The role of CD200 in immunity to B cell lymphoma

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ABSTRACT

CD200 is a transmembrane protein broadly expressed on a variety of cell types, which delivers immunoregulatory signals through binding to receptors (CD200Rs) expressed on monocytes/myeloid cells and T lymphocytes. Signals delivered through the CD200:CD200R axis have been shown to play an important role in the regulation of anti-tumor immunity, and overexpression of CD200 has been reported in a number of malignancies, including CLL, as well as on cancer stem cells. We investigated the effect of CD200 blockade in vitro on a generation of CTL responses against a poorly immunogenic CD200+ lymphoma cell line and fresh cells obtained from CLL patients using anti-CD200 mAb and CD200-specific siRNAs. Suppression of functional expression of CD200 augmented killing of the CD200+ cells, as well as production of the inflammatory cytokines IFN-γ and TNF-α by effector PBMCs. Killing was mediated by CD8+ cytotoxic T cells, and CD4+ T cells play an important role in CD200-mediated suppression of CTL responses. Our data suggest that CD200 blockade may represent a novel approach to clinical treatment of CLL. J. Leukoc. Biol. 88: 000–000; 2010.

Introduction

The differentiation and activation of B cells involve multiple processes that regulate gene rearrangement, proliferation, and apoptosis. When these are disrupted, malignancies often occur, including lymphomas and CLL [1]. Complete cure of both diseases with conventional chemotherapy remains extremely rare. Although T cell-mediated anti-tumor immune responses have the potential to eliminate tumor cells, CLL and lymphoma cells are inherently poorly immunogenic, rendering T cell-based immunotherapies ineffective [2]. Various techniques have been used to try to improve immunogenicity of CLL cells, including the use of IL-2 and TLR agonists [3]. Immunoregulatory molecules are known to play critical roles in regulating T cell-mediated immunotherapy, and manipulation of immunoregulatory pathways may be an important alternative method to improve the efficacy of such treatments.

One immunoregulatory molecule, CD200, has been shown to be overexpressed in a number of malignancies, including renal carcinoma, colon carcinoma, ovarian carcinoma, melanoma, AML, multiple myeloma, and CLL [4–8]. In AML, cell surface CD200 expression on malignant cells is correlated with poor prognosis [8]. CD200 has also been reported recently to be a cancer stem cell marker [9]. The regulatory function of CD200 is delivered through binding to a receptor, CD200R, expressed on cells of the myeloid lineage and T lymphocytes [10].

A regulatory function for CD200 in tumor immunity was suggested following studies that showed that infusion of a soluble form of CD200, CD200Fc, into EL4 thymoma-bearing C57B/6 mice enhanced tumor growth [11]. Anti-CD200 mAb have been reported recently to abrogate growth of CD200-transduced RAJI and Namalwa cells in NOD-SCID mice [12]. In addition, Pallasch et al. [13] demonstrated that CD200 expression on CLL cells had inhibitory effects on the proliferation of autologous effector T cells, and CD200 blockade, using a rat anti-CD200 mAb, produced a reduction in the number of CD25+CD4+forkhead box p3 regulatory T cells in vitro. In the case of CLL, no correlation has been reported between CD200 surface expression and other CLL prognostic markers, such as CD38 expression, Ig heavy-chain variable region mutational status, and Binet staging system [13], and indeed, the independent prognostic value of CD200 expression remains unknown.

In a model system that used a poorly immunogenic lymphoma cell line with constitutive CD200 levels or CD200+ primary CLL cells, we show below that blockade of CD200 by mAb or down-regulation of CD200 by specific silencers augmented anti-tumor CTL responses in vitro. CD4+ T cells from...
spleenocytes of individual CLL patients expressed CD200R, consistent with the hypothesis that CD200 overexpression on tumor cells themselves may mediate immunosuppression in CLL.

Treatments of primary CLL cells with a TLR7 agonist, alone or in combination with phorbol esters and IL-2, have been reported to enhance the immunogenicity of CLL cells and increase their killing by effector T cells [3, 14]. We report below that this treatment also reduced CD200 expression significantly on CLL cells and imply that down-regulation of CD200 expression on tumor cells may improve immunogenicity of CLL and lymphoma cells and enhance the efficacy of cell-based immunotherapies.

MATERIALS AND METHODS

Cells

hPBMCs were isolated from heparin-treated whole blood of healthy volunteer donors using Ficoll-Paque PLUS gradients (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Five independent volunteer donors were used on multiple occasions throughout the studies described. PBMCs were used in CTL assays immediately after isolation. Two human cell lines propagated from non-Hodgkin’s lymphomas were grown in suspension in AIM-V medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS (Hyclone, Logan, UT, USA) [15]. CD5+/CD19+ primary CLL cells were purified from the fresh blood of consenting CLL patients as described previously [16]. CLL spleens were obtained after splenectomy. Single cell suspensions from CLL spleens were obtained by standard protocols. All protocols were approved by Institutional Review Boards. hCD200-transfected HEK293 cells were obtained from Genetec (Quebec, Canada). Cells were grown in selection medium DMEM-F12, supplemented with 1 ug/ml G418 and 10% FBS.

Antibodies

The rat anti-hCD200 mAb 1B9 and 5A9 were described previously [17]. 3H4, which showed no immunoreactivity against cell surface CD200 (data not shown), was used as an isotype control in CTL assays. A polyclonal rabbit anti-hCD200 serum was generated following immunization of rabbits with CD200Fc and subsequent boosting. 1 ml of anti-hCD200 serum and CD200-absorbed serum samples were subsequently used in MLC assays at designated dilutions.

RT-PCR and real-time PCR

RNA was extracted from cells using TRIZOL reagent, and cDNA was obtained using OligoDT primers (Invitrogen). To detect CD200 mRNA level, the following primer pair was designed to detect ~100 bp amplicons: forward, AATACCTTTGGTTTTGGGAAGATCT; reverse, GGTTGCTTCTA-GAGAATTTGTAAGTA. Primer mixes for GAPDH and TATA box-binding protein were purchased from Qiagen and used as housekeeping genes for normalization of CD200 gene expression level. All primers were used in regular PCR and real-time PCR. For real-time PCR experiments, 50 ng cDNA was used per reaction.

siRNA transfection

Three commercial CD200 siRNA, designated CD200 siRNA #1, CD200 siRNA #4, and CD200 siRNA #6, were obtained from Qiagen. Two control siRNAs, a positive control GAPDH silencer and a negative control, were purchased from Ambion (Austin, TX, USA) for use in silencing experiments. Lymphoma cells (7.5×10^6) were transfected with 2 ug siRNA using lipofectamine2000 (Invitrogen) as a transfection reagent at a 1:6 ratio. Transfection was performed in triplicate in 12-well plates according to the manufacturer’s instructions. Cells were harvested for RNA at 48 h and for protein at 72 h after transfection. In some experiments, cells were used in CTL assays as stimulator cells 72 h after transfection.

CTL assays

PBs (1.2×10^6) were stimulated with mitomycin C-treated Ly5 or Ly2 cells at a 15:1 responder:stimulator ratio in 96-well plates. In some wells, 8 ug 1B9 rat anti-hCD200 mAb was added for functional neutralization of CD200 expression. Supernatants were harvested from each well 18 and 42 h after stimulation to assay for cytokines. After 6 days, fresh lymphoma cells were labeled overnight with [3H]Tdr at 37°C and washed three times in PBS, and 1 × 10^4 cells were added to each well in the 96-well plate. The plate was harvested for [3H]Tdr analysis at 18 h. All assays were performed in triplicate, and geometric means were used in quantitation of CTL activity. Cytotoxic killing of lymphoma cells was calculated from the [3H]Tdr remaining in cells with reference to unstimulated controls and the total counts added in the targets. All results shown were obtained from a minimum of three independent experiments. Where CD5+/CD19+ primary CLL cells were used as stimulators, ^51Cr release assays were performed to assess killing. At 7 days after stimulation, with unstimulated PBL cells set up as negative controls, ^51Cr-labeled CLL cells were added into each well as killing targets, and ^51Cr release was assessed in supernatant at 6 h after addition of ^51Cr-labeled CLL targets. CLL cells from three different patients were used as targets for the same PBL effectors in three independent experiments. In experiments where CD4+ or CD8+ T cells were depleted, depletion was performed using EasySep immunomagnetic cell
selection kits (StemCell Technologies, Vancouver, BC, Canada) as per the manufacturer’s instructions.

**ELISA**

Supernatant samples harvested from CTL assays were assayed for TNF-α, IFN-γ, TGF-β, IL-4, IL-6, IL-10, and IL-12 using ELISA kits purchased from eBioscience (San Diego, CA, USA), as per the manufacturer’s instruction. A standard curve was obtained in each assay to quantify cytokine present in the supernatant.

**Activation of CLL cells**

Purified CLL cells (2×10^6) were cultured in serum-free AIM-V medium plus 2-ME (Sigma-Aldrich, St. Louis, MO, USA) in 24-well plates at 37°C in 5% CO₂ in the presence or absence of the following immunomodulators: TLR-7 agonist Imiquimod (LKT Laboratories, St. Paul, MN, USA), PMA (Sigma-Aldrich), and hrIL-2. Imiquimod and PMA powders were reconstituted in DMSO as 1 mg/ml and 10 mg/ml stock solutions. For activation of CLL cells, Imiquimod, PMA, and IL-2 were used at a final concentration of 3 ug/ml, 30 ng/ml, and 500 U/ml, respectively. At 24 and 48 h after stimulation, cells were harvested, and cell surface expressions of CD200, CD83, and CD5 were determined by FACS. Up-regulation of CD83 expression was used as an indicator for response to stimulation.

**Statistical analyses**

P values for all experimental data were obtained using the Student’s t-test to determine the significance between sample means.

**RESULTS**

**Comparison of CD200 expression on primary CLL cells and two independent human lymphoma cell lines**

To explore the effect of CD200 expression on induction of anti-tumor immunity in vitro, we first characterized expression of CD200 using a number of independent isolates of primary CLL cells and established cell lines using PCR, Western blots, and FACS analysis.

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**TABLE 1. CLL Patient Characteristics of Individuals Used in Study**

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4 Rai stage: 0, Lymphocytosis; I, with adenopathy; II, with hepatosplenomegaly; III, with anemia; IV, with thrombocytopenia.

4 WBC: White blood cell count (×10^6 cells/ml) in the peripheral blood.

4 CVP: Cyclophosphamide/Vincristine/Prednisone; CHOP, Cyclophosphamide/Oncovin/Prednisone/Doxorubicin; C, Cyclophosphamide; P, Prednisone; F, fludarabine; R, rituximab; S, solumedrol; PC, Prednisone followed by Cyclophosphamide; SR, Solumedrol followed by Rituximab; CX2, 2 Courses of Cyclophosphamide treatment.

4 T12, Trisomy 12; na, not available.

4 Cells from indicated patients were used as stimulators in CTL assays (see Fig 6A).

4 Cells from indicated patients were used in activation experiments (see Fig 7). Cells from all patients in this table were stained for CD200 (Fig. A), with the exception of Patients I and II (g).

4 Spleens were obtained from indicated patients after splenectomy. Corresponding splenocytes were analyzed for CD200R expression (see Fig 6D).
and FACS. Although essentially all primary tumor cells \(n=25\); Table 1) analyzed in this study stained positive for CD200, as compared with the respective isotype controls, the MFI of the staining varied widely (Fig. 1A). Heterogeneity in the CD200 cell surface expression level was not correlated independently with any of the various clinical parameters of CLL analyzed, including Rai disease stage, CD38 expression, and cytogenetic status. To date, there are no data, to our knowledge, directly addressing the issue of altered CD200 cell surface expression with disease progression. In the two lymphoma cell lines studied, Ly5 cells showed CD200 expression levels comparable with that of primary CLL cells, and Ly2 cells failed to stain for CD200 (Fig. 1B). Results from FACS studies were confirmed using Western blot analyses, in which CD200 was detected as a band at approximately 48 kDa in lysates of hCD200-transfected HEK293 cells and Ly5 cells, but not Ly2 cells (Fig. 1C). CD200 expression on Ly5 cells, on the other hand, remained consistently high after prolonged in vitro passage (>20; data not shown). Ly5 and Ly2 cells were used in the functional studies described below, designed to investigate the functional consequences of the presence of CD200 expression on tumor cell-induced immunity.

### The effect of CD200 blockade in the killing of CD200\(^+\) Ly5 and CD200\(^-\) Ly2 cells

Different epitopes of hCD200 are recognized by independently derived rat anti-hCD200 mAb [17]. Among this panel of rat anti-human mAb (all IgG2a), 1B9 and 5A9 recognized the extracellular domain of CD200 (Fig. 1B), and another mAb, 3H4, failed to stain any of the CD200\(^+\) cells identified by 1B9 (data not shown). We have used 3H4 as an isotype control in the experiments discussed below.

We explored the effect of addition of 1B9, 5A9, or 3H4 in vitro on MLCs using hPBL from healthy blood donors as effector cells and mitomycin C-treated CD200\(^+\) Ly5 and CD200\(^-\) Ly2 cells as stimulators. \(^{3}\)H\(\text{TdR}\)-based cytotoxicity assays were performed.
performed 7 days after stimulation at three different E:T ratios to assess the effect of CD200 blockade on the killing of Ly5 and Ly2 cells by activated effectors. Data shown in the figures below are for E:T ratios of 10:1. Figure 2A shows pooled results from nine independent experiments using PBL stimulators from five different donors. Ly5 and Ly2 cells were poorly immunogenic when used alone, and optimal lysis was only ~6%. Addition of the 1B9 anti-CD200 mAb, but not of 5A9, produced an approximate fivefold increase in the killing of CD200+ Ly5 cells and no significant change in the killing of CD200+ Ly2 cells (data not shown). The isotype control antibody 3H4 failed to augment killing of Ly5 or Ly2 cells (Fig. 2B).

The enhanced lysis seen using 1B9 was relatively independent of the PBMC effector source and occurred even after pretreatment of tumor cells (but not PBMC) with mAb (data not shown), consistent with the primary target, CD200, expressed on the Ly5 tumor cells themselves. Moreover, the killing of Ly5 cells was abrogated following depletion of CD8+ cells, suggesting that CD8+ cytotoxic T cells are likely involved in tumor killing in this system (Fig. 2C). Interestingly, when CD4+ T cells were depleted, lysis of Ly5 cells increased threefold, even without CD200 blockade (Fig. 2C), which may be taken to reflect an intrinsic, autoregulatory role for CD4+ cell subsets.

Functional inhibition of expression of CD200 in Ly5 lymphoma cells by siRNA

As an alternate approach to modifying functional CD200 expression on tumor cells, we used synthetic siRNAs to downregulate CD200 expression. Three independent, commercial siRNAs were examined for their ability to modify CD200 expression at the mRNA (Fig. 3A) and protein level (Fig. 3B). Optimal silencing was seen using siRNAs #4 and #6 (Fig. 3). Western blotting and FACS analysis were used to monitor knockdown of CD200 at the protein level following siRNA transfection. By Western blots, incomplete silencing was observed. Similarly, cell surface level CD200 expression on Ly5 cells was reduced by >50% at 24 h after transfection of siRNA #4 and #6 by FACS (data not shown).

We compared the relative increase in induction of CTL by Ly5 cells in vitro using siRNAs or anti-CD200 mAb to decrease functional CD200 expression on tumor stimulator cells. As shown in Figure 4, 1B9 and anti-CD200 siRNAs #4 and #6 augmented induction of CTL for lymphoma cells in vitro (Fig. 4; one of three such studies). Consistent with data in Figure 2 using CD200 blockade by mAb, neither of the two siRNAs modulated the killing of Ly2 cells. These data confirm that functional inhibition of CD200 expression on tumor cells, by mAb or siRNA silencing, augments generation of anti-tumor immunity in vitro.

Augmented cytokine production in MLCs following decreased CD200 expression

In addition to exploring whether anti-CD200 or CD200 siRNAs could alter induction of CTL in vitro in MLCs with tumor cells, we asked whether these same reagents would alter cytokine production in vitro. Supernatants from PBMCs stimulated with Ly5 or Ly2 cells were collected at 18 and 42 h after stimulation, with typical data (one of four such studies) shown in Figure 5 (Fig. 5A shows TNF-α production; Fig. 5B, IFN-γ production). Consistent with the data in Figure 2, minimal induc-
tion of cytokine production occurred using Ly2 cells as stimulator, and there was no further augmentation using anti-CD200 mAb. In contrast, although Ly5 cells induced minimal cytokine production in the absence of additional manipulation, inclusion of anti-CD200 mAb or pretreatment of tumor cells with siRNAs augmented induction of TNF-α and IFN-γ. Again, these effects were not seen using control mAb (3H4) or siRNAs (Fig. 5). CD200 blockade did not affect the production level of a number of other cytokines, including IL-4, IL-6, IL10, IL-12, and TGF-β. Moreover, the changes in TNF-α and IFN-γ levels were not observed in the absence of stimulation by Ly5 cells (data not shown).

Augmented killing of primary CLL cells by CD200 blockade

Immunodeficiency is one of the clinical hallmarks of CLL. T cells from CLL patients generally show Th2 polarization and express low levels of CD80, CD86, and CD154 [18]. As CLL cells express high levels of CD200, the CD200:CD200R axis may be an important pathway involved in suppression of T cell activity by CLL cells. We thus examined the effect of CD200 blockade on the killing of primary CLL cells using 1B9 (the mAb with the most profound effect in the studies described above). Effector PBL from two different donors was stimulated

Figure 3. Silencing of CD200 expression by specific oligodeoxynucleotides. Ly5 cells were transfected with 2ug of the CD200 siRNAs #1, #4, or #6. All silencers were designed by Qiagen. A control using lipofectamine treatment but no siRNA was included. Cells were assayed at 48 h (RNA) or 72 h (protein) following transfection. (A) CD200 mRNA level as shown by real-time PCR. CD200 silencer #1 showed no effect. (B) Expression of CD200 protein was decreased after transfection with silencers #4 and #6, but not #1.

Figure 4. siRNAs for CD200 enhanced cytotoxic killing of Ly5 cells to a similar degree as that achieved using anti-CD200 mAb 1B9 (**, P<0.05, with both CD200 silencers). The negative control silencer (#1) had no effect.
targets, indicating involvement of CD8 cells prior to stimulation resulted in minimal killing of CLL targets. Interestingly, depletion of CD4 cells (derived from Donor 1) showed only low levels of killing of all CLL targets (Fig. 6B). However, regardless of the quantitative level of killing, CD200 blockade (but not control 3H4 antibody) increased killing of all CLL targets for both effector populations. As an adjunctive approach, we also investigated whether CLL serum, which we have found in independent studies to be capable of suppressing human allogeneic CTL immune responses in vitro (unpublished), lost this suppressive capacity after passage over a CD200 immunoadsorbent column. Data in Figure 6C show that CTL activity (measured in human allogeneic MLCs at Day 6) was inhibited by CLL serum but that this inhibition was attenuated following absorption of CD200 from the serum, again consistent with an important role for CD200 (in this case, in soluble form in CLL serum) in suppressing T cell-mediated immunity.

Figure 5. Modulation of cytokine production in MLCs using anti-CD200 mAb or CD200-specific siRNAs. Supernatants were harvested 18 and 42 h after stimulation and assayed at 1:5 and 1:10 dilution, respectively, for (A) TNF-α (18 and 42 h supernatant) and (B) IFN-γ (18 h supernatant). Suppression of functional CD200 expression by mAb 1B9 or the CD200-specific silencers #4 and #6 augmented production of TNF-α and IFN-γ by responder PBLs. Neither mAb nor siRNAs affected production of TNF-α or IFN-γ when PBLs were stimulated with Ly2 cells.

with mitomycin C-treated primary CLL cells from three different CLL patients (see Table 1), and killing in this case was assessed using a 51Cr release assay. Interestingly, effector cells derived from Donor 1 showed only low levels of killing of all three CLL targets (Fig. 6A; data are shown as mean±SD for killing of all three CLL targets), whereas effector cells derived from Donor 2 killed all targets to a greater degree (data not shown). However, regardless of the quantitative level of killing, CD200 blockade (but not control 3H4 antibody) increased killing of all CLL targets for both effector populations. As was observed for killing of Ly5 cells, depletion of CD8+ T cells prior to stimulation resulted in minimal killing of CLL targets, indicating involvement of CD8+ effector cells in CLL killing (Fig. 6B). Interestingly, depletion of CD4+ T cells alone was sufficient to augment killing of Ly5 targets in the absence of CD200 blockade (Fig. 2C), and augmented killing of CLL targets was seen only using CD200 blockade and depletion of CD4+ T cells (Fig. 6B). We speculate that this may reflect the involvement of different effector populations for the two target populations studied.

As CD200 induces immunoregulation following binding to a receptor CD200R, receptor expression was examined on cells harvested from the spleens of two patients who had undergone splenectomy for clinical reasons associated with disease treatment (Patients I and II; see Table 1). Greater than 90% of cells were CD19+ CD5+ CLL cells in the spleen of Patient I, whereas T cells constituted >50% of all cells from the spleen of Patient II (Fig. 6, D and E). Despite these differences in cellular constitution, >90% of CD4+ T cells in both spleen populations stained positive for CD200R, and only a minor population of CD8+ T cells (>1%) expressed CD200R (Fig. 6D). Splenic CD5+ CLL cells, on the other hand, did not show detectable levels of CD200R. A direct comparison of CD200R expression on splenic and circulating CD4+ T cells and CLL cells in the same patients could not be made, as peripheral blood from the two splenectomized patients was not available at the time of study. However, unlike CD200 (Fig. 6E), CD200R was never detected on CD5+ CLL cells in spleen (Fig. 6D) or peripheral blood (data not shown).

As an adjunctive approach, we also investigated whether CLL serum, which we have found in independent studies to be capable of suppressing human allogeneic CTL immune responses in vitro (unpublished), lost this suppressive capacity after passage over a CD200 immunoadsorbent column. Data in Figure 6C show that CTL activity (measured in human allogeneic MLCs at Day 6) was inhibited by CLL serum but that this inhibition was attenuated following absorption of CD200 from the serum, again consistent with an important role for CD200 (in this case, in soluble form in CLL serum) in suppressing T cell-mediated immunity.

Association of down-regulated CD200 expression with increased immunogenicity of CLL cells

Treatments of primary CLL cells with immunomodulators, such as TLR7 agonists, IL-2, and PKC agonists, have been shown to improve immunogenicity of CLL cells in vitro, potentially by increasing expression of costimulatory molecules and rendering them more effective targets for lymphokine-activated killer cells [3, 14, 16]. As cell surface expression of CD200 provides immunosuppressive signals that counter the effect of costimulatory molecules, we asked whether treatments designed to modulate immunogenicity of CLL cells would have a concomitant effect on CD200 expression. Primary CLL cells from five patients (see Table 1) at different stages of disease (Rai Stages II–IV) were treated with a TLR7 agonist of the imidazoquinoline family, Imiquimod, alone or in combination with hrIL-2 and PMA for 24 h, and then assessed for cell surface CD200 and CD5 expression by FACS. Data from stimulation of CLL cells from Patient 61 are shown as represented below (Fig. 7).

PMA and Imiquimod treatments reduced CD200 expression on CLL cells significantly in all patients tested, and expression of the CLL surface marker CD5 remained relatively un-
changed (Fig. 7). Expression of CD83, a costimulatory molecule and an activation marker, was also increased in response to PMA and Imiquimod, showing that the CLL cells were in “activated” states following treatment. PMA-induced CD200 down-regulation was observed in all CLL cells, and Imiquimod-induced CD200 down-regulation was observed in cells from four out of five patients (data not shown). IL-2, on the other hand, had no effect on CD200 expression and produced minimal increase in CD83 expression (Fig. 7). In agreement with previous reports, in which CLL cells were shown to exhibit a heterogeneous response to PMA and Imiquimod in the up-regulation of CD83, CD80, and CD86 expressions [3], the effect of these two stimuli on CD200 expression also varied among patients (data not shown). Reduction in CD200 expression was most pronounced by concomitant treatment of PMA, Imiquimod, and IL-2, which as reported previously [3], also

Figure 6. CD200 blockade augments killing of primary CLL cells by allogenic effector PBLs. Effector PBLs were stimulated by mitomycin C-treated primary CLL cells in the presence of rat IgG or 1B9. Killing was measured 7 days after stimulation by 51Cr release assay. (A) Killing was shown as an average of three independent experiments using three different CLL targets and one PBL effector. Unstimulated PBLs were used as negative controls. (B) Depletion of CD4⁺ T cell from MLCs further augmented killing of CLL targets using CD200 blockade, and CD4⁺ T cell depletion alone had no effect on CLL killing. Depletion of CD8⁺ T cells reduced the killing of CLL cells to levels akin to those seen with unstimulated PBLs, even in the presence of 1B9. (C) Effect of CD200 absorption on immunosuppression in hMLCs using CLL patient serum: CD200 was absorbed from CLL serum by overnight incubation of pooled CLL serum (obtained from 15 donors) with 1B9-conjugated Sepharose beads. CLL serum before (CLL) or after (absorbed) CD200 absorption was added to hMLC at the indicated dilutions; results show percent lysis of 51Cr-labeled target cells at a 30:1 E:T ratio in 6 h. CLL serum suppressed MLC reactivity in a dose-dependent manner compared with controls (P<0.05), but this inhibition was lost after absorption. (D) Expression of CD200R on cells gated on CD5, CD8, and CD4. Of the three populations, over 90% of CD4⁺ T cells stained positive for CD200R using cells from CLL spleen of both CLL patients, and neither CD8⁺ T cells nor CLL (CD5⁺) cells expressed detectable levels of CD200R. (E) Expression of CD200 on splenic CLL cells: CLL populations, as determined by the cell surface markers CD19 and CD5. CD5⁺CD19⁺ CLL cells from both CLL spleen populations express high levels of CD200.
resulted in the greatest increase in CD83 expression. The presence of IL-2 in combination with PMA and Imiquimod, although causing further augmentation of CD83 expression, had no effect on CD200 down-regulation.

**DISCUSSION**

Immunomodulatory molecules contributing to negative signaling of T cells are thought to play a pivotal role in regulating anti-tumor responses and tumor progression in human malignancies. As examples, altered expression of immunomodulatory molecules of the B7 family, B7-H1, B7-H3, and B7-H4, have been detected in lung, prostate, ovarian, and kidney carcinomas and neuroblastoma [19]. In prostate cancer and clear cell renal carcinoma, B7-H3 overexpression on tumor cells is associated with poor prognosis [20, 21]. In ovarian cancer, the serum B7-H4 level has been identified as another marker that predicts poor prognosis [22]. Functional blockade of these immunomodulatory molecules might thus provide a novel therapy for such malignancies. Indeed, blockade of CTLA4, a negative regulator of T cells, using a fully humanized antibody, is currently under development in Phase III clinical trials in patients with advanced melanoma and other malignancies [23].
In B cell malignancies, including lymphomas and CLL, the tumor cells themselves are known to be poorly immunogenic, despite the expression of high levels of MHC molecules and tumor antigens [24, 25]. A number of strategies have been investigated to develop clinically applicable methodologies to enhance the immunogenicity of CLL cells [26, 27]. For example, transduction of CLL cells with the CD40 ligand has been shown to enhance antigen-specific recognition of tumor cells by autologous T cells in vitro [27]. Various attempts have also been made to improve efficacy of vaccines targeting CLL-specific antigens [28, 29].

Given the dominant nature of immunomodulatory signals, the stimulation of costimulatory molecules on tumor cells alone may not be sufficient to overcome the poor immunogenicity of the tumor. Expression of CD200, a known immunoregulatory molecule, has been reported on CLL and lymphoma cells (ref. [10]; see also Fig. 1A). Although the CD200 cell surface expression level does not seem to correlate with other CLL clinical markers, it remains unknown whether CD200 expression levels on CLL cells vary in response to treatment or during disease progression. Our results described herein and data from other groups support the hypothesis that CD200 expression on tumor cells might be one of the contributors for the poor immunogenicity of leukemic/lymphoma cells. Blockade of functional CD200 expression would thus provide a promising approach to enhance immunogenicity of such tumor cells. In support of this hypothesis, Kretz-Rommel et al. [12] demonstrated recently that blockade of CD200 using specific humanized mAb enhanced anti-tumor responses using hPBMCs and tumor cells artificially transfected with a CD200 lentiviral vector.

A drawback to the study reported by Kretz-Rommel et al. [12] is that lentiviral transfection often produces protein expression levels not reflective of those seen physiologically. Accordingly, we have been interested in induction of tumor responses directed against primary CLL cells isolated from peripheral blood of patients, as well as a B-lymphoma cell line, Ly5, which constitutively expresses CD200 at levels parallelizing those expressed by primary CLL cells. A CD200+ cell line, Ly2, was used as a control. CTL assays using these two cell lines as targets showed that although both cell lines were poorly immunogenic, killing of CD200+ Ly5 cells and indeed, of primary CLL cells, but not CD200+ Ly2 cells, was augmented greater than fivefold by the presence of a rat anti-hCD200 mAb 1B9 when compared with an isotype control antibody, 3H4.

Although CD200 is expressed on normal B cells, and its expression is increased on T cells upon activation, the effect of the CD200 blockade was PBMC donor-independent (unpublished observations; see also ref. [30]) and appears to represent targeting of CD200 expressed on tumor cells. Antibody-mediated CD200 blockade, as a means of enhancing CTL responses, was affected by the CD200 epitopes targeted, as another CD200-specific mAb 5A9 produced much less augmentation of CTL induction than 1B9, despite equivalent staining of Ly5 cells in FACS by 5A9 and 1B9 (Fig. 1B). This is consistent with previous data indicating heterogeneity in the activity of different anti-CD200 mAb in different functional assays [17]. Thus, biochemical and functional characterization of the epitopes recognized by anti-CD200 mAb is of crucial importance in the design of CD200-specific mAb therapies.

CD200 blockade by 1B9- and CD200-specific siRNAs enhanced production of TNF-α and IFN-γ in vitro from effector cells, suggesting that CD200 blockade may affect anti-tumor immunity through other (cytokine-mediated) mechanisms [31]. The data described used two independent CD200 siRNAs (#4 and #6), both of which showed specific knockdown of CD200 at RNA and protein levels. Interestingly, CD200 knockdown by siRNA #6 resulted in higher production of TNF-α and IFN-γ, despite similar augmentation of the CTL response to Ly5 cells after transfection with both silencers (#4 and #6). This may simply reflect the difference in the effector populations responsible for activity in these two assays.

The in vitro killing of tumor cells in our CTL assays was mediated by CD8+ cytotoxic effector cells, as demonstrated by the minimal CTL response to Ly5 and primary CLL cells when CD8+ T cells were depleted from responder populations. NK cells, which have been shown to express CD200R [32], could also potentially play a role in the killing of tumor targets, particularly in assays with CLL targets, where killing was not abrogated completely after CD8 depletion (Fig. 6B). In our hands, ~30% of the PBMCs stained with anti-CD56 mAb in FACS analysis before cultures. We were unable to detect statistically significant changes in the percent of cells stained with anti-CD56 mAb following culture (immediately before assaying lytic activity), with levels 2–3% in all groups, and levels of CD4+/CD8+ cells in nondepleted (by anti-CD4/CD8) cultures were <10%. We presume the equivalent and low survival of CD56+ cells following various cell depletion strategies reflects the absence of production of mediators (cytokines) from CD4+ and/or CD8+ cells in tumor cell-stimulated cultures, which could contribute to NK survival/growth in vitro. In addition, no significant changes in CD56+ cells were seen in cultures incubated with anti-CD200 mAb (data not shown). We conclude that the differential killing activity seen following the manipulations shown in Figure 6 is best explained by our hypothesis that CD8+ cells are the primary effector population assayed.

Interestingly, the killing of Ly5 and CLL cells was affected significantly by the absence of CD4+ effectors. Although depletion of CD4+ T cells was sufficient to enhance killing of Ly5 cells (Fig. 2C), CD4 depletion augmented the killing of CLL cells, only in the presence of CD200 blockade (Fig. 6B). We interpret these data as suggestive of the involvement of CD4+ T cells in regulation of killing, directly (as a regulatory cell population itself; note, we have not independently assessed the effect of depletion of CD25+ cells in these assays) or indirectly, acting to affect the activity of other regulatory cells. The exact mechanism(s) involved remain unexplored.

CLL cell-mediated T cell defects have been well documented. Recently, the formation of immunological synapses between CLL cells and autologous T cells was shown to be impaired [33]. This impairment appears to be CLL-dependent, as incubation of CLL cells with allogenic T cells also led to failure in formation of normal immunologic synapse between CLL cells and normal T cells. The expression of activation markers on T cells was also impaired after incubation with CLL cells in a mechanism involving direct cell-cell contact as well as soluble factors secreted by CLL cells.
Further evidence for an important role of the CD200:CD200R axis in CLL was supported by the high frequency of CD200R−CD4+ T cells in the spleen of CLL patients as detected by FACS analyses (Fig. 6D). CD8+ T cells and CLL cells, on the other hand, showed no detectable level of CD200R expression, consistent with the hypothesis that the primary target for CD200-mediated, immunosuppressive signals represents CD4+ and/or other CD200R+ (but non-CD8+) cells. It remains to be determined whether CD200R−CD4+ T cells and CD200+ CLL cells exist in close proximity in vivo in CLL microenvironments. However, the observation that CD200R-expressing CD4+ T cells and CD200-expressing CLL cells are present in the same micro-environment (spleen, in this case) supports a model, in which CD200-mediated suppression of CD200R−CD4+ T cells is, in part, at least responsible for the Th2 cytokine polarization and diminished CD8+ cytotoxic T cell function observed in CLL patients. The ability of anti-CD200 to augment lysis of fresh CLL cells following CD4 depletion may suggest a role for such (anti-)CD200 therapy in CLL, alongside treatment with, e.g., flutaramine and alemtuzumab, both of which have a significant ability to kill CD4+ cells [35, 36]. A potential concern for immunotherapies targeting the CD200:CD200R pathway is autoimmunity, as this pathway has been shown to play important regulatory roles in a number of autoimmunity models in rodents, including collagen-induced arthritis and experimental allergic encephalomyelitis [37–39]. In vivo models of CLL will be needed to address such safety/efficacy questions. It also remains open to speculation whether CD200 blockade may even enhance treatments such as allogenic bone marrow transplantation, in which killing of tumor cells is mediated by allogenic T cells.

Treatment with imidazoquinolines, a family of TLR7 agonists, along with IL-2 and PKC agonists, has also been considered as a means to improve immunogenicity of CLL cells [2]. In vitro treatment of CLL cells with these immunomodulators is effective in transforming CLL cells to a dendritic cell-like phenotype with high expression of costimulatory molecules, production of inflammatory cytokines, and the ability to stimulate T cell proliferation, at least in vitro [3, 14, 16]. We found that expression of CD200 on the surface of CLL cells was down-regulated in response to Imiquimod or PMA, with an optimal decrease observed following combined use of Imiquimod and PMA. IL-2 treatment did not affect CD200 cell surface expression on CLL cells. Given that reversal of CD200-mediated suppression does not seem to require complete abrogation of CD200 cell surface expression (see Figs. 3 and 4 using siRNAs), this reduction of CD200 expression on tumor cells achieved by Imiquimod and PMA may represent a key feature of their immunomodulatory activities. As PMA and Imiquimod are global activators of multiple pathways, further investigations are required to evaluate the contribution of altered CD200 expression to the biological effects produced by these agents. However, our data provide evidence for the potential of therapies targeting the CD200:CD200R axis, in combination with treatments to enhance immunogenicity of tumor cells, as a mechanism to augment anti-tumor responses.

Whether the down-regulation of CD200 expression in response to PMA and Imiquimod is mediated through transcriptional control or mechanisms involving ectodomain cleavage by proteases is currently unknown. CD200 is a known activator of a disintegrin and metalloproteinase domain family of proteases and is responsible for the inducible shedding of a number of cytokines and chemokines, including TNF, TNFRI, IL-6R, and CX3CL1 [40–42]. It is thus possible that CD200 down-regulation following PMA stimulation involves proteolytic cleavage of cell surface CD200, and preliminary observations using in vitro studies of CLL cells support this hypothesis [43]. Such a shedding event might also contribute to the existence of a soluble, immunosuppressive form of CD200 in CLL serum (see Fig. 6C).

In sum, we have shown that CD200-mediated immunosuppression is an important mechanism used by CLL cells as a means to inhibit anti-tumor immune responses. We predict that inhibition of CD200 expression on tumor cells in general may have important clinical implications in developing novel immunotherapies.

AUTHORSHIP

Karrie K. Wong contributed to all research work, the design of studies, and writing of the manuscript. Ismat Khatri contributed to biochemistry and FACS analyses. Suchinta Shaha contributed to CLL cell preparation. David E. Spaner contributed to the design of all studies and writing of the manuscript. Reginald M. Gorczynski contributed to research, the design of all studies, and writing of the manuscript.

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