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The Killer's Kiss: The many functions of NK cell immunological synapses

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Abstract

Natural Killer (NK) cells comprise a subset of lymphocytes involved in protection against microbial pathogens and tumors. NK cells recognize host cells that are missing MHC class I molecules and eliminate them through localized delivery of lytic granules. The majority of NK cell effector functions require direct cell to cell contact. Binding to a target cell is accompanied by creation of complex structures at the cell-cell interface known as immunological synapses. Recent studies have contributed immensely to characterization several types of NK cell immunological synapse and understanding of the variety of processes originating at this intriguing place. The emerging picture illustrates NK cell immune synapses as the sites of highly complex regulation of NK cell activity.

Introduction

Natural killer (NK) cells play a very important role in host defense against viral infections and tumorigenesis [1,2]. They directly contribute to the removal of pathogens through their cytotoxic activity. Though primarily considered as an integral part of the innate immune response, they also contribute to generation of the specific adaptive immune response through production of chemokines and cytokines, such as interferon γ or TNFα, and by activating dendritic cells [2-5].

The activity of NK cells is regulated by a balance between a variety of germline-encoded inhibitory or activating cell surface receptors [6,7]. Inhibitory receptors are characterized by the presence of cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) and can be generally divided into two groups of molecules: i) immunoglobulin superfamily (e.g. KIR, LIR, siglecs) or ii) C-type lectin domain family (e.g. NKG2A/CD94) proteins. Activating receptors can also be divided into two groups: i) receptors that interact with immunoreceptor tyrosine-based activating motif (ITAM)-bearing polypeptides (i.e. CD3ζ, DAP12, FcRγ), including CD16, NKG2C/CD94 or natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, and ii) receptors interacting with non-ITAM-bearing proteins, for example NKG2D, 2B4 or CD2 [6-8]. Activation of NK cells, mediated by activation receptors recognizing their ligands expressed on the surface of target cells, triggers a complex and highly regulated response leading to cytolytic granule release resulting in death of a target cell. Conversely, interaction of inhibitory receptors with their ligands negatively regulates NK cell activity [6,

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8-10]. Activation and inhibition take place at the specialized contact sites between NK cells and target cells, known as the immunological synapse (IS).

In this review we will highlight recent reports providing insight into morphology and function of the NK cell immunological synapse (NKIS). We will focus on the human NKIS and discuss how those findings contribute to the understanding of the role of the NKIS in NK cell function.

The immunological synapse

An encounter between the immune system cell and other host cell may results in generation of a specialized interface at the cell-cell contact point that is often regarded as the IS. However, the definition of the IS as a simple intercellular contact involving an immune cell is very vague. Based on an elegant definition of the immunological synapse proposed by DM Davis [11], the immunological synapse in this review is defined as a contact between two cells, at least one of them being a cell of the immune system (e.g. NK cell) that results in segregation of proteins at the cell-cell interface into micrometer-scale three-dimensional domains.

The first observation of the immunological synapse was reported for APC and T cell interactions [12]. Although it is believed that the NKIS resembles that of T cells, important differences between NK and T cell IS occur. For example, NK cells use a wide variety of germline-encoded activating receptors for signaling at the IS, T cells do not form inhibitory synapses and the localization of several essential proteins (e.g. LAT, ezrin, CD43, CD45, CD2) differ significantly between NK and T cell synapses [7,13-15]. Furthermore, NK cells have the ability to form distinct types of synapses.

The activating NK cell immunological synapse (aNKIS)

The interaction between NK cell and a susceptible MHC I-negative (i.e. tumor or virus-infected) target cell results in generation of an aNKIS. The formation of the aNKIS is a sequential process [9,14,16], requiring several steps: i) contact and adhesion, ii) receptor ligation and segregation linked to initial signaling, iii) actin cytoskeleton rearrangements (tight conjugation), iv) further receptor clustering and sustained signaling (signal amplification), v) microtubule cytoskeleton rearrangements (MTOC polarization), vi) granule polarization and degranulation, and vii) IS dissasembly (Figure 1).

Upon initial contact, adhesion molecules such as LFA-1 and Mac-1 quickly segregate into a peripheral supramolecular activation cluster (pSMAC) [14*,17**,18], where they contribute to tight conjugation between NK and target cell. Interestingly, LFA-1 plays a more complex role, as it functions in inducing initial phosphorylation of signaling molecules, for example Src, Vav-1, LAT, SLP76, ZAP70, JNK, ERK1/2, PKC [19-21] and also signals the later granule polarization to the NKIS [20,22,23]. Importantly, LFA-1 also induces an early activation signal for actin polymerization that results in accumulation of filamentous (F-)actin at the pSMAC [16,17**,19,21,22]. Other molecules, including talin [17,18], ezrin-radixin-moesin (ERM) proteins [13,24] or WASp [16] also accumulate at the pSMAC and contribute to F-actin accumulation. Actin polymerization and accumulation at the aNKIS is indispensable for NK cell cytotoxicity, as disruption of the actin cytoskeleton results in severe impairment of NK cell activity [14**,16,22].

Whereas adhesion and co-stimulatory molecules segregate at the pSMAC, the activating receptors accumulate in a central supramolecular activation cluster (cSMAC), where combinations of synergistic activating receptors facilitate cytotoxic responses [8,25**]. The engagement of activating receptors leads to recruitment of molecules important in signal transduction, for example Src, Lck, Fyn, Syk, ZAP70, SLP76, Vav-1, PLCγ, PKCθ, BLNK, LAT, 2B4, Pyk2, SHP-1, forming within minutes a signalosome at the cSMAC [9**,

17**-19,26-28]. The activating receptor-induced formation of the signalosome at the cSMAC results in initiation of signaling cascades leading to signal amplification and different effector functions, such as cytotoxicity and cytokine secretion [6,10]. Two major pathways are involved in cytolytic responses: PI3K-ERK2 [21,29,30] and PLCγ – JNK [31], and their activation leads to MTOC polarization to the aNKIS. Following MTOC polarization, perforin and granzyme-containing granules are delivered to the target cell at the cSMAC of the aNKIS. Interestingly, it is not clear yet whether lytic granules are transported exclusively along MTOC-derived microtubules, or also utilize a second, Golgi-derived set of microtubules [32].

The last steps, lytic granule exocytosis, are still ill-defined in NK cells. Contact with target cell stimulates quick *de novo* synthesis of lytic granules and enhances their Ca^{2+}-dependent exocytosis [33**]. However, it is not clear whether MTOC can directly deliver lytic granules to the plasma membrane in NK cells, as happens in T cells [34]. Exocytosis in NK cells requires the actin cytoskeleton, as knock-down of CIP4, a protein that links microtubule and the actin network through its ability to bind microtubules and WASp, severely impairs MTOC polarization and cytotoxicity [35*]. Moreover, it appears that lytic granules are transferred from microtubules to the cortical actin cytoskeleton, since disruption of expression of F-actin-associated proteins, for example WASp, WIP and, importantly, myosin IIA prevents granule transport and exocytosis [14*,16,36-38].

Also, the protein machinery involved in lytic granule exocytosis is poorly understood in NK cells. It is plausible to assume that Rab27a and Munc13-4 play similar role in granule tethering and priming in NK cells as they do in T cells [39], since NK cells from Munc13-4-deficient patients have deficient degranulation [40,42]. The fusion of lytic granules with plasma membrane at the aNKIS is mediated by syntaxin 11 [41,42] and VAMP-7 [43], but other SNARE proteins are likely to be involved, as degranulation of syntaxin 11-deficient NK cells can be partially restored by IL-2 stimulation [41]. Following fusion with plasma membrane and release of the content of lytic granules, perforin forms pores in the target cell membrane, allowing granzymes to enter the target cell. Granzymes, in turn, induce the death of target cells through caspase-dependent or independent activation of apoptosis [44].

After completion of its effector function, the NK cell detaches from a target cell and the NKIS is disassembled. This step is the least known and we are lacking information regarding any mechanism regulating aNKIS disassembly. However, detachment of NK from target cells often results in formation of long membranous tubes, termed connective structures or nanotubes [18,45]. A growing number of reports indicates that formation of nanotubes has functional consequences, for example formation of transient synapses, spreading of calcium fluxes or distribution of receptors that allow pathogen entry, or possibly immune surveillance [45-47]. Interestingly, protein transfer is not limited only to nanotubes or disassembly of the aNKIS. Membrane fragments and proteins are exchanged between NK and target cell soon after conjugate formation [48]. The transfer is bidirectional, NK cell receptors are transferred to target cells and NK cell receptor ligands are transferred from targets to NK cells [18,46, 49-52]. Membrane capture takes place at the aNKIS and is an active process, depending on ATP, calcium flux, actin polymerization and activity of Src and PKC kinases, and appears to be related to specific recognition of ligands by NK cell receptors [48]. Membrane capture may serve the maintenance of the IS and regaining membrane that was lost during exocytosis. In agreement, KIR inhibitory signaling prevents exocytosis and membrane capturing [48]. The bidirectional transfer correlates with decreased activity or fratricide of NK cells and thus may provide one of the regulatory mechanisms of NK cell activity [51,52]. Additionally, transfer at the IS can contribute to pathogen spreading as evidenced by uptake of CD21 by NK cells from EBV-infected cells and their subsequent infection with EBV [53] and transfer of poliovirus receptor to CD96-positive NK cells [54].

The inhibitory NK cell immunological synapse (iNKIS)

The encounter between NK cell and MHC-I positive (i.e. normal, healthy) target cell results in generation of the immunological synapse that is dominated by inhibitory interactions and, hence, is termed an iNKIS. It is distinct from the aNKIS, lacks large cytoskletal rearrangements characteristic of the aNKIS and its role is to prevent NK cell activation and killing of the target cell. Contrary to the aNKIS, formation of the iNKIS is independent of ATP, lipid rafts and actin cytoskeleton, and is driven by thermodynamic processes [55-61].

Following the contact with target cell, inhibitory receptors bound to their ligands cluster at the iNKIS. Ligand binding alone is sufficient to induce KIR clustering and this process is independent of receptor signaling ability [56,60*]. However it requires divalent cations, particularly Zn^{2+} [55,56]. The source of the energy for movement of molecules to and/or at the synaptic region is not clear. However, Zn^{2+} initiates a multimerization of KIR that may be a clue to a role for Zn^{2+} in inducing movement in the absence of ATP [62]. Co^{2+} also induces dimerization but not multimerization of KIR [63]. Interestingly, the amount of inhibitory receptors at the iNKIS is constant and proportional to their overall level of expression, and their content do not increase in time [57**,60*]. Initially, inhibitory receptors cluster in the middle area of the iNKIS while adhesion molecules localize at the perimeter of the iNKIS [60*], the areas known, respectively, as a central and peripheral supramolecular inhibition cluster (cSMIC and pSMIC) [49]. With time inhibitory receptors disperse throughout the iNKIS or form distinct clusters, while LFA-1 moves toward the center of the iNKIS and segregates from NK cell receptors [55,57**,58,60*,64**]. In fact, the iNKIS is a very dynamic structure with HLA-C/KIR patterns changing and interchanging over time [49], a process that depends on the density of MHC-I and inhibitory receptors [57**]. Interestingly, the density of ligand/inhibitory receptors on the cell surface influences not only receptor organization but also NK cell cytotoxicity. There is a certain level of MHC-I expression required for inhibition of cytotoxicity [57**,65]. However *in vivo*, the number of inhibitory receptors as well as the density of inhibitory receptor ligands on the cell surface is high enough to maximally engage all inhibitory receptors [57**,65]. Therefore, under such conditions, the inhibitory signal is dominant and overcomes NK cell activation when both activating and inhibitory receptors are co-engaged at the same time [57**,61,65-67].

Ligation of KIR and NKG2A inhibitory receptors results in their quick phosphorylation [36, 58,60*,61,64**] and subsequent recruitment of protein tyrosine phosphatases, SHP-1 and SHP-2, to the cSMIC area [17**,27,58,60*,61,68]. In turn, phosphatase-mediated selective dephosphorylation of cellular targets disrupts early signaling events, including phosphorylation of activating receptor and proximal signaling molecules [15,17**,27,65,66, 69], cytoskeletal rearrangements [15,36,61,69], as well as impairs integrin-mediated adhesion [68,70]. As the end result lipid rafts are excluded from the iNKIS [15,56,59-61,65,71], signaling complexes are not assembled [9**,17**,27] and there is no large-scale cytoskeletal remodeling (Figure 1).

Interestingly, even though there is an abundance of inhibitory receptors at the iNKIS, their phosphorylation is limited to a small fraction that clusters in distinct microdomains at the cSMIC and does not spread outside of the iNKIS [64**]. Thus, the spatial confinement of inhibitory receptor activation allows for focusing of the inhibitory signal at the cell-cell contact site where activating signals could also be triggered, thus providing a mechanism for prompt deactivation of the NK cell. Moreover, spatial limitation of the dominant inhibitory signal explains the ability of NK cells to form activating and inhibitory synapses at the same time, even in an excess of MHC-I-postive target cells [17**].

Other NK cell immunological synapses

Formation of distinct activating and inhibitory synapses are the hallmark of NK cell activity. New reports, however, demonstrate that contact of NK cell with another cell in the organism is not a simple kill or not kill matter. Although our knowledge of these processes is still limited, we are constantly learning more about the ability of NK cells to form different types of immunologic synapses.

a) Synapses between NK and dendritic cells (DC-NK IS)

Recently, dendritic cells (DC) have been shown to affect functions and activity of resting NK cells. In response to DC stimulation, NK cells are activated [3,4,72,73**], proliferate [74-76], produce cytokines [73**,74,77,78] and increase their cytolytic potential [73**]. DC activation of NK cells may be mediated by the NKp30 receptor, but not through NKp40 or NKp46 [72,79]. Reciprocally, NK cells affect DC maturation, cytokine production and survival [72,77,78]. Importantly, the cross-talk between DC and NK cell is mediated by direct contact and delivery of DC-derived cytokines (e.g. IL-12, IL-15, IL-18) and requires formation of a DC-NK IS [73**-75**,77,78].

Only mature DC can readily form stable conjugates with NK cells. In contrast to the aNKIS, adhesion between cells is not dependent on LFA-1, but rather requires interactions of DC-SIGN and LFA-3 with ICAMs [73**,75**], and of the CX3CL1 chemokine on mature DC with CX3CR1 on the NK cell [80]. Conjugation between mature DC and NK cell is accompanied by increase of calcium flux in NK cells [73**,75**,78], followed by increase of the cell-cell contact site and formation of the synapse [73**]. Adhesion molecules, talin and F-actin accumulate at a peripheral area, while lipid rafts cluster in a central part of the DC-NK IS [73**,75**]. Formation of the DC-NK IS is dependent on actin cytoskeleton and raft accumulation, as well as microtubule integrity, and blocking of raft coalescence or actin polymerization inhibits accumulation of phosphorylated proteins at the DC-NK IS [73**]. Following the formation of the DC-NK IS, the MTOC in the DC polarizes towards the IS, allowing for localized delivery of interleukins to the NK cell [73**,75**,78].

Interestingly, inhibitory receptors also accumulate at the DC-NK IS [75**,80]. KIR and NKG2A molecules were demonstrated to quickly form multifocal patterns in the central area of the DC-NK IS, before actin polymerization and accumulation [75**]. High level of MHC I on DC and expression of inhibitory receptors on NK cells protect mature DC from lysis [72,81]. Intriguingly, the amount of KIR at the cell-cell interface decreases in time, a phenomenon dependent on CX3CL1. CX3CL1 plays a critical role in KIR ditribution at the DC-NKIS and blocking of the inhibitory signal, as the presence of CX3CL1 on mature DC blocks KIR phosphorylation, lipid raft coalescence, recruitment of SHP-1 to the DC-NKIS, and inhibits of formation of a multiprotein complex involved in actin cytoskeletal rearrangements in the NK cell. CX3CL1 signaling regulates DC-NKIS organization and allows for DC-mediated activation of NK cells, despite the interaction between inhibitory receptors on NK cell and their ligands on DC [80].

Thus, the DC-NK IS has a regulatory character and characteristics of both activating and inhibitory IS.

b) Synapses between NK cells and macrophages (M-NK IS)

Another synapse of the regulatory type is formed between macrophages and NK cells, with accumulation of 2B4 and a moderate increase of F-actin at the M-NK IS. Following the cell-cell contact macrophages, similarly to DC, stimulate NK cell proliferation, cytokine production

and enhance NK cell cytolytic activity. In contrast to DC, however, NK cell activation is mediated by 2B4 and not through NKp30 [79].

Interestingly, a contact between NK cells and macrophages activated with high doses of LPS results in formation of cytolytic rather than regulatory IS, characterized by robust actin polymerization with formation of F-actin ring, accumulation of adhesion molecules in the pSMAC area, and clustering of NKG2D, DAP10, CD3ζ in the cSMAC. This interaction leads to NKG2D-mediated killing of the macrophage, providing evidence that NK cells eliminate overstimulated macrophages [79].

c) Decidual NK cell synapses (dNK IS)

A specific subset of NK cells is present in human decidua [82,83]. Although dNK cells express a variety of activating receptors and their lytic machinery is functional, they are not cytotoxic [84]. Intriguingly, dNK cells form conjugates with target cells, similarly to peripheral blood NK cells. They accumulate F-actin, as well as CD2 and LFA-1 in the peripheral area of the IS. However, they do not polarize either MTOC or lytic granules toward the contact site with target cell and, thus, are cytotoxic-incompetent [84]. It is yet unknown whether the dNK IS is of the immature activating type (i.e. arrested at some stage of development/activation) or regulatory type.

Conclusions

Recent advances in the field clearly illustrate that the NKIS is very complex and dynamic structure serving not only cytotoxicity, but rather allowing NK cell to communicate and regulate its own activity. While encounter with a pathogenic MHC I-negative cell results in formation of the aNKIS, followed by activation of the NK cell and delivery of “the kiss of death”, encounter with MHC I-positive cell promotes formation of synapses that serve a broad-range regulation of NK cell function. Depending on the environment, NK cell can be either inhibited or stimulated to proliferate, produce cytokines and control other cells of the immune system (e.g. antigen presenting cells). The cross-talk at the immunological synapse formed between NK and an encountered cell allows fine-tuning of immune surveillance.

Though our understanding of NKIS structure and function has grown in recent years, many questions still remain unanswered. For example, what protein machineries are specifically involved in MTOC movement toward the IS and release of lytic granules? What is so special about the uterine NK cell synapse that prevents these cells from killing trophoblast cells? Are there other types of synapses formed by NK cells? What is the organization and kind of synapses formed between NK cells themselves? The challenges are great and it will be extremely interesting to follow upcoming studies to create a more detailed and complete image of this fascinating structure that has a fundamental role in regulation of NK cell activity.

Glossary of terms

ITAM, immunoreceptor tyrosine-based activation motif. A conserved YxxL/I sequence in the intracellular tail of signal-transducing cell surface proteins. Typically, two of these sequences are separated by 6 – 12 amino acids ($YxxL/Ix_{(6-12)}YxxL/I$). Following the ligand binding by the cell surface receptor, the ITAM motifs are phosphorylated on tyrosine residues by Src family kinases and recruit and activate cellular kinases involved in activating signaling pathways of the cell. In NK cells the majority of activating receptors lacks ITAM motifs but interacts with ITAM-bearing polypeptides: CD3ζ, FcRγ and DAP12, thus initiating the signal transduction.; ITIM, immunoreceptor tyrosine-based inhibitory motif. A conserved I/V/LxYxxI/V/L sequence found in the cytoplasmic part of many inhibitory receptors. Upon interaction of an inhibitory receptor with its ligand, the ITIM motifs become phosphorylated

by Src family of kinases, allowing them to interact with phosphatases such as the phosphotyrosine phosphatases SHP-1 and SHP-2, or the inositol-phosphatase SHIP. The phosphatases recruited to ITIM motifs prevent activation of molecules involved in the signal transduction in the cell.; MHC, major histocompatibility complex. A complex of genes encoding proteins involved in antigen presentation and immune responses. MHC proteins are classified as class I, class II and class III. Class I molecules (MHC I) are cell surface heterodimers consisting of an a chain associated with β_2-microglobulin and are present on all nucleated cells. Class II proteins are cell surface heterodimers comprised of an a and b chain and are present on antigen presenting cells. Class III molecules are distinct from class I and II and include complement components as well as tumor necrosis factor a and b.; KIR, killer-cell immunoglobulin-like receptors. A family of cell surface glycoproteins, expressed by all NK cells and a subset of T cells, that interacts with MHC I molecules. KIR proteins are classified according to the number of the extracellular immunoglobulin domains (2D or 3D) and by the presence of either long (L) or short (S) intracellular domain. KIR molecules with the long cytoplasmic domain are capable of induction of inhibitory signals and early termination of NK cell activity upon ligand binding via one or two ITIM motifs located in their cytoplasmic domain. Less common KIR proteins with the short cytoplasmic tail, lacking the ITIM motifs, are involved in signaling activation.; Lytic granules, a unique type of lysosomes present in NK cells and cytotoxic T cells that is secreted in a contact-dependent manner, in response to lymphocyte activation. Following the release of lytic granules at the cell-cell contact interface, the pore forming protein perforin, serine proteases (granzymes) and Fas ligand contained in the granules mediate the death of a target cell.; Dendritic cells, a subset of immune cells with characteristic long branched protrusions resembling the dendrites of nerve cells. They are found in the lymph nodes, thymus, spleen, skin and other tissues. They act as specialized antigen presenting cells, as their main function is to process antigenic material and present it on their cell surface to other immune cells.; Macrophages, mononuclear leukocytes functioning in both the innate and adaptive immune response. Their function is to phagocytose pathogens and cellular debris, and to stimulate other cells of the immune system to respond to a pathogen.; Decidual NK cells, a unique subset of natural killer cells present in the endometrium. They are the dominant type of immune cells in the uterine mucosa during formation of the placenta and play essential role in ensuring a normal blood supply to the fetus and placenta during pregnancy..

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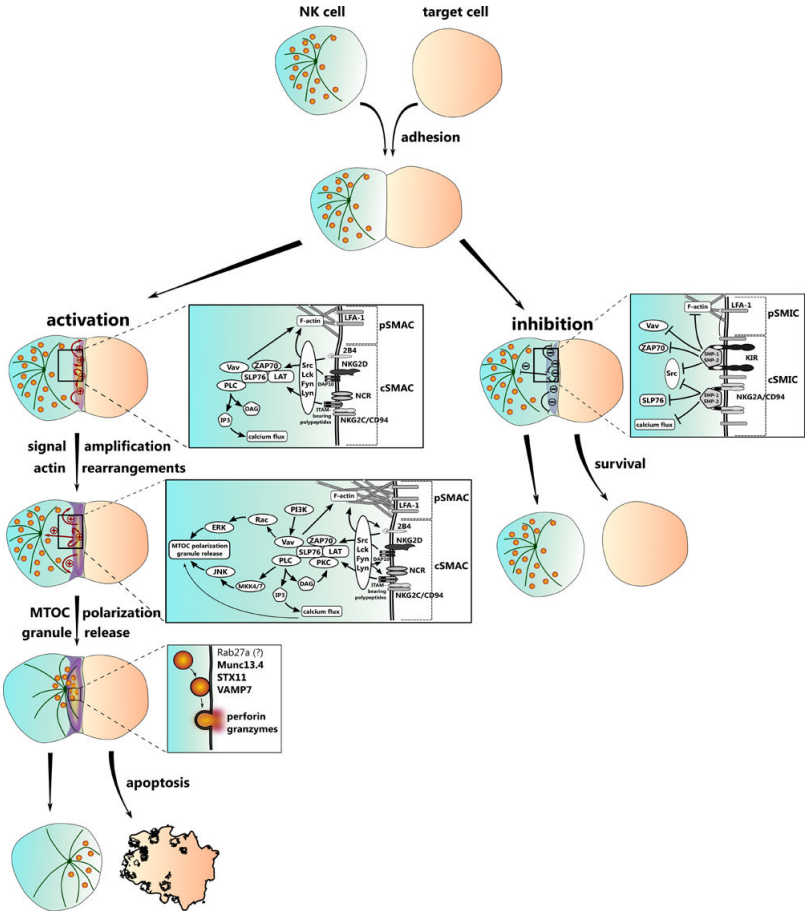

Figure 1. The NK cell immunological synapse is formed in distinct stages

The encounter between the NK and a target cell results in adhesion and conjugate formation (top). The balance between activating and inhibitory receptor signaling on the cell-cell interface decides the outcome of the interaction. The lack of MHC I molecules on the target cell, caused by viral infection or tumorigenesis, favors formation of the activating NKIS (left). Engagement of NK cell activating receptors by their ligands induces phosphorylation of membrane proximal signaling molecules and initial wave of actin cytoskeleton rearrangements. This, in turn, leads to more stable conjugation by creation of an F-actin ring in the pSMAC area and formation of a signalosome comprised of many signaling and adapter molecules in the cSMAC. Thus, a positive feedback loop is generated causing signal amplification and sustained signaling that stimulates robust actin polymerization and polarization of the MTOC to the activating NKIS. Lytic granules, containing perforin and granzymes, are transported along microtubule tracks and with MTOC translocation they are delivered to the cSMAC, where they are subsequently released. Perforin makes pores in the membrane of target cell, allowing granzymes to enter the cell and induce apoptosis. After induction of target cell lysis, the NK cell detaches from its target and can search for another target.

Conversely, the presence of MHC I on the surface of target cell results in ligation of NK cell receptors that are capable of dominant inhibitory signaling and formation of the inhibitory NKIS (right). Engagement of inhibitory receptors leads to quick disruption of activation signaling by phosphatase-mediated dephosphorylation of membrane proximal signaling molecules (or even possibly macromolecular structures) and blocking of the signalosome formation. This prevents large scale actin cytoskeleton rearrangements and inhibits MTOC and

lytic granule polarization, resulting in survival of the target cell. The diagrams represent only selected molecules. The drawings are not to scale.