BiTEs: bispecific antibody constructs with unique anti-tumor activity

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Bispecific T-cell engager molecules (BiTEs) constitute a class of bispecific single-chain antibodies for the polyclonal activation and redirection of cytotoxic T cells against pathogenic target cells. BiTEs combine a unique set of properties that have not yet been reported for any other kind of bispecific antibody construct, namely extraordinary potency and efficacy against target cells at low T-cell numbers without the need for T-cell co-stimulation. Here we review novel insights into the mechanism of BiTE action, which help to explain the unique features of BiTEs, as well as data from various animal models demonstrating the outstanding therapeutic potential of BiTEs for the treatment of malignant diseases.

T cells as prime enemies of tumor cells

The idea of engaging T cells for tumor cell elimination by bispecific antibodies is one of the most compelling concepts in new anti-cancer drug development [1,2]. This is because T cells are the most abundant type of immune cells, are present in blood, lymph and all organs, are extremely motile and are equipped with the most effective arsenal of cellular toxins against tumor cells in the body. A large part of T cells in the body are cytotoxic by virtue of containing granules with a highly toxic payload of cell-death-inducing proteolytic enzymes (called granzymes) and pore-forming proteins (perforin). The pivotal role of cytotoxic T cells in controlling tumor growth is impressively demonstrated by the broad range of strategies employed by tumor cells to evade T-cell recognition and action. Evasion mechanisms found with tumor cells act at each possible level of the complex target recognition and activation pathways of the T cells. The natural course of tumor cell lysis by a specific T cell is depicted in Figure 1. In the following, a few examples are given to demonstrate the versatility of such mechanisms.

Tumor cells can alter the subunit composition of proteasomes, which are essential for the proper processing of tumor-derived peptide antigens to be presented to T cells [3–5]. At the next level, tumor cells can lose intracellular peptide transporters (TAP-1 and -2), which are necessary for peptide antigen transport into the endoplasmic reticulum and subsequent loading onto major histocompatibility class (MHC) I molecules [6,7]. MHC class I molecules that present peptide antigens to T cells on the tumor cell surface are frequently downregulated on tumor cells [8–11]. Even alleles and entire chromosome pieces encoding their polymorphic genes can get lost in tumor cells. Likewise, there exists a selection pressure to lose expression of the MHC class I-associated β_2-microglobulin protein, which is required for the surface transport of MHC class I molecules [12]. Without MHC class I molecules, β2-microglobulin or tumor-associated peptide antigens, T cells can no longer recognize, by their specific T-cell receptors, the tumor cells as 'foreign' targets. Other evasion strategies involve the secretion by tumor cells of soluble forms of cell adhesion molecules (ICAM-1) that

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neutralize complementary recognition molecules (LFA-1) on T cells [13]. Tumor cells can express the same membrane-bound enzyme (cathepsin B) that T cells use to protect themselves from a deadly backfire of their toxic payload [14], and they can express a potent serine protease inhibitor (PtdIns-9/SPI-6) of the fast-acting cell-death-inducing T-cell enzyme granzyme B [15]. Further evasion strategies of tumor cells are the expression of signaling proteins, which can trigger apoptosis or anergy of T cells (FasL and PD-1) [15–17], and the secretion of immunomodulatory cytokines, such as TGF-β, IL-10 and IL-4 [18–20], all of which interfere with differentiation of cytotoxic T cells. Recently, tumor cells were found to upregulate an enzyme degrading the essential amino acid tryptophan [21,22]. T cells exposed to the tryptophan 'sink' of the tumor cells will be depleted of the nutrient and, as a consequence, become tolerant.

It is obvious that a multitude of evasion phenotypes can be selected through the cytotoxic action of T cells from the large number of malignant cells that are present in a tumor or disseminated in the body. Such evasion mechanisms, particularly if combined, enable subpopulations of tumor cells to evade T cell elimination effectively. Those tumor cells that are protected best against T cells will ultimately dominate and grow out into a tumor mass. It is thus not surprising to find T cell-resistant tumor cells, particularly among late-stage cancers [23], explaining the limited clinical efficacy of vaccination strategies [24]. At the same time, this situation lends support to the development of T-cell therapies that can potentially circumvent tumor cell evasion and redirect polyclonal T cells against cancer cells that have lost T-cell recognition molecules or managed to pacify bystanding cytotoxic T cells.

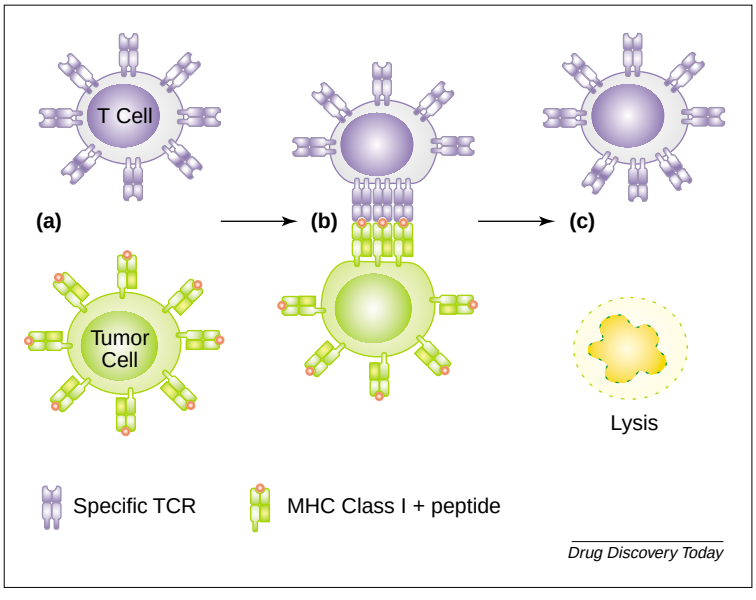

FIGURE 1

Physiological lysis of a tumor cell by a T cell. (a) A T cell recognizes, via its specific T cell receptors (TCR), a tumor cell displaying MHC class I complexes loaded with a tumor cell-specific peptide antigen. **(b)** A synapse is formed, which results in **(c)** lysis of the tumor cell.

Natural killer (NK) cells are closely related to cytotoxic T cells and share the same arsenal of toxins. NK cells are activated by tumor cells, having lost MHC class I expression, or are decorated by certain classes of antibodies with specificity for tumor-associated antigens [25,26]. However, it seems that NK cells – although being effective with small tumors – are overwhelmed by larger tumor masses and are inferior to T cells with respect to number, motility, tissue penetration and vigor of activation and proliferation.

Long history of bispecific antibodies

For almost two decades, researchers have crafted artificial antibodies that can physically crosslink, by their two different arms, T cells with tumor cells [27]. Such bispecific antibodies were also in need of one additional property, that is, the capacity to potently trigger the deadly T-cell machinery upon transient T cell–tumor cell contact, irrespective of T-cell receptor specificity. There are no naturally occurring proteins combining these two properties, therefore, different types of recombinant bispecific proteins have been designed, expressed and tested. Most of these were derived from monoclonal antibodies and substantially differ in architecture, size and specificity for the antigens recognized on T and tumor cell surfaces. Amongst others, CD2 and framework sequences of T-cell receptor α and β chains have been used as T cell targets but most experience has been collected with antibodies against the ε chain of the CD3 complex. CD3 seems particularly well-suited because it is present on all T cells and provides a potent triggering mechanism. Nevertheless, experience with different anti-CD3 bispecific molecules – including quadromas, diabodies and chemical heteroconjugates, as well as recombinant constructs using selected heterodimerization domains – showed that targeting CD3 is not necessarily sufficient to achieve maximal T-cell activation.

Most constructs experienced at least some sort of limitation [2,27,28]. One was the need for T-cell co-stimulation or some kind of pre-activation of T cells by other agents. Another limitation was the need for a large excess of T cells over target cells, suggesting that only a small subpopulation of T cells was activated successfully by the bispecific antibody. Furthermore, with most formats relatively high concentrations of bispecific antibodies were needed to achieve half-maximal target cell lysis; low microgram to high nanogram per milliliter concentrations were required, which is in the same order of magnitude as needed for *in vitro* tumor cell lysis by cytotoxic IgG1 monoclonal antibodies. As known from marketed IgG1 antibodies for oncology indications [29], this level of efficacy imposes a high burden on the development of an effective production process for bispecific antibodies. Therefore, the efficacy of conventional bispecific antibody formats is primarily limited by suboptimal T-cell recruitment and activation and issues of production.

BiTE: a bispecific format with exceptional properties

BiTE molecules constitute a type of bispecific anti-CD3 antibody that can obviously overcome limitations encountered by other bispecific antibodies [2,27,28]. BiTEs combine the minimal binding domains (Fv fragments) of two different monoclonal antibodies on one polypeptide chain of ~55 kDa. They employ single-chain antibodies [30], which have been shown to retain binding activity when arranged in tandem [31]. The structural relationship of a BiTE to its parental monoclonal antibodies is depicted in Figure 2. The two single-chain Fv fragments in BiTEs are fused by a short flexible linker enabling free rotation and kinking of the two arms. This particular design might be crucial for the efficient induction of T-cell activation because it enables optimal interaction with target epitopes on two opposing cell membranes. Moreover, the small size of BiTEs ensures close proximity of T cell and target cell membranes. This could be important to actively displace negative regulatory proteins from the forming lytic synapse, as has been shown for the tyrosine phosphatase CD45 [32]. The flexible tandem arrangement of single-chain bispecific antibodies by appropriate linker sequences, proper folding and expression in mammalian cell culture and use of parental antibodies with particular target-binding properties might explain the particular properties of BiTEs. The following functional properties are being used to define the BiTE class of bispecific antibodies:

- A 100–10,000 fold higher efficacy in tumor cell lysis relative to other CD3-bispecific formats and monoclonal IgG1 antibodies.
- An ability to induce target cell elimination by unstimulated peripheral T cells without the need for extra T cell co-stimuli or T cell pre-activation regimens.
- Serial target cell lysis by individual T cells explaining efficacy at low ratios of T cells to target cells.
- Formation of lytic immunological synapses with high frequency.
- Potent activation of caspases 3 and 7 in tumor cells.
- Strictly target cell-dependent, polyclonal activation of most $CD4^+$ and $CD8^+$ T cells.
- High protein stability and homogeneity (no glycosylation).
- Ease of production by mammalian cell culture as secreted protein.

The characteristics of BiTEs also rely on the choice of the tumor-associated antigen and the respective monoclonal antibodies used for constructing the two single-chain antibody arms. A CD19-specific BiTE was characterized in great detail [33–37]. Several BiTEs directed against the pan-carcinoma antigen epithelial cell adhesion molecule (Ep-CAM) were also made and analyzed in great detail [32,38,39]. All Ep-CAM-specific BiTEs share the essential properties with the CD19-specific BiTE, showing that the above listed properties are a class phenomena. A variety of BiTEs made against other cancer and non-cancer targets also showed reactivation of unstimulated T cells and half-maximal tumor lysis *in vitro* at or below 10 ng ml^{-1}.

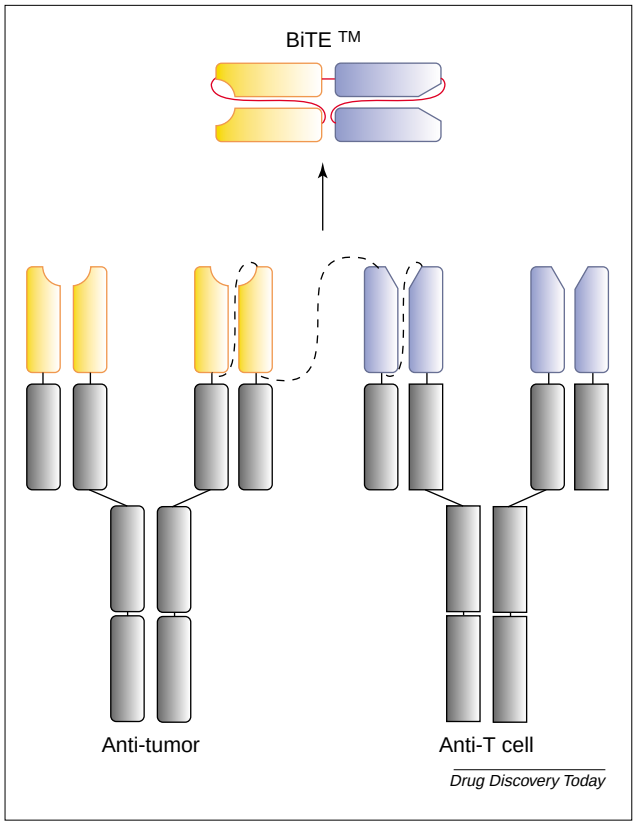

FIGURE 2

Construction of a BiTE from two different monoclonal antibodies. The variable domains of two monoclonal antibodies recognizing either tumor or T cell are genetically linked, as indicated by dotted lines. As a result, a single polypeptide chain is produced in which two single-chain antibodies are flexibly linked (BiTE).

The literature describes properties of several other single-chain bispecific antibodies [40–43]. Side-by-side comparison in various assay systems would be required to investigate and fully understand the differences to the functional properties as defined earlier for BiTE molecules.

Insights into BiTE mode-of-action

The mechanism of BiTE action has been studied in considerable detail over the past four years. In the following sections, three areas of such research will be discussed: the first is the mechanism of redirected target cell lysis, the second is the polyclonal activation of T cells and the third deals with the contact zone BiTEs induce between tumor cells and T cells, the so-called immunological lytic synapse. Insight into all three areas is needed to understand the particular properties of BiTEs and differences to the properties of other bispecific antibody formats as are reported in the literature.

Redirected target cell lysis by BiTE

Redirected target cell lysis by BiTEs has been studied using a variety of assays [32–39]. These include the classical 51-chromium release assay and the release of non-radioactive calcein-AM from pre-loaded tumor cells. Likewise, various fluorescence-activated cell sorting (FACS) based assays were used, for example, to monitor the nuclear

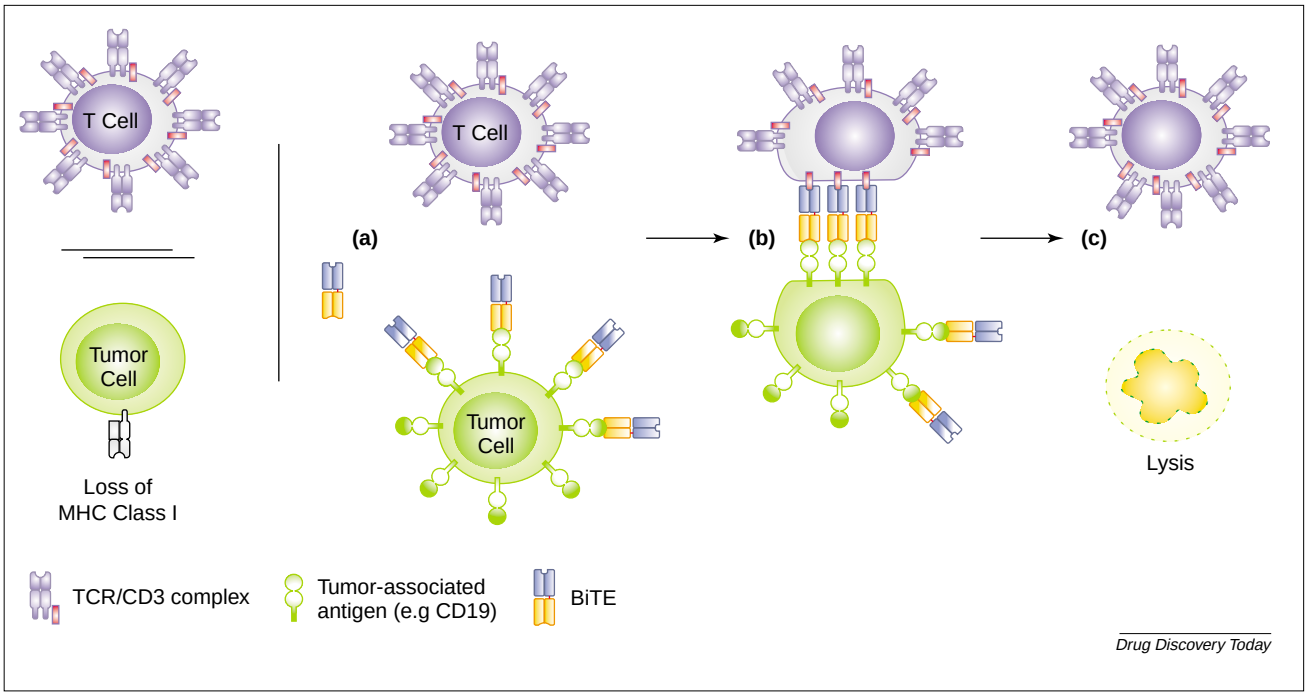

FIGURE 3

BiTE-induced lysis of a tumor cell by a T cell. Tumor cells frequently lose MHC class I expression or are deficient for peptide antigen processing. Consequently, such tumor cells are no longer recognized by cytotoxic T cells (left picture). **(a)** BiTEs recognize with one arm a cell surface protein on tumor cells of the kind recognized by monoclonal antibody therapies. **(b)** With the other arm, BiTEs recognize the CD3 complex associated with T cell receptors, leading to formation of a synapse. **(c)** This also leads to potent lysis of the tumor cell. Note that neither a specific T cell receptor nor a MHC class I–peptide complex is required for BiTE-mediated tumor cell lysis (compare to Figure 1).

uptake of propidium iodide by lysed tumor cells, or the decrease of the viable target cell population, or combinations thereof. Results from the various assays showed a remarkable congruence in dose–response analyses with respect to half maximal activity and sigmoidal curve shape. This suggests that all assays were essentially monitoring the same event, that is, the elimination of tumor cells by BiTE-activated T cells. No tumor cell lysis by BiTEs was ever observed in the absence of T cells or in the presence of a BiTE molecule that binds solely to T cells and not to target cells. Elimination of an MHC class I-negative tumor cell line by BiTE-activated T cells could also be shown [32] (see Figure 3; compare to Figure 1).

Half maximal cell lysis (EC_{50}) by BiTEs typically ranges between 10 and 10,000 pg ml^{-1} (i.e. between 180 femto- and picomolar). EC_{50} values can be influenced by a variety of parameters, one being the origin of T cells. Immortalized cytotoxic T-cell clones show the highest lytic potency, whereas T cells from human donors show a considerable variation [35]. However, donor variation of EC_{50} values is reduced and efficacy is generally increased upon prolonged *in vitro* incubation of human peripheral T cells with BiTEs. Another factor affecting BiTE potency is the nature of the target cell line. Using the same T cell source, various tumor cell lines show different EC_{50} values and overall cell lysis. This might reflect differences in target antigen density or different degrees of resistance to pro-apoptotic signals. Intriguingly, binding affinities of BiTEs have only a weak and unpredictable impact on BiTE potency. The affinity of BiTEs for CD3 on T cells is typically in the range 10^{-7}–10^{-8} M and thus several orders of magnitude above concentrations for EC_{50} [35]. The affinity for the tumor target of BiTEs can also vary between 10^{-8} and 10^{-9} M without much effect on EC_{50} values. The lowest BiTE concentrations observed so far inducing *in vitro* cell lysis were around 1 pg ml^{-1} (18 femtomolar), suggesting that only a low two-digit number of BiTE molecules bound between T and target cells occasionally is sufficient to induce redirected lysis.

The proteolytic activation of caspases 3 and 7 in tumor cells indicates that BiTE-activated T cells delivered granzyme B, a direct activator of caspases, into the tumor cell cytoplasm. Delivery of granzymes is thought to require the action of perforin, a pore-forming protein of cytotoxic T cells, which is dependent on calcium [44]. A calcium chelator could fully protect tumor cells from BiTE-induced redirected lysis [45], suggesting that the perforin–granzyme system is central for BiTE activity. The FasL–Fas receptor system has also been implicated in T-cell-mediated cytotoxicity. One study with a CD19-specific BiTE suggested that the Fas receptor–FasL system made only a minor contribution to BiTE activity [45].

Can tumor cells resist BiTE-activated cell lysis?

To date, a great variety of target cells have been tested for redirected lysis by BiTE-activated T cells. No fully BiTE-resistant human tumor cell line expressing the target

antigen has been identified so far. Intriguingly, transfected rodent cell lines expressing a human target antigen can also be potently eliminated by BiTE-activated human T cells [36,39]. This shows that BiTEs can redirect human T cells across species barriers, supporting the notion that BiTE-activated T cells do not need matching MHC class I molecules or costimulatory molecules on target cells but solely require expression of the target antigen. This is corroborated by the observation that K562 erythroleukemia cells entirely lacking MHC class I molecules are also efficiently eliminated by BiTE-activated T cells – provided that they express a transfected gene encoding the Ep-CAM target antigen [32]. Major evasion mechanisms of tumor cells, such as the loss of MHC class I expression, might thus be overcome by BiTE therapeutics.

Do BiTEs need additional T-cell stimuli for redirected tumor cell lysis?

All available evidence suggests that BiTEs do not need additional T-cell stimuli for redirected tumor cell lysis. First, published studies on BiTEs typically used unstimulated peripheral human T lymphocytes. The resting state of T effector cells was verified by the absence of expression of activation markers and cell adhesion molecules, and the lack of cytokine secretion and proliferation. The effects of anti-CD28 and exogenously added IL-2 on redirected target cell lysis were tested directly [35,39,46]. The impact by anti-CD28 antibodies was only modest, if at all; and IL-2 could only enhance cytotoxicity if T cells were incubated for several days with the cytokine. The reason why BiTEs potently activate T cells without an apparent need for costimulatory and mitogenic T cell stimuli is not well understood. It is possible that BiTE-induced synapses between T and target cells enable an optimal stimulation of T cells.

How many tumor cells can one T cell kill?

The high effector-to-target (E:T) ratios required by other bispecific antibodies suggest that only a few T cells contribute to target cell lysis. We have shown recently that BiTEs can lead *in vitro* to a complete target cell elimination at an E:T ratio of 1:5 over 24 h [37]. This can be explained either by T cells lysing tumor cells in an act of serial killing that involves a direct contact, or by the release of diffusible cytotoxic mediators. Strong support for the former mechanism came from video-assisted microscopy. It was observed that single, non-specific T cells in a lawn of tumor cells were highly motile and permanently screening surfaces of tumor cells. When BiTEs were added, T cells changed their behavior and made prolonged contacts with tumor cells, however, without forming large clusters of T and target cells that would trap T cells. As a consequence of the prolonged interaction with BiTE-activated T cells, tumor cells underwent nuclear fragmentation and membrane blebbing as morphological signs of apoptotic cell death. Single T cells stayed motile and could therefore undergo multiple rounds of such killing events during the observation period, directly demonstrating that BiTEs can induce serial killing by T cells. Tumor cell lysis was strictly dependent on a previous interaction with a T cell that lasted for at least several minutes. Target cells in the vicinity that were not contacted by T cells, or added cells that did not carry the target antigen for the respective BiTE, showed no signs of apoptosis. Therefore, it seems unlikely that diffusible factors made a major contribution to target cell lysis but rather that lytic synapse formation is a prerequisite for target cell lysis by BiTE-activated T cells.

T-cell activation by BiTE

BiTEs have a high potential to activate resting T cells. However, T-cell activation by BiTEs is only seen in the presence of target cells expressing the respective antigen [47]. BiTEs cannot activate the lytic potential of T cells by just binding with their anti-CD3 arm to the CD3 complex even at concentrations exceeding EC_{50} values for redirected lysis by several thousand-fold. This unique property might be related to the monomeric nature of BiTEs. Monovalent binding to the CD3 complex by BiTEs is apparently not sufficient to lead to a full-blown T-cell activation. Only when BiTEs are arrayed on the surface of a target cell (i.e. are presented to T cells in a polyvalent form) do they cause robust T-cell signaling, redirected lysis and a cascade of subsequent events.

T cells start expressing the early activation marker CD69 and the late marker CD25, which is subsequently shed by T cells, but only when activated by BiTEs in the context of target cells. The same is true for the upregulation of cell adhesion molecules on T cells, including CD2 and LFA-1. T cells activated *in vitro* by a BiTE start secreting a host of cytokines, including TNF-α, IFN-gamma, IL-6, IL-2, IL-4 and IL-10. Finally, T cells are sent into S phase, as demonstrated by a dramatic increase in T-cell number. Consistent with a polyclonal activation of T cells via CD3, the vast majority of $CD8^+$ and $CD4^+$ T cells from peripheral blood start expressing CD69 and CD25 in response to BiTE stimulation. However, when it comes to proliferation there is a bias towards higher proliferation rates of $CD8^+$ T cells. BiTEs thus have the potential to act as potent T-cell growth factors. As expected, cytotoxic $CD8^+$ T cells made the major contribution to redirected cell lysis by BiTEs. However, with some delay, also $CD4^+$ T cells could contribute significantly [39], which could be related to their robust upregulation of granzyme B expression in response to BiTE stimulation.

In conclusion, BiTEs that are presented to T cells via target cells appear to be sufficient to trigger T-cell activation at all levels: new surface expression of activation markers and cell adhesion molecules, secretion of cytokines and induction of cytolytic activity and cell proliferation. BiTE-induced T cell activation is polyclonal and can lead to the engagement of a high percentage of peripheral $CD8^+$ and $CD4^+$ T cells in redirected tumor cell lysis.

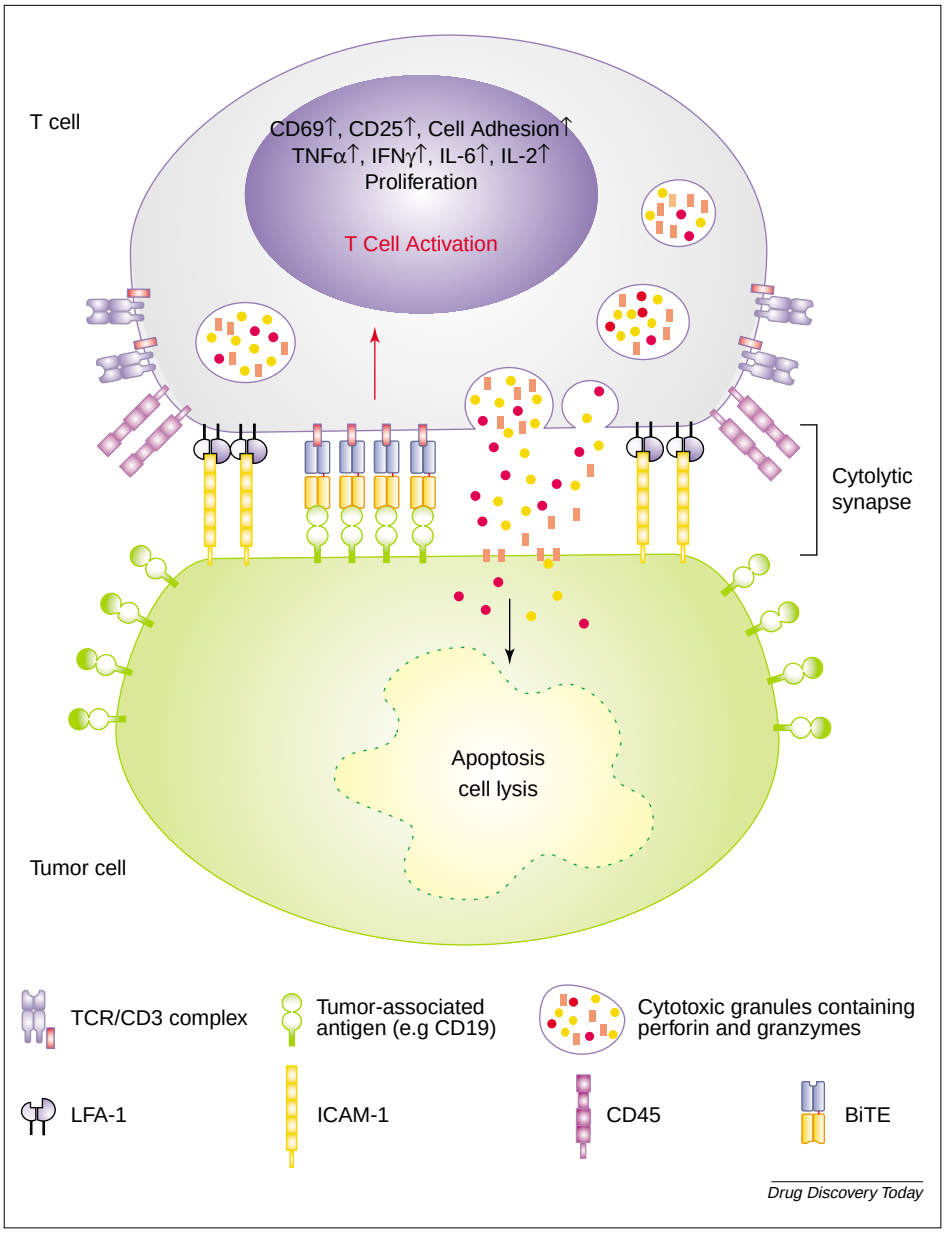

FIGURE 4

Model of a BiTE-induced cytolytic synapse. BiTEs force T cells and tumor cells to come in close contact. The resulting synapse shows all the hallmarks of a synapse formed by T cell receptor–MHC class I–peptide-induced synapses. An adhesion ring involving interaction between LFA-1 on T cells and ICAM-1 on tumor cells is formed. A signaling domain is formed, potently activating T cells to express adhesion molecules and cytokines and to proliferate. A secretory domain is formed, releasing cytotoxic granule content into the target cell. As a consequence, the tumor cell undergoes programmed cell death and lysis. The negative regulatory tyrosine phosphatase CD45 becomes excluded from the forming BiTE-induced synapse.

Lytic synapse formation by BiTE

In recent years, immunological synapses have been recognized as key subcellular structures for specific T-cell function and activation [48,49]. Regular lytic synapses form between cytotoxic T cells and target cells and are induced through the interaction of a specific T-cell receptor (TCR) with a corresponding peptide–MHC class I complex on target cells. Lytic synapses are characterized by two discrete functional domains located in the center of the contact zone formed between T and target cell membranes. Although the signaling domain is responsible for T-cell activation, the secretory domain is where the cytotoxic granule content of the T cell is delivered precisely into the target cell. The two central domains are surrounded by a ring-like domain formed by interaction of LFA-1 and ICAM-1 adhesion molecules ensuring that the deadly cocktail of the T cell remains confined to the attached target cell.

Using immunofluorescence labeling and confocal microscopy techniques, the structure and composition of regular lytic synapses was compared to that of synapses induced by BiTE molecules within the same T cell–tumor cell system [32]. BiTE-induced synapses were induced with high frequency and were indistinguishable in composition, subdomain arrangement and size from regular synapses induced upon addition of a T-cell antigen peptide in the proper MHC class I–TCR context. Lytic synapses could also be induced by BiTEs on a MHC class I-negative tumor cell line leading to robust cell lysis. These findings show that BiTEs trigger a regular mechanism of T-cell killing involving formation of lytic immunological synapses, which is independent on TCR specificity of T cells and the expression of MHC class I molecules on target cells. The reason for the outstanding potency and costimulus independence of BiTEs could thus lie in a highly effective formation of lytic synapses. Figure 4 shows a model of a BiTE-induced cytolytic synapse.

Therapeutic potential of BiTEs

MT103, a CD19-specific BiTE (also called bscCD19xCD3), is currently in a dose-escalating Phase I trial. Although it is too early to assess the therapeutic potential of BiTEs in humans, *ex vivo* experiments and many *in vivo* models have consistently shown a high anti-tumor activity of BiTEs. MT103 showed a high *ex vivo* response rate with blood samples from B cell lymphoma patients [34] and was efficacious in a severe combined immunodeficiency (NOD/SCID) mouse model [46]. Subcutaneous tumor growth was prevented in all animals with five intravenous doses of 100 ng MT103 in the presence of unstimulated human T cells. In chimpanzees, the only relevant animal species identified, repeated 2 h treatments with doses as low as 0.1 μg kg^{-1} MT103 caused a cumulative depletion of peripheral B cells and a transient activation of T cells [50].

Three different Ep-CAM-specific BiTEs were so far tested in NOD/SCID mouse models against subcutaneously growing human colon carcinoma cells [36,51]. For a supply of human T effector cells, tumor cells had to be mixed with unstimulated human peripheral blood lymphocytes at a ratio as low as 1:1. All three BiTEs showed strong anti-tumor activity in that they prevented tumor outgrowth with 5 daily i.v. injections of 0.1–1 μg BiTE in all animals. Where tested, Ep-CAM-specific BiTEs could also eradicate established subcutaneous tumors with sizes between 100 and 200 mm^3, which required slightly higher BiTE doses of 1–10 μg BiTE given daily. This *in vivo* activity did not require costimulatory agents of any kind or the preactivation of T cells. Ep-CAM-specific BiTEs were also capable of eradicating human ovarian cancer tissue in NOD/SCID mice [36]. Apparently, this activity of BiTEs relied on reactivation of the endogenous human T cells carried along by the metastatic tissue. An Ep-CAM-specific BiTE with a murine-specific anti-CD3 portion was highly active in an immunocompetent mouse model against both subcutaneous tumor growth and against disseminated tumor cells growing out in the lung [51]. This is the first model showing that endogenous T cells of the mouse are activated by BiTEs without costimulation and can also successfully eliminate disseminated syngeneic tumor cells transfected with human Ep-CAM.

Possible limitations

Unlike other bispecific antibodies, BiTEs apparently do not require costimulatory agents for activation of T cells. It is currently unclear whether this independence is holding up for BiTEs directed against other target antigens and target cells. *In vivo* studies with primates and immunodeficient as well as immunocompetent mouse models showed that various Ep-CAM- and CD19-specific BiTEs are exceptional in that they do not require co-treatment with, for example, CD28 antibodies to show high biological activity.

Another issue of BiTEs could be a frequently reported instability of certain single-chain antibodies. Current data from long-term *in vitro* stability studies, animal models and clinical trials suggest that the single-chain antibodies selected for constructing the current versions of BiTEs are highly stable proteins. Moreover, stringent stability criteria are being applied for the selection of single-chain antibodies to be used in BiTE constructs.

The relatively short half-life of BiTEs in the order of several hours [36,50] requires an appropriate dosing schedule and formulation to assure a continuous activation of T cells against target cells. Efficacy, pharmacokinetic and pharmacodynamic data from primate and mouse models and from Phase I clinical studies with MT103 will provide guidance for further optimization of dosing, route of administration and formulation for BiTEs in general.

Although there is evidence that intravenously administered BiTEs can reach and act upon solid subcutaneous tumor xenografts premixed with human lymphocytes, the question is whether, under physiological conditions, T cells are present in sufficient number within tumor tissue to sustain BiTE activity. There are numerous studies showing that essentially all human cancers contain tumor-infiltrated lymphocytes (TILs) and, in particular, T cells [52,53]. However, T cells derived from tumors – even if tumor-specific – are frequently no longer responsive [54]. The question, therefore, is whether BiTEs can reactivate TILs and overcome immune evasion mechanisms as described previously. Our finding that BiTEs are active against human metastatic tissue in immunodeficient mice [36] suggests that tumor-resident human T cells were reactivated and present in sufficient number to eliminate the xenograft. Clearly, more studies of this kind are required, in which tumor elimination is investigated at the cellular level by immunohistochemistry.

The development of BiTEs against late-stage carcinoma provides an enormous challenge. BiTEs need to penetrate well into tumor tissue and there reactivate tumor-resident T cells, which might have been tolerized or anergized by tumor cells. Tumor cells will present to BiTE-activated T cells with various T-cell evasion mechanisms and at unfavorable E:T ratios. At the same time, healthy cells expressing the target antigen should not be harmed by BiTE-activated T cells. This requires a careful selection of tumor-associated target antigens and the establishment of appropriate and predictive preclinical models to assess the therapeutic window of new BiTE molecules.

Conclusions

This review shows that substantial progress has been made in elucidating the mode-of-action and therapeutic potential of BiTEs *in vitro* and in animal models. Further challenges in preclinical BiTE research are to investigate the impact of various immune evasion mechanisms of tumor cells on BiTE efficacy, to analyze in more detail the contribution of various T-cell subpopulations to BiTE activity, and to establish animal models that properly reflect the therapeutic window of BiTEs.

Thus far, BiTEs fulfill all properties that are desirable for cytotoxic, bispecific antibodies. They seem to be active under the challenging conditions typically found *in vivo*, most importantly, low ratios of T cells to tumor cells and low drug concentration in target tissue. The exceptional potency of BiTEs and acceptable levels of production in mammalian cells suggest that there will be no problem with drug supply should material be required for large patient populations. No simultaneous administration of co-stimulatory reagents is needed, therefore, BiTE development is simpler than for other bispecific antibodies. In the future, new therapeutic areas beyond cancer will be explored for the application of BiTEs. For example, the selective elimination of activated immune cell subpopulations in inflammatory lesions by redirected T cells could provide a compelling therapeutic concept.

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