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SYMPOSIUM-IN-WRITING PAPER

Natural killer cell immunosenescence in acute myeloid leukaemia patients: new targets for immunotherapeutic strategies?

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Abstract Several age-associated changes in natural killer (NK) cell phenotype have been reported that contribute to the defective NK cell response observed in elderly patients. A remodelling of the NK cell compartment occurs in the elderly with a reduction in the output of immature CD56^{bright} cells and an accumulation of highly differentiated CD56^{dim} NK cells. Acute myeloid leukaemia (AML) is generally a disease of older adults. NK cells in AML patients show diminished expression of several activating receptors that contribute to impaired NK cell function and, in consequence, to AML blast escape from NK cell immunosurveillance. In AML patients, phenotypic changes in NK cells have been correlated with disease progression and survival. NK cell-based immunotherapy has emerged as a possibility for the treatment of AML patients. The understanding of age-associated alterations in NK cells is therefore necessary to define adequate therapeutic strategies in older AML patients.

Keywords AML · Ageing · DNAM-1 · NK cells · NKp46 · NKp30

Abbreviations

AML	Acute myeloid leukaemia
AML-NK	Acute myeloid leukaemia patient NK cells
BiKEs	Bispecific killer engagers
CAR	Chimeric antigen receptor
CMV	Cytomegalovirus
DNAM-1	DNAX accessory molecule-1
HLA	Human leucocyte antigen
IL	Interleukin
ILCs	Innate lymphoid cells
IFN	Interferon
KIRs	Killer cell immunoglobulin-like receptors
LAK	Lymphokine-activated killer
LILRs	Leucocyte immunoglobulin-like receptors
MHC	Major histocompatibility complex
MICA	MHC class I-related protein A
MICB	MHC class I-related protein B
NCRs	Natural cytotoxicity receptors
NEACT	Non-engrafting alloreactive cellular therapy
NK	Natural killer
NKG2D	NK group 2, member D
PBMCs	Peripheral blood mononuclear cells
TNF	Tumour necrosis factor
TriKEs	Trispecific killer engagers
ULBP	UL-16 binding protein

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Introduction

Natural killer (NK) cells were initially described in the 1970s as large lymphocytes, different from T and B cells, with high cytotoxic capacity against tumour cells without the requirement of previous sensitisation [26, 32, 33]. Nowadays it is well known that NK cells also play an important immunoregulatory function by the secretion of cytokines. In the recent classification of innate lymphoid cells (ILCs), NK cells are the prototypical type I ILCs playing a key role in mediating early immune responses against virus-infected cells and tumours [3, 53, 79].

NK cell activation depends on the balance between activating receptor signalling and inhibitory receptor signalling. The most important inhibitory receptors on NK cells are major histocompatibility complex (MHC) class I specific and include glycoproteins of the immunoglobulin superfamily such as killer cell immunoglobulin-like receptors (KIRs) and leucocyte immunoglobulin-like receptors (LILRs) and C-type lectin-like glycoproteins such as CD94/NKG2A [38]. These receptors recognize self-MHC class I molecules expressed by normal cells preventing their lysis by NK cells. Expression of MHC class I molecules is frequently found diminished in tumour cells as well as in virus-infected cells, and consequently the inhibitory signal will decrease promoting NK cell cytotoxicity of these target cells [1]. Together with low signalling through inhibitory receptors, activation of NK cells also requires signalling through activating receptors after recognition on target cells of their specific ligands.

Here, we summarize the current state of knowledge on NK cell function in acute myeloid leukaemia (AML) patients focusing on those age-associated effects on NK cell phenotype and functionality. Finally, we review immunotherapeutic strategies that can open new treatment possibilities in elderly AML patients.

Natural killer cells: subpopulations, receptors and functions

Human NK cells are large granular lymphocytes representing 10–15 % of circulating lymphocytes. They are usually identified by the absence of T and B cell receptors and in humans by the expression of CD56 and/or CD16. Thus, according to CD56 expression, two subsets of human NK cells have been identified, $CD56^{bright}$ NK cells that are characterized by high cytokine production and low cytotoxic capacity in contrast to $CD56^{dim}$ NK cells that are highly cytotoxic and can also produce cytokines such as interferon (IFN)-γ after activation. $CD56^{bright}$ NK cells have been recently considered as immature NK cells, precursors of $CD56^{dim}$ NK cells [55]. A minor subset of dysfunctional NK cells characterized by the expression of CD16 and the absence of CD56 has been described to be increased in different clinical conditions such as HIV infection and in ageing [12, 50, 75, 82].

The discovery of MHC class I-specific inhibitory receptors, supporting the "missing self" hypothesis proposed by Kärre [42], and the identification of activating receptors on NK cells represented an essential advance in the understanding of NK cell activation [38, 52, 55].

One of the best-studied NK cell-activating receptors is NKG2D (NK group 2, member D) a C-type lectin-like receptor identified in 1991. NKG2D is expressed on human NK cells, on $CD8^+$ T cells and also, in some pathological conditions, on $CD4^+$ T cells. In humans, NKG2D binds to the MHC class I-related proteins A and B (MICA and MICB, respectively) and to glycoproteins that bind human cytomegalovirus (CMV) UL-16 protein (ULBP1-6). NKG2D ligands are usually induced by cellular stress and have been found expressed in different tumours [64, 86].

A significant progress was the identification of three activating receptors (NKp30, NKp44 and NKp46) that were collectively named natural cytotoxicity receptors (NCR). NCR belong to the Ig superfamily and were originally considered as unique NK cell-specific receptors [54]. However, recent discoveries of lymphoid subsets have demonstrated that these NCRs can also be expressed on other ILCs as well as on T cell subsets (γδ T cells, intestinal intraepithelial lymphocytes, in vitro IL-15-activated T cells, NKT cells) [28]. Based on NKp30 and NKp46 expression, NK cells are classified as NCR^{bright} ($NKp30^{bright}$ $NKp46^{bright}$), NCR^{dull} ($NKp30^{dull}$ $NKp46^{dull}$) and $NCR^{discordant}$ ($NKp30^{bright}$ $NKp46^{dull}$ or $NKp30^{dull}$ $NKp46^{bright}$). Interestingly, the majority of NK cells from healthy young individuals show an NCR^{bright} phenotype in contrast to the increased NCR^{dull} or $NCR^{discordant}$ phenotypes described in healthy elderly donors and in AML patients [14, 21, 69, 70]. Cellular ligands for NCR have remained elusive for a long time. Recently, proliferating cell nuclear antigen (PCNA) has been identified as an NKp44 ligand and HLA-B-associated transcript 3 (BAT3), B7-H6 and CMV pp65 tegument protein as NKp30 ligands suggesting that the NCRs are a class of pattern recognition receptors recognizing damage-associated molecular patterns related to cellular stress [27, 35].

DNAX accessory molecule-1, DNAM-1, is a glycoprotein of the Ig superfamily that is involved in adhesion and signalling. DNAM-1 is expressed on NK cells, T cells, monocytes and a subset of B cells [72]. Its role in NK cell-mediated cytotoxicity varies according to the target cells analysed and usually acts as a costimulatory molecule requiring the cooperation with other activation receptors to induce lysis of target cells [7]. DNAM-1 ligands, CD112 (nectin 2) and CD155 (poliovirus receptor, PVR), are

expressed in a variety of tumour cells, and it has been proposed that their expression is regulated by cellular stress. TACTILE (CD96) and the inhibitory receptor TIGIT have been described to bind DNAM-1 ligands and regulate NK cell function [16].

Once NK cell activation threshold is overcome, NK cells exert different effector functions against target cells including cytotoxicity mediated by perforin and granzymes and FASL–FAS interactions and cytokine production such as IFN-γ and tumour necrosis factor (TNF)-α that play an important role in the regulation of the immune responses [38, 55]. Due to the recent advances in NK cell biology and effector functions, these cells have emerged as potential candidates for cancer immunotherapy originally for the treatment of haematopoietic malignancies such as leukaemia based on the graft-versus-leukaemia response in haematopoietic stem cell transplantation of mismatched KIR/KIR ligands and, more recently, for the treatment of solid tumours.

NK cells in the elderly

Immunosenescence is defined as an age-related dysregulation of the immune response due to internal factors (such as a limited replicative potential of haematopoietic stem cells or thymus involution) or external factors (antigenic stress, such as chronic virus infection, cancer, autoimmune diseases, inflammatory diseases or transplantation).

NK cells constitute an important component of the innate immune response with capacity to kill tumour cells and virus-infected cells. Ageing is associated with changes in the frequency, phenotype and function of NK cell subsets [10, 22, 75].

Age-associated changes in the frequency of NK cells

Several studies have been published regarding changes in the frequency of NK cell subpopulations with age. An increase in the percentage [6, 12, 22, 46] and absolute number [6, 18] of total NK cells has been described in healthy ageing, associated with a decrease in immature $CD56^{bright}$ NK cells [6, 13, 22], both $CD56^{bright}CD16^{-}$ and $CD56^{bright}CD16^{+}$ NK cells [12], and a decline, maintenance or expansion of the more mature $CD56^{dim}CD16^{+}$ NK cell subset [6, 12, 25, 37, 78]. Recently, it has also been reported an increase in the percentage of $CD56^{-}CD16^{+}$ NK cells from healthy individuals with age [12]. The decrease in the $CD56^{bright}$ NK cell subpopulation in the elderly supports that ageing is associated with a decrease in the output of immature $CD56^{bright}$ cells from the bone marrow and an expansion of mature long-lived $CD56^{dim}$ NK cells [75].

The phenotype of NK cells changes with ageing

The analysis of receptor expression on NK cells has shown a significant decline of the activating receptors NKp30, NKp46 and DNAM-1 in the elderly [2, 39]. It has also been reported a high percentage of elderly people showing the NCR^{dull} or $NCR^{discordant}$ phenotypes [78]. Other studies of the effect of age on the expression of inhibitory receptors on NK cells (such as KIR receptors or CD94/NKG2A) showed discrepant results. While some authors did not demonstrate significant differences in the expression of KIR and CD94/NKG2A receptors on NK cells from young and elderly donors [2, 39], others showed an increased expression of KIR and a reciprocal decrease in CD94/NKG2A expression associated with age [46]. A decreased expression of KLRG-1 on NK cells from old donors has also been described [24].

It has been found that persistent viral infections, such as CMV, also may play an important role in the alterations observed on NK cells from elderly individuals. Since the majority of elderly individuals are CMV seropositive, changes produced by CMV on NK cells are difficult to differentiate from those attributed to ageing per se. In this regard, an increased expression of the activating receptor CD94/NKG2C (but no significant differences in the expression of its inhibitory counterpart CD94/NKG2A receptor) associated with CMV seropositivity has been described, not only in total NK cells [45, 56] but also in the $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$ NK cell subpopulations [12]. An increased expression of CD57 in the $CD56^{dim}$ NK cell subpopulation from elderly donors has also been reported [6]. However, a recent study has shown that CMV seropositivity, not age, is associated with an increased expression of CD57 on total NK cells, $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$ NK cells [12, 75].

Impact of ageing on NK cell function

As expected, the changes described in the phenotype of NK cells in elderly individuals also lead to altered NK cell function with a decrease in cytotoxicity at the single-cell level [76] and reduced ability of NK cells to respond and produce cytokines after stimulation [48, 51, 57]. It has also been reported a decrease in intracellular signalling of NK cells with age [47]. Decreased cell cytotoxicity against classical cellular target cells (e.g. K562 cell line) may be due to the alterations found in the expression of activating receptors that occurs in the elderly such as DNAM-1 and NCR. However, neither the expression of CD16 nor its ability to trigger antibody-dependent NK cell cytotoxicity is affected by age [39, 46, 61, 74].

There is evidence that alterations in the number and cytotoxic capacity of NK cells are associated with an

increased risk of infections and mortality, but the causes of these disorders in elderly donors are not yet fully known. It is known that older adults have a higher risk of cancer and the defects on NK cell function may also affect NK cell-mediated tumour immunosurveillance.

NK cells in AML patients

Considerable advances have been made during the last decade in the treatment of haematologic malignancies. However, disappointing results are observed when the long-term efficacy is analysed, since a significant number of patients relapse or suffer from severe chemotherapy and radiotherapy side effects. This is especially relevant in elderly AML patients because they frequently have age-related changes in renal or hepatic function that force the adjustment of chemotherapy doses, thus limiting treatment efficacy [5, 71].

NK cells are early responders of the innate immune response that can exert potent activity against AML cells without the requirement of prior sensitization, making them good candidates for cancer immunotherapy [9, 20, 43, 67].

Changes in the frequency of NK cells in AML patients

There are controversies regarding NK cell counts in AML patients. Whereas some reports describe no changes in NK cell numbers [21, 80], others suggest that the percentage of circulating NK cells is lower in AML patients compared with healthy donors ([40, 81] and Sanchez-Correa et al. unpublished data). The proportion of the $CD3^{-}CD56^{+}CD16^{-}$ NK cells was significantly higher, and the frequency of $CD3^{-}CD56^{+}CD16^{+}$ cells was significantly lower in AML patients compared with healthy donors [40, 81].

NK cell phenotype changes in AML patients

Activation of NK cells is the result of the fine balance between activating signalling and inhibitory signalling through a diverse repertoire of surface receptors. It has been described that AML blasts can be susceptible to NK cell-mediated lysis because they express different ligands for activating receptors [62, 66, 69, 70, 84]. However, NK cells are not efficient in lysing autologous AML blasts. Thus, several mechanisms have been proposed to contribute to AML blast escape from NK cell recognition [14, 21, 41, 62, 66, 69, 70, 80, 81]. These mechanisms include not only the downregulation of NK cell-activating receptors and the loss of ligands for activating receptors on the AML blasts but also the increased expression of inhibitory receptors on NK cells or their ligands on AML blasts.

In relation to the downregulation of activating receptors on NK cells, some studies have described a reduced expression of DNAM-1 [69, 70], NKp46 and NKp30 [14, 21, 70, 81] on NK cells from AML patients when compared to healthy donors (Fig. 1). Other receptors analysed were NKG2D, activating forms of KIR receptors (KIR2DS1, KIR2DS2), CD244 or CD94/NKG2C. Discrepant results concerning NKG2D expression on NK cells from AML patients have been reported showing either a reduced expression [81] or no significant differences [70, 80]. CD94/NKG2C [70, 81] and CD244 [70] receptors were significantly decreased on NK cells from AML patients. Finally, analysis of KIR2DL1/S1 and KIR2DL2/S2 showed no significant differences compared with healthy donors [80].

Both, the expression of activating receptors and the cytotoxicity of NK cells against autologous AML cells are recovered in AML patients who achieve complete remission supporting the idea that the decreased expression of NK cell-activating receptors leading to cell dysfunction is the consequence of continuous contact with their ligands expressed on leukaemic blasts [21, 80]. Thus, we have previously shown that the expression of CD112 on AML blasts correlates with the loss of DNAM-1 on NK cells further reinforcing that chronic ligand exposure can induce a reduced NK cell-activating receptor expression in AML patients [69]. In addition, AML blasts obtained from patients with an NCR^{low} phenotype are susceptible to NK

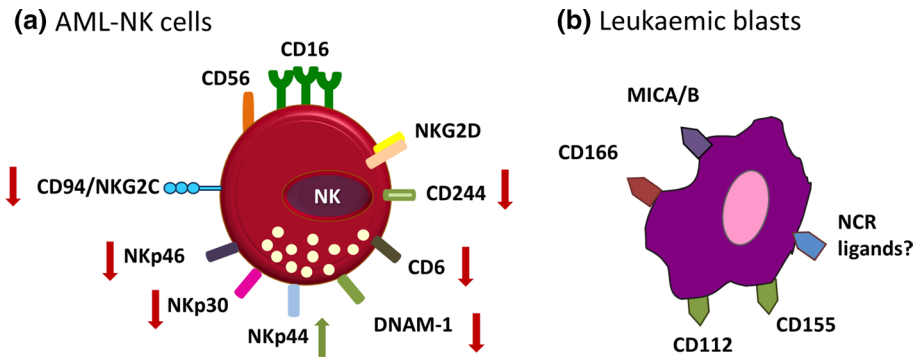

Fig. 1 NK cell-activating receptors and their ligands in AML. Schematic representation of **a** the expression of activating receptors on NK cells from AML patients (AML–NK) and **b** their ligands on leukaemic cells

cell-mediated lysis using NCR^{bright} NK cells, suggesting once more that AML blasts can be lysed by NK cells. Altogether, based on these observations it has been proposed that at some stage during AML development, NK cells turn out to be overwhelmed by the increasing number of leukaemic blasts, and their functionality goes down [14, 21, 31, 69, 73].

There are very few studies analysing the expression of inhibitory receptors on NK cells in AML patients. Stringaris et al. [80] described an increase in the frequency of NK cells expressing NKG2A in AML patients compared with healthy controls, while the analysis of KIR2DL1/S1, KIR2DL2/S2 and KIR3DL1 showed no differences. However, we did not find significant differences on the level of expression (MFI) of NKG2A within $CD94^+$ NK cells in AML patients [70].

Changes in the function of NK cells in AML patients

NK cells in AML patients show a significantly reduced cytotoxicity and CD107a degranulation against autologous leukaemic blasts [14, 80, 81] or K562 cell line [80, 81] compared with healthy donors. In addition, redirected lysis against the FcR + P815 murine cell line is also found decreased in AML patients [14, 21].

As indicated above, several alterations can be responsible for the diminished NK cell function observed in AML patients including the decreased expression of several activating receptors [14, 21, 69, 81], an altered expression of cytotoxic molecules (e.g. perforin, granzymes), low levels of ligands for major activating receptors on leukaemic blast surface [58] and high levels of HLA class I molecules on AML blasts [70]. Thus, it is well known that NK cell activation is regulated by the interactions of NK cell inhibitory receptors with HLA class I molecules on target cells; however, high levels of activating receptor ligands on the targets can counterbalance this inhibition [84]. In general, NK cells from AML patients are not efficient in the lysis of autologous blasts even when HLA class I antibodies are included in the assay to block inhibitory signals. This suggests that AML blast resistance to NK-mediated lysis is due to a defective activation of NK cells as the expression of NK cell-activating receptors is reduced in AML patients. In patients achieving complete remission, NK cell phenotype and function are found normalized, indicating that the presence of AML blasts is responsible for these NK cell defects [21, 80].

In AML patients, NK cells also show an impaired production of IFN-γ that was correlated with their response to autologous blasts [8, 80] or K562 cell line [80]. TNF-α secretion is also reduced on NK cells from AML patients, and together with NKG2A overexpression, these parameters were associated with failure to achieve remission [80].

NK cells in elderly AML patients compared with younger AML patients

AML is the most common type of acute leukaemia diagnosed in adult patients. The incidence of AML increases with advancing age, and a majority of AML cases occur in patients aged 65 years or older with a median age of 67 years at diagnosis [19, 30]. Ageing implies many adverse features such as the refractoriness of the disease and the frailty of this population contributing as a whole to the low rate of survival observed in elderly AML patients [29].

Therefore, age-associated immunosenescence can affect recognition and killing of AML blasts [11, 22, 70, 77]. As described above, age-associated alterations observed on NK cells include low per-cell cytotoxic capacity and altered cytokine production [17, 22, 60, 70, 77]. Concerning NCR, we have found a decline in the expression of NKp30 and NKp46 in AML patients [69, 70].

Since NK cell phenotype and function are influenced by age, we strongly recommend that all studies on NK cells in AML patients should take into account the age of the patients in order to differentiate age-associated from AML-associated alterations in NK cells. This consideration will allow the identification of new prognosis markers and help to develop NK cell-based therapies leading to enhance NK cell elimination of AML blasts in elderly patients. According to this, we have previously described a reduced expression of NKp30 and NKp46 on NK cells from AML patients younger than 65 years compared with age-matched healthy donors. In elderly AML patients, the expression of NKp46 was decreased, whereas no differences were found in the expression of NKp30 compared with healthy elderly donors. It is important to note that both activating receptors are downregulated in healthy elderly donors compared with younger healthy donors [69].

In a similar way, DNAM-1 expression on NK cells from AML patients is significantly decreased in patients less than 65 years of age. No differences are found in elderly patients given that DNAM-1 expression is reduced on NK cells from healthy elderly donors. A decrease in CD244 expression was found on NK cells from AML patients in both groups of age compared with healthy donors [69], suggesting that this reduction is associated with AML.

Finally, it is important to highlight that some studies have correlated NCR expression with survival. These studies describe that AML patients with high expression of NKp30 or NKp46 survive longer than patients with reduced expression of these receptors ([21] and Bergua et al. unpublished data). This observation suggests that the NCR phenotype may influence patient outcome directly through an anti-leukaemia effect or indirectly through the control of infectious diseases that also contribute to patient survival.

Altogether, these data suggest that the decreased NK cell function observed in AML patients is partially due to the decrease in the expression of activating receptors, an effect that is more pronounced in elderly patients. A better understanding of the contribution of age-associated NK cell senescence to tumour immunosurveillance in AML patients is required.

NK cell-based immunotherapy for haematologic malignancies

The clinical outcome of patients with refractory or relapsed AML is frequently very poor, and despite the development of new therapies for AML, the treatment of this group of patients remains unsolved. The use of NK cells and cytokine-induced killer cells in immunotherapy of haematologic malignancies has been proposed [63].

The first clinical trials to harness the antitumor properties of NK cells in humans were focused on the use of autologous IL-2-induced lymphokine activated killers (LAK) in combination with IL-2 and reported a clinical response in patients with solid tumours. This therapy was discarded due to the high toxicity and the lack of superiority compared with IL-2 treatment alone [83] and to the finding that in vivo administration of IL-2 induces the expansion not only of NK cells but also of regulatory T cells, although this effect could be avoided by the use of IL-15. Moreover, autologous NK cells do not lyse cancer cells unless MHC class I antigens are downregulated [34].

An additional limitation of using autologous NK cells from cancer patients for immunotherapy is that these cells are frequently hyporesponsive due to the downregulation of activating receptors that is even more accentuated in elderly patients as discussed above. In order to evaluate cytokine-induced recovery of NK cell-activating receptors in AML patients, we have performed in vitro studies analysing the effect of IL-15 on NK cells from AML patients. In Fig. 2, we show a representative example of NK cells from an elderly AML patient, with low expression of several activating receptors, which were incubated in vitro with IL-15. We observed that IL-15 increased the expression of NK activating receptors (Fig. 2a) and, more important, enhanced NK cell degranulation against the K562 erythroleukaemia cell line (Fig. 2b). These results indicate that, even in elderly patients, NK cell function can be enhanced by cytokines.

In AML, HLA-haploidentical adoptively transferred NK cells expand after lymphodepleting chemotherapy and clearance of blasts is correlated with the persistence and in vivo expansion of NK cells [4]. However, NK cell recognition of self-MHC class I molecules can inhibit killing of leukaemic blasts. Supporting these findings, many clinical studies have suggested a correlation between NK allorecognition and the outcome of allohaematopoietic stem cell transplantation (HSCT). Thus, in allo-HSCT, it has been demonstrated that NK cells can destroy "non-self" cells (e.g. leukaemic blasts), contributing to the graft-versus-leukaemia effect, provided that a KIR/KIR ligand mismatch exists that leads to graft-versus-leukaemia effect [44].

Curti et al. [15] reported the feasibility of adoptive transfer of alloreactive haploidentical KIR ligand-mismatched NK cells in elderly high-risk AML patients with best results in patients with molecular relapse of morphological complete remission. The clinical trials exploiting NK cell-mediated anti-tumour activity using HLA-haploidentical HSCT to cure high-risk acute leukaemia showed that alloreactive NK cells express inhibitory KIR specific for HLA alleles that are not present in the recipient and activating KIR recognizing recipient HLA ligands. Thus, independent of the clinical setting, NK-mediated tumour cell eradication depends on the presence of activating receptors including activating KIR and other HLA non-specific receptors.

There are several limitations for the use of adoptive transfer of NK cells, either autologous or allogeneic, including limited survival of NK cells, low activity due to inhibitory receptors or low signalling through activating receptors (e.g. shedding of ligands or downregulation of activating receptors). A better understanding of the complex balance between inhibitory receptor signalling and activating receptor signalling and how different activating receptors cooperate to activate NK cell cytotoxicity is still required for the development of new strategies aimed to select the more effective NK cells for immunotherapy in AML (Fig. 3).

Thus, several innovative strategies aimed to redirect NK cell cytotoxicity using bispecific and trispecific killer engagers (BiKEs and TriKEs, respectively) constructed using a single-chain variable fragment of a monoclonal antibody against CD16 and one (BiKEs) or two (TriKEs) variable single-chain fragments against tumour-associated antigens have been developed. BiKEs and TriKEs trigger NK cell activation through CD16. An enhanced killing of tumour cells was observed when these agents were used in combination with an inhibitor of ADAM17 to prevent CD16 shedding after NK cell activation [65, 85, 87]. In refractory AML, CD16xCD33 BiKEs overcame inhibitory signalling through KIRs and triggered NK cell cytotoxicity and cytokine production [85]. Interestingly, reactivation of CMV after HSCT induced maturation of NK cells and amplified BiKE-mediated triggering trough CD16 [49].

NK cells expressing chimeric antigen receptors (CAR) have been engineered demonstrating an increased NK cell cytotoxicity in vitro. In contrast to CAR-T cells, the use of CAR-NK cells has been mainly restricted to preclinical studies [44]. CAR-modified primary NK cells directed against

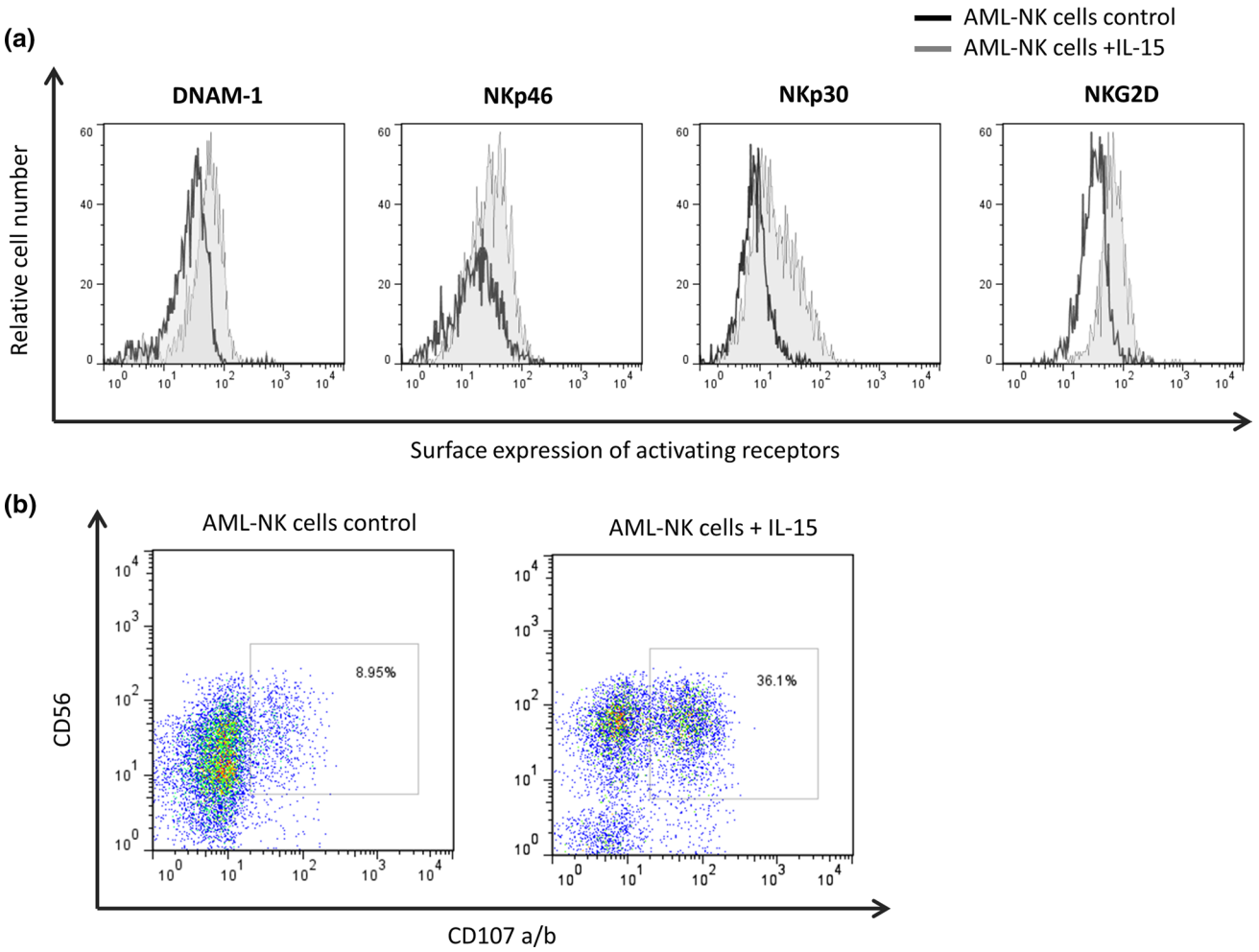

Fig. 2 IL-15 modulates the surface expression of activating receptors in NK cells and restores their functionality. A representative example from an elderly AML patient (82 years) showing the effect of in vitro incubation of NK cells with IL-15 (100 ng/ml): **a** expression of activating receptors on NK cells. *Black lines* represent expression levels of activating receptors in resting NK cells from an AML patient (AML-NK) at diagnosis, and *grey-filled* histograms indicate expression levels of activating receptors in AML-NK cells after incubation with IL-15. **b** Analysis of AML-NK cell degranulation against K562 cell line before and after preincubation of NK cells with IL-15

CD19, CD20, CD244 or HER2 and the use of the NK92 cell line expressing CAR have been reported in preclinical studies. In contrast to long-lived CAR-T cells, the use of mature NK cells with a limited lifespan might not require the incorporation of suicide genes to destroy CAR-effector cells, a possibility that will require further studies [23].

The expansion of a large number of NK cells from a small fraction of blood mononuclear cells remains challenging. Recently, a particle-based NK cell expansion technology has been developed using plasma membrane particles derived from the K562 cell line engineered to express IL-15 and CD137. This technology induces the selective expansion of large numbers of NK cells with high cytotoxic capacity against leukaemic cell lines and leukaemia blasts, facilitating the expansion of NK cells for clinical use [59]. The development of methods for the expansion of large numbers of NK cells from blood or pluripotent progenitors may contribute to the feasibility of adoptive transfer clinical trials.

A new promising strategy for AML is the infusions of HLA-mismatched leucocytes, termed non-engrafting alloreactive cellular therapy (NEACT). In elderly patients, NEACT was associated with increased survival from 10 to 39 % when administered following chemotherapy and complete remissions were observed in one-third of patients with relapsed or chemorefractory disease. Both NK cell alloreactivity and T cell alloreactivity generate anti-leukaemic effects; however, long-term disease control requires recipient-derived T cell responses against tumour-associated antigens. Several variables that may affect the NEACT effect are the release of proinflammatory cytokines such as IL-6 and the choice of different pretreatment chemotherapy [36]. Further analysis of cytokine release after

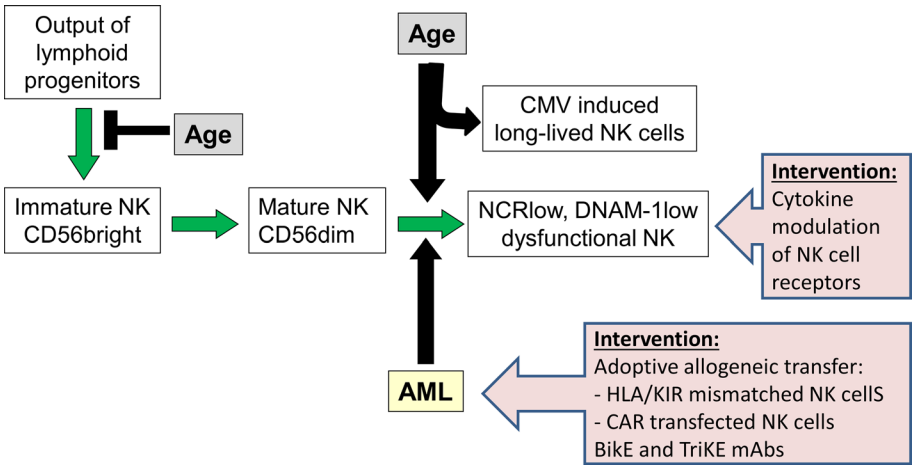

Fig. 3 Schematic representation of the effect of ageing and AML on NK cell phenotype and function and possible NK-based immunotherapy strategies. Ageing is associated with a decrease in the production of new immature $CD56^{bright}$ NK cells from bone marrow common progenitors. CMV chronic infection is associated with an expansion of a memory-like long-lived NK cell subset characterized by the expression of the activating receptor CD94/NKG2C and CD57. Ageing and AML are associated with a decreased expression of NCRs and DNAM-1 that contributes to low cytotoxic capacity of NK cells. In elderly AML patients, age-associated changes on NK cells limit their capacity to eliminate leukaemic blasts. Interventions to improve NK cell function include cytokine-induced recovery of activating receptors and adoptive transfer of allogeneic NK cells selected on the basis of HLA/KIR mismatch. Novel strategies propose the use of NK cells expressing chimeric activating receptors (CAR) and the use of bi- or trispecific antibodies aimed to redirect NK cell-mediated lysis (BiKE and TriKE)

NEACT and its correlation with disease progression and survival is required. We have reported altered cytokine profiles in AML patients including increased levels of IL-6 that inversely correlated with survival [68] and may affect NEACT effectiveness. In addition, donor selection, the characteristics of infused cells and the use of repeated infusions are questions to be addressed [36].

In conclusion, a better understanding of age-associated alterations in NK cells and the advances in our knowledge of how NK cells recognize haematologic malignancies has opened promising perspectives for the use of NK cells in the immunotherapy of elderly AML patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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