Superior Activity of Fusion Protein scFvRit:sFasL over Cotreatment with Rituximab and Fas Agonists

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Abstract
The clinical efficacy of the CD20-specific chimeric monoclonal antibody rituximab is significantly hampered by intrinsic or acquired resistance to therapy. Rituximab activates antibody-dependent cellular cytotoxicity/complement-dependent cytotoxicity–dependent lysis but also induces apoptosis by cross-linking of its target antigen CD20. Recent reports indicate that this apoptotic activity of rituximab can be synergized by cotreatment with Fas agonists. Here, we report on a strategy designed to exploit and optimize the synergy between rituximab and Fas signaling by genetically fusing a rituximab-derived antibody fragment to soluble Fas ligand (sFasL). The resultant fusion protein, designated scFvRit:sFasL, potently induced CD20-restricted apoptosis in a panel of malignant B-cell lines (10 of 11) and primary patient-derived malignant B cells (two of two non–Hodgkin lymphoma and five of six B cell chronic lymphocytic leukemia). scFvRit:sFasL efficiently activated CD20 and Fas apoptotic signaling, resulting in a far superior proapoptotic activity compared with cotreatment with rituximab and Fas agonists. scFvRit:sFasL lacked activity toward normal human B cells and also lacked systemic toxicity in nude mice with no elevation of aspartate aminotransferase and alanine aminotransferase levels or liver caspase-3 activity. In conclusion, scFvRit:sFasL efficiently activates CD20 and Fas-apoptotic signaling and may be useful for the elimination of malignant B cells. [Cancer Res 2008;68(2):597–604]

Introduction
The tumoricidal activity of the CD20-specific chimeric monoclonal antibody (mAb) rituximab predominately depends on the presence of fully functional immune effector mechanisms in the treated patient, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (1). In addition, part of the tumoricidal activity of rituximab has been attributed to the direct activation of apoptosis via cross-linking of cell surface–expressed CD20 on malignant B cells (2).

Rituximab has markedly improved clinical responses in non–Hodgkin lymphoma (NHL). Unfortunately, ~50% of patients with aggressive B-NHL have primary refractory disease or develop relapses after treatment with rituximab and chemotherapy, whereas the efficacy of rituximab is limited in B cell chronic lymphocytic leukemia (B-CLL) patients (3). Thus, the development of alternative rituximab-based approaches with enhanced tumoricidal activity is warranted, especially toward rituximab-resistant malignant B cells that currently escape from therapy. In this respect, it has recently been shown that rituximab-mediated CD20 apoptotic signaling synergizes the proapoptotic activity of various death receptor agonists. For instance, cotreatment with rituximab and recombinant human sTRAIL synergistically activates apoptosis (4). Analogously, we and others have recently shown that cotreatment with rituximab and an agonistic anti-Fas mAb also synergistically activates apoptosis in malignant B cells (5, 6). Importantly, although clinical use of anti-Fas antibodies is deemed impossible due to liver toxicity (7–9), the potential feasibility of exploiting Fas apoptotic signaling using soluble Fas ligand (sFasL) has recently been reestablished (10, 11).

Homotrimeric sFasL is nontoxic to normal cells but also lacks tumoricidal activity (12). In contrast, sFasL hexamers and secondary aggregated sFasL trimers are highly active toward tumor cells but are also toxic to liver cells (13–15). Thus, to exploit activation of Fas apoptotic signaling for cancer therapy, therapeutic strategies have to meet the prerequisite of strictly localized activation of Fas at the tumor cell surface. This prerequisite can be met by genetic fusion of sFasL to a tumor-selective scFv antibody fragment, yielding a trimeric scFv:sFasL fusion protein (10, 11). Like sFasL trimers, soluble trimeric scFv:sFasL fusion proteins are inactive or poorly active. However, they acquire strong tumoricidal activity after specific binding to a preselected cell surface–expressed target antigen.

Here, we generated a fusion protein comprising a rituximab-derived antibody fragment (scFvRit) genetically fused to sFasL, scFvRitsFasL, with the aim of exploiting and optimizing the recently reported synergy between CD20 and Fas apoptotic signaling. Importantly, scFvRitsFasL activated both CD20 and Fas apoptotic signaling, resulting in a far superior proapoptotic activity compared with cotreatment with rituximab and Fas agonists. scFvRitsFasL potently induced CD20-restricted apoptosis in a panel of malignant B-cell lines and primary patient-derived malignant B cells (two of two NHL and five of six B-CLL). In contrast, scFvRitsFasL lacked activity toward normal human B cells and lacked systemic toxicity in nude mice. In conclusion, scFvRitsFasL efficiently activates CD20 and Fas apoptotic signaling and may be useful for the elimination of malignant B cells.
Materials and Methods

Reagents and mAbs. Actinomycin D (stock solution, 2 mg/mL in ethanol) and FasL-neutralizing mAb Alf2.1 were from Sigma (Sigma-Aldrich Chemie B.V.). The caspase-8 inhibitor zIETD-FMK (stock solution, 10 mg/mL in DMSO) and the total caspase inhibitor zVAD-FMK (stock solution, 10 mg/mL in DMSO) were from Calbiochem (VWR International B.V.). Working solutions of all reagents were prepared by serial dilutions in standard culture medium. Anti–caspase-8 antibody was from Cell Signaling Technology. Anti-Fas mAb 7C11 was from Beckman Coulter. Rituximab is a chimeric mAb, comprising human IgG1-derived constant domains and VH and VL domains derived from the CD20-specific murine mAb 2B8. Rituximab was provided by Roche Nederland B.V. Secondary cross-linking polyclonal goat anti-human (GnHu) antibody was from Jackson Immunoresearch Laboratories. CD19-TRITC was from EUSA, NALM-6, and Raji. CD20-positive B-cell lines PR-1 and Z-138 were gifts of Prof. Dr. Martin Dyer (Medical Research Council Toxicology Unit, University of Leicester). The CD20-negative T-cell line MOLT16 was a gift of Prof. Dr. Martin Gramatzki (Division of Stem Cell and Immunotherapy, University of Leicester). BJAB, Ramos, JY, CA5-I, Jiyoyo, and BJAB-Nexins. Loss of mitochondrial membrane potential (ΔΨm) was analyzed by flow cytometry using the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes) as previously described (11). Immunoblot analysis of caspase-8 activation was performed as described previously (17). Caspase-8 and caspase-3 activities were assessed using luminescent assays Caspase Glo 8 and Caspase Glo 3/7 (Promega Benelux B.V.). Luminescence was quantified using an ELISA plate reader.

Differential quantification of apoptosis in target and bystander cells in mixed culture experiments. CD20-positive target cells were labeled with red fluorescent dye DiI (Molecular protems) by incubating cells (1.0 × 10⁶ cells/mL) for 5 min at 37°C in serum-free medium in-frame linker sequence. In the downstream multiple cloning site of pEEl4, a PCR-truncated 539-bp DNA fragment encoding the extracellular domain of human sFasL was inserted. The upstream multiple cloning site of pEEl4 was used to insert DNA fragment SfiI-scFvRit-NotI after digestion with restriction enzymes SfiI and NotI and standard DNA ligation reaction, yielding plasmid pEEl4-scFvRitsFasl.

Eukaryotic production of scFvRitsFasl. Fusion protein scFvRitsFasl was produced using CHO-K1 cells and the glutamine synthetase selection/amplication system, essentially as previously described (18). Briefly, CHO-K1 cells were transfected with pEEl4-scFvRitsFasl using Fugene-6 reagent (Roche Diagnostics). Transfectant CHO-K1 cells with amplified expression of scFvRitsFasl were subjected to single-cell cloning using a high-speed cell sorter (Cytomation). Subsequently, individual clones were assessed for stable secretion of scFvRitsFasl using a FasL-specific ELISA according to manufacturer’s recommendations (10P’s BVBA) yielding a CHO-K1 production cell line stably secreting 1.3 mg/L scFvRitsFasl. scFvRitsFasl-containing supernatant was harvested by centrifugation (10,000 × g, 10 min), protein was purified using affinity tag purification and size exclusion chromatography was used to isolate homotrimeric scFvRitsFasl.

CD20-specific binding of scFvRitsFasl. CD20-specific binding of scFvRitsFasl was analyzed by incubating CD20-positive BJAB cells (1.0 × 10⁶ cells) with scFvRitsFasl (1.34 μg/mL) in the presence or absence of peptide Rp10-L (20 mmol/L) or parental mAb rituximab (5 μg/mL). CD20-specific binding was analyzed by flow cytometry using PE-conjugated anti-FasL mAb. Incubations were performed for 45 min at 0°C and were followed by two washes with serum-free medium.

CD20-restricted induction of apoptosis by scFvRitsFasl. Tumor cells were treated with 0.25 × 10⁶ per well in a 48-well plate and, unless indicated otherwise, were treated for 16 h with the indicated concentrations of scFvRitsFasl. In the presence or absence of rituximab (5 μg/mL), mAb Alf2.1 (1 μg/mL), zIETD-FMK (10 μmol/L), or zVAD-FMK (10 μmol/L). Assay used to assess apoptosis was Annexin V/propidium iodide staining for phosphatidyl serine exposure on the outer cell membrane; flow cytometric analysis of exposure of phosphatidyl serine on the outer membrane was performed using an Annexin V–FITC/propidium iodide kit (Nexins). Loss of mitochondrial membrane potential (ΔΨm) was analyzed with the cell-permeant green-fluorescent lipophilic dye DiO6C (Molecular Probes) as previously described (11). Immunoblot analysis of caspase-8 activation was performed as described previously (17). Caspase-8 and caspase-3 activities were assessed using luminescent assays Caspase-Glo 8 and Caspase-Glo 3/7 (Promega Benelux B.V.). Luminescence was quantified using an ELISA plate reader.

Differential quantification of apoptosis in target and bystander cells in mixed culture experiments. CD20-positive target cells were labeled with red fluorescent dye DiI (Molecular probes) by incubating cells (1.0 × 10⁶ cells/mL) for 5 min at 37°C in serum-free medium

### Table 1. Patient characteristics and percentage of specific induction of apoptosis by scFvRitsFasl

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Diagnosis</th>
<th>Gender</th>
<th>Leucocytes (×10⁶)</th>
<th>Lymphocytes (%)</th>
<th>CD19+ (%)</th>
<th>CD20+ (%)</th>
<th>CD38</th>
<th>Specific induction of apoptosis by scFvRitsFasl %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NHL</td>
<td>f</td>
<td>5</td>
<td>33</td>
<td>26</td>
<td>26</td>
<td>Positive</td>
<td>58.90%</td>
</tr>
<tr>
<td>2</td>
<td>NHL</td>
<td>f</td>
<td>9</td>
<td>72</td>
<td>68</td>
<td>69</td>
<td>n.d.</td>
<td>19.60%</td>
</tr>
<tr>
<td>3</td>
<td>B-CLL</td>
<td>m</td>
<td>10</td>
<td>67</td>
<td>71</td>
<td>70</td>
<td>Positive</td>
<td>45.10%</td>
</tr>
<tr>
<td>4</td>
<td>B-CLL</td>
<td>m</td>
<td>87</td>
<td>87</td>
<td>84</td>
<td>71</td>
<td>Positive</td>
<td>3.00%</td>
</tr>
<tr>
<td>5</td>
<td>B-CLL</td>
<td>m</td>
<td>50</td>
<td>92</td>
<td>93</td>
<td>88</td>
<td>Negative</td>
<td>35.90%</td>
</tr>
<tr>
<td>6</td>
<td>B-CLL</td>
<td>f</td>
<td>74</td>
<td>90</td>
<td>87</td>
<td>92</td>
<td>Positive</td>
<td>44.80%</td>
</tr>
<tr>
<td>7</td>
<td>B-CLL</td>
<td>m</td>
<td>112</td>
<td>96</td>
<td>87</td>
<td>64</td>
<td>Negative</td>
<td>27.40%</td>
</tr>
<tr>
<td>8</td>
<td>B-CLL</td>
<td>m</td>
<td>110</td>
<td>95</td>
<td>90</td>
<td>49</td>
<td>Positive</td>
<td>50.30%</td>
</tr>
</tbody>
</table>

Abbreviations: f, female; m, male; n.d., not detected.
containing 5 μmol/L DiI, after which cells were washed thrice with standard medium. For experiments, Dil-labeled target and nonlabeled bystander cells were mixed at indicated ratios (final cell concentration of 0.5 × 10^6 cells per well in a 48-well plate). Apoptosis was separately evaluated in target and bystander cells by phosphatidyl serine exposure to the outer cell membrane or by ΔΨm.

Quantification of the effect of cotreatment of cells with rituximab and anti-Fas mAb 7C11. The effect of combination treatment of cells with rituximab and anti-Fas mAb 7C11 was quantified using the cooperativity index (CI). CI was calculated as (sum of apoptosis induced by single-agent treatment) / (apoptosis induced by cotreatment). CI was qualified as follows: CI < 0.9, synergy; 0.9 < CI < 1.1, additive; CI > 1.1, antagonistic.

CD20-restricted induction of apoptosis in patient-derived leukemic cells. Leukocytes from patients with B-CLL (n = 6) and NHL (n = 2) were obtained after informed consent (for individual characteristics, see Table 1). For experiments, lymphocytes were isolated and plated in a 48-well plate at a concentration of 2 × 10^6 cells/mL, after which cells were treated for 16 h with scFvRits:fasL (8 ng/mL). Apoptosis was determined by Annexin V/propidium iodide staining. Specific apoptosis was calculated using the formula (experimental apoptosis — spontaneous apoptosis) / (100 — spontaneous apoptosis) × 100%.

Assessment of in vivo toxicity of scFvRits:fasL. Nude mice were intraocularly injected with scFvRits:fasL (30 μg). Flag-tagged fasL (2.5 μg), secondarily cross-liked with anti-Flag mAb M2 (25 μg) and anti-Flag mAb alone (25 μg). Mice were continuously monitored for changes in behavior. After 24 h, mice were sacrificed, and organs and blood were collected. Isolated blood was used to determine aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels as a measure of tissue damage. Isolated livers were analyzed for caspase-3/caspase-7 activity using the Caspase-Glo 3/7 assay according to manufacturer’s recommendations (Promega). Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities.

Statistical analysis. Data reported are mean values of at least three independent experiments plus SE. P values were determined using two-sided, unpaired Student’s t test. A statistically significant difference was defined as P < 0.05.

Results

CD20-specific binding and induction of apoptosis by scFvRits:fasL. Incubation of CD20-positive BJAB cells with scFvRits:fasL resulted in binding to the cell surface of BJAB cells (Fig. 1 A, 2). Binding of scFvRits:fasL was strongly inhibited by molar excess of peptide Rp10-L, a peptide that neutralizes CD20-specific binding by rituximab (Fig. 1 A, 4). Similar competition data were obtained with rituximab (Fig. 1 A, 4). Of note, the high molar excess of peptide Rp10-L (>500-fold) and rituximab (>5-fold) required to block scFvRits:fasL points to a high avidity–type binding of trimeric scFvRits:fasL to CD20.

Subsequently, the apoptotic activity of scFvRits:fasL was determined on a panel of CD20-positive cell lines and the CD20-negative T-cell line MOLT16. Treatment with low concentrations of scFvRits:fasL potently induced apoptosis in all but one of the CD20-positive cell lines tested (for EC50 concentrations, see Table 2). In contrast, treatment with MOCK-scFv:fasL, targeted at the T-cell marker CD7, showed no or only minimal activity.
to the use of different Fas-agonists (i.e., anti-Fas on the one hand and sFasL on the other hand). Ramos cells were also subjected to cotreatment with rituximab and a control scFvCsFasL fusion protein with specificity for CD19. CD19 has no known or detectable apoptosis-related signaling activity. Consequently, the scFvCD19sFasL fusion protein can be used to exclusively activate Fas-dependent apoptotic signaling in a manner similar to scFvRit:sFasL. Importantly, the apoptotic activity of cotreatment with rituximab and scFvCD19sFasL did not significantly differ from the apoptotic activity observed for rituximab and anti-Fas (Fig. 2A). Moreover, dose-response curves for scFvRit:sFasL and scFvCD19sFasL on Ramos cells revealed a striking difference in their activity (Fig. 2B). ScFvRit:sFasL induced >95% apoptosis (96.9%; Fig. 2B), whereas a 30-fold higher concentration of scFvCD19sFasL only induced ~40% apoptosis (40.2%; Fig. 2B). Taken together, these results strongly point to a quantitatively superior apoptotic activity of scFvRit:sFasL that proceeds via both Fas and CD20.

**ScFvRit:sFasL activates CD20 apoptotic signaling.** Proof for the presence of CD20 apoptotic signaling by scFvRit:sFasL was obtained when various tumor cell lines were treated with scFvRit:sFasL in the presence of the FasL-neutralizing mAb Alf2.1 (Fig. 2C). Treatment of BJAB and Raji cells with scFvRit:sFasL in the presence of mAb Alf2.1 completely abrogated its apoptotic activity (BJAB, P = 0.0002, difference = 80.7 ± 9.8; Raji, P < 0.0001, difference = 46.6 ± 5.8). In contrast, mAb Alf2.1 failed to inhibit induction of apoptosis by scFvRit:sFasL in Ramos and PR-1 cells. In fact, the apoptotic activity of scFvRit:sFasL toward PR-1 cells was significantly increased upon coincubation with mAb Alf2.1 (P < 0.0001, difference = 17.3 ± 2.1). Apparently, induction of apoptosis upon treatment with scFvRit:sFasL and mAb Alf2.1 in Ramos and PR-1 cells proceeds via CD20 signaling, whereas induction of apoptosis by scFvRit:sFasL and mAb Alf2.1 in BJAB and Raji cells results from Fas signaling. Of note, these results do not exclude the presence of Fas or CD20 signaling in the respective cell lines in the absence of mAb Alf2.1.

**scFvRit:sFasL activates dual Fas and CD20 apoptotic signaling.** To assess whether dual activation of CD20 and Fas signaling could occur upon treatment with scFvRit:sFasL, Ramos

### Table 2. Characterization of CD20 and Fas expression and EC50 values of B-cell lines for scFvRit:sFasL

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>CD20 expression</th>
<th>Fas expression</th>
<th>EC50 (scFvRit:sFasL; ng/mL)</th>
<th>EC50 (scFvCD7:sFasL; ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-1</td>
<td>B-cell lymphoma</td>
<td>3,560.7</td>
<td>11.7</td>
<td>6.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BJAB</td>
<td>Burkitt lymphoma</td>
<td>1,712.1</td>
<td>8.2</td>
<td>0.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ramos</td>
<td>Burkitt lymphoma</td>
<td>1,380.2</td>
<td>4.4</td>
<td>2.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>JY</td>
<td>B lymphoblastoid</td>
<td>984.2</td>
<td>28.5</td>
<td>2.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Z-138</td>
<td>Mantle cell lymphoma</td>
<td>881.9</td>
<td>18.8</td>
<td>0.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>DEV</td>
<td>Burkitt lymphoma</td>
<td>496.4</td>
<td>32.9</td>
<td>0.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CA-5I</td>
<td>EBV immortalized</td>
<td>422.8</td>
<td>31.2</td>
<td>2.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Raji</td>
<td>Burkitt lymphoma</td>
<td>347.3</td>
<td>25.9</td>
<td>4.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Sjo</td>
<td>EBV immortalized</td>
<td>335.8</td>
<td>37.9</td>
<td>4.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Nalm-6</td>
<td>Pre-B-ALL</td>
<td>283.8</td>
<td>8.0</td>
<td>1.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Jyoyo</td>
<td>Burkitt lymphoma</td>
<td>38.8</td>
<td>25.4</td>
<td>30.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MOLT-16</td>
<td>T-ALL</td>
<td>—</td>
<td>13.5</td>
<td>&gt;100</td>
<td>3.9</td>
</tr>
</tbody>
</table>

**NOTE:** Expression levels of CD20 and Fas are represented as mean fluorescent intensity. Mean fluorescent intensity values are representatives of three independent binding experiments. For the calculation of EC50 values, listed tumor cell lines were treated with scFvRit:sFasL or scFvCD7:sFasL up to a concentration of 100 ng/mL.

In a direct comparison of scFvRit:sFasL with CD20-signaling induced by rituximab, scFvRit:sFasL showed superior apoptotic activity even when rituximab was secondarily cross-linked to enhance its proapoptotic activity (Ramos, P < 0.0001, difference = −58.3 ± 2.5; BJAB, P < 0.0001, difference = −91.6 ± 5.0; Nalm-6, P = 0.0004, difference = −55.1 ± 7.9; Fig. 1D). Of note, in most cell lines tested, rituximab-mediated apoptosis did not exceed 50% even at a concentration of 20 μg/mL (data not shown).

The apoptotic activity of scFvRit:sFasL is superior to cotreatment with rituximab and anti-Fas. To determine the efficacy of induction of apoptosis by scFvRit:sFasL, we compared scFvRit:sFasL treatment with the recently reported synergistic combination of rituximab and anti-Fas mAb 7C11. Cotreatment of Ramos cells with optimal concentrations of rituximab (5 μg/mL) and mAb 7C11 (2 μg/mL) synergistically induced apoptosis (CI, 0.79; Fig. 2A). Strikingly, treatment with only 5 ng/mL of scFvRit:sFasL induced apoptosis in over 95% of tumor cells, indicative of a quantitatively superior apoptotic activity of this agent (P < 0.0001, difference = −52.7 ± 4.9; Fig. 2A).

To exclude that this difference in apoptotic activity was due to the use of different Fas-agonists (i.e., anti-Fas on the one hand and sFasL on the other hand), Ramos cells were also subjected to cotreatment with rituximab and a control scFvCsFasL fusion protein with specificity for CD19. CD19 has no known or detectable apoptosis-related signaling activity. Consequently, the scFvCD19sFasL fusion protein can be used to exclusively activate Fas-dependent apoptotic signaling in a manner similar to scFvRit:sFasL. Importantly, the apoptotic activity of cotreatment with rituximab and scFvCD19sFasL did not significantly differ from the apoptotic activity observed for rituximab and anti-Fas (Fig. 2A). Moreover, dose-response curves for scFvRit:sFasL and scFvCD19sFasL on Ramos cells revealed a striking difference in their activity (Fig. 2B). ScFvRit:sFasL induced >95% apoptosis (96.9%; Fig. 2B), whereas a 30-fold higher concentration of scFvCD19sFasL only induced ~40% apoptosis (40.2%; Fig. 2B). Taken together, these results strongly point to a quantitatively superior apoptotic activity of scFvRit:sFasL that proceeds via both Fas and CD20.
cells were treated with scFvRit:sFasL. At early time points, scFvRit:sFasL induced typical Fas-associated apoptotic features, including procaspase-8 processing within 3 h and initiator caspase-8 and effector caspase-3 proteolytic activity within 6 h (Fig. 2D). Despite early caspase-8 processing, caspase-8 inhibitor zIETD-FMK or overexpression of the caspase-8 inhibitor cFLIP, did not significantly block apoptosis after 24 h of treatment (Fig. 3A and B, respectively). Similarly, overexpression of effector caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP) failed to inhibit apoptosis induced by scFvRit:sFasL (Fig. 3D). In contrast, overexpression of the mitochondrial antiapoptotic protein Bcl-2 abrogated the activity of scFvRit:sFasL (Fig. 3C).

Subsequent time course experiments with Ramos cells revealed that the apoptotic activity of scFvRit:sFasL was comparable with the activity of scFvCD19:sFasL during the initial 6 h of treatment (open squares and open triangles, respectively; Fig. 3D), most likely as a result of the activation of Fas signaling by both reagents. In contrast, at later time points, scFvRit:sFasL-induced apoptosis proceeded to >90%, whereas induction of apoptosis by scFvCD19:sFasL did not further increase after 6 h and reached a maximum of only 40%. In a similar time-course experiment with Ramos cFLIP, cells, the apoptotic activity of scFvRit:sFasL only became apparent ~6 h after start of treatment (Fig. 3D, solid squares), whereas the apoptotic activity of scFvCD19:sFasL was fully abrogated (Fig. 3D, solid triangles). Together, these results strongly suggest that scFvRit:sFasL can simultaneously activate Fas-apoptotic signaling, apparent within the first 6 h in Ramos cells, and CD20-apoptotic signaling, apparent at later time points.

Of note, treatment with rituximab and anti-Fas or scFvCD19:sFasL was abrogated by caspase-8 inhibition or overexpression of XIAP (Fig. 3A and B), which suggests that Fas-mediated caspase signaling is critical for apoptosis induced by rituximab and anti-Fas, as well as scFvCD19:sFasL.

**scFvRit:sFasL induces potent apoptosis in primary patient-derived tumor B cells.** Treatment of primary malignant B cells of leukemic NHL and B-CLL patients with scFvRit:sFasL resulted in specific induction of apoptosis in two of two NHL and seven of eight B-CLL (Table 2). For the patient-derived B-CLL cells, the median induction of apoptosis by scFvRit:sFasL was 40.4% (interquartile range, 15.2-50.3%). Induction of apoptosis by scFvRit:sFasL was dose dependent (Fig. 4A) and exceeded the induction of apoptosis by scFvCD19:sFasL (P = 0.0083, difference = 30.2 ± 4.8; Fig. 4B). Of note, cell surface expression of CD19 was higher than CD20 (data not shown), suggesting that the difference in activity is not due to preferential or higher binding of scFvRit:sFasL.

**scFvRit:sFasL lacks toxicity in nude mice.** Previously, Samel et al. reported that a homotrimeric scFv:sFasL fusion protein, targeted to the stroma marker FAP, is nontoxic for murine liver in vivo (10). Indeed, treatment of nude mice with a high amount of scFvRit:sFasL (30 A g per mouse) did not reveal any liver toxicity, as evidenced by normal blood AST levels, ALT levels, and a lack of liver caspase-3/caspase-7 activity (Fig. 4C). In contrast, treatment with

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**Figure 2.** scFvRit:sFasL shows superior apoptotic activity compared with cotreatment with rituximab and anti-Fas. A, Ramos cells were treated for 24 h with rituximab, anti-Fas, rituximab + anti-Fas, scFvCD19:sFasL, rituximab + scFvCD19:sFasL, or scFvRit:sFasL. B, Ramos cells were treated with an increasing concentration of scFvRit:sFasL or scFvCD19:sFasL. C, BJAB, Raji, Ramos, and PR-1 cells were treated with scFvRit:sFasL in the presence or absence of FasL-neutralizing mAb Alf2.1. D, Ramos cells were treated for the indicated time points with scFvRit:sFasL (5 ng/mL), after which caspase-8 processing and activation was assessed by immunoblotting for caspase-8 and Caspase-Glo 8 assay and caspase-3 activation with Caspase-Glo 3/7 assay. In A–C, apoptosis was assessed by ΔΨ. **, P < 0.0001; n.s., not significant.
secondarily cross-linked sFasL was associated with severe toxicity in mice with >30-fold increase in AST-levels (P = 0.0027, difference = 1.3 x 10^5 ± 1.9 x 10^4), a >140-fold increase in ALT-levels (P < 0.0001, difference = 1.3 x 10^5 ± 3.7 x 10^3), and a 15-fold increase in caspase-3/caspase-7 activity (P = 0.0065, difference = 37.3 ± 7.2).

**scFvRit:sFasL lacks toxicity toward purified normal B cells and PBLs.** Treatment with rituximab induces a massive depletion of normal B cells (19), which is most likely a result of activation of antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity by the Fc domain of rituximab. Because scFvRitsFasL does not contain an Fc domain but does activate CD20-signaling, we subsequently assessed whether the simultaneous binding to CD20 and Fas induced unwanted apoptotic effects in purified normal CD20-positive B cells. However, scFvRitsFasL did not significantly induce apoptosis in these cells (medium, 15.5%; scFvRitsFasL, 24.0%; Fig. 4D).

Binding of scFvRitsFasL to normal B cells may lead to an innocent bystander effect toward other human blood cells, e.g., T cells. However, treatment of PBLs, containing ~10% B cells, with scFvRitsFasL did not reveal significant apoptotic activity in any of the normal blood cell types assessed (medium, 15.7%; scFvRitsFasL, 18.3%; Fig. 4D).

**Discussion**

Here, we reported on a novel strategy designed to exploit and optimize the synergy between rituximab-induced CD20 and Fas apoptotic signaling by genetically fusing a rituximab-derived antibody fragment to sFasL. Selective and high affinity binding of the resultant scFvRitsFasL fusion protein to CD20 leads to its accretion at the tumor cell surface and to concomitant potent apoptotic activity in B-cell lines and primary NHL and B-CLL patient-derived B-cell tumor cells at low picomolar concentrations. The antitumor activity of scFvRitsFasL depends on binding to cell surface CD20, because cotreatment with parental mAb rituximab strongly inhibited the induction of apoptosis by scFvRitsFasL. Interestingly, there is no clear correlation between the expression levels of either CD20 or Fas and the proapoptotic activity of scFvRitsFasL (Table 1). Thus, although CD20-specific accretion to the tumor cell surface is required for the proapoptotic activity of scFvRitsFasL, the sensitivity of each respective cell line is most likely a result of not only CD20 and Fas expression, but also of the intricate interplay of a variety of intracellular modulators of apoptosis, for instance cFLIPL and proapoptotic or antiapoptotic Bcl-2 family members.

A distinguishing feature of scFvRitsFasL is that it does not only induce Fas apoptotic signaling but also induces apoptosis via a CD20-dependent signaling pathway. The specific binding to CD20 and the simultaneous monocellular or bicellular binding to Fas confers potent CD20 signaling activity to scFvRitsFasL. This is exemplified by the fact that the CD20-mediated signaling of scFvRitsFasL was detected at picomolar concentrations, whereas activation of CD20 signaling by rituximab typically required micromolar concentrations. Although not investigated here, this
efficient CD20 signaling could be a result of scFvRit:sFasL–induced hypercross-linking of CD20. Hypercross-linking of CD20 by a dextran polymer of rituximab has recently been shown to strongly potentiate CD20-mediated induction of apoptosis (20). In line with this hypothesis, the proapoptotic activity of scFvRit:sFasL on PR-1 cells was further increased upon coincubation with mAb Alf2.1. Apparently, secondary cross-linking of PR-1 cells further potentiates CD20 signaling. Such a potentiation of apoptosis is an effect also known to occur upon secondary cross-linking of rituximab (21).

Intriguingly, CD20 apoptotic signaling induced by scFvRit:sFasL did not critically depend on caspase signaling. In contrast, apoptosis by secondarily cross-linked rituximab or by cotreatment with rituximab and anti-Fas did depend on caspase signaling. Apparently, CD20 signaling can vary markedly, depending on the manner of cross-linking, e.g., experimental secondary cross-linking of the Fc domain of rituximab versus cross-linking of CD20 by simultaneous CD20 and Fas binding of scFvRit:sFasL. Based on the fact that caspase activity is not required while Bcl-2 overexpression blocks scFvRit:sFasL activity, it seems likely that DNases released from the mitochondria may be involved in CD20 apoptotic signaling by scFvRit:sFasL.

Primary refractory disease or the development of relapses after rituximab treatment has been attributed to several mechanisms, including intrinsic or acquired resistance to complement-dependent cytotoxicity due to overexpression of CD55 and CD59 (22–24). Because scFvRit:sFasL directly induces apoptosis, which solely requires the cross-linking of CD20 and Fas, scFvRit:sFasL may be particularly relevant for patients with poor or absent complement-dependent cytotoxicity. In addition, treatment with rituximab has been proposed to lead to the development of CD20-negative relapses in some patients, possibly due to tumor cell heterogeneity (25, 26). For scFvRit:sFasL, the reciprocal activation of Fas by CD20-immobilized scFvRit:sFasL opens up the possibility to induce apoptosis in neighboring FasL-sensitive leukemic cells that have lost CD20 expression. In our mixed culture experiments, bystander tumor cells were eliminated at target to bystander ratio as low as 1:99. This potent bystander effect of scFvRit:sFasL may help to prevent the occurrence of CD20-negative relapses.

Initial reports on Fas agonists described severe toxic effects toward murine hepatocytes. More recently, this toxicity has been ascribed to multimers or aggregates of sFasL, whereas homotrimeric sFasL is nontoxic to liver cells. In an initial screening for toxicity, treatment with up to 30 μg of scFvRit:sFasL did not induce liver damage in nude mice, which indicates that homotrimeric scFvRit:sFasL is inactive and nontoxic toward normal cells. Moreover, scFvRit:sFasL was devoid of apoptotic activity toward normal human purified B cells and PBLs in vitro. Obviously, a
future in-depth toxicologic evaluation in, e.g., a human CD20 transgenic animal, as well as pharmacokinetic studies, are required to accurately assess the applicability of scFvRitsFasL.

In conclusion, the simultaneous and synergistic activation of CD20 and Fas apoptotic signaling by scFvRitsFasL provides a novel and promising therapeutic approach for the elimination of malignant B cells.

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Superior Activity of Fusion Protein scFvRit:sFasL over Cotreatment with Rituximab and Fas Agonists

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