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Natural Killer Cells for Therapy of Leukemia

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Summary
Clinical application of natural killer (NK) cells against leukemia is an area of intense investigation. In human leukocyte antigen-mismatched allogeneic hematopoietic stem cell transplantations (HSCT), alloreactive NK cells exert powerful anti-leukemic activity in preventing relapse in the absence of graft-versus-host disease, particularly in acute myeloid leukemia patients. Adoptive transfer of donor NK cells post-HSCT or in non-transplant scenarios may be superior to the currently widely used unmanipulated donor lymphocyte infusion. This concept could be further improved through transfusion of activated NK cells. Significant progress has been made in good manufacturing practice (GMP)-compliant large-scale production of stimulated effectors. However, inherent limitations remain. These include differing yields and compositions of the end-product due to donor variability and inefficient means for cryopreservation. Moreover, the impact of the various novel activation strategies on NK cell biology and in vivo behavior are barely understood. In contrast, reproduction of the third-party NK-92 drug from a cryostored GMP-compliant master cell bank is straightforward and efficient. Safety for the application of this highly cytotoxic cell line was demonstrated in first clinical trials. This novel 'off-the-shelf' product could become a treatment option for a broad patient population. For specific tumor targeting chimeric-antigen-receptor-engineered NK-92 cells have been designed.

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Introduction

Natural killer (NK) cells are important effectors of the innate immune system belonging to the recently defined family of 'innate lymphoid cells' [1, 2]. They develop in the bone marrow from common lymphoid progenitors and are generally characterized by surface expression of the neural cell adhesion molecule CD56 (NCAM) and lack of expression of the T-cell receptor CD3. NK cell cytotoxicity is tightly regulated by an array of surface receptors with inhibitory or activating signaling functions in a non-major histocompatibility complex(MHC)-restricted manner. Since antigen priming is not required for NK cell action, these cells are able to rapidly kill transformed cells. Attacks against healthy tissues, on the other hand, are prevented through human leukocyte antigen (HLA) class I ligand-induced effector inhibition. Thus, NK cells are able to distinguish 'self' from 'non-self'. Consequently, tumor cells or virally infected cells, which frequently down-regulate HLA expression levels to escape a T-cell response become targets for NK cell lysis due to 'missing self'. Classical HLA-A, HLA-B, and HLA-C molecules are cognate ligands for an allelic family of NK cell receptors, termed killer cell immunoglobulin-like receptors (KIRs). The number and kind of KIR family genes define the KIR haplotype of an individual. However, KIR genes are inherited independently from the MHC class I genes, and not every NK cell in the population expresses the entire KIR repertoire. To ensure 'self-tolerance', NK cells are 'educated' or 'licensed' during their development [3]. They gain functional competence through a maturation process involving interactions between KIR receptors and their respective HLA ligands. Importantly, a lack of such interactions, in the absence of inhibitory receptors or a matching ligand, leaves such cells hypo-responsive [4]. NK cells express another important inhibitory receptor, the heterodimer CD94 / natural killer group (NKG) 2A. NKG2A binds to the non-classical MHC class I molecule HLA-E. Interestingly, approximately 13% of circulating peripheral blood NK cells seems to lack both inhibitory KIRs and

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NKG2A expression. Thus, a minor fraction of peripheral blood NK cells remains hypo-responsive [5].

It is now also well established that additional signals, mediated through activation receptors, are imperative to induce a NK cell cytolytic attack. Important activating receptors include additional NKG2 group members, the homodimer NKG2D and the heterodimer CD94/NKG2C and furthermore the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46. Among the ligands recognized by activating receptors, known to date, stress-induced ligands expressed by distressed cells play an important role. NKG2D for example binds to non-classical MHC molecules, the major histocompatibility complex class I chain-related protein A (MICA) A and MICB and UL16-binding proteins (ULBPs). ULBPs have been detected on different tumors, including leukemia [6]. Another group of activating receptors comprises activating variants of KIR receptors, also referred to as aKIRs [7]. A promising role for aKIRs in preventing disease relapse in transplant patients with leukemia has been recently discovered [8].

NK cells have been exploited as immunotherapeutic agents since several decades [9, 10]. Their spontaneous cytotoxicity, potentially directed against a broad range of malignancies and infectious diseases ('non-self'), renders NK cells promising candidates for clinical applications. In this review, we summarize work done on NK cells and leukemia, starting from the role of NK cells in immune surveillance against leukemogenesis and their anti-leukemic activity in preventing relapse post allogeneic transplant. We then review the results of clinical studies using NK cells as adoptive therapy and emerging novel strategies exploiting NK cells in therapy of leukemia.

Association between KIR-HLA and Leukemia

KIR gene polymorphism may play a role in predisposition to leukemia. This has in particular been observed in acute lymphoblastic leukemia (ALL). One case-control study in Canadian children with and without B-cell ALL (B-ALL) showed that harboring a higher number of activating KIR genes is associated with reduced risk for developing B-ALL in these children [11]. Another study involving 320 pediatric B-ALL patients revealed that expression of the HLA-C-encoded supertypic epitope C2, which constitutes a high-affinity ligand for the inhibitory NK cell receptor KIR2DL1, was significantly increased in such patients [12]. A correlation could be established between increasing numbers of C2 alleles and a higher incidence of late relapse (>2.5 years). Thus, interaction of KIRs with HLA-C in NK cell immunosurveillance poses a risk factor in childhood ALL [12, 13]. Such association has also been reported for acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL), where a significantly higher frequency of the inhibitory KIR phenotype, related to the high prevalence of the inhibitory KIR2DL2, was found in leukemic patients compared to controls [14]. These observations suggest a possible role of NK surveillance in leukemogenesis.

NK Cells and Hematopoietic Stem Cell Transplantation for Leukemia

Hematopoietic stem cell transplantation (HSCT) is nowadays a well-established medical treatment option for hematologic malignancies, including the 4 main leukemia types ALL, AML, CLL, and CML [15]. Allogeneic HSCT (allo-HSCT) has curative potential essentially through the immune-mediated graft-versus-leukemia (GvL) effect [16]. In contrast to total-body irradiation or chemotherapies, the immune effectors also eradicate malignant stem cells, thus minimizing the risk for disease relapse. However, its major complication is graft-versus host disease (GvHD) caused by alloreactive T cells attacking healthy host tissues [15].

The role of NK cells in allo-HSCT was first observed in haploidentical transplants, which involved extensive T-cell depletion of three-loci mismatched hematopoietic stem cell grafts, thus enabling successful transplantation across the MHC barrier. In the absence of drugs given for GvHD prophylaxis, together with 'megadoses' of T-cell-depleted grafts, NK cells rapidly recovered and played an important role in immune reconstitution as well as exerted powerful anti-leukemic activity [17]. In a landmark study in 2002, Velardi's group demonstrated the role for donor-versus-recipient NK cell alloreactivity in transplantation outcome [18]. Donor NK cell alloreactivity protected 57 AML and 35 ALL patients against GvHD and graft rejection in haplotype-mismatched family donor transplantations. Most importantly, KIR ligand incompatibilities in graft-versus-host direction reduced the probability of AML disease relapse at 5 years to 0%, compared to 75% in patients where HLA class I alleles matched the donor KIR repertoire. The probability of event-free-survival at 5 years increased from 5% in the absence of KIR ligand incompatibilities to 60% in their presence. However, no such benefits were observed in ALL patients. Lack of ALL susceptibility to NK cell killing is consistent with in vitro and in vivo findings [19, 20] and is most likely a consequence of missing activating ligands [21]. Furthermore, the size of the alloreactive NK cell subset is of relevance. Thus, the KIR gene polymorphism needs to be taken into account for the selection of the best fitting stem cell donors [13]. The field of haploidentical transplantations is rapidly growing and may likely become a leading treatment platform in the near future [22].

One interesting finding indirectly supporting the anti-leukemic activity of NK cells post allo-HSCT was the observation that patients who developed cytomegalovirus (CMV) reactivation/infection post allo-HSCT have lower relapse rates [23]. Patients experiencing CMV reactivation among 674 allogeneic HSCT recipients were protected from leukemia relapse and experienced superior disease-free survival [24]. Similar findings were reported from a study involving 101 ALL and 42 AML pediatric patients [25]. In these patients, NK cells matured rapidly into cytotoxic $CD56^{dim}$ KIR+ NKG2A– cells as a result of response to stimulatory signals provided by CMV. In particular there was significant expansion of a NK cell subset with high surface levels of the CD94/NKG2C receptor [23, 26]. The development into 'memory'-like long-lived NK cells with adaptive immune properties was indicated.

NK Cell Infusion as Adoptive Immunotherapy for Leukemia

Based on the above observations on the anti-leukemic activity of NK cells, it is logical to consider adoptive transfer of NK cells for treatment of leukemia. In contrast to unmanipulated donor lymphocyte infusion (DLI), NK cells have the potential to exert potent anti-tumor effects toward susceptible leukemias in HLA-haploidentical allo-HSCT and yet GvHD and graft rejection could be obviated through NK cell lysis of residual host dendritic and T cells. Non-hematopoietic healthy host tissues are spared from NK alloreactivity likely accounted by a lack of activating ligands [27, 28].

Donor NK cell infusion has been explored in place of the currently widely practiced unmanipulated DLI, which may be superior especially in donor-recipient combinations where NK alloreactivity may be expected to exert anti-leukemic effect. Donor NK cell infusion following HSCT could potentially reduce relapse and protect from opportunistic viral infections. NK-DLI was first demonstrated to be safe and feasible in a pilot study in 5 high-risk myeloid leukemia patients (4 AML, 1 CML). NK cells were purified from donor leukapheresis products through a two-step immunomagnetic enrichment process using CD3 T-cell depletion followed by CD56 NK cell selection. A median NK cell dose of 1.61×10^7/kg NK cells post HSCT was well tolerated, and no GvHD was observed [29]. Similar results were obtained in 30 patients receiving up to 3 infusions of 1-step CD56 immunomagnetically selected NK cells 8 weeks after transplant [30]. NK-DLI can also be generated from granulocyte-colony-stimulating factor-mobilized CD34+ progenitor cells. Six weeks culture of magnetically enriched CD34+ cells yielded a median dose of $9{,}28 \times 10^6$/kg NK cells from 1 leukapheresis product. Infusion without further T-cell depletion (1% contamination) into 14 leukemia patients (11 AML, 1 ALL and 2 myelodysplastic syndrome patients) 6–7 weeks post-transplant was generally well tolerated. GvHD did occur in a fraction of patients, which might have been a late consequence of the haplo-HSCT [31]. In a 2-center clinical phase II trial, a median dose of $1{,}21 \times 10^7$/kg of purified NK cells was given to 16 high-risk leukemia patients on days +3, +40, and +100 after transplantation. In a 5.8-year follow-up, 4/16 patients were still alive [32]. Optimal dosage and timing of application to enhance the NK cell-mediated anti-tumor effect will need to be determined in subsequent studies.

Extrapolating the theoretical benefit of NK alloreactivity to the non-transplant setting is conceptually appealing, with the possibility of further leukemic control by cell-mediated mechanisms without the toxicity of transplant. Feasibility of this concept was clearly demonstrated in the NKAML pilot study involving 10 AML pediatric patients in first complete remission after lymphodepleting chemotherapy [33]. A median haploidentical NK cell dose of 2.9×10^7 cells/kg stimulated with an adjuvant IL-2 therapy was well tolerated. NK cells expanded and engrafted transiently giving a 2-year event-free survival of 100% [33]. In 13 elderly high-risk AML patients, alloreactive effectors could be detected in the blood stream at day 10 after transfusion of highly purified NK cells and in some cases in the bone marrow [34]. Strikingly, expansion of adoptively transferred alloreactive NK cells in the patient has also been described as a consequence of elevated endogenous levels of the activating cytokine IL-15 [35]. In this study, there were no GvHD complications, and 5 out of 19 AML poor-prognosis patients entered complete remission. Further measures such as depletion of immunosuppressive T regulatory cells through IL-2 diphteria fusion protein treatment in addition to lymphodepleting chemotherapy regimens has been successful in promoting transient in vivo expansion of the mismatched NK cells, resulting in improved remission and 1-year disease-free survival in patients with refractory AML [36]. Additional manipulation of haploidentical NK cells such as priming with tumor lysate has been studied in a phase I clinical trial in high-risk AML patients, with possibly some clinical efficacy observed [37]. One concern with mismatched NK cells is the potential risk of marrow aplasia, presumably due to alloreactivity against the mismatched host hematopoietic cells, which has been observed in cases where there were prolonged NK cell engraftment [33, 37]. Exploration of alloreactive NK cells in non-transplant scenarios is also being studied for the treatment of other hematological malignancies such as lymphoma [38] and multiple myeloma [39].

Activated NK Cells for Leukemia Treatment

The anti-leukemic potency of NK cells may be further augmented through transfusion of activated effectors. A phase I/II clinical comparison between IL-2-activated NK-DLI (aNK-DLI) and unstimulated NK-DLI in pediatric leukemia patients pointed toward an enhanced effector trafficking potential for activated NK cells [40]. Thus, activated NK cells may exert greater immunotherapeutic effects compared to unstimulated cells. Recent advances in cell selection technologies and cell activation modes as well as refined culture media allow routine good manufacturing practice(GMP)-compliant large-scale productions of stimulated effectors [41–47]. Clinical NK cell doses, generally aimed for 5×10^6 NK cells/ kg to 10^7 NK cells/kg or even up to 10^8 NK cells/kg, can be reached [41, 44, 47, 48]. However, significant donor variability exists with regard to the achievable NK cell harvest and the composition of the end-product in terms of NK cell subpopulations [41, 48]. Automatization of NK cell expansions in specifically designed bioreactors, such as G-Rex-flasks (Wilson Wolf Manufacturing, Minneapolis, MN, USA) or the WAVE bioreactor (GE Healthcare Life Sciences, Piscataway, NJ, USA) is a means to further facilitate the NK cell expansion process and increase product yield [44, 47, 49]. Sources other than leukapheresis products, such as umbilical cord blood, are also being tested as starting material [50].

A crucial factor in these often complicated production protocols is the means by which activation of the NK cell is attained. Various very diverse methods have been described. These include for example addition of cytokines, such as IL-2 or IL-15 [9, 45, 51, 52], triggering through a lethally irradiated genetically modified feeder cell line expressing NK stimulatory 4-1BB ligand and IL-15 (K562-mb15-41BBL) [53], through an irradiated Epstein-Barr virus-

Table 1. Completed and ongoing clinical NK-92 (activated NK, formerly NeukoplastTM) trials

Reference	Clinical trial phase	Diseases	Number of patients	NK-92 dose ($\times 10^9/m^2$)	Total number of NK-92 cells infused ($\times 10^9$)	Responses / OS, days
T. Tonn et al. [59, 63]	phase I single-center	advanced cancers: PNET, soft tissue sarcoma, rhabdomyosarcoma, osteosarcoma, CLL-transformed, adrenal carcinoma, SCLC, soft tissue sarcoma, medulloblastoma, colorectal cancer, NSCLC, B-NHL	15	0.85–10	2.3–42.4	PD; MR; SD OS: 13–801
S. Arai et al. [65]	phase I single-center	advanced renal cell cancer or melanoma	12	0.1–3	max. 9 ($\times m^2$ body surface)	PD; MR; SD; MinR OS: 101 to >1,450
ClinicalTrials.gov NCT00990717	phase I single-center	hematological malignancies in relapse after autologous SCT: leukemia, lymphoma, myeloma, Hodgkin's disease	study currently completing estimated enrollment: 15	1–5	available upon final data collection; max. 54 ($\times m^2$ body surface)	available upon final data analysis
ClinicalTrials.gov NCT00900809	phase I single-center	refractory or relapsed AML	study ongoing estimated enrollment: 18	1–5	available upon final data collection; max. 9 ($\times m^2$ body surface)	available upon final data collection and analysis
ClinicalTrials.gov NCT02465957	phase II multi-center	stage IIIB MCC and stage IV MCC	study currently recruiting estimated enrollment: 24	2	available upon final data collection; max. 32 ($\times m^2$ body surface)	available upon final data collection and analysis

OS = Overall survival; PD = progressive disease; SD = stable disease; MR = mixed responses; MinR = minor responses; PNET = primitive neuroectodermal tumor; CLL = chronic lymphocytic leukemia; SCLC = small cell lung cancer; NSCLC = non-small cell lung cancer; NHL = non-Hodgkin lymphoma; SCT = stem cell transplantation; AML = acute myeloid leukemia; MCC = Merkel cell carcinoma.

transformed B-cell line EBV-TM-LCL [54], or a tumor cell lysate from CTV-1 leukemia cells (DSMZ) [37]. Furthermore, alloreactive single-KIR-NK selection and expansion to more specifically target HLA-mismatched leukemic blasts have also been described [55]. Nonetheless, we are only at the beginning of our understanding on how these often very complex NK cell manipulations alter NK effector biology and in vivo behavior. For instance, notable differences in genetic profiles of K562-mb15-41BBL-stimulated cells compared to controls were described [53]. Also, changes in surface receptor expression, such as upregulation of activating receptors triggered by cytokines, were found [44, 56]. Most recently, severe acute GvHD was reported in 5 of 9 post-HSCT patients – likely as a consequence of aNK-DLI, which was generated employing IL-15 plus 4-1BBL(+)IL-15Ralpha(+) artificial antigen-presenting cells as stimulants [57].

Another hurdle is the observed functional impairment of cryopreserved expanded NK cells after thawing. Short-term IL-2 treatment was necessary for the cells to reinstate potency [47, 54]. Yet, product manipulation after completed release testing is non-compliant with the stringent quality control requirements. Batch storage of a product with quality control tests done for each batch before release, however, will satisfy these prerequisites and make it possible for repeated NK effector infusions or cell banking.

NK-92 – A Third-Party NK Cell Drug

GMP-compliant banking of a clinical NK cell product promises to revolutionize cellular therapy into an 'off-the-shelf' product, a vision that has so far only been tested for the IL-2-dependent permanent NK cell line NK-92 (NantKwest, Culver City, CA, USA) [58]. The cryopreserved master-cell bank tested negative for infectious blood pathogens, viral particles as well as bacterial, fungal or mycoplasma contaminants [59]. NK-92 has been extensively characterized for its phenotypical and functional properties. It is distinguished by a superior cytotoxic potential and a lack of almost all inhibitory KIR receptors [19, 60, 61]. Optimized culture conditions have been established [62]. After initial cell inoculation of culture bags, no further media additions were required and clinical doses could be yielded within a few days [59, 63, 64]. A maximal expand-

able dose of 10^{10} cells/m^2 body surface was considered achievable in the established culture system [63]. Clinical phase I/II testing also involving leukemia patients, among other diseases, demonstrated feasibility and safety for the treatment with irradiated NK-92 cells. A maximum dosage of 10^{10} NK-92 cells/m^2 was given [59, 63, 65]. Table 1 provides an overview of completed and ongoing clinical trials involving NK-92. Future trial results are warranted for clinical efficacy evaluation. This novel concept for clinical usage of permanent NK cell lines may further be extended to other suitable candidates. Studies for such purpose have so far only been initiated with the highly cytotoxic NK cell line KHYG-1, which could potentially qualify as an alternative in the future [66–69]. Moreover, NK-92 cells designed to express the Fc receptor CD16 (FcgammaRIIIa) are enabled to kill through the mechanism of antibody-dependent cell-mediated cytotoxicity [70, 71], with the prospective to augment antibody therapy in the future. To further increase efficacy and directing it specifically to the tumor site, NK-92 has been modified to express a number of different chimeric antigen receptors (CARs). These include targeting CD19 or CD20 to overcome resistance to B-cell leukemia [72, 73] among others [74–77]. CD19-CAR-engineered NK-92 cells, for instance, effectively killed CD19-expressing B-precursor leukemia cell lines and lymphoblasts from leukemia patients, which were otherwise resistant or showed only minor sensitivity to unmodified NK-92 cells [78]. Another excellent example for selective tumor targeting is the NK-92 cell line engineered to express an ErbB2-specific CAR, which has recently demonstrated potent anti-glioblastoma activity in preclinical in vitro and in vivo models [79]. The potency of CAR-expressing effector cells has been demonstrated by the highly active autologous CD19 CAR T cells (CTL019), which showed striking efficacy in CLL and ALL patients [80, 81]. Long-term remission could be shown in large patient cohorts [82]. However, novel effective strategies to manage severe toxicities associated with CAR-T-cell therapies, such as cytokine release syndrome, are warranted [83]. Multiplex genome-edited large-scale manufacture of universal T cells may provide a means in overcoming limitations of the current personalized CAR-T-cell therapies thereby broadening applicability [84]. A major advantage for the NK-92 drug remains in its ease of clinical-scale production, allowing keeping operational costs at a minimum [59, 64]. Thus, clinical testing of the novel NK-92-CAR products is imperative to estimate their true potential and for decision-making among the alternative treatment options.

Quite naturally a significant role for transfusion services in this field of new cellular therapies arises, considering their long-standing expertise in classical blood productions, quality control, storage facilities and transport logistics, and with regard to their extensive clinical network [85, 86]. Thus, an association between blood centers and GMP-clean room facilities would be of benefit, since production steps of these novel cell preparations are not fully restricted to closed systems [87]. However, high operational expenditures for such facilities need to be taken into account [86]. Marketing of such 'advanced medicinal products', is a complex process which in Europe is overseen by the European Medicines Agency [85, 88]. Hence, practicability and cost factors pose limits to the implementation of such new types of personalized medicines in blood centers. It is conceivable that banking of a standardized third-party NK cell product, such as NK-92, may be more workable with broader applicability.

Conclusions

The field of NK cell therapy against leukemia is emerging, and much progress has been made. However, still little is known to date about the fate of NK cells after transfusion, their persistence in the patient, and the duration of engraftment. The risks associated with clinical usage of artificially activated NK cells require careful evaluation, and close patient monitoring after infusion is warranted. Costs for the often very complex GMP manufacture and regulatory matters limit application of advanced-therapy medicinal NK cell products to a wider patient population and involvement of transfusion centers in the production process. Clinical applications and stable engineering of potent NK cell lines, such as NK-92, could pave the way to standardized leukemia treatments and possibly also to those in solid tumors. As with the various other forms of adoptive cellular therapy currently being intensively studied, the exact place for NK cells in the treatment armamentarium for leukemia remains to be defined but prospect appears promising.

Disclosure Statement

The authors declare no conflict of interest.

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