CD52 is a 21-kDa to 28-kDa glycosyl-phosphatidylinositol anchored membrane glycoprotein (1) that is highly expressed on all normal B and T lymphocytes, monocytes, macrophages, eosinophils, natural killer cells, and dendritic cells (2–5). Hemato-
mechanisms, such as complement-dependent cytotoxicity (CDC; refs. 14–16) and/or antibody-dependent cellular cytotoxicity (ADCC; refs. 17–19) by virtue of its IgG1 Fc region. Two recent studies have shown that this agent induced enhanced apoptosis in primary CLL cells in vitro, alone or in combination with a cross-linking anti-Fc antibody, in the absence of complement or immune effector cells, through a nonclassic caspase-independent pathway (20, 21). There is also evidence to suggest that alemtuzumab may trigger caspase-dependent cell death in CLL cells (22, 23), as well as B-lymphoid cell lines (24) and Ramos cell lines (21). Contrasting with these, another study has shown that alemtuzumab alone did not induce apoptosis in serum-free medium (14).

Despite its extensive use, alemtuzumab has shown limitations in clinical use in B-cell malignancies because of its cytotoxic activity on T and other cell types, resulting in immunocompromised state associated with susceptibility to secondary infections. To overcome this, a concerted effort is being made to improve the existing therapeutic agents directed against CD52 and to delineate the mechanisms by which CD52-targeted reagents mediate cytotoxicity (25). Detailed analysis of in vivo and in vitro mechanisms to elucidate CD52-mediated killing of malignant B cells and testing of novel combination strategies have been limited due to the absence of valuable cell lines and animal models. Although many of in vitro maintained lymphoid-derived tumor cells express CD52, the level of expression is very low and the stability of the expression is unpredictable. To overcome this and to develop a model system to evaluate the CD52-directed antibody and immunoliposomal formulations containing cytotoxic agents for targeted delivery, we describe isolation and evaluation of a Raji-Burkitt’s lymphoma B-cell line that stably expresses high level of CD52 (CD52high Raji) in vitro and in vivo. The functional integrity of the expressed CD52 molecule was shown using alemtuzumab, which can promote rapid cell death of the CD52high cells through the same immunologic mechanisms, such as CDC, ADCC, and direct apoptosis active in primary CLL cells. Furthermore, CD52high Raji cells can be specifically targeted by alemtuzumab-coated immunoliposomes comparable with primary B-CLL cells, making this cell line model suitable for studying the targeted delivery of antibody-conjugated liposomal drug carriers. In vivo usefulness for the CD52high cell line for evaluating therapeutic efficacy of CD52-directed antibody is shown using a xenograft SCID mouse model of disseminated leukemia/lymphoma.

Materials and Methods

**Raji cell isolation.** Several clones of Raji Burkitt’s lymphoma B cells expressing different levels of CD52 were selected by limiting dilution. Briefly, Raji cells (American Type Culture Collection) were plated in 96-well plates at three and one cells per well. Resulting clones were tested for surface expression of CD52 by flow cytometry, and promising candidates were recloned in the same manner. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L l-glutamine, and penicillin (100 units/mL) streptomycin (100 μg/mL; Invitrogen) at 37°C, 5% CO2, and high humidity.

**CD52 isolation.** Peripheral blood was obtained from patients with B-CLL through the CLL Research Consortium from Ohio State University. Written, informed consent was obtained to procure blood samples from CLL patients according to the Declaration of Helsinki and approved by Ohio State University Institutional Review Board. Primary CD19-positive cells were isolated using Rosette-Sep (StemCell Technologies). Cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L l-glutamine, and penicillin (100 units/mL) streptomycin (100 μg/mL; Invitrogen) at 37°C, 5% CO2, and high humidity.

**Immunophenotyping studies.** Cells (1 × 10^6) were washed twice in PBS (Invitrogen) and stained with fluorochrome-labeled antibodies specific for human CD52 (Serotec), CD19, CD23, FcγRIB receptor (CD32), HLA-DR, CD50, CD20, CD37 (BD Biosciences), CD55, CD59 (Invitrogen), and CD40 (Beckman-Coulter) for 30 min at 4°C. The cells were rinsed twice in PBS and analyzed by fluorescence-activated cell sorting (FACS) on a Beckman Coulter EPICS XL (Beckman Coulter). Ten thousand events were collected under list mode and analyzed using Windows Multiple Document Interface for Flow Cytometry analysis program.

**Assessment of antibody-binding capacity by Quantum Simply Cellular Method.** Quantitative analysis of CD52 was done using the Quantum Simply Cellular Microbeads kit (Bangs Laboratories) according to manufacturer’s instructions. The kit consists of a mixture of four microsphere populations coated with different amounts of goat anti-human immunoglobulin with a precalibrated antibody-binding capacity (ABC). Briefly, the beads were stained with labeled antihuman CD52 antibody, and based on the different ABC, each type of microbeads bound a known amount of antibody. By plotting each population’s fluorescence intensity versus its assigned ABC value, a standard ABC curve was generated. Cells were stained with antihuman CD52 antibody and analyzed in a similar method by flow cytometry. QuickCal software was used to convert the mean fluorescence intensity of each cell sample to the number of molecules of antigen expressed per cell. For each sample, the ABC value of the isotypic control was subtracted from the ABC value of the positive cells (26, 27).

**p53 mutational analysis.** Mutations for the p53 gene were assessed by sequencing genomic DNA. Briefly, genomic DNA was extracted using the QiAmp kit according to the manufacturer’s instructions (Qiagen). Each p53 exon was amplified individually from genomic DNA and subjected to automated sequencing by standard methods at Ohio State University (Comprehensive Cancer Center) Sequencing Core facility. The sequence was then compared with reported p53 sequence (Genbank accession no. AB017815).

**Cytogenetic analysis.** Exponentially growing cells were treated with 0.77 μg/mL colcemid (Invitrogen) for 2 h at 37°C in the presence of 5% CO2 and then fixed using standard laboratory procedures. Briefly, cell suspension was spun down for 10 min at 1,000 rpm, the supernatant was discarded, and the cell pellet was slowly resuspended in 5 mL of 0.075 mol/L KCl. After incubation at 37°C, the cell suspension was spun down as above and cells were resuspended in freshly prepared Carnoy fixative (3:1, methanol/acetic acid) stored at 4°C for 30 min to overnight and then washed twice with fresh fixative. The cell suspension was then dropped onto precleaned, warm, wet slides. The slides were aged at 90°C for 1 h, banded with trypsin, and stained with Wright stain (Fisher Scientific). Banded metaphases were analyzed using a Zeiss Axioskop 40 microscope. For each cell line, 10 metaphases were karyotyped using an Applied Imaging Karyotyping System. Human cytogenetic nomenclature (ISCN, 1995; ref. 28) was used to describe karyotypes.

**Analysis of direct cytotoxicity.** Cells (1 × 10^6 cells/mL) were treated with alemtuzumab (anti-CD52), rituximab (anti-CD20), or trastuzumab (anti-HER2) at a concentration of 10 μg/mL. The cross-linker, goat anti-human IgG (Fc specific; Jackson ImmunoResearch Laboratories), was added to the cell suspension 5 min after adding the primary antibodies at a concentration of 50 μg/mL. In addition, a group of samples...
with no treatment was collected as media control. The apoptosis of cells at 24 and 48 h posttreatment was measured using Annexin V–FITC/propidium iodide staining followed by FACS analysis according to the supplier’s instructions (BD Biosciences). Results were expressed as percentage of total positive cells over media control [% positive cells = (% Annexin V and/or propidium iodide–positive cells of treatment cell sample) - (% Annexin V and/or propidium iodide–positive cells of media control)]. FACS analysis was done using EPICS XL cytometer (Beckman-Coulter).

**Cytotoxicity assay**

- For CDC assay, Raji cell clones at 10⁶ cells/mL were suspended in media alone, media with 30% human serum, or media with antibodies (10⁻⁴ g/mL). Cells were incubated with media alone or in the presence of various antibodies (10 µg/mL) at 37°C for 30 min. Unbound antibody was washed off, and the cells were plated at 1 x 10⁶ cells per well. Effector cells (peripheral blood mononuclear cells from healthy donors) were then added to the plates at indicated effector-to-target ratios. After 4-h incubation, supernatants were removed and counted in a Gamma counter. The percentage of specific cell lysis was determined by % lysis = 100 × (ER - SR)/(MR - SR). ER, SR, and MR represent experimental, spontaneous, and maximum release respectively. Data were normalized to the media control.

**Preparation of immunoliposome**

- 3'-[N-[(N,N'-Dimethylamino-ethane)-carbamoyl]-cholesterol and distearoyl phosphatidylethanolamine (DSPE)–polyethylene glycol (PEG; molecular weight, 2,000 Da)–maleimide were purchased from Avanti Polar Lipids, Inc. Methoxy-DSPE-PEG and Egg phosphatidylcholine were obtained from Lipoic. 2-Iminothiolane (Traut’s reagent), 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent), and other chemicals were purchased from Sigma Chemical Co. A carboxyfluorescein (FAM)–terminus modified ODN (6) FAM-TAC CGC GTG CGA CCC TCT) was custom synthesized by Alpha DNA, Inc. An ethanol dilution method was modified and used to prepare the FAM-ODN loaded immunoliposomes (29). Briefly, protamine sulfate in citrate acid (20 mmol/L, pH 4) was mixed with lipids composed of (3'-[N-[(N,N'-Dimethylaminoethanato)-carbamoyl]-cholesterol/Egg phosphatidylcholine/PEG-DSPE molar ratio = 33.5:65:1.5) at lipids/protamine mass ratio of 12.5:0.3, followed by addition of oligonucleotide in citrate acid (20 mmol/L, pH 4) at oligonucleotide/lipid/protamine weight ratio of 1:1.25:0.3. The complexes were then dialyzed against citrate acid (20 mmol/L, pH 4) for 1 h and then further dialyzed against HBS buffer [145 mmol/L NaCl, 20 mmol/L HEPES (pH 7.4)] overnight, using a DispoDialyzer (Spectrum Laboratories) with a molecular weight cutoff of 10,000 Da. Liposome size distribution was analyzed on a NICHROM Particle Sizer Model 370 (Particle Sizing Systems). Volume-weighted analysis showed particle size of ~54 nm. A post-insertion method was adopted to incorporate antibody ligands into preformed liposomes containing FAM-ODN. In this method, alemtuzumab (anti-CD52) was reacted with 20× Traut’s reagent (2 h, room temperature) to yield thioldiated antibody (anti-CD52-SH). The anti-CD52-SH was then reacted to micelles of maleimide–PEG-DSPE at a molar ratio of 1:10 and then incubated with FAM-ODN–loaded liposomes for 1 h at 37°C. Targeted liposomes with alemtuzumab–PEG-DSPE–to–lipid ratios of 1:500 (0.2 mol%) was prepared. Nontargeted control liposomes were prepared by coupling the isotype control antibody trastuzumab to the liposomes using the same method.

**Quantitative reverse transcription–PCR assay**

- RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s directions. DNase treated, and converted to cDNA with SuperScript First-Strand Synthesis System for reverse transcription–PCR (Invitrogen). Real-time PCR was performed using the LC FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics) in a Roche Lightcycler. Each sample was assayed in triplicate, and the experiments were repeated twice. The housekeeping gene TATA-binding protein was used for normalization purposes due to its lowest variability among different types of lymphoid cells (30). The following primer sets were used: CD52 (121 bp) 5'-CACGCTCTCCGTTATCCTGACG-3', 5'-GCCCGAAGAAAGGAAA-3' and TATA-binding protein (170 bp) 5'-AAGGACCACAGAGTGGAAGAC-3', 5'-GGTTGGTTGGTGACGAC-3'. Reaction conditions were 95°C × 10 min, 40 cycles of 95°C × 30 s, 64°C × 30 s, 72°C × 1 min. The amplicon size was confirmed by 1% agarose gel electrophoresis. Real-time PCR was run in parallel with both CD52 and TATA-binding protein primers to normalize data to the housekeeping gene. The expression of CD52 relative to the housekeeping gene TATA-binding protein was calculated by plotting the Ct (cycle number), and the average relative expression for each group was determined using the comparative method (2⁻∆∆Ct; ref. 31).

**Development of a disseminated leukemia-lymphoma model using Raji cell clones**

- Female CB17 SCID mice (Taconic Farm), 4 to 6 weeks of age, were housed in pathogen-free, isolated cages. Raji cell clones frozen in cryovials (1 x 10⁶ cells per vial) and stored in liquid nitrogen (-180°C) were thawed 10 days before the in vivo engraftment. To ensure the consistency of engraftment, cells were examined for the viability by Annexin V–FITC/propidium iodide staining followed by FACS analysis. CD52/CD19/CD20 expression, and in vitro sensitivity to antibody treatments on the same day of inoculation. Only cells with >90% viability (as evidenced by Annexin V+/propidium iodide) were used for injection. Cells were resuspended in 0.9% sodium chloride for injection (USP, Hospira, Inc.) at a density of 1 x 10⁶ cells/mL at room temperature, and 200 µL (2 x 10⁵ cells) were inoculated through tail vein using a mouse tail illuminator (Braintree Scientific). Tissue samples obtained from tumor-bearing SCID mice that showed early signs of paralysis were submitted to Ohio State University Pathology Core Facility for histologic analysis. The presence of disease was confirmed by the presence of human leukemic cells in the tissue sections. Bone marrow
cells (1 × 10^6/mL) flushed from femurs with cold PBS within 0 to 3 days after hind-limb paralysis were stained with PE-labeled anti-human CD52 and FITC-labeled anti-CD19 to confirm the existence of human leukemia cells. Antibody treatment was started 3 days postinoculation of target Raji cells. Alemtuzumab dissolved in PBS (1 mg/mL) was injected i.v. via tail vein and maintained every other day schedule for 2 weeks (5 mg/kg/injection, seven injections each mouse). Placebo (saline) and isotype control (trastuzumab) were given at the same schedule and dose. Animals were monitored daily for signs of illness and sacrificed immediately if hind-limb paralysis, respiratory distress, or 30% body weight loss was noted. Body weight was measured once every week. Survival time (paralysis time) was used as primary end point for evaluation.

All animal experiments were carried out under protocols approved by Ohio State University Institutional Laboratory Animal Care and Use Committee.

Statistical analysis of data. All analyses were done by statisticians in Center for Biostatistics, Ohio State University. Mixed effects models were fitted to the cytotoxicity data. In all cases, the primary hypothesis tests were for interactions between cell type and conditions. Random effects associated with the interactions were always included in the models to ensure that error was not underestimated.

Kaplan-Meier estimates of the survival function for both treatments (alemtuzumab versus trastuzumab) and engraftments (CD52high Raji clone 48, XY, der(4)t(1;4)(p36.1;q35), +7, 72 CCC) were plotted, and the median survival times for (alemtuzumab versus trastuzumab) and engraftments (CD52high Raji clone 99(36) 100(237) 99(308) 99(205) 93(89) 36(666) 100(520) 100(48) 100(347) 100(105) 97(11) 97(13)) are reported in Table 1. Phenotypically, these lines seem to be similar to the parental Raji B-cell line expressing comparable high level of human leukocyte antigens HLA-DR, CD19, CD20, CD37, CD40, and CD32. Compared with the parental and CD52low clones, the CD52high clone expresses higher levels of CD52 (227,538 versus 21,280 ABC) in higher percentage of cells (99% versus 10%). The CD52 mRNA level, as assessed by real-time reverse transcription – PCR analysis of RNA, indicated a 1.8-fold increase in CD52 mRNA in CD52high clone compared with CD52low clone (data not shown). In addition, the levels of expression of other glycosyl-phosphatidylinositol–linked proteins, such as CD55 and CD59, showed variation between clones in a manner directly associated to the expression of CD52 (99 ± 1%, 99 ± 1%). Unlike other B-cell lines, this pattern of surface antigen expression remained stable with continuous cell culture over several months (data not shown).

Molecular cytogenetic characterization of the isolated clones were done to confirm the lineage connection to Raji Burkitt’s lymphoma as well as to exclude possible contamination by other cell lines. The chromosomal rearrangements of the cell lines are shown in Table 2.

The Raji genome is relatively stable in these sublines, which all share three of the structural aberrations previously described, including the t(8;14) translocation, typical in

### Table 1. Surface immunostaining of Raji cell clones

<table>
<thead>
<tr>
<th>Raji cell clone</th>
<th>%CD52 (MFI)</th>
<th>%CD20 (MFI)</th>
<th>%CD55 (MFI)</th>
<th>%CD59 (MFI)</th>
<th>%CD32 (MFI)</th>
<th>%CD40 (MFI)</th>
<th>%CD19 (MFI)</th>
<th>%CD37 (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental Raji cell line</td>
<td>43 (4)</td>
<td>100 (161)</td>
<td>20 (92)</td>
<td>29 (64)</td>
<td>2 (30)</td>
<td>85 (952)</td>
<td>100 (945)</td>
<td>100 (39)</td>
</tr>
<tr>
<td>CD52low Raji clone</td>
<td>10 (2)</td>
<td>100 (300)</td>
<td>24 (163)</td>
<td>23 (117)</td>
<td>2 (34)</td>
<td>41 (590)</td>
<td>100 (520)</td>
<td>100 (48)</td>
</tr>
<tr>
<td>CD52high Raji clone</td>
<td>99 (36)</td>
<td>100 (237)</td>
<td>99 (308)</td>
<td>99 (205)</td>
<td>93 (89)</td>
<td>36 (666)</td>
<td>100 (480)</td>
<td>100 (48)</td>
</tr>
</tbody>
</table>

Abbreviation: MFI, mean fluorescence intensity.

### Table 2. Genetic characterization of Raji cell clones

<table>
<thead>
<tr>
<th>Raji cell clone</th>
<th>Chromosomal rearrangement</th>
<th>p53 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>47, XY, der(1)t(1;6)(p36.3;q13)ins(6;?)(q13;?), der(4)t(4;1)(q35;p36.11), +7, 72 CCC–CGC</td>
<td>Pro–Arg</td>
</tr>
<tr>
<td>CD52low Raji clone</td>
<td>48, XY, der(4)t(1;4)(p36.1;q35), ins(6;?)(q13;?), +7, 72 CCC–CGC</td>
<td>Pro–Arg</td>
</tr>
<tr>
<td>CD52high Raji clone</td>
<td>49, XY, der(4)t(1;4)(p36.1;q35), ins(6;?)(q13;?), +7, 72 CCC–CGC</td>
<td>Pro–Arg</td>
</tr>
</tbody>
</table>
Burkitt’s leukemia/lymphoma, and one numerical chromosomal aberration (+7; ref. 32). In addition, consistent with the previous report, we were able to identify in all the clones the arginine/proline polymorphism in the p53 gene at position 72 (33) and two missense mutations causing amino acid substitutions at residues 213 and 234 in the p53 gene (34) as summarized in Table 2.

**CD52**<sub>high</sub> Raji cells are susceptible to CDC. To determine if the CD52<sub>high</sub> Raji cells are sensitive to CDC, we tested the effect of alemtuzumab on the parental, CD52<sub>high</sub>, and CD52<sub>low</sub> Raji clones in the presence or absence of human serum as source of complement. Treatments with alemtuzumab but not trastuzumab at 10 μg/mL in the presence of 30% human serum resulted in cytotoxicity in CD52<sub>high</sub> Raji cells. Results indicated that alemtuzumab mediates CDC on the CD52<sub>high</sub> clone (19.8 ± 7.6%) but not on the CD52<sub>low</sub> Raji clone (3 ± 2.3%, P < 0.0001) or the parental Raji clone (10.9 ± 4.9%, P < 0.0001; Fig. 2). No significant cytotoxicity was observed in any of the cell types tested with heat-inactivated human serum which lacks active complement.

![Fig. 2](image_url)

**Fig. 2.** Alemtuzumab-mediated cytotoxicity of Raji clones. Top, differential CDC in Raji clones (A) and primary B-CLL cells (B). Parental, CD52<sub>low</sub>, and CD52<sub>high</sub> Raji cell clones and B-CLL cells were incubated with 10 μg/mL of alemtuzumab or trastuzumab in the presence of 30% of either active or heat-inactivated human serum as a source of complement. Relative cytotoxicity was determined after 1 h of incubation at 37°C by Annexin V/propidium iodide staining. Alemtuzumab induced higher cytotoxicity on CD52<sub>high</sub> compared with CD52<sub>low</sub> Raji cell clone; *, P < 0.0001. Middle, differential ADCC in Raji clones (C) and primary B-CLL cells (D). ADCC was measured using freshly isolated peripheral blood mononuclear cells from normal volunteers and Raji cell clones at 12.5:1, 25:1, 50:1 effector-to-target ratio (E/T) in presence or absence of 10 μg/mL alemtuzumab. Columns, average of triplicate wells and were representative of three independent experiments; bars, SD. The overall CD52<sub>high</sub> versus CD52<sub>low</sub> cytotoxicity was significantly higher for alemtuzumab (P = 0.0236). Bottom, differential cytotoxicity in Raji clones (E) and primary B-CLL cells (F). Direct cytotoxicity. Raji cell clones were incubated with 10 μg/mL of alemtuzumab or trastuzumab with or without 50 μg/mL cross-linking goat anti-human Fc antibody (αFc). The percentage of apoptosis was determined by Annexin V/propidium iodide staining after 24 h. For alemtuzumab, there was significant synergy with cross-linking for CD52<sub>high</sub> versus CD52<sub>low</sub> (P < 0.0001).
CD52<sup>high</sup> Raji cells are sensitive to ADCC. We further investigated whether CD52<sup>high</sup> Raji clone also showed a higher sensitivity to alemtuzumab-mediated ADCC compared with CD52<sup>low</sup> Raji clone, using peripheral blood mononuclear cells as effector cells (Fig. 2). Standard ⁵¹Cr release assays were done at different effector-to-target ratios. First, we compared the overall CD52<sup>high</sup> versus CD52<sup>low</sup> cytotoxicity (averaging over all of the effector/target ratios) between the alemtuzumab and trastuzumab conditions. Whereas the relative cytotoxicity was significantly higher for CD52<sup>high</sup> compared with CD52<sup>low</sup> with alemtuzumab (P = 0.0236) and not significantly different with trastuzumab (P = 0.9763). However, the comparison of the CD52<sup>high</sup> versus CD52<sup>low</sup> difference between alemtuzumab and trastuzumab was only borderline significant (P = 0.0521) based on the interaction test. In addition, we did not find any strong differences in effector concentration trends between the CD52<sup>low</sup> and CD52<sup>high</sup> cell lines with alemtuzumab (P = 0.0844).

Direct cytotoxicity. To determine if CD52<sup>high</sup> Raji clone exhibited increased sensitivity to alemtuzumab-mediated direct cytotoxicity, parental, CD52<sup>high</sup>, and CD52<sup>low</sup> Raji clones were subjected to alemtuzumab or trastuzumab treatment in media without plasma or effector cells. Alemtuzumab treatment of CD52<sup>high</sup> Raji clone resulted in 80 ± 1% cell death compared with 19.5 ± 1.1% and 17.5 ± 1%, respectively, in parental and CD52<sup>low</sup> Raji clones after 24 h of treatment in the presence of a secondary cross-linking antibody (P < 0.0001; Fig. 2). A significant synergy with cross-linking for CD52<sup>high</sup> versus CD52<sup>low</sup> was found. Specifically, without cross-linking, the average percentage of cytotoxicity for CD52<sup>high</sup> was 16.4% higher than for CD52<sup>low</sup>, and with the addition of cross-linking, the difference between CD52<sup>high</sup> and CD52<sup>low</sup> increased by 63%. Extended incubation up to 48 h resulted in a similar trend (data not shown).

In vivo applicability of CD52<sup>high</sup> Raji cells for evaluation of CD52-targeted therapy. To evaluate the expression stability of CD52 molecules and the suitability of the Raji variants for in vivo evaluation of CD52-targeted reagents, we established xenograft leukemia/lymphoma SCID mouse models using CD52<sup>high</sup> and CD52<sup>low</sup> Raji clones. Inoculation of each of the cell lines showed tumorigenic activity in mice characterized by symptomatic metastasis at multiple sites, including central nervous system, liver, and lung, resulting in a progressive body weight loss, as well as hind-limb paralysis, followed by death of nearly 100% mice from massive tumor burden 17 to 30 days postinoculation. Mice inoculated with CD52<sup>high</sup> and CD52<sup>low</sup> Raji cells were randomly divided into three groups and treated with alemtuzumab, trastuzumab, or saline solution at an early stage of disease, i.e., on day 3 after cell inoculation. All the CD52<sup>low</sup> Raji cells—inoculated mice treated with saline (data not shown) or trastuzumab died within 18 to 22 days (median survival times 20 days), whereas >93% of the CD52<sup>high</sup> Raji cells—inoculated mice receiving the same treatment died within 20 to 40 days (median survival times 34 days; Fig. 3A).

As expected, a significant engraftment treatment interaction was found (P = 0.0024) demonstrating a difference in the effect of alemtuzumab compared with trastuzumab, depending on the cell type given (CD52<sup>high</sup> versus CD52<sup>low</sup>). For CD52<sup>low</sup> clone, no difference was found between trastuzumab-treated and alemtuzumab-treated groups (P = 0.6410; hazard ratio, close to 1) indicating that alemtuzumab had no noticeable effect on death, which further indicated the lack of CD52 expression in these cells. However, for CD52<sup>high</sup> clone, the hazard ratio is 0.12, indicating that alemtuzumab decreases the rate of death by roughly eight times (1/0.12) compared with trastuzumab (P = 0.0005). Mice inoculated with CD52<sup>high</sup> Raji clones and treated with alemtuzumab survived >80 days longer (note that only 29% of the CD52<sup>high</sup> Raji mice given alemtuzumab died by the end of the study, so we cannot provide an estimate of the median survival time for that group) than mice inoculated with CD52<sup>low</sup> Raji clones receiving the same treatment. Tissue obtained from sacrificed trastuzumab-treated tumor-bearing SCID mice showed massive distribution of neoplastic cells in multiple sites, including liver, kidney, mesenteric...
and tracheobronchial lymph node, and spinal cord (Fig. 3B). Corresponding tissue obtained from alemtuzumab-treated CD52<sup>high</sup> cells–inoculated mice showed absence of neoplastic cells except for spinal cord where a few residual neoplastic cells (Fig. 3C) were observed in the meninges compared with control mice.

Bone marrow obtained within 0 to 3 days of hind-limb paralysis from trastuzumab-treated mice that had received CD52<sup>high</sup> Raji cell inoculation had a 10% human CD19<sup>+</sup>/CD52<sup>+</sup> cell population (Fig. 3D). In contrast, no human CD19<sup>+</sup>/CD52<sup>+</sup> cell population (<0.5%) was observed in alemtuzumab-treated CD52<sup>high</sup> Raji–inoculated mice (Fig. 3E). Analysis of bone marrow cells from similarly treated CD52<sup>low</sup> Raji clone–inoculated mice exhibited presence of human CD19<sup>+</sup>/CD52<sup>+</sup> cells (data not shown).

**Targeted delivery of alemtuzumab-conjugated immunoliposomal oligodeoxyribonucleotide to CD52<sup>high</sup> Raji cells and primary B-CLL cells.** The above-described properties of CD52<sup>high</sup> clone resembled several features of primary B-CLL cells, including susceptibility to alemtuzumab-mediated direct cytotoxicity, CDC, and ADCC functions and in vivo sensitivity as described previously by us and others (20, 21). To determine the possible use of this model for CD52-targeted delivery of therapeutic agents, the delivery of FAM-labeled oligodeoxyribonucleotide (FAM-ODN) containing liposomes was compared in Raji CD52<sup>high</sup> and primary B-CLL cells in vitro. CD52<sup>high</sup> Raji clones or primary B-CLL cells were incubated for 1 h at 37°C with either alemtuzumab-coated or trastuzumab-coated immunoliposomal FAM-ODN for 1 h at 37°C, washed thrice with PBS and visualized on a Nikon fluorescence microscope (magnification, 400×). Analysis of unstained cells for both the cell type was included as a control.

**Effect of rituximab on CD52<sup>high</sup> Raji cells.** The surface staining analyses of the isolated clones revealed the presence...
of other molecules, such as CD20, CD40, and CD37, that can be targeted by other B-cell specific antibodies and provide a model for such therapeutics. To further investigate the possibility of extending the use of the Raji-Burkitt’s lymphoma model to the evaluation of immunotherapeutic agents targeting molecules different from CD52, the in vitro and in vivo preliminary effects of the anti-CD20 monoclonal antibody rituximab were evaluated. Despite comparable percentage of CD20-expressing cells in CD52<sup>high</sup> and CD52<sup>low</sup> Raji clones (i.e., all the clones expressed CD20 in >99% cells), rituximab mediated 2-fold higher levels of CDC on the CD52<sup>low</sup> Raji clones (80 ± 15% versus 35 ± 16%), perhaps due to the presence on this clone of lower levels of complement resistance surface proteins, such as CD55 and CD59, that inhibit CDC on tumor cells (Fig. 5A; refs. 35, 36).

The direct cytotoxic effect of rituximab upon parental (66 ± 3%) and CD52<sup>high</sup>(60 ± 4%) Raji clones were 2-fold higher compared with the CD52<sup>low</sup> Raji clones (33 ± 3%; Fig. 5C). CD52<sup>high</sup> Raji clone showed the highest susceptibility (57 ± 11%) to rituximab-mediated ADCC when compared with the parental and CD52<sup>low</sup> Raji clone, respectively 22% ± 15% and 36% ± 15% at the highest E/T ratio (50:1; Fig. 5B). Interestingly, consistent with in vitro studies, preliminary study using CD52<sup>high</sup> Raji clone-inoculated mice showed a higher sensitivity to rituximab treatment (50% survival at 100 days after injection) compared with the CD52<sup>low</sup> Raji clone-injected mice that received the same treatment, in which all mice died within 40 to 60 days after inoculation (data not shown). The increased capability of rituximab to mediate ADCC of the CD52<sup>high</sup> clone compared with CD52<sup>low</sup> and parental clones could be explained by the fact that CD52<sup>high</sup> clone, but not CD52<sup>low</sup> and parental clones, expresses high-level CD50 (ICAM3) mRNA as detected by microarray analysis (data not shown) and surface expression confirmed by flow cytometry. Surface expressed ICAM3 on target cells serves as an important ligand for LFA1 expressed on natural killer cells in the initiation of the immune response (37). Although the differential effect of ICAM3 may explain the differential effects of CD20-mediated ADCC, the molecular changes that attribute to the differences in direct cytotoxic effect observed for CD52<sup>high</sup> and parental cell line compared with CD52<sup>low</sup> remain to be determined.

### Discussion

Herein, we have established Raji cell line models expressing high or low levels of CD52 useful for evaluating both in vitro and in vivo effects of CD52-targeted antibody reagents, such as alemtuzumab. By limiting dilution of the Raji Burkitt’s lymphoma cell line, which expresses high levels of CD52 only in a small percentage of cells (43%), we isolated clones expressing either high or low levels of CD52 in >90% of the cell population and comparable high level of HLA-DR, CD19, CD40, CD32, and CD20. The pattern of surface expression of CD52, as well as other glycosyl-phosphatidylinositol–linked protein was found stable with continuous cell culture over several months. The surface level of CD52 was found directly related to the CD52 mRNA level as real-time PCR analysis of RNA-exhibited 1.8-fold increases in CD52 mRNA in the CD52<sup>high</sup> clone. The data clearly show that alemtuzumab, as well as rituximab, can induce in vitro cell lysis in CD52<sup>high</sup> Raji cells. Furthermore, these cells are sensitive to complement or antibody-mediated cytotoxicity. In addition, both CD52-targeted and CD20-targeted antibodies induced direct cytotoxicity in the presence of a cross-linking antibody. Clearly,
the CD52 density was important in alemtuzumab-mediated killing, as it was able to induce cytotoxic effects in vitro on the CD52\textsuperscript{high} clone but not on the parental and CD52\textsuperscript{low} Raji clone. Despite the ability of alemtuzumab to mediate comparable levels of CDC, natural killer cell–mediated ADCC, and direct cytotoxicity in CD52\textsuperscript{high} Raji clones and CD19\(^+\) B cells from CLL patients (Fig. 2B), the biochemical basis of alemtuzumab-mediated direct cytotoxicity remains to be established. The CD52\textsuperscript{high} Raji model described here will serve as an amenable tool for biochemical characterization of alemtuzumab-mediated cytotoxicity. It is interesting to note that despite comparable high percentage of CD20-positive cells, the CD52\textsuperscript{high} clone showed a particularly limited sensitivity to rituximab-mediated CDC compared with CD52\textsuperscript{low} clone. This could be attributed to the lower density of CD20 antigen on the high expression clone. Studies of haptons have, in fact, indicated that increasing the density of the target molecule determine increased sensitivity to complement mediated lysis (38–40). It is also possible that the increased levels of expression of complement inhibitory proteins, such as CD55 and CD59, in CD52\textsuperscript{high} Raji clone cells may contribute to the reduced CDC effects. This is inconsistent with the inhibition of monoclonal antibody–mediated CDC on tumor cells by CD55 and CD59 (35). Further support comes from the limited sensitivity of CD52\textsuperscript{high} clone to alemtuzumab-mediated CDC compared with increased direct cytotoxicity and ADCC. Therefore, ADCC and direct cytotoxicity represent, at least in this model, the major mechanisms of alemtuzumab-mediated killing in vitro, and these may act synergistically in vivo. Alemtuzumab treatment in CD52\textsuperscript{high}-injected mice increased significantly the median survival (71% of the mice were still alive by the end of the study). Human CD19\(^+\)/CD52\textsuperscript{low} cells were found in bone marrow from trastuzumab-treated, but not alemtuzumab-treated, CD52\textsuperscript{high}-injected mice after 24 days in vivo. Consistent with the elimination of alemtuzumab-targeted cells, no sign of hCD19\(^+\)/CD52\textsuperscript{low} cells were found in bone marrow of similarly treated CD52\textsuperscript{low} inoculated mice. In addition to the bare antibody, the CD52\textsuperscript{high} Raji clone can serve as a model for other CD52-targeted therapeutics, such as alemtuzumab immunoliposomes loaded with a therapeutic agent.

Alemtuzumab has been shown to be very effective in eliminating CD52\textsuperscript{+} cells and is currently used to target leukemic B and T cells, as well as in immunosuppressive treatment (41–44). Despite extensive use of alemtuzumab in clinical trials, the mechanism of its action remains to be clarified, due mostly to the lack of reproducible model cell lines and animal models to study the in vitro and in vivo effect of this monoclonal antibody. In fact, many of in vitro maintained lymphoid-derived tumor cells only express CD52 at very low levels (1) and the stability of the molecule in vivo is less predictable. Recently a xenotransplant model of multiple myeloma in NOD/SCID mice using KMS-11 cells has been developed to investigate the in vivo activity of alemtuzumab. In this experimental condition, probably due to the low expression of CD52 on KMS-11 cells, alemtuzumab treatment initiated at an early stage of disease substantially delayed the in vivo growth of myeloma cells rather than eradicating the disease (45).

The Raji Burkitt’s lymphoma cell clones expressing high levels of CD52 stably both in vitro and in vivo will overcome the limitations of previously described Raji-xenograft models that showed drastic modulation of the expression of the CD52 molecules in vivo. The stable CD52\textsuperscript{high} B-cell lines and the disseminated leukemia-lymphoma mouse model described here can serve as an excellent system for in vitro and in vivo therapeutic and mechanistic evaluation of existing and novel antibodies directed against CD52 molecule and antibody-based therapeutic agents, such as immunoliposomes.

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