HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2017 August 15.

Published in final edited form as:

J Immunol. 2016 August 15; 197(4): 1489–1497. doi:10.4049/jimmunol.1600052.

A critical role of CD200R signaling in limiting the growth and metastasis of CD200 positive melanoma

Jin-Qing Liu[1], **Fatemeh Talebian**[1], **Lisha Wu**[1,2], **Zhihao Liu**[1,2], **Ming-Song Li**[2], **Laichu Wu**[3], **Jianmin Zhu**[4], **Joseph Markowitz**[1], **William E. Carson III**[1], **Sujit Basu**[1], and **Xue-Feng Bai**[*,1,4]

[1]Department of Pathology and Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA

[2]Department of Gastroenterology, Guangdong Provincial Key Laboratory of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou, China

[3]Davis Medical Research Center, 480 Medical Center Drive, Columbus, OH, USA

[4]Pediatric Translational Medicine Institute, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai, China

Abstract

CD200 is a cell surface glycoprotein that functions through engaging CD200 receptor on cells of the myeloid lineage and inhibits their functions. Expression of CD200 has been implicated in a variety of human cancer cells including melanoma cells. However, its roles in tumor growth and immunity are not clearly understood. In this study, we have used CD200R-deficient mice and the B16 tumor model to evaluate this issue. We found that CD200R-deficient mice exhibited accelerated growth of CD200-positive, but not CD200-negative B16 tumors. Strikingly, CD200R-deficient mice receiving CD200-positive B16 cells intravenously exhibited massive tumor growth in multiple organs including liver, lung, kidney and peritoneal cavity, while the growth of the same tumors in wild type mice is limited. CD200-positive tumors grown in CD200R-deficient mice contained higher numbers of $CD11b^{+}Ly6C^{+}$ myeloid cells, exhibited increased expression of VEGF and HIF-1α genes with increased angiogenesis and showed significantly reduced infiltration of $CD4^{+}$ and $CD8^{+}$ T cells, presumably due to reduced expression of T cell chemokines such as CXCL9 and CXCL16. The Liver from CD200R-deficient mice under metastatic growth of CD200-positive tumors contained significantly increased numbers of $CD11b^{+}Gr1^{-}$ myeloid cells, $FoxP3^{+}$ regulatory T cells and reduced numbers of NK cells. Liver T cells also had reduced capacity in the production of IFN-γ or TNF-α. Taken together, we have revealed a critical role of CD200R signaling in limiting the growth and metastasis of CD200 positive tumors. Targeting CD200R signaling may thus has a potential to interfere with the metastatic growth of CD200-positive tumors like melanoma.

[*]Correspondence should be addressed to: Xue-Feng Bai, Department of Pathology and Comprehensive Cancer Center, Ohio State University Medical Center, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, Phone: 614-292 8649, Fax: 614-292 7072, Xue-Feng.Bai@osumc.edu.

Introduction

Tumor-associated inflammation and immune responses are key components in the tumor microenvironment (TME) that regulates tumor growth, progression and metastasis (1). Tumor associated myeloid cells (TAMC) are a groups of cells that not only play key roles in inducing tumor angiogenesis (2, 3), activating invasion/metastasis (4, 5), but also regulate tumor specific T cell responses (6). Thus, identification and characterization of key pathways that can regulate TAMCs are of critical importance for developing cancer immunotherapy. In this regard, our recent mouse studies (7, 8) suggest that CD200-CD200 receptor (CD200R) interaction may be important for the growth and metastasis of tumors such as melanoma.

CD200 (also known as OX-2) is a member of the Ig super family of proteins. It contains two extracellular immunoglobulin domains and a small intracellular domain with no known signaling motif (9). CD200 is expressed in a variety of normal tissues (10-13) and the lymphoid cells including B lymphocytes and activated T cells (14). CD200R, the cognate ligand for CD200, is also an IgSF protein (15). The expression pattern of mouse and human CD200R is similar, with strong expression in macrophages, neutrophils and mast cells (16). Triggering CD200R suppresses myeloid cell activity in vitro and engagement of CD200R by CD200 inhibits their activation (17). Unlike most of the IgSF receptors, CD200R lacks ITIM domains (18). However, its 67 AA cytoplasmic tail contains 3 tyrosine residues and the third tyrosine residue is located within a NPXY motif, which is phosphorylated upon ligation of the CD200 receptor (19). This leads to the recruitment and phosphorylation of Dok-2 and 1, which then bind to RasGAP and SHIP (19-21). In macrophages and mast cells, this cascade has been shown to inhibit the phosphorylation of ERK, P38 and JNK (20). CD200R signaling in macrophage appears to limit autoimmune inflammation in animal models of multiple sclerosis and arthritis (22) and lung injury caused by viral infection (23), as CD200-deficient mice were found to have a significantly increased disease severity due to hyper activation of macrophages. CD200R-deficient mice were also shown to be more susceptible to arthritis, presumably due to enhanced functions of macrophages (24). These findings indicate that CD200-CD200R interaction is mainly involved in limiting the cellular functions of myeloid lineages of cells.

Expression of CD200 has been found in multiple types of cancer (25-27) including melanoma (28). It is generally considered that expression of CD200 on cancer cells has a pro-tumor effect (26-31). However, this conclusion was made mainly from experiments using allogenic models, our previous studies using B16 melanoma and J558 plasmacytoma mouse syngeneic models suggest that tumor expressed CD200 may inhibit tumor growth and metastasis via shaping tumor microenvironment (7, 8). In this study, we have further evaluated the role of CD200R signaling in tumor growth and metastasis using CD200R-deficient mice. We found that CD200R-deficient mice exhibited accelerated growth of CD200-positive, but not CD200-negative B16 tumors. Strikingly, CD200R-deficient mice receiving CD200-positive B16 tumor cells intravenously exhibited massive tumor growth in multiple organs such as liver, lung, kidney and peritoneal cavity, while the growth of the same tumor in wild type mice is limited. Thus, we have revealed a critical role of CD200R signaling in limiting the growth and metastasis of CD200 positive tumors.

Materials and Methods

Mice

C57BL/6 mice were purchased from Jackson laboratories. CD200R$^{-/-}$ mice were generated via a contract with Taconic Farms. The exon 1 of CD200R gene was targeted in the ES cells of 129/Sv-C57BL6 mice and the CD200R mutated mice were breed into C57BL/6 (12 generations) strain. All mice were maintained and cared for in The Ohio State University (OSU) laboratory animal facilities which are fully accredited by OSU Institutional Animal Care and Use Committee (IACUC).

Generation of CD200 positive and negative B16 cells

B16.F10 melanoma cells were transfected with a pcDNA3 (Invitrogen) expression vector containing the full length of the mouse CD200 cDNA or a control pcDNA3 expression vector to generate CD200-positive or CD200-negative B16 cells as previously described (7, 8). The resulting hygromycin-resistant cells were selected for CD200 expression using flow cytometry. The generated CD200-positive (B16-CD200) or CD200-negative (B16-Ctrl) cells were maintained in RPMI 1640 medium (Gibco) supplemented with 5% FBS and 1% Penicillin/ Streptomycin.

Establishment of subcutaneous and metastatic tumors

To establish subcutaneous tumors in C57BL/6 and CD200R$^{-/-}$ C57BL6 mice, 1×10^5 B16-CD200 or B16-Ctrl cells/mouse were used for the subcutaneous injection. Development of tumors was monitored and tumors were measured for length (a) and width (b) every three days using a digital caliper. Tumor volumes were calculated as $ab^2/2$ (32). To establish metastatic tumors, each mouse was injected with 1×10^5 B16-CD200 or B16-Ctrl cells via the tail vein. Mice were monitored for up to 3-4 weeks. At the end of the experiments, mice were sacrificed and different organs were collected, weighed or underwent various analyses.

Real time PCR

Quantitative real-time PCR was performed using an ABI 7900-HT sequence system (PE Applied Biosystems) with the QuantiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer's instructions. PCR was done using previously determined conditions (33). The following primers were used for amplifying specific genes: mArg1: 5'-ACAACCAGCTCTGGGAATCT-3' (forward) and 5'-TGTACACGATGTCTTTGGCA-3' (reverse); mCCL2: 5'-GGCTGGAGAGCTACAAGAGG-3' (forward) and 5'-ATGTCTGGACCCATTCCTTC-3' (reverse); mCCL22: 5'-AACCTTCTTGCTCCTCTGGA-3'(forward) and 5'-CTTTGTGGTCCCATATGCTG-3' (reverse); mCXCL9: 5'-GAACTCAGCTCTGCCATGAA-3' (forward) and 5'-GCATCGTGCATTCCTTATCA-3'(reverse); mCXCL10: 5'-GCTGCAACTGCATCCATATC-3'(forward) and 5'-TTTCATCGTGGCAATGATCT-3' (reverse); mCXCL16: 5'-CCAGTGGGTCCGTGAACTA-3'(forward) and 5'-GGTACTGGCTTGAGGCAAAT-3' (reverse); mHIF1α: 5'-TCAAGTTGGAACTGGTGGAA-3' (forward) and 5'-TATAGGGAGCCAGCATCTCC-3' (reverse); mMMP9: 5'-TAAGGACGGCAAATTTGGTT-3' (forward) and 5'-

CTTTAGTGGTGCAGGCAGAG-3' (reverse); mMIF: 5'-CCACCATGCCTATGTTCATC-3'(forward) and 5'-GTGCACTGCGATGTACTGTG-3' (reverse); m IL-1β: 5'-CACTACAGGCTCCGAGATGA-3' (forward) and 5'-TTTGTCGTTGCTTGGTTCTC-3'; mIL4.F: 5'-TGT ACC AGG AGC CAT ATC CA-3' (forward) and mIL4.R: 5'-TTC TTC GTT GCT GTG AGG AC-3' (reverse); mIL-6: 5'-ACTTCACAAGTCGGAGGCTT-3' (forward) and 5'-TCTGCAAGTGCATCATCGT-3' (reverse); mNOS2: 5'-ACCTTGTTCAGCTACGCCTT-3' (forward) and 5'-CATTCCCAAATGTGCTTGTC-3' (reverse); mTNF-α: 5'-ATG AGA AGT TCC CAA ATG GC-3' (forward) and 5'-CTC CAC TTG GTG GTT TGC TA-3'(reverse); mTSP-1: 5'-TGGAGATGGAATCCTCAATG-3'(forward) and 5'-CAGCTGGTCTGGATTGTGTT-3' (reverse); mVEGF: 5'-AGAGAGCAACATCACCATGC-3' (forward) and 5'-GGTCTGCATTCACATCTGCT-3' (reverse). The HPRT gene was simultaneously amplified as endogenous control. The primers were 5'-AGCCTAAGATGAGCGCAAGT-3' (forward) and 5'-TTACTAGGCAGATGGCCACA-3' (reverse). Each sample was assayed in triplicate and the experiments were repeated twice. The relative gene expression was calculated by plotting the C*t* (cycle number) and the average relative expression for each group was determined using the comparative method ($2^{-\Delta\Delta Ct}$).

Antibodies and flow cytometry

For CD200 and CD200R staining, PE-labeled anti-CD200 (clone OX-90) and FITC-labeled anti-CD200R (OX-110) antibodies (Serotech) were used. FITC-, PE-, APC- or PercP-labeled antibodies to CD4, CD8α, CD11b, Gr1, Ly6G, Ly6C, DX5, IFN-γ, TNF-α, FoxP3 and isotype-matched control antibodies were purchased from BD Biosciences. Cells were incubated with antibodies in 0.1 M PBS (pH7.4) supplemented with 1% FCS and 0.1% sodium azide on ice for 30 minutes. Cells were then washed three times and fixed in 1% paraformaldehyde followed by flow cytometry analysis. Intracellular cytokine staining procedure was the same as we described (7). Data were analyzed using the flowjo software (Tree Star, Inc., OR).

Immunohistochemistry and Immunofluorescence

Immunohistochemistry (IHC) was used for the staining of frozen tissue sections of human melanoma biopsy samples. Immunostaining were performed on cancer tissues using 4 μg/ml of mouse anti-human CD200 mAb (OX-104, eBioscience) for 120 min. Ab binding was detected using anti-mouse peroxidase conjugated EnVision reagent and diaminobenzidine as chromogen. Sections were also counterstained with Mayer hematoxylin.

Immunofluorescence staining was performed on mouse tumors. Briefly, established mouse tumors were harvested and frozen in Tissue-Tek OCT media (Sakura Finetek), and 10-μm-thick slices were prepared. Tissue sections were fixed in ice cold acetone for 30 seconds and were then stained with the corresponding fluorescent antibodies overnight at 4°C. The antibodies used for fluorescence staining of tumor sections were FITC-anti-CD31 and FITC-anti-VEGF. After washing with phosphate-buffered saline (PBS), slides were mounted with DAPI-containing Vectashield mounting medium (Vector Laboratories) and were examined and photographed on an inverted three-color fluorescence microscope system (Nikon Ti-U). Images were analyzed and quantified using the ImageJ software (NIH).

Statistical analysis

Student's t test was used to compare tumor size and number differences between two groups. For comparison of mice survival, Kaplan-Myeier survival analysis and log-rank test were used (version 10.0, SPSS, Inc., Chicago, IL). A p value less than 0.05 was considered significant.

Ethic Statement—This study and experimental protocols were approved by OSU IACUC with permit number 2008A0093-R2.

Results

1. Expression of CD200 in melanoma cells alters tumor microenvironment

Recent studies have revealed that CD200 is frequently expressed on human cancer cells such as melanoma cells (28). By IHC staining of frozen sections of melanoma biopsy tissues from patients revealed that melanoma cells either extensively express CD200 or are CD200-negative (Fig.1). It has been suggested that overexpression of CD200 on melanoma cells inhibits T cell responses to melanoma, thereby promoting melanoma tumor growth and metastasis (28). However, we have previously (8) shown that expression of CD200 in B16 melanoma cells inhibits the tumor growth and metastasis in syngenic immune competent mice. We hypothesized that tumor expressed CD200 interacts with CD200R on tumor associated myeloid cells and inhibits their function, thereby shaping tumor microenvironment (TME), which can directly or indirectly affect T cell responses in tumors. To determine if this is the case in the melanoma tumor microenvironment, we injected B16-CD200 or B16-Ctrl cells (Fig.2A) into C57BL/6 mice subcutaneously (s.c.). As shown in Fig.2B, expression of CD200 significantly inhibited tumor growth in C57BL/6 mice. We analyzed the cellular components of CD200-positive versus CD200-negative tumors, and found that CD200-positive tumors contained significantly higher numbers of $CD4^+$ and $CD8^+$ T cells compared to CD200-negative tumors (Fig.2C). T cell subset analysis revealed that tumor infiltrating $CD4^+$ T cells are mainly $FoxP3^+$ Treg cells and IFN-γ producing Th1 cells, while the proportion of Treg and Th1 cells did not show significant difference between CD200-positive and CD200-negative tumors (Fig.2D). In contrast, we found that the proportion of $CD8^+IFN\text{-}\gamma^+$ T cells was significantly increased in CD200-positive tumors (Fig.2D). For the myeloid compartment, we observed significantly decreased numbers of $CD11b^+$, in particular $CD11b^+Ly6C^+$ myeloid cells (Fig.2C). Thus, expression of CD200 in melanoma cells significantly altered the cellular components of tumors and enhanced tumor-specific T cell responses.

2. A critical role of CD200R signaling in limiting CD200-positive melanoma growth and metastasis

To determine if the inhibition of tumor growth or metastasis of melanoma expressed CD200 was through CD200R, we have generated $CD200R^{-/-}$ mice. As depicted in Fig.3A, the exon 1 of CD200R gene was targeted in the ES cells of 129/Sv-C57BL6 mice and the CD200R mutated mice were breed into C57BL/6 (12 generations) strains. By comparison of WT and $CD200R^{-/-}$ mice, we found that CD200R was differentially expressed in $CD11b^+$ myeloid cells from different organs (Fig.3B). However, both $CD4^+$ and $CD8^+$ T cells in the

peripheral lymphoid organs are lack of CD200R expression (Fig.3B). To test if lack of CD200R signaling affects tumor growth and progression, we injected B16-CD200 melanoma cells into CD200R$^{-/-}$ and their wild type littermates s.c. Accelerated tumor growth was observed in CD200R$^{-/-}$ mice (Fig.4A). However, CD200-negative B16 tumor grew similarly in WT and CD200R$^{-/-}$ mice (Fig.4B), and B16-CD200 and B16-Ctrl tumors also grew similarly in CD200R$^{-/-}$ mice (Fig.4C). Thus, the growth difference of CD200-positive tumors between WT and CD200R$^{-/-}$ mice is CD200R-dependent. To test if CD200R-deficiency affects melanoma metastasis, B16-CD200 melanoma cells were injected into CD200R$^{-/-}$ and control mice i.v. nineteen days after melanoma cell injection, CD200R$^{-/-}$ mice exhibited increased numbers of tumor foci in the abdominal cavity and organs such as livers and lungs (Fig.4D), such that increased weights of livers and lungs from CD200R$^{-/-}$ mice were observed. In some cases, extensive tumor formation in the kidneys was also observed (Fig.4D). Thus, CD200R signaling appears to play a critical role in limiting CD200-positive melanoma tumor growth and metastasis.

3. Increased expansion of myeloid cells and angiogenesis in CD200R-deficient tumors

Since CD200R is mainly expressed in the myeloid lineage of cells, we hypothesized that lack of CD200R signaling in the tumor microenvironment would affect the growth of CD200-positive tumors through affecting myeloid cells. We first examined if the myeloid populations were different between B16-CD200 tumors grown in WT and CD200R$^{-/-}$ mice. As shown in Fig.5A, B16-CD200 tumors grown in CD200R$^{-/-}$ mice contained significantly increased numbers of myeloid cells, which was dominated by the expansion of CD11b^{+}Gr1^{+} cells, followed by CD11b^{+}Ly6C^{+} and CD11b^{+}Ly6C^{-} cells. CD11b^{-}Gr1^{+} neutrophils were also increased. Gene expression analysis revealed that the expressions of VEGF, HIF1α and macrophage migration inhibitory factor (MIF) genes were elevated in CD200R-negative tumors. CD200R-deficiency does not significantly affect the expression of Thrombospondin 1 (TSP-1), IL-1β and other M1/M2 genes such as MMP9, CCL2, IL-4, IL-6, Arg1 and NOS2 (Fig.5B). However, the expression of TNF-α gene was significantly reduced in CD200R-negative tumors (Fig.5B). Immunofluorescence staining of tumor sections revealed that CD200R-deficient B16-CD200 tumors contained higher density of CD31^{+} blood vessels (Fig.5C). Thus, CD200R-deficiency leads to the increased accumulation of myeloid cell populations and tumor angiogenesis.

4. CD200R-deficiency leads to decreased T cell responses in CD200-positive tumors

Since we have found that CD200-positive B16 melanoma grew much faster in CD200R$^{-/-}$ mice, we further investigated if the enhanced tumor growth of B16-CD200 tumors in CD200R$^{-/-}$ mice was due to reduced T cell responses in the tumors. B16-CD200 cells (1×10^5/mouse) were injected into cohorts (n=5) of WT or CD200R$^{-/-}$ mice s.c. Established tumors were disassociated and analyzed by flow cytometry. As shown in Fig.6A, we found that B16-CD200 tumors from CD200R$^{-/-}$ mice contained significantly reduced numbers of CD4^{+} and CD8^{+} T cells compared to B16-CD200 tumors from WT mice. However, CD200R-deficiency does not appear to affect the production of cytokines such as IFN-γ and TNF-α by CD8^{+} (Fig.6B, 6D) and CD4^{+} (Fig.6C, 6D) T cells. The proportion of FoxP3^{+} Treg cells were also similar between B16-CD200 tumors grown in WT and CD200R$^{-/-}$ mice (Fig.6B, 6D). Gene expression analysis revealed that CD200R-deficient tumors expressed

significantly lower levels of CXCL9 and CXCL16, and elevated levels of CCL22 (Fig.6E). Thus, CD200R signaling appears mainly to affect the accumulation of $CD4^+$ and $CD8^+$ T cells in the tumor microenvironment.

Since CD200R-deficiency had a particularly significant impact on B16-CD200 tumor formation in the liver, we tested if the T cell responses in the liver were different between WT and $CD200R^{-/-}$ mice during tumor growth. B16-CD200 cells were injected into cohorts (n=5) of WT and $CD200R^{-/-}$ mice i.v. Nineteen days after tumor cell injection mice were sacrificed, and liver leukocytes were isolated followed by flow cytometry analyses. We found that livers of $CD200R^{-/-}$ mice contained significantly elevated numbers of $CD11b^+Gr1^-$ myeloid cells (Fig.7A), slightly reduced total $CD4^+$ T cells (Fig.7B) and significantly increased $FoxP3^+$ Treg cells (Fig.7C). $CD4^+IFN\gamma^+$ (Fig.7C) and $CD8^+TNF\alpha^+$ (Fig.7D) cells were also reduced in the livers of $CD200R^{-/-}$ mice bearing B16-CD200 tumors compared to WT mice bearing B16-CD200 tumors. Thus, CD200R-deficiency leads to increased Treg expansion and reduced T cell responses in the liver during the growth of B16-CD200 tumors. Since NK cells were implicated in melanoma cell liver colonization (34-36), we also examined the numbers of $DX5^+$ NK cells in the livers of $CD200R^{-/-}$ and WT mice bearing B16-CD200 tumors. As shown in Fig.7E, we found that livers of $CD200R^{-/-}$ mice bearing B16-CD200 tumors had significantly reduced NK cells compared to livers of WT mice, while the IFN-γ productivity was not significantly different between NK cells in livers of WT and CD200R-deficient mice (Fig.7F).

Discussion

In this study, using the CD200R-deficient mouse model, we have further revealed that CD200R signaling plays a critical role in limiting the growth of CD200-positive, but not CD200-negative melanoma tumors. CD200R-deficiency resulted in altered tumor microenvironment, leading to increased tumor angiogenesis and reduced T cell infiltration and/or effector functions.

Expression of CD200 has been implicated in a variety of human cancer cells including melanoma cells (28) and has been thought to play pro-tumor effects via directly inhibiting tumor reactive T cells (25, 28, 29, 31). However, the fact that CD200R is mainly expressed in tumor associated myeloid cells, but not mainly on T cells in TME (7) suggests that tumor expressed CD200 mainly acts through interacting with myeloid cells in TME. In syngeneic mouse studies, it has been shown in some slow-growth tumor models that CD200 signal, either derived from tumor cells or host cells, inhibited anti-tumor immune responses (37-40). However, the results from these studies are more mixed, and none of these studies used the more aggressive mouse melanoma model. Thus, the discrepancy of previously published results with this study suggests that the role of CD200 in tumor immunity may differ in different tumor types (41), and may be associated with the tumor aggressiveness and its ability to induce inflammation, as suggested by a recent study (42) using CD200R-deficient mice.

We have recently demonstrated that tumor associated myeloid cells express high levels of CD200R, and they are susceptible to CD200-mediated inhibition (7) in a CD200R-

dependent manner (8). In this study, we found that CD200 on melanoma cells can significantly alter the tumor associated myeloid cell populations. In wild type mice, B16-CD200 tumors contained significantly lower proportions of $CD11b^{+}Ly6C^{+}$ myeloid cells (Fig.2). In subcutaneously grown B16-CD200 tumors, CD200R-deficiency resulted in increased proportions of both $Ly6C^{+}$ and $Ly6C^{-}$ $CD11b^{+}$ myeloid cells and $CD11b^{-}$ neutrophils (Fig.5A); while in the metastatic grown B16-CD200 tumors such as those in the liver, significantly increased $CD11b^{+}Gr1^{-}$ myeloid cells were observed (Fig.7A). Thus, it appears that expression of CD200 on melanoma cells can potentially affect all the sub-populations of tumor associated myeloid cells in TME, depending on the locations of tumors.

Tumor associated myeloid cells such as $Ly6C^{-}$ myeloid-derived suppressor cells (MDSC) and $CD11b^{+}Ly6C^{+}$ cells play key roles in inducing tumor angiogenesis (2, 3). In this study, we demonstrated that in $CD200R^{-/-}$ mice B16-CD200 tumor formation and metastasis were greatly accelerated (Fig.4). Our study also revealed increased expression of VEGF, HIF-1α and MIF genes (Fig.5B) and CD31-positive vasculatures (Fig.5C) in CD200R-deficient B16-CD200 tumors. Thus, we hypothesize that lack of CD200R signaling in myeloid cells results in their accumulation and activation in the TME, which in turn leads to increased production an array of pro-angiogenic products (3, 43-45) such as VEGF, leading to increased tumor angiogenesis and tumor growth/metastasis. Indeed, a recent study (46) revealed that lack of CD200R signaling in bone marrow derived macrophages exhibited increased expression of VEGF, arginase 1 and IL-1β in response to PGE2, which increased their ability to mediate angiogenesis. Moreover, this study also showed that laser-induced choroidal neovascularization was also enhanced in CD200R-deficient mice. In this study, the mechanism of CD200R-regulating VEGF expression remains unsolved. Since the expressions of IL-1β and TNF-α genes were reduced in CD200R-deficient tumors, it is unlikely that these factors are responsible for the increased expression of VEGF and HIF1α. Nevertheless, our study provided the first evidence that CD200R signaling is involved in inhibiting angiogenesis in the tumor microenvironment.

Although CD200R expression was mainly found in macrophages and neutrophils, studies also revealed lower levels of CD200R expression in dendritic cells (DC) and some subset of T cells (16, 41, 47). CD200R signaling was shown to induce indoleamine 2,3-dioxygenase (IDO) expression in plasmacytoid DC, initiating an immunosuppressive pathway of Tryptophan (48). Thus, it is anticipated that T cell responses and T cell effector functions are also affected by CD200R signaling. Indeed, in $CD200^{-/-}$ mice, autoantigen specific T cell responses were found to be normal (22, 24). However, CTL responses in $CD200^{-/-}$ mice were found to be elevated in influenza viral infection models (23, 49). CD200 signaling has also been shown to be required for the induction of T cell tolerance (40, 50). Thus, CD200R signaling in DC and T cells appear to inhibit T cell responses. However, in this study, we found that in WT mice, B16-CD200 tumors contained more $CD4^{+}$ and $CD8^{+}$ T cells compared with B16-ctrl tumors (Fig.2); while CD200R-deficient B16-CD200 tumors contained significantly reduced numbers of $CD4^{+}$ and $CD8^{+}$ T cells compared to B16-CD200 tumors in WT mice (Fig.6A). Although the proportions of IFN-γ and TNF-α producing $CD4^{+}$ and $CD8^{+}$ T cells were similar (Fig.6), the overall numbers of effector T cells in tumors are reduced due to the global reduction of effector T cells. Thus, it appears

that tumor CD200-mediated CD200R signaling enhances T cell infiltration in CD200-positive tumors. Since we found that TILs do not normally express CD200R, while TAMCs (MDSC and TAMs) express high levels of CD200R (7), we hypothesize that tumor expressed CD200 directly interacts with CD200R in TAMCs and differentially regulates their expression of key chemokines (51) such as CXCL9, CXCL16 and possibly CCL22 (Fig.6E), and thereby regulating T cell infiltration into tumors.

Intravenously injected melanoma cells do not normally grow in the organs such as liver, abdominal cavity and kidney, as demonstrated by the tumor growth pattern in B6 mice (Fig. 4). However, in the CD200R-deficient mice, we observed massive metastatic growth of CD200-positive melanomas in these locations (Fig.4). These results suggest that CD200R signaling may play more significant role in limiting metastasis of CD200-positive tumors to these sites. This phenomenon may be explained by the higher expression CD200R in myeloid cells at these anatomical locations (Fig.3B). Additionally, we hypothesize that the local immune responses are uniquely altered by the absence of CD200R signaling. In the liver case, NK cells were implicated in melanoma cell liver colonization (34-36). Indeed, in addition to reduced T responses and elevated Treg response, we also observed significantly reduced NK cells in CD200R-deficient livers undergoing metastatic tumor growth (Fig.7). Although these results could explain why CD200R-deficient liver is sensitive to metastatic melanoma growth, the detailed mechanisms of how CD200R-deficiency affects liver local NK responses remain to be studied.

Taken together, we have found a critical role of CD200R signaling in limiting the growth and metastasis of CD200 positive tumors. Tumor expressed CD200 may do so by directly interacting with TAMCs (MDSC, TAMs), which differentially regulates their production of VEGF and the expression of key chemokines such as CXCL9 and CXCL16 that attract T cells, affecting tumor angiogenesis and T cell infiltration into tumors. More metastatic growth of CD200-positive melanoma to some anatomical locations and organs such as liver in CD200R-deficient mice suggest that CD200R signaling is more important for immune responses in these organs. Given the important roles of CD200-CD200R interaction in regulating tumor associated myeloid cells and in inhibiting tumor formation and metastasis, enhancing CD200R signaling in tumors should provide an option for the immunotherapy of human cancer. Our successful treatment of B16 lung metastasis using a triggering anti-CD200R mAb (8) proves that this approach may be feasible.

Acknowledgments

Funding support: This study was supported in part by a grant from the National Cancer Institute (R01CA138427) and a Pelotonia Idea Award from The Ohio State University Comprehensive Cancer Center.

References

1. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008; 454:436–444. [PubMed: 18650914]
2. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell. 2004; 6:409–421. [PubMed: 15488763]

3. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer. 2008; 8:618–631. [PubMed: 18633355]
4. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010; 141:39–51. [PubMed: 20371344]
5. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev. 2006; 25:315–322. [PubMed: 16967326]
6. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009; 9:162–174. [PubMed: 19197294]
7. Wang L, Liu JQ, Talebian F, El-Omrani HY, Khattabi M, Yu L, Bai XF. Tumor expression of CD200 inhibits IL-10 production by tumor-associated myeloid cells and prevents tumor immune evasion of CTL therapy. Eur J Immunol. 2010; 40:2569–2579. [PubMed: 20662098]
8. Talebian F, Liu JQ, Liu Z, Khattabi M, He Y, Ganju R, Bai XF. Melanoma cell expression of CD200 inhibits tumor formation and lung metastasis via inhibition of myeloid cell functions. PLoS One. 2012; 7:e31442. [PubMed: 22319630]
9. Barclay AN, Clark MJ, McCaughan GW. Neuronal/lymphoid membrane glycoprotein MRC OX-2 is a member of the immunoglobulin superfamily with a light-chain-like structure. Biochem Soc Symp. 1986; 51:149–157. [PubMed: 2880589]
10. Koning N, Swaab DF, Hoek RM, Huitinga I. Distribution of the immune inhibitory molecules CD200 and CD200R in the normal central nervous system and multiple sclerosis lesions suggests neuron-glia and glia-glia interactions. J Neuropathol Exp Neurol. 2009; 68:159–167. [PubMed: 19151626]
11. Ragheb R, Abrahams S, Beecroft R, Hu J, Ni J, Ramakrishna V, Yu G, Gorczynski RM. Preparation and functional properties of monoclonal antibodies to human, mouse and rat OX-2. Immunol Lett. 1999; 68:311–315. [PubMed: 10424437]
12. Dick AD, Broderick C, Forrester JV, Wright GJ. Distribution of OX2 antigen and OX2 receptor within retina. Invest Ophthalmol Vis Sci. 2001; 42:170–176. [PubMed: 11133863]
13. Rosenblum MD, Olasz EB, Yancey KB, Woodliff JE, Lazarova Z, Gerber KA, Truitt RL. Expression of CD200 on epithelial cells of the murine hair follicle: a role in tissue-specific immune tolerance? J Invest Dermatol. 2004; 123:880–887. [PubMed: 15482475]
14. Wright GJ, Jones M, Puklavec MJ, Brown MH, Barclay AN. The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. Immunology. 2001; 102:173–179. [PubMed: 11260322]
15. Barclay AN, Wright GJ, Brooke G, Brown MH. CD200 and membrane protein interactions in the control of myeloid cells. Trends Immunol. 2002; 23:285–290. [PubMed: 12072366]
16. Wright GJ, Cherwinski H, Foster-Cuevas M, Brooke G, Puklavec MJ, Bigler M, Song Y, Jenmalm M, Gorman D, McClanahan T, Liu MR, Brown MH, Sedgwick JD, Phillips JH, Barclay AN. Characterization of the CD200 receptor family in mice and humans and their interactions with CD200. J Immunol. 2003; 171:3034–3046. [PubMed: 12960329]
17. Jenmalm MC, Cherwinski H, Bowman EP, Phillips JH, Sedgwick JD. Regulation of myeloid cell function through the CD200 receptor. J Immunol. 2006; 176:191–199. [PubMed: 16365410]
18. Zhang S, Cherwinski H, Sedgwick JD, Phillips JH. Molecular mechanisms of CD200 inhibition of mast cell activation. J Immunol. 2004; 173:6786–6793. [PubMed: 15557172]
19. Mihrshahi R, Barclay AN, Brown MH. Essential roles for Dok2 and RasGAP in CD200 receptor-mediated regulation of human myeloid cells. J Immunol. 2009; 183:4879–4886. [PubMed: 19786546]
20. Minas K, Liversidge J. Is the CD200/CD200 receptor interaction more than just a myeloid cell inhibitory signal? Crit Rev Immunol. 2006; 26:213–230. [PubMed: 16928187]
21. Mihrshahi R, Brown MH. Downstream of tyrosine kinase 1 and 2 play opposing roles in CD200 receptor signaling. J Immunol. 2010; 185:7216–7222. [PubMed: 21078907]
22. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, Blom B, Homola ME, Streit WJ, Brown MH, Barclay AN, Sedgwick JD. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science. 2000; 290:1768–1771. [PubMed: 11099416]

23. Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, Gwyer E, Sedgwick JD, Barclay AN, Hussell T. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. Nat Immunol. 2008; 9:1074–1083. [PubMed: 18660812]
24. Simelyte E, Alzabin S, Boudakov I, Williams R. CD200R1 regulates the severity of arthritis but has minimal impact on the adaptive immune response. Clin Exp Immunol. 2010; 162:163–168. [PubMed: 20735439]
25. McWhirter JR, Kretz-Rommel A, Saven A, Maruyama T, Potter KN, Mockridge CI, Ravey EP, Qin F, Bowdish KS. Antibodies selected from combinatorial libraries block a tumor antigen that plays a key role in immunomodulation. Proc Natl Acad Sci U S A. 2006; 103:1041–1046. [PubMed: 16418292]
26. Moreaux J, Hose D, Reme T, Jourdan E, Hundemer M, Legouffe E, Moine P, Bourin P, Moos M, Corre J, Mohler T, De Vos J, Rossi JF, Goldschmidt H, Klein B. CD200 is a new prognostic factor in multiple myeloma. Blood. 2006; 108:4194–4197. [PubMed: 16946299]
27. Tonks A, Hills R, White P, Rosie B, Mills KI, Burnett AK, Darley RL. CD200 as a prognostic factor in acute myeloid leukaemia. Leukemia. 2007; 21:566–568. [PubMed: 17252007]
28. Petermann KB, Rozenberg GI, Zedek D, Groben P, McKinnon K, Buehler C, Kim WY, Shields JM, Penland S, Bear JE, Thomas NE, Serody JS, Sharpless NE. CD200 is induced by ERK and is a potential therapeutic target in melanoma. J Clin Invest. 2007; 117:3922–3929. [PubMed: 18008004]
29. Kretz-Rommel A, Qin F, Dakappagari N, Ravey EP, McWhirter J, Oltean D, Frederickson S, Maruyama T, Wild MA, Nolan MJ, Wu D, Springhorn J, Bowdish KS. CD200 expression on tumor cells suppresses antitumor immunity: new approaches to cancer immunotherapy. J Immunol. 2007; 178:5595–5605. [PubMed: 17442942]
30. Kretz-Rommel A, Qin F, Dakappagari N, Cofiell R, Faas SJ, Bowdish KS. Blockade of CD200 in the presence or absence of antibody effector function: implications for anti-CD200 therapy. J Immunol. 2008; 180:699–705. [PubMed: 18178807]
31. Stumpfova M, Ratner D, Desciak EB, Eliezri YD, Owens DM. The immunosuppressive surface ligand CD200 augments the metastatic capacity of squamous cell carcinoma. Cancer Res. 2010; 70:2962–2972. [PubMed: 20332223]
32. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol. 1989; 24:148–154. [PubMed: 2544306]
33. Bai XF, Liu J, Li O, Zheng P, Liu Y. Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes. J Clin Invest. 2003; 111:1487–1496. [PubMed: 12750398]
34. Gorelik E, Wiltrout RH, Okumura K, Habu S, Herberman RB. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. Int J Cancer. 1982; 30:107–112. [PubMed: 7118294]
35. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Yamaguchi N, Yagita H, Okumura K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth. Cell Immunol. 2001; 214:194–200. [PubMed: 12088418]
36. Dobos J, Mohos A, Tovari J, Raso E, Lorincz T, Zadori G, Timar J, Ladanyi A. Sex-dependent liver colonization of human melanoma in SCID mice--role of host defense mechanisms. Clin Exp Metastasis. 2013; 30:497–506. [PubMed: 23203681]
37. Gorczynski RM, Chen Z, Hu J, Kai Y, Lei J. Evidence of a role for CD200 in regulation of immune rejection of leukaemic tumour cells in C57BL/6 mice. Clin Exp Immunol. 2001; 126:220–229. [PubMed: 11703364]
38. Gorczynski RM, Clark DA, Erin N, Khatri I. Role of CD200 expression in regulation of metastasis of EMT6 tumor cells in mice. Breast Cancer Res Treat. 2011; 130:49–60. [PubMed: 21165772]
39. Gorczynski RM, Chen Z, Diao J, Khatri I, Wong K, Yu K, Behnke J. Breast cancer cell CD200 expression regulates immune response to EMT6 tumor cells in mice. Breast Cancer Res Treat. 2010; 123:405–415. [PubMed: 19953316]
40. Rygiel TP, Karnam G, Goverse G, van der Marel AP, Greuter MJ, van Schaarenburg RA, Visser WF, Brenkman AB, Molenaar R, Hoek RM, Mebius RE, Meyaard L. CD200-CD200R signaling

suppresses anti-tumor responses independently of CD200 expression on the tumor. Oncogene. 2012; 31:2979–2988. [PubMed: 22020332]

41. Rygiel TP, Meyaard L. CD200R signaling in tumor tolerance and inflammation: A tricky balance. Curr Opin Immunol. 2012; 24:233–238. [PubMed: 22264927]
42. Erin N, Podnos A, Tanriover G, Duymus O, Cote E, Khatri I, Gorczynski RM. Bidirectional effect of CD200 on breast cancer development and metastasis, with ultimate outcome determined by tumor aggressiveness and a cancer-induced inflammatory response. Oncogene. 2015; 34:3860–3870. [PubMed: 25263452]
43. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol. 2009; 86:1065–1073. [PubMed: 19741157]
44. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. Trends Immunol. 2012; 33:119–126. [PubMed: 22277903]
45. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res. 2006; 66:605–612. [PubMed: 16423985]
46. Horie S, Robbie SJ, Liu J, Wu WK, Ali RR, Bainbridge JW, Nicholson LB, Mochizuki M, Dick AD, Copland DA. CD200R signaling inhibits pro-angiogenic gene expression by macrophages and suppresses choroidal neovascularization. Sci Rep. 2013; 3:3072. [PubMed: 24170042]
47. Rijkers ES, de Ruiter T, Baridi A, Veninga H, Hoek RM, Meyaard L. The inhibitory CD200R is differentially expressed on human and mouse T and B lymphocytes. Mol Immunol. 2008; 45:1126–1135. [PubMed: 17714785]
48. Fallarino F, Asselin-Paturel C, Vacca C, Bianchi R, Gizzi S, Fioretti MC, Trinchieri G, Grohmann U, Puccetti P. Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. J Immunol. 2004; 173:3748–3754. [PubMed: 15356121]
49. Rygiel TP, Rijkers ES, de Ruiter T, Stolte EH, van der Valk M, Rimmelzwaan GF, Boon L, van Loon AM, Coenjaerts FE, Hoek RM, Tesselaar K, Meyaard L. Lack of CD200 enhances pathological T cell responses during influenza infection. J Immunol. 2009; 183:1990–1996. [PubMed: 19587022]
50. Gorczynski RM, Chen Z, He W, Khatri I, Sun Y, Yu K, Boudakov I. Expression of a CD200 transgene is necessary for induction but not maintenance of tolerance to cardiac and skin allografts. J Immunol. 2009; 183:1560–1568. [PubMed: 19592654]
51. Franciszkiewicz K, Boissonnas A, Boutet M, Combadiere C, Mami-Chouaib F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. Cancer Res. 2012; 72:6325–6332. [PubMed: 23222302]

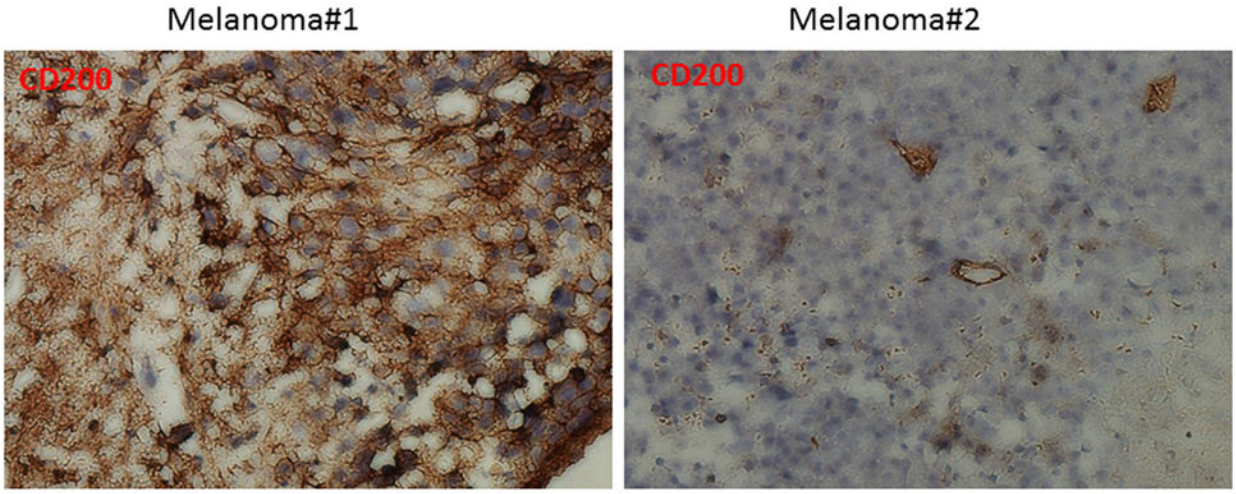

Fig.1.

IHC analyses of CD200 expression in human melanoma samples. Left image (**A**) shows a melanoma sample with overexpressed CD200 in tumor cells while the image in the right (**B**) shows a melanoma sample whose tumor cells do not express CD200. Frozen sections were used for the IHC staining. Anti-human CD200 mAb (OX-104) was purchased from eBioscience. The original images were 200 X.

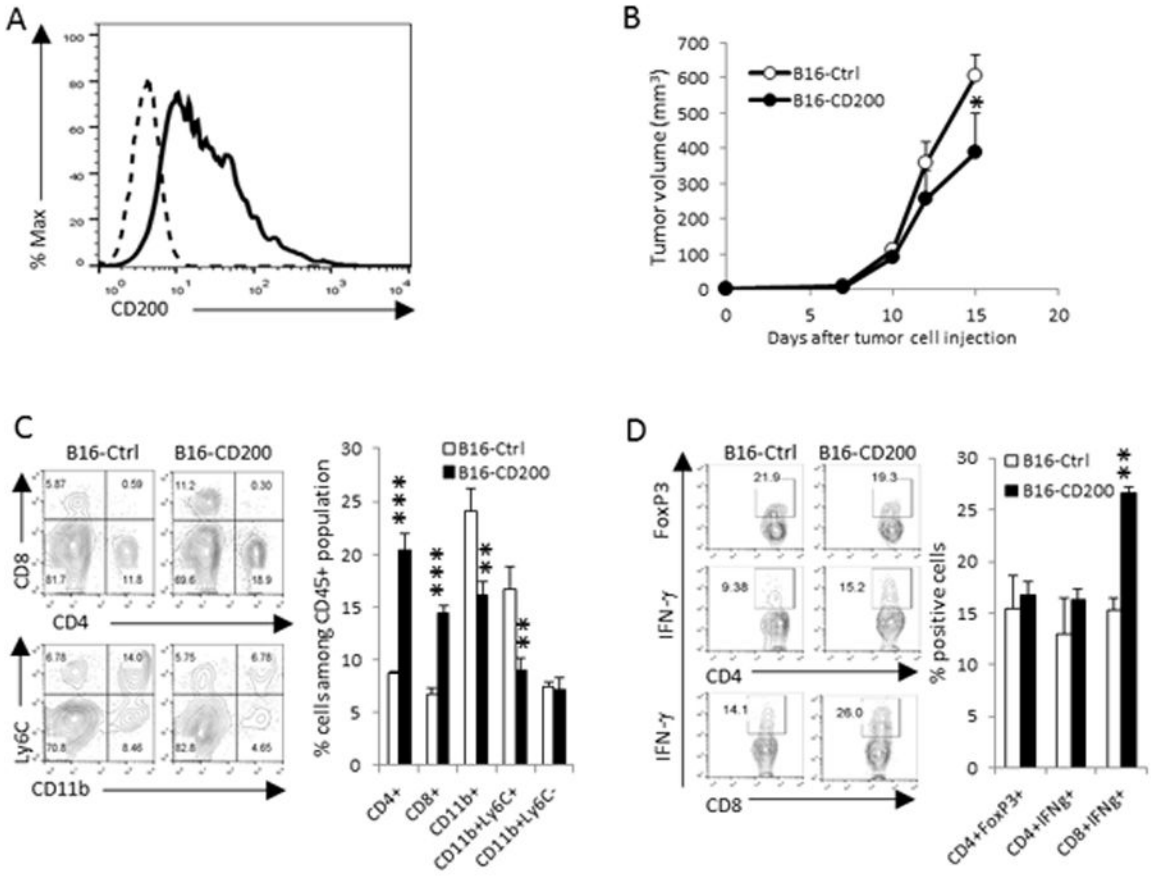

Fig.2.

Melanoma cell expression of CD200 inhibits tumor growth and alters tumor microenvironment. (**A**) B16.F10 melanoma cells were transfected with a CD200 expression plasmid or a control plasmid to generate CD200-positive (B16-CD200) or CD200-negative (B16-Ctrl) cells. Flow cytometery was used to analyze CD200 expression on B16-Ctrl (dotted line) or B16-CD200 (solid line) cells. (**B**) 1×10^5 of B16-Ctrl or B16-CD200 cells were injected into each C57BL6 mouse subcutaneously. The tumor growth was observed over time. *P<0.05 by student's t test. (**C**) Flow cytometry analysis of tumor infiltrating leukocytes was performed on disassociated tumor cells. (**D**) T cell subsets and activation status were analyzed by flow cytometry. Five mice per group were included in each group and data represents three experiments with similar results. **P<0.01 and ***P<0.001 by student's t test.

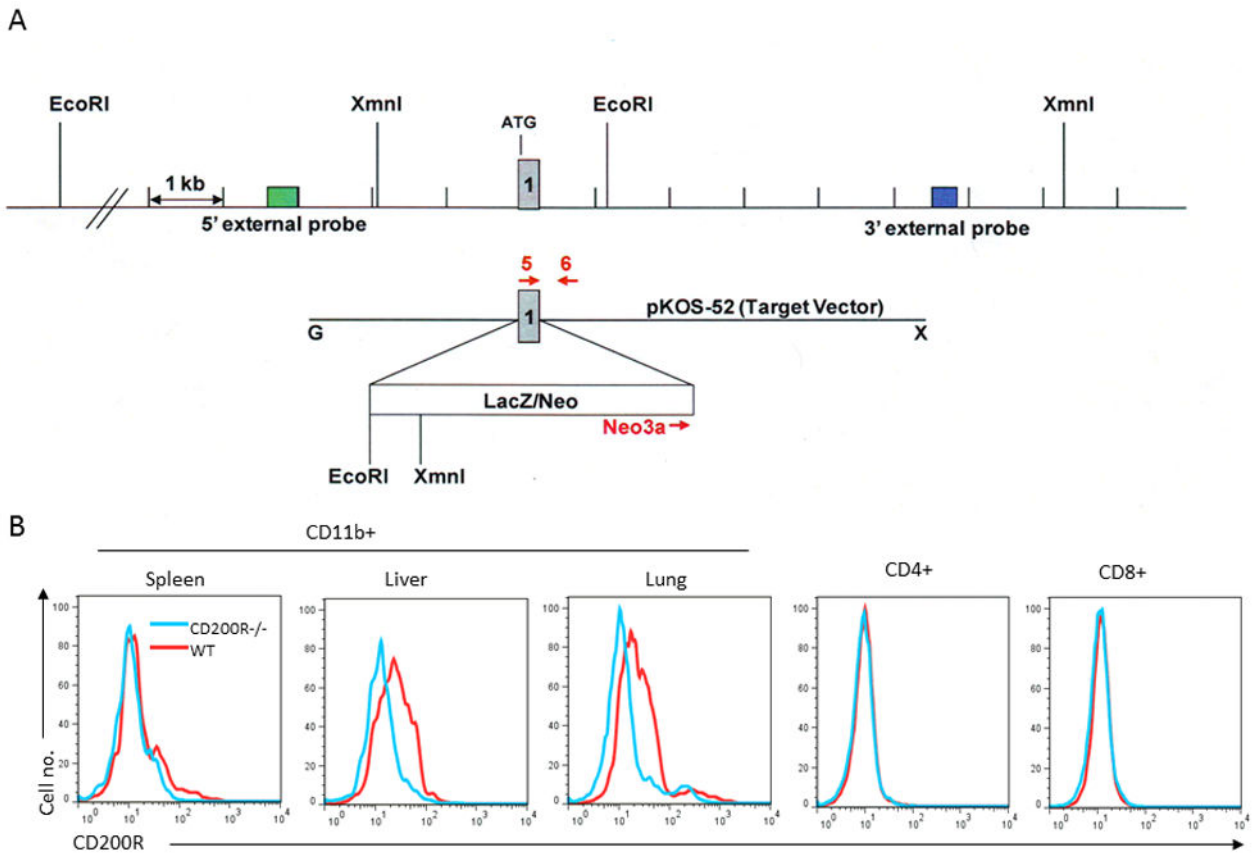

Fig.3.

Generation of CD200R$^{-/-}$ mice. (**A**) Diagram shows the targeting strategy for the generation of CD200R$^{-/-}$ mice. **(B)** CD200R expression on myeloid cells and T cells from different organs were analyzed by flow cytometry. Spleen CD4^{+} and CD8^{+} T cells were analyzed for the expression of CD200R.

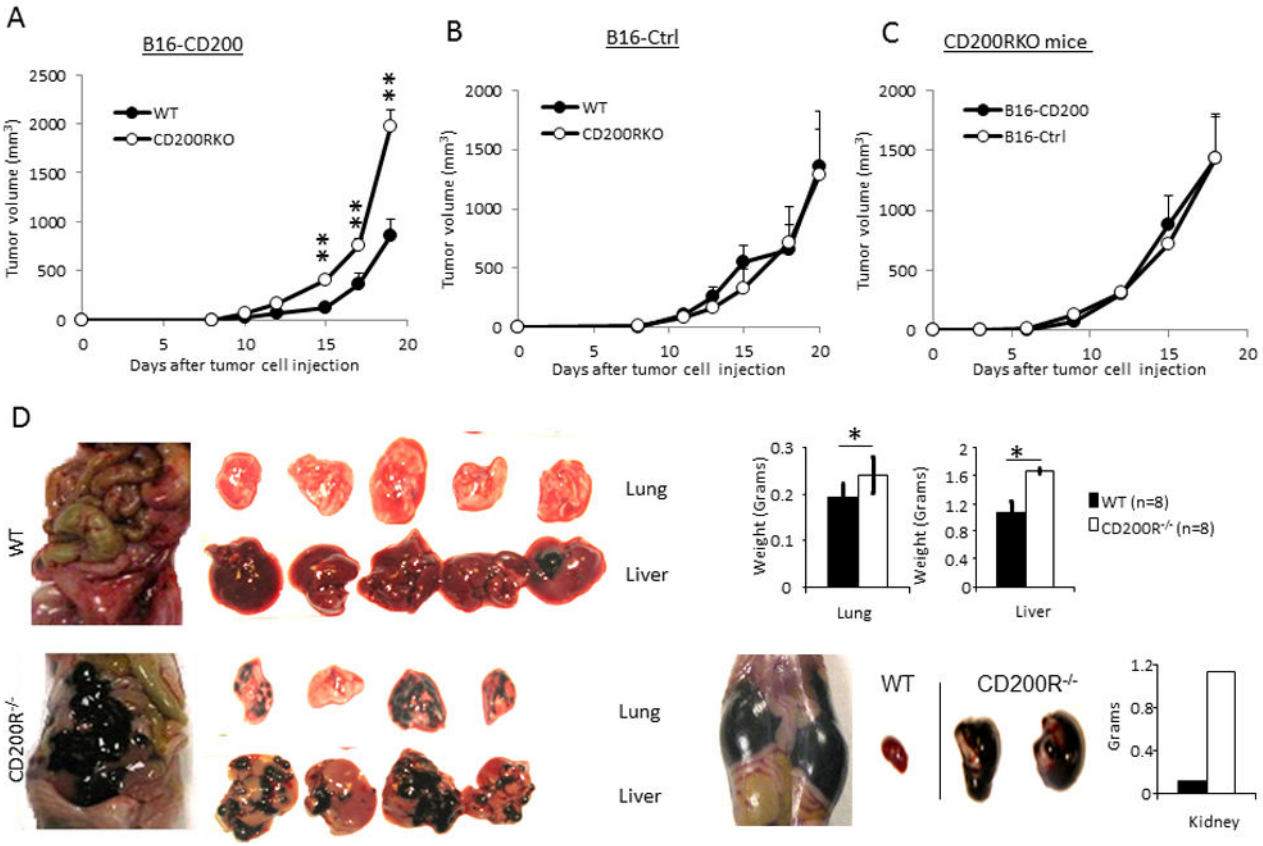

Fig.4.

CD200R-deficient mice had accelerated in situ and metastatic growth of B16-CD200 tumors. 1×10^5 B16-CD200 cells (**A**) or B16-Ctrl cells (**B**) were injected into each CD200R$^{-/-}$ mice and their wild type breeding littermates (WT) s.c. and tumor growth was observed. (**C**) B16-ctrl and B16-CD200 tumors grew similarly in CD200R$^{-/-}$ mice. 1×10^5 B16-CD200 cells or B16-Ctrl cells were injected into each CD200R$^{-/-}$ mice s.c. to observe tumor growth. (**D**) 1×10^5 B16-CD200 cells were injected into each CD200R$^{-/-}$ mice and control WT littermates i.v. Nineteen days after melanoma cell injection mice were sacrificed. Representative mice and melanoma metastasis to the lungs, livers and kidneys are shown. The weights of lungs, livers and kidneys were quantified. *: $p<0.05$ and **$p<0.01$ by student's t test. Data shown represents three (**A-C**) and five (**D**) experiments with similar results. Five mice per group were used for the experiments shown in A-C.

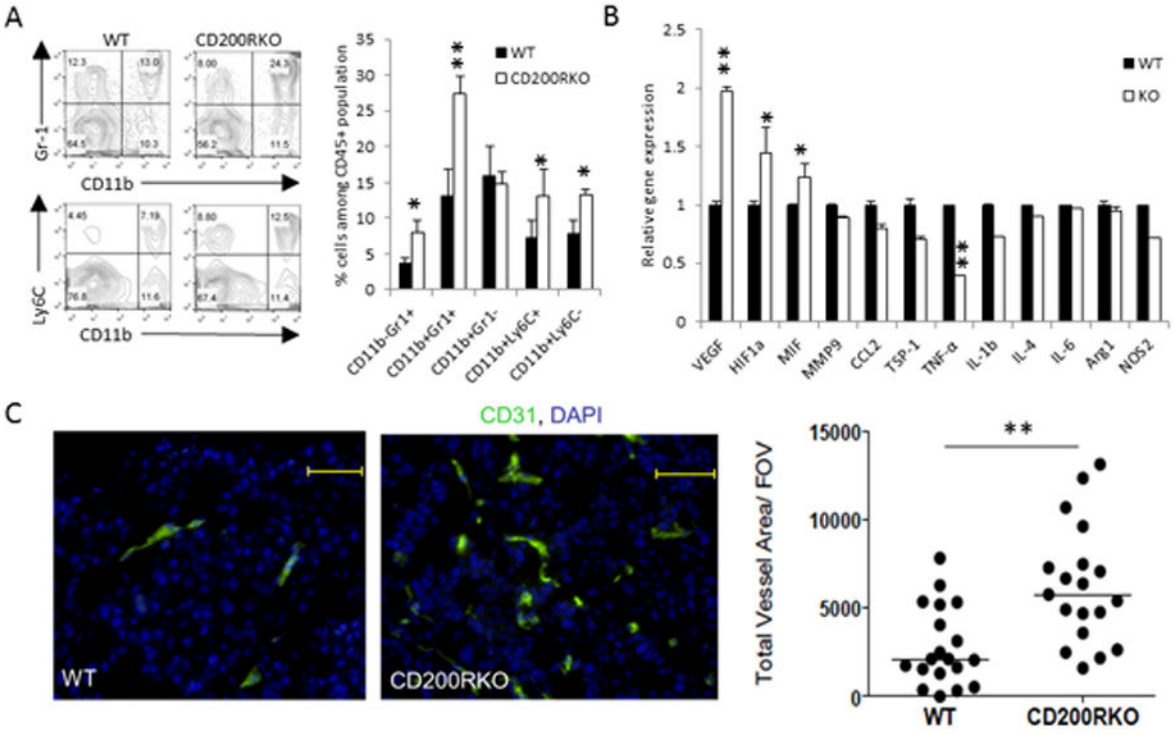

Fig.5.

Increased tumor angiogenesis in CD200R-deficient tumors. B16-CD200 cells (1×10^5/ mouse) were injected into cohorts (n=5) of WT or CD200R$^{-/-}$ mice s.c. (**A**) Flow cytometry analyses of myeloid cells from B16-CD200 tumors grown in C57BL6 or CD200R$^{-/-}$ mice. Representative flow cytometry analyses of myeloid cells in tumors and summary of their different subsets are shown. Established tumors were also analyzed for the expression of a number of genes by qRT-PCR (**B**) and histological immunofluorescence staining for CD31 (**C**). CD31$^+$ cells were quantified from images captured under a fluorescence microscope and quantified using the ImageJ software. Five tumors from each group were analyzed. *P<0.05; **P<0.01 by student's t test.

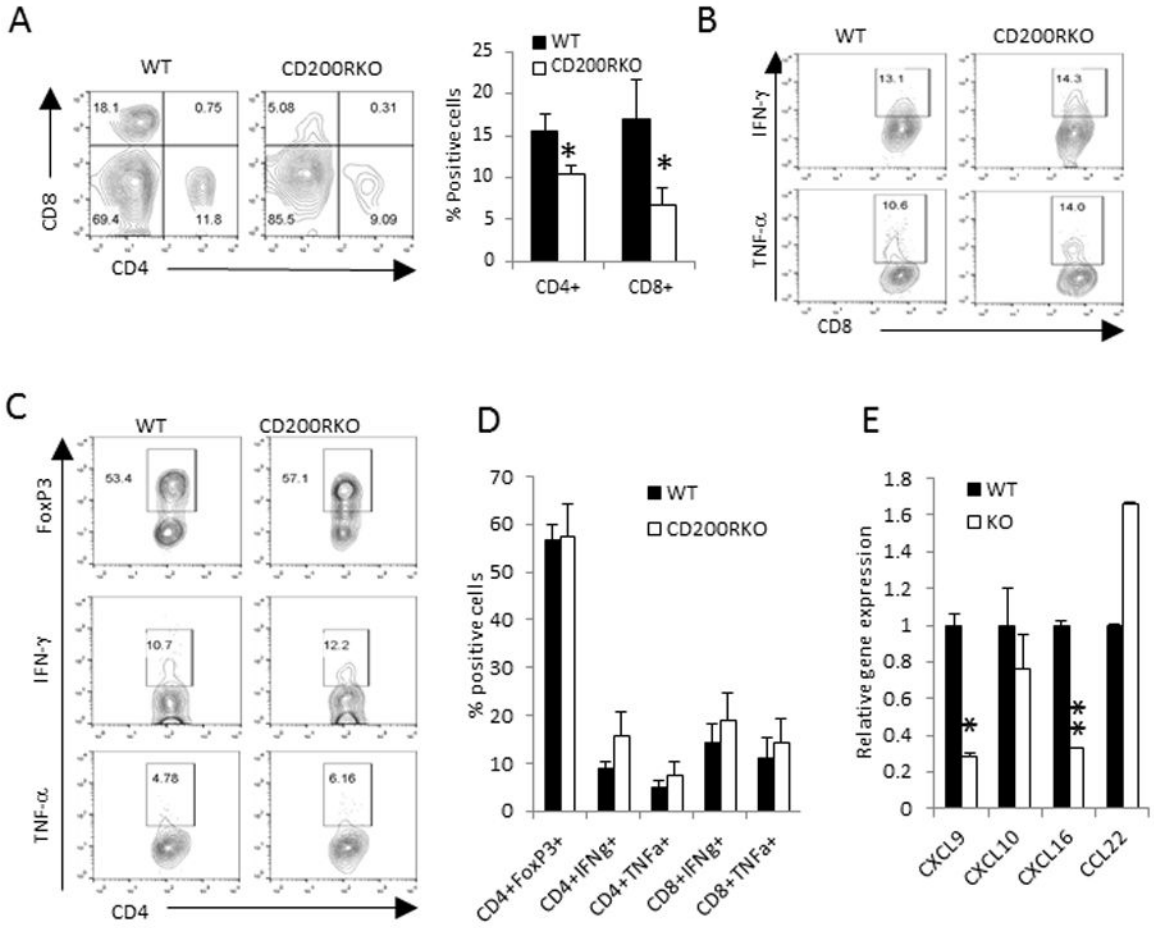

Fig.6.

Flow cytometry analyses of tumor infiltrating lymphocytes (TIL) from subcutaneously grown B16-CD200 tumors in CD200R$^{-/-}$ and WT mice. B16-CD200 cells (1×10^5/mouse) were injected into cohorts (n=5) of WT and CD200R$^{-/-}$ mice s.c. Established tumors with a tumor size about 1 cm in diameter were disassociated followed by flow cytometry analyses. (**A**) Disassociated tumors were stained for CD45, CD4 and CD8 followed by flow cytometry analysis. Data shown were gated on CD45$^+$ cells and summary of data of five tumors from each group are shown. *P<0.05 and **P<0.01 by student's t test. Intracellular staining and flow cytometry were used to analyze CD8$^+$ (**B**) and CD4$^+$ (**C**) T cells for the expression of IFN-γ, TNF-α, and FoxP3, and (**D**) dada shown are plotted from 5 tumors in each group. (**E**) Expression of chemokine genes in B16-CD200 tumors from WT and CD200R$^{-/-}$ mice were quantified by qPCR. Five tumors from each group were analyzed. *P<0.05 and **P<0.01 by the student's t test.

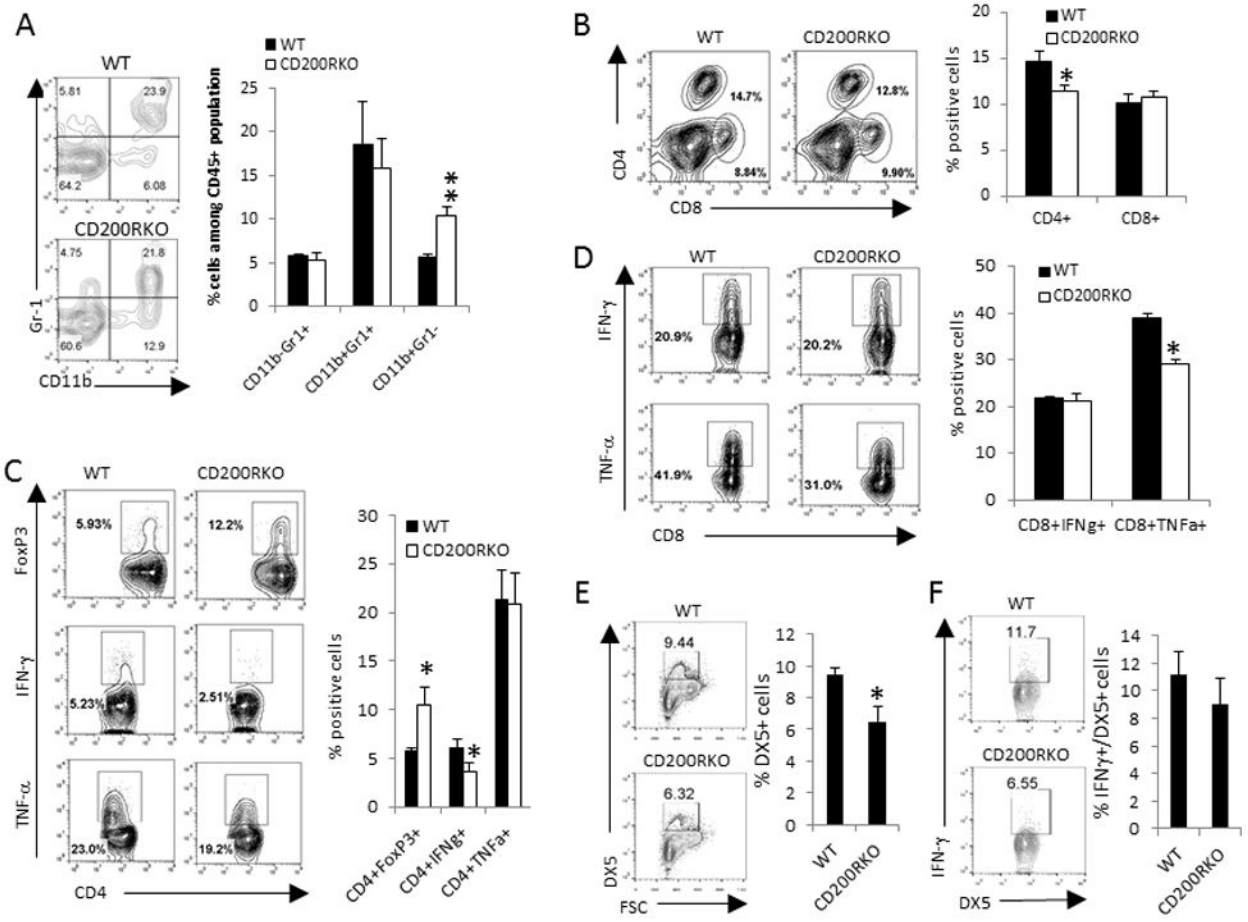

Fig.7.

Flow cytometry analyses of leukocytes from the livers of CD200-deficient and WT mice underwent metastatic growth of B16-CD200 tumors. B16-CD200 cells (1×10^5/mouse) were injected into cohorts (n=5) of WT and CD200R$^{-/-}$ mice i.v. Nineteen days after tumor cell injection mice were sacrificed, and liver leukocytes were isolated followed by flow cytometry analyses. Leukocytes from livers were stained for CD45, CD11b and Gr1 (**A**) or CD45, CD4 and CD8 (**B**) or DX5 (**E**) followed by flow cytometry analysis. Data shown were gated on CD45$^+$ cells and summary of data of five samples from each group are shown. Intracellular staining and flow cytometry were used to analyze the liver CD4$^+$ (**C**), CD8$^+$ (**D**) T cells and DX5$^+$ NK cells (**F**) for the expression of IFN-γ, TNF-α, or FoxP3. Data shown are summary of 5 samples from each group and representing two experiments with similar results. *P<0.05; **P<0.01 by student's t test.