A Recombinant Bispecific Single-Chain Fragment Variable Specific for HLA Class II and FcαRI (CD89) Recruits Polymorphonuclear Neutrophils for Efficient Lysis of Malignant B Lymphoid Cells

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*J Immunol* published online 30 December 2009

http://www.jimmunol.org/content/early/2009/12/30/jimmunol.0902033
A Recombinant Bispecific Single-Chain Fragment Variable Specific for HLA Class II and FcεRI (CD89) Recruits Polymorphonuclear Neutrophils for Efficient Lysis of Malignant B Lymphoid Cells

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Bispecific Abs offer new perspectives for cancer immunotherapy. In this study, we describe a recombinant bispecific single-chain fragment variable (bscFv) directed against FcεRI (CD89) on polymorphonuclear neutrophils (PMNs) or monocytes/macrophages and HLA class II on lymphoma target cells. FcεRI and HLA class II-directed single-chain fragment variable (scFv) fragments were isolated from phage display libraries, established from the hybridomas A77 and F3.3, respectively. The two scFv molecules were connected with a 20 aa linker. After expression in SF21 insect cells and chromatographic purification, the bispecific molecule showed specific binding to both Ags at KD values of 148 ± 42 nM and 113 ± 25 nM for the anti-FcεRI and anti-HLA class II scFv components in the bsscFv, respectively. In Ab-dependent cytotoxicity assays with PMNs as effectors and a series of lymphoma-derived cell lines (ARR-77, RAJI, REH, NALM-6, RS4;11), the bsscFv was significantly more cytotoxic than the parental murine IgG1 and its chimeric IgG1 derivative. When targeting primary tumor cell isolates from six patients with B cell malignancies, the killing capacity of the (FcεRI × HLA class II) bsscFv compared favorably to conventional HLA class II mAb. Importantly, the cell lines NALM-6 and RS4;11, as well as two primary tumor cell isolates, were exclusively lysed by the bsscFv. To our knowledge, this is the first report of an FcεRI-directed bsscFv effectively recruiting PMNs for redirected cytotoxicity against human B cell malignancies. Our data show that an (FcεRI × HLA class II) bsscFv is an interesting candidate for further engineering of small, modular immunopharmaceuticals.

The Journal of Immunology, 2010, 184: 000–000.

In the last decade, mAbs against tumor-associated Ags have gained a significant role as an additional therapeutic option in the field of immunotherapy for an increasing number of patients with malignancies (1). Malignant B-lymphoid cells almost represent ideal targets for Ab-based immunotherapy, because they are readily accessible for circulating mAb and express a wide range of well-defined target Ags. Apart from CD20 (2), the most prominent example, mAb or mAb-derived small modular immunopharmaceuticals with specificity for additional target Ags, such as CD19, CD22, CD52 (alemtuzumab) or HLA class II, have been tested in clinical trials or approved (3, 4). HLA class II Abs, including Lym-1 and Hu1D10 (apolizumab) have been moved to the clinic and have been evaluated in phase I and II clinical trials in lymphoma patients, where they demonstrated therapeutic efficacy (5). Based on promising preclinical data, Hu1D10 was evaluated in a pilot study in combination with G-CSF for recruiting neutrophils as effector cells in relapsed lymphoma patients (6). Considerable safety concerns were initially raised, because HLA class II Abs showed to be potent in activating human complement and because HLA class II expression is not lymphoma-restricted (7). However, the toxicity of the Ab was manageable through the use of slow to moderate infusion rates (8).

In general, Abs produce a direct antitumor activity by blocking molecules associated with cell proliferation or tumor progression (e.g., growth factor receptors or cell adhesion molecules important for tumor metastasis) and by induction of apoptosis. In addition, Abs can work indirectly by complement dependent cytotoxicity (CDC) as well as Ab-dependent cell-mediated cytotoxicity (ADCC). HLA class II Abs, rituximab or trastuzumab, effectively trigger ADCC, CDC, apoptosis, and growth inhibition in vitro (1, 9). However, the relevance of these mechanisms in vivo still requires further investigation.

Human IgGl Abs, such as rituximab and trastuzumab, interact efficiently with all three classes of Fcγ receptors (FcγRI, FcγRII, and FcγRIII). They are highly effective in triggering ADCC by mononuclear effector cells, and they often show a prolonged plasma half-life ($t_{1/2}$)
because of binding to FcRn (10). Studies in transgenic and other mouse models have supported the importance of the Fc receptor-mediated mechanisms of action. In knockout mice, in which the signaling machinery of FcR was genetically disrupted, rituximab and trastuzumab showed reduced therapeutic efficacy, indicating a dominant role for FcR-mediated effector mechanisms. However, mAbs also bind Fc receptors on noncytotoxic cells (such as FcγRI on platelets and B cells), Fc receptors that do not activate cytotoxic cells (such as FcγRIIb on polymorphonuclear leukocytes), and Fc receptors, which even inhibit effector cell activation (such as FcγIIb). In FcγRIIB-deficient mice, trastuzumab and rituximab induced far stronger ADCC than in wild-type mice (11, 12). The responsiveness of patients with non-Hodgkin’s lymphoma to rituximab was further correlated with polymorphisms in FcγRIIIa and FcγRIIa, which influence binding of mAbs to immune effector cells. However, the exact role of FcγR in vivo is still controversial (13-15).

In order to selectively recruit activating FcR, bispecific Abs (bsAbs) specifically binding to defined FcR epitopes were generated. The first approach was the fusion of two different Ab-secreting cells to a quadroma (quadroma technology), followed by the chemical conjugation of two different mAbs or Ab fragments. Both technologies require elaborate purification procedures and suffer from reduced production yields. Recombinant bsAbs offer the advantage of modular flexibility and large scale production. bsAbs simultaneously bind to a target Ag on tumor cells and to selected trigger molecules on effector cells. Moreover, bsAbs can be selected to recognize and activate FcR outside of the Ig binding site, thereby circumventing competition with natural Ig. These characteristics facilitate efficient recruitment of individual effector cell populations (16).

In addition to T cells and NK cells, FcR-bearing myeloid cells represent potential effector cell populations. The most numerous cytotoxic effector cells in humans are polymorphonuclear neutrophils (PMNs). Although in recent years PMNs have been increasingly recognized as playing important roles in immunosurveillance against malignant tumors (17), data from in vivo studies investigating this population of effector cells are rare. A SCID mouse model, neutrophils not only migrated and infiltrated cancerous tissues, but also actively contributed to the in vivo antitumor activity of rituximab, probably via induction of ADCC (18). In a phase I study of the bsAb MDX-H210 (anti FcγRI x anti HER-2/neu) the cytotoxic capabilities of neutrophil granulocytes were enhanced during G-CSF treatment (19).

In previous studies, we investigated the in vitro capacity of PMNs to lyse malignant B cell lines using Abs and Ab derivatives to a range of different B cell Ags. PMNs were consistently unable to lyse malignant B cell lines via CD19 or CD37-directed chemically linked bsAbs. Interestingly, chemically-cross-linked bsAb directed against FcR and HLA class II, as well as chimeric mouse/human-directed Ab of IgA isotype were highly effective in triggering tumor cell killing by PMNs (23). Therefore, we have generated a recombinant bispecific single-chain fragment variable (bsscFv), consisting of two single-chain fragment variables (scFvs) directed against HLA class II on tumor targets and FcR on PMNs. We characterized the binding of this fusion protein to effector and tumor cells and its capacity to induce effector cell-mediated lysis of lymphoma cell lines as well as primary isolates from lymphoma patients.

Materials and Methods

Mab and Ab constructs

HLA class II hybridoma F3.3 (murine Ig G1 [mIgG1]) was provided by Tenovus Research Laboratory (Southampton, U.K.). The hybridoma cell line A77 (FcγRI, CD89, mIgG1) was provided by Medarex (Amannalle, NJ). Generation of mouse/human chimeric F3.3 (chimeric Ig G1 [chIgG1]) Ab against HLA class II was performed as described (23). The mAb used for detection of recombinant bsscFv were Penta-His (Qiagen, Hilden, Germany), FITC-coupled goat anti-mouse IgG (Dako, Hamburg, Germany) and HRP-coupled goat anti-mouse IgG (Dako).

Culture of eukaryotic cell lines

Baby hamster kidney (BHK)-21 cells and murine fibroblast L66 cells were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine (RF10+ medium). BHK-21 cells, stably transfected with expression constructs for a human CD89 cDNA and the common FcγR γ-chain, were cultured in RF10+ medium. Selection pressure for the continued expression of the CD89 cDNA construct was maintained by adding 400 μg/ml geneticin (InVitrogen) and 10 μM puromycin (Sigma-Aldrich, St. Louis, MO). The continued expression of the FcγR-γ-chain construct, L66 cells, stably transfected with a cDNA expression construct coding for the human α-chain of HLA DR, were cultured under selection pressure by adding 600 μg/ml geneticin to the RF10+ medium. The malignant B cell lines RS4;11 (t4;11 acute lymphoblastic leukemia [ALL]), NALM-6 (pre-B ALL; German Collection of Microorganisms and Cell Lines, Braunschweig, Germany), REH (ALL), RAJI (Burkitt’s lymphoma), and ARJ-77 (mature B cell line; the last three cell lines were obtained from the American Type Culture Collection, Rockville, MD) were kept in RF10+ medium. All malignant cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO2. SF21 insect cells (Invitrogen) were cultured in SF-900 II medium (Invitrogen) containing 50 μg/ml penicillin and 50 μg/ml streptomycin at 27°C in a humidified incubator.

Bacterial strains and plasmids

Escherichia coli KL1-Blue (Strategene, Amsterdam, The Netherlands) was used for the amplification of plasmids and cloning, and E. coli TG1 (from Dr. G. Winter, Medical Research Council, Cambridge, U.K.) was used for screening of Ab libraries. Libraries were generated in the phagemid vector pAK100, and pAK400 (both from Dr. A. Plückthun, University of Zurich, Switzerland) was used for the expression of soluble scFv (24) in E. coli HB2151 (from Dr. G. Winter, Medical Research Council, Cambridge, U.K.). The vector pFastBAC1 (Invitrogen) was used for expression in SF21 insect cells.

Cloning of variable Ig domains

The cloning of variable light chain (VL) and variable heavy chain (VH) domains from the hybridoma A77 and F3.3 was performed as described (24, 25).

Construction of the bsscFv

The secretion leader sequence from the metilin gene of the honeybee was amplified and cloned into the plasmid pFASTBAC1 thereby creating the vector pFASTBAC1-Mel (26). Sequences for a Gly, Ser, Ile flexible linker and for the anti-HLA class II scFv were cloned from the pSecTag2HygroC-F3.3 × SDH (as an EcoRI and Pmel fragment) sited into pFASTBAC1-Mel, linearized with EcoRI and Stul, thus generating the vector pFASTBAC1-Mel-F3.3. To introduce the anti-CD89 scFv, the vector pAK400-A77 was digested with Sfil. The resulting scFv was cloned into the vector pFASTBAC1-Mel-F3.3.
linearized with SfiI, thus creating the final expression vector pFASTBAC1-Mel-A77 x F3.3. The construct was sequenced by using the ABI PRISM sequencing kit (Applied Biosystems, Foster City, CA).

Expression and purification of the bsscFv

The recombinant bacmid-construct was generated by transforming 1 µg of the expression vector pFASTBAC1-Mel-A77 x F3.3 into the bacterial strain DH10Bac (Invitrogen). Preparation of recombinant bacmid DNA and transfection of SF21 cells was performed following the manufacturer’s guidelines (Invitrogen). After 10 d, culture supernatants containing baculovirus were collected and titrated by endpoint dilution. For expression of the bsscFv, SF21 cells were infected with recombinant baculovirus with a multiplicity of infection of five. After 10 d, culture supernatant was collected and dialyzed at 4˚C against a 5000-fold excess of buffer containing 50 mmol/l NaH2PO4, 300 mmol/l NaCl, and 10 mmol/l imidazole (pH, 8.0).

The secretion leader sequence from the melittin gene of the honeybee (Mel) is followed by the Fc domain of IgG1. The c-myc tag is added to facilitate detection of the product by western blot analysis. The c-myc- and a hexahistidyl-tag (His6) for detection and purification. The c-myc tag is added to facilitate detection of the product by western blot analysis.

The 6x His-tagged bsscFv was purified by affinity chromatography with Ni-NTA agarose beads and finally dialyzed against PBS.

Negative control Ab was TIB92 (mIgG2a; American Type Culture Collection) and the bsscFv was detected with Penta-His Ab (Qiagen) according to the manufacturer’s instructions. Immunoreactive proteins were visualized by chemiluminescence detection using HRP-conjugated goat anti-mouse Igs (Dako) and ECL Western blotting detection reagents (Amersham, Little Chalfont, U.K.), the bsscFv was detected with Penta-His Ab (Qiagen) and finally dialyzed against PBS.

NaDodSO4 PAG and immunoblot analysis

Purified preparations of the bsscFv were analyzed by SDS-PAGE under reducing conditions. Gels were stained with Coomassie brilliant blue R250 (Sigma-Aldrich). After transfer onto polyvinylidene difluoride membranes (Amersham, Little Chalfont, U.K.), the bsscFv was detected with Penta-His Ab (Qiagen) according to the manufacturer’s instructions. Immunoreactive proteins were visualized by chemiluminescence detection using HRP-conjugated goat anti-mouse Igs (Dako) and ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence analysis

Target cell lines (3 x 10^5 cells) were incubated with purified bsscFv (1 µg/ml) for 30 min at 4˚C. Cells were washed with PBS containing 0.1% BSA and 7 mmol/l Na-azide (PBA) and incubated with 20 µl Penta-His Ab (Qiagen) at 200 ng/ml for 30 min at 4˚C. Unbound Abs were then removed by washing with PBA, and the cells were stained with FITC-labeled F(ab’)2 fragments of polyclonal goat anti-mouse Abs (Jackson Immunoresearch, West Grove, PA) for 30 min at 4˚C. After washing with PBA, cells were resuspended in PBS and analyzed with a flow cytometer (Coulter Epics Profile, Brea, CA). Negative control Ab was TIB92 (mIgG2a; American Type Culture Collection).

For each patient sample or cell line, respectively, the relative fluorescence intensity (RFI) was calculated as the ratio of mean linear fluorescence intensity of relevant to irrelevant, isotype-controlled Abs.

Determination of affinity constants for scFv components by flow cytometry

K_d values were determined by flow cytometry using published procedures (26). The highest mean fluorescence value was set to 100%, and all data points were normalized. Experiments were repeated four times and arithmetic mean values are reported. The K_d values were calculated using a nonlinear regression curve fit. Values and graphic analysis were generated using GraphPad Prism Software (GraphPad Software, San Diego, CA).

Isolation of mononuclear and neutrophilic effector cells and primary human leukemic cells

Experiments reported here were approved by the Ethics Committee of the University of Erlangen-Nuremberg (Erlangen, Germany) in accordance with the Declaration of Helsinki. After informed consent was obtained, 20 ml peripheral blood was drawn from randomly selected healthy volunteers or patients with leukemic B cell malignancies. Mononuclear cells (MNCs) and PMN cells were isolated as described (20). Citrate anticoagulated blood was layered over a discontinuous gradient consisting of 70% and 62% Percoll (Seromed, Berlin, Germany). After centrifugation, neutrophils were collected at the interphase between the two Percoll layers and MNCs from the serum/Percoll interface. Remaining Es were removed by hypotonic lysis. Purity of MNC and PMN preparations was >95%, as determined by cytopsins analyses. Viability of cells was tested by trypan blue exclusion and was >95%. For analysis of primary human leukemic cells, blood from patients was layered over a Ficoll (Biotech, Dreieich, Germany) gradient. After centrifugation, MNCs were isolated from the interface. Remaining Es were removed by hypotonic lysis. Viability of cells was tested by trypan blue exclusion.

ADCC assays

ADCC assays were performed as described (20). Target cells were labeled with 200 µCi (7.4 MBq) ^51Cr for 2 h. After extensive washing with RF10^− medium, cells were adjusted to 10^5 per milliliter. Whole blood, plasma, or isolated effector cells (50 µl), sensitizing Abs at indicated concentrations, and RF10^− medium were added to round-bottomed microtiter plates (NUNC, Langenselbold, Germany). For assays with isolated PMNs, RF10^− medium was supplemented with GM-CSF at a final concentration of 50 U/ml. GM-CSF does not significantly increase FcRI expression, but stimulates killing by individual effector cells (27). Experiments were started by adding target cells (50 µl), resulting in a final volume of 200 µl and an effector target (E/T) cell ratio of 40:1 with isolated effector cells. After 3 h at 37˚C, assays were stopped by centrifugation, and ^51Cr release from triplicates was measured in cpm in a scintillation counter. Percentage of specific cellular lysis was calculated with the following formula:

\[ \% \text{ specific lysis} = \left( \frac{\text{experimental cpm} - \text{basal cpm}}{\text{maximal cpm} - \text{basal cpm}} \right) \times 100 \]

with maximal ^51Cr release determined by adding perchloric acid (3% final concentration) to target cells and basal release measured in the absence of sensitizing Abs and effector cells. Ab-independent cytotoxicity (effectors

FIGURE 1. Design (A) and expression (B) of the bsscFv (FcoRI × HLA class II). The promoter derives from the baculovirus polyhedrin gene (Phed). The secretion leader sequence from the melittin gene of the honeybee (Mel) is followed by the FcoRI scFv and the HLA class II scFv, both in V_L-linker-V_H orientation. L represents the 20 aa flexible linker (Gly4Ser)4, c-myc- and a hexahistidyl-tag (His6) for detection and purification. B, Purity and integrity of the recombinant bsscFv (FcoRI × HLA class II). After expression in SF21 insect cells, the recombinant bsscFv (FcoRI × HLA class II) was purified by affinity chromatography with Ni-NTA agarose beads and analyzed by SDS-PAGE under reducing conditions. Gels were either stained with Coomassie brilliant blue or blotted onto nitrocellulose (immunoblot). Recombinant bsscFv was detected with anti-histidine Ab.
without target Ab) was observed in whole blood assays and with mononuclear effector cells, but not with PMN.

Statistical analysis

Group data are reported as mean ± SEM. Differences between groups were analyzed by unpaired (or paired, when appropriate) Student t test. Significance was accepted at p < 0.05. Levels of statistical significance are indicated.

Results

Generation and characterization of the recombinant bsscFv (FcαRI × HLA class II)

For this study, a recombinant bsscFv, directed against HLA class II on B cells and FcαRI on PMNs as effector cells was constructed. The corresponding scFv fragments were obtained by subcloning the hybridomas F3.3 and A77, respectively, using phage display technology, as described (24). Specific binding of scFv fragments to target Ags was determined by immunofluorescence experiments. First attempts to express the bsscFv in bacterial (E. coli, pET27b+ expression vector system) or mammalian cell culture (CHO/DHFR−, 293 T and BHK, pSecTag2HygroC expression vector system) were not successful. BsscFv expressed in E. coli was exclusively found in the insoluble protein fraction most likely as inclusion bodies. Refolding procedures remained unsuccessful. After expression in mammalian expression systems, only very small quantities of ∼5 μg/l culture were produced. To improve expression levels in mammalian expression systems, the order of the FcαRI and HLA class II scFv in the bsscFv construct (FcαRI × HLA class II) was changed to a bsscFv (HLA class II × FcαRI). However, doing so reversed arrangement of scFv but did not result in greater expression yields. The order of V₁/V₄ domains was inverted to generate a V₉/V₄L orientation, but again without any improvement of expression yields. As expression systems in insect cells showed promising results for other recombinant Ab-derivatives (28), a baculoviral expression system was tested.

Consequently, the scFvs were cloned into the pFASTBAC1-Mel vector (see Materials and Methods). At the 3′-end, the expression cassette contains coding sequences for myc and His6 tags for detection and purification (Fig. 1A). The bsscFv was expressed in SF21 insect cells, using baculoviral vectors, and purified from the culture supernatants by affinity chromatography with Ni-NTA agarose beads. In SDS-PAGE experiments, the bsscFv migrated close to the calculated mass of 59 kDa. No signs of degradation were detected. The yield of the purified bsscFv ranged from 60–100 μg/l supernatant (Fig. 1B). Expression levels were similar to those reported for a bsscFv directed against CD16 using the same HLA class II scFv (26). BsscFv, produced in insect cells, was used in this study for the initial analysis of the recombinant molecule and for functional assays.
To test whether the scFv components embedded in the bsscFv would still bind to their respective target Ags, flow cytometric analysis was performed. As a result, both scFv components reacted with cells transfected with FcRI or HLA-DR, but not with the corresponding nontransfected cells, indicating that both scFv retained their ability to bind specifically to their target Ags when contained in the fusion protein (Fig. 2A–D). The recombinant bsscFv reacted with FcRI on PMN and HLA class II on RAJI cells, which were used as effector cells and target cells, respectively, in chromium release assays (Fig. 2E, 2F).

**Binding affinities of scFv components in the bsscFv**

The apparent affinities for binding of the bsscFv to cell surface FcRI and HLA class II were determined by calibrated flow cytometry (26) using FcRI-transfected BHK or HLA class II-positive RAJI cells, respectively. The bsscFv bound to FcRI with a $K_D$ of $148 \pm 42$ nM (Fig. 3A) and to HLA class II with a $K_D$ of $113 \pm 25$ nM (Fig. 3B), which was in close agreement with the $K_D$ value obtained for the same scFv in a bsscFv molecule of identical format with specificities for CD16 and HLA class II ($137 \pm 34$ nM) (26). Thus, the affinity of the HLA class II scFv remained unchanged, even after fusion with different partners in the bsscFv format.

The recombinant bsscFv (FcRI × HLA class II) effectively mediates ADCC with PMN as effector cells.

To test whether PMNs are a suitable population of effector cells for the bsscFv, whole blood from healthy donors was fractionated into plasma, mononuclear, and granulocytic effector cells, respectively.

**FIGURE 5.** The recombinant bsscFv (FcRI × HLA class II) mediates lysis of malignant human B cell lines representing different maturation stages. Lysis of (A) ARH-77 (mature B lymphoid cells), (B) RAJI (Burkitt’s lymphoma), (C) REH (ALL), (D) NALM-6 (pre-B-ALL) or (E) RS4;11 (pro-B-ALL with t(4;11) translocation) was measured in standard chromium release assays. BsscFv (●) was compared with a monoclonal murine F3.3 (mIgG1; ○) and a chimeric F3.3 (chlgG1; triangles, grey lines) Ab, directed against HLA class II. Isolated human PMNs were used as effector cells at an E/T ratio of 40:1. The bsscFv triggered significant, concentration-dependent lysis of all five cell lines, mIgG1 and chlgG1 HLA class II mAb mediated killing of all cell lines except for the immature B cell lines RS4;11 and NALM-6. Data are presented as mean percentage specific lysis ± SEM from at least six independent experiments. *Significant ($p < 0.05$) lysis of mAb or bsscFv compared with effector cells without added Ab. **Significantly enhanced cytotoxicity of bsscFv compared with chlgG1 HLA class II and mIgG1 HLA class II mAb. F. Correlation between HLA class II expression level (RFI) and specific cytotoxicity mediated by bsscFv (FcRI × HLA class II). Percent specific lysis represents maximum cytotoxicity from data shown in A–E. Results are expressed as RFI, which was calculated for each cell line as the ratio of mean linear fluorescence intensity of relevant to irrelevant, isotype controlled Abs.
specific lysis, respectively. The cell lines showed heterogeneous target Ag expression. Maximum target cell lysis induced by the bsscFv was closely correlated with the expression level of HLA class II ($r^2 = 0.94$; log RFI saturation kinetics; Fig. 5F). For the bsscFv, Ab concentration at half maximal cell lysis (EC$_{50}$) was significantly lower than for the chIgG1 and the mIgG1 HLA class II mAb, when ARH-77 were used as target cells (EC$_{50}$ of 0.9 nM for the bsscFv [95% confidence interval (CI) of 0.7–1.2]; EC$_{50}$ of 4.5 nM [95% CI of 3.0–6.6] for the chIgG1; and 2.6 nM [95% CI of 1.7–4.0] for the mIgG1; $p = 0.001$, respectively, $p$ for difference of log EC$_{50}$).

The recombinant bsscFv (Fc$a$RI $\times$ HLA class II) mediates lysis of primary human leukemic cells

In general, it is more difficult to induce lysis of primary tumor cells than of lymphoma cell lines. Therefore, recombinant bsscFv, mlgG1, and chlgG1 HLA class II mAb were compared in ADCC assays against primary tumor cells from five patients with B cell chronic lymphocytic leukemia (B-CLL) and one patient with common acute lymphoblastic leukemia (Fig. 6A–F). Patient characteristics are shown in Table I. With isolated PMN from healthy donors as effectors, the recombinant bsscFv showed strong cytotoxic activity against all samples, whereas considerably lower cell killing or complete failure to induce target cell lysis was observed with mlgG1 and chlgG1 HLA class II mAb. Importantly, two primary samples did not show any lysis by the conventional mAb, whereas the bsscFv killed these tumor samples with up to 50% specific lysis.

Discussion

In this study, a novel bsscFv (Fc$a$RI $\times$ HLA class II) was generated to recruit and activate PMNs as effector cells for lysis of HLA class II-positive malignant tumor cells. This work demonstrated that a recombinant bsscFv directed against Fc$a$RI and HLA class II was effective in killing human B-lymphoid cell lines and primary tumor cells. Fractionation of whole blood into PMN, MNC, and plasma containing complement demonstrated that PMNs are the predominant effector cell population for ADCC with the bsscFv directed against Fc$a$RI.

HLA class II has long been recognized as an interesting target Ag for lymphoma therapy (9). In particular, pan-DR Abs such as F3.3 (also used in the current study) proved to be effective in vitro. However, the expression of HLA class II is not restricted to malignant B cells, but it is also found on normal B cells, activated T cells, activated endothelial cells, and dendritic cells. HLA class II is homogeneously expressed at high levels on a broad spectrum of hematologic malignancies and is more resistant to modulation than other Ags. PMNs as effector cells require high target Ag surface densities to be effective (29). In previous studies, HLA class II was a suitable target Ag in combination with PMNs as effector cells (30). However, in this study, the cell lines NALM-6 and RS4;11 were highly resistant to PMN-mediated ADCC with Fc$\gamma$R-triggering Abs directed against HLA class II. One explanation for this observation might be that NALM-6 and RS4;11 express lower levels of HLA class II, compared with the other cell lines. In addition RS4;11 is a pro-B cell line, which was established from a patient with ALL carrying the t(4;11)(q21;q23) chromosomal translocation. These leukemias are difficult to treat by standard chemotherapy. Accordingly, this cell line is highly refractory to apoptosis induction by conventional chemotherapeutics and CD95 Abs (31), reflecting a different sensitivity of the cells to proapoptotic signals. ADCC induced by PMNs generally occurs through the induction of apoptosis in the target cells (32). Interestingly, our bispecific construct, targeting the identical epitope on HLA class II as the Fc$\gamma$R-directed Abs, was able to eliminate these highly resistant cell lines, although they

**FIGURE 6.** Killing of primary human leukemic cells by the recombinant bsscFv. HLA DR-positive patient samples (A–F) were isolated and Ab-mediated lysis of the cells was evaluated in 3-h $^{51}$Cr-release assays using the recombinant bsscFv (Fc$a$RI $\times$ HLA class II) (○) or the corresponding murine (○) or chimeric (triangles) IgG Abs. The assays were performed with PMNs as effector cells from healthy donors at an E/T ratio of 40:1. The recombinant bsscFv mediated effective lysis of all samples. The killing capacity of the (Fc$a$RI $\times$ HLA class II) bsscFv compared favorably with the mlgG1 and chlgG1 HLA class II mAb.
expressed lower Ag densities. This property may be of clinical relevance, because highly variable target Ag expression can be found on tumor cells and derived metastases. Experiments with primary tumor cell isolates showing heterogeneous HLA class II expression, confirm these data. The killing capacity of the FcαRI × HLA class II bsscFv again compared favorably with the mlgG1 and chlgG1 HLA class II mAb, and high lysis rates were mostly correlated with high target Ag expression (Table I).

Our results are in accordance with previous observations that PMNs are unable to lyse malignant B-lymphoid cells with CD20-directed Abs triggering FcyR, but effectively mediate ADCC with FcαRI-directed CD20 Abs (22). Consequently, these data confirm that triggering FcαRI instead of FcyR on PMNs might lead to an improved activation of PMNs or the induction of alternative pathways to apoptosis in the target cells (33).

Another explanation for the superiority of the bsscFv could be that the small size of the bsscFv enables more intimate cell conjugation between PMNs and target cells, thereby providing optimal prerequisites for the formation of an immunological synapse—a phenomenon that is associated with successful PMN-mediated ADCC (34). This explanation was also offered for another single-chain Ab construct of the same format, directed against CD19 and CD3 on cytotoxic T cells (bispecific T cell engager molecules), which was highly effective in first clinical trials (4).

However, a bsscFv directed against CD19 and FcαRI that we constructed on the basis of the bsscFv (FcαRI × HLA class II), failed to mediate target cell lysis with PMNs as effector cells, although target cells were stained positive for CD19 expression, and the bsscFv showed specific binding to both Ags (unpublished data). These data reflect the different demands of individual effector populations for effective target cell killing. Consequently, for PMNs the nature of the target Ag or associated surface molecules (adhesion molecules) might represent the outstanding parameters (35).

A gain in affinity or avidity for the target Ag, as well as for the FcγRs, can enhance the in vitro ADCC of bispecific therapeutic scFv-fusion proteins (36). In our experiments, a high cytotoxicity activity was already triggered by monovalent binding and relatively low Ab affinities of both scFv components in the bsscFv. For CD3 as a trigger molecule on T cells, it has been shown that a weaker binding to the trigger molecule resulted in a more decisive level of effective T cell activation and cytotoxicity than a stronger level of effective T cell activation and cytotoxicity (37).

BsscFv-induced lysis of target cells was exclusively mediated by PMNs. No lysis was observed with plasma or MNCs. This finding is in contrast to human IgG1, the format of Ab used by almost all approved Ab drugs, which at least in vitro mediates target cell killing mainly by recruiting NK cells as effector cells or by complement fixation. For rituximab, first-infusion reactions were correlated with the activation of complement (38), whereas the therapeutic contribution of CDC to the antitumor activity of rituximab in patients remains to be investigated (39, 40). Our Ab construct, which does not activate human complement, might be an attractive alternative for clinical applications.

This bsscFv is a first step to investigate this target Ag/trigger molecule combination in a recombinant molecule and confirms data from a corresponding chemically cross-linked bsAb (22). Naturally occurring IgA binds to the EC1 domain of FcεRI, whereas A77, the hybridoma Ab used to generate the scFv against FcεRI in our bispecific molecule, binds in the EC2 domain of the receptor and consequently cannot be blocked by serum IgA (41).

For the treatment of residual disease, especially in the case of transplanted and therapeutically pretreated patients, PMNs appear to be an attractive effector cell population, because they are rapidly reconstituted within 3–4 wk after transplantation. At this early point, they already show cytotoxic functions, whereas T cells need several months and longer to reconstitute after HSCT (42). Furthermore, circulating PMNs can be easily expanded and activated by application of G-CSF or GM-CSF, respectively (6).

In conclusion, the results of our study underline the importance of FcεRI as a potent trigger molecule on PMN and HLA class II as suitable target Ag on malignant B cells. This bsscFv format represents a promising prototype of a recombinant bsAb construct, which we will further engineer to increase expression rate, selectivity for malignant B cells, and in vivo $t_{1/2}$.

Acknowledgments

We thank S. Moi and B. Bock for technical assistance, Dr. A. Plüchthun for the scFv vectors, Dr. G. Winter for E. coli TG1, Drs. M. Glennie and J.G. van de Winkel for the F3.3 hybridoma and the A77 hybridoma, and Dr. L. Weiner for HLA-DR transfected L66 cells.

Disclosures

The authors have no financial conflicts of interest.

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Table I. Characterization of patient samples

| Table I. Characterization of patient samples |
|---------------------------------------------|
| Patient | Patient | Patient | Patient | Patient | Patient |
|---------|---------|---------|---------|---------|---------|
| Age (y), sex | 71, male | 77, male | 67, female | 76, male | 70, male | 50, male |
| Diagnosis | B-CLL | B-CLL | B-CLL | B-CLL | B-CLL | c-ALL |
| Stage | Binet A | Binet A | Binet C | Binet A | Binet A | High risk |
| Cytogenetics | del 13q14, IgVH mut | del 13q14, IgVH mut | ND | ND | ND | ND |
| HLA DR expression (RFI) | 89.2 | 138.9 | 45.9 | 36.2 | 20.6 | 312.8 |

*Results are expressed as RFI, which was calculated for each sample as the ratio of mean linear fluorescence intensity of relevant to irrelevant isotype controlled Abs. c-ALL, common acute lymphoblastic leukemia; ND, not tested.
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