[Frontiers in Bioscience 13, 956-965, January 1, 2008]

Regulation of NK cell activity by 2B4, NTB-A and CRACC

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1. ABSTRACT

2B4, NTB-A and CRACC are members of the recently defined family of SLAM-related receptors. Here we review the role of these receptors for the regulation of Natural Killer cell function and describe the current knowledge about the signal transduction of these receptors. Finally, we critically analyze some controversial data about the function of 2B4 in mouse and man.

2. INTRODUCTION

Natural Killer (NK) cells are important for an effective immune response against transformed or virally infected cells and against certain pathogens (1-3). These functions are mediated by cellular cytotoxicity, cytokine secretion and the interaction with other immune cells. The activity of NK cells is regulated by a balance of positive and negative signals, which are transmitted by various surface receptors (4,5). Several inhibitory receptors recognizing self MHC class I antigens are responsible for the tolerance of NK cells against healthy autologous cells. These receptors include members of the KIR receptors in humans and Ly49 receptors in mice as well as the CD94/NKG2A receptor in both species. However, in recent years it became clear that also non-MHC class I recognizing inhibitory receptors play an important role in the regulation of NK cell function and tolerance (6). NK cell activation is mediated by a wide array of different surface receptors as well as cytokines. Important activating NK cell receptors include NKp30 (CD337), NKp44 (CD336), NKp46 (CD335), NKG2D (CD314), and DNAM-1 (CD226) (4,5). Here we focus on the role of 2B4 (CD244), NTB-A and CRACC (CD319) for the regulation of NK cell activity. These receptors belong to a recently described family of surface receptors called SLAM-related receptors (SRR) (7-10), which plays an important role in the fine-tuning of immune responses.

3. THE SLAM-RELATED RECEPTOR FAMILY

The SLAM-related receptor (SRR) family comprises six surface molecules belonging to the

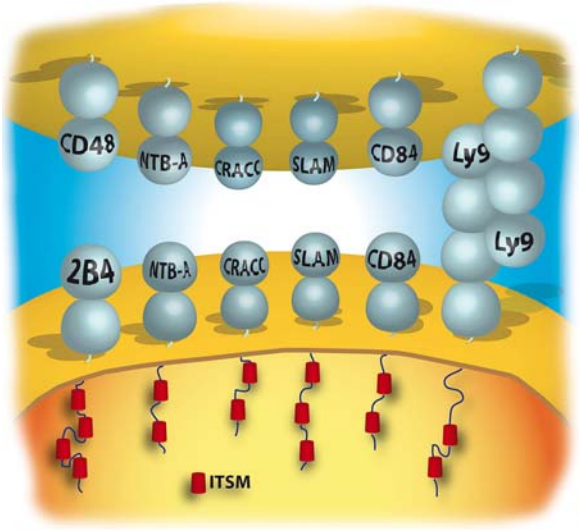

Figure 1. The SLAM-related receptor family and their ligands. Depicted are the six members of the SLAM-related receptor family with their cytoplasmic tails containing the ITSM signaling domains. On the opposing cell the corresponding ligands are shown.

immunoglobulin (Ig) super-family of receptors (Figure 1). Members of this family are SLAM (CD150), 2B4 (CD244), NTB-A (Ly-108), CRACC (CS1, CD319), CD84, and Ly-9 (CD229) (7-10). The SRR genes are located on the long arm of human chromosome 1 (1q21-24), and on mouse chromosome 1 (1H2) with a similar organization in both species. The homology in sequence and location indicates that SLAM family members arose through successive duplications of a common ancestral gene. The SRR proteins are type I transmembrane receptors with an amino-terminal V-type and a membrane proximal C2-type Ig-like domain in the extracellular region, and a cytoplasmic domain of 70-180 amino acids. Ly-9 is the only exception with its four Ig-like domains in a V-C2-V-C2 arrangement.

The expression of SRR was first discovered in T and NK cells but B lymphocytes, macrophages, monocytes, dendritic cells, platelets, granulocytes, and hematopoietic stem and progenitor cells also express various SRR (7-10). SRR family members are homophilic (11-16) and thereby represent their own ligands with the exception of 2B4, which interacts with CD48, a glycosylphosphatidylinositol-anchored molecule with broad expression in the hematopoietic system (17,18) (Figure 1). Analysis of several crystal structures and mutational analysis demonstrates that the amino-terminal V-type Ig-like domain is essential for ligand binding and specificity of the receptor (19-21).

The cytoplasmic domains of the SRR contain several immunoreceptor tyrosine-based switch motifs (ITSM), which are essential for signaling and receptor function (Figure 1). The ITSM can bind to a novel family of adapter signaling molecules consisting of three members: SAP (SH2D1A or DSHP), EAT2 and ERT (22). These cytoplasmic molecules consist of a Src homology 2 (SH2) domain and a short carboxyl-terminal extension. The important role of SRR and the adapter molecule SAP in immunity was revealed when the SAP gene was found to be mutated in around 50-70% of cases of X-linked lymphoproliferative disease (XLP), a human immunodeficiency disease characterized by a dysregulated immune response to infection by Epstein-Barr virus (EBV) (23). The dysregulated immune response leads to uncontrolled expansion of B cells, T cells and monocytes causing splenomegaly and excessive production of inflammatory cytokines. The pathology leads to death within 1-2 months of infection. This underscores the importance of functional SRR for a regulated immune response.

Human mature NK cells express the SRR family members 2B4, NTB-A, and CRACC. We will therefore focus in the following on the function of these receptors for NK cell activity.

4. FUNCTION OF 2B4, NTB-A AND CRACC IN NK CELLS

2B4 was first identified as an activating NK cell receptor in mice and later also in humans (24,25). Engagement of 2B4 results in the activation and polarization of the NK cell cytolytic machinery. This process is initiated by the interaction with its ligand CD48, recruitment of 2B4 into lipid rafts (26), phosphorylation of the cytoplasmic ITSMs (27) and interaction with SAP and other signaling molecules (28,29). This initiates a signaling cascade leading to the formation of the immunological synapse and the targeted release of perforin and granzyme B from cytolytic granules (26,30-32). Interaction of 2B4 on human NK cells with its ligand CD48 on target cells or 2B4 cross-linking by antibodies also induces the release of cytokines such as IFN-gamma and TNF alpha (24,25,29,33-36). While triggering of 2B4 may be sufficient by itself to stimulate IL-2 activated NK cells, it can also very effectively co-stimulate the activity of other activating NK cell receptors such as NKp30, NKp46, NKG2D, CD16 and others (37-39).

2B4-mediated activation of NK cell activity can be completely blocked by co-engagement of inhibitory receptors such as KIR2DL1 or CD94/NKG2A (26,27,40). As the ligand for 2B4 is ubiquitously expressed in the hematopoietic system, the control of 2B4-mediated NK cell activation by inhibitory receptors ensures the self-tolerance of NK cells. However, it also enables NK cells to be activated through 2B4 by transformed or infected hematopoietic cells that lost MHC class I expression. Furthermore, CD48 can be up-regulated for example by EBV infection, which would result in increased NK cell stimulation and activation through 2B4 (41,42). Triggering of 2B4 on human NK cells induces a strong down-modulation of 2B4 surface expression (43). This leads to decreased 2B4-mediated NK cell activation and cytotoxicity and may therefore be another mechanism for the fine-tuning of NK cell activity (43).

In NK cells from XLP patients the absence of functional SAP leads to defective 2B4 function. While some reports demonstrated a lack of 2B4-mediated NK cell activation in XLP NK cells (44-46), one report showed that 2B4 mediates an inhibitory signal in these patients (41). Also in non-pathological situations, human 2B4 can act as an inhibitory receptor. In NK cell precursors, which lack inhibitory receptors for self-MHC class I, 2B4 transmits inhibitory signals, thereby ensuring the self-tolerance of these cells (47,48). Interestingly, NK cell precursors were shown to exhibit low or absent SAP expression. Further, 2B4 activation on mature NK cells isolated from human lymph nodes can inhibit IFN-gamma production (49), suggesting that 2B4 may have opposing functions on NK cells from peripheral blood and secondary lymphoid organs. Moreover, despite normal expression pattern of activating and inhibitory receptors (50,51), triggering of 2B4 was shown to inhibit NK cell function in human decidua (50), which may also be due to a low expression level of SAP (51). In resting NK cells SAP expression is very low (52) and 2B4 stimulation can only trigger NK cell cytotoxicity when co-stimulated with additional activating NK cell receptors (37,39). Stimulation of NK cells with IL-2 and IL-12 was shown to increase SAP expression (52). In these 'primed' NK cells engagement of 2B4 alone is now sufficient to induce NK cell cytotoxicity, demonstrating a correlation between the regulated SAP expression and the function of 2B4 (52).

In mouse NK cells the function of 2B4 is unclear at the moment. While originally, 2B4 was identified as an activating receptor in mouse NK cells (24), recent studies suggest that murine 2B4 may in fact function as an inhibitory receptor. This is discussed in more detail in section 7. As CD48, the ligand for 2B4, is present on all NK cells, it is interesting to speculate that the interaction between these molecules may have functional consequences for NK cell activity. Studies on human and murine NK cells demonstrated that IL-2-induced proliferation is reduced when either anti-2B4 or anti-CD48 antibodies are added (25,53,54). However, another study did not find any changed NK cells proliferation or activity when the interaction between 2B4 and CD48 were blocked (36). It is therefore unclear how much the interaction of 2B4 and CD48 among NK cells contributes to NK cell function. However, a recent report showed that macrophage expressed CD48 can stimulate NK cell expressed 2B4 leading to increased activity against tumor targets (55).

Interestingly, 2B4 can also function as an activating NK cell ligand. 2B4-expressing target cells can efficiently stimulate NK cell cytotoxicity and IFN-gamma production via interaction with NK cell CD48 (56). This is in line with the observation that 2B4 expressing NK cells can stimulate T cell functions through the interaction with T cell expressed CD48 as discussed in section 5.

Much less is known about the function of NTB-A and CRACC on NK cells. Triggering of NTB-A on human NK cells by antibody-mediated cross-linking, NTB-A fusion proteins, or homophilic interaction with NTB-A-expressing cells stimulates cytotoxicity as well as IFN-gamma and TNF alpha production (12,36,57). NTB-A is therefore a positive regulator of NK cell function. Similar to 2B4, NTB-A can effectively co-stimulate other activating NK cell signals (57). Interestingly, as shown for 2B4, the stimulatory function of NTB-A is also defective in XLP patients and triggering of NTB-A in these SAP-deficient NK cells results in strong inhibition of NK cell function (57).

Although CRACC is widely expressed on lymphoid cells, its only known function to date is restricted to NK cells. CRACC acts as an activating receptor enhancing NK cell cytotoxicity (15,36,58). Interestingly, the function of CRACC seems to be independent of SAP and may therefore not be impaired in XLP patients (58).

5. FUNCTION OF 2B4, NTB-A AND CRACC IN OTHER IMMUNE CELLS

The expression of 2B4, NTB-A and CRACC is not only limited to NK cells. Therefore, we would like to briefly summarize the function of these receptors on other immune cells. 2B4 expression is also found on basophils, eosinophils, a proportion of CD8 peripheral T cells (approx. 50%), few CD8 thymocytes, γδ T cells and resting monocytes. CD4+ T cells and neutrophils do not express 2B4 but about 10% of splenic B cells in mouse are 2B4 positive (9,59,60). The expression of 2B4 can be induced on CD8 and CD4 T cells through activation and after infection with LCMV or MCMV (24,25,61).

Cross-linking of 2B4 on eosinophils elicits a significant release of peroxidase and the production of IFN-gamma and IL-4 (62). This demonstrates that 2B4 can also function as an activating receptor in other immune cells. However, the engagement of 2B4 on CD8 T cells has been shown to trigger only moderate target cell lysis in a non-MHC restricted manner and no cytokine production (25,35). Instead, 2B4 effectively co-stimulates T cells when triggered through the T cell receptor. The interaction between 2B4 and CD48 on target cells may increase adhesion between effector and target cells. This is supported by a recent report showing that CD48 expressing target cells lead to the polarization of 2B4 and perforin to the immune synapse of 2B4 positive CD8 T cells. Additionally, blockade of CD48-2B4 interaction can suppress T cell-mediated graft rejection (63-65).

NK cells can control adaptive immunity by augmenting proliferation of neighboring T cells through direct cellular contact. This effect was shown to occur through interactions between 2B4 on NK cells and CD48 on T cells (53,54). Similarly, the interaction of 2B4 and CD48 among neighboring T cells can augment T cell functions through a stimulating effect of CD48 engagement (63,66). NK cell expressed 2B4 can also function as a stimulating ligand for NK-B cell interactions, where the stimulation of CD48 on B cells induces switch recombination to IgG2a (67).

Interestingly, 2B4 is also expressed by multipotent hematopoietic progenitors and by more restricted multipotent hematopoietic progenitor cells which also show

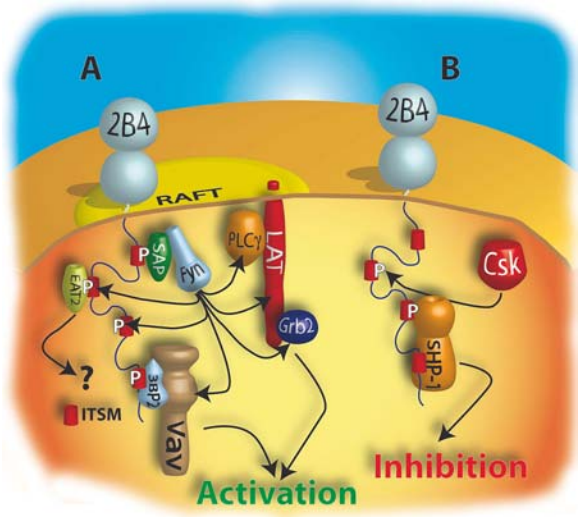

Figure 2. Signal transduction of the 2B4 receptor in NK cells. (A) Early signaling events in 2B4-mediated NK cell activation. (B) Possible mechanism for 2B4-mediated NK cell inhibition in the absence of functional SAP e.g. in XLP patients. See text for details.

CD48 expression (68). The combinatorial expression of 2B4, CD48 and SLAM is the only marker to precisely distinguish stem and progenitor cells. This suggests that these receptors may also play a role in hematopoietic progenitor function and leukocyte development. Interestingly, the development of NKT cells is defective in XLP patients and SAP-deficient mice, suggesting that the SAP-dependent signals of one or more SRR are essential for this process (69). However, individual knock-out mice for SLAM, 2B4, NTB-A, CD48 and Ly9 are not affected in their development of NKT cells (10).

NTB-A is expressed universally on NK cells, T and B lymphocytes and on eosinophils (57,70). NTB-A is a positive regulator of T cell functions. Ligation of NTB-A on human or mouse CD4 T cells modestly increases TCR–mediated proliferation and IFN-gamma secretion (71). Experiments using antibodies to engage NTB-A suggested that NTB-A induces Th1 immune responses (71). Injection of an NTB-A fusion protein, which is thought to block NTB-A function, delayed the onset of EAE, a Th1 mediated immune disorder (71). However, mice with a deletion of exon 1 and 2 of the NTB-A gene showed reduced IL-4 production by CD4 T cells and normal IFN-gamma production (70). This would suggest a role of NTB-A in the induction of Th2 responses. Further studies are needed to clarify the function of NTB-A in T helper cell polarization. An interesting insight into the importance of NTB-A in immune regulation comes from studies of autoimmunity-related genes in mouse strains. The susceptibility locus for the autoimmune disease lupus erythematosus on murine chromosome 1 contains the genes for the SRR family (72). A polymorphism in the gene encoding for NTB-A is likely to be one factor that contributes to autoimmune disease. The lupus associated NTB-A allele may be associated with modified signaling responses of T cells in lupus susceptible mice (72). Additionally, the normal NTB-A gene was found to sensitize immature B cells to deletion and RAG re-expression, whereas the lupus-associated allele did not (73). This suggests that NTB-A is an important regulator of T cell responses and tolerance checkpoints in B cells.

CRACC is expressed by NK cells, CD8 T cells, a small subset of CD4 T cells, mature dendritic cells and B cells (58,74). There is currently no information on the function of CRACC in cells other than NK cells. One can only speculate that CRACC may play an active role in B cells since CD40 stimulation leads to CRACC up-regulation on a small subset of peripheral B cells (58).

6. SIGNALING OF 2B4, NTB-A AND CRACC

Of all SRR the signaling of the 2B4 receptor is examined best (Figure 2). Upon the engagement of 2B4 by antibodies or CD48 expressing target cells the receptor is recruited to lipid rafts and its ITSM are phosphorylated by Src-family kinases leading to the recruitment of the adapter molecule SAP (26,27,29,75,76). Raft recruitment is essential for the phosphorylation of 2B4 (26). Although SAP has been reported to bind to the non-phosphorylated ITSM of SLAM, this is not the case with 2B4 (28,77). While SAP can associate with all four ITSM of 2B4, it has been shown that interaction with the membrane proximal ITSM is sufficient for 2B4 signaling. (28). The adapter molecule EAT-2 is also recruited to the phosphorylated 2B4 receptor, but only little is known about its function (78). Both adapter molecules consist of one single SH2-domain and a small c-terminal tail (77,79,80). Human EAT-2 carries one tyrosine in this tail, but in contrast to murine EAT-2, no phosphorylation has been observed (81,82). ITSM-bound SAP mediates signal transduction by recruiting the Src-kinase FynT. FynT binds to SAP in an unusual SH2-SH3-domain interaction involving the residue Arg 87 on SAP (83-85). This interaction is essential for SAP function, as mutations of Arg 87 abolish 2B4 signaling completely (76,86). FynT recruitment leads to an increased phosphorylation of 2B4 (28). The importance of SAP for 2B4 signaling is evident from XLP patients where 2B4 is unable to mediate cytotoxicity in the absence of functional SAP (41,44-46). Furthermore, 2B4 signaling is abolished in *sap* and *fyn* knock-out mice (87).

As described above, 2B4 can mediate inhibitory rather than activating functions in the absence of SAP (41). This could be due to the binding of the phosphatases SHIP, SHP-1 and SHP-2 to the phosphorylated third ITSM of 2B4, leading to negative signaling (28,29) (Figure 2B). Due to competitive binding these molecules are displaced by SAP, explaining the lack of negative signaling by 2B4 under normal conditions. It is unclear if 2B4 is still recruited to lipid rafts in the absence of SAP and if Src-family kinases are still responsible for the phosphorylation of the ITSM when 2B4 mediates negative signaling. Interestingly, the 2B4 ITSM can also be phosphorylated by the kinase Csk that can associate with 2B4 as well (28). Csk is known to inhibit the activity of Src-family kinases, which could be another mechanism of negative signaling mediated by 2B4.

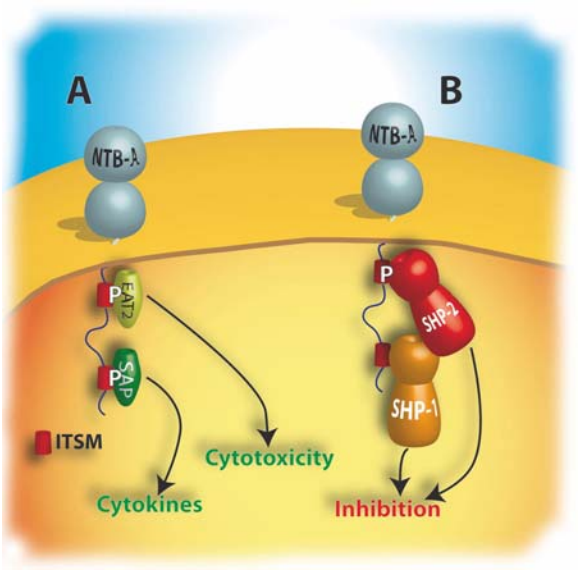

Figure 3. Signal transduction of the NTB-A receptor in NK cells. (A) EAT-2 and SAP mediate different events after NTB-A-mediated NK cell activation. (B) Possible mechanism for NTB-A-mediated NK cell inhibition in XLP patients. See text for details.

Another adapter protein that can bind to phosphorylated 2B4 is 3BP2, which associates with the fourth ITSM (Figure 2A) (88). Phosphorylated 3BP2 interacts with the signaling molecules Vav-1, LAT and PLC-gamma (88,89). Stimulation of 2B4 leads to phosphorylation of LAT, Vav-1, PLC-gamma, c-Cbl, Grb2 and SHIP (27,76,90). The Vav-1 signal results in increased phosphorylation of ERK (88). Inhibition of PLC-gamma abrogates 2B4-mediated cell lysis (91). Some reports describe a direct interaction between 2B4 and the adapter molecule LAT (90,92), while others did not find such an association (76). One explanation for this fact might be that the two molecules are immunoprecipitated together because of their affinity to lipid rafts.

The signaling of 2B4 is also regulated on the level of protein expression. The expression of SAP is low in freshly isolated, i.e. resting NK cells and increases after activation of these cells through IL-2 or IL-12, thus resulting in enhanced 2B4 signaling (52). Upon engagement of the 2B4 receptor, its surface expression is down-modulated by receptor internalization, and the expression of the 2B4 gene is reduced by inhibitory action at an ets-element in its promoter (43,93). These negative feedback mechanisms will likely limit excessive 2B4-mediated NK cell activation.

Less is known about the signaling of the receptor NTB-A (Figure 3). Similar to 2B4 it is phosphorylated upon engagement and recruits the adapter molecules SAP and EAT-2 (57,71,82). EAT-2 associates with the membrane proximal ITSM, while SAP adheres to the C-terminal one (82) (Figure 3A). The signaling pathways from each of the two ITSM contribute to different functions. The EAT-2-binding ITSM was reported to be essential for NTB-A-mediated cytotoxicity while experiments with SAP knock down cells showed reduced cytokine production after NTB-A stimulation (82). This indicates that the SAP-binding ITSM is responsible for cytokine production. Interestingly, the opposite results have been reported from experiments with XLP-NK cells, which showed decreased cytotoxicity and normal cytokine production in response to NTB-A engagement (11,57). In contrast to 2B4 no association of 3BP2 with NTB-A has been observed (88).

NTB-A has also been reported to interact with the phosphatases SHP-1 and 2. While SHP-1 can be found in complex with NTB-A regardless of its phosphorylation state, SHP-2 associates after pervanadate treatment (57). As for the 2B4 receptor, this might be the basis of negative signaling by NTB-A in the absence of functional SAP expression as reported for NK cells from XLP patients (Figure 3B).

CRACC is phosphorylated after ligation and recruits the adapter EAT-2, which promotes CRACC phosphorylation through a Src-kinase (91). Although CRACC does not bind 3BP2 (88), the CRACC signal causes phosphorylation of PLC-gamma1 and 2, Akt and c-Cbl. The phosphorylation of Vav-1 and SHIP is increased to a lesser extent (91). There are contradicting results concerning the ability of human CRACC to recruit SAP (58,91,94). But SAP does not seem to be important for CRACC signaling as XLP NK cells show no reduction in CRACC-mediated cytotoxicity (58).

7. DIFFERENCES IN 2B4 FUNCTION IN MOUSE AND MAN

There are some differences between murine and human NK cells regarding expression and function of SRR. Murine NK cells express only 2B4 and CRACC whereas the NTB-A homologue Ly108 is not expressed (61,94,95). More importantly, contradicting results have been reported on 2B4 function in the murine system. Cross-linking 2B4 in *in vitro* experiments on murine NK cells suggested activating properties resulting in the induction of cytotoxicity and cytokine production similar to the human receptor (61). But murine NK cells lyse certain CD48+ tumor cells less efficiently than their CD48- counterparts. If 2B4-CD48 interaction is blocked with antibodies, this difference is abolished, pointing to a negative signaling through 2B4 (96,97). The inhibitory signal mediated by 2B4 in these experiments seems to be independent of SAP, as the same results were obtained with NK cells from SAP knock-out mice (96). Experiments using 2B4 knock-out mice point again to an inhibitory function of 2B4. The clearance of injected CD48+ tumor cells in these mice is increased compared to wild type mice. When NK cells from these mice were transduced with 2B4 the lysis of CD48+ target cells was reduced compared to CD48- targets (96,97). Interestingly, the 2B4 KO phenotype shows some gender specificities: In experiments with metastatic melanoma cells only male 2B4 KO mice show a better rejection of CD48+ tumor cells, while female mice fail to

reject both CD48+ and CD48- cells. Although the rejection is NK cell dependent, this defect in female KO mice is not NK cell intrinsic, as NK cell cytotoxicity is not impaired *in vitro* (98).

However, similar to early reports, which indicated activating functions of murine 2B4, a recent report challenges the notion that murine 2B4 is an inhibitory receptor. *In vitro* and *in vivo* experiments using different tumor cell lines as target cells demonstrated that CD48 expression enhances lysis of these targets. Similar to the situation in the human system, this activating 2B4 signal was only turned into an inhibitory signal in SAP knock-out mice (87).

At the presence, the reason for the contradictory results and the basis for the SAP independent negative signaling of murine 2B4 is unclear. One difference between human and murine NK cells lies in the expression and function of adapter molecules. In mice exists a third adapter molecule called ERT, which shows high similarity to EAT-2 (81). Murine EAT-2 and ERT carry two tyrosines that can be phosphorylated while human EAT-2 contains only one tyrosine, which does not seem to be phosphorylated. Experiments with NK cells from knock-out mice pointed to an inhibitory effect of murine EAT-2 and ERT, which might be one explanation for the SAP independent negative signaling of murine 2B4 (81). NK cells from EAT-2 and ERT knock-out mice showed increased cytotoxicity against a variety of target cells, not only mediated by 2B4, but also by NKG2D and CD16.

One possible explanation for the contradictory results in the murine system comes from a recent report by Taniguchi and colleagues (99). This study demonstrates that the negative signaling by 2B4 is necessary to prevent the fratricide of activated NK cells. In the absence of 2B4 signaling, activated NK cells attack and kill each other, resulting in reduced NK cell proliferation and cytotoxicity. This would suggest that in earlier studies, in which 2B4 function was blocked, the observed reduced NK cell function was not due to a lack of 2B4-mediated NK cell activation but due to the induction of NK cell fratricide. However, it remains to be seen if NK cell fratricide is the only reason for the contradictory results.

8. PERSPECTIVE

It is clearly established that the SRR family of surface molecules plays an important role in the regulation of an effective immune response. Defective SRR function can lead to immunodeficiencies and autoimmunity. In recent years we have greatly advanced our understanding of the function of 2B4, NTB-A, CRACC and the other members of the SRR family in immune responses. However, there are still several open questions, which will need to be answered in order to better understand the role of these receptors in a regulated immune response. Future studies will need to further address the role of SRR in the cross-talk between different immune cells and how this influences their activity. Another challenge is the reconciliation of contradictory results obtained from *in vitro* and *in vivo* experiments investigating the role of NTB-A and other SRR in driving Th1 or Th2 dominated immune responses. Finally, we need to clearly define the differences in the function of 2B4 and other SRR between mouse and man. This is essential for any possible translation of findings from animal experiments to the human system. This will be important for any future therapeutic manipulation of SRR function in autoimmune diseases and other de-regulated immune responses.

9. ACKNOWLEDGEMENT

The work in our laboratory is supported by the Deutsche Forschungsgemeinschaft (SFB405, A13), the BioFuture program of the Bundesministerium für Bildung und Forschung and the Deutsche Krebshilfe.

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Abbreviations: EBV: Epstein-Barr virus; IFN: Interferon; Ig: Immunoglobulin; ITSM: immunoreceptor tyrosine-based switch motif; KIR: killer cell Ig-like receptor; MHC: major histocompatibility complex; SH2: Src-homology 2 domain; SRR: SLAM-related receptor; TNF: tumor necrosis factor; XLP: X-linked lymphoproliferative disease.

Key Words: Natural Killer Cells, Signal Transduction, Lymphocyte Activation, Immune Regulation, Surface Receptor, Cellular Cytotoxicity, Review

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