Tumor Therapy with Bispecific Antibody: The Targeting and Triggering Steps Can Be Separated Employing a CD2-Based Strategy

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For tumor therapy with unprimed effector cells, we developed a novel combination of a CD2 × tumor Ag bispecific targeting Ab and an anti-CD2 triggering Ab. These Ab constructs were derived from two novel CD2 mAbs, termed M1 and M2 that, together, but not individually activate T cells. Unlike many other CD2 Abs, M1 and M2 do not interfere with TCR/CD3 triggering nor do they inhibit binding of CD2 to its ligand CD58, thus preserving the physiological functions of these important effector cell molecules. M2 was chemically conjugated with an Ab recognizing the epidermal growth factor-receptor (EGF-R). Incubation of unprimed peripheral blood mononuclear cells with the bispecific $F(ab')_2$ construct (*M2xEGF-R*) in the presence of trigger Ab M1 led to efficient selective lysis of EGF-R-positive targets by CTL and NK cells. Importantly, the need for trigger Ab M1 for effector cell stimulation allowed to separate targeting from triggering steps in vitro and should thus enable to focus immune responses to sites of target Ag expression in vivo. ***The Journal of Immunology,*** **1999, 163: 2064–2072.**

One concept to circumvent impaired effector-cell/tumor-cell interactions in malignant disease relates to the use of bispecific mAbs (BsAbs)[3] that can simultaneously bind tumor cells and effector cells and activate the latter in a diseased organ. Several surface receptors expressed by immune effector cells have been suggested as targets for BsAbs such as Fc receptors on macrophages and neutrophils, the TCR/CD3 complex, and the CD2 and CD28 coreceptors (1). CD2 offers the advantage of being strongly expressed by all T lymphocytes and virtually all NK cells independent of their differentiation and activation stage (2). Potential effector cells expressing CD2 can be activated by pairs of monoclonal CD2 Abs, which leads to a vigorous proliferative response, massive cytokine production, and expression of cytolytic effector functions of both T lymphocytes and NK cells (3, 4).

In this paper we report on functional studies based on a novel mitogenic combination of CD2 mAbs, termed M1 and M2, respectively. Neither M1 nor M2 interact with the CD58 binding site of human CD2 and thus do not block CD2-mediated adhesion processes. In addition, both Abs are strongly reactive with resting T cells and, when used in combination, can trigger unprimed pre-effector cells to undergo differentiation into cytolytic effectors. M2 was chemically conjugated with an Ab fragment directed against the epidermal growth-factor receptor (EGF-R) yielding the bispecific Ab (BsAb) M2xEGF-R. If BsAb M2xEGF-R is applied together with M1, effector cells are induced to selectively kill EGF-R-positive tumor targets. Employing such a combination of BsAb plus triggering Ab, it should be possible to delineate a treatment strategy in which the targeting and activation steps can be separated. This could help to focus the cytolytic potential to sites of EGF-R expressing tumor targets and at the same time should avoid lymphocyte activation at unwanted sites and thus minimalize systemic side effects of immunotherapy in vivo.

Materials and Methods

Cells

Human PBMC were prepared by Ficoll-Hypaque- (Pharmacia, Uppsala, Sweden) density centrifugation of heparinized whole blood. Resting human T cells were prepared as described in (5, 6) and were >95% reactive with CD3 mAbs. The following cell lines were used: human Jurkat T cell lymphoma line JM-P1 (7), human epidermoid carcinoma line A 431 (8), and human erythro-leukemia line K 562 (9). C8161 is a nonpigmented human melanoma cell line isolated from a recurrent malignant melanoma (10). This cell line was kindly provided by Dr. M. J. C. Hendrix (University of Arizona, Tucson, AZ). The cells were maintained in DMEM supplemented with 10% FCS and 2 mM L-glutamine. Single cell suspensions were derived after treatment with 0.5 mM EDTA. The T cell clone C3F2 (11) (1×10^6 cells) was grown in the presence of 40 U/ml recombinant human IL-2 (Biotest, Dreieich, Germany), 1×10^6 freshly prepared human PBMC (irradiated with 60 Gy), and 1.5×10^5 EBV-transformed B-lymphoblastoid LAZ 509 cells (irradiated with 60 Gy). After 7–10 days, no live PBMC and LAZ 509 cells could be detected, and the T cell clone was used for cytotoxicity assays. Tumor infiltrating lymphocytes (TIL) have been prepared as described previously from freshly resected melanoma tissue (12) and have been stimulated via mixed lymphocyte tumor cell culture (MLTC) (13). In short, the freshly isolated lymphocytes were cocultured with autologous irradiated melanoma bulk culture tumor cells in RPMI 1640 medium supplemented with 20 U/ml IL-2 (Genzyme, Cambridge, MA) and 10% FCS. Restimulation of the bulk culture was done on day 12. MLTC derived bulk culture TILs have been used to perform cytotoxicity assays.

Monoclonal Abs

Abs M1 (=AICD2.M1, IgG1), M2 (=AICD2.M2, IgG1), and AICD2.5 (IgG1) were produced in our laboratory. In short: BALB/c mice were immunized with a sonicated lysate of Sf9 cells transfected with human CD2. Spleen cells isolated from immunized animals were fused with Ag8-PAI (provided by M. Kramer, Institut für Immunologie, Heidelberg, Germany).

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[3] Abbreviations used in this paper: BsAb, bispecific Ab; EGF-R, epidermal growth factor-receptor; TA, tumor Ag; TIL, tumor infiltrating lymphocyte; MLTC, mixed lymphocyte tumor culture; TNB, thio-bis-2-nitro-benzoic acid.

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HAT/HT selection was done according to standard protocols. Screening of CD2 specific hybridoma supernatants was done by measuring Ab binding to Jurkat T cells in cytometry analyses. Hybridomas have been subcloned twice. Other CD2 mAbs, OKT11 (IgG1; Ortho, Neckargemünd, Germany), $T11_2$ (1OLD4C1, IgG2A), $T11_3$ (1 $MONO_2A6$, IgG3; $T11_2$, and $T11_3$ were kindly provided by Dr. E. Reinherz, Dana-Farber Cancer Institute, Boston, MA), ICRFCD2.4 (IgM), ICRFCD2.5 (IgG2A), and ICRFCD2.8 (IgG2A; ICRF mAbs were kindly provided by Dr. M. Crumpton, Imperial Cancer Research Fund, London, U.K.). Further mAbs were CD3 mAb OKT3 (IgG1; Ortho), CD58 mAb AICD58.5 (IgG2A (14)), and EGF-R mAb 425 (IgG2A (15)). Abs were used as protein A-purified murine Ig unless indicated otherwise.

Preparation of BsAb and Ab fragments

BsAb M2xEGF-R was generated by chemical recombination of Fab′ fragments of CD2 mAb M2 and EGF-R mAb 425 as described by Brennan et al. (16). Briefly, Ab M2 and mAb 425 were converted into $F(ab')_2$ fragments by limited proteolysis with pepsin. $F(ab')_2$ fragments were purified by chromatography on protein A Sepharose. Fab′ fragments were generated by a mild reduction with DTT (0.5 mM; Merck, Darmstadt, Germany). The CD2-specific Fab′ fragments were modified with 5,5′-dithio-bis-2-nitro-benzoic acid (DTNB, Merck). Subsequently, bispecific $F(ab')_2$ fragments were obtained by conjugation of the Fab′-TNB (thio-bis-2-nitro-benzoic acid) derivative with the hinge-SH groups of the mAb 425-derived Fab′ fragments. Purification of intermediate products (Fab′, Fab′-TNB) as well as the final purification of BsAb was performed by gel filtration on a Superdex 200 column (Pharmacia). Purity of the bispecific conjugate was assessed by 10% nonreducing SDS-PAGE and by chromatography on a hydroxylapatite column. The $F(ab')_2$ fragments of the triggering Ab M1 were generated by limited proteolysis with papain. Fragments were purified as described.

Sheep erythrocyte rosetting assays

The CD2 molecule of human T cells binds to the CD58 molecule (T11TS) on sheep erythrocytes, thus causing the formation of rosettes of erythrocytes around T cells. Rosetting can be reduced by binding of certain CD2 mAbs to the CD2 molecule. Human T cells (2×10^5) were incubated with the respective mAb for 30 min at room temperature in a volume of 100 μl. Subsequently, 2×10^7 sheep erythrocytes were added in a volume of 400 μl. The cell mixture was sedimented for 5 min at $230 \times g$ and incubated for 1 h at 37°C in a 6% CO_2 atmosphere. Following cautious resuspension, a solution of T cell-staining crystal violet was added to facilitate counting. At least 300 T cells per culture were counted microscopically and were considered as rosetted when bound by at least three erythrocytes.

Proliferation assays

Cells were incubated at 37°C in a 6% CO_2 atmosphere for 72 h in the presence of the indicated Abs in round-bottomed microtiter plates in 200 μl RPMI 1640 medium (Life Technologies, Paisley, Scotland), supplemented with 10% FCS, 100 IU/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 4 mM L-glutamine (Life Technologies). In some of the experiments, immunoaffinity purified recombinant human CD58 (17) was added to the cultures. Subsequently, cells were pulsed with 37 kBq [^{3}H]thymidine and harvested following 16 h of additional incubation.

Flow cytometry and analysis of CD2 modulation

Flow cytometry studies of Ag recognition by mono- and bispecific Abs were conducted as described elsewhere (18). Studies of CD2 modulation were performed as follows. Each well of a flat-bottom 24-well plate received 1×10^6 freshly prepared PBMC and the respective Abs in the concentrations as indicated. Cultures with BsAb were additionally mixed with 2×10^5 A 431 cells. All cultures had a final volume of 2 ml. Cultures were incubated for 16 h at 37°C in a 6% CO_2 atmosphere to allow modulation before they were washed two times and incubated for 30 min at 4°C with biotinylated CD2 mAb ICRFCD2.8 (10 μg/ml) for detection of surface-CD2. The negative control sample was incubated with culture medium instead. Following two washes cells were stained for 15 min at 4°C with steptavidin-FITC conjugate (Becton Dickinson, San Jose, CA), washed again, and analyzed on an EPICS Profile flow cytometer (Coulter Electronics, Hialeah, FL).

Cytotoxicity assays

Cytotoxicity with T cell clones as effector cells was determined in standard ^{51}Cr-release assays in round bottomed microtiter plates. Each well (triplicate cultures) received the respective Abs, effector cells, and ^{51}Cr-labeled-target cells at the indicated concentration or number to give a total volume of 200 μl of supplemented RPMI medium (see proliferation assays). Six wells containing target cells in culture medium only were used for determining spontaneous ^{51}Cr-release (SR). Six wells containing target cells in 1% Nonidet P-40 detergent were used for determining maximum release (MR). Cell mixtures were sedimented ($230 \times g$, 5 min) and incubated at 37°C in a 6% CO_2 atmosphere for 4 h. Finally, cells were pelleted ($450 \times g$, 10 min), and 100 μl of supernatant was collected from each well to determine Chromium release in a gamma counter (LKB-Wallac, Stockholm, Sweden). The percentage specific lysis was calculated as: $100 \times$ [(experimental ^{51}Cr release $-$ SR)/(MR $-$ SR)]. In experiments with PBMC as effector cells, these were incubated for 72 h with the respective Abs before ^{51}Cr-labeled A 431 target cells were added and mixed with the effector cells. In some experiments unlabeled NK-sensitive K 562 cells were added additionally. Cell mixtures were sedimented ($230 \times g$, 5 min) and submitted to a 4-h assay as described above. In assays with TILs, cells were incubated for the indicated times. Bispecific and monospecific Abs were added simultaneously or in two steps before supernatants were collected after a total culturing time of 18 h.

Results

Anti-CD2 mAbs with a novel combination of characteristics

For the design of CD2 $\times$ EGF-R-specific BsAbs we produced anti-CD2 mAbs with a combination of favorable characteristics. These mAbs (termed AICD2.M1 and AICD2.M2, abbreviated M1 and M2) are specific for human CD2 as assessed by binding to recombinant CD2 in a sandwich ELISA (data not shown). In competitive binding ELISAs, the mAbs M1 and M2 cross-blocked with the established anti-CD2 mAb $T11_3$ (3) in binding to recombinant CD2 (Fig. 1*B*). However, mAbs M1 and M2 differ from mAb $T11_3$ in that they strongly bind to CD2 on resting human T cells (Fig. 1*A*), whereas the $T11_3$ mAb binds to its CD2 epitope only upon T cell activation (3).

A well-recognized physiological function of the CD2 glycoprotein is to mediate cell-cell adhesion by binding to its ligand CD58 (19, 20). Competitive binding of the majority of CD2 mAbs, including mAb OKT11, blocks the interaction between CD2 and CD58 (Fig. 1 and Ref. 21). In contrast, the CD2 mAbs M1 and M2 neither when applied individually nor in combination exhibit such interference as judged from sheep erythrocyte rosetting assays (Fig. 1*C*). In this respect they resemble the CD2R Ab $T11_3$ (3).

Fig. 2*A* shows that the combination of mAbs M1 and M2 induces proliferation of PBMC as well as of purified T cells, whereas, individually, the Abs have no mitogenic effect. In the presence of suboptimal concentrations of mAbs M1 and M2, addition of recombinant CD58 strongly enhances this proliferative response (Fig. 2*B*). CD58 specificity of costimulation was proven by the addition of an anti-CD58 mAb, which abrogated the functional effect of recombinant CD58.

Fig. 2*C* shows that several of the anti-CD2 mAbs tested, including mAbs $T11_2$ and $T11_3$, inhibit the in vitro activation of PBMC via the TCR/CD3 complex. mAb OKT11 was not included in this assay but had previously been reported to inhibit CD3-driven proliferation of T cells as well (21). In contrast, the mAbs M1 and M2 do not interfere with the TCR/CD3-driven proliferation of PBMC (Fig. 2*C*).

Production of BsAb M2xEGF-R

The CD2 specific mAb M2 as well as EGF-R mAb 425 were cleaved into $F(ab')_2$ and converted into Fab′ fragments. Conjugation of Fab′ fragments of mAb 425 and TNB-derivatives of M2

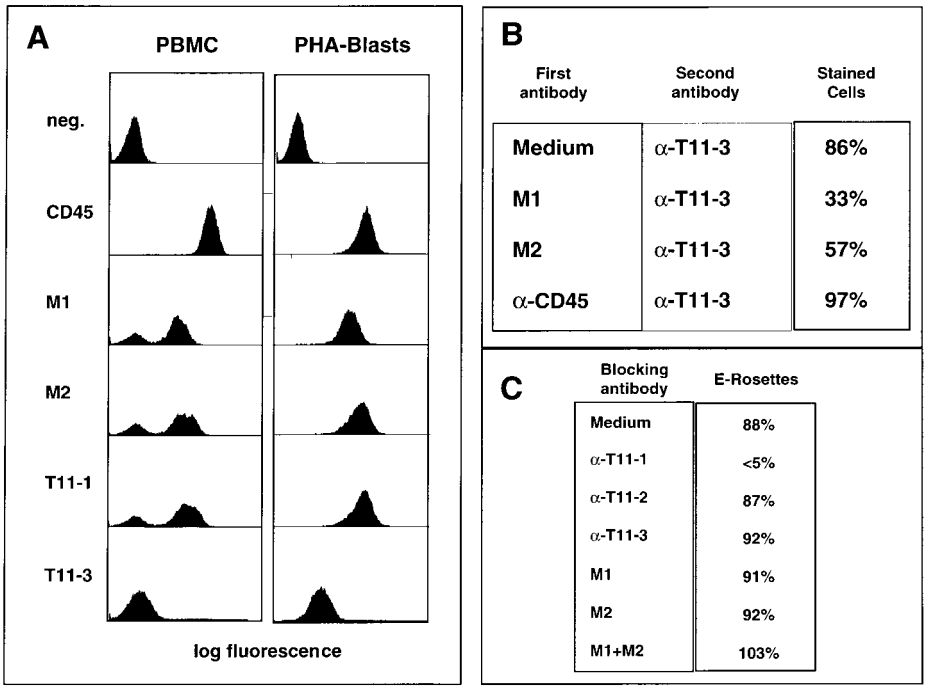

FIGURE 1. *A*, PBMC or 6-day human PHA-blasts where incubated with saturating concentrations of individual mAbs as indicated in the figure. Subsequently, goat anti-mouse IgG-FITC was added an the samples analyzed on a FACSCalibur flow cytometer. *B*, Human T lymphoblasts were incubated with saturating concentrations of mAbs or medium for 60 min at 4°C (first Ab). Subsequently, the samples were washed, a saturating concentration of mAb T11–3 was added to each sample, and they were incubated at 4°C for 30 min. Samples were then washed again and incubated with goat anti-mouse IgG3-FITC (note that $T11_3$ is of the IgG3 isotype whereas all other mAbs employed here are IgG1). Individual samples where analyzed on a FACSCalibur flow cytometer. *C*, Parental CD2 mAbs M1 and M2 do not interfere with the CD2-CD58 interaction in the erythrocyte rosetting of human T cells. Human T cells (2×10^5) were incubated for 30 min with CD2 mAbs ($T11_2$ and $T11_3$, 1:500-dilution of ascites; all other mAbs, purified Abs at final concentrations of 20 μg/ml. When M1 and M2 were applied in combination, the final total Ab concentration was also 20 μg/ml. Note that all purified–blocking Abs ($T11_1$) were equally effective at concentrations of 2 μg/ml. In addition, all mAb sources had previously been tested for saturated binding to T cells). Sheep erythrocytes (2×10^7) were added to the cultures. The cell mixtures were pelleted, incubated for 1 h, and cautiously resuspended. At least 300 T cells were counted per culture and were considered as rosetted when bound by at least three erythrocytes. Values are means of three independent experiments ± SD.

Fab′ fragments resulted in CD2 × EGF-R bispecific $F(ab')_2$ fragments (BsAb M2xEGF-R). A yield of purified BsAb of 20–30% based on the $F(ab')_2$ starting material was achieved and is demonstrated in Fig. 3. High purity of the bispecific conjugate was demonstrated by chromatography on hydroxylapatite (data not shown) as well as in nonreducing SDS-PAGE (Fig. 3).

Binding properties and CD2-modulation by BsAb M2xEGF-R

Flow cytometry studies showed that BsAb M2xEGF-R bound to both the CD2-positive T cell line JM-P1 as well as to the EGF-R-positive epidermoid carcinoma line A 431 (Fig. 4). As expected from the monovalent CD2 binding of the bispecific conjugate, JM-P1 staining by BsAb M2xEGF-R was weaker as compared with staining by the parental CD2 mAb M2 $(Fab')_2$. When compared with the parental EGF-R mAb 425 $(Fab')_2$, however, staining of A 431 cells was only moderately reduced.

Engagement of the CD2 glycoprotein by CD2 specific Abs can lead to its modulation. This is shown here for the incubation of PBMC with an anti-CD2 mAb of the IgM isotype (compare modulated cells in Fig. 5*C* with unmodulated cells in Fig. 5*B*). Modulation could also be expected in a "targeting" situation in which effector and target cells bind to each other via BsAbs and oligomerize CD2. A strong modulation of the CD2 receptor would not allow repeated binding of effector cells to targets nor would a second challenge with BsAb be able to target and activate such effector cells. Therefore, we tested whether the CD2 molecule remains detectable on the surface of effector cells following incubation with BsAb M2xEGF-R and M1 $F(ab')_2$ in the presence of A431 target cells. Fig. 5*D* shows that modulation of CD2 in PBMC effector cells during a 16-h incubation in vitro was moderate. The CD2 molecules remained detectable with a reduction of the available surface CD2 of about 41%.

BsAb M2xEGF-R mediates EGF-R-specific target cell lysis by a cytotoxic T cell clone

To induce lysis of tumor cells, BsAb M2xEGF-R has to mediate binding of effector cells to target cells (targeting) and to activate effector cells to trigger their cytolytic function. To investigate the targeting properties of BsAbs, cytotoxicity assays were conducted employing a cytotoxic $CD8^+$ T cell clone (C3F2) (11). Before assays, C3F2 cells were cultured in the presence of IL-2 and feeder cells (irradiated PBMC plus EBV-transformed B cells). Since in such a differentiation state further activation is not required for cytolytic action, incubation with BsAb alone should be sufficient to achieve tumor cell lysis. Fig. 6 shows that, whereas the parental mAbs M2 $(Fab')_2$ and 425 $(Fab')_2$ were unable to mediate A431 cell lysis, BsAb M2xEGF-R induced strong cytotoxicity indicating efficient targeting to the effector cells. Furthermore, lysis of A 431 cells was due to the cytolytic function of the effector cells and not to a direct toxic effect of BsAb M2xEGF-R on the targets since the

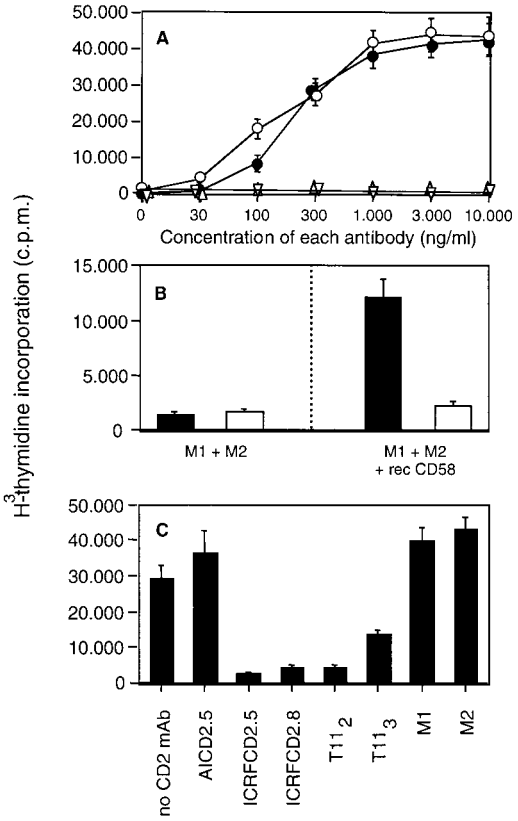

FIGURE 2. Performance of M1 and M2 in CD2- and CD3-driven proliferation of PBMC and T cells. *A*, The combination of M1 and M2 induces proliferation of PBMC and T cells. PBMC (○) and purified T cells (●) were incubated with a combination of mAbs M1 and M2 in different concentrations. When given individually, neither mAb M1 (△) nor M2 (▽) could induce proliferation of PBMC cultures. *B*, Suboptimal stimulation by M1 and M2 allows further costimulation by CD58. Purified T cells were incubated with M1 plus M2 (50 ng/ml) (bars to the left). Additionally, recombinant CD58 (150 ng/ml) was added to some of the cultures (bars to the right). T cells were grown in the absence (filled columns) or presence (open columns) of CD58 mAb AICD58.5 (5 μg/ml). *C*, M1 and M2 do not inhibit the CD3-driven proliferation of T cells. Cultures of PBMC were incubated with 25 μl of Sepharose-coupled CD3 mAb OKT3 and the respective CD2 mAb. CD2 mAbs were added as ascites ($T11_2$, 1:2000 dilution; $T11_3$, 1:4000 dilution) or in purified form (10 μg/ml, all other mAbs). All cultures in *A–C* (5×10^4 cells each) were grown for 72 h, pulsed with 37kBq [^{3}H]thymidine, and harvested following 16 h of additional incubation. Values represent means of triplicate cultures ± SD.

incubation of A431 cells with the BsAb in the absence of effector cells did not induce lysis (data not shown).

Specificity of BsAb-mediated targeting was tested by adding an excess of EGF-R mAb 425 $(Fab')_2$ to cultures of C3F2 T cells, target cells and BsAb. Fig. 6 shows that the addition of EGF-R mAb led to an almost total inhibition of A 431 cell lysis thus proving EGF-R specificity. Furthermore, the inserted diagram in Fig. 6 demonstrates that BsAb M2xEGF-R, even at a high concentration of 250 ng/ml, did not induce lysis of the EGF-R-negative target K562 by C3F2.

BsAb M2xEGF-R plus trigger Ab M1 activate resting PBMC for target cell lysis

In a further set of experiments, we investigated whether the combination of BsAb M2xEGF-R and $M1(Fab')_2$ would be able to activate resting PBMC to undergo proliferation. As shown in Fig. 7, this is indeed the case. When added individually, however, these reagents exerted no stimulatory effect.

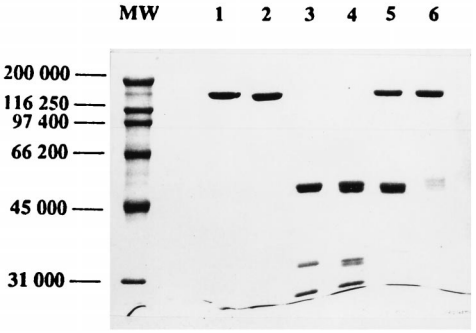

FIGURE 3. SDS-PAGE of intermediate and final products of BsAb production. $F(ab')_2$ fragments derived from mAb 425 (*lane 1*), $F(ab')_2$ fragments derived from mAb M2 (*lane 2*), Fab′ fragments derived from mAb 425 (*lane 3*), Fab′-TNB fragments derived from mAb M2 (*lane 4*), BsAb M2xEGF-R after conjugation (*lane 5*) and after purification by gel filtration (*lane 6*) were run on a 10% SDS polyacrylamide gel under nonreducing conditions.

Activation of PBMC by BsAb M2xEGF-R plus trigger Ab M1 $(Fab')_2$ as observed in proliferation assays also induced the cytolytic effector function in resting T cells: Fig. 8 shows that this combination of Abs was capable of activating PBMC to lyse EGF-R-positive A 431 cells. Again individual Abs were not able to mediate tumor cell lysis. To test the BsAb-mediated contribution to cytolysis by $CD2^+$ NK cells, which represent a subset of PBMC, unlabeled NK target cells (K 562) were added to the mixed cultures of PBMC and labeled A 431 cells (cold target inhibition). Fig. 8 shows that the addition of K562 cells approximately halved the specific lysis of A 431 cells indicating a considerable contribution of NK cells to the BsAb-mediated cytotoxic activity of PBMC.

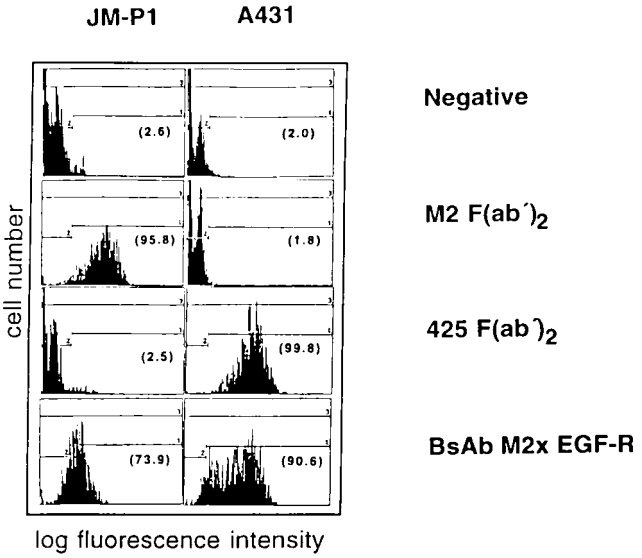

FIGURE 4. Ag recognition by BsAb M2xEGF-R and parental mAbs. A concentration of 1×10^{-7} M of the indicated Abs was used to incubate CD2-positive JM-P1 T cells and EGF-R-positive A 431 epidermoid carcinoma cells. Cells were analyzed on a flow cytometer. As a negative control cells were incubated with secondary goat anti-mouse FITC conjugate only. Numbers in brackets indicate the percentages of positively stained cells.

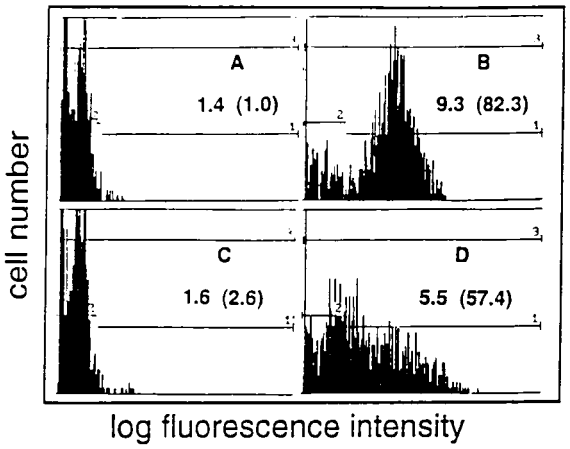

FIGURE 5. BsAb M2xEGF-R causes limited modulation of the CD2 molecule. Freshly prepared PBMC were incubated for 16 h at 37°C with culture medium (*A* and *B*), CD2 mAb ICRFCD2.4 (IgM, 1:400 dilution of ascites) (*C*), BsAb M2xEGF-R (50 μg/ml), mAb M1 F(ab′)$_2$ (50 μg/ml), and A 431 cells (*D*). The PBMC/A 431 cell ratio in *D* was 5:1. Cultures (except *A*) were then incubated with biotinylated CD2 mAb ICRFCD2.8 (10 μg/ml), which could not be blocked by the Abs used in the previous incubation. Negative control (*A*) was incubated with culture medium instead. Cells were stained with steptavidin-FITC conjugate and analyzed on a flow cytometer. Numbers without brackets represent mean fluorescence intensities in arbitrary units. Numbers in brackets show the percentages of positively stained cells.

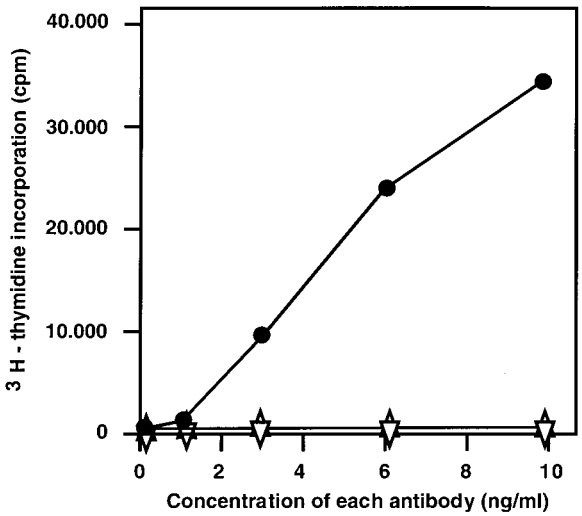

FIGURE 7. BsAb M2xEGF-R induces proliferation of PBMC when complemented by trigger Ab M1 F(ab′)$_2$. PBMC (5×10^4) were cultured for 72 h in the presence of M2xEGF-R + M1 F(ab′)$_2$ (●), M2xEGF-R (△), or M1 F(ab′)$_2$ (▽). Cells were pulsed with 37 kBq [^{3}H]thymidine and harvested following 16 h of additional incubation. Values represent means of triplicate cultures ± SD.

BsAb M2xEGF-R plus trigger Ab M1 mediate cytotoxicity in a two-step protocol

In a final set of experiments, we investigated whether it is possible to target effector cells to tumor cells in a first step by giving BsAb alone and to trigger the former in a second step, i.e., 12 h later by M1 (Fab′)$_2$ to kill. We made use of a human allogeneic system employing the EGF-R-positive C8161 melanoma cell line as target along with MLTC-derived freshly prepared tumor infiltrating lymphocytes. Fig. 9*A* shows that dose dependently the combination of BsAb M2xEGF-R plus M1 (Fab′)$_2$, when added simultaneously, induced tumor cell lysis while the individual reagents did not. The efficiency of this TIL driven melanoma specific lysis was similar as for the PBMC/A431 system situation (compare Fig. 8). Importantly, virtually analogous results were obtained when the addition of BsAb M2xEGF-R and triggering-mAb M1 F(ab′)$_2$ was separated by 12 h (Fig. 9*B*). This result opens up the possibility to employ the CD2-based approach delineated here to separate targeting from triggering steps in a potential therapeutic setting.

Discussion

Novel anti-CD2 mAbs were produced by selection for mitogenicity for T cells as well as for noninterference with additional intrinsic T cell functions. The combination of mAbs M1 and M2 was

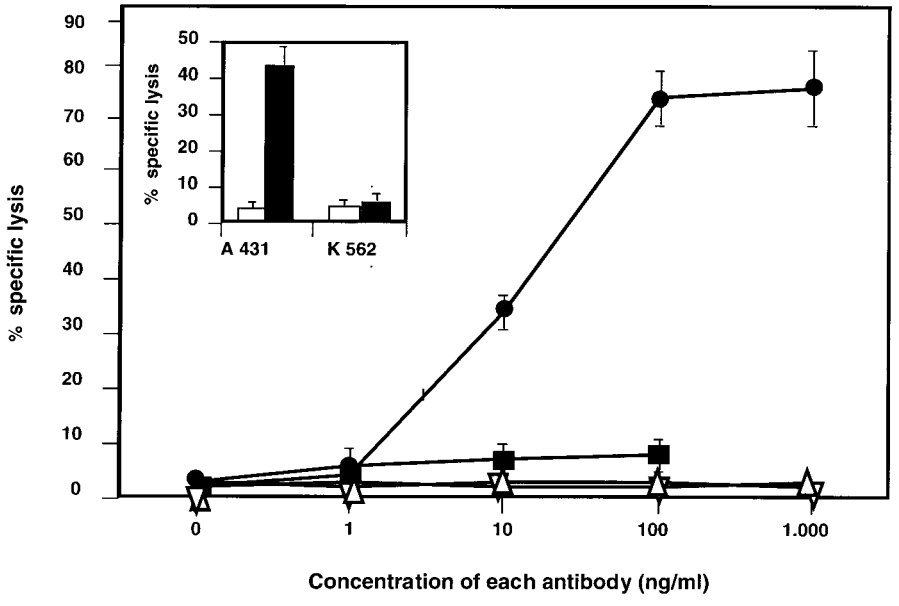

FIGURE 6. BsAb M2xEGF-R mediates EGF-R-specific lysis of target cells by the cytotoxic T cell clone C3F2. ^{51}Cr-labeled A 431 target cells (2×10^3) were incubated with 2×10^4 C3F2 T cells and the following Abs: BsAb M2xEGF-R (●), CD2 mAb M2 F(ab′)$_2$ (△), EGF-R mAb 425 F(ab′)$_2$ (▽), and BsAb M2xEGF-R plus mAb 425 F(ab′)$_2$ (50 μg/ml) (■). *Inset*, C3F2 effector cells and targets (A 431 and K 562 cells) were cultured in a ratio of 3:1 without Ab (open columns) or in the presence of an optimal concentration of the BsAb M2xEGF-R (250 ng/ml) (filled columns, compare dose-response curve (●)). Data are expressed as percentage of specific lysis in a 4-h assay and represent means of triplicate cultures ± SD.

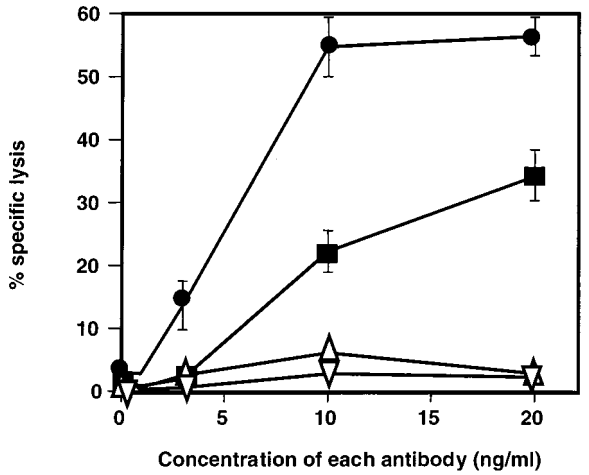

FIGURE 8. Activation of PBMC by BsAb M2xEGF-R plus mAb M1 $F(ab')_2$ induces cytotoxicity against EGF-R-positive target cells. Cultures of 5×10^4 PBMC were grown for 72 h in the presence of various Abs: M2xEGF-R + M1 $F(ab')_2$ (●) and (■), M2xEGF-R(△), and M1 $F(ab')_2$ (▽). Subsequently, 5×10^3 ^{51}Cr-labeled A 431 cells were added. Additionally, some of the cultures were mixed with 8×10^4 unlabeled NK-sensitive K 562 cells (■). Specific lysis of A 431 cells was determined in a 4-h assay. Values represent means of triplicate cultures ± SD.

selected and proved to be highly mitogenic for human T cells and PBMC. The requirement of two different CD2 mAbs to activate human T cells is a common characteristic of mitogenic CD2 mAbs (22) and represents the basis for a novel concept of BsAb-mediated tumor therapy in which the targeting and triggering steps can be separated.

MAbs M1 and M2 exhibited cross-blocking with the previously described comitogenic CD2 mAb $T11_3$ in binding to the CD2 molecule. However, mAbs M1 and M2 recognize the CD2 molecule on resting T cells, in contrast to $T11_3$ (3), suggesting that the novel mAbs and mAb $T11_3$ bind to overlapping but not identical epitopes (23). Recognition of CD2 on resting T cells is a prerequisite for their use as parental mAbs to form bispecific conjugates that are designed to target unprimed effector cells to particular sites in vivo.

The novel CD2 mAbs were tested for noninterference with the CD2-CD58 interaction. The rationale behind this was that bispecific Abs used in, e.g., cancer treatment should not block important T cell functions involving the CD2-CD58 interaction, such as cell-cell adhesion, recirculation of T-lymphocytes as well as costimulation in the activation of T cells through CD2 (17, 19, 24). Unlike mAb OKT11 (which had been used as parental CD2 mAb for a trispecific CD2 × CD3 × TA construct) (25) and several other CD2 specific Abs, mAbs M1 and M2 did not interfere with the CD2-CD58 interaction. Consequently, recombinant CD58 proved to be able to exert its costimulatory function in the presence of parental CD2 mAbs M1 and M2. Thus, in cancer therapy, two effects can be expected: T cells that have bound therapeutic Abs of M1 or M2 specificity but have not yet found access to tumor cells would still possess their CD2-mediated adhesion functions. Secondly, T cells which have bound to tumor cells via a CD2 × TA bispecific conjugate may receive an additional stimulus from binding of CD58 expressed on the tumor cells that would augment their cytotoxic activity.

In this regard, unlike several other costimulatory ligands (CD80, CD86, CD54), CD58 is expressed in the majority of tumor cells of various derivations investigated so far (26) and therefore could enhance the Ab-driven activation of different types of effector cells.

CD2 mAb OKT11when applied in unconjugated form blocks T cell activation via the TCR/CD3 complex (21) through an as yet unknown mechanism whereas as mentioned above (25) in the form of a $T11_1$ × CD3 × target cell trispecific Ab strongly enhances lysis. We have examined both parental CD2 Abs M1 and M2 and other CD2 mAbs for interference with this kind of activation. In contrast to mAbs $T11_2$, $T11_3$, and a series of additional CD2 mAbs, M1 and M2 did not reduce TCR/CD3-driven proliferation and were even slightly comitogenic. As long as the precise molecular mechanisms underlying lymphocyte triggering by mAbs are not elucidated, explanations regarding receptor clustering processes or intracellular events that might be responsible for these

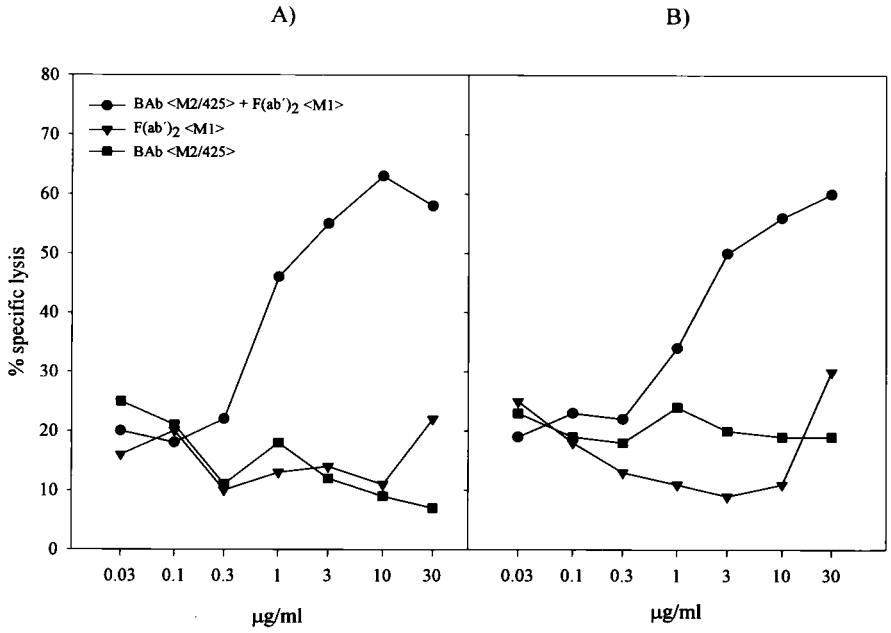

FIGURE 9. BsAb M2xEGF-R plus trigger Ab M1 mediate cytotoxicity in a two-step protocol. MLTC-derived tumor infiltrating lymphocytes (E:T ratio, 20:1) were incubated with ^{51}Cr-labeled C8161 melanoma cells (2×10^3). *A*, BsAb and M1 $F(ab')_2$ were added simultaneously (●). *B*, BsAb was given at time zero and trigger Ab M1 $F(ab')_2$ was added 12 h later (●). Cromium release was tested after an additional 6 h of culture. Neither BsAb M2xEGF-R (■) nor M1 $F(ab')_2$ (▲) induced a remarkable response when given individually and measured after 18 h.

phenomena will remain pure speculations. However, at least one point can me made: there is ample experience from studies on large Ab panels, e.g., CD clusters that have clearly demonstrated that functional Ab activities often relate to molecular features of the Abs themselves (which in spite of extensive investigations on their avidities, isotypes, or molecules/epitopes to which they bind are far from being understood). In this regard it should be mentioned that one of our criteria for the selection of M1 and M2 was that they should not interfere with the CD3 initiated mode of activation in vitro so that negative side effects such as immunosuppression during in vivo application of M1- and M2-derived $(Fab')_2$ fragments and BsAbs would become rather unlikely. Perhaps more importantly, when compared with the widely used targeting to the CD3 complex, the M1- and M2-based targeting of the CD2 molecule may have a critical advantage: previous in vitro studies have shown that binding of CD3 × TA BsAbs to CTL can lead to the inability of these cells to carry out a TCR-specific target cell lysis (27). This phenomenon may be due to inhibition of activation via the TCR/CD3 complex. Alternatively, a CD3 × TA BsAb-mediated reduction of surface expression of the TCR/CD3 complex (modulation) (28) is possible, which would lead to an inability to bind MHC/Ag complexes. Moreover, exposure of T cells to CD3 mAb alone can induce anergy (29). Indeed, we have observed that a CD3 × EGF-R BsAb leads to a strong loss of cell surface expression of the TCR/CD3 complex (our unpublished data). These unwanted reactions can be excluded for BsAbs with M1- or M2-specificity because these mAbs neither inhibit activation via TCR/CD3 (Fig. 2C) nor do they modulate the TCR/CD3 complex (data not shown).

With regard to the Ag on target cells, the EGF-R was chosen. The EGF-R has been reported to be highly over-expressed on malignant cells like epidermal and colorectal carcinoma cells (30) besides its normal expression on endothelial cells, fibroblasts, glial cells, and other cell types. Its high over-expression on certain tumors should allow to find a range of Ab concentrations that cause preferential damage to malignant cells. Moreover, unlike many Ags that are more specific for malignant cells, the EGF-R does not bear the disadvantage of a strong heterogeneity of expression within a given tumor (31).

Binding of particular Abs to triggering receptors can result in their total loss from the cell surface through modulation (32–34). In our experiments, coincubation of PBMC with M2 × EGF-R, trigger Ab $M1(Fab')_2$, and A 431 target cells (imitating the potential targeting situation in vivo) led to a 41% reduction of the cell surface density of CD2. Maintained CD2 expression may enable T cells to bind further BsAb if a therapy with repeated administration of BsAb (35) is chosen.

Cytotoxicity assays with a $CD8^+$ T cell clone showed that BsAb M2xEGF-R was able to target T cells to EGF-R-positive A 431 tumor cells with the bispecific conjugate being active at nanogram quantities. Inhibition of target cell lysis by the addition of EGF-R mAb as well as unreactivity toward an EGF-R negative cell proved the specificity of M2xEGF-R -mediated targeting.

The performance of M2xEGF-R in the activation of unprimed effector cells was tested in both proliferation and cytotoxicity assays. In these assays the BsAb has to be supplemented by the trigger Ab $M1(Fab')_2$ to achieve activation. Proliferation assays showed that the M2 moiety in BsAb M2xEGF-R maintained its ability to stimulate lymphocytes in concert with $M1(Fab')_2$.

Cytotoxicity assays showed that stimulation by M2xEGF-R and $M1(Fab')_2$ induced cytotoxic activity in human PBMC against EGF-R-positive target cells at Ab concentrations similar to those inducing proliferation. No previous study analyzing the performance of a combination of CD2 × TA BsAb and CD2-trigger-Ab (36–38) had demonstrated that such a combination of Abs can induce cytotoxicity in resting PBMC. Given that the majority of NK cells express functional CD2 molecules, it was not unexpected that the cytolytic activity of PBMC was to ~50% due to NK activity as judged from cold target inhibition assays using the standard NK target K562.

The ability of the Ab combination M2xEGF-R plus $M1(Fab)_2$ to activate and recruit NK cells for tumor cell neutralization should be considered an important advantage over the function of BsAbs with CD3 specificity. The simultaneous recruitment of T and NK cells as indicated by our in vitro experiments may provide the basis of an enhanced NK activity because T lymphocytes can strongly support NK functions by secreting IL-2.

Further differences between CD2- and CD3-directed BsAbs exist: In CD3 × TA $F(ab')_2$ BsAbs the monovalency of the anti-CD3 moiety may cause problems because effective CD3-mediated T cell activation is dependent on cross-linking of CD3 molecules (39). In accordance with this fact, triggering of T cells with monovalent reagents requires cross-linking of CD3 molecules by the simultaneous binding of BsAbs to CD3 on T cells and Ags on tumor cells (40). Often tumor cells have reduced surface densities of tumor Ags due to immunoselection (41) or modulation (42). This may cause insufficient cross-linking of the CD3 molecules on T cells if BsAbs are chosen that are specific for such tumor Ags. Activation of T cells via CD2, however, can be achieved by the use of soluble monoclonal CD2 mAbs (Ref. 3 and Fig. 1*A*) or BsAb M2xEGF-R plus mAb $M1(Fab)_2$ without cross-linking (Fig. 7). Thus, here, cross-linking cannot become a limiting factor for T cell activation.

The "two signal hypothesis" of T cell activation (43) demands a second signal delivered by binding of ligands to accessory TCRs in addition to the first signal delivered via the TCR/CD3 complex. However, ligands which normally deliver such second signals, like the molecules of the B7 family, are not expressed on many solid tumors (44). Thus, second signals may not be sufficiently available in the case of tumors that would limit the application of CD3 × TA BsAbs. In such cases the combination of a CD2 × TA BsAb and trigger CD2 mAb may be more efficient because binding of two different CD2 Abs provides both signals required for T cell activation (45). Moreover the majority of malignant cells investigated in our laboratory express at least some CD58 molecules which amplify the CD2-dependent mode of T cell and NK cell activation (26).

The requirement of two Abs for activation of T cells via CD2 opens the perspective of a two-step concept of tumor therapy. Lymphocytes may be loaded ex vivo with BsAb M2xEGF-R before their injection into the blood stream. Alternatively BsAb M2xEGF-R could be administered i.v. Ab-coated lymphocytes or BsAb M2xEGF-R should then be targeted to tumor sites. Importantly, effector cells are not activated during this "targeting phase" because they lack binding of the second CD2 Ab. The length of the targeting phase can be optimized by preceding biodistribution analyses (currently performed in tumor xenotransplanted mice) in which the time of maximal tumor infiltration by BsAb-armed lymphocytes can be determined. In this work a time range of up to 12 h has been tested in vitro (Fig. 9). The 12-h time range is far off the overall half-life of murine $F(ab')_2$ Ab fragments in blood which is 6–8 h in experimental animals (46, 47). Thus, most of the circulating BsAb is expected to be either localized or cleared by the time the trigger Ab is given. In the second step ("activation phase") trigger Ab $M1(Fab)_2$ will be delivered which should activate only those lymphocytes that have been targeted by BsAb M2xEGF-R, thus allowing a preferential local activation. In this concept, BsAb-mediated side effects should be reduced by injecting the trigger Ab

following a targeting phase of optimal length. Moreover, in contrast to a previously proposed strategy using a bispecific CD2 × TA Ab with a $T11_3$ moiety (36), the present concept based on M1 and M2 does not need ex vivo activation of CD2-positive lymphocytes before reinjection.

For the clinical application of CD2 × TA BsAbs a study by Riethmüller et al. (48) may be considered in which a mAb was used to target disseminated tumor cells in patients with colorectal cancer who had undergone curative surgery. The easy access to unshielded disseminated tumor cells for Abs as well as for effector cells may represent a favorable situation for the application of CD2 × TA BsAbs like M2xEGF-R especially when considering the biodistribution pattern of NK cells. In solid tumors these cells are found in rather small numbers (49), whereas disseminated cells may be ideal targets for BsAb-redirected NK cells. Finally, besides their therapeutic application in vivo M1 plus M2xEGF-R Abs could be valuable for ex vivo tumor cell purging.

To prepare future in vivo studies, Abs M2xEGF-R and $M1(Fab)_2$ have been successfully tested for their in vitro performance in two-step cytotoxicity assays (Fig. 9) and for their in vitro function in autologous systems (W. Strittmatter, C. Jäggle, and S. Matzku, unpublished data). Furthermore, the kinetics of biodistribution of M2xEGF-R have been determined in experimental animals to gain data on the optimal period of time needed for targeting by the BsAb (W. Strittmatter, C. Jaggle, and S. Matzku, manuscript in preparation). Mice transgenic for human CD2 (23, 50) exist and can be employed to determine the in vivo performance of M1/M2-based combinations of CD2 × TA BsAbs and $F(ab')_2$ trigger Abs for tumor neutralization.

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References

1. van de Winkel, J. G. J., B. Bast, and G. C. de Gast. 1997. Immunotherapeutic potential of bispecific antibodies. *Immunol. Today 18:562.*
2. Barclay, A. N., M. H. Brown, S. K. A. Law, McKnight, A. J., Tomlinson, M. G, and P. A. van der Merwe. 1997. *The Leukocyte Antigen Facts Book*. 2nd Ed. Academic Press, San Diego.
3. Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1984. An alternative pathway of T cell activation: a functional role for the 50 kDa T11 sheep erythrocyte receptor protein. *Cell 36:897.*
4. Siliciano, R. F., J. C. Pratt, R. E. Schmidt, J. Ritz, and E. L. Reinherz. 1985. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature 317:428.*
5. Schraven, B., M. Roux, B. Hutmacher, and S.C. Meuer. 1989. Triggering of the alternative pathway of human T cell activation involves members of the T200 family of glycoproteins. *Eur. J. Immunol. 19:397.*
6. Samstag, Y., A. Bader, and S. C. Meuer. 1991. A serine phosphatase is involved in CD2-mediated activation of human T lymphocytes and natural killer cells. *J. Immunol. 147:788.*
7. Schneider, U., H. U. Schwenk, and G. Bornkamm. 1977. Characterization of EBV-genome negative "null" and T cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int. J. Cancer 19:621.*
8. Haigler, H., J. F. Ash, S. J. Singer, and S. Cohen. 1978. Visualization of fluorescence of binding and internalization of epidermal growth factor in human carcinoma cells A431. *Proc. Natl. Acad. Sci. USA 75:3317.*
9. Lozzio, C. B., and B. B. Lozzio. 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood 45:321.*
10. Welch, D. R., J. E. Bisi, B. E. Miller, D. Conaway, E. A. Seftor, K. H. Yohem, L. B. Gilmore, R. E. Seftor, M. Nakajima, and M. J. Hendrix. 1991. Characterization of a highly invasive and spontaneously metastatic human malignant melanoma cell line. *Int. J. Cancer 47:227.*
11. Deusch, K., U. Moebius, K.-H. Meyer zum Büschenfelde, and S. C. Meuer. 1986. T lymphocyte control of autoreactivity: analysis with human T-cell clones and limiting dilution culture. *Eur. J. Immunol. 16:1433.*
12. Shimizu, Y., E. Weidmann, S. Iwatsuki, R. B. Herberman, and T. L. Whiteside. 1991. Characterization of human autotumor-reactive T-cell clones obtained from tumor-infiltrating lymphocytes in liver metastasis of gastric carcinoma. *Cancer Res. 51:6153.*
13. Knuth, A., T. Wölfel, E. Klehmann, T. Boon, and K.-H. Meyer zum Buschenfelde. 1989. Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. *Proc. Natl. Acad. Sci. USA 86:2804.*
14. Dengler, T. J., J. C. Hoffmann, P. Knolle, M. Albert-Wolf, M. Roux, R. Wallich, and S. C. Meuer. 1992. Structural and functional epitopes of the human adhesion receptor CD58 (LFA-3). *Eur. J. Immunol. 22:2809.*
15. Rodeck, U., M. Herlyn, C. Molthoff, and et.al. 1987. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects. *Cancer Res. 47:3692.*
16. Brennan, M., P. F. Davidson, and H. Paulus. 1985. Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science 229:81.*
17. Albert-Wolf, M., S. C. Meuer, and R. Wallich. 1991. Dual function of recombinant human CD58: inhibition of T cell adhesion and activation via the CD2 pathway. *Int. Immunol. 3:1335.*
18. Endler-Jobst, B., B. Schraven, B. Hutmacher, and S. C. Meuer. 1991. Human T cell responses to IL-1 and IL-6 are dependent on signals mediated through CD2. *J. Immunol. 146:1736.*
19. Hünig, T. 1985. The cell surface molecule recognized by the erythrocyte receptor of T lymphocytes. *J. Exp. Med. 162:890.*
20. Plunkett, M. L., M. E. Sanders, P. Selvaraj, M. L. Dustin, and T. A. Springer. 1987. Rosetting of human T lymphocytes with autologous erythrocytes. *J. Exp. Med. 165:664.*
21. Van Wauwe, J., J. Goosens, W. Decock, P. Kung, and G. Goldstein. 1981. Suppression of human T-cell mitogenesis and E-rosette formation by the monoclonal antibody OKT11A. *Immunology 44:865.*
22. Valentin, H., S. Huet, J. Morizet, and A. Bernard. 1989. The delivery of distinct activation signals to T-cells via CD2. In *Leukocyte Typing IV*. W. Knapp, ed. Oxford University Press, Oxford, p. 272.
23. Wild, M. K., A. M. Verhagen, S. C. Meuer, and B. Schraven. 1997. The receptor function of CD2 in human CD2 transgenic mice is based on highly conserved associations with signal transduction molecules. *Cell. Immunol. 180:168.*
24. Tiefenthaler, G., T. Hünig, M.L. Dustin, T. A. Springer, and S. C. Meuer. 1987. Purified lymphocyte function-associated antigen-3 and T11 target structure are active in CD2-mediated T cell stimulation. *Eur. J. Immunol. 17:1847.*
25. Tutt, A., G. T. Stevenson, and M. J. Glennie. 1991. Trispecific $F(ab')_3$ derivatives that use cooperative signaling via the TCR/CD3 complex and CD2 to activate and redirect resting cytotoxic T cells. *J. Immunol. 147:60.*
26. Lindauer, M., W. Rudy, B. Gückel, M. v. Knebel-Doeberitz, S. C. Meuer, and U. Moebius. 1998. Gene transfer of costimulatory molecules into a human colorectal cancer cell line: requirement of CD54, CD80 and class II MHC expression for enhanced immunogenicity. *Immunology 93:390.*
27. Segal, D. M., J. H. Qian, D. Mezzanzanica, N. A. Garrido, J. A. Titus, S. M. Andrew, A. J. T. George, C. R. Jost, P. Perez, and J. R. Wunderlich. 1992. Targeting of anti-tumor responses with bispecific antibodies. *Immunobiology 185:390.*
28. Jacobs, N., A. Mazzoni, D. Mezzanzanica, D. R. Negri, O. Valota, M. I. Colnaghi, M. Moutschen, J. Boniver, and S. Canevari. 1997. Efficiency of T cell triggering by anti-CD3 monoclonal antibodies (mAb) with potential usefulness in bispecific mAb generation. *Cancer Immunol. Immunother. 44:257.*
29. Bonnefoy-Berard, N., and J. P. Revillard. 1996. Mechanisms of immunosuppression induced by antithymocyte globulins and OKT3. *J. Heart Lung Transplant. 15:435.*
30. Carpenter, G., and S. Cohen. 1979. Epidermal growth factor. *Annu. Rev. Biochem. 48:193.*
31. Fanger, M. W., P. M. Morganelli, and P. M. Guyre. 1992. Bispecific antibodies. *Crit. Rev. Immunol. 12:101.*
32. Lin, J., R. W. Yon, K. D. Chavin, L. Qin, J. Woodward, Y. Ding, H. Yagita, and J. S. Bromberg. 1995. Anti-CD2 monoclonal antibody-induced receptor changes: down modulation of cell surface CD2. *Transplantation 59:1162.*
33. Meuer, S. C., R. E. Hussey, D. A. Cantrell, J. C. Hodgdon, S. F. Schlossman, K. A. Smith, and E. L. Reinherz. 1984. Triggering of the T3-Ti antigen receptor complex results in clonal T cell proliferation via an interleukin 2 dependent autocrine pathway. *Proc. Natl. Acad. Sci. USA 81:1509.*
34. Gückel, B., C. Berek, M. Lutz, P. Altevogt, V. Schirrmacher, and B. A. Kyewski. 1991. Anti-CD2 antibodies induce T cell unresponsiveness in vivo. *J. Exp. Med. 174:957.*
35. Weiner, G. J., S. A. Kostelny, J. R. Hillstrom, M. S. Cole, B. K. Link, S. L. Wang, and J. Yun Tso. 1994. The role of T cell activation in anti-CD3 × antitumor bispecific antibody therapy. *J. Immunol. 152:2385.*
36. Scott, C. F., J. M. Lambert, R. S. Kalish, C. Morimoto, and S. F. Schlossman. 1988. Human T cells can be directed to lyse tumor targets through the alternative activation/T11-E rosette receptor pathway. *J. Immunol. 140:8.*
37. Scott, C. F., W. A. Blättler, J. M. Lambert, R. S. Kalish, C. Morimoto, and S. F. Schlossman. 1988. Requirements for the construction of antibody heterodimers for the direction of lysis of tumors by human T cells. *J. Clin. Invest. 81:1427.*
38. Scott, C. F., S. Bolender, G. D. McIntyre, J. Holldack, J. M. Lambert, Y. P. Venkatesh, C. Morimoto, J. Ritz, and S. F. Schlossman. 1989. Activation of human cytolytic cells through CD2/T11: Comparison of the requirements for induction and direction of lysis of tumor targets by T cells and NK cells. *J. Immunol. 142:4105.*

39. Meuer, S.C., M. Hauer, K. Deusch, U. Moebius, and K.-H. Meyer zum Büschenfelde. 1987. Two pathways of T cell activation. *Behring Inst. Mitt. 81:15.*
40. Roosnek, E., and A. Lanzavecchia. 1989. Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. *J. Exp. Med. 170:297.*
41. Urban, J.L., M. L. Kripke, and H. Schreiber. 1986. Stepwise immunological selection of antigenic variants during tumor growth. *J. Immunol. 137:3036.*
42. Schreiber, H. 1989. Tumor immunology. In *Fundamental Immunology,* 2nd Ed. W. E. Paul, ed. Raven Press, New York, p, 923.
43. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science 169:1042.*
44. Guinan, E. C., J. G. Gribben, V. A. Boussiotis, G. J. Freeman, and L. M. Nadler. 1994. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood 84:3261.*
45. Meuer, S. C., B. Schraven, and Y. Samstag. 1994. An "alternative" pathway of T cell activation. *Int. Arch. Allergy Immunol. 104:216.*
46. Pimm, M. V., S. M. Andrew, R. W. Baldwin. 1989. Blood and tissue kinetics of radiolabeled anti-CEA monoclonal antibody and $F(ab')_2$ and Fab fragments in nude mice with human tumour xenografts: implications for tumor imaging and radioimmunotherapy. *Nucl. Med. Commun.* 10:585.
47. Molthoff, C. F., J. J. Calame, H. M. Pinedo, and E. Boven. 1991. Human ovarian cancer xenografts in nude mice: characterization and analysis of antigen expression. *Int. J. Cancer 47:72.*
48. Riethmüller, G., E. Schneider-Gädicke, G. Schlimok, W. Schmiegel, R. Raab, K. Höffken, and R. Gruber. 1994. Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. *Lancet. 343:1177.*
49. Whiteside, T. L., and G. Parmiani. 1994. Tumor-infiltrating lymphocytes: their phenotype, functions and clinical use. *Cancer Immunol. Immunother. 39:15.*
50. Lang, G., D. Wotton, M. J. Owen, W. A. Sewell, M. H. Brown, D. Y. Mason, M. J. Crumpton, and D. Kioussis. 1988. The structure of the human CD2 gene and its expression in transgenic mice. *EMBO J. 7:1675.*