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Blockade of CD200 in the Presence or Absence of Antibody Effector Function: Implications for Anti-CD200 Therapy

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CD200 is an immunosuppressive molecule overexpressed in multiple hematologic malignancies such as B cell chronic lymphocytic leukemia, multiple myeloma, and acute myeloid leukemia. We previously demonstrated that up-regulation of CD200 on tumor cells suppresses antitumor immune responses and that antagonistic anti-human CD200 mAbs enabled human PBMC-mediated tumor growth inhibition in xenograft NOD/SCID human (hu)-mouse models. Ab variants with effector function (IgG1 constant region (G1)) or without effector function (IgG2/G4 fusion constant region (G2G4)) exhibited high antitumor activity in a human tumor xenograft model in which CD200 was expressed. In this report, we seek to select the best candidate to move forward into the clinic and begin to decipher the mechanisms of tumor cell killing by comparing anti-CD200-G1 vs anti-CD200-G2G4 in two related animal models. In a CD200-expressing xenograft NOD/SCID hu-mouse model where CD200 ligand/receptor interactions are already established before initiating treatment, we find that anti-CD200-G1 is a less effective Ab compared with anti-CD200-G2G4. Separately, in a model that evaluates the effect of the Abs on the immune cell component of the xenograft NOD/SCID hu-mouse model distinctly from the effects of binding to CD200 on tumor cells, we find that the administration of anti-CD200-G1 Abs completely abolished human PBMC-mediated tumor growth inhibition. Along with supporting in vitro studies, our data indicate that anti-CD200-G1 Abs efficiently mediate Ab-dependent cellular cytotoxicity of activated T cells, critical cells involved in immune-mediated killing. These studies suggest important implications regarding the selection of the constant region in anti-CD200 immunotherapy of cancer patients. ***The Journal of Immunology,*** **2008, 180: 699–705.**

The immunosuppressive molecule CD200 (1, 2) is up-regulated on B cells in all patients with B cell chronic lymphocytic leukemia (3), and the up-regulation of CD200 correlates with a negative prognosis in multiple myeloma (4) and acute myeloid leukemia (5). CD200 is a type 1a membrane protein related to the B7 family of costimulatory receptors with two extracellular Ig superfamily domains, a single transmembrane region, and a short cytoplasmic tail with no known signaling motifs (6). It is normally expressed on thymocytes, T and B lymphocytes, some dendritic cells, neurons, kidney glomeruli, syncytiotrophoblasts, and endothelial cells (7). We previously demonstrated that the presence of human CD200 on Namalwa tumor cells (Burkitt's lymphoma cell line) coinjected with human PBMCs (hPBMCs)[2] into NOD/SCID mice prevents hPBMCs from eradicating the cancer cells. Administration of antagonistic anti-CD200 Abs blocked tumor growth in this model (8), suggesting possible use in cancer therapy.

A number of mAbs have been successfully used in cancer therapy such as rituximab (anti-CD20) in non-Hodgkin's lymphoma (9), alemtuzumab (anti-CD52) in chronic B cell leukemia (10), trastuzumab (anti-human epidermal growth factor receptor 2) in breast cancer (11), and bevacizumab (anti-vascular endothelial growth factor) (12) and cetuximab (anti-epidermal growth factor receptor) (13) in colorectal cancer. Although these Abs have shown clinical effectiveness for many years, the critical in vivo mechanisms of action are still in question (14, 15) but most likely include direct effects on target cells via signaling mechanisms and by blocking growth factors, as well as mediating effector function through the constant region (C region) of the Ab.

Because CD200 does not have a signaling domain it appears that anti-CD200 Abs do not directly affect targeted tumor cells, and we demonstrated that the anti-CD200 mAbs, as expected, did not inhibit tumor cell proliferation or directly induce cell death (8). Therefore, antagonistic anti-CD200 Abs are expected to exert their effect by blocking immune suppression and, depending on the C region of the Ab, by potentially mediating Ab-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Our previous studies demonstrated that in an immediate treatment model where the first dose of Ab was injected at the same time as the tumor cells and effector hPBMCs, the effects of anti-CD200-IgG1 constant region (G1) compared with those of anti-CD200-IgG2/G4 fusion constant region (G2G4) were nearly indistinguishable and confirm that a blockade of CD200 immune suppression by an effectorless anti-CD200 mAb is sufficient to achieve high antitumor activity.

To gain more insight into the mechanism of action of our anti-CD200 Abs in the xenograft NOD/SCID human (hu)-mouse model and to provide more information in support of one Ab C region over the other in the context of anti-CD200 therapy in the clinic, we evaluated both anti-CD200-G1 and anti-CD200-G2G4 Abs in two additional tumor models, a delayed treatment model and a model where the tumor xenograft lacks CD200 expression. These

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[2] Abbreviations used in this paper: hPBMC, human PBMC; ADCC, Ab-dependent cellular cytotoxicity; CDC, complement dependent cytotoxicity; C region, constant region; G1, IgG1 C region; G2G4, IgG2/G4 fusion C region; hu, human; mOKT3, mouse OKT3.

studies, along with supporting in vitro data, indicate that mechanisms of cell killing provide clues that may be critical to the success of CD200 blockade in cancer. We demonstrate that a blockade of CD200 in the presence or absence of effector function has implications for cancer therapy and support the use of anti-CD200-G2G4 in the clinic.

Materials and Methods

Mice

Four- to six-week-old NOD.CB17-*Prkdc*^{scid}/J (NOD/SCID) mice were obtained from The Jackson Laboratory. Animals were housed at Perry Scientific. All procedures and protocols were approved by the Perry Scientific Institutional Animal Care and Use Committee.

Anti-CD200 mAbs

Engineering of chimeric and humanized anti-CD200 mAbs with either an IgG1 (G1) or an IgG2/G4 fusion C region (G2G4) have been described previously (8) and designated chB7-G1 and chB7-G2G4 to denote chimeric anti-CD200 Abs derived from anti-CD200 Fab B7 or hB7V2-G1 and hB7V2-G2G4 to denote humanized variants derived from anti-CD200 Fab B7. Chimeric and humanized Abs recognize the same epitope with only slight differences in affinity and show similar efficacy in all experiments where variants with the same C region were evaluated. Anti-CD200 Abs and the negative control mAb ALXN4100, recognizing anthrax toxin, were produced in serum-free medium by stable Chinese hamster ovary clones containing the appropriate Ab construct. Ab in supernatant was purified by HPLC on protein A columns. Ab concentrations were determined by OD measurements. Endotoxin levels were below the level of detection as demonstrated by the *Limulus* amebocyte lysate (LAL) test (Cambrex).

Isolation of hPBMCs

hPBMCs were obtained from normal healthy donors after informed consent by density gradient centrifugation of heparinized whole blood using the Accuspin System. Fifteen milliliters of Histopaque-1077 (Sigma-Aldrich) was added to each Accuspin tube (Sigma-Aldrich), which was then centrifuged at 700 × *g* for 2 min so that the Histopaque was allowed to pass through the frit. Thirty milliliters of whole blood was layered over the frit and the tubes were centrifuged for 15 min at 900 × *g* at room temperature with no brake. The hPBMC interface was collected and mononuclear cells were washed twice in RPMI 1640 medium or phosphate buffer with centrifugation at 200–250 × *g* for 10 min.

Transfer of cells into NOD/SCID mice

Mice were uniquely numbered with ear tags and were randomized into the required number of treatment groups based on their body weight on the day before cell injection. Four million tumor cells and 2–10 × 10^6 fresh hPBMCs in a total volume of 200 μl of RPMI 1640 with 10% FCS (RPMIc) were injected s.c. in a shaved area on the back of each mouse.

Ab dose, schedule, and administration

In immediate treatment models, one-tenth of the dose of Ab indicated was mixed with 4 × 10^6 tumor cells and 5–10 × 10^6 hPBMCs in a total volume of 200 μl and administered s.c. Animals subsequently received six doses of Ab as indicated, administered i.v. two times a week for 3 wk. In delayed treatment models, Ab was administered i.v. two times per week for 3 wk, starting 7 days after tumor cell and hPBMC injection.

Tumor volume measurements

Tumor length and width were measured using a caliper three times per week by personnel blinded to the group design. Tumor volumes were calculated as follows: (length × width × width)/2. If a second tumor occurred in a given mouse, both tumor volumes were measured and their volumes were added together. Tumor growth inhibition was calculated by 100 − ((mean tumor volume anti-CD200 Ab treated groups/mean tumor volume control mAb-treated groups) × 100).

Statistics

Tumor volume data are presented as mean ± SEM. Differences between treatment groups were determined by the Wilcoxon Rank sum test once tumor size in the control group exceeded 450 mm^3, with $p < 0.05$ considered significant. The statistical analysis was performed by T Walker Consulting.

Isolation of human CD3$^+$ T cells

hPBMCs were obtained from normal healthy donors as described above and passaged over a human T cell enrichment column (HTCC-5; R&D Systems) according to the manufacturer's instructions to obtain purified CD3$^+$ T cells. Eluted cells were washed, counted, and resuspended in complete RPMI 1640 medium (Mediatech) containing 5% heat-inactivated single donor serum, 2 mM L-glutamine (Mediatech), 10 mM HEPES (Mediatech) and 1% penicillin/streptomycin (Mediatech).

PHA activation of hPBMCs

hPBMCs from healthy human donors were cultured in 6-well plates (Falcon) at a concentration of 2 × 10^6 cells/ml in complete medium in the absence or presence of 5–10 μg/ml PHA (Sigma-Aldrich). Cells were maintained for 72 h at 37°C in a humidified incubator containing 5% CO_2. At the end of the culture period, the cells were either chromated for use as targets in ADCC or for flow cytometric analysis.

Activation of CD3$^+$ T cells with plate-bound mouse OKT3 (mOKT3)

The wells of 12-well plates were coated by overnight incubation at 4°C with 10 μg/ml mOKT3 (Ortho Pharmaceuticals) diluted in PBS. Residual Ab was removed and the plates were gently rinsed with PBS. Purified CD3$^+$ T cells, isolated as described above, were added to the plates at a final concentration of 2 × 10^6/well in RPMI 1640 containing 5% heat-inactivated single donor serum, 2 mM L-glutamine, 10 mM HEPES, and penicillin/streptomycin. Cells were maintained for 72 h at 37°C in a humidified incubator containing 5% CO_2.

ADCC assay

ADCC assays were performed using ^{51}Cr-labeled mOKT3-activated CD3$^+$ or PHA-activated hPBMC target cells and CD56$^+$ effector cells. Cells were chromated by the addition of 125 μCi of ^{51}Cr (PerkinElmer) per 10^6 cells for 2 h at 37°C. Labeled cells were harvested, washed in RPMI 1640 containing 5% heat-inactivated single donor serum, and resuspended at a final concentration of 2 × 10^5 cells/ml in the same medium. CD56$^+$ effector cells were isolated from preparations of hPBMCs by positive selection with anti-CD56-conjugated magnetic beads (Miltenyi Biotech). CD56$^+$ effector cells were resuspended at 5 × 10^6 cells/ml (for an E:T cell ratio of 50:1), 2.5 × 10^6 cells/ml (for an E:T cell ratio of 25:1), or 10^6 cells/ml (for an E:T cell ratio of 10:1) and were distributed (100 μl/well) to wells containing the target cells. Ten-fold dilutions of the indicated anti-CD200 Abs were added to the effectors and targets at final concentrations of 10, 1, 0.1, and 0.01 μg/ml. Assay controls included the following: 1) effectors and targets in the absence of Ab (0 Ab); 2) target cells in the absence of effectors (spontaneous lysis); and 3) targets incubated with 0.25% SDS (maximum release). All cell culture conditions were performed in triplicate. Cells were incubated at 37°C for 4 h in a humidified incubator containing 5% CO_2. At the end of the culture period, the plates were centrifuged to pellet the cells and 150 μl of cell supernatant was transferred to scintillation vials and counted in a gamma scintillation counter (Wallac). The results are expressed as the percentage of specific lysis according to the following formula: (mean sample cpm − mean spontaneous lysis)/(mean maximum lysis − mean spontaneous lysis) × 100.

CDC assay

PHA-activated hPBMCs were washed 3–4 times and plated in V-bottom microtiter plates at cell concentrations of 4–5 × 10^5 per well. Cells were incubated with the indicated concentrations of Abs in a total volume of 50 μl for 20 to 30 min at 4°C. After one wash, 50–100 μl of 20% C9-depleted serum (Calbiochem/EMD Biosciences) was added to each well and cells were incubated for 20–30 min at 4°C. Cells were then washed twice and incubated with 100 μl of sheep anti-human C3:FITC-conjugated Ab (MP Biomedicals) (diluted 1/200) for 30 min at 4°C. After 2–3 washes, cells were resuspended in 200–400 μl of HBSS$^+$ and transferred to FACS tubes. Five to ten thousand events from each sample were collected on a FACSCalibur flow cytometer (BD Biosciences). The percentage of C3 positive events among total cells or among gated viable cells based upon forward and side scatter profiles was calculated using CellQuest software (BD Biosciences).

Flow cytometry

hPBMCs or isolated cell fractions (10^6/ml) were incubated with 50 μl of the following Abs diluted 1/5 in FACS buffer (Dulbecco's PBS without calcium and magnesium and containing 2% FBS and 0.02% sodium azide) for 20–30 min at 4°C: FITC-conjugated anti-human CD25 (BD Biosciences);

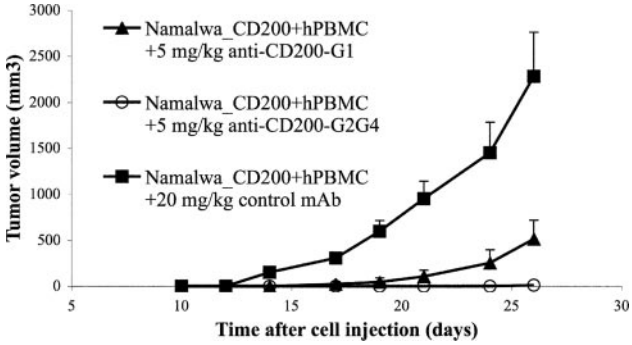

FIGURE 1. The anti-CD200-G2G4 mAb has higher antitumor activity than the anti-CD200-G1 mAb. Mice injected s.c. with 4×10^6 Namalwa_CD200 cells and 8×10^6 hPBMCs were treated i.v. twice per week starting 7 days after cell injection with anti-CD200-G1 mAb (hB7V2-G1), anti-CD200-G2G4 mAb (chB7-G2G4), or a negative control mAb. Ten of 11 mice in the control group, six of 11 mice in the anti-CD200-G1 group, and two of 11 mice in the anti-CD200-G2G4 group developed tumors by the end of the study. Error bars represent mean ± SEM; $n = 11$ per group.

PE-conjugated anti-human CD200 (Serotec); and allophycocyanin-conjugated anti-human CD3 (BD Biosciences). As a control, some cells were also incubated with FITC-conjugated murine IgG1 Ab (BD Biosciences), PE-conjugated murine IgG1 (BD Biosciences), or allophycocyanin-conjugated murine IgG1 (BD Biosciences). After washing cells twice with PBS, cells were resuspended in 200 μl of PBS and analyzed by flow cytometry using a FACSCalibur and CellQuest Software (BD Biosciences). The results are expressed as the percentage of total positive cells.

Results

Anti-tumor activity of anti-CD200-G1 vs anti-CD200-G2G4 Ab in a CD200-expressing tumor model

We previously developed a tumor model in which administration of 5–10 $\times$ 10^6 hPBMCs to NOD/SCID mice injected simultaneously with Namalwa tumor cells (Burkitt's lymphoma cell line; Ref. 16) lacking CD200 expression inhibited tumor growth compared with what was observed in mice in the absence of hPBMCs (8). In contrast, when the Namalwa tumor cells were engineered to express human CD200 and injected with hPBMCs, CD200 expressed on the surface of tumor cells prevented the hPBMCs from eradicating the cancer cells, indicating that CD200 has immunosuppressive activity in this model. The effect was specific to

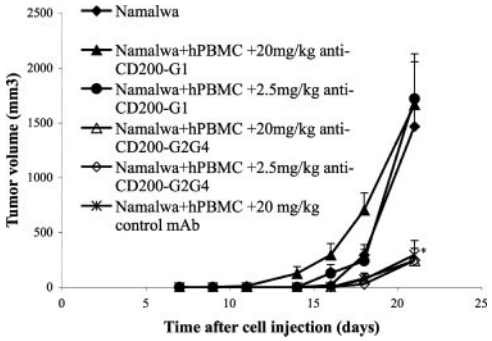

FIGURE 2. Anti-CD200-G1 mAb, but not anti-CD200-G2G4 mAb, inhibits hPBMC-mediated tumor growth inhibition of Namalwa cells lacking CD200 expression. Mice injected s.c. with 4×10^6 Namalwa cells and 8×10^6 hPBMCs were treated i.v. twice per week with anti-CD200-G1 mAb (hB7V2-G1), anti-CD200-G2G4 mAb (chB7-G2G4), or negative control mAb. Treatment was initiated with one-tenth of a dose mixed with cells at time of s.c. cell injection. Error bars represent mean ± SEM; $*$, $p < 0.05$ compared with anti-CD200-G1 mAb groups as evaluated by Student's t test ($n = 10$ per group).

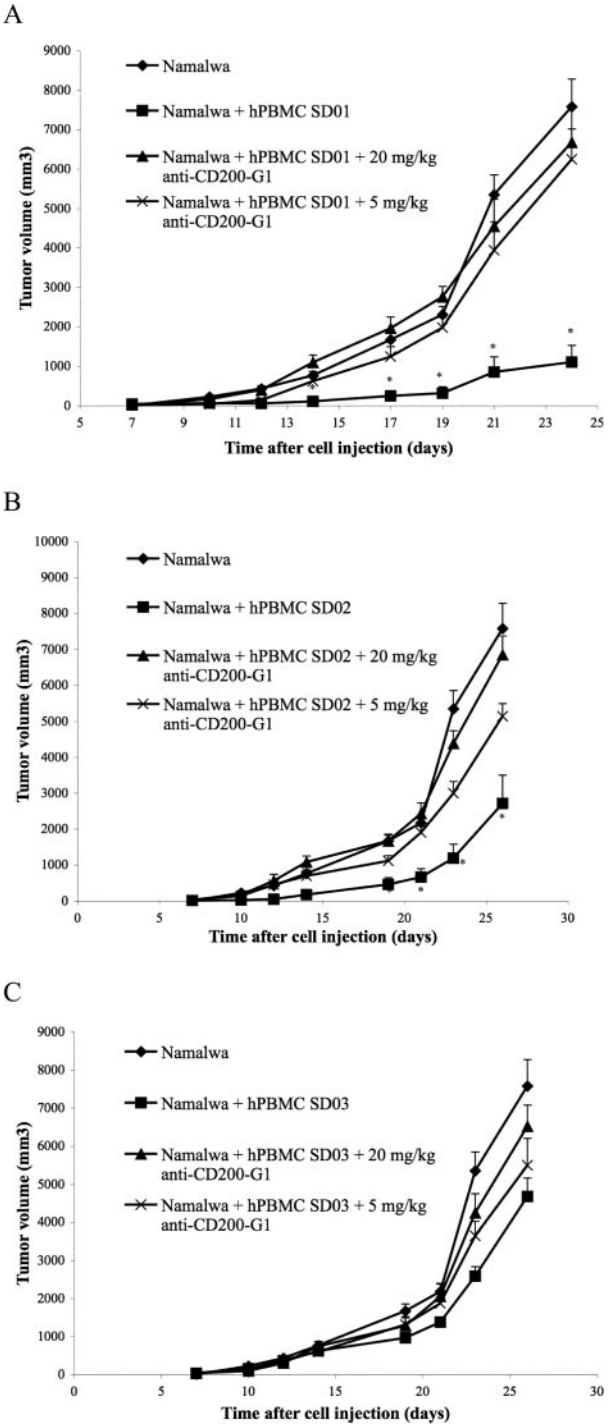

FIGURE 3. Blockade of hPBMC-mediated tumor growth inhibition of Namalwa cells lacking CD200 expression by anti-CD200-G1 mAb is not hPBMC donor dependent. Mice injected s.c. with 4×10^6 Namalwa cells and 10×10^6 hPBMCs from donor SD01 (*A*), donor SD02 (*B*) and donor SD03 (*C*) were treated i.v. twice per week with anti-CD200-G1 mAb (chB7-G2G4) or vehicle control. Treatment was initiated with one-tenth of a dose mixed with cells at time of s.c. cell injection. Error bars represent mean ± SEM; $n = 11$ per group. $*$, $p < 0.05$ compared with all other groups as evaluated by Student's t test ($n = 10$ per group).

CD200, because treatment with anti-CD200 Abs was highly effective in inhibiting the growth of Namalwa_CD200 tumors in NOD/SCID hu-mice by >90%. To differentiate tumor cell eradication via Ab blocking effects from tumor cell eradication through Ab effector function, we generated C region variants of chimeric

Table I. *CD200 expression on hPBMC populations*

Cell Type	Percentage of Cells Stained (n = No. of Donors)	Staining Intensity[a]
$CD19^+$ (B cells)	41–90 ($n = 20$)	Medium
$CD3^+$ (T cells; freshly isolated)	11–16 ($n = 10$)	Low
$CD56^+CD16^+$ (NK cells)	<3 ($n = 2$)	Low
$CD14^+$ (monocytes)	0 ($n = 4$)	NA
$CD41^+CD61^+CD62^{low}$ (resting platelets)	0 ($n = 2$)	NA
$CD41^+CD61^+CD62^{high}$ (activated platelets)	0 ($n = 2$)	NA
$CD11c^+$ (myeloid and dendritic cells)	3–9 ($n = 5$)	Low
$CD34^+$ (T cells; freshly isolated)	3–11 ($n = 3$)	Low-medium

[a] Low, Geometric mean fluorescence (GMF) < 50; medium, GMF > 50 < 100; NA, Not available.

and humanized anti-CD200 Abs. The first set of variants contains an IgG1 C region that can mediate ADCC, and the second set of variants contains an IgG2/G4 fusion C region (17) that cannot mediate ADCC or CDC. All variants tested showed high antitumor activity when treatment was started at the time of cell injection (8), demonstrating that although ADCC mechanisms may contribute, merely blocking CD200 ligand/receptor interactions is sufficient. To mimic the cancer patient setting more closely where ligand/receptor interactions are already in place before treatment initiation, we now compared the ability of the C region variants to inhibit tumor growth in the Namalwa_CD200 model with Ab treatment initiated 7 days after tumor cell and hPBMC injection (Fig. 1). Antitumor activity of both C region variants of anti-CD200 Abs was highly significant compared with the ALXN4100-treated group ($p < 0.001$ from day 15 throughout the end of the study). However, in contrast to what might be expected intuitively, treatment with a 5 mg/kg anti-CD200-G2G4 dose resulted in more substantial tumor growth inhibition (99.5% tumor growth inhibition at day 26 compared with the negative control mAb-treated control group) than treatment with a 5 mg/kg anti-CD200-G1 dose (77.56% tumor growth inhibition at day 26 compared with the negative control mAb -treated control group). The difference in antitumor activity between these two groups at the end of the study on day 26 reached statistical significance ($p < 0.03$). These results suggest a detrimental effect of effector function contributed by a G1 C region when an anti-CD200 Ab binds to its target.

Anti-CD200-G1 Ab blocks hPBMC-mediated tumor cell killing in a tumor model lacking tumor CD200 expression

One might predict that the added component of ADCC activity on tumor cells would yield a more potent Ab, but CD200 is also known to be expressed on populations of human immune cells; thus, the difference noted could be due to Ab binding to immune cells. To distinguish the effects of anti-CD200 on immune cells from the anti-CD200 blocking effects on tumor cells, we again set up the xenograft NOD/SCID hu-mouse model, but this time with Namalwa tumor cells that did not express CD200. Therefore in this model, we look specifically at the potential of anti-CD200-G1 vs anti-CD200-G2G4 Abs to compromise immune cell rejection of the tumor. As expected, based on earlier experiments (8) an injection of hPBMCs inhibited the growth of Namalwa tumor cells that lack CD200 (Fig. 2) by ~80%. Administration of 20 mg/kg or 2.5 mg/kg anti-CD200-G2G4 Ab did not affect the hPBMC-mediated tumor growth inhibition. In stark contrast, the group treated with a 20 mg/kg dose of anti-CD200-G1 Ab demonstrated a significant negative effect on hPBMC-mediated tumor growth inhibition ($p < 0.05$ at days 18 and 21). At the end of the study (day 21), tumor growth was not inhibited at all compared with the group that received no hPBMCs. Even the lower dose (2.5 mg/kg) of anti-CD200-G1 had a significant negative impact on tumor growth inhibition, with no tumor growth inhibition at the end of the study ($p < 0.03$). Median tumor volumes in the anti-CD200-G1-treated groups significantly differed from those in the groups treated with anti-CD200-G2G4 (20 mg/kg dose groups, $p < 0.002$; 2.5 mg/kg dose groups, $p < 0.006$) at the end of the study. Because we previously demonstrated that tumor cell killing in this model is mediated by T cells (8), one explanation could be the elimination of the critical effector population via anti-CD200-G1 effector mechanisms.

The effects of anti-CD200-G1 Abs are not donor specific

A second study evaluated potential hPBMC donor differences in relation to the negative effect of 5 or 20 mg/kg doses of anti-CD200-G1 on hPBMC-mediated tumor growth inhibition in the Namalwa tumor model where tumors lack CD200 expression by using three different hPBMC donors. hPBMCs from all three donors significantly ($p < 0.005$) inhibited tumor growth of Namalwa cells as expected (Fig. 3). hPBMC-mediated tumor growth inhibition varied between 38 and 85% depending on the donor in the absence of the anti-CD200 mAb, whereas in the anti-CD200-G1-treated groups human PBL-mediated tumor growth inhibition was largely abolished. Differences between the vehicle-treated hPBMCs and anti-CD200-treated hPBMC groups reached statistical significance ($p < 0.001$) in the groups that received hPBMCs from donors SD01 or SD02. Donor SD03 did not mediate strong tumor growth inhibition (only 38%), and therefore differences between mice that received vehicle or anti-CD200 mAb did not reach statistical significance ($p > 0.05$). Taken together, this study confirmed the negative effect of 5 or 20 mg/kg doses of anti-CD200-G1 Ab on hPBMC-mediated tumor growth inhibition in the Namalwa tumor model lacking CD200 expression on the tumor and suggest that this effect is not dependent on the hPBMC donor.

CD200 expression on freshly isolated hPBMCs and activated T cells

Because there is no difference in the variable region among the two Ab variants evaluated in the animal models, it appears that the IgG1 version has a detrimental effect on antitumor activity based on its ability to mediate effector function. In addition to its presence on tumor cells, CD200 is also expressed on immune cells (7). We evaluated CD200 expression on immune cells in more detail by flow cytometric analysis. In freshly isolated hPBMCs, CD200 expression was detected on the surface of the majority of B cells (Table I). Fifteen to 20% of $CD3^+$ T cells also showed CD200 staining, but of lower intensity. CD200 expression was very low or absent on $CD56^+$ NK cells, $CD11c^+$ dendritic cells, and $CD14^+$ monocytes and platelets.

Activation of T cells resulted in increased CD200 expression (Table II). PHA activation of freshly isolated T cells resulted in

Table II. *Flow cytometric analysis of CD200 expression on activated T cells*

Cell Type	Percentage of Cells Stained (n = No. of Donors)	Staining Intensity[a]
$CD3^+$ (freshly isolated)	11–16 (n = 10)	Low
$CD3^+$ (PHA activated)	100 (n = 5)	High
$CD3^+$ (OKT3 activated)	12–20 (n = 5)	Medium

[a] Low, Geometric mean fluorescence (GMF) < 50; medium, GMF > 50 < 100; high, GMF > 100.

high expression of CD200 on all T cells compared with low expression on 11–16% of T cells in freshly isolated T cells. Anti-CD3 (OKT3) treatment also resulted in up-regulation of CD200, although the percentage of $CD200^+$ cells remained the same compared with freshly isolated cells. These results indicate that CD200 is a marker of activated T cells.

ADCC activity of anti-CD200-G1

Based on elevated expression of CD200 on activated T cells, differences between the IgG1 and IgG2/G4 variants in antitumor activity in hu-SCID models might be related to the ability of the IgG1 Abs to mediate the killing of activated human T cells. We evaluated the ability of the anti-CD200-G1 and anti-CD200-G2G4 Abs to mediate ADCC in vitro using activated human $CD3^+$ cells as target cells. As shown in Fig. 4, mOKT3-activated T cells were not lysed by autologous NK cells in the absence of added Ab, while the addition of increasing concentrations of anti-CD200-G1 resulted in efficient target cell killing by ADCC. By contrast, the anti-CD200-G2G4 Ab was inactive in these assays as expected for Abs bearing the fusion IgG2/G4 C region.

We also evaluated the anti-CD200 Abs for their ability to activate complement in vitro. PHA-activated hPBMCs were used as target cells for complement deposition. The cells were sensitized by incubation with various preparations of anti-CD200 or control Ab (mOKT3) and then incubated with C9-deficient serum as a source of human complement proteins. Activation of the complement cascade was revealed by the deposition of the complement fragment C3 on the cell surface. As shown in Table III, the positive control murine anti-CD3 Ab (mOKT3) mediated efficient complement deposition on this cell population, with 67% to >85% of cells demonstrating cell surface C3 deposition following sensitization with Ab concentrations of 0.5–50 μg/ml. Neither anti-CD200-G1 nor anti-CD200-G2G4 demonstrated significant complement deposition above that observed in the absence of Ab. These data suggest that most likely the observed differences in antitumor activity between the IgG1 and IgG2/G4 versions of anti-CD200 Abs are based on the ADCC of activated T cells. Thus, Ab blockade of CD200 with an anti-CD200-G1 Ab impairs CTL activity and may have significant implications on the selection of the appropriate C region for anti-CD200 therapy in the clinic.

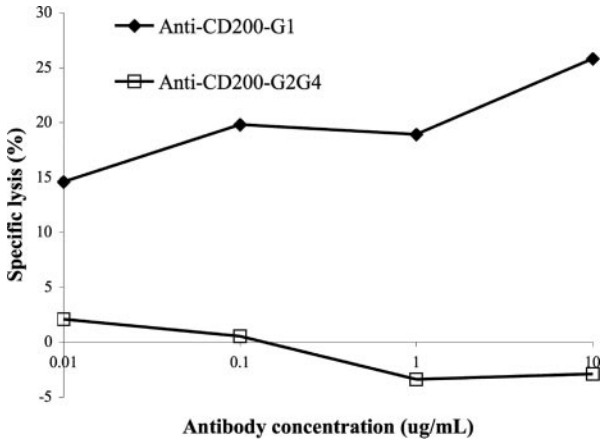

FIGURE 4. Human T cells activated through TCR signaling serve as sensitive targets for anti-CD200-G1- but not anti-CD200-G2G4-mediated ADCC. One typical experiment of five is shown evaluating anti-CD200 Abs hB7V2-G1 and hB7V2-G2G4. Background lysis in the absence of Ab amounts to 2.2%.

Table III. *Anti-CD200 antibodies do not mediate C3 deposition on PHA-activated hPBMC*[a]

Ab Concentration (μg/ml)	Fraction of Cells Reactive with Antihuman C3 following mAb Sensitization (%)		
	Anti-CD200-G2G4	Anti-CD200-G1	mOKT3
50	16.05	16.44	78.13
5.0	14.94	16.72	80.00
0.5	15.36	15.88	76.39
0	15.37	15.25	16.84

[a] Data are expressed as the percentage of viable (gated) cells positive for C3 staining, comparing humanized variants hB7V2-G2G4 with hB7V2-G1. hPBMC from a single donor were used. The experiment was performed three times using different donors with similar results.

Discussion

The present study demonstrates that the lack of effector function of therapeutic anti-CD200 mAbs is critical for optimal antitumor activity. Furthermore, our data indicate that CD200 is a marker of activated T cells and that anti-CD200-G1 mAbs appear to very effectively eliminate activated T cells.

Engineering of the effector function of Abs to optimize therapeutic efficacy has been the goal of many groups. ADCC and CDC activity can be initiated by the binding of FcγR and C1q, respectively, to IgG/Ag complexes. Various sites critical for such interactions have been identified in the Fc region of mAbs and have been altered to enhance or diminish ADCC or CDC activity (18–22). For cancer therapies, efficient effector functions have been shown to be crucial for the majority of Abs such as anti-CD20 (14, 23), anti-CD52 (10), anti-human epidermal growth factor receptor 2 (24), and anti-epidermal growth factor receptor (25). However, in other indications such as the use of OKT3 (anti-CD3) in the transplantation setting, complement activation properties are linked to the release of cytokines associated with an acute clinical syndrome (26), and Abs without effector function are preferred. In our studies, we compared an anti-CD200 mAb with an IgG1 C region to the anti-CD200 mAb variant that contains a hybrid IgG2/G4 human C region designed to minimize effector function. The human IgG2 Ab isotype is known to be a poor binder of Fc receptors (18), while the human IgG4 isotype is a poor activator of the complement cascade (27). The IgG2/G4 hybrid C region includes the CH1 region and the hinge regions of human IgG2 fused to the CH2 and CH3 regions of human IgG4 and was shown to lack the ability to bind Fc receptor and to activate complement (17, 28). Furthermore, eculizumab, an anti-C5 mAb that has been successfully used in the clinic for the treatment of paroxysmal nocturnal hemoglobinuria, contains the same IgG2/G4 fusion C region described herein. The anti-CD200-G2G4 mAbs evaluated show very high antitumor activity in models using CD200-positive tumor cells, presumably without the detrimental elimination of activated T cells. Although both the chimeric and humanized anti-CD200-G1 Abs evaluated demonstrate good antitumor activity in models using CD200-positive tumor cells, their efficacy might be

primarily based on the ADCC of CD200-positive tumor cells rather than blocking immune suppression; thus, in the absence of CD200 on tumor cells, effector cells are targeted instead. Although we cannot recover human immune cells in sufficient numbers in our NOD/SCID hu-mouse model to formally demonstrate that T cells are indeed eliminated in mice that received anti-CD200-G1 mAb, we have previously shown T cell dependence of antitumor efficacy in this model, and the current studies demonstrate that anti-CD200-G1 mAbs effectively kill activated T cells in vitro. However, we cannot rule out that the anti-CD200-G1 Ab has other detrimental effects such as, e.g., cross-linking of negative regulatory cell populations. Regardless of the underlying mechanism of the detrimental effect of the G1 Ab, in addition to demonstrating therapeutic superiority in cancer models, anti-CD200 mAbs with a G2G4 C region might also provide a better safety profile than an IgG1 variant because CD200 is expressed on normal cells such as vascular endothelial cells, neural cells, and glomerular cells (7), thus posing a potential target for ADCC.

Blockade of CD200 immune suppression by the anti-CD200-G2G4 Ab resulted in very high antitumor activity and the majority of mice were tumor-free at the end of the study, indicating that an anti-CD200-G2G4 Ab might be a very potent immunotherapeutic Ab. A number of immunomodulatory mAbs are currently in development. Some of these Abs are agonistic ligands for surface receptors involved in the activation of lymphocytes and/or APCs, whereas others are antagonists of mechanisms that normally limit the intensity of immune responses. Several mAbs of this category have been described as displaying in vivo antitumor activity in mouse models. Only the anti-CTLA-4 (CD152) mAb has entered clinical trials with very promising results (29–31), but strong preclinical effects have been described for anti-CD40 (32), anti-CD137 (clone 4-1BB) (33), anti-B7-H1-targeting Abs (34), anti-B7-H4-targeting Abs (35), and regulatory T cell-depleting Abs (36), indicating the importance of this novel class of therapeutics. This new group of antitumor agents holds promise either on their own or with the additive effects of conventional cancer therapies. Furthermore, the combination of blocking negative regulators of T cell immunity combined with vaccines may help to direct the immune response toward the tumor, enhancing clinical efficacy and perhaps reducing treatment-related adverse events (37).

Although CD200 is present at low levels on freshly isolated T cells, it is up-regulated on activated T cells. Similar increase in cell surface expression has been observed for other negative immune regulators upon T cell activation such as CTLA-4 (37). An anti-CD200-G1 mAb that does not block CD200/CD200R interaction might be more specific in eliminating activated T cells than the currently used anti-CD3 mAbs in the transplantation setting.

Taken together, CD200 mAbs have therapeutic promise, and the expression profile of CD200 provides the opportunity to clinically explore effectorless mAbs or mAbs with effector function in different indications.

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Disclosures

All authors received stock options as employees of Alexion.

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