INTRODUCTION

Despite recent advances in conventional therapy, many types of cancer still have a poor prognosis. Immunotherapeutic approaches for combating minimal residual disease have therefore attracted much interest in the past years. Specifically, efforts have been made to mount systemic T-cell responses against tumor-associated antigens (TAAs) by using various vaccination protocols. Basically, effective T-cell activation requires both a specific signal mediated by the cognate recognition of a peptide major histocompatibility complex (MHC) through a specific T-cell receptor (TCR) and costimulatory signals that are delivered by antigen-presenting cells (APCs), for example, by their expression of CD80 and CD86. Dendritic cells (DCs) are the most potent APCs capable of presenting peptides and providing the requisite costimulatory signals, which is the precondition for inducing an immunologic memory (1). Numerous antitumor vaccination protocols have been elaborated on the basis of transfer of DCs that were generated in vitro and pulsed with tumor-derived proteins or peptides or transduced with TAA-encoding gene constructs (2–4). However, immunization against a single antigen can result in selection of antigen loss mutants and is therefore inferior to polyvalent, whole-cell–based immunization strategies where even yet-unidentified antigens can be included (5–9).

In contrast to DCs, bispecific antibodies (bsAbs) activate T cells by cross-linking CD3 on T cells with TAAs expressed on the tumor cell surface, thereby bypassing the need for specific interaction between a TCR and a peptide-MHC complex (10,11). Although the first activation signal can be provided independently of DCs, DC-dependent costimulatory signals are missing, if the bsAb constructs used are devoid of the immunoglobulin (Ig) Fc region. This drawback is obviated by trifunctional bsAbs (trAbs) containing two binding arms of different specificities and an intact Fc region (12,13). The latter is able to recruit and to stimulate APCs via activating Fcγ receptors (FcγRs), giving rise to a reciprocal stimulation of DCs and T cells. DCs provide cytokines and costimulatory molecules and thereby foster efficient tumor cell killing through naive T cells (12). Furthermore, it is anticipated that the trifunctional bsAb bound to FcγRs of DCs via the Ig Fc region will allow TAAs from lysed tumor cells to be readily internalized by DCs. Processing
of TAAs and presentation of immunogenic peptides through activated DCs will lead to the induction of a long-lasting T-cell memory (14).

A long-term vaccination effect seems to be exerted by trAbs because, in a mouse model, specific tumor-protective T cells recognizing a variety of TAAs were found after treatment with a trifunctional bsAb, but not with its F(ab′)2 counterpart (15). This suggests that accessory cells may play a crucial role for trAb-induced T-cell memory in vivo. In the present study, we dissect the interplay of different immune cells during trAb-mediated antitumor reactions in an in vitro system. We show that DCs play a decisive role in T-cell activation and tumor cell elimination induced by a trAb. Based on these findings, we established a novel immunotherapeutic approach that considerably improved the therapeutic potential of a trifunctional bsAb by combining bsAb treatment with simultaneous delivery of exogenous DCs.

MATERIALS AND METHODS

Cell Lines, Preparation of T Cells and Generation of DCs

B78-D14 melanoma is derived from B16F0, a cell line with C57BL/6 background, by transfection of genes coding for β1,4-N-acetylgalactosaminyltransferase and α-sialyltransferase, inducing the expression of the disialogangliosides GD2 and GD3 (16,17). B16-EpCAM is a B16 variant expressing the EpCAM antigen (14). The latter cells were used as controls because they were not recognized by the therapeutic trAb Surek but could be detected in fluorescence-activated cell sorting (FACS) analyses by virtue of their transgene expression. Cell lines were cultured in RPMI 1640 medium supplemented with 2% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and streptomycin, 50 μmol/L 2-mercaptoethanol, sodium pyruvate and nonessential amino acids in the presence of 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (18). Medium was replaced every 2 d. DC maturation was optionally performed at d 7 by adding 1 μg/mL lipopolysaccharide for 16 h. DCs were characterized by FACS analyses by using monoclonal antibodies against CD11b (M1/70; BioLegend, San Diego, CA, USA), CD11c (HL3; BD, Heidelberg, Germany), MHC II (MS/114.15.2; eBioscience, San Diego, CA, USA), CD83 (Michel-19; BioLegend), CD80 (16-10A1; BD), CD86 (GL1; BD) and CD40 (3/23; BioLegend).

T cells were enriched from sterile spleen suspensions from C57BL/6 mice by negative panning. Briefly, tissue culture dishes (TPP, Trasadingen, Switzerland) were coated with 5 μg/mL anti-mouse B220 (TIB146), anti-mouse IgM (HB88) and anti-mouse κ (HB58; all antibodies provided by E Kremmer, Munich) in 4 mL sterile carbonate buffer (0.1 mol/L, pH 9.5) overnight at 4°C; 4 × 107 spleen cells in 4 mL standard medium were added per dish and incubated for 1 h at 4°C. Nonadherent cells were analyzed by FACS by using monoclonal antibodies (mAbs) against CD19 (dD5; Serotec, Puchheim, Germany), CD3 (500A2; eBioscience), CD11b (M1/70; BioLegend) and NK1.1 (PK136; BD). Purity of T cells was >80%.

Bispecific Ig Constructs

The trifunctional bsAb Surek is derived from the parental Abs 17A2 (anti-mouse CD3, rat IgG2b) and Me361 (anti-GD2, mouse IgG2a) (19). Surek was generated by quadroma technology and purified by one-step affinity chromatography (20). bsF(ab′)2 fragments were produced by digestion of Surek with pepsin (14).

Coculture Experiments

For analyzing T-cell activation and cytokine production, 5 × 105 DCs, 1 × 106 enriched T cells and 1 × 105 B78-D14 cells were cocultured in 1 mL standard medium with or without 20 μg/mL Surek in 24-well plates. Control groups contained 1 × 105 B16-EpCAM cells. T cells were analyzed by FACS staining by using mAbs against CD69 (H1.2F3; eBioscience), CD62L (Mel14; BD), CD4 (Rm4-5; BD), CD8 (53-6.7; BD) and TCRαβ (17A2; eBioscience). Th1/Th2 cytokines in culture supernatants were analyzed by using the Bio-Plex™ cytokine assay system (Bio-Rad, Munich, Germany).

For analyzing Surek-induced tumor cell killing, 5 × 105 DCs, 1 × 106 enriched T cells and 1 × 105 B78-D14 or B16-EpCAM cells were cocultured in 1 mL standard medium with or without 20 μg/mL Surek in 24-well plates. Tumor cell killing was analyzed by FACS staining by using propidium iodide (PI) to exclude dead cells. Tumor cells were additionally stained with antibody Me361 (19) binding to GD2 or with antibody C215 (21) binding to EpCAM and a fluorescence-labeled secondary rat antimouse IgG (H+L) F(ab′), fragment (Jackson Laboratories, West Grove, PA, USA). To determine cell numbers, we used CountBright™ Absolute Counting Beads (Invitrogen/Life Technologies Ltd, Paisley, UK) according to the manufacturer’s instructions. Microscopic images of cultures were made by using an Axiovert 200M microscope, an AxiosCam MRm camera and the AxioVision Rel. 4.8 software (Carl Zeiss MicroImaging, Göttingen, Germany). Alternatively, tumor cell killing was assessed in XTT assays where 4 × 104 B78-D14 cells were coincubated with 2 × 105 enriched T cells in the presence or absence of 5 × 104 immature DCs and with different concentrations of Surek or Surek-bsF(ab′)2. After 3 d, effector cells were removed by washing, and remaining adherent tumor cells were stained by using the XTT proliferation kit II (Roche Diagnostics, Mannheim, Germany). Cell killing in percentage was calculated as follows: [(absorbance tumor cells – absorbance sample)/(absorbance tumor cells – absorbance effector cells)] × 100. GraphPad Prism software (version...
Activation and Proliferation In Vitro

DCs Enhance trAb-Induced T-Cell Activation and Proliferation In Vitro

The murine cell line B78-D14 was generated by engineering B16 melanoma cells to express the gangliosides GD2 and GD3, which are promising target antigens for cancer immunotherapy (16). The trAb Surek binds to mouse CD3 on T cells as well as to the ganglioside GD2. Surek mediates elimination of B78-D14 cells in vivo in a dose- and T cell–dependent manner and induces a long-lasting polyvalent antitumor T-cell response despite low binding affinity to GD2 (15,22).

To elucidate whether DCs promote trAb-dependent T-cell activation, we cocultured B78-D14 cells either with T cells alone or with T cells and syngeneic BM-derived DCs in the presence or absence of Surek in vitro. Surek was able to activate CD8+ as well as CD4+ T cells already after 24 h, as evidenced by upregulation of the early activation marker CD69 (Figure 1A). The combination of T cells with Surek and DCs led to an even stronger upregulation of CD69 on T cells than Surek alone and furthermore encompassed a significant downregulation of CD62L. The latter effect was only seen in those groups that contained T cells, Surek and DCs (Figure 1B). In control groups without Surek, T cells showed no upregulation of CD69 and no downregulation of CD62L irrespective of whether or not DCs were present (Figures 1A, B). In cultures containing DCs and Surek, CD8+ T cells were not only activated, but also showed elevated cell numbers after a time period of 6 d (Figure 1C). By contrast, CD4+ T cell numbers did not significantly increase (Figure 1D), suggesting that the additional signals provided by DCs in the presence of trAb may stimulate survival and proliferation of CD8+ T cells only. These results are in line with our previous findings demonstrating the activation and proliferation of mainly CD8+ T cells induced by Surek in vivo (15). Thus, the combination of DCs, T cells and trAb in this in vitro model appropriately reflects the in vivo situation.

It was anticipated that T-cell activation required trAb binding to CD3 but not simultaneous engagement of the tumor-specific binding arm of Surek. Therefore, the coculture experiments were repeated by using melanoma cells that did not express the GD2 antigen targeted by Surek. In this setting, T-cell activation and proliferation were indeed nearly identical to the results obtained with B78-D14 cells. Similarly, the T cell–stimulating effect of DCs was also independent of tumor-specific binding of Surek (Supplementary Figure S1). Most likely, the tumor specificity of Surek is not required for activat-
ing T cells, but only for redirecting their cytotoxic activity toward the target cells. This result was confirmed by in vitro killing assays (see below).

**Mixed Th1/Th2 Response In Vitro**

After immunizing mice with Surek, a typical Th1-promoting cytokine pattern was found in vivo that included interleukin (IL)-12 expressed by DCs and interferon (IFN)-γ derived from T cells. Th2 cytokines were also produced, which is compatible with the requirement of Th2 cells for priming Th1 responses (15). We then tested whether Surek induces a similar T-cell–activating cytokine profile in cultures containing T cells and DCs. Already after 1 d, high levels of IL-12 in cocultures containing T cells and DCs and Surek were found (Figure 2A), which is a hallmark of DC activation. With regard to T cell–derived cytokines, enhanced levels of the Th1-related cytokines IL-2 and IFN-γ as well as GM-CSF and a small but significant increase of the Th2 cytokines IL-4, IL-5 and IL-10 (Figure 2B) compared with the control groups were detected. These data also indicate a strong T-cell activation induced by Surek at early time points. Because of the anti-CD3 activity of Surek, the same cytokine patterns were found in cultures containing irrelevant tumor cells that were not recognized by Surek (Supplementary Figure S2). Taken together, strong T-cell activation, as evidenced by expression of surface activation markers and by cytokine release, strictly depends on the presence of both DCs and trifunctional bsAb in this setting.

**Efficient Tumor Cell Elimination Depends on the Interaction Between trAb, T Cells and DCs**

Having shown that the combination of T cells, DCs and tumor cells redirected to each other by a trifunctional bsAb is most efficient in activating DCs and T cells, we wondered whether this is also mirrored by the potential to eliminate tumor cells. To analyze Surek-induced tumor cell killing, we cocultured T cells, DCs and live B78-D14 cells in the presence of Surek and analyzed cell death by FACS staining at different time points. Compared to controls, where one component was missing in each case, significantly less viable B78-D14 cells were detected in the group containing T cells, DCs and Surek at d 4 of coculture (Figure 3A) and later (not shown). In contrast, growth of the target cells expressing an irrelevant transgene was not affected. This result indicates that specific binding of the trAb to the tumor antigen stipulates the redirection of the cytotoxic T-cell activity toward the target cell. Because viable tumor cells might have lost GD2 expression and might have been overlooked in the FACS analyses, living, adherently growing B78-D14 cells were also visualized microscopically. Adherently growing B78-D14 cells were present in all groups except for the group containing T cells, DCs and Surek, where almost no adherent cells were detected at d 6 (Figure 3B). Furthermore, no outgrowth of residual tumor cells could be observed, even after more than 14 d (not shown). Thus, it was formally demonstrated that Surek is capable of redirecting naive T cells, but only for redirecting their cytotoxic activity toward the target cells. This result was confirmed by in vitro killing assays (see below).

![Figure 2. Cytokine patterns in cocultures containing T cells, DCs and Surek. After coculturing B78-D14 cells with DCs and T cells with or without 20 μg/mL Surek, cytokine levels were analyzed by using the Bioplex™ system. All groups contained B78-D14 cells. T cells, DCs and Surek were added as indicated. Supernatants were collected at d 1, 2 and 6. Means and standard deviations from three experiments are shown. (A) Th1-related cytokines GM-CSF, IL-2, IFN-γ and IL-12p70 in culture supernatants. (B) Th2 cytokines IL-4, IL-5 and IL-10 in culture supernatants.](image-url)
ROLE OF DCs IN TUMOR THERAPY BY TRIFUNCTIONAL bsAb

T cells to induce tumor cell killing in the presence and with the help of DCs. Importantly, redirection of T cells by the trAb without addition of DCs was not sufficient to induce tumor cell killing (Figure 3A), a fact underscoring the importance of APC activation by the Fc part of intact bsAbs. The relevance of the Fc part was also evaluated in an XTT killing assay, which showed that the F(ab′)2 fragment of Surek was not capable of inducing significant lysis of B78-D14, even in the presence of DCs (Figure 3C). In summary, DCs, representing an important type of FcR+ cells, appear to have high potential to promote Surek-induced T-cell activation, proliferation of CD8+ T cells and tumor cell killing.

**Combination of trAb with Exogenous DCs Increases the Therapeutic Efficacy**

On the basis of our findings in vitro, we reasoned that the synergism between trAb and DCs might be exploited to improve the efficacy of trAb therapy in vivo. Therefore, we inoculated mice with B78-D14 cells and started treatment 2 d later with a low dose of Surek alone or in combination with BM-derived syngeneic DCs. As shown in Figure 4A, the combination of Surek with DCs significantly ameliorated the therapeutic outcome compared with therapy with trAb alone.

To examine the relevance of trAb binding to DCs for the therapeutic effect in vivo, therapy experiments were done under mAb-mediated blocking of FcγRs. The complete reversal of the DC-mediated bonus effect in this setting (Figure 4A) confirms the mechanism that involves trAb-binding to FcγRs of exogenous DCs.

Mice that had been rescued from tumor growth also survived a rechallenge with the parental B16 melanoma, from which B78-D14 was derived (Figure 4A). This result indicates the induction of a long-lasting T-cell memory that, in the effector phase, does not depend on the expression of GD2 targeted by Surek. T cells induced by bsAb immunization rather recognize a variety of other tumor-derived antigens.

In contrast, no significant survival benefit was seen in any therapy group that was treated with Surek and B16-EpCAM cells, which were not recognized by Surek (Figure 4B). This result is in accordance with the finding that growth of B16-EpCAM could not be inhibited in vitro (Figure 3A), although T-cell activation and proliferation were also induced when trAbs only bound to T cells. Altogether, our data demonstrate that the combination therapy with a trifunctional bsAb and syngeneic DCs is clearly superior to bsAb treatment alone.

**DISCUSSION**

Trifunctional bsAbs directed against T cells and tumor cells simultaneously activate T cells and accessory cells in contrast to bispecific F(ab′)2 fragments, which can only bind to one type of immune cell (12). TrAbs that are composed of mouse IgG2a and rat IgG2b preferentially bind to activating (FcγRI, IIa and III) and only marginally to inhibitory (FcγRIIB) human FcγR (13,23). Several data indicate that this result also holds true for murine FcγR (14,15). It is postu-
lated that the formation of a so-called "triple-cell complex" mediated by trAbs leads to activation of different types of naive immune cells, resulting in more effective tumor cell killing and generation of an immunologic memory (12–14,24). Additionally, trAbs of this isotype combination can be easily produced in sufficient amounts by quadroma technology and purified in a one-step procedure (20). In clinical studies, the therapeutic success of trAbs has been strikingly demonstrated (25,26).

In the present study, we used a murine melanoma model to investigate the relevance of different immune cell types for the antitumor effects of a trAb. The trAb Surek is directed against mouse CD3 and the ganglioside GD2, which is an attractive target antigen for immunotherapy of human malignancies such as small cell lung cancer, melanoma, neuroblastoma or glioma (27,28). Human T cells against melanoma were effectively activated by a trAb directed against GD2 and human CD3 in vitro (19).

By coculturing melanoma cells with purified T cells, BM-derived DCs and Surek, we observed that T cells are activated most effectively when all three cell types are put together in the presence of trAb (Figures 1A, B). Without DCs, T-cell activation was less pronounced, with T cells showing decreased upregulation of CD69 and no downregulation of CD62L (Figures 1A, B). Addition of DCs furthermore induced strong proliferation of CD8+ and less proliferation of CD4+ T cells in the presence of Surek (Figures 1C, D). The reason for this differential proliferation is not yet clear. Moreover, in long-term cultures beyond d 12, only CD8+ cells survived in significant amounts (not shown).

The activation status and the proliferation of the T cells in the cocultures (Figure 1) were reflected by the cytokine patterns found in the supernatants (Figure 2). The highest cytokine production was observed in those cocultures where T cells, DCs, tumor cells and Surek were combined. High levels of the Th1 cytokines IL-2, IFN-γ, GM-CSF and IL-12, and significantly enhanced levels of the Th2 cytokines IL-4, IL-5 and IL-10 compared with the control groups, were detected. The induction of a Th1 response is desirable because Th1 cells but not Th2 cells are effective in tumor rejection (29).

The concomitant expression of Th2 cytokines was not surprising, since it has been shown before that a sustained Th1 response depends on the induction of Th2 cytokines as well (30,31).

The efficacy of T-cell activation by trAbs in the presence of DCs was further demonstrated by the elimination of tumor cells in cocultures containing DCs and Surek. By contrast, no elimination could be detected in groups where either Surek or DCs were missing (Figure 3). This strict dependence of trAb-mediated tumor cell killing on DCs was not observed for other bispecific Ab constructs (14). We assume that this is a hallmark of Surek, which is due to the low affinity to the GD2 antigen (22).

By using irrelevant tumor cells (Supplementary Figures S1, S2) or by omitting the target cells (not shown), we showed that T-cell activation by Surek did not depend on specific recognition of GD2. We assume that the tumor specificity of Surek is not necessary for activating T cells but only for redirecting their cytotoxic activity toward the tumor cells, which was indeed confirmed by in vitro killing assays (Figure 3A) and by in vivo challenge with the irrelevant melanoma (Figure 4B). Although the experiments were performed in vitro and therefore represented an artificial situation, off-site T-cell activation and cytokine release could be a concern in the future clinical development. However, clinical data from the development of catumaxomab and ertumaxomab suggest that a
low starting dose and subsequent increased dosages prevent such undesirable effects (32,33).

T-cell activation, tumor cell elimination as well as the cytokine patterns in vitro exactly paralleled the observations we made in vivo, where antitumor T-cell responses correlated with activation of CD11c+ cells and a mixed Th1/Th2 profile in sera of immunized mice. Memory responses depending on tumor-specific, cytolytic T cells were only generated when the trAb (that is, the bsAb with an intact Fc part) was used (15). Taken together, the data are in accordance with the assumption that trAbs efficiently work by forming a triple-cell complex (12) and underscore the advantage of the trAb, which is able to activate accessory immune cells. Activated accessory cells, in turn, provide additional signals that are required for effective T-cell induction (34,35) apart from the “first” priming signal that is conveyed by binding of the trAb to CD3. This concept was confirmed by the inability of Surek-F(ab′)2 to mediate tumor cell killing in vitro, even in the presence of DCs (Figure 3C) and by the inability of DCs to enhance Surek-mediated survival in the presence of an FcR-blocking mAb (Figure 4A).

A prerequisite for inducing systemic antitumor T-cell responses is the efficient antigen uptake by APCs. Antigen internalization can be done by phagocytosis, pinocytosis or (most efficiently) adsorptive endocytosis (36). TrAbs bind to tumor cells and to activating FcR on APCs and may therefore facilitate engulfment of tumor antigens by adsorptive endocytosis. The uptake of multiple antigens derived from the targeted tumor by accessory cells and their presentation toward T lymphocytes may explain the polyclonal T-cell responses induced by Surek in vivo (15). This is a major advantage of trAbs, since polyvalent immunization makes escape variants less likely.

The dependence of the trAb-mediated effects on interactions with CD11c+ cells suggested that the antitumor effect in vivo could be further improved by combining trAb treatment with DC therapy. We showed that the combination of Surek with exogenous BM-derived DCs is indeed more potent in curing mice than the trAb alone (Figure 4A). In combination with DCs, it is possible to improve survival to 100% with only two injections of 10 μg trAb. As a monotherapy, Surek has to be delivered at a cumulative dosage that is at least 7.5 times higher to achieve the same outcome (not shown).

Thus, combination with DCs may reduce the required therapeutic dosage. The combination therapy takes advantage of using preactivated DCs, which might activate T cells more effectively than endogenous, trAb-targeted DCs. For use in the clinic, further in vitro amendments of the exogenous DCs seem feasible (for example, their transfection with TAA-encoding RNAs or induction of high IL-12 expression by using appropriate differentiation protocols [37]). On the other hand, this approach is an improvement compared with conventional DC-based vaccination protocols because DCs precoated with trAbs will be more readily directed to the target cells.

**CONCLUSION**

The data demonstrate that an interplay among trAbs, T cells, DCs and the target cells determines the therapeutic potential of the antibody constructs. Thus, the combined use of trAbs together with DCs offers advantages to trAb treatment alone and is a promising option for cancer treatment in humans.

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**DISCLOSURE**

H Lindhofer is the CEO of Trion Pharma and the inventor or coinventor of several trifunctional antibody patents.

**REFERENCES**