Review Article

Role of Common-Gamma Chain Cytokines in NK Cell Development and Function: Perspectives for Immunotherapy

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NK cells are components of the innate immunity system and play an important role as a first-line defense mechanism against viral infections and in tumor immune surveillance. Their development and their functional activities are controlled by several factors among which cytokines sharing the usage of the common cytokine-receptor gamma chain play a pivotal role. In particular, IL-2, IL-7, IL-15, and IL-21 are the members of this family predominantly involved in NK cell biology. In this paper, we will address their role in NK cell ontogeny, regulation of functional activities, development of specialized cell subsets, and acquisition of memory-like functions. Finally, the potential application of these cytokines as recombinant molecules to NK cell-based immunotherapy approaches will be discussed.

1. Background: The Common-Gamma Chain Cytokine Family

Cytokines are soluble mediators of intercellular signals and play an essential role in the activation and regulation of both adaptive and innate immunity. In particular, the family of cytokines, sharing the common cytokine-receptor gamma-chain (γ_c or CD132) in their receptor complexes, consists of several members with a similar four alpha-helix bundle structure. This family comprises interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21, which display functional redundancy in the regulation of the immune response and in the homeostasis of the lymphoid system but have also specific functions [1]. Each of these cytokines binds to a specific high affinity receptor complex formed by a cytokine-specific α chain and the γ_c [2, 3] (Figure 1). Different from other members of this family, IL-2 and IL-15 can bind with high affinity to heterotrimeric receptor complexes, which consist of IL-2Rα (CD25) or IL15-Rα, respectively, and of

IL-2Rβ (CD122) and γ_c chains [4, 5]. The γ_c is an essential component of the receptors of all these cytokines, as it associates to the Janus tyrosine-kinase (JAK)-3, which is required for signal transduction [6]. JAK-3 phosphorylates different downstream signal transducer and activator of transcription (STAT) molecules, in relationship to the type of the receptor complex involved (Figure 1). Thus, IL-4 predominantly signals through STAT-6, whereas IL-2, IL-7, IL-9, and IL-15 mainly activate STAT-5, and IL-21 acts through STAT-3 and STAT1 [1, 7]. The activation of different STAT proteins by JAK-3 is related to its ability to phosphorylate the intracytoplasmic tail of different receptor chains. Tyrosine-phosphorylation of aminoacidic motifs in cytokine-specific receptor molecules generates docking sites for specific unphosphorylated STAT monomers, which are recruited to the receptor complex through their SH-2 domains [8]. Upon tyrosine phosphorylation by JAK-3, phosphorylated STAT molecules dimerize and migrate into the nucleus, where they bind to STAT-sensitive regulatory elements and control
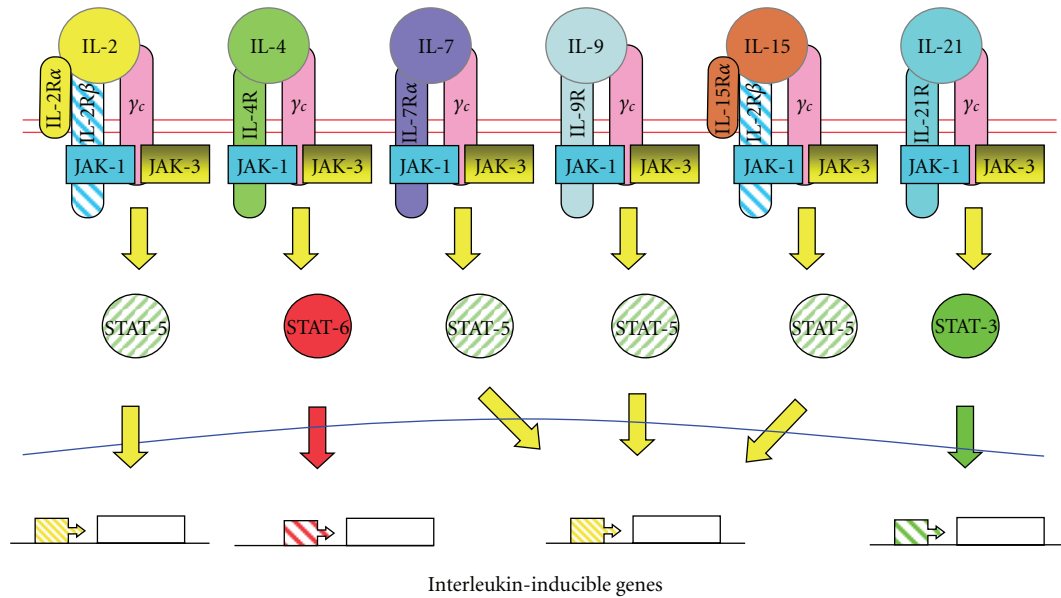
FIGURE 1: The family of cytokines sharing the common cytokine-receptor γ_c in their receptor complexes. Each cytokine binds to a specific α chain, which forms a receptor complex with the γ_c. In case of IL-2 and IL-15, trimeric high affinity complexes, which include common IL-2Rβ and γ_c chains, can be formed. Each receptor complex mediates signal transduction through JAK1 and/or JAK3 and different STAT molecules. Tyr-phosphorylated STAT dimers regulate transcription of specific cytokine-sensitive genes.

transcription of specific genes. Both the γ_c and JAK3 are essential for the function of all cytokine receptors of this family and are required for the development of the lymphoid cell system. Indeed, genetic defects of γ_c or JAK-3 results in a severe combined immune deficiency (SCID) characterized by the lack of T, B, and NK cells in both mice and humans [9]. In addition to JAK-3, some receptor complexes also activate JAK-1, which predominantly phosphorylates STAT-1 molecules.

IL-2 is the firstly identified member of this cytokine family, and its gene was originally cloned on the basis of the T-cell growth factor (TCGF) activity of this cytokine [10, 11]. Besides its TCGF activity, IL-2 upregulates NK cell proliferation and function, induces lymphokine-activated killer (LAK) activity, and also mediates activated B cell proliferation and Ig production [12]. IL-4 plays an important role in T helper (Th)2 cell development and function, in the regulation of B cell responses, and particularly in IgE production. Therefore, it is involved in allergic diseases and defense against parasitic infections [13]. Few effects of IL-4 have been reported on NK cells. Recent data indicate that IL-4 downregulates the expression of the activating receptor NKG2D in mouse NK cells, thus inhibiting NKG2D-dependent killing in vitro and in vivo [14]. In addition, IL-4 can also inhibit Ly49 receptors expression [15], suggesting a functional role in the innate immunity response. Finally, human NK cells cultured for short term with IL-4 did not release interferon (IFN)-γ and showed no cytolytic activity in response to stimulation through NKp46-activating receptor. In contrast, IL-12-cultured NK cells released IFN-γ and displayed strong cytolytic activity against tumor cells or immature dendritic cells (DC). These data suggest that IL-4

may negatively influence the NK/DC cross-talk, impair Th1 priming, and favor tolerogenic or Th2 responses in humans [16]. Different from the two previous cytokines, which mainly regulate the immune response, IL-7 is fundamental for the homeostasis of the immune system, as it regulates T, B, and NK lymphoid cell development [17]. Indeed, IL-7 is produced by thymic and bone marrow epithelial and stromal cells and by reticular cells in peripheral lymphoid tissues. IL-7 supports differentiation of hematopoietic stem cells into lymphoid progenitor cells and proliferation and survival of lymphoid precursor cells in the bone marrow and in the thymus. In addition, it stimulates survival of naive and memory T cells in the periphery. The crucial role of IL-7 in lymphoid cell development is clearly evidenced by the T and B-cell deficient SCID phenotype of patients and mice with genetic defects of the IL-7Rα (CD127) chain [16, 18]. Although IL-7Rα-deficient patients and mice do not have NK cell deficiency, several data indicate that IL-7 is involved in the development of specific subsets of NK cells [19–24]. IL-9 is a proinflammatory cytokine released by activated CD4$^+$ T cells and mediates activation of eosinophils, mast-cells, and bronchial epithelial cells, thus playing a relevant role in asthma [25]. However, IL-9 appears not involved in NK cell regulation. IL-15, instead, plays a pivotal role in NK cell biology. This cytokine shares several functional activities of IL-2 due to the promiscuous usage not only of the γ_c, but also of the IL-2Rβ chain [4, 12, 26]. However, differently from IL-2, IL-15 is expressed in several tissues and it is produced by different nonlymphoid cell types such as monocytes, DC and stromal cells of the bone marrow and thymus [5]. IL-15 produced in bone marrow, thymus, and secondary lymphoid organs is a crucial element to drive the development and

survival of NK cells [27–29] and of certain subsets of T cells [27, 28]. Finally, IL-21, the most recently identified member of this cytokine family [30] was originally discovered as the ligand of an IL-2Rβ-related orphan receptor [31], now termed IL-21R. IL-21 can costimulate the proliferation of T, B, and NK cells and promotes the terminal differentiation of IL-15 activated NK cells and of activated B cells into plasma cells [32]. In addition, IL-21 mediates apoptosis of partially activated normal B cells [33] and of certain human neoplastic B cells [34–37].

This paper will focus on the role of IL-2, IL-7, IL-15, and IL-21 in NK cell development and function and will discuss the possible relevance of NK activation by these cytokines in cancer immunotherapy.

2. Distinct Role of IL-2 and IL-15 in NK Cell Biology

IL-2 and IL-15 share several functional properties in relationship to the use of two promiscuous receptor components (IL-2Rβ and γ$_c$) and common signaling pathways. Indeed, both cytokines stimulate the proliferation, survival, and functional activities of NK cells and activated T and B cells. Nonetheless, the two cytokines have also specific functions, which are related to the different cellular distribution and functional properties of the IL-2Rα and IL-15Rα chains and the to the distinct cellular origin and regulation of IL-2 and IL-15 production [12].

IL-2 may act through two types of receptor complexes: the high affinity trimeric receptor formed by IL-2Rα, IL-2Rβ, and γ$_c$ and an intermediate affinity dimeric receptor formed by the IL-2Rβ and γ$_c$. While the high affinity trimeric receptor is expressed on activated T and NK cells, the intermediate receptor is constitutively expressed on CD3$^-$CD56$^+$CD16$^+$ NK cells [38] and on minor subsets of T cells, which can directly respond to high concentrations of IL-2. The constitutive expression of functional IL-2R accounts for the induction of LAK cells, displaying broad non-MHC restricted cytolytic activity against different types of tumor cells by short-term culture of PBMC in IL-2-containing medium [39]. Indeed, LAK cells are predominantly represented by IL-2-activated NK cells and by a subset of T cells [40]. However, a subset of human CD3$^-$CD56^{bright}CD16$^-$ NK cells constitutively express high affinity IL-2 receptors and may respond to low IL-2 concentrations [41, 42].

The IL-2 and IL-15 receptor α chains display remarkable differences. IL-2Rα alone has a low affinity for IL-2 and is devoid of signaling properties although it is required for the generation of high-affinity trimeric IL-2R complexes. IL-2Rα expression is induced in T cells upon activation; however, it is constitutively expressed at high levels on immune suppressive CD4$^+$CD25$^+$FoxP3$^+$ regulatory T (Treg) cells [43]. Indeed, IL-2 plays a specific role in immune regulation and in peripheral tolerance [44], as it is involved in the maintenance and fitness of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells [45, 46]. In addition, IL-2 participates in activation-induced cell death of T cells, which limits T cell responses [47]. The primary role of IL-2 in immune regulation is evidenced by the study of mice defective of IL-2 [48] or of IL-2Rα [49] genes, which develop a lymphoproliferative disorder associated with autoimmunity and impaired Treg function. The genetic defect of the IL-2Rβ chain results in autoimmunity in mice and also in a unique NK-deficient immunophenotype in mice [50] and humans [51]. In addition, an NK cell defect is part of the SCID phenotype in humans [52] and mice [53] bearing genetic defects of the γ$_c$. Altogether, these studies indicated that IL-2 or IL-2Rα are not necessary for NK cell development, although the IL-2Rβ and the γ$_c$ are strictly required, suggesting a role for IL-15. Indeed, IL-15 and its specific receptor IL-15Rα are essential for the generation and maintenance of NK cells, as IL-15 mediates the development of NK cells from committed NK cell precursors, promotes the differentiation of immature NK cells, and supports the survival of mature NK cells in the peripheral lymphoid organs [54–56].

Different from IL-2Rα, IL-15Rα alone has a high affinity for IL-15 and is constitutively expressed in several lymphoid and nonlymphoid cell types [57, 58]. The study of IL-15- [28] or IL-15Rα-KO [27] mice, which have similar NK-deficient phenotypes, confirmed that the IL-15/IL-15Rα system has an unique essential role in the development and survival of NK cells and of certain subsets of T cells such as NKT cells and intestinal intraepithelial CD8αα$^+$ T cells. In addition, IL-15Rα deficient mice have a reduced CD8$^+$CD44^{high} memory T cell pool, indicating a critical role of this receptor in the maintenance of a CD8 memory.

Similar to IL-2, IL-15 can mediate conversion of poorly cytolytic resting NK cells into highly cytolytic effector NK cells, which acquire enhanced antitumor activity [59]. Resting murine NK cells contain abundant granzyme A, but little granzyme B or perforin while the mRNAs for all three genes are highly expressed. IL-2 or IL-15 mediate a dramatic increase in granzyme B and perforin proteins without altering their mRNA abundance. These data suggest that these cytokines can mediate the removal of a block of perforin and granzyme B mRNA translation that prevents resting NK cells to be fully cytotoxic [60].

Several evidences indicate that IL-15 supports NK cell survival [12, 29] and is more potent than IL-2 in this respect. The antiapoptotic effects of IL-15 on murine NK cells are mediated through the inhibition of Bim expression by different mechanisms involving Erk-1/2 phosphorylation or the phosphatidylinositol-3-OH kinase (PI(3)K)-dependent inactivation of the transcription factor Foxo3a. In addition, IL-15 also promotes NK cells survival by the upregulation of Mcl-1, a molecule that was previously reported to be required for the development and survival of T and B lymphocytes [61].

Recent observations indicate that IL-2 or IL-15-activated NK cells display a different sensitivity to corticosteroids. The corticosteroid methylprednisolone inhibited the surface expression of the activating receptors NKp30 and NKp44 and cytolytic activity in IL-2- or IL-15-cultured human NK cells. However, proliferation and survival were inhibited in IL-2- but not in IL-15-cultured NK cells. Moreover, methylprednisolone inhibited activation of STAT-1, STAT-3, and STAT-5 in IL-2-cultured NK cells but only partially in

IL-15-cultured NK cells. This study indicates a distinct ability of IL-15-cultured NK cells to survive to steroid treatment, an observation that is important in immune disorders requiring this drug [62].

Other important differences between IL-2 and IL-15 concern their regulation of expression and secretion. IL-2 gene is transcriptionally activated in Th cells in response to antigen presentation, and then activated Th cell release soluble IL-2, which can activate lymphoid cells expressing high or intermediate affinity receptor complexes. By contrast, IL-15 gene is constitutively expressed in several normal cell types, such as monocytes, DC, stromal cells, epithelial cells and also in some human neoplastic cells [5, 63]. Two IL-15 mRNA isoforms generated by alternative splicing have been identified in human cells: one encodes for a short signal peptide (SSP)-IL-15 and the other for a long-signal peptide (LSP)-IL-15 isoform [64, 65]. The two isoforms show a different intracellular trafficking, as the SSP-IL-15 predominantly localize to the cytoplasm and nucleus, whereas the LSP-IL-15 enters in the endoplasmic reticulum [66]. As a consequence the SSP-IL-15 isoform is not secreted and may have regulatory intracellular functions, whereas the LSP-IL-15, which is usually more expressed, can be potentially exported outside the cell. Nonetheless, it appeared that only very limited amounts of IL-15 are secreted by IL-15 mRNA-expressing cells, possibly in relationship to its low translational rate, to inefficient secretion, and/or to putative retention motifs in the COOH terminus [65–68]. Thus, multiple mechanisms may regulate IL-15 production and secretion.

3. Role of Cytokines in NK Cell Interactions in Secondary Lymphoid Organs

The expression of IL-15 in stromal cells of primary and secondary lymphoid organs and the deficiency of NK cells in mice with genetic defects of the IL-15/IL-15R system indicate a primary role of IL-15 in NK cell development and homeostasis [69–71]. Nonetheless, several data support an important role of a DC/NK cell cross-talk, primarily mediated by cytokines, in the immune response and defense against pathogens in mice [72] and humans [73]. In bacterial infections the interaction between human NK and DC cells may lead to NK cell proliferation, activation, and cytolytic activity [73].

Stimulation in vitro with IL-15, IL-2, IL-1 or IL-18 or engagement of activating receptors (i.e., CD16 or NKG2D) in combination with IL-12 induces production of IFN-γ and of other proinflammatory cytokines and chemokines, such as tumor-necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-1α by human NK cells [74, 75]. It is of note that cytokines that co-activate NK cells may be produced by monocytes/macrophages or DC and, in this context, it has been shown that DCs colocalize with NK cells in the T cell areas of lymph nodes. Membrane-bound IL-15 is highly exspressed on human DCs activated through CD40 engagement, and is essential for NK cell proliferation and survival. Thus, secondary lymphoid organs are important sites for DC/NK cell interactions [76].

Human CD3$^-$CD56$^+$ NKp46$^+$ NK cells are a heterogeneous cell population. The CD56^{dim}CD16$^+$ cells, predominant in peripheral blood, were originally described as strong cytotoxic effectors with low ability to produce cytokines, by contrast, the CD56^{bright}CD16$^-$ NK cell subset, the most represented in secondary lymphoid organs, has been reported to be the most potent producer of IFN-γ but provided of low cytolytic activity [77]. A recent study of De Maria et al. [78] reevaluated the functional capabilities of the two major human NK subsets and showed that NKp46 and NKp30-mediated stimulation induces an early (at 2–4 h from stimulation) and abundant production of IFN-γ by CD56^{dim}CD16$^+$ NK cells in relationship to the constitutive expression of IFN-γ mRNA. However, IFN-γ production was transient and no more cytokine production by this cell subset is observed after 16 h from stimulation, when CD56^{bright}CD16$^{+/-}$ cells begin to release IFN-γ. IFN-γ production by the two NK subsets is also observed upon exposure to cytokine combinations such as IL-12+IL-2 or IL-12+IL-15. In this case, CD56^{dim}CD16$^+$ NK cells show early but persistent response to stimulation. Therefore, CD56^{bright}CD16$^+$ NK cells may play an important role in the early phases of innate responses and in the cross-talk with DC.

Different from CD56^{dim}, CD56^{bright}NK cells constitutively express the high-affinity heterotrimeric IL-2 receptor complex [41, 42] and can, therefore, respond to picomolar concentrations of IL-2 produced by Th cells. Costimulation of CD56^{bright} human NK cells with IL-2 and IL-12 produced by DC triggers the production of IFN-γ [75]. IFN-γ can further activate DCs and influence the polarization of the Th cell response to Th1. In addition, activated NK cells also produce TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF), which support DC differentiation and maturation. Therefore, CD56^{bright} NK cells may link the innate- and antigen-specific immune response, through an NK/DC cross-talk, and shape the adaptive immune response [79, 80].

4. Involvement of Trans-Presentation in IL-15 Activity

The lack of detectable IL-15 secretion, in contrast with its trafficking from the secretory compartment to the early endosomes in some IL-15-expressing human melanoma cells, suggested a possible juxtacrine activity of IL-15. In this early model, IL-15-mediated effects required cell to cell proximity (Figure 2) and could be blocked by an anti-IL-15 monoclonal antibody (mAb) [81]. Further studies showed that IL-15 is expressed as membrane-bound molecule in IFN-γ-stimulated human monocytes, which are capable to stimulate T cell proliferation [82]. In addition, IL-15-mediated proliferation of mouse T cells, triggered by Poly I:C, did not require IL-15Rα on the T cells but instead was dependent on the IL-15Rα present on surrounding cells [83]. These findings could be later explained by the
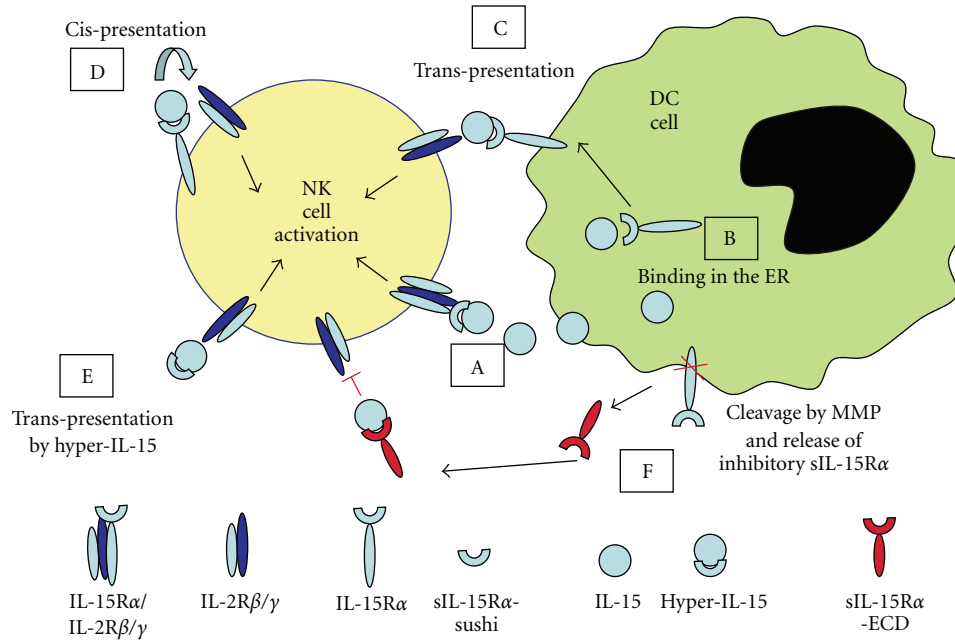
Figure 2: Different mechanisms of IL-15 action. (a): Low levels of secreted IL-15 can bind to high affinity trimeric receptors through a juxtacrine mechanism. (b): IL-15 binds to the high-affinity IL-15Rα chain within the endoplasmic reticulum and is then shuttled to the cell membrane as a complex. (c): IL-15Rα can trans-present IL-15 to an apposing IL-2Rβ/$γ_c^+$ NK cell through cell contact. (d): The possibility of cis-presentation by IL-15Rα/IL-15 membrane complex to an IL-2Rβ/$γ_c$ heterodimer on the same NK cell is shown. (e): A soluble sushi domain-IL-15Rα bound to IL-15 can perform trans-presentation in a soluble form. (f): Soluble IL-15Rα extracellular domain generated by metalloprotease cleavage can bind soluble IL-15 and block its function.

trans-presentation of IL-15 through IL-15Rα. For trans-presentation, exogenous IL-15 binds to cell surface IL-15Rα chain, the complex is internalized, recycled at the cell membrane and trans-presented to apposing T cells expressing IL-2Rβ/$γ_c$ heterodimers (Figure 2(c)) [84]. Alternatively, endogenously produced LSP-IL-15 binds into the endoplasmic reticulum with IL-15Rα, is exported onto the cell surface of human spleen fibroblasts as a complex with the IL-15Rα chain (Figures 2(b) and 2(c)). The trans-presentation of IL-15 to bystander haematopoietic progenitors commits these cells to the NK differentiation pathway [85]. Similar results have been reported in murine bone marrow DC, where IL-15Rα/IL-15 cell membrane complexes activate NK cells via trans-presentation [86]. The study of in vivo models further supported the role of trans-presentation in peripheral NK cell activation and survival. Indeed, adoptive transfer of normal NK cells into mice lacking the IL-15Rα results in the rapid disappearance of these cells. Conversely, IL-15Rα-deficient NK cells survive upon transfer in normal but not in IL-15Rα-deficient mice. Collectively these data show that IL-15Rα expression on surrounding cells is crucial for the survival of peripheral NK cells, while IL-15Rα expression on NK cells is not involved [87]. The finding that bone marrow progenitors from IL-15Rα$^{-/-}$ mice cultured with IL-7, stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (FLT3L), followed by IL-15, differentiate into CD94/NKG2$^+$ NK cells, which lacked Ly49 expression, suggest that IL-15Rα on NK cell precursors is not required for NK cell development but is required for their Ly-49 expression [88].

IL-15 and IL-15Rα have a broad tissue distribution, although their expression is not always coincident, raising the question about the cell type(s) involved in trans-presentation. IL-15 trans-presented by DC has been shown to activate NK cells both in vitro and in vivo [89]. In addition, a study suggested that both hematopoietic cells, such as DC and macrophages and nonhematopoietic cells, including stromal cells and epithelial cells are involved in trans-presentation of IL-15 to human NK cells [90]. Moreover, mice lacking IL-15Rα on macrophages, DCs, or on both, exhibit equivalent defects in NK cell homeostasis and activation, whereas only the expression on macrophages was important for the development of memory CD8$^+$ T cell responses [89].

Besides the relevance of cell-bound IL-15Rα in IL-15 trans-presentation, it has been hypothesized that IL-15Rα may also act as a cis-presenting molecule [83, 91]. In this model, the membrane IL-15Rα/IL-15 complex presents IL-15 to neighbour IL-2Rβ/$γ_c$ complexes on the same cell (Figure 2(d)). In this context, previous data showed that human CD3$^+$ or CD3$^-$ neoplastic lymphoid cells from lymphoproliferative disorder of large granular lymphocytes (LDGL) express surface bound IL-15 [92]. As these cells express IL-15Rα and proliferate to exogenous IL-15 stimulation, it is possible that they may bind IL-15 in vivo and store it on the cell membrane, thus allowing sustained stimulatory effects of endogenous IL-15 through cis-presentation. Nonetheless, the possible source of the in vivo bound IL-15 remains to be determined. The fact that IL-15 may not only

support neoplastic NK cell proliferation but may also play a role in the pathogenesis of LDGL was also suggested by the observation of spontaneous LDGL-like leukemias occurring in mice with transgenic overexpression of IL-15 [93].

In addition to trans- or cis-presentation of IL-15 by IL-15Rα, other forms of membrane-bound IL-15, independent from IL-15Rα, have been described. One of these IL-15 forms has been identified on human monocytes and may behave not only as a ligand but also as a signaling molecule. Thus, membrane-bound IL-15 may produce a reverse signaling that results in cellular adhesion and production of inflammatory cytokines [94]. Also, human haematopoietic progenitors derived from peripheral blood, but not from other sources, express an IL-15Rα-independent membrane-bound IL-15, which mediates reciprocal intercellular signals. This reciprocal trans-presentation induces the in vitro generation of a novel subset of mature noncytolytic NK cells (NKireg) that display regulatory functions and express the immunosuppressive molecule HLA-G. Remarkably, a small subset of NKp46$^+$HLA-G$^+$IL-10$^+$ is detected within freshly isolated decidual NK cells, suggesting that these cells could represent an in vivo counterpart of the in vitro-generated NKireg cells [95]. In addition, these NKireg precursors maintain along their differentiation process in vitro the expression of a membrane-IL-15 able to deliver a bidirectional signal. Indeed, the soluble IL-15Rα chain, upon binding with membrane-IL-15, triggers a reverse signal leading to the appearance of an adherent subset with DC morphology. These cells may represent a terminally differentiated population, since they do not proliferate, display both specific NK (NKp46) and myeloid dendritic (CD1a and BDCA1) markers as well as cytokine production and functions, illustrating another possible chapter of the NK/DC functional interplay [96]. Nonetheless, the in vivo significance of these cells has not yet been explored.

Recent evidences also indicate that soluble forms of IL-15 receptors can be generated by either alternative splicing or by in vivo cleavage of surface IL-15Rα through metalloproteinase-driven mechanisms [97, 98]. Such natural soluble forms of IL-15Rα corresponding to the extracellular domain behave either as a high-affinity IL-15 antagonists (Figure 2(f)) or as superagonist, depending on the isoform involved. Indeed, soluble IL-15R$\alpha\Delta$3 isoform bound to recombinant IL-15 generates a soluble complex, "hyper-IL-15", displaying a 100-fold higher biological activity on IL-2Rβ/γ_c^+ target cells than that exerted by the soluble cytokine [85]. Moreover, a recombinant, soluble sushi domain of IL-15Rα, capable to bind IL-15 with high affinity, is a potent IL-15 agonist and enhances the binding and the biological effects of IL-15 mediated through the IL-2β/γ heterodimer (Figure 2(e)). Nonetheless, the possible in vivo sources of similar "hyper-IL-15" forms and their potential role in the innate and adaptive immune response remains to be determined. In addition, fusion proteins consisting of IL-15 and IL-15Rα-sushi domain linked by a flexible aminoacid sequence (RLI) are even more potent stimulators of NK and T cells [99]. The use of RLI or of soluble IL-15 on a T cell line expressing both IL-15Rα/IL-2Rβ/γ_c trimeric and IL-2Rβ/γ_c dimeric complexes allowed to study the dynamics of cis-presentation (by IL-15) or trans-presentation (by RLI). IL-15 cis-presentation induced fast and transient activation, while trans-presentation mediated slower and more persistent responses [100].

5. Role of Cytokines in NK "Memory-Like" Responses

In specific immunity, the expansion of antigen-specific memory cells and persistent antibody production in response to pathogen's antigen challenge provide enhanced protection against the same pathogen upon a subsequent exposure. By contrast, NK cells are generally thought to be "naturally active" cells, which constitutively display effector functions against infected or transformed cells [101] and are incapable of adapting their responsiveness and of maintaining a memory of a first pathogen encounter in subsequent challenges. However, it is well known that freshly isolated NK cells show low effector functions in terms of cytolytic activity and cytokine production when incubated in vitro with tumor target cells. Several evidences indicate that NK cells can modify their behavior in response to environmental stimuli and can even show memory-like responses in mouse models [102, 103]. For example, recombinase activation gene (RAG)$^{-/-}$ mice (lacking both T and B cells) but not γ_c-deficient mice (lacking also NK cells) can develop delayed-type hypersensitivity (DTH) reactions to hapten. This response was specifically mediated by a subset of liver NK cells expressing Ly49C (an inhibitory receptor for self MHC molecules). In addition, NK cells from sensitized mice develop a specific memory-like function, since their adoptive transfer into naive mice can mediate DTH to the same hapten [104].

Besides this type of memory, which appears to be linked to expression of a specific type of receptor, NK cells may change their way of responding to stimuli following their exposure to specific cytokines [105]. Thus, NK cells from RAG$^{-/-}$ mice, activated for short-term with a cytokine cocktail consisting of IL-12, IL-18, and IL-15, produce high levels of IFN-γ in vitro. Upon subsequent transfer into naïve hosts, these cells can be detected up to three weeks later when they are returned similar to resting NK cells, as they do not display constitutive production of IFN-γ nor enhanced lytic properties. However, they produce significantly more IFN-γ than naïve cells when restimulated. These data suggest that NK cells retain memory of prior cytokine activation. In addition, this memory-like function appeared as an inheritable characteristic, as cytokine-activated NK cells proliferated once injected in mice [105].

As already mentioned, DC can interact with NK cells and activate them via trans-presented IL-15 in vitro [86]. Subsequent findings in a mouse model indicate that similar to T cells, NK cells need to be primed by contact with DC to achieve a full ability to respond to pathogen in vivo and this priming is mediated by IL-15 [106]. Upon engagement of Toll-like receptor by pathogen ligands in the periphery, NK cells migrate into regional lymph nodes where their interact with DCs. NK cell priming required IFN-mediated activation

of CD11c$^+$ DCs, which subsequently trans-present IL-15 to NK cells. Thus priming of NK cells results in arming, as IL-15 can confer full cytolytic properties to NK cells [60]. NK cells become then effector cells and migrate the periphery, where they can efficiently respond to pathogens. Priming and arming is a short-term response finalized to effector functions needed to eliminate pathogens. However, it has been proposed that upon priming and arming NK cells can further develop into memory-like NK cells, which do not produce cytokines or display lytic properties but maintain the capacity to respond to a subsequent pathogen challenge in a more vigorous way than naïve NK cells [102].

Thus, several evidences suggest that NK cells are capable to mediate memory-like responses in different murine models, although the relevance of such responses in humans is still to be defined.

6. Role of IL-7 in NK Cell Subset Development

In addition to IL-15, which is strictly required for the generation of all NK cells [21, 28], other cytokines have been involved in the development of specific NK cell subsets in different organs. In fact, it is clear that NK cells derive from CD34$^+$ hematopoietic progenitor cells originating in the bone marrow. However, early NK precursors can migrate to different organs, where they differentiate towards mature NK cells. Thus, the development of NK cells takes place not only in the bone marrow, but also in the thymus [107, 108], in secondary lymphoid organs [109] and in mucosae-associated lymphoid tissue in the gut in mice and humans [23, 110–112]. Although most mature circulating NK cells do not express IL-7Rα, experimental evidences have been provided that thymic murine NK1.1$^+$ cells express IL-7Rα and that IL-7 is required for the homeostasis of these thymic NK cells [108]. Indeed, differently from classical NK cells, the development of thymic-derived NK cells is dependent upon IL-7 and GATA-3 transcription factor [108]. This peculiar subset is characterized by low cytotoxic activity but high cytokine secretion potential.

A subset of CD56$^+$NKp44$^+$ cells has been identified in human tonsils, and mucosae-associated lymphoid tissues of the gut. Since these cells secrete IL-22 in response to IL-23, they were termed "NK-22" cells [112]. Similar to Th17 cells, NK-22 cells express the transcription factors retinoic-acid-related orphan receptor RORγt and aryl hydrocarbon receptor but do not produce IL-17. IL-7 supports the NK-22 cell survival and maintains the ability to secrete IL-22 in response to IL-23 stimulation. In addition, the combination of IL-7 with IL-1β or IL-2 also mediates NK-22 proliferation, indicating a synergistic effect of IL-7 with these cytokines [22]. The use of IL-1β and/or IL-2 altered the cytokine profile of NK22 cells, suggesting their functional plasticity. Indeed, IL-1β induced constitutive IL-22 secretion, while IL-2 reduced secretion of IL-22 and induced production of IFN-γ.

A murine equivalent of NK-22 cells has been identified in gut-associated lymphoid tissues [20] on the basis of their expression of the natural cytotoxicity receptor

(NCR) NKp46, a specific marker of NK cells from several species [113]. This subset was phenotyically characterized as NKp46$^+$IL-7Rα^+RORγt$^+$ and depends for its development on IL-7, RORγt, and intestinal microbial flora [20, 110]. Different from classical NK cells, the development of these mucosal-associated NK cells does not require on IL-15 or IL-2Rβ. Altogether, these data suggest that IL-22–producing NKp46$^+$IL-7Rα^+RORγt$^+$ cells and classical NK cells develop through different pathways under the control of different cytokines [20].

Since intestinal mouse CD3$^-$NKp46$^+$ and human NK-22 cells have some features of immature NK cells, they may possibly represent NK cell precursors that develop locally into specialized NK cell subsets, under the influence of a specific cytokine milieu and microbial product stimulation [23, 111]. In addition, a population of IL-22-producing immature NK cells showing a CD34$^-$IL-7Rα^+CD161$^+$CD9$^-$ surface phenotype, which do not produce IFN-γ and lack of cytolytic activity, has been described in human secondary lymphoid organs [114].

A recent report showed that rare human CD34$^+$ hematopoietic progenitors develop into NK cells in vitro in the presence of cytokines, such as IL-7, IL-15, SCF, and FLT3L. Moreover, the addition of hydrocortisone and stromal cells enhanced the frequency of progenitor cells that could develop into killer cell Ig-like receptor (KIR)$^+$NK cells [115]. These data suggest that NK cells can be derived from precursor cells committed to the myeloid lineage. This latter point is also supported by the existence of human CD14$^+$ myeloid-like cells within cord blood behaving as a novel progenitor for NK cells. Indeed, this CD14$^+$ myeloid-like subset can be redirected into NK differentiation in the presence of IL-15 and then generates mature functional NK cells [116].

CD34$^+$ hematopoietic precursors in human decidua were recently found to express IL-2Rβ, IL-7Rα, and mRNA for E4BP4 and ID2 transcription factors involved in NK cell development [117]. These data suggested that decidual CD34$^+$ cells are precursors committed to the NK cell lineage. In fact, these cells differentiate into functional IL-8- and IL-22-producing CD56^{bright}CD16$^-$KIR$^{+/-}$ NK cells in the presence of growth factors (including IL-15 and IL-7) or upon coculture with decidual stromal cells.

7. IL-21 as a Regulator of NK Cell Responses

IL-21 is produced by CD4$^+$ T cells in response to antigen presentation by DC during the adaptive immune response. At this stage, murine NK cells have been already activated by IL-12 and IL-15 produced by DC and then IL-21 can further support their proliferation and induce their functional maturation into potent effector cells with large granular lymphocyte morphology [118]. Thus, IL-21 upregulates the expression of CD16, the Fc-γRIII required for in antibody-dependent cellular cytotoxicity (ADCC), costimulates the secretion of IFN-γ, and upregulates the expression of granzyme and perforins. Similarly, IL-21 potentiates human NK cell ADCC activity and their ability to secrete cytokines

in response to antibody-coated tumor target cells [119]. IL-21 is also capable to boost NK-mediated ADCC in NK cells with defective cytotoxic properties, such as those of head-and-neck cancer patients [120]. However, different from IL-2 and IL-15, IL-21 alone does not mediate NK cell proliferation though at low concentrations, it costimulates the mitogenic effect of IL-2 or IL-15 [121, 122]. Although it is clear that IL-21 is an important regulator of NK cell functions [30], IL-21 may display positive and negative effects on NK cells, in relationship to their activation/maturation stage and species of origin. In fact, remarkable differences in IL-21 activity have been observed in mouse and human NK cells. Although IL-21 costimulates several functional properties of IL-15-activated murine NK cells, such as IFN-γ production and cytotoxicity, it does not support their survival. Instead, IL-21, at high concentrations, limits the proliferation of NK cells mediated by IL-15 and promotes an apoptotic program. Thus, it has been proposed that IL-21 mediates the transition of an early innate towards an adaptive response in the mouse, through the elimination of terminally differentiated NK cells and the induction of cytotoxic T lymphocyte (CTL) memory responses [123]. Differently, IL-21 alone stimulates the cytolytic activity of freshly isolated, peripheral human NK cells and the combination of low concentrations IL-21 plus IL-15 costimulates the expansion of CD56$^+$CD16$^+$ NK cells, which develop strong effector functions [121]. Possible explanations for these discrepancies may relate not only to species differences but also to the concentrations of IL-21 and IL-15 that were used in the different experiments or on the timing and activation state of NK cells.

NKG2D is an important activation receptor on mouse and human NK cells and triggers cytotoxicity upon engagement with ligands, such as antibodies or cell surface ligands. These effects are mediated through DNAX-activating protein of 10 kDa (DAP10). Data in a mouse tumor model showed that IL-21 is able to enhance tumor rejection through an NKG2D-dependent mechanism [124] as NKG2D-blocking inhibited the antitumor activity and cytotoxicity of IL-21 activated NK cells. On the opposite, treatment of human NK and CD8$^+$ T cells with IL-21 in combination with IL-2 reduces the cell surface expression of NKG2D and its ability to trigger cytotoxicity, relative to cells treated with IL-2 alone [125]. IL-21-induced downregulation of NKG2D is related to inhibition of DAP10 gene transcription. However, IL-21 induced the expression of the NK activation receptors NKp30 and 2B4, suggesting that IL-21 modulates human NK cell functions and their target specificity by altering the expression levels of different activation/costimulatory receptors.

IL-21 also induced an accelerated development of NK cells from human cord blood CD34$^+$ haematopoietic progenitor cells, in concert with IL-7, IL-15, and SCF. Indeed IL-21 costimulates the expression of NKp46 and NKp30 triggering receptors, CD94/NKG2A inhibitory receptor, KIRs, CD2, and CD16, typical of mature NK cells and the acquisition of cytotoxic activity [126]. In addition, also rare CD34$^-$lineage$^-$ cells cultured with Flt3-L, SCF proliferated in response to IL-15 and IL-21 and acquired a KIR$^-$CD56$^+$CD16$^{-/+}$ lymphoid phenotype, consistent with pseudomature NK cells. These cells secreted IFN-γ, GM-CSF

and MIP-1α, and displayed cell surface CD107a upon contact with NK-sensitive targets [127]. Thus IL-21 may possibly have a role in the development of NK cells although the study of IL-21 KO mice indicated that IL-21 is not strictly necessary for the differentiation of NK cells from progenitors [56].

8. Perspectives for Cancer Immunotherapy

IL-2 represents a milestone in the history of the immunotherapy of cancer and is still clinically used for the treatment of advanced melanoma and renal carcinoma. The induction of some long-lasting remissions in metastatic patients treated with recombinant (r)IL-2 alone or in combination with LAK cells provided an important proof of principle that activation of the immune system may result in tumor rejection even in patients with bulky disease [128]. However, these effects were observed in a minor subset of patients and the treatment showed a remarkable toxicity, predominantly related to "vascular leak syndrome" [129] and adverse effects on the heart [128]. The availability of novel recombinant cytokines may offer new possibilities for cancer immunotherapy [130, 131].

The functional properties of IL-15 have suggested the use for this cytokine in tumor immunotherapy [12]. It is hoped that IL-15 may display lower toxic effects than IL-2 and provide similar immune-enhancing effects on tumor-reactive T and NK cells. In early experiments, tumor cells transduced with a modified IL-15 cDNA, allowing enhanced IL-15 secretion, showed reduced tumorigenic potential in immunodeficient [132] or in syngeneic mice [133], through the recruitment of NK cells and/or CTLs. In addition, IL-15-transduced tumor cells, administered as vaccine, reduced the incidence of experimental metastases in syngeneic mice. Administration of rIL-15 has also been shown to display antitumor activity in murine tumor models with a lower toxicity than rIL-2 [134]. Finally, plasmid gene transfer of IL-15 trough an hydrodynamic method increases the number and function of NK and IFN-producing killer DC cells in mice [135].

In a preclinical study, human rIL-15, administered intravenously daily for 12 days to rhesus macaques, showed both short- and long-lasting effects on lymphoid cell homeostasis. A transient lymphopenia preceded a clearcut increase in NK and memory CD8$^+$ T cells in the peripheral blood. An inverted CD4/CD8 T-cell ratio was observed as result of CD8$^+$ T cell expansion. By day 48, homeostasis appears restored throughout the body, with the exception of the maintenance of an inverted CD4/CD8 ratio in lymph nodes [136]. A phase I study of intravenous rIL-15 in adults with refractory metastatic melanoma and renal cancer has been recently started and is currently recruiting participants. The objectives of this study are the evaluation of the safety and efficacy of rIL-15 and to examine how the body processes the infused cytokine [NCT01021059].

Since the scaling up of IL-15 production for clinical purposes has been technically difficult, the possible usage of hyper IL-15 in clinical settings of cancer immunotherapy can be envisaged. The potential advantages are that hyper-IL-15

would act in lower doses than IL-15, as it is a more potent activator of the immune system than IL-15 on a molar basis. Interestingly, the potential development of hyper-IL-15 in NK-based immunotherapy is also illustrated by data from Kroemer et al. [137] in a skin transplant in Rag$^{-/-}$ mice. Resting NK cells did not reject skin allografts, while hyper-IL-15-stimulated NK cells mediated acute skin allograft rejection in the absence of T and B cells.

The transfer of NK cells is an emerging strategy for cancer immunotherapy, particularly in haematologic neoplasia. A pivotal role in the antileukemic effects of allogeneic NK cell transfer is played by KIRs, which are inhibitory receptors for HLA class I molecules [138]. The engagement of a KIR expressed on the NK cell surface by the appropriate HLA class I allele on a target cell produces an inhibitory signal to the NK cell activation resulting in target cell protection. In T cell-depleted haploidentical hematopoietic stem cell transplantation (haplo-HSCT) donor NK cells may express KIR(s) that do not recognize the HLA-class I alleles present on recipient's cells. In this "KIR-mismatch" setting, these "alloreactive" NK cells efficiently lyse leukemic cells and generate a strong graft versus leukemia effect, which contributes in eradicating residual disease. In addition, alloreactive NK cells eliminate residual host dendritic cells, thus preventing graft-versus-host disease [138].

Because NK cells are a fraction of peripheral blood mononuclear cells the development of methods to produce large numbers of functional NK cells could be useful to optimize NK-based therapies. Coculture of NK cells with K562 leukemia cells, genetically modified to express membrane-bound IL-15 and 41BBL, allowed a 20-fold expansion of CD56$^+$CD3$^-$ NK cells from peripheral blood but induced no proliferation of T cells. The expanded NK cells were potent effectors against acute myeloid leukemia cells (AML) in vitro and eradicated AML in xenograft models in immunodeficient mice. This method provide a new platform for expanding activated NK cells for cell therapy of cancer [139].

In view of its immune-enhancing functions, also IL-21 has been considered as a good candidate for cancer immunotherapy. In addition, IL-21, differently from IL-2 is unable to mediate the proliferation of activated CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in vitro [140] although Treg cells express IL-21R gene. Nonetheless also IL-21 has shown immune regulatory functions related to the induction of IL-10 production, which inhibits the immune response by acting at several levels [141]. Although this may represent a potential drawback, it is almost likely that each immune enhancing cytokine possess its own negative regulatory mechanisms, to prevent exaggerated responses and autoimmune reactivity.

Several studies in murine tumor models has shown that IL-21 is endowed with antitumor properties [142, 143], which can be mediated by NK, T, or B cell-dependent responses, in relationship to the experimental model considered.

Different types of tumor cells, genetically modified to produce IL-21, form small tumors when injected into syngeneic mice and are then eventually rejected by an IL-21-driven immune reaction, which is followed by immunity to tumor antigens [144]. In addition, human pancreatic cancer cells transduced with murine IL-21 gene are rejected when xenografted in immune-deficient mice through activation of NK cells [145]. In a syngeneic model of mammary adenocarcinoma, tumor-released IL-21 induced the local recruitment of both CD8$^+$ and NK cells and the production of IFN-γ and of IFN-γ-dependent CXC chemokines, which mediated local antiangiogenic effects [144]. IL-21-transduced tumor cells were also effective when used as a vaccine to treat metastatic tumors [140, 146]. However, in the mammary adenocarcinoma model, the therapeutic effect was partial and could be synergistically enhanced by targeting CD4$^+$CD25$^+$ Treg cells by an anti-CD25 mAb [140]. Thus, Treg cells appeared to limit not only CTL- but also NK-mediated responses by tumor-released IL-21. The antitumor effects of IL-21 therapy did not require Th cells, suggesting that IL-21 may bypass the requirement of Th cells for the induction of CTL and NK responses. In a neuroblastoma syngeneic model the efficacy of IL-21-based immunotherapy was even enhanced by transient CD4$^+$ T cell depletion, which also resulted in the elimination of CD4$^+$ Treg cells [147].

Another approach has been the direct gene transfer of IL-21 in vivo through a plasmid-based hydrodynamic system, which results in sustained IL-21 levels and NK-dependent antitumor effects in syngeneic tumor models [148]. The coinjection of IL-15- and IL-21-encoding plasmids in mice bearing lymphoma produced cooperative effects of tumor rejection [149]. Also, the injection of plasmids encoding for an IL-21/IgFc chimeric protein mediated antitumor effects in melanoma-bearing syngeneic mice, through the induction of an NK-mediated response [150]. Other studies combined IL-21 protein administration with antibody treatments. In view of the ability of IL-21 to enhance ADCC activity by NK cells, rIL-21 has been combined with an anti-Her2/neu antibody to treat mice bearing Her2/neu$^+$ tumors. This combination showed a synergistic antitumor effect through an IFN-γ-dependent mechanism [119].

Altogether preclinical studies led to the design of clinical trials in cancer patients. Several clinical studies of rIL-21 monotherapy or combining IL-21 with other drugs are now ongoing in different type of cancers. Phase I studies in melanoma and renal cancer have been already concluded and showed that IL-21 has an acceptable toxicity profile and does not induce vascular-leak syndrome by repeated iv infusion [151, 152] or subcutaneous administration [153]. IL-21 induced increased levels of soluble CD25 and upregulated IFN-γ, perforin, and granzyme B expression in circulating CD8$^+$ T cells and NK cells, indicating cytotoxic lymphocyte activation. By i.v., IL-21 induced a dose-dependent decrease in circulating NK and T cells, followed by a return to baseline in resting periods. Objective responses and disease stabilizations were observed and were also confirmed in an initial phase II clinical study [154], which also suggested an increase in progression-free survival.

Besides immune-enhancing activities, IL-21 mediates apoptosis of specific B cell malignancies such as chronic lymphocytic leukemia [34, 35] and follicular [37] or diffuse large B cell lymphoma [36]. CD4$^+$CD25$^+$ regulatory T cells are

expanded in solid and hematological malignances including CLL and the use of IL-21 instead of IL-2 may contribute to limit this expansion [155]. For this reason and for the ability of IL-21 to enhance ADCC activity by NK cells, a phase I trial of rIL-21 combined with the anti-CD20 mAb rituximab has been designed in chemotherapy refractory/relapsed non-Hodgkin's lymphoma. Acceptable toxicity, several objective responses, and disease stabilizations were reported in a preliminary analysis of data [156]. Overall data from early clinical trials indicate that IL-21 warrants further testing, particularly in multimodality therapy regimens for cancer.

IL-21 also plays a role in controlling chronic viral infections and its serum levels were reduced in HIV-infected persons, which display defective NK activity. Two recent reports indicated that IL-21 enhanced viability, HIV-specific ADCC, IFN-γ secretion, and cytotoxic functions of NK cells from HIV-infected persons [157, 158]. In addition, the IL-21-activated NK cells inhibit viral replication in cocultured CD4$^+$ T cells. These data suggest that that IL-21 could represent a potential tool for immunotherapy or as adjuvant for vaccines in HIV-infected patients.

The systemic administration of cytokines at high doses frequently results in toxicities, and the amount of cytokine effectively delivered at the tumor site is generally low. Tumor cells genetically modified to secrete cytokines may elicit potent immune responses upon injection in syngeneic mice, without systemic effects, due to a high local concentration of cytokine. Although cytokine gene transfer procedures have shown some promising effects in easily accessible tumors (such as subcutaneous melanoma metastases) [159], it cannot be easily applied in case of systemic metastases. Another possibility to reduce systemic toxicity and reach high cytokine concentrations at the tumor site is based on the targeted delivery of cytokines, through the generation of fusion proteins formed by a recombinant antibody linked to a cytokine. These antibody/cytokine chimeras, defined as immunocytokines, have shown promising results in animal models [160]. An example of such immunocytokines is L19-IL-2 [161], which was obtained by chimerization of IL-2 with a single-chain human antibody specific for an oncofetal fibronectin isoform of the tumor extracellular matrix. L19-IL-2 was capable to accumulate at sites of neoangiogenesis in tumors, to determine the recruitment of T and NK and to induce tumor regression in both syngeneic and nude mice. Therefore, L19-IL-2 has entered several clinical trials in different types of tumors and a phase I study was recently concluded [NCT01058538] [162]. This study showed that L19-IL-2 can be safely and repeatedly administered in advanced solid tumours and preliminary evaluation suggests clinical activity in patients with metastatic renal carcinoma.

In a murine neuroblastoma model, the therapeutic effect of an immunocytokine consisting of an anti-GD2 antibody linked to IL-2 was dependent on NK cells [163]. In addition, most human neuroblastomas express low levels of HLA class I [164] and express ligands for NCR activating receptors, thus representing potential targets for NK-based therapies [165]. A phase II trial of the humanized anti-GD2 monoclonal antibody linked to human IL-2 (hu14.18-IL2) was recently performed on relapsed/refractory neuroblastoma patients. To explore the role of NK cells in this treatment patients were genotyped for KIR, HLA, and FcR alleles. The presence of a KIR/KIR-ligand mismatch was signficantly associated with response/improvement to immunocytokine, and there was a trend towards a higher response rate in patients with the FcγR2A 131-H/H genotype than other genotypes. These findings are strongly suggestive for a role of NK cells in clinical responses to hu14.18-IL2 cytokine treatment in relapsed/refractory neuroblastoma patients [166].

9. Conclusions

In conclusion, several evidences indicate that IL-2, IL-7, IL-15, and IL-21 play important roles in NK cell biology and that, in spite of some redundancy, each cytokine has clearly distinct functions. In addition, they may differentially act on subset of NK cells, whose phenotypic and functional heterogeneity is now well established. Moreover, these cytokines allow to manipulate and expand NK cells in vitro to generate populations with increased effector functions or to directly boost NK cell activity in vivo. Thus, these cytokines may represent potentially relevant tools for NK-based immunotherapy strategies in diseases such as leukemias, solid tumors, and AIDS. In this context, the development of novel strategies of cytokine targeted delivery or the use of synergistic combinations with antibodies or of different cytokines may offer the possibility to maximize their therapeutic potential.

Acknowledgment

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