Retargeting Bispecific Antibody BIS20x3Costimulation in the Efficacy of the T Cell The Role of B Cell-Mediated T Cell

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The Role of B Cell-Mediated T Cell Costimulation in
the Efficacy of the T Cell Retargeting Bispecific Antibody BIS20x3

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In this study, we investigated the role of the naturally occurring B cell-mediated T cell costimulation in the antitumor efficacy of the bispecific Ab BIS20x3. BIS20x3 has a dual specificity for both CD20 and CD3 and has previously been shown to effectively direct the lytic potential of cytolytic T cells toward malignant, CD20+ B cells. BIS20x3 instigated T cell-B cell interaction caused a dose-dependent activation of T cells that was 30 times stronger when compared with T cell activation induced by monovalent anti-CD3 Abs. The activation of T cells by BIS20x3 and B cells appeared functional and resulted in the rapid induction of high lytic potential in freshly isolated peripheral T cells. BIS20x3-mediated T cell-B cell interaction resulted in a significant up-regulation of ICAM-1 on B cells and the activation of T cells was found to be dependent on the interaction of ICAM-1 with LFA-1 and trans-activation by the NF-κB pathway. Also, the lytic potential of freshly isolated T cells activated via BIS20x3 appeared to be dependent on NF-κB signaling in the target B cells. Interestingly, the costimulatory signaling effects described in this study appeared specifically related to the targeting against CD20 because targeting against CD19, by a CD3xCD19-directed bispecific Ab, was significantly less effective in inducing T cell activation and T cell-mediated B cell lysis. Together these results demonstrate that the malignant B cells actively contribute to their own demise upon CD20-directed bispecific Ab-mediated T cell targeting. The Journal of Immunology, 2004, 173: 6009–6016.

To improve the response rate of anti-CD20 Ab therapy we developed a bispecific Ab (BsAb) named BIS20x3. BIS20x3 combines the Ag specificity for CD20 on B cells with the Ag specificity for CD3ε on T cells, within one Ab molecule (16). In contrast to rituximab, this BsAb uses the patients T cell compartment for its antitumor effect by activating and retargeting T cells to CD20+ B cells, independent of the TCR and MHC specificity of the T cells.

Previously we demonstrated that BIS20x3 induces efficient T cell activation and allows retargeting of T cells to CD20-positive B cells, resulting in efficient killing of CD20+ B cells. In addition to this, BIS20x3-mediated cross-linking of CD20 at the cell surface of B cells induced significant apoptosis in the B cell lymphoma cell line Ramos (16). In the present study we investigated the mechanism of T cell activation mediated by BIS20x3 and B cells. “Full blown” T cell activation via BIS20x3 targeted B cells appeared to involve integrin-mediated costimulation of the T cells and required a functional NF-κB pathway in the B cells. In addition, this B cell-mediated costimulation of T cells was specifically related to the targeting against CD20. Together, these data demonstrated that B cells are not just passive targets for T cell retargeting therapy. Instead, BIS20x3 allows active participation of the CD20+ target B cells in the functional activation of T cells and this ultimately resulted in the effective BsAb-mediated eradication of the malignant B cells.

Materials and Methods

Abs and reagents

BIS20x3 was produced and purified by IQ Products (Groningen, The Netherlands) as previously described (16). The immunoreactivity of both binding moieties within BIS20x3 was characterized using flow cytometric analysis, using secondary Abs against the murine IgG1 (CD20) and IgG2b (CD3) chains within the BsAb that were purchased from IQ Products. The

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presence of these IgG1 and IgG2b chains and the homogeneity of the preparation were also demonstrated using gel electrophoresis of the H chain and L chain (data not shown). The anti-CD3ε Ab 37-6673 (17) was a kind gift from Dr. R. A. van Lier (Academic Medical Centre, Amsterdam, The Netherlands). The hybridoma cell line B-ly1 producing the IgG1 anti-CD20 Ab B-ly1 (18) was generated in our department. F(ab)\textsubscript{2} of GaM-IgG Abs (GaM) used for cross-linking of BIS20x3 or anti-CD3 Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-LFA-1 (CD11a) Ab (clone L15) was a kind gift from Dr. C. G. Figdor (Department of Tumor Immunology, University of Nijmegen, Nijmegen, The Netherlands). The anti-ICAM-1 (CD54) Ab (clone Hu5/3) was provided by Dr. M. A. Gimbrone, Jr. (Department of Pathology, Brigham and Woman’s Hospital, Boston, MA). The CD3xCD95 BsAb was kindly provided by Dr. G. Moldenhauer (Department of Molecular Immunology, Deutsche Krebsforschungszentrum, Heidelberg, Germany) (19). Commercial aliphophycocyanin-, FITC-, or PE-conjugated Abs (against CD3, CD20, and CD54) used for FACS analysis were obtained from IQ Products. Hygromycin B was obtained from Invitrogen Life Technologies (Breda, The Netherlands), calcein-AM from Molecular Probes (Leiden, The Netherlands), and recombinant human IL-2 from EuroCetus (Amsterdam, The Netherlands). Lymphoprep was purchased from Nycomed (Oslo, Norway), Lympho-kwik (T) from One Lambda (Canoga Park, CA), serum- and phenol-free medium (X-VIVO) from BioWhittaker (Verviers, Belgium) and Triton X-100 from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Cells

The CD20\textsuperscript{+} Burkitt’s lymphoma cell line Ramos was obtained from the American Type Culture Collection (Manassas, VA). Ramos-IkB\textsubscript{A} (Ramos cells transduced with the nondegradable inhibitor of NF-κB; IkB\textsubscript{A}) and Ramos\textsubscript{IκB\textsubscript{A}} (Ramos cells transduced with an empty L2RS vector as a control) were generated by retroviral transduction as previously described (20). The CD20\textsuperscript{+} cell line Raji (Dr. M. Little, University of Heidelberg, Heidelberg, Germany) and Daudi (Dr. C. J. M. Melief, University Medical Center, Leiden, The Netherlands), the EBV-immortalized human B cell line JY (Dr. L. Bakker, Department Tumor Immunology, Nijmegen, The Netherlands) and the T cell leukemia cell line Jurkat AM/T (Dr. P. Schirer, University Hospital Leiden, The Netherlands) were cultured in RPMI 1640 culture medium (containing 25 mM HEPES and L-glutamine, supplemented with 14% heat-inactivated FCS from Bodinck (Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 mM 2-ME, and 0.1 mg/ml gentamicin sulfate obtained from BioWhittaker, Verviers, Belgium). Human B cell line JY (Dr. L. Bakker, Department Tumor Immunology, Nijmegen, The Netherlands) and the T cell leukemia cell line Jurkat AM/T (Dr. P. Schirer, University Hospital Leiden, The Netherlands) were cultured in RPMI 1640 culture medium (containing 25 mM HEPES and L-glutamine, supplemented with 14% heat-inactivated FCS from Bodinck (Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 mM 2-ME, and 0.1 mg/ml gentamicin sulfate obtained from BioWhittaker, Verviers, Belgium). The anti-ICAM-1 (CD54) Ab (clone Hu5/3) was produced by the hybridoma cell line B-ly1 producing the IgG1 anti-ICAM-1 Ab (clone L15). Ramos cells were similarly preincubated with various concentrations of the blocking anti-LFA-1 Ab, clone L15. Ramos cells were significantly preincubated with various concentrations of the blocking anti-ICAM-1 Ab, clone Hu5/3. An isotype-matched sham-Ab was used as a control. T cell activation was analyzed at various time points up to 24 h by detection of luciferase activity using the Luciferase Assay System (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. Luminescence was measured on the Anthos Lucy Microplate Luminometer and Photometer (Labtech International, Ringmer, U.K.). Experiments were performed in triplicate and luciferase levels were corrected for levels obtained in medium control samples.

Detection of T cell activation

T cell activation was determined using the Jurkat T cell subline Jurkat AM/T as previously described (16, 21). Briefly, in each experiment 1.0 × 10⁶ Jurkat AM/T cells were activated in 1.0 ml of medium at 37°C, using the stimuli described, in the presence or absence of 0.2 × 10⁶ B cells (Ramos, Ramos-IkB\textsubscript{A}, Ramos-IkB\textsubscript{A}, Raji, Daudi, or JY). The B cells were characterized by flow cytometry for CD20 and CD54 expression. To block the LFA-1-ICAM-1 interaction, Jurkat AM/T cells were preincubated for 30 min at 37°C with various concentrations of the blocking anti-LFA-1 Ab, clone L15. Ramos cells were similarly preincubated with various concentrations of blocking Ab and L15. Ramos cells were characterized by flow cytometry for CD53 and CD25 expression and used in a cytotoxicity assay to measure lytic capacity. The anti-CD3ε Ab 37-6673 (17) was a kind gift from Dr. R. A. van Lier (Academic Medical Centre, Amsterdam, The Netherlands). All cell lines were maintained at 37°C in a humidified atmosphere (Verviers, Belgium) and Triton X-100. All cell lines were maintained at 37°C in a humidified atmosphere (Verviers, Belgium). 0.5 mM 2-ME, and 0.1 mg/ml gentamicin sulfate obtained from BioWhittaker, Verviers, Belgium). 0.5 mM 2-ME, and 0.1 mg/ml gentamicin sulfate obtained from BioWhittaker, Verviers, Belgium). Lymphoprep was purchased from Nycomed (Oslo, Norway), Lympho-kwik (T) from One Lambda (Canoga Park, CA), serum- and phenol-free medium (X-VIVO) from BioWhittaker (Verviers, Belgium) and Triton X-100 from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Cytotoxicity assays

Cytotoxicity of the BIS20x3 retargeted T cells was determined using the calcein-release assay as described (22). Briefly, target cells were resuspended in X-VIVO medium at a concentration of 2 × 10⁶ cells/ml and incubated with 8 μM calcein-AM for 40 min, after which extracellular calcein-AM was removed by washing. After 2 h incubation in the absence or presence of 0.5 μg/ml BsAb, cells were pelleted by centrifugation and the supernatant was transferred to a new 96-well plate. Calcein fluorescence in the supernatant was determined using a Bio-Tek FL500 fluorescence plate reader (Bio-Tek Instruments, Burlington, VT; excitation at 485 nm, emission at 530 nm). The percentage of cytotoxicity was calculated from the formula: $\frac{F_{\text{sample lysis}} - F_{\text{spontaneous release}}}{F_{\text{maximal lysis}} - F_{\text{spontaneous release}}} \times 100$. Maximal lysis values were obtained by addition of 0.5% Triton X-100 to labeled target cells.

Results

Resting T cells are activated by BIS20x3 in the presence of B cells

To investigate whether BIS20x3 can induce T cell activation without the use of additional costimulation, PBMC-derived T cells were incubated with irradiated Ramos cells in the absence or presence of BIS20x3. As shown in Fig. 1A, significant up-regulation of the activation marker CD25 was noted on T cells incubated with Ramos cells in the presence of BIS20x3. No up-regulation of CD25 was noted on T cells incubated with Ramos cells in the absence of BIS20x3. Incubation of isolated T cells with Ramos B cells in the presence of BIS20x3 resulted in the formation of large aggregates of T cells around the B cells along with a typical change in morphology associated with activation of the T cells (Fig. 1B). To assess the cytolytic potential of the T cells, fresh calcein-AM-labeled Ramos cells were added to the T cells, which had been preincubated with irradiated Ramos B cells and BIS20x3 for 48 h. As shown in Fig. 1C, T cell priming in the presence of BIS20x3 induced significant cytolytic potential resulting in the induction of a 45% lysis of target cell death at an E:T cell ratio of 30:1.

CD3 signaling in Jurkat AM/T cells induced by BIS20x3

To demonstrate the capacity of BIS20x3 to activate T cells via CD3ε cross-linking, the NFAT-luciferase reporter T cell line Jurkat AM/T was used, in which T cell activation can be determined quantitatively by measuring luciferase activity. Fig. 2A shows an increase in T cell activation upon binding of increasing concentrations of BIS20x3 to CD3 on the surface of Jurkat AM/T cells in the presence of GaM fragments, resulting in a 3-fold increase in luciferase activation. To attain cell-mediated CD3 cross-linking via the BsAb, CD20\textsuperscript{+} Ramos cells were added to the Jurkat AM/T cells in the presence of BIS20x3. Again, a dose-dependent increase in T cell activation was observed upon stimulation with increasing amounts of BIS20x3 (Fig. 2B). Notably, the maximum level of T cell activation was 25-fold higher than the activation levels obtained with the same concentration of BIS20x3 (1.0 μg/ml) cross-linked by GaM fragments alone. Interestingly, using saturating concentrations of BIS20x3, T cell activation decreased, indicating that the interaction between B cells and T cells, as shown in Fig. 1B, contributed significantly to the efficiency of T cell activation. Similar to Ramos, three other CD20\textsuperscript{+} B cell lines, Raji, JY, and Daudi also mediated T cell activation in a BIS20x3 restricted manner (data not shown).

PBMC and T cell isolation and activation

PBMC were isolated from heparinized whole blood by density centrifugation using Lymphoprep. T cells were purified from the isolated PBMC using Lympho-kwik (T), according to the protocol provided by the manufacturer. For activation, T cells were incubated for 48 h with or without BIS20x3 in the presence of 50 Gy irradiated Ramos cells and recombinant human IL-2. After BIS20x3-mediated activation, the T cells were characterized by flow cytometry for CD3 and CD25 expression and used in a cytotoxicity assay to measure lytic capacity.
The effects of differential CD3 cross-linking on the levels of T cell activation

To further investigate the cause of the significant activation of T cells, different forms of CD3 ligation were compared. Soluble monospecific anti-CD3 Abs minimally increased the level of activation in Jurkat AM/T cells during the first 8 h, whereas for Jurkat AM/T cells in plates precoated with anti-CD3 Abs T cell activation kept increasing for at least 24 h (Fig. 3A). However, coated anti-CD3 Abs caused T cell activation at a level far below the level that could be reached via stimulation through BIS20x3 and Ramos cells. To demonstrate that the stimulatory effect of BIS20x3 on T cells was not just the additive result of simultaneous CD3 and CD20 signaling, Jurkat AM/T cells were incubated with both B-ly1 (the parental anti-CD20 Ab) and 37-6673 (the parental anti-CD3 Ab), in the presence of Ramos cells (Fig. 3B). Cross-linking the anti-CD20 Ab B-ly1 on Ramos cells was not sufficient to induce T cell activation. In concordance with the results
described in Figs. 2 and 3A, the cross-linked monoclonal anti-CD3 Ab could only induce minor levels of T cell activation. To mimic the dual binding properties of BIS20x3, both parental Abs and cross-linking GaM fragments were added simultaneously to the Ramos/Jurkat AM/T mixture, thus bridging both cell lines via GaM fragments. This led to an increased activation of the T cells, especially when high (10 μg/ml) concentrations of the Abs were used. However, as shown in Fig. 3B, BIS20x3 induced a 15-fold more effective T cell activation. These results demonstrate that the efficient activation by cross-linking was not merely caused by CD3 cross-linking on T cells in the presence of B cells. The close cell-cell interaction between the B cell and T cell as induced by BIS20x3 binding seems a prerequisite for this effect.

**LFA-1-ICAM I interaction is involved in T cell costimulation induced by BIS20x3 and Ramos cells**

We hypothesized that the mechanism of efficient T cell activation via BIS20x3 takes place through the natural interaction of costimulatory molecules such as LFA-1 and ICAM-1 present on T cells and B cells. It has been described that costimulatory molecules in B cells are up-regulated upon activation of the B cell (23, 24). We first determined whether BIS20x3-mediated activation of B cells influenced expression levels of ICAM-1 on the surface of these cells. Fig. 4A shows that Ramos cells display a constitutive expression of ICAM-1 (CD54), which could be significantly up-regulated through activation with BIS20x3 and Jurkat AM/T cells. Similar increases in ICAM-1 expression were observed in Raji,
DAUDI, AND JY CELLS (DATA NOT SHOWN). THE INDUCTION OF COSTIMULATORY MOLECULES ON B CELLS SUGGESTED AN ACTIVE ROLE FOR THE BIS20X3-TARGETED B CELLS IN THE ACTIVATION OF T CELLS.

Next, we investigated the functional significance of the involvement of ICAM-1. Fig. 4B shows that inhibition of the LFA-1/ICAM-1 interaction by blocking Abs reduced T cell activation to 50% using anti-LFA-1 Abs and to 40% for the anti-ICAM-1 blocking Abs. An isotype-matched control Ab did not decrease T cell activation.

Discussion

The chimeric Ab rituximab is the most effective anti-cancer Ab described to date. Still, rituximab therapy is not curative and virtually all patients relapse. It is observed that relapsed lymphomas express higher levels of complement inhibitory proteins or deficient Ab-dependent tumor cell lysis. These findings suggest that therapeutic outcome may improve if the need for the complement system can be circumvented and/or the lytic hit delivered to the target cells can be enforced. To meet these objectives we designed BIS20x3, which is not dependent on components of the humoral immune response but derives its anti-tumor effect from the cytoidal activity of retargeted T cells in a CD20-restricted manner resulting in an efficient eradication of CD20+ B lymphocytes.

A number of BsAb formats have been described over the last decade and in vivo antitumor activity has been shown in some cases in experimental clinical treatment settings (29–31). Many
studies have described the necessity of costimulation as a prerequisite for effective BsAb-mediated T cell retargeting, posing a limitation with respect to the clinical effectiveness of many of the BsAbs described to date (32, 33). One significant advantage of BIS20x3 lies in the fact that BIS20x3 targets B cells, which are considered professional APCs and are thus intrinsically capable of relaying costimulatory signals to T cells. We have demonstrated that cell-cell interactions between Ramos B cells and T cells, mediated through binding of BIS20x3, leads to a highly efficient activation of the T cells (Figs. 1–3). This is not a finding unique for Ramos cells, but was noted in several B cell lines, suggesting that these B cell lymphoma cell lines, and presumably B cell lymphomas in general, have the capacity of triggering T cells with costimulatory signals.

We had two alternative hypotheses for the efficient T cell activation by BIS20x3. First, the possibility existed that the length of the CD3 stimulus determined the activation state of the T cells. It has been shown that specific binding of an Ab to CD3 leads to internalization of the TCR/CD3 Ab complex and eventually to the extinction of the T cell activation signal (34). Simultaneous binding of BIS20x3 to B cells could probably prevent TCR/CD3 internalization, thus leading to longer stimulation and higher levels of T cell activation. Indeed, coating of monovalent anti-CD3 Abs resulted in increased activation of T cells when compared with soluble anti-CD3 Abs. However, this activation did not reach the efficiency by which BIS20x3 was shown to activate T cells in the presence of B cells. This suggested that, although the mechanism of CD3 binding and cross-linking is important for CD3-mediated T cell activation, in the case of BIS20x3 other mechanisms of T cell triggering play a major role.

The second hypothesis was that, upon binding of BIS20x3, B cells and T cells converge and costimulatory molecules such as the integrins LFA-1 and ICAM-1 may interact, leading to reciprocal signaling within both the B cells and T cells. The interaction of these costimulatory molecules may be allowed to take place in a physiological manner because the membrane distance, necessary for these interactions, matches the predicted distance between cells bridged by BIS20x3. Normally, the T cell Ag receptor interacts with MHC-bound Ag, which leads to an intercellular membrane distance of ~7 nm (35–37). IgG molecules (as for example BIS20x3) are known to have a spatial distance between their Ab binding sites of 5.5–7 nm (38). Therefore, the bridge between the T cells and B cells formed by BIS20x3 spans the same distance as in a physiological T cell-APC interaction.

**FIGURE 5.** T cell activation by B cells in the presence of BIS20x3 involves NF-κB and is CD20 dependent. A, Jurkat AM/T cells were cultured in the presence of Ramos or Ramos-IkB or Ramos LZRS cells in a 5:1 ratio and BIS20x3 (0.5 μg/ml) in a total volume of 1.0 ml. As an additional control, RamosLZRS (empty vector) cells were cultured with the T cells and BIS20x3 for 24 h. Luciferase activity was measured and corrected for medium control samples. The mean level of luciferase activation is displayed for each time point and condition along with the SD. B, Jurkat AM/T cells were incubated for 20 h with Ramos cells in a 5:1 ratio in the presence of the BsAbs BIS20x3 or CD3xCD19 in concentrations increasing from 0.01 to 1.0 μg/ml. Luciferase activity was measured and corrected for medium control samples. The mean levels of luciferase activation along with the SD are displayed. C, T cells isolated from PBLs were prestimulated as described with 0.5 μg/ml BIS20x3 or CD3xCD19 Abs and irradiated Ramos or Ramos-IkB or Ramos-LZRS as costimulatory cells. After preincubation, new calcein-AM-labeled Ramos and Ramos-IkB or Ramos-LZRS cells were added to the T cells in a 30:1 E:T ratio and incubated with fresh BsAbs (0.5 μg/ml) for 2 h. Lysis of Ramos or Ramos-IkB or Ramos-LZRS cells was determined by measuring calcein fluorescence in the supernatant. The percentage lysis obtained in cells cultured with either BIS20x3 or CD3xCD19 is corrected for the percentage lysis found in matching samples without BsAb. The mean result of three independent assays is displayed along with the SEM.
The initial purpose of BIS20x3 is to attract T cells to malignant B cells and simultaneously induce T cell activation via CD3 triggering, thus circumventing TCR restriction and mimicking the activation of the natural immune synapse. CD20 is described to interact with MHC class II on various B cell lines (39, 40). This suggests that CD20 might be part of the larger supramolecular activation cluster complex, which is critically involved in MHC/TCR signaling and T cell activation. We hypothesized that BIS20x3 binding to CD20 is expected to induce costimulatory molecules to interact with their counterparts in the vicinity of the TCR, thereby efficiently enhancing the activation of the T cell.

We have shown that ICAM-1 expression is increased upon CD20 stimulation and we demonstrated that the interaction of ICAM-1 and LFA-1 contributes significantly to the efficient activation of T cells (Fig. 4). An important implication of this finding is that the malignant target B cells play an active role in the activation of the T cells resulting in the concomitant eradication of the B cells themselves.

As it is known that activation of B cells (via the B cell receptor, CD40, or MHC class II) or the expression of costimulatory molecules can be regulated by NF-κB signaling (25, 28, 41, 42), we investigated whether the BIS20x3-mediated costimulatory signals were also under control of the NF-κB pathway. Pathways that regulate NF-κB-mediated gene transcription have been described to be involved in CD20 signaling (43). For example, the Src family protein tyrosine kinase signaling pathway is described to be shared in several B cell activation routes including CD20 and NF-κB signaling (40, 44–47). We demonstrated that the BIS20x3-mediated costimulatory effects observed in the activated Ramos B cells are under the control of NF-κB transactivation. In addition, we showed that Jurkat AM/T cells were less efficiently activated using Ramos-1KbB3 cells (Fig. 5A). Also the lytic capacity of freshly isolated T cells was significantly decreased upon preincubation with BIS20x3 and Ramos-1KbB3 cells instead of “normal” Ramos cells. These results underline the importance of signaling through the NF-κB pathway in the target B cells when BIS20x3 is applied (Fig. 5C).

To investigate whether the BIS20x3 incited costimulatory capacity of B cells was restricted to CD20 targeting, another B cell-directed BsAb, CD3xCD19, was used. Both BsAbs are directed against the CD3ε chain of the TCR-CD3 complex and stimulate T cells with similar characteristics and kinetics. As such the CD3 binding parts of the two BsAbs were considered comparable in physiological terms. We demonstrated that the CD3xCD19 BsAb induced significantly less T cell activation and, as a result, did not yield the cytolytic potential in resting T cells as obtained with BIS20x3 (Fig. 5, B and C). This suggested that CD20 targeting BsAbs are more efficient in eliciting costimulatory signals on B cells. These findings were not merely the result of different expression rates of CD19 and CD20 molecules on the surface of the Ramos cells, because we found that the level of Jurkat AM/T cell activation was not associated with the number of CD20 molecules on various B cell lines (data not shown). This indicated that variation in the level of T cell activation is not a direct quantitative effect of the amount of targeted CD20 molecules on the surface of B cells. Also the affinity of Ag binding by the BsAbs does not seem to be intervening with our findings, considering that saturating concentrations BsAbs are used (1.0 μg/ml or higher) resulting in significant differences between CD20 and CD19 targeting. However it cannot be excluded that under nonsaturating conditions, differences in affinity between both BsAbs might be a cofactor in cellular costimulatory effects. In contrast to CD20, binding of Abs to CD19 leads to the internalization of CD19, which suggests that in a clinical setting such BsAbs may not be available for T cell targeting. CD19-targeting Abs might therefore be considered as a preferred reagent for delivery of cytotoxic agents rather than for T cell retargeting therapy (48–50). Moreover, it was described that in vivo antitumor effects of unconjugated anti-CD20 mAbs are superior to those of anti-CD19 mAbs (51). Because BIS20x3 targets to CD20 instead of CD19, it is likely that this BsAb could perform well in clinical settings without the need for additionally applied costimulatory reagents such as CD28 Abs. In vivo comparison of CD3xCD19 Abs with BIS20x3 should be performed to prove the hypothesized higher clinical efficacy of BIS20x3 over CD3xCD19.

Upon transformation and progression of malignancies unique tumor Ags may develop and antitumor-specific blocking Abs could interfere with therapeutic Ab binding or be involved in tumor proliferation. However, to our knowledge this phenomenon has not as yet been demonstrated to occur in the case of CD20-directed therapies such as with rituximab that may relate to the fact that the CD20 receptor neither is a unique tumor Ag nor is it known to be associated with transformation or tumor progression. No studies exist that show either interference of anti-CD20 binding or functional hindrance of anti-CD20-directed treatment modalities by CD20-directed blocking host Abs. We therefore do not expect this phenomenon to be a major problem for the clinical applicability of a CD20 targeting BsAb like BIS20x3.

In conclusion, the results described show that BIS20x3 is a very potent reagent for the activation and retargeting of T cells to CD20+ malignant B cells. We demonstrate for the first time that targeting against CD20 via BIS20x3 induces a process of NF-κB-dependent costimulation, which allows efficient and functional T cell activation. The role of B cells as professional APCs is thus efficiently used by BIS20x3, which makes this Ab a particularly promising new candidate for the treatment of B cell malignancies and warrants further investigation of this treatment modality in experimental clinical treatment settings.

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