Published OnlineFirst October 17, 2019; DOI: 10.1158/1078-0432.CCR-19-2234

CD200 Checkpoint Reversal: A Novel Approach to Immunotherapy

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ABSTRACT

Purpose: Advances in immunotherapy have revolutionized care for some patients with cancer. However, current checkpoint inhibitors are associated with significant toxicity and yield poor responses for patients with central nervous system tumors, calling into question whether cancer immunotherapy can be applied to glioblastoma multiforme. We determined that targeting the CD200 activation receptors (CD200AR) of the CD200 checkpoint with a peptide inhibitor (CD200AR-L) overcomes tumor-induced immunosuppression. We have shown the clinical efficacy of the CD200AR-L in a trial in companion dogs with spontaneous high-grade glioma. Addition of the peptide to autologous tumor lysate vaccines significantly increased the median overall survival to 12.7 months relative to tumor lysate vaccines alone, 6.36 months.

Experimental Design: This study was developed to elucidate the mechanism of the CD200ARs and develop a humanized peptide inhibitor. We developed macrophage cell lines with each of four CD200ARs knocked out to determine their binding specificity and functional response. Using proteomics, we developed humanized CD200AR-L to explore their effects on cytokine/chemokine response, dendritic cell maturation and CMV pp65 antigen response in human $CD14^+$ cells. GMP-grade peptide was further validated for activity.

Results: We demonstrated that the CD200AR-L specifically targets a CD200AR complex. Moreover, we developed and validated a humanized CD200AR-L for inducing chemokine response, stimulating immature dendritic cell differentiation and significantly enhanced an antigen-specific response, and determined that the use of the CD200AR-L downregulated the expression of CD200 inhibitory and PD-1 receptors.

Conclusions: These results support consideration of a CD200AR-L as a novel platform for immunotherapy against multiple cancers including glioblastoma multiforme.

Introduction

The discovery of immune checkpoints and their inhibition ("checkpoint blockade") is a recently developed modality for the treatment of cancer that has truly revolutionized care for some patients (1, 2). Current FDA-approved checkpoint inhibitors are mAbs that can extend survival in patients with selected solid tumors such as melanoma. However, many solid tumors respond poorly to checkpoint inhibitors. This includes glioblastoma multiforme (GBM; ref. 3), an incurable primary central nervous system (CNS) tumor with a median overall survival of 14.6 months with the current standard of care (4, 5). Combinations of inhibitors to target multiple immune checkpoint pathways have been employed in an effort to significantly enhance survival. Unfortunately, these combinations can cause severe immune-related adverse events, often leading to treatment discontinuation or morbidity and mortality (6–9).

The CD200 immune checkpoint causes suppression of the secretion of proinflammatory cytokines, including IL2 and IFNγ (10, 11), and increases production of myeloid-derived suppressor cells (12) and T regulatory cells (12–14) resulting in compromised antitumor activity. Previously, we discovered the following mechanisms employed by the CD200 protein for immunosuppression: (i) it is upregulated in GBM-associated endothelial cells creating an immunologic barrier around the tumor microenvironment (10); and (ii) it is shed from tumors (12, 15) and interacts with the CD200 inhibitory receptor (CD200R1) on immune cells both in the tumor microenvironment and draining lymph nodes (10, 15). Our research focuses on the development of a therapeutic agent that targets the CD200 immune checkpoint regulatory system, which is known to modulate an immune response through CD200R1 (10, 12).

However, in addition to the inhibitory CD200R1, there is a series of activation receptors (CD200AR2, 3, 4, and 5) in mice (16). By using specific synthetic peptide ligands (CD200AR-L) that we identified through protein sequencing and structural analyses of CD200 (10), we developed a peptide-based strategy to engage these activation receptors on immune cells (10) and demonstrated that the inhibitory effects of CD200 protein can be surmounted by selectively engaging CD200ARs (11, 17). This was accomplished using specific CD200AR-L that we identified through protein sequencing and structural analyses of the native CD200 protein (10). The ability to overpower the suppressive effects of CD200 is lost when using a scrambled CD200AR-L or CD200ARKO mice, demonstrating that these peptides mimic active sites within the CD200 protein to modulate CD200AR activity resulting in immune stimulation (10, 12).

We tested the efficacy of the CD200AR-L in companion dogs with spontaneous high-grade glioma using a canine-specific peptide (18). In this study, intradermal injections of the canine CD200AR-L prior to and during administration of autologous tumor lysate vaccines

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

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Clin Cancer Res 2020;26:232–41

doi: 10.1158/1078-0432.CCR-19-2234

Published OnlineFirst October 17, 2019; DOI: 10.1158/1078-0432.CCR-19-2234

Translational Relevance

This report evaluates the ability to modulate the CD200 immune checkpoint by employing synthetic peptides directed as ligands to its paired immune activation receptor. We previously reported the presence of CD200 in the sera and tumor vasculature of patients with glioblastoma multiforme (GBM). We have also shown that a canine CD200AR-L extends the lives of companion dogs with high-grade glioma. The data we present here show that the human CD200AR-L directed to the CD200 activation receptor on $CD14^+$ cells activates immune upregulation through induction of cytokine response and dendritic cell differentiation. In addition, hCD200AR-L downregulates the inhibitory CD200 and PD-1 receptors. These findings provide a basis to evaluate hCD200AR-L as a novel immune therapy for patients with GBM. Downregulation of PD-1 suggests that hCD200ARL may also obviate the need for PD1- and PD-L1–directed therapies for GBM and other malignancies.

significantly enhanced the efficacy of this immunotherapeutic modality, doubling the median overall survival time compared with dogs receiving tumor lysate vaccines alone (18). We found the therapeutic effect of CD200AR-L compelling enough to translate these findings into the human clinical setting. Herein, we describe the binding of CD200AR-L to specific CD200ARs on antigen-presenting cells (APC) resulting in immune activation. We also describe the development of specific human CD200AR-Ls that enhance the ability of human APCs to initiate an antigen-specific response.

Materials and Methods

Transfection

Cells from the macrophage cell line, Raw 264.7, were incubated in RPMI1640 supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C until confluent. Upon confluency, transfection was performed using the Neon electroporation system (Thermo Fisher Scientific). A total of 5×10^4 cells were harvested and incubated in 10-mL Neon Buffer R with 1 μL (1 μg/μL) of Clean-Cap Cas9 mRNA (TriLink Biotechnologies) and 1-μL (100 pmol/μL) CRISPR evolution sgRNA Synthego (Synthego) for 2 minutes. Following incubation, cells were placed in a Neo electroporator at 1,720 pulse voltage, 10 pulse width, and two pulse numbers. Two days after transfection, cells were analyzed by PCR to validate the deletion of each CD200AR.

Immunofluorescence cell-binding assay

A total of 5×10^4 macrophages were grown in a Lab-Tek II 8 chamber slide in 200-mL RPMI containing 10% calf serum and 1% penicillin/streptomycin. At approximately 70% confluency, cells were washed twice with 1× PBS, pulsed with 10 μmol/L biotinylated CD200AR-L for an hour, fixed in 4% paraformaldehyde for 20 minutes at room temperature, then incubated with streptavidin Alexa Fluor 568 conjugate (Thermo Fisher Scientific) for 1 hour, washed with 1× PBS, and stained with 1-μg DAPI. Imaging was performed using an Inverted Ti-E Deconvolution Microscope System (Nikon Instruments Inc.).

Peptide synthesis

Human peptides (P1: IVTWQKKKAVSPENM, P2: NITLEDEGCYMCLFN, P3: VTFSENHGVVIQPAY and P4: CLFNTFGFGKISGTA) were synthesized (Thermo Fisher Scientific; **Fig. 2A**). The purity of the peptides was >95% and each peptide was modified by N-terminal acetylation and C-terminal amidation to enhance their stability.

Cytokine measurements

A total of 5×10^5 human $CD14^+$ cells were isolated from peripheral blood mononuclear cells (PBMC) using anti-CD14 beads (BD Biosciences) with a typical yield of ≥70% recovery and ≥90% purity. Cells were pulsed with 2 μmol/L of each CD200AR-L, P1, 2, 3, or 4 and incubated for 48 hours. The supernatants were then analyzed by bead array for cytokine production (BD Biosciences).

Dendritic cell differentiation

$CD14^+$ cells were purified from cytomegalovirus positive (CMV^+) HLA-$A2^+$ lymphocyte packs (American Red Cross) as described above. Approximately 8×10^8 cells were cultured in polystyrene tissue culture flasks at 37°C in 5% CO_2. GM-CSF (25 ng/mL) and IL4 (40 ng/mL) were added on days 3 and 5 to derive immature dendritic cells (iDC).

Cytomegalovirus assay

iDCs (5×10^5) were pulsed with 10-μg cytomegalovirus (CMV) antigen peptide $pp65_{495–503}$ (NLVPMVATV) and cultured as described above. iDCs were washed three times and coincubated with $CD8^+$ T cells from CMV^+ donors (5×10^5). PBMCs from CMV^- donors were used as a negative control. Supernatants were collected after 48 hours of incubation and analyzed for IFNγ production by cytometric bead array (BD Biosciences).

NanoString gene expression analysis

Total RNA from $CD14^+$ cells was sent to New Zealand Genomics Limited to measure the expression of genes that are differentially expressed during inflammation (nCounter GX, NanoString Technologies). Briefly, total RNA was extracted from $CD14^+$ cells (MagJET RNA kit, Thermo Fisher Scientific) using the protocol adapted for tissue (KingFisher Duo machine, Thermo Fisher Scientific). RNA samples were then quantified (Qubit 2.0 fluorometer, Thermo Fisher Scientific) and subjected to RNA integrity analysis (2100 Bioanalyzer, Agilent Technologies). Probes for the genes encoding CD44 (NM_001001392.1), NANOG (NM_024865.2), OCT4 (NM_002701.4), STAT3 (NM_139276.2), and the housekeeping genes glucuronidase beta (GUSB; NM_000181.1), clathrin heavy chain (CLTC; NM_4859.2), and hypoxanthine phosphoribosyltransferase 1 (NM_000194.1) were designed and manufactured by NanoString Technologies.

Expression data obtained with NanoString GX were analyzed using nSolver Analysis Software 3.0 (nanostring.com/products/nSolver) using default settings and normalized to housekeeping genes. nSolver performed cluster analysis and generated heatmaps using Java Treeview Version: 1.1.6r4. Pathway analysis was performed using PathCards Pathway Unification Database (pathcards.genecards.org; ref. 19). Student *t* tests were used to determine significant differences among groups ($P < 0.05$).

Results

Murine CD200AR-L binds CD200AR2&3 and CD200AR3&4 to activate APCs

The CD200 checkpoint modulates immune responses through paired receptors; an inhibitory receptor (CD200R1; ref. 20) and several activation receptors (CD200ARs). Two CD200ARs are expressed on human immune cells and four on murine cells (CD200AR2–5;

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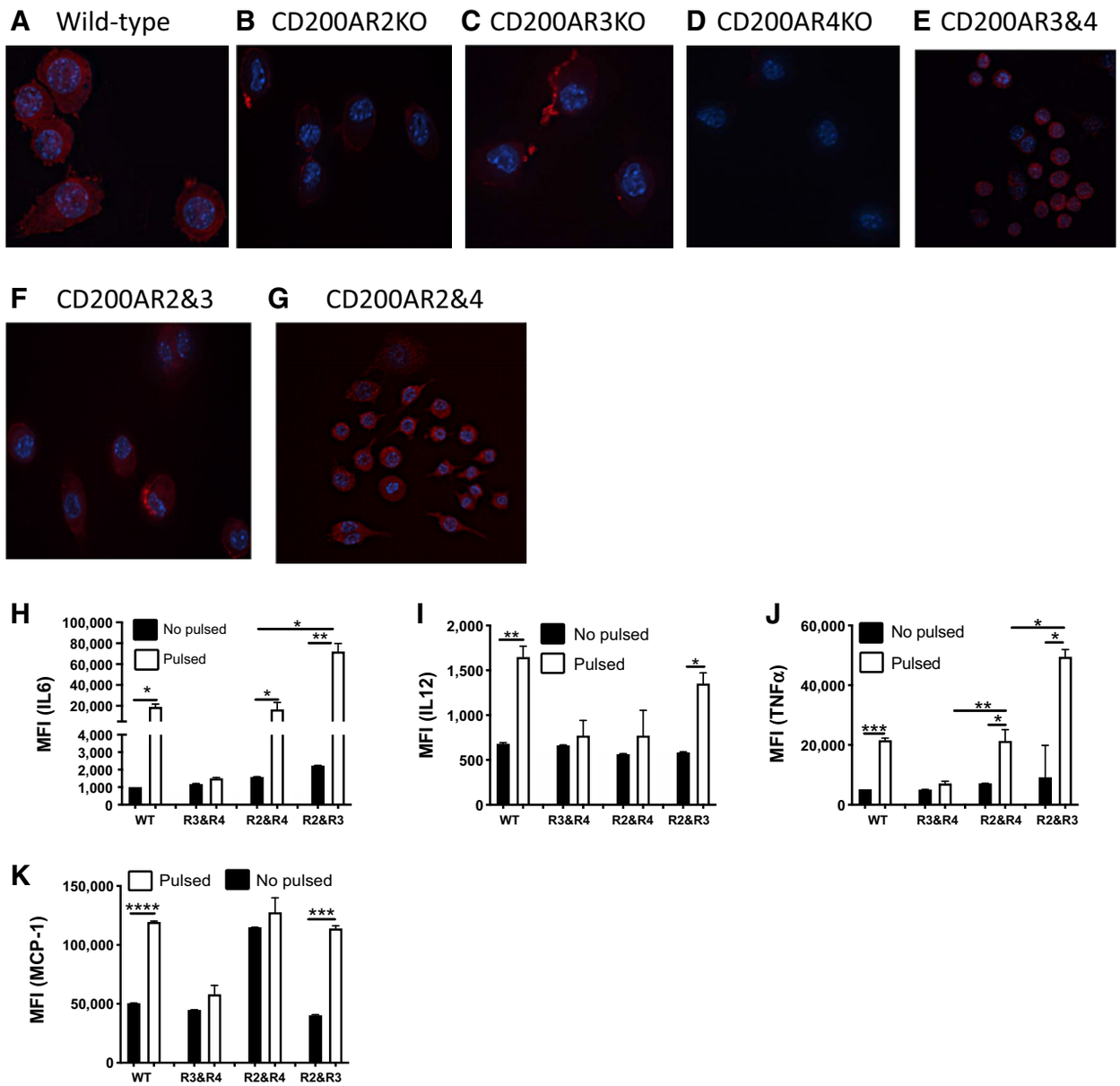

Figure 1.

CD200AR-L binds to CD200AR complexes. Wild-type (**A**), CD200AR2KO (**B**), CD200AR3KO (**C**), CD200AR4KO (**D**), CD200AR3&4 expressing (**E**), CD200AR2&3 expressing (**F**), and CD200AR2&4–expressing macrophages (**G**) were pulsed with fluorescently labeled CD200AR-L and assessed by microscopy. Cells were pulsed with the unlabeled CD200AR-L, incubated for 48 hours, and supernatants were analyzed for alterations in IL6 (**H**), IL12 (**I**), TNFα (**J**), and MCP-1 production (**K**). Nonpulsed cells with the same receptors were used as controls. Error bars, SD ($n = 3$/group; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$ by t test).

refs. 11, 21). Although interactions between CD200 and the inhibitory receptor have been characterized, the natural ligands for the activation receptors and the molecular signaling that results from ligation remain unknown. We have demonstrated that targeting CD200ARs may represent a promising approach for immunotherapy by enhancing an antiglioma response in induced murine and spontaneous canine models with the addition of CD200AR-L to autologous tumor lysate vaccination. However, we wished to establish a better understanding of the mechanisms involved in targeting CD200ARs before translation to patients with human glioblastoma. To achieve this, a murine macrophage cell line was pulsed with a fluorescently labeled murine CD200AR-L (**Fig. 1A**) to validate binding. Using CRISPR, we created macrophage cell lines expressing single or combinations of CD200ARs. To achieve this, macrophage cells first had either CD200R1 or one of the CD200ARs knocked out (Supplementary Fig. S1A). These single knockout cell lines were then used to develop dual receptor knockout cell lines, which then had further receptor knockouts to create cell lines expressing a single CD200 receptor (Supplementary Figs. S1B–S1F). Cells were validated by PCR to validate gene removal using wild-type cells as a positive control. All

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receptor knockouts were sequenced to validate gene removal (Supplementary Figs. S1A, S1G, and S1H). Cells with receptor 1, 3, and 4 knockout were validated by flow cytometry; however, there is no commercially available anti-CD200AR2 antibody. Relative to wild-type cells, reduced peptide binding was seen on CD200AR2KO and CD200AR3KO and no peptide binding was observed on CD200AR4KO cells. However, we subsequently demonstrated strong peptide binding on cells expressing CD200AR2&3, CD200AR3&4, and CD200AR2&4 (**Fig. 1A–G**).

We next wanted to determine the functional effects of ligand binding to the different CD200ARs. Cell lines were pulsed with the murine CD200AR-L and supernatants were analyzed for cytokine production. These experiments correlated with the binding experiments in that the pulsed cells expressing CD200ARs 2&3 and 2&4 had a significant increase in IL6 and TNFα production, and those expressing CD200AR2&3 had a significant increase in IL12 and MCP-1 compared with the unpulsed controls (**Fig. 1H–K**). These experiments demonstrated that the CD200AR-L targets activating receptor complexes, specifically CD200AR2&3 and CD200AR3&4, to activate APCs.

Design and identification of inhibitory peptides against CD200AR-L

Because we were now confident that we were targeting activation receptors with the peptide ligands, we sought to develop human-specific CD200AR-Ls for clinical use. Previous analysis of regions of CD200 that interact with CD200ARs revealed four regions with significant homology among the human, canine, and murine proteins (**Fig. 2A**). Four CD200AR-L peptides termed P1–4 were generated. To determine whether these peptides activated human APCs as we previously observed for the murine CD200-mimic peptides (10), purified human $CD14^+$ cells were pulsed with each of the four CD200AR-L peptides and supernatants were analyzed for immunostimulatory cytokines. We observed a significant increase in IL1β ($P = 0.0126$, $P = 0.0364$, $P = 0.0022$, $P = 0.008$) and TNFα ($P = 0.0146$, $P = 0.0007$, $P = 0.0002$, and $P = 0.0082$) in $CD14^+$ cells pulsed with P1, P2, P3, or P4, respectively, compared with unpulsed controls (**Fig. 2B** and **C**). To determine an antigen-specific response, we used a CMV model in which T cells from CMV^+ donors are primed with the CMV antigen pp65. Pulsing iDCs with CMV antigen pp65 and each of the CD200AR-L peptides elicited a significant antigen-specific response exemplified by IFNγ production ($P = 0.034$, $P = 0.033$, $P = 0.0042$ and $P = 0.020$; P1-4 respectively) compared with pulsing with pp65 alone (**Fig. 2D**).

We next conducted an alanine scanning experiment that is designed to identify the specific amino acid residues responsible for the peptide's conformation, stability, and function. Alanine is sequentially substituted for each nonalanine residue in each of the four peptides. The corresponding change in epitope activity was measured to identify the peptide that induced maximal immune stimulation of APCs. Sixty-one

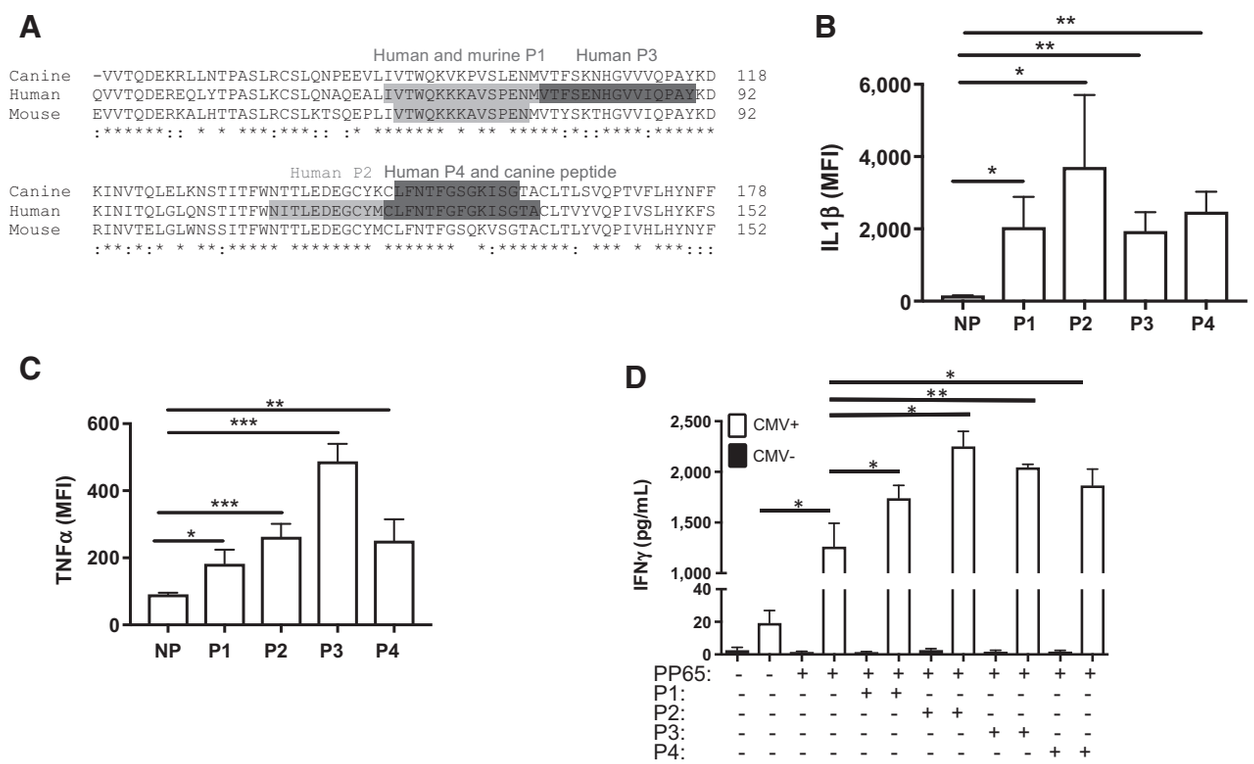

Figure 2.

Targeting CD200ARs stimulates APCs. **A,** Amino acid sequences of canine, human, and murine CD200 protein showing the homology of the various CD200 peptides. $CD14^+$ cells were pulsed with peptides 1–4 and incubated for 48 hours. Nonpulsed cells were used as a control. Supernatants were harvested and analyzed for IL1β (**B**) and TNFα (**C**). **D**, iDCs were pulsed with the CMV antigen pp65 ± peptides 1–4. Cells were washed and autologous T cells were added and incubated for another 48 hours. Supernatants were harvested and analyzed for IFNγ production. Error bars, SD ($n = 3$/group; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test between treatment groups). Data from cells of three separate healthy donors.

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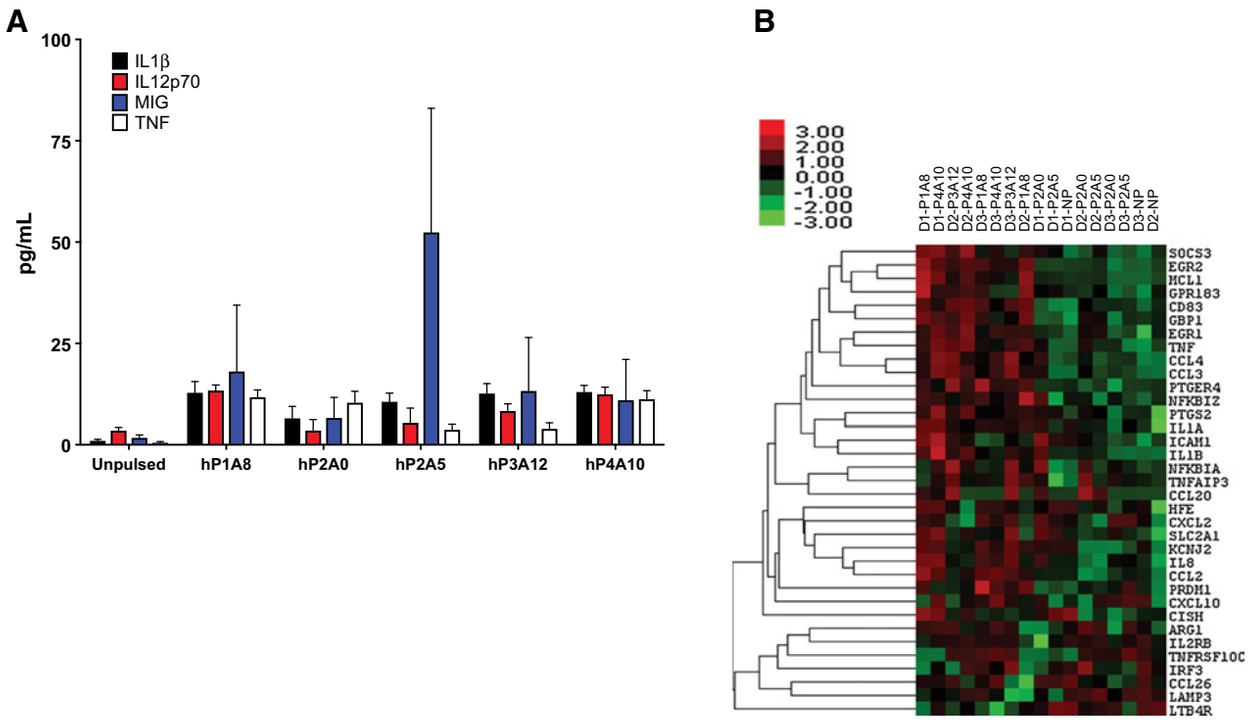

Figure 3.

Alanine substitutions enhance antigen-presenting cell stimulation. **A,** $CD14^+$ cells were pulsed with equimolar ratio of peptides, P1A8, P2A0, P2A5, P3A12, and P4A10, and incubated for 48 hours. Supernatants were harvested and analyzed for IFN1β, IL12p70, MIG, and TNFα production. **B,** $CD14^+$ cells were incubated with each peptide for 1 hour and then RNA was harvested and analyzed by NanoSight for alterations in immune-related transcripts. Pulsed cells were normalized to a nonpulsed (NP) control to derive a heatmap by nSolver using Java Treeview. Cluster analysis of the 35 genes that showed significant expression changes in one or more of the treated samples when compared to the NP controls (excludes the outlier D1-P3A12). Error bars, SD ($n = 3$ donors run in triplicate).

Table 1. Pulsed and nonpulsed CD14 cells were analyzed by IPA analysis for upregulation of the TNF pathway.

Pathways	Gene upregulation
LDL oxidation in atherogenesis	*CCL2, CCL3, ICAM1, IL1B, TNF*
Immune response, MIF-mediated glucocorticoid regulation	*ICAM1, IL8, NFKBIA, PTGS2, TNF*
EBV LMP1 signaling	*CCL20, IL8, NFKBIA, TNF*
Type II interferon signaling (IFNγ)	*CXCL10, GBP1, ICAM1, IL1B, SOCS3*
Cytokines and inflammatory response	*CXCL2, IL1A, IL1B, TNF*
Canonical NFκB pathway	*NFKBIA, TNF, TNFAIP3*
IL10 pathway	*IL1A, IL1B, SOCS3, TNF*
IL15 signaling pathways and their primary biological effects in different immune cell types	*CCL3, CCL4, TNF*
Chemokine superfamily pathway: human/mouse ligand–receptor interactions	*CCL2, CCL20, CCL3, CCL4, CXCL10, CXCL2, IL8*
Chemokine superfamily pathway: human/mouse ligand–receptor interactions	*CCL2, ICAM1, IL1B, IL8, TNF*
TNF signaling pathway	*CCL2, CCL20, CXCL10, CXCL2, ICAM1, IL1B, NFKBIA, PTGS2, SOCS3, TNF, TNFAIP3*

alanine-substituted peptides were created and purified $CD14^+$ cells were pulsed with each peptide to determine their response as measured by cytokine release. This led to the identification of five peptides, P1A8 (designation for peptide, P1, with alanine substitution of the 8th residue), P2A0, P2A5, P3A12, and P4A10 that stimulated maximal secretion of inflammatory cytokines by $CD14^+$ cells.

The effects of these peptides on a broader set of immunostimulatory cytokines, including IL12p70, MIG, and TNF, were then measured in pulsed $CD14^+$ cells (**Fig. 3A**). In all instances, significant cytokine induction was observed after treatment with each of the five peptides. To further characterize the effect of these peptides on $CD14^+$ cells, NanoString analysis was performed. Consistent with our previous observations with murine cells that CD200 exposure suppresses TNF signaling in APCs and CD200-mimic peptides reverse that effect, the human peptides, P1A8, P2A0, and P4A10, induced a notable increase in mRNA expression of cytokines associated with the TNF signaling pathway (**Table 1**). These results were recapitulated using a NanoString platform designed to detect the mRNA expression of TNF-regulated cytokines (**Fig. 3B**). The three peptides (P1A8, P2A0, and P4A10) that consistently induced potent upregulation of mRNA expression of TNF associated cytokines were-selected for subsequent analysis.

Targeting CD200ARs enhances DC differentiation

The NanoString analysis suggested that stimulation of monocytes with P1A8, P2A0, and P4A10 induced expression of many genes

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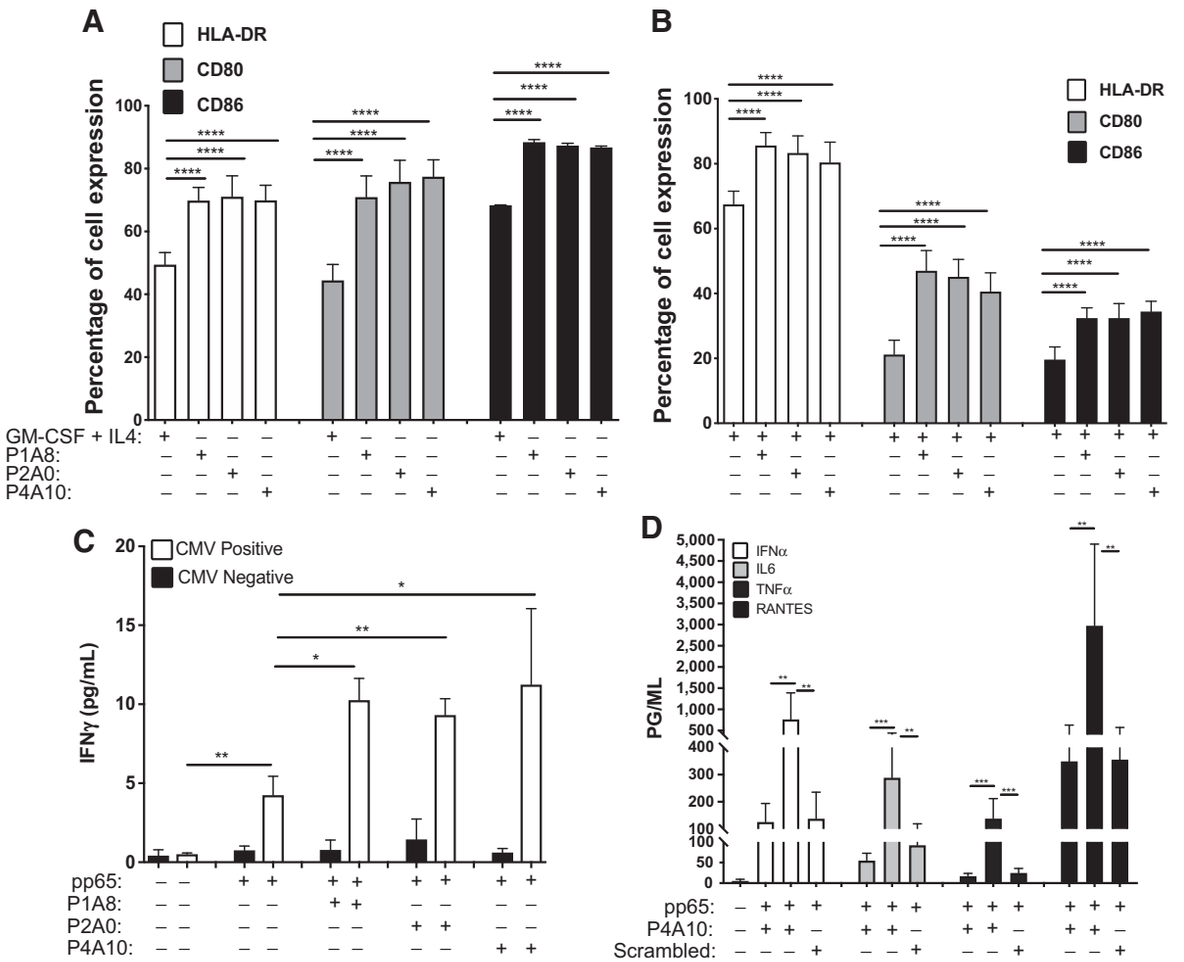

Figure 4.

Targeting CD200ARs enhances DC maturation. CD14-purified cells were pulsed with GM-CSF + IL4 or equimolar ratios of peptide P1A8, P2A0, or P4A10 (**A**) or GM-CSF + IL4 with equimolar ratios of peptide P1A8, P2A0, or P4A10 (**B**) and incubated for 48 hours. Cells were harvested and phenotyped for CD80, CD86, and HLA-DR. **C,** Immature dendritic cells were pulsed with the CMV antigen pp65 ± equimolar ratios of peptide P1A8, P2A0, or P4A10. Cells were washed and autologous T cells were added and incubated for another 48 hours. Supernatants were analyzed for IFNγ production. **D,** GM-CSF + IL4–derived iDCs were pulsed the CMV antigen pp65 ± P4A10. Cells were washed, autologous T cells were added and incubated for another 48 hours. Supernatants were analyzed for IFNγ, IL6, TNFα, and RANTE. Error bars, SD ($n = 3$ donors each run in triplicate; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test).

implicated in DC maturation (**Table 1**). To substantiate this observation, $CD14^+$ cells isolated from healthy human donors were pulsed with GM-CSF + IL4 or one of the peptides, P1A8, P2A0, or P4A10. These studies demonstrate that hCD200AR-Ls induce the differentiation of CD14 cells into iDCs. This population of cells has decreased CD14 expression and increased expression of costimulatory molecules, CD80/86 and HLA-DR, compared with cells treated with GM-CSF + IL4 ($P < 0.0001$; **Fig. 4A**). Moreover, we observed synergistic upregulation of CD80/86 and HLA-DR when $CD14^+$ cells were incubated with GM-CSF + IL4 and each of the peptides ($P < 0.0001$; **Fig. 4B**). These results show that P1A8, P2A0, and P4A10 pulsing enhances differentiation of $CD14^+$ monocytes from healthy donors into iDCs ready for antigen priming.

To assess the effects of the peptides on an antigen-specific response, GM-CSF + IL4–induced iDCs were pulsed with the CMV antigen pp65 with and without an equimolar ratio of P1A8, P2A0, or P4A10. Autologous T cells were incubated with the iDCs for 48 hours and IFNγ was measured in supernatants. pp65 exposure increased T-cell IFNγ production approximately 4-fold (**Fig. 4C**). Addition of P1A8, P2A0, or P4A10 with pp65 induced an additional two- to threefold increase in IFNγ release demonstrating that these peptides can enhance DC induction of human T-cell antigen-specific response. We tested the effect of P4A10, the most potent of the three peptides, on T-cell secretion of inflammatory cytokines after pp65 antigen presentation by dendritic cells. We observed that DCs pulsed with P4A10 induced an increase in secretion of IFNγ, IL6, TNFα, and RANTES by 5.4-, 5.6-, 16.6-, and 16.3-fold, respectively, by T cells. Pulsing with a scrambled peptide as a control failed to enhance the pp65 response (**Fig. 4D**). These results show that P4A10 enhanced the ability of APCs, specifically iDCs, to induce a T-cell–mediated immune response.

Validation of the GMP peptide

Because of issues with the stability of P4A10, the peptide that induced the most potent responses in canine, we opted to produce

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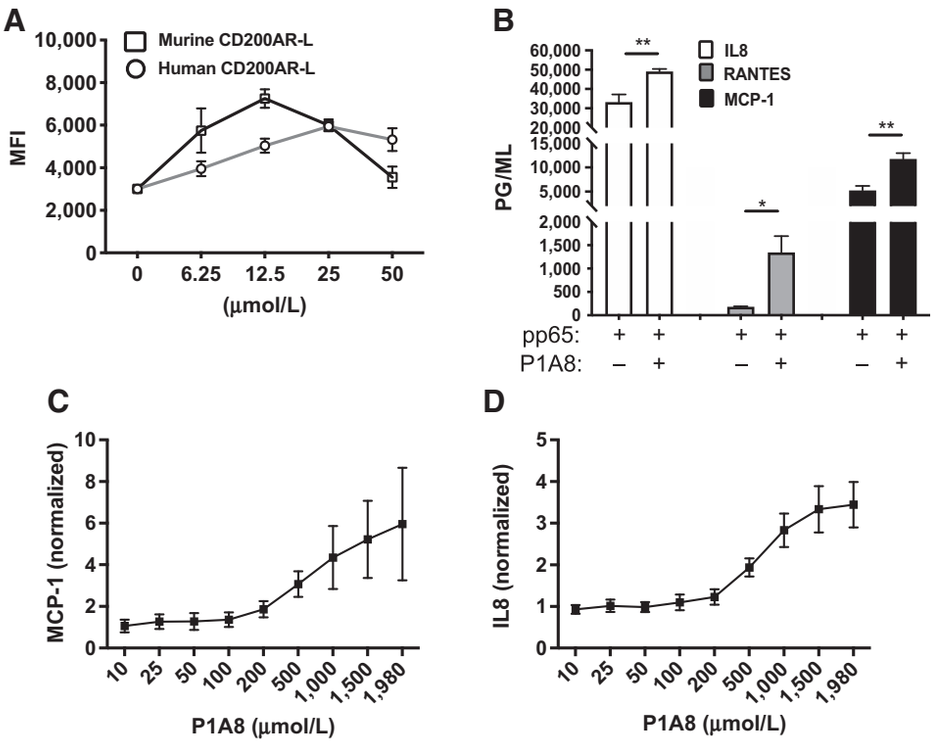

Figure 5.
Validation of the GMP peptide. **A,** HEK293 cells were pulsed with the murine P1A12 and human P1A8 fluorescently labeled CD200AR-L and analyzed for binding. **B,** GM-CSF + IL4–derived iDCs were pulsed the CMV antigen pp65 ± P1A8. Cells were washed, autologous T cells were added back, and incubated for a further 48 hours. Supernatants were analyzed for IFNγ production. $CD14^+$ cells were pulsed with different concentrations of P1A8 and analyzed for MCP-1 (**C**) and IL8 production (**D**). Cells were normalized to nonpulsed cells ($n = 3$ donors run in triplicate; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test).

P1A8 for translation into a phase I clinical trial in patients with GBM. The murine correlate of P1A8 showed the greatest efficacy in our murine survival model. To insure the GMP-grade peptide retained activity following production and formulation for vialing, we compared its binding kinetics to that of the murine peptide to HEK293 cells and showed no significant differences (**Fig. 5A**). Next, we tested the GMP peptide using a human CMV model to assess an anti-pp65 response and demonstrated that the

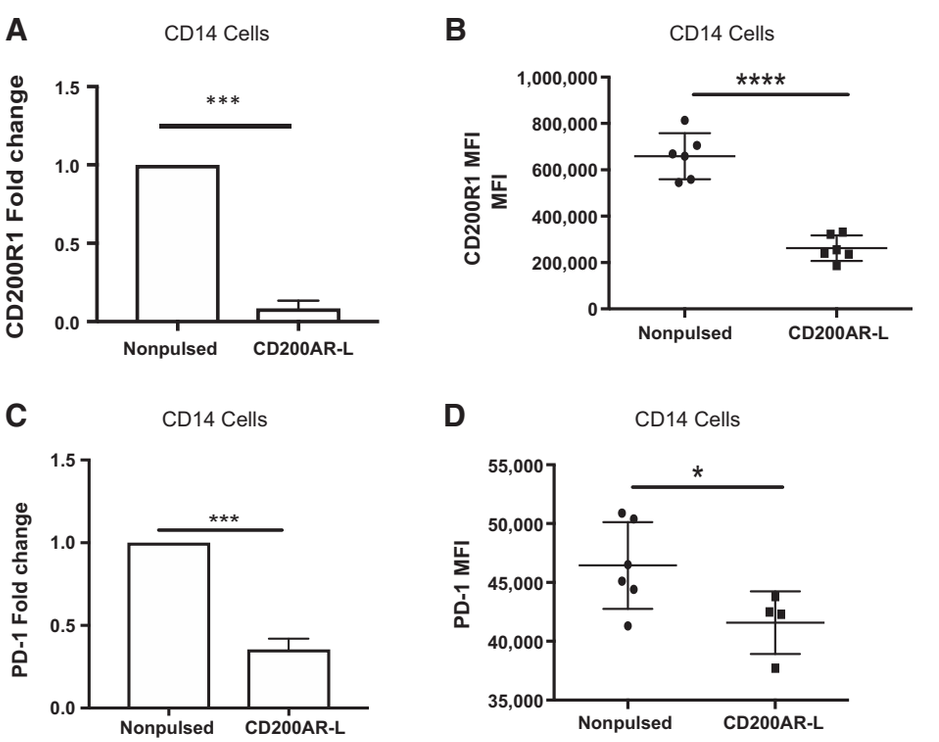

Figure 6.
Inhibitory receptor, CD200R1, is downregulated by CD200AR-L. $CD14^+$ cells were pulsed with CD200AR-L and analyzed for changes in CD200R1 transcription (**A**) and protein levels by flow cytometry (**B**). $CD14^+$ cells were pulsed with CD200AR-L and analyzed for changes in PD-1 transcription (**C**) and protein levels (**D**) by flow cytometry ($n = 3$ donors run in triplicate; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test).

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manufactured peptide maintained its ability to enhance an antigen-specific response (**Fig. 5B**). Finally, we assessed the binding kinetics of the GMP-grade peptide to CD14 cells and found a dose response of cytokine induction that peaked at approximately 1,500 μmol/L (**Fig. 5C** and **D**). The results from all of these experiments confirm maintenance of immunostimulatory activity of the GMP-grade peptide.

APCs primed with P1A8 downregulate the expression of CD200R1

We have demonstrated that targeting CD200ARs activates the immune system, in part, by overpowering the suppressive effects of CD200. To gain a better understanding of this mechanism, we pulsed human CD14 cells with GMP-grade P1A8. In **Fig. 6A** and **B**, we show that this treatment decreased the expression of the inhibitory receptor, CD200R1. Therefore, APCs in the draining lymph nodes or glioblastoma microenvironment that have been exposed to our peptide should be resistant to the effects of soluble CD200 from the tumor. This suppression was not observed in mock-treated control reactions. Interestingly, downregulation of PD-1 on APCs was also observed ($P = 0.005$; **Fig. 6C** and **D**). These results open the possibility of overcoming CNS immunosuppression through modulation occurring outside of the CNS.

Discussion

Immune checkpoint inhibitors are currently at the forefront of developing immunotherapies (22, 23). The most clinically successful have been those against CTL-associated protein 4 (CTLA4), programmed cell death 1 receptor (PD-1) and its ligand, programmed cell death ligand 1 (PD-L1). However, tumors can inhibit the antitumor immune response through multiple checkpoints hindering the use of these inhibitors as monotherapy. This is particularly critical for high-grade malignant brain tumors with a relatively low mutational burden and/or low immunogenicity. Therefore, multiple checkpoint inhibitors are often used concomitantly to enhance survival, but this practice frequently causes serious immune-related adverse events (6, 7).

Currently, no single FDA-approved checkpoint inhibitor has demonstrated significant efficacy in patients with high-grade glioma. Here, we explore an alternate paradigm of immune checkpoint inhibition at the site of autologous tumor vaccine inoculation outside of the CNS. Our previous studies provided compelling evidence that the CD200 immune checkpoint protein in tumor lysate suppresses the ability of APCs to trigger an effective antitumor immune response through locally recruited T cells (10, 12).

Several rigorous studies have provided evidence that targeting the CD200 checkpoint enhances immunotherapy (24–28). In the most advanced of these studies, a mAb against CD200, ALXN6000, was evaluated in a clinical trial (NCT00648739) initiated in 2008 for patients with relapsed or refractory B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma. The results of this study that was terminated before completion were recently published that showed some efficacy of ALXN6000 treatment in patients with B-CLL, but progressive disease in all patients with multiple myeloma (29).

We believe that there are problems associated with the use of an anti-CD200 antibody for GBM, some of which are exemplified by the Alexion results: (i) antibodies fail to cross the blood–brain barrier, which limits their efficacy in CNS tumors; (ii) multiple cells including neurons and immune cells express CD200 (30, 31); therefore, the use of an anti-CD200 antibody might cause off-target toxicity and decrease effectiveness. Alexion reported that in patients with B-CLL, 95% of the patients with B-CLL had as much as a 98% reduction of $CD200^{+}CD4^{+}$ T cells. The loss of these immune cells may create an immunocompromised condition and may be the reason that only one achieved a durable partial response while most of the patients had stable disease. Although they reported CD200 expression on B-CLL cells was greatly reduced in 67% of patients, CD200 is secreted from tumors (32) and this parameter does not correlate with tumor reduction.

We chose to develop peptide ligands to target the CD200-activating receptors on APCs. Peptides have the ability to penetrate further into tissue (33) and have higher activity per unit mass, greater stability, and reduced potential for nonspecific binding that may result in decreased toxicity (34). Despite having clearly demonstrated clinical efficacy of a synthetic peptide ligand, the mechanism that modulated an immune response through activation receptor binding was unknown. We developed three murine CD200AR-Ls that demonstrated different survival rates in a murine glioma model. The murine ligand, P1, that was predicted to bind primarily to CD200AR4 enhanced survival in our murine glioma model, whereas other ligands predicted to bind primarily to CD200AR2 and CD200AR3 enhanced survival in our murine breast tumor model, but had no efficacy in the glioma model (12). Because our interest was primarily in the role of activation receptors (CD200ARs), we focused cell lines expressing CD200AR2–4. We observed that cells expressing certain CD200AR combinations, specifically 2&3 and 3&4, responded to stimulation by the P1 ligand. In contrast, cells expressing other receptor combinations, including 1, 2, and 3, 1, 3, and 4, or 2, 3, and 4, failed to bind P1 and had no increase TNFα production, and the CD200AR2&4 cell line bound P1, but had no concomitant increase TNFα production. We suggest that ligation of the various ARs produces different immune responses, a phenomenon that is currently under investigation in our laboratory. These data lead to the hypothesis that the activation receptors (CD200ARs) function as complexes to modulate immune activation. This could explain our observation that targeting different CD200ARs induced different survival benefits in our breast carcinoma and glioma murine models (12).

Our studies provide compelling data that the presence of the CD200 protein in brain tumor lysates suppresses the capacity of local APCs to activate recruited T cells and trigger an effective antitumor immune response (10, 12). We built on this earlier observation and tested peptides in an attempt to target CD200-mediated immunosuppression and successfully reversed the immunosuppressive effect of CD200 in murine studies.

While murine brain tumor models have yielded valuable insights into the etiology of glioblastoma, the vast majority of novel therapies that showed enormous promise in these models subsequently failed in clinical studies. Recent attention has been focused on companion dogs as a translational model due to their strong anatomic and physiologic similarities to humans and the sheer number of pet dogs that are diagnosed and managed with cancer each year (35–37). Strong similarities have been shown between the canine and human genome, especially with respect to gene families associated with cancer. These combined factors suggest cancer in companion dogs as a viable model for preclinical human cancer research including brain tumors (38–40). Because of our success in the canine CD200 trial (41), the human CD200AR-L, P4A10, most analogous to the canine CD200AR-L, was initially selected for a human phase I trial. However, the charges within this peptide made it difficult to scale up for GMP production, therefore

Published OnlineFirst October 17, 2019; DOI: 10.1158/1078-0432.CCR-19-2234

we chose P1A8, analogue of the murine peptide that provided the greatest survival benefit in our murine studies. Our human *in vitro* studies demonstrated that hP1A8 enhanced DC differentiation, maturation, and cytokine production, as well as an antigen-specific T-cell response. In addition to cell activation, we also demonstrated downregulation of the inhibitory receptor, CD200R1. This is important because CD200 secreted by the tumor suppresses the ability to mount an antitumor response due to binding of CD200/CD200R1 on immune cells and CD200 is upregulated in the tumor-associated vascular endothelium (as evidenced in our earlier studies; ref. 10) limiting the ability of immune cells to extravasate into the tumor microenvironment in response to immunotherapy. We believe downregulation of CD200R1 will allow immune cells to move into the tumor microenvironment from the tumor vasculature. Moreover, the downregulation of both CD200R1 and PD-L1 should render immune cells resistant to tumor-induced suppression in the tumor microenvironment. We hypothesize that the significant survival response seen in the canine preclinical trial is due to the ability of the CD200AR-L peptide to override the suppressive effects of multiple immune checkpoints.

Disclosure of Potential Conflicts of Interest

G.E. Pluhar is an advisory board member/unpaid consultant for OX2 Therapeutics. D.A. Largaespada is an employee/paid consultant for Surrogen, Inc., reports receiving commercial research grants from Genentech, and holds ownership interest (including patents) in NeoClone Biotechnology, ImmuSoft, formerly Discovery Genomics, Inc., and Recombinetics, Inc. C.L. Moertel and M.R. Olin are employees/paid consultants for and hold ownership interest (including patents) in OX2 Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Xiong, E. Ampudia Mesias, G.E. Pluhar, M.R. Olin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Ampudia Mesias, G.E. Pluhar, S.K. Rathe, D.A. Largaespada, Y.Y. Sham, C.L. Moertel
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.L. Moertel, M.R. Olin
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Acknowledgments

We thank the following people for their technical assistance and critique of this work: Mark Sanders, Director of the University Imaging Center, for his help with the **Fig. 1**, Dr. Francisco Puerta-Martinez Andrew Janeiro with their help in the laboratory. We also acknowledge a generous gift from Robert and Corinne Ferris, Randy Shaver Research and Community Fund, Children's Cancer Research foundation, Humor to Fight the Tumor, and Love Your Melon. M.R. Olin and C.L. Moertel have financial involvement with OX2 Therapeutics involving materials discussed in the manuscript.

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Received July 8, 2019; revised August 25, 2019; accepted October 14, 2019; published first October 17, 2019.

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Published OnlineFirst October 17, 2019; DOI: 10.1158/1078-0432.CCR-19-2234

Clinical Cancer Research

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Clin Cancer Res 2020;26:232-241. Published OnlineFirst October 17, 2019.