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Review Article

BiTE: a new class of antibodies that recruit T-cells

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Abstract

Certain bispecific antibodies hold promise for redirecting cytotoxic T-cells, the body's most effective killer cells, against malignant cells. This cannot be achieved by conventional monoclonal antibodies because T-cells do not express antibody-binding Fcγ receptors. Many bispecific antibody formats have been developed over the past 20 years that bind with one arm to an activating surface receptor on T-cells and with the other arm to a tumor-associated antigen, but only a few have made it into clinical trials. This article reviews a novel class of bispecific antibodies, known as BiTE antibodies, in terms of their mode of action, *in vitro* and *in vivo* characteristics and clinical activity. It appears that BiTE antibodies have overcome many of the issues of previous bispecific antibody formats regarding the need for T-cell pre- or co-stimulation, low potency of redirected lysis, or nonconditional T-cell activation. Monotherapy with a BiTE antibody directed against CD19 has provided impressive results in the treatment of refractory non-Hodgkin's lymphoma (NHL) patients, showing depletion of peripheral B lymphoma cells, confirmed partial and complete responses, clearance of bone marrow from tumor cells and reduction of splenomegaly. We describe how BiTE antibodies are evolving into a new class of antibody-based therapeutics, with several new members in various stages of preclinical development.

Introduction

Cytotoxic T-cells have an enormous capacity to eradicate pathogenic cells in the body. This is evident from the highly efficient clearance of virus-infected cells by specific T-cells in infectious diseases, but also from the impressive antitumor responses occasionally observed when skin or renal cancer patients are treated with anti-CTLA-4 antibodies (1) or after adoptive T-cell transfer (2). For such therapies and all other vaccination approaches (3), specific T-cell clones that bear T-cell receptors specific for non-self peptide antigens presented by malignant target cells need to be activated and expanded. The high degree of specificity for T-cell recognition and the need for antigen presentation by professional antigen-presenting cells (APCs) is, on the one hand, a safeguard tightly controlling the body's most effective weapon against tumors, but on the other hand, the many control elements of T-cell recognition introduce multiple Achilles' heels that can impede T-cell responses against tumor cells.

There are many ways for malignant cells to escape T-cell recognition (4, 5). A prime strategy involves a substantial alteration in tumor cells of the machinery involved in generating and presenting peptide antigens to T-cells. This can include: 1) the loss or downmodulation of MHC class I molecules; 2) a change in proteasome subunits; 3) the loss of TAP (transporter associated with antigen processing) transporters; and 4) the loss of β_2-microglobulin. A second strategy is the expression by tumor cells of proteins that negatively affect T-cell survival, differentiation or activation. While such proteins normally help to balance T-cell responses, they are used by cancer cells to anergize or tolerize tumor-infiltrated T-cells. Examples are the secretion by tumor cells of the immunomodulatory cytokines transforming growth factor β (TGF-β), IL-10 or IL-4, the expression of ligands for the negative regulatory PD-1 and Fas receptors on T-cells, or the expression of an enzyme locally degrading tryptophan, which tolerizes T-cells via depletion of the essential amino acid. The multitude of immune escape mechanisms may explain the limited response rates of therapies activating specific T-cell responses, such as

various vaccination approaches, anti-CTLA-4 antibodies and adoptive T-cell transfer.

One way of overcoming immune escape mechanisms while still harnessing the power of T-cells is the use of bispecific antibodies. Such antibodies have been extensively reviewed in previous articles (6-11). As their name suggests, they combine two different antigen-binding specificities in one protein molecule. By binding with one arm specifically to CD3, an invariant signaling component of all T-cell receptors, and with the other arm to a frequently expressed surface antigen on target cells, bispecific antibodies strive to functionally replace the highly specific T-cell receptor/peptide/MHC class I complex that naturally serves for target cell recognition and initation of target cell lysis. By doing so, bispecific antibodies are able to activate T-cells independently of a specific T-cell receptor, the vulnerable antigen presentation machinery, and of co-stimulatory signals needed for naïve T-cell priming. Another intriguing aspect of such bispecific antibodies is that they can principally recruit every T-cell in the body regardless of T-cell receptor specificity. We therefore refer to their action as 'polyclonal' T-cell engagement (12). Lastly, with the help of bispecific antibodies, T-cells are able to recognize target cells by a cell-surface antigen, as is the case for conventional immunoglobulins. This gives T-cells a new mode of target cell recognition that no longer relies on intracellular processing and surface presentation of peptide antigens.

The low potency of redirected lysis by many bispecific antibody formats suggests that a mimicry of the specific T-cell receptor/MHC class I/peptide interaction has not been well achieved in the past. In many cases, redirected lysis *in vitro* required μg/ml concentrations of bispecific antibodies and a high excess of T effector cells over target cells, known as the E:T ratio (13-15). While many bispecific antibodies provided basic proof of principle, they were in most cases not suitable for formal pharmaceutical development, generally due to issues of production and lack of potency.

In the present review article, we focus on one kind of bispecific antibodies, known as BiTE antibodies, that, by a combination of unique properties, can closely mimic a natural cytotoxic T-cell response. BiTE antibodies have previously been reviewed in detail in comparison to other bispecific antibody formats (12-14, 16, 17). Here, we focus on new insights into the mode of action of BiTE antibodies, describe the properties of new BiTE antibodies under development and review interim data from an ongoing clinical study with the CD19/CD3-bispecific BiTE antibody MT103/MEDI-538.

Structure and production

BiTE antibodies are constructed from two different single-chain antibodies (scFvs) that are flexibly linked by a nonimmunogenic peptide. Single-chain antibodies are among the smallest binding domains that can be derived from a monoclonal antibody. They are constructed by connecting the variable heavy- and light-chain domains via a flexible peptide linker. The basic design of a BiTE antibody and its structural relationship to its two parental IgG molecules is depicted in Figure 1.

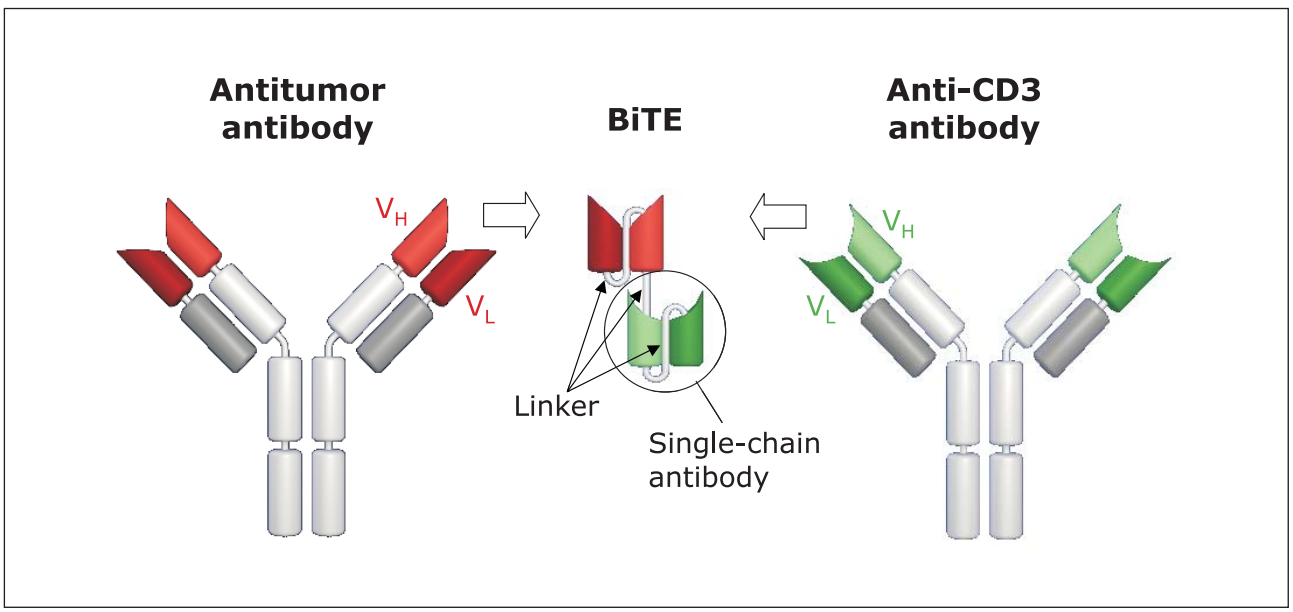

Fig. 1. Structural relationship of a BiTE antibody to its parental monoclonal antibodies. Generation of a BiTE antibody is principally shown. Note that only variable V_H and V_L domains of antibodies are used in BiTE antibodies. In a first step, linkers are used to connect V_H and V_L domains into single-chain antibodies. In a second step, a linker combines two single-chain antibodies to form a BiTE antibody molecule. Recombinant DNA technology is used to combine four different gene products (two V_H and two V_L domains from two distinct antibodies) into one gene product. The BiTE antibody forms a single nonglycosylated polypeptide, which has approximately a third of the size of a conventional monoclonal antibody.

All currently developed BiTE antibodies have the target antigen-binding scFv in the *N*-terminal and the CD3-targeting scFv in the *C*-terminal position. BiTE antibodies form monomeric polypeptides with average molecular weights between 55 and 60 kDa, are not glycosylated and are highly stable. They can be purified to homogeneity using conventional chromatography steps.

Currently, all BiTE antibodies are produced as secreted proteins in eukaryotic cell culture systems similar to those used for manufacturing the majority of conventional monoclonal antibody therapies. Due to their high potency, BiTE antibodies are administered at much lower doses than conventional antibodies.

Unique mode of action

The particular biological activity of BiTE antibodies relates to their simultaneous binding to two distinct cell types —a T-cell and a target cell. This principle, which is distinct from that of conventional monoclonal antibodies, is depicted in Figure 2. Monoclonal antibodies can only recruit immune effector cells expressing Fc receptors (such as natural killer cells, granulocytes or macrophages), while they have no reach to T-cells, which are the most potent killer cells in the organism. BiTE antibodies use the CD3 complex to specifically recruit T-cells. Conventional monoclonal antibodies and BiTE antibodies may share the same target antigen on tumor cells.

It is not desirable that binding of a BiTE antibody to tumor cells or T-cells alone result in a biological effect. On the tumor cell side, binding of the BiTE antibody alone should, for instance, not trigger target antigen internalization leading to a reduction of available surface target prior to an encounter with a cytotoxic T-cell. Internalization, which typically requires crosslinking of a surface molecule, may be avoided by monovalent binding of the BiTE antibody to the antigen. On the T-cell side, the BiTE antibody should not trigger T-cell activation in the absence of a target cell, or internalization of CD3.

While target antigen internalization following BiTE antibody binding has not been studied in detail, experimental data suggest that monovalent CD3 binding by BiTE antibodies alone does not activate T-cells (18). In the absence of target cells, even BiTE antibody concentrations that exceed those for half-maximal redirected lysis by 5 orders of magnitude did not trigger expression of the activation markers CD69 and CD25 on T-cells, release of cytokines or cell cycling. Only in the presence

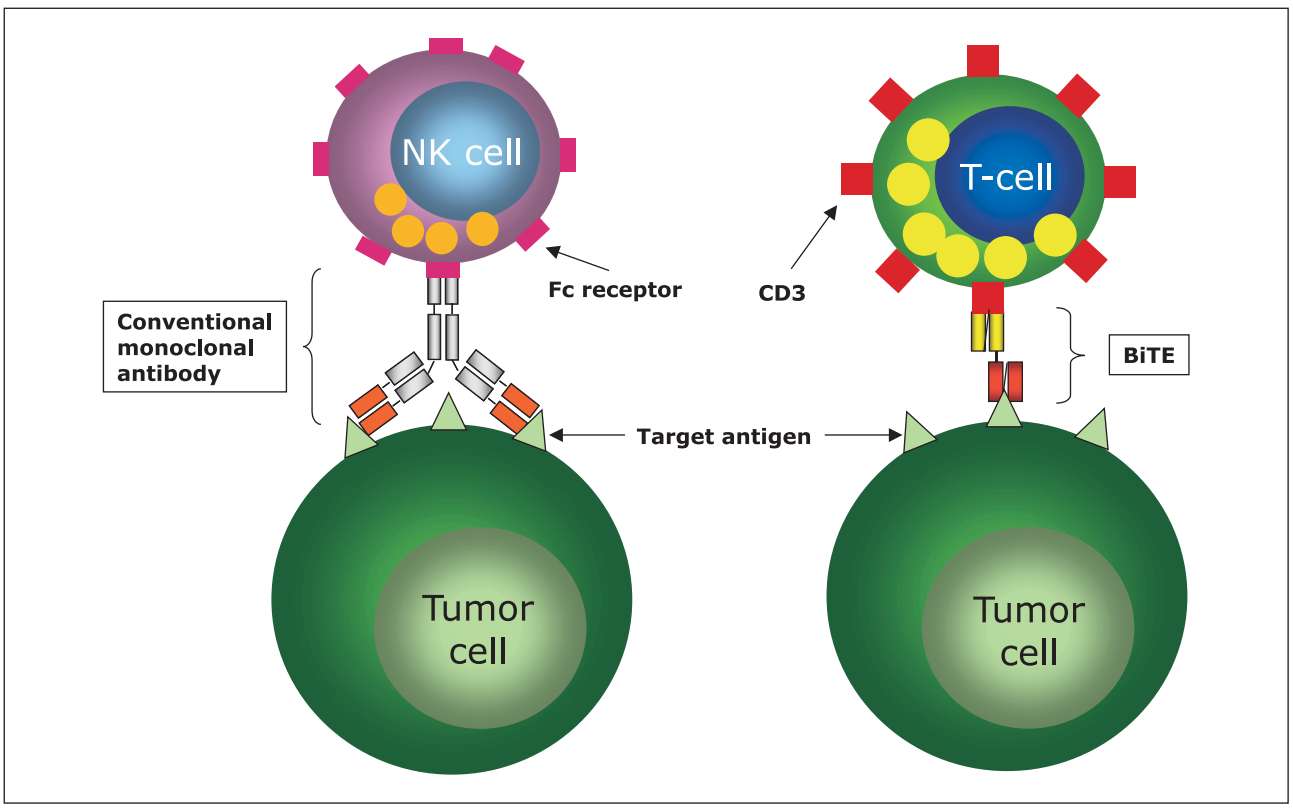

Fig. 2. Difference in mode of action between a conventional cytotoxic monoclonal antibody and a BiTE antibody. A conventional monoclonal IgG_1 antibody (*e.g.,* trastuzumab, cetuximab or rituximab) can only recruit immune effector cells expressing Fcγ receptors (*i.e.*, CD16, CD32, CD64), but not cytotoxic T-cells. Most important for antibody-dependent cellular cytotoxicity (ADCC) in humans appear to be natural killer (NK) cells expressing CD16. BiTE antibodies are designed to recruit immune effector cells expressing CD3, which by definition are T-cells. T-cells are regarded as the most powerful killer cells in the organism. As depicted, conventional antibodies and BiTE antibodies can use the same target antigen on tumor cells but bind with distinct valency.

of both T-cells and target cells can BiTE antibodies potently activate T-cells and cause redirected lysis of target cells at low picomolar concentrations (19, 20). Once a few BiTE antibodies establish a first contact between T-cells and target cells, additional BiTE antibodies may enter the forming synapse. Simultaneous binding of two different antigens by BiTE antibodies in the cleft will be of high avidity, which will further promote and stabilize the cytolytic synapse.

The particular mode of action of BiTE antibodies is depicted in Figure 3, where four sequential stages of redirected target cell lysis are outlined: 1) BiTE antibody binding to tumor and T-cells (Fig. 3A); 2) formation of a cytolytic synapse that delivers toxins into the tumor cell with simultaneous activation of the T-cell (Fig. 3B); 3) separation of the activated T-cell from the disintegrating tumor cell (Fig. 3C); and 4) lysis of the next tumor cell while the T-cell is recharging its toxins and proliferating (Fig. 3D).

In many aspects, the mode of action of BiTE antibodies is not any different from that of regular cytotoxic T-cell activation as naturally governed by specific T-cell receptor/peptide antigen/MHC class I recognition. The 'regular' elements of a BiTE antibody-induced T-cell response include the following.

- *Highly specific recognition of target cells by T-cells.* We have shown that a single amino acid substitution in an epitope of a target antigen that abrogates the binding of BiTE antibodies can completely prevent target cell lysis and T-cell activation (18). With this high degree of specificity, surface antigen recognition by BiTE antibodies is as stringent as peptide antigen recognition by T-cell receptors.
- *Extremely potent redirected target cell lysis.* With preactivated T-cells, certain BiTE antibodies can trigger half-maximal redirected target cell lysis at concentrations as low as 1 pg/ml, reflecting the involvement of

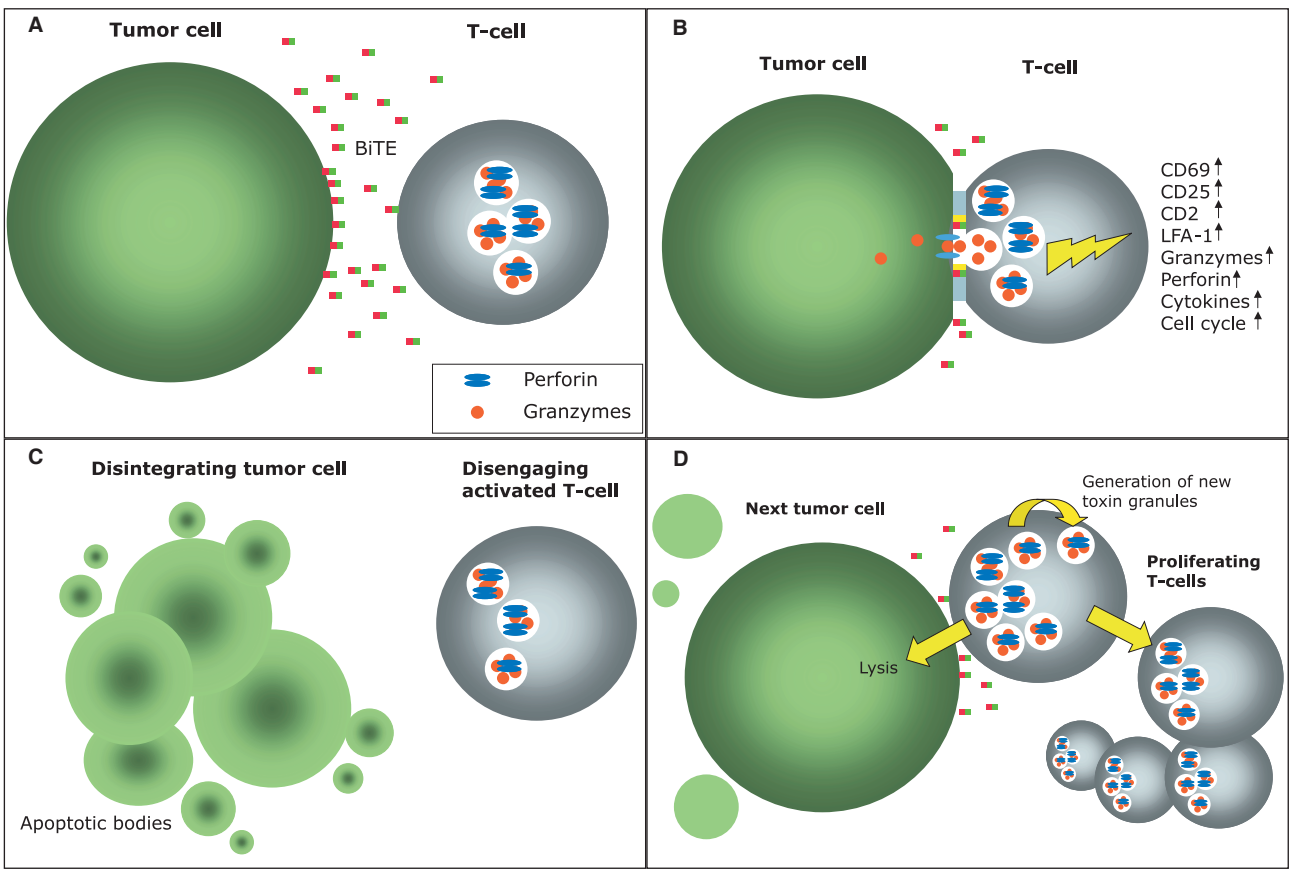

Fig. 3. Proposed mode of action of a BiTE antibody. (**A**) BiTE antibodies bind to tumor cells. Binding to unstimulated T-cells is of low affinity. (**B**) BiTE antibodies induce the formation of a cytolytic synapse between tumor and T-cells. After discharge of the cytotoxic granule content by the T-cell into the synaptic cleft, perforin pores form in the tumor cell membrane and apoptosis-inducing granzymes are delivered into the cytoplasm of tumor cells. At the same time, the T-cell is being fully activated, leading to upregulation of T-cell activation markers (CD69 and CD25), cell adhesion molecules (CD2 and LFA-1), cytotoxic granule proteins (granzymes and perforin), inflammatory cytokines and cell cycling. (**C**) Perforin and granzymes trigger death in tumor cells. As a consequence, the tumor cell disintegrates and the remaining apoptotic bodies are cleared by macrophages. The activated T-cell disengages and is ready for the next round of cell lysis. (**D**) The BiTE antibody-activated T-cell is now in serial killing mode. It constantly recharges perforin and granzymes, proliferates and can serially lyse tumor cells. An army of activated T-cells is formed that may over time outnumber tumor cells.

only a very few BiTE antibodies in synapse formation. This is comparable to specific effector T-cell clones, which only need single-digit numbers of peptide antigen/MHC class I complexes to initiate target cell lysis (21). It therefore appears that BiTE antibodies are able to optimally activate cytotoxic T-cells, as seen in a natural setting.

- *Formation of a cytolytic synapse.* BiTE antibody-induced cytolytic synapses are structurally indistinguishable from those synapses formed between a specific T-cell clone and a target cell line expressing the proper peptide antigen/MHC class I complex (22). Cytolytic synapses formed by T-cells have been described as essential subcellular structures for precise and economic delivery of the cytotoxic granule content of T-cells into target cells (23). Like with natural cytolytic synapses, we have observed that BiTE antibody-activated T-cells pick up membrane proteins from their target cells as a consequence of transient plasma membrane fusions occurring during serial killing (22).
- *Employment of perforin and granzymes for redirected target cell lysis.* Perforin and granzymes are major cytotoxic components of T-cell granules (24). The involvement of perforin, a calcium-dependent protein pore, is evident from the almost complete inhibition of BiTE antibody-induced target cell lysis by the extracellular calcium chelator EGTA (ethylene glycol tetraacetic acid) (19, unpublished data).
- *Upregulation of granzyme B and perforin expression.* This is a means to recharge new granules in $CD8^+$ T-cells with cytotoxic proteins for subsequent rounds of killing. Even $CD4^+$ T-cells show an upregulation of granzyme B (25) and perforin expression (unpublished data) upon BiTE antibody stimulation, explaining why $CD4^+$ T-cells can likewise contribute to BiTE antibody-induced redirected lysis, but with delayed kinetics.
- *Target cell-dependent activation of T-cells.* BiTE antibodies induce upregulation of the T-cell activation markers CD69 and CD25, cell adhesion molecules such as LFA-1 and CD2, and the release by T-cells of inflammatory cytokines such as tumor necrosis factor α (TNF-α), interferon gamma (IFN-γ) and IL-6 (18, 26). These events are, however, only seen in the presence of target cells.
- *Induction of T-cell proliferation.* This response was most prominent with $CD8^+$ T-cells, suggesting that the cytolytic synapses formed by this T-cell subpopulation provide the strongest proliferative stimulus (25). There is no evidence that BiTE antibody-stimulated T-cells undergo apoptosis. To the contrary, T-cell numbers dramatically increase during prolonged BiTE antibody stimulation *in vitro*.
- *Repeated cycles of target cell lysis.* Studies using videomicroscopy and cytotoxicity assays at very low E:T ratios demonstrated serial killing by individual BiTE-activated T-cells (27), as is the case for naturally activated cytotoxic T-cells.

This summary suggests that BiTE antibodies are capable of inducing target cell lysis by cytotoxic T-cells in a fashion that closely resembles the natural response governed by specific T-cell receptor/peptide antigen/MHC class I recognition. However, there are also certain differences in target cell lysis and T-cell activation between a BiTE antibody-induced and a natural cytotoxic T-cell response. The BiTE antibody-induced cytotoxic T-cell response has the following unique features.

- *Independence from specific T-cell receptors.* For T-cell activation and target cell lysis, BiTE antibodies solely require the common signaling CD3 subunit shared by all T-cell receptors. Monovalent binding of the CD3 subunit by BiTE antibodies does not apparently elicit significant signal-transducing events in T-cells. Rather, T-cell activation by BiTE antibodies requires the presence of a target cell that, in the presence of BiTE antibodies, allows the simultaneous stimulation of many CD3 molecules on the T-cell.
- *No need of extra stimuli for redirected lysis.* BiTE antibodies can activate unstimulated T-cells on their own. The independence from co-stimulation by CD28 may relate to the observation that the population of effector memory $CD8^+$ T-cells, which can lack CD28, is making the largest contribution to target cell lysis (unpublished data). The independence from co-stimulatory signals is also shown by redirected lysis via human T-cells of hamster cell lines expressing a human target antigen (25).
- *No requirement for peptide antigen presentation.* It has been demonstrated that human cells lacking expression of MHC class I molecules are efficiently lysed by BiTE antibody-activated human T-cells (18), as are rodent cells with no matching MHC class I molecules (25). It can therefore be expected that other components of peptide antigen processing and presentation machinery will likewise not be necessary for BiTE antibody-mediated target cell lysis.
- *Ignorance toward major immune escape mechanisms of tumor cells.* As described above, major strategies of cancer cells to evade T-cell recognition involve either impairment of antigen presentation or expression of T-cell-inhibitory proteins. The evasion mechanisms studied thus far for their impact on BiTE antibody activity are loss of MHC class I (22) and the presence of TGF-β (unpublished data). Both could not prevent tumor cell lysis by BiTE antibody-activated T-cells. Future studies will explore the impact of other immune escape mechanisms on BiTE antibody-mediated cytotoxicity.
- *Selective engagement of effector memory T-cells.* T-cell therapies currently developed for cancer treatment, such as vaccines (3), anti-CTLA-4 antibodies (1) or T-cell transfer technologies (2), require tumor-specific T-cell clones, which arise from the repertoire of naïve T-cells following tumor antigen presentation. As a consequence, autoreactive T-cell clones may also develop, which can cause autoimmune diseases. This has been observed with anti-CTLA-4 antibodies, where hypophysitis, thyroiditis, colitis and dermatitis can accompany clinical responses (1). In contrast, BiTE

antibodies do not promote priming of naïve T-cells in the absence of CD28 co-stimulation (28), but selectively recruit for target cell lysis effector memory T-cells (unpublished data), which should not be self-reactive.

- *Exclusive use of surface antigens for T-cell recognition.* $CD8^+$ T-cells typically recognize target cells by short peptides displayed on MHC class I molecules. Peptide antigens can be fragments of cytoplasmic, nuclear or membrane proteins. In contrast, BiTE antibodies make T-cells exclusively recognize extracellular membrane-bound antigens. These are typically large proteins or carbohydrate structures. This way, BiTE antibodies allow T-cells to gain a unique target specificity, like conventional monoclonal antibodies.

There are a number of prerequisites that need to be fulfilled for BiTE antibody action: 1) BiTE antibodies need to penetrate into the tumor; 2) the target antigen needs to be present and ideally accessible on every tumor cell; 3) T-cells need to be present in the tumor tissue or find easy access from the outside; and 4) T-cells and the tumor must physically meet in the presence of BiTE antibodies.

With a size of only 55-60 kDa, BiTE antibodies are well suited to penetrate into tumor tissue, as has been shown for similarly sized antibody fragments (29). We have selected target antigens for BiTE antibodies that are very frequently expressed on tumor cells, and in some cases have been identified to be expressed on so-called cancer stem cells (see below). BiTE antibodies do not depend on particular T-cell clones, but rather all cytotoxic T-cells in the body can potentially contribute to their action. Serial lysis and T-cell proliferation at the site of redirected lysis may further ensure that even very low E:T ratios, as often found inside tumors, do not pose a limitation for BiTE antibody efficacy. Progressive elimination of tumor cells and invasion and local proliferation of T-cells will over time lead to a beneficial increase in E:T ratios inside the tumor, which may accelerate redirected lysis. Cytokines concomitantly released by locally activated T-cells, such as IFN-γ, have antitumor activity on their own by, *e.g.*, being antiangiogenic or attracting and activating other immune cells, such as macrophages. Comprehensive T-cell activation via CD3 signaling, which in the case of BiTE antibodies is dependent on the presence of target cells, may reactivate tumor-resident T-cells (25), some of which may even be specific for a tumor antigen.

Two types of studies suggest that peripheral cytotoxic T-cells can enter solid tumors and that there may be no requirement for the presence of tumor-resident T-cells for tumor eradication. One study in mice followed the movement and cytotoxic activity of a fluorescently labeled tumor-specific T-cell clone in a subcutaneous tumor model by intravital microscopy (30). The labeled T-cells were observed to enter the tumor from the outside, deeply penetrate tumor tissue and ultimately eradicate the established tumor. A control tumor not expressing the peptide antigen on the opposite flank of the mouse was not affected and no penetration by T-cells was observed. The other observation comes from adoptive T-cell transfer. In this technology, immune cells are prepared from tumor tissue followed by *ex vivo* expansion of T-cells to large numbers (up to 10^{11} cells) using cytokine stimulation. Expanded autologous T-cell cultures are then re-infused into late-stage metastatic melanoma patients who have received prior chemotherapy for depletion of regulatory T-cells and other immune cells that could create a cytokine sink. In the presence of high-dose IL-2, an overall response rate of 50% has been observed with this approach, and some patients with metastatic melanoma showed complete responses (2). Two cases of tumor regression have also been observed with infused T-cells expressing a transfected cDNA encoding a T-cell receptor with specificity for a melanoma-associated peptide antigen (31), providing proof of concept that cytotoxic T-cells used as monotherapy have the capacity for treating late-stage disease.

Antitumor activity in animal models

BiTE antibodies have been assessed in various animal models. BiTE antibodies specific for CD19, EpCAM (CD326), the receptor tyrosine kinase EphA2 (also known as ECK) and carcinoembryonic antigen (CEA) have all shown potent control of tumor outgrowth in mouse xenograft models (25, 32-35), and in some cases eradication of small tumors (25, 33). In these models, immunodeficient NOD/SCID mice received s.c. inoculations with human tumor cell lines expressing the respective target antigens. Because the BiTE antibodies are specific for human CD3, freshly isolated human peripheral blood mononuclear cells (PBMCs) or purified human T-cells must be co-administered with tumor cells. Control treatments typically include tumor cells in the absence of T-cells, vehicle instead of BiTE antibody or the use of control BiTE antibodies that are of unrelated specificity in either the CD3- or target antigen-binding arm, or both.

While these mouse models are suitable for impressively demonstrating the *in vivo* antitumor activity and potency of BiTE antibody development candidates, they are not suited to address issues of safety and therapeutic window because BiTE antibodies will not activate mouse T-cells and will not bind mouse orthologues of target antigens potentially expressed on normal tissues. Moreover, human T-cells have a rather short half-life following i.v. engraftment in mice (*i.e.,* approximately 11 h) (34), which is why tumors in mice can only be treated shortly after inoculation while they still contain sufficient numbers of human effector T-cells. Only rapidly growing tumors, such as from the colon cancer line SW480, previously allowed testing of BiTE antibody efficacy with established tumors of between 50 and 200 mm^3 in size. High efficacy of BiTE antibodies in such models was observed with daily i.v. injection of doses as low as 0.1 μg (25, 33). Unstimulated human PBMCs or T-cells were used in these studies, showing that BiTE antibodies can fully activate resting human T-cells for redirected lysis not only *in vitro* but also *in vivo*. This is in contrast to various other bispecific anti-

bodies tested in similar animal models, which required co-administration of IL-2 or anti-CD28 antibodies, preconditioning of T-cells and high E:T ratios (*e.g.*, 36).

Another model using immunodeficient NOD/SCID mice tested the efficacy of two different EpCAM-specific BiTE antibodies against authentic human metastatic tissue (25, 33). The tumor tissue was derived by debulking from the peritoneal cavity of metastatic ovarian cancer patients, rapidly transferred to the animal test site and tumor pieces of 50 mm^3 were prepared and propagated under the skin of 16 NOD/SCID mice per patient sample. Tumors from 3 of 6 patients were accepted by mice. In cases of acceptance, 8 tumor-bearing mice were treated with the EpCAM BiTE antibody and 8 mice with a control BiTE antibody by 3 cycles of 5 daily i.v. injections. Tumor rejection or reduction in size was observed with all patient samples tested. This suggested that BiTE antibodies administered i.v. did reach T-cells residing in subcutaneous human tumors, reactivated those T-cells and unleashed their antitumor activity. The EpCAM BiTE antibody was also active against isolated human ovarian cancer cells in culture (37).

A different type of mouse model was used to evaluate BiTE antibodies that have specificity for human target antigens and for CD3 on murine T-cells. These BiTE antibodies are called 'hybrid' BiTE antibodies and allow efficacy experiments in immunocompetent animals without supplementation of human T-cells. In order to prevent rejection in these mice, tumor cell lines need to be syngeneic. Data for subcutaneously growing syngeneic tumors transfected to express human EpCAM have shown potent and dose-dependent BiTE antibody inhibition of tumor outgrowth (38), indicating that peripheral mouse T-cells can enter tumors and control their growth. In another model, tumor cells were administered i.v., in which case tumors developed in the lung from locally trapped single tumor cells (38). Treatment with EpCAM-specific hybrid BiTE antibodies completely prevented lung tumor development when given daily from the start of tumor cell injection, and in the majority of animals when given 5 days after the start of tumor cell injection. While in such animal models side effects from mouse T-cell activation may be assessed, the consequence of target antigen being present not only on the tumor, but also on normal tissue, remains elusive. No adverse events have been observed to date upon treatment of mice with hybrid BiTE antibodies.

A fourth type of mouse model was used to assess a BiTE antibody that is specific for both CD3 of mouse T-cells and the murine orthologue of the target antigen. This BiTE antibody, called muS110, recognizes murine EpCAM (CD326) and has been investigated in great detail for both efficacy and therapeutic window in immunocompetent BALB/c mice (39). EpCAM was shown by immunohistochemical analysis to be similarly expressed in a wide variety of normal tissues from humans and mice. In both an orthotopic breast cancer and a lung tumor model, muS110 showed antitumor activity at daily i.v. or s.c. doses between 2 and 10 μg/kg. These treatments were well tolerated for several weeks, indicating a robust therapeutic window and little or no recognition by muS110 of EpCAM expressed on vital organs such as lung, pancreas, colon and liver (bile ducts). The dose of muS110 could be escalated in mice up to 400 μg/kg. Dose-limiting side effects were increased levels of inflammatory cytokines resulting in piloerection, reduced body weight, motility and body temperature. Side effects were significantly reduced by either glucocorticoid co-treatment or by a week-long adaptation period with a low dose of muS110. Of note, histopathological examination of mice did not detect any damage by muS110 to EpCAM-expressing normal tissues. muS110 has been developed as a surrogate molecule for the human specific EpCAM BiTE antibody MT110 as part of its preclinical development program. muS110 and MT110 had similar binding affinities, potency of redirected lysis and *in vivo* activity when data from the respective assay systems were compared.

In summary, all animal models developed to date to test a variety of different BiTE antibodies revealed significant antitumor efficacy with unstimulated endogenous or transferred human T-cells. In those models where side effects were assessable, they could be attributed to reversible cytokine release.

Because of its very limited species crossreactivity, the CD19/CD3-bispecific BiTE antibody MT103/MEDI-538 (40) has been investigated in chimpanzees as part of its preclinical development (41). T- and B-cells of this species showed very similar reactivity to MT103 compared to human T- and B-cells. Weekly 2-h i.v. infusions led over 5 weeks to a cumulative depletion of peripheral $CD19^+$ B-cells. The first infusion of MT103 in chimpanzees caused a transient increase in cytokine levels, which was much reduced or absent upon subsequent weekly short-term infusions, consistent with a so-called 'first-dose effect' and T-cell adaptation.

Clinical proof of concept

MT103/MEDI-538 is being tested in an ongoing phase I clinical trial in Europe for safety and tolerability, pharmacodynamics and pharmacokinetics, and signs of clinical activity in non-Hodgkin's lymphoma (NHL) patients. Interim results showing clinical activity of the CD19/CD3-bispecific BiTE antibody have been presented at two international conferences (42-44). CD19 was selected as a target because it is being used as the standard marker for identification of all human B-cell malignancies, with the exception of multiple myeloma. In contrast to the CD20 target, which is recognized by the antibody therapies Rituxan® (rituximab), Zevalin® and Bexxar®, CD19 is more frequently expressed on various B-cell malignancies, in particular acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

In the current trial, MT103/MEDI-538 is administered by continuous i.v. infusion to late-stage NHL patients whose lymphomas are resistant or refractory to standard therapies, including the anti-CD20 antibody rituximab. Earlier clinical studies have shown that repeated bolus

infusions of the BiTE antibody are associated with cycles of T-cell activation and inactivation, and with various adverse reactions, including fully reversible central nervous system (CNS) events, primarily somnolence, confusion, aphasia and cerebellar symptoms, in approximately 30% of patients (unpublished observations). In order to obtain sustained T-cell activation and to mitigate CNS effects, administration was changed to a continuous infusion regimen. On this regimen, MT103/MEDI-538 is given for up to 8 weeks on an outpatient basis using a belt-worn pump after an initial hospitalization to monitor for adverse events. Continuous i.v. administration leads to a constant serum level of MT103, which may support uninterrupted cytotoxic T-cell activity.

Interim results reported at the annual meetings of the European Hematology Association and American Society of Hematology in 2006 (42, 43) showed confirmed clinical responses with MT103/MEDI-538 monotherapy according to Cheson criteria. Responses were first noted at a dose of 15 μg/m^2/day. Among 8 patients treated at this dose level, 1 complete and 2 partial responses were observed. The remaining evaluable patients either had a minor response (n=1), stable disease (n=3) or progressive disease (n=1). No dose-limiting toxicity was reached at the time of reporting. Other proof for clinical activity of MT103/MEDI-538 included complete and lasting depletion of circulating B-cells at doses of 5 μg/m^2/day and higher, and normalization of spleen size in a patient with splenomegaly at a dose of 15 μg/m^2/day. Of particular interest was the observation that, of 5 patients with infiltrated bone marrow, 2 showed a reduction and 3 a complete clearance of tumor cells in bone marrow biopsies at a dose of 15 μg/m^2/day. This suggests that the BiTE antibody may also be suitable for treating B-cell malignancies other than NHL that frequently show bone marrow infiltration, such as ALL and CLL.

While at higher doses peripheral B-cells disappeared during the first days of infusion for the entire treatment period, T-cells first eclipsed but then reappeared during administration of the BiTE antibody. In many cases, T-cell counts increased severalfold over the baseline level determined at the beginning of treatment. As further reported at the American Society of Hematology meeting (44), analysis of T-cell subpopulations showed that CD8$^+$ and CD4$^+$ effector memory T-cells made up the bulk of expanded peripheral T-cells, while counts of naïve and central memory T-cells remained more or less constant. This pharmacodynamic finding in patients mirrors the situation in cell culture assays, where effector memory T-cells showed the strongest proliferative response to BiTE antibody stimulation and made the largest contribution to redirected lysis. It is tempting to speculate that the effector memory T-cell response triggered in patients by the BiTE antibody MT103/MEDI-538 is a direct consequence of the deadly encounter of this T-cell population with tumor B-cells in target tissue. After cessation of treatment, overall T-cell counts and activation status of T-cells returned to normal values within a few days, suggesting that BiTE activity is self-limiting.

Major side effects associated with MT103/MEDI-538 treatment were lymphopenia and leukopenia, which are thought to be related to its mode of action, *i.e.*, sustained B-cell depletion, transient redistribution of T-cells and activity in bone marrow. Initial pyrexia was well controlled by standard medication. Of note, no overt or dose-dependent cytokine production was measured in patients receiving doses up to 15 μg/m^2/day, but this may have been mitigated by steroid pretreatment. Occasionally, transient IL-10 and IL-6 peaks were observed. Infectious complications did not exceed the expected rate of infections observed in the studied patient population. Importantly, administration by continuous infusion was associated with a significantly lower incidence of CNS effects.

These initial results from this ongoing clinical trial in late-stage NHL patients show that BiTE antibodies recapitulate in patients many of the features observed in cell culture assays and animal models. These include highly potent cytotoxic activity against CD19$^+$ target cells (as present in blood, lymph nodes, bone marrow and spleen) and selective activation and expansion of effector memory T-cells. Moreover, the results provide clinical proof of concept for bispecific antibodies of the BiTE antibody class.

Novel platform for development of antibody-based therapeutics

This last section describes three more BiTE antibodies that are in preclinical development. It will show that the particular features of the BiTE antibody MT103/MEDI-538 are not restricted to recognition of CD19 as a target antigen, but are applicable to a wide range of target antigens. Bispecific antibodies of the BiTE class therefore constitute a novel platform for generating cytotoxic antibody-based therapies.

EpCAM-specific BiTE antibody

The characteristics of the EpCAM/CD3-bispecific antibody MT110 have recently been described in detail (25). The EpCAM-specific BiTE antibody is active against a variety of human carcinoma lines expressing the target antigen. EpCAM (CD326) is very frequently and highly expressed on the majority of human adenocarcinoma and several squamous cell carcinoma cells (45), and is considered one of the best-characterized tumor-associated antigens known (46). Recent studies have shown that EpCAM is a signaling molecule that can upregulate nuclear expression of the proto-oncogene c-Myc and cyclins (47). When overexpressed in quiescent cells, EpCAM induces cell proliferation, growth factor independence and growth of colonies in soft agar, which are hallmarks of oncogenic proteins. When EpCAM expression is knocked down by specific small interfering RNA (siRNA) in breast cancer cells, such cells cease to proliferate, migrate and be invasive (48). This oncogenic signaling of EpCAM may explain why EpCAM overexpression correlates with poor overall survival in a number of human

malignancies, including breast and ovarian cancer (49). EpCAM has recently been found to be expressed on so-called 'cancer stem cells' (50), which opens up the possibility for MT110 to eliminate the presumed culprits responsible for tumor relapse after therapy (51). EpCAM has already been employed as a target antigen in several antibody-based therapeutic approaches, including a human antibody (52), an immunotoxin and a trispecific antibody (see 46).

A concern with EpCAM is its expression on most normal epithelial tissues. However, treatment of mice with the murine EpCAM/CD3-bispecific BiTE antibody muS110 showed a robust therapeutic window, indicating that EpCAM on normal tissue is not accessible while EpCAM on tumor cells is targeted by muS110 and allows their redirected lysis by BiTE antibody-activated T-cells (39). A number of mechanisms may contribute to this therapeutic window: 1) tight association of EpCAM in normal cells with other proteins, such as tetraspanins, CD44 and claudin-7, which may be reduced or absent in tumor cells; 2) sequestration of EpCAM on normal tissue within intercellular boundaries; and 3) poor access of T-cells to well-organized epithelial cells embedded in an intact extracellular matrix.

The EpCAM BiTE antibody MT110 will soon be tested in a phase I study for safety and early signs of efficacy.

EphA2-specific BiTE antibody

This BiTE antibody recognizes EphA2, which is a member of a large family of tyrosine kinase receptors. High levels of EphA2 expression have been observed on carcinomas of the breast, ovary, lung, colon, esophagus, prostate and kidney (53). Like EpCAM, EphA2 is also expressed on normal tissues. However, the kinase may be sequestered in intercellular boundaries where it is complexed with membrane-bound ligands, called ephrins. On tumor cells, EphA2 function is altered, leading to a diffuse distribution on cells and increased accessibility to antibody-based therapies (54). Potent BiTE antibodies have been developed that spare normal EphA2-expressing cells forming intact cell layers but eliminate malignant cells growing more diffusely by redirected lysis (33). The study also shows complete target cell lysis by EphA2-specific BiTE antibodies, activity at low E:T ratios, high specificity of lysis and a significant correlation between EphA2 target antigen density on cells and the potency of redirected lysis by EphA2-specific BiTE antibodies.

CEA-specific BiTE antibody

This type of BiTE antibodies recognize CEA (CD66e), which is among the best-characterized and longest known tumor-associated antigens (55). CEA is expressed on a substantial proportion of carcinomas of the colon, lung, pancreas, stomach, ovary, uterus and breast. Like EpCAM and EphA2, CEA is also expressed on normal epithelial tissues of colon, stomach, esophagus, sweat glands, prostate and cervix. In contrast to tumor tissue, CEA is expressed on normal epithelia in a highly polarized fashion on the apical side, where the antigen may not be accessible to systemically administered antibodies or T-cells. CEA has already been employed as a target antigen in several antibody-based therapeutic approaches, including antibodies conjugated with toxins and prodrug-converting enzymes. A series of CEA-specific BiTE antibodies have been constructed that trigger highly potent and specific redirected lysis of CEA-expressing cancer cell lines and human CEA-transfected rodent cell lines by human T-cells (35). A subset of CEA-specific BiTE antibodies have the unique feature of being insensitive in their lytic activity to high concentrations of soluble CEA, as are found in the serum of advanced cancer patients, where they are used as a prognostic marker.

EpCAM-, EphA2- and CEA-specific BiTE antibodies all showed potent inhibition of tumor growth in respective animal models using immunodeficient NOD/SCID mice and, where tested as 'hybrid' BiTE antibodies, in immunocompetent mice.

Outlook

Data from an ongoing phase I dose-escalation study with the anti-CD19 BiTE antibody MT103/MEDI-538 in heavily pretreated NHL patients showed encouraging results and provided clinical proof of concept for the BiTE antibody technology. 'Liquid' tumors from lymphomas and leukemias have also been shown to respond well to monotherapy with conventional monoclonal antibodies. High response rates have been seen with the anti-CD20 MAb Rituxan® or the anti-CD52 MAb alemtuzumab (Campath®), both of which largely act via antibody-dependent cellular cytotoxicity (ADCC). ADCC is related to the mode of action of BiTE antibodies, with the key difference that ADCC uses Fcγ receptor-positive cytotoxic immune cells, such as natural killer (NK) cells, while BiTE antibodies exclusively recruit T-cells. In contrast to liquid tumors, solid tumors in patients with adenocarcinoma or squamous cell carcinoma typically do not respond well to monotherapy with conventional antibodies, suggesting a fundamental difference in responsiveness to immunotherapies between solid and liquid tumors. This may relate to issues of tumor penetration, the presence and accessibility of immune effector cells to tumors, and accrued anti-apoptotic and immune escape mechanisms. Future clinical trials with the EpCAM-specific BiTE antibody MT110 will test the efficacy of the BiTE platform against solid tumors, and may reveal whether BiTE antibody-activated T-cells are better suited than monoclonal antibody-activated NK cells for the treatment of solid tumors.

Because of its novel and unique mode of action, BiTE antibody therapy may be advantageously combined in solid tumor settings with established treatments, such as standard chemotherapies or novel targeted therapies. Lastly, the 'seek and destroy' mechanism inherent to BiTE antibody therapeutics may be particularly suitable to treat cancer as a consolidation therapy after debulking of tumors by surgery, chemotherapy or radiation. In this sit-

uation of minimal residual disease, both resting and proliferating tumor cells may be better accessible and thereby more vulnerable to attack by BiTE antibody-activated cytotoxic T-cells. On the other hand, chemotherapy, which primarily targets rapidly dividing tumor cells, may not be successful in eradicating residual cancer stem cells having a low proliferation index but high tumorigenic potential.

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