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Recombinant Antibodies to Arm Cytotoxic Lymphocytes in Cancer Immunotherapy

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Keywords
Recombinant antibodies · Immuno-engager · TandAb · Bispecific antibodies · ADCC · CD16 · Cellular therapy · Immuno-oncology

Summary
Immunotherapy has the potential to support and expand the body's own armamentarium of immune effector functions, which have been circumvented during malignant transformation and establishment of cancer and is presently considered to be the most promising treatment option for cancer patients. Recombinant antibody technologies have led to a multitude of novel antibody formats, which are in clinical development and hold great promise for future therapies. Among these formats, bispecific antibodies are extremely versatile due to their high efficacy to recruit and activate anti-tumoral immune effector cells, their excellent safety profile, and the opportunity for use in combination with cellular therapies. This review article summarizes the latest developments in cancer immunotherapy using immuno-engagers for recruiting T cells and NK cells to the tumor site. In addition to antibody formats, malignant cell targets, and immune cell targets, opportunities for combination therapies, including check point inhibitors, cytokines and adoptive transfer of immune cells, will be summarized and discussed.

Introduction

To protect against pathological alterations such as infections, parasites and cancer, vertebrates have evolved a complex network of innate and adaptive immune effector mechanisms. These comprise soluble factors such as toxins, antibodies, chemokines, and several types of immune cells with discrete functions such as phagocytosis and targeted cytotoxicity. Due to the body's permanent exposure to potentially harmful environmental substances, pathogens, commensal bacteria and malignantly transformed cells, maintenance of its homeostasis represents a challenge, which requires the concerted action of a large variety of different immune effector functions. Moreover, pathogens and malignantly transformed cells can actively outsmart the immune system and escape from immunological selection pressure by adaptation, even during an ongoing immune response. The dynamic interplay of pathogens and malignantly transformed cells with the immune system is referred to as 'immuno-editing'. The process of immuno-editing can be divided into three phases: elimination, equilibrium, and escape [1]. According to this model, pathogens and malignantly transformed cells are eradicated instantaneously (elimination), coexist for some time with the body's defense armamentarium (equilibrium), and, if eradication cannot be achieved, evade immunosurveillance (escape), allowing for persistence and, consequently, establishment of a potentially life-threatening disease condition.

Current approaches to treat persistent infections and cancer aim either at restoration of the equilibrium phase, thus transforming the pathological condition into a chronic but stable disease, or, ideally, at restoration of the elimination phase, thereby curing the patient. Immuno-surveillance of parasites, infected tissue, and malignantly transformed cells crucially depends on NK cells and cytotoxic T cells (CTLs), which specifically kill target cells after the polarized release of cytotoxic granules. Therefore, it is not surprising that both cell types are subject to numerous immune evasion strategies which have evolved over time and result in the disarming or sequestration of immune cells from the pathological lesion. Conversely, targeted therapies aim at improved recruitment and activation of cytotoxic NK cells and CTLs to the site of infection or malignant alteration.

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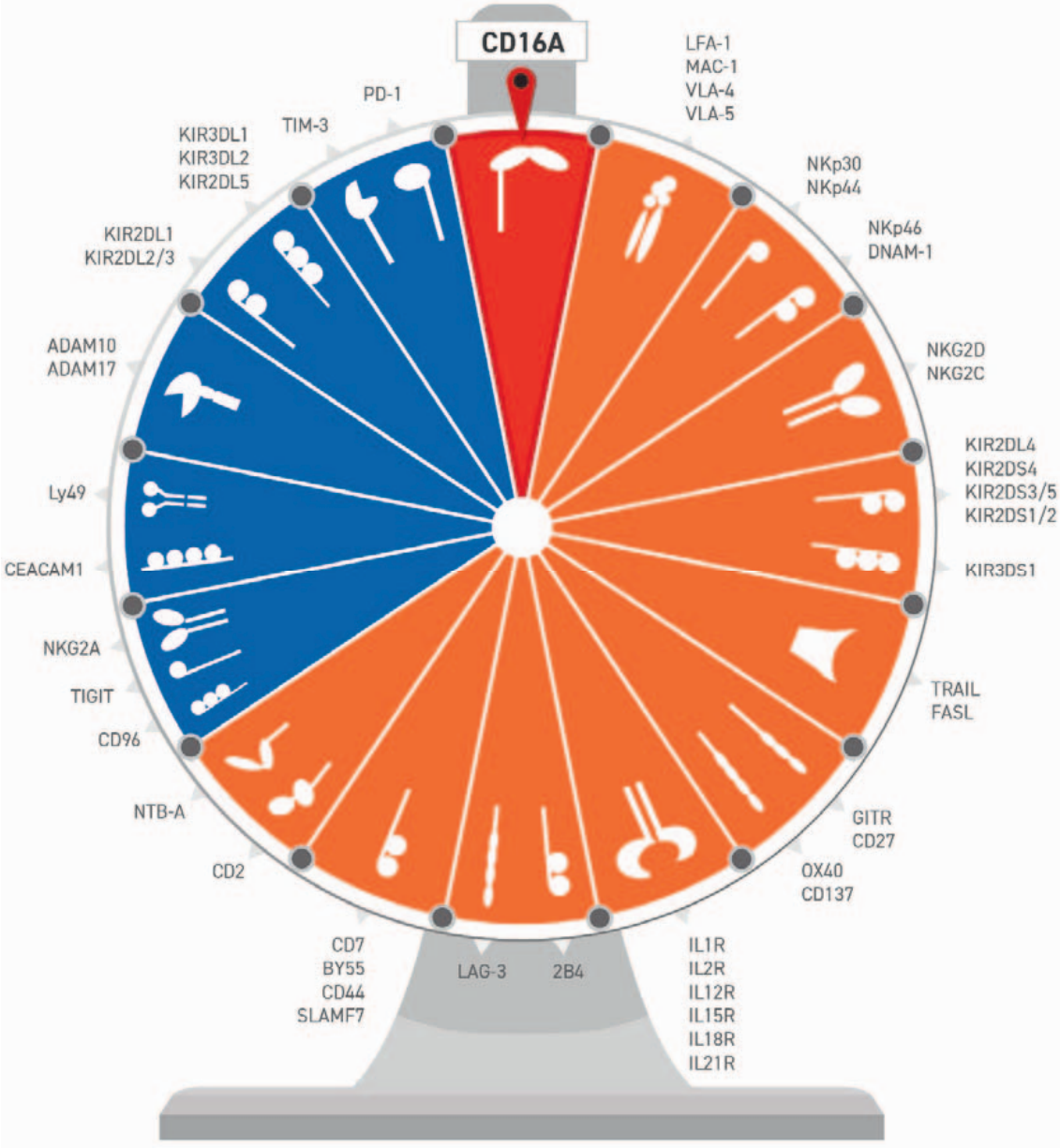


Fig. 1. Major inhibitory and activating NK cell receptors. Inhibitory receptors (blue) are important for self/non-self-discrimination. The net input of individual or several activating receptors (orange/red) triggers cytotoxicity of NK cells towards target cells. NK cell activation can be initiated by loss of inhibitory signaling, e. g. upon downregulation/loss of HLA molecules on the plasma membrane of target cells ('missing self'), and/or (over)expression of stress-induced ligands on target cells which trigger signaling of activating NK cell receptors ('induced self'). CD16A = Fc-gamma RIII-alpha; LFA-1 = complement receptor C3 subunit beta; MAC-1 = macrophage integrin VLA-4 = integrin alpha-4; VLA-5 = integrin alpha-5; NKp30 = natural cytotoxicity triggering receptor 3; NKp44 = natural cytotoxicity triggering receptor 2; NKp46 = natural cytotoxicity triggering receptor 1; DNAM-1 = DNAX accessory molecule 1; NKG2D = killer cell lectin-like receptor subfamily K member 1; NKG2C = killer cell lectin-like receptor subfamily C = member 2; KIR2DL = killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail; KIR2DS = killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; FASL = Fas ligand; GITR = glucocorticoid-induced TNFR-related protein; CD27 = tumor necrosis factor receptor superfamily member 7; OX40 = tumor necrosis factor receptor superfamily member 4; CD137 = tumor necrosis factor receptor superfamily member 9; ILXR = interleukin X receptor (X indicates number of interleukin); 2B4 = NK cell type I receptor protein 2B4; LAG-3 = lymphocyte-activation gene 3; CD7 = T-cell leukemia antigen; BY55 = natural killer cell receptor BY55; CD44 = GP90 lymphocyte homing/adhesion receptor; SLAMF7 = SLAM family member 7; CD2 = T-cell surface antigen CD2; NTB-A = SLAM family member 6; CD96 = T-cell surface protein tactile; TIGIT = T cell immunoreceptor with Ig and ITIM domains; NKG2A = killer cell lectin-like receptor subfamily C member 1; CEACAM1 = carcinoembryonic antigen-related cell adhesion molecule 1; Ly49 = killer cell lectin-like receptor subfamily A = member 1; ADAM = disintegrin and metalloproteinase domain-containing protein; TIM-3 = T-cell immunoglobulin and mucin domain-containing protein 3; PD-1 = programmed cell death protein 1.

NK Cells in Cancer Immuno-Surveillance

Even though recent reports have attributed adaptive features to NK cells, they are a part of innate immunity due to the expression of germline-encoded receptors [2, 3]. NK cells are distributed throughout the body, but are enriched in the bone marrow, liver, blood, spleen, and lymph nodes. Phenotypically, NK cells are defined by the presence of the cellular markers CD56 and NKp46 (NCR1, CD335), and the absence of T-cell-specific (CD3 and TCR) and B-cell-specific markers (CD19). Furthermore, NK cells are discriminated on the basis of two principal subsets: $CD56^{bright}$ CD16– NK cells, which represent the predominant species in lymphoid organs and are generally characterized by high cytokine production, and $CD56^{dim}$ CD16+ NK cells, which are the predominant

species in peripheral blood and are regarded as highly cytotoxic [3]. This simplistic categorization was challenged by previous reports suggesting a much broader spectrum of phenotypic and functional diversity due to stochastic distribution of receptors to individual NK cells and additional shaping by epigenetic modification, DNA methylation, and environmental influences [4]. Adding another level of plasticity to the NK cell population, it is currently under debate whether $CD56^{bright}$ cells differentiate into $CD56^{dim}$ cells [5] or whether $CD56^{dim}$ CD16+ NK cells develop from a different progenitor than $CD56^{bright}$ CD16– NK cells, T cells, B cells, or myeloid cells [6].

$CD56^{bright}$ NK cells are characterized by the absence of CD16 and KIR expression and their potency to secrete immunomodulatory cytokines. Even though resting peripheral blood $CD56^{bright}$ cells are poorly cytotoxic, they display a tremendous proliferative capacity in response to cytokines such as IL-2. In contrast, CD3– $CD56^{dim}$ NK cells express high levels of CD16A and KIR, are highly cytotoxic and are capable of rapid and strong production of IFN-γ following activation [7].

NK cell cytotoxicity is governed by the net result of signaling through inhibitory and activating receptors recognizing self and non-self or altered-self structures on target cells. Among many others (fig. 1), prominent activating NK cell receptors in humans comprise the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, NKG2D, DNAM-1 as well as CD16A [8].

NK cell engagement and activation can be enhanced or triggered by antibodies. Those antibodies can block ligand binding to inhibitory receptors ('antagonist antibodies', e.g. KIR) expressed on NK cells or enhance the function of activating receptors ('agonist antibodies', e.g. CD137), by mimicking the respective ligand function. This type of engagement does not necessarily lead to a direct activation but can have a supportive effect in a very complex signaling network towards activation and degranulation. Conversely, antibodies directed against CD16A [9] or CD16A and B [10, 11] have the potential to directly trigger an activating pathway and degranulation. Another strategy to support and maintain NK cell activity is the inhibition of ADAM17 activity [12, 13]. ADAM17 is a metalloproteinase and a potential key player in immuno-oncology [14] that is expressed on NK and cancer cells. Among many other functions, ADAM17 is responsible for the shedding of CD16A. Based on these NK cell characteristics, different NK cell-engaging and/or -activating antibodies are currently in clinical development (table 1). Prominent examples of antagonist antibodies are lirilumab (anti-KIR [15, 16]) and monalizumab (anti-NKG2A [17]) and of agonistic antibodies urelumab and utomilumab (anti-CD137 [18, 19]).

Following activation, NK cells release pre-formed granules into the immunological synapse at the NK target interface by exocytosis. These granules contain pore-forming perforin, granzymes, and IFN-γ mRNA, which trigger caspase-dependent and caspase-independent apoptosis of target cells and production of IFN-γ [20, 21]. Alternatively, target cell apoptosis can be initiated by Fas ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the plasma membrane of NK cells or released from cytotoxic

Table 1. Overview of antibodies for NK-cell engagement and/or activation

Targets	Antibody or programs	Format/Isotype	Mode of action	Clinical phase	Company/references
ADAM-17	D1 (A12)	hIgG1	enzyme inhibition	preclinical	[12, 13]
CD137 (4-1BB)	Urelumab, Utolimumab	hIgG4, hIgG2	agonist	I, II	BMS, Pfizer/ [136]
CD27	Varlilumab (CDX-1127)	hIgG1	agonist	II	Celldex Therapeutics/ [136, 137]
CEACAM1	DIATHIS1	scFv	antagonist	preclinical	[103, 138, 139]
CTLA-4	Ipilimumab	hIgG1	agonist	approved	BMS/ [140]
GITR	TRX518, INCAGN1876	hIgG1	agonist	I–II	Leap Therapeutics, Agenus/ [141, 142]
KIR2D-L1,-L2, -L3;	Lirilumab, IPH2101	hIgG4	antagonist	I–II	Innate Pharma/ [15]
KIR3D-L2	IPH4102	hIgG4	antagonist	I	Innate Pharma/ [143]
LAG-3	TSR-033	hIgG4	antagonist	preclinical	Tesaro/ [144, 145]
NKG2A	Monalizumab, IPH2201	hIgG4	antagonist	I–II	Innate Pharma/ [17]
OX40	MOXR0916, INCAGN1949	hIgG1	agonist	II	Genentech, Agenus/ [136, 137, 142]
PD-1	Nivolumab, Prembrolizumab	hIgG4	antagonist	approved	BMS, Merck & Co./ [146, 147]
PD-L1	Atezolizumab, Avelumab, Durvalumab	hIgG4	antagonist	approved I–III	Roche/ [146, 147] Merck KGaA, Pfizer AZ, MedImmune
SLAMF7 (CS-1)	Elotuzumab	hIgG1	agonist	approved	BMS, Abbvie /[148]
TIGIT	MTIG7192A, BMS-986207	hIgG1	antagonist	I–II	Genentech, BMS/[149, 150];
TIM-3	TSR-022	hIgG4	antagonist	I	Tesaro/ [151]

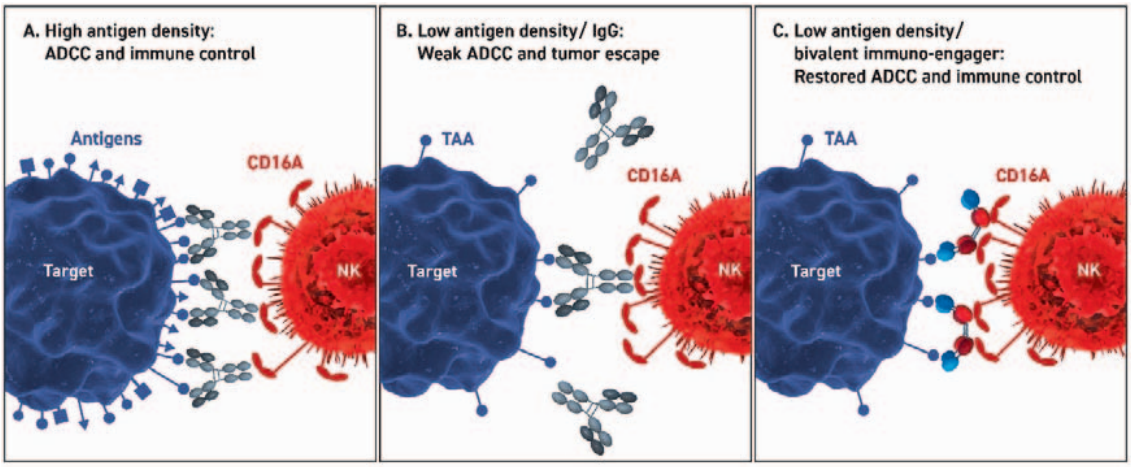


Fig. 2. Scenarios of antibody-mediated immune cell and target cell engagement. Engagement is shown at high antigen density (**A**) and at low antigen density with IgG-based (**B**) and recombinant bispecific tetravalent immuno-engagers (**C**). **A** At high antigen densities on the target cell (e.g. virus infection, blue) a polyclonal antibody response is initiated leading to saturating opsonization of the target cell and robust ADCC upon Fc binding to CD16A on the NK cell (red). **B** At limiting antigen densities on the target cell (e.g. malignantly transformed cells), an insufficient degree of opsonization of the target cell by IgG molecules leads to a low level of ADCC and thus tumor immune escape due to few low affinity interactions between the Fc domains and CD16A. **C** At limiting antigen densities on the target cell (see B) bispecific tetravalent immuno-engagers enable robust ADCC and immune control due to multivalent and apparent high affinity binding to CD16A.

granules which recognize Fas or TRAIL receptors on the target cell. [22]. Importantly, NK cells can be triggered by antibody-dependent cellular cytotoxicity (ADCC) after recognition of antibody-opsonized target cells via CD16A (see below).

Even though NK cells were discovered more than four decades ago, it is only now that their potential in targeted cancer immunotherapy is exploited in clinical settings. Several lines of evidence demonstrate a strong impact of NK cells on cancer immuno-surveillance: i) Based on epidemiological studies, individuals with low cytotoxicity of peripheral NK cells have a higher incidence of cancer [23]. ii) NK cells are subject to numerous tumor immune escape strategies, suggesting that establishment and maintenance of a tumor-promoting microenvironment critically depends on failure of NK cell immuno-surveillance [24, 25]. iii) Many tumors depend on blocking tumor infiltration of lymphocytes to limit access for anti-tumor activity of immune cells. Consequently, tumor infiltration of NK cells is a good prognostic marker in several tumor entities including clear renal cell carcinoma, non-small cell lung cancer (NSCLC), and colorectal cancer [26–30]. iv) NK cells mediate cross-talk with the adaptive immune system and therefore have the potential to increase the potency of several immune effector arms [31–34]. Notably, since NK cell activation depends on the net-activation signal of several activating receptors, NK cell killing is potentially more redundant, making therapy resistance less likely to occur. v) Adoptive transfer of ex vivo expanded NK cells showed high efficacy in early clinical trials [35–37]. Furthermore, NK cells display many advantageous features for clinical application. Firstly, NK cells are the first lymphoid cells to repopulate after stem cell transplantation (SCT), reaching normal numbers within 1 month regardless of donor type or patient age [38–40]. Therefore, NK cells provide the first opportunity for targeted cellular anti-cancer therapy following SCT. Secondly, NK cells contribute to the graft-versus-tumor/graft-versus-leukemia effect with significantly less or even no graft-versus-host disease (GvHD) compared to allogeneic T cells [36], demonstrating a superior safety profile. Thirdly, NK cells are more easily adaptable to an allogeneic/off-the shelf approach than T cells which require an autologous setting due to otherwise fatal GvHD reactions.

Antibody-Dependent Cellular Cytotoxicity of NK Cells

The human body produces antibodies as a defense mechanism against viruses and bacteria. To protect against infectious organisms, a polyclonal IgG response is mounted, whereby multiple antigens and epitopes on infected cells and organisms are recognized. Due to the high density of these antigens, a high degree of opsonization enables multivalent binding of Fc receptors, thus compensating for the low affinities of individual IgGs, and enables strong ADCC and immune control (fig. 2). Conversely, a low density of tumor antigens targeted by monoclonal antibodies, which recognize a single epitope on a single tumor antigen, elicits a low degree of opsonization and, consequently, a limited potential to induce ADCC of immune cells. Interestingly, most monoclonal therapeutic antibodies are blocking antibodies (e.g. cetuximab and panitumumab) and were not developed for immune cell engagement. Therefore, the potential of these molecules to recruit immune cells in a therapeutic setting remains unclear. This limitation could potentially be overcome by bispecific tetravalent immuno-engagers, which mediate robust ADCC and immune control due to multivalent and apparent high affinity binding to CD16A.

A large variety of mouse and human Fc receptors have been identified which are able to bind the Fc portion of IgG antibodies. The FcγR family comprises four subclasses of receptors – FcγRI, FcγRII, FcγRIII, FcγRIV (only in mouse) – which are expressed on most hematopoietic cells, except for T cells [41, 42]. All of these receptors are activating except for the inhibitory FcγRIIb. Most notably, the activating receptor FcγRIIIa (CD16A) is the main receptor facilitating ADCC, whereas all activating FcγRs mediate anti-

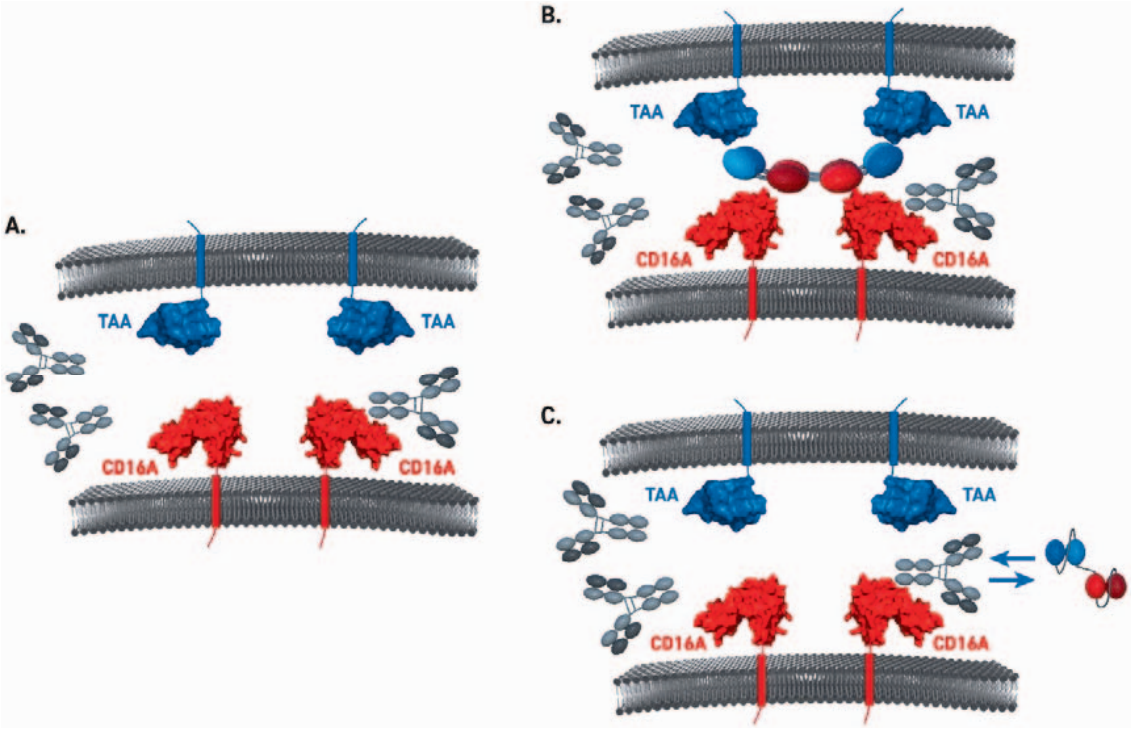


Fig. 3. Models for CD16A engagement and IgG competition. **A/B** In the ground state, CD16A on innate immune cells is occupied by polyclonal plasma IgG. This creates a threshold for Fc-based therapeutic antibodies or immuno-engagers, employing the recognition site on CD16A also bound by the Fc proportion of IgG antibodies, thus limiting therapeutic potential. **C** Tetravalent bispecific immuno-engagers, which recognize a different epitope on CD16A, are virtually unaffected by plasma IgG. This enables high affinity binding of CD16A and respective tumor antigens leading to strong ADCC and immuno-surveillance.

body-dependent cellular phagocytosis (ADCP [43]). Notably, NK cells express only FcγRIIIa but no inhibitory FcγR. For signaling, CD16A associates with homodimeric or heterodimeric ITAM-containing adapter protein complexes of FcεRI-γ chains or CD3ζ chains within the plasma membrane [44]. The affinity of antibodies for CD16A directly correlates with their ability to trigger NK cell activation, thus reducing the antibody dose required for activation [45]. CD16A is the only activating receptor triggering the cytotoxic activity of naïve human NK cells even in the absence of co-stimulatory signals [21, 46, 47].

The X-ray crystal structure of a human IgG1 Fc fragment-CD16A ectodomain complex has been solved to 3.2 Å resolution [48]. In this complex, the Fc fragment binds asymmetrically to the two Ig domains of CD16A. Residues of the Cγ2 domains and the hinge region of the Fc domain contact with residues in the membrane proximal domain 2 of CD16A and two residues in the linker connecting domain 1 and 2 of CD16A [48].

In addition to NK cells, CD16A has been reported to be expressed on monocytes, macrophages, and γ/δ T cells [49, 50]. Two allelic single nucleotide polymorphisms have been identified in human CD16A altering the amino acid in position 158, which is important for interaction with the hinge region of IgGs. The allelic frequencies of the homozygous 158 F/F and the heterozygous 158 V/F alleles are similar within the Caucasian population, ranging between 35 and 52% or 38 and 50%, whereas the homozygous 158 V/V allele is only found in 10–15% [51]. The allelic variant 158V has a higher affinity for IgG than the 158F variant and may therefore confer two advantages in IgG-mediated cancer immunotherapy: i) enhanced potential of NK cells to engage with antibody-opsonized tumor cells and ii) an increased release of granules by NK cells upon encounter with an antibody-opsonized tumor cell [52]. Expression of the allelic variant 158V has been described to correlate with positive clinical response of patients suffering from B-cell lymphoma, leukemia, breast cancer, or colorectal cancer to ADCC-mediating IgG-based therapy [53–57]. Some studies suggest using the performance of patient-derived 158V NK cells in in vitro ADCC assays with antibody-coated tumor cells as a predictive marker for the patients' response to anti-tumor therapy [58]. Notably, IgG antibodies with low or absent fucose side chains are more effective in eliciting ADCC both in vitro and in vivo when compared to conventional IgG antibodies; however, the impact on clinical efficacy has been very limited [59, 60].

IgGs can be divided into four subclasses (IgG1, IgG2, IgG3, and IgG4). Among these, IgG1 and IgG3 display the highest affinity to the two known CD16A alleles with affinities in the single digit micromolar range, whereas IgG2 and IgG4 show affinities in the higher micromolar range [61].

Notably, the binding affinities for IgG1 and IgG3 to CD16A 158V are two-fold higher than for CD16A 158F. Although a number of specific IgGs have shown ADCC-related efficacy in pre-clinical models [62], the observed micromolar affinities of IgGs for CD16A and variations in the binding affinities for different CD16A alleles are unfavorable for therapeutic application of ADCC-inducing antibodies. Moreover, due to high plasma levels of IgG (roughly 10 mg/ml), Fc-based antibody formats face competition for CD16A binding, thereby limiting CD16A occupancy and increasing the required dose of therapeutic antibody. Competition with plasma IgGs might be even more pronounced in disease conditions which are characterized by high levels of plasma IgGs such as multiple myeloma [63]. In the ground state, CD16A on innate immune cells

is occupied by polyclonal plasma IgG (fig. 3). This creates a threshold for Fc-based therapeutic antibodies or immuno-engagers which employ the recognition site on CD16A also bound by the Fc proportion of IgG antibodies [64, 65], thus limiting therapeutic potential. Importantly, tetravalent bispecific immuno-engagers, which recognize a different epitope on CD16A [9], are virtually unaffected by plasma IgG. This enables high-affinity binding of CD16A and respective tumor antigens leading to strong ADCC and immuno-surveillance.

To increase the binding affinity to CD16A and to allow for CD16A binding independent of patient genotype, a variety of different antibody formats have been developed [66–68]. These combine binding domains and, hence, different functionalities in one antibody molecule. These molecules have the potential for greater therapeutic success compared to administration of a mixture of antibodies with the same respective specificities [69–71].

Principles of Bispecific Antibody Engineering

Apart from disease settings, PK/PD relationship, patient's treatment status and regimens, the target architecture and biology should ideally be the driver for selection of the appropriate antibody format. However, this information is often unavailable or insufficient for a straightforward, fully rational antibody design approach. Thus, to select the best candidate bearing functional characteristics for optimal therapeutic intervention, extensive molecular engineering of a desired format with multiple iterations combined with high-throughput automated processes for functional screenings are required [72, 73]. The simplest bispecific molecules are built from only two antigen binding domains (ABDs) [74] that recognize different antigens. These ABDs are generally composed of Ig heavy and light variable domains of mouse [75] or human origin [76–79]. The smallest ABD units are single chain antibodies (single chain variable fragment or scFv, [80]) or the larger antigen binding fragments (Fab). Alternatively, heavy chain variable domains of camelids [81] or non-antibody-binding domains [82, 83] such as anticalins [84, 85] or DARPins [82] serve as building blocks for bispecific molecules. By linking more than two ABDs together, avidity and functionality are substantially improved as demonstrated by the bispecific tetravalent tandem diabodies (TandAb) molecule for CD3- or CD16A-specific immune effector engagement [9, 86]. Depending on the linker sequence and linker lengths [87–89], the orientation of the VL and VH domain, the number of ABDs and the number of polypeptide chains, the bispecific antibodies can adopt different formats. Prominent examples currently in clinical development include the bispecific tetravalent TandAbs [90], bispecific T-cell engagers (BiTEs [91]), bi- or trispecific killer cell engagers (BiKEs, TriKEs, [11, 92, 93], and dual-affinity re-targeting antibodies (DARTs [94]). Adopting a different strategy, two or more ABDs can be genetically fused to a 'stable' scaffold allowing further diversity of bispecific molecules. Examples are Fc- or IgG-based bispecific antibody molecules [9–97], which have recently entered clinical trials as T-cell recruiters [98].

Bispecific NK- and T-Cell Engagers in Clinical Development

A major subclass of bispecific antibodies recruits cytotoxic lymphocytes such as T and NK cells via ABDs against CD3 and CD16, respectively. For CD3 recruitment, the majority of ABDs derive from the cynomolgus monkey cross-reactive antibody clones SP34 [99] or UCHT1 [100]. In principle, NK cell recruiters are based on two different anti-CD16 domains: clone 3G8 [64, 101, 102] that is specific for both CD16A and CD16B, and clone LSIV21 [9] that recognizes a CD16A-specific epitope. Examples of bispecific cytotoxic lymphocyte engagers and different designs are the BiTE and TandAb molecules [9, 86]. Those bispecific formats are solely composed of ABDs connected to each other via flexible linkers. The formation of the final expression product is largely influenced by its linker lengths. Long linkers between heavy and light variable domains enable intra-molecular pairing of both domains as is the case for both scFv molecules the monovalent BiTE molecule. In contrast, relatively short linkers between heavy and light chain domains allow homo-dimerization of two identical polypeptide chains in a head-to-tail fashion, thereby creating a tetravalent bispecific molecule with two binding sites for each antigen as shown for the TandAb molecule. Other cytotoxic lymphocyte engager molecules that are currently under clinical development are combined with an Fc portion. Table 2 shows the bispecific cytotoxic lymphocyte engagers currently in clinical development. Most formats in development are T-cell engagers comprising an anti-CD3 domain for immune effector cell recruitment in combination with different ABDs directed against tumor-associated antigens such as CD19 [103], CEA [104, 105], CD20 [106], PSMA [107], EpCAM [108], CD33 [109], BCMA [110], GPA33 [111], P-cadherin [112], B7-H3, and Her2 [113]. The number of T-cell engagers in clinical development also illustrates the great variety of different bispecific designs, ranging from relatively small molecules to bigger scaffolds employing Fc portions. Even though, to date, T-cell engagers have been the predominant class of bispecific immune cell engagers, they have been challenged by severe side effects in recent clinical trials. Conversely, bispecific NK cell engagers, which only recently have entered the clinic, have shown efficacy and superior safety profiles in clinical [9, 86] or late-stage pre-clinical development [10, 11, 114, 115].

Enhancing NK Cell Efficacy with Cytokines

Cytokines hold great promise to improve the anti-tumor activity of NK cells. Beyond IL-2 and IL-15, which are arguably the best studied cytokines in a therapeutic setting [36], IL-12, IL-18, IL-21, and type I IFNs can be used for in vitro expansion and activation of NK cells before adoptive transfer. The combination of IL-12, IL-15, and IL-18 has recently been reported to induce a population of memory-like NK cells, which showed high efficacy in a phase I clinical trial in acute myeloid leukemia (AML) [35]. Moreover, it has been reported that stimulation of NK cells in vitro with IL-2 or

Table 2. Overview of bi-specific T-cell engagers in clinical development*

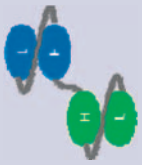
Bispecific Format	Compound/Program	Target combination	Clinical phase (indication)	Company [references]
BiTE 	Blinatumomab AMG103 MT103	CD19xCD3	Market (ALL)	Amgen [103]
	MT111, AMG211, Medi565	CEAxCD3	I (GC, ADC)	Amgen [104]
	Pasotuxizumab MT112 BAY2010112	PSMAxCD3	I (PC)	Bayer
	Solitomab MT110 AMG 110	EPCAMxCD3	I (CRC, GIC, LC and ST)	Amgen [108]
	AMG330	CD33xCD3	I (AML)	Amgen [109]
	AMG420, BI 836909	BCMAxCD3	I (MM)	Boehringer Ingelheim, Amgen [110]
TandAb 	AFM11	CD19xCD3	I (NHL, ALL)	Affimed [86]
	AMV564	CD33xCD3	II (AML)	Amphivena [152]
Crossmab 	RG7802 (2+1)	CEAxCD3	I (CEA+ ST)	Roche [105]
DART 	MGD006 S80880	CD123xCD3	I (AML)	Macrogenics, Servier [153]
	MGD007	GPA33xCD3	I (CR)	Macrogenics, Servier [111]
	PF06671008	P cadherin CD3	I (ST)	Pfizer, Macrogenics [112]
Fc-DART 	MGD009 (Fc-DART)	B7H3 CD3	I (ST)	Macrogenics
	Duvortuxizumab, MGD 011, JNJ64052781 (Fc-DART)	CD19 CD3	II (ST)	Macrogenics, Janssen [154]

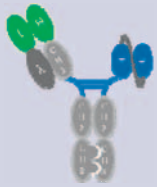
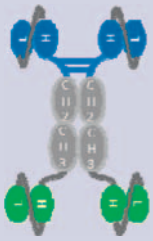
Table 2 continued on next page

IL-15 for several days resulted in NK cell activation and killing of malignantly transformed cells, which were initially resistant to killing by naïve NK cells [116].

IL-2 and IL-15 bind to the shared dimeric receptor complex comprised of the IL2ββ (15Rβ, CD122) and γ_c (CD132) chains with only nanomolar affinity thus requiring relatively high concentrations of cytokines for activation. Alternatively, a high-affinity trimeric IL-2 receptor comprised of the IL-2Rαα (CD25) and $\beta\gamma_c$ chain binds to IL-2 with picomolar affinity. CD25 is constitutively expressed by $CD56^{bright}$ NK cells and Tregs; however, its expression requires induction by cytokine stimulation on $CD56^{dim}$ cells [117–120]. IL-2 has been extensively studied in cancer patients and was demonstrated to be toxic at effective doses. Only few clinical responses were described after monotherapy as high-affinity binding of IL-2 to Tregs results in extensive expansion, sequestration of IL-2, and inhibition of NK cell responses [121]. Interestingly, despite these undesirable side effects, combination therapy of IL-2 with anti-tumor monoclonal antibodies was shown to be safe and demonstrated ADCC-related clinical response in some neuroblastoma and melanoma patients [122].

In contrast, IL-15 does not activate Tregs. IL-15 is trans-presented to the intermediate affinity receptor complex IL-15R$\beta\gamma_c$ on

Table 2. Continued

Bispecific Format	Compound/Program	Target combination	Clinical phase (indication)	Company [references]
cLC / hetero-H-chain IgG 	MCLA117	CLEC12A CD3	I/II (AML)	Merus [155]
	REGN1979	CD20 CD3	I (B-cell malignancy)	Regeneron [106]
	ERY974	GPC3 CD3	I (ST)	Chugai
IgG assembled from half antibodies bsmAb 	RG7828, BTCT 4465A (KiH)	CD20xCD3	I (NHL, CLL)	Genentech [156]
	JNJ 63709178 Duobody	CD123 CD3	I (AML)	Janssen, Genmab [157]
scFv-Fc-(Fab) -fusion 	Xmab14045 Fab-scFv-Fc	CD123 CD3	I (AML)	Xencor, Novartis,
	GBR1302 Fab-scFv-Fc	Her2 CD3	I (Her2+ tumors)	Glenmark [113]
scFv-Fc-(Fab) -fusion 	MOR 209, ES414 ScFv2-Fc-scFv2	PSMA CD3	I (PC)	Morphosys, Emergent Biosolutions [107]

ADC = Adenocarcinoma, ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphocytic leukemia; CRC = colorectal cancer; GC = gastric cancer; GIC = gastrointestinal cancer; LC = lung cancer; MM = multiple myeloma; NH = non-Hodgkin lymphoma; PC = prostate cancer; ST = solid tumors

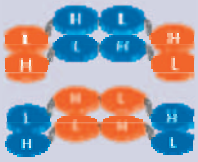
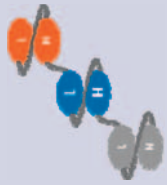
*Symbols for the respective bi- or trispecific antibody molecules are: Variable light chain domains (L) and variable heavy chain domains (H) are shown for CD3 (green) and for CD16 (orange) as well as for other antigen-binding domains (blue or grey). For the IL-15 TriKE molecule, the IL-15 domain is shown as black square.

NK cells by high-affinity receptor chain IL-15α, which is expressed on dendritic cells and macrophages [123]. Ligation induces NK cell differentiation and proliferation as well as cytotoxicity of the NK cells via JAK1, JAK3, and STAT5 signaling [124]. Deletion of IL-15, its receptor, or its downstream signaling proteins results in NK cell lymphopenia [125]. The IL-15α chain can also be secreted and endocytosed, and subsequently trans-present IL-15 to other cells [126, 127].

In non-Hodgkin's lymphoma, high concentrations of serum IL-15 following autologous hematopoietic stem cell transplantation are associated with better survival [128]. Moreover, in a phase I clinical trial in patients suffering from metastatic malignancies, daily infusion of recombinant human IL-15 induced NK cell proliferation, albeit, without objective response [129]. Several clinical studies with recombinant human IL-15 are currently ongoing in melanoma, renal cell carcinoma (NCT01021059, NCT01369888), and advanced cancers (NCT01572493, NCT01727076). Another clinical study deals with the support of NK cells after adoptive transfer in leukemia patients (NCT01385423).

Combination of bispecific NK cell-based immuno-engagers with IL-15 stimulation might be a promising therapeutic concept for various malignancies as it combines tumor targeting with improved differentiation, proliferation, and activation of immune cells. Therefore, this combination therapy might be particularly interesting in the context of solid tumors, which inhibit activation and infiltration of NK cells, which is otherwise a positive prognos-

Table 3. Overview of bi-specific NK-cell engagers in clinical development*

Structure	Compound	Target combination	Clinical phase (indication)	Company [references]
TandAb 	AFM13	CD30 x CD16A	II (HL)	Affimed [9]
	AFM24	EGFR x CD16A	preclinical	Affimed
	AFM26	BCMA x CD16A	preclinical	Affimed
BiKE 	n.d.	CD33 x CD16	preclinical	[92, 114, 115, 158]
IL-15 TriKE 	n.d.	CD33xIL-15xCD16	preclinical	Oxis Biotech [92, 114, 115, 158]
TriKE 	n.d.	CD19xCD22xCD16	preclinical	[92, 114, 115, 158, 159]

HL = Hodgkin lymphoma; n.d. = not determined.
*Symbols for the respective bi- or trispecific antibody molecules are: Variable light chain domains (L) and variable heavy chain domains (H) are shown for CD3 (green) and for CD16 (orange) as well as for other antigen-binding domains (blue or grey). For the IL-15 TriKE molecule, the IL-15 domain is shown as black square.

tic marker in renal cell carcinoma, NSCLC, and colorectal cancer [26, 27, 28, 29, 30]. Due to the poor bioavailability of IL-15, its short plasma half-life of <40 min, and an unfavorable relation between required dosing and toxicity, the potential of recombinant IL-15 is limited [130]. In contrast, fusion proteins combining IL-15 and its high-affinity receptor subunit IL-15α display reduced renal clearance, increased potency, increased plasma half-life, and increased retention times in lymphoid tissues when compared to IL-15 alone.

Two molecules employing IL-15 and IL-15Rα combinations are currently being tested in in phase I/II mono and combination trials in multiple myeloma, non-Hodgkin's lymphoma, and solid tumors (NCT02452268, NCT02099539, NCT03003728, NCT02523469, NCT03022825, NCT02138734, NCT01946789, NCT02559674, NCT02384954) [119, 131].

To improve NK cell numbers and anti-tumor activity, a variety of protocols to expand cells ex vivo have been established using NK cells derived from either the patient (autologous setting) or from a healthy donor (allogeneic setting) (fig. 4). The most established source for NK cells is peripheral blood, with current efforts exploring the suitability of NK cells derived from bone marrow, umbilical cord blood, human embryonic stem cells, or induced pluripotent stem cells [132]. Enrichment of NK cells is usually achieved by magnetic depletion of T and B cells with or without additional selection based on CD56.

Preferably, autologous NK cells are used for cell transfer as these are less likely to promote autoimmune reactions. Indeed, ex vivo cytokine activated and expanded autologous NK cells were shown to be safe; however, no clinical responses in cancer patients with metastatic melanoma, renal cell carcinoma or advance gastrointestinal cancer were seen [133–135]. These limitations could potentially be overcome by combination of autologous NK cells (either the patients' endogenous NK cells or reinfused autologous cells after ex vivo stimulation/expansion) in combination with a bispecific immuno-engager and optional IL-15.

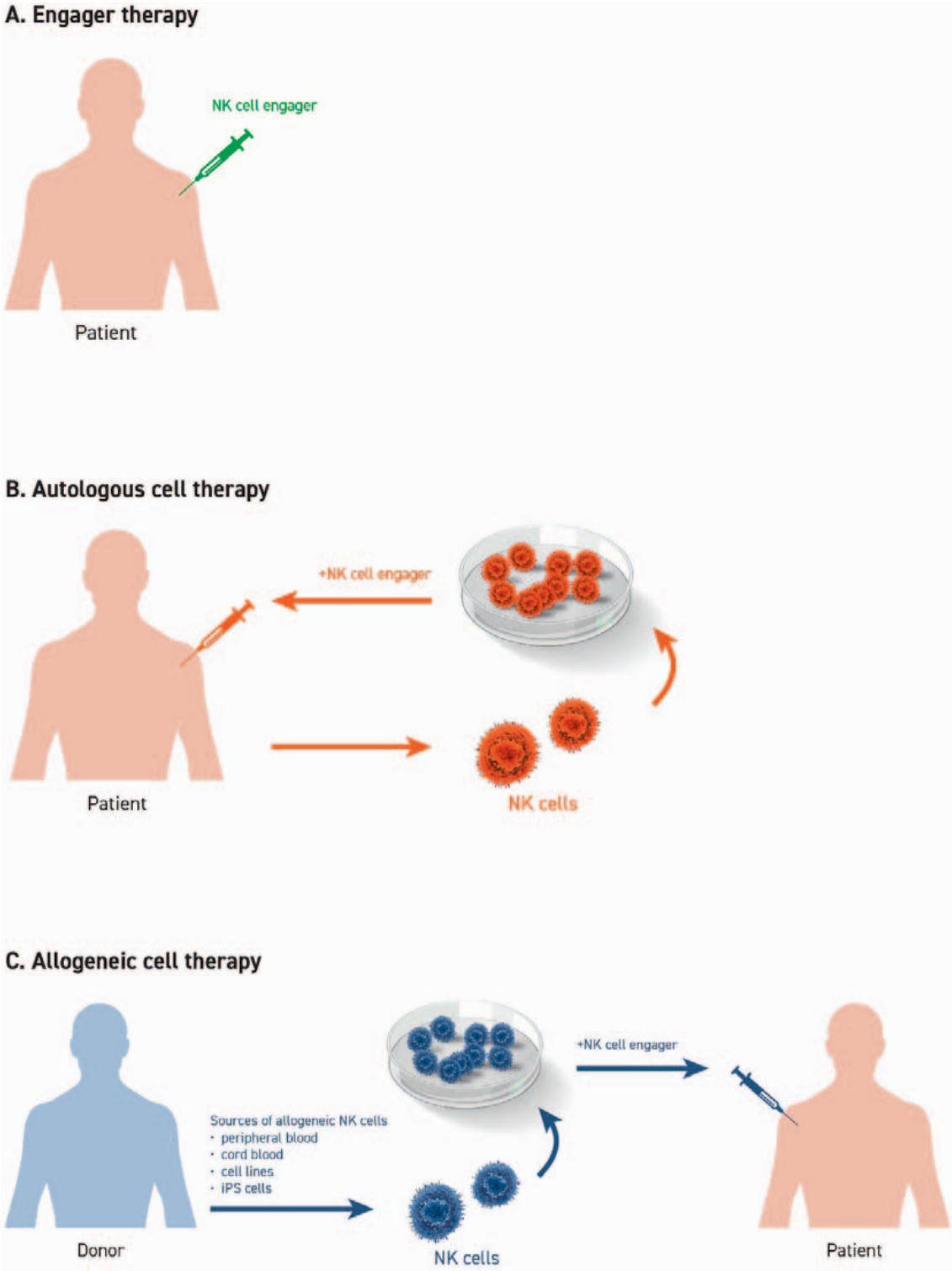


Fig. 4. Adoptive NK cells in cancer therapy. **A** A patient's own NK cells can be stimulated by monotherapy using NK cell engagers to overcome tumor immune evasion and immunosuppression. **B** Ex vivo expansion and stimulation of autologous NK cells followed by re-infusion in combination with NK cell engagers is a viable therapeutic approach providing increased numbers of activated NK cells. **C** Alternatively, NK cells can be derived from peripheral blood, cord blood or iPS cells of healthy donors (allogeneic setting) or from immortalized cell lines. After ex vivo stimulation and expansion, NK cells are infused into the patient in combination with NK cell engagers.

Conclusion and Outlook

Immunotherapy is a highly promising approach to cancer therapy. However, efficacy and safety of cellular immunotherapy based on activated or genetically engineered donor-derived immune effector cells need to be improved. Immuno-engagers enable tumor targeting and anti-tumoral cytotoxicity of transferred immune effector cells and therefore represent promising therapeutic avenues. Solid tumors including EGFR+ cancers such as breast cancer, NSCLC and head and neck squamous cell carcinoma, where immunosuppressive tumor microenvironments prevail, could particularly benefit from these therapies. Different variations and combinations of immuno-engager formats, targets on tumor cells, and targets on immune effector cells are currently being investi-

gated. Each of these has unique properties in terms of molecular weight, number of binding sites, specificity, serum half-life, and the potential to mediate cross-talk between different immune cells and must therefore be evaluated for its respective potency. Future and ongoing clinical studies will serve to identify which immuno-engager and which combination product (including cytokines, check point inhibitors and adoptively transferred cells) is best suited for the treatment of a particular cancer and addresses the need for personalized therapy.

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