Molecular mechanisms of natural killer cell regulation

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1. ABSTRACT

Natural Killer (NK) cells are important for early immune reactions against viral infections and cancer. They are regulated by a highly redundant system of different activating and inhibitory receptors. Here we summarize our current understanding about the regulation of these cells and describe how mathematical modeling and systems biology approaches can help to shed some light on the complex regulatory network that governs NK cell reactivity.

2. INTRODUCTION

The activity of Natural Killer (NK) cells was first described in 1975 (1, 2). Without knowledge about the identity of the cells performing this natural cytotoxicity, NK cells were considered for a long time as being unspecific killers. Today, the identity of NK cells is well defined using different surface markers such as CD3⁻ /NKp46⁺ or genetic profiling (3). As these cells do not possess a large repertoire of antigen-specific receptors generated by somatic recombination, NK cells are
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Figure 1. Diversity of activating NK cell receptors and their ligands. Activating NK cell receptors (bottom) and their ligands (top). Members of the immunoglobulin-family of receptors are symbolized by ovals and lectin-like receptors are symbolized by half-circles. For a detailed description see text. \( \gamma \), Fc\( \gamma \) chain; \( \zeta \), CD3\( \zeta \) chain; Y, Tyrosine-based signaling motif distinct from ITAM or ITSM.

considered part of the innate immune system. The effector functions of NK cells can easily be summarized as cellular cytotoxicity and the production of cytokines and chemokines. However, in the 35 years since their discovery it became clear that NK cells are no unspecific innate killers, but represent a highly regulated, divers population of lymphocytes that fulfills an important role in early immune reactions and that engages in complex interactions with other immune cells. Various studies in humans and mice have demonstrated an important role of NK cells in the immune surveillance against transformed cells (4, 5). A 11 year follow-up study demonstrated that low NK cell activity is associated with an increased risk of developing cancer (6). Furthermore, NK cells are important for early immune reactions against viral infections such as cytomegalovirus, herpes simplex, influenza, poxvirus and others (7). Rare cases of selective NK cell deficiencies in humans result in overwhelming fatal infections during early childhood (8). In addition, NK cells play an important role during pregnancy (9), in regulating antigen-specific T and B cell responses and they have an impact on the function of DC, macrophages and neutrophils (10). Furthermore, NK cells are involved in mucosal immunity through the production of IL-22 (11), although the origin of these NK cell-like cells is currently debated. In line with these diverse functions it is not surprising that the process of NK cell regulation is highly complex. Here we will summarize the current knowledge of the molecular mechanisms that regulate NK cell activity. The emphasis of this review will be on receptor-mediated signaling pathways resulting in NK cell cytotoxicity.

3. NK CELL ACTIVATION

Although the name “Natural Killer” suggests that NK cells are like loaded guns and therefore ready-to-go, recent studies have demonstrated that NK cells do require priming by IL-15 presented by DCs or macrophages for their functional competence (12, 13). Therefore, like T or B cells, NK cells have to undergo a maturation process. But unlike these adaptive lymphocytes, who mainly rely on a single antigen receptor for their activation, NK cells can be stimulated through a large variety of different receptors. Some of these activating NK cell receptors are NKG2D, the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, members of the SLAM-family of receptors such as 2B4, NTB-A and CRACC, as well as DNAM-1, NKp80, NKp65 and the Fc\( \gamma \) receptor CD16 (14, 15) (Figure 1).

Several of these receptors can recognize multiple different ligands, adding even more complexity to the system (Figure 1).

Ligands for NKG2D are up-regulated on infected, stressed or transformed cells and include the MHC class I homologous proteins MICA, MICB and in humans the proteins ULBP1-6 that are structurally related to MHC class I (16). Cellular ligands for NKp46 and NKp44 are still unknown, while NKp30 recognizes B7-H6, which is expressed on certain tumor cells (17). 2B4 recognizes CD48, which is widely expressed in the hematopoietic system, and NTB-A and CRACC are homophilic (18). DNAM-1 binds to CD155 and CD112, which are expressed
Figure 2. Membrane proximal signaling events induced by the different classes of activating receptors. (left panel) The YINM motif of the NKG2D signaling adaptor DAP10 is phosphorylated by Src family kinases (SFK) and can recruit PI3K and Vav1 via the adaptor Grb2. (middle panel) The ITSM motifs of SLAM-related receptors are phosphorylated by SFKs and then recruit the adapters SAP and EAT2. (right panel) Phosphorylation of ITAMs by SFKs induce the recruitment of the kinases Syk and Zap70. See text for a more detailed description of the signaling pathways induced by these receptors. Solid arrows represent activation events and dashed arrows represent recruitment events.

NKG2D signaling

ITSM signaling

ITAM signaling

3.1. ITAM-based receptors

Several activating NK cell receptors signal via immunoreceptor tyrosine-based activation motif (ITAM)-containing partner chains. In this respect, the activation via CD16, Nkp30, or Nkp46, which signal via CD3ζ and/or FcRγ, or Nkp44, which signals via DAP12, is similar to what is known from T cell receptor or B cell receptor signaling pathways. In short, receptor engagement induces Src-family kinase-dependent phosphorylation of the ITAM sequence (Figure 2). This enables the recruitment of the kinases ZAP70 or Syk to the phosphorylated ITAM, resulting in the phosphorylation of the trans-membrane adapter molecules LAT and NTAL and the cytosolic adapters SLP76 and 3BP2. This induces the assembly of signaling complexes via the recruitment, phosphorylation and activation of signaling molecules such as phosphatidylinositol-3-OH kinase (PI3K), phospholipase C (PLC-γ1 and PLC-γ2) and Vav1, 2, 3.

3.2. NKG2D

NKG2D can also signal via DAP12 in mice (22), which would follow the above described signaling pathway. However, in humans NKG2D couples exclusively to DAP10 (23), which contains a different tyrosine-based signaling motif (YINM) (Figure 2). This can be phosphorylated by Src-family kinases and recruit PI3K or the adapter Grb2, which binds Vav1 (24). As the binding sites overlap, one DAP10 molecule can either interact with PI3K or Grb2. However, NKG2D is a hexameric complex consisting of one NKG2D dimer associated with two DAP10 dimers (25). Therefore, both signaling pathways can be initiated by one receptor complex. Vav1 recruitment and phosphorylation results in the phosphorylation of SLP-76 and PLC-γ2 and leads to actin reorganization via the activation of small G proteins of the Rho family such as Rac-1 or Cdc42 (26-28), and to polarization of the microtubule organizing center (MTOC) toward target cells (24). As a result, Vav1 deficient NK cells are defective in NKG2D-mediated cytotoxicity (29, 30). PI3K activation results in the production of phosphatidylinositol-3,4,5-trisphosphate, facilitating the membrane recruitment of Tec-family kinases and further supporting the recruitment of PLC-γ, Grb2 and Vav1 (31). Therefore, PI3K and Grb2 binding to DAP10 are both necessary for efficient NKG2D-mediated Ca2+ flux (24). Additionally, Vav1 is necessary for efficient PI3K activity downstream of NKG2D (30). This demonstrates that activation of PI3K and Vav1 are not separate events induced by the recruitment of either signaling molecule to DAP10, but that these two signaling pathways are interconnected and are likely both initiated by a single NKG2D receptor complex. However, in contrast to ITAM-dependent receptors, signaling of human NKG2D is independent of LAT and NTAL and does not require Syk or ZAP70 kinases (32-34). However, NKG2D-mediated cytokine production is defective in mice lacking Syk family kinases (34).

3.3. SLAM-family receptors

Signaling of the SLAM-family receptors 2B4, NTB-A and CRACC is again different from the events initiated by ITAM-coupled receptors or by NKG2D (18). These receptors do not couple to any signaling partner chains, but possess their own tyrosine-based signaling motifs...
motif in their cytoplasmic tail (Figure 2). This so-called immunoreceptor tyrosine-based switch motif (ITSM) can be phosphorylated by Src-family kinases and recruit the small SH2 domain containing adapter molecules SAP (SH2D1A), EAT-2 (SH2D1B), or ERT (SH2D1C) (35-38). Association of SAP is essential for the signal delivered by 2B4 and NTB-A. SAP is mutated in patients suffering from X-linked lymphoproliferative disease (XLP) resulting in defective 2B4 and NTB-A function in these patients (39-43). SAP can recruit the Src family kinase Fyn through an untypical SH2-SH3 domain interaction (44, 45), which is likely the basis for the positive signal transmitted by SAP-dependent receptors. As a result, 2B4 function is defective in Fyn deficient mice (46). Signaling through 2B4 further involves the cytosolic adapter 3BP2 and results in the phosphorylation and activation of LAT, PLC-γ1 and Vav1 (47-51). In contrast, CRACC function is independent of SAP and seems to rely on the association with EAT-2 (52, 53). EAT-2 also plays a role in the signaling of NTB-A (36, 54). However, it is unclear how 2B4 can transmit a positive signal. While EAT-2 may also be able to recruit Src-family kinases (55) another report has demonstrated that murine EAT-2 can have an inhibitory effect through the phosphorylation of two Tyrosines in its C-terminal region (38). However, this inhibitory signaling may depend on the genetic background, as another report has found activating properties of EAT-1 and ERT in C57BL/6 mice (56). Another controversy in field of SLAM-family receptor signaling is the fact that these receptors may transmit inhibitory signals. This could be due to the ability of the ITSM to also bind the phosphatases SHP-1, SHP-2 or SHIP instead of SAP (37). Inhibitory function of 2B4 has mostly been reported in mice (38, 57), although other reports demonstrate an activating function of mouse 2B4 (46, 54). These differences could be explained by the experimental systems used as the amount of 2B4 and SAP expression as well as the strength of receptor cross-linking can determine if 2B4 is activating or inhibitory (58).

3.4. Complexity and robustness of NK cell activation

The signaling induced by other activating NK cell receptors such as DNAM-1, Nkp80 or Nkp65 is again different from the events described above (15). In addition, integrin signaling is also important for NK cell function, as LFA-1 has been shown to be important for the polarization of the lytic granules (59). Different receptors can also synergize in the activation of resting human NK cells. While some receptors are very effective co-activators in combination with other receptors (e.g. NKG2D and 2B4 or Nkp46 and DNAM-1), other receptor combinations merely show an additive effect (60). One possible mechanism responsible for this synergistic effect is the enhanced activation of Vav1 (61). Due to this multitude of different activating receptors in NK cells, the receptor proximal signals can be very complex and divers, depending on which receptors are triggered by a specific target cell. This makes NK cell activation a highly redundant and therefore robust process. In support of this robustness, genetic deletion of Syk and ZAP70 does not significantly affect NK cell development or cytotoxicity (62). Similarly, NK cells are not significantly impaired by the deletion of all ITAM containing transmembrane adapters (33) and NK cell cytotoxicity is not affected by the absence of CD45 (63-65).

3.5. Common signaling pathways in NK cell activation

Most studies only investigate the signals initiated by one receptor. We are therefore only beginning to understand the complexity and cross-talk of signals induced by triggering combinations of different receptors by the various ligands expressed on a target cell. However, there are also common downstream signaling pathways induced by all activating NK cell receptors. Ca2+ flux induced by the activity of PLC-γ is essential for the exocytosis of lytic granules. PLC-γ1 and PLC-γ2 are differentially used by different activating receptors and have non-redundant roles in NK cells (66, 67). Deletion of PLC-γ2 results in defective NK cell cytotoxicity (68, 69). Influx of extracellular Ca2+ via the calcium release-activated calcium channel ORAI1 is essential for NK cell degranulation and cytotoxicity (70). ERK activation, stimulated through a PI3K – Rac1 – MEK pathway is also important for granule polarization and release (71) and inhibition of JNK can similarly affect this process (72).

Actin polymerization plays an essential role during NK cell activation. It is involved in the clustering of activating receptors, the formation of the immunological synapse between NK and target cells and may also play a role in granule polarization and degranulation. It is therefore not surprising that patients with a defect in Wiskott-Aldrich syndrome protein (WASp), a protein involved in actin polymerization, show impaired synapse formation and NK cell cytotoxicity (73, 74). Similarly, pharmacological inhibition of actin polymerization interferes with the clustering of activating receptors and inhibits NK cell activation (75, 76). Actin dynamics in NK cells are regulates by small G proteins of the Rho family such as Rac-1 or Cdc42, which in turn are activated by the guanine nucleotide exchange factor Vav (26-28). NK cells express Vav1, Vav2 and Vav3 and use these different isoforms depending on the activating receptor triggered. NKG2D/DAP10 and 2B4 signaling induces Vav1 phosphorylation, whereas Vav2 and Vav3 are activated by ITAM-coupled receptors (29, 50, 77, 78). Also integrin signaling can result in Vav1 activation (79). Therefore, the early interaction between NK and target cells through integrins and activating receptors will lead to Vav activation, resulting in actin reorganization. This induces the clustering of activating receptors in the immunological synapse and facilitates their recruitment into specialized membrane domains, often referred to as lipid rafts (76, 80-82). Lipid rafts or membrane microdomains are known to play an important role in membrane organization, lipid sorting and signal transduction (83). Through clustering and accumulation in membrane microdomains the activating receptors are brought into close proximity to each other in an area that is enriched in Src-family kinases (84). This ligand-induced proximity is believed to be the basis for the triggering of activating receptors.

NK cell activation is in many aspects similar to the events known from the stimulation of B or T cells through the BCR or the TCR, respectively, especially in the
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case of ITAM-based NK cell receptors. However, in contrast to these lymphocytes, NK cells can be activated by many different receptors, which can cooperate with each other and which induce different receptor proximal signaling events. In addition, NK cell regulation is not only dependent on activating receptor signaling, but also influenced to a great extend by the signals from inhibitory receptors.

4. NK CELL INHIBITION

As activating NK cell receptors recognize also ligands on normal, healthy cells, the activation of effector functions has to be tightly controlled to avoid autoactivity. NK cells therefore express various inhibitory receptors that can counteract activation. The first, and probably most important mechanism of NK cell inhibition that was discovered in the 1980s is based on the recognition of MHC class I molecules on cells resistant to lysis by NK cells (85, 86). It is remarkable, that even though these receptors are very heterogeneous and fast evolving, the basic principle is conserved among species and most of the receptors have the same signaling properties.

4.1. Inhibitory receptors

The first inhibitory NK cell receptors described were the C-type lectin like Ly49 receptors in the mouse (87) now also called killer cell lectin-like receptor family a (Klra). Ly49 receptors are a family of type II transmembrane proteins that recognize MHC class I proteins. Some family members have inhibitory function as they carry an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic tail. Other Ly49 receptors instead interact via a charged amino acid in their transmembrane domain with the ITAM-containing adapter DAP-12. These receptors are therefore activating and their ligands are not restricted to MHC class I but they can also recognize MHC class I homologue viral ligands (88, 89). As an example, the recognition of the mouse cytomegalovirus protein m157 leads to control of the viral infection in mice expressing the respective receptor Ly49H.

The first inhibitory receptors identified on human NK cells were killer cell Ig-like receptors (KIR) (90, 91). The KIR receptors can be divided in two groups, based on their extracellular domains: they either contain two (KIR2D) or three (KIR3D) Ig-like domains. Furthermore, similar to Ly49 receptors, they are distinguished by the length of their cytoplasmic tail. Those with a long (L) cytoplasmic tail (KIR2DL and KIR3DL) contain an ITIM and function as inhibitory NK cell receptors, while those with a short (S) cytoplasmic tail (KIR2DS and KIR3DS) deliver activating signals by coupling to DAP12. KIR receptors specifically recognize certain human leukocyte antigen (HLA)- A, -B or -C allotypes, but unlike T cells, this is not peptide specific although the peptide can contribute to KIR binding (92, 93). NK cells also express inhibitory receptors of the C-type lectin-like family, which are conserved between mice and human: NKG2 members forming heterodimers with CD94. The NKG2/CD94 complex specifically binds the non-classical MHC class I molecule HLA-E in human (94) and the functional homologue Qa-1 in mice (95). HLA-E and Qa-1 present the leader peptide of classical MHC class I molecules. Surface expression of HLA-E and Qa-1 is therefore a marker for the overall MHC class I expression. The NKG2/CD94 receptor complex also comes in inhibitory and activating forms: NKG2A and B contain an ITIM in their cytoplasmic domain whereas NKG2C and E couple to DAP12 and can deliver activating signals.

NK cells also express other inhibitory receptors. LILRB1 binds to a conserved region of HLA class I and can inhibit NK cell activation (96). Siglec-7 and -9 (CD328 and CD329) are expressed on NK cells (97) and may be responsible for mediating the tolerance of NK cells towards healthy MHC class I low or negative cells, like cells of the nervous system (98). Other receptors that can inhibit NK cell functions are LAIR-1 (CD305) which binds to collagen (99), KLRG1 which binds to cadherins (100, 101), CEACAM-1 (BGP, CD66a) which is homophilic (102), PILR which binds to CD99 (103, 104), CD300a (IRp60) which has no known ligand (105), and NKR-P1 (KLRB1, CD161) which binds to human LLT1 (106, 107). This complex receptor repertoire makes it hard to study the importance of individual receptors and their interplay under physiological conditions.

4.2. ITIM signaling

Although NK cells possess many different inhibitory receptors, the signaling of most of these receptors is similar and is based on ITIM sequences in their cytoplasmic tail (91). These ITIMs become tyrosine phosphorylated upon ligand binding (108), resulting in the recruitment of the phosphatases SHP-1 and SHP-2 (109, 110) (Figure 3). Different ITIM-containing receptors display a relative selectivity for the individual phosphatases (91). This binding increases phosphatase activity (111), which might further be stabilized by phosphorylation (112). For KIR2DL1 it has been shown that association of β-arrestin 2 results in enhanced recruitment of SHP-1 and SHP-2 (113), but the underlying mechanism and how this contributes to NK cell inhibition is still unknown. Furthermore, the C-terminal Src kinase (Csk) can bind to the ITIM of e.g. human LILR (114). Csk phosphorylates the inhibitory tyrosine of Src family kinases, thereby stabilizing the closed, inactive conformation.

The only direct target of KIR-associated SHP-1 that could be identified so far is the guanine nucleotide exchange factor Vav1 (115). By blocking Vav phosphorylation inhibitory receptors could therefore effectively interfere with actin polymerization, the clustering of activating receptors, formation of the immunological synapse and many of the downstream signals that have been described above (Figure 3). In addition, KIR or CD94-NKG2A engagement results in the phosphorylation of the small adapter Crk leading to an active disassembly of complexes between Crk the scaffold protein c-Cbl and the guanine nucleotide exchange factor C3G (116). These complexes are involved in actin remodeling and LFA-1-mediated adhesion. Active
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disassembly of these complexes through the induced phosphorylation of Crk may therefore be another signaling function of ITIM-containing receptors and would contribute to their inhibitory effect on actin-dependent activation processes.

4.3. NK cell education

The inhibitory receptors are expressed in a seemingly random manner on NK cells, although there is emerging evidence that expression of KIRs is not completely stochastic (117). For the Ly49 gene cluster a probabilistic binary transcriptional switch has been described that provides a mechanism for selective and stable activation of Ly49 transcripts in a subpopulation of cells (118). Furthermore, the repertoire of inhibitory Ly49 receptors is skewed in mice with different MHC class I backgrounds, suggesting an additional regulation of Ly49 expression by the presence or absence of their respective ligands (119). As inhibitory receptors were thought to be necessary to maintain self-tolerance, the hypothesis was that every NK cell has to express ‘at least one’ self-specific KIR or NKG2A (120). But in humans and mice lacking the expression of MHC class I NK cells are not auto-reactive, despite the absence of inhibitory ligands (121, 122). In contrast, they are hypo-responsive. Similarly, the subset of mature NK cells in humans or mice, which does not express an inhibitory receptor for self-MHC class I is also hypo-responsive (123-125). These findings led to the hypothesis, that NK cells undergo some form of education to ensure the proper reactivity of these cells. Either NK cells are non- or hypo-responsive and need the inhibitory signal to become activated (licensing or arming model) or they are initially auto-reactive, but get anergic in the absence of inhibitory signal (disarming model) (126, 127).

Recently it has been demonstrated in mice with various MHC class I backgrounds that this education of NK cells even is dose-dependent. The more MHC class I alleles are expressed in the host, the higher is the reactivity of NK cells to recent findings demonstrating that the responsiveness of mouse NK cells can change depending on their MHC class I environment and is therefore not cell intrinsic (131, 132).

5. SIGNAL INTEGRATION IN NK CELLS

One major difference between NK cells and other lymphocytes is that NK cell regulation is critically dependent on activating and inhibitory signaling. Therefore, NK cells are ideally suited to investigate how these opposing signals are integrated.

5.1. What do inhibitory receptors inhibit?

Inhibitory receptors can influence many different aspects of NK cell activation. As one of the first steps, firm adhesion to target cells is affected (133). NK cell adhesion to target cells is dependent on integrins. Integrin activity is regulated by inside-out signaling, a process by which activating NK cell receptors can induce the high affinity conformation of LFA-1. Inhibitory receptors can block these inside-out signals mediated by different activating receptors (134). This may be connected with the effect of inhibitory signaling on the early signal transduction of activating receptors. Inhibitory receptors control the engagement of membrane microdomains (‘lipid rafts’) to the immunological synapse (81, 82). This prevents the recruitment of activating receptors such as 2B4 and NKG2D in these membrane domains (76, 80), thereby affecting the receptor phosphorylation (50). These results show, that inhibitory receptors can interfere with the earliest stages of NK cell activation.

Another prominent effect of inhibitory signaling is its influence on actin dynamics. Inhibitory receptors prevent the accumulation of actin at the immunological synapse (135). Actin dynamics are essential for the formation of activating synapses and the clustering of activating receptors. It is therefore not surprising that inhibitory receptors can interfere with many signaling steps necessary for NK cell cytotoxicity, including the phosphorylation of various signaling molecules, Ca$^{2+}$ flux, the polarization of lytic granules and ultimately degranulation (136). One central question is if inhibitory receptors interfere with all these events individually, or if these effects are the result of one specific upstream event, which is selectively targeted by inhibitory receptors. In fact, inhibition seems to be a very precise and selective event. An NK cell that is inhibited via its attachment to a resistant target cell may simultaneously bind and kill another susceptible target cell (137). This demonstrates that although inhibitory signals can prevent many types of effector responses, they do not globally inhibit NK cell functions, but rather restrict their effect within the area close to an attached target cell.

5.2. Integration of activating and inhibitory signals: The Vav1 Hypothesis

How can inhibitory receptors effectively control NK cell activation mediated by many different activating and co-activating receptors, while restricting their effect to a confined region within the NK cell? Vav1 has been identified as a direct target for SHP-1 when recruited by an inhibitory NK cell receptor (115). This may therefore represent the first step at which activating and inhibitory signals converge. Vav plays an important role in Rho-family GTPase-mediated actin polymerization, synapse formation, clustering of activating receptors and is essential for NK cell cytotoxicity (29, 78). Early phosphorylation of Vav by different activating receptors or through the engagement of LFA-1 (79) would induce actin polymerization necessary for the clustering of activating receptors and the formation of the immunological synapse (Figure 3). The signals by these activating receptors would further enhance Vav phosphorylation through a positive feed-back loop, ultimately resulting in full NK cell activation and degranulation. By blocking this early Vav phosphorylation, inhibitory receptors could effectively interfere with this positive feed-back loop and would prevent polarization of activating receptors and signaling.
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Figure 3. Model for the integration of activating and inhibitory signals. Engagement of activating receptors induces the phosphorylation of Vav, resulting in the actin-dependent clustering of activating receptors and their recruitment to lipid rafts. This leads to more Vav phosphorylation, inducing a positive feed-back loop, ultimately resulting in NK cell cytotoxicity. Activation of SHP-1 by the engagement of inhibitory receptors results in the de-phosphorylation of Vav. Additionally, though Abl-dependent phosphorylation of the adapter Crk the activating Cbl–Crk–C3G complex is disassembled. These events block the actin remodeling necessary for the positive feed-back loop of activating receptors, thereby effectively controlling NK cell activation. Solid arrows represent activation events, dashed arrows represent recruitment events and blunt arrows represent inhibition events.

components towards the target. This mechanism would be consistent with the observation that polarization is the main target of inhibition (138). Additionally, by inducing the phosphorylation of Crk (116) and thereby inhibiting complexes between Crk, c-Cbl and C3G, which are also regulators of actin dynamics, inhibitory receptors would further block these actin-dependent processes. This mechanism could explain how inhibitory receptors can control NK cell activation mediated by many different receptors, coupling to diverse signaling pathways. By affecting local Vav phosphorylation and thereby actin polymerization, the inhibitory signal would be confined to a region within the NK cell, thereby still allowing the killing of other sensitive targets (137). However, such a central role of Vav in the inhibition of NK cell activity has not been formally proven. Therefore, it cannot be excluded that inhibitory receptors initiate also other events that act in parallel of Vav dephosphorylation in order to control NK cell reactivity.

6. MATHEMATICAL APPROACHES TO NK CELL REGULATION

T cell activation is regulated by a complex network of signals initiated through the TCR and costimulatory molecules such as CD28. Mathematical modeling and systems biology approaches have greatly contributed to the understanding of these complex networks (see other articles in this issue). As outlined above, NK cell activation is even more complex, as it is regulated by many different receptors, which are coupled to diverse signaling pathways and act in synergy, and which are influenced by inhibitory signaling. In addition, individual NK cell populations seem to differ significantly in their effector functions (139, 140). Up to now we have mostly used classical methods such as biochemistry, functional assays and flow cytometry to investigate NK cell regulation. However, these methods have their limitations when it comes to complex regulatory networks. Therefore, mathematical modeling and systems biology approaches are needed to get further insight into the complex field of NK cell regulation.

In a purely theoretical approach one study has developed a detailed molecular model of NK cell activation, incorporating membrane-proximal signals and affinities of receptor-ligand interactions (141). This approach suggested that Vav and Erk activation are digital in nature on the single-cell level, even in the absence of any positive feedback. Such digital behavior of Erk phosphorylation matches previous results with T cells (142), where opposing feedback loops of positive (Erk) and negative (SHP-1) signals created this response.

Another study used an ‘ensemble modeling’ approach (143) combined with experimental verification to investigate the interplay of activating and inhibitory signals in NK cells (144). This study experimentally confirmed a digital Vav phosphorylation and de-phosphorylation induced by activating and inhibitory signaling, respectively.
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The mathematical modeling revealed that the association of Src-family kinases with activating but not with inhibitory receptors was essential for such a response. While this might suggest a direct association between activating NK cell receptors and Src-family kinases, it could also support the known role of membrane microdomains in NK cell activation (76, 80-82). As these domains are enriched in Src-family kinases, the recruitment of activating receptors to these domains might be the important event, which brings kinases and receptors in close proximity. Mathematical modeling further suggested that Vav phosphorylation may indeed be the signaling event where activating and inhibitory signals are integrated (144). The digital nature of Vav phosphorylation (141, 144) could explain how these opposing signals are integrated to come to a yes or no decision about NK cell activity, resulting in the survival or the death of an attached target cell.

Another field where mathematical modeling has been used to understand NK cell biology is the acquisition of inhibitory receptors by developing NK cells. As the recognition of self-MHC class I is so important for the development and functional maturation of NK cells, the question arose, if inhibitory receptors are expressed in a stochastic manner on individual cells. In mice, differences in MHC class I expression result in a different distribution and frequency of Ly49 receptor combinations (145, 146). This could be explained by selection of NK cells after random expression of Ly49 receptors, or by the sequential expression of Ly49 receptors until maturation is possible (147). Comparing experimental data with mathematical simulations of both possibilities favored the selection of mature NK cells (148, 149). Following the same idea, Andersson and colleagues analyzed a human cohort for KIR expression (117). Their results argue against both previous theories, revealing that KIR acquisition probabilities are mostly independent of self MHC class I expression, but could be based on genetically predisposed expression probabilities. This might reflect a difference in inhibitory receptor acquisition between human and mouse NK cells.

Inhibitory receptors have a great impact on NK cell responsiveness through the process of NK cell education. As described above, the molecular basis for this process is still debated. Theoretical studies have suggested that inhibitory receptors could indeed transmit positive signals under certain circumstances (141, 144). This could contribute to the licensing of NK cells, during which it was suggested that inhibitory receptors have a positive impact on NK cell maturation (126). While these are interesting concepts, they will have to be verified in experimental settings.

7. FUTURE CHALLENGES

The reactivity of mature NK cells is regulated by positive and negative signals generated by diverse receptors. This complicated signaling network is more complex and redundant than the one in T lymphocytes. However, while the understanding of T cell signaling has greatly benefited from mathematical modeling and systems biology approaches, surprisingly few studies use such approaches to study NK cell regulation. These technologies harbor a great potential for the understanding of NK cell signaling and regulation. We can therefore be hopeful that we will learn much more about NK cell regulation in future studies.

Complex regulatory networks do not only dictate the reactivity of mature NK cells. They also influence NK cell development and education. In addition, recent studies have demonstrated the existence of long-lived NK cells that can mediate memory responses (150). It is completely unknown how activating and inhibitory signals differ between memory and conventional NK cells. Therefore, while we have learned so much about the function, regulation and the biology of NK cells in the past 30 years, there are still many more open questions that will need to be answered. Mathematical modeling and systems biology approaches may be the only way to really understand these complex and redundant immune cells.

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