Research Article

Cancer Immunology Research

Antitumor Activity of TLR7 Is Potentiated by CD200R Antibody Leading to Changes in the Tumor Microenvironment

Zofia Pilch[1], Katarzyna Tonecka[1], Agata Braniewska[1,2], Zuzanna Sas[1,2], Marcin Skorzynski[1], Louis Boon[3], Jakub Golab[1,4], Linde Meyaard[5,6], and Tomasz P. Rygiel[1]

Abstract

Stimulation of Toll-like receptor 7 (TLR7) activates myeloid cells and boosts the immune response. Previously, we have shown that stimulation of the inhibitory CD200 receptor (CD200R) suppresses TLR7 signaling and that the absence of CD200R signaling leads to a decreased number of papillomas in mice. Here, we investigated the effects of agonistic anti-CD200R on the antitumor activity of a TLR7 agonist (R848) in a syngeneic mouse tumor model. Intratumoral administration of R848 inhibited the growth of the CT26 colon carcinoma and simultaneously decreased CD200R expression in tumor-infiltrating immune cells. The antitumor effects of R848 were potentiated by anti-CD200R. Successfully treated mice were resistant to rechallenge with the same tumor cells. However, the immediate antitumor effects were independent of lymphocytes, because treatment efficacy was similar in wild-type and *Rag1*^{tm1Mom} mice. Administration of R848, particularly in combination with anti-CD200R, changed the phenotype of intratumoral myeloid cells. The infiltration with immature MHC-II^{+} macrophages decreased and in parallel monocytes and immature MHC-II^{-} macrophages increased. Combined treatment decreased the expression of the macrophage markers F4/80, CD206, CD86, CD115, and the ability to produce IL1β, suggesting a shift in the composition of intratumor myeloid cells. Adoptively transferred CD11b^{+} myeloid cells, isolated from the tumors of mice treated with R848 and anti-CD200R, inhibited tumor outgrowth in recipient mice. We conclude that administration of agonistic anti-CD200R improves the antitumor effects of TLR7 signaling and changes the local tumor microenvironment, which becomes less supportive of tumor progression. *Cancer Immunol Res; 6(8); 930–40. ©2018 AACR.*

Introduction

Cancer progression is associated with tumor infiltration by myeloid cells, including undifferentiated monocytes and granulocytes, also known as myeloid-derived suppressor cells (MDSCs), as well as macrophages at various stages of maturation. Macrophages are functionally plastic and can differentiate into a number of phenotypically and functionally different subsets. On the extremes of these phenotypes are proinflammatory macrophages (M1), exerting antitumor activities, and immunoregulatory (M2) macrophages, also referred to as alternatively activated macrophages (1). M1 macrophages exert microbicidal and tumoricidal effects and secrete proinflammatory cytokines such as IL1β, IL6, TNFα, IL12, and IL23 (2), thus polarizing the antitumor immune response toward a T_H1 profile and stimulating the cytotoxic activity of CD8^{+} T lymphocytes and NK cells. The effector functions of M1 macrophages are supported by upregulated production of superoxide anions and nitrogen radicals (3). On the other hand, M2 macrophages provide a favorable microenvironment for tumor growth. They produce anti-inflammatory molecules such as IL10, TGFβ1, and IL1 receptor antagonist (IL1RA), several isoforms of the vascular endothelial growth factor (VEGF) and multiple matrix metalloproteases that remodel the extracellular microenvironment. Additionally, these cells produce enzymes such as indoleamine-2,3-dioxygenase (IDO) and arginase-1 that are involved in the downregulation of the adaptive immune response (4, 5). Tumor-associated macrophages (TAMs) have an M2-like phenotype with the upregulated expression of the mannose receptor (CD206). M2 cells express CD200R. The immune inhibitory receptor CD200R limits the immune response in various tissues, thereby restricting reactivity to innocuous antigens, especially at mucosal surfaces (6). CD200R signaling is triggered by binding to its ligand CD200 and controlling cell functions (e.g., monocyte/macrophage), in both mouse and human systems (7). CD200–CD200R inhibitory signaling can modulate inflammation and thereby influence the antitumor immune response (8). Earlier, we showed that lack of CD200 expression reduces the number of chemically induced endogenous skin tumors (9). Furthermore,

[1]Department of Immunology, Medical University of Warsaw, Warsaw, Poland. [2]School of Molecular Medicine, Medical University of Warsaw, Warsaw, Poland. [3]Bioceros BV, Utrecht, the Netherlands. [4]Centre for Preclinical Research and Technology, Medical University of Warsaw, Warsaw, Poland. [5]Laboratory of Translational Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht, the Netherlands. [6]Oncode Institute, University Medical Center Utrecht, Utrecht, the Netherlands.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

Corresponding Author: Tomasz P. Rygiel, Medical University of Warsaw, 1A Banacha Str., F building, Warsaw 02-097, Poland. Phone: 48-22-5992175; Fax: 48-22-5992194; E-mail: trygiel@wum.edu.pl

doi: 10.1158/2326-6066.CIR-17-0454

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blockade of CD200–CD200R interaction, with monoclonal antibodies, leads to the suppression of tumor growth in immune-competent mice (10). In our previous work, we revealed that lack of CD200 expression enhances TLR7-dependent antiviral responses (11).

TLRs activate the immune response by recognizing pathogen-associated molecular patterns and endogenous damage-associated molecular patterns released from stressed or dying cells. Intracellular TLR7 and TLR8 (not expressed in mice) bind viral ssRNA (single-stranded RNA), whereas TLR9 interacts with unmethylated CpG DNA from bacteria and some viruses (12). However, stimulation of TLR7/TLR8 leads to the activation of NF-κB, increased tumor cell survival and chemoresistance in human lung cancer cells (13). Clinical data indicate that TLR7/TLR8 agonists exert antitumor effects in skin malignancies, including melanoma and squamous cell carcinoma, with complete remissions occurring in subsets of patients (14). TLR9 and TLR7 agonists inhibit syngeneic tumor growth in mouse models (15, 16). Intratumoral application of TLR7/8 agonist modifies the tumor microenvironment from tumor promoting to tumor inhibiting by shifting the phenotype of tumor-infiltrating macrophages from predominantly M2 to M1 (17). Stimulation of human monocytic MDSC cells with a TLR7/8 agonist skews their polarization toward tumoricidal M1-like macrophages (18). However, prolonged triggering of TLR can induce a state of immune unresponsiveness (19). A protocol in which administration of TLR7 is irregular prevents TLR unresponsiveness and leads to antitumor effects (15) whereas the systemic application of TLR7 agonist R848 limits the suppressive capacity of MDSC cells, but has a limited effect on tumor growth (20). Considering these findings, our aim was to investigate the effects of agonistic anti-CD200R on the antitumor activity of TLR7 agonist in a syngeneic mouse tumor model.

Materials and Methods

Mice, cell lines, and compounds

BALB/c mice were purchased from the SPF-unit of the Mossakowski Medical Research Centre (Warsaw, Poland). BALB/c mice (females 8–12 weeks of age) were housed in the non-SPF facility of the Medical University of Warsaw. Immunodeficient *Rag1^tm1Mom^* mice (BALB/c background, females 12 weeks old) were purchased from The Jackson Laboratory and were housed in SPF facility of Medical University of Warsaw. All *in vivo* experiments were performed in accordance with the legal guidelines and approved by the Local Ethics Committee. All cancer cell lines were obtained from the ATCC. Cells were regularly checked for mycoplasma infection and used only when negative. The mouse colon carcinoma cells CT26 were cultured in RPMI 1640 (Thermo Fisher Scientific), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Hyclone) and penicillin/streptomycin (P/S; Sigma-Aldrich). For all experiments cells, were never cultured for longer than 2 weeks. Resiquimod (R848), LPS, and class B CpG oligonucleotide (ODN 1862) were obtained from Invivogen and dissolved in LAL reagent water.

Tumor therapy

CT26 (1.5×10^5) cells in 30 μL of PBS:Matrigel Growth Factor Reduced (Corning, LifeSciences) mixture (1:1) were injected subcutaneously into the right thigh. Treatment with R848 was started when tumor volume reached approximately 100 mm^3 (normally on day 8–9 after inoculation of tumor cells). Before any treatment, mice were randomized to different groups. When indicated, mice received intratumoral injections of 10 μL R848 (10 μg) or PBS four times at 0, 4, 6, and 24 hours, the treatment cycle was repeated 5 days later according to the protocol described by Bourquin and colleagues (15). CD200R antibodies (clone OX110, BioLegend, 123912) or isotype control Ig (clone RTK2758, BioLegend, 400565) were injected intravenously, 100 μg per dose, every 3 days, starting one day before first R848 administration. Tumor volume was measured with calipers and tumor volume was calculated according to the formula: $[mm^3] = (\text{length [mm]}) \times (\text{width [mm]})^2/2$. At the end of the experiment, mice were sacrificed and spleens, inguinal lymph nodes, blood, and tumors were used for further analysis. Successfully treated mice were rechallenged with the second inoculation into their contralateral thigh with CT26 cells (1.5×10^5).

Cell isolation and adoptive transfer of $CD11b^+$ cells

$CD11b^+$ cells were isolated from tumors obtained from female *Rag1^tm1Mom^* mice. Donor mice were treated with R848 alone or in combination with anti-CD200R, or R848 + isotype Ig. $CD11b^+$ cells were purified using the EasySep Mouse CD11b Positive Selection Kit II (STEMCELL Technologies, ref: 18970). The positive fraction of the cells was suspended in sterile saline and counted using an automatic cell counter (EVE Automated Cell Counter, NanoEnTek) and the trypan blue exclusion method. $CD11b^+$ cells (the purity confirmed by flow cytometry ~90%), were resuspend in appropriate volume of PBS and combined with CT26 cells. CT26 cells (1.5×10^5) and $CD11b^+$ cells (4.5×10^5) were injected subcutaneously into the right thigh in 30 μL of PBS: Matrigel Growth Factor Reduced (Corning, LifeSciences) mixture (1:1). Tumor volume was measured with calipers and tumor volume was calculated according to the formula: $[mm^3] = (\text{length [mm]}) \times (\text{width [mm]})^2/2$.

Isolation and culture of bone marrow–derived macrophages (BMDMs)

Bone marrow cells were isolated from femurs and tibias of female BALB/c mice and were cultured in DMEM:F12 GlutaMAX (Thermo Fisher Scientific) medium supplemented with 10% FCS, M-CSF (50 ng/mL, eBioscience), and P/S. When indicated, cells were stimulated with LPS (1 μg/mL), R848 (2.5 μg/mL), or ODN1862 (1 μg/mL), at day 6 and 8. To study the effect of treatment combinations and in experiments with cocultures of BMDM cells with CT26, bone marrow cells (3.0×10^5) were plated into 6-well plates. From day 5, medium was supplemented with antibodies (1 μg/mL): anti-CD200R or isotype control. On day 6, cells were stimulated with R848 (2.5 μg/mL). At day 8, culture medium was refreshed and new R848 and antibodies were added for additional 24 hours. For coculture experiments, culture inserts with CT26 were placed into wells with 4-day-old BMDM cells cultures. For the measurement of CT26 viability, culture inserts were transferred to a new plate and cell viability was measured with the MTT Cell Proliferation Assay. The recommended volume of MTT (of 5 mg/mL stock) was added to the wells. After 3 hours of incubation at 37°C, a solubilization buffer was added to each well (10% SDS, 0.01 N HCl) and incubated overnight at 37°C. The absorbance was read at 590 nm with a reference filter of 620 nm.

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Real-time PCR and ELISA

Total cellular RNA was isolated from BMDM cells using TRIzol reagent (Life Technologies), and cDNA was synthesized using High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) according to the manufacturer's recommendation. Expression levels of CD200R (catalog no. 4331182, Assay ID: Mm00491164_m1) and GAPDH (catalog no. 4331182, Assay ID: Mm99999915_g1) were determined using commercial TaqMan Gene Expression Assay primer sets (Thermo Fisher Scientific). Reactions were carried out in triplicates using the LightCycler 480 Instrument II (Roche Life Science). The amount of IFNα and IL6 in blood serum was determined using appropriate ELISA kits (eBioscience), with detection done using VICTOR Multilabel Plate Reader.

Flow cytometry

Tumors were cut into small pieces, digested for 30 minutes at 37°C using Collagenase type IV (600 U; Sigma-Aldrich) and DNAse (400U, Sigma-Aldrich). Next, tissues were dissociated using a gentleMACS dissociator and filtered through a 100-μm cell strainer, washed with PBS containing 2 mmol/L EDTA and 1% FCS, centrifuged and stained. Spleens and lymph nodes were forced through the cell strainer (70 μm) and cells were centrifuged (500 × *g*) at 4°C. When necessary, erythrocytes were lysed using buffer containing 155 mmol/L NH_4Cl, 10 mmol/L NaH_2CO_3, and 0.1 mmol/L EDTA, pH 7.3. For staining, cells were blocked in 5% normal rat serum and stained with fluorescently labeled monoclonal antibodies anti-CD11b (M1/70, 53-0112-82), anti-CD11c (N418, 48-0114-82), anti-Ly6C (HK1.4, 17-5932-80), anti-CD200R (OX110, 12-5201-82), anti-CD86 (GL1, 17-0862-82), anti-CD115 (AFS98, 53-1152-82), IL1β (NJTEN3, 17-7114-80), anti-MHC-II (M5/114.15.2, 25-5321-80), (eBioscience), anti-CD206 (c068cd, 141708), anti-F4/80 (BM8, 123118; BioLegend), anti-CD45.2 (104, 562129), anti-IL6 (MP5-20F3, 554401), anti-Gr-1 (RB6-8C5, 552093; BD Pharmingen). For the intracellular staining of IL1β, cells were first stimulated with LPS (1 μg/mL) for 6 hours, whereas for IFNγ cells, were stimulated with PMA (LC Laboratories)/ionomycin (Thermo Fisher Scientific), and GolgiStop (BD Pharmingen) for the last 5 hours and subsequently stained using Intracellular Fixation and Permeabilization Buffer Set (eBioscience) according to the manufacturer's instructions.

Results

TLR7 ligand exerts antitumor effects and downregulates CD200R and CD200 expression

As prolonged TLR7 signaling leads to immune unresponsiveness, we employed a treatment protocol with the synthetic TLR7 agonist R848, according to the protocol of Bourquin and colleagues (15). R848 administered in two cycles consisting of four intratumor injections, significantly reduced CT26 colon carcinoma growth in BALB/c mice (Fig. 1A and B). TLR7 stimulation resulted in reduced CD200R expression in tumor-infiltrating immune cells ($CD45^+$), but not in immune cells derived from tumor draining lymph nodes and spleen, indicating local rather than systemic effect of R848 (Fig. 1C). Two cycles of asynchronous application of R848 decreased CD200R expression on total tumor $CD45^+$ cells, total myeloid cells ($CD11b^+$), and macrophages ($CD11b^+F4/80^+$) but not monocytes ($CD11b^+Ly6C^+$; Fig. 1D). In contrast, one cycle of R848 treatment did not decrease CD200R expression on any of the investigated populations (Fig. 1E). In BMDMs repetitively stimulated with R848 *in vitro*, less CD200R mRNA was observed (Fig. 1F). Less CD200R protein was also observed in BMDM cells stimulated with R848 (Fig. 1G). To investigate if CD200R downregulation is specific to TLR7 stimulation, we compared the effects of TLR7 (R848), TLR4 (LPS), and TLR9 (ODN) agonists on BMDM cells. Stimulation of each of these receptors significantly decreased cell-surface expression of CD200R protein (Fig. 1H). Moreover, the expression of CD200 (the ligand of CD200R) in BMDM cells was decreased by TLR7 and TLR9 agonists, whereas stimulation with TLR4 agonist increased CD200 expression (Fig. 1I), indicating that this protein is differentially regulated by various TLRs and that R848 stimulation downregulates both CD200R and its ligand, thereby inhibiting their signaling.

The combination of R848 and anti-CD200R enhances the antitumor effect of R848

To investigate the therapeutic potential of the modulation of CD200R signaling in combination with TLR7 stimulation, we combined R848 treatment with agonistic anti-CD200R in the mouse colon carcinoma CT26 model. Mice received anti-CD200R (OX110) or isotype control Ig intravenously with or without R848 treatment (Fig. 2A). Administration of antibody alone had no significant effect on tumor growth (Fig. 2B and C). Two cycles of R848 application significantly reduced tumor volume, and the combination of R848 with anti-CD200R further decreased tumor volume when compared with mice treated with R848 and isotype control Ig. This indicates that anti-CD200R exerts antitumor effects only in combination with TLR7 stimulation. Next, tumor-free mice from the groups receiving R848 or R848 combined with anti-CD200R were rechallenged with the same tumor cells (CT26). All tumor-free mice proved to be resistant to the tumor rechallenge (Fig. 2D), suggesting development of immune memory after the successful primary treatment.

Next, we analyzed the percentages of intratumoral $CD3^+$ T cells after the treatment. Administration of R848 with or without antibodies significantly decreased the percentage of $CD3^+$ T cells from 8% to around 2% of $CD45^+$ cells (Fig. 2E). Upon stimulation with PMA/ionomycin, intratumoral T cells produced more IFNγ in all groups treated with R848 (Fig. 2F and G). To address whether the therapeutic effects of the treatment with R848 and anti-CD200R are dependent on lymphocytes, we performed a therapeutic experiment in immunodeficient $Rag1^{tm1Mom}$ mice. As in immunocompetent mice (Fig. 2B), treatment with R848 (and isotype antibody) resulted in decreased tumor growth. Administration of anti-CD200R further potentiated these effects (Fig. 2H). This shows that T and B cells are not necessary for the tumor growth inhibition mediated by the therapeutic combination of R848 and anti-CD200R.

R848 increases inflammation systemically, independently of CD200R antibody

Proinflammatory serum cytokine levels were measured 24 hours after initiation of each cycle of R848 administration. We did not observe significant differences in IFNα concentrations between treatments (Fig. 3A). This probably indicates that the enhanced secretion of IFNα upon TLR7 effect is temporary and, even with repetitive stimulations, is not detectable in the circulation after 24 hours. In contrast, serum levels of IL6 were significantly increased after each cycle of R848 administration (Fig. 3B). This suggests a more persistent induction of IL6 production upon TLR7 stimulation, in comparison with IFNα.

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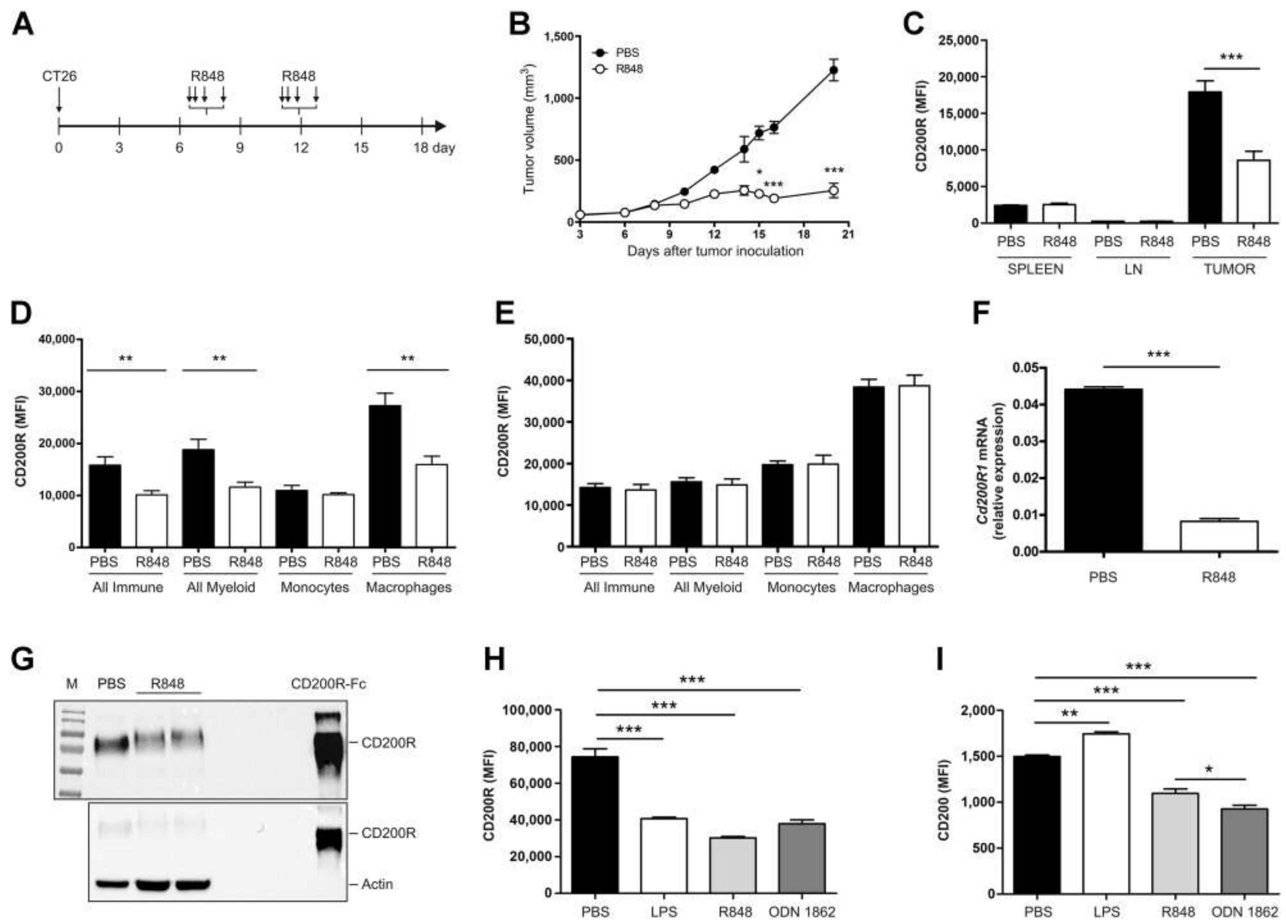

Figure 1.

Intratumor administration of R848 decreases CT26 tumor size**. A,** Treatment scheme of CT26 tumors: R848 was injected intratumorally in two cycles (days 9 and 13 after inoculation of tumor cells) at 0, 4, 6, and 24 hours. **B,** Tumor volumes in mice treated with R848 or PBS, $n = 8$. **C,** CD200R expression measured by flow cytometry on immune (CD45$^+$) cells in dissociated organs—spleen, tumor draining lymph nodes (LN) and tumor. Organs were collected at day 17 after inoculation of CT26 cells, $n = 7$–8. CD200R expression on tumor-infiltrating immune cells from mice treated with double (**D**) and single (**E**) cycle of R848 and control mice, $n = 7$–8. **B–D**, Reproduced three times. Significance was calculated with *t* test. *, $P < 0.05$; ***, $P < 0.001$. Total expression of CD200R mRNA measured by RT-PCR (**F**) and protein level measured by Western blot (**G**) in BMDM cells stimulated with R848. CD200R (**H**) and CD200 (**I**) expression in BMDM cells stimulated with TLR4, 7, and 9 agonists analyzed by flow cytometry, $n = 4$. **E** and **F,** Performed once. **G–H,** Reproduced three times. Significance was calculated with one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Experiments are repeated from two to four times.

Simultaneously, we observed an increase in the percentage of blood monocytes (CD11b$^+$Ly6C^{high}Ly6G^{int}MHC-II$^-$) from around 10% of the leukocytes in controls or in mice treated with anti-CD200R to 70% to 80% in R848-treated mice (Fig. 3C and D). These results indicate that local intratumoral R848 injection also exerts systemic inflammatory effects that are not affected by the combination with anti-CD200R.

R848 and anti-CD200R treatment modifies the phenotype of tumor-infiltrating myeloid cells

To investigate the influence of myeloid cells on the growth of CT26 cancer cells, we cocultured BMDM with CT26 cells in a transwell setup. When cocultured, BMDM increased the number of metabolically viable CT26 cells by 60% to 70% in comparison with CT26 cultured alone (Supplementary Fig. S1A). R848 neutralized the effects of BMDM cells by decreasing the number of viable CT26 cells, bringing it down to almost the levels of controls, regardless of the presence of CD200R antibodies or control Ig (Supplementary Fig. S1A). R848 had no effect on CT26 cells cultured alone without myeloid cells (Supplementary Fig. S1B). To get a better understanding of the R848 effects in combination with anti-CD200R on the tumor microenvironment, we performed flow cytometric analysis of the tumor-infiltrating immune cells 24 hours after the last dose of R848. Treatment with R848, with or without anti-CD200R, decreased the viability of immune cells to around 30% (Fig. 4A). Whereas CD45$^-$ nonimmune cells within the tumor were not as affected, R848 reduced their viability to around 60% (Fig. 4B). The percentage of myeloid cells in the pool of total viable immune cells was similar (70%–80%) in all treatment groups (Fig. 4C). However, the treatment decreased the expression of a number of macrophage markers, including CD206 (a M2/TAM marker), and CD86 (a macrophage activation marker) by approximately 75%, and M-CSF receptor (CD115) by about 30% of untreated cells (Fig. 4D–F). The expression of the macrophage marker F4/80 was reduced by R848 alone (by 70%), and this reduction was even stronger in mice treated with the

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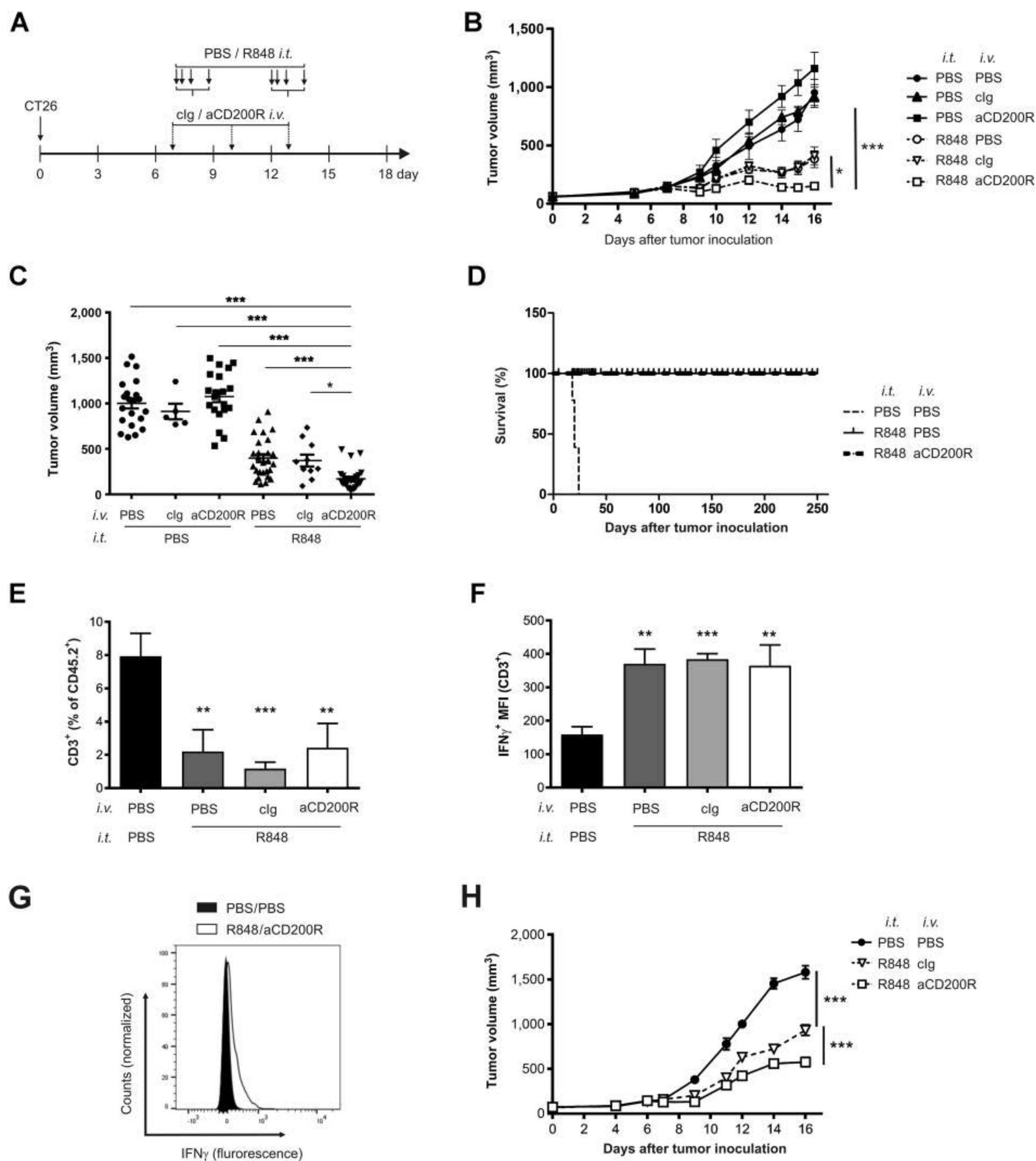

Figure 2.

CD200R antibody improves antitumor effects of R848. **A,** Treatment scheme of CT26 tumor-bearing mice, R848 was injected intratumorally in two cycles as described in Fig. 1A. Short arrows, CD200R antibody (aCD200R) and control Ig (cIg; doted arrows) were injected intravenously at days 8, 11, and 14 after CT26 inoculation. **B,** Tumor volumes in mice treated with R848 and/or anti-CD200R, $n = 5$–8. The experiment was reproduced three times. **C,** Tumor volumes on the last (16) day of experiment. Mice were pooled from 3 independent experiments, $n = 5$–27. **D,** Survival of mice free of tumors after R848 or anti-CD200R/R848 that were rechallenged with CT26 in the opposite leg. Controls were naïve mice that received the same CT26 inoculum. Mice were sacrificed if any of the tumor diameters reached 15 mm, $n = 4$–6. **E,** Percentage of $CD3^+$ T lymphocytes in intratumoral immune cells, $n = 7$–9. **F,** Production of IFNγ in intratumoral $CD3^+$ T lymphocytes upon stimulation with PMA/ionomycin, $n = 7$–9. **G,** Examples of IFNγ expression in R848/anti-CD200R and control mice. **H,** Tumor (CT26) volumes in immunodeficient *Rag1*^{tm1Mom} mice treated according to scheme from **A**, $n = 6$–9. **D–H,** Performed once. Significance was calculated with one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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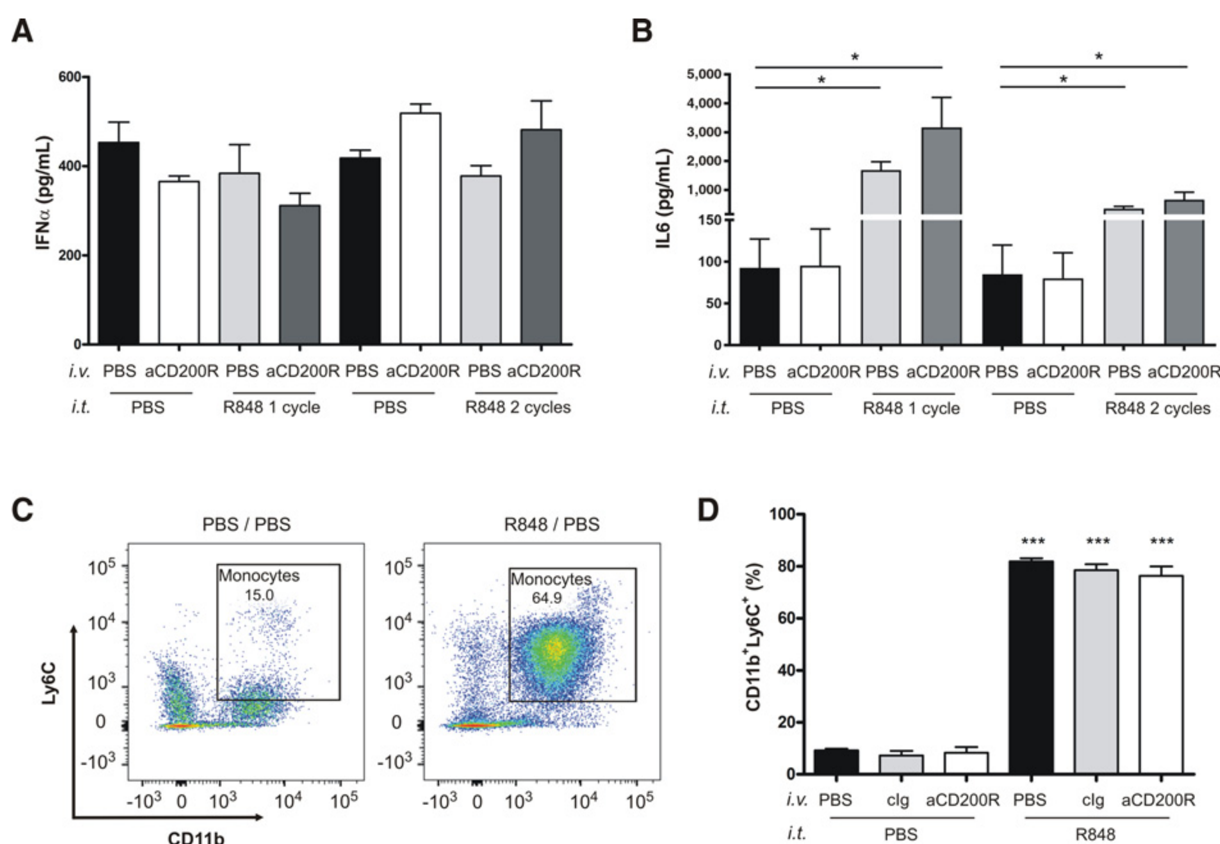

Figure 3.

R848 increases systemic inflammation. Concentration of IFNα (**A**) and IL6 (**B**) was measured in the serum 24 hours after the first and the second R848 treatment. Days 9, 13 after CT26 inoculation, $n = 7$–9. **A–B,** Performed once. Flow cytometry analysis of monocytes (CD11b$^+$Ly6C^{hi}) in leukocytes of treated mice after second R848 cycle. Representative examples (**C**) and graph (**D**), $n = 4$–6. **C–D,** Reproduced three times. When not indicated otherwise, significance was calculated in comparison with the PBS-only control group using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

combination of R848 and anti-CD200R (Fig. 4G and H). Additionally, we measured IL1β production by LPS-stimulated tumor-derived myeloid cells *ex vivo*. Production of IL1β was significantly decreased in the cells isolated from mice that received R848 and this effect was even further potentiated by the addition of anti-CD200R (Fig. 4I and J). These results indicate that treatment with R848 modulates both the phenotype and the function of tumor-infiltrating myeloid cells, and that these effects are potentiated by the combined administration of anti-CD200R.

R848 and anti-CD200R treatment changes the composition of tumor-infiltrating myeloid cells

We next analyzed populations of intratumoral myeloid cells by flow cytometry, according to the gating strategy described by Laoui and colleagues (21). First, CD11b$^+$ cells were distinguished from live cells and neutrophils (Ly6G^{hi}) were excluded. Subsequently, five populations at various stages of differentiation were identified: Ly6C^{hi}MHC-II$^-$ monocytes, Ly6C^{int}MHC-II$^-$ immature macrophages (MHC-II$^-$ Imm Mφ), Ly6C$^{hi/int}$MHC-II$^+$ immature macrophages (MHC-II$^+$ Imm Mφ), Ly6C$^-$ MHC-II$^-$ TAMs (MHC-II$^-$ TAM), and Ly6C$^-$MHC-II$^+$ TAMs (MHC-II$^+$TAM) according to strategy (Supplementary Fig. S2). Treatment with R848, particularly in combination with anti-CD200R, significantly increased the fraction of intratumor Ly6C^{hi}MHC-II$^-$ monocytes (Fig. 5A) and MHC-II$^-$ Imm Mφ (Fig. 5B). This was accompanied by decreased percentages of MHC-II$^+$ Imm Mφ cells, which were the most common in control tumors (Fig. 5C). Neither population of mature TAMs (MHC-II$^+$ TAM nor MHC-II$^-$ TAM) was significantly affected (Fig. 5D and E). Thus, the treatment with R848 and anti-CD200R changed the myeloid tumor environment from a dominant MHC-II$^+$ Imm Mφ population to mainly monocytic/MHC-II$^-$ Imm Mφ (Fig. 5F).

The combination of R848 and anti-CD200R changes macrophage activity

We next sought to investigate whether the changes in the phenotype of tumor myeloid cells are associated with the inhibition of tumor outgrowth after the therapy with R848 or the combination of R848 with CD200R antibodies. We isolated CD11b$^+$ myeloid cells from tumors at day 15 (Fig. 6A), and inoculated them subcutaneously together with CT26 cells into naïve mice at a 3:1 ratio (CD11b$^+$: CT26). The growth of tumors coinoculated with CD11b$^+$ cells isolated from mice treated with R848 and anti-CD200R was significantly slower than those coinoculated with myeloid cells isolated from control mice (Fig. 6B), with two out of seven tumors regressing entirely in this group. The growth of tumors coinoculated with CD11b$^+$ cells isolated from tumors that received R848 with isotype Ig was faster than in

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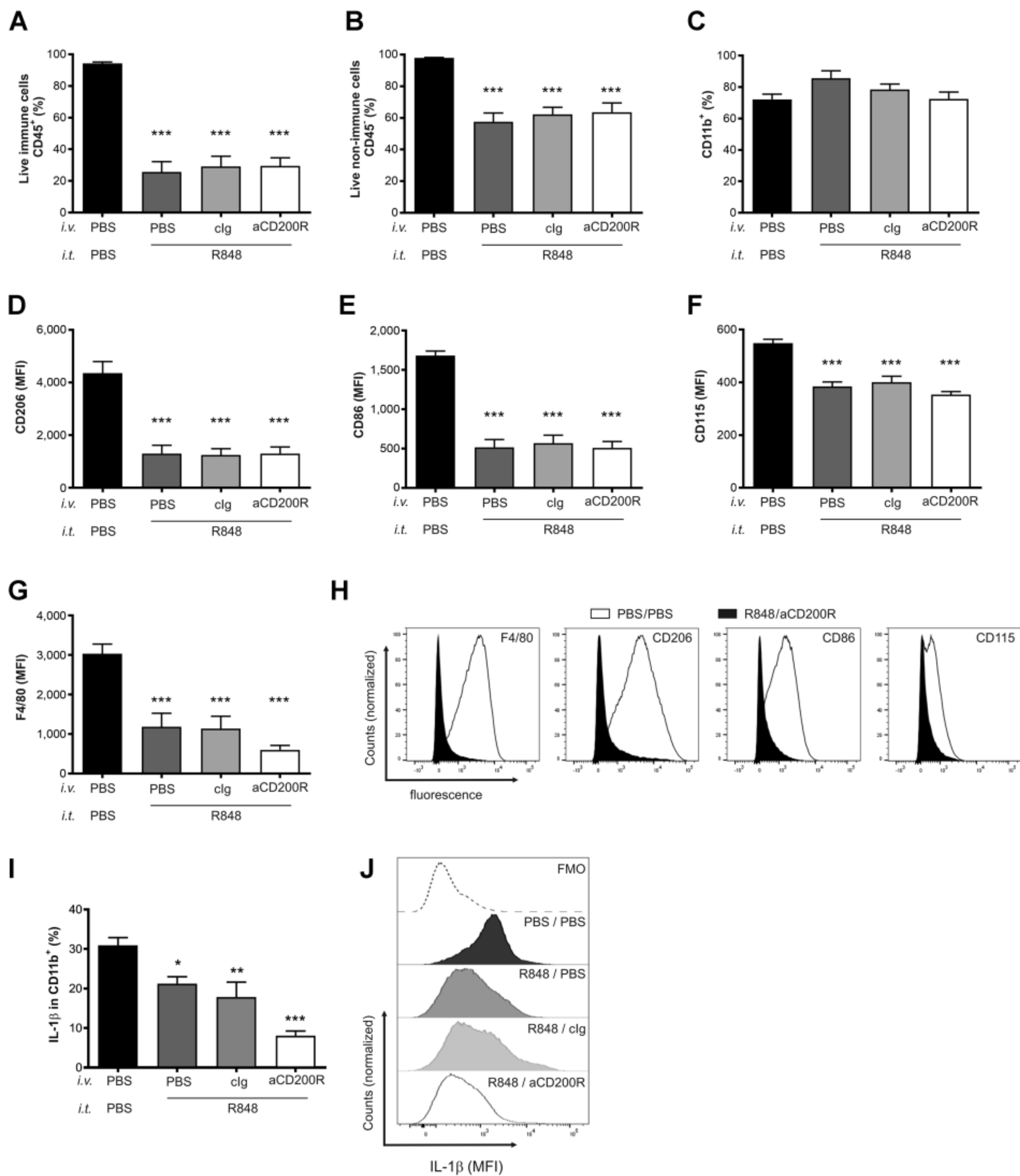

Figure 4.

Combination of R848 and anti-CD200R affects activation of tumor-infiltrating myeloid cells. Single-cell suspensions of CT26 tumors were analyzed by flow cytometry (24 hours after the therapy). Percentage of viable immune cells CD45.2$^+$ (**A**) and nonimmune cells (**B**). **C,** Percentage of intratumoral myeloid cells (CD11b$^+$). Expression of macrophage markers was analyzed in the CD11b$^+$ cell population: (**D**) CD206, (**E**) CD86, (**F**) CD115, (**G**) F4/80, $n = 8–9$. **H,** Examples of expression of the F4/80, CD206, CD86, and CD115 in R848/anti-CD200R and control mice. **I,** Production of IL1β in intratumoral CD11b$^+$ cell, upon stimulation with LPS, $n = 8–9$. **J,** Examples of IL1β expression in all treatment groups. **A–H,** Reproduced two times. **I** and **J,** Performed once. When not indicated otherwise, significance was calculated in comparison with the PBS-only control group using one-way ANOVA. *, $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$.

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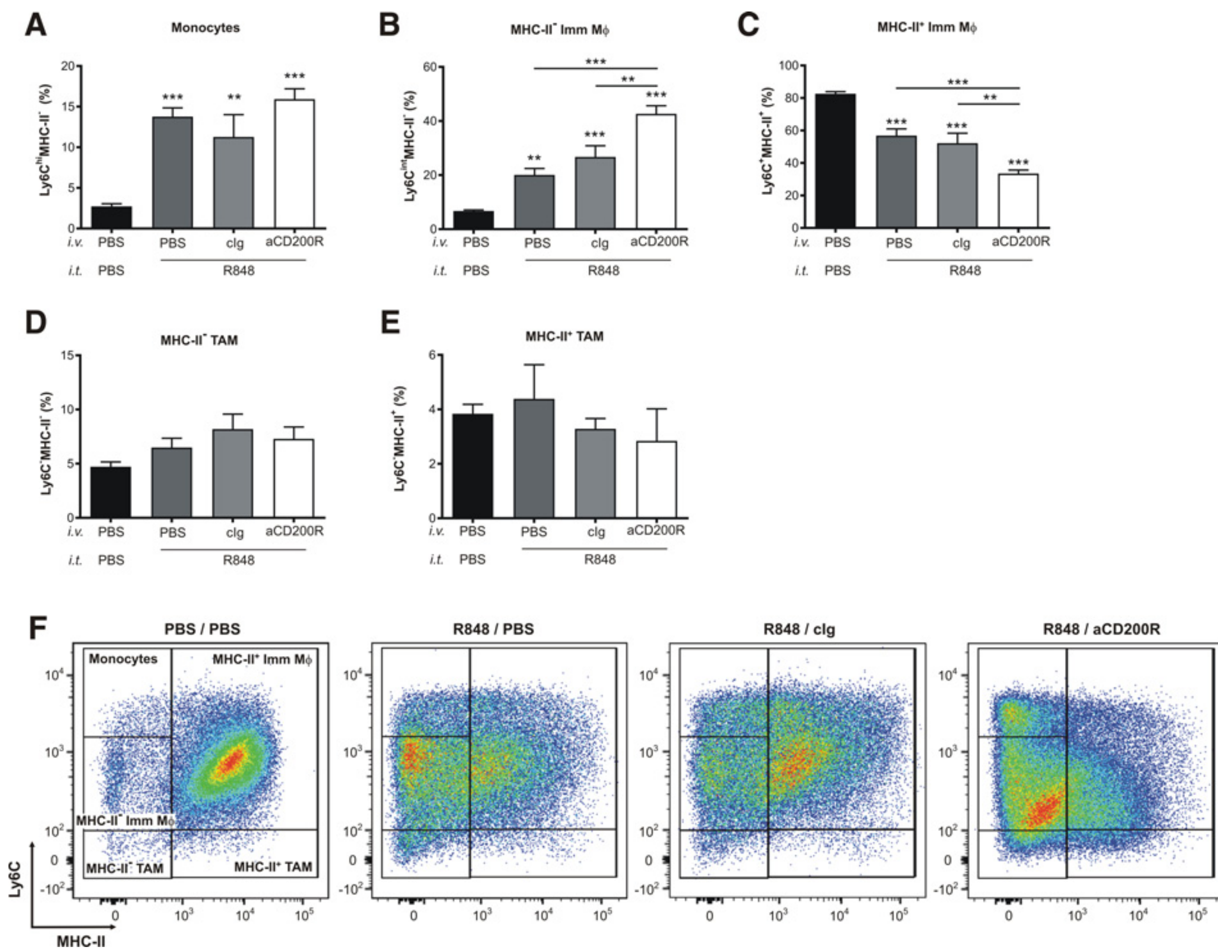

Figure 5.

Treatment with R848 and anti-CD200R leads to changes in the composition of intratumor myeloid cells. Fractions of intratumor myeloid cells ($CD11b^+$). **A,** $Ly6C^{hi}MHC\text{-}II^-$ monocytes; **B,** immature macrophages ($MHC\text{-}II^-$ Imm Mφ); **C,** $Ly6C^{hi/int}MHC\text{-}II^+$ immature macrophages ($MHC\text{-}II^+$ Imm Mφ); **D,** $Ly6C^-MHC\text{-}II^-$ TAMs ($MHC\text{-}II^-$ TAM); **E,** $Ly6C^-MHC\text{-}II^+$ TAMs ($MHC\text{-}II^+$TAM). **F,** Example scatterplots of intratumoral myeloid cells showing gating for all above populations, from all treatment groups. **A–F,** Reproduced two times. When not indicated otherwise, significance was calculated in comparison with the PBS-only control group using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

controls. Similarly, a 72-hour incubation of BMDM cells with R848 plus anti-CD200R conferred on these cells the ability to slightly, although statistically insignificantly, suppress the growth of CT26 tumors when coinoculated into naïve mice (Fig. 6C and D). The results of these experiments indicate that the combination of R848 and anti-CD200R changes the phenotype of tumor-infiltrating myeloid cells, thereby suppressing tumor growth.

Discussion

In this study, we investigated the effects of anti-CD200R on the antitumor activity of a TLR7 agonist (R848) in a mouse syngeneic tumor model of colon carcinoma. In the tumors of animals treated with R848, particularly in combination with anti-CD200R, we noticed a decrease in the percentage of $MHC\text{-}II^+$ immature macrophages that was accompanied by an increase in the percentage of monocytes and $MHC\text{-}II^-$ immature macrophages. Furthermore, the differentiation status of the tumor myeloid cells in the tumor was shifted by the combined R848/anti-CD200R treatment, as revealed by downregulation of F4/80, CD206, CD86, and CD115. Additionally, the treatment with R848/anti-CD200R decreased the ability of intratumoral myeloid cells to produce IL1β. Previously, we and others have shown that absence of the CD200R signaling pathway results in decreased outgrowth of endogenous (papilloma) and syngeneic tumors (EMT6; refs. 9, 10, 23). In the current study, we show that intravenous application of agonistic anti-CD200R has no effect on tumor growth or on the tumor environment.

Stimulation of TLRs, particularly those such as TLR7/8 that are responsible for viral recognition, is associated with induction of proinflammatory cytokines. Once triggered, these receptors shift the immune response toward cell-mediated immunity, which inhibits tumor growth (15, 16). TLR7 stimulation induces the production of type I IFNs (α, β; ref. 12). Earlier, we showed that production of IFNα is regulated by CD200R signaling during mouse hepatitis virus (MHV) infection (11). Here, we did not observe changes in IFNα concentration in the serum of mice undergoing R848 stimulation. This may be due

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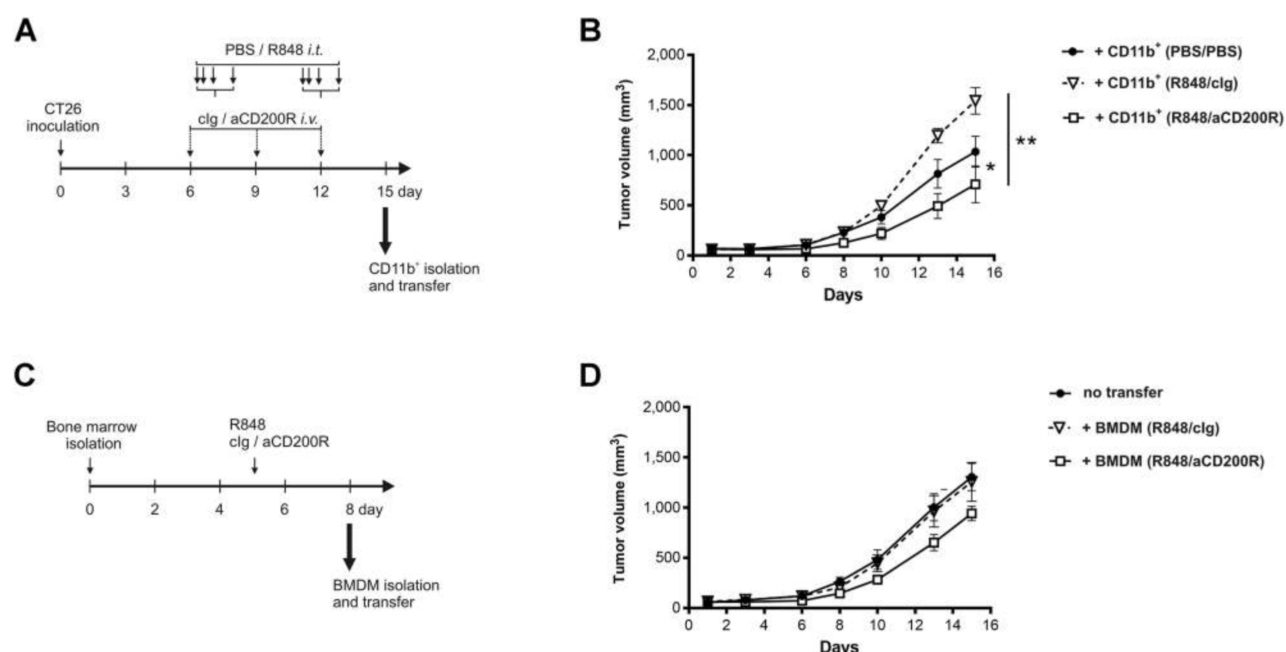

Figure 6.

CT26 tumor growth is affected by adoptively transferred myeloid cells. Intratumoral CD11b$^+$ cells were isolated from mice that received treatment as shown in **A** and inoculated with CT26 cells (ration 3:1) to naïve mice. **B,** Tumor growth after inoculation of CT26: CD11b$^+$ cell mixture (ratio 1:3), $n = 6$–7. CD11b$^+$ cells were isolated from mice that received three different variants of the treatment. Prior to the adoptive transfer, BMDM cells were stimulated with R848/isotype or R848/anti-CD200R as shown in **C. D,** Tumor growth-control tumors or tumors inoculated with CT26:BMDM cell mixture (ratio 1:3), $n = 6$–7. **A–D,** Performed once. Significance was calculated using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$.

to the timing of blood sampling after initial stimulation (24 hours). In our earlier studies in the MHV-infection model, we noticed an induction of IFNα 4 hours after infection (11). The kinetics of IFNα production stimulation driven by prolonged presence of a virus probably differ from the kinetics driven by administration of a synthetic ligand (11).

The TLR7-dependent nucleic acid "danger" signal may signify viral infection or local cell death thus triggering the retention of Ly6C$^+$ monocytes by the endothelium. Monocytes infiltrate tissues upon TLR7 activation dependent on CX3CR1 and αMβ2 integrin expression (23) or upon CCR2 activation (24). The shift in composition of tumor-infiltrating myeloid cells, particularly of monocyte/macrophage subsets, affects tumor development. Tumor-infiltrating Ly6C^{low} monocytes/immature macrophages exert antitumor effects, inhibiting metastasis through the regulation of NK-cell recruitment and activity (23). TLR7-stimulated production of cytokines, especially type I IFNs, are necessary for CCR2-dependent monocyte accumulation during inflammation (24). Additionally, type I IFNs inhibit maturation of Ly6C^{hi} monocytes to macrophages and upregulation of the F4/80 or CD115 expression (24). Similarly, in our study, R848–an inducer of type I IFNs production, particularly if delivered in combination with anti-CD200R–inhibits maturation of intratumoral monocytes and decreases expression of F4/80, CD86, CD206, and CD115.

TAMs provide an inflammatory microenvironment that promotes breast cancer progression. This effect is associated with elevated IL1β. IL1β blockade or inactivation of inflammasome reduced tumor growth and metastasis in several preclinical cancer models (25). This is in accordance with our observation that the efficacy of tumor-inhibitory treatment with R848/anti-CD200R is accompanied by a decrease in the capacity of intratumoral myeloid cells to produce IL1β.

Treatment with R848, independently of anti-CD200R, inhibits tumor outgrowth and shapes intratumoral microenvironment, at the same time allowing for development of lymphocyte responses. However, involvement of NK cells in this antitumor responses cannot be ruled out. Several mice (treated with R848 alone or with R848 and CD200R antibody) were tumor-free for more than 50 days and proved to be resistant to rechallenge with CT26 cells inoculated on the contralateral site. Thus, the proinflammatory effect of TLR7 stimulation supports the development of antitumor adaptive responses and leads to development of immune memory. However, the immediate tumor inhibitory effects seem to be independent of adaptive immunity, because the treatment with R848 and anti-CD200R decreased tumor growth in immunodeficient mice as much as it did in immunocompetent mice.

Spinetti and colleagues studied the effects of R848 on myeloid cell activity in the CT26 model (20). Double subcutaneous injection of 25 μg of R848, on the opposite side of the body than the tumor, caused a substantial decrease in the percentage of CD11b$^+$Gr-1^{int} cells in the tumor, spleen, blood, and bone marrow. Additionally, the authors showed that R848 stimulated the expression of myeloid-maturation markers as F4/80, CD80, and MHC-II (20). On the other hand, in our model, intratumoral injection of R848 increased the percentage of monocytes and decreased expression of mature-macrophage markers such as F4/80 and CD206. These two models differ in the route of R848 administration, which apparently matters for this effect. It would

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be interesting to investigate the effect of subcutaneous R848 application on differentiation and activation of myeloid cells in the tumor microenvironment.

We observed a shift in the percentage of various populations of tumor-infiltrating myeloid cells. In control tumors, the predominant population consisted of MHC-II^{+} immature macrophages. In mice treated with R848 alone or in combination with anti-CD200R, the tumors were mainly infiltrated with Ly6C^{hi}MHC-II^{-} monocytes and MHC-II^{-} immature macrophages. These changes were accompanied by extensive cell death within the myeloid cell population. Thus, we hypothesize that depletion of intratumoral immune and myeloid cells by R848 leaves an empty niche that is filled by cells arriving from the bone marrow and that the activity of these newly recruited cells can be further modulated by anti-CD200R. The latter concept is supported by the observation that BMDM cells incubated *ex vivo* with R848 have suppressed tumor-promoting activity. Moreover, R848 alone does not reach therapeutic effect because CD11b^{+} cells isolated from tumors treated with the combination of R848 and anti-CD200R, but not the cells from the tumors of mice treated with R848 alone, transferred antitumor activity to naïve CT26-bearing mice. Treatment with anti-CD200R alone did not have an antitumor effect.

The timing of our therapy, in which anti-CD200R was applied earlier than R848, was likely essential. Anti-CD200R would have time to trigger CD200R before its later downregulation by the second cycle of R848 (Fig. 1D and E). Triggering CD200R on tumor myeloid cells such as macrophages likely results in inhibition of their tumor-promoting effects. After the second R848 cycle, CD200R expression is downregulated and the tumor microenvironment is shifted to a more proinflammatory state. In this scenario, the combination therapy (R848/anti-CD200R) works through two independent pathways, firstly inhibiting TAMs and secondly inducing antitumor inflammation.

Based on these findings, we propose that modulation of CD200R in combination with TLR7 stimulation might be considered as an antitumor therapy with long-lasting effects on the tumor microenvironment.

Disclosure of Potential Conflicts of Interest

T.P. Rygiel has ownership interest in a patent application for Cellis Sp. z o.o. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Z. Pilch, J. Golab, L. Meyaard, T.P. Rygiel
Development of methodology: Z. Pilch, J. Golab, T.P. Rygiel
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Pilch, K. Tonecka, A. Braniewska, Z. Sas, M. Skorzynski, L. Boon, J. Golab
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Pilch, J. Golab, T.P. Rygiel
Writing, review, and/or revision of the manuscript: Z. Pilch, L. Boon, J. Golab, L. Meyaard, T.P. Rygiel
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Tonecka, A. Braniewska, Z. Sas, M. Skorzynski
Study supervision: Z. Pilch, J. Golab, T.P. Rygiel

Acknowledgments

This work was cofinanced by the Polish Ministry of Science and Higher Education under grant no. 0454/IP1/2013/72 (to T.P. Rygiel) and by the Foundation for Polish Science under grant no. TEAM TECH/2016-1/8 (to T.P. Rygiel).

We thank Kavita Ramji for the editorial support.

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Received August 21, 2017; revised March 13, 2018; accepted May 4, 2018; published first July 18, 2018.

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