



Underground Adaptation to a Hostile Environment: Acute Myeloid Leukemia vs. Natural Killer Cells

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Acute myeloid leukemia (AML) is a heterogeneous group of malignancies which incidence increases with age. The disease affects the differentiation of hematopoietic stem or precursor cells in the bone marrow and can be related to abnormal cytogenetic and/or specific mutational patterns. AML blasts can be sensitive to natural killer (NK) cell antitumor response. However, NK cells are frequently defective in AML patients leading to tumor escape. NK cell defects affect not only the expression of the activating NK receptors, including the natural cytotoxicity receptors, the NK group 2, member D, and the DNAX accessory molecule-1, but also cytotoxicity and IFN- γ release. Such perturbations in NK cell physiology could be related to the adaptation of the AML to the immune pressure and more generally to patient's clinical features. Various mechanisms are potentially involved in the inhibition of NK-cell functions in AML, including defects in the normal lymphopoiesis, reduced expression of activating receptors through cell-to-cell contacts, and production of immunosuppressive soluble agents by leukemic blasts. Therefore, the continuous cross-talk between AML and NK cells participates to the leukemia immune escape and eventually to patient's relapse. Methods to restore or stimulate NK cells seem to be attractive strategies to treat patients once the complete remission is achieved. Moreover, our capacity in stimulating the NK cell functions could lead to the development of preemptive strategies to eliminate leukemia-initiating cells before the emergence of the disease in elderly individuals presenting preleukemic mutations in hematopoietic stem cells.

Keywords: natural killer cells, acute myeloid leukemia, immunoediting, natural killer receptors, immune escape of cancer, aging and cancer

INTRODUCTION

In recent years, the field of cancer immunology has known a growing interest due to development of innovative therapeutic strategies in various malignant pathologies. Since the first hypothesis by P. Ehrlich at the beginning of the twentieth century suggesting that the organism could defend itself against tumor cells (1), through the "Immunosurveillance" theory developed by Burnett (2) and Thomas (3) in the late 1950s, and into the more recent "three Es of the immunoediting" suggested

by Schreiber et al. (4); scientists and clinicians learnt that not only cancers were capable of inhibiting the tumor-specific immune response but also host immune cells could potentially be restored or manipulated to eliminate tumor cells. Therefore, therapeutic strategies combining conventional chemotherapy treatments and reinforcement of the self anticancer immunity appear as very promising. Recent successes in the use of immune checkpoint inhibitors to restore the T-cell response against solid tumors are in favor of such approaches (5). Interestingly, the observation that immune cells need external interventions to recover an activity against the autologous tumors demonstrates, as a negative, the adaptation process engaged by tumor cells in order to expand despite the patient's immune system. Leukemic diseases are particularly suitable to study the dialog with the immune system, as they develop in the same bone marrow (BM) environment as normal hematopoiesis, are well molecularly characterized and also because they invade the organism through the circulation network, so directly in contact with the circulating immune cells.

ACUTE MYELOID LEUKEMIA: A LONG-TERM MALIGNANT PROCESS, SIDE EFFECT OF AGING

Acute myeloid leukemia (AML) is a heterogeneous group of diseases characterized by the proliferation of a hematopoietic progenitor clone blocked in its differentiation (6, 7). The blockage can concern each maturation step of the myeloid precursors, including granulocytic, monocytic, megakaryocytic, and erythroid precursors. In general, the disease develops in the BM, and the presence of malignant clones inhibits the normal hematopoiesis not only by reducing space available for healthy hematopoietic stem cells (HSCs) but also by direct inhibition (8). This inhibition leads to marrow failure associated with cytopenia. The annual overall incidence of the disease is 3.8 cases per 100,000 adults in western countries, but it increases to 15 cases per 100,000 for elderly over 60 years (9). Many advances have been made in the molecular characterization of the disease and the evaluation of molecular markers in specific cytogenetic AML subsets is now a standard procedure for patient's diagnosis and risk stratification (9, 10). Moreover, attempts were also developed to categorize AML based on mutation profiling (11–13) or on gene expression profiling, associated or not with recurrent acquired mutations identified in routine diagnosis (14–17). Finally, a new paradigm is taking form in our understanding of the connection between aging and leukemia with the identification of recurrent mutations in genes involved in the epigenetic regulation of the HSCs genome (*DNMT3A*, *TET2*, and *ASXL1*), acquired with age in healthy HSC, and leading to clonal hematopoiesis associated with increases in the risk of hematological cancer, including AML (18–20). The demonstration that healthy HSC could integrate mutations originally identified in AML is coherent with the identification of leukemia stem cell (21), with the potential to initiate a malignant clone at the origin of the disease. Indeed, preleukemic HSCs, defined as a pool of HSC with recurrent *DNMT3A* mutations but without the additional mutations observed in AML blasts, were found in AML patients (22).

Altogether, these observations are in favor of the hypothesis that HSCs accumulate somatic mutations and give rise to AML-initiating cells following a clonal selection process (23) at diagnosis and also after relapse (24). This long duration of the malignant development process, in parallel with patient's aging, questions the nature of the stimuli leading to this evolution, why particular successive mutations are required to ensure AML survival and proliferation, and how the organism's environment, including the immune system, can deal with the emerging preleukemic and leukemic cells.

THE NATURAL KILLER CELL: A MAJOR ANTITUMOR EFFECTOR CELL

Among the different immune partners, natural killer (NK) cells were defined, at the time of their discovery, as being capable to directly eliminate tumor cells (25–28). NK cells are lymphocytes from the innate immunity, therefore characterized by the absence of rearranged antigen-specific receptors, such as T-cell or B-cell receptors. This population was recently assigned to a newly described family of innate lymphocytes, comprising various innate lymphoid cells (ILCs) (29). Innate lymphocyte populations show some analogies with the subdivision observed for the T-lymphocytes family with the CD8⁺ cytotoxic T-cells, and the Th1, Th2, and Th17 CD4⁺ T-cells. Similarly, conventional NK cells constitute the cytotoxic innate lymphocytes with capacities to eliminate infected or transformed target cells, whereas ILC subsets are capable to support the development of the local immune response through the production of cytokines, such as IFN- γ (ILC1 subset), IL-5 and IL-13 (ILC2 subset), or IL-17 and/or IL-22 (ILC3 subset). NK cells were first categorized as type 1 cells such as Th1 cells because of their capacity to produce IFN- γ , but the expression of perforin and granzymes authorized to distinguish the cytotoxic ILC, i.e., the NK cell subsets, and the helper ILC1 (30). This role sharing could suggest that innate and adaptive lymphocyte populations can interact and support each other to initiate and sustain the immune response (31).

Natural killer cells represent 5–10% of the blood lymphocytes. Two major NK cell subsets are present in blood and secondary lymphoid organs (32). The CD56^{dim}CD16⁺ NK cells constitute the vast majority of NK cells in blood (90–95%). They are highly cytotoxic but can also produce significant amounts of cytokines, such as IFN- γ and TNF- α , after stimulation by a sensitive target (33). The expression of the Fc γ RIII CD16 ensures the capacity for NK cells in mediating the antibody-dependent cellular cytotoxicity (ADCC). By contrast, the CD56^{bright}CD16^{low/-} NK cell subpopulation is mainly found in lymph nodes whereas they represent about 10% of blood NK cells (32, 34). The CD56^{bright} NK cells store less intracellular cytolytic vesicles containing perforin and granzymes than their counterpart, but they can secrete large amounts of cytokines in response to an inflammatory environment (32). In addition to the cytokine-mediated triggering, NK cell functions are regulated by a balance between inhibitory and activating signals provided through regulatory receptors on the cell surface (35).

NK Cell Functions Are Tightly Regulated

Natural killer cells are tightly regulated by numerous receptors that either trigger or inhibit the cell's functions. To allow the distinction between healthy and abnormal cells (i.e., infected or tumor "stressed" cells) is the ultimate goal of this balance. Indeed, NK cells detect modified target cells that display perturbations in the expression of surface ligands (35).

Through the recognition of some HLA class-I molecules on the target cell, receptors, such as some of the killer immunoglobulin-like receptors (KIRs) or the lectin heterodimer CD94/natural killer group 2, member A (NKG2A), inhibit NK cell functions. Originally described as the "Missing self" theory (36), the physiological function of these receptors is to detect loss or reduction of the class-I antigen-presenting molecules on the surface of tumor cells, a frequent alteration observed in cancer cells (37) and viral infections (38) at the origin of the escape from T-cell-mediated immunity. The absence of HLA class-I molecules on the target cell surface will therefore lead to an absence of inhibition of the NK cell functions. However, chronic exposure of NK cells to HLA class-I loss tumor variants can also lead to NK cell anergy as an escape mechanism. Such anergy can be reversed in presence of IL-12 and IL-18 (39).

Optimal NK cell triggering will also require activation signals provided by activating receptors that detect ligands on the target. A majority of cancers of all cell types express, at variable levels, stress-induced molecules, including the MHC class-I-related chains A and B (MICA/B) and the UL16-binding proteins (ULBPs) (40). These proteins are recognized by the activating lectin-homodimer NK group 2, member D (NKG2D) receptor on NK cells, resulting in the elimination of the tumor (41). Importantly, ataxia telangiectasia, mutated (ATM), and ATM- and Rad3-related (ATR) protein kinases activation as a response to DNA damage can stimulate NKG2D-ligands (NKG2D-L) surface expression (42). Other pathways regulate the expression of certain NKG2D-L [reviewed in Ref. (43)] and participate in leukemic physiology, including alterations in the microRNAs repertoire (44), the heat shock stress pathway (45), which can induce MICA and MICB gene expression, or the activation of the PI3K pathway that is often constitutively activated in leukemia (46) and can stimulate the expression of the mouse NKG2D-L RAE-1. Stress signals associated with DNA damage response, including reactive oxygen species (ROS), can also promote the expression of the poliovirus receptor (PVR or CD155) recognized by the activating receptor DNAX accessory molecule-1 (DNAM-1) (CD226) (47, 48). Interestingly, the DNAM-1 ligands PVR and Nectin-2 were observed on many cancers and DNAM-1 can collaborate with other activating NK cell receptors to mediate killing of tumor cells (49, 50). The natural cytotoxicity receptors (NCRs), such as NKp30, NKp44, and NKp46, were also implicated in the recognition of tumors and notably AML (51, 52) even if ligands are expressed at low levels (53). B7-H6, a ligand for NKp30, and MLL5, from which a short isoform is recognized by NKp44, can be expressed on AML blasts (54, 55). AML cells are also recognized by NKp46 (56), but the ligands involved have not yet been identified. Additional receptors or coreceptors can induce NK cell activation in a cell-to-cell interaction with a target, including the adhesion molecule lymphocyte function-associated antigen-1 (LFA-1),

and the signaling lymphocytic activation molecule (SLAM) family receptors, such as 2B4 (CD244), CRACC (CD319), or NTB-A (57). LFA-1 will bind to the intercellular adhesion molecules-1 (ICAM-1 or CD54) expressed on most AML cells (56). The SLAM receptors will be involved in homotypic interactions, except 2B4 (CD244), which will recognize CD48. To date, CD48 was the only SLAM family receptor frequently observed on AML cells (52).

A Direct Role for NK Cells in the Antitumor Immune Response

The original identification of NK cells as tumor killers has been abundantly confirmed in a wide variety of cancers. The higher susceptibility of murine models lacking NK cells to spontaneous or carcinogen [methylcholanthrene (MCA)]-induced tumors was in favor of a direct role of these lymphocytes in the elimination of malignant cells (4). Numerous *in vitro* and *in vivo* models demonstrated the direct implication of perforin together with IFN- γ produced by cytotoxic cells, including NK cells (58, 59), or of the activating NK receptors (41, 50, 60). In addition, the death receptor pathways involving the Fas-ligand (FasL) receptor and the TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, both induced on NK cells by either IFN- γ or IFN- α/β , were also involved in the antitumor function of NK cells (61, 62). In human, an indirect evidence for a role for NK cells *in vivo* came from the observation of the association between the natural cytotoxicity quantified in blood and the risk of cancer (63). The positive correlation between solid tumor infiltrating NK cells and good prognosis also suggests that NK cells could directly eliminate tumor cells *in vivo* (4). Importantly, in addition to a direct cytotoxicity, NK cells promote the antitumor response through the production of IFN- γ , which is required for the early phase of Th1 priming and polarization in the lymph node (64) and also for the stimulation and polarization of macrophages (65).

AML ESCAPE FROM NK CELLS: IMMEDIATE AND LONG-TERM PROCESSES

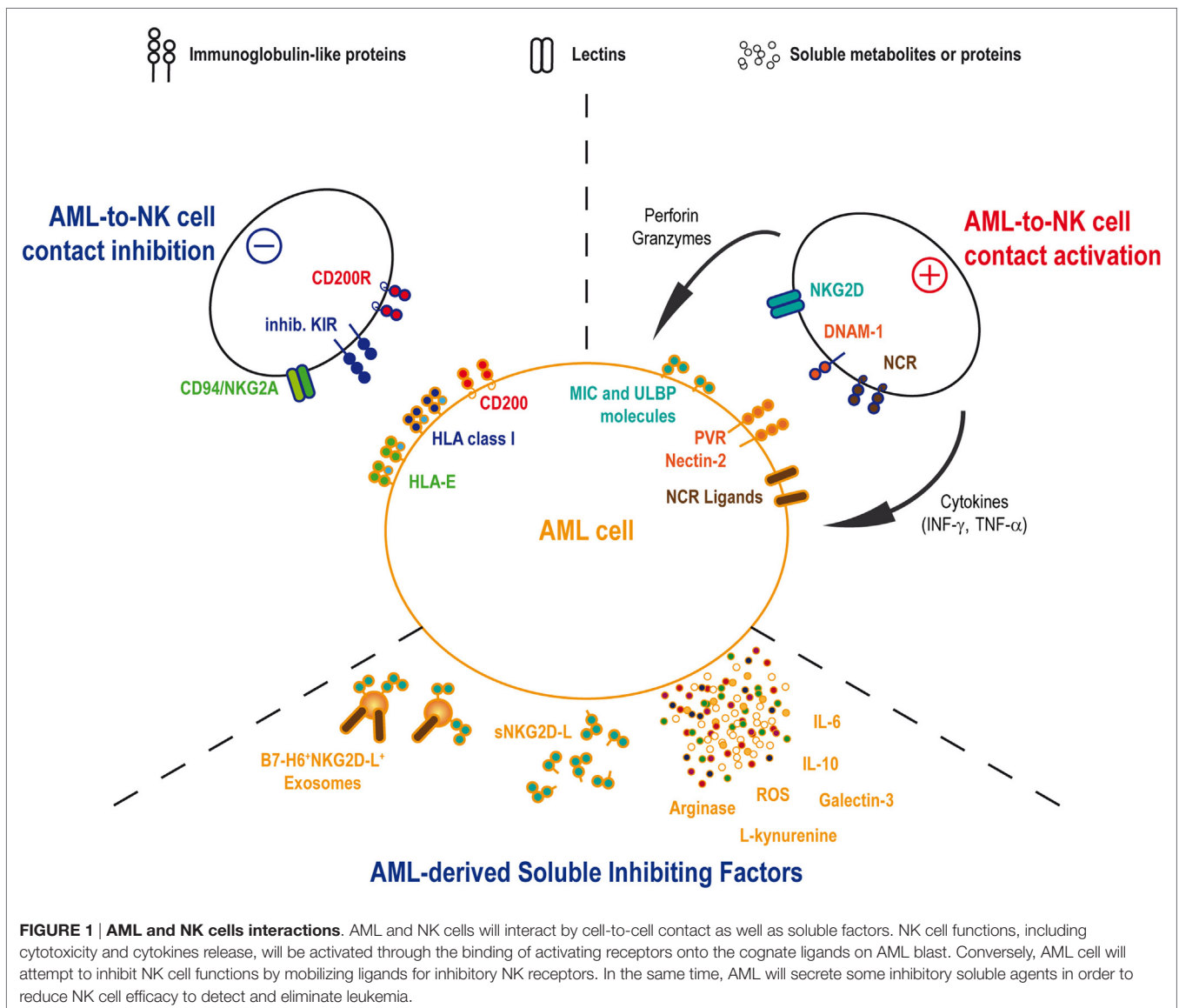
In vivo AML sensitivity to NK cell-mediated cytotoxicity has been shown by Ruggeri et al. in patients treated by haploidentical HSC transplantation (66). This team reported a lower incidence of relapse in patients transplanted with graft containing alloreactive NK-cell clones against recipient cells. By contrast, the absence of such NK-cell incompatibility was associated with a high relapse rate (66, 67). This observation was based on the existence of KIR/KIR-ligand (i.e., HLA class-I molecules) mismatches in the responsive donor/recipient pairs. The absence of cognate ligands for the inhibitory KIRs allowed NK cell activation by AML cells and elimination of the leukemic target. This illustration of the "missing-self" theory (36) found its counterpart in the observation that the activating KIR2DS1 can also provide a significant reduction of AML relapse in donor/recipient pairs where recipient expresses specific HLA-C ligands (68). According to these results, the selection of the donor may be of importance in order to optimize the graft-vs.-leukemia effect expected from the HSC transplantation. Therefore, haploidentical and umbilical cord

blood transplantations would be the most suitable transplantation settings to find KIR/KIR-ligands mismatches. A few studies analyzed the role of KIR/KIR-ligand mismatch in cord blood transplantation setting with contradictory results either demonstrating the advantage of such treatment in myeloid leukemia (69) or, by contrast, showing a higher risk of acute graft-vs.-host disease without curative advantages after reduced intensity conditioning (70). Cytomegalovirus (CMV) infection or reactivation in transplanted patients could explain these discrepancies, as CMV-driven NK cell expansion and maturation could participate in the reduction of the relapse risk (71). Clinical trials using infusions of IL-2-activated haploidentical NK cells in AML patients showed encouraging results with *in vivo* expansions of donor NK cells and complete remissions (CR) in certain patients, suggesting an antitumor NK-cell-mediated immune response (72, 73). However, patient's autologous NK cells often show defects at diagnosis. Activating receptors, such as DNAM-1, and the NCR,

such as NKp30 and NKp46, present reduced expression levels on NK cell surfaces in a large proportion of patients (74–76). The inhibitory receptor CD94/NKG2A can also be upregulated on patient NK cells (77). In parallel to the phenotypic perturbations, cytotoxic activity and IFN- γ release are also decreased (76–78). These defects were associated with pejorative outcomes, including increased relapse risk and/or reduced overall survival. Interestingly, NK cell phenotype and function are normalized after chemotherapy treatment, underlying the role for AML blasts in decreasing NK cell's abilities (75, 76). Altogether, these observations imply that AML inhibits the autologous NK cell response through several mechanisms (Figure 1).

Leukemogenesis and Immune Escape

Tumor microenvironment plays a critical role in the inhibition of the antitumor immune response (79). Leukemogenesis occurs mainly in the BM, the primary site for the healthy hematopoiesis.



This suggests that leukemia BM environment could modify the immune cell differentiation process and also that healthy immune populations may influence leukemogenesis. Such interference was demonstrated in myelodysplastic syndromes (MDS), a heterogeneous group of myeloid disorders displaying low to high risk to give rise to secondary AML (80). As observed in AML patients, blood NK cells from MDS patients show severe defects with downregulation of activating receptors, including NKG2D and DNAM-1 (81), reduced cytotoxic activity (81, 82), and increased apoptosis rate in response to IL-2 stimulation associated with reduced proliferation *in vitro* (82). Importantly, increased NK cell defects were also associated with high-risk MDS, characterized by higher International Prognostic Scoring System (IPSS) Score, presence of excess blasts, abnormal karyotype, and hypercellularity (83). BM NK cells in MDS patients are also deeply affected by the disease, suggesting that MDS BM environment could play a role in those defects (81–83). In AML, BM environment influences healthy hematopoiesis by affecting BM cell populations. Notably, BM stromal cells show low proliferative rate as well as genetic and epigenetic alterations (84). Similar numbers of healthy CD34⁺CD38⁻ HSC were found in the BM of AML patients and healthy individuals. However, normal CD34⁺CD38⁺ progenitors were found reduced in BM of AML patients likely resulting from a differentiation block at the HSC-progenitor progression (8). Consequently, similar to MDS, NK cell differentiation in the BM seems to be affected by AML. A recent work by Vasold et al. suggested a role for mesenchymal stromal cells and hypoxia in the reduction of NK cell cytotoxic activity against autologous AML blasts highlighting the importance of the BM stroma in the emergence of abnormal mature immune cells in the peripheral blood (85). Connected to this observation, we recently described that AML cell transcriptional program is intimately associated with the deepness of the NK cell defects (76). NK cell deficiencies were associated with an increased risk of relapse. AML blast transcripts coding for proteins involved in cytokine/cytokine receptor and chemokine pathways were found severely diminished in patients with defective NK cells. In return, IFN- γ production by CD56^{bright} NK cells is almost abrogated at diagnosis in those patients (76). Altogether, these observations would indicate that AML blasts can modify BM environment, including stromal, precursor, and mature immune cell populations. In addition, the immune pressure, notably provided by NK cells, could influence the AML transcription program leading to AML escape and a more pejorative outcome.

Cell-to-Cell Contact and Defective Immunological Synapse

Interactions between immune cells and targets constitute a multi-step process where first immune cells build conjugates with the target partner and then initiate a reorganization of proteins localized at the membrane at the cell-to-cell contact point [reviewed in Ref. (86)]. This includes actin mesh polymerization and centrosomes polarization (87). The supramolecular activation cluster (SMAC) recruits regulating NK receptors, in parallel with ligands on the target cell, together with costimulatory and adhesion molecules in order to integrate and amplify intracellular signaling. NK cells

will therefore polarize cytolytic vesicles toward the immunological synapse (IS) (i.e., the target cell) and secrete perforin and granzymes within the intercellular space (86). At the resolution of the synapse, NK cells express low levels of activating receptors and need a period of time to regain their full function (88).

Even if leukemic cells are sensitive to allogeneic NK-cell-mediated lysis, alterations in the expression of some activating receptors, including NCR and DNAM-1, on the autologous NK cells suggest that NK-to-AML interactions can be defective in patients. Such observation has been made for T cells with a reduction in actin polarization and phosphotyrosine signaling in T-cell/AML blast conjugates (89). Reduction of NKR expression by autologous NK cells was partially associated with the presence of cognate ligands on AML cells as *in vitro* coculture of NK cells with AML cell lines or primary blasts decreases the NKR levels (56, 90). However, this phenomenon is not proportional to the ligand expression levels on the target, suggesting additional mechanisms controlling the profound NKR downregulation observed *in vivo*. When studying the IS between NK and AML cells, NK cells showed defects in the polarization of their cytolytic granules toward the IS against AML blasts (56). Consequently, defects in NKR expression may also be the result of a continuous exposure to ligands in incomplete cytotoxic synapses against AML blasts, leading to an exhaustion of the NK-cell cytotoxic activity. Therefore, NK cell cytotoxic activity is progressively switched off, whereas AML cells survive and proliferate. Whether the decrease in IFN- γ production observed in patients is also the result of perturbations in the IS or in downstream NKR signaling pathways requires further studies. The molecular mechanisms responsible of this cytotoxic defect need also to be identified. Inhibitory KIRs can reduce the autologous anti-AML response as demonstrated in autologous HSC transplantations (91). Nevertheless, inhibitory KIRs likely play a very early role in the building of the IS during polymerization of the actin network and would be only marginally implicated at later stages to inhibit the lytic granules' polarization (86). Other receptors could be good candidates in the negative regulation of NK functions, such as CD96, as demonstrated by the higher resistance of *Cd96*^{-/-} mice to solid tumors (92). On the other side, CD200 (also named OX2) expressed at high levels on some AML has been recently identified as a suppressor of patient's NK cell cytotoxic and cytokine secretion functions in the antitumor response (93).

Soluble Molecules

In addition to cell-to-cell contact-based inhibition, NK cells' functions are inhibited by various soluble molecules, including soluble ligands of regulatory NK receptors, cytokines, such as TGF- β or IL-10, metabolic compounds, such as ROS, and tryptophan or arginine catabolites.

Some AML clones have adopted strategies to inhibit NK cells with specific soluble compounds. To date, NKG2D and NKp30 were the main targets described for such inhibition. Soluble ligands can be released by tumor cells into the extracellular environment. NKG2D-ligands (NKG2D-L) and the NKp30 ligands B7-H6 are cleaved by metalloproteinases, either matrix metalloproteinases (MMP) or A disintegrin and metalloproteinases

(ADAMs) (94–99). Soluble NKG2D-L was found in the serum of patients with solid tumors or hematological cancers. Moreover, soluble MICA release is regulated by chaperones, such as ERp5, modulating MICA structure with the support of the heat shock protein GRP78 to induce conformational changes allowing its cleavage (100, 101). Despite a high heterogeneity in the surface expression of NKG2D-L by AML, the majority of patients shows soluble forms of MICA, MICB, or ULBP2, alone or more often in combination with up to five soluble NKG2D-L (102). As observed in solid tumors (103), the presence of soluble NKG2D-L in AML patients' serum is associated with a reduction of the surface NKG2D expression leading to a decrease in NKG2D-mediated NK-cell's activity (102). Importantly, a recent work by Deng et al. showed that the soluble form of MULT-1, a mouse high-affinity NKG2D-L, released by tumors, can stimulate NK-cell cytolytic function, and induce tumor rejection in mice (104). Activating soluble NKG2D-L was not identified in human yet, but such molecule, if it exists, could be of interest for inducing antitumor NK-cell function.

B7-H6 shedding by solid tumors seems to induce a reduction of the NKp30-mediated tumor cell recognition by NK-cells (96). In contrast to soluble NKG2D-L, this process would be due to a reduction of the B7-H6 expression on the tumor cell surface rather than to a direct inhibition of the NKp30 expression on the NK cell. No observation of B7-H6 shedding by AML has been made yet but the expression of B7-H6 on AML cells together with the reduction of expression of NKp30 on patient's NK cells would justify studying this pathway in AML patients.

Soluble NKG2D and NKp30 ligands can be also released in the serum bound to tumor-derived exosomes (TEX) (105, 106). Exosomes from solid tumors or leukemia/lymphoma cells can present MICA, MICB, and ULBP molecules leading to an inhibition of the NKG2D-mediated NK cell activation (105, 107). In the same way, a reduction of NKp30 can be observed when NK-cells are incubated with B7-H6 positive exosomes produced by myeloid subsets in inflammatory conditions (108). By contrast, exosomes carrying BAG6, another NKp30 ligand, are necessary to activate NK cells in order to eliminate chronic lymphocytic leukemia (CLL) cells, whereas soluble BAG6 lead to tumor evasion (106). In addition, soluble galectin-3 produced by solid tumor cells works as an inhibitory ligand of NKp30 (109). In line with this observation, higher levels of galectin-3 gene expression in BM are an independent unfavorable prognostic factor for overall survival in patients with AML (110). Altogether, these observations would suggest that soluble or exosomes bound NKp30 ligands could also interfere, in parallel with other soluble ligands, with NK cell functions in AML patients.

Imbalance in serum cytokines can be responsible for perturbations in the regulation of the antitumor response. In contrast to reduced TGF- β levels in plasma of AML patients as compared to healthy individuals (75, 111), IL-10 was found significantly higher together with the proinflammatory cytokines IL-6 and TNF- α . Curiously, high levels of IL-6 and low levels of IL-10 are associated with poor outcome (111). Even if such cytokine environment is probably more related to the AML physiology, it still can influence immune cell properties. Indeed, high levels of IL-6 were shown to impair perforin and granzyme B expression

and reduce NK cell cytotoxic activity in individuals with autoimmune diseases (112), with heart failure (113), and cancer patients treated with recombinant IL-6 (114).

In parallel, high levels of small immunosuppressive molecules, side products of the leukemic cell metabolism, can be released by AML blasts. ROS participate to NK-cell defects in the expression of activating receptors, such as NKp46 and NKG2D (115). Interestingly, they are highly produced by AML with specific mutation patterns, such as activating mutations in RAS family members or FLT3/ITD mutations (116, 117). Arginine metabolism is also enhanced in AML blasts leading to an immunosuppressive environment. High levels of production of active arginase II by AML blasts can induce an accumulation of this enzyme in the plasma of patients, resulting in significant inhibition of lymphocyte proliferation (118). In addition, the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) is also expressed by leukemic blasts, whereas it is absent from normal hematopoietic CD34⁺ stem cells (119). IDO catalyzes tryptophan degradation by producing L-kynurenine, which can directly affect NK cell phenotype and cytolytic function through the inhibition of the cytokine-induced upregulation of NKp46 and NKG2D (120). In addition, IDO activity can stimulate the emergence of CD4⁺CD25^{high} regulatory T-cells (Tregs) (121) capable of inhibiting NK cell functions by TGF- β release (122) or IL-2 starvation (123).

AML AND CONVENTIONAL TREATMENTS: RESISTANCE TO CHEMOTHERAPIES

Acute myeloid leukemia treatment by conventional chemotherapy eliminates tumor blasts and leads to the achievement of CR in 70–80% of patients younger than 65 years (6). Elimination of circulating AML blasts allows the recovery of NK cell phenotype and functions (75, 76), and a sustained autologous NK cell activity can support a continued CR (124). However, at least half of patients will eventually relapse (6). A major limitation for success in chemotherapy of AML is dominance of drug-resistant subpopulations of cells. AML cells also can achieve the resistance phenotype through modification of multiple and diverse pathways, such as inactivation of the mitochondrial apoptotic machinery, decreased expression of proapoptotic agents, upregulation of antiapoptotic molecules, and promotion of drug efflux. Although daunorubicin (DNR) is the most efficient and widely used anthracycline to treat AML, resistance to this drug remains a critical problem (125–127). In this regard, the intrinsic and acquired resistance of AML to drug treatment remains a fundamental challenge for improving patient outcome. One of the consequences of acquisition of drug resistance by leukemic cells is the appearance of cross-resistance against immune effector cells. We have recently demonstrated that the acquisition of resistance to DNR resulted in the acquisition of cross-resistance to NK cell-mediated cytotoxicity. MiR microarray analysis revealed that this cross-resistance was associated with miR-181a downregulation and the subsequent upregulation of MAP3K10 and MAP2K1 tyrosine kinases and the BCL-2 (BCL-2 and MCL-1) family. These studies point to a determinant role of miR-181a in the

sensitization of leukemic resistant cells to DNR and NK cells and suggest that miR-181a may provide a promising option for the treatment of immuno- and chemoresistant blasts (128).

In contrast, previous studies showed that NKG2D-L can be upregulated on the AML cell surface after treatment with various molecules, including the histone deacetylase inhibitor valproic acid (VPA) (129, 130). In the same way, the administration of all-*trans*-retinoic acid (ATRA) can also increase the NKG2D-L expression levels on acute promyelocytic leukemia, a particular subtype of AML with a *PML-RARA* gene fusion (130). Importantly, such increased expression of NKG2D-L cannot be observed in patients treated with chemotherapy in absence of ATRA or VPA (130). These observations suggest that chemotherapy can stimulate an anti-AML NK-cell-mediated response. Recently, we showed that cytarabine-resistant cells become more susceptible to NK-mediated cell lysis as compared to parental cytarabine-sensitive cells. The increased susceptibility correlates with the induction of ULBP 1/2/3 and NKG2D-ligands on target cells by a mechanism involving *c-Myc* induction (131). These studies could help to improve the efficacy of NK-cell-based therapy that allows for better designing of NK-based immunotherapy.

IMMUNOTHERAPEUTIC STRATEGIES

Given the sensitivity of AML to NK-cell-mediated lysis, strategies to enhance or restore NK cell functions in patients could be of interest besides conventional chemotherapy. Numerous methods have been developed during the last few years in order to either modulate immunity against tumors using immunomodulatory drugs (IMiDs) or cytokines or to specifically target or activate NK cells against leukemia cells. Such treatments, used alone or in combination with chemotherapy, aim to eliminate chemoresistant tumor cells.

Immunomodulatory Drugs

Immunomodulatory drugs are structural and functional analogs of thalidomide (132). To date, two molecules have been approved in MDS, multiple myeloma (MM), or mantle cell lymphoma (MCL): lenalidomide and pomalidomide. Alone (for MDS or MCL) or in combination with dexamethasone (MM), as a second or third line of treatment, IMiDs improve both time-to-progression and overall survival of patients. Several studies have also explored the synergistic effect of IMiDs with rituximab for the treatment of CLL (133) or MCL (134). Beyond their direct effect on cancer cell proliferation and angiogenesis, these molecules stimulate antitumor effectors, including B, T, and NK cells (135). Hence, by their broad range of effects, IMiDs represent a novel strategy for immunotherapy as evidenced by numerous ongoing clinical trials, in many cancer settings, including AML (136).

In the case of NK cells, IMiDs increase the expression of activating receptors, notably NCR (137, 138). These molecules induce expansion of NK cells as confirmed by immunomonitoring studies in several clinical trials (137, 139, 140). Enhanced NK cell ADCC or natural cytotoxicity is largely mediated *via* IL-2 produced by T cells (141). In addition, we have recently shown that lenalidomide enhances tumor cell recognition by NK cells by improving the stability of the immune synapse (56).

Finally, IMiDs increase tumor infiltration by NK cells in murine models (142).

Cytokines

Several cytokines of the IL-2 family are essential for NK cell survival, expansion, and activation, but so far, only IL-2 has an approval for clinical use. In the family of IL-2, IL-15 and IL-21 share some characteristics such as activation and proliferation of NK cells, and the common γ -chain for their receptor (143). IL-2 is able to induce expression of NKG2D, NKp44, and NKp46 on NK cells (49, 82, 144). In MDS, however, following *in vitro* IL-2 stimulation, NK cells do not recover a normal cytolytic activity when compared to healthy volunteers (82). Moreover, IL-2 fails to induce NK cell proliferation compared to healthy volunteers, but rather increases the rate of apoptotic NK cells (82). So far, the therapeutic use of IL-2 for the treatment of hematological malignancies has been hampered by a peripheral toxicity (145) and an unwanted expansion of Tregs (146). Conclusions of clinical trials report modest antitumor activity when used as a monotherapy. Therefore, the use of IL-2, especially at high doses, might be restricted to *ex vivo* expansion of NK cells given problems of *in vivo* toxicity (145).

In contrast to IL-2, IL-15 and IL-21 may represent a better alternative because these cytokines do not expand Tregs. Accordingly, many clinical trials currently aim to demonstrate an efficient NK cell-mediated antitumor response with *in vivo* or *ex vivo* IL-15-expanded NK cells in AML. Hence, in the absence of clinical approval for IL-15, several groups are testing the possibility to expand NK cells *in vitro* before reinfusion into patients.

IL-15 plays a major role in the proliferation, differentiation, survival, and functions of T and NK cells (147, 148). *Ex vivo* exposure of NK cells from AML patients to IL-15 enhance NKp30, NKp46, NKG2D, and NKG2C surface expression. Accordingly, this increase of receptor expression correlated with an enhanced natural cytotoxicity against autologous AML cells (147, 149). In addition, in hematological malignancies, low levels of circulating IL-15 after BM transplantation are predictive of risk of relapse (150). In line, NK cell recovery in stem cell transplantation is strongly correlated with plasmatic concentrations of IL-15 (149).

The serum concentration of IL-15 increases dramatically following administration of cytotoxic agents (147, 150). For some authors, this elevation of serum IL-15 could be related to the depletion of lymphoid populations that normally consume circulating IL-15 or to inflammation induced by chemotherapy (149). *In vivo* injections of the IL-15/IL-15R α heterodimer result in significant expansion of $\gamma\delta$, CD8⁺ T, and NK cells (148). Recently, this cytokine has become available for use in early phase clinical trials as an alternative to IL-2 (147, 148). IL-15 is currently assessed as a therapy for various solid tumors, including refractory metastatic melanoma, metastatic renal cell cancer.

IL-21 has been proven safe in phase I clinical trials with signs of clinical activity (151). IL-21 stimulation of NK cells mainly results in enhanced NK cell functions. *Ex vivo*, IL-21 is capable of inducing NK cell maturation and stimulates the production of IFN- γ and cytotoxic properties of NK cells (152–154). Several clinical trials have reported the effect of IL-21 therapy on immune system after administration in patients with metastatic

melanoma and renal cell carcinoma (151). Although NK and T cell numbers were temporarily decreased during administration of IL-21, the cells had higher expression of CXCR3, hyaluronan-mediated motility receptor (HMMR), IFN- γ , perforin, and granzyme. In addition, NK cells from patients displayed an enhanced cytotoxicity capacity (151). These results were confirmed in a phase II trial for metastatic melanoma (155). In the absence of clinical approval, IL-15 and IL-21 are also used *ex vivo* to expand and activate NK cells for further infusion in patients. NK cells stimulated *ex vivo* by the leukemic cell line K562 expressing membrane-bound IL-15 or IL-21 showed a strong proliferation and cytolytic activity with a higher proliferation rate and an increase in telomere length for IL-21-activated NK cells (156). NK cells expanded *ex vivo* by membrane-bound IL-15 are currently infused into MDS or AML patients (phase I clinical trial #NCT02123836).

Bi- and Trispecific Killer Cell Engager

Several monoclonal antibodies (mAbs) directed against tumor antigens have been generated and are currently used in the clinics. The most famous therapeutic mAbs remains the anti-CD20 rituximab, which is widely used to B-cell-related diseases and cancers. Several mechanisms of action have been identified and one of these is the recognition of the Fc part of the human or humanized IgG1 or IgG3 isotypes by CD16 expressed by NK cells and myeloid cells. Upon engagement of CD16, the cells are activated and kill the targeted cells. Unfortunately, several studies have shown that the polymorphism of CD16 and the engineering process may alter ADCC efficacy. Bispecific killer cell engagers (BiKEs) are engineered antibodies with dual specificity, for a tumor antigen like CD19 or CD20 for B-cell-related diseases and CD16 targeting NK cells. The anti-CD16 part of the BiKEs bypasses the disadvantages of classical mAbs (157). For instance, AFM13, a BiKE targeting CD19 and CD16, has been recently tested in phase I and II trials (#NCT01221571 and #NCT02321592, respectively) in non-Hodgkin lymphoma.

In vitro studies demonstrated that CD33 \times CD16 BiKEs trigger NK cell activation against AML cell lines and primary targets through CD16 signaling, leading to cytokine and chemokine production (158). As a consequence, significant increases in NK cell cytolytic activity led to induction of target cell apoptosis at high and low target to effector ratios. In a study based on NK cell isolation from patients with MDS, authors showed that CD33 \times CD16 BiKE potently activates blood and marrow MDS–NK cells at all disease stages to lyse CD33⁺ MDS and CD33⁺ myeloid-derived suppressor cells (MDSCs) targets (159). Noteworthy, MGD006, a CD123 \times CD3 BiKE is tested in a phase I trial (#NCT02152956), confirming the current explosive attention to BiKEs as potent therapeutic tools for AML and other cancers. In the same way, a CD30 \times CD16 bispecific tetraivalent chimeric antibody (TandAb) was used in a phase I clinical trial in patients with relapsed or refractory Hodgkin lymphoma showing good tolerance and tumor targeting (160). However, with respect to AML treatment, the use of BiKEs remains limited due to the heterogeneity of tumor antigen in this disease.

Recently, several new reagents were developed in attempt to enhance the targeting of malignant cells. Gleason et al. have

generated a trispecific mAb (TriKE) directed against CD19, CD20, and CD16 (159). This TriKE, efficiently engaged NK cells against CD19⁺CD20⁺ leukemic targets, as proven by a strong cytotoxicity and IFN- γ production. To increase NK-cell activating properties, Miller et al. have developed a TriKE targeting CD33 and CD16 that contains IL-15 (161). This reagent not only mediates CD16 directed cytotoxicity against CD33⁺ leukemic cells but also sustains NK-cell activation and persistence by the IL-15 linker.

In conclusion, targeted cellular immunotherapy with BiKEs and TriKEs are promising approaches in terms of effector cell retargeting and induction of efficient antitumor response and are currently being developed and evaluated for targeting of various malignancies (162, 163).

Antibodies Directed against NK Cell Inhibitory Receptors

Among strategies to improve the recognition of tumor cells by NK cells, blocking the inhibitory interactions is appealing. Inhibitory molecules, such as KIR and NKG2A, are expressed at the surface of NK cells and inhibit NK cell activation *via* their ligands (HLA-C and HLA-E, respectively). In the case of cancer patients, expression of KIR and NKG2A, as well as expression of their ligands at the surface of tumor cells, has been described in several solid cancers and leukemias (164–166). Subsequently, activation of NK cells is likely prevented and leads to NK-mediated immune evasion. Inhibition of these mechanisms by blocking antibodies is currently being assessed.

IPH2101 is a fully human IgG4 that blocks the interaction between the major subset of KIR (KIR2DL1, KIR2DL2, and KIR2DL3) and their cognate ligands (167, 168). A second generation of anti-KIR mAb, lirilumab (IPH2102/BMS-986015) with a stabilized hinge was generated (167, 168).

In vitro studies showed that IPH2101 augments NK cell-mediated lysis of KIR-ligand matched tumor cells and enhances NK cell-mediated ADCC against antibody-bound tumors (168–170). The therapeutic potential of IPH2101 has also been demonstrated in preclinical mouse models (171, 172), which have formed the basis for clinical trials evaluating IPH2101 in patients with cancer (173). Blocking NK inhibition with the anti-KIR IPH2101 antibody has been proven to be safe in early phase clinical trials in patients with AML and MM (174, 175) and enhances *ex vivo* NK cell cytotoxicity against MM cells (175).

However, a phase II study in MM patients did not reveal lasting objective responses (173). IPH2101 has also been assessed *in vitro* in combination with lenalidomide and potentiates NK-cell cytotoxicity toward autologous myeloma cells. This combination is currently being tested in a phase I clinical trial in MM patients (#NCT01217203) (176). The second generation anti-KIR lirilumab was also shown to synergize with Lenalidomide to increase NK cytotoxicity of myeloma patients treated with Daratumumab (anti-CD38) (177). *In vitro* and *in vivo* lirilumab enhances NK activity against CD20⁺ lymphoma cells (167). With respect to AML, lirilumab is currently tested in patients in CR for long-term maintenance (#NCT01687387), and for the treatment of patients with refractory/relapsed AML (#NCT02399917). Although safe,

this therapeutic mAb did not induce impressive clinical improvement so far. First of all, cytotoxic effectors expressing KIRs (NK cells, $\alpha\beta$ CD8⁺, and $\gamma\delta$ T cells) use other inhibitory KIRs that are not targeted by lirilumab (NKG2A, KIR3DL, and CD85j/ILT2). In an autologous setting, it is likely that these cells may still remain tolerant to leukemic cells. In addition, lirilumab also recognizes KIR2DS1 and KIR2DS2; blocking these receptors may, in contrast, unfavor tumor cell clearance (168).

Noteworthy, a newly engineered mAb directed against NKG2A has been generated by Innate Pharma (IPH2201) and is currently tested in clinical trials (phase I/II) in ovarian cancer, squamous cell carcinoma, and refractory CLL (#NCT02459301, #NCT02331875, and #NCT02557516, respectively). It is tempting to speculate on the efficacy of this new reagent in AML treatment, as these cells are expected to express HLA-E.

Other inhibitory receptors, including PD-1, LAG-3, or TIM-3, usually classified as “inhibitory checkpoint receptors” may influence NK cell activity. The increasing interest for the PD-1/PD-1-ligands axis on T-cells in cancer therapy legitimated the analysis of PD-1 expression on NK cells in various pathological situations. In a mouse model of glioblastoma, NK cell functions against mouse glioma stem cells and the survival of the mice can be ameliorated by blocking either PD-1 or the PD-1 ligand B7-H1 (also named PD-L1) (178). In human, data describing a role for PD-1 in the regulation of NK cells are relatively scarce. Wiesmayr et al. observed the expression of PD-1 on NK cells in pediatric patients with post-transplantation lymphoproliferative disorders caused by EBV infection. The presence of PD-1 was associated with a reduced expression of NKP46 and NKG2D and NK cell function impairment, and blocking the PD-1 inhibitory pathway could restore IFN- γ secretion (179). The PD-1/PD-L1 axis was also involved in the modulation of NK-cell functions against MM (180). Interestingly, IFN- γ can induce the expression of PD-L1 on AML cells leading to the inhibition of the antileukemic response mediated by T-lymphocytes (181) and NK cells (182). Therefore, the anti-PD-1 mAb Nivolumab is tested in a phase II clinical trial in AML patients in remission with high risk of relapse (#NCT02532231).

Chimeric Antigen Receptor–NK Cells

Another strategy to improve antitumor immunity has arisen from recent advances in cell genetic modification that have allowed the specific targeting of tumor cells by cytotoxic effectors. Most of the tools generated are chimeric antigen receptor (CAR)-T cells, i.e., T cells engineered to express a receptor for tumor antigen (for instance, CD19 in the case of B-cell leukemia) coupled to activate signaling adaptors. The few clinical trials with CAR-T cells have obtained somewhat promising results that should be strengthened by other studies. Genetic modification of NK cells has been more recently performed, but not yet with myeloid tumor specificity. For instance, Töpfer et al. generated NK cell lines or primary NK cells targeting PSCA, a prostate cancer antigen (183). As expected, these cells react against PSCA positive tumor cell lines by secreting IFN- γ and killing these target cells. In line with this observation, several others have

been designed, based on NK-92 or other NK cell lines (184–187). This new strategy is promising although the costs may remain a serious limitation.

CONCLUSION

Acute myeloid leukemia is the most common myeloid leukemia, usually treated with a combination of anthracyclines and cytarabine in a first attempt to achieve CR. The consolidation phase of the treatment aims to prolong CR and eventually to cure the disease. However, disease heterogeneity (cytogenetic and mutation profile, deepness of BM failure, resistance to treatment) and patient's general condition (age, secondary AML) led to an unfavorable prognosis for many patients. Over time, AML develops various mechanisms to protect itself from the patient's immune system and more precisely from NK cells. The long-term coexistence of leukemia-initiating cells, and then tumor blasts, with NK cells, first in the BM and later in the periphery, can explain the emergence of NK cell defects together with immunoresistant AML cells. The antitumor function of NK cells, demonstrated after allogeneic HSC transplantation, justifies developing methods in order to restore, stimulate, or induce NK cell activity in AML patients. Treatments combining the elimination of the peripheral leukemic blasts using conventional chemotherapy, together with the chemoresistant leukemia-initiating cells, targeted by immune mediators, including NK cells, appear very attractive. However, we could consider the opportunity to stimulate NK cell antileukemic functions before the emergence of the disease. Indeed, the recent observation of preleukemic mutations in healthy elderly individuals' HSC questions the capacity of the immune system to eliminate or tolerate the presence of leukemia-initiating cells in the BM at advanced age. In that context, stimulating the immunosurveillance mediated by NK cells could be a promising preemptive strategy against AML.

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Conflict of Interest Statement: DO is a founder and shareholder of Imcheck Therapeutics (Marseille, France). The other authors declare no competing financial interests.

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