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Lymphoma (JL Muñoz, Section Editor)

The Future of Natural Killer Cell Immunotherapy for B Cell Non-Hodgkin Lymphoma (B Cell NHL)

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Opinion statement

Natural killer (NK) cells have played a critical—if largely unrecognized or ignored—role in the treatment of B cell non-Hodgkin lymphoma (NHL) since the introduction of CD20-directed immunotherapy with rituximab as a cornerstone of therapy over 25 years ago. Engagement with NK cells leading to lysis of NHL targets through antibody-dependent cellular cytotoxicity (ADCC) is a critical component of rituximab's mechanism of action. Despite this important role, the only aspect of B cell NHL therapy that has been adopted as standard therapy that even indirectly augments or restores NK cell function is the

introduction of obinutuzumab, a CD20 antibody with enhanced ability to engage with NK cells. However, over the last 5 years, adoptive immunotherapy with effector lymphocytes of B cell NHL has experienced tremendous growth, with five different CAR T cell products now licensed by the FDA, four of which target CD19 and have approved indications for some subtype of B cell NHL—axicabtagene ciloleucel, brexucabtagene autoleucel, lisocabtagene maraleucel, and tisagenlecleucel. These T cell-based immunotherapies essentially mimic the recognition, activation pathway, and cytotoxic machinery of a CD19 antibody engaging NK cells and lymphoma targets. Despite their efficacy, these T cell-based immunotherapies have been difficult to implement because they require 4–6 weeks of manufacture, are costly, and have significant toxicities. This renewed interest in the potential of cellular immunity—and the manufacturing, supply chain, and administration logistics that have been addressed with these new agents—have ignited a new wave of enthusiasm for NK cell-directed therapies in NHL. With high safety profiles and proven anti-lymphoma efficacy, one or more new NK cell-directed modalities are certain to be introduced into the standard toolbox of NHL therapy within the next few years, be it function-enhancing cytokine muteins, multi-domain NK cell engagers, or adoptive therapy with expanded or genetically modified NK cells.

Introduction

Approximately 90,000 new cases of lymphoma are diagnosed in the USA per year, approximately 90% of which (estimated 81,560 in 2021 per American Cancer Society) are non-Hodgkin lymphoma (NHL), and the majority of these are B cell NHL [1–3]. While the survival rate for newly diagnosed children and adolescents with B cell NHL treated with chemotherapy and antibody-based regimens has more than doubled from the 1970s to the early 2000s (45% to > 90%) [1], the prognosis is dismal in patients with relapsed/refractory B cell NHL [4, 5, 6••, 7, 8, 9•]. Novel approaches with CD19- and BCMA-targeted CAR T cellular immunotherapy have recently been approved by the FDA for patients with relapsed/refractory B cell NHL including CD19 CAR T cells [10, 11••, 12••, 13••]. However, treatment with CAR T cells is complicated by high cost, manufacturing logistics, and toxicity.

In contrast, NK cells have similar cytotoxic effector mechanisms as T cells but appear to have a broader safety profile and are more amenable to generating allogeneic ready-to-infuse (a.k.a. "off-the-shelf") products. Like T cells, NK cells can also be genetically modified for antigen-specific targeting [3]. Liu et al. recently reported the safety and efficacy of cord blood derived CD19 CAR NK cells in patients with relapsed/refractory CD19 B-cell NHL and chronic lymphocytic leukemia (CLL) [13••]. We now summarize the clinical and preclinical experience with NK cells and CAR NK cells in B-cell NHL.

Endogenous NK cells in B-cell NHL

NK cells are innate lymphocytes that play a key role in the recognition of cells that are cancerous or virus-infected. NK cell activation or inhibition (tolerance) is determined by an integrated balance of signals from NK cell activating and inhibitory receptors binding to their corresponding ligands on target cells [14] (Fig. 1). As part of our first line of defense, NK cells exert their effector function directly via cellular cytotoxicity and indirectly via proinflammatory cytokine

secretion. In the last few decades, NK cells have moved to the forefront of immune oncology for several reasons. First, improved understanding of NK cell biology, discovery of new NK cell sources, and advances in NK cell culture techniques have made it possible to expand and activate NK cells for adoptive cell therapy. In addition, in contrast to T cells, NK cells do not depend on

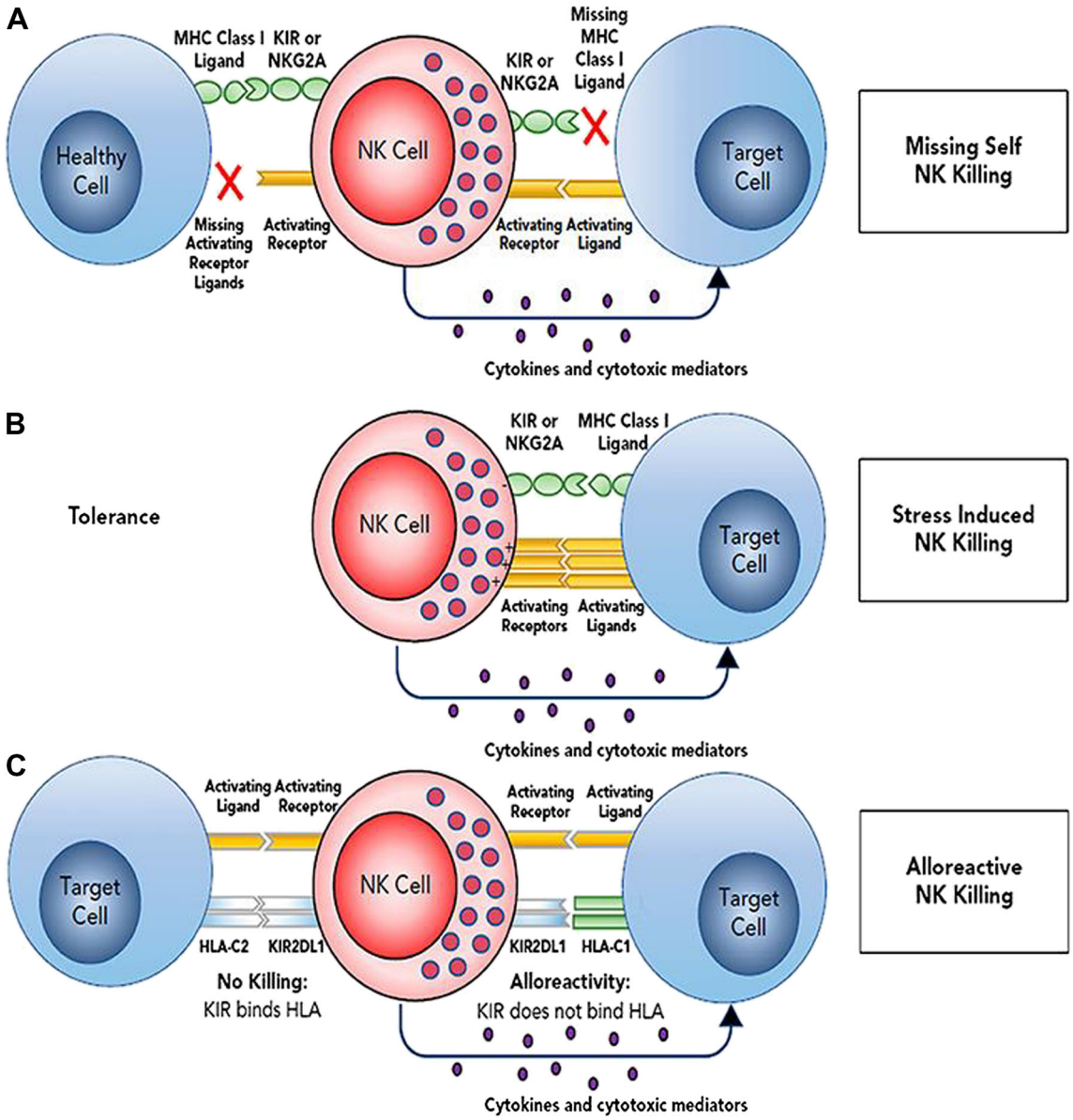

Fig. 1. NK cell recognition of target cells. NK cell effector function is dependent on a balance of activating and inhibitory signals to distinguish between healthy cells (tolerance) and cancer or virally infected cells. The absence of MHC Class I ligand (missing self, observed with HLA downregulation or induced with KIR-ligand mismatch in HLA mismatched recipient/donor pairs) combined with upregulation of stress-induced activating receptor ligands leads to target recognition, NK cell activation, and cytotoxicity. Reproduced with permission from: Cooley S, Parham P, Miller JS. Strategies to activate NK cells to prevent relapse and induce remission following hematopoietic stem cell transplantation. Blood. 2018;131(10):1053-62. doi: 10.1182/blood-2017-08-752170.

antigen presentation but instead utilize a balance of inhibitory and activating cell receptors that recognize self and stress ligands to determine effector function. Without the need for antigen presentation, NK cells can be ubiquitously effective even in cancers where tumor specific antigens remain elusive. Finally, allogeneic NK cell therapy is safe with no reports of dose limiting toxicities including graft versus host disease (GVHD), even with minimal HLA matching [15–17].

In patients with lymphoma, decreased number and function of NK cells portends a poor prognosis [18–20, 21•]. Methods of immune evasion in the lymphoma tumor microenvironment (TME) include immune checkpoints, hypoxia-induced immune modulation, and aberrant NK cell receptor/ligand expression [22–27]. Similar to the well described graft-versus tumor effect in hematopoietic stem cell transplant (HSCT) for leukemia, early and robust NK cell recovery after autologous and allogeneic HSCT in lymphoma is associated with improved survival [28–32]. To facilitate improved NK cell function after autologous HSCT in lymphoma, several studies administered low dose recombinant IL-2 as maintenance immunotherapy to prevent relapse [33–35]. In a study of relapsed/refractory NHL patients undergoing autologous HSCT, 10 patients received low dose recombinant IL-2 (rIL2) as maintenance therapy for 12 months after autologous HSCT. Following rIL2 therapy, peripheral blood (PB) samples from patients had a significant increase in NK cell number, function, and CD16-mediated ADCC compared to their baseline number and function before therapy [33]. None of the 10 patients treated on the protocol had relapse of their disease, with a median follow up of 16 months from the start of rIL2. Interestingly, two patients who still had residual disease after HSCT showed complete resolution of the residual disease while receiving rIL2 therapy. A similar study by Miller et al. using low dose IL-2 after autologous HSCT in patients with lymphoma and breast cancer also demonstrated a more than 10-fold increase in PB NK cells with enhanced cytotoxicity against resistant cell lines [35]. To build off of these early studies, clinical trials utilizing adoptive transfer of autologous ex vivo-activated NK cells or lymphokine-activated killer cells in patients with lymphoma emerged with only modest activity [34, 36–40] (Table 1). In addition, although feasible, the use of autologous NK cells was costly, often required more than one apheresis procedure, and NK cell doses were limited to around 10^7/kg [44]. To improve clinical efficacy, more recent studies have utilized highly functional expanded NK cells and/or allogeneic NK cell sources to facilitate the graft versus tumor effect. Yang et al. demonstrated the safety of expanded random healthy donor PB NK cells for treatment of malignant lymphoma and other solid tumors [42]. Multiple doses of up to 3×10^7/kg were administered with no dose limiting toxicities and no GVHD. Clinical responses, however, were limited with only 8/17 patients with stable disease as best response overall response and no complete responses. Although the safe use of donor derived NK cells represented progress in the field, alloreactive NK cells alone did not appear to be enough to eliminate bulk disease.

Targeting NK cells to NHL with antibodies

Antibody-based therapy has become a critical part of the treatment landscape in hematologic malignancies in the past few decades and several monoclonal

Table 1. Published results of adoptive NK cell therapy for lymphoma

Lymphoma type	NK Cell product	NK Cell source	NK cell dose	Lymphodepleting chemotherapy	Combination therapy	Outcomes	Author	Year
HL (n = 3), NHL (n = 8), Breast Cancer (n = 1)	Expanded	Autologous	6.8×10^8–4×10^{10} NK Cells for 1 dose	None	IL-2	Not Reported	Lister et al. [36]	1995
B cell NHL (n = 6)	Overnight IL-2 Activation	Haploidentical	2×10^6–40×10^6/kg for 1 dose	Fludarabine, Cyclophosphamide	IL-2 + rituximab	2 CR, 2 PR	Bachanova et al. [41]	2010
NHL (n = 6) HL (n = 2) MM (5)	Overnight IL-2 Activation	Haploidentical	1×10^5–2×10^7/kg for 1 dose	None		Primary endpoint safety, 8/13 in remission	Klingemann et al. [40]	2013
NHL (n = 2) and Advanced Solid Tumors (n = 18)	Expanded	Unrelated healthy donor	1×10^6–3×10^7/kg for 1–3 doses	None		8/17 SD	Yang et al. [42]	2016
NHL (n = 15)	Overnight IL-2 Activation	Haploidentical	0.5–3.27×10^7/kg for 1 dose	Fludarabine, Cyclophosphamide, Methylprednisolone	IL-2 + rituximab	4/15 ORR, 2/15 CR	Bachanova et al. [21]	2018
NHL (n = 9)	Expanded	Autologous	1×10^6–1×10^7/kg for 1 dose	none	rituximab	7/9 with CR	Tanaka et al. [43]	2020

NK natural killer, *HL* Hodgkin lymphoma, *NHL* non-Hodgkin lymphoma, *IL-2* interleukin-2, *CR* complete response, *PR* partial response, *MM* multiple myeloma

antibodies have been FDA approved that target the lymphoma-specific antigens CD19 (loncastuximab tesirine and tafasitimab-cxix), CD20 (rituximab, obinutuzumab, ofatumumab, ibritumomab tiuxetan), CD30 (brentuximab vedotin), CD52 (alemtuzumab), CD38 (daratumumab, isatuximab), CD79b (polatuzumab vedotin), and CCR4 (mogamulizumab). One of the mechanisms by which antibodies mediate tumor cell lysis is through ADCC by NK cells. NK cells recognize the Fc portion of antibodies bound to the surface of target cells via the Fc-gamma receptor III (CD16). Optimal efficacy of antibody therapy depends on high number and function of NK cells. In patients with DLBCL and follicular lymphoma treated with anti-CD20 monoclonal antibodies, low pre-treatment NK cell count was associated with shorter progression free survival and decreased overall survival compared to patients with higher pre-treatment NK cells [45]. NK cell ADCC has been exploited in antibody therapy by systemic cytokine stimulation of endogenous NK cells [46–50] or in combination with adoptive NK cell therapy [43, 51]. Autologous cytokine-expanded NK cells were combined with chemotherapy and rituximab in 9 patients with relapsed CD20-positive lymphoma patients to enhance ADCC [43]. A single dose of escalating expanded NK cells (1×10^6/kg, 3×10^6/kg, and 10×10^6/kg) was given on the day after rituximab. Complete responses were observed in 7/9 patients and there was a significant increase in PB NK cells and cytolytic activity in all patients two weeks after infusion. In a phase II trial, patients with relapsed/refractory $CD20^+$ NHL were given IL-2-activated haploidentical PB NK cells after lymphodepleting chemotherapy [21•]. A single dose of 0.5–3.27 × 10^7 NK cells/kg was given in combination with IL-2 every other day × 6 doses and weekly rituximab × 4 doses. The NK cells were well tolerated and elicited responses in 4/14 evaluable patients, including 2 complete responses. Importantly, the authors noted improved NK cell persistence and effector function in patients with higher endogenous IL-15 at the time of NK cell infusion highlighting the importance of in vivo cytokine stimulation. Based on this and similar observations, subsequent studies utilizing IL-15 alone or in combination with adoptive NK cell infusion are being investigated. ALT-803 is an IL-15 super agonist complex developed to mimic physiologic trans-presentation of IL-15 and prolong the half-life. A phase I study of ALT-803 in 33 adult patients with hematologic malignancies who relapsed after allogeneic HSCT demonstrated that the cytokine therapy was safe and significantly increased NK and $CD8^+$ T cell number and function [52]. Although only 4 patients had objective responses (1 CR, 1 PR, 2 SD) after 5 doses of ALT-803, the enhanced immune milieu and tolerability has led to further trials of ALT-803 and related compounds combined with rituximab [53] and/or adoptive NK cell therapy, including in lymphoma (NCT02890758) (Table 2).

Targeting NK cells to NHL with CARs

With the success of CD19 CAR T cell therapy in hematologic malignancies, there has been a push to develop CAR NK cells targeting a wide variety of tumor antigens. Currently, the FDA-approved CAR T cell products are manufactured from autologous T cells due to the risk of GVHD from the native T cell receptor in allogeneic CAR T cells. Manufacturing CAR T cells on a patient-by-patient basis is costly, time consuming, and often fails due to poor T cell function in

Table 2. Clinical trials utilizing adoptive NK cell therapy for lymphoma

NK Cell Source	Combination Therapy	Disease	Phase	NCT Number	Status	Country
Umbilical Cord Blood NK Cells	AFM13	CD30+ HL and NHL	Phase I	NCT04074746	Recruiting	USA
Haploidentical NK Cell Enriched DLI	Haploidentical HSCT	NHL, HL, MM, CLL	Phase I	NCT03524235	Recruiting	USA
Umbilical Cord Blood NK Cells	rituximab, Autologous HSCT	NHL	Phase II	NCT03019640	Recruiting	USA
Universal Donor Expanded NK Cells	ALT803	HL, NHL, AML, MDS, ALL, CML, CLL, Solid Tumors	Phase I	NCT02890758	Recruiting	USA
Natural Killer Cells (Source Unspecified)	rituximab	B Cell Lymphoma	Phase I/II	NCT02843061	Completed	China
Umbilical Cord Blood NK Cells	Umbilical cord blood HSCT +/- rituximab	HL, NHL AML, ALL, MDS, CML	Phase II	NCT02727803	Recruiting	USA
Haploidentical NK Cells	Autologous HSCT	Lymphoma, Neuroblastoma, Solid Tumors	Phase I	NCT02130869	Completed	USA
Haploidentical NK Cell Enriched DLI	Haploidentical HSCT	Lymphoma, AML, ALL, MDS, Neuroblastoma, Rhabdomyosarcoma	Phase I/II	NCT01386619	Completed	Germany, Switzerland
Donor Derived Expanded NK Cells	Allogeneic HSCT	Lymphoma, Leukemia	Phase I	NCT01287104	Completed	USA
NK-92 Cell Line		NHL, HL, Leukemia, Myeloma	Phase I	NCT00990717	Completed	Canada
Donor Derived NK Cells	Allogeneic HSCT	Lymphoma, Leukemia, MM, MDS, Brain Tumors, Soid Tumors	Phase I/II	NCT00823524	Completed	Korea
Donor Derived NK Cells	Allogeneic HSCT	NHL, HL, ALL, AML, MDS, CLL, CML, Myeloma	Phase I/II	NCT00789776	Completed	USA
Haploidentical NK Cells	Lymphodepleting chemotherapy and IL-2	NHL, ALL, AML, MDS, CML, JMML	Phase I	NCT00697671	Completed	USA
Haploidentical NK Cells	Autologous HSCT	Lymphoma, Leukemia, Myeloma	Phase I	NCT00660166	Completed	USA
Haploidentical NK Cells	Lymphodepleting chemotherapy and IL-2	T Cell LLy, ALL, AML, JMML, MDS	Phase I	NCT00640796	Completed	USA
Haploidentical NK Cell Enriched DLI	Haploidentical HSCT	Lymphoma	Phase I	NCT00586703	Completed	USA
Matched Family Donor NK Cell Enriched DLI	Matched Family Donor HSCT	Lymphoma	Phase I	NCT00586690	Completed	USA
Donor Derived NK or T Cells	Allogeneic HSCT with alemtuzumab	Lymphoma, Leukemia	Phase II	NCT00536978	Completed	USA
Donor Derived NK Cells	Allogeneic HSCT, GM-CSF, rituximab	CD20 + NHL, CLL	Phase I	NCT00383994	Completed	USA

Table 2. (Continued)

NK Cell Source	Combination Therapy	Disease	Phase	NCT Number	Status	Country
Donor Derived NK Cells	Lymphodepleting chemotherapy, rituximab, IL-2	CD20 + NHL, CLL	Phase I/II	NCT00625729	Terminated	USA
Umbilical Cord Blood NK cells	Lymphodepleting chemotherapy, Lenalidomide, rituximab	Leukemia, Lymphoma	Phase I	NCT02280525	Active, Not Recruiting	USA
Umbilical Cord Blood NK cells	Umbilical cord blood HSCT +/− rituximab	HL, NHL, ALL, AML, MDS, CML, CLL, Myeloma	Phase I	NCT01619761	Active, Not Recruiting	USA

NK natural killer, *NCT* national clinical trial, *HSCT* hematopoietic stem cell transplant, *HL* Hodgkin lymphoma, *NHL* non-Hodgkin lymphoma, *AML* acute myelogenous leukemia, *MDS* myelodysplastic syndrome, *ALL* acute lymphoblastic leukemia, *CML* chronic myelogenous leukemia, *LLy* lymphoblastic lymphoma, *JMML* juvenile myelomonocytic leukemia, *IL-2* interleukin-2, *CR* complete response, *PR* partial response, *MM* multiple myeloma, *CLL* chronic lymphocytic leukemia, *DLI* donor lymphocyte infusion, *GM-CSF* granulocyte-monocyte colony-stimulating factor

heavily pretreated cancer patients. CARs usually contain a single chain variable fragment from a monoclonal antibody, a transmembrane hinge region, a signaling domain such as CD3-zeta, and one or more co-stimulatory domains such as CD28, 4-1BB, or 2B4 (CD244) [54, 55] (Fig. 2). Since NK cells utilize many of the same signaling domains and activation pathways as T cells, NK cells can also be redirected to specifically target tumor antigens by the introduction of a CAR, which then permanently recapitulates the targeting function of an antibody and activation pathways for cytotoxicity. Moreover, the addition of a CAR does not abrogate the NK cell's innate recognition of cancer through endogenous receptors. One clear advantage of NK cells is the ability to use donor derived, "off-the-shelf" NK cells that minimize or eliminate the need for HLA matching without the risk of GVHD. Recently, Liu et al. developed a novel cord blood derived, IL-15 expressing, CD19 CAR NK cells with an inducible caspase 9 suicide gene (iC9/CAR.19/IL-15 CB NK cells) [56]. In a phase I/II trial, 11 patients with relapsed/refractory CD19-positive cancers were given escalating doses of these CD19 CAR NK cells after lymphodepleting chemotherapy with fludarabine and cyclophosphamide [13••]. Of 11 patients treated (5 with CLL, 4 with NHL), 8 patients (73%) had an objective response, including 7 patients with a complete response (Fig. 3). The NK cell infusions were safe with no CRS, neurologic toxicity, or GVHD, and the NK cells were detectable (by assaying for the vector transgene) for at least 12 months after infusion. Progress in the field of genetic modification of NK cells has opened the door for true "off-the-shelf" CAR NK cell therapy. Additional

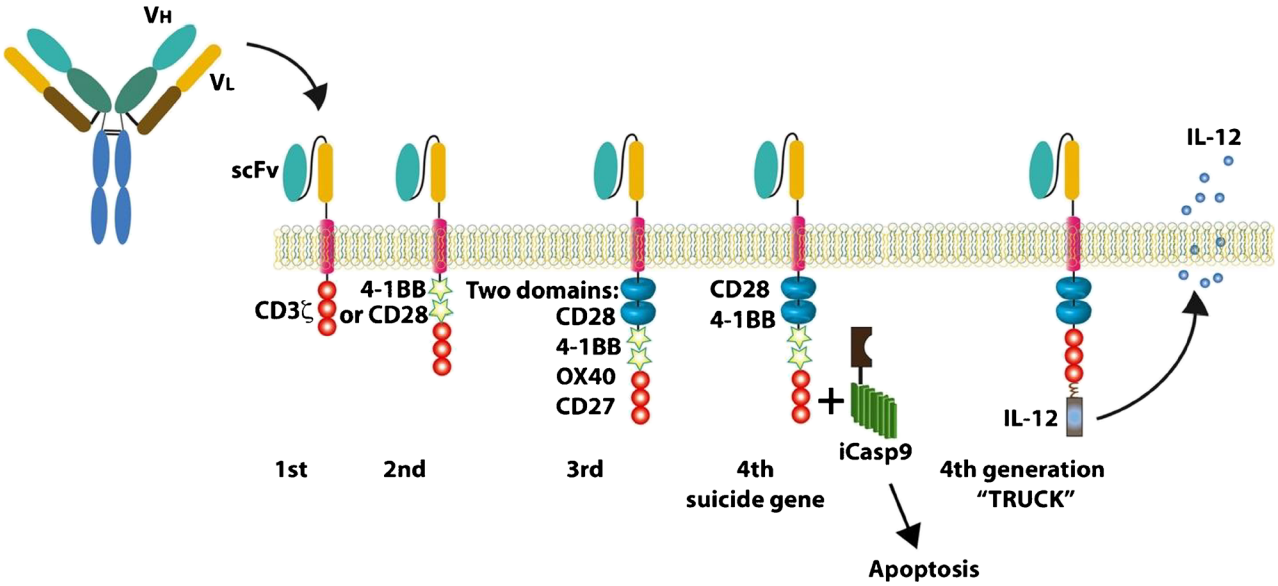

Fig. 2. Chimeric antigen receptor (CAR) structure. CAR constructs typically consist of an extracellular antigen binding domain (single chain variable fragment (scFv)), a transmembrane domain, and an intracellular signaling domain comprised of a stimulatory domain without (first generation) or with one (second generation) or more (third generation) costimulatory domains. Fourth generation CARs include additional elements such as cytokine secretion or inducible suicide genes. Reproduced with permission from: Barth MJ, Chu Y, Hanley PJ, Cairo MS. Immunotherapeutic approaches for the treatment of childhood, adolescent and young adult non-Hodgkin lymphoma. Br J Haematol. 2016;173(4):597-616. doi: 10.1111/bjh.14078.

CAR NK cell targets for lymphoma currently under clinical investigation include CD19, CD22, and CD7 (Table 3).

Sources of NK or CAR NK cells for adoptive NK cell-based immunotherapy for B cell NHL

There are numerous donor sources for isolation, purifying, and targeted NK cells for adoptive NK cell-based immunotherapy. These include autologous NK cells from patients, allogeneic NK cells from peripheral blood, umbilical cord blood, CD34 hematopoietic stem cells, embryonic stem cells, and induced pluripotent stem cells, and leukemia- or lymphoma-derived NK cell lines [34, 57–69]. An extensive review of the pros, cons, and experience to date with these NK cell sources has recently been published [70].

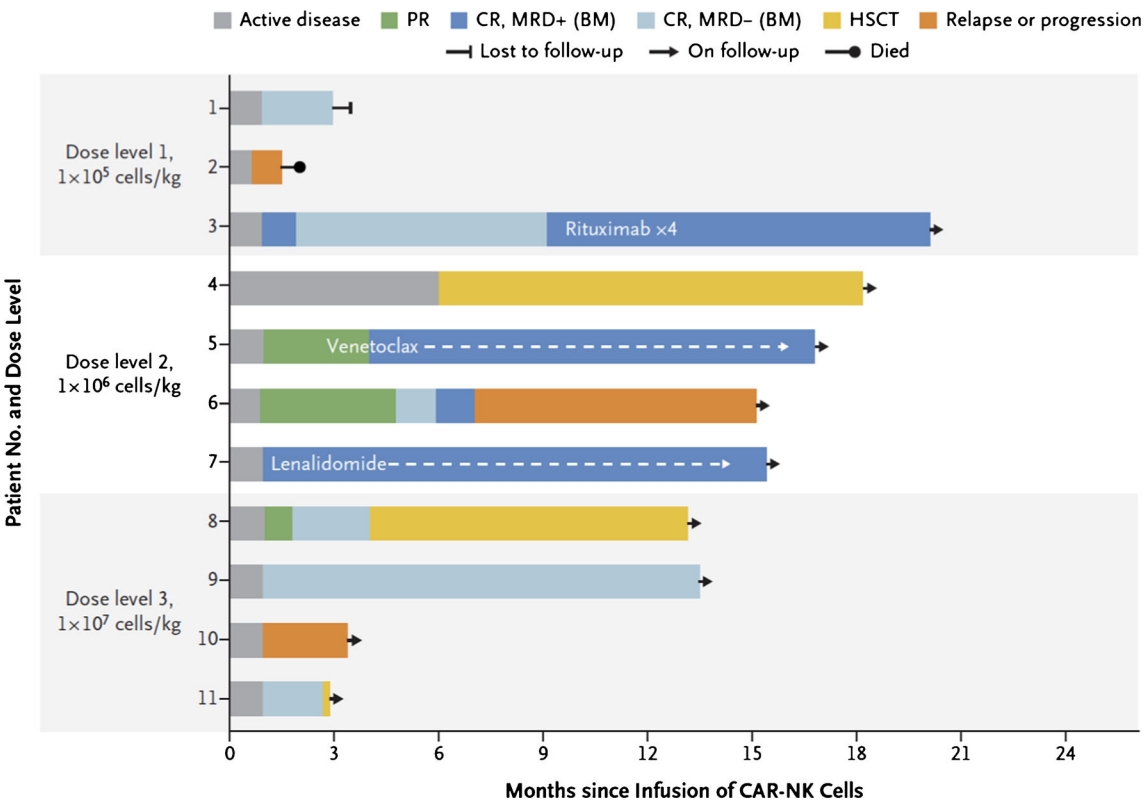

Fig. 3. Clinical responses to CD19 CAR-NK therapy for CD19 positive lymphoid malignancies. Clinical outcomes of 11 patients treated with cord blood derived, IL-15 expressing, CD19 CAR NK Cell with an inducible caspase 9 suicide gene (iC9/CAR.19/IL-15 CB NK cells). The legend denotes partial response (PR), complete response (CR), minimal residual disease (MRD), and hematopoietic stem cell transplantation (HSCT). Reproduced with permission from: Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, et al. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. N Engl J Med. 2020;382(6):545-53. doi: 10.1056/NEJMoa1910607.

Table 3. CAR-NK clinical trials for lymphoma

Target Antigen	Tumor Type	Type of NK Cell	Phase	NCT Number	Status	Country
CD19	NHL	Not Listed	Early Phase I	NCT04639739	Not yet recruiting	China
CD19	ALL, CLL, Mantle Cell and Follicular Lymphoma	NK-92	Phase I/II	NCT02892695	Unknown	China
CD19	ALL, NHL, CLL	Cord Blood	Phase I	NCT04796675	Recruiting	China
CD19	ALL, NHL, CLL	Cord Blood	Phase I/II	NCT03056339	Recruiting	USA
CD19	ALL, CLL, B Cell Lymphoma	Cord Blood	Phase I	NCT04796688	Recruiting	China
CD19	B cell lymphoma, Mantle cell and Follicular Lymphoma	Cord Blood	Phase I/II	NCT03579927	Withdrawn	USA
CD19	B Cell Lymphoma, CLL	iPSC	Phase I	NCT04245722	Recruiting	USA
CD19	B Cell Lymphoma	Not Listed	Early Phase I	NCT03690310	Not yet recruiting	China
CD19/CD22	B cell Lymphoma	Not Listed	Early Phase I	NCT03824964	Unknown	China
CD22	B Cell Lymphoma	Not Listed	Early Phase I	NCT03692767	Not yet recruiting	China
CD7	AML, T-ALL, T-LLy, NK/T-LLy	NK-92	Phase I/II	NCT02742727	Unknown	China

CAR chimeric antigen receptor, *NK* natural killer, *NCT* national clinical trial, *NHL* non-Hodgkin lymphoma, *AML* acute myelogenous leukemia, *ALL* acute lymphoblastic leukemia, *LLy* lymphoblastic lymphoma, *CLL* chronic lymphocytic leukemia

Ex vivo NK cell expansion for adoptive NK cell-based immunotherapy for B cell NHL

Ex vivo NK expansion with feeder cells for B cell NHL

To overcome the limitation of small number of active NK cells in the donor PB, NK cells can be ex vivo activated and expanded with feeder cells such as irradiated PBMCs [71–73], Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) [74], or gene-modified cell lines such as K562 [75–78]. Preclinical studies demonstrated that highly cytotoxic NK cells can be expanded with irradiated and activated autologous PBMCs to efficiently kill lymphoma cells in vitro and in mice xenografted with human lymphoma cells [71–73]. These NK cells expanded with irradiated autologous PBMC are suitable for allogeneic transfer without the risk of graft-versus-host disease induction [71–73]. Our group and others have successfully expanded active PB NK cells or CB NK cells in vitro by co-culture with irradiated EBV-LCL, or with K562 cells expressing transfected cell-membrane bound IL-15 and 4-1BBL to kill leukemia and B-NHL [74–77]. Lee and colleagues have developed a novel feeder cell line what was engineered to express membrane bound IL-21 and 4-1BB ligand (K562-mbIL21-41BBL) to expand PB NK or CB NK ex-vivo [78]. This method resulted in over 35,000-fold increase in NK cells in 3 weeks while avoiding telomere shortening and NK cell senescence and significant increase in NK cell functional activation against lymphoma (Fig. 4) [78].

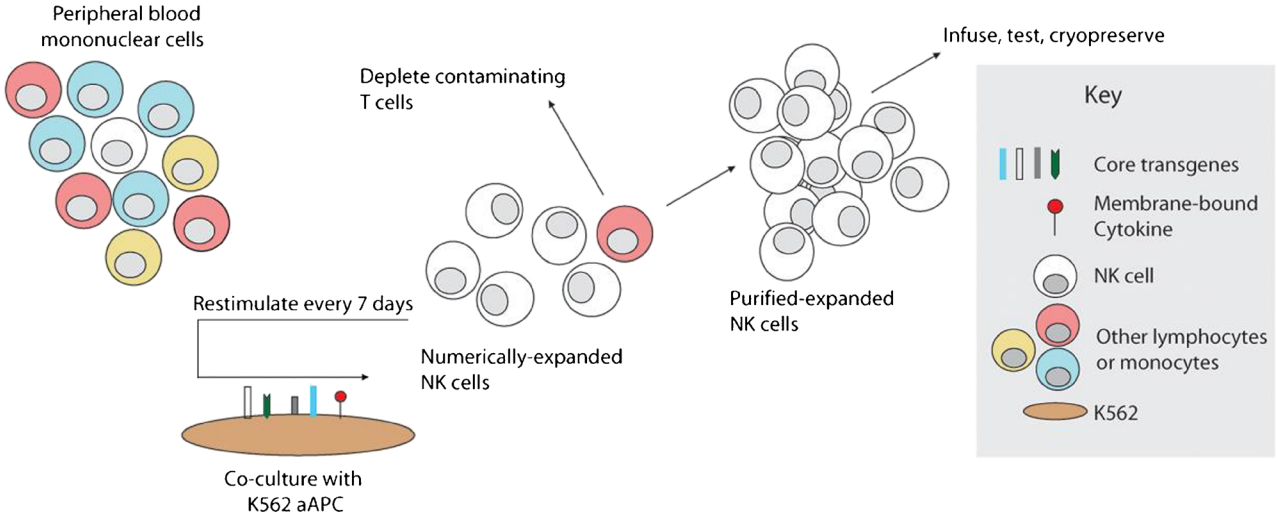

Fig. 4. Schema for NK cell manufacturing with genetically-engineered feeder cells. Feeder cells were produced by genetic modification of K562 to express costimulatory molecules and membrane-bound cytokines. To expand NK cells *ex vivo*, unfractionated PBMC are stimulated weekly with irradiated PBMC, inducing rapid proliferation of NK cells and a variable degree of non-specific expansion of T cells. Contaminating T cells may be depleted before or during expansion, and the remaining purified NK cells may be stimulated weekly by the artificial antigen-presenting cells as needed to obtain sufficient numbers. Expanded NK cells may be used directly or cryopreserved for future use. Reproduced with permission from: Denman CJ, Senyukov VV, Somanchi SS et al. (2012), Membrane-Bound IL-21 promotes sustained *ex vivo* proliferation of human Natural Killer cells. PLoS ONE 7(1): e30264. doi: 10.1371/journal.pone.0030264.

Ex-vivo NK cell expansion without feeder cells for B cell NHL

NK cells can also be ex vivo activated and expanded with feeder-free systems. Clinical responses were observed in 4 of 6 B cell NHL patients who were administered rituximab and haploidentical donor PB NK cells activated with IL2 [41]. However, due to host Treg proliferation stimulated by the IL2, the donor NK cell expansion was inhibited in the peripheral blood [41]. Nicotinamide (NAM) is a form of Vitamin B3. Preclinical studies showed that NAM enhanced expansion (60-80 fold) of functional donor NK cells in feeder-free cultures stimulated with IL-2 and IL-15 for 2 weeks and the expanded NK cells with NAM displayed the increased in vitro cytotoxicity against Burkitt lymphoma (BL) cells, in vivo homing, and survival in immunodeficient mice [79]. Additionally, NK cells can be expanded with a feeder-free, particle-based approach, which uses plasma membrane particles (PM-particles) derived from K562-mbIL15-41BBL or K562-mbIL21-4-1BBL cell lines [80, 81], but preclinical studies in B cell NHL have not yet been reported.

Preclinical studies of CAR engineered NK cells for B cell NHL

CAR NK therapy is promising and may provide some advantages over CAR T cells such as low risk of on-target/off-tumor toxicity to normal tissues, reduced risk for GVHD, and reduced frequency and severity of cytokine release

syndrome (CRS) or immune effector cell-associated neurotoxicity (ICANS) [55]. NK cells can be engineered to express CAR through several technologies such as transposons, messenger ribonucleic acid (mRNA)-mediated gene delivery, lentiviruses, and CRISPR/AAV targeted gene insertion [82]. We and others have engineered expanded PB NK (exPBNK) cells with anti-CD19 CAR or anti-CD20 CAR utilizing retroviral integration or CAR mRNA electroporation to target B cell malignancies including B-NHL [83, 84]. We previously reported that anti-CD20 CAR exPBNK cells had significantly enhanced in vitro cytotoxicity against BL, limit BL tumor metastasis, and extended the survival of NSG mice xenografted with human BL cells [84]. The combination of anti-CD20 CAR exPBNK cells with a histone deacetylase inhibitor romidepsin, which enhanced expression of NKG2D ligands on the surface of BL, significantly killed BL, reduced tumor burden, and extended the survival in NSG mice xenografted with human BL cells [85]. Similarly, engineering the NK-like lymphoma cell line NK92 with an anti-CD19 CAR significantly increased in vitro cytotoxicity and prominently induced apoptosis in rituximab- and obinutuzumab-resistant cell lines and patient-derived cells, and delayed tumor growth in B-NHL xenografts [86]. Liu et al. genetically modified NK cells expanded from cord blood (CB) cells with a retroviral vector (iC9/CAR.19/IL-15) [56]. Their preclinical studies demonstrated that iC9/CAR.19/IL-15 CB NK cells efficiently killed $CD19^+$ primary leukemia cells and Raji in vitro and significantly prolonged the survival in a xenograft Raji lymphoma murine model [56]. Emerging preclinical evidence shows that IL-12 and IL-18 plus IL-15 can induce murine and human NK cells with long-lasting memory-like functionality [87]. These cytokine-induced memory-like (CIML) NK cells display increased proliferative capacity, prolonged persistence in vivo, and superior functionality in killing of rituximab-coating Raji lymphoma cells compared with control NK cells in vitro, and substantially reduced growth of established lymphoma tumors in mice [88–90]. Anti-CD19 CAR-modified CIML NK cells displayed significantly increased interferon-γ (IFNγ) secretion and cytotoxicity against NK-resistant lymphoma lines and primary lymphoma tumor cells, and significantly reduced lymphoma burden and significantly improved the survival in human lymphoma xenograft models [91]. Goodridge et al. developed a CAR NK product FT596, derived from a master induced pleuripotent stem cell (iPSC) line engineered to uniformly express anti-CD19-CAR, an enhanced functioning high-affinity, non-cleavable CD16, and a recombinant fusion of IL-15 and IL-15 receptor alpha (IL-15RF) [92]. FT596 showed significantly enhanced clearance of Raji tumor cells in combination with rituximab in a Raji xenografted mouse model [92]. Furthermore, utilizing an allogenic human CD34 engrafted NSG mouse model, FT596 demonstrated improved survival and safety over primary CAR19 T cells, either as a monotherapy or as a combination therapy with rituximab against Raji tumor cells [92]. These preclinical findings have provided the preliminary results to conduct a Phase I dose-finding study of FT596 as monotherapy and in combination with rituximab or obinutuzumab in subjects with relapsed/refractory B cell lymphoma or CLL (NCT04245722).

Preclinical studies of combinatorial therapies of NK cells for B cell NHL

A large number of preclinical studies of combination therapies incorporating NK cells for B cell NHL have been reported (Table 4), a number of which are summarized below.

Combinatorial therapy of NK cells with a novel type II anti-CD20 antibody Obinutuzumab

Obinutuzumab is a humanized, type II anti-CD20 monoclonal antibody glycoengineered to enhance Fc receptor affinity and has been approved for the treatment of patients with previously untreated advanced-stage follicular lymphoma [100, 101]. Our preclinical studies demonstrated that obinutuzmab has significantly enhanced ADCC compared to rituximab and induced apoptosis in BL in vitro, and the combination of obinutuzumab with exPBNK significantly enhanced overall survival of NSG mice xenografted with Raji tumor cells as compared to the combination of rituximab with exPBNK [93]. Our group is currently conducting a clinical trial to evaluate the safety and response rate of obinutuzumab as a single agent alone and in combination with ifosfamide, carboplatin and etoposide (O-ICE) without exogenous NK cells in children, adolescents and young adults with recurrent refractory $CD20^+$ mature B-NHL including BL (NCT02393157).

Combinatorial therapy of NK cells with bispecific or trispecific killer engagers

Bispecific killer engagers (BiKEs) or trispecific killer engagers (TriKEs) are designed to have one "arm" binding to CD16 on NK cells and the other one or two "arms" targeting to the specific antigen(s) on the tumor cells [102]. The engager substitutes for traditional antibody-Fc interactions in mediating the immunological synapse between tumor cells and NK cells to stimulate NK activation and killing [102]. Gleason and colleagues developed an anti-CD16/CD19 BiKE and an anti-CD16/CD19/CD22 TriKE, and showed that they trigger NK cell activation through direct signaling of CD16 to secrete lytic granules and induce BL tumor death via a caspase-3 apoptosis pathway [94•]. A TriKE designated 161519 was developed combining the cytokine IL-15 with the anti-CD16 scFv and the anti-CD19 scFv (98) in order to link cytokine signaling with antigen-specific NK cell activation. Preclinical studies demonstrated that this novel 161519 TriKE induced more degranulation and IFNγ production in NK cells, resulting in the highest level of Raji cell death as compared with rituximab, 1619 BiKE, or controls [95], indicating the potential immunotherapeutic value of 161519 TriKE in B cell NHL.

Combinatorial therapy of NK cells with IL15 superagonist (N-803) and rituximab

IL-15 shares similar functions with IL-2 but has a distinct advantage over IL-2 for cancer immunotherapy due to its minimal binding to the low-affinity IL-2 receptor CD25, resulting in a lack of effect on Tregs [103]. N-803 is an IL-15 superagonist (originally named ALT-803) that consists of a high-affinity

Table 4. Preclinical studies of human NK therapy for lymphoma

NK source	Activation and expansion method	Genetically engineered	Engineering method	Combination	Lymphoma subtype	Study stage	Year	Ref
NK-92	IL-2	No		N/A	Human BL	Preclinical	1994	[59]
haNK	N/A	Yes (CD16-158V and erIL2)	Transfection/ insertion	rituximab	Human CD20+ lymphoma	Preclinical	2016	[60]
CB	Anti-CD3, IL2, IL7, IL12	No	No	N/A	Human BL	Preclinical	2009	[64]
hESC	NK differentiation medium (IL15, IL3, IL7, SCF, Flt3L) and irradiated AFT-24 cells	No	No	rituximab	Human BL	Preclinical	2005	[67]
iPSC	N/A	Yes (ADAM17 knockdown)	CRISPR/Cas9	rituximab	Human BL	Preclinical	2020	[69]
PB	IL12, IL15, IL18	No	No	rituximab	Human BL	Preclinical	2017	[88–90]
PB	NAM, IL2, IL15	No	No	No	Human BL	Preclinical	2011	[79]
PB	irradiated autologous PBMCs	No	No	No	Human BL	Preclinical	2013, 2013, 2017	[71–73]
PB	irradiated k562-mbIL15-41BBL	No	No	No	Human BL	Preclinical	2013	[77]
CB	irradiated k562-mbIL15-41BBL	No	No	No	Human BL DLBCL	Preclinical	2017	[76]
PB	irradiated k562-mbIL21-41BBL	No	No	No	Human BL	Preclinical	2012	[78]
PB	irradiated k562-mbIL15-41BBL	Yes (anti-CD19 CAR)	mRNA electroporation	No	Human BL	Preclinical	2012	[83]
PB	irradiated k562-mbIL15-41BBL	Yes (anti-CD20 CAR)	mRNA electroporation	No	rituximab sensitive and resistant human BL	Preclinical	2015	[84]
PB	irradiated k562-mbIL15-41BBL	Yes (anti-CD20 CAR)	mRNA electroporation	romidepsin	rituximab sensitive and resistant human BL	Preclinical	2017	[85]

Table 4. (Continued)

NK source	Activation and expansion method	Genetically engineered	Engineering method	Combination	Lymphoma subtype	Study stage	Year	Ref
NK-92	IL2	Yes (anti-CD19 CAR)	Lentiviral transduction	No	Human BL, DLBCL	Preclinical	2020	[86]
CB	irradiated k562-mbIL21-41BBL	Yes (anti-CD19 CAR-IL15-iC9)	Retroviral transdduction	No	Human BL	Preclinical	2019	[56]
PB	IL12, IL15, IL18	Yes (anti-CD19 CAR)	N/A	No	Human BL	Preclinical	2020	[91]
iPSC (FT596)	N/A	Yes (anti-CD19 CAR-hnCD16-IL15RF)	N/A	rituximab	Human BL	Preclinical	2019	[92]
PB	irradiated k562-mbIL15-41BBL	No	No	Obinutuzumab	Human BL	Preclinical	2015	[93]
PB	No	No	No	CD19/CD16 BiKE CD19/CD22/CD16 TriKE	Human BL	Preclinical	2012	[94•]
PB	No	No	No	161519 TriKE	Human BL	Preclinical	2012	[95]
PB	No	No	No	CD30/CD16A (AFM13) tandem diabody	Human HL	Preclinical	2014	[96]
PB	No	No	No	rituximab	Human BL FL	Preclinical	2016	[97]
PB	No	No	No	N-820	Human BL	Preclinical	2016	[98]
PB	irradiated k562-mbIL21-41BBL	No	No	N-820	rituximab sensitive and resistant human BL	Preclinical	2020	[99]

CB cord blood; *PB* peripheral blood; *hESC* human embryonic stem cell; *SCF* stem cell factor; *Flt3L* Flt3 ligand; *iPSC* induced pluripotent stem cell; *ML* memory-like; *NAM* nicotinamide; *iC9* inducible caspase-9; *CAR* chimeric antigen receptor; *hnCD16* high-affinity, non-cleavable CD16; *BL* Burkitt lymphoma; *FL* follicular lymphoma; *mRNA* messenger ribonucleic acid; *Ref*. references

interleukin-15 mutein (IL-15N72D) and a dimeric IL-15 receptor alpha (IL-15Rα)/Fc fusion protein [104]. N-803 has at least 25 times the activity of the wild type IL-15 in vivo and a significantly longer serum half-life in vivo than wild-type IL-15 (25 h vs. < 40 min) [104, 105]. The study from Rosario et al. showed that the combination of N-803 with rituximab significantly increased the expression of granzyme B and perforin, IFNγ production, and ADCC of human NK cells against BL cell lines or primary follicular lymphoma cells [97]. The study supports the future clinical investigation of N-803 plus NK cells and anti-CD20 mAbs in patients with aggressive B cell NHL.

Combinatorial therapy of NK cells with N-820, a novel antibody-N-803 fusion

N-820 was generated by fusing four single-chains of rituximab to the N terminus of N-803 [98]. N-820 activated primary NK cells to enhance ADCC and induced apoptosis of B cell NHL in vitro and in BL xenografted NSG mice [98]. N-820 also significantly enhanced the cytotoxicity of exPBNK against rituximab-sensitive and -resistant BL cells in vitro and in BL xenografted NSG mice in vivo, as compared to controls [99]. Our study and others suggest that N-820 is an attractive novel agent to be combined with NK therapy for $CD20^{+}$ relapsed/refractory B-NHL [99].

Future directions

Although much is now established regarding the importance of NK cell number and function in the context of cancer survival, the parameters that define the optimal NK cell with respect to phenotype, function, proliferative potential, and long-term persistence are not well defined. In vitro assays that accurately reflect in vivo conditions, tumor microenvironment, trafficking, and cross-talk with other immune cells are much needed, and fully murine models have only partially filled this gap because of significant differences between mouse and human NK cell biology. It is also important to recognize that the definition of "optimal" may vary between different cancers and with different combination therapies.

This baseline understanding of optimal will be important in defining differences—pro and con—between the various starting material/sources from which NK cells are generated, expansion methods, genetic modification methods, and cytokine adjuvants. With several new options now enabling engineering of NK cells after decades of difficulty in this area, the wide variety of targeting domains, signaling and cytokine combinations, and cell sources literally provide hundreds of potential CAR NK products that are now feasible and therefor will need to be tested in robust models for each disease.

In addition, the combinations with exogenous cytokines, engagers, immune modulators, and checkpoint inhibition will result in many new options for patients that need careful investigation. To achieve the best outcome quickly, clinical investigation will require cooperation, intelligent trial design, and implementation of multi-cohort, basket, or surrogate endpoints.

Lastly, this review has focused on the relevance of NK cells in the treatment of B cell NHL. Several antibodies have been developed for T cell NHL (e.g., alemtuzumab (anti-CD52), brentuximab (anti-CD30), and mogalizumab (anti-CXCR4)), all of which mediate at least part of their anti-lymphoma efficacy

through ADCC. Thus, approaches to enhancing NK cell number and activity may also be highly relevant for T cell NHL.

Summary

NK cell-based immunotherapy holds tremendous promise for patients with B-NHL. A robust, carefully designed, and centrally coordinated systematic investigation of the available modalities should lead to significantly improved outcomes in the near future.

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Declarations

Human and Animal Rights and Informed Consent
No human or animal studies were performed for the preparation of this manuscript.

Conflict of Interest
YC and ML have no conflict of interest to declare. MC is a consultant to Jazz Pharmaceuticals and NEKTAR and serve on the speaker bureau of Jazz Pharmaceuticals, Servier, Amgen, Sanofi and Sobi. DL reports stock ownership in Courier Therapeutics, has received consulting fees and stock options in Caribou Biosciences, and has received research grants, stock ownership, royalties, and consulting fees from Kiadis Pharma.

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