Local adenoviral delivery of soluble CD200R-Ig enhances antitumor immunity by inhibiting CD200-β-catenin-driven M2 macrophage

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CD200 is known as an immune checkpoint molecule that inhibits innate immune cell activation. Using a head and neck squamous cell carcinoma (HNSCC) model, we sought to determine whether localized delivery of adenovirus-expressing sCD200R1-Ig, the soluble extracellular domain of CD200R1, enhances antitumor immunity. Mouse-derived bone marrow cells and M1/M2-like macrophages were cocultured with tumor cells and analyzed for macrophage polarization. As an in vivo model, C57BL/6 mice were subcutaneously injected with MEER/CD200High cells, CD200-overexpressing mouse HNSCC cells. Adenovirus-expressing sCD200R1-Ig (Ad5sCD200R1) was designed, and its effect was tested. Components in the tumor-immune microenvironment (TIME) were quantified using flow cytometry. CD200 promoted tumor growth and induced the expression of immune-related genes, especially macrophage colony-stimulating factor (M-CSF). Interestingly, CD200 induced M2-like polarization both in vitro and in vivo. Consequently, CD200 recruited more regulatory T (Treg) cells and fewer CD8+ effector T cells. These effects were effectively abolished by local injection of Ad5sCD200R1. These protumor effects of CD200 were driven through the β-catenin/NF-κB/M-CSF axis. CD200 upregulated PD-L1, and the combined targeting of CD200 and PD-1 thus showed synergy. The immune checkpoint CD200 upregulated immune-related genes through β-catenin signaling, reprogrammed the TIME, and exerted protumor effects. Ad5sCD200R1 injection could be an effective targeted strategy to enhance antitumor immunoediting.

INTRODUCTION

Immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA4 antibodies opened a new era of antitumor immunotherapy by showing notable clinical benefits in the treatment of solid cancers.1–4 Most current immune checkpoint inhibitors, including those directed against more recent targets such as LAG3 and TIGIT, aim to enhance antitumor T cell activation rather than innate immune cell activation. Among these molecules, CD200 is known as an immune checkpoint molecule that inhibits innate immune cell activation. CD200 is expressed on the surface of various tumor cell types.5,6 Its receptor, CD200R1, is mostly expressed on myeloid cells, including macrophages.7,8 The CD200-CD200R1 interaction delivers an inhibitory signal to myeloid cells, leading to their inactivation, and also regulates myeloid-derived suppressor cell (MDSC) expansion in the pancreatic ductal adenocarcinoma (PDAC), creating immune-suppressive microenvironment.10 Therefore, CD200 represents an innate arm of immune checkpoints that tumor cells utilize for immune evasion, and it has been suggested as a promising immunotherapeutic target. Accordingly, CD200-blocking strategies, including anti-CD200 antibodies, have been reported by several groups.10,11 Although antibody-based immune checkpoint inhibitors attract attention, their systemic use is always accompanied by a certain degree of autoimmunity via enhancement of general systemic immune responses. Anti-CTLA4 antibody therapy shows the most evident autoimmune side effects. Anti-PD-1 antibody therapy is also accompanied by some degree of autoimmunity, although the effect

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is milder than that of anti-CTLA4 therapy. Thus, localized delivery of immune checkpoint inhibitors is an alternative strategy to enhance antitumor immunity with minimal autoimmune side effects. We previously reported that local treatment with PD1-Ig-expressing adenovirus could augment antitumor immunity by strengthening CD8+ T cell reactivity.12 In accordance, localized delivery of CD200-blocking moieties such as soluble CD200R1-Ig (sCD200R1-Ig) may enhance antitumor immunity by targeting myeloid cells rather than T cells.

On the other hand, CD200 itself is known to deliver a protumoral intracellular signal in tumor cells. For example, we previously showed that CD200 induced epithelial-mesenchymal transition (EMT) in head and neck squamous cell carcinoma (HNSCC) cells.13 However, it is not well known whether this inward CD200 signal in tumor cells grants tumor cells immunosuppressive properties. To study the immunomodulatory role of CD200 in tumor cells and to evaluate the effect of localized sCD200R1-Ig delivery, we adopted a syngeneic mouse tumor model of HNSCC.

In this study, we elucidated that CD200 stimulates the β-catenin/NF-κB/macrophage colony-stimulating factor (M-CSF) axis in tumor cells, which promotes M2 macrophage differentiation in the tumor immune microenvironment (TIME). Local inhibition of CD200 signaling by treatment with sCD200R1-Ig-expressing adenovirus effectively abolished the activity of this pathway, induced a switch from M2 to M1 polarization in macrophages, and showed profound therapeutic efficacy. Furthermore, we found that CD200 upregulates PD-L1 expression on tumor cells and that combined treatment with sCD200R1-Ig adenovirus and anti-PD1 antibody further potentiated the antitumor effect. Thus, inhibiting both checkpoint molecules suppressing T cells and myeloid cells by localized adenovirus delivery is a promising strategy to maximize antitumor immunity.

RESULTS

CD200 expression promotes HNSCC tumorigenesis

The Cancer Genome Atlas (TCGA) database analysis of HNSCC patients showed that the expression level of CD200 was closely associated with the degree of histological progression of tumors,13 and CD200-overexpressing murine macrophage cells were tumorigenic in vivo.13 Hence, we hypothesized that CD200 on cancer cells may contribute to tumor initiation and progression and attempted to investigate whether CD200 can change the TIME by driving cytotoxic plasmic intracellular signaling. We stably transfected MEER cells, murine HNSCC cells from C57/BL6 mice, with a CD200-expression plasmid and selected a clone (MEER/CD200High) that expressed a high level of CD20012 (Figure S1A) but did not show any difference in proliferation in vitro compared to that of the control cell line (MEER/control) (Figure S1B). In contrast to this normal growth rate in vitro, MEER/control cells inoculated into syngeneic B6 mice did not show significant growth in vivo. In contrast, MEER/CD200High cells grew exponentially (Figure S1C). These results imply that CD200 on tumor cells may generate a tumor microenvironment favorable for tumor growth in vivo.

Inhibition of tumor growth by CD200 neutralization

To confirm that the rapid tumor growth of MEER/CD200High cells is caused directly by CD200, we designed sCD200R1-Ig, the extracellular domain of the CD200 binding partner CD200R1 fused with the fragment crystallizable region (Fc) domain of mouse IgG2a, for neutralization of CD200. For delivery of sCD200R1-Ig to MEER/CD200High cells, a replication-deficient adenovirus harboring the sCD200R1-Ig gene under the control of the EF1α promoter (Ad5sCD200R1) was constructed (Figure 1A). We assumed that MEER/CD200High cells transduced with Ad5sCD200RI would generate and secrete sCD200R1-Ig proteins and that the secreted sCD200R1-Ig might bind to MEER/CD200High cells in an autocrine or paracrine manner to block CD200.

As expected, sCD200R1-Ig proteins were detected in lysates and culture supernatants of MEER/CD200High cells transduced with Ad5sCD200RI (MOI, 10 or 100), whereas these proteins were not detected in cells transduced with empty adenovirus (Ad5MOCK) (Figure 1B). The specific binding activity of the secreted sCD200R1-Ig protein was confirmed by detection of cell-bound sCD200R1-Ig on MEER/CD200High cells but not on MEER/control cells after treatment of those cells with the culture supernatant of the transduced cells (Figure 1C). Next, to evaluate whether the binding of sCD200R1 to CD200 can lead to tumor growth suppression, MEER/CD200High tumors were subcutaneously established and injected with AdsCD200RI. Ad5sCD200RII effectively inhibited tumor growth, as shown in the left panel of Figure 1D. This growth inhibition was already evident within several days after the first virus injection, indicating the rapidity of the effect (Figure 1D, right panel). These data imply that the CD200-CD200R1 axis could be a potential target for suppressing the growth of CD200-expressing HNSCC tumors.

Abundance of M2-like macrophages in CD200-overexpressing tumors

Although several published reports have noted that CD200 expression on tumor cells enhances tumor growth, the underlying mechanisms are largely unknown except for the assumption that CD200 engages CD200R1 on myeloid cells to inhibit their activation.11,14,15 Furthermore, it is also unclear whether this inward CD200 signal in tumor cells endows these cells with their immunomodulatory capacity. Thus, we tried to identify tumor-intrinsic immunomodulatory factors in CD200-expressing HNSCC cells. For this purpose, the overexpressed genes in MEER/CD200High cells were investigated by RNA sequencing (RNA-seq). The transcript fold changes (criteria: p < 0.05, fold change > 1.5, normalized read count (RC) (log2) > 4) between MEER/CD200High and MEER/control cells were analyzed in the gene set of the “immune response” category.16 HNSCC tumors expressing CD200 in TCGA database were selected and explored with the RNA-seq data for the “immune response” category gene set in CD200-expressing HNSCC cells. This analysis showed that MEER/CD200High cells shared 322 genes with the human CD200+ HNSCC group (Figure 2A, top panel) and that 12 genes were related to the immune response (Figure 2A, bottom panels). Interestingly, among those 12 genes was CSF1, also called M-CSF. The increased expression of M-CSF in MEER/CD200High cells compared with MEER/control
cells was also confirmed at the protein level (Figure 2B). M-CSF is a cytokine involved in monocyte/macrophage differentiation and, more importantly, contributes to tumor-promoting M2 polarization of macrophages in the tumor microenvironment.17,18 Thus, it is conceivable that in addition to the outward effects of CD200 on CD200R1 on macrophages, inward CD200 signaling in tumor cells grants them the capacity for macrophage skewing toward a more protumor phenotype. M-CSF may be one of the readouts of this macrophage-skewing capacity of CD200-expressing tumor cells. To evaluate this possibility in vivo, the F4/80+CD206− (M1-like)/F4/80+CD206+ (M2-like) macrophage polarization ratio in tumor-infiltrating macrophages was analyzed in MEER/CD200High tumor-bearing mice. As the tumors grew, the macrophage phenotype underwent a significant shift toward an M2-like phenotype, supporting our hypothesis (Figure 2C).

**Increased M2-like macrophages in the presence of MEER/CD200High cells in vitro**

To test the M2-skewing capacity of CD200-expressing tumors more directly, we cocultured undifferentiated bone marrow cells (BMCs) with MEER/CD200High cells and examined whether MEER/CD200High cells affect macrophage polarization during in vitro macrophage differentiation. First, the macrophage differentiation potential of BMCs was verified by cytokine-induced differentiation and polarization assays. BMCs were successfully differentiated into M1-like macrophages (F4/80+CD200R1+CD206−) by GM-CSF + interferon (IFN)-γ or into M2-like macrophages (F4/80+CD200R1+CD206+) by M-CSF + interleukin (IL)-4. The polarity of the resulting macrophages was further confirmed by assessment of nitric oxide production (Figures S2A and S2B). Thus, these BMCs were fully capable of differentiation into macrophage-lineage cells. Then, when these BMCs were cocultured with MEER/CD200High cells (M1/M2: 7.2) more dramatically induced M2 polarization than did MEER/control cells (M1/M2: 25.2) (Figure 3A). To further evaluate the M2-polarizing potential of MEER/CD200High cells, tumor cells were cocultured with predifferentiated M2-like macrophages (M1/M2: 25.1) (Figure 3B). However, there was no change in MEER/control (M1/M2: 0.44) and MEER/CD200High cells (M1/M2: 0.45) when we used predifferentiated M2-like macrophages (Figure 3C). Thus, MEER/CD200High cells acquired M2-polarizing capacity via expression of CD200 in vivo and in vitro.
The therapeutic effect of Ad5sCD200R1 is mediated by M1 polarization enhancement

Next, we evaluated whether the therapeutic effect of Ad5sCD200R1 is dependent on the blockade of M2 polarization of tumor macrophages by MEER/CD200\textsuperscript{High} cells and the resulting facilitation of antitumor M1 polarization of macrophages. Subcutaneously growing MEER/CD200\textsuperscript{High} tumors were injected with Ad5sCD200R1 or Ad5-MOCK, and the M1/M2 ratio of tumor-infiltrating macrophages was assessed after 11 days. Ad5sCD200R1-treated tumors showed a significant reduction in the M2-like macrophage proportion compared to that in Ad5MOCK-treated tumors, resulting in a higher M1/M2 ratio in the Ad5sCD200R1-treated group than in the Ad5MOCK-treated group (Figure 4A). These results were consistent with the immunohistochemical staining of M1-like macrophages (F4/80\textsuperscript{+}iNOS\textsuperscript{+}) (Figure 4B).

Then, to verify the direct involvement of sCD200R1-Ig in the correction of M2 polarization, we evaluated the effect of sCD200R1-Ig treatment on macrophage differentiation and polarization mediated by MEER/CD200\textsuperscript{High} cells \textit{in vitro}. When sCD200R1-Ig, obtained via Ad5sCD200R1 transduction, was added to the \textit{in vitro} culture systems used in the experiment described above (Figure 4), M2 polarization was partially reversed to M1 polarization by sCD200R1-Ig treatment in both BMCs and differentiated M1-like macrophages cultured with MEER/CD200\textsuperscript{High} cells (Figure 4C, D). Of interest, even for predifferentiated M2-like macrophages cocultured with MEER/CD200\textsuperscript{High} cells, which were not able to be further polarized toward an M2 phenotype due to their strong M2 polarization, sCD200R1-Ig slightly but statistically significantly reversed the polarization of M2-like macrophages to an M1-like phenotype (M1/M2: 0.44 to 0.54) (Figure 4E). Therefore, adenoviral delivery of sCD200R1-Ig efficiently inhibited M2 polarization mediated by CD200 on tumor cells and facilitated M1 polarization.

Finally, to confirm that the inhibition of tumor growth by CD200 neutralization is caused by enhanced reactivity of macrophages, we depleted macrophages using clodronate. The enhanced tumor-
suppressive effect of Ad5sCD200R1 was almost completely abolished in macrophage-depleted mice (Figure 4F), further supporting the role of M1-skewed macrophages in this therapeutic setting.

Enhancement of M-CSF expression through the CD200/b-catenin interaction

We then explored the molecular mechanism by which CD200 on tumor cells can deliver intracellular signals leading to the acquisition of M2-polarizing capacity, as represented by enhanced M-CSF production, and tested whether sCD200R1-Ig could regulate this process. Since we observed that MEER/CD200High cells produced more M-CSF than MEER/control cells (Figure 2B), we further confirmed the influence of CD200 on M-CSF production by blocking CD200 in MEER/CD200High cells. First, Ad5sCD200R1-infected MEER/CD200High cells produced less M-CSF mRNA, probably via secretion of sCD200R1-Ig (Figure 5A). Consistent with this finding, CD200 siRNA (Figure 5B, left panel) or purified sCD200R1-Ig (Figure 5B, right panel) treatment reduced the M-CSF transcript abundance in MEER/CD200High cells. Next, we tried to identify a CD200 signaling pathway responsible for enhancing M-CSF transcription. It was reported that the cytoplasmic tail of CD200 is cleaved by g-secretase and translocates to the nucleus.20 We previously showed that the cleaved CD200 cytoplasmic tail interacts with b-catenin and contributes to EMT in human HNSCC cells.12 Therefore, we investigated whether the CD200-b-catenin interaction also occurs in murine MEER/CD200High cells and is utilized for M-CSF production, which has not been studied before. We transfected MEER/control cells with the plasmid encoding the cytoplasmic tail (CD200/C-terminal) of CD200 fused with a 3xFLAG tag and performed a coimmunoprecipitation assay, which confirmed the binding of the CD200 cytoplasmic tail to b-catenin (Figure 5C). This interaction was confirmed in both the cytosol and nucleus (Figure 5D). Moreover, the expression of the b-catenin target genes of c-MYC and S100A4 was increased (Figure 5E). Interestingly, the S100A4/RAGE signaling pathway is known to activate NF-kB,19 which is reported to regulate M-CSF transcription.20 Thus, we assessed the activation of the NF-kB pathway and found that CD200 overexpression resulted in a decrease in nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBa) phosphorylation and an increase in p65 and IKK phosphorylation in MEER/CD200High cells and subsequent enhancement of NF-kB transcriptional activity (Figures 5F and 5G). Consistent with this finding, NF-kB activation in MEER/CD200High cells was inhibited by treatment with CD200 siRNA and purified sCD200R1-Ig (Figures 5H and 5I). Inhibition of nuclear translocation of phosphorylated NF-kB by treatment with CD200 siRNA and Ad5sCD200R1 was also confirmed (Figure 5J). These data demonstrated that the interaction of the CD200 cytoplasmic tail with b-catenin may upregulate the production of the cytokine M-CSF through the NF-kB pathway, which is activated by the S100A4/RAGE pathway. Ad5sCD200R1 transduction and subsequent sCD200R1-Ig secretion can block this signaling to downregulate M-CSF production.
T cell responses are necessary for the therapeutic effect of Ad5sCD200R1

M1 polarization of macrophages in the tumor microenvironment not only potentiates the inflammatory properties of macrophages but also enhances T cell infiltration and activation. Thus, M1 skewing by Ad5sCD200R1 treatment may alter tumor-infiltrating T cell responses. When we examined the tumor-infiltrating T cell population in MEER/CD200High tumor-bearing mice, Ad5sCD200R1-treated MEER/CD200High tumor tissue contained a very abundant T cell receptor (TCR)+ cell population (Figure 6A) and a greater number of perforin+/IFN-γ+ effector CD8+ T cells (Figure 6B) than the Ad5-MOCK group. To evaluate whether this enhancement of T cell responses is necessary for the therapeutic efficacy of Ad5sCD200R1, we depleted CD8 T cells in the MEER/CD200High tumor model using an anti-CD8 depleting antibody. The inhibitory effect of Ad5sCD200R1 on tumor growth was nearly abolished by depletion of CD8+ T cells (Figure 6C). Consistent with this finding, the anti-tumor effect of Ad5sCD200R1 was not observed in T cell-deficient nude mice inoculated with MEER/CD200High tumor cells (Figure 6D). In addition, the population of regulatory T (Treg) cells were monitored using C57BL/6-Tg (Foxp3-GFP)90Pkraj/J transgenic mice implanted with MEER/CD200High tumor (Figure 6E). Tumors treated
Anti-tumor effects of dual blockade of PD1 and CD200

One way that the finding that CD200 blockade enhances T cell responses can be interpreted is that CD200 may potentiate the expression of T cell-inhibiting immune checkpoint molecules such as PD-L1. To test this hypothesis, we treated MEER/CD200High cells with IFN-γ in vitro and examined PD-L1 expression. Surprisingly, PD-L1 expression was upregulated to a far greater extent in MEER/CD200High cells than in MEER/control cells (Figure 7A). Since MEER/control cells did not grow well in vivo, it was difficult to analyze PD-L1 expression on MEER/control cells in vivo. However, approximately 20% of MEER/CD200High cells expressed PD-L1 when grown subcutaneously (Figure S3), whereas in-vitro-cultured MEER/CD200High cells did not express PD-L1 (Figure 7A). Thus, CD200 may also affect PD-L1-mediated T cell inhibition.

Finally, to evaluate the potential synergistic effects of CD200- and PD-1-targeted combination therapies, Ad5sCD200R1 and anti-mouse PD1 antibody were co-administered in the MEER/CD200High mouse model. Tumor suppression was additively enhanced by this combination treatment of Ad5sCD200R1 and anti-mouse PD1 antibody (4/5 mice were tumor-free) compared with each monotherapy (Figures 7B and S4). Hence, local inhibition of innate immune molecule by scCD200R1-Ig may be an attractive strategy for enhancing anti-PD1 immunotherapeutic efficacy.

DISCUSSION

The immune checkpoint function of CD200 has been identified mainly in hematologic cancers such as acute myeloid leukemia (AML) but infrequently in solid cancers. Additionally, CD200 stimulated the β-catenin/NFκB/M-CSF axis in tumor cells, leading to M2 macrophage polarization in the HNSCC model in this study, and it modulated cytokine signaling in MDSC in the PDAC model, resulting in MDSC expansion. Inhibition of CD200 by local injection of adenovirus expressing scCD200R1-Ig effectively abolished the activity of this pathway, induced M1-like polarization, and thus had significant therapeutic efficacy. Furthermore, we showed a dramatic increase in PD-L1 expression in MEER/CD200High cells after IFN-γ treatment in vitro. In line with the reported correlation in AML, our data showed a similar pattern in a solid cancer, HNSCC. Considering that NF-κB and interferon regulatory factor (IRF) modulate PD-L1 expression, there could be shared signaling pathways linking PD-L1 and CD200. Accordingly, combined targeting of CD200 and PD-1 synergistically inhibited the growth of MEER/CD200High tumors. Combined targeting of these molecules by local injection of scCD200R1-Ig expressing adenovirus and anti-PD1 antibody effectively inhibited the growth of MEER/CD200High tumors. This finding implies that antibodies inhibiting the PD-1/PD-L1 interaction would potentiate antitumoral effects with scCD200R1-Ig-expressing adenovirus.

HNSCC often occurs on mucosal surfaces of the larynx, throat, lips, mouth, nose, and salivary glands. The locoregional nature of HNSCC makes it accessible for both intratumoral injection and tissue biopsy. For this reason, it is one of the cancers in which adenovirus-based gene therapy is most frequently attempted. Here, the adenovirus Ad5sCD200R1, targeting CD200, was constructed to eventually attenuate tumor growth based on the observation that the adenovirus is the classical backbone for various gene therapies for HNSCC. We observed that local injection of Ad5sCD200R1 effectively inhibited the growth of MEER/CD200High tumors and decreased M2-like macrophage polarization. This growth inhibition was abolished by macrophage depletion.

Beyond the previously shown nonimmunological function of CD200 in inducing EMT in HNSCC cells, we initially sought to determine whether targeting CD200 affects tumor growth by modulating the TIME using the same model of HNSCC. Our mouse model exhibited increased M-CSF expression in CD200-overexpressing cells, similar to...
the pattern observed in TCGA data. Furthermore, the interspecies homology of CD200 between humans and mice in the DNA (81.7%) and protein (77.6%) sequences could imply similarity between the mouse model and clinical conditions. We initially hypothesized that CD200 induces tumor growth by driving M2-like polarization through increased expression of M-CSF. In line with our prediction and a previous report, BMCs and M1-like macrophages (F4/80⁺CD206⁻) cocultured with MEER/CD200⁺ cells were polarized into M2-like macrophages (F4/80⁺CD206⁺). Furthermore, this effect was abrogated by neutralizing sCD200R1-Ig. Interestingly, sCD200R1-Ig treatment polarized even M2-like macrophages to M1-like macrophages, although weakly. Indeed, the tumor suppression mediated by Ad5scCD200R1 in cultures with MEER/CD200⁺ cells could be mainly due to the inhibition of CD200-driven M2-like polarization and the induction of M1-like macrophage polarization in the TIME. In this study, we showed that the promotive effect of CD200 on protumor M2-like polarization is mediated through the β-catenin/S100a4-RAGE/NF-kB/M-CSF axis in tumor cells. In parallel, PD-L1 expression was so dramatically induced by IFN-γ in a CD200-rich environment in vitro that combined targeting of PD1 cells and perforin/IFN-γ effector T cells but also CD4⁺CD25⁺ regulatory T cells might be diverse critical players.

Taken together, our findings indicated that in solid cancers such as HNSCC, the myeloid immune checkpoint molecule CD200 exclusively induced M2-like polarization in the TIME by binding β-catenin and stimulating the S100A4-RAGE/NF-kB/M-CSF axis in tumor cells. In parallel, PD-L1 expression was so dramatically induced by IFN-γ in a CD200-rich environment in vitro that combined targeting of PD1
and CD200 by local injection of Ad5sCD200R1 adenovirus potentiated the antitumor effect. Extrapolation from replication-deficient adenoviruses expressing sCD200R1-Ig suggests that the effects of replication-competent adenoviruses such as ONYX-01527 could be potentiated by simultaneous expression of sCD200R1-Ig in clinical settings.

MATERIALS AND METHODS

Cells and mice
Immortalized mouse tonsillar epithelium with E6, E7, and Ras (MEER) cells were generated and used to establish a murine model for HPV16+ HNSCC. MEER/CD200High cells were generated by stably transfecting cells with the pUNO1.mouseCD200 plasmid, which is a subclone of clone #13 used in our previous publication (InvivoGen, San Diego, CA, USA). MEER/control, MEER/CD200High, HEK293, and HEK293T cells were cultured in DMEM containing 10% fetal bovine serum (FBS; GE Healthcare, Chicago, IL, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Six- to eight-week-old female BALB/c-nude and C57BL/6 mice were purchased from OrientBio (Sungnam, Korea). All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Cancer Center, Goyang, Korea.

Analysis of TCGA data
Total RNA of MEER/CD200High cells was extracted using an RNaseasy Mini Kit (QIAGEN, Hilden, Germany). The normalized read counts from MEER cell mRNA expression data were obtained using the Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA). Fold change values were calculated for “immune response” category genes that were differentially expressed in CD200High versus control cells (fold change > 1.5, p < 0.05). TCGA mRNAseq data for 522 patients with HNSCC were downloaded from cBioPortal. We then compared the genes with significantly increased expression levels after CD200 overexpression between the datasets (MEER CD200High cells versus TCGA data). A p value < 0.05 and a fold change > 1.5 were considered to indicate a statistically significant difference in expression.

Coculture of bone-marrow-derived macrophages and MEER cells
BMCs were harvested from the femurs and tibias of female C57BL/6 mice, dispersed into RPMI 1640 medium, and cultured in RPMI 1640 medium containing 10% (w/v) FBS, 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and either GM-CSF or M-CSF. Recombinant GM-CSF or M-CSF (10 ng/mL) was used for differentiation into M1 or M2 macrophages. The medium was changed every other day for 7 days. For polarization into the M1-like or M2-like phenotype, M1-like or M2-like macrophages were stimulated with 50 ng/mL IFN-γ or 10 ng/mL IL-4. MEER/control or MEER/CD200High cells (2 × 10⁴) were seeded and cocultured with 2 × 10⁵ cells) were cocultured with 2 × 10⁴ MEER/control or MEER/CD200High cells for 3 days. These cocultures were exposed to 4 μg of purified sCD200R1-Ig for 3 days to neutralize CD200.

Adenovirus construction
Ad5sCD200R1 was constructed with AdenoZAP™ 1.2 kits for truncation of E1 and E3 (OD260, Boise, ID, USA). The construct containing the extracellular domain of mouse CD200 receptor 1 fused with mouse Fc1 was called sCD200R1-Ig. To generate sCD200R1-Ig, the extracellular domain of mouse CD200R1 (OriGene, Montgomery County, MD, USA) was amplified by PCR using two primers: 5’-GAA TTC GCC ACC ATG TTT TGC TTT TGG-3’ and 5’-CAA TGG CTC CTC CTC CTC GTA ATG ATT GGT T-3’. The amplified
product was inserted into the EcoRI/XcoI site in pFUSE-mIgG2A.Fc1, which contains the EF1α promoter, Fc1 of mIgG2A, and the SV40 poly A sequence (InvivoGen, San Diego, CA, USA), resulting in the fusion of scCD200R1 with Fc1 of IgG2a. Then, EF1α-scCD200R1 was subcloned into the NotI/EcoRV site in a viral shuttle vector, pZAP1.1 (OD260, Boise, ID, USA), to generate pZAP1.1.EF1α-scCD200R1.Fc1. To construct Ad5scCD200R1, pZAP1.1.EF1α-scCD200R1.Fc1 was digested with DraiI/PacI/Clal, ligated with RightZAP1.2 (OD260, Boise, ID, USA), and transfected into HEK293 cells. The control adenoviruses Ad5MOCK was prepared and used as previously described.

**Western blot analysis, flow cytometry, and immunohistochemistry**

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Antibody-antigen complexes on PVDF membranes were quantified using ImageQuant software (Molecular Dynamics, San Diego, CA, USA). Antibodies specific for the following proteins were used: phosphorylated p65 (Cell Signaling Technology, Danvers, MA, USA); phosphorylated IκBα (Ser32/36) (Cell Signaling Technology); phosphorylated IκKα/β (Ser176/180) (Cell Signaling Technology); CD200 (R&D Systems, Minneapolis, MN, USA); CD200R1 (R&D Systems, Minneapolis, MN, USA), S100A4 (R&D Systems); c-MYC (Cell Signaling Technology); RAGE (R&D Systems); β-catenin (Merck, Palo Alto, CA, USA); M-CSF and R&D Systems); and β-actin (Santa Cruz, TX, USA). For flow cytometric analysis, all cells (1 × 10^7) or dissociated tumors were incubated for 15 minutes in the dark with an anti-mouse CD16/CD32 antibody (BD Biosciences, San Jose, CA, USA). MEER/CD200^High cells, M1/M2-like macrophages, regulatory T cells, CD4^5/CD4^+ T cells, and CD4^5/CD8^+ T cells were detected with PE-conjugated anti-CD200 (BD Biosciences), PE-conjugated anti-F4/80, FITC-conjugated anti-CD206, FITC-conjugated anti-TCR Vα2, APC-conjugated anti-IFN-γ, PE-conjugated anti-Perforin, PE-conjugated anti-CD45, FITC-conjugated anti-CD4, and FITC-conjugated anti-CD8 (BioLegend, San Diego, CA, USA) antibodies. All cells were washed with FACS buffer (0.2% BSA, 0.1% sodium azide, and 2 mM EDTA). Data were acquired with a FACSVersa flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star, OR, USA). For immunofluorescence staining of phosphorylated p65, 1 × 10^4 cells were seeded onto cover glasses in 12-well plates and blocked with PBS containing 3% BSA. Then, a rabbit polyclonal antibody against phosphorylated p65 (Cell Signaling Technologies) was incubated with cells overnight at 4°C and rinsed with PBS containing 0.05% tween 20. Cells were incubated with rhodamine-conjugated anti-rabbit antibodies and a phalloidin-conjugated anti-F-actin compound (Life Technologies, Grand Island, NY, USA) for 2 h at room temperature. Fluorescence images were acquired using a confocal microscope.

**Immunoprecipitation assays**

Cell lysates (obtained after transfection of the pCMV-3AG-3a-EV or pCMV-3AG-3a-CD200 construct) containing 1 mg of protein were precleared by incubation with 40 μL of protein-A/G linked agarose beads (Santa Cruz) for 1 h at 4°C. After the beads were pelleted by centrifugation, the supernatant was incubated with 40 μL of anti-FLAG M2 affinity gel (Sigma-Aldrich) overnight at 4°C. After incubation, the beads were washed 3 times in RIPA buffer before being dissolved in SDS-PAGE loading buffer. Then, western blot analysis was performed. For fractionation of cellular extracts, MEER cells were transfected with the pCMV vector or pCMV-mouseCD200 vector. Nuclear and cytoplasmic extracts were prepared as described previously.21

**Transient transfection and PCR analysis**

Cells were seeded at 2 × 10^5 cells/well and transiently transfected with a small interfering RNA (siRNA) targeting mouse CD200 or a scrambled siRNA (Origene Technologies, Rockville, MD, USA). For each transfection, 20 pmol of siRNA in 500 μL of serum-free Opti-MEM mixed with 7 μL of Lipofectamine RNAiMAX (Invitrogen) was used. Total RNA was extracted from each cell line using TRIzol (Invitrogen) according to the manufacturer’s protocols. cDNA synthesis was performed in a solution with a total volume of 10 μl using QIAGEN Omniscript RT kits (QIAGEN). PCR was performed using primers specific for mouse CD200 (5’T-AAA CAT CCC AGG AAC CCT TG-3’ and 5’T-TGT CTT TGT AGG CAG GCT GG-3’), M-CSF (5’-CAG CTG CTT CCA CAA GGA GGA CT-3’ and 5’T-TCA TGG AAA GTT CCG ACA CA-3’), and GAPDH (5’-CCA CCC TGT TGC TGT AG-3’ and 5’-CCC ACT CTT CCA CTT TGC AT-3’) with the following thermal cycling conditions: preheating for 10 minutes at 95°C, 30 cycles of amplification for 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and final extension for 10 minutes at 72°C. All measurements were performed in triplicate.

**Animal experiments**

Female C57BL/6 mice and C57BL/6-Tg (Foxp3-GFP)/90Pkraji/J (The Jackson Laboratory, Bar Harbor, ME, USA) of 6 to 8 weeks old were inoculated subcutaneously with 1 × 10^8 MEER/CD200^High or MEER/CD200^Low control cells. When tumors were palpable (approximately day 10 to 12), 5 × 10^6 PFUs of adenovirus were injected intratumorally 3 times at 4-day intervals. Tumors were harvested from the mice after euthanasia, and tumor tissues were then dissociated using a tumor dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For macrophage depletion studies, 1.4 mg of a clodronate liposome formulation, Clophosome (FormuMax Scientific, Sunnyvale, CA, USA), was intraperitoneally injected before the first injection of adenovirus and was then administered (0.7 mg) every 4 days for a total of three treatments. For CD8+ T cell depletion studies, an anti-CD8 depletion antibody (clone 2.43) was injected intraperitoneally one day before virus injection and was then administered (500 μg) 6 times at 5-day intervals. For combination therapy of AdsCD200R1 with anti-PD1 antibodies, anti-mouse PD-1 (Bioxcell, Lebanon, NH, USA) was injected intraperitoneally (400 μg) 4 times at 4-day intervals. Tumor volumes were determined using the following formula: tumor volume (mm^3) = length × width^2 × 0.5236.

**Statistical analysis**

Comparisons between two groups were made using two-tailed paired t tests or unpaired t tests. Two-tailed p values <0.05 were considered.
significant. STATA/SE version 10.1 software (StataCorp LP, College Station, TX, USA) was used for analyses.

SUPPLEMENTAL INFORMATION
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Institute of Laboratory Animal Resources (ILAR) guide.

AUTHOR CONTRIBUTIONS
A.-R.G., J.-M.J., H.-G.K., S.-J.K., J.-K.K., and S.-P.S. prepared Figures 1, 2, 3, 4, 5, 6, and 7 by performing most of experiments reported in this manuscript. Y.-S.B. and E.-J.P. put forward idea of the paper. S.-J.L.,
Y.-S.J., and K.C. wrote the main manuscript text. All authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests

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