

Insights into Seven and Single Transmembrane-Spanning Domain Receptors and Their Signaling Pathways in Human Natural Killer Cells

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Abstract—Human natural killer (NK) cells are important cells of the innate immune system. These cells perform two prominent functions: the first is recognizing and destroying virally infected cells and transformed cells; the second is secreting various cytokines that shape up the innate and adaptive immune re-

sponses. For these cells to perform these activities, they express different sets of receptors. The receptors used by NK cells to extravasate into sites of injury belong to the seven transmembrane (7TM) family of receptors, which characteristically bind heterotrimeric G proteins. These receptors allow NK cells to sense the chemotactic gradients and activate second messengers, which aid NK cells in polarizing and migrating toward the sites of injured tissues. In addition, these receptors determine how and why human resting NK cells are mainly found in the bloodstream, whereas activated NK cells extravasate into inflammatory sites. Receptors for chemokines and lysophospholipids belong to the 7TM family. On the other hand, NK cells recognize invading or transformed cells through

another set of receptors that belong to the single transmembrane-spanning domain family. These receptors are either inhibitory or activating. Inhibitory receptors contain the immune receptor tyrosine-based inhibitory motif, and activating receptors belong to either those that associate with adaptor molecules containing the immune receptor tyrosine-based activating motif (ITAM) or those that associate with adaptor molecules containing motifs other than ITAM. This article will describe the nature of these receptors and examine the intracellular signaling pathways induced in NK cells after ligating both types of receptors. These pathways are crucial for NK cell biology, development, and functions.

I. Introduction

A. Natural Killer Cells

Human natural killer (NK)¹ cells make up approximately 10 to 15% of total blood lymphoid cells. NK cells perform two major functions: the first is recognizing and lysing tumor cells and virally infected cells (French and Yokoyama, 2003; Wu and Lanier, 2003); the second is regulating the innate and adaptive immune responses by secreting CCL3, CCL4, CCL5, or XCL1 chemokines (Taub et al., 1995; Inngjerdigen et al., 2001) or cytokines such as granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , or IFN- γ (Biron et al., 1999; Cooper et al., 2001). In the circulation, human NK cells are classified into two major subsets: those that express CD16 and low CD56 [known as CD16⁺ (or high) CD56^{dim}] and those that express CD56 but low or no CD16 [known as CD16⁻ (or low) CD56^{bright}]. The latter subset makes up approximately 10 to 20% of total NK cells, secretes IFN- γ and other cytokines, and has less cytolytic activity, whereas the CD16^{high}CD56^{dim} subset

makes up approximately 80 to 90% of blood NK cells, secretes cytokines with low intensity, and is highly cytolytic.

For any cell type, including NK cells, to perform their biological activities, they must activate various intracellular signaling molecules. These molecules are transduced through the activation of either seven transmembrane (7TM)-spanning domain receptors, also known as G protein-coupled receptors (GPCRs), or through receptors composed of a single transmembrane (1TM)-spanning domain, which in most cases associate with adaptor molecules.

B. Heterotrimeric Guanine Nucleotide-Binding (G) Proteins

Heterotrimeric G proteins are composed of three subunits: α , β , and γ . Approximately 20 α and several β and γ subunits have been reported (Hamm, 1998). The α subunits belong to four subfamilies: 1) α_s (α_s and α_{olf}); 2) α_q (α_q , α_{11} , α_{14} , α_{15} , and α_{16}); 3) α_i (α_{i1} , α_{i2} , α_{i3} , α_{o1} , α_{o2} , α_z , α_t , α_{gus} , α_{con} , and α_{rod}); and 4) α_{12} (α_{12} and α_{13}). Members of $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_z$ are insensitive to bacterial toxins, and members of $G\alpha_i$ or $G\alpha_s$ are sensitive to treatment with these toxins. For example, cholera toxin ADP-ribosylates the arginine (D) residue in the carboxy terminal of the α subunit of G_s , and pertussis toxin (PTX) ADP-ribosylates the cysteine (C) residue in the carboxy terminal of the α subunit of G_i or G_o (Barritt and Gregory, 1997). $G\alpha_s$ activates adenylyl cyclase, resulting in the accumulation of cAMP; $G\alpha_i$ inhibits the accumulation of cAMP; $G\alpha_q$ activates phospholipase C β , resulting in the hydrolysis of phosphatidylinositol bisphosphate and the generation of diacylglyceride (DAG), an activator of protein kinase C and inositol 1,4,5 trisphosphate; $G\alpha_o$ activates Ca²⁺, Cl⁻, or K⁺ ion channels; and $G\alpha_z$ is involved in the inhibition of cAMP production or cellular proliferation (Clapham, 1996).

The α subunit of the heterotrimeric G proteins is anchored in the plasma membranes through its lipid modification, α_s is palmitoylated, α_i is palmitoylated and myristoylated, and α_q is myristoylated (Wedegaertner et al., 1995), whereas the γ subunit is either farnesylated

¹ Abbreviations: NK, natural killer; IFN, interferon; 7TM, seven transmembrane; GPCRs, G protein-coupled receptor; 1TM, single transmembrane; PTX, pertussis toxin; DAG, diacylglyceride; GRK, G protein-coupled receptor kinase; PH, pleckstrin homology; MAPK, mitogen-activated protein kinase(s); GEF, guanine nucleotide exchange factor; ERK, extracellular signal-regulated protein kinase(s); MEK, MAPK/ERK kinase; Sos, son of sevenless; Grb2, growth factor receptor-bound protein 2; SH, Src homology; PI3K, phosphoinositide 3 kinase; PI(3,4)P₂, phosphatidylinositol 3,4 bisphosphate; PI(3,4,5)P₃, phosphatidylinositol 3,4,5 trisphosphate; PTEN, 3'-phosphatase and tensin homolog; WASP, Wiskott-Aldrich syndrome protein; IL, interleukin; LPL, lysophospholipid; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; PLC, phospholipase C; LPC, lysophosphatidylcholine; MHC, major histocompatibility complex; Ig, immunoglobulin; KIR, killer inhibitory receptor; HLA, histocompatibility leukocyte antigen; ITIM, immune receptor tyrosine-based inhibitory motif; SHP, SH-containing tyrosine phosphatase; DAP, DNAX adaptor protein; ITAM, immune receptor tyrosine-based activating motif; SYK, spleen tyrosine kinase; ZAP, ξ -associated protein; SLP, SH2 domain-containing leukocyte phosphoprotein; LAT, linker for activation of T cell; NC, natural cytotoxicity; ITSM, immunoreceptor tyrosine-based switch motif; ULBP, UL-16 binding protein; FTY720, 2-amino-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride; DBL, diffuse B-cell lymphoma; OGR1, ovarian G protein-coupled receptor-1; SPC, sphingosylphosphorylcholine.

or geranylgeranylated and binds the membranes through its carboxy helix (Lambright et al., 1996). The $\beta\gamma$ complex binds switch II (or the junction between switch I and II) regions of the α subunit (Sprang, 1997). Upon binding of the ligands, conformational changes occur in GPCRs, allowing the separation of the 3TM and the 6TM loops of the 7TM receptors, which creates a pocket and results in a higher-affinity binding of the α subunit to these receptors (Bourne, 1997; Maghazachi and Al-Aoukaty, 1998). Consequently, conformational changes occur in the α subunit of G proteins, leading to the exposure of the switch II region to the membranes and allowing GTP, which is abundant in these membranes to bind this region. When this occurs, the GTP displaces the $\beta\gamma$ subunit of the heterotrimer from the α subunit, leading to the release of the $\beta\gamma$ complex and GTP- α subunit that activate various intracellular effector molecules. To turn off the activation of G proteins, the α subunit that possesses an intrinsic GTPase activity digests the GTP into GDP, resulting in the reassociation of the α subunit with the $\beta\gamma$ complex and hence reverting to the inactive state where the heterotrimer binds the GPCRs (Fig. 1). Another pathway to desensitize GPCRs is via the phosphorylation of these receptors. Many second messengers and kinases have been described that phosphorylate these receptors; among them is the G protein-coupled receptor kinases (GRKs). GRKs are serine/threonine kinases that phosphorylate the carboxy terminal of GPCRs rich in these residues. These kinases can be recruited to the membranes through the binding of their pleckstrin homology (PH) domains with the β subunit of G proteins (Wang et al., 1994; Inglese et

al., 1995). Phosphorylation of GPCRs by GRKs results in the recruitment of β -arrestins, which bind phosphorylated receptors and uncouple G proteins from these receptors. Through their proline-rich segment found in the amino terminal, β -arrestins recruit and activate the nonreceptor tyrosine kinase c-Src by binding their SH3 domains (Luttrell et al., 1999). c-Src then phosphorylates dynamin, which facilitates the endocytosis of GPCRs into clathrin-coated pits (Fig. 1). Although this pathway generally results in desensitization, other reports showed that the same pathway leads to the activation of mitogen-activated protein kinases (MAPK) (Ahn et al., 1999; Luttrell et al., 1999).

C. Small G Proteins

In addition to the heterotrimeric G proteins, small G proteins also known as GTPases have been described. GTPases exist in an inactive GDP-bound form, and they exchange GDP for GTP very slowly due to the low dissociation rate of GDP. GTPases include Ras, Rab, Ran, Rho, and Arf. All GTPases have two switch regions known as switch I and switch II because conformational changes occur within these regions during GDP-GTP exchange, an activity facilitated by guanine nucleotide exchange factors (GEFs). TIAM1, VAV, and DBL are GEFs for Rac; GEF p115, lymphoid blast crisis, VAV, and DBL are GEFs for RhoA; and Cdc24/25, VAV, and DBL are GEFs for Cdc42. It is noteworthy that all GEFs contain one or more PH domains that associate with diffuse B-cell lymphoma-homolog domains that possess GTP exchange capability (Soisson et al., 1998). The PH domains are present in more than 100 proteins that

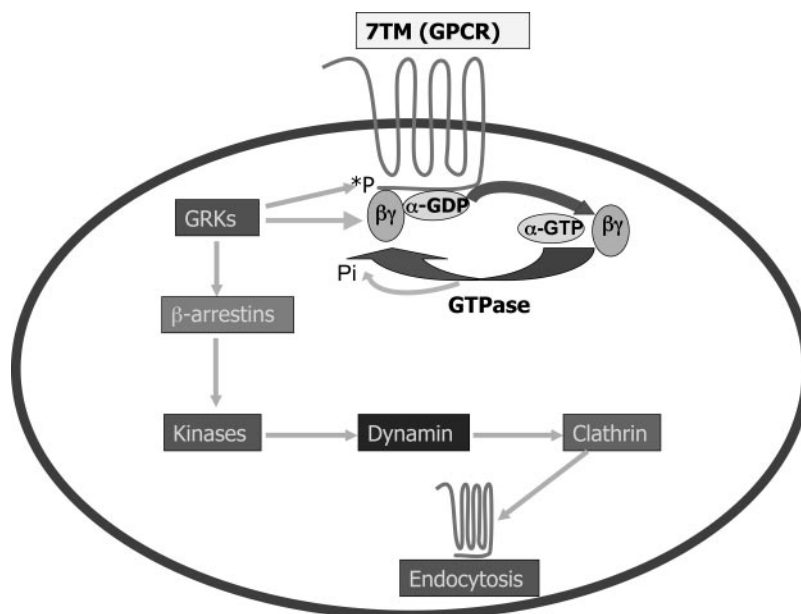


FIG. 1. Activation and desensitization of the heterotrimeric G proteins. Upon stimulating the GPCRs, conformational changes occur in these receptors that allow the exchange of GDP with GTP, resulting in the dissociation of the $\beta\gamma$ subunit from the α -GTP. To desensitize this activation process, GTPase present in the α subunit hydrolyzes GTP into GDP, reverting into inactive GDP bound form. Desensitization also occurs when GRKs are recruited by the $\beta\gamma$ subunit. GRKs phosphorylate the carboxy terminal of GPCR leading to the recruitment of β -arrestins, c-Src kinase, dynamin, and clathrin, which eventually leads to the endocytosis of GPCR. Of note, this desensitization process may also lead to MAPK activation.

share limited amino acid homology, although the tertiary structure of these domains is the same in all of these proteins (Lemmon and Ferguson, 2000). The PH domains comprise two orthogonal antiparallel β sheets of three and four strands with an amphipathic C-terminal α helix that is closed at one end. Although each GEF is unique in its structure and function, GEFs have a common feature by invading the switch regions of small G proteins, exposing those areas in the GTPase that bind GTP (Sprang and Coleman, 1998). This subsequently leads to the activation of GTPases, which in turn bind several effector molecules and induce multiple biological activities, among them being cell motility.

II. Signal Transduction Pathways Induced upon Ligand Binding

A. Mitogen-Activated Protein Kinases

Members of the MAPK such as extracellular signal regulated-protein kinases (ERK) 1 and 2 phosphorylate various nuclear binding proteins, such as, among others, c-Jun/Fos, c-Myc, and ELK-1, resulting in gene expression, mRNA, and protein synthesis. ERK1 and ERK2 are phosphorylated by another kinase known as MAPK kinase (MAPK/ERK kinase or MEK1/2), which is phosphorylated by a serine/threonine kinase known as Raf-1 (MAPK kinase kinase). Raf-1 is a substrate of the small GTP-binding protein p21^{ras} (Ras). The latter is activated by a homolog of son of sevenless (Sos)-1, which is recruited to the cell membrane by an adaptor protein called growth factor receptor-bound protein 2 (Grb2), a 23-kDa protein having three Src homology (SH) domains. Through its SH3 domain, it binds the carboxy terminal of Sos, and through its SH2 domain, it binds the adaptor molecule Shc, which exists in three different forms: 46, 52, and 66 kDa. Dimerization of various receptors activates Shc, resulting in the activation of the Ras pathway. Other members of the MAPK include those activated by stress, such as DNA-damaging agents (Robinson and Cobb, 1997). The p38 kinase is another kinase activated by MEK3 or MEK6 and phosphorylates the transcription factor CHOP/GADD153 (C-EBP homologous protein/growth arrest and DNA damage-inducible protein). Stress induces other kinases such as Jun/Sapk, which is activated by MEK4, and promotes apoptosis.

B. Phosphoinositide 3 Kinase

Phosphoinositide 3 kinase (PI3K) is present in three different forms: I, II, and III. These isoforms phosphorylate the D3 position of phosphatidylinositol to produce phosphatidylinositol 3 phosphate, phosphatidylinositol 3,4 bisphosphate [PI(3,4)P₂], or phosphatidylinositol 3,4,5 trisphosphate [PI(3,4,5)P₃] (Wymann et al., 2000; Curnock et al., 2002). PI3K I is present in mammals and divided into PI3K IA and PI3K IB. PI3K IA comprises a catalytic subunit (p110 α , β , or δ) that is associated with

a regulatory p85 subunit. The latter possesses an SH2 domain that binds the tyrosine-phosphorylated receptors upon activation. On the other hand, PI3K IB expresses the catalytic p110 γ , which lacks the SH2 domain and does not bind tyrosine-phosphorylated receptors but instead has a motif that binds the $\beta\gamma$ subunit of G proteins (Stoyanov et al., 1995). PI(3,4,5)P₃ is hydrolyzed by either Src homology SH2-containing inositol polyphosphate 5-phosphatase into PI(3,4)P₂ or by another enzyme known as 3'-phosphatase and tensin homolog (PTEN), which hydrolyzes it into PI(4,5)P₂ (Rickert et al., 2000). Class II PI3K contains the C2 domain, which phosphorylates phosphoinositide or PI(4)P (Curnock et al., 2002). The products of PI3K bind PH domains, resulting in the recruitment of PH domain-containing molecules into the plasma membranes. A known example of this transfer is protein kinase B (also known as Akt), which is recruited into the membranes upon ligation of the receptor by growth factors (Chung et al., 2001). In addition, PI(3,4,5)P₃-dependent protein kinase-1 is recruited into the membranes through the binding of its PH domains to PI(3,4,5)P₃. Consequently, PI(3,4,5)P₃-dependent protein kinase-1 phosphorylates Akt, which performs many important roles, among them being inhibition of apoptosis.

III. Cell Movement

A. Role for GTPases

Motile cells are engaged in two steps: contraction leading to cell movement and relaxation leading to cell arrest. During these processes, the cells form filopodia (finger-like structure extensions) and lamellipodia (web-like structure extensions). Filopodia and lamellipodia are important for cell spreading on the migrating surfaces and are controlled by the GTPases Cdc42 and Rac, respectively. Rac and Cdc42 bind p21-activated kinase, which contains a motif known as Cdc42/Rac-interacting protein. In addition, these GTPases bind WASP (Wiskott-Aldrich syndrome protein) (Bishop and Hall, 2000; Ridley, 2001, 2003) and activate insulin receptor kinase p53 and the WASP family verprolin-homology protein/suppressor of cyclic AMP receptor protein, which, through the WASP-conserved verprolin-homology, cofilin-homology, acidic domain, activates the Arp2/3 (Fig. 2). Rho (Ras homolog), which activates Rho kinase, plays a prominent role in phosphorylating myosin light chain and promoting cellular contraction (Fig. 2). Of note, most if not all GTPases are under the control of guanine nucleotide dissociation inhibitors, which keep GTPases in a GDP-bound form and hence guard against unwanted activation of these proteins (Bishop and Hall, 2000; Ridley, 2001).

B. Mechanisms Regulating Cell Motility

Although eukaryotic cells are equipped for spatial sensing between the leading edge and rear end, most

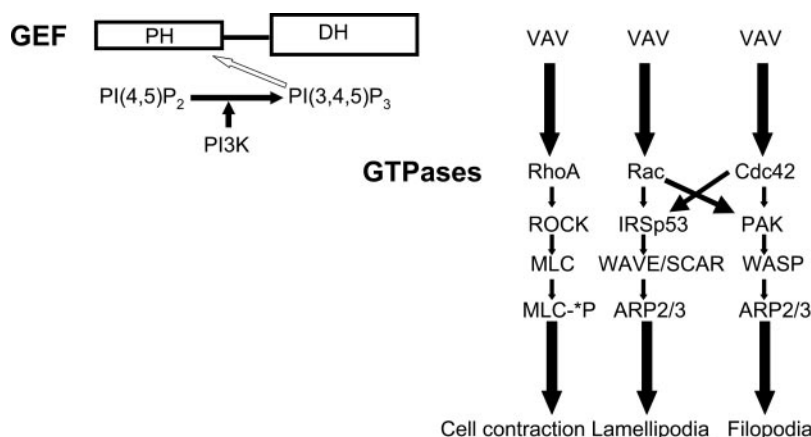


FIG. 2. GTPase signaling pathways. GEF, which facilitates the GDP/GTP exchange in GTPases, contains PH and diffuse B cell lymphoma-homolog domains, as well as other domains not shown here. Upon the phosphorylation of PI(3,4)P₂ by PI3K, PI(3,4,5)P₃ is generated. This phospholipid binds the PH domain present in GEF such as VAV (as well as in all other GEFs). VAV activates GTPases such as RhoA, Rac, and Cdc42, resulting in cellular contraction and the formation of lamellipodia and filopodia.

activities linked to motility occur at the leading edge, whereas the trailing edge of the cells forms sites for attachment to the substratum and adherence to other cells (Iijima and Devreotes, 2002; Van Haastert and Devreotes, 2004). When the cells sense the chemotactic gradients, they orient their leading edge toward the chemotactic peptides, forming filopodia and lamellipodia and reorganizing the cytoskeleton. Cell movement starts by recruitment of the leading edge of the PH domain-containing proteins, followed by rapid recruitment of PI3K γ , which associates with this complex (Hirsch et al., 2000; Maghazachi, 2000; Wymann et al., 2000). This rapid accumulation of PI3K, PI(3,4,5)P₃, and Rac/Cdc42 at the leading edge is linked with the activation of RhoA/Rho kinase at the trailing edge, allowing the cells to contract. The accumulation of PI(3,4,5)P₃ is controlled by PTEN, and in PTEN knockout cells there is increased production of phosphoinositides, corroborating the continuous recruitment of PH domain-containing proteins into the membranes, which results in high abnormal motile morphology (Iijima and Devreotes, 2002; Devreotes and Janetopoulos, 2003; Van Haastert and Devreotes, 2004). Hence, the distribution of various molecules to the leading edge is important in allowing the cells to sense the chemoattractant gradients and to orient themselves toward these sites, thereby providing local stimulation, whereas PTEN accumulating at the rear end provides a global inhibition (Devreotes and Janetopoulos, 2003).

IV. Natural Killer Cells Express Seven Transmembrane Chemokine Receptors

This section will deal with the mechanisms of NK cell chemotaxis; however, a more detailed explanation for the extravasation of NK cells into sites of inflammation will be provided at the end of this article.

A. Chemokines

For NK cells to perform their activities, they must extravasate into various tissues either to destroy infected and transformed cells or to secrete regulatory cytokines, which aid T helper cells in polarization. In addition, NK cells recruit other cell types into the injured tissues by secreting chemokines. Chemokines are responsible for allergic disorders, autoimmune diseases, and ischemia associated with the infiltration of leukocytes. These mediators also play important roles in the inflammatory conditions associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. Chemokines are classified into four subfamilies: CXC (α chemokines), CC (β chemokines), C (γ chemokines), and CX₃C (δ chemokines). Chemokines are also divided into constitutive/homeostatic and inflammatory/inducible categories based on their physiology and receptor binding (Rodriguez-Frade et al., 2001; Maghazachi, 2003). In addition, six CXC, ten CC, one C, and one CX₃C chemokine receptors have been reported (Murphy et al., 2000). The major G protein attributed to be coupled to chemokine receptor is G α _{i1} because in most cases PTX inhibits the biological activities induced by chemokines. However, anti-G α _q also inhibits this response, suggesting that PTX-insensitive G proteins are involved in chemokine-induced chemotaxis and accumulation of intracellular calcium in NK cells (Maghazachi et al., 1997). In other studies, it was observed that G α _q but not G α _i mediates the chemotaxis of various cell types toward chemokines (Soede et al., 2000, 2001; Mellado et al., 2001).

B. Effects of Chemokines on Natural Killer Cell Chemotaxis

CXCL8 induces the chemokinesis of IL-2-activated NK cells (Sebok et al., 1993), whereas CXCL1, CXCL10, CXCL12, CCL1, CCL2, CCL4, CCL5, CCL6, CCL7,

CCL8, CCL11, CCL17, CCL19, CCL20, XCL1, and CX₃CL induce the chemotaxis of human NK cells (Al-lavena et al., 1994; Maghazachi et al., 1994, 1997; Taub et al., 1995; Bianchi et al., 1996; Loetscher et al., 1996; Godiska et al., 1997; Maghazachi, 1997; Al-Aoukaty et al., 1998; Inngjerdingen et al., 2000, 2001; Fraticelli et al., 2001). Importantly, human resting NK cells express receptors for the constitutive or constitutive/inflammatory chemokine receptors such as CXCR4, CCR4, CCR7, and CX₃CR1, with low expression of the inflammatory chemokine receptors CXCR3 and CCR1 (Inngjerdingen et al., 2001; Maghazachi, 2003).

C. Mechanisms Controlling Human Natural Killer Cell Chemotaxis

It was observed that chemokine receptors are promiscuously coupled to various subtypes of G proteins in NK cells (Al-Aoukaty et al., 1996). Such coupling is important since chemokines induce various activities in NK cells, which include chemotaxis, mobilization of intracellular calcium, and lysis of tumor cells (Taub et al., 1995; Loetscher et al., 1996; Maghazachi and Al-Aoukaty, 1998; Maghazachi, 2000) (Fig. 3). The important question of how chemokines induce the chemotaxis of NK cells is partially resolved. It turns out that the conditions governing NK cell chemotaxis are the same as in other cell types where PI3K γ and PH domain-containing proteins play significant roles. The PI3K inhibitor wortmannin as well as inhibitory antibodies to PI3K γ inhibit C, CC, and CXC chemokine-induced NK cell chemotaxis (Al-Aoukaty et al., 1999; Maghazachi, 2000). Stimulating NK cells with CXCL10, CXCL12, CCL2, CCL5, or XCL leads to the release of the G $\beta\gamma$ subunit, which associates with PH domain-containing molecules, and

this is followed by the recruitment of PI3K γ forming a ternary complex in the membranes of NK cells. Recruited PI3K γ subsequently generates PI(3,4,5)P₃, which binds other PH-domain containing proteins (Al-Aoukaty et al., 1999; Maghazachi, 2000). These findings demonstrate that PI3K γ is essential for chemokine-induced NK cell chemotaxis.

Surprisingly, it was observed that neutrophils in PI3K γ knockout animals can still migrate, albeit with low efficiency (Wymann et al., 2000), suggesting that other pathways may compensate for the lack of PI3K γ . Whether NK cells lack migratory behavior in PI3K γ knockout animals is not known. However, we observed that CXCL12 activates Lck, which is essential for NK cell chemotaxis (Inngjerdingen et al., 2002). The correlation between Lck and NK cell migration is still not resolved, but results from other groups showed that G α_s can activate Lck (Ma et al., 2000), suggesting that similar coupling may take place in NK cells. In addition, our unpublished observations indicate that PI3K γ associates with Lck in NK cells after stimulating these cells with CXCL12, as determined by immunoblot analysis. Once Lck is activated, it phosphorylates GEFs such as VAV, resulting in initiating the GTPases cascade and inducing NK cell polarization and chemotaxis. This scenario suggests that the product of PI3K γ , i.e., PI(3,4,5)P₃, may not be a limiting factor in these processes, because other molecules may compensate for its deficiency. Accordingly, there may be three pathways that could control NK cell chemotaxis. These are a) the release of the $\beta\gamma$ subunit that recruits and activates PI3K γ , which generates PI(3,4,5)P₃ that binds PH domains present in GEFs and results in the activation of small GTPases such as Cdc42 and Rac; b) the activation

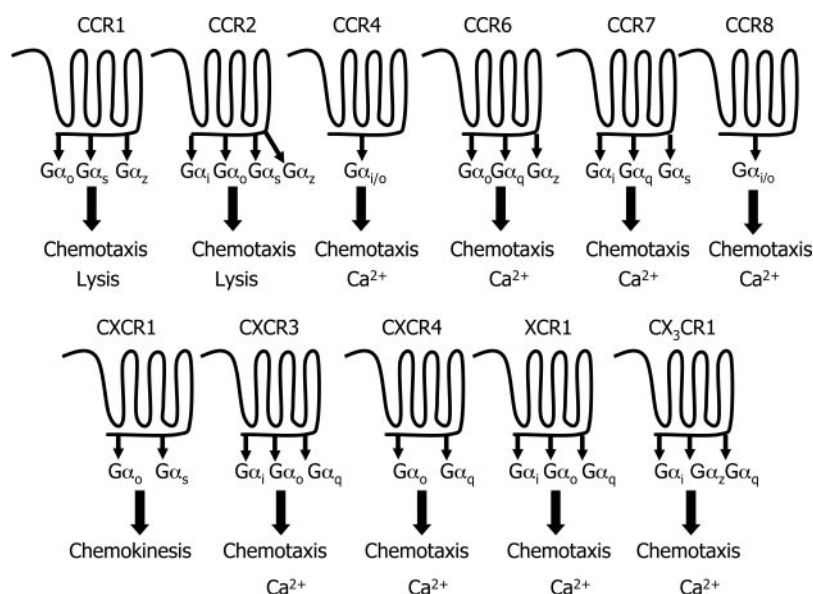


FIG. 3. Promiscuous coupling of chemokine receptors to multiple heterotrimeric G proteins in human IL-2-activated NK cells. Members of the CC, CXC, XC, and CX₃C chemokine receptors are coupled to several heterotrimeric G proteins, resulting in NK cells performing several activities, including chemotaxis, intracellular calcium mobilization, and tumor cell lysis.

of Lck by PI3K γ , which phosphorylates VAV and results in the activation of Cdc42 and Rac; and c) the possibility of G proteins other than G $_i$ (for example G $_s$) binding and activating Lck independently of PI3K γ , resulting in the activation of Cdc42 and Rac and consequently the cells being able to form filopodia and lamellipodia. To terminate pathway a, PTEN hydrolyzes PI(3,4,5)P $_3$ into PI(4,5)P $_2$. However, PTEN does not terminate pathways b and c induced by PI3K γ - or G $_s$ -Lck-VAV-Cdc42-Rac pathway (Fig. 4).

V. Natural Killer Cells Express Seven Transmembrane Lysophospholipid Receptors

A. Lysophospholipids

Members of the lysophospholipids (LPLs) are classified into several family members. The most extensively studied are lysophosphatidic acid (LPA), a glycerophospholipid that is characterized by having a glycerol backbone, and sphingosine 1-phosphate (S1P), a sphingophospholipid that is characterized by having a sphingoid backbone (Pyne and Pyne, 2000; Perry and Hanun, 2001; Mills and Moolenaar, 2003; Anliker and Chun, 2004). LPA is generated by the conversion of lysophosphatidylcholine by LPLD, also known as autotoxin (Mills and Moolenaar, 2003). In addition, phosphatidic acid that is generated by the phosphorylation of diacylglycerol by diacylglyceride kinase is hydrolyzed to LPA by phospholipase A $_2$ (PLA $_2$). S1P is generated by the conversion of sphingomyelin into ceramide by sphingomyelinase, ceramide into sphingosine by ceramidase, and sphingosine into S1P by sphingosine kinase (Fig. 5).

LPA and S1P are secreted by platelets and constitute a major part of serum and plasma (Yatomi et al., 1997a; Sano et al., 2002).

S1P induces endothelial cell migration (Lee et al., 1999; Kimura et al., 2000), human umbilical vein endothelial cell proliferation (Hisano et al., 1999), platelet activation (Yatomi et al., 1997b), calcium mobilization (Meyer zu Heringdorf et al., 1998), invasion of primitive hematopoietic cells into stromal cell layers of the bone marrow (Yanai et al., 2000), angiogenesis (Lee et al., 1999), phosphorylation of ERK2 in airway smooth muscle cells (Rakhit et al., 1999), and accumulation of integrins at the focal sites through Rho activation (Windh et al., 1999). S1P also inhibits adenylyl cyclase, ERK1 activation (Lee et al., 1996), tumor cell invasion (Sadahira et al., 1992), and apoptosis (Radeff-Huang et al., 2004). LPA is secreted by ovarian cancer cells (Fang et al., 2002; Tanyi et al., 2003), prostate cancer cells (Xie et al., 2002), multiple myeloma (Sasagawa et al., 1999), and many other cancer cell types. In fact, it was suggested that LPA is a marker for ovarian cancer patients since it is highly increased in the serum and ascitic fluids of women with this disease (Xu et al., 1998), with the level in these patients capable of reaching up to 500 μ M. Furthermore, LPA induces the release of angiogenic factors such as vascular endothelial growth factor (Hu et al., 2001) and is involved in neovascularization and tumor growth and survival. Similar to S1P, LPA is a pleiotropic lipid that induces focal adhesion assembly (Sawada et al., 2002), activates the TIAM/Rac pathway (Van Leeuwen et al., 2003), induces RhoA activation through G $\alpha_{12/13}$ in neuronal cells (Kranenburg et al.,

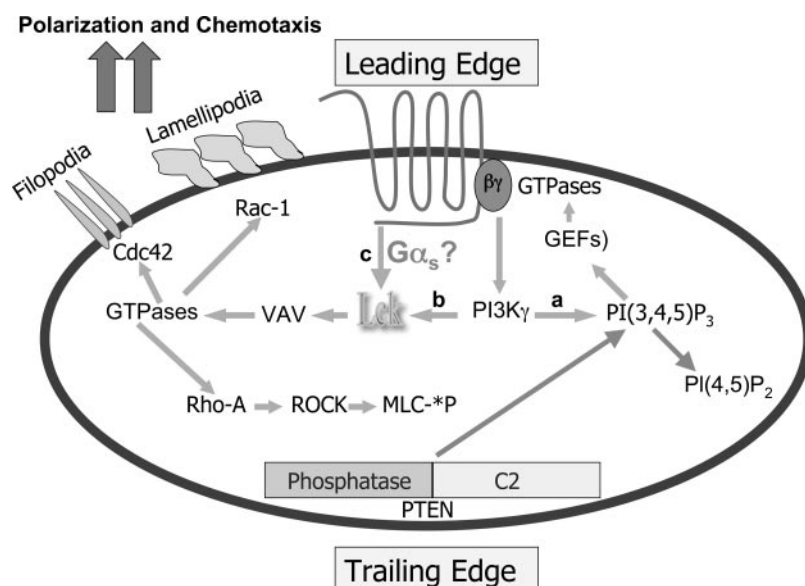


FIG. 4. Spatial distribution of molecules governing NK cell chemotaxis. In this model, molecules responsible for the polarization of NK cells accumulate at the leading edge of motile NK cells. Three different pathways can result in the activation of Rac/lamellipodia, Cdc42/filopodia, and RhoA/cell contraction formation in NK cells. At the rear end of the cell, PTEN, which is composed of a phosphatase and C2 domain, hydrolyzes the PI3K product, i.e., PI(3,4,5)P $_3$ into PI(4,5)P $_2$, and consequently terminates pathway a (see text for explanation). Lck is placed in the center since activation of this kinase may lead to NK cell polarization and chemotaxis either dependently or independently of PI3K γ .

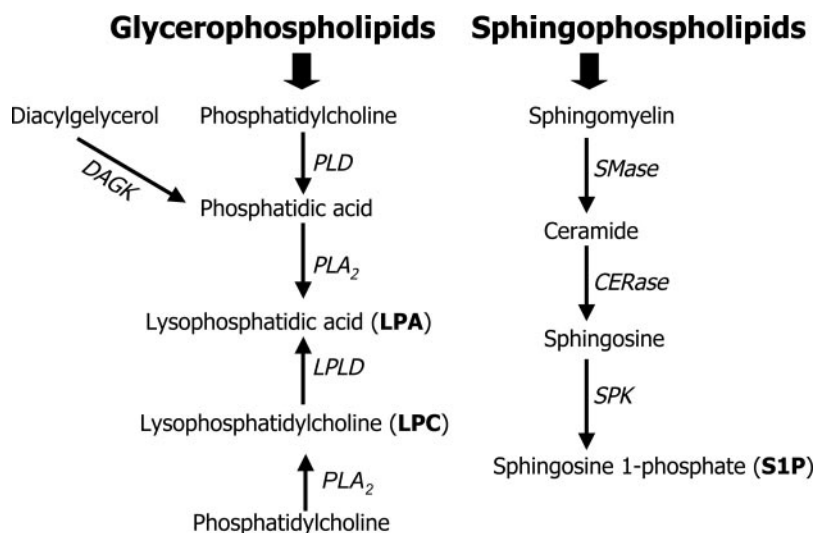


FIG. 5. Generation of LPLs. These are classified into glycerophospholipids and sphingophospholipids. The enzymes responsible for the generation of these lipids are shown in italics. CERase, ceramidase; DAGK, diacylglyceride kinase; PLA₂, phospholipase A₂; PLD, phospholipase D; SMase, sphingomyelinase; SPK, sphingosine kinase. Arrows show the sequence of generating a particular lipid. Bold characters show the lipids described in this paper.

1999), induces the expression of a cyclooxygenase gene through the activation of Cdc42 and Rac (Hahn et al., 2002), induces the mobilization of $[Ca^{2+}]_i$ in monocytic cell lines (Lee et al., 2004), regulates cell spreading through the activation of Rac and Cdc42 (Ueda et al., 2001), and inhibits apoptosis (Radeff-Huang et al., 2004).

The receptors for lysophospholipids belong to the 7TM family of receptors. Those that bind S1P are known as S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅, whereas those that bind LPA are known as LPA₁, LPA₂, LPA₃, and LPA₄ (Chun et al., 2002). Similar to all other GPCRs, receptors for S1P have the conserved cysteine (C) residue, which is important for palmitoylation (Sanchez and Hla, 2004). However, they have glutamic acid (E) residue instead of the aspartic acid (D) residue found in other GPCRs. Several techniques have been used to delineate the nature of G proteins coupled to S1P receptors. These include coimmunoprecipitating S1P receptors with G proteins in transfected cells (Lee et al., 1996), GTP γ S binding in Sf9 or EK293 cells transfected with S1P receptors and G proteins (Windh et al., 1999), or transfecting cells lacking endogenous S1P receptors with these receptors and with chimeric G proteins (Ancellin and Hla, 1999), among many other techniques. The results of these studies demonstrate that S1P₁ is coupled to the PTX-sensitive $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, and G_{α_o} but not to other G proteins (Pyne and Pyne, 2000; Radeff-Huang et al., 2004). S1P₂ and S1P₃ are coupled to G_{α_i} as well as to the PTX-insensitive $G_{\alpha_{q11}}$, $G_{\alpha_{i2}}$, and $G_{\alpha_{i3}}$ (Windh et al., 1999; Siehler and Manning, 2002); S1P₄ is coupled to $G_{\alpha_{i/o}}$ (Van Brocklyn et al., 2000) or to G_{α_q} (Pyne and Pyne, 2000); and S1P₅ is coupled to $G_{\alpha_{i/o}}$ and $G_{\alpha_{i2}}$ but not to G_{α_s} or $G_{\alpha_{q11}}$ (Malek et al., 2001).

B. Sphingosine 1-Phosphate Induces Human Natural Killer Cell Chemotaxis through G Proteins and Phosphoinositide 3 Kinase

Human IL-2-activated NK cells express mRNA for S1P₁, S1P₄, and S1P₅, the receptors for S1P. Other potential receptors for S1P such as GPR3, GPR6, and GPR12 (Uhlenbrock et al., 2002; Ignatov et al., 2003) were not examined in these cells. We observed that $G_{\alpha_{i2}}$, G_{α_o} , $G_{\alpha_{i3}}$, G_{α_s} , and $G_{\alpha_{q11}}$ mediate NK cell chemotaxis induced by S1P (Kveberg et al., 2002). The effect of $G_{\alpha_{i3}}$ confirms the results of Windh et al. (1999), who observed that $G_{\alpha_{i3}}$ mediates the activity of S1P in Sf9 or human embryonic kidney 293 cells. However, the coupling of S1P receptors to G_{α_s} is not yet resolved, although in transfected cells it was observed that S1P induces the synthesis of cAMP (Kon et al., 1999). Our results suggest that G_{α_s} may be directly coupled to S1P receptors in NK cells or that there may be a switch between G_{α_i} and G_{α_s} after stimulating NK cells with S1P, similar to the switch occurring among G_{α_i} and G_{α_s} upon perturbation of the β_2 -adrenergic receptor (Daaka et al., 1997). In addition, antibodies to the PTX-sensitive G_{α_i} and PTX-insensitive $G_{\alpha_{q11}}$ inhibit S1P-induced NK cell chemotaxis, reinforcing the switch hypothesis between various G proteins upon activation of S1P receptors in NK cells. A switch among G_{α_s} and $G_{\alpha_{q11}}$ has been reported upon signaling by the 7TM prostacyclin receptor (Lawler et al., 2001). Alternatively, a cross-talk among members of the G protein subunits may take place in NK cells treated with lysosphingolipids, similar to the cross-talk observed between $G_{\alpha_{q11}}$ and G_{α_i} in platelets (Gratacap et al., 2001).

The PI3K inhibitors wortmannin and LY294002 inhibit S1P-induced ERK activation (Rakhit et al., 1999),

or S1P-induced endothelial cell migration (Morales-Ruiz et al., 2001). Similarly, we observed that wortmannin and LY294002 as well as blocking antibody to PI3K γ inhibit S1P-induced NK cell chemotaxis (Kveberg et al., 2002), whereas anti-PI3K β was without effect. In contrast, S1P recruits PI3K β isozyme into NK cell membranes, suggesting that although this isoform is not involved in chemotaxis, it is activated by S1P. Hence, it can be concluded that PI3K β is not involved in mediating cellular chemotaxis but instead may induce other functions in the cells, one of them possibly being the generation of nitric oxide (Igarashi and Michel, 2001).

What are the mechanisms of S1P-induced NK cell chemotaxis? It is plausible that phosphorylation and activation of Akt may take place after the addition of S1P and that Akt may phosphorylate S1P $_1$, leading to the activation of the chemotaxis response. This is based on the findings of Lee et al. (2001), who observed that Akt phosphorylates the Thr²³⁶ of the 3TM loop of S1P $_1$, which results in Rac activation. In this scenario, stimulating NK cells with S1P leads to the activation of PI3K γ , which recruits Akt, resulting in the activation of Rac/chemotaxis pathway. In addition, PI3K γ can lead to NK cell chemotaxis by activating GEFs/GTPases, similar to the effect of this kinase in chemokine-induced NK cell chemotaxis (see above). On the other hand, the activation of PI3K β may lead to the generation of exogenous nitric-oxide synthase (Fig. 6), although this pathway has not yet been examined in NK cells.

C. Lysophosphatidic Acid Induces Human Natural Killer Cell Chemotaxis through G Proteins

LPA $_1$, one of the receptors of LPA, signals through G $\alpha_{i/o}$ in addition to PTX-insensitive G proteins (Fukushima et al., 1998), whereas the mobilization of calcium induced by LPA upon ligating LPA $_2$ and LPA $_3$ in insect cells is insensitive to PTX (Bandoh et al., 1999). How-

ever, calcium mobilization induced by LPA in LPA $_1$ -transfected cells is maintained through PTX-sensitive G α_i only, whereas the same response is mediated by PTX-sensitive G α_i and PTX-insensitive G α_q upon ligating LPA $_2$ by LPA (An et al., 1998). In addition, G α_i mediates LPA-induced Rac1 activation (Van Leeuwen et al., 2003). LPA $_2$ activates Rho and induces cytoskeleton rearrangement through G $\alpha_{12/13}$, whereas LPA $_3$ activates PLC through G α_q (Ye et al., 2002). Coupling of LPA $_3$ to G α_q and G $\alpha_{12/13}$ has been noted in other systems (Anliker and Chun, 2004). In Swiss 3T3 cells, G α_{13} and not G α_{12} mediates LPA-induced Rho activation (Gohla et al., 1998). It can be concluded that LPA activates Rac, which is involved in cell adhesion and spreading, and RhoA, which is involved in cell contraction, leading to cell migration and chemotaxis. In polarized T helper 1 and 2 cells, LPA induces [Ca²⁺]_i through activation of PTX-insensitive G proteins, whereas differential effects were observed when chemotaxis is measured in these cells. LPA induces T helper 2 cell chemotaxis through PTX-sensitive G proteins, but similar activity was insensitive to PTX in T helper 1 cells (Wang et al., 2004).

Activated human NK cells express LPA $_1$ and LPA $_2$, the receptors for LPA, and these cells are chemotaxed toward LPA (Jin et al., 2003). This activity is inhibited by prior treatment of the cells with PTX, suggesting that heterotrimeric G $\alpha_{i/o}$ protein is involved in the chemotactic response. Furthermore, LPA induces the mobilization of intracellular calcium in NK cells, an effect that is partially inhibited by PTX, suggesting that [Ca²⁺]_i is mediated by both PTX-sensitive and -insensitive G proteins. These findings determined that LPA exerts distinct effects in various cell types, and that in activated NK cells LPA $_1$ is involved in both chemotaxis and calcium mobilization, whereas LPA $_2$ is involved in calcium mobilization only (Fig. 7). Of note, resting NK cells do

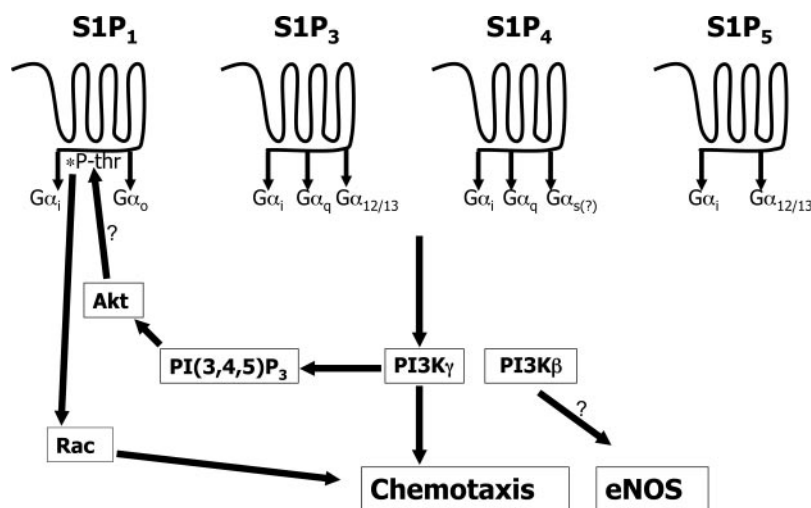


FIG. 6. Coupling of receptors for S1P to multiple heterotrimeric G proteins in human IL-2-activated NK cells. S1P activates the PI3K γ pathway in these cells, resulting in the generation of PI(3,4,5)P $_3$, which may activate Akt, resulting in the generation of Rac and the induction of chemotaxis, although this has not yet been proven in NK cells. In addition, by binding GEFs, PI(3,4,5)P $_3$ may activate the GTPase Rac. Question marks suggest hypothetical pathways that have not yet been examined in NK cells.

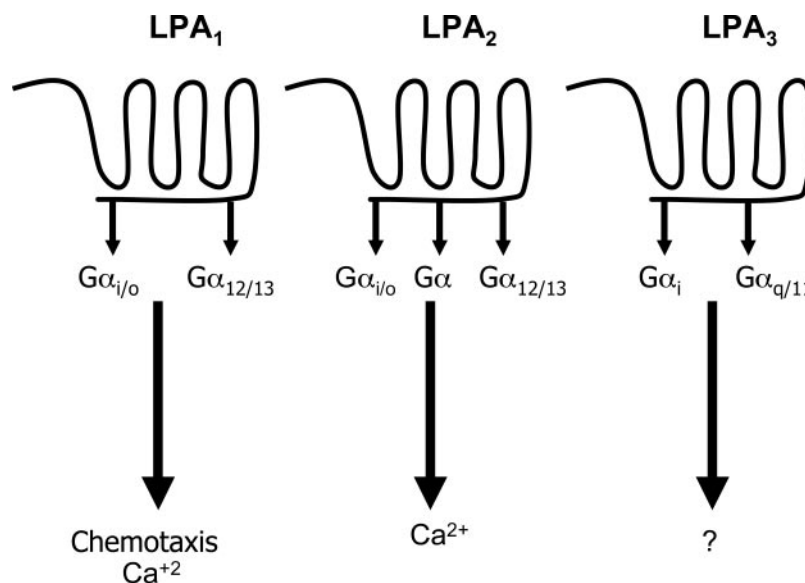


FIG. 7. Coupling of receptors for LPA (LPA₁ and LPA₂) to multiple heterotrimeric G proteins in NK cells. Similar to S1P, the effect of LPA results in the induction of chemotaxis and intracellular calcium mobilization in IL-2-activated NK cells. The expression, coupling, and signaling of LPA₃ are not yet clear.

not express LPA₃, but IL-2-activated NK cells may express this receptor (A. A. Maghazachi, unpublished observations). Therefore, the expression of this receptor and its signaling pathways are not yet resolved in NK cells. In addition, the expression and signaling pathways of the recently described LPA₄ (Noguchi et al., 2003) has not yet been examined in NK cells.

D. Effect of Lysophosphatidylcholine on Human Natural Killer Cell Chemotaxis

Lysophosphatidylcholine (LPC) is a phosphorylcholine-containing lipid generated by the conversion of phosphatidylcholine into LPC by PLA₂ (Fig. 5). LPC is increased in the serum of ovarian cancer patients (Okita et al., 1997) and is highly important for the induction of atherosclerosis because it binds oxidized low-density lipoprotein at the artery wall (Jing et al., 2000; Lusis 2000). This lipid causes inflammation during atherosclerosis (Gupta et al., 1997), induces T cell proliferation in the presence of diacylglyceride and calcium ionophore (Asaoka et al., 1992), and enhances the chemotaxis of human T cells (McMurray et al., 1993). Surprisingly, LPC induces cell arrest in a PTX-insensitive pathway, but it enhances cell growth in a PTX-sensitive pathway (Xu, 2002). We recently observed that LPC enhances the chemotaxis of resting NK cells; however, this effect is diminished in activated NK cells, and both PTX-sensitive and insensitive pathways regulate this activity of LPC (Jin et al., 2005).

VI. Single Transmembrane-Spanning Domain Receptors in Natural Killer Cells

A. Overview

To lyse their target cells, NK cells must express receptors that recognize these target cells. In late 1980s

and early 1990s, a plethora of NK receptors were discovered. Surprisingly, these were mostly inhibitory receptors; notwithstanding, it was clear that NK cells destroy tumor cells or virally infected cells (Ortaldo and Longo, 1988). The reason for this is because investigators searched for inhibitory receptors to prove the validity of the “missing self-hypothesis” (Ljunggren and Karre, 1990), which forms the foundation for discovering NK cell receptors. In subsequent studies, many activating receptors in NK cells were discovered. This article will deal with receptors expressed in human NK cells only, although excellent work has been done in mice to delineate the receptor expression and their signaling pathways. As indicated above, NK cell receptors can be either inhibitory or activating. All these receptors belong to 1TM-spanning domain receptors, with the inhibitory receptors existing as monomers and transductive inhibitory signals, whereas most activating receptors lack signaling abilities but associate with adaptor molecules that have signaling motifs.

B. Inhibitory Natural Killer Cell Receptors

Each NK cell expresses one or two inhibitory receptors, ensuring that under physiological conditions NK cell is inhibited upon ligating self-MHC molecules, which guards against autoimmunity. These receptors either have immunoglobulin (Ig) or C-type lectin-like extracellular domains. The Ig domain receptors bind group 1 or group 2 HLA-C, HLA-B, or HLA-A gene products (Moretta et al., 1993, 1994; Litwin et al., 1994; Colonna and Samaridis, 1995; Wagtmann et al., 1995; Dohring et al., 1996; Pende et al., 1996; Vitale et al., 1996). In general, the receptors that recognize HLA-C contain two Ig extracellular domains (2D) and have long (L) cytoplasmic tails. KIR2DL1 recognizes group 2

HLA-C, which possesses asparagine (N) at position 77 and lysine (K) at position 80 of the α helix of the MHC molecule. These include HLA-Cw2, HLA-Cw4, HLA-Cw5, and HLA-Cw6. On the other hand, KIR2DL2 recognizes group 1 HLA-C, which possesses serine (S) at position 77 and asparagine (N) at position 80 of the α helix of MHC. The latter group includes HLA-Cw1, HLA-Cw3, HLA-Cw7, and HLA-Cw8. KIR2DL3 shares the same recognition strategy as KIR2DL2. KIR3DL1 recognizes gene products of HLA-Bw4, whereas KIR3DL2 recognizes gene products of HLA-A3 and HLA-A11 (Bottino et al., 2004). The C-type lectin-like domain-containing inhibitory receptors include the heterodimer of CD94/NKG2A, which recognizes gene products of HLA-E (Borrego et al., 1998; Lee et al., 1998). HLA-E has a limited polymorphism since it binds peptides of the leader sequence of most HLA class I molecules and consequently is transported to the cell membrane. Therefore, HLA-E is ubiquitously expressed in most tissues. All inhibitory receptors have a common motif in their cytoplasmic tail composed of V/IxYxxL/V (x is any amino acid), also known as immune receptor tyrosine-based inhibitory motif (ITIM) (Daeron and Vivier, 1999; Colucci et al., 2002). Upon triggering these receptors with MHC molecules, ITIM is phosphorylated, resulting in the recruitment of phosphatases known as SH-containing tyrosine phosphatase 1 and 2 (SHP-1 and SHP-2). These phosphatases inhibit the function of the activating receptors by dephosphorylating molecules such as VAV (Long, 1999; Stebbins et al., 2003). Figure 8 shows the inhibitory receptors and their intracellular signaling pathways.

C. ITAM-Based Activating Receptors

ITAM-based activating receptors are characterized by their lack of long cytoplasmic tails; to be effective, they associate with other molecules that can transduce their

signals. An adapter molecule known as DNAX adaptor protein (DAP)-12, which has a negatively charged aspartic acid (D) residue, binds positively charged residues present in these activating receptors (Lanier et al., 1998). DAP-12 has an activating motif composed of YxxL-x6-8-YxxL, known as immune receptor tyrosine-based activating motif (ITAM). Upon activation, kinases such as Lck and Fyn phosphorylate ITAM, resulting in the recruitment of spleen tyrosine kinase (SYK) or ξ -associated protein (ZAP)-70. SYK and ZAP-70 kinases initiate the activation process by recruiting and scaffolding many activating molecules such as the Shc-Grb2-Sos-Ras-Raf-MEK-ERK pathway. In addition, the SH2 domain-containing leukocyte phosphoprotein-76 (SLP-76) and the 36-kDa linker for activation of T cells (LAT) are substrates for ZAP (Clements et al., 1998; Zhang et al., 1998). Upon phosphorylation, LAT and SLP-76 recruit SH-2 domain-containing proteins such as PLC γ or Grb2. Consequently, complexes composed of SLP-76-PLC γ -DAG (inositol trisphosphate) or the LAT/(SLP-76)-VAV-Rac-Pak-MEK-ERK pathway is generated upon stimulating NK cells (Fig. 9). In addition, PI3K activates the VAV-Rac-Pak-MEK-ERK pathway in human NK cells, leading to NK cell lysis of tumor target cells (Jiang et al., 2000).

Receptors associated with DAP-12 include those that have extracellular Ig domains such as KIR2DS1, which recognizes group 2 HLA-C molecules, and KIR2DS2, which recognizes group 1 HLA-C molecules (Moretta et al., 2001; Colucci et al., 2003; Farag et al., 2003; Bottino et al., 2004; Zompi and Colucci, 2005). Another member of the activating receptors known as CD94/NKG2C/E that also recognizes MHC gene product but has a C-type lectin-like molecule instead of having Ig extracellular domains has been described. This receptor recognizes HLA-E, associates with DAP-12, and transmits its activity similar to KIR2DS1/KIR2DS2 (Fig. 9).

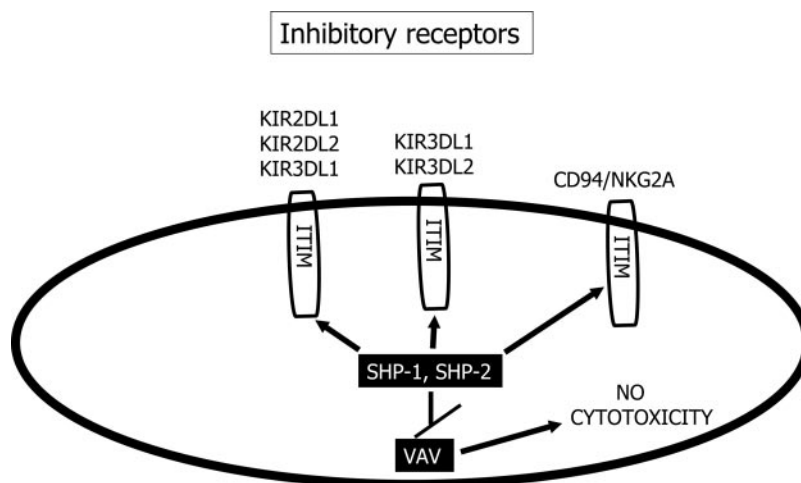


FIG. 8. Inhibitory NK cell receptors. These receptors contain either Ig domains (KIR2DL1, KIR2DL2, KIR3DL1, and KIR3DL2) or C-type lectin (CD94/NKG2A) in their extracellular domains. All these receptors bind MHC class I molecules. Similarly, they all contain ITIM in their cytoplasmic domain. Upon activation, ITIMs recruit the phosphatases SHP-1 and SHP-2, which dephosphorylate VAV, resulting in inhibiting the activation responses.

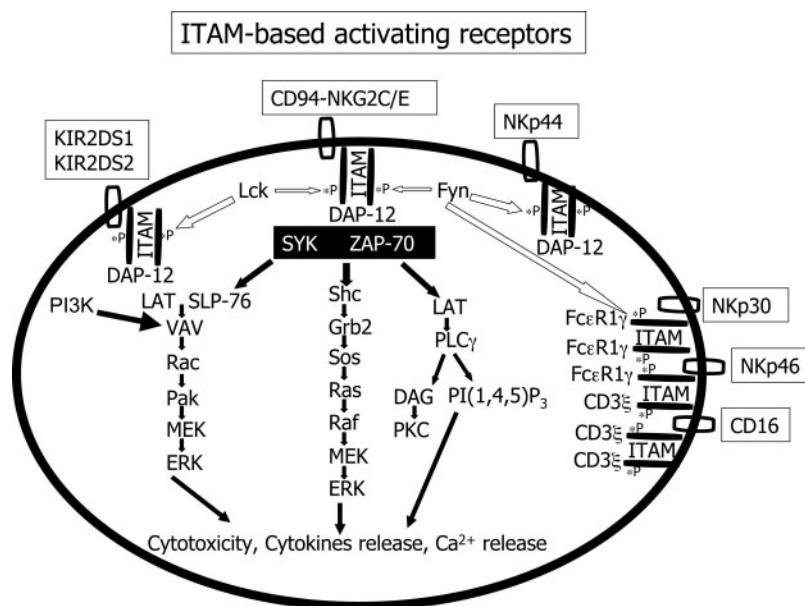


FIG. 9. Signaling pathways induced by activating receptors that associate with adaptor molecules containing the ITAM. These receptors are characterized by having short cytoplasmic domains that associate with adaptor molecules containing ITAM. After stimulating NK cells, ITAM is phosphorylated by Lck and Fyn kinases, leading to the recruitment of SYK and ZAP-70, resulting in activating various signaling cascades. The ITAM present in molecules associated with the receptors shown on the right side (NKp30, NKp46, and CD16) is phosphorylated by Fyn and not Lck kinase. Adaptor molecules, whether DAP-12, Fc ϵ R1 γ , or CD3 ξ , associate with SYK and ZAP-70, leading to the activation of downstream activating pathways, which results in NK cell cytotoxicity, cytokine release, and calcium mobilization. White arrows show the phosphorylation of ITAM by Lck/Fyn; black arrows demonstrate the signaling molecules downstream of SYK/ZAP-70.

Activating receptors that are different from the above in the sense that they do not recognize MHC gene products have been characterized and are collectively known as natural cytotoxicity (NC) receptors. These include NKp44, which is up-regulated upon activating NK cells with IL-2 or IL-15 (Vitale et al., 1998). This receptor binds the DAP-12 molecule and signals similarly to other activating receptors that associate with DAP-12. Two other receptors in this family, NKp30 and NKp46, are ubiquitously expressed on all NK cells (Pende et al., 1999; Pessino et al., 1998). Although the ligands for NKp30, NKp44, and NKp46 have not yet been reported, NKp30 and NKp46 have a positively charged lysine (K) residue in their cytoplasmic short tails that noncovalently associates with ITAM-based adaptor molecules through the aspartic (D) residue found in these adaptor molecules. Similar to DAP-12, these adaptor molecules contain the ITAM. These adaptors are composed of either a homodimer of Fc ϵ R1 γ -Fc ϵ R1 γ , a homodimer of CD3 ξ -CD3 ξ , or a heterodimer of Fc ϵ R1 γ -CD3 ξ . Upon phosphorylation by Fyn kinase, these ITAM-based motifs recruit SYK or ZAP-70, resulting in the activation of various intracellular stimulatory molecules similar to those transmitted by DAP-12 (Fig. 9).

Receptors that are also associated with Fc ϵ R1 γ or CD3 ξ adaptors include Fc γ RIII (known as CD16), which is expressed on most resting human blood NK cells (Lanier et al., 1989). CD16 binds immune complexes and mediates antibody-dependent cell-mediated cytotoxicity. This receptor activates various intracellular signaling molecules, which, similar to other ITAM-based adap-

tors, results in the recruitment of ZAP-70 and SYK and leads to the activation of the ERK, inositol 1,4,5 trisphosphate, and protein kinase C pathways (Ting et al., 1995). The phosphorylation of VAV due to the engagement of the CD16 molecule has been previously reported, and abrogation of VAV expression (Billadeau et al., 1998), or inhibition of the GTPase Rac (Galandrini et al., 1996) leads to inhibition of NK cell mediated killing, or to reduced polarization and binding to target cells. These findings demonstrate crucial effects for GEFs/GTPases in NK cell functions. In addition activation of human NK cells by ligating the CD16 molecules results in the phosphorylation of Shc, and its association with Grb2, leading to the eventual activation of Ras and the MEK/ERK pathway (Fig. 9).

D. Non-ITAM-Based Activating Receptors

There are several different receptors that belong to this category. The most extensively studied include NKG2D, LFA-1, and 2B4 (CD244).

1. *NKG2D*. NKG2D is a C-type lectin stimulatory receptor that recognizes cells that express molecules due to stress, including tumor cells. In humans, these molecules are known as UL-16-binding proteins (ULBP-1, ULBP-2, and ULBP-3) and MHC class I chain-related A/B antigens. Only one form of NKG2D (NKG2D-long) has been described in humans (Moretta et al., 2001; Colucci et al., 2002; Farag et al., 2003; Raulet, 2003), where its cytoplasmic domain through the positively charged arginine (R) residue associates with the negatively charged aspartic acid (D) residue found in the

adaptor molecule known as DAP-10 (Wu et al., 1999). DAP-10 has a YxxM (YNIM) motif that binds PI3K through the SH2 domain of the regulatory subunit (p85) upon phosphorylation. DAP-10 also binds Grb2 through its SH2 domain. Grb2 activates the Sos-Ras-Raf-MEK-ERK pathway, whereas PI3K generates PI(3,4,5)P₃, which activates the VAV-Rac-Pak-MEK-ERK pathway (Fig. 10).

2. *LFA-1*. LFA-1, a member of $\alpha_L\beta_2$ integrin molecules that bind intracellular cell adhesion molecule-1 on the target cells, is important for adhesion of NK cells to these target cells. It has been shown that the perturbation of LFA-1 results in the activation of the VAV-ERK pathway, resulting in natural cytotoxicity. Furthermore, the proline-rich tyrosine kinase 2 is activated upon stimulating β_2 integrins, resulting in the activation of ERK pathway and NK cell-mediated cytotoxicity (Gismondi et al., 2003).

3. *2B4 (CD244)*. 2B4 (CD244), which binds CD48 and contains the TxYxxV/I motif [immunoreceptor tyrosine-based switch motif (ITSM)], has been described as an activating receptor (Nakajima et al., 1999; Chuang et al., 2001). The ITSM recruits the signaling lymphocyte activation molecule-associated protein. Triggering 2B4 results in the phosphorylation of LAT and the recruitment of PLC γ and Grb2, which activate various intracellular activating molecules (Fig. 10). Of note, a mutation in the signaling lymphocyte activation molecule-associated protein results in the recruitment of SHP rather than activating molecules. This pathway inhibits NK cell lysis of Epstein Barr virus-infected cells, resulting in the development of X-linked lymphoproliferative disease.

The final outcome of the activation process, whether through ITAM-based or non-ITAM-based activating receptors, results in the enhancement of NK cell cytotoxicity, both natural and antibody-dependent cell-mediated cytotoxicity, cytokine secretion, and in most cases, mobilization of [Ca²⁺]_i. However, distinction among cytotoxicity and IFN- γ secretion has been noted in few circumstances. For example, KIR2DL4, which recognizes the HLA-G molecule, stimulates the production of IFN- γ but inhibits NK cell cytotoxicity (Rajagopalan et al., 2001).

VII. Sojourn of Human Natural Killer Cells into Inflammatory Sites, and Their Development

A. Introduction

Two lingering issues regarding human NK cells have not yet been resolved: the first is their migration into diseased tissues; the second is their development. With T cells, there are differences in the expression of chemokine receptors. For example, T central memory cells express CCR7 and L-selectin and migrate into the lymph nodes, whereas the other cell types termed T effector memory cells lack CCR7 but perform most of the effector functions (Sallusto et al., 1999). Recent work demonstrates that T cells entering the blood circulation from the thymus are facilitated by S1P₁; antagonizing this receptor by the immunosuppressive drug FTY720 results in the inability of mature single-positive T cells to egress into blood (Matloubian et al., 2004). Similarly, S1P₁-deficient T cells entering secondary lymphoid tissues were unable to egress into other tissues, including inflammatory sites (Brinkmann et al., 2004). These re-

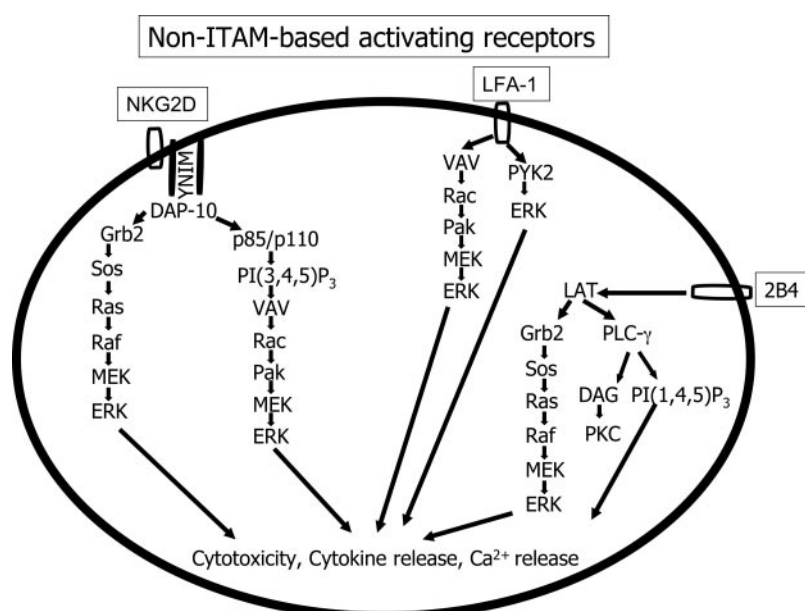


FIG. 10. Signaling pathways induced by activating receptors associated with adaptors or other molecules not having ITAM. NKG2D is associated with the adaptor molecule containing YNIM known as DAP-10, which activates the p85/PI3K pathway or the Grb2 pathway. See text for a detailed explanation of the other receptors.

sults suggest that S1P₁ is important in maintaining T cells in the blood circulation and that circulating T cells must down-regulate this receptor to migrate into secondary lymphoid tissues. In fact, T cells down-regulate S1P₁ early after immunization and are consequently sequestered in the lymph nodes, but 3 days later they acquire S1P₁ and consequently return to the bloodstream (Brinkmann et al., 2004; Matloubian et al., 2004).

B. Natural Killer Cell Extravasation into Inflamed Sites

Resting human NK cells express receptors for the homeostatic chemokine receptors CCR7 and CXCR4, as well as for the homeostatic/inflammatory chemokine receptors CCR4 and CX₃CR1. For NK cells to migrate into the sites of inflammation, they must up-regulate the inflammatory chemokine receptors, a process that occurs at the surface of the endothelium and is aided by the combined activation of NK cells with inflammatory cytokines such as IL-2 and by perturbation of adhesion molecules (Maghazachi, 2003, 2005). Although human IL-2-activated NK cells express S1P₁ and S1P₄ by reverse transcription-polymerase chain reaction analysis, flow cytometric analysis showed that 80% resting CD16^{high}, 40% resting CD16^{low}, and only 10% IL-2-activated NK cells express S1P₁. In contrast, resting CD16^{high} and CD16^{low} have no protein expression of S1P₄, whereas more than 40% of IL-2-activated NK cells express this receptor (A. A. Maghazachi, unpublished observations). Similarly, resting CD16⁻, resting CD16⁺, and IL-2-activated NK cells variably express LPA₂, whereas LPA₁ is only expressed on activated NK cells (A. A. Maghazachi, unpublished results). In addition, G2A is expressed on more resting NK cells than activated NK cells (Jin et al., 2005), but the ligand for this receptor is still controversial (Witte et al., 2005). To summarize, resting NK cells express CCR7, CXCR4, CCR4, CX₃CR1, S1P₁, LPA₂, and G2A. Because chemokines are not found freely in the circulation but are instead immobilized on the surface of the endothelial cells, it can be extrapolated that chemokines do not play a major role in maintaining NK cells in the circulation. Instead, S1P, LPA, and LPC, which are found physiologically at 1, 20, and 200 μM, respectively, play a prominent role in sustaining these cells in the bloodstream. On the other hand, activated NK cells that extravasate into inflamed sites acquire different phenotypic expression. IL-2-activated NK cells up-regulate the expression of inflammatory chemokine receptors, receptors for lysophospholipids such as S1P₄ and LPA₁, as well as other receptors not discussed here, such as OGR1, the receptor for sphingosylphosphorylcholine (SPC), and PAFR, the receptor for platelet-activating factor (Jin et al., 2005). At the same time, activated NK cells down-regulate the expression of S1P₁, suggesting that S1P₁ plays an im-

portant role in maintaining resting NK cells in the circulation. The down-regulation of S1P₁ allows activated NK cells to extravasate into the sites of inflammation. Furthermore, these cells have the machinery that aids them in this process. For example, they express the inflammatory chemokine receptors CCR1, CCR2, CCR8, CXCR1, CXCR3, as well as OGR1, PAFR, LPA₁, and S1P₄. The model described in Fig. 11 explains why only activated and not resting NK cells are found at the sites of inflammation (Dalbeth and Callan, 2002; Maghazachi, 2003, 2005; Dalbeth et al., 2004).

C. Human Natural Killer Cell Development

Although the development of mouse NK cells is clear (Yokoyama et al., 2004), the same can not be said about human NK cells. As indicated above, peripheral blood human NK cells are divided into CD16^{high}CD56^{dim} cells, which also express CD94/NKG2⁺, KIR⁺, NC⁺, perforin⁺, and CD16^{low}CD56^{bright} cells that are CD94/NKG2⁺, KIR⁻ (or low), NC^{low}, and perforin⁻ (Moretta, 2002; Della Chiesa et al., 2003; Farag et al., 2003), whereas activated NK cells express different phenotypes than resting NK cells (Maghazachi, 2005). To explain the presence of these different subsets of NK cells, two models were put forward. The first indicates that there are two different subsets of resting NK cells (Colucci et al., 2003; Ferlazzo and Munz, 2004); the second indicates that development and activation of the same NK cell subset is the predominant feature rather than NK cells being generated from two unrelated cell subsets (Perussia et al., 2005). In the latter model, immature NK cells secrete type 2 cytokines (IL-5 and IL-13) and express the CD161 but not CD56 phenotype. Upon activation with IL-12, these cells develop into intermediary cells that secrete both type 1 (IFN-γ) and type 2 (IL-13) cytokines. Upon further activation, these cells become mature NK cells that secrete only type 1 (IFN-γ) cytokine and acquire the CD16^{low}CD56^{bright} phenotype (Loza and Perussia, 2001, 2004).

Our findings support the model of development and activation from a single progenitor rather than from two different subsets of NK cells. Our results indicate that resting CD16^{high}CD56^{dim} NK cells show high expression of S1P₁ and G2A and that, upon activation with IL-2, these cells develop into activated NK cells (CD16^{low}CD56^{bright}) that have high expression of the inflammatory chemokine receptors and lysophospholipid receptors. Upon cessation of the inflammatory reactions, some of these cells that may have the ability to survive may return to the circulation, forming 10% of the semiresting NK cells found in the bloodstream (Fig. 11). These semiresting cells express CD16^{low}CD56^{bright} and have intermediate expression of receptors for LPC, SPC, LPA, and S1P compared with resting CD16^{high}CD56^{dim} and activated NK cells (Maghazachi, 2003, 2005; Jin et al., 2005). Hence, activation and maturation at the surface of the inflamed endothelium and inside inflammatory sites facilitate the gen-

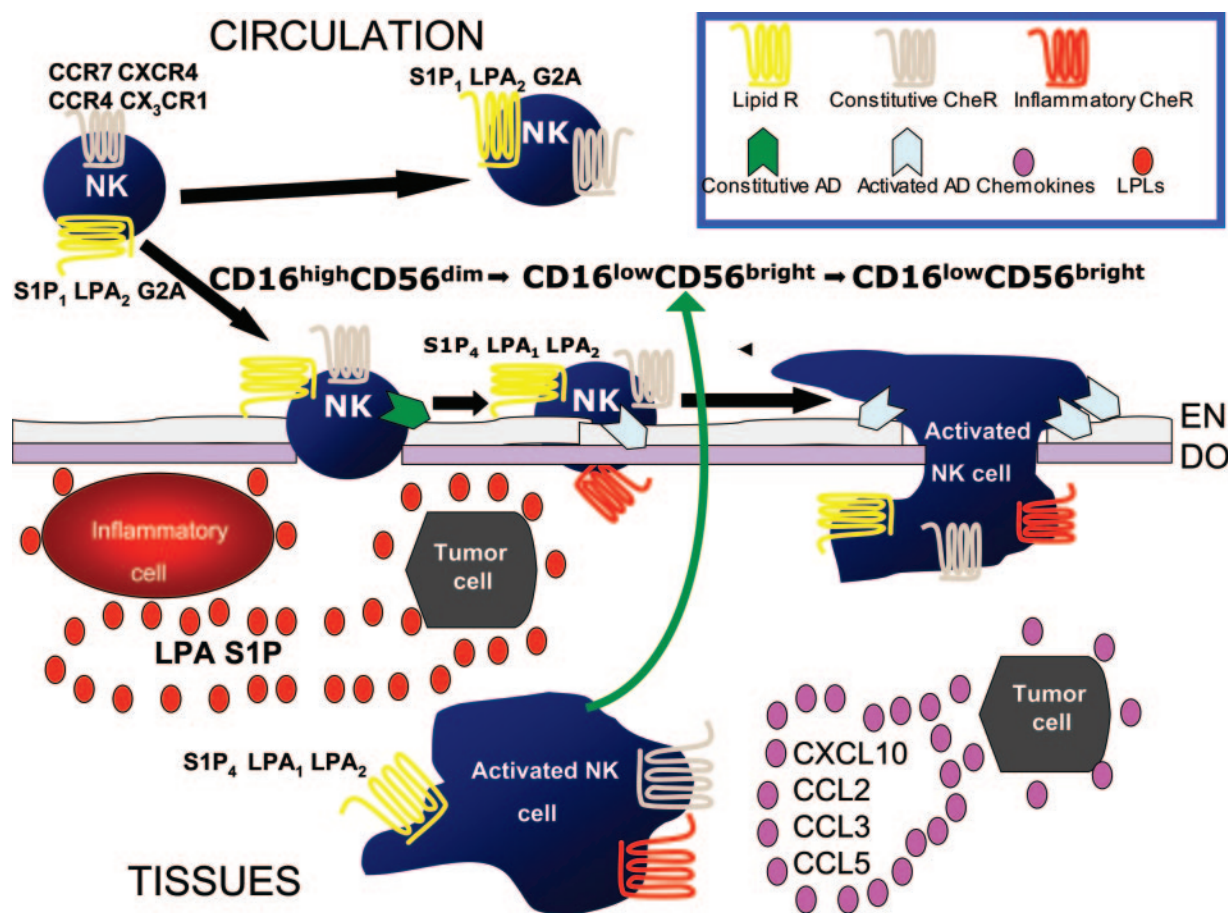


FIG. 11. Compartmentations and development of human NK cells. Most resting human NK cells express $CD16^{\text{high}}CD56^{\text{dim}}$, constitutive chemokine receptors, as well as $S1P_1$, LPA_2 , and $G2A$. Most of these cells are not circulatory, and they stay in the bloodstream. However, certain numbers of these cells move to the sites of inflammation, where they start to down-regulate $CD16$ and up-regulate $CD56$, inflammatory chemokine receptors, $S1P_4$, LPA_1 , and other receptors. These cells extravasate into inflammatory sites and migrate toward the concentration gradients of either chemokines or lysophospholipids secreted by tumor cells (in case of tumors) or inflammatory cells (in case of infections). Some of these cells may return to the periphery, forming the intermediate subset (semiresting) NK cells (green arrow), although this has not yet been proven. ENDO, endothelium.

eration of the activated and intermediate subsets (semiresting) of NK cells. In summary, our model suggests a developmental relationship among the three subsets of NK cells in the following sequence: resting $CD16^{\text{high}}CD56^{\text{low or dim}}$ $CCR4^+ CCR7^+ CXCR4^+ CX_3CR1^+ S1P_1^+ LPA_2^+ G2A^{\text{high}}$ \rightarrow activated $CD16^{\text{low}}CD56^{\text{high}}$ $CCR1^+ CCR2^+ CCR4^+ CCR7^+ CCR8^+ CX_3CR1^+ CXCR1^+ CXCR3^+ S1P_4^+ LPA_1^+ LPA_2^+ OGR1^+ PAFR^+$ \rightarrow intermediate (semiresting) $CD16^{\text{low}}CD56^{\text{high}}$ $CCR4^+ CCR7^+ CXCR4^+ CX_3CR1^+ S1P_1^+ LPA_2^+ G2A^{\text{intermediate}}$ (Fig. 11).

VIII. Concluding Remarks

Human NK cells are antitumor/antiviral effectors. Recent studies showed that these cells are highly efficacious in treating patients with acute myeloid leukemia (Velardi et al., 2002). Although highly cytolytic for transformed or virally infected cells, NK cells spare normal cells from destruction. In the last 10 years, we have learned a great deal about how NK cells discriminate between self- and nonself-MHC molecules. Human NK cells express a plethora of single transmembrane-spanning domain receptors that bind MHC molecules. Some

of these receptors are inhibitory, ensuring that NK cells do not respond to self-MHC molecules and, therefore, guard against autoimmunity. Other receptors are activating, and they ensure that NK cells mount a robust response upon interaction with tumor or virally infected cells. The signal transduction pathways induced by inhibitory and activating receptors are also different. For example, the inhibitory receptors have long cytoplasmic domains and contain the ITIM motifs that transduce inhibitory signals, resulting in preventing NK cells from killing their targets, whereas the activating receptors have short cytoplasmic domains and associate with adaptor molecules that either contain or do not contain the ITAM motif. The final outcome is activation of gene expression; mRNA and protein synthesis, which control cytokine secretion; mobilization of intracellular calcium; and lysis of target cells. However, there are still several aspects that have not been resolved, such as the ligands for the natural cytotoxicity receptors and the influence of soluble mediators on the regulation of these receptors or their ligands. This last issue is highly important since NK cells live in a milieu that are rich in soluble media-

tors such as cytokines, chemokines, and lysolipids that influence NK cell behavior.

For NK cells to perform their activities, such as lysing target cells and secreting cytokines, they must extravasate into inflammatory sites. To facilitate their extravasation, human NK cells express seven transmembrane-spanning domain receptors (GPCRs). These receptors play a prominent role in maintaining resting NK cells in the circulation and in recruiting activated NK cells into the sites of diseases. Chemokines are the major chemoattractants for these cells. However, it is still not clear whether chemokine receptors expressed in activated NK cells are responsible for their accumulation at the sites of metastases. In addition, it is not known whether chemokines and/or chemokine receptors are important for the cytolytic process of NK cells. Receptors for LPLs such as LPA, S1P, and SPC are also expressed on these cells, and these lipids are exquisite for the compartmentations observed with NK cells. Both types of chemoattractants (i.e., chemokines and LPLs) induce various intracellular signaling molecules such as PI3K and Lck that activate members of the GTPases necessary for NK cell orientation toward the chemoattractants, reshaping the cytoskeleton, and forming filopodia and lamellipodia.

It is not yet clear why lysophospholipids such as LPA, which is increased in patients with ovarian cancer and is considered to be a marker for patients with this disease, chemoattract NK cells. A plausible explanation is that tumor cells might have developed a strategy to recruit cells that secrete cytokines (NK cells secrete IFN- γ and other cytokines) necessary for the growth of the tumor cells. However, for the tumor cells to survive, they must down-regulate the cytolytic activity of the antitumor effector cells. In addition, LPLs may facilitate the interaction of NK cells with other cell types that secrete growth factors such as dendritic cells, which could facilitate the growth of tumors. Alternatively, LPLs secreted by tumor cells may inhibit NK cell lysis of dendritic cells, resulting in either activating suppressor cells or facilitating the secretion of IL-10; both are important for the survival of tumor cells. These issues have not yet been resolved, but they will be exciting questions to consider investigating. Although success was reported using activated NK cells by certain cancer patients, in most cases this immunotherapeutic modality failed. The reasons for this could be due to our incomplete understanding of NK cell biology and because we may have underestimated the capability of tumor cells to survive, even when surrounded by antitumor effector cells. The knowledge gained from understanding the compartmentations and functional activities of NK cells may result in developing modalities and drugs to treat various diseases.

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