Trifunctional Bispecific Antibodies Induce Tumor-Specific T Cells and Elicit a Vaccination Effect

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Abstract

A major goal of tumor immunotherapy is the induction of long-lasting systemic T-cell immunity. Bispecific antibodies (bsAbs) that lack the immunoglobulin Fc region confer T–cell-mediated killing of tumor cells but do not induce long-term memory. In contrast, trifunctional bsAbs comprise an appropriate Fc region and, therefore, not only recruit T cells but also accessory cells that bear activating Fcγ receptors (FcγR), providing additional T–cell-activating signals and securing presentation of tumor-derived antigens to T cells. In this study, we show that trifunctional bsAbs induce a polyvalent T-cell response and, therefore, a vaccination effect. Mice were treated with melanoma cells and with a trifunctional bsAb directed against the melanoma target antigen ganglioside GD2 in addition to murine CD3. The trifunctional bsAb activated dendritic cells and induced a systemic immune response that was not replicated by treatment with the $F(ab')_2$-counterpart lacking the Fc region. Restimulation of spleen and lymph node cells *in vitro* yielded T-cell lines that specifically produced interferon-γ in response to tumor. In addition, trifunctional bsAb-induced T cells recognized various specific peptides derived from melanoma-associated antigens. Moreover, these polyvalent responses proved to be tumor-suppressive and could not be induced by the corresponding $bsF(ab')_2$-fragment. Taken together, our findings provide preclinical proof of concept that trifunctional bsAbs can induce tumor-specific T cells with defined antigen specificity. *Cancer Res; 72(16); 3958–66. ©2012 AACR.*

Introduction

Despite intense efforts to improve treatment of cancer, many malignancies are still incurable. Innovative immunologic modalities for treating patients in a minimal residual disease situation have, therefore, attracted much interest. A promising approach is antibody (Ab)-mediated therapy. In the past 20 years, more than 30 Abs and immunoglobulin (Ig) derivatives have been approved for a huge variety of indications (1). As responses to Ab therapy are often only partial, different approaches are currently pursued to improve the therapeutic efficacy, for example, by optimizing Ab structure (2).

Bispecific Abs (bsAb) are promising tools for eliminating disseminated tumor cells (3, 4). These constructs contain 2 different binding arms that are directed against a tumor-associated antigen (TAA) and a surface molecule expressed on immunologic effector cells, respectively (5). BsAbs are able to redirect immune cells to the tumor site and induce specific tumor cell killing. Effector cells recruited by bsAbs may be T cells (6), natural killer cells (7), or Fcγ-receptor (FcγR) I^+ cells (8, 9).

Appropriate activation of T cells requires several signals normally delivered by antigen-presenting cells (APC). The first signal is provided by the peptide–MHC complex interacting with the T-cell receptor; further signals are mediated by cytokines or costimulatory molecules such as B7 (CD80) expressed by APCs (10, 11). BsAbs that bind to CD3 on T cells can deliver the first T–cell-activating signal, but do not provide the additional signals, if they are devoid of the Ig Fc region. In contrast, bsAbs that do comprise an appropriate intact Fc part, additionally recruit accessory cells bearing activating FcγRI, IIa, or III, thus forming a "tri-cell-complex" (12). Intact, "trifunctional" bsAbs are, therefore, capable of triggering several signals needed for T-cell activation. Further, it is anticipated that APCs recruited *via* Fc–FcγR interaction phagocytose and process antigens derived from lysed tumor cells and subsequently present immunogenic peptides to T cells (13).

Therefore, trifunctional bsAbs not only directly eliminate tumor cells but also presumably exert a vaccinating effect (14). However, a formal proof of tumor-reactive T cells induced by such constructs is missing and the peptide specificities of those T cells are unknown. In this study, we set out to isolate the T cells elicited by immunization with trifunctional bsAbs in a mouse model. We investigated the tumor reactivity of the T cells and their tumor-protective potential, and we defined TAAs that are recognized by these

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T cells. As a model tumor, we used the B78-D14, a murine melanoma derived from B16F0 that is engineered to express the ganglioside GD2 (15), which is an attractive target for immunotherapy of small cell lung cancer and of malignancies of neuroectodermal origin such as neuroblastoma, glioma, or melanoma in humans (16, 17). A trifunctional bsAb directed against GD2 and human CD3 was shown to effectively activate human T cells against melanoma *in vitro* (18). For studying bsAb-mediated vaccination, the trifunctional bsAb Surek was generated that cross-links GD2 with the murine CD3 antigen.

Materials and Methods

Cell lines

The C57BL/6-derived melanoma cell line B16F0 (19, 20) was obtained from the American Tissue Type Culture Collection and cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 2 mmol/L L-glutamine, nonessential amino acids, sodium pyruvate, antibiotics, and 50 μmol/L 2-mercaptoethanol. The cell line B78-D14 (kindly provided by J.C. Becker) is derived from B16F0 by transfection with genes encoding β-1,4-N-acetylgalactosaminyltransferase and α-sialyltransferase, inducing the expression of the disialogangliosides GD2 and GD3 (15, 21). B78-D14 cells were cultured in RPMI-1640 supplemented with 8% fetal calf serum, 2 mmol/L L-glutamine, 0.4 mg/mL G418, 0.5 mg/mL hygromycin B, sodium pyruvate, and nonessential amino acids. The identity of the cell lines was regularly confirmed on the basis of cell morphology, *in vivo* growth behavior, and the expression of selected antigens.

BsAb constructs

Surek is a trifunctional bsAb derived from the parental Abs 17A2 (anti-mouse CD3, rat IgG2b) and Me361 (anti-GD2, mouse IgG2a; ref. 18). Surek was generated by quadroma technology and purified as described previously (22). Surek-bsF(ab′)$_2$ was produced by digestion of Surek with pepsin (14).

Animal studies

Animals were kept under specific pathogen-free conditions in our animal facility. C57BL/6 mice were purchased from Taconic (Ry, Denmark). All animal experiments were approved by *Regierung von Oberbayern*. Typical experiments were performed with groups of 5 to 6 female animals and repeated up to 5 times. Statistical analyses were done using the log-rank test.

For *in vivo* T-cell activation and proliferation assays, mice were inoculated with 10^5 irradiated B78-D14 cells (100Gy) and 10 μg Surek intraperitoneally (i.p.). Control mice received irradiated B78-D14 cells alone or PBS. After 48 hours, spleens and lymph nodes were taken for fluorescence activated cell sorting (FACS) analyses. Alternately, 1.5 mg bromodesoxyuridine (BrdUrd) was injected i.p. and spleen and lymph node samples were collected 3 hours later.

For immunization, mice were injected i.p. with 10^5 irradiated B78-D14 cells and 10 μg Surek or equimolar amounts of the bsF(ab′)$_2$-fragment or the parental Abs on days 0 and 14. Control mice received either irradiated B78-D14 cells alone or PBS. Spleen, lymph nodes, and sera were taken at day 21. BsAb-induced antitumor immunity was tested by challenge of immunized mice with 3×10^3 B16F0 cells i.p. at day 21.

To examine the tumor-protective potential of T cells *in vivo*, 10^6 T cells from *in vitro* cultures after 3 rounds of restimulation (see below) were injected i.p. together with 3×10^3 B16F0 cells. In addition, 24×10^3 IU interleukin-2 (IL-2; Novartis) were injected i.p. for 5 days, starting on the day of T-cell transfer.

T-cell assays

For the assays, 5×10^6 spleen and lymph node cells from immunized mice were stimulated with 5×10^5 irradiated B78-D14 cells in the presence of 30 U/mL IL-2 (Amersham-Pharmacia, Freiburg, Germany). After 1 week of *in vitro* culturing, the surviving cells of the lymph node and spleen suspension were mainly CD3$^+$ T cells. Then, 10^6 of these T cells were restimulated with 10^6 irradiated syngeneic splenocytes (30 Gy) and 5×10^5 irradiated B78-D14 cells and IL-2. Additional stimulation rounds followed after 7-days intervals. Alternatively, restimulation was done using 5×10^6 wild-type (WT) splenocytes loaded with peptides mentioned below without addition of IL-2 for 7 days.

The 24-hour-readout assays, which were conducted either after organ isolation or after 7-day restimulation rounds, were done by coincubating 2×10^5 syngeneic splenocytes and 5×10^4 B78-D14 or B16F0 cells with 10^5 responder cells for 24 hours.

To determine peptide specificities, 10^5 irradiated syngeneic splenocytes were pulsed with 1 μg peptide for 2 hours and then coincubated with 10^5 *in-vitro* restimulated T cells for 24 hours, followed by measurement of interferon (IFN)-γ in supernatants. The following peptides (PSL) were used: HNTQYCNL (MAGE-A5$_{5-12}$; ref. 23), LGITYDGM (MAGE-AX$_{169-176}$; ref. 23), KYMCNSSCM (p53$_{232-240}$; ref. 24), EGSRNQDWL (gp100$_{25-33}$; ref. 25), and SVYDFFVWL (Trp2$_{180-188}$; ref. 24).

Cytokine quantitation

IFN-γ concentrations in supernatants were determined by ELISA (Becton Dickinson) according to the manufacturer's instructions. Additional cytokines in culture supernatants as well as Th1/Th2 cytokines in sera of mice were analyzed by using the Bio-Plex cytokine assay (Bio-Rad).

Flow cytometry

The following directly labeled Abs were used (Becton Dickinson): anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD4 (RM4-5), anti-CD11c (HL3), anti-CD69 (H1.2F3), anti-CD62L (MEL-14), anti-CD86 (PO3), anti-CD83 (Michel-19), and anti-CD80 (16-10A1). Intracellular staining with anti-IFN-γ (XMG-1.2; BioLegend) and anti-IL-12 (C15.6; Becton Dickinson) was carried out after 4 hours of stimulation with PMA/ionomycin and Brefeldin A (eBioscience). For BrdUrd labeling, the fluorescein-isothiocyanate BrdU Flow Kit (Becton Dickinson) was used according to the manufacturer's instructions. To stain for Trp2-specific CD8$^+$ cells, a phycoerythrine-labeled Pro5 MHC Pentamer (ProImmune) was used. Data acquisition and analyses were done using a BD LSR II Flow Cytometer (Becton Dickinson) and

the BD FACSDiva and FlowJo analysis software (TreeStar Inc.).

Cytotoxicity assay

Cell-mediated lysis was quantitated in a standard 4 hours ^{51}Cr-release assay. Trp2 or HY (FNSNRANSS; ref. 26) peptide-loaded or unloaded syngeneic bone marrow-derived mature dendritic cells (DC) were used as target cells. These were labeled with ^{51}Cr and incubated with effector cells from T-cell cultures at varying effector-target ratios.

Reverse-transcription polymerase chain reaction

Total RNA was isolated by using Trireagent (Biozol) according to the manufacturer's instructions. Then, 1 μg RNA was reversely transcribed using an oligo(dT)$_{15}$ primer and avian myeloblastosis virus reverse transcriptase (Roche Diagnostics). TAA expression was analyzed using the LightCycler 2.0 system (LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit; Roche Diagnostics) and the following primers: MAGE-A5, 5′-agggacattgtggactcagc (forward), 5′-aatcacagcagggaggacac (reverse); MAGE-AX, 5′-gaggcctctgagtgcttgaa (forward), 5′-acctggggttagaagggaaa (reverse); p53, 5′-agagaccgccgtacagaaga (forward), 5′-ctgtagcatgggcatccttt (reverse); gp100, 5′-gcaccc-caacttgttgttcct (forward), 5′-gtgctaccatgtggcatttg (reverse); Trp2, 5′-agcagacggaacactggact (forward), 5′-gcatctgtggaaggg-ttgtt (reverse).

Results

T-cell activation induced by the trifunctional bsAb Surek *in vivo*

The trifunctional bsAb Surek is directed against the ganglioside GD2 expressed by B78-D14 melanoma cells and CD3 on murine T cells, respectively. Surek is capable of eliminating B78-D14 cells *in vivo* (27). To evaluate bsAb-dependent tumor immunization, we first analyzed the activation status of T lymphocytes in animals receiving either B78-D14 cells alone or B78-D14 combined with Surek. Enhanced levels of intracellular IFN-γ were detected in CD8$^+$ T cells 48 hours after injection in the bsAb-treated group as compared with control mice (Fig. 1A). In addition, these cells showed upregulated expression of CD69 and downregulated CD62L (Fig. 1B). CD4$^+$ T cells exhibited the same signs of activation, albeit to a lesser extent (not shown). At the same time point, the CD4/CD8 ratio significantly shifted toward CD8$^+$ cells in spleens of animals treated with melanoma cells and Surek (Fig. 1C). As this may be due to enhanced proliferation of CD8$^+$ T cells, we analyzed BrdUrd incorporation in the different T-cell subsets *in vivo*. Then, 48 hours after injection, a strong proliferation of CD8$^+$ T cells and a slightly enhanced proliferation of CD4$^+$ T cells was seen in the bsAb-treated group as compared with the controls (Fig. 1D). These data indicate a strong bsAb-induced activation of T cells *in vivo*.

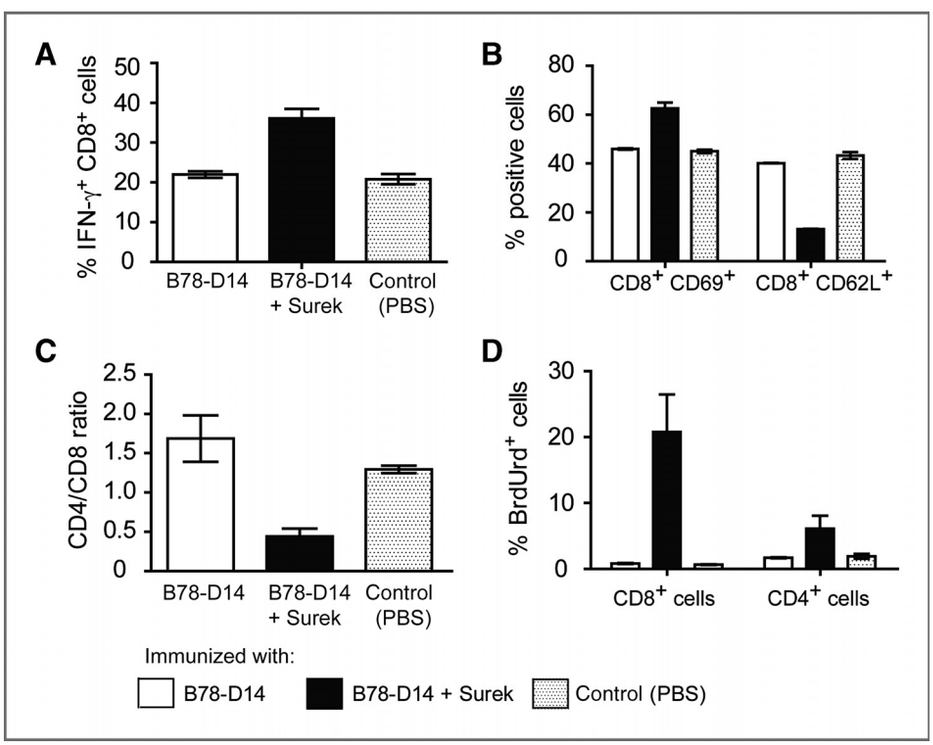

Figure 1. Activation of T cells after immunization with the trifunctional bsAb Surek. Mice received 10 μg Surek together with 10^5 irradiated B78-D14 cells i.p. or tumor cells alone or PBS. After 48 hours, T cells were analyzed in spleens. A, intracellular labeling of IFN-γ in CD8$^+$ T cells after treatment with bsAb and tumor cells or tumor cells alone or PBS. B, staining of the activation markers CD69 and CD62L on CD8$^+$ T cells. Signs of activation including IFN-γ expression were less pronounced in the CD4$^+$ T-cell population. C, ratio of CD4$^+$ and CD8$^+$ T-cell numbers in spleens of differentially treated animals. D, proliferation of T cells as measured by BrdUrd incorporation in spleens after treatment. All panels show means and standard deviations from at least 4 individual mice. The differences detected in the group treated with Surek and B78-D14 compared with the control groups are significant with $P < 0.01$ (Mann-Whitney *U*). The differences between the control group and the group treated with tumor cells alone are not significant.

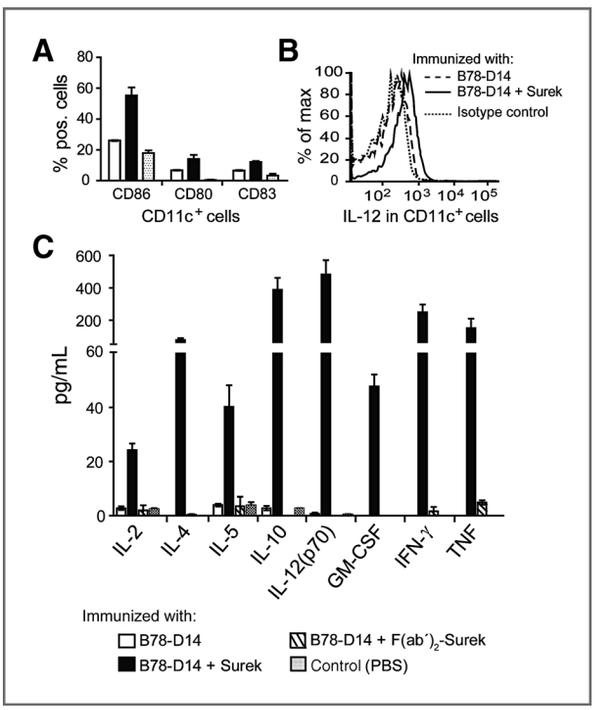

Figure 2. Activation of $CD11c^+$ cells and cytokine expression induced by trifunctional bsAb. A, staining of the activation markers CD86, CD80, and CD83 on $CD11c^+$ spleen cells 48 hours after treatment of mice with Surek and irradiated B78-D14 cells or with tumor cells alone. Means and standard deviations from 4 mice. B, expression of IL-12 in $CD11c^+$ cells from spleens as determined by intracellular FACS staining 48 hours after treatment of mice. Typical result from 4 experiments. C, cytokine concentrations in sera were measured in a Bioplex assay 1 week following delivery of the indicated reagents. Means and standard deviations from 5 mice. A similar pattern was seen after 48 hours.

Activation of DCs by Surek *in vivo*

As trifunctional bsAbs not only bind to T-cell antigens but also to activating FcγRs of accessory cells, we investigated the activation status of DCs after treatment with Surek. Indeed, DCs showed a more mature phenotype 48 hours following injection of Surek and tumor cells as compared with the control groups, thus indicating bsAb-mediated DC activation (Fig. 2A). Activated DCs provide further stimulatory signals for T cells and thereby determine the Th1/Th2 balance. The elevated IFN-γ levels found in T cells (Fig. 1A) are indicative for a Th1/Tc1 bias having occurred after treatment with Surek. To investigate how DCs may contribute to this bias, we analyzed cytokine expression of DCs by intracellular staining. Already 48 hours after bsAb administration, $CD11c^+$ cells were activated by Surek to express augmented amounts of IL-12 (Fig. 2B), which is a typical Th1-inducing cytokine. In sera of bsAb-treated mice, increased levels of the Th1-associated cytokines IL-12, IFN-γ, IL-2, GM-CSF, and TNF, but also of the Th2-related cytokines IL-4, IL-5, and IL-10 were found (Fig. 2C). This was not surprising, because the induction of Th1 responses also requires the expression of Th2 cytokines (see Discussion).

To unequivocally show the significance of the Fc part of the bsAb for immune activation, we also treated mice with a Surek-derived $F(ab')_2$ fragment and B78-D14 cells. No enhanced cytokine levels could be detected in $F(ab')_2$ fragment-treated mice (Fig. 2C), indicating that the intact bsAb is superior to the $F(ab')_2$ fragment in terms of inducing a systemic immune response.

Tumor-reactive T cells are induced after treatment with trifunctional bsAb

The recruitment of DCs is likely to be associated with their engulfment of tumor debris and presentation of tumor-derived peptides toward T cells, which is the prerequisite for a long-lasting T-cell memory. To investigate whether tumor-reactive T cells are induced by bsAb treatment and what is their antigen specificity, we immunized mice twice with B78-D14 cells and Surek. Control mice either received B78-D14 cells alone or PBS. One week after the second immunization, spleen and lymph node cells were isolated and restimulated with B78-D14 cells *in vitro*, each restimulation round lasting 1 week. In contrast to the control groups, high levels of IFN-γ were found in the group immunized with Surek after restimulation (shown for 1 and for 2 stimulation rounds in Fig. 3A). The IFN-γ detected in supernatants was almost exclusively derived from $CD8^+$ T cells, as was shown by intracellular cytokine staining (Fig. 3B).When, after restimulation, T-cell reactivity was tested in 24-hour readout assays using different target cells, IFN-γ was only released in the presence of tumor cells, while there was no response against syngeneic, normal spleen cells (Fig. 3C). This indicates that the response was tumor-specific.

It has been shown before that $bsF(ab')_2$ fragments as well as the combination of the parental Abs show lytic activity against tumor cells (14), but their ability to induce long-lasting memory responses remained elusive. We, therefore, compared the induction of tumor-specific T cells after immunization with $bsF(ab')_2$-Surek, a mixture of the parental Abs (17A2 and Me361) and intact Surek. Testing of reactivity against B78-D14 and B16F0 cells in a 24-hour readout assay revealed a markedly reduced IFN-γ production in the groups having received $bsF(ab')_2$-Surek or the parental Abs as compared with the group immunized with Surek (Fig. 3D). The data show that tumor-reactive T cells are induced by trifunctional bsAbs and that these constructs are clearly superior to $bsF(ab')_2$ fragments in terms of inducing tumor-specific T cells. Further, it turned out that tumor recognition by Surek-induced T cells was independent of the expression of GD2, because untransfected B16F0 cells were equally recognized (Fig. 3D).

T cells induced by trifunctional bsAbs recognize specific tumor-associated antigens

As T cells isolated from Surek-immunized mice and restimulated *in vitro* did not significantly differ with regard to their reactivity against transfected B78-D14 and WT B16F0 melanoma cells, respectively, the ganglioside recognized by Surek appears not to play a critical role as target for the induced T-cell immunity (Fig. 3D). This raised the question as to what is the nature of the melanoma antigens recognized by these T cells. Several antigens have been described that frequently occur in malignant melanoma and that may serve as rejection antigens. In our attempt to define antigens recognized by bsAb-induced T cells, we initially examined the expression of some well-defined immunogenic antigens in B78-D14 and B16F0 cells by

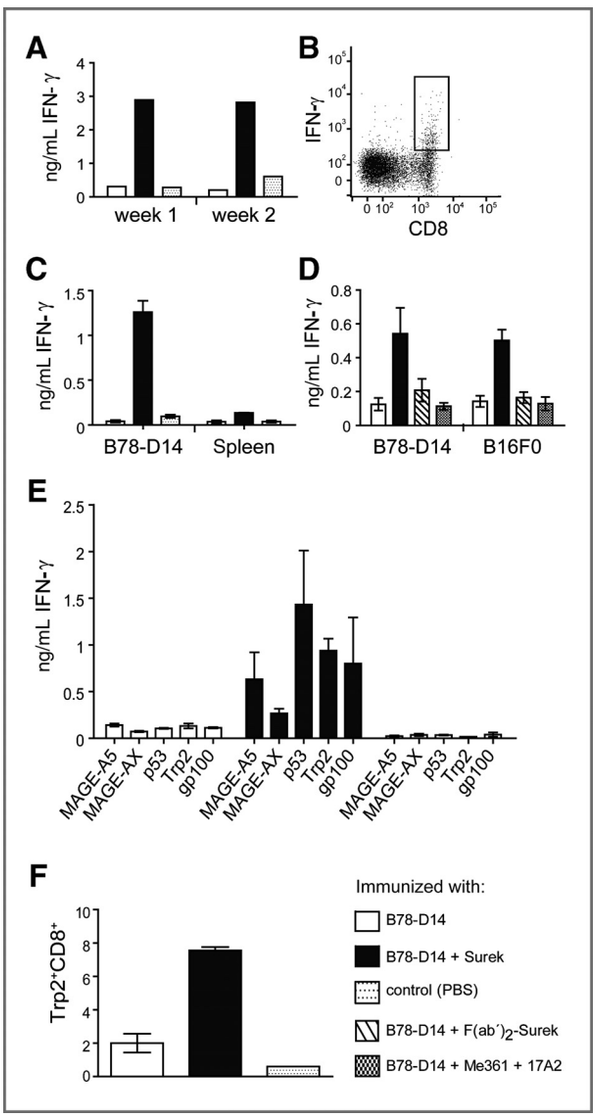

Figure 3. Identification of Surek-induced tumor-reactive T cells and peptide specificities of T cells. Splenocytes of mice that were immunized as indicated were restimulated *in vitro* with irradiated B78-D14 cells in 1-week intervals as described in Materials and Methods. Immunized groups contained 5 mice and immunization and T-cell assays were repeated 5 times. A, IFN-γ levels measured in supernatants of restimulation assays after 1 or 2 restimulation rounds. Typical result from 2 identical experiments. B, IFN-γ detected in restimulation assays is almost exclusively derived from $CD8^+$ cells, as shown by intracellular FACS labeling. The rectangle denotes the IFN-γ-expressing $CD8^+$ T cells. C, T cells after 2 rounds of restimulation were tested in a 24–hour-readout assay for IFN-γ secretion using B78-D14 cells or normal splenocytes as targets. D, comparison of antitumor reactivities of T cells that were induced *in vivo* by trifunctional Surek, bsF(ab′)$_2$-Surek fragment, or a mixture of the parental Abs together with B78-D14 and from animals immunized with B78-D14 alone. Reactivity was tested in a 24-hour-readout assay using B78-D14 cells or the parental B16F0 melanoma cell line. E, T cells after 1 round of restimulation were tested for IFN-γ production by incubating for 24 hours with APCs that were pulsed with specific peptides. F, frequency of Trp2-specific $CD8^+$ cells as measured by Trp2-multimer staining of cells from immunized mice after 1 round of restimulation. Means and standard deviations from 3 experiments are shown.

using real-time PCR (RT-PCR). Expression of MAGE, p53, gp100, and of the tyrosinase-related protein 2 (Trp2) in both cell lines could be verified (data not shown), and appropriate peptides were selected from these antigens (23–25, 28).

To evaluate if these TAAs are targets for Surek-induced T cells, T cells from animals immunized with B78-D14 and Surek were restimulated *in vitro* and subsequently examined for IFN-γ secretion in response to splenocytes that were loaded with the selected peptides. Specific recognition of 2 peptides from the MAGE-A family (MAGE-$A5_{5-12}$ and MAGE-$AX_{169-176}$) and of the peptides $p53_{232-240}$, $gp100_{25-33}$, and $Trp2_{180-188}$ was observed (Fig. 3E). As expected, T cells from the group immunized with B78-D14 cells alone and from the control group treated with PBS virtually showed no reactivity against these peptides.

The frequency of peptide-specific $CD8^+$ T cells was exemplarily estimated for 1 epitope. Therefore, T cells were restimulated *in vitro* with B78-D14 and stained with Trp2-specific MHC-I pentamers. After 1 week, about 8% $Trp2^+$ cells were detected in the $CD8^+$ population in the group immunized with Surek (Fig. 3F).

Taken together, the data formally prove that trifunctional bsAbs but not the parental mAbs nor the corresponding bsF(ab′)$_2$ fragments induce tumor-reactive T cells that are capable of recognizing specific TAAs derived from the bsAb-targeted tumor.

BsAb-induced T cells are functionally active

In addition, we confirmed the peptide reactivity of the T cells in a functional readout assay. Given the high frequency of Trp2-specific T cells (Fig. 3F), we selected this specificity for evaluating cytotoxicity *in vitro*. T cells were isolated from mice immunized with B78-D14 and Surek or with B78-D14 alone and were restimulated with peptides. Only after immunization with Surek and B78-D14, T cells were cytolytic against Trp2-pulsed DCs but not against unloaded DCs or DCs loaded with an irrelevant HY peptide (Fig. 4A).

The functional capacity was tested *in vivo* by T-cell transfer to naïve animals along with a lethal challenge of B16F0 melanoma cells. Approximately 70% of the mice survived (Fig. 4B). In contrast, all animals injected with T cells that were derived from mice treated with melanoma cells alone succumbed to tumor growth. Tumor protection was not only conveyed by T-cell transfer to naïve mice but was also established in those mice that had been immunized with Surek (Fig. 4C). The rejection of B16F0, which is lacking the bsAb-targeted antigen GD2, also indicates that this antigen does not play a critical role in Surek-induced antitumor immunity, but indicates that Surek induces a polyvalent immune response. These data show that treatment with intact trifunctional bsAbs generates a T-cell immunity that is not only TAA-specific but also provides effective tumor protection *in vivo*.

Discussion

Trifunctional bsAbs have not only been described as effective immunologic agents *in vitro* (12) and in mouse tumor models (29), but have also proven successful in cancer immunotherapy in humans (30–32). While bispecific F(ab′)$_2$ fragments redirect only 1 type of effector cells, for example, T

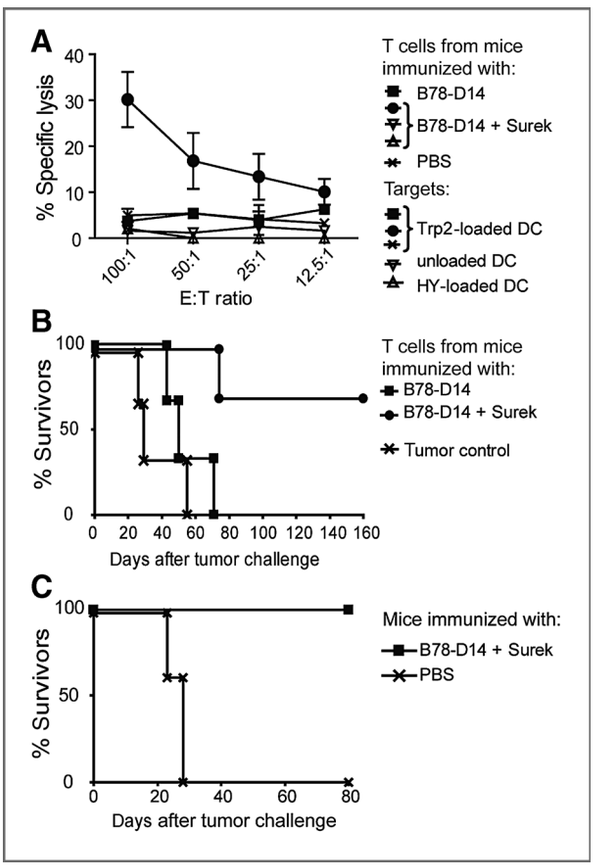

Figure 4. Functional characterization of Surek-induced T cells. T cells were generated by immunizing mice with Surek and B78-D14 cells or with tumor cells alone and subsequent restimulation *in vitro*. A, splenic cells of immunized mice were restimulated for 7 days with irradiated splenocytes loaded with peptides that were identified as targets for bsAb-induced immunity (Fig. 3E). DCs that were loaded with the indicated peptides were used as targets in a standard ^{51}Cr release assay. Means and standard deviations from 3 identical experiments. B, splenic cells were restimulated with irradiated B78-D14 cells for 3 weeks and 1×10^6 restimulated cells were adoptively transferred to naïve animals together with a lethal challenge of B16F0 cells. A total of 9 mice were included in 3 transfer experiments. The survival benefit provided by Surek-induced T cells is significant with $P < 0.05$ (log-rank). C, mice were immunized twice with Surek and irradiated B78-D14 cells and received a lethal dose of viable 3×10^3 B16F0 cells 1 week later. Controls received no immunization before tumor challenge. Similar results were obtained when a tumor rechallenge was given to mice that had received Surek and live tumor cells even 6 months previously (27).

lymphocytes, to malignant cells, trifunctional bsAbs additionally recruit APCs and natural killer cells via their intact Ig Fc part. Through simultaneous activation of different effector mechanisms in a "tri-cell complex" (12), disseminated tumor cells can effectively be killed (14). An important issue for constructing trifunctional bsAbs is the use of the isotype combination mouse IgG2a and rat IgG2b, because it was shown that this combination mediates interaction with activating, but not inhibitory, human FcγR (33). Several data described in this and in previous work indicate a similar FcγR binding of this isotype combination in the mouse (14). As described earlier, these subclasses also enable the production of high and affordable amounts of bsAbs due to a species-restricted preferential pairing of Ig heavy and light chains of corresponding specificities (22). Thus, correctly assembled bsAbs can easily be generated from quadroma supernatants by using a 1-step purification method (22), circumventing the cumbersome procedures that have hitherto limited the production of bsAbs on a clinical scale (3).

Presentation of tumor-derived antigens by APCs that are activated by trifunctional bsAbs elicits a long-lasting antitumor T-cell response. We characterized this immune response by using the transplantable B16-derived B78-D14 mouse melanoma cell line and the trifunctional bsAb Surek. The latter is directed against GD2, a ganglioside that is expressed in human small cell lung cancer, glioma or melanoma and whose potential as target structure for tumor immunotherapy has been shown *in vitro* and in mouse tumor models (34–36). In particular, Ig-IL-2 fusion proteins (21, 37–39) and bsAbs (18) turned out to be effective reagents for initiating immune responses against tumor cells expressing this ganglioside. Thus, B78-D14 and the surrogate antibody Surek provide an appropriate animal model for establishing bsAb-mediated therapy of various types of human cancer.

We could show that treatment with Surek and irradiated tumor cells induces an immunologic memory. In addition, therapeutic and vaccination effects *in vivo* were shown by combining trifunctional bsAbs against EpCAM or GD2 with live tumor cells (14, 27). Further, human cancer patients treated with trifunctional bsAbs showed immune responses against antigens, which were not targeted by the therapeutic bsAb (40).

In mice, Surek induced proliferation of T cells that showed an activated phenotype and enhanced IFN-γ production. Although in other tumor models depletion experiments *in vivo* revealed that bsAb-mediated tumor elimination is equally dependent on both $CD8^+$ and $CD4^+$ T cells (14), $CD8^+$ T cells showed a more pronounced expression of activation-associated surface molecules and of IFN-γ as well as stronger proliferation than $CD4^+$ T cells. In addition, the differential IFN-γ expression in $CD8^+$ and $CD4^+$ cells was reflected in the restimulation assays *in vitro* (Fig. 3B). The significance of this difference in the light of the previous *in-vivo* findings is not clear and awaits further elucidation. As expected, DCs expressed high levels of IL-12 following bsAb treatment, which supports the contention that DCs are being activated in the "tri-cell complex" and are able to endorse antitumor T cells.

It is generally accepted that effective antitumor immunity requires Th1/Tc1 rather than Th2/Tc2 responses (41, 42). The IFN-γ levels measured in T cells and in sera of Surek-treated animals and the IL-12 expression detected in DCs (Fig. 1A, 2B and 2C) indicate a bias toward a Th1/Tc1 response. The finding of Th2 cytokines like IL-4, IL-5, and IL-10 in the sera (Fig. 2C) is not in contrast to this notion, because an efficient Th1 response is dependent on Th2 cytokines as well (42, 43). Thus, a Th1-dependent antitumor vaccination fails when mice are devoid of IL-4 (42). In addition, it has been reported that Th1 cells may shift to expression of Th2 cytokines as a means of "self-limitation" (44).

In this study, the immunizing effect of a trifunctional bsAb was unambiguously shown for the first time by isolation of T

cells from bsAb-treated mice. After restimulation *in vitro*, T cells were identified that specifically recognized the B78-D14 and the parental B16F0 melanoma but not syngeneic splenocytes. We only found tumor-specific T cells in the cultures derived from mice that were immunized with Surek and B78-D14 before restimulation, but not in the controls originating from animals that only received tumor cells or PBS. Of note, the T cells from the latter groups had no therapeutic potential in contrast to those that originated from animals immunized with Surek *in vivo*. Even when Surek was added to the *in vitro* cultures to compensate for *in vivo* administration, no tumor-specific T cells could be detected in T-cell cultures of non-immunized mice (data not shown). This is in accordance with another tumor model described earlier where an *in vivo* priming step was a compulsory requirement for generating T cells with prolonged survival time and tumor-protective potential (42). A possible explanation may be a unique cytokine milieu encountered *in vivo* upon immunization (42).

The reactivity of our bsAb-induced T cells against the parental B16F0 melanoma indicated that the ganglioside recognized by Surek played no pivotal role as target for the ensuing cellular response. To define specific antigens recognized by bsAb-induced T cells, some tumor-associated antigens were exemplarily selected. Indeed, reactivity against peptides derived from gp100, p53, Trp2, and members of the MAGE-A family was found. Further, we could identify Trp2-specific $CD8^+$ T cells by staining with a Trp2-specific MHC-I pentamer (Fig. 3E and 3F) and in a cytotoxicity assay (Fig. 4A). These data indicate that treatment with the trifunctional bsAb mounted a polyvalent cellular response. It has been reported by our group and others that polyvalent antitumor immunity is superior to monoclonal responses (42, 45, 46). Therefore, the immunizing effect of trifunctional bsAbs provides the invaluable advantage that tumor immune escape, for example, by selection of antigen loss variants, is less likely to occur.

The tumor-suppressive function of the bsAb-induced T cells was shown by adoptive transfer into naïve animals, which is the most rigorous readout system for assessing T-cell antitumor effector functions. The parental tumor cell line B16F0, which lacks the bsAb-targeted ganglioside but is supposed to express a similar antigen pattern as the transfected cell line B78-D14, was rejected after adoptive T-cell transfer (Fig. 4B). This further indicates that the antigen GD2 does not play a critical role in Surek-induced antitumor immunity, but on the contrary, that Surek treatment induces a polyvalent immune response.

The induction of a T-cell response was recently also shown for the anti-human CD20 mAb Rituximab in patients (47) and in a mouse model (48). However, in the latter study, cellular immunity was dependent on the expression of the Ab target CD20 on the tumor cells used for rechallenge (48). This indicates the induction of a monovalent immune response by the mAb, which entails a higher risk of tumor escape than the polyvalent immune response found in our system.

Further, our data indicate that the trifunctional bsAb was clearly superior to the parental mAbs and to the corresponding $bsF(ab')_2$ fragments. In contrast to the intact bsAb, immunization with the $F(ab')_2$ fragment or with a combination of the parental mAbs did not lead to detectable specific T-cell responses against B78-D14 or B16F0 melanoma after *in vitro* restimulation (Fig. 3D). Consistent with the missing T-cell response *in vitro*, no enhanced cytokine levels were detected in sera of mice treated with the $F(ab')_2$ fragment (Fig. 2C). In accordance with these findings, the therapeutic potential of $bsF(ab')_2$ fragments *in vivo* was significantly reduced in comparison to trifunctional bsAbs (14).

Taken together, our data formally prove the induction of a T-cell response by a trifunctional bsAb and define the specificity of these T cells. The study provides insights of clinical relevance, because treatment of patients with trifunctional bsAbs may induce long-lasting antitumor responses besides the efficient direct destruction of tumor cells, thus opening new therapeutic options for treating cancer.

Disclosure of Potential Conflicts of Interest

H.L. is the CEO of Trion Pharma and the inventor or co-inventor of several trifunctional antibody patents. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: H. Lindhofer, R. Mocikat
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Eissler
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Eissler, P. Ruf, J. Mysliwietz, R. Mocikat
Writing, review, and/or revision of the mansucript: N. Eissler, J. Mysliwietz, H. Lindhofer, R. Mocikat
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Ruf, R. Mocikat
Review of the manuscript: P. Ruf
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