Harnessing the Fcμ Receptor for Potent and Selective Cytotoxic Therapy of Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy in need of new, effective, and safe therapies. The recently identified IgM receptor FcμR is overexpressed on malignant B cells in CLL and mediates the rapid internalization and lysosomal shuttling of IgM via its Fc fragment (Fcμ). To exploit this internalization and trafficking pathway for targeted drug delivery, we engineered an IgM-derived protein scaffold (FcμR) and linked it with the cytotoxic agent monomethylauristatin F. This FcμR-drug conjugate was selectively toxic for FcμR-expressing cell lines in vitro and for CLL cells but not autologous normal T cells ex vivo. Notably, the cytotoxic activity of the FcμR-drug conjugate was maintained in CLL cells carrying a 17p deletion, which predicts resistance to standard chemotherapy. Next, we tested the possible therapeutic application of the FcμR-drug conjugate in immunodeficient NOD/SCID/IL-2Rγnull (NSG) mice engrafted with peripheral blood cells from patients with leukemia. Three intravenous injections of the FcμR-drug conjugate over a 10-day period were well tolerated and selectively killed the human CLL cells but not the coengrafted autologous human T cells. In summary, we developed a novel strategy for targeted cytotoxic therapy of CLL based on the unique properties of FcμR. FcμR-targeted drug delivery showed potent and specific therapeutic activity in CLL, thus providing proof of concept for FcμR as a valuable therapeutic target in CLL and for IgM-based antibody–drug conjugates as a new targeting platform. Cancer Res; 74(24); 7510–20. ©2014 AACR.

Introduction

On the basis of their ability to selectively deliver highly cytotoxic drugs to tumor cells, antibody–drug conjugates (ADCs) are among the most promising next-generation antibody therapeutics for cancer therapy (1, 2). The promise of ADCs is the targeted delivery of a potent cytotoxic drug selectively into tumor cells, thereby minimizing toxicity toward normal cells. The Food and Drug Administration (FDA) approval of brentuximab vedotin for the therapy of Hodgkin lymphoma and anaplastic large cell lymphoma in 2011 and of trastuzumab emtansine for HER2 metastatic breast cancer in 2013 were milestones that established the therapeutic utility of ADCs (3, 4). Brentuximab vedotin consists of a chimeric mouse/human anti-human CD30 monoclonal antibody (mAb) in IgG1 format as the carrier protein conjugated to monomethylauristatin E (MMAE) as the cytotoxic payload (5). MMAE is a synthetic antitubulin agent active at subnanomolar concentrations. Each MMAE drug is linked to the antibody molecule through a linker that harbors a valine–citrulline–para-aminobenzylcarbamate (Val–Cit–PABC) linker that is cleaved by lysosomal proteases such as cathepsin B. The linker also contains a maleimide group that reacts with thiol groups in the IgG1 hinge region. This random conjugation results in an ADC mixture with an average drug-to-antibody ratio (DAR) of 4±1 (range, 0.1 to 8.1). Trastuzumab emtansine is based on the humanized anti-human HER2 mAb trastuzumab randomly conjugated to an average of 3.5 maytansinoid drugs through the ε-amino group of lysine (Lys) using a noncleavable linker (6). This new generation of ADCs has demonstrated two important treatment advances; first, patients who relapse or are refractory to first-line therapy can be rescued using targeted cytotoxic drug delivery; and second, an ADC can replace systemic cytotoxic therapy demonstrating higher efficacy with lower toxicity (7, 8).

Chronic lymphocytic leukemia (CLL), the most common leukemia in Western countries, is characterized by the
accumulation of mature monoclonal B cells in the blood, bone marrow, spleen, and lymph nodes. The median age at diagnosis is 75 years, precluding use of allogeneic hematopoietic stem cell transplantation, the only curative treatment option, in the majority of patients. Current first-line treatment is chemoinmunotherapy with an anti-CD20 mAb and an alkylating agent with or without the addition of a purine analog (9–11). One of the most commonly used regimens for younger patients is the combination of fludarabine, cyclophosphamide, and rituximab (FCR; refs. 11, 12). Although initial response rates are high, most patients relapse. FCR is less active in high-risk disease, less tolerable in elderly or frail patients, and increases the risk of infections. Fludarabine and cyclophosphamide are also toxic for normal T cells and myeloid cells, leading to often long-lasting cytopenias. In contrast, the anti-CD20 mAbs rituximab, ofatumumab, and obinutuzumab selectively target B cells (13). These mAbs induce cell death mostly through immunologic effector mechanisms such as complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (13, 14). As single agents, the efficacy of anti-CD20 mAbs in CLL is limited and they are used primarily in combination regimens. Notably, CD20 is expressed at higher levels on normal B cells than on CLL cells (15), and anti-CD20 mAbs efficiently kill normal B cells, potentially contributing to life-threatening viral, bacterial, and fungal infections (16). Recently, kinase inhibitors that target B-cell receptor (BCR) signal transduction have shown impressive clinical activity in CLL (17–19). However, while well tolerated as single agents, these inhibitors achieve mostly partial responses and have to be taken continuously. In fact, even after years of treatment with a kinase inhibitor, residual disease is easily detected in virtually all patients and resistant clones emerge that lead to relapse. Thus, there is a great clinical need for targeted cytotoxic agents that could be combined with kinase inhibitors to eradicate the disease.

ADCs are also currently being tested as treatment options for CLL. A phase II clinical trial (NCT01461538) is investigating brentuximab vedotin in CD30+ malignancies, including CLL. In addition, a recently launched phase I clinical trial (NCT01290549) is based on an analogous ADC that targets CD79B of the BCR complex in non-Hodgkin lymphoma and CLL. Notably, none of the cell-surface antigens targeted by ADCs in clinical trials are overexpressed in CLL. On the contrary, CD79B was not detected on the tumor cells of 43% of the patients with CLL tested, while it is expressed on normal B cells (20), and CD30 is expressed at higher levels on activated normal B and T cells compared with CLL cells (21, 22). Thus, the current panel of clinically investigated ADCs for CLL does not bypass immunosuppression, underscoring the need for pursuing new ADCs that selectively target CLL.

The Fc receptor for IgM (FcμR), also known as TOSO or Fas apoptotic inhibitory molecule 3 (FAIMS), is highly expressed on CLL cells (23–26). FcμR is an approximately 60-kDa type I single-pass transmembrane protein whose expression in normal human cells and tissues is virtually restricted to the lymphoid lineage (23, 27). FcμR-deficient mice are viable and exhibit normal development (28–30). However, they have reduced numbers of marginal zone B cells (29), and FcμR-deficient B cells are less responsive to BCR stimulation and more readily undergo apoptosis. FcμR is overexpressed on the CLL B cells compared with B cells from normal donors and compared with the nonclonal T cells in the blood of patients with CLL. FcμR may play a role in the pathogenesis of CLL, possibly by contributing to concomitant BCR and Toll-like receptor (TLR) activation that could enhance leukemic cell proliferation and survival (26, 31, 32). However, exactly what functional role, if any, FcμR has in the pathogenesis of CLL remains to be defined.

Fc receptors are highly effective in targeting specific molecules for binding to and internalization into select target cells. The existence of a variety of distinct Fc receptors with restricted cellular expression provides the basis for selective targeting approaches. For example, FcγRIII on dendritic cells has been used for internalization of vaccine components (33). However, delivering cytotoxic agents through Fc receptor–mediated internalization as anticancer therapy has not been established. In previous work, we have shown that FcμR on CLL cells is functional, rapidly internalizes IgM, and delivers it to the lysosome where it is degraded (26). In fact, more than 50% of IgM bound to the CLL cell surface is internalized via FcμR within 1 minute, and internalization is complete by 5 minutes. We hypothesized that the overexpression of FcμR on CLL cells and its ability to internalize bound IgM could be used for targeted delivery of antileukemic therapy.

Here, we describe the derivation of an FcμR-targeting strategy that can deliver a cytotoxic payload selectively into CLL cells and show that it has antitumor activity against primary human tumor cells in vivo. On the basis of the work by Kuhagawa and colleagues (23), we knew that binding of IgM to FcμR critically depends on the integrity of sequences in the Fc region of IgM (Fcμ) in particular the Cμ2–4 domains. We therefore engineered a protein scaffold consisting of Fcμ and chose a site-directed conjugation technology we have previously developed (34, 35). In this approach, the introduction of a C-terminal selenocysteine (Sec) makes site-specific as opposed to random conjugation of the payload possible, thereby preserving the structure of critical protein domains.

Materials and Methods

Supplementary Materials and Methods describe the (i) cloning, expression, and purification of Fcμ-Sec; (ii) conjugation of Fcμ-Sec; (iii) SDS-PAGE, Western blotting, and gel filtration chromatography; (iv) synthesis of MMAF compounds 1a and 1b; and (v) comparison of treatment naïve and previously treated patients.

Primary cells and cell lines

With written informed consent, peripheral blood was collected from patients with treatment-naïve CLL at the Clinical Center, National Institutes of Health (Bethesda, MD; www.clinicaltrials.gov identifier NCT00923507). Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation (Lymphocyte Separation Medium; MP Biomedicals) and cultured in serum-free AIM-V medium (Life Technologies). Mantle cell lymphoma (MCL) cell lines Mino, JeKo-1, and HBL-2 were grown in RPMI-1640 medium (Life Technologies) and HeLa cells in DMEM (Lonza) supplemented with 10% (v/v)
fetal bovine serum (FBS; HyClone), 2 mmol/L l-glutamine, 1,000 U/mL penicillin G, and 100 mg/mL streptomycin (all from Life Technologies).

Confocal immunofluorescence microscopy

We monitored Fcγ–Sec internalization as previously described (26). HeLa cells stably transfected with FcγR were grown on coverslips, incubated for 15 minutes at 4°C with Cj3–Cj4–Sec, washed with ice-cold PBS, and incubated at 37°C with DMEM (Lonza) for 30 or 120 minutes. Cells were then fixed with 3% (w/v) paraformaldehyde (Electron Microscopy Sciences), washed with PBS, and incubated in Staining Buffer (0.05% (w/v) saponin, 10 mmol/L glycine, and 5% (v/v) PBS in PBS) for 15 minutes. Cells were costained with rabbit anti-human LAMP-1 polyclonal antibodies (pAb; Abcam) for 1 hour, washed twice with PBS, and incubated with donkey anti–human Fcγ pAbs conjugated to DyLight 488 and donkey anti–rabbit IgG pAbs conjugated to Cy5 (all from Jackson Immunoresearch Laboratories). Cells were then washed twice with PBS and labeled for 5 minutes at room temperature with 1 μg/mL Hoechst 33258 (Life Technologies) diluted in Staining Buffer. Subsequently, coverslips were washed twice with PBS, mounted with Fluoromount-G (SouthernBiotech), and visualized by confocal microscopy. Images were acquired using a Leica TCS SP5 laser scanning confocal microscope (LAS AF software) using the HCX PLAPO 63X objective (numerical aperture, 1.4). Images were processed with Adobe Photoshop and analyzed using the same settings.

Flow cytometry

Mino, JeKo-1, and HBL-2 cells were stained with Cj3–Cj4–Sec/DyLight 488 or Cj2–Cj3–Cj4–Sec/DyLight 488. FcγR expression in cryopreserved PBMC samples from 30 patients with CLL was measured with a mouse anti-human FcγR mAb (Abnova) and a FITC-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich) as previously described (26). B cells and normal T cells were distinguished with an APC-conjugated antibody (Sigma-Aldrich) as previously described (26). B cells and normal T cells were distinguished with an APC-conjugated antibody (Sigma-Aldrich) as previously described (26). B cells and normal T cells were distinguished with an APC-conjugated antibody (Sigma-Aldrich) as previously described (26). B cells and normal T cells were distinguished with an APC-conjugated antibody (Sigma-Aldrich) as previously described (26).

Ex vivo cytotoxicity assays

Cytotoxicity toward primary malignant B cells and primary normal T cells ex vivo was measured by flow cytometry using TO-PRO-3 and Annexin V staining. Briefly, cryopreserved PBMC samples from 30 different patients with CLL (5 × 10^5 cells in 100-μL serum-free AIM-V medium) were incubated with 100 nmol/L compound 1a, Cj2–Cj3–Cj4–Sec/compound 1b conjugate, Cj2–Cj3–Cj4–Sec alone, or were left untreated for 1 hour at 37°C, washed and then cytotoxicity against malignant B cells and normal T cells was evaluated by flow cytometry using PE-conjugated Annexin V, a PE–Cj5–conjugated mouse anti-human CD19 mAb, a FITC-conjugated mouse anti-human CD3 mAb (all from BD Biosciences), and TO-PRO-3 (Invitrogen) according to the manufacturer’s instructions.

Circulatory half-life

Two groups of two NOG/SCID/IL-2Rγnull (NSG) mice (JAX strain 5557; The Jackson Laboratory) were injected i.v. (tail vein) or i.p. with 5 mg/kg of the Fcγ–carrier protein (Cj2–Cj3–Cj4–Sec). One control mouse was injected with PBS. Blood was collected via the tail vein at 0.5, 24, 48, and 72 hours after injection and serum was isolated. Sera were diluted 25-fold in 1% (w/v) BSA in PBS and analyzed by sandwich ELISA. To do this, 100 ng of rabbit anti-human Fcγ pAbs (Jackson Immunoresearch Laboratories) was coated on a 96-well Costar 3690 plate (Corning). After blocking with 3% (w/v) BSA in PBS, the diluted sera or purified Cj2–Cj3–Cj4–Sec as standard were added to duplicate wells, washed 10 times with PBS, and incubated with a mouse anti-His, mAb conjugated to horseradish peroxidase (GenScript). All steps were carried out for 1 hour at 37°C. The plate was washed 10 times with PBS and colorimetric detection was performed using 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS; Roche) as substrate according to the manufacturer’s instructions. Absorbance values were measured at 405 nm using a Victor3 plate reader (PerkinElmer).

NSG/CLL xenograft model

The NSG/CLL xenograft model was first introduced by Bagnara and colleagues (36). We used a modified protocol established in our laboratory that replicates important aspects of CLL biology and that we have successfully used in drug studies (37). Mice were housed and handled in accordance with the guidelines set by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute (Bethesda, MD). All animal experiments were carried out on an approved animal protocol. Thirty NSG mice were preconditioned with an i.p. injection of 25 mg/kg busulfan (Otsuka America Pharmaceutical) and on the following day (day 1) i.v. injected with 5 × 10^7 PBMC from 1 out of 4 patients with CLL. All mice were bled on day 4 and PBMC were isolated by gradient centrifugation using Lymphocyte Separation Medium. On day 4, 6, and 8, different cohorts of mice were i.v. injected with 10 mg/kg Cj2–Cj3–Cj4–Sec carrier protein or Cj2–Cj3–Cj4–Sec/compound 1b conjugate or an equal volume of PBS. On day 11, retro-orbital puncture bleeds and spleens were harvested from all mice. PBMC were isolated as above. Spleens were
homogenized using the gentleMACS Dissociator (Miltenyi Biotec). The homogenate was filtered through a 70-μm nylon cell strainer (BD Biosciences) and washed with ACK Lysing Buffer (Quality Biological) to remove erythrocytes and platelets. PBMC and splenocytes were stained with a mouse anti-human CD45 mAb conjugated to PE-Cy5, a mouse anti-human CD3 mAb conjugated to FITC, PE-conjugated Annexin V (all from BD Biosciences), and TO-PRO-3 (Life Technologies), and then analyzed by flow cytometry as described above. Normalized titers of human lymphocyte populations in PBMC isolated on days 4 and 11 were determined with 5.0- to 5.9-μm AccuCount-Blank Particles (Spherotech) according to the manufacturer’s instructions.

Results

**Generation and characterization of Fcµ–Sec**

To be able to target a payload to FcµR, we first generated IgM-derived protein scaffolds consisting of two (Cµ3–Cµ4) or three (Cµ2–Cµ3–Cµ4) of the C-terminal constant domains of human IgM. In the expression plasmids, the Fcµ-encoding sequence was followed by a TGA stop codon, a sequence directing a hexa-histidine (His6) tag, a TAA stop codon, and finally a downstream Sec incorporation sequence (SECIS). In the presence of SECIS, the C-terminal codon instructs the translational insertion of Sec (Supplementary Fig. S1A), with a diminished propensity to form stable and defined multimers (Supplementary Fig. S1B and data not shown). Indicating successful incorporation of Sec (35), the His6 tag was detected in both purified Fcµ–Sec proteins (Supplementary Fig. SIC).

Binding, internalization, and trafficking of human IgM by HeLa cells transiently transfected with human FcµR cDNA was previously shown by flow cytometry and confocal immunofluorescence microscopy (26). We confirmed that purified Cµ3–Cµ4–Sec selectively bound to FcµR-expressing, but not wild-type HeLa cells, followed by rapid internalization and

Figure 1. Fcµ–Sec engineering and structural formulas of auristatin F derivatives. Schematic of the Fcµ–Sec expression cassettes, which encode two (A) or three (B) C-terminal constant domains of the heavy chain of human IgM (gray). A C-terminal Sec (red) followed by a His6 tag (black) was introduced by combining a TGA stop codon with a 3′-untranslated region (3′-UTR) that contains a SECIS element. The dominant product of these expression cassettes is pentameric and hexameric (shown) proteins with a single Sec–His6–displaying C-terminus. C, we synthesized a tertiary butyl ester of the tubulin polymerization inhibitor MMAF (compound 1a) and its maleimidocaproyl derivative (compound 1b) for site-specific conjugation to the unique selenol group in Fcµ–Sec.

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trafficking to lysosomes (Fig. 2). Thus, as described previously (23), the Fc fragment of IgM is sufficient for FcµR binding, internalization, and trafficking. We also confirmed the binding of both purified Fcµ–Sec proteins to the FcµR-expressing MCL cell line, Mino, using Fcµ–Sec proteins that we labeled in a Sec-dependent reaction with a maleimide derivative of DyLight 488. Two other MCL cell lines, JeKo-1 and HBL-2, which do not express FcµR, did not bind Fcµ–Sec/DyLight 488 (Fig. 3A and data not shown).

**Generation and in vitro investigations of Fcµ–drug conjugate**

As the cytotoxic payload, we chose the tubulin polymerization inhibitor monomethylauristatin F (MMAF). MMAF differs from MMAE (the drug component of brentuximab vedotin) by having a C-terminal phenylalanine (40). As free drug control, we synthesized a tertiary butyl ester of MMAF (MMAF–tBu; compound 1a), which freely diffuses into cells through the plasma membrane (40), and for Fcµ–Sec conjugation, we synthesized a maleimide derivative with a stable alkyl linker (maleimidocaproyl–MMAF–tBu; compound 1b; Fig. 1C).

To test the potency and specificity of free versus conjugated drug, we used MCL cell lines Mino (FcµR-positive) and HBL-2 (FcµR-negative; Fig. 3A). After exposure for 1 hour and chase incubation for 71 hours, the free drug potently killed both Mino and HBL-2 cells with IC₅₀ values of 0.26 and 1.35 nmol/L, respectively (Fig. 3A). As expected, the Fcµ–drug conjugate was less potent than the freely diffusing drug. Under the same conditions, the Fcµ–Sec/MMAF conjugate killed Mino and HBL-2 cells with IC₅₀ values of 43 nmol/L and 223 μmol/L, respectively (Fig. 3B). Thus, FcµR-positive Mino cells were approximately 5,200 times more sensitive to targeted MMAF than FcµR-negative HBL-2 cells. Taking the different sensitivities to free drug into account, we observed a 1,000-fold increase in potency when FcµR, the target of the carrier protein, was present.

**Ex vivo and in vivo investigations of Fcµ–drug conjugate**

Next, we tested whether the Fcµ–Sec/MMAF conjugate can selectively kill primary CLL cells ex vivo. FcµR expression on cryopreserved PBMC from 30 patients with CLL (Supplementary Table S1) was measured by flow cytometry. In patients with CLL, there are very few or no normal B cells in circulation; therefore, >99% of CD19⁺ cells are CLL cells (31). As expected, virtually all CLL cells (CD19⁺) and much fewer T cells (CD3⁺) expressed FcµR (Fig. 4). These findings are consistent with previously published observations that FcµR is overexpressed in CLL and that some T cells, especially when activated, can also express FcµR (23), albeit at low cell-surface levels (24). FcµR expression was equally high in treatment-naïve and previously treated patients (Supplementary Fig. S4), which indicates for wide clinical applicability of the FcµR-targeting strategy.

The same 30 CLL PBMC samples were then treated in parallel with 100-nmol/L free MMAF or the Fcµ–Sec/MMAF conjugate.
conjugate for 1 hour, washed, and then analyzed for cell death 47 hours later by flow cytometry using TO-PRO-3 and Annexin V staining. Clearly, malignant B cells were effectively killed by both free and targeted MMAF, whereas normal T cells were only killed by free MMAF (Fig. 5A). In fact, the average viability of CLL B cells was reduced from >90% in controls to <20% in Fcµm–drug–treated cultures, while T-cell viability was unchanged. The Fcµm–Sec carrier protein alone did not affect cell viability. This exquisitely selective cytotoxicity and the stark differential expression of FcµR between malignant B cells and T cells provide substantial support to the idea that Fcµ–drug conjugates can be used to selectively deliver cytotoxic payloads to target cells without harming bystander cells (Fig. 5B). The Fcµ–drug conjugate was equally effective against tumor cells with high-risk molecular features (expression of an unmutated IGVH gene and/or a deletion of chromosome 17p) and those without these characteristics (Fig. 5C and D).

In preparation for the investigation of the Fcµ–drug conjugate in vivo, we assessed the circulatory half-life of the Fcµm–Sec carrier protein in NSG mice. After i.v. or i.p. injection of 100 mg (5 mg/kg) Fcµ–Sec in 100-μL isotonic saline, the serum

Figure 3. In vitro cytotoxicity of Fcµ–drug conjugate. A, FcµR is expressed in MCL cell line, Mino, but not HBL-2, as previously shown (26). Western blot analysis of cell lysates probed with anti-FcµR and p97 for loading control (inset). Viability of Mino (red circles) and HBL-2 cells (purple squares) exposed to free MMAF compound 1a was determined using a colorimetric assay. B, the same experiment was carried out with MMAF compound 1b conjugated to C6–C7–C8–Sec (Fcµ–Sec/drug).

Figure 4. Cell-surface expression of FcµR on PBMC samples from patients with CLL. PBMC samples from 30 untreated patients with CLL were analyzed for cell-surface expression of FcµR by flow cytometry. A, histograms comparing FcµR expression (red) by CD19+ malignant B cells (right) and autologous CD3+ T cells (left) of a representative sample. The percentages of FcµR+ cells are indicated in red. The isotype control is shown in blue. B, the specific expression of FcµR by CD3+ T cells and CD19+ malignant B cells is plotted for all 30 PBMC samples (for color-code and patient characteristics; see Supplementary Table S1). A paired two-tailed t test was used to calculate P.
and the other half were left untreated. On day 11, mice were i.v. injected with 10 mg/kg FcSec (Supplementary Table S1). On days 4, 6, and 8 half of the mice were injected with cells from 1 out of 4 different patients with CLL tumor cells, and up to 10% T cells). Each set of mice was studied using the FcµSec/drug conjugate. Cell viability was determined by flow cytometry using TO-PRO-3 staining. A, Fcµ-Sec/drug conjugate-mediated cytotoxicity of CD19+ malignant B cells (left) and autologous CD3+ T cells (right) in PBMC samples from patients with CLL (Supplementary Table S1). Paired two-tailed t tests were used to calculate P. B, cytotoxicity of the Fcµ-Sec/drug conjugate and expression of FcµR are highly correlated (Spearman correlation, r = 0.8431 and P < 0.0001). C and D, the Fcµ-Sec/drug conjugate is equally effective against tumor cells with adverse prognostic features. C, comparison of cytotoxicity against cells with or without a deletion of chromosome 17p (del17p and no del17p, respectively). D, comparison of cytotoxicity against cells of the IGHV-mutated (M-CLL) and of the IGHV-unmutated CLL subtype (U-CLL).

Figure 5. Ex vivo cytotoxicity of Fcµ–drug conjugate. PBMC from 30 patients with CLL were left untreated (control) or treated with (i) 100 nmol/L Cyt2–Cyt3–Cyt4–Sec (Fcµ–Sec), (ii) 100 nmol/L free MMAF compound 1a (free drug), or (iii) 100 nmol/L MMAF compound 1b conjugated to Cyt2–Cyt3–Cyt4–Sec (Fcµ–Sec/drug). Cell viability was determined by flow cytometry using TO-PRO-3 staining. A, Fcµ–Sec/drug conjugate-mediated cytotoxicity of CD19+ malignant B cells (left) and autologous CD3+ T cells (right) in PBMC samples from patients with CLL (Supplementary Table S1). Paired two-tailed t tests were used to calculate P. B, cytotoxicity of the Fcµ–Sec/drug conjugate and expression of FcµR are highly correlated (Spearman correlation, r = 0.8431 and P < 0.0001). C and D, the Fcµ–Sec/drug conjugate is equally effective against tumor cells with adverse prognostic features. C, comparison of cytotoxicity against cells with or without a deletion of chromosome 17p (del17p and no del17p, respectively). D, comparison of cytotoxicity against cells of the IGHV-mutated (M-CLL) and of the IGHV-unmutated CLL subtype (U-CLL).

concentration was measured by a sandwich ELISA. Regardless of the route of injection, the peak serum concentration ranged between 8 and 10 µg/mL and the circulatory half-life was approximately 18 hours (Supplementary Fig. S2). In comparison, the reported circulatory half-life of mouse IgM in mice ranges between 24 and 30 hours (41). Thus, despite their differences in molecular weight and composition, FcSec and IgM share similar circulatory half-lives.

We chose the recently described NSG/CLL xenograft model to evaluate the activity of the Fcµ–drug conjugate in vivo. In these immunodeficient mice, xenografted human CLL cells and the coinjected autologous T cells engraft in the murine blood and spleen (36, 37). Thirty NSG mice were preconditioned with busulfan and on the following day (day 1) i.v. injected with 5 × 10^7 PBMC (typically composed of ~90% tumor cells, and up to 10% T cells). Each set of mice was injected with cells from 1 out of 4 different patients with CLL (Supplementary Table S1). On days 4, 6, and 8 half of the mice were i.v. injected with 10 mg/kg Fcµ–Sec/MMAF conjugate and the other half were left untreated. On day 11, mice were terminally bled by retro-orbital puncture and spleens were harvested. PBMC prepared from mouse blood before (day 4) and 72 hours after the last injection of the Fcµ–drug conjugate (day 11) were stained with anti-human CD45 (to identify human cells), CD19, and CD3 mAbs to distinguish malignant B cells (CD45+CD19+) and normal T cells (CD45+CD3+). The viability of the xenografted human cells was assessed by flow cytometry using Annexin V and TO-PRO-3 staining.

To quantify the reduction in tumor burden on treatment with the Fcµ–Sec/MMAF conjugate, we measured a number of different endpoints. First, we determined the leukemic cell count in the blood using flow cytometry and counting beads. There was a substantial decrease in circulating malignant B cells in treated mice compared with controls (Fig. 6A). The average leukemic cell count over the treatment period of 1 week decreased by 74.4 ± 6.6 compared with untreated mice (P < 0.0001). In contrast, there was no change in the number of circulating T cells (Fig. 6A). To be able to quantify the tumor burden in the tissue, we determined the number of live malignant B cells relative to the total number of nucleated...
cells in single-cell suspensions prepared from spleens. The majority of these nucleated cells are murine cells that have been used successfully as internal reference in other studies (36, 37). The tumor burden in spleens decreased on average by $64.9 \pm 9.7$% on treatment with the Fcµ–Sec/MMAF conjugate ($P < 0.0001$) but again there was no effect on T-cell numbers (Fig. 6B). The unconjugated Fcµ–Sec carrier protein alone had no effect on the viability of CLL cells nor T cells (Supplementary Fig. S3A and S3B). Taken together, these in vivo studies revealed substantial reductions of the total tumor burden in both blood ($\sim 74\%$) and spleen ($\sim 65\%$) of mice treated with Fcµ–drug conjugate (Fig. 6C). Consistent with the desired selectivity of this targeted approach, there was no effect on T cells neither in blood nor spleen (Fig. 6C). Notably, none of the mice showed signs of toxicity, and the viability of murine blood cells was not reduced in treated as compared with control mice (data not shown).

Discussion

FcµR is overexpressed in CLL and mediates the rapid internalization of IgM by malignant B cells and its trafficking to lysosomes (24–26). In this study, we used an Fcµ–drug conjugate to exploit FcµR for targeted drug delivery. In contrast to conventional ADCs, our targeting platform is a recombinant protein scaffold designed to mimic binding of the natural ligand and not a mAb. In other respects, our Fcµ–drug conjugate is built on important design principles shared with the recently FDA-approved ADCs brentuximab vedotin and trastuzumab emtansine (1–8). Specifically, the target antigen FcµR is overexpressed on tumor cells and rapidly internalized upon ligand binding; the Fcµ carrier protein was engineered with a C-terminal Sec residue for site-specific conjugation to the potent antitubulin agent MMAF; finally, use of a noncleavable linker that can minimize systemic drug release is possible because internalized FcµR travels to the lysosome where the carrier protein is degraded releasing the cytotoxic payload. The resulting Fcµ–Sec/MMAF conjugate selectively killed FcµR-expressing malignant B cells in vitro, ex vivo, and in vivo. Accordingly, our study provides proof-of-concept for FcµR as a therapeutic target in CLL and for Fcµ carrier proteins as new targeting devices.

As the carrier protein, we tested two formats of the Fc fragment of IgM carrying a C-terminal Sec residue. Both Cµ3–Cµ4–Sec and Cµ2–Cµ3–Cµ4–Sec were expressed, purified, and conjugated in high yield, and both mediated selective drug delivery in vitro. However, for the subsequent ex vivo and in vivo studies, we focused on the larger Cµ2–Cµ3–Cµ4–Sec, as it more closely resembled IgM with respect to the formation of stable pentamers and hexamers, which are required for high-affinity binding to the target molecule (23). Despite their differences in molecular weight and composition, Cµ2–Cµ3–

Figure 6. In vivo cytotoxicity of the Fcµ–Sec/drug conjugate. Thirty NSG mice were injected i.v. on day 1 with $5 \times 10^7$ PBMC from four different patients with CLL and treated on days 4, 6, and 8 with 10 mg/kg Fcµ–Sec/drug conjugate in PBS or with PBS alone (control) by i.v. injection. On day 11, the absolute numbers of live (TO-PRO-3–negative) CD19+ malignant B cells and autologous CDS+ T cells in blood and spleen were quantified by flow cytometry. Two-way ANOVA was used to compare cell numbers in treated and control mice. A, the leukemic cell count in the blood of the xenografted NSG mice was significantly reduced by Fcµ–Sec/drug, while there was no change in T-cell numbers. B, live CD19+ malignant B cells relative to the total number of live nucleated cells in the spleen were also greatly reduced by Fcµ–Sec/drug. Again, T cells were unchanged. Note that while the human cells engraft in the spleen, the majority of nucleated cells are of murine origin. C, the column graph summarizes the effect of the Fcµ–Sec/drug conjugate on tumor burden (left) and T cells (right). Shown is the mean ± SEM.
Cμ4–Sec and IgM shared similar circulatory half-lives (~1 day), which are relatively short compared with IgG (>7 days), but long compared with scFv and other antibody fragments (<1 hour). Although providing sufficient time for on-target toxicity, a circulatory half-life of approximately 1 day may diminish off-target toxicity. We conclude that the pharmacokinetic properties of Cμ2–Cμ3–Cμ4–Sec are suitable for targeted drug delivery.

The rationale for using our Sec technology (34, 35) was to enable site-specific as opposed to random attachment of the drug payload. Site-specific conjugation minimizes interference with functional domains of the Fcμ scaffold and has been shown to increase the therapeutic index compared with conventional drug conjugation strategies (42, 43). Given a Sec insertion rate of approximately 10% (35), the dominant fraction of the purified Fcμ–Sec pentamers and hexamers contains one C-terminal Sec residue, affording a drug-to-carrier ratio of 1:1. Although this proved to be sufficient for killing malignant B cells in vitro, ex vivo, and in vivo, it is lower than the ratio of 4:1 considered ideal for ADCs generated using non–site-specific techniques (2). Although the lower drug-to-carrier ratio may decrease the potency of the current Fcμ–drug conjugate, improvements in site-specific conjugation technologies that increase the number of drug molecules per carrier could further increase the potency of the Fcμ–drug conjugate. For example, we have recently shown that proteins carrying two C-terminal Sec residues can be conjugated with two drug molecules (44).

The linker between carrier protein and payload has to be stable to minimize systemic toxicity while at the same time ensuring effective intracellular drug release. Drug release from noncleavable linkers, such as the alky linker, used here and in trastuzumab emtansine requires antibody degradation within lysosomes (45). The use of this technology in the targeting of FcμRl is made possible by the effective delivery of the internalized complex to the lysosome where it is degraded (26, 46).

FcμR is the only Ig receptor that exclusively binds IgM (23). In addition, the polymeric Ig receptor (PIGR) and the FcεRI/α receptor (FCAMR) bind and internalize both IgM and IgA. Whereas binding of IgA and IgM to PIGR requires the J chain, which is not present in our Fcμ–drug conjugates, FCAMR can mediate IgA and IgM binding in the absence of the J chain (47, 48). As is the case for FcμR, the expression of FCAMR is virtually restricted to specialized immune cells, including follicular B cells and follicular dendritic cells of germinal centers (49). Nonetheless, an assessment of on-target and off-target toxicities of Fcμ–drug conjugates in preclinical and clinical investigations will have to take both FcμR and FCAMR expression by normal cells into consideration.

For the in vivo studies, we chose a recently established adoptive transfer model of human primary PBMC from patients with CLL injected into NSG mice, which recapitulates key aspects of tumor biology as seen in patients (36, 37). Both malignant B cells and the corresponding autologous T cells of the patient engraft in the blood and spleen of the mouse, the latter demonstrating tumor cell aggregates reminiscent of human lymph nodes. Thus, the model allows testing of potency and selectivity of the Fcμ–drug conjugate in a partially humanized environment. Furthermore, variability in tumor biology among individual patients is, at least partially, reproduced by injecting cells from different patients into separate cohorts of mice. A caveat of this model is that the survival of the mice is not determined by tumor progression and that over time T-cell expansion dominates and can lead to the demise of the animals. We therefore chose the impact of the Fcμ–drug conjugate on tumor burden as the clinical endpoint.

Here, we demonstrated effectiveness and selectivity of Fcμ–drug conjugates by comparing the effect on malignant B cells and normal T cells from patients with CLL side-by-side ex vivo and in vivo. With only three injections of the Fcμ–Sec/MMAF over 1 week, we consistently obtained objective responses quantified as >60% reduction in tumor burden. Notably, the tumor samples studied here were primarily from patients with high-risk disease characterized by advanced Rai stage and the expression of unmutated IGHV genes, an indicator of more rapid disease progression and reduced benefit from standard treatment. The potent activity of Fcμ–Sec/MMAF against tumor cells from these high-risk patients is promising. Using this partially humanized xenograft model, we could also verify a degree of selectivity in vivo; while CLL B cells were killed, we did not observe a decrease in the viability of the autologous T cells. This is consistent with the fact that the malignant B cells overexpress the FcμR while the normal T cells of the patients with CLL as well as the normal B and normal T cells of healthy donors have considerably lower expression levels (24, 26). This suggests that FcμR targeting with ADCs will be less damaging to normal cells and tissues than current treatment options in CLL.

Collectively, our study provides proof-of-concept for the therapeutic targeting of the recently identified FcμR with a novel IgM-derived protein–drug conjugate. We established the utility of lead components and compositions of Fcμ–drug conjugates that provide opportunities for further optimization of FcμR-targeted drug delivery and translation of this approach into the clinic.

Disclosure of Potential Conflicts of Interest
C. Rader, T.R. Burke Jr, and J.D. Thomas have ownership interest in the U.S. patent application 20100104510. No potential conflicts of interest were disclosed by the other authors.

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