Acute myeloid leukemia targets for bispecific antibodies

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Despite substantial gains in our understanding of the genomics of acute myelogenous leukemia (AML), patient survival remains unsatisfactory especially among the older age group. T cell-based therapy of lymphoblastic leukemia is rapidly advancing; however, its application in AML is still lagging behind. Bispecific antibodies can redirect polyclonal effector cells to engage chosen targets on leukemia blasts. When the effector cells are natural-killer cells, both antibody-dependent and antibody-independent mechanisms could be exploited. When the effectors are T cells, direct tumor cytotoxicity can be engaged followed by a potential vaccination effect. In this review, we summarize the AML-associated tumor targets and the bispecific antibodies that have been studied. The potentials and limitations of each of these systems will be discussed.

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INTRODUCTION

Acute myeloid leukemia (AML), characterized by the infiltration of bone marrow, blood, and other tissues by malignant cells of the myeloid lineage is the most common acute hematologic malignancy of adults. In patients diagnosed before 60 years of age, AML is curable in 35–40% of cases, whereas only 5–15% of those presenting later in life can be cured.1 The treatment of AML that has remained essentially unchanged over the last three decades consists of intensive induction therapy followed by hematopoietic stem cell transplantation (HSCT). Many novel therapeutic agents, both small molecules targeting signaling pathways and immunologics are actively being investigated as salvage therapies or as alternatives to the standard of care. One class of immunotherapeutic agents is that of bispecific antibodies.

Bispecific antibodies combine the binding specificities and biologic functions of two antibodies into one molecule, one for a tumor-associated surface antigen, and the other for a surface antigen on the effector cells, such as T cells or natural-killer (NK) cells. Through the dual specificities, tumor cells are brought into close proximity to the effectors. Most importantly, if binding to the second specificity is agonistic, the cytotoxic functions of effectors can be activated at close proximity to the leukemic cells. Various combinations of whole antibodies and their fragments have yielded more than 60 different formats of such AML bispecific antibodies (examples in Figure 1).2 The immunologic properties and clinical potentials of each of these AML-associated targets are summarized in Table 1. Besides, a list of clinical trials investigating bispecific antibodies in myeloid leukemia is mentioned in Table 2. Characteristics of the bispecific antibodies (molecular weight, affinity, EC50 and parental clone) are summarized in Table 3.

In this review, we summarize those AML targets for which bispecific antibodies have been developed, in descending order of clinical relevance: CD33, CD123, Wilms’ tumor protein (WT1), CD13, CD15, CD30, CD45, CD47, C-type lectin-like molecule 1 (CLL1), Fms-like tyrosine kinase 3 (FLT-3) and angiogenic growth factors.

CD33

CD33 or Siglec-3, a 67-kDa glycoprotein, is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family, which in turn belongs to the immunoglobulin superfamily. The extracellular domain of CD33 comprises one amino-terminal sialic acid-binding V-type and one C2-type immunoglobulin-like domain connected by a helical transmembrane sequence to a cytoplasmic tail containing two immunoreceptor tyrosine-based inhibitor motifs (ITIM). Phosphorylation of the tyrosine residues by the Src family kinases is involved in the recruitment and activation of the Src homology-2 (SH2) domain-containing tyrosine phosphatases SHP1 and SHP2.3

CD33 is a receptor that appears during commitment of the hematopoietic stem cell to the myelomonocytic lineage. It is expressed on myeloid progenitors, monocytes, myeloid dendritic cells and less so, on macrophages and granulocytes.3 Although it is restricted mainly to the myeloid lineage, low levels of CD33 expression has been reported on some lymphoid cells, including the earliest precursors of human fetal thymocytes and human CD34(+) postnatal thymocytes.4 In addition, activated NK cells and T cells can also express CD33.5 CD33 is expressed on the majority of AML cells and the level of CD33 seems to correlate with the disease prognosis.5

The first bispecific T-cell engager (BiTE) developed against CD33 is AMG 330 (Amgen Inc., Thousand Oaks, CA, USA), which binds to a linear epitope containing the amino acid sequence IPYYDKN in the CD33 V-type domain.7–9 This BiTE activates and expands T cells from autologous clinical samples of patients with AML and mediates in vitro lysis of primary AML and normal myeloid cells in a dose dependent manner at concentrations as low as 1 ng/ml (EC50 = 0.35–2.7 pm).8,10 However, it is noteworthy that activated T and NK cells can also express CD33.5 CD33-independent activation with an anti-CD19 BiTE led to CD33 neo-expression on a minor subset (< 6%) of T cells, associated with their fratricide but with minimal effect on AML-directed cytotoxic T-cell function.9 Importantly, soluble CD33 found in the blood of some patients with AML does not affect the AMG 330-mediated T-cell activation or cytotoxicity.9 Antibody-mediated endocytosis of antigens could...
reduce the availability of cell surface targets for antibody therapy. In contrast to bivalent anti-CD33 IgG antibodies, AMG 330 is not endocytosed and does not modulate surface expression of CD33. In addition, the function of this BiTE is neither affected by common CD33 single nucleotide polymorphisms nor by the adenosine triphosphate-binding cassette transporter activity. Although the majority of primary AML cells do not express PD-L1, it can be upregulated by the proinflammatory pathway. The addition of anti-PD-1/PD-L1 antibodies restored T-cell cytokine release during the activation of T-cells by AMG 330.

To better understand the immunobiology of AMG 330 BiTE, human leukemic cell lines were engineered to express the inhibitory (PD-L1 and PD-L2) or activating (CD80 and CD86) ligands to interact with their respective receptors (that is, CD28 and PD-1). It was found that the expression of PD-L1 and PD-L2 inhibitory molecules on target cells reduced the in vitro cytotoxicity of AMG 330 BiTE in the presence of healthy donor T cells at low effectortarget (E:T) ratios. This inhibition correlated with the level of ligand expression on target cells. On the other hand, CD80 and CD86 activating molecules expressed on tumor cells increased the cytolytic activity of AMG 330. Treatment with anti-CD28 activating antibodies enhanced the cytotoxicity of AMG 330 compared with samples taken from patients at the time of first diagnosis. These data suggest the existence of possible resistance mechanisms among AML blasts.

A second bispecific antibody format against CD3 and CD33 attaches the VH of an anti-CD3 to the VL of an anti-CD33 antibody, whereas connecting the VH of the anti-CD33 to the VL of the anti-CD3 antibody, with the two tandem single-chain Fv fragments (scFv) joined by a glycine-serine (G4S)5 linker. The resulting construct was initially complicated by the formation of fragmented nonfunctional proteins because of DNA recombination triggered by sequence homology. This problem was resolved by rearranging the variable regions in a way such that the variable heavy (VH) of CD33 and VH of CD3 were linked to their cognate variable light (VL) regions. In addition, the (G4S)5 linker was replaced by a new helical sequence (amino acid sequence: EKEALKKIIEDQQESLNK) in joining the CD33 and CD3 binding moieties. The newly generated CD33 × CD3 single-chain bispecific tandem fragment variable (scBsTaFv) construct was able to lyse CD33-expressing target cells in the presence of peripheral blood mononuclear cells (PBMC) obtained from healthy donors. When a green fluorescent protein was incorporated into the bispecific antibody, the formation of immunological synapse between T cells and target cells was visualized. When the CD33-CD3 scBsTaFv bispecific antibody was further humanized, it
Table 1. Advantages and disadvantages of AML-associated antigens for antibody development (for a cartoon representation of each bispecific antibody format, please see Figure 1)

| Antigen | Advantages | Disadvantages | Bispecific formats |
|---------|------------|---------------|--------------------|
| CD33 | Expressed on the majority of myeloid blasts and leukemia stem cells (LSC)\(^{3,17}\) | Not expressed on all myeloid blasts or myeloid stem cells\(^{117}\) | Tandem double and triple scFv (BiTE, ScBsTaFv, bsscFv, TandAb, BtIKe, TrIKe, sctb), chemical conjugates |
| | Not expressed or expressed at lower levels on normal HSCs\(^{3,17}\) | Expressed on HSCs in some studies\(^{121}\) | |
| | High CD33 expression is associated with high-risk mutations and inversely associated with low-risk mutations\(^{3,139}\) | Expressed on activated natural killer (NK) cells and T cells\(^{5,9}\) | |
| | High CD33 expression correlated with inferior disease features and outcome\(^{19}\) | Modulation (decreased surface expression) of surface CD33 expression upon bivalent anti-CD33 antibody treatment\(^{16,122}\) | |
| | Expressed on immunosuppressive myeloid derived suppressor cells that are associated with extramedullary infiltration and detection of minimal residual disease\(^{120}\) | Circulating CD33 might interfere with the anti-CD33 antibodies\(^{3}\) | |
| | Expresses on peripheral blood monocytes, dendritic cells and granulocytes\(^{142}\) | Treatment-related myelosuppression (neutropenia and thrombocytopenia)\(^{124}\) | |
| | Related low abundance on cell surface | Relatively low abundance on cell surface\(^{125}\) | |
| CD123 | Expressed on the majority of myeloid blasts and LSCs\(^{44,129}\) | Not expressed on myeloid blasts or myeloid stem cells\(^{49}\) | Tandem double and triple scFv (sctb), DART, BtIF |
| | Not expressed or expressed at lower levels on normal HSCs\(^{38,121}\) | A chimeric antigen receptor that was made based on CD123 antibody significantly reduced B cells, platelets and myeloid cells in an animal model\(^{128}\) | |
| | Absent on T cells\(^{127}\) | | |
| | Associated with lower complete remission and poorer survival rates\(^{39}\) | | |
| | Associates with higher proliferation and more resistance to apoptosis of AML cells\(^{19}\) | | |
| WT1 | Expressed on the majority of myeloid blasts and LSCs\(^{44,129}\) | HLA-restricted expression of WT1 limits the application of each anti-WT1 antibody to one special HLA haplotype\(^{130}\) | Tandem double scFv (BITE) |
| | Not expressed or expressed at extremely low levels in a small population of CD34(+)cells in bone marrow\(^{29}\) | Not expressed on all myeloid blasts or myeloid stem cells\(^{129}\) | Chemical conjugation (Fab' fragments) |
| | Higher WT1 gene expression is associated with lower complete remission and decreased survival\(^{45,130}\) | Low cell surface density of HLA-WT1-peptide complexes\(^{46}\) | |
| | Expressed on peripheral blood monocytes, dendritic cells and granulocytes\(^{142}\) | Expressed in normal tissues\(^{131}\) | |
| | Anti-CD13 monoclonal antibodies can induce apoptosis in AML cells | Expressed in normal tissues and cells including monocytes, granulocytes, capillary endothelium, nephron convoluted tubules, bile ducts, pancreas, skin, small intestine and liver\(^{133}\) | |
| CD13 | Expressed on the majority of myeloid blasts and LSCs\(^{132}\) | Not expressed on all myeloid blasts\(^{132}\) | Chemical conjugation (Fab conjugated with whole IgM) |
| | Expressed at higher levels on AML stem cells than on normal HSCs | Not expressed on HSCs\(^{121}\) | |
| | Anti-CD13 monoclonal antibodies can induce apoptosis in AML cells | Not expressed on HSCs\(^{121}\) | |
| CD15 | Expressed on the majority of AML cells\(^{134}\) | Not expressed on HSCs\(^{121}\) | T and Ab |
| | Expressed on some NK cells, T cells, monocytes, neutrophils, eosinophils and neurons\(^{32,135}\) | Expressed on some NK cells, T cells, monocytes, neutrophils, eosinophils and neurons\(^{32,135}\) | |
| | Not expressed in more than 50% of activated T cells\(^{136}\) | Not expressed on activated T cells\(^{55}\) | |
| | Not secreted upon cleavage of the extracellular domain\(^{98,59}\) | Not secreted upon cleavage of the extracellular domain\(^{98,59}\) | |
| CD45 | Not internalized upon antibody ligation\(^{139}\) | Not expressed on all LSCs\(^{141}\) | Chemical conjugation (Fab fragments) |
| | Expressed on most AML cells\(^{140}\) | Expressed on all hematopoietic cells except mature red blood cells and platelets\(^{51}\) | |
| | Not expressed outside hematopoietic system | | |
| CD47 | Is a universal target in human cancers CD47 upregulation on leukemic cells allows them to evade macrophage killing\(^{69}\) | Expressed on the majority of normal tissues\(^{63}\) | DVD-Ig |
| | Overexpressed on AML stem cells than on their normal hematopoietic counterparts\(^{65}\) | Expressed on normal tissues\(^{63}\) | |
| | Overexpression of CD47 on AML cells is associated with shortened survival\(^{65}\) | CD47 expression on normal tissues may generate an antigen sink preventing the therapeutic antibody to reach its target on AML cells\(^{66}\) | |
| CLL1 | Expressed on the majority of myeloid blasts and LSCs\(^{142}\) | Expressed on peripheral blood monocytes, dendritic cells and granulocytes\(^{142}\) | Chemical conjugation (Fab fragments) |
| | Not expressed on normal tissues\(^{142}\) | Relatively low abundance on cell surface\(^{142}\) | |
| | Not expressed on normal hematopoietic stem cells\(^{51,142}\) | Antigen modulation upon ligation with anti-CLL1 antibody\(^{142}\) | |
| | | Not expressed on all AML cells\(^{142}\) | |
Table 1. (Continued)

| Antigen             | Advantages                                                                 | Disadvantages                                                                 | Bispecific formats                  |
|---------------------|----------------------------------------------------------------------------|------------------------------------------------------------------------------|-------------------------------------|
| FLT-3               | 143.19 Expression on the majority of AML samples                          | 212.29 Not expressed on all AML cells                                          | Tandem double scFv (BiTE), Fabsc     |
| VEGF-A, Ang-2       | 144.19 VEGF-A and Ang-2 are overexpressed on the                          | 144.19 Not expressed on all AML cells                                          | CrossMab, chemical conjugation       |
|                     | 143.19 Expression on LSCs is higher than on normal hematopoietic cells     | 144.19 Are secreted from the cells so T-cell responses cannot be redirected    |                                     |
|                     | 143.19 Expression on LSCs is higher than on normal hematopoietic cells     | 144.19 Are secreted from the cells so T-cell responses cannot be redirected    |                                     |
|                     | 144.19 Anti-angiogenic therapy might control disease                      | 144.19 Are secreted from the cells so T-cell responses cannot be redirected    |                                     |
|                     | 144.19 Expression on VEGF-A and Ang-2 on AML is                           | 144.19 Are secreted from the cells so T-cell responses cannot be redirected    |                                     |
|                     | associated with negative outcome                                          | 144.19 Are secreted from the cells so T-cell responses cannot be redirected    |                                     |

retained its ability to arm T cells to kill AML blasts at picomolar concentrations (EC50 = 15 pm). When tested by

forming assays, the multilineage reconstitution potential of hematopoietic stem cells treated with the humanized antibody

was not compromised. To further improve the potency of this bispecific antibody, 4-1BB ligand (4-1BBL) co-stimulatory molecule

was incorporated into the construct in a modular manner. The novel modular system outperformed the original humanized

CD33 × CD3 scBsTaFv in killing CD33low AML cells at low E:T ratios in vitro. One obstacle in the use of these small-size bispecific antibody fragments is their rapid clearance through kidney, necessitating their continuous infusion for up to several weeks. To tackle this problem, mesenchymal stromal cells (MSC) were engineered to

continuously secrete the humanized CD33-CD3 scBsTaFv, and to express 4-1BBL for T-cell co-stimulation. T cells, AML cells and

scBsTaFv-engineered MSC cells were co-cultured for 24 h before injection into immunodeficient mice. Results showed that

antibody-engineered MSC cells could redirect T cells toward AML cells and extend the survival of leukemic mice. Further

experiments will be necessary to assess whether this therapeutic modality can target neoplastic cells in established leukemia animal models, and to determine the optimal delivery method for the engineered MSCs, as well as to investigate if MSCs can home to tumor sites and activate T cells without inducing immunosuppressive effects.

Recently, a panel of bispecific tandem diabodies (TandAb) specific for CD3 and CD33 have been generated of which one (AMV-564) was chosen for further clinical development. The AML lysis mediated by this construct in the presence of T cells depended on antibody dose and E:T ratio. Furthermore, the TandAb-induced cytoxicity was similar against AML samples from newly-diagnosed patients, or from those with refractory/relapsed leukemia. No correlation was found between the suppressive properties, and are significant in patients

with MDS.

A further improvement of BiKE attempted to improve in vivo expansion of NK cells. Instead of using interleukin-2 (IL2), which induces regulatory T cells (Tregs) that suppress NK-cell function, interleukin-15 (IL15) can be used to enhance NK-cell homeostasis in a trispecific killer-cell engager (TriKE). Here, the human IL15 sequence, flanked by two linkers, was inserted between the scFvs of the CD16 × CD33 BiKE. The IL15 TriKE induced similar NK-cell mediated killing when compared with a mixture of IL15 and BiKE. In a murine AML xenograft model using human NK cells, TriKE was superior to BiKE. Instead of the IL15 sequence, a third scFv can be inserted to create a triple scFv antibody. Two anti-CD33 scFv fragments attached in tandem via a flexible 20-amino acid linker (Gly4Ser6) to a single anti-CD16 scFv (sctb: single-chain Fv triplebody) was compared with the single anti-CD33 scFv construct (bsscFv: bispecific single-chain Fv). CD33 avidity of the scFv was 3.5 fold higher when compared with that of the bsscFv, even though their affinity was comparable. Both antibody constructs in the presence of human PBMC mediated lysis of CD33(+) AML cell lines with EC50 values in picomolar ranges (212–426 pm for bsscFv, 1.8–18 pm for
scTB). Importantly, the scTB was 10–200-fold more potent than the bsFcFv in killing human leukemic cell lines. The logic of this enhancement was extended to targeting more than one tumor antigen on the surface of cancer cells. For acute leukemia with mixed-lineage phenotypes, a scTB with dual target specificities (anti-CD33 scFv and anti-CD19 scFv) was attached to an anti-CD16 scFv. Compared with the CD33 × CD16 or CD19 × CD16 bsFcFv, the CD33 × CD16 × CD19 scTB redirected cytotoxicity of MNCs against a double-positive cell line at 23 and 1.4 fold lower concentrations, respectively.

In another series of experiments three scTB antibodies were generated. Two of the scTB antibodies targeted a single antigen on leukemia cells (either CD33 or CD123), a BiFab antibody was generated by conjugating the antigen-binding fragments (Fab) of two antibodies using bio-orthogonal chemical linkers.

### Table 2. Clinical trials involving bispecific antibodies in AML

| Name                        | Format          | Phase | Endpoint                        | Sponsor                                           | Study population          | Status             |
|-----------------------------|-----------------|-------|---------------------------------|---------------------------------------------------|---------------------------|--------------------|
| JNJ-63709178                | CD123 × CD3 DuoBody | 1     | Safety and efficacy             | Janssen Research & Development, LLC               | Relapsed/refractory AML   | Suspended         |
| AMG 330                     | CD33 × CD3 Tandem scFv (BiTE) | 1     | Safety                          | Amgen                                             | Relapsed/refractory AML   | Suspended         |
| MGD006                      | CD123xCD3 DART   | 1     | Safety                          | MacroGenics                                       | Relapsed/refractory AML, MDS | Recruiting        |

Abbreviations: AML, acute myeloid leukemia; BiTE, bispecific T-cell engager; DART, dual-affinity re-targeting; MDS, myelodysplastic syndrome. DuoBody (Genmab, Copenhagen, Denmark) is the commercialized name of the controlled Fab-arm exchange (cFAE) platform to generate IgG1 monovalent bispecific antibodies.

### Table 3. Characteristics of bispecific antibodies generated for AML

| MW (kDa) | Affinity (Kd) | EC50 pM | Clone          | Effector (CD3 and so on) | Target (CD33 and so on) | Parent clone | Bispecific format |
|----------|---------------|--------|----------------|--------------------------|------------------------|--------------|-------------------|
| CD33 × CD3 BITE AMG 330       | 55     | 5.1    | —              | 8                        | 0.4–3                  | —            | DRB2 (CD33)       |
| CD33 × CD16 chemically conjugated | 60     | —      | 0.1            | 10–100                   | 15                     | 251 (CD33), 3G8 (CD16) |
| CD16 × 33 BITE                | 55     | 20     | (scFv)         | —                        | —                      | —            | NM3E2 (CD16)      |
| CD33 × CD16 × CD33 scTB²⁹     | 90     | 45.1 ± 4.3 | —            | 7.9 ± 1.1                 | 1.8–18                | K132 (CD33), 3G8 (CD16) |
| CD33 × CD16 × CD19 scTB³⁰     | 90     | 49.2 ± 5.1 | —            | CD33 (29.1 ± 1.9)         | 7.2 ± 2               | K132 (CD33), 3G8 (CD16) |
| CD123 × CD16 × CD33 scTB⁵¹    | 90     | 21.7 ± 1.8 | —            | CD33 (17.8 ± 2.2)         | 21–118                | K132 (CD33), 3G8 (CD16), CD123 (from phage)⁴¹ |
| CD123 × CD3 BiTE⁵¹,¹⁵²         | 140    | 10     | 0.01–0.05      | 0.1                      | 10                    | 12F1 (CD123), UCHT1 (CD3) |
| CD123 × CD123 DART⁶²          | 59     | 9 ± 2.3 | —              | 0.13 ± 0.01              | 0.17                  | 7G3 (CD123)    |
| CD123 × CD16 bsFcFv²⁴         | 60     | 49.5 ± 5 | —              | 4.5–101                  | 211–364              | 3G8 (CD16), CD123 (from phage)⁴¹ |
| CD15 × CD66 chemically        | —      | —      | 0.1            | —                        | —                      | —            | PM81 (CD15), 32 (CD64) |
| conjugated²⁴                 |        |        |                | —                        | —                      | —            |                   |
| CD33 × CD64 chemically        | —      | —      | 0.1            | —                        | —                      | —            | H22 (CD64), 251 (CD33) |
| conjugated²⁴                 |        |        |                | —                        | —                      | —            |                   |
| CD16 × CD33 chemically        | —      | —      | 0.1            | —                        | —                      | —            | 3G8 (CD16), 251 (CD33) |
| conjugated³³                 |        |        |                | —                        | —                      | —            |                   |
| CD3 × CD33 modular re-targeting system¹⁹ | — | — | 0.1 | — | — | MT-301 (CD3), DRB2 (CD33) |
| CD33 × CD3 BiFab⁶⁸            | ~100   | —      | 7              | Similar to their parental clones | 25–445               | Mutated hM195 (CD33), and UCHT1 (CD3) |
| anti-CD3 Fab³ anti-CD13 Fab   | 100–110 | —      | —              | —                        | —                      | —            | OKT3 (CD3), My7 (CD13) |
| chemically conjugated³¹       |        |        |                | —                        | —                      | —            |                   |
| CD30 × CD16A TandAb⁶⁰         | 105–110 | 0.39   | 17.2           | 9.3                      | 35,800                | LSV21 (CD16), HRS-3 (CD30) |
| CD30 × CD16A bispecific diabody⁶⁰ | 60     | 35     | 17.2           | 751                      | 194,700               | LSV21 (CD16), HRS-3 (CD30) |
| WT1 × CD3 BITE⁴⁶,⁴⁷           | —      | —      | —              | —                        | 0.2                   | —            | ESK1 (WT1), L2K (CD3) |
| CD20 × CD47 DVD-Ig            | —      | —      | CD47 (3.1)     | CD47<sub>mut</sub> (48–60) | 2.88                  | —            | CD20 (CD20), B6H12.2 (CD47) |
| CLL1 × CD3 BiFab⁴⁶⁸           | ~100   | —      | 6.1            | Similar to their parental clones | 2.1–41               | —            | Mutated 107.5 (CLLI), and UCHT1 (CD3) |
| FLT-3 × CD3 BITE⁷¹            | —      | —      | —              | —                        | —                      | —            | 4G8 (FLT-3), UCHT1 (CD3), OKT3 (CD3), BMA031 (CD3) |
| FLT-3 × CD3 Fabsc¹⁷           | 87     | —      | —              | —                        | —                      | —            |                   |
| VEGF × Ang-2 CrossMab⁸⁰,⁸¹     | —      | —      | Bevacizumab (< 0.1) | Bevacizumab (Ang-2) (0.2) | —                      | —            | Bevacizumab = humanized A.4.6.1 (VEGF) and LC06 (Ang-2) |

*Bifab antibodies were generated by conjugating the antigen-binding fragments (Fab) of two antibodies using bio-orthogonal chemical linkers.
whereas the third scFv targeted both leukemia antigens. Although both single and double antigen formats were able to mediate lysis of AML targets in the presence of PBMC, the latter was more potent, showing the advantage of simultaneous targeting of more than one tumor antigen.31

CD64 (FcγRII), which is expressed on monocytes, macrophages and neutrophils, has an important role in antibody-dependent cell mediated cytotoxicity (ADCC). A bispeci antibody was generated by chemical conjugation of CD33 and CD64 and was able to redirect cytotoxic CD33+ monocytes toward CD64+ AML cells. IFNγ, granulocyte-colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) increased the expression of CD64 on monocytes and enhanced their cytotoxic potential.32 Treatment of target cells with IFNγ also increased their killing by the bispeci antibody.33 Although the treatment of AML cells with monospecific anti-CD33 or anti-CD64 antibodies for 48 h inhibited AML cell growth, the inhibitory potency mediated by the bispeci molecule was higher. Furthermore, the bispeci antibody augmented the anti-tumor effects of monocytes in a murine tumor model.34

CD123

CD123 is the alpha subunit of the interleukin-3 (IL-3) receptor. This 75 kDa single-pass type-I membrane protein contains three extracellular, one transmembrane and one intracellular domain. It is expressed on monocytes, B cells, megakaryocytes, plasmacytoid dendritic cells, and hematopoietic stem and progenitor cells. CD123 expression in B lymphoid and myeloid progenitors is high, whereas erythroid progenitors and primitive hematopoietic cells express either low or undetectable levels.36 Overexpression of CD123 can promote cell proliferation and may induce leukemogenesis.37 CD123 was once thought to be an AML stem cell marker.38 AML cells with high CD123 expression had higher proliferation and were more resistant to apoptosis induced by growth factor deprivation. Higher expression level of CD123 among AML blasts is associated with lower complete remission rates and poorer overall survival.39

To redirect T cells toward CD123 leukemic cells, the scFv of an anti-CD123 antibody (CD123scFv) was fused to the N terminus of the truncated human IgG1-Fc (CH2-CH3) connected in turn to an anti-CD3 scFv (CD3 scFv) at its C terminus, a platform named bispeci scFv immunofusion (Bif). The C-terminal location of the CD3 scFv reduced its affinity for CD3+ T cells by two orders of magnitude. The Bif was able to mediate lysis of CD33(+)/CD123+ target cells by T cells at low picomolar EC50 (EC50 = 8 pm).40 Another bispeci tandem scFv construct was built comprising the scFv fragments of anti-CD123 and anti-CD16 antibodies. The bispeci construct at picomolar concentrations (EC50 = 211–364 pm) trig-gered the lysis of leukemic cells in the presence of PBMC.41 As mentioned earlier, a CD16 scFv-based scb against CD123 demonstrated potent anti-leukemic activity, but inferior to a similar but double-targeting scbt simultaneously targeting CD33 and CD123.42

A dual-affinity re-targeting (DART) molecule named MGD006 (MacroGenics, Rockville, MD, USA), was built using two independent chains, one consisting of the humanized VL of anti-CD3 attached in tandem to the VH of anti-CD123, and the other consisting of the VL of anti-CD13 attached to the VH of anti-CD3 antibody. The two polypeptides were attached via disulfide bonds between the C terminus of the VH fragments. This DART molecule was able to activate and expand T cells and suppress AML xenografts in mice reconstituted with human PBMC. Because of the small size of the molecule (59 kD), continuous infusion over seven days was necessary. In cynomolgus macaques, MGD006 produced transient IL-6 release and reversible decrease in the red blood cell mass.42 In humanized NSG mice, MGD006 was effective against xenografts derived from a leukemic cell line or from primary AML samples.43 A first in human study is ongoing in AML patients (ClinicalTrials.gov identifier NCT02152956).

WT1

WT1 is a zinc finger transcription factor that has important roles in cell survival and development. Several isoforms of WT1 exist, each with specific inhibitory or activating functions on a variety of important genes and cellular pathways. Mutations in the WT1 gene have been associated with human diseases. In AML, WT1 is considered an oncogene and its expression was found on the majority of AML samples.44 Importantly, higher WT1 gene expression is associated with lower complete remission rates and decreased survival.45 Most therapeutic antibodies recognize intact antigens expressed on the cell surface; however, the majority of leukemia-associated antigens are intracellular. When degraded, their peptides are presented on human leukocyte antigens (HLA) as peptide–MHC (major histocompatibility complex) complexes on the cell surface. Recently, a BiTE construct comprised of the scFv fragments of antibodies specific for CD3 and WT1 epitope RMF in the context of HLA-A*02:01 (WT1-BiTE) was made.46 This BiTE could bind to WT1(+) and HLA-A*02:01(+) AML targets and activate T cells to proliferate and kill the leukemic cells in vitro and in immunodeficient mice. No binding to CD34(+) cells was found. Furthermore, WT1-BiTE therapy induced long-term T-cell responses against tumor-associated epitopes other than WT1 because of epitope spreading, which could greatly enhance the therapeutic efficacy of this bispecific construct. However, cross reactivity of these T-cell receptor (TCR)-like antibodies with other peptides on other HLA antigens could be a major hurdle for clinical development.47

CD13

CD13, also known as aminopeptidase-N or gp150, is a myeloid membrane-bound zinc-dependent metalloprotease with an extracellular enzymatic moiety that cleaves N-terminal amino acid residues from oligopeptides.38 CD13 contains an extracellular domain joined via a helical transmembrane sequence to a small cytoplasmic domain. CD13 is the alpha subunit of the interleukin-3 (IL-3) receptor. This gene have been associated with human diseases. In AML, CD13 is important genes and cellular pathways. Mutations in the WT1 gene have been associated with human diseases. In AML, WT1 is considered an oncogene and its expression was found on the majority of AML samples.44 Importantly, higher WT1 gene expression is associated with lower complete remission rates and decreased survival.45 Most therapeutic antibodies recognize intact antigens expressed on the cell surface; however, the majority of leukemia-associated antigens are intracellular. When degraded, their peptides are presented on human leukocyte antigens (HLA) as peptide–MHC (major histocompatibility complex) complexes on the cell surface. Recently, a BiTE construct comprised of the scFv fragments of antibodies specific for CD3 and WT1 epitope RMF in the context of HLA-A*02:01 (WT1-BiTE) was made.46 This BiTE could bind to WT1(+) and HLA-A*02:01(+) AML targets and activate T cells to proliferate and kill the leukemic cells in vitro and in immunodeficient mice. No binding to CD34(+) cells was found. Furthermore, WT1-BiTE therapy induced long-term T-cell responses against tumor-associated epitopes other than WT1 because of epitope spreading, which could greatly enhance the therapeutic efficacy of this bispecific construct. However, cross reactivity of these T-cell receptor (TCR)-like antibodies with other peptides on other HLA antigens could be a major hurdle for clinical development.47

CD15

CD15, also called Lewis x antigen, is a carbohydrate expressed on the majority of AML cells and on some NK cells, T cells, monocytes, neutrophils and eosinophils.52 CD15 expression on AML blast was associated with favorable prognosis such as continuous complete remission and longer survival.53 In an attempt to design a bispecific antibody, the fragment of antigen-binding (Fab) of an anti-CD64 antibody was chemically conjugated with whole IgM monoclonal antibody against CD15. This bispecific construct was able to redirect in vitro cytotoxicity of IFNγ-activated monocyes toward CD15(+) AML cells in the presence of human serum as a source of complement. The antibody conjugate was assessed in a phase I clinical trial of four patients with CD15(+) cancer. Six doses of the drug over a period

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of two weeks were administered. In a patient with AML, a 30–60% transient reduction in peripheral blasts was observed when peak serum level of the antibody was as low as 50 ng/mL. No toxicity was reported.\(^5^4\)

**CD30**

CD30, member 8 (TNFRSF8) of the tumor necrosis factor receptor superfamily, is a 120-kDa type-I transmembrane glycoprotein. It is expressed on resting human CD8(+) T cells, activated B and T cells and their leukemias and lymphomas. CD30 has a co-stimulatory, mitogenic and activation effects on T cells.\(^5^5\) CD30 is also expressed with variable intensity on 36\(^6^1\) to 50\(^5^7\)% of patient AML and its expression is associated with FLT-3-ITD mutations and leucocytosis.\(^5^7\) Upon cleavage from the extracellular domain, CD30 is released as a soluble form that can be detected in the blood of patients with Hodgkin and non-Hodgkin lymphoma and it is associated with poorer prognosis.\(^5^8\),\(^5^9\) A TandAb containing two binding sites for CD30 and CD16A has been generated. Unlike an anti-CD30 IgG, the TandAb did not bind to the non-activating CD16B receptor. Furthermore, binding of the TandAb to CD30(+) target cells was higher than that of the anti-CD30 IgG, resulting in improved killing of target cells. Although not tested against AML blasts, this TandAb exhibited cytotoxicity against CD30(+) non-Hodgkin lymphoma cells. In the absence of CD30(+) target cells, TandAb did not activate NK cells,\(^6^0\) an important consideration for the clinical application of such bispecific antibodies.

**CD45**

CD45 is a protein tyrosine phosphatase enzyme expressed in various isoforms. It contains an extracellular sequence connected via a helical transmembrane domain to a cytoplasmic tail. CD45 is expressed on all hematopoietic cells except mature red blood cells and platelets.\(^6^1\) A recombinant bispecific antibody was generated comprising a scFv against the radiometal complex with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, a metal chelating agent) and a CD45 binding moiety containing the light chain of an anti-CD45 antibody and the heavy chain of rituximab.\(^6^2\) In vivo targeting was performed in human-AML-bearing mice after injecting the antibody construct, followed by administering a clearing agent after 22 h and finally injecting the yttrium-90-labeled DOTA. Biodistribution studies revealed favorable antibody retention in tumor sites, whereas the normal organs showed minimum uptake. The therapeutic potential of this bispecific agent was not reported.

**CD47**

CD47 is a 50-kDa glycoprotein belonging to the immunoglobulin superfamily. It contains an extracellular IgV domain next to a five-fold-passing transmembrane domain and a short cytoplasmic sequence.\(^6^3\) CD47 binding to signal regulatory protein-\(\alpha\) expressed on dendritic cells and macrophages delivers a signal which inhibits phagocytosis.\(^6^4\) CD47 is a leukemia stem cell (LSC) marker and its overexpression in AML has been associated with poor survival.\(^6^5\) Recently, a bispecific antibody was generated to target CD47 and CD20 to treat lymphoma. Here, the C terminus of the VH and VL of an anti-CD20 antibody were joined by short peptide linkers to the N terminus of the respective chains in the anti-CD47 IgG to generate a bispecific dual-variable-domain immunoglobulin (DVD-Ig) format. Affinity of the DVD-Ig relative to the parental monoclonal antibody for CD47 was diminished 15–20-fold. In comparison to a 12-amino acid linker, DVD-Ig using the shorter 5-amino acid linker showed weaker binding to CD47. Fortunately this lower affinity allowed the 5-amino acid linker form to prefer the double-positive CD47(+)/CD20(+) cells and avoid being trapped in a CD47 antigen sink. The bispecific short-linker construct improved the survival of lymphoma-bearing mice and recapitulated the therapeutic benefit of anti-CD47 and anti-CD20 antibody combination therapy.\(^6^6\)

**CLL1**

CLL1, also known as C-type lectin domain family 12 member A (CLEC12A), is a pan-myeloid antigen containing an extracellular, a transmembrane and a cytoplasmic domain. CLL1 is expressed on myeloid cells and peripheral blood monocytes, dendritic cells and granulocytes. Thanks to its absence on normal hematopoietic stem cells and its expression on LSC, CLL1 was explored as a marker for minimal residual disease in AML.\(^6^7\) To test the effect of targeting CLL1 in AML, the Fab fragments of anti-CLL1 and anti-CD3 antibodies were site-specifically modified using the unnatural amino acid, p-acetylphenylalanine and then conjugated to generate a bispecific Fab (BiFab). Compared with a BiFab against CD33 and CD3 generated using the same methodology, the CLL1-CD3 antibody-mediated stronger in vitro cytotoxicity and superior in vivo anti-tumor activity in immunodeficient mice with established leukemia xenografts receiving expanded T cells.\(^6^8\) Another laboratory also successfully demonstrated the in vitro functionality of a CLL1-CD3 BiTE in the presence of T cells against AML cells.\(^6^9\)

**FLT-3**

FLT-3, also known as CD135, is a receptor-type tyrosine kinase and has an important role in the development of hematopoietic progenitor cells and dendritic cells. FLT-3 contains an extracellular sequence with an Ig-like C2-type domain, a helical transmembrane part and a cytoplasmic domain with protein kinase activity. FLT-3 is expressed on committed lymphoid and myeloid progenitors and on monocytes.\(^7^0\),\(^7^1\) Activating FLT-3 mutations are present in about 30% of patients with AML.\(^7^2\) Furthermore, the level of FLT-3 expression on AML cells is increased and is associated with higher percentage of bone marrow blasts and high leukocyte counts.\(^7^0\),\(^7^3\) Two bispecific antibody formats have been designed to redirect T cells towards FLT-3(+)AML cells. One of the constructs, termed Fabsc, contained an anti-FLT-3 Fab fused to a scFv specific for CD3 via a CH2 linker modified to prevent dimerization and binding to Fc receptors. In the other construct, scFv fragments of the two antibodies were fused together using a BiTE format. Whereas both constructs were able to lyse FLT-3(+)-target cells, the Fabsc format had higher affinity for FLT-3, lower aggregate formation and superior production yield.\(^7^1\)

**ANGIOGENIC GROWTH FACTORS**

Neoangiogenesis has a crucial role in pathogenesis of cancers including AML. The expression of two angiogenic agents, vascular endothelial growth factor-A (VEGF-A) and the angiopoietin-2 (Ang-2) on AML cells is associated with poorer patient outcome.\(^7^4\),\(^7^5\) Circulating levels of Ang-2 and VEGF are also strong predictors of poor survival in AML patients.\(^6^4\),\(^6^6\)–\(^6^8\) The expression of VEGF-A and its receptors on AML cells is increased, raising the possibility of an autocrine proliferation mechanism in AML.\(^7^6\) Bispecific antibodies have been generated to simultaneously target VEGF-A and Ang-2. In one approach called CrossMab technology, a heterodimeric IgG1-based bispecific antibody with monovalent binding moieties for VEGF-A and Ang-2 antigens was constructed. In this platform, monomeric heavy chains of anti-VEGF-A and Ang-2 antibodies are preferentially paired via the Knobs-into-holes methodology to make a heterodimeric heavy chain, whereas the correct pairing of light chains is guaranteed by crossover of the heavy chain constant-1 (CH1) and light chain constant (CL) domains.\(^8^0\) Although not yet tested against AML targets, this CrossMab antibody showed in vivo functionality against various cell lines.
including breast, lung, prostate, gastric, pancreatic and colon cancer, inhibited hematogenous spread of tumor cells, and was able to suppress corneal angiogenesis in mice.\textsuperscript{10,81} Using another approach, so-called bispecific CoxX-bodies were chemically synthesized by first linking the chemically synthesized VEGF and Ang-2 binding peptides by means of an azetidinone linker and then fusing these heterodimeric peptides to a scaffold antibody in a site-specific manner. \textit{In vivo} functionality studies showed that the CoxX-body imparted anti-angiogenic effects and reduced the growth of colon, breast and skin cancer xenografts.\textsuperscript{82}

**PERSPECTIVE AND CONCLUSIONS**

T lymphocytes are highly efficient professional killers proven successful clinically in a variety of human cancer types. Although this recognized potential of T cells is just beginning to be exploited for immunotherapy of leukemia, NK cells offer unique opportunities in the AML setting, both in terms of its trafficking pattern and its functional competency. In contrast to solid cancers leukemia is generally a blood borne disease where NK and T cells have an easier access to the cancer cells. Since leukemic blasts are mainly present in the bone marrow and blood, effector cells have better access to cancer cells. Unlike T cells, NK cells do not penetrate all tissues.\textsuperscript{93} Hence, although T-cell-bispecific antibodies may be necessary for solid tumors, both NK and T-cell bispecifics could be used for blood born malignancies such as leukemia. The important role of NK cells and the killer-cell immunoglobulin-like receptor (KIR)-ligand interactions in preventing relapse following allogeneic transplantation for AML is well documented.\textsuperscript{84} As higher E:T ratio improves the function of bispecific antibodies,\textsuperscript{10,13,85} a robust endogenous effector population or the adoptive transfer of exogenous effector cells should increase the efficacy of these therapies. Application of allogeneic effector cells as an off-the-shelf immunotherapy has been extensively investigated after myeloablative preconditioning.\textsuperscript{96} With age, thymic function involutes and if T cells are further damaged by myeloablative conditioning,\textsuperscript{87} they need to be resuscitated with growth factors, such as interleukin-7, interleukin-15, keratinocyte growth factor or sex steroid hormone inhibition.\textsuperscript{88} Alternatively, the administration of allogeneic precursor T cells could improve T and NK-cell reconstitution without inducing graft-versus-host disease (GvHD) at least in mouse models;\textsuperscript{99,98} however, their potential for human application is still uncertain. Unlike T cells, NK-cell recovery does not require the thymus, and is relatively fast after dose-intensive or myeloablative chemotherapy.\textsuperscript{90} Unlike allogeneic T cells that induce GvHD, allogeneic NK cells do not generate and may even reduce GvHD.\textsuperscript{91} Hence, administering such NK cells to enhance effector-target ratio should be clinically safe. Although CD3 is a proven activating receptor on T cells for bispecific antibodies, the clinical utility of agonistic receptors other than CD16 on NK cells is still investigational. NKG2D is one such activating receptor\textsuperscript{92} and MICA, MICB, ULBP1-6 are the other known activating ligands.\textsuperscript{93-95} Activating NK cells using anti-CD16 antibodies allows selective activation of FcRII (CD16), unlike IgGs which stimulate all Fc receptors of the activating (CD16,CD32A, CD64) and inhibitory classes (for example, CD32B). In addition, activation of CD16 through specific anti-CD16 antibody bypasses the genetic polymorphism among patients with differential FcR affinity for IgGs.\textsuperscript{60,96,97} With increasing sophistication in the genetic engineering of antibodies, multi-specific formats to include antibodies that will neutralize the inhibitory receptors may be possible, including inhibitory KIRs (2DL2/2DL3, 3DL1),\textsuperscript{98,99} PD-1 (B7-H1),\textsuperscript{100} and B7-H3.\textsuperscript{101}

Selecting the optimal leukemia-associated target antigen is as critical as choosing the proper class of effector cells or the particular antibody platform. Because of the importance of LSC in the recurrence of AML, bispecific antibody constructs need to engage antigens expressed on LSCs. Some of the potential LSC candidate antigens for generation of bispecific antibodies have been discussed by other investigators, including CD44,\textsuperscript{102} CD52,\textsuperscript{103} CD96,\textsuperscript{104} CD300f\textsuperscript{105} and TIM-3.\textsuperscript{106} A subset of leukemia called biphenotypic or mixed-lineage leukemia are composed of a heterogeneous mixtures of lymphoid and myeloid blasts. In addition, lineage switching could happen \textit{de novo} in neonates, or over the natural course of the disease or following ralapse after treatment.\textsuperscript{107} Bispecific antibodies simultaneously targeting CD3 (on T cells) and a single marker shared by both lymphoid and myeloid stem cells (for example, CD9/96s,\textsuperscript{108,109}) or trispecific antibodies targeting two separate markers on the leukemia blast (CD33 for myeloid and CD19 for lymphoid) could prevent escape of biphenotypic LSC. Furthermore, the carbohydrate antigen Lewis Y (CD174) has been introduced as a new marker for AML blasts and has been targeted with CARs in a clinical trial.\textsuperscript{110} Development of bispecific antibodies against this antigen in AML merits attention. CD33 may have a theoretical advantage over other myeloid markers for building bispecific antibodies since it carries on myeloid suppressor cells, an important population in the tumor microenvironment that impacts host T-cell immunity.\textsuperscript{111}

Antigen density on leukemic cells can affect antibody binding and effectiveness of bispecific antibodies. When antigens are expressed at low levels, like WT1-peptide-MHC complexes, higher-affinity antibodies may be necessary for optimal cytotoxicity.\textsuperscript{112} However, in the case of antigens with abundant expression on tumor cells, standard-affinity antibodies should be adequate for killing leukemic cells while sparing normal cells with low levels of the antigen. In this context, high affinity could be counterproductive given the sequestration of such antibodies by antigen sinks in normal tissues or by affinity barriers.\textsuperscript{113,114} Several bispecific antibody platforms have been tested in AML. Each platform has its unique strengths and weaknesses. BiTEs, diabodies and DARTs have small molecular size (~50 kD) below the renal clearance threshold. Although suitable for imaging because of their fast clearance from the blood, continuous infusion is necessary for their therapeutic applications. Other formats, such as Fc-fusion proteins or IgG-scFv have molecular sizes above the renal threshold and hence longer serum half-lives. None of the platforms presented in this review are able to cross the blood brain barrier, which permits only certain molecules with small molecular weights (~600 Da) and water–lipid partition coefficients, unless the bispecific antibody is piggybacked onto transcytosis pathways such as the transferrin receptor.\textsuperscript{115} In addition to their size, the valency of these platforms could be important for their biologic effect. Higher avidity for their cognate antigen usually translates into better binding and cytotoxicity, although cross-linking antigens can also lead to internalization of some antigens (for example, CD33). Bispecific antibodies that engage T cells stimulate the release of proinflammatory cytokines which act like a double-edged sword. Such cytokines can increase the anti-leukemia efficacy by direct cytotoxicity and by activation and recruitment of immune cells into the tumor site. However, they also increase the risk of adverse effects including the life-threatening cytokine release syndrome.\textsuperscript{116} Moreover, lessons learned from bone marrow transplantation would suggest that application of bispecific antibodies at the time of minimal residual disease should maximize the chance of cure besides reducing the risk of tumor lysis syndrome or the accompanying cytokine storm.

Generally, antibodies recognize cell surface antigens. Nonetheless, as the majority of leukemia-associated antigens are intracellular proteins whose peptide epitopes are presented outwardly in the context of HLA molecules, developing bispecific antibodies against these peptide–HLA complexes can unveil a treasure trove of potential novel tumor-specific targets. However, the field of TCR-like antibody is still in its infancy and many challenges remain. Cross reactivity of such antibodies with other peptides and with other HLA molecules could risk adverse side effects. Furthermore, as these antibodies only recognize antigens
in the context of HLA molecules, they can only be applied in patients with specific HLA types.

Although not covered in this review, highly encouraging results of chimeric antigen receptor (CAR)-armed T cells in lymphoblastic leukemia have been widely published. Yet the cost and complexity of cell preparation protocols, as well neurotoxicity could hamper the universal application of CAR technology beyond specialized centers with well-trained cytotherapy staff. In comparison, bispecific antibodies could be more cost-effectively generated in pharmaceutical scales for conventional transport and distribution with less geographic restrictions. Despite these advantages, the future of bispecific antibodies for AML will depend highly on their clinical efficacy and toxicity as more patients are being treated with these modalities.

CONFLICT OF INTEREST

MSKCC and NK Cheung have financial interest in Y-mabs Therapeutics Inc.

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REFERENCES

1. Dohner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. N Engl J Med 2015; 373: 1136–1152.
2. Spiess C, Zhai QT, Carter PJ. Alternative molecular formats and therapeutic applications for bispecific antibodies. Mol Immunol 2015; 67: 95–106.
3. Laszlo GS, Estey EH, Walter RB. The past and future of CD33 as therapeutic target in acute myeloid leukemia. Blood Rev 2014; 28: 143–153.
4. Marquez C, Trigueros C, Franco JM, Ramiro AR, Carrasco YR, Lopez-Botet M et al. Identification of a common developmental pathway for thymic natural killer cells and dendritic cells. Blood 1998; 91: 2760–2771.
5. Hernandez-Caselles T, Martinez-Esparza M, Perez-Oliva AB, Quintanilla-Cecconi AM, Garcia-Alonso A, Alvarez-Lopez DMR et al. A study of CD33 (SIGLEC3) antigen expression and function on activated human T and NK cells: two isoforms of CD33 are generated by alternative splicing. J Exp Med 2006; 79: 46–58.
6. Krupka C, Kufer P, Kischel R, Zugmaier G, Bogeohle J, Kohnhke T et al. CD33 target validation and sustained depletion of AML blasts in long-term cultures by the bispecific T-cell-engaging antibody AMG 330. Blood 2014; 123: 356–365.
7. Walter RB. Biting back: BiTE antibodies as a promising therapy for acute myeloid leukemia. Blood Rev 2011; 5: 1549–1557.
8. Laszlo GS, Estey EH, Walter RB. The past and future of CD33 as therapeutic target in acute myeloid leukemia. Blood Rev 2014; 28: 143–153.
9. Aigner M, Feulner J, Schaffer S, Kischel R, Kufer P, Schneider K et al. T lymphocytes can be effectively recruited for ex vivo and in vivo lysis of AML blasts by a novel CD33/CD3-bispecific BiTE antibody construct. Leukemia 2013; 27: 1107–1115.
10. Friedrich M, Henn A, Raum T, Bajtus M, Matthes K, Hendrich L et al. Preclinical characterization of AMG 330, a CD3/CD33-bispecific T-cell-engaging antibody with potential for treatment of acute myelogenous leukemia. Mol Cancer Ther 2014; 13: 1549–1557.
11. Laszlo GS, Gudgeon CJ, Harrington KH, Dell’Arlinga J, Newhall KJ, Means GD et al. Cellular determinants for preclinical activity of a novel CD33/CD3 bispecific T-cell engager (BiTE) antibody, AMG 330, against human AML. Blood 2014; 123: 554–561.
12. Laszlo GS, Gudgeon CJ, Harrington KH, Walter RB. T-cell ligands modulate the cytolytic activity of the CD33/CD3 BiTE antibody construct, AMG 330. Blood Cancer J 2015; 5.
13. Krupka C, Kufer P, Kischel R, Zugmaier G, Lichtenegger FS, Kohnhke T et al. Blockade of the PD-1/PD-L1 axis augments lysis of AML cells by the CD33/CD3 BiTE antibody construct AMG 330: reversing a T-cell-induced immune escape mechanism. Leukemia 2015; 30: 484–491.
14. Harrington KH, Gudgeon CJ, Laszlo GS, Newhall KJ, Sinclair AM, Franken SR et al. The broad anti-AML activity of the CD33/CD3 BiTE antibody construct, AMG 330, is impacted by disease stage and risk. PLoS One 2015; 10: e0135945.
Blood Cancer Journal

38 Jordan CT, Uphchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL et al. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. Leukemia 2000; 14: 1777–1784.

39 Testa U, Riccioni R, Milidi S, Coccia E, Stellacci E, Samoggia P et al. Elevated expression of IL-3 R alpha in acute myelogenous leukemia is associated with enhanced blast proliferation, increased cellularity, and poor prognosis. Blood 2002; 100: 2890–2896.

40 Kuo SR, Wong L, Liu JS. Engineering a CD123xCD3 bispecific scFv immunofusion for the treatment of leukemia and elimination of leukemia stem cells. Protein Eng Des Sel. 2012; 25: 561–569.

41 Stein C, Kellner C, Kugler M, Reff N, Mentz K, Schwenkert M et al. Novel conjugates of single-chain Fv antibody fragments specific for stem cell antigen CD123 mediate potent death of acute myeloid leukemia cells. Br J Haematol 2004; 128: 879–890.

42 Chichili GR, Huang L, Li H, Burke S, He L, Tang Q et al. A CD3xCD123 bispecific DAR for redirecting host T cells to myelogeneous leukemia: Preclinical activity and safety in nonhuman primates. Sci Trans Med 2015; 7: 289ra82.

43 Al-Husseini M, Rettg MP, Ritchey JK, Karpova D, Uly GL, Eisenberg LG et al. Targeting CD123 in acute myeloid leukemia using a T-cell-directed dual-affinity retargeting platform. Blood 2016; 127: 122–131.

44 Mennes HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S et al. Presence of Wilms-Tumor gene (WT1) transcripts and the WT1 nuclear-protein in the majority of human acute leukemias. Leukemia 1995; 9: 1060–1067.

45 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H et al. as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 1994; 84: 3071–3079.

46 Tao D, Pankov D, Scott A, Korontzis T, Zakhaleva V, Xu Y et al. Therapeutic bispecific T-cell engager antibody targeting the intracellular oncoprotein WT1. Nat Biotechnol 2015; 33: 1079–1086.

47 Ataie N, Xiang J, Cheng N, Brea EJ, Lu W, Scheinberg DA et al. Presence of a TCR-mimic antibody with target predicts pharmacogenetics. J Mol Biol 2016; 428: 194–205.

48 Ashmun RA, Look AT. Metalloprotease activity of CD13/aminopeptidase N on the surface of human myeloid cells. Blood 1990; 75: 462–469.

49 Mina-Osorio P. The moonlighting enzyme CD13: old and new functions to target. J Hematol 2016; 7: 879–895.

50 Jordan CT, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL et al. The novel AML stem cell associated antigen CLL-1 aids in discrimination of acute myeloid leukemia. Blood 1999; 93: 3717–3721.

51 Ataie N, Xiang J, Cheng N, Brea EJ, Lu W, Scheinberg DA et al. Structure of a TCR-mimic antibody with target predicts pharmacogenetics. J Mol Biol 2016; 428: 194–205.

52 Stein C, Kellner C, Kugler M, Reff N, Mentz K, Schwenkert M et al. Novel conjugates of single-chain Fv antibody fragments specific for stem cell antigen CD123 mediate potent death of acute myeloid leukemia cells. Br J Haematol 2004; 128: 879–890.

53 Chichili GR, Huang L, Li H, Burke S, He L, Tang Q et al. A CD3xCD123 bispecific DAR for redirecting host T cells to myelogeneous leukemia: Preclinical activity and safety in nonhuman primates. Sci Trans Med 2015; 7: 289ra82.

54 Al-Husseini M, Rettg MP, Ritchey JK, Karpova D, Uly GL, Eisenberg LG et al. Targeting CD123 in acute myeloid leukemia using a T-cell-directed dual-affinity retargeting platform. Blood 2016; 127: 122–131.

55 Mennes HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S et al. Presence of Wilms-Tumor gene (WT1) transcripts and the WT1 nuclear-protein in the majority of human acute leukemias. Leukemia 1995; 9: 1060–1067.

56 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H et al. as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 1994; 84: 3071–3079.

57 Tao D, Pankov D, Scott A, Korontzis T, Zakhaleva V, Xu Y et al. Therapeutic bispecific T-cell engager antibody targeting the intracellular oncoprotein WT1. Nat Biotechnol 2015; 33: 1079–1086.

58 Ataie N, Xiang J, Cheng N, Brea EJ, Lu W, Scheinberg DA et al. Structure of a TCR-mimic antibody with target predicts pharmacogenetics. J Mol Biol 2016; 428: 194–205.

59 Ashmun RA, Look AT. Metalloprotease activity of CD13/aminopeptidase N on the surface of human myeloid cells. Blood 1990; 75: 462–469.

60 Mina-Osorio P. The moonlighting enzyme CD13: old and new functions to target. J Hematol 2016; 7: 879–895.

AML targets for bispecific antibodies 8 So Hseine and NK Cheung
87 Fry TJ, Mackall CL. Immune reconstitution following hematopoietic progenitor cell transplantation: challenges for the future. Bone Marrow Transplant 2005; 35: S53–S57.

88 Chaudhry MS, Velardi E, Dudakov JA, van den Brink MR. Thymus: the next (re) generation. Immunol Rev 2016; 271: 56–71.

89 Zakrzewski JL, Suh D, Markley JC, Smith OM, King C, Goldberg GL et al. Tumor immunotherapy across MHC barriers using allogeneic T-cell precursors. Nat Biotechnol 2008; 26: 453–461.

90 Petersen SL, Ryder LP, Bjork P, Madsen HD, Heilmann C, Jacobsen N et al. A comparison of T-, B- and NK-cell reconstitution following conventional or nonmyeloablative conditioning and transplantation with bone marrow or peripheral blood stem cells from human leucocyte antigen identical sibling donors. Bone Marrow Transplant 2003; 32: 65–72.

91 Ruggieri L, Capannelli M, Urbani E, Peruccio K, Shlomchik WD, Tosti A et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002; 295: 2097–2101.

92 Chang YH, Connolly J, Shimasaki N, Mimura K, Kono K, Campana D. A chimeric receptor with NGK2D specificity enhances natural killer cell activation and killing of tumor cells. Cancer Res 2013; 73: 1777–1786.

93 Keller C, Gunther A, Humpe A, Repp R, Klauss K, Deier S et al. Enhancing natural killer cell-mediated lysis of lymphoma cells by combining therapeutic antibodies with CD20-specific immunoglobulins engaging NGK2D or Nk3p. Oncoimmunology 2016; 5: e1058459.

94 Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W et al. ULBP, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytototoxicity through the NGK2D receptor. Immunity 2001; 14: 123–133.

95 Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL et al. Targeting of CD44 for antibody enhancement of anti-lymphoma activity of natural killer cells as a model of anti-lymphoma therapy. Cancer Res 2006; 62: 5475–5483.

96 Petersen SL, Ryder LP, Bjork P, Madsen HO, Heilmann C, Jacobsen N et al. Antagonist antibodies against IREM-1: potential for targeted therapy of AML. Blood 2004; 103: 453–461.

97 Baker EJ, Grunewald S, van Rhijn CM, Oskam E, Fleuren GJ, Warnaar SO, Litvinov SV. The immunophenotype of 325 adult acute leukemias: relevance for immunotherapy of carcinomas. Br J Cancer 1998; 78: 478–483.

98 Adams GP, Schier R, McCull AM, Simmons HH, Horak EM, Alpough RK et al. High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules. Cancer Res 2001; 61: 4750–4755.

99 Feinstein JN, van Osdl W. Early intervention in cancer using monoclonal antibodies and other biological ligands: micropharmacology and the "binding site barrier". Cancer Res 1992; 52: 2747–2751.

100 Yu YJ, Atwal JK, Zhang Y, Tong RK, Wildsmith KR, Tan C et al. Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. Sci Trans Med 2014; 6: 261ra154.

101 Teachey DT, Rheingold SR, Maude SL, Zugmaier G, Barrett DM, Seif AE et al. Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. Blood 2013; 121: 5154–5157.

102 Hauswirth AW, Florian S, Prinz D, Sotlar K, Krauth MT, Fritsch G et al. Expression of the target receptor CD33 in CD34+(+CD38−)/CD123+(+) AML stem cells. Eur J Clin Invest 2007; 37: 73–82.

103 Laszlo GS, Estey EH, Walter RB. The past and future of CD33 as therapeutic target in acute myeloid leukemia. Blood Rev 2014; 28: 113–150.

104 Patrick JA, Alonzo TA, Loken M, Gerbing RB, Ho PA, Bernstein IE et al. Correlation of CD33 expression level with disease characteristics and response to gemtuzumab ozogamicin containing chemotherapy in childhood AML. Blood 2012; 119: 3705–3711.

105 Sun H, Li Y, Zhang ZF, Yu J, Li L, Zhang BC et al. Increase in myeloid-derived suppressor cells (MDSCs) associated with minimal residual disease (MRD) detection in adult acute myeloid leukemia. Int J Hematol 2015; 102: 579–586.

106 Tauszig DC, Pearce DJ, Simpson C, Rohtahiner AZ, Lister TA, Kelly G et al. Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. Blood 2005; 106: 4086–4092.

107 van der Velden VHJ, te Meerveld JG, Hoogeveen PG, Bernstein IE, Hautsmuller AB, Berger MS et al. Targeting of the CD33-calicheamicin immunocugnate Mylotarg (CMA-676) in acute myeloid leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells. Blood 2001; 97: 3197–3204.

108 Abdoor A, Yeh CH, Kantarjian H, O'Brien S, Bruey J, Giles F et al. Circulating CD33 and its clinical value in acute leukemia. Exp Hematol 2010; 38: 462–471.

109 Larson RA, Sievers EL, Stadtmann EA, Lowenberg B, Estey EH, Dombret H et al. Final report of the efficacy and safety of gemtuzumab ozogamicin (Mylotarg) in patients with CD33-positive acute myeloid leukemia in first recurrence. Cancer 2005; 104: 1442–1452.

1010 Walter RB, Appelbaum FR, Estey EH, Bernstein IE. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. Blood 2012; 119: 6196–6208.

1011 Munoz L, Normedde JF, Lopez O, Carnerie MJ, Bellido M, Aventin A et al. Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. Hematologica 2001; 86: 1261–1269.

1012 Sato N, Caux C, Kitamura T, Watanabe Y, Arai K, Banchereau J et al. Expression and factor-dependent modulation of the interleukin-3 receptor subunits on human hematopoietic cells. Blood 1993; 82: 752–761.

1013 Gill S, Tassign SR, Ruella M, Shrestova O, Li Y, Porter DL et al. Preclinical targeting of human acute myeloid leukemia and myeloaiblation using chimeric antigen receptor-modified T cells. Blood 2014; 123: 2343–2354.

1014 Hosen N, Shirakata T, Nishida S, Yanagihara M, Tsuboi A, Kawakami M et al. The Wilms' tumor gene WT1 mRNA in acute myeloid leukemias is associated with a worse long-term outcome. Blood 1997; 90: 1217–1225.

1015 Ramani P, Cowell JK. The expression pattern of Wilms' tumour gene (WT1) product in normal tissues and paediatric renal tumours. J Pathol 1996; 179: 162–168.

1016 Thallhammer-Scherrer R, Mitterbauer G, Simonitsch I, Jaeger U, Lechner K, Schneider B et al. The immunophenotype of 325 adult acute leukemias: relationship to morphologic and molecular classification and proposal for a minimal

1017 Ritchie DS, Neevon PJ, Khot A, Peinert S, Tai T, Tainton K et al. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. Mol Ther 2013; 21: 2122–2129.

1018 Zhang H, Li ZL, Ye SB, Ouyang LY, Chen YS, He J et al. Myeloid-derived suppressor cells inhibit T cell proliferation in human extranodal NK/T cell lymphoma: a novel prognostic indicator. Cancer Immunol Immunother 2015; 64: 1587–1599.

1019 Velders MP, van Rijn CM, Oskam E, Fleurens GJ, Warana SO, Litvinov SV. The impact of antigen density and antibody affinity on antibody-dependent cellular cytotoxicity: relevance for immunotherapy of carcinomas. Br J Cancer 1998; 78: 478–483.
screening program highly predictive for lineage discrimination. Am J Clin Pathol 2002; 117: 380–389.

133 Dixon J, Kaklamanis L, Turley H, Hickson ID, Leek RD, Harris AL et al. Expression of aminopeptidase-n (CD 13) in normal tissues and malignant neoplasms of epithelial and lymphoid origin. J Clin Pathol 1994; 47: 43–47.

134 Griffin JD, Davis R, Nelson DA, Davey FR, Mayer RJ, Schiffer C et al. Use of surface marker analysis to predict outcome of adult acute myeloblastic-leukemia. Blood 1986; 68: 1232–1241.

135 Buttler D, Mai JK, Ashwell KW, Andressen C. Clonogenic CD15 immunoreactive radial glial cells from the developing human lateral ganglionic eminence. Curr Pharm Biotechnol 2013; 14: 29–35.

136 Chadburn A, Inghirami G, Knowles DM. The kinetics and temporal expression of T-cell activation-associated antigens CD15 (LeuM1), CD30 (Ki-1), EMA, and CD11c (LeuM5) by benign activated T cells. Hematol Pathol 1992; 6: 193–202.

137 Deutsch E, Cheung NKO. Non-coordinating buffers. I. Synthesis and characterization of water soluble derivatives of 2,6-di-tert-butyl-pyridine. J Org Chem 1973; 38: 1123.

138 The Human Protein Atlas. http://www.proteinatlas.org/ENSG00000120949–TNFRSF8/tissue. 2016. Available from: http://www.proteinatlas.org/ENSG00000120949–TNFRSF8/tissue.

139 Vanderjagt RHC, Badger CC, Appelbaum FR, Press OW, Matthews DC, Eary JF et al. Localization of radiolabeled antinmyeloid antibodies in a human acute-leukemia xenograft tumor-model. Cancer Res 1992; 52: 89–94.

140 Caldwell CW, Patterson WP, Toalson BD, Yesus YW. Surface and cytoplasmic expression of Cd45 antigen isoforms in normal and malignant myeloid cell-differentiation. Am J Clin Pathol 1991; 95: 180–187.

141 Witte KE, Ahlers J, Schafer I, Andre M, Kerst G, Scheel-Walter HG et al. High proportion of leukemic stem cells at diagnosis is correlated with unfavorable prognosis in childhood acute myeloid leukemia. Pediatr Hemat Oncol 2011; 28: 91–99.

142 Bakker ABH, van den Oudenhoven S, Bakker AQ, Feller N, van Meijer M, Bia JA et al. C-type lectin-like molecule-1: A novel myeloid cell surface marker associated with acute myeloid leukemia. Cancer Res 2004; 64: 8443–8450.

143 Levis M, Small D. FLT3: It Does matter in leukemia. Leukemia 2003; 17: 1738–1752.

144 Trujillo A, McGee C, Cogle CR. Angiogenesis in acute myeloid leukemia and opportunities for novel therapies. Journal of oncology. 2012; 2012: 128608.

145 Labrijn AF, Meesters JJ, de Goeyj BE, van den Bremer ET, Neijssen J van Kampen MD et al. Efficient generation of stable bispecific IgG1 by controlled Fab-arm-targeting. Proc Natl Acad Sci USA: 2013; 110: 5145–5150.

146 Bachmann M, Stamova S. Anti-CD33 antibodies and use thereof for immunotoxin targeting in treating CD33-associated illnesses. Google Patents 2014.

147 Stamova S, Cartellieri M, Feldmann A, Arndt C, Koristka S, Bartsch H et al. Unexpected recombinations in single chain bispecific anti-CD3-anti-CD33 anti-bodies can be avoided by a novel linker module. Mol Immunol 2011; 49: 474–482.

148 Wiernik A, Foley B, Zhang B, Verneris MR, Warlick E, Gleason MK et al. Targeting natural killer cells to acute myeloid leukemia in vitro with a CD16 x 33 bispecific killer cell engager and ADAM17 inhibition. Clin Cancer Res 2013; 19: 3844–3855.

149 McCall AM, Adams GP, Amoroso AR, Nielsen UB, Zhang L, Honak E et al. Isolation and characterization of an anti-CD16 single-chain Fv fragment and construction of an anti-HER2/neu/anti-CD16 bispecific scFv that triggers CD16-dependent tumor cytolysis. Mol Immunol 1999; 36: 433–445.

150 Vallera DA, Felices M, McElmurry R, McCullar V, Zhou X, Schmohl JU et al. IL15 trispecific killer engagers (TriKE) make natural killer cells specific to CD33+ targets while also inducing persistence, in vivo expansion, and enhanced functionality. Clin Cancer Res 2016; 22: 3440–3450.

151 Kuo SR, Wong L, Liu JS. Engineering a CD123xCD3 bispecific antibody to target CD33-associated illnesses. Google Patents 2014.

152 Kuo SR, Alfano RW, Frankel AE, Liu JS. Antibody internalization after cell surface antigen binding is critical for immunotoxin development. Bioconjug Chem 2009; 20: 1975–1982.