Targeting chronic lymphocytic leukemia cells with a humanized monoclonal antibody specific for CD44

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Edited* by Dennis A. Carson, University of California at San Diego, La Jolla, CA, and approved March 4, 2013 (received for review December 20, 2012)

Chronic lymphocytic leukemia (CLL) cells express high levels of CD44, a cell-surface glycoprotein receptor for hyaluronic acid. We found that a humanized mAb specific for CD44 (RG7356) was directly cytotoxic for leukemia B cells, but had little effect on normal B cells. Moreover, RG7356 could induce CLL cells that expressed the zeta-associated protein of 70 kDa (ZAP-70) to undergo caspase-dependent apoptosis, independent of complement or cytotoxic effector cells. The cytotoxic effect of this mAb was not mitigated when the CLL cells were cocultured with mesenchymal stromal cells (MSCs) or hyaluronic acid or when they were stimulated via ligation of the B-cell receptor with anti-μ. RG7356 induced rapid internalization of CD44 on CLL cells at 37 °C, resulting in reduced expression of ZAP-70, which we found was complexed with CD44. Administration of this mAb at a concentration of 1 mg/kg to immune-deficient mice engrafted with human CLL cells resulted in complete clearance of engrafted leukemia cells. These studies indicate that this mAb might have therapeutic activity, particularly in patients with CLL that express ZAP-70.

cell survival | preclinical studies | animal model | antibody therapy

B-cell chronic lymphocytic leukemia (CLL) is characterized by the clonal expansion of mature, antigen-stimulated CD5+/CD23+ B cells in blood, secondary lymphoid tissues, and marrow (1). Most of the circulating CLL cells in patients are arrested in the G0/G1 phase of the cell cycle and express high levels of antiapoptotic proteins (2). CLL therefore has been characterized as a process of defective apoptosis, rather than increased proliferation. However, despite their apparent longevity in vivo, CLL cells undergo spontaneous and drug-induced apoptosis in vitro, unless rescued by monocyte-derived Nurse-like cells (NLCs), follicular dendritic cells, or mesenchymal stromal cells (MSCs) (3–6). Thus, it has been postulated that CLL cells receive survival signals from these accessory cells, which constitute part of the CLL B-cell microenvironment in secondary lymphoid tissues and marrow (6). These survival signals can inhibit spontaneous or drug-induced apoptosis, particularly for CLL cells that express unmutated Ig heavy-chain variable genes (IGHVs) and/or the zeta-associated protein of 70 kDa (ZAP-70), which typically is not expressed by normal B cells (7). Patients with leukemia cells that possess such characteristics typically have a relatively short interval from diagnosis to initial therapy compared with patients with CLL cells that express mutated IGHVs or that lack expression of ZAP-70 (8–12).

One of the survival signals received by leukemia cells may be mediated via CD44, a surface glycoprotein receptor for the nonsulfated glycosaminoglycan hyaluronic acid (HA), which typically is found in the microenvironment of lymphoid tissues (13). CLL cells express high levels of CD44, particularly those that express unmutated IGHVs and/or ZAP-70 (14). Upon binding HA in the extracellular matrix, CD44 activates the phosphoinositol 3-kinase (PI3K)/AKT and MAPK/ERK pathways and induces increased expression of antiapoptotic proteins (e.g., myeloid cell leukemia 1), which can promote CLL-cell survival (14). Conceivably, such prosurvival signaling also might occur in vivo, in which case CD44 might be a good target for therapy.

In this study, we evaluated the expression level of surface CD44 on CLL cells and examined the activity of a humanized anti-CD44 monoclonal antibody (mAb; RG7356; Roche) (15) on leukemia cells in vitro and in vivo.

Results

Expression of CD44 on CLL Cells. CLL cells from 59 patients and B cells from 25 healthy donors each were examined for expression of CD44. Expression of CD44 was detected on both CLL cells and normal B cells by flow cytometry, but not on EW36, a human B-cell lymphoma cell line (Fig. 1*A*). Immunoblot analyses demonstrated that RG7356 reacted with each of various CD44 isoforms present on normal B cells or CLL cells (Fig. 1*B* and Fig. S1). The median of the mean fluorescence intensity (MFI) ratio (median MFIR) for CD44 detected on the surface of each normal-B-cell population (125.1) was not significantly different from that of the median MFIR for CLL cells (131.9) (Fig. 1*C Right*). However, the median MFIR for CD44 on CLL B cells that used unmutated IGHV, or that expressed ZAP-70, was significantly higher than the median MFIR for CD44 on CLL cells that used mutated IGHV genes (Fig. 1*C Center*; median MFIR = 161.2 vs. 118.5, respectively; $P = 0.013$) or that were ZAP-70 negative ($ZAP\text{-}70^{Neg}$) (Fig. 1*C Left*; median MFIR = 161.2 vs. 118.7, respectively; $P = 0.019$).

RG7356 Directly Induces Apoptosis of CLL Cells That Express ZAP-70. We examined whether CLL cells were sensitive to treatment with RG7356. CLL cells from each of 28 patients (16 $ZAP\text{-}70^{Pos}$ and 12 $ZAP\text{-}70^{Neg}$) or blood mononuclear cells from 6 healthy donors were incubated with various concentrations of RG7356 for various times at 37 °C in RPMI 1640 full medium. Induction of apoptosis was analyzed by flow cytometry after staining the cells with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and propidium iodide (PI) (Fig. 2*A*). CLL cells that were $ZAP\text{-}70^{Pos}$ appeared more sensitive to the cytotoxic activity of RG7356 than $ZAP\text{-}70^{Neg}$ CLL cells, even though the latter were selected for having expression levels of CD44 that were comparable with the $ZAP\text{-}70^{Pos}$ CLL cells (Fig. 2*E* and Fig. S2). For example, treatment of $ZAP\text{-}70^{Pos}$ CLL cells for 24 h with ≥2 μg/mL RG7356 caused significant loss in the cell viability relative to control IgG-treated cells, whereas concentrations of ≥10 μg/mL were required

Author contributions: S.Z., C.C.N.W., J.-F.F., B.C., L.C., and T.J.K. designed research; S.Z., C.C.N.W., J.-F.F., B.C., L.C., L.Z., R.W., and F.L. performed research; L.R. and S.W. contributed new reagents/analytic tools; S.Z., C.C.N.W., J.-F.F., B.C., L.C., L.R., and T.J.K. analyzed data; and S.Z. and T.J.K. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221841110/-/DCSupplemental.

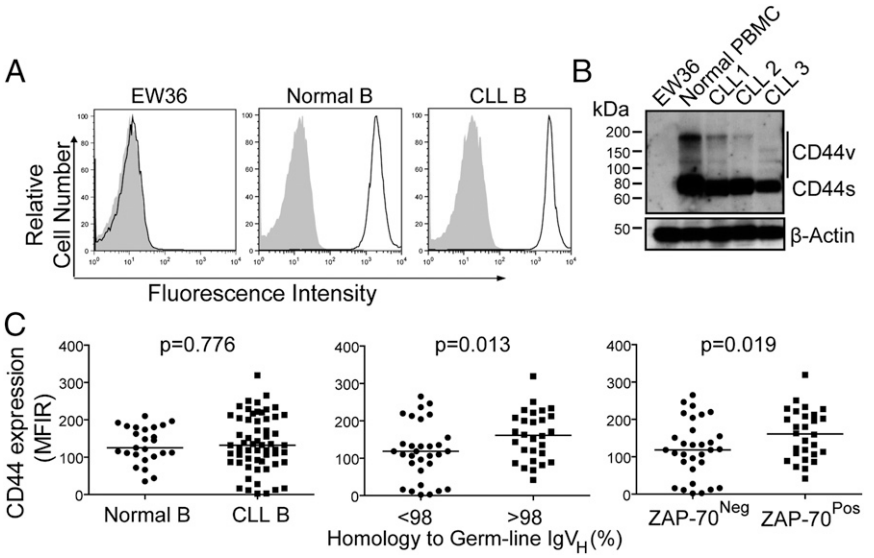

Fig. 1. High-level expression of CD44 on CLL B cells associates with features of aggressive disease. (*A*) Fluorescence histograms of a human lymphoma B-cell line (EW36), normal B cells, and CLL B cells stained with Alexa 647-conjugated RG7356 mAb (open histograms) or control mAb (filled histograms). (*B*) Immunoblot analysis of lysates from EW36, peripheral blood mononuclear cells (PBMCs) from healthy adult, or CLL patients using RG7356 mAb specific for human CD44 or Ab for β-Actin (CLL1, CLL2, CLL3). (*C*) PBMCs from healthy adult ($n = 25$) or patients with CLL ($n = 59$) stained with Alexa 647-conjugated RG7356 mAb or control mAb as in *A*. MFIR is obtained by dividing the MFI of RG7356 mAb staining by the MFI of control mAb staining. (*Left*) Each dot represents the expression of CD44 from an individual CLL patient sample gated on $CD19^{Pos}CD5^{Pos}$ B cells or a healthy adult sample gated on $CD19^{Pos}$ B cells. (*Center* and *Right*) CD44 expression levels were correlated with clinical features of CLL according to the extent of somatic mutations in IgV_H genes (*Center*) or to the level of ZAP-70 expression (*Right*) as described (12). The line indicates the median CD44 expression level by each group. $P < 0.05$ indicates statistical significance of the differences in the collective CD44 expression between the two groups, as calculated using the Student *t* test.

to significantly reduce the relative cell viability of $ZAP\text{-}70^{Neg}$ CLL cells (Fig. 2*B*). Furthermore, treatment of CLL cells with saturating amounts of RG7356 (e.g., 50 μg/mL) caused significant loss in viability of $ZAP\text{-}70^{Pos}$ CLL cells relative to control IgG-treated cells at ≥12 h, but not until ≥24 h for $ZAP\text{-}70^{Neg}$ CLL cells (Fig. 2*C*). Moreover, the relative cytotoxic activity of saturating amounts of RG7356 for CLL cells at 24 h appeared proportionately associated with the level of ZAP-70 expressed by individual CLL-cell populations (Fig. 2*F*; Pearson R = −0.5345; $P = 0.0034$). In contrast, RG7356 did not reduce the viability of normal B cells relative to that of cells treated with control IgG, even at concentrations of ≥50 μg/mL and for time periods of up to 48 h (Fig. 2 *C* and *D*).

We also examined the cytotoxic activity for CLL cells of IgG4_SPLE, a mAb of the IgG4 subclass that has the same Fab-binding domain of RG7356. In addition, we generated F(ab)′2 from RG7356 and examined its ability to direct killing of CLL cells in vitro. We found that either IgG4_SPLE or the F(ab)′2 of RG7356 could induce significant killing of CLL relative to that of control human IgG or F(ab)′2 (Fig. S3), indicating that RG7356 had cytotoxic activity for CLL cells that was independent of Fc-dependent immune-effector mechanisms.

Apoptosis Induced by RG7356 Is Caspase-Dependent and Not Mitigated by Accessory Cells. CLL cells were treated with RG7356 or control IgG for 48 h and then assessed for viability by flow cytometry and for poly(ADP–ribose) polymerase (PARP) cleavage by immunoblot analysis. CLL cells treated with RG7356 had significantly greater proportions of Annexin V-positive apoptotic cells than did CLL cells treated with control IgG (Fig. S4). Furthermore, CLL cells treated with RG7356 had detectable PARP cleavage, which was not detected in lysates of control-treated cells (Fig. 3*A*). The pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) could inhibit apoptosis of RG7356-treated CLL cells in a dose-dependent fashion (Fig. 3*B*), indicating that apoptosis induced by RG7356 was caspase-dependent.

We examined whether accessory cells or growth/survival factors postulated to exist in the microenvironment (16) could inhibit apoptosis of CLL cells induced by RG7356. $ZAP\text{-}70^{Pos}$ CLL cells treated with RG7356 had rapid and significant loss in relative cell viability when treated alone or in combination with MSCs; ∼50% of the RG7356-treated CLL cells were dead by 48 h (Fig. 4). In contrast, RG7356 did not induce $ZAP\text{-}70^{Neg}$ CLL cells to undergo apoptosis with or without MSCs.

Effect of HA on CLL Cells in Vitro. MSCs express HA synthases as well as HA (17, 18), which is a principal ligand of CD44. As such, we investigated whether HA could influence the survival of CLL cells. CLL cells were cultured for 24 h, with or without 50 μg/mL HA, and then stained with $DiOC_6$/PI before flow cytometry. Treatment of CLL cells with HA significantly enhanced the viability of $ZAP\text{-}70^{Pos}$ CLL cells (Fig. 5*A Right*). In contrast, HA had little or no effect on the viability of most $ZAP\text{-}70^{Neg}$ CLL cell populations.

HA induced rapid phosphorylation of AKT in 5–10 min, as assessed by a phosphorylation AKT (p-AKT)/total AKT-specific ELISA (Fig. 5*B*). HA-induced phosphorylation of AKT was observed primarily in $ZAP\text{-}70^{Pos}$ CLL cells, although all cases expressed similar levels of CD44 (Fig. S2). Nevertheless, treatment of $ZAP\text{-}70^{Pos}$ CLL cells with RG7356 inhibited the capacity of HA to induce increases in p-AKT or to enhance cell survival (Fig. 5 *C* and *D*).

RG7356 Induces Down-Modulation of CD44 and ZAP-70 In CLL Cells. We incubated CLL cells with Alexa 647-conjugated RG7356 and observed rapid internalization of cell-surface CD44 within 10 min at 37 °C. Moreover, the MFI of cells stained with Alexa 647-conjugated RG7356 was reduced by >40% after 2 h at 37 °C (Fig. 6*A*). In contrast, treatment with RG7356 did not cause any reduction in the levels of surface IgM (sIgM) at any time, up to 24 h in culture (Fig. S5 *A* and *B*).

Immunoblot analysis revealed that treatment with RG7356 for 48 h caused significant reduction in the levels of CD44 of either $ZAP\text{-}70^{Pos}$ or $ZAP\text{-}70^{Neg}$ CLL cells (Fig. 6*B* and Fig. S5*C*). Treatment with RG7356 for 6–12 h reduced the level of detectable ZAP-70 in $ZAP\text{-}70^{Pos}$ CLL cells by 30–50%, as assessed by flow cytometry (Fig. 6*C* and Fig. S5*D*). Immunoprecipitation of CLL-cell lysates with RG7356 revealed that ZAP-70 was associated with CD44 (Fig. 6*D*), suggesting that ZAP-70 may be involved in CD44 survival signaling in CLL cells. Indeed, treatment with RG7356 disrupted the ZAP-70/CD44 complex (Fig. 6*E*). Subsequently, RG7356 disrupted the capacity of sIgM ligation with anti-μ to induce intracellular

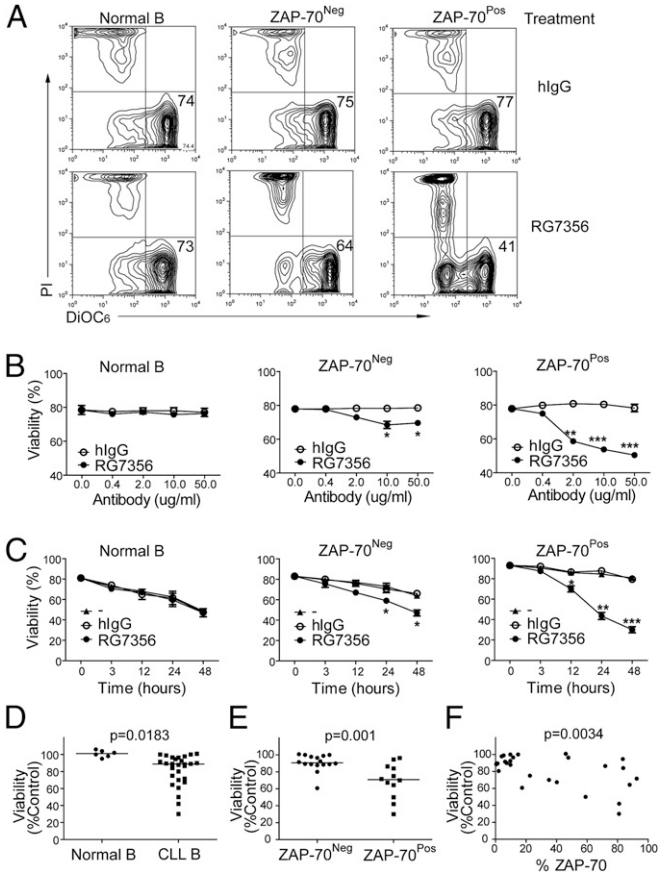

Fig. 2. RG7356 directly induces apoptosis of ZAP-70^{Pos} CLL cells in vitro. CLL cells or normal PBMCs were cultured in the presence of RG7356 or human IgG (hIgG) control mAb at the indicated concentrations and time period. The cells were harvested and stained with $DiOC_6$/PI to measure viability by flow cytometry. Normal PBMCs were also stained for CD19 expression to evaluate cell death in the B-cell population. (*A*) Contour maps from the flow-cytometric analysis of two representative CLL samples incubated with 50 μg/mL mAb for 24 h. The relative $DiOC_6$ and PI fluorescence intensities are depicted on the x and y axis, respectively. The cells that are $DiOC_6$ bright and PI negative (PI^{Neg}/DiOC6^{Hi} in the lower right quadrant) are viable; such cells were used for the generation of plots shown below. (*B*) CLL cells separated according to their expression of ZAP-70 or normal PBMCs were cultured with increasing concentrations of mAb and harvested 24 h later for analysis. (*C*) Cells were cultured in the presence or absence of 50 μg/mL mAb and harvested at the times indicated for analysis. (*D*) Each dot represents the relative viability of cells from one patient cultured with 50 μg/mL RG7356 mAb for 24 h. The percentage of viable cells has been normalized to the viability of control mAb-treated cells. The line indicates the median viability of cells treated with RG7356 mAb by the group. $n = 6$ for normal and $n = 28$ for CLL cells (*E*) The percent viable cells remaining following CD44 mAb exposure depicted in *D* are presented in function of ZAP-70 status, using the standard 20% expression as a cutoff. $P = 0.001$ (Student's *t* test). (*F*) The percentages of viable cells following treatment with 50 μg/mL RG7356 depicted in *D* are plotted with respect to the percentages of CLL cells found to express ZAP-70 for each sample. (Pearson R = −0.5345; $P = 0.0034$; $n = 28$). The statistical significance in *B* and *C* was analyzed by using Student's *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

calcium flux, an indicator of B cell receptor (BCR) signaling (Fig. 6*F*). Also, CLL cells treated with RG7356 had significant reductions in viability relative to that of CLL cells treated with control IgG, regardless of whether the leukemia cells were stimulated by sIgM ligation via anti-μ (Fig. 6*G*). Moreover, treatment with anti-μ lost its capacity to enhance the viability of CLL cells following treatment with RG7356 (Fig. 6*G*).

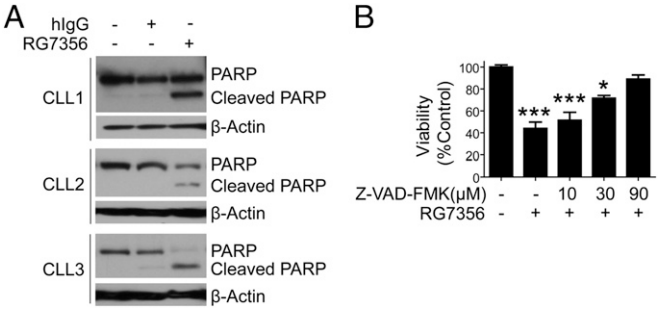

Fig. 3. RG7356 mAb-mediated apoptosis of CLL cells is caspase-dependent. CLL cells were cultured for 48 h with 50 μg/mL RG7356 mAb or hIgG control Ab. (*A*) Cell lysates were harvested and analyzed by immunoblot analysis for cleavage of PARP. β-actin was used as loading control. (*B*) Cells were treated with RG7356 with or without a pan-caspase inhibitor, Z-VAD-FMK, at various concentrations for 48 h. The cells' viability was analyzed by flow cytometry. Statistical significance was determined by using Dunnett's multiple comparison test. *$P < 0.05$; ***$P < 0.001$.

RG7356 Can Direct Clearance of CLL Xenografts. We established xenografts of human CLL cells in the peritoneal cavity of immunodeficient Rag2/common-gamma-chain knockout mice (Rag2$^{-/-}$γc$^{-/-}$), which subsequently were treated with control Ig or RG7356. ZAP-70^{Pos} CLL cells were more sensitive to treatment with RG7356 than ZAP-70^{Neg} CLL cells; the viability and yield of ZAP-70^{Pos} CLL cells were affected by doses as small as 0.01 mg per kg of body weight (Fig. 7*A*). Nevertheless, both ZAP-70^{Neg} and ZAP-70^{Pos} CLL xenografts were sensitive to treatment with RG7356 at higher doses; >90% of the CLL cells were cleared from mice treated with 1 mg/kg RG7356, regardless of whether or not the CLL cells were ZAP-70^{Neg} or ZAP-70^{Pos} (Fig. 7*B*).

RG7356 Can Direct Ab-Dependent Cell Phagocytosis. Although Rag2$^{-/-}$γc$^{-/-}$ mice are deficient in B, T, and natural killer cells, they still possess macrophages in the peritoneal cavity that might account for the noted clearance of ZAP-70^{Neg} CLL following treatment with RG7356. To examine for this possibility, we cultured ZAP-70^{Neg} CLL cells or isolated normal B cells from healthy donors either alone or with macrophages in medium containing either 1 or 10 μg/mL of RG7356, rituximab, or control IgG. After 3 h of incubation, the CLL cells cultured in medium

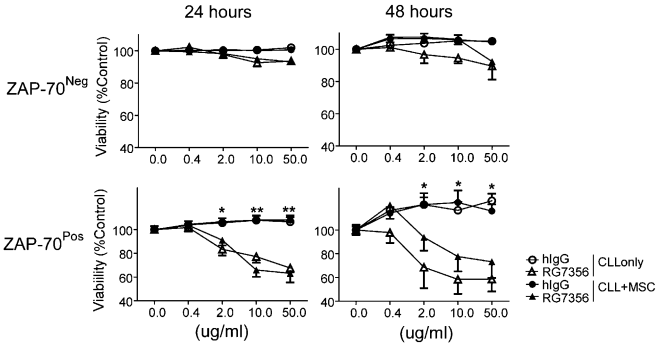

Fig. 4. RG7356 induces apoptosis of ZAP-70^{POS} CLL cells, even in the presence of MSCs. CLL cells cultured either alone or in the presence of MSCs were treated with 50 μg/mL RG7356 or control hIgG at the concentrations indicated for 24 or 48 h. The viability of the CLL cells was assessed by using flow cytometry. Data were normalized to the population of PI^{Neg}/DiOC$_6^{Hi}$ at time point 0 as 100% viability. Results shown are the mean (±SEM) of triplicate samples from each of three different patients from each group. An asterisk (*) indicates a statistically significant difference between cells treated with RG7356 vs. hIgG (paired Student's *t* test). *$P < 0.05$ and **$P < 0.01$.

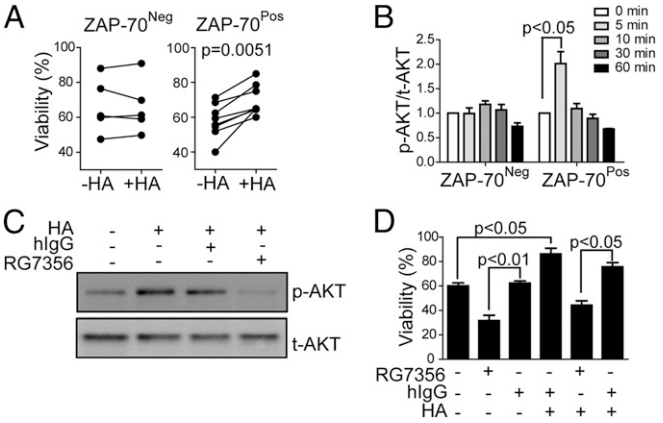

Fig. 5. RG7356 mAb blocks HA-induced AKT phosphorylation and survival in CLL cells. (*A*) CLL cells from ZAP-70^{Neg} CLL (n = 5) or ZAP-70^{Pos} CLL (n = 7) samples were incubated with or without HA (50 μg/mL) for 24 h, and cell viability was analyzed by flow cytometry. The data shown depict the percent of viable cells for each patient tested. (*B*) Cell lysates were harvested at different time points from CLL samples (n = 3, ZAP-70^{Neg} or ZAP-70^{Pos}) stimulated with HA (50 μg/mL) and analyzed by a p-AKT/total AKT (t-AKT)-specific ELISA. Results shown are mean ± SD of the level of p-AKT normalized to t-AKT at different time points relative to that of pretreatment (0 min). $P < 0.05$ indicates statistical significance of differences analyzed using paired Student's t test. (*C*) ZAP-70^{POS} CLL samples were pretreated with or without RG7356 (50 μg/mL) for 20 min, then stimulated with HA (50 μg/mL) for 5 min. Cells were lysed and analyzed by immunoblot for the expression of p-AKT or t-AKT. (*D*) ZAP-70^{POS} CLL cells were incubated with or without 50 μg/mL RG7356 mAb and with or without 50 μg/mL HA for 24 h and then were analyzed for viability by flow cytometry. The percent of viable cells is shown. Statistical significance was determined by one-way ANOVA following Tukey's multiple comparison test.

containing either RG7356 or rituximab had significantly lower viability when cocultured with peritoneal macrophages (Fig. 8, gray bars) than when cultured alone or with control IgG in the presence of macrophages. However, we did not observe significant reductions in the viability of normal blood B cells when cocultured with such macrophages in the presence of 10 μg/mL RG7356 (Fig. S6). Conversely, RG7356 did not appear to direct complement-mediated cytotoxicity of CLL cells, in contrast to what we observed with rituximab (Fig. 8, black bars).

Discussion

We found that a humanized anti-CD44 mAb (RG7356) could directly induce ZAP-70^{Pos} CLL cells to undergo caspase-dependent apoptosis. This activity was not dependent on complement or immune-effector cells, but rather was induced by Ab ligation of CD44, which might affect growth/survival signaling for CLL cells (19). Consistent with this notion, we observed that the F(ab)′2 of RG7356, or a derivative of RG7356 with an IgG4 Fc, also could direct significant killing of ZAP-70^{Pos} CLL cells (Fig. S3). In contrast, rituximab did not have this effect on CLL cells (Fig. S3).

Sensitivity to RG7356 was not predicated solely on the expression level of CD44 by CLL cells. ZAP-70^{Neg} CLL cells that expressed comparable levels of CD44 as ZAP-70^{Pos} CLL cells were relatively resistant to the cytotoxic effects of this mAb. Moreover, CD44 is expressed on a variety of normal tissues, including hematopoietic tissues and normal B cells (14, 20), which apparently also are resistant to the direct cytotoxic effects of RG7356.

Instead, the direct cytotoxic effects of this mAb suggests that CD44 signaling in ZAP-70^{Pos} CLL cells is qualitatively distinct from that of ZAP-70^{Neg} CLL or normal B cells. Prior studies found that CD44 could activate PI3K/AKT and MAPK/ERK upon binding its principle ligand HA, thereby enhancing CLL-cell survival (14). Consistent with this observation, we found that HA induced rapid phosphorylation of AKT in ZAP-70^{Pos} CLL cells. However, phosphorylation of AKT was not observed upon

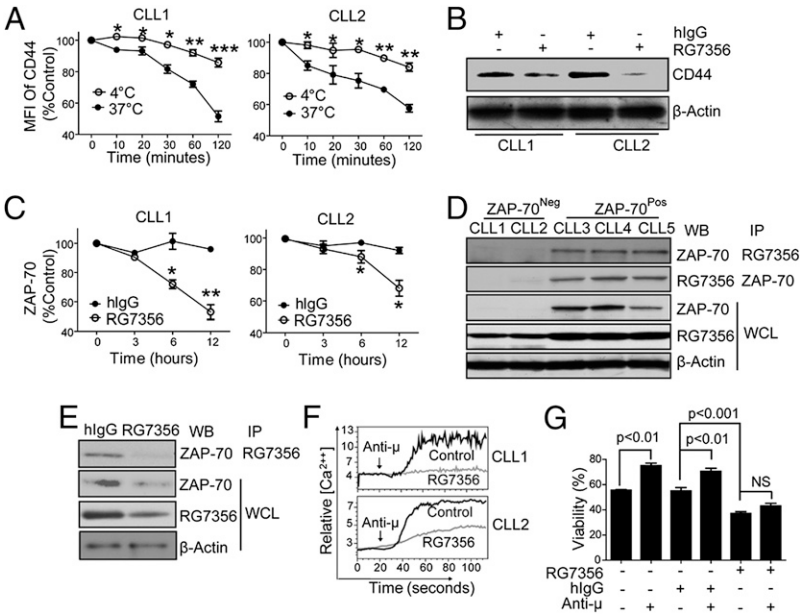

Fig. 6. RG7356 down-modulates CD44 and ZAP-70 in CLL, disrupts the CD44-ZAP-70 complex, and inhibits BCR signaling. (*A*) Internalization of RG7356 mAb by CLL cells. CLL cells were stained with Alexa 647-conjugated RG7356 at either 4 °C or 37 °C and analyzed at indicated time points by flow cytometry. Data are presented as MFI of stained cells normalized with respect to the MFI of stained cells examined after 20 min staining at 4 °C as 100%. (*B*) Down-modulation of CD44 protein following treatment with RG7356. CLL cells were treated with either hIgG or RG7356d (50 μg/mL) for 48 h, and cell lysates were analyzed by immunoblot. (*C*) Decreased ZAP-70 protein levels following CD44 mAb treatment. CLL cells were incubated with RG7356 mAb or control Ab for the indicated time period, stained with Alexa 488-conjugated anti– ZAP-70 Ab, and analyzed by flow cytometry. Shown are two representative CLL samples that were ZAP-70^{Pos}. (*D*) CD44 physically associates with ZAP-70 in CLL cells. Protein lysates of CLL cells from different patients were immunoprecipitated (IP) with either RG7356 or anti-ZAP-70 Ab. The bound products or whole-cell lysates (WCL) were probed by immunoblot using the Abs indicated in the WB column. (*E*) Down-modulation of CD44 and ZAP-70 protein complex following RG7356 mAb treatment. ZAP-70^{Pos} CLL cells were treated with RG7356 (50 μg/mL) or hIgG and subsequently lysed for immunoprecipitation (IP) with RG7356 mAb, which then was examined by immunoblot analyses with the indicated Abs. (*F*) Treatment of RG7356 reduced IgM-induced calcium flux in CLL cells. ZAP-70P^{Pos} CLL samples were first labeled with the fluorescent calcium indicator Fluo-4AM following treatment with either RG7356 (50 μg/mL) or hIgG control Ab for 12 h, then stimulated with anti-μ. The fluorescence intensity was recorded over time by FACS. The lines represent the changes in fluorescence intensity (on the y axis) over time (x axis) for control CLL cells (black line) or cells preincubated with RG7356 (gray line). An arrow indicates the time when anti-μ was added. (*G*) RG7356 mitigates IgM-induced survival of CLL cells. ZAP-70^{Pos} CLL samples were incubated with or without 50 μg/mL RG7356 or hIgG with or without treatment with anti-μ (10 μg/mL) for 48 h. Cell viability was analyzed by flow cytometry. Representative data were shown from one of the three patient samples tested. Each bar depicts the mean proportion of viable cells from triplicates. Error bar indicates SEM. Statistical significance was analyzed by using the one-way ANOVA test following Tukey's multiple comparison test. NS, no significant difference.

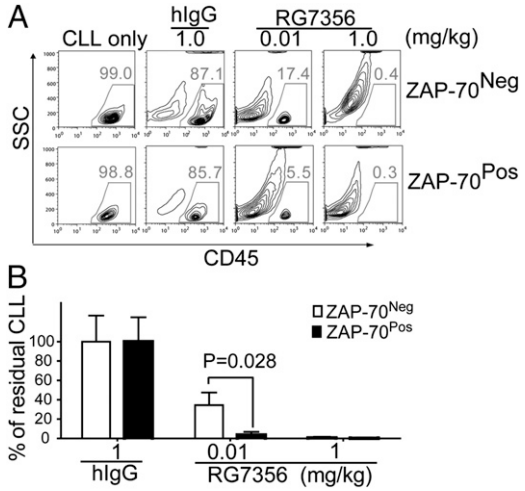

Fig. 7. RG7356 directs clearance of CLL cells in vivo. CLL cells were injected to the peritoneal cavity of $Rag2^{-/-}\gamma c^{-/-}$ mice 1 d before treatment with mAb. Peritoneal lavage was collected 7 d after cell injection and subjected to residual CLL determination by cell counting and flow cytometry analysis following staining with mAb specific for CD5, CD19, and CD45. (*A*) Contour plots of two representative CLL samples treated with either low- or high-dose mAb. Cells on the lower right gates are human CLL cells, and those numbers were used to generate the bar graph shown in *B*. (*B*) Each bar in the graph represents percentage of residual CLL cells harvested form mice after treatment with different concentrations of RG7356, normalized with respect to that cells harvested from mice treated with control hIgG, which was to 100%. Data shown are mean ± SEM from three different patients with $n = 3$ in each group. *P* indicates a statistically significant difference between RG7356-treated ZAP-70^{Pos} and RG7356-treated ZAP-70^{Neg} samples, as per Student's *t* test.

HA treatment in ZAP-70^{Neg} CLL cells, suggesting that ZAP-70 is involved in mediating such CD44 signaling. Furthermore, both signaling and survival induced by anti-μ in ZAP-70^{Pos} CLL cells were attenuated by RG7356, revealing a potential cross-talk between CD44 and ZAP-70 in BCR signaling, which also might contribute in part to the cytotoxic activity of this mAb for ZAP-70^{Pos} CLL cells.

Furthermore, prior studies found that CD44 on CLL cells could form a supramolecular complex with other surface proteins, namely, CD38, CD49d, and matrix metalloproteinase-9 (MMP-9) (21). CLL cells that are ZAP-70^{Pos} experience dynamic expression of CD38 (22–24), a plasma-membrane ectoenzyme that can interact with a ligand on endothelial cells (CD31) to initiate signaling that leads to enhanced leukemia-cell proliferation and/or survival (25). Also, expression of CD38 and ZAP-70 identifies CLL cells with enhanced migration to the chemokine CXCL12 (26), suggesting a functional link between these two proteins. CD49d is the alpha subunit that complexes with CD29 to form the α4β1 integrin known as very late antigen 4, which can interact with CD44 and also bind to its ligand, vascular cell adhesion molecule 1, to promote the viability of CLL cells through the activation of NF-κB (27, 28). MMP-9 is a type IV collagenase that apparently plays a critical role in facilitating the infiltration of CLL cells into the lymphoid tissues, where they can find growth and survival signaling mediated by cells in the nodal microenvironment (29, 30). Finally, as with ZAP-70, high-level CLL-cell expression of CD44 (31), CD49d (32–34), or MMP-9 (35) has been linked to adverse outcome in CLL. Moreover, studies have found that these proteins coassociate on the plasma membrane, particularly of CLL cells of patients with aggressive disease (21). The disruption of this complex by RG7356 also may factor in the capacity of this mAb to induce apoptosis of ZAP-70^{Pos} CLL cells.

RG7356 also induced rapid internalization of CD44 on CLL cells, resulting in reduced expression of ZAP-70, which we found was complexed with CD44. Our study found that ZAP-70 (like CD38, CD49d, or MMP-9) could physically associate with CD44 in CLL cells. Because RG7356 can down-modulate surface CD44 expression, these CD44-associated proteins may be down-modulated, as observed for ZAP-70. In any case, the reduction of ZAP-70 could impair BCR signaling (36, 37), which might account for some of the cytotoxic effects of this mAb, as noted for other inhibitors of ZAP-70 expression in CLL (38). Also, the capacity of this mAb to down-modulate CD44 and its associated proteins could render the leukemia cell resistant to the survival factors elaborated by accessory cells in the microenvironment. Consistent with this notion are the observations that the cytotoxic activity of RG7356 for CLL cells is not mitigated by co-culture with MSCs or HA.

Targeting CD44 by using 0.01 mg/kg RG7356 caused striking clearance of ZAP-70^{Pos} CLL cells and partial clearance of ZAP-70^{Neg} CLL cells, reflecting the dependence of ZAP-70 expression for direct cytotoxicity. However, treatment with single dose of 1 mg/kg anti-CD44 mAb resulted in almost complete clearance of engrafted CLL cells, regardless of whether they expressed ZAP-70 or had functional p53, implying that this mAb might be effective in the treatment of patients' chemotherapy-resistant disease. The noted clearance of even ZAP-70^{Neg} CLL cells in this model also suggests that mechanism(s) other than direct cytotoxicity might be involved in the clearance of leukemia cells in vivo. Consistent with this notion is our finding that RG7356 can direct Ab-dependent cellular phagocytosis of ZAP-70^{Neg} or ZAP-70^{Pos} CLL cells in vitro and, presumably, in vivo.

Because CD44 apparently is expressed on putative cancer stem cells (39), RG7356 currently is being evaluated in clinical trials involving patients with solid-tumor malignancies. Because CD44 also can be found on normal tissues, patients treated with this mAb will need to be closely monitored for nonspecific toxicity. Nevertheless, the selective toxicity of RG7356 for CLL cells demonstrated in this study should encourage the clinical evaluation of this mAb also in treatment of patients with this disease.

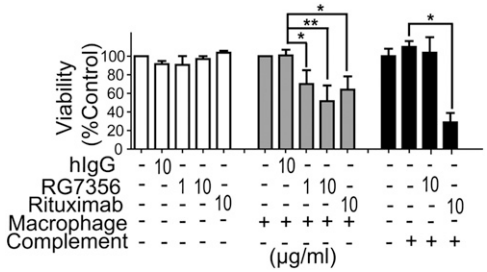

Fig. 8. RG7356 mAb can direct phagocytosis of CLL cells, but not complement-mediated cytotoxicity. CLL samples were incubated with RG7356, hIgG, or rituximab at the concentrations indicated for 30 min on ice. The cells then were incubated at 37 °C for an additional 3 h, either alone (open bars) or with macrophages (gray bars) at 1:5 target cell to macrophage ratio or with rabbit complement (black bars). Cells were collected, stained, and analyzed for viable CLL cells by flow cytometry. Data shown are mean ± SEM from triplicate samples of each of five different patients per group, normalized to the corresponding control samples, which were listed as 100% viability. $*P < 0.05$; $**P < 0.01$ (one-way ANOVA following Tukey's multiple comparison test).

Materials and Methods

Samples were collected from patients evaluated at University of California at San Diego (UCSD) Moores Cancer Center after they provided written informed consent on a protocol approved by the Institutional Review Board of UCSD, in accordance with the Declaration of Helsinki. All patients fulfilled diagnostic criteria for CLL. More than 90% of the isolated blood mononuclear cells from patients with CLL were $CD19^{Pos}/CD5^{Pos}$ cells, as assessed by flow cytometry. ZAP-70 expression and IGHV mutational status were

assessed, as described (12). Blood mononuclear cells were isolated from buffy coat samples that we obtained from the San Diego Blood Bank by density centrifugation with Ficoll-Hypaque (Pharmacia). A detailed description of the reagents, cellular assays, ELISAs, and animal studies are available in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Catriona Jamieson, MD, PhD (University of California at San Diego Moores Cancer Center) for providing RAG2$^{-/-}\gamma c^{-/-}$ mice. This work was supported in part by National Institutes of Health Grant P01-CA081534 and the University of California at San Diego Moores Cancer Center Blood Cancer Research Fund.

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