Formation and function of the lytic NK-cell immunological synapse

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Abstract | The natural killer (NK)-cell immunological synapse is the dynamic interface formed between an NK cell and its target cell. Formation of the NK-cell immunological synapse involves several distinct stages, from the initiation of contact with a target cell to the directed delivery of lytic-granule contents for target-cell lysis. Progression through the individual stages is regulated, and this tight regulation underlies the precision with which NK cells select and kill susceptible target cells (including virally infected cells and cancerous cells) that they encounter during their routine surveillance of the body.

'Danger' signals
Agents that alert the immune system to danger and thereby promote the generation of immune responses. Danger signals can be associated with microbial invaders (exogenous danger signals), and can also be produced or expressed by damaged cells (endogenous danger signals).

Natural killer (NK) cells are lymphocytes of the innate immune system that elicit effector functions following the ligation of germline-encoded receptors. In humans, NK cells are able to deliver a response immediately after recognizing specific signals, including stress signals, 'danger' signals or signals from molecules of foreign origin. They were originally described as having rapid cell-mediated cytotoxicity. However, they are now also known to promote slower receptor-mediated apoptosis, to provide contact-dependent co-stimulation and to efficiently produce soluble mediators, including cytokines. NK cells therefore participate in the defence against infections, the regulation of immune responses and the surveillance of stressed or cancerous cells (reviewed in REF. 1). Although these functions are not unique to NK cells, they use NK-cell-specific receptor systems, and the ability to mediate effector functions rapidly without the need to develop further is a key distinguishing feature between mature NK cells and cytotoxic T lymphocytes (CTLs), both of which are efficient at mediating cytotoxicity.

NK-cell cytotoxicity involves the secretion of cytolytic effector molecules from specialized organelles that are known as lytic granules. Much of what is known about this process is derived from studies of CTLs and this has provided guidance for subsequent studies of NK cells. However, there are several important differences between NK cells and CTLs, and so the processes leading to cytotoxicity by the two cell types should not be assumed to be identical. For example, the lytic granules are preformed in resting human NK cells but not in CTLs. As a result, the cytolytic process in NK cells needs to be well regulated, and this might involve additional or enhanced mechanisms for controlling the secretion of lytic-granule contents in NK cells compared with the process in CTLs. Indeed, direct comparisons between the cytolytic process in NK cells and CTLs have identified important kinetic and mechanistic differences[2].

The induction of many NK-cell effector functions, including cytotoxicity, requires that the NK cell contacts its target cell. This ensures precise targeting of the cytolytic process to a single diseased cell in a tissue without affecting its neighbouring cells. The events that occur following the interaction between a cytolytic cell and its target cell have been well studied. They include the delivery and secretion of cytolytic effector molecules at the interface that is formed between the cytotoxic cell and its target cell through a process known as directed secretion.

Our understanding of directed secretion for cytotoxicity has been advanced by the discovery of the immunological synapse. The immunological synapse was originally defined in the late 1990s[3,4] as the crucial junction between a T cell and an antigen-presenting cell (APC) at which T-cell receptors (TCRs) interact with MHC molecules. Subsequent studies extended these observations and identified relevant immunological synapses between different types of immune cell, as well as between immune cells and non-immune cells. Thus, an immunological synapse can be defined as the orderly rearrangement of molecules in an immune cell at the interface with another cell. Numerous molecules have been identified as participating in the immunological synapse, including receptors, signalling molecules, cytoskeletal elements and cellular organelles. Some studies suggest that these molecules accumulate in distinct regions in an activating immunological synapse to form a supramolecular activation cluster (SMAC), which may be segregated into peripheral (pSMAC) and central

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Microclusters
Discrete collections of molecules at the immunological synapse that can move in the plasma membrane and generate signals. They are smaller than and exclusive of the central supramolecular activation cluster, which can represent a coalescence of multiple microclusters.

Perforin
A component of cytolytic granules that participates in the permeabilization of plasma membranes, thereby allowing granzymes and other cytotoxic components to enter or be taken up by target cells.

(cSMAC) zones. However, the purpose of this molecular patterning is debatable, as functions typically attributed to the SMAC can be induced in its absence under some circumstances[5]. Furthermore, single molecule studies and kinetic studies of the immunological synapse have shown that, in some settings, the engagement of individual receptors[6] or the involvement of microclusters of cell-surface and signalling molecules can support cell activation without the need for a mature synapse[7,8]. Therefore, the importance of larger scale accumulations and distinct segregation of molecules at the synapse is currently being re-evaluated.

Although debate regarding the role of the immunological synapse in enabling immune responses continues, several of its potential functions seem relevant and worth considering (BOX 1). Many of these functions were originally assigned to the immunological synapse between a T helper cell and an APC, but they also apply to the NK-cell synapse. Certain aspects, however, are more relevant to NK cells, such as the directed secretion of lytic granules for cytotoxicity.

Lytic granules are hybrid organelles that are specialized for the secretion of the lytic effector molecules which reside in them (BOX 2). For an NK cell to mediate cytotoxicity, lytic granules are emptied onto a target cell at a prototypical mature lytic synapse (FIG. 1). In the mature synapse, filamentous actin (F-actin) and adhesion receptors accumulate and are thought to form a ring in the pSMAC through which perforin and other lytic-granule contents are secreted. Domains in the lytic synapse that contain specific signalling molecules or secretory machinery have been described in CTLs[9], but may not always be required for CTL-mediated cytotoxicity[6]. Similar molecule distribution patterns have been observed in NK cells, albeit inconsistently[10–12], and many aspects of NK-cell synapse organization have not been elucidated. However, lytic granules are large organelles and must traverse dense F-actin networks at the synapse, and actin reorganization is required for their release. Therefore, synapse formation has the specific function in NK cells of enabling cytotoxicity.

Box 1 | Proposed functions of the immunological synapse

There are numerous functions ascribed to the immunological synapse, many of which are relevant to natural killer (NK) cells.

Ligand recognition. The immunological synapse creates a crucial zone where ligands can be recognized accurately by their cognate receptors among many non-relevant ligands.

Signal amplification and integration. Whether in large clusters at the central supramolecular activation cluster (cSMAC) or in discrete microclusters that are located in the periphery, important signalling molecules localize with receptors at the synapse. Innovative studies in NK cells have described functional signalling at these sites[40], as well as functional integration of signals[11].

Co-stimulation. Co-stimulatory ligands, such as CD40 ligand[116] and OX40L ligand[117], that are expressed by NK cells can bind receptors on adaptive immune cells to facilitate immune responses. *In vivo*, NK cells have been shown to engage with other innate cells and T cells simultaneously[118]. The innate cell presumably induces the expression of NK-cell co-stimulatory ligands, which are then recognized by adjoining T cells.

Cytotoxicity. The close approximation of an NK cell and its target cell that is conferred by the immunological synapse ensures precise targeting of cytotoxicity[31] and can protect neighbouring cells from the damaging effects of cytotoxic mediators.

Directed secretion. The arrangement of molecules at the synapse can create a conduit through which cellular components can be secreted[9]. Linkage between the synapse and intracellular transport machinery allows the focused secretion of small and large components, including lytic granules[21] and cytokines[119].

Multidirectional secretion. Activation signals originating from molecules in the synapse can induce secretion at sites away from the synapse[21,50]. This might be useful following the recognition of IgG-coated particles by NK-cell FcγRIII (low-affinity Fc receptor for IgG) for defence against infections with extracellular pathogens and induction of local inflammation.

Protein transfer. Cell-surface proteins can be transferred from the NK-cell to the target-cell membrane through the immunological synapse[51,120–124] to induce target-cell signal generation and to protect NK cells from fratricide[69,125].

Cell-fate determination. In cytotoxic T lymphocytes, the immunological synapse coordinates the polarization and unequal partitioning of cell components before cell division, which leads to distinct fates for the daughter cells[126]. This has not yet been defined in NK cells.

Inhibition of activation. The immunological synapse affords the opportunity to specifically ligate inhibitory receptors and prevent cell activation (see BOX 3).

Signal termination. Molecular patterns at the immunological synapse might facilitate NK-cell receptor internalization and/or terminate their productive signalling[127]. This might be important in recycling cytotoxic function or creating a refractory period, as defined in T cells[39,67].

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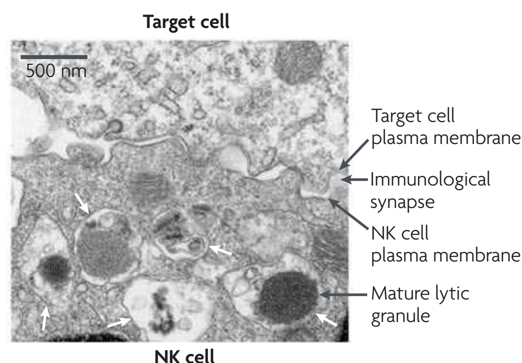
Box 2 | **NK-cell lytic granules**

The lytic granules that are found in natural killer (NK) cells are secretory lysosomes that have characteristics of both the cellular lysosomal compartment and specialized secretory machinery (reviewed in REF. 128). They arise from the fusion of endosomes with specific secretory components from the trans-Golgi network and therefore are dual-function organelles — that is, specialized for secretory and destructive activities. Important regulators of lytic-granule maturation are the adaptor protein 3 (AP3) complex and lysosomal trafficking regulator (LYST) protein, which regulate the incorporation of lysosomal proteins from the Golgi and lysosomes, respectively. Components that are sorted into the lytic granules include granzymes A and B, which are transported from the trans-Golgi network by the mannose-6-phosphate receptor, as well as CD95 (also known as FAS ligand), granulysin and perforin. Mannose-6-phosphate-receptor-independent sorting mechanisms have recently been described and are probably also relevant to NK-cell lytic-granule maturation[129].



The formation of secretory lysosomes in NK cells is a multistep process and is similar to that in cytotoxic T cells. However, there are probably important differences between the processes in the two cell types, as the generation of T-cell lytic granules only occurs after cell activation. By contrast, secretory lysosomes are preformed and maintained in resting human NK cells[130]. In addition, NK cells contain various related organelles with distinct morphology, including mature lytic granules, lytic-granule precursors, multivesicular bodies and late endosomes (see figure of an electron micrograph of the synapse between an *ex vivo* NK cell and a K562 target cell; arrows indicate lytic granules and their precursor forms). These can polarize to the immunological synapse (as shown) where their contents can be secreted into the cleft that forms between the NK cell and the target cell.

This Review describes the specific steps that lead to the formation and function of the NK-cell lytic synapse. Recent evidence has defined a sequence of events that has some surprising linearity and a series of important checkpoints. A better appreciation of this process and of its regulation will help to define and control NK-cell cytotoxic ability.

Stages of NK-cell lytic-synapse formation

The formation of a mature and functional NK-cell lytic synapse can be divided into a series of discrete stages: the initiation stage, the effector stage and the termination stage (FIG. 2). Each of these stages can be further subdivided into multiple steps, some of which have been defined in a linear sequence with clear prerequisites and therefore occur in series and not in parallel. Together, these processes enable the delivery of lytic granules to the synapse, followed by the association and subsequent fusion of lytic granules to the NK-cell membrane, which results in the release of their contents onto the target cell. Because lytic granules exist in resting NK cells before activation, each stage must be controlled to prevent accidental release of cytotoxic mediators and to enable rapid directed secretion at the appropriate moment.

Initiation stage. The initial stage in the formation of a lytic synapse includes establishing a close cell–cell association, initial intracellular signalling and adherence of the NK cell to its target cell (FIG. 2). Although these steps remain in part theoretical, experimental evidence that favours the occurrence of such events is accumulating. In the first step, the NK cell either intentionally or accidentally encounters its potential target cell. This can be in response to chemotactic signals, which enable NK-cell localization in the organism to sites where their effector functions are required (reviewed in REF. 13).

The first contact between an NK cell and its target is a cellular association that may be similar to tethering. Although studies of the NK-cell synapse have not firmly established the exact molecules involved, they may include the selectin family members, as has been shown in *in vivo* studies of NK-cell localization[14]. They may also include the recognition of sialyl Lewis X by CD2 on NK cells[15], as CD2 has been shown to accumulate rapidly at the developing NK-cell synapse[12]. Identification of the molecules that are involved in the initial contact between an NK cell and its target cell is challenging, as the *in vitro* approaches that are used to study the immunological synapse may reduce the requirements for this particular step. The early interactions could then result in a longer-lasting association that leads to initial adhesion. These events probably contribute to NK-cell activation, as receptors that are potentially engaged at this point, such as CD2, may participate in activation signalling[16].

The next step in the formation of the lytic synapse is firm adhesion, which is facilitated by receptor–ligand interactions of higher affinity. The integrin family of adhesion molecules is important in firm adhesion. Some of the integrins that are expressed by NK cells are well studied and include lymphocyte function-associated antigen 1 (LFA1; CD11a–CD18) and macrophage receptor 1 (MAC1; CD11b–CD18). Although these integrins rapidly cluster at the NK-cell synapse following initiation[12,17,18], they probably function in adhesion and participate in signalling (even in resting NK cells[19]) before their rearrangement to the synapse[20]. Integrin signalling can fully activate some NK cells[19] and partially activate others[21]. Although integrin signalling contributes to the maturation of the synapse, it is less likely to be involved in the commitment of a NK cell to cytotoxicity at this early stage of synapse formation.

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Under physiological conditions, subsets of more potent NK-cell activating receptors, such as the natural cytotoxicity receptor family, are probably engaged at this stage by ligands on the target cell, owing to their diffuse cell-surface distribution. These interactions provide additional signals that might support early NK-cell activation. The function of these activating receptors, however, is probably distinct from that of TCRs at the CTL immunological synapse. In favour of this, a TCR can associate with up to six CD3 molecules that contain a total of ten immunoreceptor tyrosine-based activation motifs (ITAMs), whereas NK-cell activating receptors couple with fewer ITAM-containing molecules. So, the ligation of an individual TCR complex could theoretically provide more potent signals for synapse formation and function than those provided by NK-cell activating receptor ligation. This may contribute to the observed rapid triggering of TCRs and subsequent CTL activation that occurs following binding to just a few peptide–MHC complexes[6], and may not apply to NK cells.

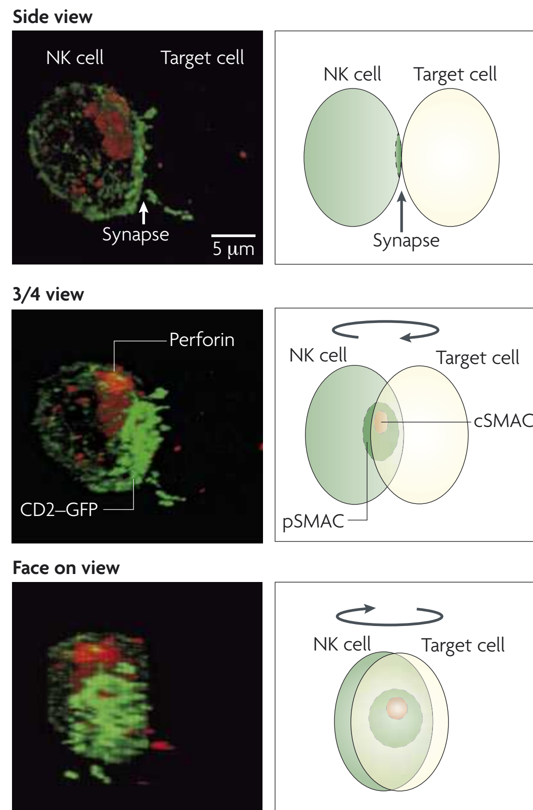

Figure 1 | **The prototypical mature NK-cell lytic synapse.** The mature natural killer (NK)-cell lytic synapse is defined by the formation of a supramolecular activation cluster (SMAC) at the interface between the NK cell and the target cell to which lytic granules polarize. The prototypical version of this synapse contains a central SMAC (cSMAC) that includes a secretory domain through which lytic granules may traverse. Confocal microscopy images show a human NK cell (YTS cell line) that expresses a CD2–GFP (green fluorescent protein) fusion protein making contact with a target cell (Epstein–Barr-virus-transformed B-cell line). The cell–cell conjugates were fixed, permeabilized and evaluated for the presence of perforin (red) using a monoclonal antibody. Perforin is contained in lytic granules and therefore can be used as a marker for them; the distribution of CD2 under normal conditions parallels filamentous actin at the mature synapse[12]. Microscopy images were produced by serial acquisition of optical slices along the *z*-axis and subsequent three-dimensional reconstruction *in silico*; the side view, 3/4 view and face on view of the cell–cell interaction are shown. An interactive version of this figure is available online (see Supplementary information S1 (figure)). pSMAC, peripheral SMAC.

Importantly, these initial steps in the formation of the NK-cell lytic synapse probably occur before molecular patterning or polarization are evident and are completed quickly. Whether the NK cell progresses to molecular reorganization at the synapse seems to depend on the level of signals received through inhibitory receptors, such as killer-cell immunoglobulin-like receptors, which can establish the so-called inhibitory synapse (BOX 3). Such regulation ensures that NK cells effectively carry out their surveillance function, by leaving most cells undisturbed while being ready to destroy cells that are diseased. The NK-cell inhibitory synapse is especially elegant in that it directly interferes with the ability of the lytic synapse to progress past the initiation stage[22–24].

Effector stage. After target-cell recognition and synapse initiation has occurred, and in the absence of overriding inhibitory signals, reorganization of the immunological synapse can proceed. The key steps of the effector stage can allow the following functions: formation of a stable NK-cell–target-cell interface that has a cleft into which cytolytic molecules are secreted; recruitment of lytic granules from throughout the cell to the synapse; clearance of a conduit in the NK-cell cortex through which lytic granules are thought to be directed to the cell membrane; and fusion of the lytic-granule membrane with the plasma membrane for the release of the lytic-granule contents. The exact sequence of events is debatable, but several observations support some linearity.

An early stage in the commitment to lytic-synapse formation is actin reorganization. This involves the formation of F-actin networks from the cellular pool of monomeric globular actin (G-actin) — a step that was first observed in polarized NK cells in 1983 using fluorescently labelled phalloidin (which binds junctions between approximated actin subunits)[25]. F-actin reorganization at the NK-cell synapse occurs downstream of activating-receptor-induced VAV1 activity[19,26] and depends on Wiskott–Aldrich syndrome protein (WASP)[27], which is a part of the protein unit that promotes F-actin branching. Accordingly, in the absence of WASP, or in the presence of actin inhibitors, F-actin accumulation at the synapse and NK-cell cytotoxicity are decreased[12,27–29]. The WASP-dependent reorganization of F-actin at this stage is also important for the characteristic changes in the shape of the NK cell that occur soon after synapse formation[2,12,27,29] (J.S.O., unpublished observations).

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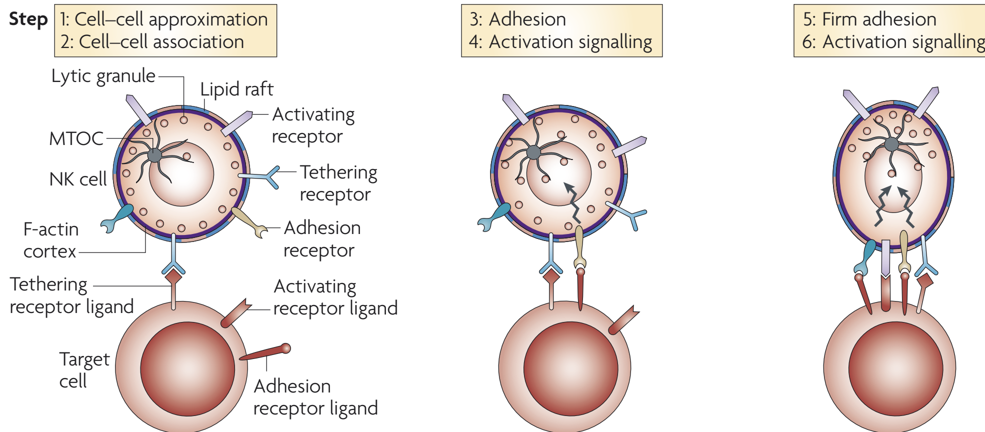

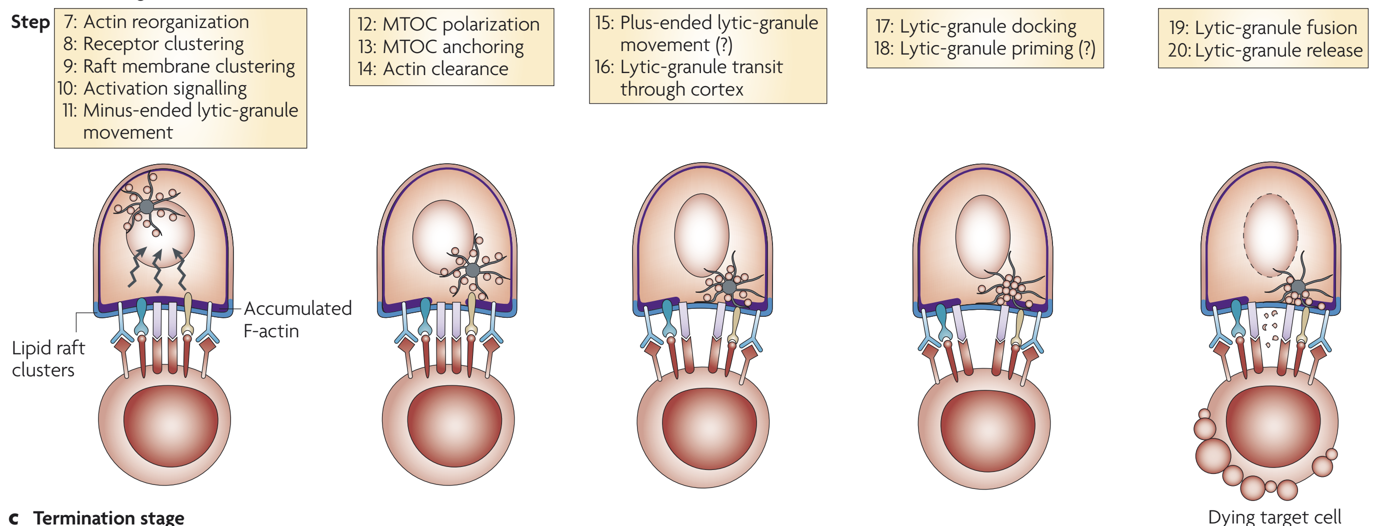

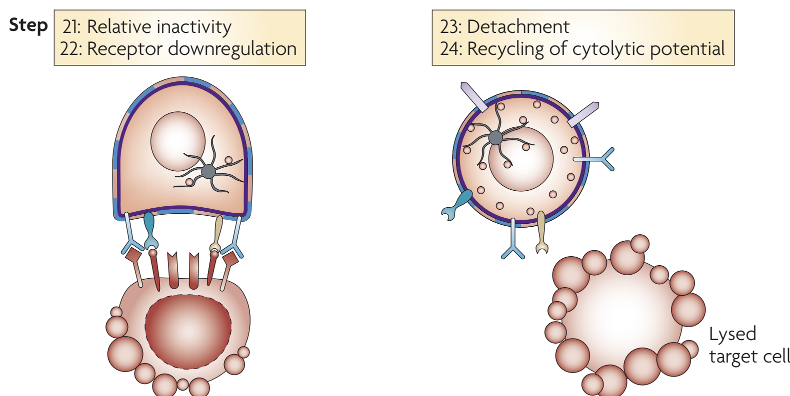

Figure 2 | **Model for sequential stages in the formation and function of the NK-cell lytic synapse.** It is proposed that the formation of a functional natural killer (NK)-cell lytic synapse can be divided into three main stages — initiation (**a**), effector (**b**) and termination (**c**) — that are each subdivided into multiple steps. Important steps that are proposed to occur in the initiation stage include adhesion and initial signalling for cell activation. In the effector stage, key steps include actin reorganization, receptor clustering, polarization of the microtubule-organizing centre (MTOC) and lytic granules, and lytic-granule fusion with the plasma membrane. The crucial steps of the termination stage are proposed to include a period of inactivity and detachment. The specific time required to progress through the various stages varies and is likely to be a feature of the given target cell and the activation state of the NK cell. A linear progression between certain steps (such as the requirement of actin reorganization for MTOC polarization) has been proven experimentally, whereas the linearity between other steps remains hypothetical at present. The inhibitory synapse (see BOX 3) has been shown to halt the progression of lytic-synapse formation by interfering with the late steps of the initiation stage (activation signalling) and early steps in the effector stage (actin reorganization and receptor clustering). F-actin, filamentous actin.

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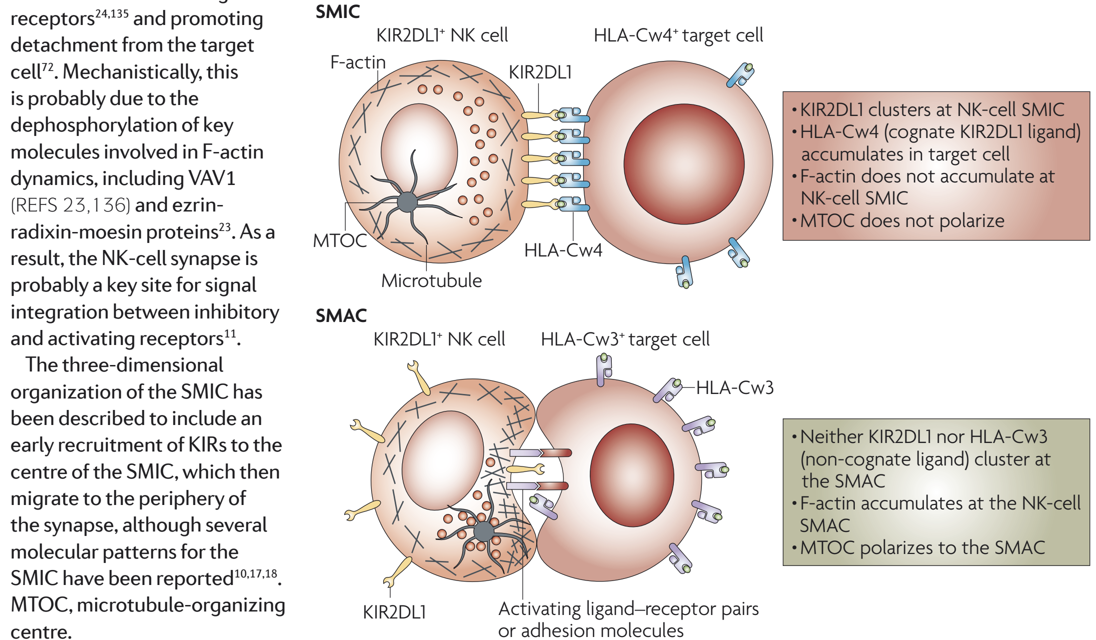
Box 3 | The inhibitory immunological synapse in NK cells

An important structure besides the lytic synapse that can form between a natural killer (NK) cell and another cell is the inhibitory synapse[17]. Because resting NK cells contain lytic granules and express germline-encoded activating receptors, a robust mechanism for restraining the formation of a lytic synapse is essential to avoid inadvertent cytolysis. NK cells express a family of inhibitory receptors that recognize determinants of self and prevent the lysis of healthy cells. This inhibitory activity is mediated through the formation of an inhibitory synapse. Here, inhibitory receptors such as the killer-cell immunoglobulin-like receptors (KIRs), which contain long cytoplasmic tails with immunoreceptor tyrosine-based inhibitory motifs, engage their ligands and induce the activity of phosphatases such as SHP1 (SRC-homology-2-domain-containing protein tyrosine phosphatase 1)[40,131]. The clustering of KIRs and associated components at the inhibitory synapse has been termed the supramolecular inhibitory cluster (SMIC)[17] (see figure). The SMIC is distinguished from the NK-cell supramolecular activation cluster (SMAC) in that it excludes lipid raft membrane domains[37,132–134], does not accumulate substantial filamentous actin (F-actin)[31] and contains inhibitory signalling molecules such as SHP1 (REFS 10,18,131,134). The function of the SMIC in preventing SMAC formation is achieved by preventing actin reorganization[22,23], blocking the recruitment of activating receptors[24,135] and promoting detachment from the target cell[72]. Mechanistically, this is probably due to the dephosphorylation of key molecules involved in F-actin dynamics, including VAV1 (REFS 23,136) and ezrin-radixin-moesin proteins[23]. As a result, the NK-cell synapse is probably a key site for signal integration between inhibitory and activating receptors[11].

The three-dimensional organization of the SMIC has been described to include an early recruitment of KIRs to the centre of the SMIC, which then migrate to the periphery of the synapse, although several molecular patterns for the SMIC have been reported[10,17,18]. MTOC, microtubule-organizing centre.



Lipid rafts
Liquid-ordered membrane domains that are enriched in sphingolipids and cholesterol, and also contain glycophosphatidylinositol-anchored receptors. They can provide ordered structure to the lipid bilayer and have the ability to include or exclude specific signalling molecules and complexes. Given that most techniques for studying lipid rafts are indirect (such as cell-membrane binding of cholera toxin, cholesterol sequestration using nonspecific reagents and cell fractionation based on detergent sensitivity), their physiological relevance and function remain controversial.

Microtubule-organizing centre
(MTOC). A structure that is found in all plant and animal cells from which microtubules radiate. The two most important types of MTOC are the basal bodies that are associated with cilia, and the centrosome, which is composed of γ-tubulin-ring complexes for microtubule nucleation.

The events that occur at the same time as actin reorganization have not yet been fully elucidated, but they include receptor clustering, lipid-raft aggregation, further activation signalling and lytic-granule redistribution. Although many receptors might be present in the synapse at the initial stages, some accumulate rapidly after cell conjugation, and these include those that are important in both cell adhesion and triggering of cytotoxicity. Of these, CD11a, CD11b and CD2 do not seem to cluster in the absence of actin reorganization[12], thereby providing some evidence for linearity in synapse formation. The mechanisms underlying actin-dependent receptor clustering in NK cells are not clear, but the ezrin, radixin and moesin (ERM) family of proteins are present in the NK-cell lytic synapse[23,30,31] and are known to function in lateral receptor motility in other cell types[32–35].

A related issue is that of lipid rafts. Whether these membrane domains represent discrete entities or more fluid collections of molecules is unclear, but they have been shown to aggregate at the NK-cell lytic synapse (reviewed in REF. 36) and their integrity is probably required for cytotoxicity, as shown in experiments using the nonspecific cholesterol-sequestering reagent β-methylcyclodextrin[37]. The existence and function of lipid rafts, unfortunately, have been defined using indirect methods, such as cholera toxin binding to the cell membrane, detergent-resistant biochemical fractionation and cholesterol sequestration, and therefore remain controversial. Similar to receptor clustering, however, lipid-raft aggregation at the NK-cell synapse depends on actin polymerization[23], which further supports a linear progression through the early effector steps of synapse formation.

It has been shown that receptor clustering at the NK-cell lytic synapse is important for the induction of robust signalling in NK cells[38]. In T cells, TCR signalling stems from microclusters of TCR molecules that move from the pSMAC to the cSMAC as activation proceeds[39]. Although functional microclusters have been identified in NK cells, these have only been investigated at the supramolecular inhibitory cluster[40]. So, it is unclear whether the level of signalling organization of the pSMAC and cSMAC that is observed in T cells can be similarly applied to the NK-cell lytic synapse.

Another requirement for effector function of the NK-cell lytic synapse is the polarization of lytic granules to the synapse. This begins with movement of the granules along microtubules to the microtubule-organizing centre (MTOC). Owing to the minus ends of

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microtubules being present at the MTOC, lytic granules require a minus-ended-directed motor, such as dynein, to move along microtubules. The exact motor used, however, has not been defined. While lytic granules aggregate around the MTOC, the MTOC also begins to polarize towards the immunological synapse. Signals that are required for MTOC polarization include ERK (extracellular-signal-regulated kinase) phosphorylation, VAV1 activation and PYK2 (protein tyrosine kinase 2) activity in NK cells[26,41,42]. In T cells, MTOC polarization also requires CDC42 (cell-division cycle 42)[43] and activation of a signalling platform comprising ZAP70 (ζ-chain-associated protein kinase of 70 kDa), SLP76 (SRC-homology-2-domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T cells)[44], as well as the function of the formins Diaphanous-1 and Formin-like-1 (REF. 45). The molecular motors used for the propulsion or the force-generating steps that are required to pull the MTOC to the NK-cell synapse have not been defined. CDC42-interacting protein 4 (CIP4; also known as TRIP10), which interacts with tubulin, CDC42 and WASP, localizes to the MTOC after synapse formation and is required for complete MTOC polarization[46]. Unlike CDC42 in T cells[42] or VAV1 in NK cells[26], however, reducing CIP4 function does not impair actin reorganization in NK cells[46]. Although the mechanism underlying CIP4 function is not known, it might actively participate in MTOC motility or it might help to anchor the MTOC to the NK-cell synapse by interacting with WASP or CDC42. Polarization of the MTOC in NK cells also probably depends on the appropriate insertion of the plus ends of microtubules into the accumulated F-actin at the synapse, which in T cells has been shown to be mediated through ADAP (adhesion- and degranulation-promoting adaptor protein)[48] and could function to apply force to the MTOC as the synapse reorganizes. In NK cells, the integrity and reorganization of the F-actin network is required for MTOC and lytic-granule polarization to the synapse, as blockade of actin function prevents such polarization[2,12,26], thereby further defining a linear series of events in synapse formation.

Requirements for cytokine secretion at the synapse are less well established but have been shown to require WASP in T cells[49] and can occur in both a directed and multidirectional manner[50]. However, there are several important differences between the cellular mechanisms underlying secretion of cytokines and lytic-granule contents, and the ability to separate the two events is probably crucial for achieving specificity in immune responses.

Although actin function is required for MTOC and lytic-granule polarization, a discrete region of the F-actin network needs to be disassembled to create a conduit through which the granules gain access to the plasma membrane. The function of these conduits remains hypothetical, however, large channels of 1–4 μm in diameter are often observed in the cSMAC of a prototypical NK-cell lytic synapse[12,18,51,52]. Theoretically, these conduits could also be smaller channels that are just large enough to allow the transit of the ~500 nm diameter lytic granules. The mechanism responsible for this targeted F-actin disassembly is unknown, but it seems to be independent of MTOC polarization, as actin clearance occurs normally in NK cells that are treated with microtubule depolymerizing agents[12]. So, this step occurs before MTOC polarization in the sequence that leads to synapse maturation.

Following MTOC polarization at the synapse, there are several ways in which the lytic granules might proceed through the conduit in the cortical F-actin. Originally, lytic granules were thought to traffic to the plasma membrane using plus-ended microtubule motors following their delivery to the synapse by the MTOC. Consistent with this, lytic granules have been shown *in vitro* to undergo plus-ended microtubule motility using kinesin[53]. Recently however, the MTOC in CTLs was shown to associate closely with the synapse and to directly deliver the lytic granules, without the need of additional plus-ended granule motility along individual microtubules[54]. The transit of lytic granules, or even the MTOC together with the granules, through clearances in the F-actin network in NK cells might require additional motor functions. Myosin II is a candidate protein for this function. Indeed, NK cells in which myosin II function is inhibited biochemically or in which myosin II expression is downregulated by small interfering RNAs form a mature lytic synapse with polarized MTOC and lytic granules, but do not degranulate[52]. Lytic-granule approximation to the membrane after polarization is therefore another important linear step in synapse maturation.

Once the lytic granules have traversed the F-actin and can interact with the plasma membrane, there are probably several final steps in the effector stage of NK-cell lytic-synapse formation. On the basis of concepts defined in neurological synapses and observations made in CTLs, these are predicted to include the docking of lytic granules to the synapse and their priming for membrane fusion. After vesicle priming, the membrane of the lytic granules could then fuse with the plasma membrane, causing the release of granule contents into the cleft that is formed between the two interacting cells. Although all of these events occur in the confined space of the synapse, individual steps can be visualized using electron microscopy[55] and fluorescent imaging techniques, such as total-internal reflection fluorescence microscopy[56].

Insights from CTLs are informative in defining these steps in NK cells, which at the moment are largely hypothetical. In CTLs, lytic-granule docking to the synapse requires members of the RAB family of small GTPases, which are important regulators of vesicle trafficking and compartmentalization (reviewed in REF. 57). RAB proteins undergo post-translational prenylation to gain hydrophobicity, which allows them to link to a cargo and facilitate their interaction with a target membrane. RAB27A carries out this function for the docking of lytic granules in CTLs[58]. Furthermore, active RAB27A can interact with key effector molecules that enable vesicle priming for fusion with the plasma membrane. In NK cells, MUNC13-4 (also known as UNC13D) has been shown to interact with RAB27A[59] and is probably responsible for vesicle priming. Interestingly, MUNC13-4 can be derived from distinct vesicles through a process of regulated fusion with developing

Vesicle priming
The process of preparing, through the acquisition of biochemical attributes, a secretory vesicle (such as a lytic granule) for fusion with the inner leaflet of the membrane.

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lytic granules, which suggests the existence of an independent step and, therefore, of additional components for regulating this stage of synapse formation.

Probable targets of lytic-granule-associated MUNC13-4 are members of the SNARE (soluble *N*-ethylmaleimide-sensitive-factor accessory-protein receptor) family. SNARE proteins act in a coordinated manner to facilitate the fusion of two distinct membranes (reviewed in REF. 60). SNARE proteins that are present on vesicles are termed v-SNAREs and those present on target membranes are termed t-SNAREs; an interaction between v-SNAREs and t-SNAREs is required for membrane fusion. Although direct interactions between MUNC13-4 and SNAREs have not been shown in NK cells, homologous proteins in other cell types have been shown to interact and mediate fusion[61,62]. The only SNARE components that have been identified so far to be required for lytic function in NK cells are the t-SNAREs VAMP7 (vesicle-associated membrane protein 7) (REF. 63) and syntaxin-11 (REFS 64,65). The v-SNARE syntaxin-7 is present in NK-cell lytic granules and is likely to participate in membrane fusion[66]. The regulation of SNAREs and other proteins of these late effector stages will probably prove to be crucial in the fine-tuning of the NK-cell lytic synapse.

Termination stage. Termination stages of the NK-cell lytic synapse refer to those that occur after the lytic-granule contents have been secreted. Although the sequence of events is hypothetical at present, they are known to include a period of inactivity and downmodulation of the accumulated activating receptors that is followed by the detachment of NK cells from the target cell and the recycling of their cytolytic capacity (FIG. 2). The cleft that is formed at the lytic synapse between the NK cell and the target cell creates a protected pocket of up to 55 nm deep that is present 45 minutes after conjugation[31]. It is unclear how long this cleft is stable for, but it probably remains intact during a period of relative inactivity after the granules have been released. The cleft and this delay in progression to subsequent stages may serve to increase the concentration of the lytic effector molecules that are exposed to the target cell while protecting neighbouring cells from exposure to these damaging molecules; this may be an important function of the lytic synapse *in vivo*.

After this time has elapsed, the synapse function is downmodulated. In T cells, this is achieved by TCR internalization through the cSMAC[39,67] and involves localized TCR ubiquitylation, which suggests that the receptors are targeted for degradation[68]. In NK cells, downregulation of the activating receptor NKG2D (natural-killer group 2, member D) from the synapse has been observed under some circumstances[69] and may also apply to other receptors, such as 2B4 and NKp46 (REFS 70,71). The purpose of the downregulation of NK-cell activating receptors and the mechanisms involved may be different from those underlying TCR downmodulation. Receptor downregulation at the NK-cell synapse could potentially occur at any point after signal generation is complete, but the persistence of certain activating receptors at the SMAC at late time points following conjugation[12,51] suggests that it might be a relatively late event.

Once the NK cell has carried out its cytolytic function, it can detach from the target cell and restore its ability to kill another susceptible cell. The physical process of detachment has been evaluated in the context of the NK-cell inhibitory synapse[72], and may result from reduced integrity of interactions between the F-actin cortex and the plasma membrane through dephosphorylation of ERM-protein targets[23]. Although this mechanism might also be involved in the detachment of the lytic synapse, it has not yet been defined in NK cells. There might, however, be additional active detachment processes that are similar to those that have been defined for the T-cell synapse, such as a role for protein kinase Cθ (PKCθ) in promoting synapse destabilization[73]. Indeed, PKCθ has been found to localize to the lytic synapse in NK cells[18].

After detachment, the NK cell can restore its cytotoxic potential by generating new lytic granules and re-expressing activating receptors. Early functional studies[74] suggested that some NK cells retain the capacity to form a second synapse immediately after dissipation of the first one, and more recent work defines a serial killing ability of activated NK cells[75]. This ability would require the NK cells to actively renew their functional capacity and might occur rapidly (within a few hours) given the kinetics of lytic-granule refilling that have been defined in CTLs[76]. The signals initiating the process of recycling NK-cell cytolytic capacity are not known but might be derived from those involved in cytotoxic function at the NK-cell lytic synapse. Interestingly, ligation of the activating receptor NKp30 induces rapid activation of nuclear factor-κB (NF-κB)[77], which has been shown to function as a transcription factor for the expression of the lytic-granule component perforin[78]. The physical process of detachment itself might also provide a signal for recycling in NK cells, although this notion remains hypothetical.

Human diseases affecting the NK-cell synapse

Primary immunodeficiency diseases in humans are characterized by genetic aberrations that impair immunological function, antimicrobial defence or both. Several of these diseases affect NK cells (reviewed in REFS 79,80) and an informative subset is characterized by a specific block in the stages that lead to the formation of a functional lytic synapse (TABLE 1). So far, none of the diseases in this subset has been found to exlusively impair NK cells and should affect all cytotoxic lymphocytes. So, insight into how the lytic synapse is formed in cells from patients with these diseases has been gained mostly from T-cell studies. For those considered here, however, the functional defect has at least been established in NK cells from patients with the disease. Although cytotoxic lymphocytes have crucial roles in host defence and immune regulation, there are likely to be specific contributions of NK-cell deficiency to the clinical phenotypes.

Most of these diseases can result in haemophagocytic lymphohistiocytosis (HLH)[81]. HLH represents an inappropriately robust immune response to infection (typically with herpesviruses), which results in a persistent systemic

NKG2D
(Natural-killer group 2, member D). A primary activating receptor that is encoded by the NK-cell gene complex and is expressed by all mature NK cells. It recognizes distinct families of ligands that are generally expressed only by infected, stressed or transformed cells.

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Table 1 | **Defects of the lytic synapse in NK cells from patients with genetic disease**

Disease	Gene	Protein	HLH phenotype	Effect on the lytic synapse	Step affected*	Refs‡
LAD-I	*ITGB2*	CD18	No	Decreased conjugation with target cell	3–5	87,88
WAS	*WAS*	WASP	Some	Decreased F-actin reorganization and integrin redistribution	6–10	12,27,91
CHS	*LYST*	LYST	Yes	Inability to generate normal lytic granules for trafficking to the synapse	6–10	98–103
HPS2	*AP3B1*	AP3 β-subunit	Yes	Inappropriate formation of lytic granules and movement along microtubules	6–10	106,107
GS2	*RAB27A*	RAB27A	Yes	Lytic granules move to the synapse but remain associated with microtubules	14–15	106,107
FHL3	*UNC13D*	MUNC13-4	Yes	Lytic granules move to the synapse but fail to dock and so do not achieve an intimate association with the NK-cell plasma membrane	16–17	85
FHL4	*STX11*	Syntaxin-11	Yes	Lytic granules polarize to and dock at the NK-cell plasma membrane but fail to fuse	18	64,65,114

*Step affected refers to the progress in formation of the natural killer (NK)-cell lytic synapse as depicted in FIG. 2.
‡References directly relevant to NK cells are provided, others indirectly related to NK cells are provided in the main text. *AP3B1*, adaptor-related protein complex 3, β1 subunit; AP3, adaptor protein 3; CHS, Chediak–Higashi syndrome; F-actin, filamentous actin; FHL, familial haemophagocytic lymphohistiocytosis; GS2, Griscelli syndrome type 2; HLH, haemophagocytic lymphohistiocytosis; HPS2, Hermansky–Pudlak syndrome type 2; *ITGB2*, integrin-β_2; LAD-I, leukocyte adhesion deficiency type I; *LYST*, lysosomal trafficking regulator; NK, natural killer; STX11, syntaxin-11; WAS, Wiskott–Aldrich syndrome; WASP, WAS protein.

inflammatory syndrome. This leads to the physiological symptoms of septic shock and is also associated with the pathological finding of haematophagocytosis (the ingestion of red blood cells by phagocytes). The defect in cytotoxic lymphocytes is believed to contribute to this phenotype, as the infected cells and other activated cells that promote inflammation cannot be eliminated (FIG. 3). NK cells may be most relevant to the HLH phenotype, given their localization to the marginal zones of lymphoid organs after viral infection, their innate function early in the course of infection[82] and their inherent ability to eliminate hyperactivated macrophages[83]. Although the defective NK cells are unable to eliminate infected cells by direct cytotoxicity, they can still carry out other functions, including the production of cytokines and the stimulation of inflammatory responses. This is consistent with the idea that the requirements for secretion of cytokines and lytic-granule contents at the synapse are distinct. However, the inability of NK cells to eliminate infected cells early in the infection (before T cells would have expanded) may be an important cause of HLH.

Other primary immunodeficiency diseases that impair cytolytic cell function but do not disrupt the formation of the NK-cell synapse can also result in HLH. For example, HLH occurs in patients with a mutation in the gene encoding perforin[84] and, in this setting, the disease is characterized by the normal secretion of lytic granules that are unable to mediate cytotoxicity owing to the absence of perforin[85].

The diseases that provide specific insight into the NK-cell lytic synapse are considered in two groups. Diseases in the first group affect steps that are involved in the initiation stage or the activation steps of the effector stage of synapse formation. Diseases in the second group affect steps in lytic-granule trafficking to the synapse in the effector stage.

Diseases affecting initiation or activation steps of NK-cell lytic-synapse formation. Leukocyte adhesion deficiency type I (LAD-I) results from a defect in the CD18 (β_2-integrin) component of leukocyte integrin heterodimers[86] (TABLE 1). Leukocytes from patients with LAD-I do not adhere to inflamed or activated cells properly and cannot localize effectively to tissues and sites of inflammation. This leads to increased numbers of leukocytes in the blood and susceptibility to infectious diseases. Because early steps in NK-cell synapse formation — adhesion and activation signalling — depend on integrins, NK cells from patients with LAD-I do not adhere to their target cells, which results in defective cytotoxicity[20,87–89]. LAD-I is distinguished from other diseases that are discussed here because it does not lead to HLH. This is presumably because NK cells from LAD-I patients do not form immunological synapses and are not activated through the synapse to produce cytokines.

Wiskott–Aldrich syndrome (WAS) results from a defect in actin reorganization and cell signalling in haematopoietic cells owing to WASP deficiency (reviewed in REF. 90) (TABLE 1). Patients lacking WASP expression or expressing abnormal WASP have NK cells with decreased cytolytic capacity[27,91]. Clinically, patients with WAS are susceptible to infection with herpesviruses[92] and can develop HLH[93–95], which is consistent with a functional role for WASP in NK-cell lytic-synapse formation. Accordingly, formation of the lytic synapse is abnormal in NK cells from WAS patients and is associated with decreased F-actin accumulation and adhesion-receptor clustering at the synapse[12,27,91]. This highlights a requirement for WASP in the early effector stages of lytic-synapse formation. Interestingly, exposure to interleukin-2 (IL-2) *in vitro* reverses the defect in WASP-deficient NK cells and restores lytic function and F-actin accumulation at the synapse[91,96]. This suggests that alternative

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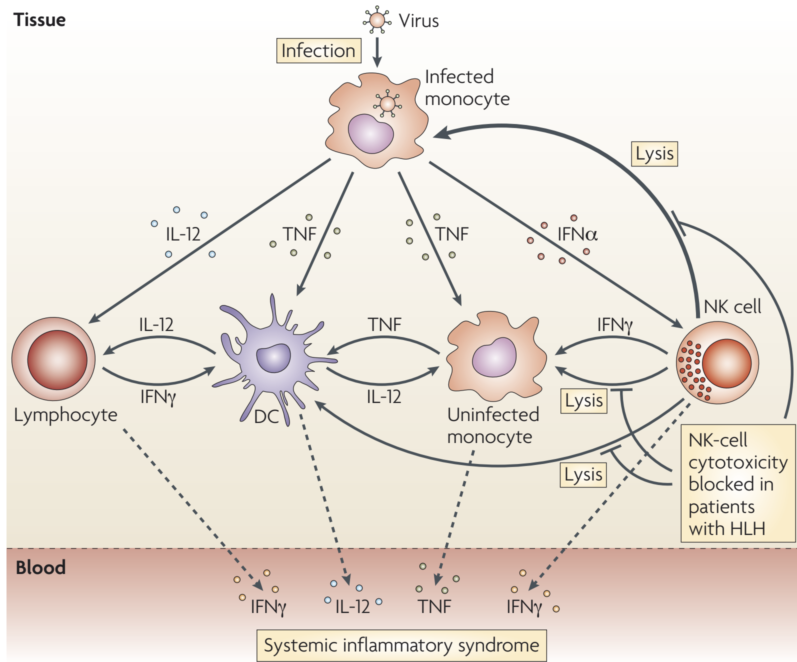

Figure 3 | **Proposed mechanism of haemophagocytic lymphohistiocytosis (HLH) immunopathogenesis owing to defective NK-cell lytic-synapse function.** Pathogenic viral infection in a normal individual leads to the production of pro-inflammatory factors, such as tumour-necrosis factor (TNF), interferon-α (IFNα) and interleukin-12 (IL-12) by the infected cell. This induces relevant responses from uninfected cells, including dendritic cells (DCs), monocytes, natural killer (NK) cells and other lymphocytes. These cells produce additional factors to further activate the induced cells and elicit the responses of others. Once induced, NK-cell cytotoxicity can help to rapidly eliminate the infected cell and serves to prevent further immune-cell activation that might be induced by the infected cell. NK-cell cytotoxicity can also eliminate other uninfected, activated monocytes and DCs to provide additional and crucial immunoregulatory function. In normal individuals, these processes usually remain localized and focused to the sites of infection. In an individual with impaired NK-cell cytotoxicity but a normal capacity for NK-cell activation, the NK cell might fail to lyse the infected cell. So, the inflammatory response continues unabated and leads to further activation of uninfected cells, as well as further pro-inflammatory activity by the induced cells. Although the infection may be localized, and the initial responding cells localized to the infection, the response amplifies and leads to an uncontrolled systemic inflammatory syndrome. The inflammatory response might control the viral replication, but without eliminating the source of the inflammation itself, the inflammatory response might not be containable.

(IL-2-inducible) pathways of actin polymerization exist in NK cells. At the baseline, however, WASP is crucial for lytic-synapse function but not for the earlier steps in synapse formation, which may be sufficient to support some pro-inflammatory functions and may occasionally result in an HLH phenotype.

Diseases affecting lytic-granule traffic to the NK-cell lytic synapse. Chediak–Higashi syndrome (CHS) and Hermansky–Pudlak syndrome type 2 (HPS2) both affect the normal formation of lytic granules and lead to the presence of 'giant' lytic granules. Both syndromes are also associated with albinism, which is caused by the aberrant function of melanocytes, the function of which is to pigment the skin through the secretion of melanosomes (an equivalent of lytic granules). CHS and HPS2 are similar in that they both result from a block in the late effector stages of NK-cell lytic-synapse function owing to a failure in the migration of the abnormal lytic granules along microtubules to the MTOC. CHS results from a mutation in the *LYST* gene, which encodes lysosomal trafficking regulator (reviewed in REF. 97) (TABLE 1). Most patients with CHS ultimately experience an 'accelerated phase' of the disease, which is an infection-induced HLH. Although not explored recently, older studies identified a defect in the cytolytic activity of NK cells from patients with CHS, despite normal NK-cell adhesion to target cells[98–102]. These defective NK cells feature abnormal giant lytic granules[103], and studies in CTLs indicate that such granules arise from the fusion of individual lytic granules[104].

HPS2 is caused by a mutation in the *AP3B1* gene (reviewed in REF. 105) (TABLE 1), which encodes the β-subunit of adaptor protein 3 (AP3) and, unlike CHS, HPS2 is associated with excessive bleeding owing to the lack of the platelet storage pool and ensuing abnormal platelet aggregation. In addition, patients with HPS2 have defective NK-cell cytotoxicity[106,107] and the HLH phenotype[107]. AP3 is required for the appropriate sorting of molecules from the Golgi into lytic granules. Similar to patients with CHS, CTLs from patients with HPS2 have enlarged granules that fail to move along microtubules[108].

Griscelli syndrome type 2 (GS2) is a third syndrome that combines albinism and immunodeficiency and is also associated with an accelerated phase of HLH. It is distinct from CHS and HPS2 in that patients have a broader range of hypopigmentation. GS2 is caused by a mutation in the *RAB27A* gene, which encodes the RAB27A small GTPase[109] (TABLE 1). NK-cell cytotoxicity is decreased in patients with GS2, but is not necessarily absent[110,111], and this phenotype can be reversed by culturing the NK cells in the presence of IL-2 (REF. 111). So, the mechanism underlying the defective lytic synapse in NK cells from patients with GS2 is also distinct from that in patients with CHS and HPS2. Studies carried out in mouse RAB27A-deficient CTLs show that lytic granules migrate and polarize towards the synapse but fail to dock at it[58]. Specifically, confocal microscopy of mouse RAB27A-deficient CTLs revealed that lytic granules were separate from the membrane. However, the reversal of this defect by IL-2 suggests there may be some redundancy in RAB27A function; RAB27B can in fact substitute for deficient RAB27A function under certain circumstances[112] and may provide a means for the activation-induced increase in lytic-granule traffic.

Familial haemophagocytic lymphohistiocytosis (FHL) types 3 and 4 are similar to GS2 but are not associated with albinism, which indicates that the affected genes are not essential in melanocytes. FHL3 is caused by a mutation in the *UNC13D* gene, which encodes MUNC13-4 (TABLE 1). Initially defined in CTLs, the lytic granules in MUNC13-4-deficient cells polarize towards the synapse and dock at the plasma membrane but do not fuse with it[113]. Defective cytolytic activity and decreased granule fusion with the plasma membrane have also been observed in MUNC13-4-deficient NK cells[85]. As described earlier, MUNC13-4 interacts with RAB27A and primes the lytic granules for SNARE-mediated fusion with the NK-cell plasma membrane at the lytic synapse.

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FHL4 is caused by mutations in the *STX11* gene, which encodes syntaxin-11 (REF. 114) (TABLE 1). Although NK cells from patients harbouring a *STX11* mutation are defective in cytotoxic activity, it was initially thought that syntaxin-11 conferred a co-stimulatory role in promoting susceptibility of monocytes to NK-cell cytotoxicity. However, subsequent studies indicated a direct role for syntaxin-11 in NK-cell degranulation[64,65]. As described earlier, syntaxin-11 is a t-SNARE in NK cells and presumably interacts with respective v-SNAREs to enable lytic-granule fusion. Importantly, the polarization of lytic granules to the synapse of NK cells from FHL4 patients without subsequent degranulation has been directly observed[65]. FHL4 patients have diverse clinical phenotypes, with some showing late onset of disease[115], which implies that syntaxin-11 mutations are hypomorphic or that there is some redundancy of the protein in enabling lytic-granule fusion. In support of some level of redundancy, IL-2 stimulation of NK cells from patients with FHL4 can restore degranulation[65], which suggests an activation-induced synthesis of proteins that complement defective syntaxin-11 function.

Concluding remarks

The NK-cell lytic synapse is formed in a series of stages that are required for cytolytic function. These include initiation, effector and termination stages that together enable the precise delivery of lytic-granule contents onto a susceptible target cell. Experimental investigations have defined some sequence to the individual steps, but they have also raised many new and unanswered questions. First, what governs the access of lytic granules from the NK-cell cytoplasm to the plasma membrane? A prerequisite conduit through the actin cortex at the synapse is probably a crucial element of cytotoxic cells, which may be generated by a mechanism as elegant as that of cytotoxicity itself. A second question relates to the regulation of the individual steps in synapse formation. Each step may have its own specific signal requirements or may simply represent a gradient emanating from strong initial signals. The former would enable the ideal fine-tuning of the cytotoxic host-defence mechanism.

Although there are important similarities between the lytic synapse in NK cells and the immunological synapse in T cells, there are also several notable known and potential distinctions. These may relate to the fact that NK cells are armed for cytotoxicity in the resting state and rely on a balance of signals from inhibitory and activating receptors to function in immunosurveillance. So, it is probable that studies of synapse regulation in NK cells will highlight mechanisms that control progression through the steps of lytic-synapse formation. Some of these may be increased or even specific to NK cells to provide fine-tuning of the innate immune response in the face of less antigen specificity compared with the adaptive immune response. Identifying these mechanisms spatially and in real time as they relate to the synapse will provide valuable insight into the control of the NK-cell cytolytic process.

Our understanding of the mechanisms of NK-cell lytic-synapse formation and function has also been obtained from the study of rare diseases that affect these processes. Investigation of the HLH phenotype, in particular, has advanced our understanding of the discrete stages of NK-cell lytic-synapse formation. Although a limited number of genes have been ascribed to the HLH phenotypes, there are many patients with defective NK-cell function and HLH for which the genetic defects are currently unknown. It is likely that further study of these individuals will uncover additional proteins that are involved in the process of NK-cell lytic-synapse formation.

Note added in proof

Recent work has shown a crucial role for HS1, the haematopoietic-cell-specific homologue of cortactin, in the late steps of the initiation stage and the early steps of the effector stage in the formation of the NK-cell lytic synapse[137]. Specifically, reduction in HS1 expression was shown to prevent full activation of integrins, optimal adhesion and actin accumulation at the synapse. This defines HS1 as a crucial early mediator of NK-cell lytic-synapse formation and function.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
CD2 | CDC42 | ezrin | IL-2 | LFA1 | MAC1 | moesin | RAB27A | radixin | STX11 | UNC13D | WASP
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
Chediak–Higashi syndrome | Griscelli syndrome type 2 | Hermansky–Pudlak syndrome type 2 | familial haemophagocytic lymphohistiocytosis | leukocyte adhesion deficiency type I | Wiskott–Aldrich syndrome

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