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Toll-Like Receptors and Natural Killer Cells

Carmen Maldonado-Bernal and David Sánchez-Herrera

Abstract

Natural killer (NK) cells represent a heterogeneous subpopulation of lymphocytes of the innate immune system with a powerful antitumor activity, a function given by a complex collection of receptors. They act synergistically to recognize, regulate, or amplify the response according to the microenvironment, thus highlighting Toll-like receptors (TLRs), a type of receptors that allows sensing evolutionarily conserved molecules of pathogens known as pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs). Those TLRs are essential to start the immune response. There is little information about the different subpopulations that form NK cells as well as their expression profile of innate immune response receptors in hematological cancers.

Keywords: Toll-like receptors, natural killer cells, innate immunity, pathogen-associated molecular patterns, damage-associated molecular patterns

1. Introduction

Natural killer (NK) cells represent a highly specialized subpopulation of lymphocytes that are part of the innate immune system, whose functions vary according to the microenvironment. NK cells are involved in the early defense against foreign cells or own cells subjected to some stress (bacterial infection, viral or tumor transformation) through the recruitment of neutrophils, macrophages, dendritic cells, or B/T lymphocytes. They induce an effective adaptive response; regulate, directly or indirectly, the activity of antigen-presenting cells (APCs); and activate T lymphocytes through the natural cytotoxic activity that characterizes them or through the production of cytokines and chemokines that generate an inflammatory environment [1, 2].

NK cells play an important role in the surveillance and suppression of tumor cells; despite the significant advances that have been reached in the last decades, it is still unknown if there is a direct relationship among the population dynamics, functionality, and the phenotype of these cells. Its role in the establishment and development of malignant hematological disorders such as acute lymphoblastic leukemia (ALL), a disease characterized by the uncontrolled proliferation of B or T lymphoid precursors, is still unknown.

2. Overview of natural killer cells and toll-like receptors

2.1 NK cells

NK cells, although they are larger and present granules in their cytoplasm, morphologically are indistinguishable from the other lymphocytes. According to different authors, they comprise from 5 to 15% [3], 20% [2, 4], or even 25% [5] of total peripheral blood mononuclear cells and are derived from a CD34⁺ hematopoietic progenitor, as are dendritic cells (DC) and B and T lymphocytes [6].

NK cells are phenotypically defined by the expression of CD56 (neural cell adhesion molecule (NCAM) and CD16a (also known as Fcγ-RIIIA), but not CD3 and CD19, which are molecules of T and B lymphocytes, respectively [7, 8]. For a long time, they were considered as the only population of non-B or T lymphocytes. It is currently accepted that NK cells are within a subgroup of the so-called innate lymphoid cells (ILCs), whose subpopulations are differentiated according to the immunophenotype, the profile of cytokines they produce, and the transcription factors they possess [9, 10].

The NK cells were classified in group 1 of the ILCs (ILC1) due to their ability to produce INF-γ, but not cytokines such as IL-4, IL-5, IL-9, IL-13, IL-17, or IL-22, characteristic of the ILC2 and ILC3 groups, respectively [9, 11, 12]. Even within the same subgroup, they differ by having cytotoxic capacity and selectively expressing the eomesodermin (EOMES) transcription factor of other ILC non-NK that also produce INF-γ [13].

NK cells maintain a pro-inflammatory environment through the release of cytokines and recruit cells of the immune system to combat infectious agents through the release of chemokines [14] and regulate the activity of dendritic cells or activated lymphocytes [1]; they are in charge of antitumor surveillance and tolerance to healthy own cells, which conditions the rejection of transplants [15] among other functions.

In recent years, it has been reported that in addition to NK cytotoxic or regulatory NK cells, there are memory NK cells [16, 17] and NK cooperators, which secrete Th1-type cytokines (NK1) and Th2 (NK2) [18]. Even, NK cells are similar to antigen-presenting cells [19, 20], although this is in controversy.

2.2 Population diversification of NK cells and their role in the immune response

According to what was compiled by Huntington et al., NK cell precursors (NKP) originate mainly in the bone marrow from hematopoietic precursor cells (HSCs), although they can also do so in organs such as the thymus from early lymphoid progenitor (ELP). These NKP can mature into competent NK cells in the bone marrow or other organs, such as the liver, spleen, thymus, and lymphoid nodules, to finally enter to the circulation [21]. They migrate to other sites such as the lung, liver, mucous membranes and skin, uterus, pancreas, joints, and central nervous system (CNS), where they can exhibit unique characteristics ranging from the increase or decrease of the expression of activation receptors or effector cytotoxic molecules to the modulation of resident immune cells (**Figure 1**) [22].

According to the expression of CD56 (NCAM), NK cells can be divided into two subpopulations: NK^{Dim} and NK^{Bright}. However, according to the relative expression of the CD56 and CD16 (FcγRIIIa) markers, their functionality, and their distribution in peripheral blood or lymphoid organs, it is possible to differentiate five subpopulations of mature NK cells (**Figure 2**).

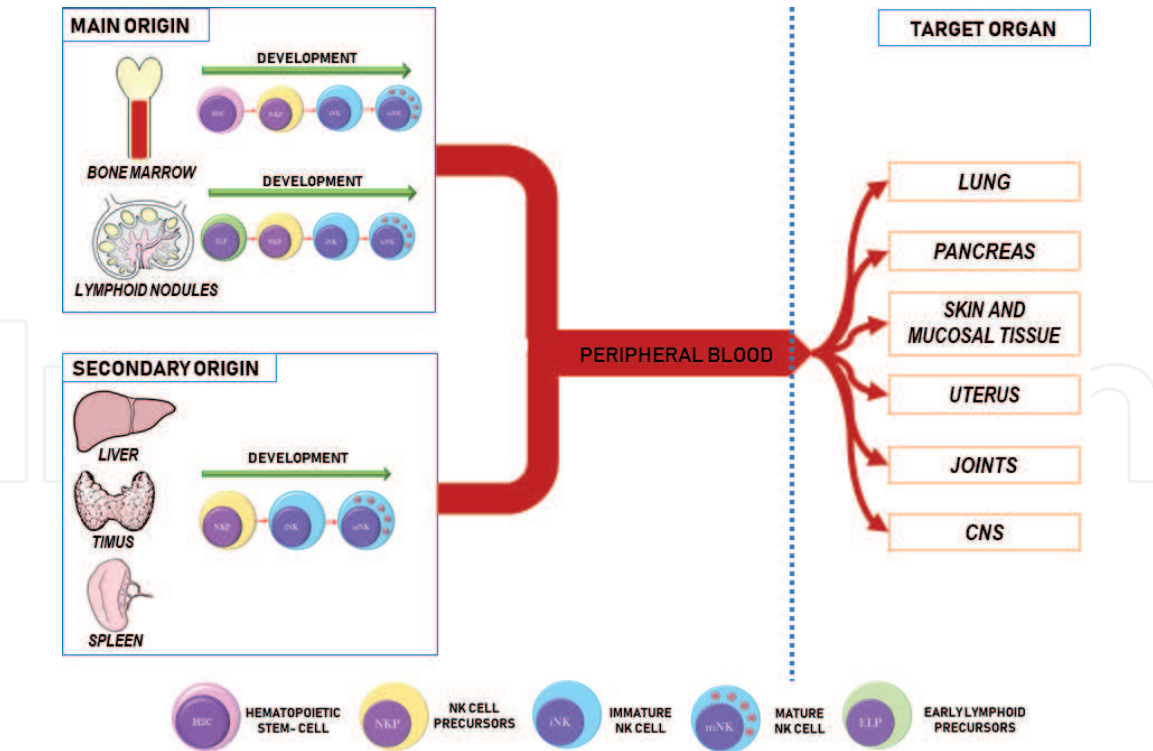


Figure 1.
Origin and distribution of NK cells in humans. The precursors of NK cell precursor (NKP) can originate from hematopoietic progenitor cells (HSC) in the bone marrow or from early lymphoid progenitor (ELP). NK cells mature mainly in the bone marrow, although the immature NKP and NK (iNK) can recirculate among the liver, spleen, and lymphoid nodes as alternative maturation sites. Mature NK cells (mNK) that leave the bone marrow reach different organs through blood circulation where they reside and modify their phenotypic and functional characteristics.

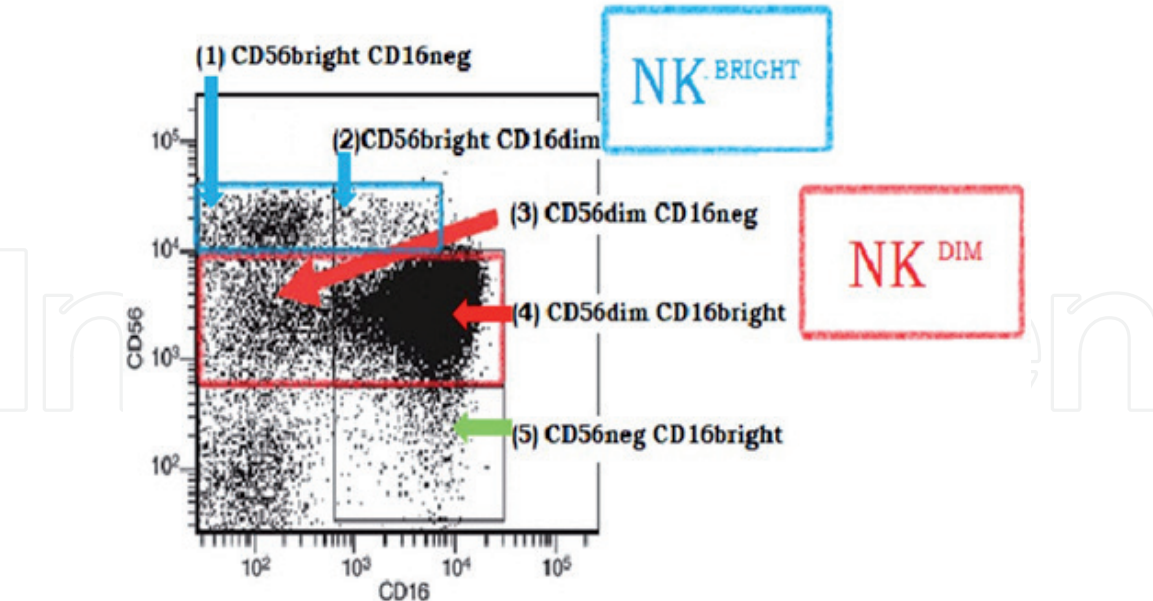


Figure 2.
Subpopulations of NK cells in peripheral blood based on the relative expression of CD56 and CD16. (1) CD56^{Bright} CD16^{Neg}, recognized for its immunoregulatory activity, represents between 50 and 70% of the CD56^{Bright} population; (2) CD56^{Bright} CD16^{Dim} represent between 30 and 50% of the CD56^{Bright} population; (3) CD56^{Dim} CD16^{Neg}; (4) CD56^{Dim} CD16^{Bright} is recognized for its cytotoxic activity; and (5) CD56^{Neg} CD16^{Bright} whose function is still unknown. Modified from [23].

The NK^{Bright} populations, also known as NK regulators, comprise about 10% of total peripheral blood NK cells. From these, about 50–70% have a [CD56^{High} CD16^{Neg}] phenotype and 30–50% a [CD56^{High}/CD16^{Low}] phenotype

[23]; they are mainly characterized by their poor cytotoxic capacity and their high capacity to secrete several types of post-activation cytokines, mainly $\text{INF-}\gamma$ but also $\text{TNF-}\beta$, IL-5, IL-10, and IL-13 [7, 8] and constitutively some chemokines such as MIP-1 α , MIP-1 β , and RANTES [24]. They also have the ability to proliferate in culture when exposed to low doses of IL-2 (picomoles) compared to the cytotoxic NK cells, which does not show an evident proliferation under the same conditions [25]. The $\text{NK}^{\text{Bright}}$ cells [$\text{CD56}^{\text{High}}$ CD16^{Neg}] are assigned an exclusively regulatory function, since their cytotoxic activity is poor and their genetic profile is directed mainly to the production of cytokines and not to the cytotoxic activity, in comparison with the NK^{Dim} , whereas the $\text{NK}^{\text{Bright}}$ subpopulation [$\text{CD56}^{\text{High}}$ CD16^{Low}] is considered more as a transition phenotype [26]; because despite having the same characteristics of the previous phenotype, it has a lower rate of cell division when stimulated with IL-2 and does not change its activity even with ligands for c-KIT [27]. It exhibits cytotoxic activity [28], and it has also been seen that it represents the highest percentage of NK cells in circulation in bone marrow transplants, until its normalization around the fourth month [29, 30]. $\text{NK}^{\text{Bright}}$ cells are usually not found in peripheral blood, bone marrow, and spleen as they are mainly distributed in secondary lymphoid nodules (parafollicular zone of T cells) [31] and tonsils [32].

On the other hand, the NK^{Dim} population represents around 90% of peripheral blood NK cells; they have a phenotype [CD56^{Low} CD16^{Neg}] whose main function has not been well established [23] and a main phenotype [CD56^{Low} $\text{CD16}^{\text{High}}$] that exhibits potent cytotoxic activity. Although they are generally poor producers of cytokines [7, 8, 33], they tend to predominate in the spleen, peripheral blood, and bone marrow [32, 34].

It is important to clarify that the behavior of each subpopulation, in terms of post-activation secretion of cytokines and chemokines, will depend largely on the stimuli they receive either by recognizing target cells (tumor or transformed) or responding to exogenous cytokines.

Compared with NK^{Dim} cells, $\text{NK}^{\text{Bright}}$ cells produce more $\text{TNF-}\alpha$ and $\text{INF-}\gamma$ after activation with PMA/ionomycin [35] or exogenous cytokines such as IL-12, IL-15, or IL-18, alone or in combination [8].

On the contrary, when it comes to a response to the recognition of target cells, NK^{Dim} cells significantly increase their production of cytokines and chemokines compared to $\text{NK}^{\text{Bright}}$, such as MCP-1 (CCL2), IL-8 (CXCL8), IP-10 (CXCL10), soluble IL-2R α (CD25), GM-CSF, and IL-5 and low levels of IL-1 β , IL-6, IL-7, IL-10, IL-12p40, IFN- α , and MIG (CXCL9). In addition, it increases the production of chemokines such as MIP-1 α (CCL3), MIP-1 β (CCL4), and RANTES (CCL5) that produce constitutively [24].

The cytotoxic activity exhibited by NK cells does not require prior sensitization to kill their target cells, since it is not dependent on the presentation of a specific antigen as in the case of CD8^+ T cells [7, 36] and can be mediated through membranolytic and/or apoptotic mechanisms (**Figure 3**).

The membranolytic mechanisms include the production of perforins, enzymes that when integrated into the cell membrane form a pore that allows water to enter and cause osmotic lysis [37]. In the past, it was believed that both $\text{NK}^{\text{Bright}}$ and NK^{Dim} cells had similar levels of perforins [38]; however, more recent studies by flow cytometry indicate that NK^{Dim} cells have at least 10 times more perforins than $\text{NK}^{\text{Bright}}$ ones [35].

Regarding apoptotic mechanisms, these can induce the death of the target cell through complex mechanisms that involve both death-inducing proteins and specific ligand-receptor interactions through one of the following routes:

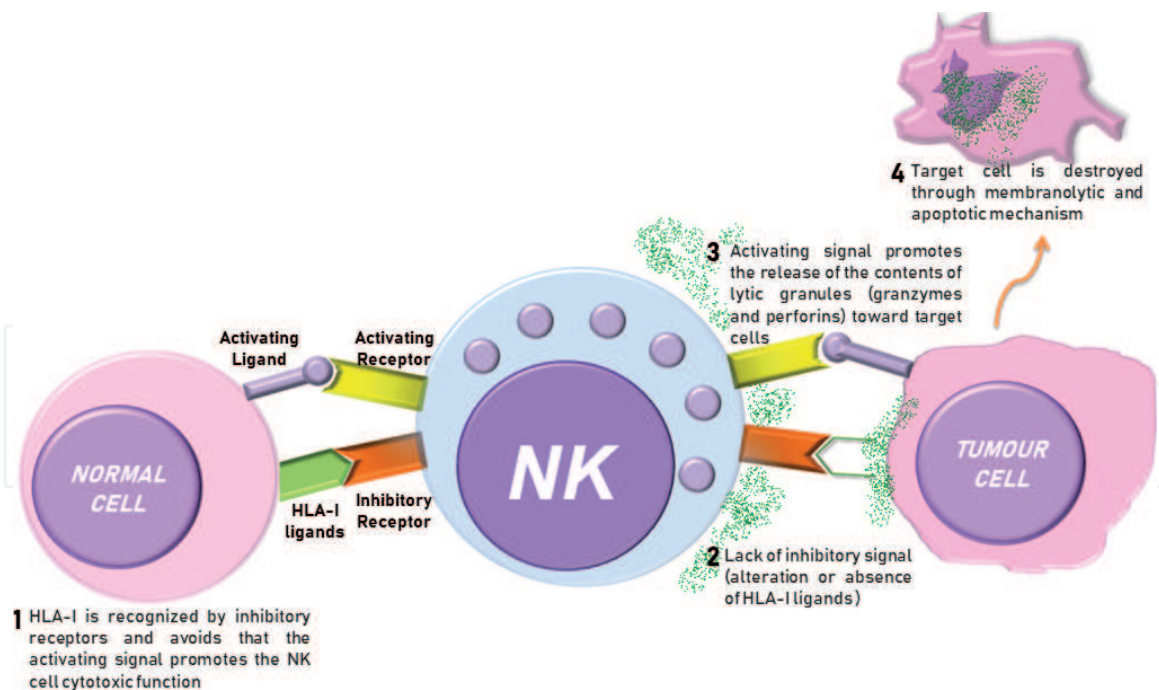


Figure 3.
 Recognition and elimination of abnormal cells by NK cells. NK cells possess the ability to discriminate normal cells of tumor or transformed cells by detecting alterations at the HLA-I level; the target cells are eliminated by membranolytic and/or apoptotic mechanisms.

2.3 Granzyme pathway

Granzymes are proteins capable of activating the apoptosis program [39] following two mechanisms. The first does not depend on the activity of caspase proteins and is mediated mainly by granzyme A, and this fraction is single-stranded DNA (ssDNA) and interferes in the repair of genetic material without producing cell lysis [40, 41], while the second promotes the activity of caspase proteins and is mediated mainly by granzyme B [42].

The NK cell presents granzymes A, B, K, and M; NK^{Dim} cells present a high expression of granzymes A and B, whereas NK^{Bright} cells mainly express granzyme K [35, 43]. There are reports that NK cells express almost exclusively granzyme M; this enzyme is capable of mediating cell death independent of the activation of caspase proteins and in the presence of perforins, without fractionating DNA or producing changes in mitochondria [44].

In mice, deficient in granzymes A/B and/or perforins, it has been seen that there is uncontrolled growth of solid tumors, which suggests that these enzymes play an important role in the immunosurveillance of tumor cells mediated by NK cells [45].

2.4 NK cell ligand binding pathway to the cell death receptors expressed by the target cell

They include Fas ligands [FasL (CD95L)-Fas (CD95)] and/or the ligand that induces apoptosis related to tumor necrosis factor α (TRAIL) [46, 47].

2.5 Antibody-dependent cellular cytotoxicity (ADCC)

NK cells express Fc γ RIIC/CD32c [48] and Fc γ RIIIA/CD16a [34]. These receptors interact with opsonized target cells, through the Fc regions of the antibodies, which combined with cellular antigens that cause the death of the target cell [49]. To through of mechanisms that involve the release of cytotoxic granules

(perforin-granzyme), or by stimulation of apoptosis through of TNF-related apoptosis-inducing ligand (TRAIL) and/or by release of pro-inflammatory cytokines that promote the activity of other cells [50].

NK^{Dim} cells with [CD56^{Low} CD16^{High}] phenotype direct this mechanism in comparison with NK^{Bright} cells. Although it has been seen that the subpopulation with [CD56^{High} CD16^{Low}] phenotype exhibits low cytotoxic activity [51], NK^{Dim} cells [CD56^{Low} CD16^{Neg}] show a higher antitumor activity against cell lines (natural cytotoxicity) than other subpopulations [52]. This is supported by other studies where it is reported that NK^{Dim} cells [CD56^{Low} CD16^{High}] lose the expression of CD16 and increase the expression of CD107a (a degranulation marker), through a disintegrin and a metalloprotease-17 (ADAM-17), to become [CD56^{Low} CD16^{Neg}] with high cytotoxic capacity [53].

The role of the [CD56^{Neg} CD16^{High}] subpopulation is still not clearly defined. It is known that it is found in a low frequency in healthy individuals. It does not express surface molecules of other lymphoid lineages and that in chronic viral diseases, such as the human immunodeficiency virus (HIV). It presents changes in the level of expression of their activity receptors, characterized by the increase in the expression of inhibitory receptors and the decrease of natural cytotoxicity receptors (NCRs), together with other effector molecules that are hardly observed in healthy people [54–56].

It is considered that the [CD56^{Neg} CD16^{High}] subpopulation is dysfunctional in terms of its lytic and antiviral activity, although it retains the ability to produce pro-inflammatory chemokines [54–56].

Zulu et al. demonstrated that the HIV induces the expansion of the negative CD56 population of NK cells through the upregulation of NKG2C receptors and the negative regulation of Siglec-7, NKG2A, and CD57 receptors [57].

2.6 Receptors of the NK cells

NK cells have signals through a wide variety of receptors that allow them to respond to different types of stimuli and grant great flexibility when exercising their effector and/or cytotoxic function.

The function of the NK cell is given by a complex collection of receptors that act in a synergistic way to recognize, regulate, or amplify the response according to the microenvironment. Thus highlighting the pattern recognition receptors (PRRs), such as Toll-like receptors or natural cytotoxicity receptors, and inhibitory killing receptors (iNKR), such as receptors that are activated during early response to pathogens, cells transformed by virus or tumor cells [58].

PRRs are a family of innate immune response receptors that recognize evolutionarily conserved microbial products whose activation favors the production of pro-inflammatory cytokines. Within the PRR group, the TLRs are the most studied, although they are not the only ones; there are also the NOD-like receptors (NLRs) and the retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) [59].

NK cells express innate immune response receptors, such as NOD2, NLRP3, TLR3, TLR7, and TLR9, and promote the production of inflammatory cytokines and chemokines that are capable of amplifying the immune response [60]. The modulation of these cells through their innate immune response receptors, mainly via TLR, has gained interest and represents a promising therapeutic alternative against conditions such as cancer. Since there have been studies for a long time that support the possibility of its use, it has been observed that when ODNs (ligands of TLR9) are intraperitoneally administered in lymphoma murine models, an effective elimination of tumor cells occurs in 80% of cases [61].

2.7 Toll-like receptors and their role in NK cells

Toll-like receptors are among the most important group of pattern recognition receptors, since they orchestrate a wide variety of activities related to the immune response.

These receptors recognize a wide variety of molecules evolutionarily conserved, associated with microorganisms, such as lipopolysaccharides, lipoproteins, mycolic acids, non-methylated DNA, and double-stranded RNA, generically known as pathogen-associated molecular patterns [62–64]. TLRs also recognize endogenous molecules called damage-associated molecular patterns, which originate from damaged cells [65] or are products of altered metabolism of transformed cells in conditions such as cancer [66, 67] and autoimmune diseases [67–70] or associated with chronic inflammation [71, 72]. They play an important role in the evolution of these conditions.

2.8 Overview of the toll-like receptors

Structurally, TLRs are type I integral glycoproteins that present an extracellular domain with leucine-rich repeats (LRRs) that are responsible for binding and discriminating ligands (PAMPs or DAMPS) present in the cellular microenvironment. They have a transmembrane domain and an intracellular Toll/interleukin (IL)-1 receptor (TIR) domain that triggers the signaling cascade via MyD88/TRIF and is highly conserved among each subfamily of TLRs [73].

There are 13 TLRs described in mammals, and 10 are found at the protein level in humans and differ according to their cellular localization and to the different PAMPs/DAMPs to which they respond. TLR11 in humans is a pseudogene, so it is not expressed [74].

The TLRs that are found mainly in the cell membrane are TLRs 1, 2, 4, 5, and 6. They sense structural components of bacteria, fungi, helminthes, or protozoa, whereas TLRs that are mainly found in intracellular compartments, such as TLRs 3, 7, 8, and 9, sense nucleic acids of viral and/or bacterial origin [73, 75] (**Figure 4**).

The stimulation of the TLRs is capable of initiating an immune response to various stimuli on its own, as well as of controlling the adaptive response through the inflammatory process with the production of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α), chemokines (IL-8, MCP-1) [76]; defensins [77]; type I interferons [78]; co-stimulation and MHC molecules [79]. The union between the different types of immune responses through the TLRs takes as a classic example the dendritic cells. They inspect their environment through the TLRs [80]; and once they detect a ligand (bacterial product, viral or stress protein), increase the expression of co-stimulatory molecules capable of stimulating T naive cells [81] and polarizes the adaptive immune response toward Th1 or Th2 profiles [82].

The cellular response through direct stimulation with TLR ligands will depend largely on the type of lineage in question. The information that is reported about activity and expression in NK cells is relatively new, and it is more associated with innate antibacterial or antiviral immune response [83], but not in cancer.

2.9 Expression of TLRs in NK cells

The expression profile of TLRs in NK cells was initially limited to the detection of mRNA. However, the results do not always reflect the expression of the protein since it is difficult to identify the receptors in the NK cell.

NK cells express most of the human TLRs reported to date, although the detection and level of mRNA expression of each receptor vary depending on the author.

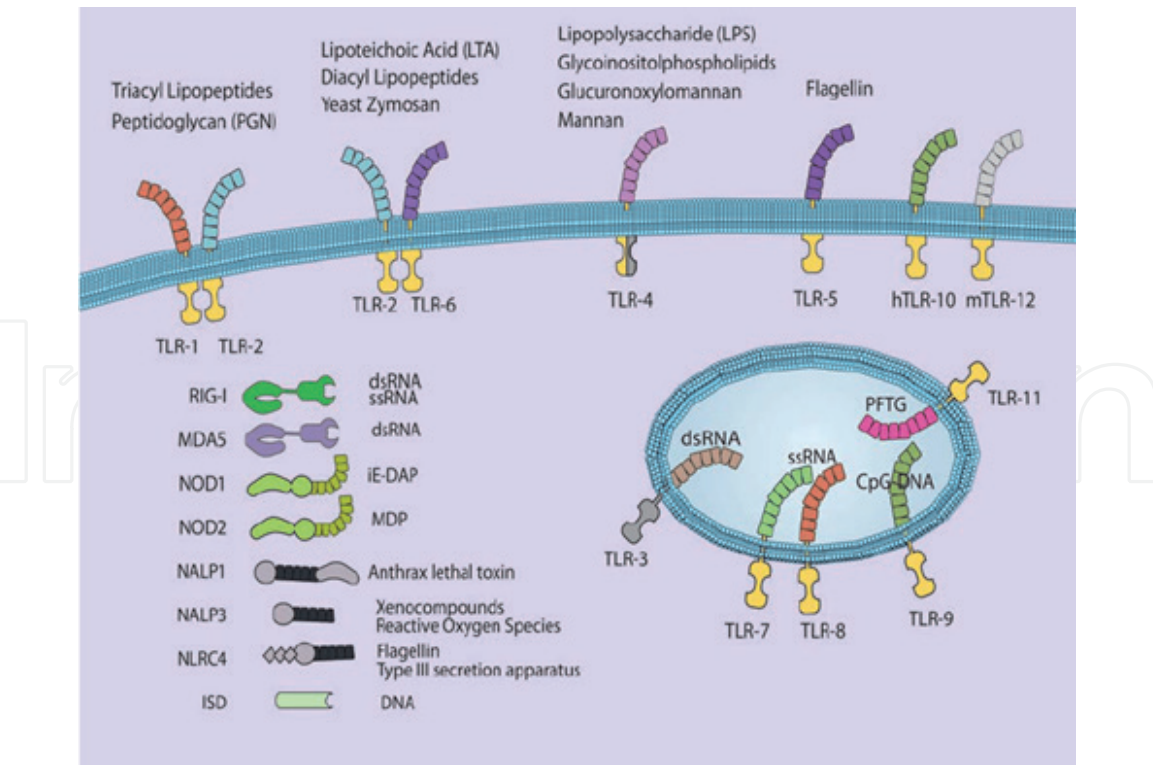


Figure 4. Toll-like receptors and their ligands. TLRs are transmembrane proteins of a glycoprotein nature that possess the ability to sense highly conserved microorganism molecules known as pathogen-associated molecular patterns (PAMPs), such as flagellin, LPS, or genetic material (ssDNA, ssRNA, dsDNA, dsRNA). In humans, 10 of the 13 TLRs present in mammals have been detected [84].

There are authors who indicate that NK cells express high levels of TLR1, followed by TLR2, TLR3, TLR5, TLR6, and low levels of TLR4 [85, 86]. Other authors agree that TLR2 and TLR3 have greater expression, followed by TLR5 and TLR6; in those studies, TLR1 mRNA was not quantified [87–89]. On the other hand, TLR7 [85, 89, 90] and TLR10 [83, 86] present levels so low that they are practically undetectable.

There is a controversy about whether or not NK cells express TLR8 [86, 87, 89] and TLR9 [86, 88], although some authors point out that the cells constitutively express mRNA of all TLRs [85, 91, 92].

NK cells have higher levels of TLR3 mRNA than any other peripheral blood mononuclear cell, such as monocytes, B and T lymphocytes, or plasmacytoid dendritic cells [85].

Through techniques such as flow cytometry, Western blot, and immunoprecipitation, it is possible to know that NK cells of healthy people have a defined TLR expression profile (**Table 1**) and the expression of receptors is independent of their activation state [58].

In addition, there are variations in the level of TLR expression within the same subpopulations of NK cells. It is accepted that both NK^{Bright} and NK^{Dim} exhibit a similar mRNA profile of TLRs, although it is not always reflected at the protein level and there is a great controversy regarding the distribution and presence of some of these receptors in both subpopulations, especially TLR2, TLR4, and TLR3.

TLR2 and TLR4 are mainly distributed on the cell surface, whereas TLR3 is generally found in intracellular vesicles [75]; however, it has been seen that in NK cells, TLR3 is expressed both within [93] and on the cell surface [94]. There are publications reporting that TLR2 and TLR4 exhibit a marked intracellular distribution [95], although other authors indicate otherwise [96, 97].

The relative amount of some TLRs may vary according to the phenotype (**Dim** or **Bright**), although expression levels appear to be higher in cells with regulatory

TLR	mRNA ¹	Protein Presence	Detection method
1	Very high	Yes	Flow cytometry [97] Western blot [97]
2	High/moderate	Yes ³	Direct activation of the TLR2-/MyD88- dependent pathway [96] Flow cytometry [95–97] Western blot
3	High/moderate	Yes	Flow cytometry [93, 94] Western blot [94]
4	Low	Yes ⁴	Flow cytometry [94, 95, 99]
5	High/moderate	Yes ⁵	S/R
6	High/moderate	Yes	Flow cytometry and Western blot [97]
7	Very low/undetectable	Yes	Flow cytometry [93, 94] Western blot [94]
8	Low ²	Yes	Flow cytometry [94] Western blot [90, 94]
9	Low ²	Yes	Flow cytometry [93, 95, 100] Western blot [100]
10	Very low/undetectable	N/R	N/R

N/R, not reported.¹The levels of relative expression are given according to what was reported by [85, 91] and refer to the comparison of expression among the 10 TLRs.
²There is a controversy whether or not they express mRNA of these TLRs, since some reports indicate that it was not possible to detect it.
³In previous studies, TLR2 could not be detected by flow cytometry or by immunoprecipitation.
⁴More recent studies indicate that it is expressed mainly as intracellular [95, 99].
⁵No reports were found indicating the presence of TLR5; however it is inferred that it is present as it responds specifically to flagellin [88, 101, 102], a molecule that it is only recognized through this receptor.

Table 1.
Expression of TLRs in human natural killer cells.

phenotype [89], which suggests that the type of response could be conditioned to promote a cytotoxic or immunomodulatory response when using one ligand or another in TLR activation assays (**Table 2**).

It has been seen that NK^{Bright} cells express more TLR1, TLR2, and TLR6 than NK^{Dim} [97], although other studies report that less than 1% of total NK cells express these three receptors [98].

NK^{Dim} cells can express, under normal conditions, more TLR4 than NK^{Bright} cells [99] although other authors seem to find no differences in the expression in TLR2, TLR4 [95], and TLR9 [95, 100].

There is no information about whether there is differential expression of TLRs 3, 5, 7, or 8, and the distribution pattern of TLR5 is not known. However, it is inferred that NK cells express it, since they respond to flagellin and there are several studies that demonstrate it [88, 101, 102]. To date there are no reports about the presence, distribution, or role of TLR10 in NK cells.

The therapeutic use of TLR ligands in the modulation of NK cells against cancer, especially in malignant hematological disorders such as leukemia, is an interesting alternative for the treatment of this type of diseases, since there are reports that reveal their therapeutic use as potential antitumor agents and as adjuvants in vaccines and other therapeutic modalities [103]. It is currently the subject of an extensive review by several research groups [104, 105].

TLR	Cellular localization	Population distribution
1	Extracellular [97]	NK ^{Bright} > NK ^{Dim} [97]
2	Extracellular [96, 97] and intracellular [95]	NK ^{Bright} > NK ^{Dim} [97]
3	Intracellular [93] and extracellular ¹ [94]	N/R
4	Extracellular [94] and intracellular ² [95, 99]	NK ^{Bright} < NK ^{Dim} [99] NK ^{Bright} = NK ^{Dim} [95]
5	N/R	N/R
6	Extracellular [97]	NK ^{Bright} > NK ^{Dim} [97]
7	Intracellular [93, 94]	N/R
8	Intracellular [94]	N/R ⁴
9	Mainly intracellular ³ [93, 95, 100]	NK ^{Bright} = NK ^{Dim} ⁵ [95]
10	N/R	N/R

N/R, not reported.¹It was found that both, in cell lines (NKL, NK92, and YT) and in NK of peripheral blood, TLR3 is expressed on the surface [94].
²Studies that are more recent indicate that it is mainly expressed as intracellular [95, 99].
³TLR9 expression exists in plasma membrane, but it is quite low compared to intracellular expression.
⁴There are no studies that determine whether there is differential expression, although it has been seen that NKBright cells are better activated with ssRNA40 than NKDim cells, suggesting that the latter have a lower expression of TLR8.
⁵In other studies, it seems that NKDim cells express more TLR9 than NKBright cells and its expression conditions the response to ligands of this TLR [100].

Table 2.
Localization and differential distribution of TLRs in human natural killer cells.

3. Conclusion

In this chapter, we included the overview of NK cells, their population diversification and role in the immune response, and their expression and role of TLRs.

Acknowledgment

We thank doctors Jacques Zimmer, MD, PhD, and Sankar Ghosh, PhD, for allowing us to use some of their figures, **Figures 2 and 4**, in this chapter of the book.

Conflict of interest

The authors declare that there is no conflict of interest.

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