FULL-LENGTH ARTICLE

Translational Research

BL-01, an Fc-bearing, tetravalent CD20 × CD5 bispecific antibody, redirects multiple immune cells to kill tumors *in vitro* and *in vivo*

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ABSTRACT

Background aims: The authors describe here a novel therapeutic strategy combining a bispecific antibody (bsAb) with cytokine-induced killer (CIK) cells.

Methods: The authors have designed, produced and purified a novel tetravalent IgG1-like CD20 × CD5 bsAb called BL-01. The bsAb is composed of a fused heavy chain and two free light chains that pair correctly to the heavy chain sequences thanks to complementary mutations in the monoclonal antibody 2 CH1/CL sequences.

Results: The authors show that BL-01 can bind specifically to CD20 and CD5 with an affinity of 4–6 nM, demonstrating correct pairing of two light chains to the fused heavy chain. The CD20 × CD5 BL-01 bsAb has a functional human IgG1 Fc and can induce up to 65% complement-dependent cytotoxicity of a CD20⁺ lymphoma cell line in the presence of human complement, similar to anti-CD20 rituximab. The bsAb also induces significant natural killer cell activation and antibody-dependent cytotoxicity of up to 25% as well as up to 65% phagocytosis by human macrophages in the presence of CD20⁺ tumor cells. The BL-01 bsAb binds to CD20 and CD5 simultaneously and can redirect CIK cells *in vitro* to kill CD20⁺ targets, increasing the cytotoxicity of CIK cells by about 3-fold. The authors finally show that the CD20 × CD5 BL-01 bsAb synergizes with CIK cells *in vivo* in controlling tumor growth and prolonging survival of nonobese diabetic/severe combined immunodeficiency mice inoculated with a patient-derived, aggressive diffuse large B-cell lymphoma xenograft.

Conclusions: The authors suggest that the efficacy of bsAb *in vivo* is due to the combined activation of innate immunity by Fc and redirection of CIK cells to kill the tumor target.

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Introduction

Some unconjugated monoclonal antibodies (mAbs), most notably the anti-CD20 rituximab, have shown impressive therapeutic activity as single anti-cancer agents [1–3]. These mAbs generally act through activation of the innate immune response, antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells, antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) [1,3,4]. Nonetheless, even effective mAbs are not curative in all patients, with

relapse and resistance intervening [5–8], due at least in part to the poor capacity of some antibodies to activate immune mechanisms and/or to exhaustion of the immune components [3,4,9].

The development of bispecific antibodies (bsAbs) that redirect immune cells, such as T, NK or granulocytes, to the tumor target is one approach to try and overcome these limitations [10–14]. Most bsAbs that have reached the clinic are T-cell-engaging molecules, such as the bispecific T-cell engager (BiTE) CD3 × CD19 blinatumomab, which activates CD3⁺ T cells to kill CD19⁺ leukemic targets [15–17]. Again, however, not all patients respond to blinatumomab, in part due to an immunosuppressed T-cell compartment caused by the underlying disease or previous chemotherapy [18–22].

A way to overcome insufficient or inactive T cells is to combine BiTEs with *in vitro*-expanded T cells derived from either the patients

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themselves or compatible donors [23,24]. Cytokine-induced killer (CIK) cells are particularly appropriate for such purposes, as they are polyclonal T cells that are easily expanded *in vitro* in large numbers under Good Manufacturing Practice conditions [25,26]. They are mostly CD3⁺CD8⁺ cells and show NK-like anti-tumor activity of their own, although this is not generally sufficient to control tumor growth *in vivo*, except in conditions of minimal residual disease [27–30]. After systemic inoculation, CIK cells localize in tumor tissue [27,31,32] and do not show significant graft-versus-host disease activity *in vivo* even in an allogeneic setting. They are widely used in phase 1/2 clinical trials for treatment of solid and hematopoietic tumors [27–30].

Since BiTEs are small molecules with short half-lives *in vivo*, acting exclusively through cytotoxic T cells, the authors aimed to design a novel strategy that would combine CIK cells with a novel, IgG-like tetravalent bsAb capable of activating innate immunity through its Fc region and also redirect CIK cells to tumor targets [33]. As a first approach, the authors chose CD20 as the tumor antigen. As a CIK antigen, the authors chose CD5 rather than CD3 since the bsAb is bivalent for each target antigen and cross-linking CD3 would likely be very toxic. By contrast, cross-linking CD5 alone has limited signaling capacity in T cells, and CD5 expression is mostly restricted to this lineage in adults [34]. The authors describe here the design and production of this novel CD20 × CD5 bsAb called BL-01 and its functional characterization *in vitro* and *in vivo*.

Methods

Antibody cloning and production

The construction of transfer vectors carrying light and heavy chains for mAb and bsAb construction was performed as described previously (see supplementary material) [33,35] based on the VH/VL sequences of anti-CD20 rituximab and anti-CD5 clone 0590 and the authors' previous patent (patent no. WO/2013/005194). The generation and cloning of recombinant baculoviruses expressing two chains (mAbs) or three chains (bsAb), antibody expression and purification were performed as described.

Cells

The study was approved by the hospital ethical committee in accordance with the Declaration of Helsinki of 1975, as revised in 2008. Peripheral blood was obtained from healthy donors after their written informed consent. Peripheral blood mononuclear cells (PBMCs) were purified by standard Ficoll-Hypaque gradient centrifugation (Seromed; Biochrom AG, Berlin, Germany). The lymphoma cell lines SU-DHL-4 (DHL-4) and JEKO-1 were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Immunofluorescence

To measure antibody binding, cells were incubated with increasing concentrations of bsAb or mAbs, washed and then labeled with human Fc-specific, fluorescein isothiocyanate-labeled mAb (Sigma-Aldrich, St Louis, MO, USA). Control antibodies were fluorescein isothiocyanate-labeled CD20 and CD5 mAbs (BD Biosciences, Franklin Lakes, NJ, USA). After washing, binding was assessed by flow cytometry using a FACSCanto II instrument (BD Biosciences). For cell–cell binding experiments, CD20⁺ BJAB and CD5⁺ Jurkat cells were labeled with PKH26 red and PKH67 green fluorescent dyes (Thermo Fisher Scientific, Waltham, MA, USA), respectively. They were then mixed 1:1 in the presence or absence of 7 nM bsAbs and the presence or absence of excess (17.5 nM) mAbs. Cell–cell binding was measured as the percentage of double-positive events by flow cytometry using a FACSCanto II instrument.

Generation of CIK cells

CIK cells were prepared as described previously [25]. Briefly, PBMCs from healthy donors were cultured in serum-free X-VIVO 15 medium

(Lonza, Basel, Switzerland) supplemented on day 0 with 1000 U/mL interferon gamma (IFN- γ) (Imukin; Boehringer Ingelheim Inc, Ingelheim am Rhein, Germany) and on day 1 with 50 ng/mL anti-CD3 mAb (OKT3; Janssen-Cilag SpA, Milano, Italy) and thereafter expanded in 500 U/mL rhIL-2 (Proleukin; Chiron BV, Amsterdam, the Netherlands).

Complement-dependent cytotoxicity

Cell lines were plated at 2×10^5 cells/well in 96-well plates in complete medium. Antibodies were added at the indicated concentration, followed 5 min later by pooled human serum to a final concentration of 25%. After 4 h of incubation at 37°C, 5 μ L 7-aminoactinomycin D (BD Biosciences) was added to each well and the percentage of 7-aminoactinomycin D-positive cells read using a flow cytometer.

NK cell activation assays

To assess NK cell degranulation, 150×10^3 PBMCs from normal donors were added to 75×10^3 target cells in the presence or absence of increasing concentrations of test or control mAbs or bsAb. After 4 h of incubation at 37°C, cells were stained with anti-CD56-allophycocyanin and anti-CD107a-phycoerythrin and degranulation measured by flow cytometry as increased percentage of CD107a⁺ cells in the CD56⁺ population.

Cytotoxicity assays with NK or CIK cells as effector cells

For cytotoxicity assays, target cells were first loaded with 3.5 μ M calcein acetoxymethyl ester (Merck Life Science, Milano, Italy). A total of 5×10^3 labeled targets were plated in 96-well plates with either freshly isolated PBMCs as a source of NK cells at a 10:1 effector:target ratio or freshly generated CIK cells at a 5:1 effector:target ratio and in the presence or absence of increasing concentrations of bsAbs or control mAbs. After 4 h of incubation at 37°C, 100 μ L of supernatant was removed from each well and fluorescence measured using a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Ortenberg, Germany).

Phagocytosis by macrophages

Monocyte-derived macrophages were generated as described [36]. Chronic lymphocytic leukemia target cells were stained with 0.1 μ M carboxyfluorescein succinimidyl ester (Thermo Fisher Scientific) and incubated with the macrophages in the presence or absence of bsAb or control mAbs. After 2 h of incubation at 37°C, cells were harvested and stained with CD19-allophycocyanin and CD11b-phycoerythrin (BD Biosciences) and analyzed by flow cytometry. Phagocytosis was defined as the percentage of CD11b⁺ cells that were carboxyfluorescein succinimidyl ester-positive and CD19[−] [37].

Cytokine induction

PBMCs from healthy donors were plated at 10^6 cells/mL in complete medium. Antibodies were added at 33 nM and cells incubated at 37°C in 5% carbon dioxide. Supernatant was collected after 2 h and 6 h and cytokine concentrations measured using a cytometric bead array human cytokine kit (BD Biosciences).

In vivo B-cell lymphoma model

The patient-derived (PDX) diffuse large B-cell lymphoma (DLBCL) model DLBCL-PER has been described previously [23]. Briefly, lymphoma cells from a patient with activated B-cell-like DLBCL treated with chemotherapy and refractory to a second line of treatment that included rituximab were collected at relapse. The cells were serially passaged intraperitoneally in CB17/severe combined immunodeficiency mice

Figure 1. Structure and purification of CD20 × CD5 bsAb BL-01. (A) The CD20 × CD5 bsAb is composed of a fused heavy chain and two free light chains. Starting from the N-terminus, the fused heavy chain is composed of the rituximab anti-CD20 VH sequence CH1-hinge1-linker and the anti-CD5 VH sequence CH1(CR3mut)-hinge2-CH2-CH3. The CH1-hinge-CH2 and CH3 sequences are from human IgG1. The two light chains are rituximab anti-CD20 VL-Ck and anti-CD5-Ck(CR3mut). (B) Monospecific and bispecific antibodies were analyzed by SDS-PAGE in reducing conditions, and heavy and light chains were detected by silver staining. The positions of the heavy and light chains are indicated. H, heavy; L, light; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (Color version of figure is available online.)

(Charles River Laboratories Italia srl, Calco, Italy) for five passages and stored at -180°C . These cells expressed relatively low levels of CD20 (see supplementary Figure S1). For immunotherapy experiments, passaged DLBCL-PER cells were inoculated intravenously at 5×10^6 cells/mouse in 6-week-old female CB17 nonobese diabetic/severe

combined immunodeficiency mice (Charles River Laboratories Italia srl). At day 5, day 15, day 27, day 40 and day 50, equipotent amounts of CD20 × CD5 and CD20 control mAbs ($75 \mu\text{g}/\text{mouse}$ and $15 \mu\text{g}/\text{mouse}$, respectively) were inoculated intravenously in groups of five mice. The following day, 20×10^6 CIK cells were

Figure 2. Specificity of binding of CD20 × CD5 BL-01 to both target antigens. The specificity of the purified CD20 × CD5 bsAb and respective anti-CD5 and anti-CD20 mAbs was tested at 10 nM using CD20⁺ and CD5⁺ single-positive cell lines as targets (BJAB and Jurkat, respectively) and anti-human Fc-FITC secondary antibody. The commercial FITC-labeled anti-CD20 and anti-CD5 mAbs were used in parallel. Flow cytometry histograms are shown for the respective cell lines and indicated antibodies. FITC, fluorescein isothiocyanate; NEG CTRL, negative control. (Color version of figure is available online.)

inoculated intravenously. Animals were killed when hind leg paralysis was observed and then autopsied.

Statistical analysis

Statistical significance was calculated using Student's *t*-test. *In vivo* survival data were analyzed by Mantel–Cox logrank test.

Results

Design and production of CD20 × CD5 BL-01 bsAb

The CD20 × CD5 bsAb was called BL-01 and was based on the authors' previously published and patented bispecific backbone consisting of three polypeptide chains [33]: a fused heavy chain carrying the two VH-CH1 domains in tandem, separated by a hinge and the flexible linker sequence VH(mAb1)-CH1'-hinge'1/linker/VH(mAb2)-CH1mutCR3-hinge2-CH2-CH3, and two different light chains expressed as separate polypeptides—VL(mAb1)-CL(1) and VL(mAb2)-CL(2)—both kappa. The correct pairing of the VL(mAb1)-CL(1) and VL(mAb2)-CL(2) light chains to the correct VH sequences takes place thanks to the introduction of pairs of complementary mutations called CR3 in the CH1(2) and CL(2)

sequences. The overall structure of the bsAb is shown in Figure 1A. The VH(mAb1)/VL(mAb1) sequences are derived from the published rituximab anti-CD20 sequence, whereas the VH(mAb2)/VL(mAb2) sequences are derived from the previously described mouse anti-CD5 antibody (clone 0490) [33]. The construction of the vectors, cloning of recombinant baculoviruses and production and purification of mAbs and bsAb were described earlier. All batches of antibodies were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 1B) and checked for consistent binding affinity using CD20⁺ and CD5⁺ cell lines and flow cytometry.

BL-01 specifically binds to cell surface CD20 and CD5 with nM affinity

The authors first verified the specificity of the mAbs and CD20 × CD5 BL-01 bsAb by flow cytometry using CD20 and CD5 single-positive cell lines (BJAB and Jurkat, respectively). The CD20 and CD5 mAbs were able to bind only to BJAB and Jurkat cell lines, as expected. CD20 × CD5 bsAb instead bound to both the BJAB and Jurkat cell lines (Figure 2). Specificity was confirmed by blocking experiments (data not shown). The authors then performed dose–response curves of the mAbs and bsAb to measure relative binding affinity of the antibodies for cell-bound antigens

Figure 3. Relative binding affinity of CD20 × CD5 BL-01 and respective mAbs for target antigens expressed by the BJAB and Jurkat cell lines. The relative affinity of the purified CD20 × CD5 bsAb and respective anti-CD5 and anti-CD20 mAbs was tested by flow cytometry using the CD20⁺ BJAB and CD5⁺ Jurkat cell lines, increasing concentrations of primary antibodies and fixed anti-human Fc-FITC secondary antibody. MFIs obtained with antibody binding to CD20 (A) and CD5 (B) are shown, as are measured relative binding affinities (EC₅₀) for each antigen (C). FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; NA, not applicable. (Color version of figure is available online.)

(Figure 3A,B). The relative binding of bsAb for CD20 was similar to that of the corresponding mAb, with only a 2- to 3-fold decrease in relative affinity (EC_{50} , 6 nM and 2.8 nM, respectively) (Figure 3C). With regard to CD5, the EC_{50} measured for the bsAb was 4 nM compared with about 0.5 nM for the isolated mAb (Figure 3B,C), therefore indicating a decreased affinity of about 8-fold. This decrease is probably due to partial steric hindrance of the Fab(2) by the Fab(1) and relatively closed structure around hinge1 and has also been observed in other constructs based on the same scaffold [33]. The affinity of the bsAb BL-01 for CD20 and CD5 BL-01 was quite balanced (6 nM and 4 nM, respectively) and therefore appropriate for further studies.

BL-01 Fc is competent for CDC, ADCC and ADCC

The authors next determined whether the Fc portion of the bsAb was functional. The authors first analyzed the capacity of the bsAb to activate CDC using the complement-sensitive cell line DHL-4 [38]. The CD20 × CD5 bsAb was able to activate complement and lyse targets efficiently in the presence of 25% human serum as a source of complement (Figure 4A). There was no significant difference in CDC observed with CD20 mAb or CD20 × CD5 bsAb using 0.7–66 nM antibodies, with up to 65% increased lysis over background at maximal antibody concentration. The authors also tested CD5⁺ cell lines in CDC assays, but no lysis was observed (data not shown), consistent with the inability of the parent anti-CD5 mAb to activate complement [33].

The authors next determined whether the CD20 × CD5 bsAb was able to induce phagocytosis by macrophages. As shown in Figure 4B, the anti-CD20 mAb and CD20 × CD5 bsAb were able to mediate efficient phagocytosis of CD20⁺ chronic lymphocytic leukemia targets, with 57–68% phagocytosing macrophages detected after 2 h at optimal doses of antibodies (2 nM).

The authors finally analyzed NK cell activation and ADCC of CD20⁺ targets. Both CD20 mAb and CD20 × CD5 bsAb were able to activate NK cell degranulation (Figure 5A) as well as ADCC of CD20⁺ BJAB targets (Figure 5B). CD20 mAb induced up to 35% killing of target at 0.08–8 nM. CD20 × CD5 bsAb BL-01 induced up to 28% killing at 0.8–8 nM. These data showed that the CD20 × CD5 bsAb was effective in mediating ADCC, albeit less efficiently than the CD20 mAb.

BL-01 redirects CIK cells to kill CD20⁺ targets

The authors first analyzed whether the bsAb was able to bind to the CD20 and CD5 antigens simultaneously and therefore bring together CD20⁺ and CD5⁺ cells. As shown in Figure 6A, flow cytometry assays showed an increase in double-positive cellular doublets containing red CD20⁺ BJAB and green CD5⁺ Jurkat T cells in the presence of CD20 × CD5 bsAb. The formation of doublets was inhibited by adding excess anti-CD20 or anti-CD5 mAbs alone or together, as expected for a bispecific binding. These data showed that BJAB can indeed bind to Jurkat in the presence of bsAb BL-01.

The authors next investigated whether the CD20 × CD5 bsAb was able to redirect the killing by CD5⁺ CIK cells to CD20⁺ target BJAB. As shown in Figure 6B, the addition of 0.7–18 nM bsAb significantly increased (approximately 3-fold) the killing of BJAB targets by CIK cells.

The authors concluded that CD20 × CD5 bsAb was able to kill CD20⁺ target cells by Fc-mediated mechanisms as well as by redirecting CIK cells to mediate target cell killing.

BL-01 acts synergistically with CIK cells in protecting mice in a PDX DLBCL xenograft model

The authors next investigated whether the CD20 × CD5 BL-01 bsAb was able to effectively protect mice from CD20⁺ tumors *in vivo*

Figure 4. CD20 × CD5 bsAb BL-01 mediates complement-dependent cytotoxicity and phagocytosis of CD20⁺ targets. (A) The CD20⁺ DHL-4 cell line was incubated in the presence of increasing concentrations of CD20 × CD5 bsAb, anti-CD20 or CTX control mAbs in the presence of 25% human serum as a source of complement. Complement-mediated lysis was measured after 4 h by flow cytometry. (B) Human *in vitro*-generated macrophages were incubated with CLL cells in the presence or absence of the indicated antibodies. Phagocytosis was measured after 2 h by flow cytometry. Statistical significance was calculated with respect to controls without mAbs. * $P < 0.05$, ** $P < 0.01$. CLL, chronic lymphocytic leukemia; CTX, cetuximab. (Color version of figure is available online.)

in the presence or absence of CIK cells. To do this, the authors used the PDX DLBCL-PER model [23,24], an aggressive DLBCL obtained from a patient who relapsed after chemotherapy and was refractory to a second-line, rituximab-containing regimen. Indeed, primary and *in vivo*-passaged DLBCL-PER cells expressed relatively low levels of CD20 (see supplementary Figure S1). DLBCL-PER cells were inoculated intravenously to best mimic the hematopoietic tumor. The bsAb or control CD20 mAb was then injected intravenously once every 10–12 days for a total of five inoculations starting 5 days after tumor cells. A total of 20×10^6 CIK cells were inoculated 1 day after each antibody treatment. As shown in Figure 7A, BL-01 alone had some therapeutic activity, increasing median survival time by about 15 days, from a median of 71 days to 86 days ($P < 0.01$). CIK cells alone had no significant therapeutic activity. However, the combination of CIK cells and bsAb increased efficacy further compared with bsAb alone, prolonging median survival by 45 days compared with vehicle, from a median of 71 days to 116 days ($P < 0.01$), and 30 days compared with bsAb alone ($P < 0.01$). Two animals in the combination group survived long term (>200 days), whereas none of the animals in the control group treated with vehicle alone or CIK cells alone survived longer than 75 days. The authors performed the same experiment in parallel with an equipotent dose of rituximab (based on

Figure 5. CD20 × CD5 bsAb BL-01 activates NK cells and mediates ADCC. PBMCs were incubated in the presence of CD20⁺ BJAB target cell lines and the presence or absence of bsAb or control mAbs at the indicated concentrations. (A) NK cell activation was measured as the percentage of CD107a on CD56⁺ cells by flow cytometry. (B) ADCC was measured by calcein release assays. Statistical significance is shown with respect to no mAb controls. **P* < 0.05. (Color version of figure is available online.)

different molecular weight and affinity for CD20). Rituximab alone had some activity, increasing median survival time by 28 days over vehicle, but the combination of CIK cells and rituximab was not significantly more active (Figure 7B). The authors concluded that CD20 × CD5 bsAb had therapeutic activity *in vivo* synergistically with CIK cells, indicating that the CD5 moiety of BL-01 was also functional *in vivo* and could effectively redirect CIK cells to CD20⁺ targets.

Analysis of possible toxicities *in vitro*

Finally, the authors wanted to verify that the CD20 × CD5 bsAb was not inducing polyclonal T-cell activation of resting T cells through CD5. To do this, the authors spiked CD20⁺ JEKO1 cells into PBMCs, added the different antibodies and analyzed the activation of CD4⁺ and CD8⁺ cells at different times. As a control, the authors used the anti-CD3 mAb OKT3, which activates T cells through the T-cell receptor. The results demonstrated that whereas the anti-CD3 antibody OKT3 induced strong activation of both CD4⁺ and CD8⁺ cells, seen as induction of CD69 or CD107a, CD20 mAb and CD20 × CD5 bsAb did not do so to any significant extent (Figure 8A–C).

The authors also analyzed cytokines, including IL-6, IL-4, IL-2, tumor necrosis factor alpha, IFN- γ and IL-10, that may be induced in PBMCs cultured in the presence of anti-CD5 parent mAb or bsAb BL-01 and compared positive and negative controls anti-CD3 (OKT3) and anti-epidermal growth factor receptor cetuximab, respectively. The authors observed clear tumor necrosis factor alpha, IL-2 and IFN- γ induction at 6 h after the addition of anti-CD3 but not anti-CD5 or CD20 × CD5 bsAb (see supplementary Figure S2).

These data suggested that binding of CD5 by bsAb and cross-linking to CD20 did not induce any evident T-cell activation or release of inflammatory cytokines by PBMCs.

Discussion

In this article, the authors describe the design, construction, purification and functional analysis *in vitro* and *in vivo* of a novel tetravalent IgG1-like bsAb directed against CD5 and CD20 called BL-01. The bsAb is based on a patented structure and platform (patent no. WO/2013/005194) [33]. The light chains, directed against two different specificities, bind to the correct region of the fused heavy chain thanks to pairs of complementary mutations within the CH1 and CL domains of the internal Fabs (Figure 1A). The Fc bears a fully functional IgG1 Fc. The authors have shown previously that these bispecific tetravalent antibodies are easily produced against a variety of specificities and that they are quite stable *in vitro* at 4°C as well as *in vivo*, presumably due to the fact that these bsAbs fold similarly to a natural IgG1 antibody and are composed nearly entirely of sequences derived from natural immunoglobulins (unpublished data from MDC) [33]. These molecules are therefore likely to be minimally immunogenic even if this aspect has not been tested directly.

The novel BL-01 CD20 × CD5 bsAb described here contains in the second position the same anti-CD5 moiety already tested in the prototype bsAb [33]. By contrast, the N-terminal Fabs were replaced by the published rituximab anti-CD20 sequences to specifically redirect CD5⁺ CIK cells to CD20⁺ tumor targets. The final structure is bivalent for each antigen, allowing for good affinity and avidity. The authors

Figure 6. BL-01 bsAb binds to CD20 and CD5 simultaneously and redirects CIK cells to mediate killing of CD20⁺ targets. (A) CD20⁺ BJAB and CD5⁺ Jurkat cells were labeled with red and green fluorescent dyes, respectively, and incubated at a 1:1 ratio in the absence or presence of 7 nM CD20 × CD5 bsAb and excess anti-CD20 and/or anti-CD5 mAbs. The percentages of double-positive events with respect to all events, after background subtraction, are shown. Statistical significance in the presence or absence of bsAb and the presence or absence of blocking mAbs is shown. (B) CIK cells were expanded *in vitro* from the PBMCs of healthy donors. After 21 days of culture, they were incubated in the presence of calcein acetoxymethyl ester-labeled CD20⁺ BJAB targets at a 5:1 effector:target ratio and in the presence or absence of increasing concentrations of CD20 × CD5 bsAb. After 4 h, BJAB lysis was measured by calcein release assay. Statistical significance is shown for the presence versus absence of CD20 × CD5 bsAb at different doses. **P* < 0.05, ***P* < 0.01. (Color version of figure is available online.)

were able to show here that the purified bsAb bound specifically to both CD20 and CD5 expressed on cell surfaces. The relative binding affinity of BL-01 for CD20 is about 6 nM and only 2- to 3-fold lower than that observed with the corresponding anti-CD20 mAb. This small decrease in affinity in the bsAb is thought to be due to the greater constriction of the N-terminal Fabs in the bsAb molecule compared with the mAb molecule (data not shown). The anti-CD5 moiety in the bsAb is more internal to the molecule (Figure 1A), a position that can result in some steric hindrance and usually induces a loss of affinity of 8- to 10-fold in the bsAb compared with the corresponding mAb [33]. Thus, the relative binding affinity of BL-01 for CD5 was about 4 nM compared with about 0.5 nM for the corresponding mAb. The authors conclude that the bsAb can bind specifically in the nanomolar range and in a balanced manner to both CD5 and CD20.

The authors next analyzed whether the Fc domain of the CD20 × CD5 BL-01 construct was capable of activating immune-mediated mechanisms in the presence of CD20⁺ tumor targets. The authors were able to show that the bsAb could indeed activate complement, NK cells and macrophages to mediate CDC, ADCC and phagocytosis, respectively. CDC using the complement-sensitive DHL-4 target and phagocytosis were not significantly different *in vitro* between CD20 control mAb and BL-01 bsAb. Parent CD5 did not activate complement, presumably due to its inability to form circular hexameric assemblies capable of binding C1q and activating complement, a phenomenon that was recently unraveled

at the molecular level with rituximab and that depends on the steric orientation of mAbs binding to their target antigens as well as Fab–Fab interactions [4,39]. The authors' data suggest that the bsAb BL-01 binds to CD20 and can form multimeric assemblies similar to rituximab, thus inducing significant complement activation. NK cell activation and ADCC were slightly less efficient with BL-01 compared with the CD20 mAb, presumably due to slightly lower affinity of bsAb for CD20 or to differences in the binding affinities of the antibodies for Fc gamma receptors (FcγRs) expressed by NK cells, such as FcγRIIIA (CD16A). It is worth noting at this stage that both the CD20 × CD5 bsAb and control anti-CD20 mAb were produced in insect cells, which generate antibodies with low fucosylation. The authors have shown previously that only 60% of glycans linked to antibodies produced in Sf9 cells are fucosylated [40] compared with 100% when antibodies are produced in CHO cells [41]. Thus, NK cell activation and ADCC could have been favored by the low fucosylation of the recombinant antibodies, similar to what is observed with low-fucose antibodies produced in mammalian cells [42–44].

The authors conclude that the data clearly demonstrate that the IgG1 Fc domain of BL-01 is functional and can interact with C1q and FcγRs to mediate CDC, ADCC and ADCP. These mechanisms are known to be important for the efficacy of unconjugated mAbs like rituximab and other anti-CD20 antibodies [6,45–48].

The authors also showed that the bsAb BL-01 was able to redirect CIK cells to kill CD20⁺ tumor targets *in vitro*. This capacity likely depends on

Figure 7. CD20 × CD5 bsAb has therapeutic activity *in vivo* and synergizes with CIK cells. NOD-SCID mice (five per group) were inoculated intravenously with 5×10^6 DLBCL-PER cells and treated every 10 days with 75 μ g bsAb or 15 μ g RTX per mouse (approximately equipotent doses based on molecular weight and CD20 affinity) starting from day +5 and 1 day later with 20×10^6 CIK cells for a total of five treatments. Survival curves for bsAb (A) and RTX (B) are shown, with measured MSTs for each group on the right. ** $P < 0.01$. CTRL, control; MST, median survival time; NOD-SCID, nonobese diabetic/severe combined immunodeficiency; RTX, rituximab. (Color version of figure is available online.)

bsAb binding simultaneously to both antigens. This was also demonstrated *in vitro* using labeled CD20⁺ and CD5⁺ cell lines. Similar observations were made previously with DR-CD5 prototype bsAb [33].

Finally, the CD20 × CD5 BL-01 bsAb was shown to have therapeutic efficacy *in vivo* in a PDX DLBCL xenograft in mice, which is relatively resistant to rituximab. In this model, control bsAb alone had some measurable therapeutic activity. CIK cells alone had little activity, whereas the combination of CIK cells and BL-01 bsAb significantly increased survival of mice. By contrast, CIK cells combined with the rituximab control antibody did not significantly enhance therapeutic activity. These data suggest that bsAb BL-01 can act by multiple mechanisms *in vivo*: via innate immunity like CD20 mAb as well as by redirecting CIK cells to targets. These mechanisms appear to be synergistic.

CIK cells have been reported to express FcγRs—in particular CD16—albeit at low levels compared with NK cells [25,49,50]. The authors cannot exclude that low levels of CD16 on CIK cells also participate in the synergistic activity of CD20 × CD5 bsAb *in vivo*, although the lack of such synergy with rituximab suggests that this is not significant and that the CD5 moiety of BL-01 bsAb is required for the effect *in vivo*.

CIK cells have been shown to have therapeutic potential in a variety of hematopoietic and solid cancers and for this reason have been quite widely used in the clinic. Furthermore, CIK cells have been shown to be able to localize to many tumors, including B-cell and solid tumors [27,31,32]. Nonetheless, their efficacy has been shown mostly in conditions of minimal residual disease, suggesting that their therapeutic potential and specificity need to be increased using combined

treatments [27–30]. The authors previously suggested combining CIK cells with BiTEs, and this possibility was also demonstrated *in vivo* using blinatumomab in PDX leukemia models [23,24]. This option, however, is limited by the fact that BiTEs have a very short half-life *in vivo* and need continuous infusion protocols. In addition, the mechanism of action of BiTEs is solely dependent on T cells since they lack the Fc portion, and this may be a disadvantage in patients with inadequate T-cell function, such as that seen following chemotherapy or as a result of induction of regulatory T cells or checkpoint inhibitors by tumor cells [19–22]. Finally, BiTEs activate T cells through the T-cell receptor, and this may potentially cause toxicity when infusing large numbers of CIK cells into patients. By contrast, the authors' proposal of combining an Fc-bearing bsAb like BL-01 offers the advantage that the bsAb can both activate innate immunity through Fc and redirect CIK cells to the target through the CD5 moiety, thus providing multiple mechanisms of tumor cell killing. It is also conceivable that the immune activation initially taking place after BL-01 binding to target cells may induce inflammatory cytokines and chemokines locally, which could potentially increase CIK cell localization to tumors. Indeed, CIK cells have been described as expressing several chemokine receptors, including CXCR3, CXCR4, CCR5, CCR6 and CCR7, some of which are inflammatory chemokine receptors [51,52]. The hypothesis that innate immunity activation by Fc may favor the homing of CIK cells to tumors and/or CIK cell cytotoxic activity is only a theory at this stage and needs to be verified. The other advantage of BL-01 over BiTEs is that its size and the presence of functional Fc should confer on it a longer half-life *in vivo*, although the exact half-life of BL-01 *in vivo* still needs to be investigated.

Figure 8. CD20 × CD5 bsAb BL-01 does not significantly activate resting T cells *in vitro*. PBMCs were spiked with the CD20⁺ JEKO1 cell line and incubated with 67 nM of the indicated antibodies. Panels A and B: Activation of CD8⁺ (A) and CD4⁺ cells (B) was measured at 6 h and 24 h by flow cytometry as induction of CD69. Panel C: Degranulation of cytotoxic CD8⁺ T cells was measured as CD107a induction. Data are the means and standard deviations of three to four experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. CTRL, control. (Color version of figure is available online.)

One concern is the fact that BL-01 binds to CD5 expressed by all T cells, which may lead to T-cell activation or cytokine storms independently of CD20 recognition. *In vitro* experiments using PBMCs into which antibodies were spiked suggested, however, that this is not the case. Furthermore, *in vivo*, the authors could not observe any distress in mice following treatment with CIK cells alone or combined with CD20 × CD5 bsAb. These data suggest that such treatment may be safe, although this will obviously need to be verified with more physiological models, such as humanized mice, which express human CD5 on T cells and have a full complement of immune cells.

Conclusions

The authors propose that the data presented are a proof of principle that the novel anti-cancer strategy of using a tetravalent bsAb recognizing a tumor antigen and CD5 to both activate innate immunity and redirect CIK cells to kill tumor cells may be an effective strategy. With regard to the BL-01 bsAb, before proceeding to clinical development, a number of steps will clearly need to be performed: humanization of both the CD20 and CD5 variable sequences as well as optimization of the relative affinities for CD20 and CD5. The present molecule shows a slightly higher affinity for CD5 than CD20. However, on a theoretical basis, the authors would favor a higher affinity for CD20 than CD5 to favor bsAb localization to the tumor before inoculation of CIK cells. VH and VL sequences derived from other anti-CD20 mAbs could also be used in the final construct, such as that

of the anti-CD20 ofatumumab, which is fully human and has strong complement activation potency and high affinity for CD20. In addition, different linker sequences between the Fab1 and Fab2 moieties could be tested to improve relative affinities of the two Fabs and perhaps optimize the redirection of CIK cells to target cells. The best molecule selected *in vitro* would then need to be investigated in humanized mouse models before clinical development. Nonetheless, if successful, this kind of approach could be extended in the future to other suitable tumor antigens.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Acquisition of data: AI, SC, MS, RV, SC, MR. Analysis and interpretation of data: JG, MDC, RA. Drafting or revising the manuscript: JG, MDC, RA, MR. All authors have approved the final article.

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Supplementary materials

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