REVIEW ARTICLE

NK cell recognition of hematopoietic cells by SLAM-SAP families

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The signaling lymphocyte activation molecule (SLAM) family of receptors (SFRs) are ubiquitously expressed on immune cells, and they regulate multiple immune events by recruiting SH2 (Src homology 2) domain-containing SAP family adapters, including SAP and its homologs, Ewing's sarcoma-associated transcript 2 (EAT-2) and EAT-2 related transducer (ERT). In human patients with X-linked lymphoproliferative (XLP) disease, which is caused by SAP mutations, SFRs alternatively bind other inhibitory SH2 domain-containing molecules to suppress immune cell activation and development. NK cells express multiple SFRs and all SAP family adapters. In recent decades, SFRs have been found to be critical for enhancing NK cell activation in response to abnormal hematopoietic cells in SAP-family-intact NK cells; however, SFRs might suppress NK cell activation in SAP-family-deficient mice or patients with XLP1. In this paper, we review how these two distinct SFR signaling pathways orchestrate NK cell activation and inhibition and highlight the importance of SFR regulation of NK cell biology and their physiological status and pathological relevance in patients with XLP1.

Keywords: SLAM; SAP; NK cells; immune signaling

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INTRODUCTION

Natural killer (NK) cells play an essential role in innate defenses against "unwanted" allogeneic bone marrow transplants or autologous cells undergoing various forms of stress, such as transformed tumorous cells and virus-infected cells. NK cells kill these cells via a sequential process, including recognition, formation of a conjugate and synapse, polarization and targeted killing. One of the most important steps is discrimination between self cells and nonself or modified-self cells, which is mediated by NK cell receptors. In contrast with adaptive T cells and B cells, NK cells have no antigen-specific TCRs or BCRs that enable this recognition. There are two distinct types of NK cell receptors, activating receptors and inhibitory receptors. Whether NK cells eliminate these abnormal cells largely depends on the signaling balance originating from these cell receptors.

The mouse inhibitory Ly49 family and the human killer inhibitory receptors (KIRs), as well as NKG2A located in the NK gene complex in both mice and humans, represent the major NK cell inhibitory receptors.[1–6] They can specifically sense the presence of MHC class I (MHC-I) molecules, which are considered self-cell markers.[7] MHC-I molecules on normal self-cells can sufficiently elicit inhibitory signaling leading to NK cell inactivation. However, the "unwanted" cells usually lose or express mismatched MHC-I and thus fail to elicit sufficient inhibitory signals. Under "missing-self" status, these cells become susceptible to NK cell-mediated lysis. However, it remains unclear whether the absence of self-MHC-I molecules is sufficient for NK cell activation. NK cells also bear many activating receptors, such as CD16, natural killer gene 2D (NKG2D), natural cytotoxicity receptors, and activating KIRs in humans (Ly49D and Ly49H in mice).[2,8,9] They execute NK cell recognition in a "modified self" manner, by which they detect stress-inducible ligands unduly expressed on tumorous or virus-infected cells.[2–4] After engagement with their respective ligands, these receptors initiate various activating signaling through noncovalent association with transmembrane immunoreceptor tyrosine-based activation motif (ITAs)-bearing signaling adapters, such as FcRγ, CD3ζ, DNAX-activating protein of 12 kD (DAP12), and DAP10 that harbor a YxxM motif.[2,8–10]

Signaling lymphocytic activation molecule (SLAM) family of receptors (SFRs) are also expressed on NK cells, and they are usually self-ligands and mediate homotypic interactions of NK cells between hematopoietic cells. SFRs are characterized by the presence of two or more immunoreceptor tyrosine-based switch motifs (ITSMs) in their cytoplasmic domain, and they regulate multiple immune events by recruiting SH2 domain-containing SAP family adapters, including SAP and its homologs EAT-2 and ERT. ERT has been found to be a nonfunctional pseudogene in humans.[11,12] SFRs alternatively bind other inhibitory molecules with the SH2 domain to suppress immune cell activation and development.[11–17] The great importance of the SLAM-SAP family in immune regulation was highlighted by the discovery that mutations of *Sh2d1a1*, which encodes for SAP, were identified in human patients with X-linked lymphoproliferative (XLP)-1 disease, which is a rare primary immunodeficiency disease affecting approximately 1–2 per 1 million males.[12,18,19] A key feature of XLP1 is the exquisite sensitivity of affected individuals to disease induced by EBV infection.[20–23] These patients develop a severe immunodeficiency syndrome that includes impaired NK-T cell development and humoral immunity.[21,23–27] NK cells express multiple SLAM

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family receptors and all SAP family adapters.[11,13,28–30] NK cells from SAP-deficient patients display impaired NK cell cytotoxicity to hematopoietic cell lines.[17,31–33] During recent decades, SFRs have been revealed to be critical for enhancing NK cell activation in response to abnormal hematopoietic cells in SAP-family-intact NK cells; however, SFRs might suppress NK cell activation in SAP-family-deficient NK cells.[17,31–34] As a consequence, SAP-deficient patients are susceptible to lymphoma partly due to compromised NK cell activation. Here, we review how these SFR signaling pathways orchestrate activation of mouse NK cells, which highlights the great importance of SFR regulation of NK cell biology and their physiological status and pathological relevance in patients with XLP1.

EXPRESSION OF SLAM-FAMILY RECEPTORS AND SAP FAMILY ADAPTERS DURING NK CELL DEVELOPMENT

SFRs include seven members: SLAM, CD48, Ly9, 2B4, CD84, and Ly108 [in mice, but natural killer T and B-cell antigen (NTB-A) in humans] and CD2-like receptor activating cytotoxic cells (CRACC).[11–13,35] The genes encoding SFRs are all located within the same locus on chromosome 1 in humans and mice.[12,13,36] Although this gene cluster is likely generated from duplication of a common ancestor gene and these receptors have the same origin, they are structurally and functionally distinct.[12,25,36,37]

With the exception of CD48, which is a glycosyl-phosphatidylinositol-anchored protein, SFRs are type I transmembrane proteins, with an extracellular segment followed by a single transmembrane region and a cytoplasmic domain. The extracellular segment is characterized by an N-terminal Ig V-like domain and a membrane-proximal C2 domain. SFRs recognize themselves, in other words, they are self-ligands, with the exception that 2B4 binds CD48. The binding specificity of SFRs is determined by the extracellular IgV domain.[37–39] In contrast with immune receptors usually bearing ITAM or immunoreceptor tyrosine-based inhibitory motifs (ITIMs), SFRs are characterized by the presence of one or more ITSMs in their cytoplasmic domain, which is also observed in the coreceptor programmed death 1 (PD1).[11,12,40]

SFRs transmit immune signaling via coupling of Src homology 2 (SH2) domain-containing adapters, including SAP, EAT-2 and ERT.[41–44] The *Sh2d1a* gene encoding SAP is positioned on the chromosome X, whereas the gene encoding EAT-2 and ERT are colocalized near the SFR gene cluster. The SAP family adapters consist almost entirely of an SH2 domain followed by a short carboxy-terminal tail. SFRs bind with SAP family adapters through their ITSMs. The structural properties of SFRs determine their binding specificity with SAP family adapters. It is not clear whether all SFRs are able to recruit all three adapters. In fact, CRACC binds EAT-2 and ERT but not SAP.[44]

SFRs are detectable only on hematopoietic lineage cells. SLAM-family members, such as SLAM, 2B4, and CD48, are differentially expressed among distinct progenitor cells in mice. Long-term hematopoietic stem cells (HSCs) express only SLAM, defined as $SLAM^+2B4^-CD48^-$ cells, whereas multipotent progenitors (MPPs) lose SLAM and acquire 2B4 expression and are defined as $SLAM^-2B4^+CD48^-$ cells.[45,46] Common lymphoid progenitors (CLPs) that are $SLAM^-2B4^+CD48^+$ start to acquire CD48 expression. Moreover, heterogeneous HSCs and MPPs can be subdivided into a hierarchy of functionally distinct subpopulations based on their expression of Ly9.[45] Although the combinatorial expression of SFRs is a putative marker to precisely distinguish hematopoietic progenitors, whether SFRs play any physiological roles is still unclear. At a minimum, mice with a combined deficiency of SFRs appear to have an intact composition of hematopoietic progenitors (unpublished data). Thus, SFRs behave as makers for distinguishing HSCs and other progenitors.

NK cell development occurs in specialized BM niches. Nearly all SFRs are expressed on mouse NK cells, but these receptors display a dynamic expression profile along with NK cell maturation. For example, a high proportion of NK cell progenitors express both SLAM and Ly108 when NK cells are committed.[28,29] During NK cell differentiation, the presence of these two SFRs gradually decreases. SLAM disappears at the terminal stage. Interestingly, Ly108 expression is highly associated with NK cell development and education; Ly108-positive NK cells are mostly "licensed" as $Ly49C/I^+$ in the B6 background.[28,29] In addition to 2B4, Ly9, and CD84, CRACC maintains a high level of expression throughout all stages.[28,29] It is also expressed on activated $CD8^+$ T cells and B cells.[12] Notably, CRACC is highly expressed on multiple myeloid cells, and thus has become a potential target for treatment of this malignancy.[47–49] Although SFRs are widely expressed and dynamically altered during NK cell differentiation, there is no strong evidence suggesting which receptors are involved in regulation of NK cell differentiation.[28,44] Individual deletion of Ly108 does not disturb NK cell development, suggesting a redundant role of SFRs.[28] This possibility is supported by the recent finding that a combined deficiency of SFRs leads to a mild change in NK cell differentiation.[29,50]

SAP family adapters are all expressed in mouse NK cells. These adapters also undergo temporal changes in their expression during NK cell development. SAP expression is lowest in NK cell precursors and gradually increases during NK cell maturation.[28,30,51,52] In contrast, a preponderance of EAT-2 over SAP is seen in immature NK cells, but a predominance of SAP over EAT-2 is observed in more differentiated NK cells.[30,51] Despite this, mice lacking all SAP family adapters preserve intact NK cell differentiation.[14,24,25,34,43] Thus, SFRs likely regulate NK cell differentiation through SAP-independent signaling, which needs to be further elucidated. Furthermore, deletion of SAP family adapters also fails to rescue the slightly altered NK cell differentiation in SFR-deficient mice, excluding the possibility of alternative signaling of SAP family signaling in NK cell maturation.[29]

SLAM-FAMILY RECEPTORS ARE HEMATOPOIETIC CELL-SPECIFIC NK CELL ACTIVATING RECEPTORS

Allogeneic bone marrow transplantation is a potentially curative treatment for a substantial proportion of patients with hematologic malignancies, including intermediate and high-risk acute myeloid leukemia (AML), recurrent chronic myeloid leukemia (CML), multiple myeloma, and lymphoma. This therapeutic effect is partly due to the action of NK cells.[53–55] Data based on clinical studies also indicate that adoptive transfer of ex vivo activated NK cells has some beneficial effects on hematopoietic malignancies.[56,57] Rejection of parental allogeneic bone marrow was reported in a phenomenon called "hybrid resistance", which refers to the failure of first-generation hybrid mice to accept semiallogeneic parental bone marrow but not other non-hematopoietic grafts.[58–60] NK cells are experimentally verified to be responsible for the rejection of bone marrow via missing-self reactivity.[61,62] Based on the theory of NK cell licensing, self-MHC-I molecule is essential for NK cell functional competence, likely through engagement with self-MHC-specific inhibitory receptors. Because F1 offspring mice carry heterozygote MHC-I alleles that are parentally inherited, two distinct NK cell subsets are presumably separately licensed by paternal and maternal MHC-I. As a result, the functional NK cells that are licensed by maternal MHC-I will reject MHC-I mismatched paternal BM transplants but not MHC-I matched maternal BM transplants, and vice versa.[4,62] However, these findings raise another important question, namely, why NK cells preferentially kill hematopoietic cells under missing-self conditions. Although NK cells can kill primary MHC-I-deficient hematopoietic cells, which presumably lack any endogenous ligands for NK cell activation. This result raises two possibilities: the

absence of self-MHC-I is sufficient for NK cell activation, or NK cells likely bear self-specific activating receptors that can specifically recognize hematopoietic cells.

Most NK cell experts typically disagree with the first possibility, and they have tried to find an activating receptor that executes NK cell rejection of hematopoietic cells. As a result, some NK cell activating receptors, such as Ly49D and NKG2D, were revealed to contribute to allogeneic bone marrow rejection and "hybrid resistance".[63–66] In hybrid resistance, Ly49D and NKG2D are necessary only for F1 (BALB/c×C57BL/6) NK cell-mediated rejection of BM from BALB/c but not C57BL/6 donors.[63–65] Blockade of NKG2D can also diminish NK-mediated rejection of hematopoietic cells. Because the identity and tissue-specificity of those ligands for NKG2D or Ly49D are not clear, particularly on primary healthy cells, these findings cannot fully explain the specificity of hybrid resistance.

In patients with XLP1, the ability of NK cells to kill hematopoietic cells is highly impaired. As a result, over 30% of these patients suffer from lymphoma following EBV infection, but not other non-hematopoietic tumors. The incidence of lymphoma is most likely correlated with NK cell and $CD8^+$ cytotoxic T lymphocytes dysfunction.[17,31–33,67,68] Mouse studies further validate these data from humans; SAP-deficient NK cells fail to eliminate hematopoietic cells.[14,28,34,69] Thus, SAP is critical for the NK cell response towards hematopoietic cells. In mice lacking all three SAP family adapters, NK cells nearly lose their ability to kill a range of hematopoietic cells, including tumor cell lines, MHC-I-deficient RMA-S cells, YAC-1 cells (which overexpress ligands for mouse NKG2D), and primary MHC-I-deficient hematopoietic cells.[34] Intriguingly, SAP-family-deficient NK cells preserve their intact ability to kill non-hematopoietic cells.[28,34,69] Therefore, these data demonstrate that SAP is compulsory for NK cell recognition of hematopoietic cells. Due to the position of the *Sh2d1a* gene on the X chromosome, SAP-deficient F1 hybrid mice can be obtained. SAP-deficient F1 hybrid mice on the BALB/c×B6 background show a pronounced defect in NK cell rejection of parental hematopoietic cells.[29] Thus, SAP is also required for NK cell-mediated "hybrid resistance".

SAP is mainly recruited downstream of SFR signaling. The determination of NK cell specificity to kill hematopoietic cells via SAP family adapters indicates that SFRs are key activating receptors specific to hematopoietic cells. Notably, SFRs are strictly detectable on hematopoietic cells, including HSCs, lymphocytes, and myeloid cells. Donor hematopoietic cells from mice lacking SFRs significantly increase the resistance to killing by wild-type NK cells.[29] However, disassociation of SFR engagement between NK cells and hematopoietic targets only moderately influences NK cell elimination. This experiment implies that other NK cell activating receptors may also be involved. Considering that SFRs are hematopoietic-specific and endogenously expressed on NK cells, these receptors may represent the first self-specific activating receptors for NK cell recognition under "missing self" conditions. The existence of these receptors also verifies the hypothesis that the absence of self-MHC-I is not sufficient for full activation of NK cells.

Much effort has been employed to increase the success of bone marrow transplantation. Blockade of host NK cell activation may facilitate engraftment. The discovery that the SLAM family acts as self-specific activating receptors and accounts for the preference of NK cells to kill hematopoietic grafts may bring a glimmer hope for therapeutic interference with bone marrow rejection. Through ectopic expression of SFR on nonhematopoietic B16 cells, multiple SFRs, including 2B4, Ly9, CRACC, and Ly108, can elicit activation of NK cells, at least in mice. Determination of the key SLAM family members that carry out NK cell recognition of hematopoietic cells needs further investigation.

Although SFRs are self-specific activating receptors during the NK cell effector process, genetic deletion of all SFR members leads to elevated NK cell responsiveness to hematopoietic cells.[29] A possible explanation is that chronic engagement of SFRs between NK cells and other hematopoietic cells may desensitize NK cell responsiveness. This suggests that in addition to self-specific inhibitory receptors, endogenous NK cell activating receptors may also regulate NK cell education, a process of NK cell functional acquisition.[70–74]

SAP-DEPENDENT SLAM FAMILY SIGNALING IN NK CELL ACTIVATION

NK cells express multiple SFR members and all SAP family adapters, and thus, engagement of SFRs between NK cells and hematopoietic targets trigger very complicated signaling, which in turn gives rise to distinct NK cell responses. SAP is highly homologous to EAT-2 and ERT, but they transmit divergent downstream signaling. The downstream molecules that bind to SFRs usually transmit signaling through SAP, EAT-2, ERT, and other SH2-domain-containing phosphatases.

An essential role of SAP in NK cell activation has been found in human XLP1 patients and SAP-deficient mice. The engagement of SFRs on mature NK cells induces tyrosine phosphorylation of ITSMs via Src family kinases. Phosphorylated ITSMs binds the SAP protein. Apart from CRACC, all other SFRs, including 2B4, Ly108 in mice (NTB-A in humans), Ly9 and CD84, are able to recruit SAP.[11–13] Nevertheless, only triggering 2B4, Ly108, or Ly9 potentiates NK cell cytotoxicity, whereas CD84 promotes NK cell activation to a lesser extent in mice. Thus far, 2B4 is one of the most well studied SFRs in NK cell activation, and its activity is highly dependent on SAP, which also contributes to human NTB-A-triggered NK cell activation. Less is known about the SAP-dependence of Ly9 signaling activity.

2B4 is expressed on virtually all NK cells and contains four ITSMs in its cytoplasmic tail.[16,75] Its engagement results in the formation of lipid rafts, where tyrosine phosphorylation of its ITSM domain is likely initiated by the enzyme Csk.[76,77] This is an early and essential step in 2B4-mediated NK cell activation. The phosphorylated ITSM of 2B4 recruits at most SAP, or other SH2 domain-containing signaling molecules, to trigger downstream signals.[14–17,69] Although SAP is able to bind to all four ITSMs of 2B4, the first ITSM is sufficient for 2B4-mediated NK cell activation, and the second ITSM may contribute to SAP-dependent 2B4 signaling.[16,75]

SAP is an SH2 domain-only containing adapter, and it must recruit other kinases to facilitate SFR signaling. In contrast with EAT-2/ERT, SAP has no signaling activity mediated by its short cytoplasmic tail. SAP has to recruit Fyn, a Src family protein tyrosine kinase (PTK), to 2B4.[14,16,69,75] Biochemical studies have revealed a direct interaction between arginine 78 (R78) in the SH2 domain of SAP and the Src homology 3 (SH3) domain of Fyn.[14,75,78,79] The recruitment of Fyn kinase promotes full phosphorylation of other ITSMs of 2B4, which results in positive feedback, enhancing SAP binding with Fyn. In addition to the Fyn kinase, SAP can also bind with other SH3-domain-containing molecules, such as Pak-interacting exchange factor (PIX), the adapter Nck, and protein kinase C-θ (PKC-θ), through the same R78 residue.[14,16,69,80–82]

Because 2B4-triggered cytotoxicity and cytokine production are compromised in both SAP-deficient and Fyn-deficient mice, the SAP-Fyn interaction must be critical for 2B4-mediated NK cell activation.[14,69] This hypothesis was further approved by SAP^{R78A} mutant mice, in which SAP cannot bind with Fyn.[14] R78A mutant NK cells also show less killing activity against hematopoietic cells, such as MHC-I-missing RMA-S. However, compared with SAP-deficient NK cells, SAP^{R78A}NK cells have only mild defects.[14] This interesting finding not only indicates that SAP-Fyn binding is critical for NK cell activation but also suggests that SFRs can transmit other SAP-independent signaling pathways, which are most likely to be inhibitory.

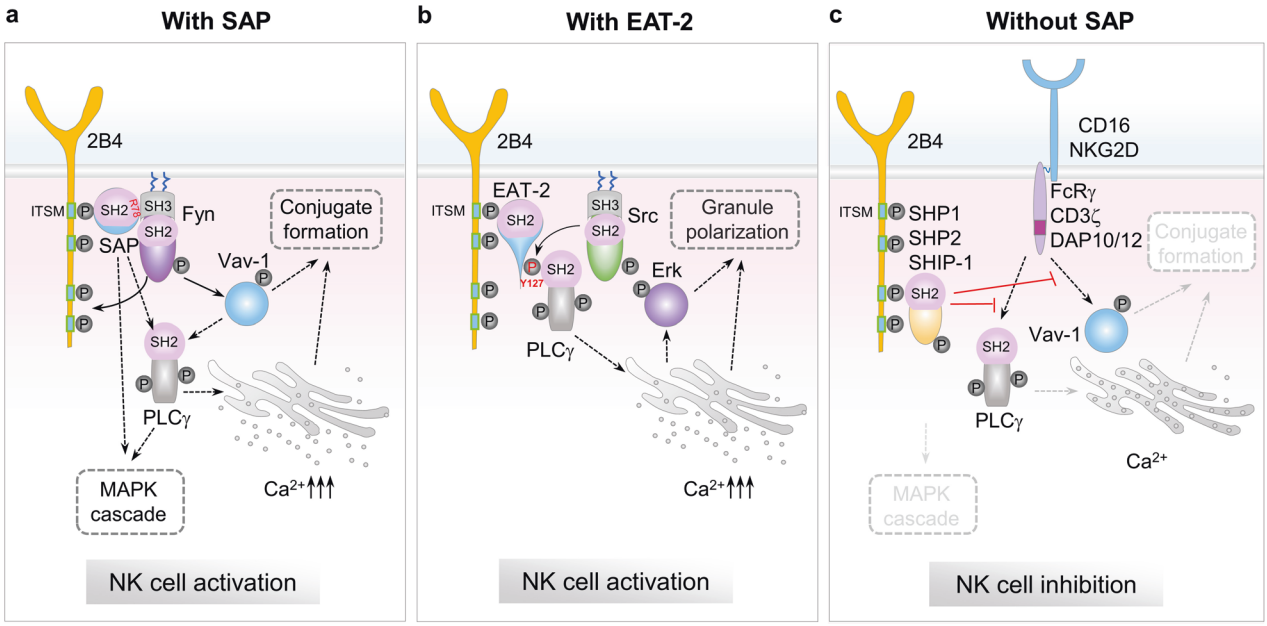

Fig. 1 SAP-family-dependent and -independent SFR signaling in NK cell activation and inhibition. Engagement of SFRs (2B4 is shown as an example) induces tyrosine phosphorylation of cytoplasmic ITSMs, likely by Src family kinases. SH2 domain-containing proteins, SAP family adapters, or other phosphatases then bind to the phosphorylated ITSM of SFR through their SH2 domain. **a** SAP-dependent SFR signaling. Through arginine 78 (R78) in the SH2 domain, SAP binds to the SH3 domain of Fyn. Fyn subsequently promotes full phosphorylation of other ITSMs in SFRs, further enhancing SAP binding with Fyn. Fyn triggers Vav-1 phosphorylation and PLCγ-mediated Ca^{2+} flux directly or indirectly, leading to augmented NK cell conjugate formation. **b** EAT-2-dependent SFR signaling. EAT-2 coupling enables phosphorylation of the tyrosine in its C-terminal tail by a Src family kinase. EAT-2 associates with PLCγ through a direct interaction between phosphorylated Tyr127 in the C-terminal tail of EAT-2 and the N-terminal SH2 domain of PLCγ, which evokes calcium flux and Erk activation, leading to NK cell granule polarization. **c** SAP-family-independent SFR signaling. In the absence of SAP family adapters, SFRs preferentially bind to other SH2 domain-containing inhibitory molecules, such as SHP-1, SHP-2, and SHIP-1, which strongly suppress CD16- or NKG2D-mediated activating signaling

2B4 promotes NK cell activation by inducing SAP-Fyn binding (Fig. 1a). Biochemically, 2B4 engagement can evoke tyrosine phosphorylation of Vav-1, an exchange factor that promotes cytoskeleton reorganization and formation of lytic synapses.[14,75,83–85] Deficiency of the family of Vav proteins, including Vav-1, −2, and −3, severely compromises NK cell activity.[86] 2B4 crosslinking-induced Vav-1 phosphorylation is highly dependent on SAP and Fyn kinase.[14,75] Importantly, Vav-1 activation is also minimal when SAP-Fyn binding is disrupted in SAP^{R78A} NK cells.[14] Consequently, the abovementioned activating signaling cascade augments the affinity of the adhesion molecule LFA-1 for intercellular adhesion molecule-1 (ICAM-1), leading to durable conjugation between NK cells and hematopoietic target cells.[14,87–89] To this end, SFRs, such as 2B4, act as activating receptors by recruiting SAP and triggering Fyn-Vav-1-mediated adhesion signaling to facilitate NK cell formation of stable conjugation with hematopoietic cells. In addition to Fyn, other signaling molecules, such as PLCγ, calcium mobilization, and MAPK pathway, can also promote 2B4-mediated NK cell activation.[14,90,91]

Although 2B4 predominantly triggers NK cell activation in a significant manner through SAP-mediated enhancement of conjugate formation, 2B4 also utilizes another mechanism to initiate intracellular signaling. Suppression of either NKp46 or the downstream ITAM-containing protein FcεRIγ substantially reduces 2B4-initiated cytotoxicity of human NK cells.[92,93] NK cells from FcεRIγ, CD3ζ, and DAP12 triple deficient NK cells have a compromised cytolysis ability against CD48-bearing target cells.[94] Moreover, a physical interaction of CD3ζ with SAP and 2B4 has been delineated.[93] In addition, the cytoplasmic adapter 3BP2 is directly associated with the fourth phosphorylated ITSM of 2B4 in human NK cells.[95–97] These data suggest that 2B4-initiated NK cell activation may utilize other adapters, thus updating the prevailing assumption that SAP-dependent SFR signaling is critical for NK cell activation. The involvement of other adapters in SFR-mediated NK cell activation needs to be validated as either direct or indirect binding.

EAT-2-DEPENDENT SLAM FAMILY SIGNALING IN NK CELL ACTIVATION

EAT-2 and ERT have very similar amino acid sequences. Distinct from SAP, both EAT-2 and ERT bear a short cytoplasmic tail, which possesses two tyrosine residues.[12,30,42–44,98] Although EAT-2 and ERT do not share the same binding site as SAP for Fyn in their intracellular domain, they can be recruited to SFRs by binding of their SH2 domain to the phosphorylated tyrosine of the SFR cytoplasmic domain.[42] This coupling enables phosphorylation of one or two tyrosine residues in their C-terminal tails, linking SFRs to downstream signals. In mice, the C-terminal tail of EAT-2 encompasses two tyrosine residues, Tyr120 and Tyr127, whereas the human EAT-2 contains only a single C-terminal Tyr127.[30,43] The capacity of EAT-2 to promote NK cell activation by SFRs requires the C-terminal Tyr127 in both species (Fig. 1b). Despite this, compared with SAP-deficient NK cells, NK cells deficient in both EAT-2 and ERT show only minor defects in NK cell activation in response to hematopoietic cells.[34,43] Thus, SAP-mediated SFR signaling may dominate NK cell activation. However, simultaneous deletion of all SAP family adapters might severely compromise the NK cell-mediated rejection of allogeneic hematopoietic cells.[34]

EAT-2 and ERT were first identified to be associated with 2B4.[43] EAT-2 serves a stimulatory function in 2B4-evoked NK cell activation. When the 2B4 ITSM tyrosine is phosphorylated by the Src family kinase Fyn and to a lesser extent by Lck, Src, and Lyn, EAT-2 directly associates with PLCγ in human NK cells, and this coupling is mediated by a direct interaction between the

phosphorylated Tyr127 in the C-terminal tail of EAT-2 and the N-terminal SH2 domain of PLCγ, which supports 2B4-triggered PLCγ tyrosine phosphorylation, calcium fluxes and then Erk activation[30,98,99] (Fig. 1b). This interaction also provokes a small increase in c-Cbl tyrosine phosphorylation, which likely creates a negative feedback network to terminate the function of EAT-2.[85,98] Unlike SAP, EAT-2 cannot evoke Fyn or Lck tyrosine phosphorylation, EAT-2 also cannot trigger activation of PI3K and the downstream protein Akt.[30] However, the effect is only limited to an enhancement of calcium flux triggered by CD16 in mouse NK cells. Although EAT-2 is dispensable for conjugate formation, the process critical for SAP-mediated NK cell activation, it promotes granule polarization towards the NK cell synapse to accelerate the effector phase of NK cell activation.[30] This effect is Tyr127-dependent and is generally mediated by the ability of EAT-2 to enhance calcium flux. Mutation of Tyr127 decreases NK cell activation.

CRACC is the only SLAM family member that maintains an activating function in NK cells of XLP1 patients, partly due to its binding specificity with EAT-2.[44,100] In addition, the action of EAT-2 in CRACC-induced NK cell activation is well-documented. Mouse CRACC contains three tyrosine residues, Tyr261, Tyr266, and Tyr281, in its cytoplasmic domain, whereas human CRACC does not contain the second tyrosine residue, Tyr266.[44] The third tyrosine (Tyr281) specifically interacts with EAT-2 and is responsible for the activating activity of CRACC, whereas Tyr261 accounts for its inhibitory effect when EAT-2 is absent in mouse NK cells. EAT-2 binds to phosphorylated Tyr281 in CRACC through its SH2 domain, linking CRACC to downstream effectors.[44] Whether CRACC tyrosine phosphorylation is dependent on EAT-2/ERT is controversial.

In human NK cells, EAT-2 seems to induce tyrosine phosphorylation of CRACC. The two tyrosine residues located in a short sequence in the carboxy-terminal portion of the SH2 domain of EAT-2 facilitate the coupling of CRACC to downstream effector proteins. This indicates that EAT-2 is involved in more distal events in the CRACC signaling pathway. Ligation of CRACC profoundly phosphorylates PLCγ1, PLCγ2, PI3K and c-Cbl and to a minor extent, Vav-1 and SHIP-1.[44,98] EAT-2 binding induces activation of the PLCγ and PI3K signaling pathways, triggering calcium flux and CRACC-mediated NK cell cytotoxicity.[44,98]

In addition to 2B4 and CRACC, EAT-2 can also link human NTB-A to downstream activation signaling. The homophilic interaction of NTB-A triggers its tyrosine phosphorylation by Src family kinases. The cytoplasmic tail of NTB-A contains three tyrosine residues, Tyr273, Tyr284, and Tyr319, and the second and third tyrosine are embedded within the consensus sequence of ITSM.[101] The second tyrosine specifically recruits EAT-2, which is essential and sufficient for NTB-A-mediated NK cell cytotoxicity, while the third tyrosine contributes to the full activation of NK cells mediated by NTB-A, likely through associating with SAP.[101] NTB-A primarily stimulates NK cell activation by enhancing the activity of PLCγ and PI3K and, to a lesser extent, the MEK kinase pathway.[101] Actin reorganization and Src kinases are also involved in the generation of NTB-A-mediated NK effector functions.

SAP-FAMILY-INDEPENDENT SLAM FAMILY SIGNALING IN NK CELL INHIBITION

The severe defect in NK cell response to hematopoietic cells in SAP-family-deficient NK cells seemingly demonstrates an important role for SAP-family-dependent SFR signaling in NK cell activation. However, several studies suggest that SFRs can also employ other SH2 domain molecules when SAP family adapters are absent from NK cells. First, SFRs can induce tyrosine phosphorylation of other SH2 domain-containing molecules in SAP-family-deficient NK cells.[14–17] Second, compared with SAP-deficient mice, NK cell activity is only mildly impaired by blocking SAP-dependent activating signaling in SAP^{R78A} mutant mice.[14] Finally, but importantly, the removal of SFRs on SAP-family-deficient NK cells can completely rescue the severe defect of NK cell rejection of MHC-I-deficient hematopoietic cells.[29] On the basis of these clues, SFRs not only can transmit the activating signaling for NK cell activation but also deliver certain inhibitory signals for NK cell inhibition in the absence of SAP family members. In other words, SAP family adapters mediate the switch-of-function through two distinct activities, active signaling molecules and natural blockers.

NK cell activity is severely impaired in SAP- but not EAT-2/ERT-deficient mice; however, ectopic expression of individual SFRs strongly mitigates the activation of EAT-2/ERT-lacking NK cells while only slightly affecting SAP-deficient NK cells.[34] Thus, NK cell dysfunction caused by SAP deficiency is likely due to the loss of SFR-mediated activating signaling and the gain of inhibitory signaling. However, EAT-2 and ERT most likely function as "natural blockers" to prevent the binding of inhibitory molecules to SFRs.[42] This probably explains the discrepancy between mice deficient in SAP and those deficient in EAT-2/ERT.

NK cells express multiple SFRs. Except for CD84, most of these SFRs are activating. Ectopic expression of CRACC, Ly9 and CD48 can enhance NK cell cytotoxicity towards nonhematopoietic B16 cells and IFN-γ production.[29,34] However, when SAP family adapters are absent, the overexpression of individual SFRs not only fails to elicit NK cell activation but also strongly suppresses NK cell activation caused by other "induced self" stimuli.[34] Although CD84 is not an activating receptor on NK cells, the engagement of CD84 can still pronouncedly dampen NK cell cytotoxicity.[34,102] Thus, all SFRs appear to be inhibitory in SAP-family-deficient NK cells. As a result, deletion of SFRs either on donor hematopoietic cells or on recipient NK cells can overcome the severe defect of SAP-deficient NK cell killing towards hematopoietic cells under "missing-self" status.[29] This finding raises many critical questions. First, if SFRs are self-specific activating receptors, how can mice with a combined deficiency in all SFRs and SAP family adapters reject allogenic hematopoietic cells? SFR deficiency only partially affects NK cell elimination of MHC-I-deficient hematopoietic cells, suggesting the presence of other NK cell activating receptors, which most likely compensate for the deficiency in SFRs. Second, which SFR member(s) contributes to the severely defective NK cell activation in patients with XLP1 is unknown. To answer this question, individual deletions of each SFR member will be useful in determining the key inhibitory SFR(s) in SAP-deficient NK cells and will aid in the treatment of NK cell dysfunction in XLP1 by enabling blockade of certain SFRs.

Genetic and biochemical analyses provide strong evidence supporting the inhibitory identity of SFRs in SAP-family-deficient NK cells. Many inhibitory SH2-domain-containing molecules, including protein phosphatase SHP1 and SHP2, the lipid phosphatase SHIP1, and Csk, can be alternatively recruited to certain phosphorylated ITSMs of SFRs.[14–17] SAP family adapters likely prevent their binding to SFRs either through competing with the same ITSMs or through occupancy hindrance (Fig. 1c).

Many indirect studies support the notion that these SH2-domain phosphatases are critical for NK cell inhibition induced by SFR. 2B4 is a well-documented SFR member that induces NK cell inhibition. Functionally, 2B4-mediated inhibition of NK cell cytotoxicity is robustly elevated in SAP-deficient NK cells.[14,17] Biochemically, the engagement of 2B4 induces tyrosine phosphorylation of SHIP-1.[14,16,75] In contrast with the first two ITSMs, which have specificity for SAP binding, the phosphorylated third ITSM of 2B4 can additionally recruit several proteins, such as SHIP-1, SHP-1, and SHP-2.[14–17] Genetic deletion of SHIP1 significantly abolishes 2B4-mediated inhibition of calcium flux and Erk phosphorylation induced by BCR crosslinking, at least in the chicken DT40 cell line.[14] This is the first genetic evidence showing

that SFRs directly couple with other inhibitory molecules to exert their inhibitory activity. In fact, combined deletion of SHP-1 and SHP-2 in DT40 cells can also slightly relieve 2B4-induced inhibition.[14] Thus, SFRs likely couple multiple SH2-domain-containing proteins to suppress various activating signaling pathways via NK cell receptors, such as CD16 and NKG2D. However, because mice lacking SHIP-1 or SHP-1 have severe defects in NK cell development or functional acquisition, it is still not clear whether SFR-mediated inhibitory molecules are involved in NK cell biology. Limited study suggests that this SAP-family-independent signaling mediated by SLAMF6 participates in mouse NK cell education.[28]

CONCLUDING REMARKS

Hematopoietic cell-specific SFRs act as self-specific endogenous activating receptors, endowing NK cell specificity for control of "unwanted" hematopoietic cellular targets. The presence of SAP family adapters can switch the features of SFRs to generate inhibitory or activating behavior, and thus, they strictly determine this important action mediated by SFRs. SAP promotes SFR-mediated NK cell activation occurs via a combined mechanism: SAP coupling of SFRs to Fyn through R78 in the SH2 domain, which triggers Vav-1 phosphorylation leading to augmented NK cell conjugate formation; SAP can also function as a natural blocker to prevent recruitment of SH2 domain-containing inhibitory molecules. EAT-2 has two distinct roles in NK cell activation: EAT-2 functions through Y127-dependent recruitment of PLCγ, which evokes calcium flux and Erk activation, and EAT-2 can also prevent SFR binding with SH2 domain-containing inhibitory molecules. In the absence of SAP, inhibitory molecules, such as SHIP-1, link with SFRs to restrain NK cell activation, which occurs in NK cells from SAP-mutated patients. Thus, studies of NK cells in SAP deficiency have shed substantial light on the requirement of the SLAM-SAP families for NK cell immunosurveillance in abnormal hematopoietic cells. This knowledge, in turn, has the potential to be translated into novel treatments for not only XLP1 patients but also individuals suffering from viral infection-related diseases and hematopoietic tumors.

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ADDITIONAL INFORMATION

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