofluorescence in WD SCC, LN Met, and Lung Met (white arrows). DAPI counterstain was conducted to visualize nuclei. Dashed line demarcates SCC keratinocytes in LN Met. B, FACs analysis of CD200R1, CD11b, and Gr-1 or MHC class II expression. The total population of CD200R1+ cells was gated and subsequently analyzed for expression of CD11b and Gr-1 or MHC class II. The percentage of the total CD200R1 pool for a single experiment is shown. Far right, murine SCC were stained with antibodies against CD200R1 (red) and CD11b (green) and counterstained with DAPI (blue). Arrows point to CD200R1+/CD11b+ (yellow) stromal MDSCs. C, left: bar graph showing the densitometric units representing GM-CSF and G-CSF levels in pRS-NS Lung Met/CD200R1+ cocultures versus pRS-CD200 Lung Met/CD200R1+ cocultures. *, statistically significant difference (GM-CSF, P = 0.049; G-CSF, P = 0.002). Right, H&E staining (top left) and G-CSF (red) and CD200 (green) immunofluorescence in murine PD SCC. Nuclei were delineated with DAPI (blue). Bars, 50 μm.
least eight different body sites, suggesting that CD200 was not selectively regulated by certain tumor microenvironments but might be a general feature of the metastatic SCC cell. Moreover, we did not observe CD200 in human or murine WD SCC, although these are highly invasive lesions, and CD200 levels were maintained in metastatic SCC cells in culture, suggesting that the regulation of CD200 is a permanent fixture of the late-invasive/metastatic cell. Finally, the observation of enhanced early survival of CD200+ cells following tail vein injection, but preceding tumor implantation, compared with CD200− cells strongly favors a key role for CD200 in metastasis. In conclusion, the onset of CD200 expression in PD SCC indicates roles for CD200 in tumor invasion and metastasis, whereas the mechanisms that underlie the induction of CD200, whether they involve a combination of tumor stromal–derived factors and genetic/epigenetic changes within invading SCC cells, remain to be determined.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We are grateful to Fiona Watt for providing the human α6 antibody and especially to Kristie Gordon for her assistance with flow cytometry.

Grant Support
NIH R01CA114014 and R21CA131897 grants.

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Received 12/01/2009; revised 01/21/2010; accepted 01/29/2010; published OnlineFirst 03/23/2010.

References
Correction: Online Publication Dates for Cancer Research April 15, 2010 Articles

The following articles in the April 15, 2010 issue of Cancer Research were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:


- **Lu ZH, Shvartsman MB, Lee AY, Shao JM, Murray MM, Kladney RD, Fan D, Krajewski S, Chiang GG, Mills GB, Arbeit JM. Mammalian target of rapamycin activator RHEB is frequently overexpressed in human carcinomas and is critical and sufficient for skin epithelial carcinogenesis. Cancer Res 2010;70:3287–98. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3467.**


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Published OnlineFirst 05/11/2010. 
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doi: 10.1158/0008-5472.CAN-10-1347
The Immunosuppressive Surface Ligand CD200 Augments the Metastatic Capacity of Squamous Cell Carcinoma

Magda Stumpfova, Desirée Ratner, Edward B. Desciak, et al.

Cancer Res 2010;70:2962-2972. Published OnlineFirst March 23, 2010.

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