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Targeting T Cells with Bispecific Antibodies for Cancer Therapy

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Abstract

Bispecific antibodies (BiAbs) offer a unique opportunity to redirect immune effector cells to kill cancer cells. BiAbs combine the benefits of different binding specificities of two monoclonal antibodies (mAbs) into a single construct. This unique feature of BiAbs enables approaches that are not possible with single mAbs. Advances in antibody engineering and antigen profiling of malignant cells have led to the development of a number of BiAb formats and their combinations for redirecting effector cells to tumor targets. There have been significant advances in the design and application of BiAbs for intravenous and local injection. The initial barrier of cytokine storm has been partially overcome by more recent constructs that have improved clinical effectiveness without dose-limiting toxicities. Since the recent revival of BiAbs, there has been multiple, ongoing, phase I/II and III trials, and some promising clinical outcomes have been reported in completed clinical studies. This review focuses on arming T cells with BiAbs to create the 'poor man's cytotoxic lymphocyte'.

1. Introduction

The advances in molecular antibody engineering and high-throughput methods for screening and identifying specific tumor antigens provide extraordinary tools for developing bispecific antibodies (BiAbs) to redirect immune cells to cancer cells. BiAbs combine the specificities of two antibodies into a single molecule, enabling the bridging of cytotoxicity-triggering receptors on an effector cell with selected surface molecules on a target cell. Targeting of two antigenic determinants was initially assessed in preclinical models, and phase I/II clinical trials were started nearly 20 years ago. However, initial clinical studies were disappointing, mainly due to low efficacy, severe adverse effects without a significant impact on the clinical outcome of disease, and the immunogenicity of the BiAbs. These shortcomings gave rise to the development of numerous formats of BiAb fragments and whole IgG molecules. The formats of BiAbs include chemical heteroconjugation of two whole monoclonal antibodies (mAbs) or fragments of mAbs, quadroma F(ab)₂, diabodies, tandem diabodies, single-chain variable fragment (scFv) antibodies, and dock-and-lock multivalent-multifunctional antibodies.^[1-4] The designs are limited only by one's imagination, with the engineering process ranging from simple chemistry to complex recombinant technology to produce BiAbs that target effector cells, drugs, prodrugs, toxins,

DNA, enzymes, anti-vascular agents, vectors, or radionuclides to tumor-associated antigens (TAAs) on malignant cells. BiAb engineering and glycoengineering can achieve the desired effector function, pharmacokinetics, and clinical outcome. The engineering of BiAb constructs to optimize tissue penetration, *in vivo* stability, targeting specificity, and binding affinity for tumor cells has been reviewed elsewhere^[5,6] and will not be discussed here. This review focuses on the use of BiAbs to redirect effector cells to target cancer cells. The preclinical approaches and their clinical translation and the pros and cons of *in vivo* infusions of BiAb versus *ex vivo* 'franking' or arming of effector cells with BiAb will be detailed in this review.

2. The Challenges of Immune Cell Therapy

2.1 Adoptive T-Cell Therapy

Adoptively transferred lymphokine-activated killer cells (LAK),^[7,8] tumor-infiltrating lymphocytes (TIL),^[9] anti-CD3-activated T cells (ATC),^[10,11] and anti-CD3/anti-CD28 co-activated T cells (COACTs)^[12-14] have been used to eliminate or reduce tumor burden in preclinical murine models. However, translating these approaches to patients has been challenging. Although results were initially encouraging in patients with malignant melanoma (MM) or renal cell carcinoma using TIL infusions,^[9,15] subsequent studies have not clearly shown improved remission or overall survival rates with these approaches. Since 1986, clinical immunologists have sought to develop preclinical models to dissect the mechanisms responsible for the lack of anti-tumor responses and to demonstrate that effector cell therapy can induce sustained memory anti-tumor responses. Clinical studies in advanced MM showed some encouraging results.^[16] Infusions of specific cytotoxic T lymphocytes (CTL) in combination with 720 000 IU of interleukin (IL)-2/kg given every 8 hours induced clinical responses 7 days after non-myeloablative chemotherapy with cyclophosphamide (60 mg/kg×2 days) and fludarabine (25 mg/m² × 5 days).^[16] A mean of 7.8×10^{10} ($2.3\text{--}13.7 \times 10^{10}$) anti-melanoma CTL were infused. Six of 13 patients had objective clinical responses and 4 of 13 (30%) patients had mixed responses. Although TIL, ATC, and COACTs can usually be expanded to large numbers, they failed to induce objective clinical responses in most clinical studies. This may be due to intrinsic T-cell defects caused by the malignancy,^[17] inadequate numbers of specific CTL, chemotherapy, or a combination of factors.

The *sine qua non* of successful immunotherapy is the allogeneic graft-vs-leukemia (GVL) effect seen after allogeneic stem cell transplant (SCT). The original observation was that SCT patients who developed chronic graft-vs-host disease (GVHD) had lower relapse rates.^[18] This GVL effect was also seen in patients who received donor lymphocyte infusions (DLIs) for relapsed chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), and other hematologic malignancies.^[19,20] DLI can induce cytogenetic and molecular remissions in patients with CML.^[20,21] A similar GVL effect was observed in patients who developed Epstein-Barr virus (EBV)-driven lymphoproliferative disorder (LPD) after SCT with a T-cell-depleted allograft.^[22] Infusions of donor-derived EBV-specific CTL induced clinical remissions in patients who had developed LPD.^[23,24] Unfortunately, DLI is less effective against AML and ALL.^[18] The use of DLI for the treatment of solid tumors remains a challenge.

2.2 Tumor Escape

Tumors evade immune surveillance by expressing low levels of tumor or human leukocyte antigens (HLA).^[25,26] Altered HLA expression has been reported in breast,^[27] prostate,^[28] colon,^[29] lung,^[30] and pancreatic^[31] cancers and MM.^[32] Furthermore, tumor-derived suppressive cytokines inhibit differentiation of myeloid cells and promote accumulation of

both myeloid and lymphoid (regulatory T [T_{reg}] cells) suppressive cells in the neoplastic bed and in the secondary lymphoid organs. T_{reg} cells, myeloid-derived suppressor cells, and tumor-associated macrophages can inhibit the cellular and humoral immune responses to cell-based therapies or vaccines. Cytokines (transforming growth factor- β , IL-10, and IL-6) secreted by tumors and suppressor cells downregulate the synthesis of T-helper type 1 (T_H1) cytokines IL-2 and interferon (IFN)- γ . The suppression of IL-2 and IFN- γ inhibits T-cell proliferation and blocks the production of perforin granules and granzyme B, which are needed for non-major histocompatibility complex (MHC)-restricted killing.^[33] The presence of suppressive cytokines is known to decrease responses to treatment with IL-2 or IFN- γ .^[34,35] Immune escape mechanisms challenge the effectiveness of natural, adoptively transferred T cells and vaccines responses. Besides tumor escape and sabotage of immune responses, tumors provide a physical barrier with a well fortified perimeter consisting of pressure gradients that is difficult for immune effectors and antibodies to infiltrate/penetrate. Redirecting T cells with BiAbs may circumvent tumor escape mechanisms.

3. Clinical Infusions of Bispecific Antibodies (BiAbs)

Since 1997, when rituximab (Rituxan®) was approved, there have been nine additional US FDA-approved mAbs for cancer therapy as of June 2011. Currently, there are more than 22 mAbs approved for clinical use by the FDA, beginning with muromonab (anti-CD3) for transplant rejection in 1986. Most indications are for organ graft rejection, anti-platelet therapy, rheumatoid arthritis, respiratory syncytial virus infections, Crohn disease, breast cancer, colon cancer, asthma, and hematologic malignancies. Unconjugated, radioimmunoconjugated, and chemoimmunoconjugated mAbs have been approved for use based on their clinical efficacy and impact through specific targeting of CD20-positive lymphomas and epidermal growth factor receptor (EGFR)- and human epidermal growth factor receptor-2 (HER2)-positive solid tumors. The FDA-approved mAbs provide a unique source of material that can be paired with anti-CD3 through heteroconjugation to create BiAbs for targeting tumor cells with T cells. Table I summarizes the ongoing or completed clinical trials of BiAbs in various cancer types.

3.1 Whole IgG-based BiAbs

Most of the original targeting strategies were designed to infuse the BiAb into the patient, with the assumption that the infused BiAb would activate and redirect immune effector cells to tumor cells *in vivo*, leading to the lysis of the tumor target. In most of the clinical trials, BiAbs have been infused as 'drugs'. Shortly after the first BiAbs were made in the early 1980s, clinical applications for targeting cancer were recognized.^[56-58] The first phase I clinical trial was conducted in patients with CD19-expressing non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL) using the anti-CD3 $\times$ anti-CD19 BiAb SHR-1.^[38] The clinical approach was to determine if SHR-1 infusions could redirect endogenous T cells to lymphomas. With the exception of thrombocytopenia, SHR-1 doses ranging from 5 to 10mg did not cause toxicity. However, no clear clinical effects were seen in chemotherapy-resistant CLL patients. Failure was thought to be related to rapid clearance of SHR-1.

The next BiAb, anti-CD3 $\times$ anti-epidermal glycoprotein 2 (EGP-2) [BIS-1], was designed to prolong *in vivo* serum half-life and was clinically effective for tumor imaging.^[59] BIS-1 was made to target carcinoma cells expressing the 38 kDa epithelial carcinoma-associated transmembrane glycoprotein EGP-2.^[38] In a phase I trial in renal cell cancer patients, intravenous infusions of BIS-1 with IL-2 induced high levels of specific cytotoxicity associated with elevated serum tumor necrosis factor (TNF)- α and IFN- γ levels.^[38] The maximum tolerated dose was reached at 5 μ g/kg, with dose-limiting toxicities (DLTs) of dyspnea, vasoconstriction, and fever. This study showed that preclinical toxicology did not predict clinical toxicities. On the other hand, injecting autologous, *ex vivo*, IL-2-activated

peripheral blood mononuclear cells (PBMCs) and BIS-1 into carcinomatous ascites or pleural effusions did not cause dose-limiting side effects.^[39] Local administration most likely avoided cytokine storm effects caused by BiAb binding to Fc-receptor-bearing cells in circulation. Fc R-bearing cells may have aggregated with BiAb-armed T cells, triggering T cells to secrete cytokines (see figure 1).

Cytokine storm has been a major limitation for strategies that use anti-CD16 (anti-Fc γ RIII) $\times$ anti-TAA BiAbs, which bind to and redirect natural killer (NK) cells and neutrophils to target tumor antigens. The anti-CD16 $\times$ anti-HER2 BiAb, 2B1, was used to target NK cells to HER2-positive tumors in a phase I clinical trial involving 15 patients. There was one complete response (CR), one partial response (PR), three minor responses, and one mixed response. Treatment induced a 100-fold increase in circulating levels of TNF α , IL-2, and IL-8 and slight increases in the levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN γ .^[60] Fourteen of 15 patients developed human anti-mouse antibody (HAMA) responses. In a phase I/II trial using the BiAb HRS-3/A9 (anti-CD16 $\times$ anti-CD30) to treat patients with refractory Hodgkin's disease, there were encouraging clinical results.^[61] In a follow-up study involving 16 patients, there was one CR, three PRs, and three patients with stable disease (SD). Even though clinical responses have been seen with whole IgG-based BiAbs, cytokine storm has been a major limitation. Since whole-cell IgG-based BiAbs showed nonspecific activation of Fc receptor-expressing innate immune cells, the next modification step was to remove the Fc portions of the BiAb constructs.

3.2 The Heterogeneous F(ab')₂ Molecule-Based BiAbs

Using the same platform as 2B1 and targeting the same epitope on HER2, a humanized anti-CD64 (Fc γ RI) Fab was chemically linked with a murine anti-HER2 Fab (anti-CD64 $\times$ anti-HER2) to produce the BiAb MDX-H210. Multiple phase I studies using MDX-H210 with various dose ranges and responses have been carried out^[2-4,62-65] and are summarized in table II.

The BiAb MDX-447 (anti-CD64 $\times$ anti-EGFR) was tested in a phase I study in 64 patients to target renal cell carcinoma or head and neck cancer. Patients received MDX-447 at dose levels ranging from 1 to 40mg/m²/week alone or in combination with G-CSF. Hypotension was dose limiting with grade III toxicity. Other toxicities included fever, hypertension, arrhythmia, allergic reaction, dyspnea, and tumor pain.^[48]

Conjugated Fab fragments of anti-CD64 $\times$ anti-CD30 (H22 $\times$ Ki-4) at doses ranging from 1 to 20mg/m²/day on days 1, 5, and 7 were given to ten patients with refractory Hodgkin's disease in a phase I dose study. Side effects included hypotension (4 of 10 patients), tachycardia (6 of 10 patients), fatigue (10 of 10 patients), and fever (2 of 10 patients). There was one CR, three PRs, and four patients with SD.^[66]

These formats of BiAbs showed some clinical responses but their efficacy in clinical trials has either been insufficient or associated with DLTs. Moreover, the serum stability, poor yield, and immunogenicity of the above-mentioned BiAb formats have restricted their use. These limitations prompted the development of a new generation of BiAbs.

4. New Generation BiAbs

4.1 Trifunctional BiAbs

The development of trifunctional antibodies (TriFabs), a new generation of whole IgG-based BiAbs, is an impressive and significant improvement on the classical quadroma approach.^[67,68] The major improvement was a preferential species-restricted heavy-/light-

chain pairing, in contrast to the random pairing in conventional mouse/mouse or rat/rat quadromas using an original subclass combination (mouse IgG2a and rat IgG2b).

The TriFAB ertumaxomab (anti-CD3 $\times$ anti-HER2 BiAb, Fresenius Biotech GmbH; Munich, Germany) was used to treat women with metastatic breast cancer in a phase I clinical trial.^[69] Ertumaxomab, which was designed with a modified Fc type I/III receptor, creates a tri-cell complex consisting of T cells, Fc-receptor-positive cells, and tumor cells.^[69] Patients who received 100 μ g of ertumaxomab showed mild and transient side effects and there was one CR, two PRs, and two patients had SD. However, a higher intravenous dose (150–200 μ g) of ertumaxomab was not well tolerated; 7 of 17 patients experienced serious adverse events and in three patients (18%), the serious adverse events were classified as drug related.^[69]

Another TriFAB, catumaxomab (anti-CD3 $\times$ anti-epithelial cell adhesion molecule [EpCAM]; Removab®; Trion Pharma, Munich, Germany), when administered intraperitoneally to 23 patients with malignant ascites in a phase I/II study of refractory ovarian cancer, resulted in a 5-log reduction in EpCAM-positive tumor cells in the ascitic fluid and had an acceptable safety profile.^[44] Catumaxomab received EU approval in 2009 for the treatment of EpCAM-positive ovarian cancer ascites, making it the first BiAb to be approved for clinical use. A phase I study using catumaxomab was done in non-small-cell lung cancer to evaluate the safety and tolerability of intravenous treatment. Grade 3 and 4 DLTs were observed at dose level IV (dexamethasone 10 mg premedication, catumaxomab 5 μ g) and V (dexamethasone 40 mg followed by catumaxomab 7.5 μ g). The maximum tolerated dose was defined as dose level III (dexamethasone 40 mg followed by catumaxomab 5 μ g).^[45] Combining the use of catumaxomab with dexamethasone reduced the cytokine side effects successfully.^[45]

A novel future application of Removab® would be to target T cells to solid tumors in patients undergoing allogeneic SCT while simultaneously preventing the development of acute GVHD (US patent 2000601154810 entitled *Treating tumor growth and metastasis by using TriFAB antibodies to reduce the risk for GVHD in allogeneic anti-tumor cell therapy*). If successful, this would be an extraordinary clinical advance for the use of a trifunctional BiAb construct in treating solid tumors in combination with an allogeneic SCT.

4.2 BiAb Format Based on Single-Chain Variable Fragment

ScFv-based bispecific T-cell engager (BiTE) represents a highly innovative advance towards the development of a new generation of BiAbs. BiTEs combine the minimal binding domains (Fv fragments) of two different mAbs on one polypeptide chain of ~ 55 kDa. Studies using the BiTE format have shown some promising clinical results. BiTE antibodies induced lysis of target antigen-expressing cells at pico- to femtomolar concentrations without the need to pre-stimulate or co-stimulate the T cells. BiTE may induce cytolytic immunological synapses between cytotoxic T cells and target cells that are similar to normal T-cell synapses.^[70,71] Phase I/II clinical trials are being performed with the BiTE antibody MT103 (anti-CD19 $\times$ anti-CD3; blintumomab).^[72-74] Ongoing or completed phase I/II studies with MT103 suggest that T cells engage and lyse tumors.^[75] In 38 patients with follicular lymphoma, mantle cell lymphoma, or CLL who received doses ranging from 0.0005 to 0.06 mg/m²/day, 11 patients had CRs. The four CRs and seven PRs occurred at doses of 0.015 mg/m²/day and higher. Seven patients who received 0.06 mg/m²/day had objective responses. Doses of 0.015 mg/m²/day and higher cleared tumor cells from the blood, lymph nodes, spleen, and bone marrow. In 9 of 11 cases with bone marrow involvement, immunohistochemical staining and flow cytometry had partial (3/11) or complete (6/11) clearance of tumor cells.^[75] A phase II trial using MT103 to treat patients with precursor B-cell acute lymphoblastic leukemia is ongoing, wherein tumor cells are

detected only by PCR.^[76] These results show that MT103 can effectively recruit and redirect T cells against both bulky and minimal residual disease in hematological malignancies;^[77] however, clinical efficacy of the BiTE BiAb format in the treatment of solid tumors has not been reported.

Several BiTE antibodies directed at CD19, EpCAM, HER2, EGFR, CD66e (or carcinoembryonic antigen [CEA] or carcinoembryonic antigen-related cell adhesion molecule 5 [CEACAM5]), CD33, EphA2, and melanoma-associated chondroitin sulfate proteoglycan (MCSP) [or high molecular weight melanoma-associated antigen] are in the developmental pipeline.^[78] The cetuximab-based BiTE antibody showed promising results in preclinical models. It prevented tumor growth of *KRAS*- and *BRAF*-mutated human colorectal cancer (CRC) xenografts at very low doses, where cetuximab showed no effect.^[79] During treatment for 3 weeks in non-human primates, complete lysis of EGFR-overexpressing cancer cells was observed. These data suggest that EGFR-specific BiTE antibodies may be an effective treatment for CRC that is not responsive to conventional antibodies.^[79,80]

The critical points in design to improve clinical effectiveness without inducing DLTs are: (i) the design of the anti-effector activating or engaging construct directed at CD3, CD16, or CD64; (ii) the modification of the Fc-portions to attenuate cytokine storm; and (iii) the use of drugs or chemotherapy that can modulate the cytokine storm and thus improve the tolerability of BiAb infusions.

5. Clinical Infusions of BiAb with Effector Cells or BiAb-Armed (Coated) Effector Cells

The critical element of a BiAb is that it takes advantage of the binding specificities of two antibodies and combines them with the powerful effector functions of cytotoxic immune cells.^[56,57,81,82] Arming ATC with anti-CD3× anti-TAA BiAb transforms every polyclonal ATC into a CTL directed at a TAA.^[83] In the early 1990s, initial studies were done using *ex vivo* expanded LAK or ATC armed with BiAbs. The general strategy took advantage of T-cell proliferation over 10–14 days so that large numbers of effector cells could be armed.

5.1 Redirecting Immune Effectors

Co-injection of autologous LAK and chemically heteroconjugated anti-CD3 × anti-glioma BiAb was first reported in 1990.^[36] BiAb-armed and -unarmed LAK were injected into the brain tumors of ten patients. Four of ten patients who received armed LAK had tumor regression and four patients had improved overall survival (OS); OS and progression-free survival (PFS) appeared to be better than in patients who received unarmed LAK cells. In a subsequent phase I trial, ATC were co-injected with anti-CD3 × anti-EGFR BiAb and anti-CD28 × anti-EGFR BiAb into an Ommaya reservoir that was connected to the surgical cavity of glioma patients. Two often patients had CRs who received 70 and 250 × 10⁶ ATC. It was remarkable that such a small number of ATC led to clinical responses; however, the infusions were associated with transient fever, nausea, headache, and the aggravation of preexisting neurologic deficits.^[84]

In the early 1990s, several phase I and II studies were conducted in which ovarian carcinoma patients were treated with intraperitoneal injections of phytohemagglutinin- or anti-CD3-ATC armed with anti-CD3 × anti-Mov28 (ovarian carcinoma-associated antigen) or anti-CD3 × anti-folate receptor (OC/TR), with encouraging clinical responses.^[85-87] The product contained both CD4+ and CD8+ T cells. In a series of advanced ovarian carcinoma patients, intraperitoneal injections of ATC armed with OC/TR induced regression in patients who had debulking laparotomies for advanced disease.^[87] The patients received two cycles

of five daily intraperitoneal doses of ATC armed with OC/TR BiAb and 0.6×10^6 IU of IL-2. Ten patients received between 4 and 9×10^9 armed ATC. Despite poor prognostic features, 7 of 26 (27%) patients had a CR (4) or PR (3). Metastatic lesions that were >2 cm disappeared after therapy. The side effects were mild to moderate fever, nausea, and emesis. However, HAMA responses developed in 84% of the patients.

In preclinical studies, we showed that ATC armed with chemically heteroconjugated anti-CD3 $\times$ anti-HER2 BiAbs (anti-CD3 $\times$ trastuzumab) [HER2Bi] could (i) kill HER2 breast,^[88] prostate,^[89] ovarian,^[90] and pancreatic^[91] cancer cell lines; (ii) secrete cytokines (IFN γ , TNF α , GM-CSF) and chemokines (RANTES [Regulated on Activation, Normal T cell Expressed and Secreted], and MIP-1 α [macrophage inflammatory protein 1 alpha]);^[92] (iii) prevent the development of tumors in severe combined immune deficiency (SCID)/Beige mice when co-injected in Winn assays or induce remission when directly injected into established prostate PC-3 xenografts;^[93] (iv) traffic to tumors in SCID/Beige mice;^[93] (v) inhibit and eliminate ovarian cancer in SCID mice;^[90] and (vi) proliferate and kill tumors multiple times without undergoing apoptosis. If *in vivo* targeting of a tumor leads to tumor lysis and release of cytokines/chemokines by T cells at the tumor site, endogenous monocytes and T cells could be recruited and activated to induce immune responses. TAAs could then be processed and presented by dendritic cells to T cells (figure 2). Table III summarizes the preclinical studies using BiAbs that redirected T cells and NK-T cells. Table I summarizes the clinical trials for cancer involving infusions of redirected T cells or *in vivo* arming of effector cells by infusions of BiAb.

Our initial phase I clinical trials involved eight infusions of HER2Bi (anti-CD3 $\times$ trastuzumab)-armed ATC given over 4 weeks in combination with low-dose IL-2 (300 000 IU/m² daily) and GM-CSF (250 μ g/m² twice weekly) starting 3 days prior to the first ATC infusion and ending 1 week after the last infusion for the treatment of patients with high-risk HER2 0-3+ stage II–IV breast cancer, hormone-refractory prostate cancer, and advanced pancreatic cancer. ATC activated with muromonab and expanded in the presence of IL-2 for 14 days were harvested, armed with 50 ng HER2Bi/ 10^6 ATC, washed, aliquoted, and cryopreserved. There were two DLTs that occurred in the dose range between 80 and 160×10^9 armed ATC per patient. One patient died of heart failure most likely due to digoxin toxicity and one patient developed a subdural hematoma associated with high blood pressure due to GM-CSF administration and armed ATC infusions. The subdural hematoma was evacuated without any neurologic sequelae. The non-dose-limiting toxicity profile of the remaining patients included chills, fever, hypotension, and fatigue. The side effects were easily managed with prophylactic antihistamines, antipyretics, and vigorous hydration. A total of 22 women with metastatic breast cancer, nine women with high-risk breast cancer, six men with hormone-refractory prostate cancer, and one patient with pancreatic cancer were treated with HER2Bi-armed ATC. Women with breast cancer and men with prostate cancer reported decreased bone pain.^[126] Circulating ATC bearing HER2Bi could be detected for up to several weeks after the infusions.^[127] T_H1 cytokines (GM-CSF, IFN- γ , TNF α , IL-2), IL-12, RANTES, and MIP-1 α and low levels of T_H2 cytokines (IL-10, IL-4) were detected in the serum during infusions.^[128]

In a phase I clinical trial in patients with refractory, resistant, or high-risk CD20-positive non-Hodgkin's lymphoma, up to a total of 80×10^9 anti-CD3 $\times$ Rituxan® (CD20Bi)-armed ATC were given in four divided doses over 4 weeks after high-dose chemotherapy and peripheral blood SCT, without DLTs.^[129]

5.2 *In Situ* Vaccination by Redirected T Cells

ATC release TNF α , IFN γ , GM-CSF, IL-4, IL-6, and IL-10 during BiAb-mediated tumor lysis in an IL-2 independent manner.^[100] Analysis of cytokine levels in patients treated with

BiAb-armed T cells showed increases in TNF and IFN but not IL-4.^[100] TNF and IFN secretion during BiAb targeting shifts the *in vivo* milieu towards a Th1 anti-tumor environment. Repeated stimulation of T cells armed with anti-CD3×anti-CD19 or anti-CD3×anti-HER2 may improve survival and enhance *in vivo* cytotoxicity.^[100,130] Our phase I trial using HER2Bi (anti-CD3 ×anti-HER2)-armed ATC showed not only a shift towards a Th1 cytokine pattern but also the secretion of RANTES, and MIP-1,^[130] which would attract endogenous antigen-presenting cells and naïve T cells to the tumor site. This process would immunize the patient's endogenous immune system against the TAAs (figure 2).

5.3 Activated T Cells Armed with BiAb Survive to Kill Again

CTL that express Fas ligand (FasL) are known to kill tumor cells and the antigen-dependent binding of T cells to tumors releases IFN, which is known to upregulate Fas expression on tumor targets.^[131] The FasL-Fas interaction between the T cells and tumor cells induces apoptosis of tumor cells; however, the tumor cells counterattack by inducing apoptosis in the effector T cells.^[132-134] This counterattack may eliminate effector T cells before the immune response begins,^[135] but T-cell receptor (TCR) stimulation can protect CD8+ T cells from CD95-mediated apoptosis.^[136] BiAb-armed ATC, upon TCR restimulation, may be resistant to activation-induced cell death (AICD) since binding to the TCR and other receptors via the BiAb-complex may mimic secondary signals.^[92] We showed that armed ATC can repeatedly kill tumor targets and not undergo AICD.^[92] The data suggest that BiAb arming of ATC may not only re-stimulate the ATC to proliferate and secrete cytokines, but also enhance *in vivo* T-cell survival.

6. Strategies to Enhance Tumor Targeting

6.1 Co-Activation and Redirecting of T Cells

Co-stimulatory signals for T-cell activation have emerged as a promising strategy for tumor immunotherapy. A recombinant single-chain BiAb, rM28, directed to a melanoma-associated proteoglycan (NG2) and the co-stimulatory CD28 molecule on T cells, induced T-cell activation, which resulted in tumor-cell killing without additional TCR/CD3 stimulation. Presentation of a CD28 antibody within a suitable recombinant, bispecific format may result in 'targeted supra-agonistic stimulation' of the CD28 molecule, which leads to effective tumor-cell killing.^[137,138] On a cautionary note, when TGN1412, a mono-specific 'superagonistic' CD28 antibody, was injected into six healthy volunteers it induced life-threatening systemic T-cell activation and severe cytokine storm^[139] that raised concerns about the use of immunomodulatory molecules. However, as rM28 is a bispecific molecule, it will not be activated in the absence of target cells, in contrast to the systemic T-cell activation seen with TGN1412 in the absence of target cells.

In addition, co-stimulation with anti-CD3/anti-CD28 and targeting with BiAb has been reported and may be critical to obtaining long-term memory responses. Co-stimulation with anti-CD28 alone or anti-CD28×anti-TAA induces enhanced signaling,^[140,141] cytokine synthesis,^[142] enhanced killing in leukemia/lymphoma models,^[95,98,105] and cytotoxicity in colon cell lines.^[143] It has also been reported that BiAb and CD28 co-stimulation induces Th1 differentiation.^[143] However, it remains unclear whether co-stimulation correlates with more effective long-term clinically relevant immune responses.

6.2 T Cells Expressing Chimeric Antibody Receptors

T cells have been transduced with genes that express chimeric scFv domain receptors, creating T cells with chimeric antibody receptors (CARs) [i.e. 'T-bodies'] to deliver lethal hits to tumors.^[144-149] Earlier constructs using retroviral vectors have been replaced with lentiviral vectors that have better transduction efficiency. Most CARs include heavy and

light chain-derived variable regions connected by peptide linkers and activating signaling chains that consist of a gamma chain and CD28 signaling chains.^[150] Although early phase I clinical trials using T-bodies were not encouraging,^[151] recent studies involving the addition of a CD28 signaling chain to the gamma or zeta chain indicate that this approach may increase the function and survival of CARs in the body. Constructs have been made to target CD19,^[152] CEA,^[153] and prostate-specific membrane antigen^[154] and have shown encouraging clinical responses.

6.3 Immunologic Space

Both preclinical and clinical studies strongly suggest that depletion of T_{reg} cells or creating immunologic space using cyclophosphamide can lead to improved anti-tumor activity.^[155-158] An early study showed that infusions of purified CD4⁺ cells induced remissions in patients with solid tumors after lymphodepleting cyclophosphamide doses ranging from 500 to 1000 mg/m².^[159]

6.4 Affinity of BiAbs

The affinity, isotype, targeted CD3 epitope, and arming dose of the BiAb may affect signaling, proliferation, cytokine synthesis, and cytotoxicity. Changing the valency may alter the ability of the anti-CD3-based BiAb to induce specific T cell functions. In a series of scFv anti-HER2 constructs with affinities ranging from 10⁻¹¹ to 10⁻⁷, increasing binding affinity led to increasing cytotoxicity.^[160]

6.5 Ex Vivo BiAb-Armed ATC versus BiAb Infusions

Activated and fresh unactivated T cells can be armed with BiAbs to target tumors in a non-MHC-restricted manner.^[161] Fresh T cells in PBMCs can be armed with BiAb, inducing proliferation and cytotoxicity.^[161] Most investigators choose to infuse BiAb instead of performing *ex vivo* expansion of T cells followed by arming with BiAb to induce anti-tumor cytotoxicity. We adopted the *ex vivo* expansion and arming approach to avoid *in vivo* activation of a very large number of endogenous T cells.^[88] Infusing BiAb alone results in substantially more BiAb (micrograms to milligrams per kg) being infused into patients compared with arming ATC with 25–50ng BiAb/million cells. Infusing free BiAb would result in binding to all circulating T cells, tumor targets, and Fc-receptor-bearing cells immediately after the infusion, potentially leading to the development of a cytokine storm. The BiTE format with significantly reduced doses of 0.015 mg/m²/day given as a continuous infusion may have overcome the cytokine storm limitation of infusing BiAb alone.^[75] Obviously, the binding to effector cells and tumor cells will be dependent upon the affinity of each arm of the BiAb construct and the Fc-binding ability of the Fc-portion of the BiAb.

In addition, immobilized BiAb on the surface of T cells, NK cells, monocytes, or neutrophils may resist clearance from the circulation, whereas circulating single-chain antibodies, diabodies, minibodies, leucine-zippered antibodies, or ‘knobs-into-holes’ constructs are cleared more rapidly than effector cells.^[5] The arming concentration of the BiAb to re-activate T cells upon tumor engagement may be critical. Overloading ATC with BiAb may trigger or induce activation-induced cell death.

7. Conclusions

The use of BiAbs for redirecting immune effector cells shows promise. As the understanding of the interactions between cancer stem cells and the cells involved in the inflammatory response to tumors improves, cell- and BiAb-based engineering will enable construction of customized BiAb molecules for optimal targeting of specific tumors. The

key considerations for successful manipulation of immunologic responses are (i) the mode and state of activation of the effector cells; (ii) the binding affinity of the BiAb to the effector cells; (iii) the presence and functional capacity of regulatory or suppressor cells; (iv) the type of BiAb construct (chemically conjugated whole or fragments of antibodies, Fab2, scFv, trifunctional, BiTE etc.); (v) the presence of competing decoy antigens; (vi) tumor antigen modulation after BiAb engagement; (vii) the rate and route of delivering the BiAb alone or as armed effector cells; and (viii) the type of tumor and the overall immunologic state of the patient. All in all, antibody engineering, immunologic approaches and concepts on a single platform offer excitement and promise in targeting cancer.

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Fig. 1.

Fc-receptor binding to immune cells, leading to cytokine storm. Bispecific antibodies (BiAbs) alone can bind to Fc R on natural killer (NK) cells, mast cells, and monocytes and induce the release of cytokines/chemokines leading to the cytokine storm. The figure shows how the interaction between the Fc-portions on a BiAb can occur via available Fc-receptors on NK cells, mast cells, and monocytes.

Fig. 2.

In situ immunization of the endogenous immune system. Armed, activated T cells engage the tumor by targeting tumor-associated antigens (TAAs) on the tumor. The targeting process induces T helper-1 (T_h1) cytokine secretion and the release of TAAs from the tumor. Differentiated dendritic cells (DCs), which are induced by interleukin (IL)-12 produced by monocytes, process the released TAAs and present them to endogenous naïve T cells recruited by chemokine release, leading to local *in situ* immunization that becomes systemic immunization.

Table I

Clinical trials using bispecific antibodies (BiAbs)

Targets	BiAb	INNs/trade names (company)	Effector cells	Clinical outcome	References
Glomas	Anti-CD3×anti-glioma		LAK	DLTs	36
Ovarian carcinoma	Anti-OCAA×anti-CD3	OC/TR	T cells	DLTs	37
NHL	Anti-CD3×anti-CD19	SHR-1	T cells	No clear clinical responses	38
Lung cancer, renal cell cancer	Anti-CD3×anti-EGP-2	BIS-1	T cells	DLTs	39
Hodgkin's disease	Anti-CD16×anti-CD30	HRS-3/A9	NK cells	No clear-cut dose side effect or dose response	40
AML	CD64×CD33	251-22	Monocyte/macrophage	DLTs	41
Breast and prostate cancer	Anti-CD64×anti-HER2	MDX-210; MDX-H210	Monocyte/macrophage	DLTs	42,43
Malignant ascites in ovarian, gastric, colon, breast	Anti-CD3×anti-EpCAM	Removab® (Trion Pharma)	T cells	Objective clinical responses (market approved 2009)	44
Gastric, lung, colorectal carcinomas	Anti-CD3×anti-EpCAM	Removab® (Trion Pharma)	T cells	Objective clinical responses	45
HER2+ tumors	Anti-CD16×anti-HER2	2B1	NK cells	DLTs/cytokine storm	46,47
Solid tumors	Anti-CD64×anti-EGFR	MDX-447	Monocyte/macrophage	DLTs/cytokine storm	48
NHL	Anti-CD3×anti-CD20	CD20BiAb	T cells	Phase I	49
Metastatic breast cancer	Anti-CD3×anti-HER2	HER2BiAb	T cells	Phase II	50
Ovarian carcinoma	Anti-CD3×anti-EpCAM	Removab (Trion Pharma)	T cells	Phase II	51
Metastatic breast cancer	Anti-CD3×anti-HER2	Ertumaxomab (Trion Pharma)	T cells	Phase I/II	52
Metastatic melanoma	Anti-CD28×MAPG	rM28	T cells	Phase I/II	53
NHL	Anti-CD3×anti-CD19	Blinatumomab (Micromet)	T cells	Phase I/II	54
B-ALL	Anti-CD3×anti-CD19	Blinatumomab (Micromet)	T cells	Phase I/II	55
Relapsed/refractory B-ALL	Anti-CD3×anti-CD19	Blinatumomab (Micromet)	T cells	Phase II	55

ALL=acute lymphocytic leukemia; AML=acute myelogenous leukemia; B-ALL= precursor B-cell ALL; DLTs =dose-limiting toxicities; EGFR=epidermal growth factor receptor; EGP2= epithelial glycoprotein 2; EpCAM= epithelial cell adhesion molecule; HER2=human EGFR-2; INN = international non-proprietary name; LAK =lymphokine-activated killer cells; MAPG= melanoma-associated proteoglycan; NHL= non-Hodgkin's lymphoma; NK cells=natural killer cells; OCAA= ovarian cancer-associated antigen.

Table II
Phase I clinical trials using the bispecific antibody MDX-H210 (anti-CD64 ×anti-HER2)

Indication (no. of pts)	Dosage	Clinical outcome	Adverse effects	References
Refractory breast and ovarian cancer (10)	0.35 to 18mg/m ²	1 PR and 1 mixed response	'Flu-like' symptoms, chest pain, dyspnea, fever, chills, myalgias, fatigue, hypotension	2,62
Prostate cancer (6)	1 to 8mg/m ²	5 of 6 pts had stable PSA levels for over 40 days and decreases in HER2 levels	'Flu-like' symptoms, chest pain, dyspnea, fever, chills, myalgias, fatigue, hypotension	3,63
Advanced prostate cancer (25)	5 µg/kg/day 4 days/week for 6 weeks	20 of 25 pts had >50% decrease in PSA levels (median duration 128 days)	Therapy stopped in 2 pts who developed heart failure, dyspnea, and an allergic reaction	4,64
Prostate cancer (13)	1 to 20mg/m ² , in combination with GM-CSF	Of 11 evaluable pts, 1 near-PR, 6 SD, 3 PD	5 of 11 pts developed HAMAs	65

EGFR=epidermal growth factor receptor; GM-CSF=granulocyte-macrophage colony-stimulating factor; HAMAs=human anti-mouse antibodies; HER2= human EGFR-2; PD =progressive disease; PR = partial response; PSA =prostate-specific antigen; pts= patients; SD= stable disease.

Table III
Preclinical studies of anti-CD3× anti-tumor-associated antigen bispecific antibodies (BiAbs)

Tumor antigen	Year	BiAb	Tumor	References
AMOC-31	1993	Anti-CD3×anti-AMOC-31	Carcinomas expressing the 40kDa membrane-bound glycoprotein AMOC-31	94
B-cell idiotype	1996	Anti-CD3 ×anti-idiotype	Idiotype on BCL1 lymphoma in Balb/c mice	95
CA125	2006	Anti-CD3×anti-CA125	Ovarian carcinomas	90
CA19-9	1997	Anti-CD3 ×anti-CA19-9	CA19-9	96
CD13	1993	Anti-CD3×anti-CD13	CD13 ⁺ acute myeloid leukemia	97
CD19	1993	Anti-CD3×anti-CD19	Leukemic B cells	98,99
CD19	1992	Anti-CD3×anti-CD19	Malignant B cells	98,100-103
CD20	2005	Anti-CD3×anti-CD20	NHL	104
CD20	2005	Anti-CD3×anti-CD20	Multiple myeloma	104
CD30	1993	Anti-CD3×anti-CD30	Hodgkin's lymphoma	105-107
CEA	1997	Anti-CD3×anti-CEA	Human CEA-expressing cells	108
EGFR	1995	Anti-CD3×anti-EGFR	Glioma, neoplastic keratinocytes	109
EGFR	2006	Anti-CD3×anti-EGFR	Colon, head and neck, and lung	110
EpCAM	2001	Anti-CD3×anti-EpCAM	Adenocarcinomas expressing EpCAM	111
G250	1996	Anti-CD3×anti-G250	Renal cell carcinoma	112
Glioma	1990	Anti-CD3 ×anti-glioma	Human glioma	113
HER-2/neu (HER2)	1992	Anti-CD3×anti-HER2	HER2 receptor-expressing renal cell, colon, breast, and prostate carcinomas	88,89,114-118
HLA-DR beta chains	2001	Anti-CD3×anti-HLA-DR beta chains	Malignant B cells	119
kDa K29	1994	Anti-CD3 ×anti-kDaK29	Renal cell carcinoma	120
MUC1	1939	Anti-CD3×anti-MUC1	Bile duct carcinoma	121
PSA	2000	Anti-CD3×anti-PSA	PSA-expressing prostate carcinomas	122
Tenascin	1995	Anti-CD3 ×anti-tenascin	Human glioma	123
Transferrin receptors	1996	Anti-CD3 ×anti-transferrin receptor	Tumors expressing transferrin receptors	124
Tumor (F[ab] ₂)	1995	Anti-CD3 ×anti-tumor F(ab) ₂	For retargeting TIL	125

CEA=carcinoembryonic antigen; EGFR=epidermal growth factor receptor; EpCAM=epithelial cell adhesion molecule; HER2=human EGFR-2; HLA= human leukocyte antigen; NHL=non-Hodgkin's lymphoma; PSA=prostate-specific antigen; TIL=tumor-infiltrating lymphocytes.