

Role of Toll-like receptors in natural killer cell function in acute lymphoblastic leukemia (Review)

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Received August 22, 2020; Accepted December 22, 2020

DOI: 10.3892/ol.2021.13009

Abstract. Natural killer (NK) cells are specialized lymphocytes primarily involved in the response to infection and tumors. NK cells are characterized by the presence of specific surface molecules, as well as a wide repertoire of receptors that impart microenvironment-dependent effector functions. Among these receptors, Toll-like receptors (TLRs) can be activated to condition the NK response to either a cytotoxic or immunoregulatory phenotype. However, cellular function is frequently impaired during disorders such as cancer. In the last decade, it has become increasingly evident that the stimulation of NK cells is a requirement for their increased cytotoxic activity. TLR activation has been suggested as an alternative route for reestablishing the antitumor activity of NK cells. The present review summarizes the characteristics of NK cells, their receptors, the expression and function of NK cell TLRs, and their functional status in cancer, primarily acute lymphoblastic leukemia.

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Key words: natural killer cells, Toll-like receptors, acute lymphoblastic leukemia, cytotoxic phenotype, immunoregulatory phenotype

1. Overview of NK cells

Natural killer (NK) cells are considered to be a subpopulation of lymphocytic cells and, due to their granularity and size, are also regarded as long granular lymphocytes; however, NK cells are a morphologically homogeneous population indistinguishable from the total lymphocyte pool. NK cells constitute 5-15% of all mononuclear cells in the peripheral blood (1), and were initially thought to be generated only from CD34⁺ lymphoid precursors in the bone marrow (2). However, studies in mice and humans suggest that NK cells are also able to develop and mature in secondary lymphoid tissues (3).

Phenotypically, NK cells are primarily identified by the expression of CD56 (neural cell adhesion molecule) (4) and CD16 (human IgG Fc receptor III, FcγRIII), as well as the lack of CD3, T-cell receptors and CD19, typical of T and B lymphocytes (5). NK cells have recently been categorized as group 1 innate lymphoid cells, due to their ability to produce IFN-γ and selectively express the T-box eomesodermin transcription factor, which is necessary for their development and function (6).

Functionally, NK cells play an important role in immunosurveillance, since they participate in innate resistance against infected cells without prior sensitization or antigenic presentation, and also exert natural cytotoxic activity against tumor cells (7). The activation of NK cells favors the maintenance of an inflammatory environment, since they produce a number of inflammatory cytokines that recruit other immune cells (8). Similarly, NK cells serve an important role in the expansion of Th1-type responses, and are involved in hematopoiesis-associated processes (9).

The existence of a population of memory NK cells has been suggested. However, despite evidence in non-human primates (10), this NK cell function has not yet been verified in humans, and requires further investigation (11).

2. Origin of NK cells

NK cells are derived from precursors that originate from hematopoietic stem cells in the bone marrow; when stimulated with interleukin (IL)-2, IL-15 and IL-7, these precursors develop into mature NK cells (12). However, these NK precursors (NKP) can also originate from an early thymus lymphoid precursor (13). NKP are able to circulate between different

organs, maturing into functional NK cells in the bone marrow, thymus, liver, spleen and lymph nodes (12).

On reaching immune competence, NK cells leave their sites of origin and migrate to target tissues, including the lung, mucosa, intestine, liver, skin and uterus. At these sites, NK cells differentiate according to their resting or activated phenotypes, which express stimulation markers and exhibit unique functional properties (14).

3. NK cell diversification

Due to the various effector functions exhibited by NK cells in humans, the existence of subpopulations with specialized functions has been suggested. The use of monoclonal antibodies and flow cytometry has enabled the classification of NK cells according to the expression density of surface markers and their association with characteristic functions.

Based on the surface expression density of CD56, NK cells can be classified as 'bright', with high CD56 expression density, and 'dim', with low CD56 expression density. In the peripheral blood, ~90% of NK cells are CD56^{dim}, while the other 10% are CD56^{bright} (15). Furthermore, according to the relative co-expression of CD56 and CD16, NK cells can also be classified into the following five subpopulations: i) CD56^{bright}/CD16⁻, constituting 50-70% of the total CD56^{bright} population; ii) CD56^{bright}/CD16⁺, constituting 30-50% of total CD56^{bright} cells; iii) CD56^{dim}/CD16⁻; iv) CD56^{dim}/CD16⁺; and v) CD56⁻/CD16⁺ (16). These populations detected in the blood of a healthy donor by flow cytometry (Appendix S1) are presented in Fig. 1.

CD56^{bright} NK cells are considered to be immunoregulatory, as they have the ability to secrete immunomodulatory cytokines such as IFN- γ , IL-10, IL-13, TNF- β and granulocyte and monocyte colony stimulating factor at rest, with increased secretion following activation. However, the quantity and quality of these cytokines varies according to the stimulus, since interleukins such as IL-2, IL-15 and IL-18 are capable of increasing the secretion of IFN- γ , IL-10 and TNF- β (17). NK CD56^{bright} cells have a low density of perforin granules and granzymes, and exert decreased cytotoxic activity compared with CD56^{dim} cells (18). Furthermore, CD56^{bright} cells are the only NK cells that constitutively express the high-affinity IL-2 receptor, which is able to induce the proliferation of NK cells at low (picomolar) concentrations (19). CD56^{bright} cells are also capable of proliferation and self-maintenance in the presence of IL-15, without co-stimulation (20).

The expression of adhesion molecules such as CD62L (L-selectin) (21), as well as chemokine receptors, namely C-C chemokine receptor type (CCR) 7, CCR4 and C-X-C chemokine receptor type (CXCR) 3, on the surface of CD56^{bright} NK cells enables them to preferentially migrate to secondary lymphoid organs such as the tonsils and lymph nodes (22). Furthermore, the expression of CXCR1 and CXCR3 confers different migratory properties to CD56^{bright} cells, causing them to be located primarily in the spleen and bone marrow, as well as at sites of acute inflammation (16).

CD56^{bright} NK cells have been suggested to be precursors of their CD56^{dim} counterparts as, under the influence of certain cytokines, the former can decrease their expression of CD56 and increase that of CD16 (23). Also, the CD56^{bright}

subpopulation is the first to appear during reconstitution of the immune system after bone marrow transplantation. In the NK^{bright} population, CD56^{bright}/CD16⁻ cells are considered immunomodulatory due to their interaction with dendritic cells in the lymph nodes, and elevated production of IFN- γ (16). By contrast, the CD56^{bright}/CD16⁺ subpopulation is considered to be transitional, with minimal IL-2-induced proliferation and cytotoxic activity (24).

Approximately 90% of peripheral blood NK cells are NK^{dim}, of which the predominant phenotype is CD56^{dim}/CD16^{bright} (95%), with lower numbers of CD56^{dim}/CD16⁻ and CD56⁻/CD16^{bright} cells (16). Unlike their bright counterparts, NK^{dim} cells are poor cytokine producers (17). However, when activated by contact with target cells, they are capable of producing IFN- γ , TNF- α , macrophage inflammatory protein (MIP)-1 α and MIP-1 β , sometimes in higher concentrations than those generated by CD56^{bright} cells (8).

NK^{dim} cells express low surface levels of the IL-2 receptor and, therefore, have little proliferative capacity *in vitro*, even when stimulated with high concentrations of IL-2 (19) and IL-15 (20). NK^{dim} cells generally possess high cytotoxic activity due to their high expression levels of perforins, granzymes and cytolytic granules (25). Furthermore, NK^{dim} cells express high quantities of receptors that enhance their ability to respond to abnormal cells, and a high surface density of CD16 that allows them to carry out antibody-mediated cytotoxicity functions (25).

Unlike CD8 T cells, the cytotoxic activity of NK cells against target cells does not require prior activation and is independent of the expression of major histocompatibility complex (MHC) molecules. This activity can be initiated by different processes, including degranulation and antibody-dependent cytotoxicity, as well as the binding of ligands to cellular death receptors (26).

The molecular mechanisms that regulate cellular cytotoxicity may be divided into three categories: i) Recognition of the target cell; ii) formation of the immune synapse; and iii) NK-induced cell death. Following recognition and the formation of the immune synapse, cell death is induced by two primary mechanisms (27). The first mechanism involves the activation of cell death receptors present on the surface of the target cell. These receptors include the TNF-related apoptosis-inducing ligand receptor (TRAIL-R) and Fas (CD95), which are activated by TRAIL and Fas ligand (CD95L), respectively (26). The stimulation of these receptors induces activation of the caspase 8 and 10 pathways, resulting in apoptotic cell death (28). However, the predominant mechanism of cytotoxicity involves the direct release of lytic granules towards the target cell. This requires reorganization of the cytoskeleton, as well as the polarization of NK cell microtubules. NK cells also contain perforins, enzymes that when integrated into the target cell membrane form a pore through which water can enter the cell, promoting osmotic lysis (29).

Granzyme B is a critical component of NK cells, as it is a protein capable of inducing apoptosis by promoting the breakdown of peptides with aspartic acid residues (26). Once inside the target cell, granzyme B induces caspase-dependent and -independent apoptosis, which is itself dependent on initiation by the direct cleavage of caspase 8 or 3 (30).

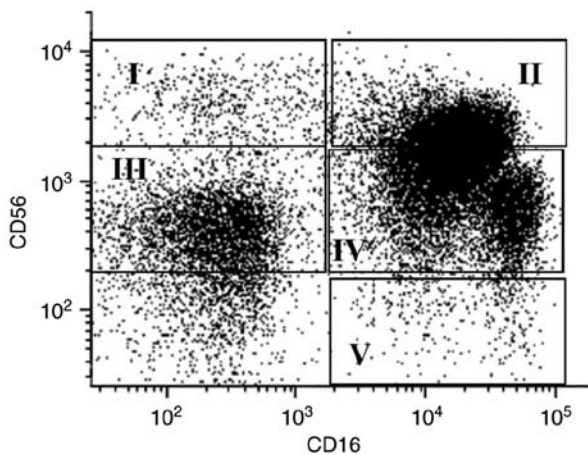


Figure 1. Flow cytometric analysis of CD56^{bright} and CD56^{dim} natural killer cell subpopulations from a healthy donor. (I) CD56^{bright}/CD16⁻, (II) CD56^{bright}/CD16⁺, (III) CD56^{dim}/CD16⁻, (IV) CD56^{dim}/CD16⁺ and (V) CD56⁻/CD16⁺.

Caspase-independent apoptosis is achieved by the induction of cytochrome release from the mitochondria, and the cleavage of the pro-apoptotic protein Bid (31). These natural cytotoxic activities are reportedly most effectively by CD56^{dim}/CD16^{bright} cells since upon activation, CD56^{dim}CD16^{bright} cells have been shown to lose their CD16 expression through metallo-protease-mediated cleavage to become CD56^{dim}CD16⁻, and express increased levels of CD107a, which has been described as a marker of degranulation (32).

As aforementioned, NK cells are capable of participating in antibody-mediated cytotoxicity, an immune mechanism by which NK cells induce the death of antibody-opsonized cells (33). Since CD56^{dim}/CD16^{bright} NK cells express a high density of CD16, this subpopulation of NK^{dim} cells is the most capable of performing this process (18). Once the target cell has been opsonized and CD16 recognizes the Fc region of immunoglobulins, cell death is induced by one of the aforementioned mechanisms (26).

4. NK cell receptors

NK cells express a variety of different receptors that allow them to interact with diverse cell subpopulations, and thus trigger different effector functions. Since NK cells do not express clonal receptors, the receptor repertoire of these cells comprises germline receptors, which have traditionally been classified as inhibitors or activators (15) (Fig. 2). These germline receptors include pattern recognition receptors (PRRs), which are discussed in more detail below.

The inhibitory receptors comprise two NK receptor superfamilies. The first family comprises the killer cell immunoglobulin-like receptors (KIRs) KIR2DL1-5 and KIR3DL1-3, which recognize classical MHC-I molecules. The other superfamily comprises lectin C-type receptors, which are heterodimers such as CD94/natural killer group 2 member A (NKG2A) that primarily recognize non-classical MHC molecules, including human leukocyte antigen (HLA)-E (34). When these receptors interact with their ligands, immunoreceptor tyrosine-based inhibitory motifs initiate signaling

cascades that prevent NK-cell activation (35). NK cells also express subfamily B member 1 of the inhibitory leukocyte immunoglobulin-like receptor family (LILRB1), which binds non-classical MHC molecules such as HLA-G (36). Under physiological conditions, NK cells are inhibited by the interaction between the aforementioned receptors and their ligands, which are present on the surface of healthy cells. By contrast, when infected with intracellular pathogens or undergoing malignant transformation, cells frequently experience a reduction or loss of MHC-I expression, and therefore, become potential NK-cell targets. This phenomenon is known as missing self-recognition (37). However, the absence of MHC-I molecules on the surface of target cells is not sufficient to trigger the activation of NK cells, as the correct balance between inhibitory and activating receptor signals is required (38).

Activating receptors, such as KIR2DS, KIR3DS, NKG2D and NKG2E, are also capable of recognizing classical MHC-I molecules, while NKG2D receptors also bind non-classical MHC-I (36). These receptors possess immunoreceptor tyrosine-based activating motifs that transduce activation signals (39).

Furthermore, NK cells express natural cytotoxicity receptors, which are involved in the preferential activation of NK cells against tumor cells (15). Among these receptors, NKp30 (CD337) and NKp46 (CD335) are expressed by resting and activated NK cells, while NKp44 (CD336) is only expressed by activated NK cells (34).

NK cells also express co-stimulation ligands, including CD40L (CD154) (40) and OX40L, which allow them to impart co-stimulatory signals to T or B cells (41). Thus, NK cells serve as a bridge between innate and adaptive immunity. Dendritic cells stimulate NK cells to provide co-stimulatory signals to T or B cells, allowing for an optimal immune response (42).

Additionally, NK cells express PRRs through which they recognize pathogen-associated molecular patterns, as well as damage-associated molecular patterns (DAMPs). PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs) (43) and RIG-I-like receptors (RLRs) (44). From the NLR family, NK cells have been shown to express NOD2 and NLRP3; when activated, these NLRs increase the cytotoxicity of NK cells against tumor cells, and upregulate the production of cytokines such as TNF- α and IFN- γ (45). With respect to RLRs, NK cells have been shown to express RIG-I and melanoma differentiation-associated protein 5 receptors, which are of great importance in antiviral defense. These receptors have been shown to serve an important role in human NK-cell functioning, increasing their production of IFN- γ as well as their antitumor activity (44).

Several studies have demonstrated the functional responses of human NK cells to stimulation with various TLR ligands (46,47). The majority of studies indicate that NK cells require the concurrent presence of pro-inflammatory cytokines to respond to TLR agonists (48).

5. Toll-like receptor expression in NK cells

Initially, the identification of TLR expression in NK cells was based on mRNA detection only. In NK cells isolated from human peripheral blood, the constitutive expression of TLR1-8 mRNA

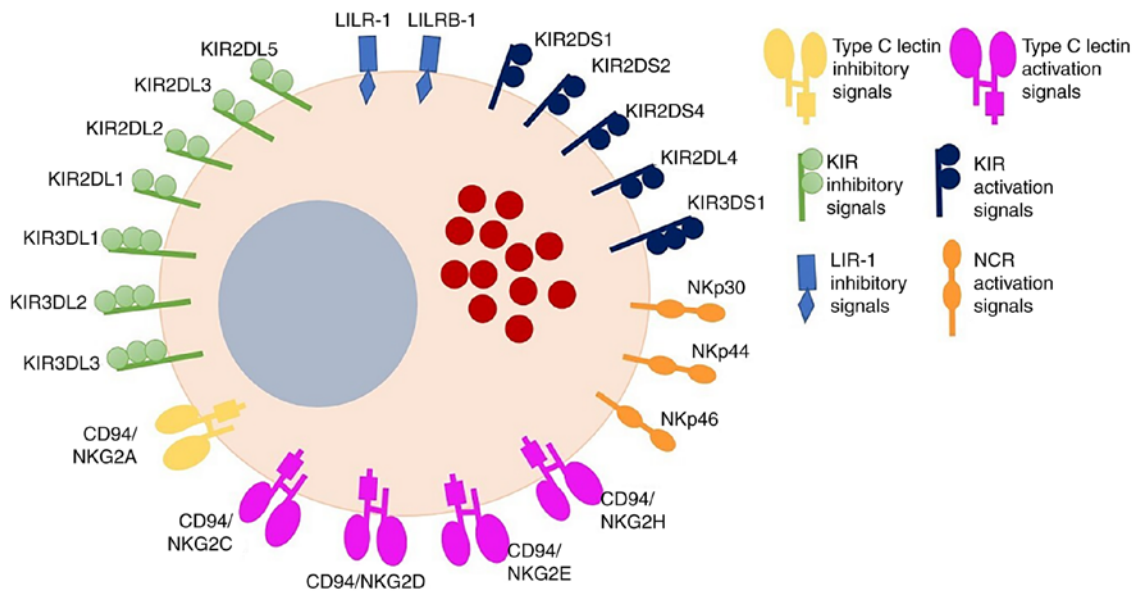


Figure 2. Inhibitory and activating NK-cell receptors, and the associated signals that are triggered subsequent to ligand-induced activation. NK, natural killer; KIR, killer cell immunoglobulin-like receptor; LIR-1, leukocyte immunoglobulin-like receptor 1; NCR, natural cytotoxicity receptor.

was observed, of which TLR2 and 3 were the most abundantly expressed. By contrast, the expression of TLR9 and 10 mRNA was found to be insignificant in CD56^{bright} and CD56^{dim} NK-cell populations (43). Furthermore, TLR2 mRNA was reported to be highly expressed, while TLR4 and 3 mRNAs were weakly expressed, and TLR8 mRNA was undetectable in resting human NK cells (49). Additionally, the mRNA levels of these four TLRs increased considerably following exposure to viral particles (49). In general, it has been observed that TLR2, 3, 5 and 6 are more frequently expressed than TLR4, 7, 8 and 9 by NK cells (50).

Regarding the responses generated by the activation of these TLRs, variations between different TLRs have been observed in NK cells *in vitro* and in samples from healthy donors. Becker *et al* (51) observed that when activated via TLR2, using lipophosphoglycan purified from leishmania, NK cells exhibited increased levels of IFN- γ , as well as the increased expression of cell surface TLR2. In 2004, Schmidt *et al* (52) determined that poly-inosinic-cytidylic acid [poly (I:C)] activates human NK cells via TLR3. In the same year, Sivori *et al* (53) demonstrated that TLR9 activation induces human NK cells to secrete IFN- γ and TNF- α .

Lauzon *et al* (50) revealed that human NK cells express TLR1-10 mRNA, and that the ligand binding of TLR2, 3, 5 and 9 stimulates the secretion of IFN- γ . In the same year, Gorski *et al* (54) concluded that TLR7 and 8 activation increases the production of IFN- γ by human NK cells. In 2007, Alter *et al* (55) observed that TLR7 and 8 activation increased the production of pro-inflammatory cytokines by human NK cells from patients with HIV-1. In 2010, Mian *et al* (56) revealed that the production of IFN- γ and TNF- α was increased via TLR4 activation in humans and mice, and in 2013, He *et al* (57) demonstrated that human and mouse NK cells could be activated via the binding of TLR1 with several miRNAs.

These observations are generalized throughout the NK subpopulations. However, relative TLR expression varies according to NK-cell phenotype. For example, TLR2 is

preferentially expressed by CD56^{bright} NK cells, and TLR3 by CD56^{dim} cells (48,58). These variations suggest that stimulation with TLR ligands imparts a cytotoxic or immunomodulatory response, depending on the ligand.

The knowledge that TLRs are expressed by NK cells (Fig. 3) has increased interest in their immune response against viral and bacterial infections, as well as their antitumor activity. However, it has been observed that the DAMP-induced activation of NK cells can only occur via complex interaction with other cells of the immune system and within the cellular microenvironment (59).

6. NK cells in cancer

As aforementioned, NK cells are the primary mediators of immunosurveillance against tumor cells (60), as they can detect changes in the expression of MHC-I molecules and eliminate cells that have undergone malignant transformation (29). Mutations that arise during malignant transformation are reflected via alterations in MHC-I expression (61), as well as the overexpression of stress molecules that can be recognized by NKG2D receptors (62). However, neoplastic cells use various mechanisms to evade antitumor activity. In an 11-year follow-up study, an association was found between the low cytotoxicity of peripheral blood NK cells and an increased risk of cancer (63). Although the infiltration of NK cells has been shown to favor tumor elimination in various carcinomas, including colorectal (64), gastric (65) and lung cancers (66), these cells are found in small quantities within the tumor (67) and exhibit changes in the expression of activating receptors (68); additionally, the tumor microenvironment favors immunosuppression (69,70), which may also explain the small number of NK cells found within the tumor itself.

7. NK cells in acute lymphoblastic leukemia

With regard to hematological cancers such as leukemia, little is known of how these disorders affect the origination of NK

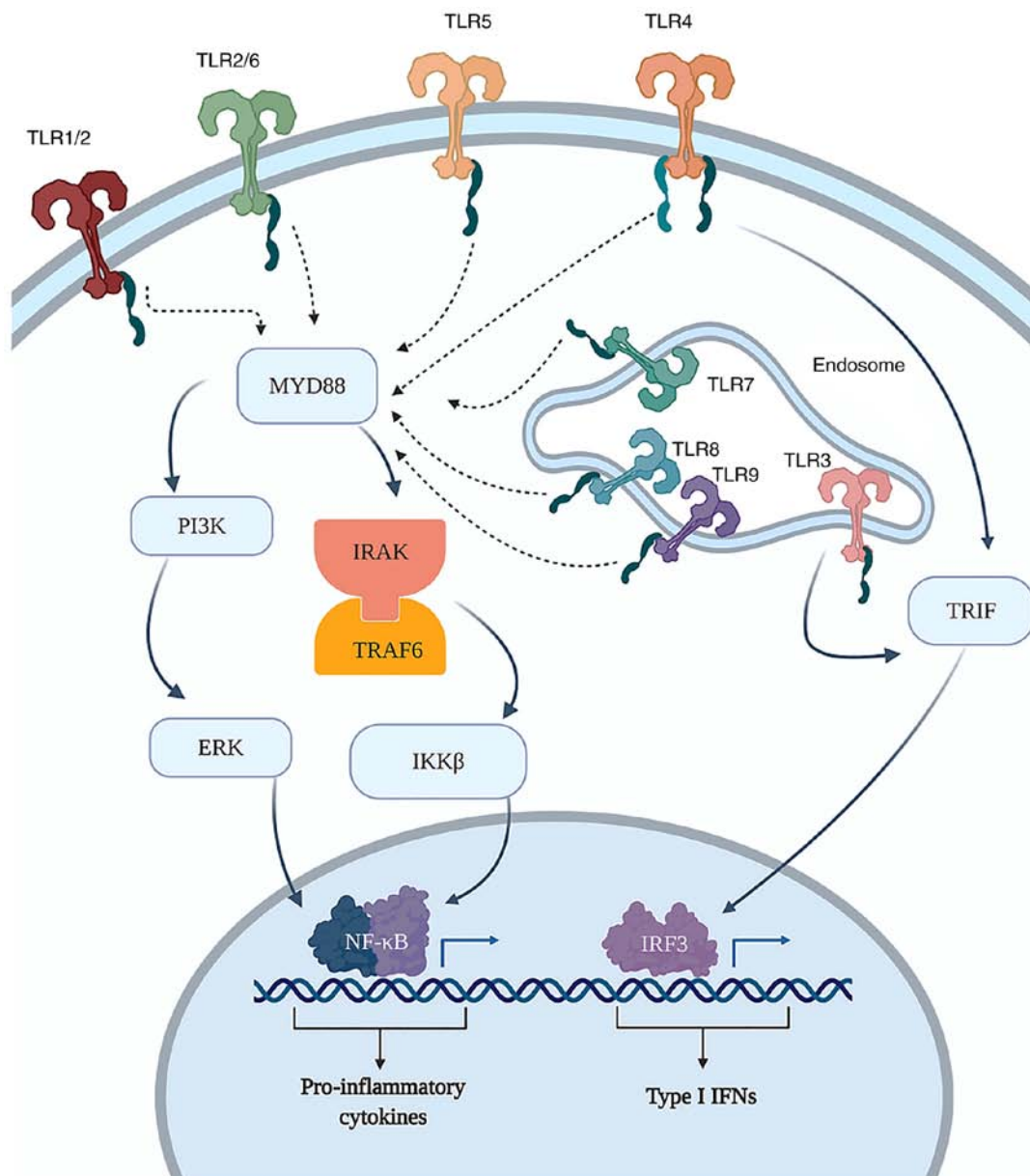


Figure 3. TLRs and their post-activation signals in NK cells based on observations in cells from healthy donors. NK, natural killer; TLR, Toll-like receptor; IRAK, interleukin-1 receptor associated kinase; TRAF6, TNF receptor-associated factor 6; TRIF, TIR-domain-containing adapter-inducing interferon- β ; IRF3, interferon regulatory factor 3.

cells, since both the neoplasia and NK cells originate from the bone marrow. On the basis of studies of patients with chronic myeloid leukemia (71,72), impaired or abnormal NK-mediated cytotoxicity, as well as the aberrant expression of NK receptors, constitute fundamental factors in the progression of the neoplasm (73).

A study of NK cells in the peripheral blood of patients when diagnosed with acute lymphoblastic leukemia (ALL) type B revealed that they exhibit TGF- β 1-mediated compromised cytotoxicity towards K562 cells and autologous blasts. Furthermore, the NK cells were found to have an inhibitory phenotype, represented by altered cell surface expression of Nkp46 and NKG2A, compared with those from healthy control subjects of the same age (74). The study also demonstrated that after remission, NK cell-mediated cytotoxicity towards K562 was recovered, but not that towards autologous

blasts, suggesting that blasts undergo successful immune editing (74).

A recent study in Mexican patients with ALL reported a reduction in the percentage of NK cells at diagnosis, as well as impaired cytotoxicity in patients with B- and T-ALL (75). Furthermore, in B-ALL, NK cell-mediated cytotoxicity in patients with leukocyte counts of $>50,000/\text{mm}^3$ was observed to be impaired compared with that in cases with lower counts. These data suggest that the abnormal effector function of NK cells is not observed equally in all pediatric patients with B-ALL, and that there may be other contributing factors to NK cell-mediated cytotoxicity (75).

Chemotherapeutics against leukemia have been shown to decrease the number and activity of NK cells during treatment. However, upon the discontinuation of treatment, it is possible that normal NK-cell levels slowly recover, although their

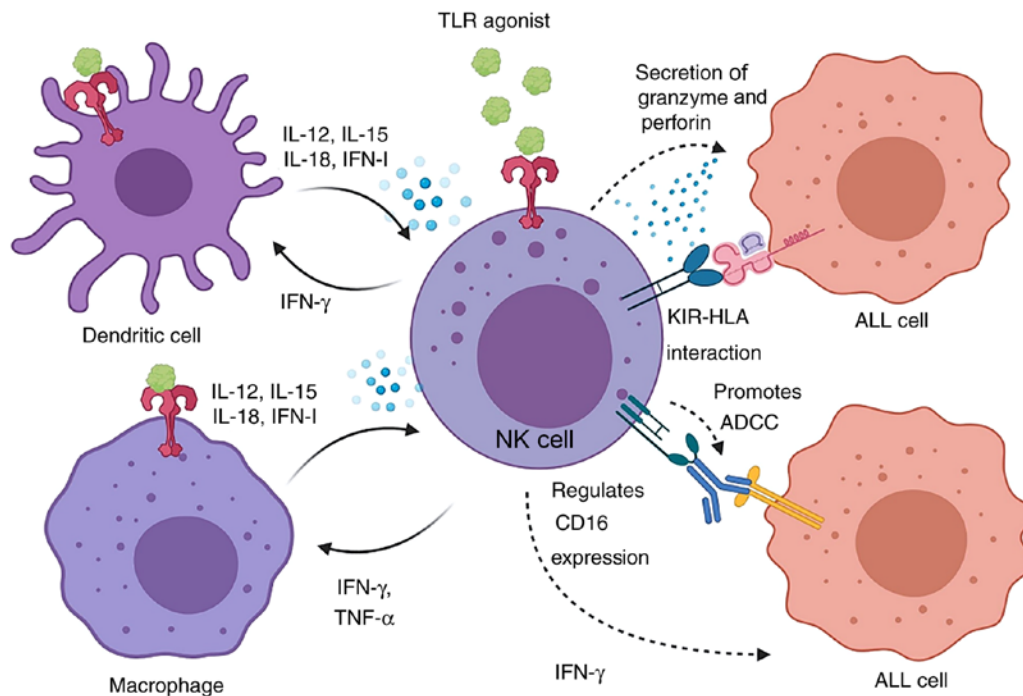


Figure 4. NK-cell activation through TLRs may trigger and enhance different mechanisms to attack to cells in ALL. The dotted arrows indicate hypothetical mechanisms, as they have been observed for NK cells with PBMCs not cancer cells. Mechanisms are based on observations from research on donor PBMC/NK cells treated with TLRs. NK, natural killer; TLR, Toll-like receptor; ALL, acute lymphoblastic leukemia; PBMC, peripheral blood mononuclear cell; IL, interleukin; ADCC, antibody-dependent cytotoxicity; KIR, killer cell immunoglobulin-like receptor; HLA, human leukocyte antigen.

cytotoxicity does not (76). Evidence of the importance of NK cells in tumor regulation has primarily been based on clinical studies of patients with leukemia who have undergone allogeneic hematopoietic stem cell transplantation (cell therapy) (77). This is a safe therapy, which avoids graft-vs.-host rejection disease (GvHD) (78).

Cellular immunotherapy, also known as adoptive cell therapy, has been accomplished by the use of engineered cells, among which chimeric antigenic receptor T (CAR-T) cells have achieved successful outcomes for B-cell lymphoblastic leukemia; a cohort study revealed that a single infusion of this therapy provided durable remission with long-term persistence in pediatric and young adult patients with relapsed or refractory B-cell ALL (79). However, the application of CAR-T cells has faced obstacles, two of which are: Patients who experience low CAR-T cell persistence or disease relapse cannot be reinfused with CAR-T cells; and the majority of patients experience cytotoxic effects (80). The development of CAR-NK cells could be an attractive solution to those issues.

As NK cells lack T-cell receptors that could cause GvHD and do not require HLA matching to be cytotoxic, CAR-NK cells have potential as cellular therapeutics. Pre-clinical studies with CAR-expressing NK cells have focused on anti-CD19 and anti-CD20 CARs targeting B cell malignancies, and have observed that CAR-NK cells are capable of increasing IFN-γ expression in response to CD19⁺ cells as well as increasing their specific cytotoxicity in xenograft models of B-ALL (81,82).

Despite their advantages over CAR T cells, CAR-NK cell-based therapies have made limited clinical progress (83). Currently, there are 19 studies registered in the clinicaltrials.gov database for the use of CAR-NK cells in patients with

cancer; 12 are trials in phase I/II that are recruiting, and two have already been completed. In the majority of these trials, the CAR-NK cells target CD19, CD22, CD33 or CD7 on hematopoietic malignancies (83).

The first large-scale trial of CAR-NK cells used CAR-NK cells derived from cord blood to treat high-risk CD19⁺ B cell malignancies (84). An anti-CD19-CD28-CD3ζ CAR was used for transduction of the cells, in addition to an IL-15 gene and inducible caspase 9 as a safety switch. In that study, 7/11 patients achieved complete remission, and showed an early expansion of CAR-NK cells compared with the non-responders. This response was achieved with no serious side effects, such as cytokine release syndrome, neurotoxicity and GvHD, even in the 5 patients with a KIR-ligand mismatch (84). Overall, the study demonstrated the initial efficacy and safety profile of this CAR-NK cell therapy. Although CAR-NK cells have multiple advantages compared with CAR-T cells, they have several challenges to overcome, such as loss of targeted antigen and tumor heterogeneity (83). Therefore, novel strategies should be sought to optimize the efficacy of CAR-based NK cell therapy.

8. TLRs' importance against acute lymphoblastic leukemia

Considering that poor cytotoxic activity of NK cells suggests an alteration in their activation, immunological strategies designed to improve the sensitivity of neoplastic cells to NK cell-mediated cytotoxicity should focus on the mechanisms by which NK cells are activated. Such mechanisms include the use of specific TLR ligands to restore NK-cell activation and cytotoxicity.

However, little information is available regarding the possibility of reactivating or modulating the functions of NK

cells *in vivo*. Although ligands exist for the stimulation of these receptors *in vitro*, it is not yet known with certainty whether they can be used as therapeutic agents in leukemia, where a reduction in the expression of TLRs has been reported in peripheral blood mononuclear cells (85).

A preclinical study demonstrated that TLR ligands can effectively activate donor NK cells to induce blast lysis (86). Therefore, they have been suggested as potential boosters for stimulating the immunological effector function of NK cells in leukemia immunotherapy. Among these ligands, the TLR3 agonist poly (I:C) demonstrated the ability to activate NK cells *in vivo*, and improved the therapeutic activity of a monoclonal antibody against human CD7 in a xenograft model of T-ALL (87). The TLR3 agonist was also demonstrated to cause changes in the expression levels of CD2, CD16/32 (FcRII/RIII), CD161 (NK1.1) and F4/80 in the host splenocyte population. It was suggested that the mechanism underlying the boost in NK cells following their activation via TLR3 in the ALL model is antibody-dependent cytotoxicity (ADCC), based on the previous observation that when cancer cells are coated by an antibody, the cell lysis is increased compared with that of cells not coated with antibody (87).

There is evidence that the TLR7 agonist R848 can boost the efficacy of obinutuzumab in CD-20⁺ lymphoma, since it has been shown to enhance non-specific NK-cell cytotoxicity (88). Since R848 and other TLR7 agonists can raise the levels of activating FcγR and reduce those inhibitory FcγRIIb, this supports the suggestion that R848 triggers ADCC as an effector mechanism. Notably, the interaction of NK cells with R848 *in vivo* induced the production of IFN-γ by the NK cells upon engagement of the Fc receptors by obinutuzumab. Furthermore, the study demonstrated that the systemic administration of R848 combined with obinutuzumab therapy enhanced the long-term survival of patients with lymphoma (88). A more recent study with an encapsulated TLR7/8 agonist also demonstrated positive immunotherapeutic effects, since strong *in vivo* cytotoxicity against the K562 leukemia cell line was achieved through NK degranulation and the release of granzyme B, and the activation of NK cells was prolonged. In addition, the TLR7/8 agonist enhanced the *in vitro* and *in vivo* ADCC of the epidermal growth factor receptor-targeting antibody cetuximab. These findings indicate the potential of the TLR7/8 agonist as a potent immunostimulatory adjuvant for antibody-based cancer immunotherapy, which acts via the promotion of NK-cell activation (89).

Although the aforementioned studies confirm that it is possible to activate NK cells through their TLRs to favor the lysis of carcinogenic cells, it is not yet possible to affirm the effects of this method in NK cells from hematological disorders such as ALL. Although there are existing clinical trials evaluating the effects of TLR stimulation on leukemia, with the clinicaltrials.gov database listing four completed clinical trials and two in the recruitment phase, only one of these has been performed in patients with ALL, with one NK-associated observation (90). This trial, reported by Ronsley *et al* (90), was a phase I pilot study, in which the regulatory effect of the TLR9 ligand GNKG168 on various genes was evaluated in three pediatric patients with ALL with residual disease following conventional therapy. GNKG168 was concluded to induce immunological changes in the mononuclear cells of patients via the negative regulation of genes (mRNAs) that

participate in the antitumor response, such as single Ig and TIR domain containing, IL-1 receptor ligand 1, CCR8, IL-7 receptor (IL7R), CD8B and CD3D. These findings suggest that inhibiting IL7R may also act as a checkpoint inhibitor of IL7R expression in the CD56^{bright} NK population, which is important for modulating the post-transplant response.

9. Conclusions

NK cells play an important role in the antitumor response. Due to the discovery that TLRs can induce the cytotoxicity and cytokine production of NK cells, TLR agonists have been proposed as potential stimulators of the antitumor effector functions of NK cells. However, further studies are required to elucidate the antitumor effects of NK cells, and understand the activation mechanisms of TLRs therein, in order to improve NK cell-mediated immunotherapy.

Acknowledgements

Not applicable.

Funding

Children's Hospital of Mexico Federico Gómez (grant no. HIM/2019/035 SSA 1618).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JGZ reviewed the literature and prepared drafts of the manuscript. CMB assisted in evaluation of the literature and finalizing manuscript for submission. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The study was ethically approved by the Research, Research Ethics and Biosafety Committees of the Children's Hospital of Mexico Federico Gómez (protocol no. HIM/2019/035). Patient consent was not required because waste blood from the blood bank of the Children's Hospital of Mexico Federico Gomez was used.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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