

CD200R1-CD200 checkpoint inhibits phagocytosis differently from SIRP α -CD47 to suppress tumor growth

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Targeting macrophage inhibitory receptors like signal regulatory protein α (SIRP α) is a promising avenue in cancer treatment. Whereas the ligand of SIRP α , CD47, is widely expressed on tumor cells, its simultaneous presence on all normal cells raises concerns about toxicity and efficacy. This study identifies CD200R1, which binds CD200 on specific tumor types and limited normal cells, as an alternative inhibitory checkpoint for phagocytosis. Blocking or removing CD200R1 from macrophages or CD200 from tumor cells increases phagocytosis and suppresses tumor growth. In humans, CD200R1 is mainly expressed in immunosuppressive macrophages and is induced by interleukin-4. Unlike SIRP α that utilizes phosphatases Src homology 2 domain phosphatase (SHP)-1 and SHP-2, CD200R1 mediates its inhibitory effect via the kinase Csk. Combined CD200R1-CD200 and SIRP α -CD47 blockade further boosts phagocytosis and reduces tumor growth of CD200-expressing tumors, compared to either blockade alone. Thus, targeting CD200R1-CD200 is a promising strategy for immune checkpoint blockade in macrophages, either alone or alongside blockade of other checkpoints.

Immunotherapy is one of the most significant advancements in anti-cancer treatment of recent years^{1,2}. Most efforts have concentrated on blocking inhibitory immune checkpoints on T cells, such as PD-1 and CTLA-4. These strategies have shown impressive results against solid tumors like melanoma and renal cancer but have had limited success with hematological malignancies. However, promising results against hematological cancers have emerged from blocking inhibitory checkpoints on phagocytes, particularly macrophages^{3,4}.

Notably, the blockade of SIRP α , an inhibitory receptor on macrophages that recruits the phosphatases SHP-1 and SHP-2, or of its ligand CD47 that is widely expressed on cancer cells, has been shown to enhance tumor cell phagocytosis and suppress tumor growth in mice^{3,4}. Phase 1 and 2 clinical trials have also suggested the anti-tumor efficacy of SIRP α -CD47 blockade in hematological malignancies such as diffuse large B cell lymphoma, follicular lymphoma, Sezary's syndrome and some solid tumors^{5–9}.

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Despite these advances, SIRP α -CD47 blockade has demonstrated limited efficacy against other tumors, including myelodysplastic syndrome and acute myelogenous leukemia, and toxicity issues such as anemia, thrombocytopenia and lymphopenia frequently arose due to CD47's broad expression on normal cells^{7,10}. Suggested approaches to address these issues are to block alternative macrophage inhibitory checkpoints or to combine SIRP α -CD47 blockade with blockade of other checkpoints to lower treatment dosage⁷.

Macrophage activation is regulated by a range of activating and inhibitory receptors^{3,4,11}. When activating receptors, such as Fc receptors (FcRs), integrin CD11b and SLAMF7, are predominantly engaged by ligands on potential target cells, macrophages are activated and exhibit phagocytosis. In contrast, prevalent engagement of inhibitory receptors like SIRP α by ligands on potential target cells suppresses activation and phagocytosis. Most, if not all, macrophage subtypes have a phagocytic capacity, including the so-called inflammatory (M1) and anti-inflammatory (M2) macrophages, especially in the context of phagocytosis inhibitory checkpoint blockade^{12,13}.

CD200R1 is a transmembrane receptor expressed on macrophages and other immune cells such as T cells, mast cells, and other myeloid cells^{14,15}. It recognizes CD200, a ligand expressed in a restricted fashion on B cell malignancies, including chronic lymphocytic leukemia (CLL) and multiple myeloma (MM), and some solid tumors like melanoma and lung cancer^{16–19}. In several tumor types, higher expression of CD200 correlated with a worse prognosis^{16–19}. Engagement of CD200R1 by CD200 was reported to inhibit T cell activation, mast cell degranulation, and Toll-like receptor (TLR) signals in myeloid cells, seemingly through recruitment of Ras-GTPase-activating protein (Ras-GAP), which inhibits the Ras-Erk pathway^{20–25}. Identification of CD200R1 as an inhibitory axis in T cells led to the testing of blocking CD200 mAb samalizumab and blocking CD200R1 mAb 23ME-00610 in phase 1 clinical trials of human cancer, with encouraging results including limited toxicity and some clinical responses^{25,26}.

While the CD200R1-CD200 interaction has been reported to influence inflammatory responses by macrophages—possibly leading to indirect consequences on phagocytosis^{14,21,27,28}—a direct impact on the phagocytosis process, in particular of cancer cells, has not been assessed. The molecular mechanism through which CD200R1 may suppress activating pro-phagocytic receptors also remains to be elucidated. Additionally, the therapeutic potential of targeting the CD200R1-CD200 pathway, alone or in combination with other modalities, including SIRP α -CD47 blockade, to enhance phagocytosis has yet to be investigated.

In this study, we discovered that the antibody-mediated blockade or loss of CD200R1 on macrophages or CD200 on tumor cells increased phagocytosis and enhanced macrophage-dependent anti-tumor responses in vivo. Acute blockade of the CD200R1-CD200 interaction resulted in a rapid (within 15 min) augmentation of actin polarization towards tumor cells, suggesting that the impact of CD200R1-CD200 on phagocytosis was direct. In humans, CD200R1 was induced by IL-4 and predominantly expressed in anti-inflammatory (M2-type) macrophages. Unlike SIRP α that acts through SHP-1 and SHP-2, CD200R1 mediated its inhibitory impact on phagocytosis by way of Csk, a protein tyrosine kinase that inactivates Src family kinases. When combined, blockade of CD200R1-CD200 and SIRP α -CD47 worked synergistically to enhance phagocytosis and inhibit tumor growth. Therefore, CD200R1 is a phagocytosis inhibitory checkpoint in macrophages that is triggered by CD200 expressed on tumor cells. Blocking this pathway may provide useful strategies for eliminating CD200-positive malignancies.

Results

CD200R1, a CD11b-interacting protein, inhibits phagocytosis

Several studies have demonstrated that inhibitory receptors, such as SIRP α and PD-1, mediate their inhibitory functions in part through

physical association, direct or indirect, with the activating receptors that they suppress^{29–31}. In macrophages, SIRP α , an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptor, closely associates with CD11b, the α M subunit of Mac-1, a key effector in phagocytosis^{30,31}. CD11b serves not only as a complement receptor but also as a co-receptor for pro-phagocytic receptors such as FcRs and SLAMF7³.

We aimed to identify CD11b-associated inhibitory receptors, beyond SIRP α , that control phagocytosis when engaged by ligands expressed on cancer cells and act through a molecular mechanism distinct from that of SIRP α . CD11b was immunoprecipitated from mouse bone marrow-derived macrophages (BMDMs), and the associated proteins were analyzed using mass spectrometry. A few inhibitory receptors were identified in CD11b immunoprecipitates from wild-type BMDMs, but not from CD18 (the β 2 subunit of Mac-1)-deficient BMDMs, which lack CD11b (Fig. 1a and Supplementary Table 1). Some of these receptors, namely SIRP α , Ly-9, CD84 and CD200R1, recognize protein ligands potentially expressed at the surface of cancer cells. Most contain ITIMs or immunoreceptor tyrosine-based switch motifs (ITSMs), including SIRP α and Ly-9, both known to inhibit phagocytosis^{3,4,32}. CD84 is related to Ly-9. The notable exception was CD200R1, which contains a NPXY (asparagine-proline-any residue-tyrosine) motif and has not been reported to suppress pro-phagocytic receptors^{14,15}. The co-immunoprecipitation of CD200R1 and CD11b was validated by protein immunoblots (Fig. 1b).

To test the involvement of CD200R1 in phagocytosis, the effect of antibody-mediated blockade of CD200R1-CD200 on phagocytosis of CD200⁺ mouse tumor cell lines, including B cell lines WEHI-231, A20 and J558, as well as breast cancer cell line TUBO, was studied (Fig. 1c and Supplementary Fig. 1a, b). Variants of these cell lines expressing the Tac molecule (human CD25) were also created, to enable opsonization by mouse IgG2a Tac mAb 7G7²⁸ (Fig. 1c and Supplementary Fig. 1b). CD200 mAb OX-90, but not a control (Ctrl) mAb, inhibited the binding of a CD200R1-Fc fusion protein to WEHI-231 cells, confirming its effectiveness as a blocking mAb (Supplementary Fig. 1c). In a 2-h-microscopy-based phagocytosis assay, OX-90 enhanced the ability of wild-type C57BL/6 mouse BMDMs to phagocytose IgG-opsonized CD200⁺ tumor cells, compared to Ctrl mAb (Fig. 1d). While WEHI-231, A20, and TUBO did not show increased phagocytosis in the absence of IgG opsonization, J558 did, as will be further discussed below (Fig. 1d).

Similar effects were observed in a pHrodo-based assay, over a wide range of co-incubation times as short as 1 h that were analyzed by Incucyte (1–4 h; Fig. 1e) or flow cytometry (2h; Supplementary Fig. 1d–f). Confocal microscopy assays revealed that blocking CD200 mAbs also augmented the proportion of macrophages displaying actin polarization around the tumor cells within 15 min of co-incubation (Fig. 1f). However, there was no increase in conjugate formation between the macrophages and the tumor cells.

The ability of CD200 blockade to enhance phagocytosis of IgG-opsonized WEHI-231 was eliminated in BMDMs lacking FcR γ (*Fcrlg*^{−/−}), confirming that phagocytosis was mediated by FcRs³⁰ (Supplementary Fig. 1g). Likewise, the increased phagocytosis of non-opsonized J558 cells was negated in BMDMs without SLAMF7 (*Slamf7*^{−/−}), validating that this response was mediated by SLAMF7²⁸ (Supplementary Fig. 1h). CD200 blockade also improved the ability of WT BMDMs to phagocytose WEHI-231 cells opsonized with complement (C3bi), which directly triggers CD11b, compared to the Ctrl mAb¹⁶ (Fig. 1g). This effect was prevented by a blocking CD11b mAb. Additionally, CD200 blockade enhanced the phagocytic capacity of mouse peritoneal and liver macrophages (Supplementary Fig. 1i). CD200R1 mAb OX-131, which blocks CD200R1 rather than CD200, also exhibited a phagocytic enhancement²⁷ (Fig. 1h).

Therefore, CD200R1 was one of a few inhibitory receptors other than SIRP α that were co-immunoprecipitated with CD11b in macrophages. Antibody-mediated blockade of CD200R1-CD200 increased phagocytosis of tumor cells initiated by several pro-phagocytic receptors, including CD11b, FcRs and SLAMF7. The rapidity of these effects suggested that the inhibitory impact of CD200R1-CD200 on the functions of pro-phagocytic receptors may be direct.

Genetic evidence that CD200R1-CD200 suppresses phagocytosis

To confirm these results, we examined the impact of ablating expression of CD200R1 in macrophages, CD200 on tumor cells, or both (Fig. 2a; Supplementary Fig. 2a,b). Like the effects seen with the CD200 mAb, *Cd200r1*^{-/-} BMDMs showed an increased phagocytosis of IgG-opsonized WEHI-231 cells in the presence of the Ctrl mAb, compared to

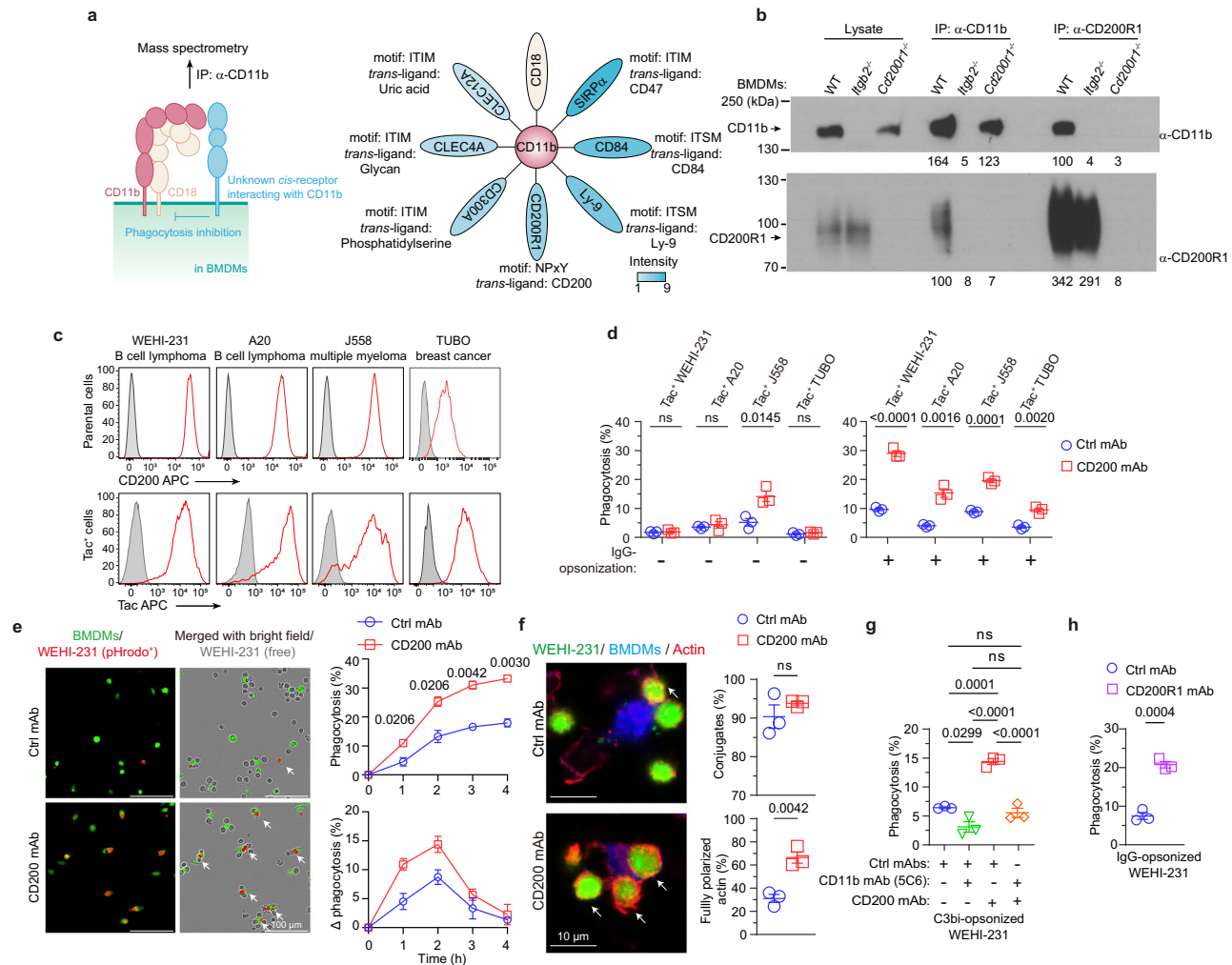


Fig. 1 | CD200R1, a CD11b-interacting protein, inhibits phagocytosis. a Mass spectrometry analysis of CD11b immunoprecipitates from BMDMs. Left: experimental workflow for isolating the CD11b-interacting proteins. Right: relative abundance of CD11b-interacting proteins, with a focus on cell surface receptors with inhibitory potential, indicated by a color gradient. ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; NPXY, asparagine, proline, any residue, tyrosine. IP, immunoprecipitation. **b** Immunoblot analysis of CD11b and CD200R1 interaction in immunoprecipitates from WT, *Itgb2*^{-/-} and *Cd200r1*^{-/-} BMDMs. Relative abundance is shown at the bottom of each panel. **c** Flow cytometry analysis of CD200 expression on parental (top) and Tac expression on Tac⁺ (bottom) mouse tumor cell lines. Red curves represent staining with CD200 or Tac mAbs. Filled curves, control (Ctrl) mAbs. **d** Microscopy-based phagocytosis assay of non-opsonized (-) or IgG-opsonized (+) Tac⁺ WEHI-231, Tac⁺ A20, Tac⁺ J558 and Tac⁺ TUBO cells by WT BMDMs, in presence of CD200 mAb OX-90 (rat IgG2a) or Ctrl mAb 2A3 (rat IgG2a). Tumor cells were opsonized with Tac mAb 7G7 (mouse IgG2a), (*n* = 3). **e** Time-course pHrodo-based phagocytosis assay using IgG-opsonized Tac⁺ WEHI-231 cells and WT BMDMs. WEHI-231 cells were labeled with pHrodo red dye, and BMDMs were labeled with CFSE. Phagocytosis over time (0-4 h) was analyzed using an IncuCyte Live Cell

Analyzer. Left: representative images at 2 h (scale bar, 100 μ m; arrows, BMDMs with engulfed tumor cells). Right: quantification of cumulative phagocytosis (top) and time-specific increase in phagocytosis (Δ phagocytosis; bottom) over 0-4 h, (*n* = 3). **f** Confocal microscope-based conjugate formation and actin polarization assay of IgG-opsonized WEHI-231 cells labeled with CFSE (green) and co-incubated with WT BMDMs labeled with Cell Trace Violet (CTV; blue), in presence of CD200 mAb or Ctrl mAb. Actin (red) was detected by β -actin mAb. Left: representative images (scale bar, 10 μ m; arrows, BMDMs with fully polarized actin). Scale, 10 μ m. Right: quantification of conjugate formation (top) and of conjugates with fully polarized actin (bottom), (*n* = 3). **g** As per Fig. 1d, except that phagocytosis of complement (C3bi)-opsonized WEHI-231 cells by WT BMDMs in the presence of blocking CD200 mAb OX-90, blocking CD11b mAb 5C6 (rat IgG2b), Ctrl mAb 2A3 or Ctrl mAb LTF-2 (rat IgG2b), (*n* = 3). **h** As per Fig. 1d, expect that phagocytosis of IgG-opsonized WEHI-231 cells by WT BMDMs in the presence of CD200R1 mAb OX-131 (mouse IgG1, Fc-silent) or Ctrl mAb (mouse IgG1, Fc-silent), (*n* = 3). Data are from three (a-h) independent experiments. Each symbol represents one mouse. Data are presented as mean $\pm$ s.e.m. Statistical analysis: two-tailed *t*-test (d-f, h) with multiple comparisons (e); One-way ANOVA test with multiple comparisons (g); ns, not significant. See also Supplementary Fig. 1 and Supplementary Table 1.

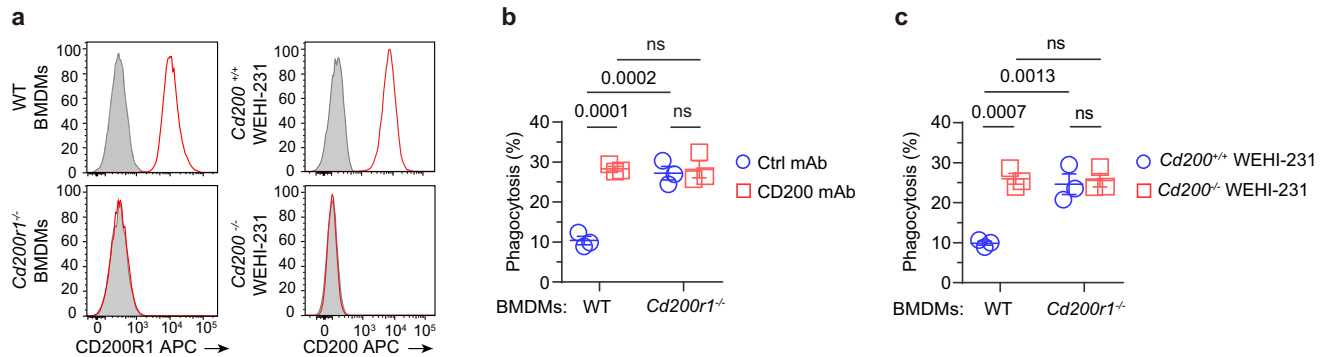


Fig. 2 | Genetic evidence that CD200R1-CD200 suppresses phagocytosis. **a** Flow cytometry analysis of CD200R1 expression (left) on WT (top) or *Cd200r1*^{-/-} (bottom) BMDMs, and CD200 expression (right) on *Cd200*^{+/+} (top) or *Cd200*^{-/-} (bottom) WEHI-231 cells, as measured by flow cytometry. Red curves, CD200R1 or CD200 mAbs. Filled curves, Ctrl mAb. **b** As per Fig. 1d, except that phagocytosis of IgG-opsonized WEHI-231 cells in the presence of WT or *Cd200r1*^{-/-} BMDMs was studied,

(*n* = 3). **c** As per Fig. 2b, except that phagocytosis of IgG-opsonized *Cd200*^{+/+} and *Cd200*^{-/-} WEHI-231 cells, in the presence of WT or *Cd200r1*^{-/-} BMDMs, (*n* = 3). Data are from three independent experiments (**a–c**). Each symbol represents one mouse. Data are mean ± s.e.m. Statistical analysis: two-way ANOVA test, with multiple comparisons (**b, c**), ns not significant. See also Supplementary Fig. 2.

WT BMDMs (Fig. 2b). There was no additional increase by adding the CD200 mAb. Furthermore, we generated a variant of WEHI-231 cells that lacked CD200 (Fig. 2a and Supplementary Fig. 2b). IgG-opsonized *Cd200*^{-/-} WEHI-231 cells elicited enhanced phagocytosis by WT BMDMs compared to *Cd200*^{+/+} WEHI-231 cells (Fig. 2c). There was no further increase when BMDMs were deficient in CD200R1.

Thus, the absence of CD200R1 on macrophages or CD200 on tumor cells enhanced phagocytosis similarly to blockade of the CD200R1-CD200 interaction.

Blockade of CD200R1-CD200 inhibits tumor growth in vivo

To assess the effects of CD200R1-CD200 blockade in vivo, we used RAG-1-deficient mice (*Rag1*^{-/-}), which lack T cells and B cells, and were inoculated with WEHI-231 or A20 cells. RAG-1-deficient mice were utilized, to prevent tumor cell rejection via T cell-mediated mechanisms, as all the available CD200⁺ tumor cell lines originated from mouse strains with a genetic background distinct from C57BL/6. In addition, this approach enabled us to avoid contributions of T cells and B cells to the anti-tumor effects of CD200R1-CD200 blockade. Once tumor growth was established, mice were treated with CD200 mAb or Ctrl mAb, plus Tac mAb 7G7 for opsonization.

Mice injected intravenously with WEHI-231 developed liver tumors (Fig. 3a–d, and Supplementary Fig. 3a). When euthanized at day 15, they displayed enlargement, white discoloration and tumor cell infiltration of the liver compared to those without tumor cell injection (Fig. 3b–d, Supplementary Fig. 3a). Compared to Ctrl mAb, treatment with CD200 mAb resulted in a notable decrease in these parameters (Fig. 3b–d). Flow cytometry analyzes of liver cells showed that the total number of WEHI-231 cells (defined as GFP⁺) was reduced by ~90% with the CD200 mAb compared to Ctrl mAb (Supplementary Fig. 3b, c). The total counts of immune cells, including macrophages, neutrophils, and NK cells, were also reduced (Supplementary Fig. 3c). Most immune cells in tumors were macrophages.

To evaluate the growth of WEHI-231 tumors in liver over time, we developed a variant of WEHI-231 that expressed luciferase (Supplementary Fig. 3a). Tumor mass was assessed by measuring luminescence. Compared to Ctrl mAb, the CD200 mAb caused a significant inhibition of tumor growth over time (Fig. 3e, f). Mouse survival was also improved (Fig. 3g). Importantly, the inhibitory effect of CD200 mAb on tumor growth was dependent on IgG opsonization of tumor cells, as it was not seen in the absence of mAb 7G7, compared to in the presence of mAb 7G7 (Supplementary Fig. 3d). It was also reliant on host macrophages, as its impact disappeared when mice were treated with clodronate-containing liposomes, which depleted >90% of liver

macrophages, compared to control liposomes (Supplementary Fig. 3e–g).

In the case of A20 cells, RAG-1-deficient mice were injected subcutaneously with tumor cells and tumor size was ascertained over time using a caliper (Fig. 3h; Supplementary Fig. 4a). CD200 mAb yielded a reduction in A20 tumor size over time, compared to Ctrl mAb (Fig. 3i). Upon euthanasia, there was a decrease in tumor weight (Fig. 3j). Lastly, there was a diminution in the numbers of tumor cells and immune cells, most of the latter being macrophages (Supplementary Fig. 4b, c).

In summary, blockade of CD200R1-CD200 with CD200 mAb suppressed mouse tumor cell growth in vivo. This effect was dependent on IgG opsonization of tumor cells and required macrophages.

Distribution of CD200R1-CD200 in normal and malignant human cells

Although *Cd200r1* is broadly expressed in mouse macrophages (www.immgen.org), whether this is also the case for human macrophages needs to be clarified. Unprimed blood monocyte-derived human macrophages exhibited low levels of CD200R1 (Fig. 4a and Supplementary Fig. 5a). However, IL-4, which induces differentiation into anti-inflammatory M2a-type macrophages³¹, augmented expression by ~5- to 10-fold. Interferon (IFN)-γ, which drives the differentiation into inflammatory M1-type macrophages, reduced expression, although the difference did not reach statistical significance³³. Little to no effects were seen with IL-10 or transforming growth factor (TGF)-β. These observations were supported by analyzes of published RNA sequencing data, in which we noted that *CD200R1* was among the most up-regulated transcripts following IL-4 treatment (Fig. 4b). They were also confirmed by our evaluation of a separate study with bulk RNA sequencing (Supplementary Fig. 5b)³⁴. Additionally, they aligned with previous studies indicating that CD200R1 is a marker for M2a-type macrophages³⁵.

To assess expression of *CD200R1* in macrophages from human tumors, we took advantage of published RNA sequencing datasets. In these studies, the authors identified several tumor-associated macrophages (TAMs) subtypes, including *SPPI*⁺ (secreted phosphoprotein 1) TAMs, *ISGL5*⁺ (interferon-secreted gene 15⁺) TAMs, *INHBA*⁺ (inhibin subunit beta A⁺) TAMs and *CIQC*⁺ (complement C1q C-chain) TAMs^{36,37} (Supplementary Fig. 5c). Amongst these, we found that *CIQC*⁺ TAMs showed the highest frequency of *CD200R1* RNA expression, compared to other subtypes such as *SPPI*⁺, *ISGL5*⁺ and *INHBA*⁺ TAMs (Fig. 4c). In contrast to *CD200R1*, *SIRPA* exhibited broader RNA expression across these various TAM subtypes (Supplementary Fig. 5d).

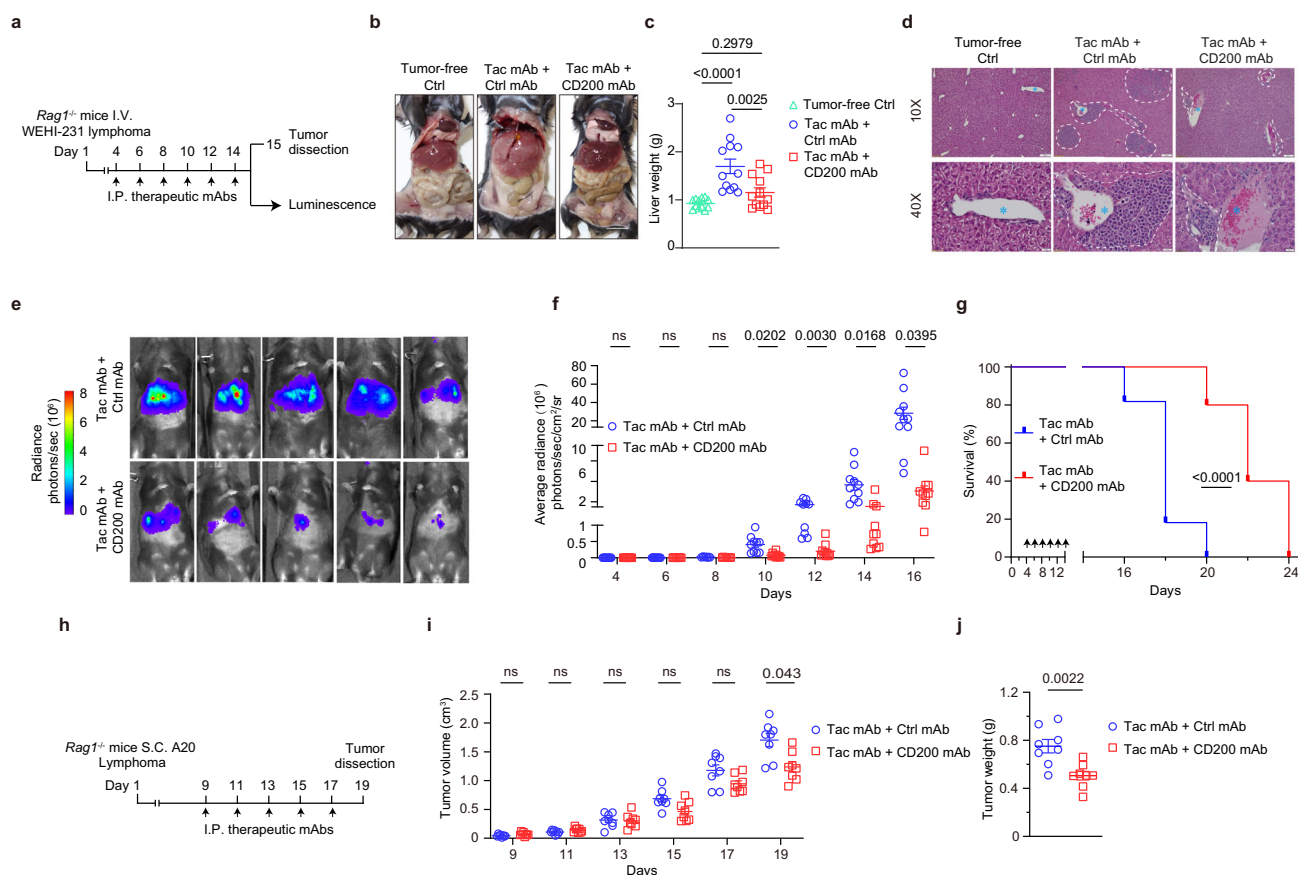


Fig. 3 | Blockade of CD200R1-CD200 inhibits tumor growth in vivo. a–g Tac⁺ WEHI-231 tumor (with or without luciferase expression) were injected intravenously into $Rag1^{-/-}$ mice, followed by intraperitoneal injection of Tac mAb combined with CD200 mAb or Ctrl mAb every 2 days. Mice were euthanized on day 15 (**b–d**) or monitored over time using luminescence, if cells were expressing luciferase (**e–g**), ($n = 12$, **b, c**, $n = 10$, **e–g**). **a** Schematic representation of the experimental workflow. I.V., intravenously; I.P., intraperitoneally. **b** Representative photographs of mice euthanized on day 15, with or without tumor injection, and treated with the indicated mAbs. Scale bar, 1 cm. **c** Liver weight of mice injected or not with tumor cells. **d** Hematoxylin and eosin staining of liver sections, showing blood vessels (blue asterisks) and tumor cell aggregates adjacent to blood vessels

(outlined in white). Scale bars, 100 μ m (10 $\times$ magnification), and 20 μ m (40 $\times$ magnification). **e** Representative luminescence images on day 12. **f** Tumor progression over time as measured by luminescence. sec, second, st, steradian. **g** Kaplan-Meier analysis of survival. **h–j** Tac⁺ A20 cells were injected subcutaneously in $Rag1^{-/-}$ mice followed by intraperitoneal injection of Tac mAb combined with CD200 mAb or Ctrl mAb, every 2 days, ($n = 8$). **h** Schematic representation of the experimental workflow. S.C, subcutaneously. **i** Tumor volume over time. **j** Tumor weight. Data are from three c or two d-j independent experiments, respectively. Each symbol represents one mouse. Data are presented as mean $\pm$ s.e.m. Statistical analysis: one-way ANOVA test, with multiple comparisons (c); two-tailed t-test (f, i, j). log-rank (Mantel-Cox) test (g). ns, not significant. See also Supplementary Figs. 3 and 4.

Of note, the authors of these datasets indicated that $CIQC^+$ TAMs had an M2 gene expression signature with a high phagocytosis score characterized by expression of *MRC1* (mannose receptor C-type 1), *CD163*, *MERTK* (MER Proto-Oncogene, tyrosine kinase) and *CIQB* (complement C1q B-chain), compared to the other subsets^{36,37} (Supplementary Fig. 5c). Gene ontology analyzes within the $CIQC^+$ TAM population also revealed that *CD200R1*-positive macrophages expressed higher levels of genes associated with phagocytosis, compared to *CD200R1*-negative macrophages (Supplementary Fig. 5e).

In the case of *CD200*, databases of human hematological tumors demonstrated that *CD200* RNA was most prevalent in B cell malignancies, such as CLL, MM, pre-B cell acute lymphoblastic leukemia (ALL), and Hodgkin's lymphoma (HL), as well as in certain T cell malignancies like angioimmunoblastic T cell lymphoma (AITL) (Fig. 4d and Supplementary Fig. 5f). In contrast, *CD47* RNA was broadly detected in all hematological tumors (Fig. 4d and Supplementary Fig. 5f). As an example of expression of *CD200* in solid tumors, *CD200* RNA was identified in tumor cells from ~50% of patients in a cohort of 14 melanoma cases, in keeping with previous

reports¹⁸ (Fig. 4e). In contrast, melanoma cells universally expressed *CD47*. Analysis of the immune cells infiltrating these melanoma tumors also revealed that macrophages expressed *CD200R1* and *SIRPA*.

In normal hematopoietic cells, *CD200* also had a restricted expression pattern. *CD200* was more highly detected in activated $CD4^+$ T cells, resting B cells and activated B cells, with no expression in red blood cells, platelets and minimal or no expression in several cell types including resting $CD4^+$ T cells, resting $CD8^+$ T cells and activated $CD8^+$ T cells (Fig. 4f). This expression pattern was confirmed by flow cytometry (Fig. 4g). By opposition, *CD47* was broadly expressed in all normal hematopoietic cell types (Fig. 4f, g).

Therefore, human *CD200R1* exhibited a narrower expression profile in macrophages compared to human *SIRPA*, being induced by IL-4 in blood-derived macrophages from healthy donors and primarily expressed in M2-type TAMs with high phagocytic capacity in human cancer tissues; likewise, human *CD200* displayed a more restricted expression pattern compared to *CD47*, being present in B cell malignancies, specific T cell malignancies, and their normal counterparts, as well as in some solid tumors like melanoma.

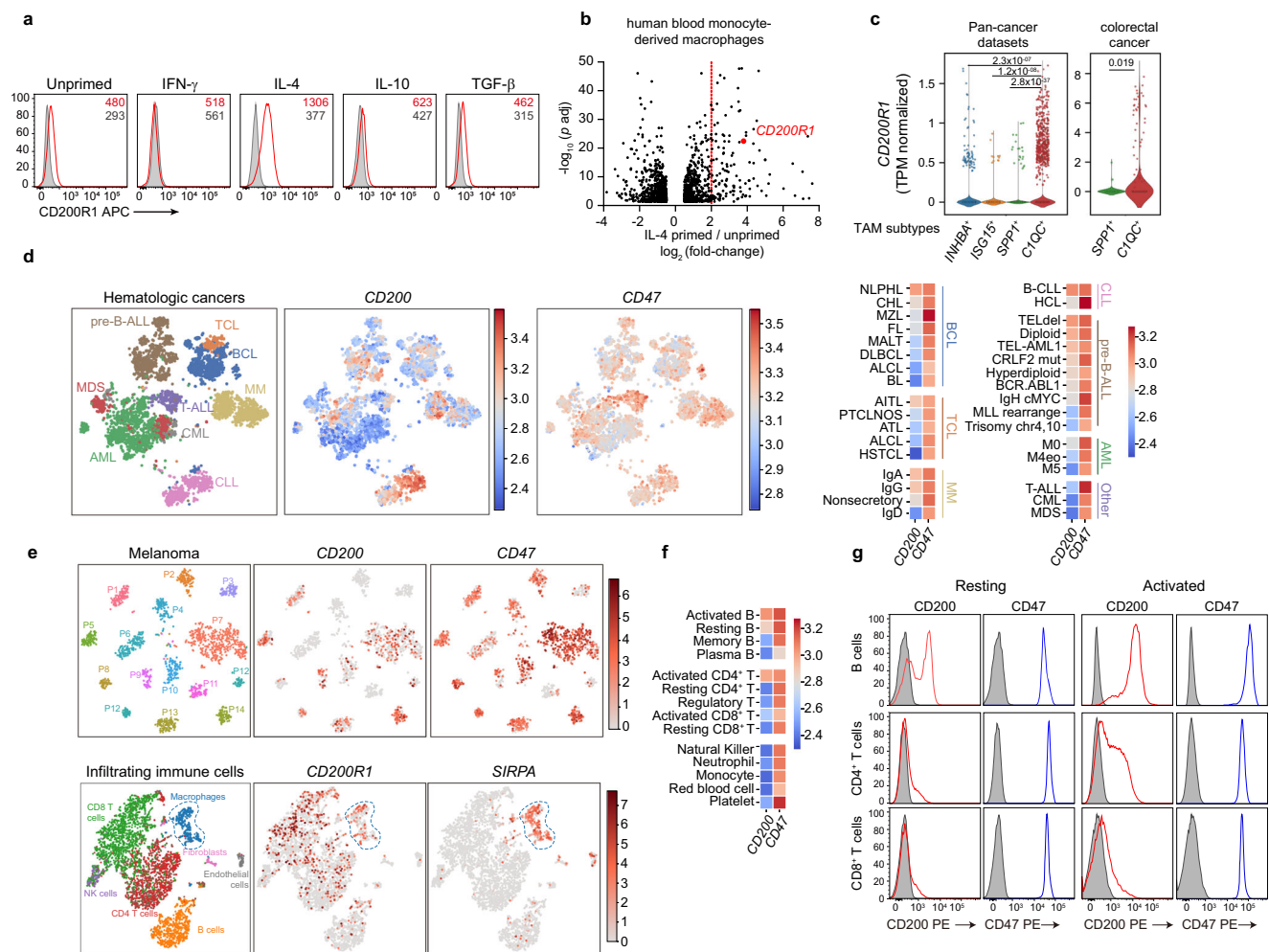


Fig. 4 | Expression of CD200R1 and CD200 in human cells. **a** Flow cytometry analysis of CD200R1 expression on human blood monocyte-derived macrophages, either unprimed or primed for 1 day with the indicated stimuli. Red curves, CD200R1 mAb. Filled curves, Ctrl mAb. Number in histograms indicated mean fluorescence intensity (MFI). **b** Fold changes of RNA expression of human blood monocyte-derived macrophages treated with or without IL-4. *CD200R1* is highlighted in red. Fold changes ($\log_2$; x-axis) and adjusted p -values ($-\log_{10}$; y-axis) are shown. The red line represents a 4-fold change threshold. Data are from dataset GSE195440. **c** Frequency and relative expression levels of *CD200R1* RNA across various human TAM subtypes, distinguished by gene expression profiles^{36,37}. Data are from single-cell RNA sequencing (scRNA-seq) datasets GSE154763³⁶ and GSE146771³⁷ of TAMs from patients with the cancers indicated in Supplementary Fig. 5c, and colon carcinoma. **d** RNA expression profiles of human hematological malignancies, determined by microarray analysis. Left: UMAP plots of samples with tumor diagnosis are shown and colored by cluster identity. Middle: *CD200* and *CD47* RNA expression levels overlaid onto the UMAP (color gradient shows relative expression). Right: mean expression of *CD200* and *CD47* RNA in common subtypes

of hematological malignancies and normal immune cells (color gradient). Data are from published dataset HEMAP³⁶ ($n = 7092$). **e** Single-cell RNA sequencing analysis of human melanoma. Left: t-SNE profiles of melanoma malignant or non-malignant cells. Malignant melanoma cells are clustered by patient sample (top), while infiltrating immune cells are clustered by cell type (bottom). The middle and right: normalized expression of selected markers overlaid onto the t-SNE space, with a color gradient reflecting relative expression. Macrophages are outlined by a blue line. Data are from published dataset GSE15978³⁷. **f** Same as **d**, except that expression of *CD200* and *CD47* RNA was analyzed in normal hematopoietic cells. **g** Flow cytometry analysis of expression of CD200 and CD47 on resting or activated human B cells, CD4⁺ T cells, and CD8⁺ T cells. Data are from three independent experiments (**a**, **g**). Each symbol represents one gene (**b**), one cell (**c**), one healthy donor or patient (**d**). Statistical analysis: Wald test followed by multiple comparisons (**b**); Kruskal–Wallis H test followed by Dunn’s post-hoc test with multiple comparisons (**c**, left); two-tailed Mann–Whitney U test (**c**, right panels). See also Supplementary Fig. 5.

Blockade of human CD200 enhances phagocytosis and reduces growth of human tumor cells

In line with the detection of *CD200* RNA in specific human tumor types, CD200 was found on the surface of several human B cell lines, including NCI-H929 (MM), SLVL (B cell lymphoma), 721.221 (B lymphoblastoid cells), and KG-1a (acute myelogenous leukemia) (Fig. 5a). Additionally, it was detected on the human melanoma cell line SK-MEL-28 and the small cell lung cancer cell line NCI-H209 (Fig. 5a). The effects of CD200 blockade by therapeutic human CD200 mAb samalizumab on phagocytosis of these cells were assessed²⁶. To ensure that samalizumab did not engage FcRs, we engineered an Fc-silent version of the mAb. When combined with

IgG-opsonization, samalizumab enhanced phagocytosis by IL-4-pre-treated human macrophages, compared to Ctrl mAb (Fig. 5b). Little to no increase was observed in the absence of IgG-opsonization (Fig. 5b). The increase in phagocytosis in response to CD200 blockade by samalizumab was nearly equivalent to that seen with CD47 blockade by CD47 mAb B6H12 (Fig. 5c).

To evaluate the impact of samalizumab on human tumor cell growth in vivo, SLVL or 721.221 cells were inoculated subcutaneously into NSG mice—deficient in T cells, B cells, and NK cells. Mice were then treated or not with samalizumab, in combination with anti-CD20 mAb rituximab for IgG opsonization. Compared to the Ctrl mAb, samalizumab significantly reduced the size and weight of tumors caused by

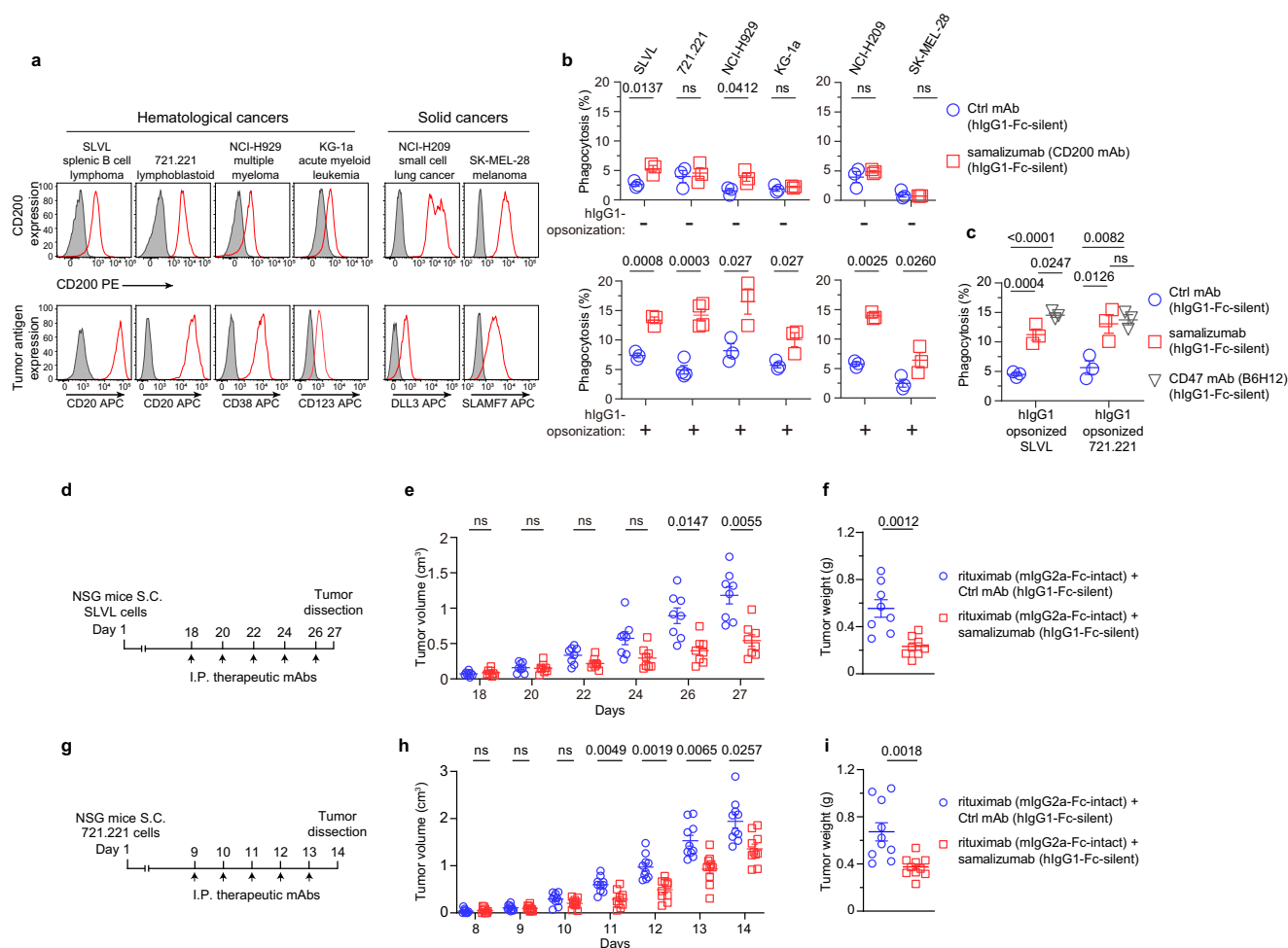


Fig. 5 | Therapeutic CD200 mAb samalizumab boosts phagocytosis and slows the growth of human tumors. **a** Flow cytometry analysis of CD200 (top) or tumor antigens (CD20, CD38, CD123, DLL3 and SLAMF7; bottom) on various human tumor cell lines. Red curves, CD200 mAb or tumor antigen-specific mAbs. Filled curves, Ctrl mAbs. **b** As per Fig. 1d, except using IL-4-primed human blood monocyte-derived macrophages and human tumor cells in the presence of samalizumab (human IgG1, Fc-silent) or Ctrl mAb MOPC21 (human IgG1, Fc-silent). IgG opsonization was performed using CD20 mAb rituximab (SLVL, 721.221), CD38 mAb daratumumab (NCI-H929), CD123 mAb talatocutumab (KG-1a), DLL3 mAb rovalpituzumab (NCI-H209), or SLAMF7 mAb elotuzumab (SK-MEL-28) ($n = 3$ or 4). **c** As per Fig. 5b, except using the indicated cell lines and samalizumab (human IgG1, Fc-silent), CD47 mAb B6H12 (human IgG1, Fc-silent), or Ctrl mAb MOPC21 (human IgG1, Fc-silent). Tumor cells were opsonized as detailed for Fig. 5b, ($n = 3$). **d–f**

Subcutaneous injection of SLVL cells in NSG mice, followed by intraperitoneal injection of rituximab (mouse IgG2a version) combined with samalizumab or Ctrl mAb every 2 days, ($n = 8$). **d** Schematic representation of the experimental workflow. **e** Tumor volume over time. **f** Tumor weight. **g–i** Subcutaneous injection of 721.221 cells in NSG mice, followed by intraperitoneal injection of rituximab (mouse IgG2a version) combined with samalizumab or Ctrl mAbs every 2 days, ($n = 10$). **g** Schematic representation of the experimental workflow. **h** Tumor volume over time. **i** Tumor weight. Data are from three to four (**a**, **b**), three (**c**), or two (**d–i**) independent experiments. Each symbol represents one human sample or one mouse. Data are presented as mean $\pm$ s.e.m. Statistical analysis: two-tailed t-test (**f**, **i**), with multiple comparisons (**b**, **c**, **e**, **h**). ns , not significant. See also Supplementary Fig. 6.

SLVL or 721.221 cells and led to a decrease in the number of tumor cells and immune cells, which consisted of macrophages and to a lesser extent neutrophils, recovered from tumor tissues (Fig. 5d–i and Supplementary Fig. 6a–h).

Put together, these results showed that CD200 mAb samalizumab augmented phagocytosis and inhibited growth of human tumor cells in vivo, when combined with tumor-opsonizing mAbs. The impact of CD200 blockade on phagocytosis of human tumor cells was quantitatively analogous to that of CD47 blockade.

CD200R1 inhibits phagocytosis via Csk

While SIRP α inhibits phagocytosis through ITIMs that recruit SHP-1 and SHP-2, CD200R1 lacks such motifs^{3,4}. Instead, CD200R1 contains three conserved cytoplasmic tyrosines, Y286, Y289, and Y297, one of which, Y297, is part of the NPXY motif (Fig. 6a)^{14,15}. When phosphorylated at Y297, the CD200R1 NPXY motif has been reported to bind the

adaptor molecules Dok-1 and Dok-2, which subsequently enlisted the enzyme Ras-GAP, to suppress mast cell degranulation and myeloid cell cytokine production^{20–24}.

To investigate how CD200R1 suppressed phagocytosis, we created mutants of mouse CD200R1 with tyrosine-to-phenylalanine (Y-to-F) substitutions at positions Y286, Y289, or Y297. Additionally, we engineered a CD200R1 variant missing most of the cytoplasmic domain (Δ intra-cytoplasmic; Δ IC)^{23,25,26,38}. All mutations, along with WT CD200R1, were introduced into *Cd200r1*^{-/-} BMDMs, where they expressed at levels approximately 10-fold higher than endogenous CD200R1 on WT BMDMs (Supplementary Fig. 7a). In comparison to *Cd200r1*^{-/-} BMDMs expressing only GFP, those transduced with WT CD200R1 exhibited reduced phagocytosis, like WT BMDMs expressing GFP alone (Fig. 6b). This inhibitory effect was alleviated by CD200 mAb OX-90. Moreover, it was abrogated by the Y286F, Y297F, and Δ IC mutations, but not by the Y289F mutation.

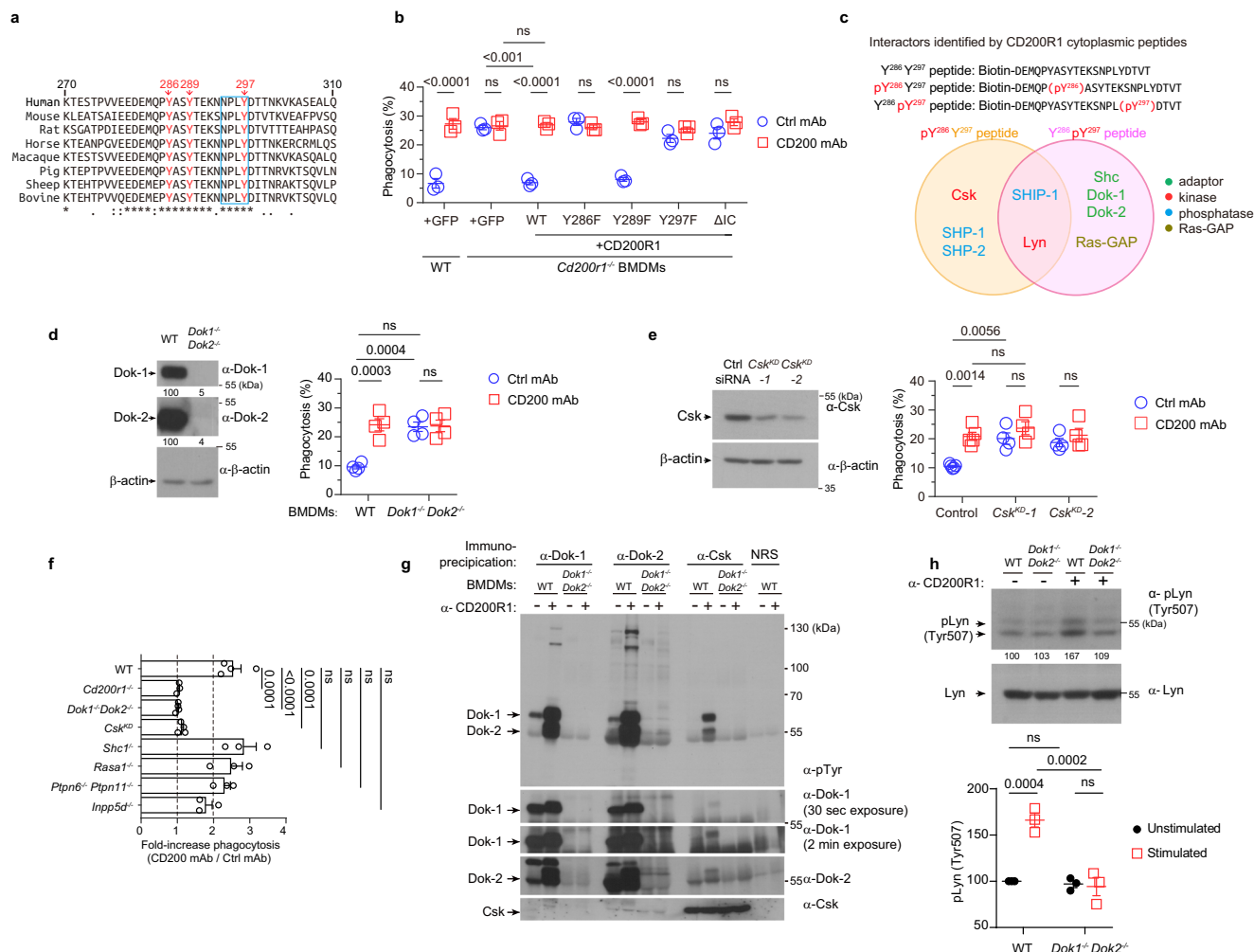


Fig. 6 | CD200R1 inhibits phagocytosis via Csk. a Partial sequences of the cytoplasmic domain of CD200R1 from different species. The conserved tyrosines (Y²⁸⁶, Y²⁸⁹, and Y²⁹⁷; based on mouse amino acid numbering) are in red, whereas the conserved NPXY motif is boxed. Identical residues are depicted by asterisks (*), while conserved and semi-conserved amino acids are highlighted by colons (;) and periods (.), respectively. **b** Phagocytosis of IgG-opsonized WEHI-231 cells by WT BMDMs expressing GFP alone, or *Cd200r1*^{-/-} BMDMs expressing GFP alone or CD200R1 variants, in the presence of CD200 mAb or Ctrl mAb, (*n* = 3). **c** Mass spectrometry analyses of cytoplasmic proteins with inhibitory potential interacting with Y²⁸⁶ or Y²⁹⁷ in pull-down assays. Peptides are depicted at the top. Interactors identified by phosphorylated peptides are shown below. Negative regulators of immune cell activation, either adaptors, kinases, phosphatases or Ras-GAP, are indicated. **d** Immunoblot analysis of Dok-1 and Dok-2 expression (left) and phagocytosis of IgG-opsonized WEHI-231 cells (right) by BMDMs from WT or *Dok1*^{-/-} *Dok2*^{-/-} mice. β-actin as loading Ctrl (left). Normalized protein abundance (in %) relative to actin is shown below the top panel, (*n* = 4). **e** Same as Fig. 6d except that WT BMDMs

transduced with Ctrl or Csk-specific siRNAs [*Csk* knockdown (*Csk*^{kd})] were used. Two different *Csk*-specific siRNAs were studied. *Csk* expression (left) and phagocytosis (right) were studied, (*n* = 4). **f** Summary of fold-changes in phagocytosis for the various genetically deficient BMDMs in response to CD200 mAb, compared to WT BMDMs, (*n* = 3 or 4). **g** WT or *Dok1*^{-/-} *Dok2*^{-/-} BMDMs were stimulated or not for 1 min with biotinylated CD200R1 mAb OX-110 and streptavidin. Cell lysates were immunoprecipitated with α-Dok-1, α-Dok-2, α-Csk, or normal rabbit serum (NRS), and probed by immunoblotting with antibodies targeting phosphotyrosine (pTyr), Dok-1, Dok-2 or Csk. **h** WT or *Dok1*^{-/-} *Dok2*^{-/-} BMDMs were stimulated or not for 30 s with biotinylated CD200R1 mAb OX-110 and streptavidin. Cell lysates were probed with α-pLyn (Tyr 507) or α-Lyn Abs (top). A quantification of multiple independent experiments is shown at the bottom, (*n* = 3). Data are from three (b, e, g, h) or four d independent experiments, two (pY²⁹⁷ peptide) and three (pY²⁸⁶ peptide) (c) independent experiments. Each symbol represents one mouse (b, d, e). Data are mean ± s.e.m. Statistical analysis: two-way ANOVA test, with multiple comparisons (b, d, e, h); One-way ANOVA test, with multiple comparisons (f). ns not significant. See also Supplementary Figs. 7, 8 and Supplementary Table 2.

To assess if Y286 and Y297 were recruiting inhibitors of phagocytosis, we used pull-down assays with synthetic peptides, phosphorylated or not, and lysates of mouse BMDMs, followed by mass spectrometry (Fig. 6c). The phosphorylated peptides, but not the unphosphorylated ones, recovered several proteins with inhibitory potential (Fig. 6c and Supplementary Table 2). These inhibitory proteins included the adaptors Dok-1, Dok-2, and Shc, Ras-GAP, the lipid phosphatase SHP-1, protein tyrosine phosphatases SHP-1, and SHP-2, and the protein tyrosine kinase Csk.

Based on these findings, we examined the phagocytic function of BMDMs genetically lacking Dok-1 and Dok-2, Shc, Ras-GAP, SHP-1, or

SHP-1 and SHP-2. We also generated *Csk*-deficient BMDMs (*Csk* knockdown; *Csk*^{kd}) by transducing WT BMDMs with *Csk*-targeting small interfering (si) RNAs. We predicted that if any of these molecules were downstream effectors of CD200R1-mediated inhibition, we would observe increased phagocytosis without CD200 blockade, in addition to a reduction in the ability of CD200 blockade to enhance phagocytosis.

Compared to Ctrl BMDMs, the deficient BMDMs exhibited defective expression of the target proteins, but normal expression of various cell surface markers (Fig. 6d, e and Supplementary Figs. 7b–g and 8a–d). BMDMs lacking Shc, Ras-GAP, or SHP-1 and SHP-2

did not show any increase in phagocytosis in the absence of CD200 blockade or any impairment in the capacity of CD200 mAb to enhance phagocytosis, compared to the Ctrl mAb (Supplementary Fig. 8a–d). While SHIP-1-deficient BMDMs exhibited a small, albeit not statistically significant, increase in phagocytosis relative to Ctrl BMDMs in the absence of CD200 blockade, a further increase was still observed with the CD200 mAb, suggesting the possible involvement of SHIP-1 in CD200R1-independent inhibition of phagocytosis (Supplementary Fig. 8c). In contrast, BMDMs lacking Dok-1 and Dok-2, or those deficient in Csk, displayed increased phagocytic activity in the absence of the CD200 blockade compared to WT BMDMs. They also lost the ability of CD200 mAb to enhance phagocytosis (Fig. 6d, e). A summary of these data is shown in Fig. 6f.

As Dok-1 and Dok-2 bind NPXY motifs and have tyrosine phosphorylation sites that can recruit Csk, we tested if these adaptors were responsible for recruiting Csk to CD200R1. To this end, we analyzed the tyrosine phosphorylation of Dok-1 and Dok-2, along with their association with Csk in response to engagement of CD200R1. Compared to unstimulated cells, WT BMDMs stimulated with non-blocking CD200R1 mAb OX-110 exhibited prominent tyrosine phosphorylation of Dok-1 and Dok-2, which were also detected in Csk immunoprecipitates (Fig. 6g). No Dok-1 or Dok-2 phosphorylation or association with Csk was observed in BMDMs lacking Dok-1 and Dok-2 (Fig. 6g). As reported in earlier studies with the adaptor PAG³⁸, although Dok-1 and Dok-2 were present in Csk immunoprecipitates, we did not reciprocally observe Csk in immunoprecipitates of Dok-1 or Dok-2. This is likely related to the poor quality of the Csk antibodies available for immunoblot analyzes. In keeping with the involvement of Csk in Dok-1- and Dok-2-mediated inhibition, the CD200R1 mAb-induced phosphorylation of Lyn at the inhibitory tyrosine, Y507, which is the target of Csk, was abolished in BMDMs lacking Dok-1 and Dok-2, in comparison to Ctrl BMDMs (Fig. 6h).

Thus, the capacity of CD200R1 to inhibit phagocytosis involved a mechanism distinct from SIRPα that required the kinase Csk, which was recruited to CD200R1 via Dok-1 and Dok-2.

Synergy between CD200R1-CD200 and SIRPα-CD47 blockade in anti-tumor immunity

Since CD200R1 and SIRPα utilized different mechanisms of inhibition, we examined if combined blockade of CD200R1-CD200 and SIRPα-CD47 cooperated to augment phagocytosis. Mouse cell lines WEHI-231, A20, and J558 expressed both CD200 and CD47, albeit in different relative amounts (Fig. 7a). Single agent blockade of CD200R1-CD200 or SIRPα-CD47 with CD200 mAb or SIRPα mAb 27, respectively, yielded an increase in phagocytosis of IgG-opsonized WEHI-231, A20, and J558 cells, compared to Ctrl mAbs (Fig. 7b). Although doubling the concentrations of the individual mAbs did not increase phagocytosis further, combined blockade of CD200 and SIRPα did.

We also assessed synergic potential in vivo, using the WEHI-231 model expressing luciferase detailed in Fig. 3, with the exception that mice were treated with CD200 mAb, SIRPα mAb, or both. When used individually, CD200R1-CD200 blockade and SIRPα-CD47 blockade inhibited tumor growth and improved survival, compared to Ctrl mAbs (Fig. 7c–f). A greater impact was seen with CD200R1-CD200 blockade, compared to SIRPα-CD47 blockade. However, a much greater suppression was noted when CD200R1-CD200 blockade and SIRPα-CD47 blockade were combined.

In addition to causing anemia and thrombocytopenia, SIRPα-CD47 blockade can induce loss of lymphocytes, including CD8⁺ chimeric antigen receptor (CAR)-T cells, thereby potentially interfering with T cell-dependent anti-tumor responses³⁹. Thus, we also compared the effects of blocking CD200R1-CD200 or SIRPα-CD47 individually on the phagocytosis of normal human T cells. In keeping with the expression patterns of CD200 and CD47 in human cells (Fig. 4f, g), blockade of SIRPα-CD47, but not of CD200R1-CD200, augmented

phagocytosis of activated CD8⁺ T cells, which expressed CD47 but little to no CD200 (Fig. 7g). Either blockade strategy augmented phagocytosis of activated CD4⁺ T cells and B cells, which expressed both CD200 and CD47 (Fig. 7g).

Thus, combined blockade of CD200R1-CD200 and SIRPα-CD47 resulted in greater phagocytosis and inhibition of tumor growth in vivo compared to blocking of either checkpoint alone; moreover, unlike SIRPα-CD47 blockade, CD200R1-CD200 blockade did not enhance phagocytosis of activated CD8⁺ T cells.

Discussion

In this study, we found that engagement of CD200R1 on macrophages by CD200 on tumor cells inhibited phagocytosis in vitro and promoted tumor growth in vivo. CD200R1 suppressed phagocytosis initiated by several pro-phagocytic receptors, including FcRs, SLAMF7, and CD11b, presumably because of physical proximity between CD200R1 and CD11b, as suggested by the co-immunoprecipitation of CD200R1 with CD11b and as reported for SIRPα^{30,31}. This inhibitory effect was demonstrated in several types of mouse macrophages, as well as in human blood monocyte-derived macrophages treated with IL-4. The effect was reversed by either antibody blockade of CD200R1 or CD200, or through genetic depletion of CD200R1 in macrophages or CD200 in tumor cells. Notably, within 15 min of acute disruption of the CD200R1-CD200 interaction, macrophages had enhanced actin polarization toward tumor cells, indicating that CD200R1's influence on phagocytosis was rapid and likely direct.

There are several notable differences between CD200R1-CD200 and SIRPα-CD47. First, in humans, the expression patterns of CD200 and CD47 differed, both in tumor cells and in normal cells. While CD47 was essentially expressed on all tumor and normal cells, CD200 was restricted to a narrower range of cells, primarily B cell malignancies, some T cell neoplasia, and solid tumors such as melanoma, lung, breast, and prostate cancer^{18,19}. Unlike CD47, CD200 was not found in red blood cells, platelets, and activated CD8⁺ T cells.

Second, expression of CD200R1 and SIRPα also showed significant differences. In human cells, CD200R1 expression was induced by IL-4 in human blood monocyte-derived macrophages, indicating its regulation by specific cytokines tied to immune responses. This regulation may be different in mice, where expression of *Cd200r1* seems to be constitutive in macrophages (www.immgen.org). In human tissues, CD200R1 was primarily identified in *CIQC*⁺ TAMs, which were described to have M2-type features with high phagocytic capacity^{36,37}. In contrast to human CD200R1, human SIRPα was constitutively expressed on blood monocyte-derived macrophages and was more uniformly present across TAMs. Although macrophages in the tumor microenvironment are often viewed as being poorly phagocytic, it should be noted that these cells, like pro-inflammatory macrophages, are capable of phagocytosis, especially when phagocytosis inhibitory checkpoints are blocked. This was already reported for SIRPα-CD47 blockade^{13,40}. This notion was further supported by our data herein showing that IL-4-treated human macrophages displayed augmented phagocytosis in response to CD200 or CD47 blockade.

Third, the expression of CD200R1 and SIRPα diverged among immune cells other than macrophages. In addition to macrophages, human CD200R1 was reported to be present in T cells, NK cells, innate lymphoid cells (ILCs), mast cells, and dendritic cells (DCs)^{41,42}. In contrast, SIRPα was additionally found in neutrophils, DCs, and NK cells. While the inhibition of tumor growth by CD200 mAbs in RAG-1-deficient mice and NSG mice indicated that the effects of CD200R1-CD200 blockade in our studies were independent of T cells, B cells, and, in NSG mice, NK cells, it remains possible that other immune cell types expressing CD200R1 will influence the overall effects of CD200R1-CD200 blockade in human patients. Along these lines, inhibitory effects of CD200R1 during T cell activation have been reported^{41,42}. While the current study focussed on macrophages and

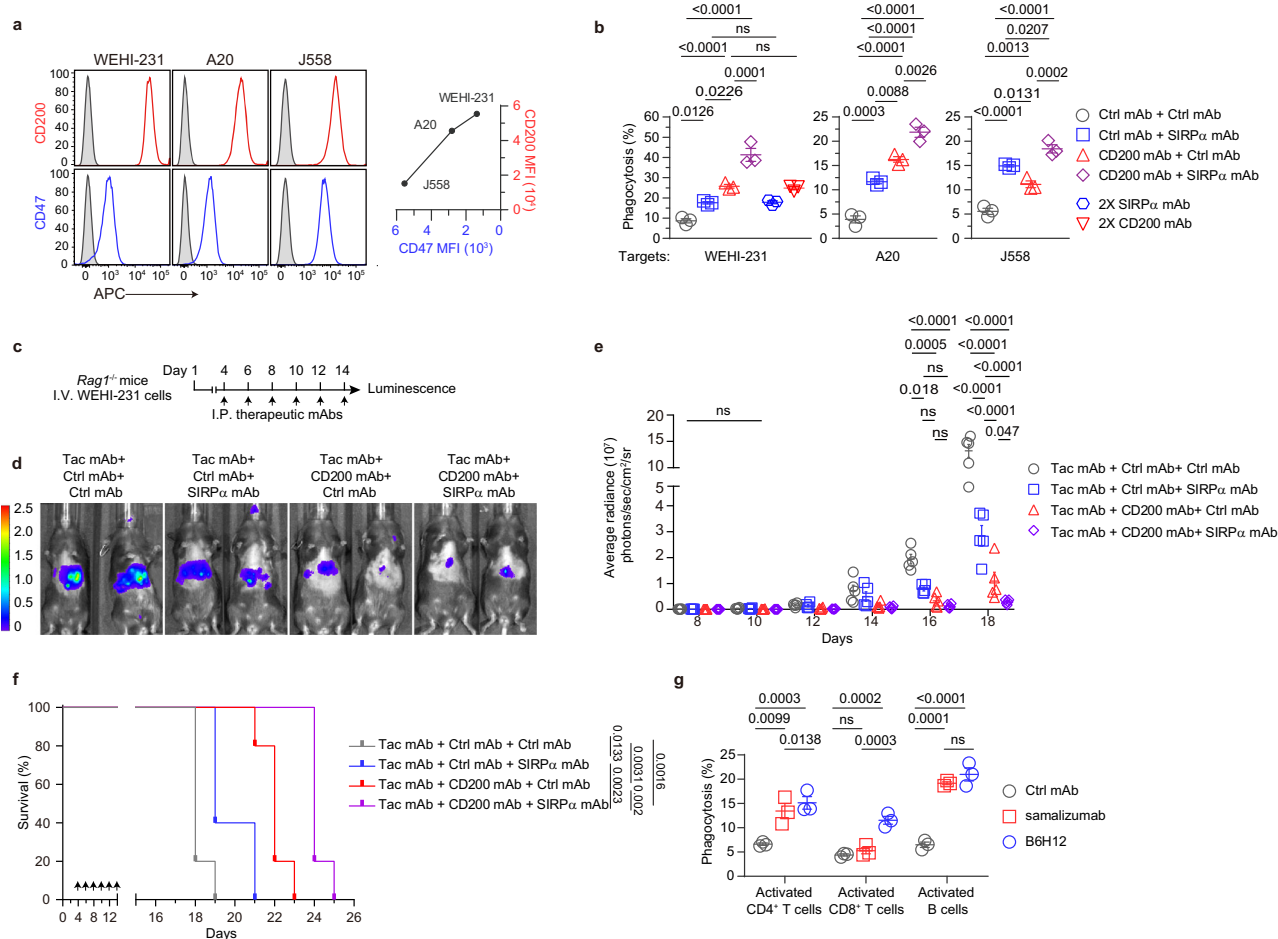


Fig. 7 | Synergy between CD200R1-CD200 and SIRPα-CD47 blockade in anti-tumor immunity. **a** Flow cytometry analysis of CD200 (red curves; top) and CD47 (lavender curves; bottom) expression on J558, A20 and WEHI-231 cells (left). Filled curves, Ctrl mAbs. The right panel shows relative expression levels of CD200 and CD47. **b** As per Fig. 1d, except that mAbs were used in combination: CD200 mAb OX-90 (rat IgG2a), SIRPα mAb 27 (mouse IgG2a, Fc-silent), Ctrl mAb 2A3 (rat IgG2a), and Ctrl mAb MOPC21 (mouse IgG2a, Fc-silent), ($n = 3$). **c–f** Luciferase⁺ Tac⁺ GFP⁺ WEHI-231 cells were injected intravenously into *Rag1*^{-/-} mice, followed by intra-peritoneal injection of Tac mAb combined with the indicated mAbs every 2 days starting from day 4, ($n = 5$). **c** Schematic representation of the experimental workflow. Tumor progression was measured over time using luminescence.

Representative photographs of mice (**d**) and quantification (**e**). **f** Kaplan-Meier curves of survival. **g** Phagocytosis of normal activated human T cells or B cells by autologous human macrophages, in the presence of samalizumab (human IgG1, Fc-silent), CD47 mAb B6H12 (human IgG1, Fc-silent) or Ctrl mAb (human IgG1, Fc-silent). T cells were not opsonized (implying phagocytosis was mediated by SLAMF7), whereas B cells were opsonized with rituximab (human IgG1, Fc-active) ($n = 3$). Data are from three (**a**, **b**, **g**) or two (**c–f**) independent experiments. Each symbol represents one mouse or donor. Data are mean $\pm$ s.e.m. Statistical analysis: two-way ANOVA test, with multiple comparisons (**b**, **e**); log-rank (Mantel-Cox) test (**f**); one-way ANOVA test, with multiple comparisons (**g**). ns, not significant.

phagocytosis, future studies, including with other mouse models and, especially, clinical trials in humans, will be needed for addressing the contributions of other macrophage functions and other types of immune cells to the responses of CD200R1-CD200 blockade in vivo.

Fourth, whereas SIRPα-CD47 suppresses phagocytosis via SHP-1 and SHP-2, CD200R1-CD200 inhibited phagocytosis through Csk⁴³. This contrasts with the ability of CD200R1 to suppress mast cell degranulation and myeloid cell cytokine production, which were reportedly regulated by Ras-GAP^{20–25}. The capability of CD200R1 to suppress phagocytosis also depended on two intracellular tyrosines in CD200R1, Y286 and Y297. Although the mechanism of action of Y286 remains to be clarified, one possibility, in keeping with the pull-down assays, is that Y286 was involved in recruiting the Src family kinase Lyn, which in turn was responsible for phosphorylating Y297, thereby enabling binding of Dok-1 and Dok-2 to phosphorylated Y297 and subsequent recruitment of Csk to Dok-1 and Dok-2. The divergence in molecular mechanisms between CD200R1 and SIRPα may explain the additive effects of combining CD200R1-CD200 and SIRPα-CD47 blockade in enhancing phagocytosis and suppressing tumor growth.

One of the questions emerging from our findings is whether Csk prevented or terminated phagocytosis. It is plausible that if Csk were already at the plasma membrane prior to engagement of a pro-phagocytic receptor, it would prevent activation before the activating signals are initiated. This is the case in T cells for PAG-associated Csk, which forms a complex prior to antigen-induced activation³⁸. However, it is conceivable that the situation is different if the recruitment of Csk to the membrane occurs in response to cell activation, as was the case for CD200R1 in macrophages. In this case, CD200R1-associated Csk may terminate already initiated activating signals.

At first glance, the several differences between CD200R1-CD200 and SIRPα-CD47 may indicate that SIRPα-CD47 blockade would be effective against a wider range of tumor types, compared to CD200R1-CD200 blockade. Nonetheless, they also imply a higher potential for toxicity with SIRPα-CD47 blockade, due to the broader distribution of CD47 on normal cells. Thus, even though CD200R1-CD200 blockade may offer clinical efficacy against a narrower spectrum of tumor types, it may also have lower toxicity. For CD200⁺ tumors, the combination of CD200R1-CD200 blockade with SIRPα-CD47 blockade may also allow

for lowering the dose of each blocking agent, thereby further reducing associated toxicities.

Despite all these distinctions, there is one key similarity between blockade of CD200R1-CD200 and blockade of SIRP α -CD47 that is therapeutically relevant. Like SIRP α -CD47 blockade, the CD200R1-CD200 blockade required the concomitant use of FcR-engaging anti-tumor antibodies to trigger phagocytosis, unless tumor cells expressed natural pro-phagocytic ligands like SLAMF7. Therefore, for most cancers, blockade of CD200R1-CD200, like that of SIRP α -CD47, will require combination therapies involving tumor-opsonizing mAbs. This is not surprising as blockade of inhibitory receptors on immune cells consistently requires co-engagement of activating receptors, either naturally or by therapeutic ligands, to evoke immune cell activation.

Data of phase 1 clinical trials with blocking CD200 or CD200R1 mAbs as T cell immune checkpoint blockers have been reported in patients with a variety of cancer types, including CLL, MM, and solid tumors^{25,26}. The mAbs were well tolerated, indicating their safety. Additionally, in the samalizumab CD200 mAb trial with CLL and MM, over 60% of patients showed a noticeable clinical response, although only a small fraction experienced a reduction in tumor volume of 50% or more, and no complete responses were recorded. Such outcomes are common for single-agent therapies, particularly if immune checkpoint blockers primarily act on macrophages rather than T cells. The simultaneous use of Fc-active anti-tumor antibodies to trigger FcRs may be needed to achieve greater efficacy in clinical trials of CD200R1-CD200 blockade.

Previously, macrophage biology was dominated by the M1/M2 paradigm, with M1 macrophages being pro-inflammatory and anti-tumoral, and M2 macrophages promoting tumor progression^{31,33}. However, single-cell RNA sequencing studies revealed that macrophages exhibit substantial heterogeneity and plasticity^{36,37}. Indeed, in many preclinical mouse tumor models, TAMs exhibit immunosuppressive, pro-tumoral phenotypes^{36,37}. However, in our models, macrophages seemingly played an anti-tumoral role, at least in the presence of phagocytic inhibitory checkpoint blockers. This is consistent with findings with other macrophage-targeting immunotherapies, such as SIRP α -CD47 blockade, which enhanced macrophage-mediated phagocytosis and suppressed tumor growth in preclinical models^{3–5}. This contrasting functionality of macrophages is also reflected in ongoing clinical trials targeting different aspects of macrophage biology. For instance, anti-CSF1R antibody therapies aim to deplete immunosuppressive TAMs, while SIRP α -CD47 blockade therapies seek to enhance macrophage-mediated phagocytosis^{3–5,44}.

In conclusion, our findings indicated that the CD200R1-CD200 checkpoint functions as a suppressor of phagocytosis in macrophages and serves as a promising macrophage target for treating CD200⁺ malignancies. Given the distinct molecular mechanism of action associated with CD200R1, the blockade of CD200R1-CD200 may be well-suited for combination with other inhibitory checkpoint blockers that target macrophages, potentially enhancing their efficacy while reducing toxicity. These insights also highlight the crucial need for sustained investigations into the mechanisms that regulate immune cell functions, which can then be better leveraged for developing effective and safer therapeutic strategies against human diseases.

Methods

Ethical statement

This research complies with all relevant institutional and national ethical regulations. All procedures involving human participants were approved by the IRCM Research Ethics Board, and informed consent was obtained from all participants. Animal studies were approved by the IRCM Animal Care Committee (protocol 2023-09) and conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

Mice

Mice lacking SLAMF7 (*Slamf7*^{−/−}) or FcRγ (*Fcgr1g*^{−/−}) were generated in our laboratory, as described in refs. 12,31,40. The following mice were obtained from The Jackson Laboratory (Bar Harbor, ME): C57BL/6J, NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/Sz), RAG-1-deficient (*Rag1*^{−/−}), CD18-deficient (*Itgb2*^{−/−}), and *Lys2-Cre*. CD200R1-deficient mice (*Cd200r1*^{−/−}), Dok-1- and Dok-2-deficient mice (*Dok1*^{−/−} *Dok2*^{−/−}), conditional SHIP-1-deficient mice (*Inpp5d*^{fl/fl}), conditional Shc-deficient mice (*Shc1*^{fl/fl}), conditional Ras-GAP-deficient mice (*Rasa1*^{fl/fl}), conditional SHP-1-deficient mice (*Ptpn6*^{fl/fl}), conditional SHP-2-deficient mice (*Ptpn11*^{fl/fl}) and *UBC-Cre-ERT2*⁺ transgenic mice were reported^{45–52}. Mice were maintained in the C57BL/6 background, unless specified. They were kept in a specific pathogen-free (SPF) environment. Either males or females were used, between 6 and 12 weeks of age. Littermates were used as controls in most experiments, except for some studies in which WT syngeneic, age and sex-matched mice were used. Housing was in Allentown PNC (positive/negative control) Model PNC 160 individually ventilated cages with Edstrom™ watering (Allentown Inc, Allentown, NJ, USA) in ventilated racks under negative pressure, ensuring a controlled environment at 20–23 °C and 40–60% humidity. An automated 12-h light/12-h dark cycle light system was used. Animal handling was performed in HEPA-filtered biosafety cabinets in a 100% filtered air ventilation room.

Cells

Mouse BMDMs and thioglycolate-elicited peritoneal macrophages were generated as described^{12,31}. In brief, BMDMs were generated in medium supplemented with 30% (vol/vol) L929 cell-conditioned medium, which served as a source of colony-stimulating factor-1 (CSF-1). To deplete genes in *UBC-creERT2* BMDMs in vitro, 0.5 μg/mL of 4 OH-tamoxifen (Sigma-Aldrich, St. Louis, MO) was added to the BMDM culture on day 2 and removed on day 3. To obtain thioglycolate-elicited peritoneal macrophages, mice were intraperitoneally injected with 1 mL of thioglycolate broth (BD Biosciences, Franklin Lakes, NJ). After 4 days, mice were euthanized, and peritoneal cells were collected via peritoneal lavage. Cells were first plated in a Petri dish for macrophage attachment. After 30 min, the suspended cells were removed, and the attached cells (macrophages) were harvested with 2 mM ethylene diamine tetra acetic acid (EDTA)-phosphate-buffered saline (PBS) and plated overnight into 24-well plates. Phagocytosis assays were performed the next day. To obtain liver macrophages, liver perfusion was done as described⁵³. Liver was then dissected and digested in medium containing collagenase (1 mg/mL; Sigma-Aldrich) and DNase I (0.03 mg/mL; Sigma-Aldrich) at 37 °C for 30 min with constant agitation. Crude liver non-parenchymal cells (LNPCs) were enriched by centrifuging the suspension at 50 × g for 3 min at room temperature (RT), followed by centrifugation at 400 × g for 10 min at RT. Liver macrophages were generated by culturing LNPCs in medium supplemented with CSF-1 for 7 days.

Human blood samples were collected from healthy donors, above 18-year-old, both male and female, through the IRCM blood donor program. All participants provided written informed consent prior to participation, as approved by the IRCM Human Ethics Board. Peripheral blood mononuclear cells (PBMCs) were differentiated into macrophages by culture for 7 days in medium supplemented with 10% human serum and 10 ng/ml CSF-1, as reported⁴⁰. Human monocyte blood-derived macrophages were either unprimed or primed with IFN-γ (100 ng/ml, PeproTech, Cranbury, NJ), IL-4 (10 ng/ml, PeproTech), IL-10 (50 ng/ml, PeproTech), or TGF-β (50 ng/ml, PeproTech) for 24 h prior to the phagocytosis assay. Human B cells, CD4⁺ T cells, and CD8⁺ T cells were isolated freshly or from frozen PBMCs using a negative selection method (STEMCELL Technologies, Vancouver, BC, Canada), achieving a purity of >90%. B cells were subsequently activated with lipopolysaccharide (LPS, 1 μg/mL; Sigma-Aldrich) for 2 days. CD4⁺ and

CD8⁺ T cells were activated with Fc-silent anti-CD3 ϵ (3 μ g/mL, OKT-3) for 2 days.

WEHI-231, A20, J558, L1210, 70z/3, BW5147.3, YAC-1, S194/5, MPC-11, P815, B16-F10, CMT-93, L929, 293 T, NCI-H209, SK-MEL-28, and Phoenix-Eco cell lines were purchased from American Type Culture Collection (Rockville, MA). KG-1a and SLVL cell lines were purchased from RIKEN BioResource Center (Kyoto, Japan). NCI-H929 cell line was purchased from European Collection of Authenticated Cell Cultures (Salisbury, UK). 721.221 cell line was purchased from International Histocompatibility Working Group (Seattle, WA). The TUBO cell line was a gift from Dr. John Stagg, University of Montreal, Montreal, Canada. All cells were negative for Mycoplasma. They were certified as authentic by commercial providers, and identity was confirmed by assaying expression of relevant cell surface markers using flow cytometry.

DNA constructs, retroviral infection, and transfections

cDNAs encoding mutated versions of CD200R1 (Y286F, Y289F, Y297F, Δ IC) were generated by overlap-extension PCR. After cloning of WT or mutated cDNAs in the indicated retroviral vectors, plasmids were transfected in Phoenix-Eco cells, and viral supernatants were recovered. Supernatants were then used for spin infection of primary cells, as described³¹. Empty plasmids were used as controls. BMDMs were infected with retroviruses (produced with pFB-GFP) encoding the indicated CD200R1 variants. GFP-positive cells were sorted after 48 h of retroviral infection and propagated for an additional 24 h in growth medium before experimentation.

WEHI-231, A20, J558, and TUBO derivatives expressing Tac (human CD25) were generated using retroviruses, encoding a cytoplasmic domain-deleted version of Tac fused to the transmembrane domain of 2B4 (cloned into pFB-neo or pFB-GFP), followed by cell sorting³¹. Polyclonal Tac-expressing cells were isolated by cell sorting, whereas monoclonal populations were established by limiting dilution. CD200-deficient (*Cd200*^{-/-}) WEHI-231 cells were generated using CRISPR-Cas9-mediated genome editing, with the guide RNA (gRNA) sequences 5' TCTCCACCTACAGCCTGATT 3' [Mm.Cas9.CD200.1AE; Integrated DNA Technologies (IDT), San Diego, CA] or 5' TTGTAGTGAAGGTGTACTAT 3' (Mm.Cas9.CD200.1AL; IDT). Cells transduced with scrambled gRNA (Alt-R® CRISPR-Cas9 Negative Control crRNA #1; IDT) were generated as control. In brief, gRNAs (10 μ M) were assembled with Cas9 nuclease (62 μ M, IDT) through incubation at RT for 15 min. Then, WEHI-231 cells (1×10^6) were resuspended in 20 μ L buffer R, containing gRNA-Cas9 complexes, and cell electroporation was done using the Neon electroporation system (1000 V, 30 ms, 2 pulses). CD200-negative cells were sorted after 48 h. Csk expression was knocked-down using two different Csk-specific short-interfering (si) RNAs (mm.Ri.Csk.13.1, mm.Ri.Csk.13.2; IDT), which were transduced in BMDMs with Lipofectamine RNAi MAX (Invitrogen, Burlington, Ontario, Canada). Universal negative control siRNAs (Sigma-Aldrich) were used as control. In brief, 2×10^5 BMDMs were plated in a 6-well-plate. Then, 4 μ L of 10 μ M siRNAs were mixed with 12 μ L Lipofectamine RNAi MAX in 200 μ L Opti-MEM and added to BMDMs. Fresh medium was added after 6 h. Cells were used for phagocytosis or immunoblotting assays after 2 days.

Antibodies and fusion proteins

Recombinant mAbs were generated as described in ref. 12. In brief, cDNAs encoding the variable fragments of heavy chain (VH) and light chain (VL) of mAbs were synthesized by GeneArt (Invitrogen). Then, VH cDNAs were cloned in-frame into the expression vector pAb-hlgG1 or pAb-mlgG2a. Fc-silent versions carried the LALAPG mutation, i.e., leucine 234-to-alanine 234; leucine 235-to-alanine 235; proline 329-to-glycine 329. These point mutations, based on human IgG1 amino acid numbering, prevent binding to FcRs. The VL cDNAs were cloned in-frame into the expression vector pAb-hlgK or pAb-mlgK. Recombinant mAbs were expressed in CHO cells and purified with protein A

Sepharose (Cytiva). Samalizumab sequences were obtained from patent US7408041B2, whereas rituximab sequences were from patent US5736137 (IDEC, C2B8). Control mAb MOPC21, CD47 mAb B6H12, and SLAMF7 mAb elotuzumab were described elsewhere¹². Coomassie blue staining of protein gels was used to verify protein production and quality.

For phagocytosis assays and in vivo assays, the following mAbs were used: CD200 mAb OX-90 (BioXCell, Lebanon, NH); CD200R1 mAb OX-131 (Absolute Antibody, Boston, MA), SIRP α mAb no. 27¹²; rat IgG2b isotype Ctrl mAb LTF-2 (BioXCell); rat IgG2a isotype Ctrl mAb 2A3 (BioXCell); Ctrl mAb MOPC-21 (in-house generated); and blocking CD11b mAb 5C6 (BioXCell). For cell opsonization, the following mAbs were used: Tac mAb 7G7 (BioXCell); human CD20 mAb rituximab biosimilar (BioXCell); human CD123 mAb talacotuzumab (MedChemExpress, Princeton, NJ); human CD38 mAb daratumumab (MedChemExpress); human DLL3 mAb rovalpituzumab (MedChemExpress). For cell stimulation, the following mAbs were used: biotinylated CD200R1 mAb OX110 (Invitrogen) or human CD3 ϵ mAb OKT-3 (hlgG1, Fc-silent). For flow cytometry, we used the following fluorophore-conjugated mAbs from Biolegend (San Diego, CA) or eBioscience (Waltham, MA): PD-L1 mAb 10 F.9G2; CD38 mAb 90; CD47 mAb miap301; CD19 mAb 6D5; F4/80 mAb BM8; CD200R1 mAb OX-110; CD200 mAb OX-90; CD45 mAb 30-F11; MHC class I H-2Kd mAb SF1-1.1.1; NK1.1 mAb PK136; Ly6G mAb 1A8; CD11a mAb M17/4; CD11b mAb M1/70; CD11c mAb N418; CD18 mAb M18/2; SIRP α mAb P84; CD64 mAb VX54-5/7.1; CD16/32 mAb 93; SLAMF7 mAb 4G2; CD20 mAb SA271G2; B220 mAb RA3-6B2; IgM mAb RMM-1; rat IgG2a isotype Ctrl mAb RTK2758; rat IgG1 isotype Ctrl mAb RTK2071; mouse IgG1 isotype Ctrl MOPC-21; Armenian hamster IgG isotype Ctrl mAb HTK888; rat IgG2b isotype Ctrl mAb RTK4530.

Immunoblotting was done using antibodies directed against Dok-1, Dok-2, Shc, Csk, SHP-1, SHP-2 or SHIP-1 Ab, which were generated in the Veillette laboratory^{38,54}; CD11b (EPRI344, Abcam, Ontario, CA), CD200R1 (AF2554, R&D Systems, Minneapolis, MN), Ras-GAP (B4F8; Santa Cruz Biotechnologies, Dallas, TX), β -actin (C4, Santa Cruz Technologies), or p-Lyn (Tyr507) (Cell Signaling Technology, Danvers, MA) or Lyn (Cell Signaling Technology).

Flow cytometry

Cells were harvested and washed with 2% fetal bovine serum (FBS)-containing PBS (flow cytometry buffer). In most cases, to prevent non-specific binding to FcRs, FcRs were blocked with a mix of mouse IgG2a (mAb 7G7) and CD16/32 mAb 2.4G2 for 30 min on ice. Afterwards, cells were stained with fluorophore-conjugated antibodies (1:200 dilution, 1×10^7 cells/ml) for 30 min on ice. After being washed with flow cytometry buffer, samples were analyzed using a Fortessa flow cytometer (BD Biosciences).

To assess blocking of the CD200R1-CD200 interaction by CD200 mAb OX-90, 2.5×10^5 WEHI-231 cells were pre-incubated with CD200 mAb or Ctrl mAb for 30 min on ice. Subsequently, cells were incubated with Fc-silent variants of CD200R1-Fc or Ctrl Fc, carrying the LALAPG mutations, and labeled with Alexa Fluor 647, following the manufacturer's instructions (Invitrogen). After incubation, cells were washed with flow cytometry buffer, and binding of the fusion proteins was analyzed by flow cytometry.

In vitro phagocytosis assays

Phagocytosis was assessed using either a microscopy-based assay or a pHrodo-based assay, as detailed³¹. For the microscopy-based assay, 5×10^4 macrophages were plated in a 24-well tissue culture plate. The following day, the culture medium was replaced with serum-free DMEM medium for 2 h. Meanwhile, the target cells were opsonized (or not) with antibodies at RT for 30 min, then labeled with 2.5 mM carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye (Invitrogen). 2.5×10^5 CFSE-labeled target cells were co-incubated with

macrophages for 2 h, in the presence of blocking or Ctrl mAb at 10 μ g/ml. Bright field and fluorescent images were captured using an inverted microscope (Carl Zeiss Axiovert S100 TV). Phagocytosis efficiency was determined as the number of macrophages containing CFSE⁺ target cells per 100 macrophages. For phagocytosis using the combination of CD200 mAb and SIRP α mAb, the 1 $\times$ concentration of each mAb was 10 μ g/ml. To opsonize target cells with complement (C3bi), WEHI-231 cells were incubated with C5-deficient human serum (Sigma-Aldrich) for 1 h at 37 °C, before the microscopy-based phagocytosis assay.

For the pHrodo-based assay, 1 $\times$ 10⁶ target cells were pre-labeled with 50 ng/ml pHrodo Red SE pH indicator (Invitrogen) in 100 μ l PBS at RT for 30 min. Subsequently, 2.5 $\times$ 10⁵ pHrodo-labeled target cells were co-incubated with macrophages for 2 h, in the presence of blocking or Ctrl mAb at 10 μ g/ml. After 2 h, target cells and macrophages were harvested with Accutase (Innovative Cell Technologies, San Diego, CA), stained with F4/80 APC-Cy7, and processed for flow cytometry. Macrophages were identified as F4/80⁺ and GFP^{intermediate} (macrophage autofluorescence). The pHrodo signal was acquired using the PE channel. Phagocytosis efficiency was calculated as the percentage of F4/80⁺ cells containing pHrodo⁺ cells. When monitoring phagocytosis over time using the Incucyte Live Cell Analyzer (Sartorius, Göttingen, Germany), 1 $\times$ 10⁶ target cells were pre-labeled with 500 ng/ml pHrodo Red SE pH indicator (Invitrogen) in 100 μ l PBS at RT for 30 min. Subsequently, 1 $\times$ 10⁵ pHrodo-labeled target cells were co-incubated with macrophages (pre-labeled with CFSE) for 4 h, in the presence of blocking antibodies or Ctrl mAb at 10 μ g/ml. Images were captured at 0, 1, 2, 3, and 4 h.

Conjugate formation and actin polarization

Conjugate formation and actin polarization assays were performed as previously described³¹. Briefly, 2 $\times$ 10⁵ BMDMs were labeled with Cell Trace Violet (CTV) and plated overnight. The following day, 1 $\times$ 10⁶ target cells were labeled with CFSE and co-incubated with BMDMs in serum-free DMEM medium in the presence of either CD200 mAb or Ctrl mAb for 15 min at 37 °C. After incubation, the dishes were washed, and cells were then fixed in PBS containing 4% paraformaldehyde solution, followed by permeabilization in PBS containing 0.1% Triton X-100. Subsequently, cells were blocked in PBS containing 5% BSA for 30 min at 4 °C. Following this, cells were washed and incubated with β -actin mAb AC-74 (Sigma-Aldrich) in PBS containing 5% BSA for 2 h at 4 °C. Subsequently, the cells were incubated with Alexa Fluor 647 anti-mIgG (Jackson ImmunoResearch Labs, West Grove, PA) for 1 h at 4 °C. Images were acquired using an LSM710 confocal microscope (Carl Zeiss). The degree of conjugation between BMDMs and target cells, relative to total BMDMs, as well as the percentage of cells with fully polarized actin at conjugation sites were quantified.

In vivo mouse tumor models

Tumor injections and treatments: For WEHI-231 and A20, pools of three clones of Tac⁺ GFP⁺ cells (1 $\times$ 10⁶ each) were injected intravenously (WEHI-231) or subcutaneously (A20) in 6–10-week-old *Rag1*^{-/-} mice. Alternatively, for WEHI-231, a polyclonal population of luciferase⁺ Tac⁺ GFP⁺ WEHI-231 cells (1 $\times$ 10⁶) was used. At the indicated times after tumor cell injection, mice received intraperitoneal injections every 2 days of Tac mAb 7G7 or Ctrl mAb (200 μ g), along with either CD200 mAb OX-90 (200 μ g), SIRP α mAb 27 (200 μ g), both mAbs, or Ctrl mAb 2A3 (200 μ g). For macrophages depletion in vivo, mice were injected I.V. with 100 μ l of clodronate liposomes or control liposomes (Liposoma BV, Amsterdam, Netherlands) every 4 days, starting from 1 day prior to tumor transplantation and until the end of the experiment.

For SLVL and 721.221 cells, cells (5 $\times$ 10⁶) were injected subcutaneously in 6–10-week-old NSG mice. Prior to injection, SLVL tumor cells were resuspended in a 1:1 mixture of PBS and pre-warmed Matrigel (Corning Life Sciences, Glendale, AZ). At the indicated times

after tumor cell injection, mice were treated with rituximab (mIgG2a variant, 200 μ g) plus Fc-silent samalizumab or control mAb (200 μ g), intraperitoneally.

Analysis: For A20, SLVL, and 721.221, tumor size was monitored with a caliper and was calculated using the formula (length $\times$ width²)/2. Mice were euthanized when tumor diameter reached 1.5 cm or more. For WEHI-231, mice were either euthanized at day 15 for liver examination or were monitored for tumor growth using luminescence. Upon euthanasia, the liver was dissected, weighed, and digested. Then, cells were fractionated by differential centrifugation, and red blood cells were lysed. After counting total cell numbers, populations of tumor and immune cells were identified by flow cytometry. For histology, liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the IRCM Histology Core Facility as described in ref. 55.

For mice injected with luciferase⁺ WEHI-231 cells, tumor progression was recorded every two days starting from day 4 post-inoculation. To this end, mice received 200 μ l of a 15 mg/mL D-luciferin substrate (PerkinElmer, Woodbridge, ON) in PBS via intraperitoneal injection, followed by imaging using the IVIS[™] Bioluminescence Imaging System (PerkinElmer). For survival analysis, mice were euthanized when the luminescence in the abdominal region reached 5 $\times$ 10⁷ photons/s/cm²/steradian.

Immunoprecipitations, immunoblots, peptide pull-down assays, and mass spectrometry

For immunoprecipitation of CD11b and CD200R1 followed by immunoblotting, BMDMs from the WT, *Cd200r1*^{-/-} and *Itgb2*^{-/-} mice were lysed by 1 $\times$ Brij99 lysis buffer (150 mM NaCl; 1% Brij99 (polyethylene [20]-oleylether); 25 mM HEPES (Gibco)), supplemented with protease and phosphatase inhibitors. The cell lysates were then centrifuged to remove insoluble material. Immunoprecipitation was performed using pre-cleared cell lysates and CD11b mAb M1/70 (Biolegend) or CD200R1 mAb OX110 (Biolegend), followed by capture with protein G sepharose (Cytiva). Beads were washed with lysis buffer containing 300 mM NaCl. For mass spectrometry, immunoprecipitates of CD11b from WT and *Itgb2*^{-/-} mice were eluted using 0.1 M glycine (pH 2.8) buffer followed by neutralizing using 1 M Tris-HCl (pH 9.0). Three technical replicates were performed for each sample, and immunoprecipitates from *Itgb2*^{-/-} mice were used as negative control.

Synthetic peptides corresponding to the cytoplasmic domain of mouse CD200R1 (residues 281 to 301), phosphorylated or not on the indicated tyrosines and containing biotin at the amino terminus, were obtained commercially (Genescript, Piscataway, NJ). For experimentation, they were conjugated to NeutrAvidin[™] Agarose (Thermo Fisher Scientific, Waltham, MA), followed by incubation with lysates of WT BMDMs in 1 $\times$ TNE buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 1% NP-40), supplemented with protease and phosphatase inhibitors. After incubation for 2 h at 4 °C, agarose beads were washed with 1 $\times$ TNE buffer, then with PBS. Bound proteins were eluted using pH 2.8, 0.1 M glycine buffer for mass spectrometry. Two or three technical replicates were performed for each sample, with proteins pulled down by unphosphorylated peptides serving as negative control.

Mass spectrometry was performed by the IRCM Proteomics Core Facility, as outlined elsewhere^{12,31}. In brief, samples were trypsin-digested and subjected to liquid chromatography-tandem mass spectrometry on an LTQ Orbitrap Velos (Thermo Fisher Scientific) equipped with a Proxeon nano electrospray ion source. A gradient of 100 min was used for liquid chromatography separation, and standard proteomics parameters were used for the mass spectrometers. Protein database searching was performed with Mascot (Matrix Science), and data analysis was conducted using the Scaffold software. Immunoprecipitates were analyzed by criteria for identifying potential CD11b interactors, included: (1) the candidates were 10-fold or greater more abundant in WT BMDMs, compared to *Itgb2*^{-/-} BMDMs; (2) normalized

Total Spectra of the protein candidates exceeded 1; (3) the candidates were identified in all of three independent experiments. Criteria for identifying potential CD200R1 peptide interactors included: (1) the candidates were 10-fold or greater more abundant with CD200R1 pY286 or pY297 peptides, compared to non-phosphorylated peptides; (2) normalized Total Spectra of the protein candidates exceeded 5; (3) the candidates were identified in all of two or three independent experiments.

To study protein phosphorylation, macrophages were stimulated or not with biotinylated CD200R1 mAb OX-110 at RT for 5 min, followed by streptavidin at 37 °C for additional 1 min. After stimulation, cells were lysed using 2× TNE buffer and analyzed by protein electrophoresis. Protein tyrosine phosphorylation was detected by immunoblotting of total cell lysates with phosphotyrosine (pTyr) mAb 4G10 (Sigma-Aldrich). Immunoprecipitation of Dok-1, Dok-2 or Csk was achieved using pre-cleared cell lysates and previously reported antibodies, which were produced in the Veillette laboratory, as reported elsewhere³⁸. Normal rabbit serum (NRS) was used as control.

Expression of specific proteins was revealed by immunoblotting with antibodies directed against Dok-1, Dok-2, Shc, Csk, SHP-1, SHP-2, or SHIP-1 Ab, which were generated in the Veillette laboratory^{38,54}; Ras-GAP (B4F8; Santa Cruz Biotechnologies, Dallas, TX); or β -actin (C4, Santa Cruz Technologies).

Published sequence data

Bulk RNA sequencing data of human blood monocyte-derived macrophages were normalized using the median of ratios method, followed by downstream analysis of differential expression conducted using PyDESeq2. For the study of *CD200R1* or *SIRPA* RNA expression in human TAMs, single-cell RNA sequencing (scRNA-seq) data of TAM subtypes in multiple human cancer types were obtained from Cheng et al.³⁶ and Zhang et al.³⁷ For Cheng et al.³⁶, only data for TAM subtypes identified in at least three human cancer types were used. *CD200R1* and *SIRPA* RNA expression were depicted using violin plots. Differentially expressed genes (DEGs) in *CD200R1*-positive versus *CD200R1*-negative *CIQC*⁺ TAMs were analyzed using the Scanpy package (v1.9.6), applying a threshold of adjusted *p*-value of <0.05 and a fold-change ≥ 1.41 . Gene Ontology (GO) enrichment analysis was performed using the gseapy package (v1.1.1).

For the study of *CD200* and *CD47* RNA expression in hematopoietic malignancies and normal cells, dataset annotation and log₂-transformed gene expression data were extracted from Dufva et al.⁵⁶ UMAP coordinates were utilized, where the color gradients for *CD200* and *CD47* represent the log₂-transformed gene expression levels for each dataset. The datasets were grouped based on tumor or cell subtypes, and the mean log₂ gene expression values for *CD200* and *CD47* were computed and depicted. In the study of human melanoma, dataset annotation and log₂-transformed transcript-per-million (TPM) normalized scRNA-seq data were from Jerby-Arnon et al.⁵⁷ The t-SNE coordinates were employed to visualize the data, with color gradients representing the log₂-transformed expression levels of *CD200* and *CD47* in malignant cells, as well as *CD200R1* and *SIRPA* in immune cell populations.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the data are available within the article and its Supplementary Information. Protein mass spectrometry raw data in this study were deposited in ProteomeXchange repository PXD063538 and PXD063540. Bulk RNA sequencing data of human blood monocyte-derived macrophages were from the Gene Expression Omnibus (GEO) database [GSE195440](#) and [GSE227737](#). Single-cell RNA sequencing

(scRNA-seq) data of TAM subtypes in multiple human cancer types were obtained from Cheng et al.³⁶ ([GSE154763](#)) and Zhang et al.³⁷ ([GSE146771](#)). Single-cell RNA sequencing of human melanoma dataset were from Jerby-Arnon et al.⁵⁷ ([GSE115978](#)). All reasonable requests for data will be fulfilled by andre.veillette@ircm.qc.ca. Source data are provided with this paper.

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Author contributions

J.L., Z.W., M.-C.Z., Z.T., J.Q., D.D., and A.V. designed experiments. J.L., Z.W., X.Q., M.-C.Z., J.Q., and J.D. performed experiments or analyzed data. T.H., P.D.K., J.A.N., and Y.Y. provided reagents. All authors interpreted the results. J.L. and A.V. wrote the manuscript. All authors commented on the manuscript. A.V. and J.A.N. provided funding.

Competing interests

The authors declare no competing interests.

Additional information

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