Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

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born in: Hamburg

Oral-examination: _____

Regulation of human lymphocytes by SLAM-related receptors

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Acknowledgements

First of all I thank Prof. Dr. Carsten Watzl. Thank you for your excellent supervision, and your helpful discussions. Your motivating optimism and your enthusiasm for scientific issues supported me very much.

Furthermore I highly appreciate that PD Dr. Ursula Klingmüller agreed to represent this work in the Faculty of Natural Science of the University Heidelberg.

I also express my gratitude towards Dr. Guido Wabnitz our cooperation partner in the T cell project. Thank you very much for your productive ideas, practical advice and for providing crucial reagents.

Then I want to thank all past and present members of the Watzllab-crew: Philipp Eissmann, Johanna Endt, Sabrina Hoffmann, Doris Urlaub, Maren Claus, Kristine Kohl, Stefanie Margraf-Schönfeld, Ewelina Miczka, Sabine Wingert, Birgitta Messmer, Mina Sandusky, Patrick Rämer, Rauf Bhat, André Cohnen, Stephan Gütgemann, and Wolfgang Merkt. Thank you for fruitful discussions, for all you taught me, and for your friendship. You helped me to keep my spirits up and made me feel at home in the lab.

I also thank the following cooperation partners that contributed to parts of this work: Dr. David Nutt, formerly at the Interdisciplinary Center for Scientific Computing, for his calculations on the NTB-A structure; Dr. Matthias Konstandin, formerly at the department of cardiology, for recruiting the patients and collecting the blood samples; and the group of PD Dr. Thomas Giese at the Institute for immunology for performing quantitative RT-PCR on our mRNA samples.

In addition, I thank all members of our institute who were involved in the recruitment of blood donors, collection of blood samples and isolation of T cells.

Furthermore I also thank all the other members of the institute who readily supported me when I asked for help or advice.

Finally, there are also persons from my private life I like to thank: My parents, my sister and my brother and all my friends for their backup.

Thank you very much!

Summary

The six members of the SLAM-related receptor family are expressed on many cell types of the immune system and play a role in fine-tuning of immune responses. In this study we investigated the SLAM-related receptors 2B4, NTB-A and CRACC.

2B4 and NTB-A are activating receptors on human natural killer (NK) cells. 2B4 binds to CD48, NTB-A is homophilic. The molecular basis for the homophilic NTB-A interaction has been identified by crystal structure analysis, but the results have not been tested in functional assays. Using mutational analysis we could show that the residues H54 and S90 are very important for functional homophilic interaction between two NTB-A molecules, whereas the residues E37 and Q88 are not.

After binding to their ligands 2B4 and NTB-A recruit the two adapter molecules SAP and EAT-2. Although many elements of 2B4 and NTB-A signaling have been described, the early events in their signal transduction are not fully understood. In this study we could show that in human natural killer cells the phosphorylation of 2B4 and NTB-A takes place independently of SAP. However, both receptors need the presence of SAP to trigger cytotoxic responses. The adapter EAT-2 does not bind to the phosphorylated receptors in the absence of SAP. This leads to the conclusion that SAP association with the receptors is the crucial prerequisite for further signaling events, including the recruitment of EAT-2.

CRACC is an activating receptor on NK cells triggering cytotoxicity and enhancing cytokine production. The receptor is also expressed on a subset of CD8-positive and few CD4-positive T cells, but the function of CRACC on these cells is unknown. In this study we describe CRACC as co-stimulatory receptor on T cells. Simultaneous engagement of the T cell receptor and CRACC induces expression of activation markers, proliferation and cytokine production. T cell-mediated cytotoxicity is not enhanced by engagement of CRACC. We found that CRACC is expressed mainly on CD8-positive memory T cells, and its expression is induced on CD8-positive T cells by activation. Therefore we suggest that CRACC co-stimulation supports the expansion of activated cells and facilitates the re-activation of memory T cells.

Furthermore we could detect CRACC expression on CD4-positive, CD28-negative T cells in patients with unstable angina pectoris. This population appears in patients with chronic inflammatory diseases and amplifies the inflammatory process. We

suggest that CRACC co-stimulation could be involved in the continuous activation of these cells, and therefore is a possible therapeutical target.

Zusammenfassung

Die SLAM verwandten Rezeptoren sind eine Rezeptorfamilie, deren sechs Mitglieder auf vielen verschiedenen Zelltypen des Immunsystems vorkommen, wo sie an der Feinabstimmung von Immunantworten beteiligt sind. In dieser Arbeit wurden die SLAM verwandten Rezeptoren 2B4, NTB-A und CRACC untersucht.

2B4 und NTB-A sind aktivierende Rezeptoren auf natürlichen Killerzellen (NK-Zellen) des Menschen. 2B4 bindet an CD48, NTB-A ist homophil. Die molekulare Grundlage für die homophile Interaktion von NTB-A wurde durch Kristallstrukturanalyse aufgeklärt, aber die Ergebnisse wurden noch nicht in Experimenten funktionell bestätigt. Durch Mutationsexperimente konnten wir zeigen, dass die Aminosäurereste H54 und S90 für die homophile Interaktion eine sehr wichtige Rolle spielen, während die Reste E37 und Q88 weniger wichtig sind.

Im aktivierten Zustand rekrutieren die Rezeptoren 2B4 und NTB-A die beiden Adaptermoleküle SAP und EAT-2. Obwohl schon viele Elemente der Signaltransduktion durch 2B4 und NTB-A beschrieben wurden, sind viele der Ereignisse am Beginn der Signalweiterleitung ungeklärt. Wir konnten zeigen, dass in humanen NK-Zellen die Phosphorylierung von 2B4 und NTB-A unabhängig von SAP stattfindet. Um eine zytotoxische Reaktion auszulösen, sind beide Rezeptoren allerdings auf SAP angewiesen. Das Adaptermolekül EAT-2 zeigt in Abwesenheit von SAP keine Bindung an die phosphorylierten Rezeptoren. Das führt zu dem Schluss, dass die Assoziation von SAP mit den phosphorylierten Rezeptoren die unerlässliche Voraussetzung für weitere Signaltransduktionsschritte ist, so auch für die Bindung von EAT-2 an die Rezeptoren.

CRACC ist ein aktivierender NK-Zellrezeptor, der Zytotoxizität und Zytokinproduktion auslöst. Außerdem wird dieser Rezeptor von einem Teil der CD8-positiven und wenigen CD4-positiven T-Zellen exprimiert, wobei seine Funktion auf diesen Zellen nicht bekannt ist. In dieser Arbeit zeigen wir, dass CRACC ein kostimulatorischer Rezeptor auf T-Zellen ist. Eine gleichzeitige Stimulation von T-Zellrezeptor und CRACC führt zur Expression von Aktivierungsmarkern, Proliferation und Zytokinproduktion. Auf die Zytotoxizität von T-Zellen hat die Aktivierung von CRACC keinen verstärkenden Einfluss. Wir konnten zeigen, dass CRACC hauptsächlich auf CD8-positiven Gedächtnis-T-Zellen exprimiert wird, und dass seine Expression auf CD8-positiven T Zellen durch Aktivierung induziert wird. Deshalb vermuten wir, dass

Kostimulation von CRACC die Vermehrung von aktivierten T-Zellen begünstigt und die Reaktivierung von Gedächtnis T-Zellen erleichtert.

Außerdem konnten wir eine Expression von CRACC auf CD4-positiven, CD28-negativen T Zellen von Patienten mit instabiler Angina pectoris feststellen. Diese Zellpopulation tritt bei Patienten mit chronischen entzündlichen Erkrankungen auf und verstärkt die Entzündungsprozesse. Wir vermuten, dass Kostimulation durch CRACC an der fortdauernden Aktivierung dieser Zellen beteiligt sein könnte und deshalb ein mögliches Ziel für neue therapeutische Ansätze darstellt.

1 Introduction

1.1 T lymphocytes and natural killer cells

1.1.1 Innate and adaptive immunity

The mammalian immune system has developed a variety of different cell types that are involved in the detection and clearance of pathogens. These cells are divided into two groups termed cells of the innate and the adaptive immune system. Cells of the innate immune system are regulated by germ line encoded receptors recognizing common structures expressed by pathogens or signals that are induced by pathogens. Therefore cells of the innate immunity can react immediately upon encounter of pathogens. Cells of the adaptive part allow the immune system to react to a multitude of molecular structures by receptors that are generated through re-arrangement of germ line encoded segments. These receptors are expressed in a clonal fashion, and cells bearing a specific receptor for the encountered pathogen have to undergo proliferation before they can mount an effective immune response. Thus, the innate immune system serves as a first line of defense keeping pathogens under control, until they can be cleared by an adaptive response.

1.1.2 Natural killer cells and T lymphocytes

Natural killer (NK) cells and T cells are representatives of the innate and the adaptive part of the immune system, respectively. Both develop from a common bipotential progenitor in the bone marrow (1) and share some properties despite their classification into innate and adaptive immune cells. T cell progenitors migrate to the thymus, where the genetic recombination of the T cell receptor gene and selection of T cells takes place. NK cells develop in the bone marrow.

The majority of lymphocytes in peripheral blood are T cells, whereas only 5 to 15 % are NK cells. T cells that emerge from the thymus are called naïve T cells, because they have not encountered their specific antigen yet. These naïve cells constantly re-circulate through secondary lymphoid organs like spleen, gut or mucosa-associated lymphoid tissues and lymph nodes, where they screen antigen-presenting cells for their specific antigen (2).

NK cells are also present in secondary lymphoid organs; about 5 % of lymphocytes in lymph nodes are NK cells. Other non-lymphoid tissues like lung and liver are also frequented by NK cells (1, 3).

While T cells can be identified by expression of the T cell receptor, human NK cells have been defined as T cell receptor-negative cells expressing CD56. Recently the receptor NKp46 has been described to be a more specific NK cell marker, as it is expressed on human and murine NK cells (4, 5).

1.1.3 Activation of T cells

T cells are characterized by the clonal expression of unique T cell receptors (TCR) that allow them to recognize specific peptide antigens. Recognition of the antigen leads to T cell activation. To be recognized by the TCR the antigenic peptides have to be bound by specialized glycoproteins called major histocompatibility complex (MHC) molecules. Polymorphy and polygeny of the MHC molecules ensure that a large variety of peptides can be presented to T cells. There are two types of MHC molecules that present antigen from different sources. Peptides derived from proteins in the cytosol are presented on the cell surface by MHC class I molecules, which are expressed on all nucleated cells. MHC class I-bound peptides are recognized by cytotoxic T cells expressing the co-receptor CD8. This enables them to detect intracellular pathogens like viruses and eliminate the infected cells. So-called antigen-presenting cells like macrophages or B cells take up antigens from extracellular pathogens and present antigenic peptides bound to MHC class II molecules. The peptides derived from extracellular antigens are loaded onto the MHC class II molecules in vesicular compartments. MHC class II-bound peptides are recognized by T cells expressing the co-receptor CD4. Therefore these T cells can provide help to antigen-presenting cells that have taken up antigen from extracellular pathogens.

The TCR consists of several proteins. A heterodimer formed by α- and β-chain recognizes the specific antigen presented by MHC molecules. This variable part is different on each T cell clone. The constant TCR component is the CD3 complex that mediates signal transduction of the TCR. It consists of one γ chain, one δ chain, two ε chains, and two ζ chains. The antigen specificity of the α- and β-chain is determined through random rearrangement of germ line encoded segments, which takes place during T cell development in the thymus. This recombination process allows generation of a vast number of TCR specificities. As the recombination process is random, the receptors can be specific for any possible peptide, including peptides derived from self-proteins. To prevent autoimmunity T cells expressing receptors that bind to self-antigens are deleted in the thymus. Because not all possible

autoantigens are expressed in the thymus, activation of naïve T cells that have not been deleted in the thymus has to be controlled in the periphery.

One mechanism of activation control is the strong dependency on a second signal in addition to TCR engagement (6, 7). Only very strong TCR signals are able to fully activate naïve T cells and induce proliferation and differentiation (8, 9). The co-stimulatory signal allows full activation of T cells at lower thresholds. The absence of a co-stimulatory signal during engagement of the TCR on naïve T cells normally results in anergy (10, 11). The co-stimulatory signals are provided only by specialized antigen-presenting cells like mature dendritic cells or B cells (12). The encounter of naïve T cells with antigen-presenting cells takes place in secondary lymphoid organs like lymph nodes, spleen or Peyer's patches in gut associated lymphoid tissue. The activation of antigen-presenting cells and expression of co-stimulatory ligands are induced by signals from the innate part of the immune system.

The interaction of the receptor CD28 on T cells with CD80 (B7.1) or CD86 (B7.2) on antigen-presenting cells is generally regarded as the primary co-stimulatory pathway, although other co-stimulatory pathways exist. The most important effects of CD28 co-stimulation are the stabilization of interleukin-2 (IL-2) mRNA and expression of the IL-2 receptor α-chain, which associates with the β and γ-chain to form the high-affinity IL-2 receptor (13, 14). Stimulation through IL-2 is crucial for T cell proliferation. Thus, co-stimulation of T cells triggers a positive feedback loop, enabling autocrine induction of proliferation. During this clonal expansion naïve T cells differentiate into effector T cells. The effector T cells leave the secondary lymphoid organs and are guided by chemokines to the site of infection. Effector functions of these cells can be triggered by engagement of the T cell receptor without co-stimulation.

The original notion was that a co-stimulatory receptor activates a distinct signaling pathway, which is needed as a second signal besides TCR signaling (15). Therefore the co-stimulating effect of CD28 was regarded as an independent signal that complements the TCR signal. In the meantime it has become evident that CD28 signaling rather enhances TCR signals than contributing qualitatively different signals (16). This is supported by the identification of many other receptors with co-stimulatory ability that do not use the same signaling pathways as CD28. These co-stimulatory receptors belong to a variety of different families (17): Another co-stimulatory receptor from the CD28-family is ICOS; other receptors like 4-1BB, OX40

and CD27 belong to the tumor necrosis factor (TNF)-family; there are co-stimulatory members of the immunoglobulin superfamily like CD2 or the SLAM-related receptors SLAM, 2B4, NTB-A and CD84; furthermore integrins, tetraspanins, members of the T cell immunoglobulin and mucin domain (TIM)-family and receptors from the super-family with scavenger receptor cystein-rich domains have been found to have co-stimulatory properties. The circumstances, under which any of these co-stimulatory receptors gain importance, still need to be investigated.

Activation of CD8-positive T cells seems to be controlled by a further mechanism, possibly because an autoimmune reaction of CD8-positive cells can cause serious damage due to their cytotoxic potential. It has been shown that they need stronger co-stimulation than CD4-positive cells (18). Activated CD4-positive T cells can stimulate dendritic cells to increase the expression level of co-stimulatory molecules on their surface, thus providing support for the activation of CD8-positive T cells (18). Because the CD4-positive T cells must recognize different peptides presented by the same antigen-presenting cell for enhancing the co-stimulation, the risk of activating self-reactive CD8-positive T cells is further reduced.

Because proliferation of activated T cells is enhanced by a positive feedback-loop, the proliferative response must be controlled by inhibitory mechanisms. One of these mechanisms is the down-regulation of CD28 expression on activated T cells (19). This down-regulation interrupts the co-stimulatory signals. In addition, activated cells express the inhibitory receptor CTLA-4. CTLA-4 binds to the same ligands on antigen-presenting cells as CD28, but with higher affinity. Thus the expression of CTLA-4 limits the proliferative response of activated T cells (20). By these means the positive feedback loop of autocrine IL-2 secretion, which is triggered by co-stimulation, is interrupted in a T cell intrinsic manner.

1.1.4 Functions of T cells

T cells are among the most versatile cells of the immune system fulfilling a variety of functions. The CD8-positive T cells that make up about one third of periphal blood T cells are capable of eliminating infected cells. They can induce apoptosis of those cells via the release of perforin and granzymes from intracellular granules or via the engagement of apoptosis-inducing death receptors on the target cell by Fas ligand or TRAIL.

About two thirds of peripheral blood T cells express CD4 and shape and coordinate innate and adaptive immune responses through secretion of cytokines and

expression of membrane associated proteins. They can differentiate into a variety of effector subsets with different functions depending mainly on the cytokines present in the microenvironment during their activation. Five different effector phenotypes of CD4-positive T cells have been characterized (21). There are four different types of helper T cells named T helper (Th) 1, 2 or 17 and follicular helper T (Tfh) cells. A fifth group of CD4-positive effector T cells are regulatory T cells (Treg). In the immature effector stage, where activated CD4-positive cells still have the potential to develop into any of these effector cell types, they are called Th0 cells. Th1 cells are characterized by their production of the cytokine interferon-γ (IFN-γ) and are involved in cellular immunity against intracellular pathogens. Development of Th1 cells is induced by IL-12, which can be secreted by macrophages or dendritic cells, and IFN-γ secreted by NK cells or other T cells. Th2 cells secrete IL-4, IL-5 and IL-13. They play a pivotal role in the humoral immune response against helminths and other extracellular pathogens. A polarization towards Th2 effector development is mediated by IL-4. Besides promotion of Th1 or Th2 responses, the cytokines IFN-γ and IL-4 suppress the differentiation of the respective counterpart. The name Th17 cells was coined after their production of IL-17. They also release IL-22 and are important for the clearance of extracellular bacteria and fungi, especially at mucosal surfaces. *In vitro* their development can be induced by transforming growth factor-β (TGF-β) in combination with the pro-inflammatory cytokines IL-6, IL-21 and IL-23. Tfh cells regulate and promote B cell responses in B cell follicles and need IL-21 for their development. Treg cells play a crucial role in the maintenance of immune tolerance and the prevention of autoimmunity, as they can suppress T cell mediated immune responses.

A minority of T cells named γδ T cells expresses a TCR generated from different germ line encoded fragments. The TCR of these cells are of lower variability and can bind to certain phosphorylated non-peptide-antigens. These antigens are often of bacterial origin, therefore γδ T cells seem to play a role in antibacterial immune responses. There is also a rare T cell subset termed natural killer T cells. They display a limited TCR diversity and recognize glycolipids bound to CD1d. Activated NKT cells can shape innate and adaptive immune responses by secretion of IFN-γ and IL-4. Because of their restricted variability in antigen recognition γδ T cells and NKT cells are considered part of the innate immune system.

1.1.5 T cell memory

One hallmark of adaptive immunity is the improved immune response upon re-encounter of pathogens. This immunological memory is established by the development of memory cells during an adaptive immune response. Upon encounter with specific antigen the proliferation of activated T cells gives rise to large numbers of effector cells that are needed for clearance of the infection. After accomplishment of their task these cells are removed by several mechanisms (22). One is the deprivation of cytokines that renders the activated effector cells susceptible to apoptosis. Another mechanism is the re-stimulation induced cell death that occurs when already activated cells are stimulated through their TCR during the contraction phase of an immune response. The re-stimulated cells then also undergo apoptosis. Some of the activated T cells differentiate into memory cells. These cells are more resistant to apoptosis and are not affected by the mechanisms of deletion. According to the current model, memory cells circulate in the periphery and maintain their numbers through homeostatic proliferation. In a simple approach, memory cells can be divided into central memory cells that re-circulate through secondary lymphoid organs like naïve T cells, and effector memory cells that stay in the periphery (23, 24). Recent reports suggest that the bone marrow provides a niche, where memory cells are maintained (25-27).

The improved immune response upon re-infection is based on several factors (28). Memory T cells specific for the respective antigens of the pathogen are more frequent than antigen-specific naïve T cells during the first infection. This gives the secondary response a broader basis. Furthermore, memory T cells can more rapidly acquire effector functions, which reduces the time-span needed to mount the secondary response. In addition, effector memory T cells circulate in the periphery and can act directly upon encounter of pathogen at the site of infection. Therefore memory T cell responses are stronger and faster than the T cell response against newly encountered pathogens.

1.1.6 Activation of NK cells

In contrast to T cells the activation of NK cells is not dependent on the specificity of one receptor, but is regulated by the interplay of activating and inhibitory germ line encoded receptors (29).

Inhibitory receptors on human NK cells are members of the family of killer cell immunoglobulin-like receptors (KIR) or members of the C-type lectin-like NKG2-

family that form heterodimers with CD94 (30). KIR bind to MHC class I molecules, but their specificity is not dependent on the MHC-bound peptide, although the peptide contributes to KIR binding (31). Specificity of KIR is determined by the allotype of the MHC molecules. NKG2/CD94 heterodimers recognize the non-classical MHC molecule HLA-E, which presents peptides derived from the leader peptides of other MHC molecules. The concept of NK cell inhibition by MHC molecules is called the detection of 'missing self' (32, 33). Down-regulation of MHC class I molecules is a common mechanism used by viruses to avoid recognition by T cells (34), and tumor cells often lose MHC expression completely (35). In contrast to healthy cells, these cells become susceptible to NK cell-mediated lysis, because they fail to provide sufficient inhibitory signals.

The inhibitory receptors seem to be expressed on NK cells in a rather random manner. In all human individuals NK cells can be found that express only KIR that recognize MHC allotypes not expressed in the respective individual or no inhibitory receptor at all. This led to the question how NK cell self-tolerance is ensured, because activation of these cells could not be controlled by expression of MHC molecules on healthy cells. Based on the finding that human NK cells expressing no inhibitory receptor are hypo-responsive to stimulation, a model of NK cell 'education' during their development was proposed (36). In this model developing NK cells can only become fully functional if they receive signals through inhibitory receptors. Recent reports show that the strength of inhibitory receptor signaling during NK cell development determines their cytotoxic potential. Experiments with murine NK cells showed that their cytotoxic potential increased with the number of different inhibitory receptors that were engaged during NK cells development (37, 38).

The activating receptors expressed on NK cells are more heterogeneous than the inhibitory receptors and not all ligands are known (29). Some of the receptors bind to molecules that are expressed ubiquitously, also on healthy cells, e.g. the members of the SLAM-related receptor family 2B4, NTB-A and CRACC that will be discussed below (39). The ligands for the C-type lectin-like receptor NKG2D are MHC class I-related chain (MIC) proteins A and B and the UL16-binding proteins (ULBP), which are expressed after DNA damage or viral infection (40, 41). The recently described ligand for NKp30 B7-H6 seems to be expressed only on tumor cells (42). For other activating NK cell receptors an interaction with viral ligands on infected cells has been reported, e.g. NKp44 and NKp46, which recognize viral hemagglutinins (43,

44). Finally, NK cells express the low affinity Fc receptor CD16 (FcγRIII), which links NK cell function to adaptive immunity. When antibodies bind to antigens on the surface of a cell, they can be recognized by NK cells via CD16 resulting in elimination of the cell expressing the antigen.

NK cells are also regulated by cytokines. IFN-α or β, IL-12, IL-15 and IL-18 activate NK cells (45). Cytokine-activated NK cells can produce cytokines in turn, and they display increased cytotoxicity due to higher perforin content of their lytic granules, increased expression of Fas-ligand and lower thresholds for activation through activating receptors (46, 47). IL-2 is also able to stimulate NK cell proliferation, cytotoxicity and to some extent cytokine secretion (48). This may happen in the lymph nodes, where NK cells could be stimulated by IL-2 produced by activated T cells (49). NK cell functions can be inhibited by TGF-β, which is produced by regulatory T cells (50-52).

Besides the control through inhibitory receptors, autoreactivity of NK cells can be limited by the need for at least two activating signals, similar to co-stimulation in T cells. One of these signals can be cytokine stimulation. IL-2-activated NK cells react to stimulation of any activating receptor. In contrast, resting NK cells cannot be activated by engagement of only one single type of receptor with the exception of CD16. It has been shown that engagement of pair-wise combinations of activating receptors is needed to trigger a response in resting cells (47).

1.1.7 Functions of NK cells

Natural killer cells play an important role in the control of infected or transformed cells (53). Due to the detection of 'missing self', viral or stress-induced ligands they can eliminate potentially dangerous cells. This is mainly mediated by direct cellular cytotoxicity, as they can induce apoptosis in target cells via the release of perforin and granzymes. Similar to cytotoxic T cells, they can also induce apoptosis by engagement of Fas or TRAIL receptors. The elimination of infected cells by NK cell possibly improves adaptive T cells responses, because dendritic cells can take up antigens from apoptotic NK cell targets and present them to T cells (54). The cytotoxic activity of NK cells seems to have also regulatory aspects. It has been shown that human NK cells can lyse immature dendritic cells, which implies that NK cells influence dendritic cell homeostasis (55). NK cells could also control inflammatory responses by deletion of over-activated macrophages, as it has been shown that activated macrophages are susceptible to NK cell cytotoxicity (56).

The second important function of NK cells is the production and secretion of cytokines. The main cytokines produced by NK cells are IFN-γ, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) (57). With their cytokine secretion NK cells not only activate cells of the innate immune system like macrophages, they can also shape the adaptive immune response (58, 59). IFN-γ and TNF-α secreted by NK cells, as well as yet-to-be-defined contact dependent signals, promote the maturation of dendritic cells that in turn can activate T cells and NK cells (55, 60). IFN-γ also drives the differentiation of activated CD4-positive T cells towards a Th1 response (61).

Two distinct subsets of human NK cells have been described that are specialized for one of the two effector functions. A small population of NK cells has a low cytotoxic potential, but can produce high amounts of cytokines. These cells can be identified by high expression levels of the NK cell marker CD56 and lack of the receptor CD16. The majority of NK cells shows lower expression of CD56, expresses CD16 and is more cytotoxic (1).

A specialized subset of NK cells is found in the human uterus during pregnancy and is therefore called uterine NK cells or uNK cells. Although they contain high amounts of granules, these NK cells are less cytotoxic and seem to have mainly regulatory functions in the decidua (62, 63). Because these cells produce angiogenic factors like angiopoietins 1 and 2, these cells are likely to play a role in vascularization of the decidua (64).

A recently identified NK cell subset in mucosa associated lymphoid tissue has been shown to be essential for mucosal homeostasis (65). These NK cells are not proficient at the classical NK cell functions cytotoxicity and IFN-γ production. Because these cells produce IL-22, a cytokine that plays a role in the maintenance of mucosal epithelia, they have been named NK-22 cells (66).

The crucial role of NK cells in the immune system is demonstrated by the severe symptoms of patients with a rare NK cell deficiency (67). These patients suffer from recurring viral and bacterial infections despite the presence of T and B cells that can mount an adaptive immune response. This underscores that immunity is mediated by the interplay between the innate and adaptive immune system.

1.2 The SLAM family of immunoglobulin like receptors

1.2.1 SLAM-related receptors

The family of SLAM-related receptors (SRR) is part of the immunoglobulin (Ig) receptor super-family. The family comprises six members, namely SLAM (CD150), 2B4 (CD244), NTB-A (Ly108 in mice), CRACC (CS1, CD319), CD84, and Ly-9 (CD229), which are expressed on cells of the hematopoietic lineage (39, 68, 69). The SRR genes are located on the long arm of chromosome 1 in humans (1q21-24), and on mouse chromosome 1 (1H2) with a similar organization in both species (39). The homology in sequence and organization of the gene loci implies that SRR genes arose from one common ancestor gene through gene duplication. All SRR are type I transmembrane receptors with an extracellular part consisting of one N-terminal V-type Ig-domain and one membrane-proximal C2-type Ig-domain (fig. 1). An exception is Ly-9, which contains four Ig-domains in the order of IgV-IgC2-IgV-IgC2. The size of the intracellular domain of SRR varies between 70 and 180 amino acids. With the exception of 2B4 all SRR are homophilic (70-75). 2B4 binds to CD48, a glycosylphosphatidylinositol-anchored membrane protein that is widely expressed on cells of the immune system and is also part of the Ig receptor super-family (76, 77). The cytosolic part of SRR contains two to four tyrosine-based signaling motifs that become phosphorylated upon receptor engagement and are the basis for SRR signaling (39, 78) (fig. 1). The tyrosine of these motifs is embedded in a consensus sequence TxYxxV/I, where x represents any amino acid. These motifs have been termed immunoreceptor tyrosine-based switch motifs (ITSM), because they can recruit different signaling molecules that promote activating or inhibitory signals (79). ITSM can bind a group of adapter molecules that consists of SLAM-associated protein (SAP, SH2D1A) and Ewings sarcoma-Fli1-activated transcript 2 (EAT-2, SH2D1B) in humans. In mice exists a third member, EAT-2-related transducer (ERT, SH2D1C), but the ERT gene in humans is only a pseudogene (80, 81). These adapter molecules are small, comprising one Src homolgy 2 (SH2) domain and a short C-terminal extension. The importance of SRR and SAP function in immunity is underscored by the finding that the severe immune disorder X-linked lymphoproliferative disease (XLP) is caused by the absence or dysfunctionality of SAP.

The mediators of inhibitory signaling that can bind to phosphorylated ITSM are the tyrosine-phosphatases SHP-1 and 2, and the inositol-phosphatase SHIP (79, 82-84).

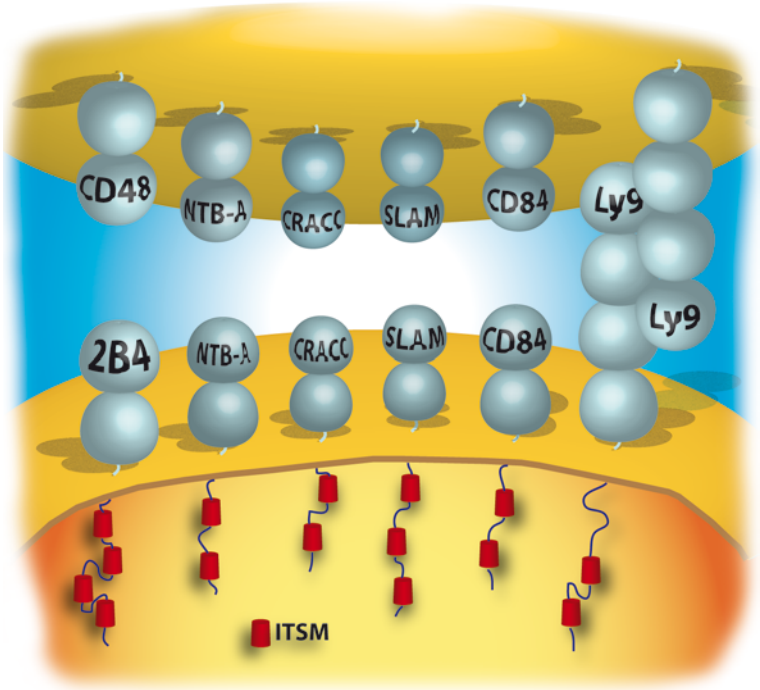

Figure 1: The family of SLAM-related receptors and their ligands
Depicted are the six members of the SLAM-related receptor family with their cytoplasmic tails containing the immunoreceptor tyrosine-based switch motifs (ITSM). The respective ligands are shown on the opposing cell surface. Picture by courtesy of Claus *et al.* (39).

1.2.2 Expression and functions of the SRR 2B4, NTB-A and CRACC

The expression of SRR is heterogeneous on different immune cells and no SRR shows expression confined to only one cell type. In addition, SLAM and CD84 are expressed on hematopoietic stem cells, and 2B4 is found on multipotent progenitor cells (85, 86). Despite the differences in expression pattern on cells of different functions the common role of the SRR family can be described as fine-regulation of immune responses.

2B4 (CD244)

2B4 is expressed on NK cells, γδ T cells, monocytes, basophils, eosinophils and some thymocytes (87-90). On human T cells the expression is confined to approximately 50 % of the CD8-positive T cells. 2B4-positive T cells display a memory cell phenotype, and 2B4 expression can be induced on human and murine CD8-positive cells by *in vitro* activation.

Its function has first been described on murine and human NK cells as a receptor triggering cytotoxicity and IFN-γ production after engagement of its ligand CD48 (88, 91-94). The ubiquitous expression of the 2B4 ligand CD48 on cells of hematopoietic origin suggests that a main function of 2B4 on NK cells is the immunosurveillance of

other immune cells. Down-regulation of MHC class I molecules on transformed cells renders these cells susceptible to 2B4-triggered NK cell cytotoxicity.

Other findings indicate that under certain circumstances human 2B4 can also play an inhibitory role on NK cells. At early stages of NK cell development the expression of activating receptors precedes the expression of inhibitory receptors and the cells gain their cytotoxic potential. In these precursor cells 2B4 has been shown to fulfill inhibitory functions ensuring self-tolerance of these cells (95). Similarly, in NK cells isolated from human lymph nodes engagement of 2B4 reduced IFN-γ production (96). A third NK cell population with inhibitory 2B4 signaling in humans are decidual NK cells during pregnancy (62, 97). The inhibitory function of 2B4 in these cases could be caused by a reduced expression of the adapter SAP (62), which is supported by the report that 2B4 mediates inhibitory signals in XLP patients with defective SAP (98).

The role of 2B4 on NK cells in mice has been the issue of controversial discussion. The first notion that 2B4 is an activating receptor on murine NK cells was challenged by the finding that mouse NK cells showed a decreased cytotoxicity against certain CD48-expressing tumor cells compared to their CD48-negative counterparts. Blocking of 2B4-CD48 interactions with antibodies abolished this difference (99, 100). Experiments using 2B4 KO mice pointed in the same direction. These mice showed an increased clearance of injected tumor cells compared to wild type mice, when the tumor cells expressed CD48 (99, 100). The inhibitory signal mediated by 2B4 in these experiments seemed to be independent of SAP, as the same results were obtained with NK cells from SAP KO mice (99). 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-positive tumor cells, while female mice fail to reject both CD48-positive and CD48-negative 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* (101). However, a recent report again supported the notion from early experiments that murine 2B4 is an activating 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 KO mice (102).

The reason for the conflicting results regarding activating or inhibitory properties of 2B4 in mice is unclear. However, a recent study provided a possible explanation for this discrepancy. It has been shown that the engagement of human and murine 2B4 has activating or inhibitory effects depending on the expression level of the receptor, the extent of receptor cross-linking and the expression levels of the adapter SAP (103). Antibody-mediated Cross-linking of 2B4 on cells with low surface expression of the receptor led to activation. In contrast, cells with high expression levels of 2B4 were inhibited, when the receptor was cross-linked. This inhibitory signaling was changed to an activating signal, when less receptor molecules on these cells were engaged. In cells expressing high levels of both 2B4 and SAP the effect of strong receptor cross-linking was also activating.

In human T cells 2B4 has been described as a co-stimulator enhancing proliferation and cytotoxicity of antigen-specific CD8-positive T cells (104, 105). Interestingly, in a study showing that NK cells can enhance antigen-specific proliferation of T cells 2B4 on NK cells served as ligand for CD48 on T cells (106).

On human eosinophils cross-linking of 2B4 elicited cytokine secretion and eosinophil-mediated cytotoxicity (90).

NTB-A (Ly108)

Human NK cells, T cells and B cells express the SRR called NK, T and B cell antigen (NTB-A) (84). In addition NTB-A has also been found on eosinophils (90, 107). In mice the expression of the NTB-A homolog Ly108 on NK cells is strain dependent (108). Therefore the function of NTB-A on NK cells has mainly been investigated in human cells. NTB-A engagement on NK cells induces cytotoxicity and production of IFN-γ and TNF-α (70, 71, 84). The analysis of NTB-A functions in NK cells from XLP patients showed that in the absence of functional SAP the cytotoxic response was not only reduced, but rather inhibited, while IFN-γ production was intact (70, 84).

On human T cells NTB-A has been shown to have a co-stimulatory potential inducing proliferation and IFN-γ production when engaged simultaneously with the TCR (109). As IFN-γ promotes development of CD4-positive cells into Th1 helper type cells, it was assumed that NTB-A plays a role in shaping of Th1 immune responses. This was supported by experiments with mice injected with NTB-A-Fc-fusion proteins that are thought to block homophilic interaction of Ly108, the murine NTB-A homolog. Treated mice displayed a reduced Th1 cytokine-induced isotype switch to IgG2a and IgG3. Furthermore the injection of fusion proteins delayed the onset of experimental

autoimmune encephalomyelitis, a Th1-mediated model for human multiple sclerosis (109). However, mice with a defective Ly108 gene displayed impaired Th2 responses characterized by a loss of IL-4 production and intact IFN-γ secretion (107). Further experiments will be needed to solve this issue.

The phenotype of mice with defective Ly108 revealed another function of the receptor in innate immunity. The mice showed increased susceptibility to bacterial infections, which was due to an impaired generation of reactive oxygen species in neutrophils (107).

Another finding that sheds a light onto NTB-A function is the connection of a polymorphism in the Ly108 gene to the autoimmune disease systemic lupus erythematosus in mice. The lupus-associated Ly108 allele may be linked with modified signaling responses of T cells in lupus-susceptible mice (110). Additionally, the normal Ly108 gene has been reported to sensitize immature B cells to deletion and RAG re-expression, whereas the lupus-associated allele did not (111). Therefore NTB-A seems to have a function in the regulation of T and B cell responses and the maintenance of self-tolerance.

CRACC (CS1, CD319)

The CD2-like receptor activating cytotoxic cells (CRACC) is expressed on NK cells, a subset of CD8-positive T cells and on few CD4-positive T cells. Despite its name it is also expressed on activated B cells and mature dendritic cells (112, 113). On NK cells CRACC has been described as an activating receptor triggering cytotoxicity (112, 114, 115) and enhancing IFN-γ production (115). CRACC-mediated NK cell cytotoxicity was not impaired in XLP patients or SAP KO mice (112, 115), because CRACC signaling seems to be mediated only by EAT-2 (114, 115). Cross-linking of CRACC on human B cells has been reported to induce proliferation and expression of cytokines, but did not induce antibody production (116).

1.2.3 XLP, a severe immune disorder caused by defective SRR signaling

XLP or Duncan's disease was first characterized by an inappropriate immune response to Epstein-Barr virus (EBV) infection (117). EBV belongs to the human γ-herpesvirus family and infects mature B cells. Infected B cells proliferate and some undergo transformation. These lymphoblasts are readily detected and eliminated in normal immunocompetent individuals (118). Although latent EBV infected B cells persist for life, they are kept under control by cytotoxic lymphocytes (119). EBV

infection is widespread in humans. In children under the age of 10 the infection is often asymptomatic and about 50 % of infections above that age result in infectious mononucleosis (120).

In contrast, up to 60 % of XLP patients develop a fatal fulminant infectious mononucleosis after EBV infection leading to death within 1 or 2 months. Other manifestations of the disease are hypogammaglobulinemia and lymphoproliferative disorders, mainly of B lymphocytes. The disease manifests usually about the age of five and the mortality rate is close to 100 % at the age of 20 (78, 121). The disease is caused by mutations in the gene encoding SAP (122, 123). The defects in lymphocyte function that lead to the pathogenesis of XLP are not fully understood, but in recent years several findings have shed a light on the underlying mechanisms.

The development of lymphocytes seems to be not impaired by SAP deficiency, as the numbers of NK, T and B cells are normal in XLP patients. Only the subset of NKT cells does not develop in these patients (124, 125). Whether the absence of these cells contributes to the pathophysiology of XLP is not known.

The humoral immune response is generally impaired in XLP patients. Their number of memory B cells is very low and no class-switch immune response is observed after booster vaccinations (126, 127), which explains the reported hypogammaglobulinemia. Recent studies revealed that these defects in antibody production are due to impaired T cell function (128).

NK cells from XLP patients display impaired cytotoxic responses (129-131). In addition EBV-specific cytotoxic CD8-positive T cells are lower in frequency and show reduced cytotoxicity against autologous EBV-infected B cells (129, 132). Recent studies gave a possible explanation for this impairment of the cytotoxic immune response: EBV-infected B cells show an up-regulated expression of CD48, the ligand for 2B4 (133). Two studies could show that the cytotoxic response of CD8-positive T cells from XLP patients against EBV-infected cells is strongly impaired by defective 2B4 signaling (134, 135). 2B4 and NTB-A-mediated cytotoxicity is also impaired in NK cells from XLP patients (84, 98, 136-138). The failure to control proliferation of EBV-infected B cells surely contributes to the massive expansion of the lymphocyte population observed in XLP patients after EBV infection.

An animal model of XLP could be created by generation of SAP KO mice, which display similar immune defects like XLP patients (139-141). SAP KO mice also have normal numbers of NK, T and B cells, but lack the NKT subset (124, 125).

Because mice are not susceptible to EBV infection, the SAP KO mice were infected with lymphocytic choriomeningitis virus (LCMV), murine γ-herpesvirus-68 or non-viral pathogens like *Toxoplasma gondii* or *Listeria major*. This makes the results less comparable to the findings in XLP patients. These mice could clear acute infections but succumbed to chronic infections because of an overwhelming response mediated by CD8-positive T cells (140). In acute infections the observed numbers of activated T cells were increased and the response of CD4-positive T cells was altered to increased IFN-γ production and reduced IL-4 and IL-10 secretion (140, 141). This skewing from a Th2 to a Th1 response is accompanied by low antibody production after infection (140-142).

Similar to the findings in XLP patients, the defective humoral immune response of SAP KO mice seems to be caused by impaired T cell function. CD4-positive T cells in these mice develop into effector cells that express molecules capable to provide T cell help to B cells, but fail to interact with B cells effectively (143). One reason for the inefficient T cell help has been shown to be a reduced duration of T-B cell contact in germinal centers of SAP-deficient mice (144). The SRR that play a role in these processes have not been identified yet.

In summary the pathophysiology of XLP is caused by a complex dysregulation of immune responses and illustrates the dependence of immune function on fine-tuning mechanisms provided by SRR.

1.2.4 Molecular mechanisms of SLAM-related receptor signaling

2B4-mediated signaling

Of all SRR the molecular mechanisms of 2B4 signaling have been examined best (fig. 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 (145, 146). Lipid raft domains are rich in kinases (147) and the Src-family kinase Lck is one possible candidate that can phosphorylate 2B4 (137). Raft recruitment has been shown to be essential for the phosphorylation of 2B4 (145). Phosphorylated ITSM of 2B4 can recruit the adapter molecules SAP and EAT-2 (83, 148, 149).

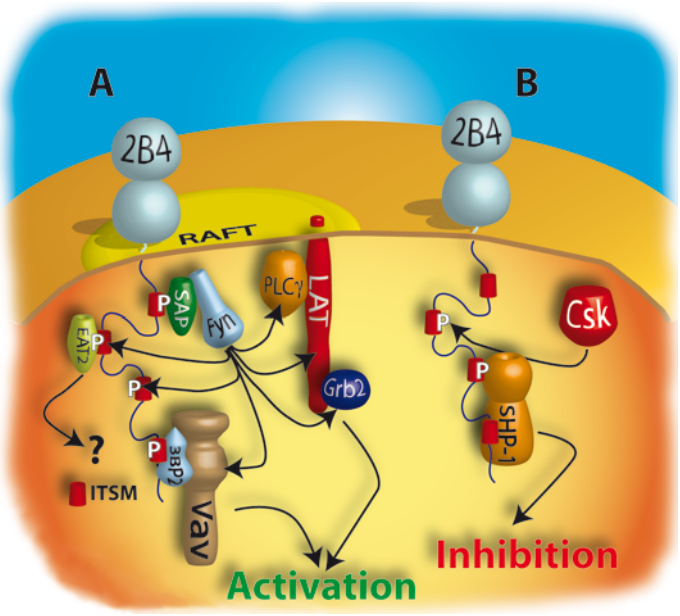

Figure 2: The model of 2B4 signal transduction
A: Early signaling events in 2B4-mediated lymphocyte activation **B:** A possible mechanism for 2B4-mediated inhibitory signals in the absence of functional SAP, e.g. in XLP patients. See text for details. Picture by courtesy of Claus *et al.* (39).

SAP can associate with all four ITSM of 2B4, but it has been shown that interaction with the membrane proximal ITSM is sufficient for 2B4 signaling (82). Only little is known about the function of the adapter protein EAT-2 (148). Both adapter molecules are about 15 kDa in size and consist of one single SH2-domain and a small C-terminal tail (148, 150, 151). The C-terminal part of human EAT-2 contains one tyrosine residue, but no phosphorylation of EAT-2 has been observed (152).

ITSM-bound SAP mediates signal transduction by recruiting the Src-family kinase FynT (153, 154) (fig. 2A). The basis for interaction between FynT and SAP was not clear until SAP was crystallized in complex with a phosphorylated ITSM and FynT. The structure revealed that FynT binds to SAP in an unusual SH2-SH3-domain interaction involving the residue arginine-87 on SAP (155). This interaction is essential for SAP function, as mutations of R87, which have been found in XLP patients, completely abolish 2B4 signaling (156, 157). Binding of FynT to SAP has been reported to increase the kinase activity of FynT, probably by preventing conformational changes into an inactive state (154, 158). FynT can also phosphorylate 2B4 (82), but there are contradicting reports whether 2B4 can be phosphorylated independently from SAP-mediated FynT recruitment (98, 102, 114, 156). The importance of SAP for 2B4 signaling becomes evident in XLP patients where 2B4-mediated cytotoxicity is abolished (98, 137, 138). Furthermore, 2B4 signaling is also abolished in SAP and Fyn KO mice (102).

Findings in the murine system suggested that EAT-2 and the closely related ERT could have inhibitory functions there (81). This hypothesis was challenged by the finding that the receptor CRACC that recruits EAT-2, but not SAP mediates activating signals in murine NK cells (115). The function as an inhibitory counterpart to SAP was also excluded by the finding that the inhibitory effects of SRR observed in SAP KO mice were also present in mice lacking all three adapters SAP, EAT-2 and ERT (108). This led to the conclusion that EAT-2 mediates activating signals through SRR in murine NK cells.

The activating function of EAT-2 in human NK cells could be comparable to the murine protein. However, there may be differences between humans and mice. In contrast to human EAT-2, the murine protein carries two tyrosine residues in its C-terminal part that can be phosphorylated (81). Interestingly, a mutated form of murine EAT-2 that could not be phosphorylated failed to mediate the activating signaling of CRACC (115), but also the inhibitory effects that have been reported (81).

As mentioned above, 2B4 can mediate inhibitory rather than activating functions in the absence of SAP (62, 95-98). This can be explained by recruitment of molecules mediating negative signals. The binding of the phosphatases SHIP, SHP-1 and SHP-2 to the phosphorylated third ITSM of 2B4 has been reported (82, 83) (fig. 2B). Under normal conditions these molecules can be displaced by SAP due to competitive binding. Thus negative signaling is suppressed and activating signaling pathways dominate. This model assumes that 2B4 can be phosphorylated in the absence of SAP. It is unclear if 2B4 is still recruited to lipid rafts in the absence of SAP. Interestingly, the 2B4 ITSM can also be phosphorylated by the kinase Csk that can associate with 2B4 as well (82). Csk is known to inhibit the activity of Src-family kinases (159), which could be another mechanism of negative signaling mediated by 2B4.

Another adapter protein that can bind to phosphorylated 2B4 is 3BP2, which has been reported to associate with the fourth ITSM (160) (fig. 2A). Phosphorylated 3BP2 interacts with the signaling molecules Vav-1, LAT and PLC-γ (160, 161). A recent report could show that association of 3BP2 with 2B4 is dependent on SAP, explaining why 3BP2-mediated signal transduction cannot compensate for the absence of SAP in XLP (162).

Stimulation of 2B4 leads to phosphorylation of LAT, Vav-1, PLC-γ, c-Cbl, Grb2 and SHIP (146, 156, 163). These molecules then propagate the signal further, initiating

the effector functions. PLC-γ cleaves phosphatidylinositol 4,5-bisphosphate (PI4,5P2) into inositol 1,4,5-trisphosphate (IP3) and 1,2-diacyl-glycerol (DAG). IP3 induces the release of Ca^{2+} from intracellular stores leading to a rise in intracellular Ca^{2+} concentration, whereas DAG activates the protein kinase C (PKC) and the Ras pathways. The Ca^{2+} release by PLC-γ is necessary for the secretion of cytotoxic granules and its importance for 2B4 signaling is shown by the finding that inhibition of PLC-γ abrogates 2B4-mediated cell lysis (114). LAT, Grb2 and Vav-1 signals are involved in activation of the mitogen activated protein kinase (MAPK) pathway, which is shown by increased phosphorylation of ERK after 2B4 engagement (160).

The signaling of 2B4 is also regulated on the level of protein expression. The expression of SAP observed in freshly isolated, i.e. resting NK cells is low and increases after activation of these cells through IL-2 or IL-12, thus resulting in enhanced 2B4 signaling (164). Engagement of the 2B4 receptor leads to down-modulation of its surface expression by receptor internalization, and the expression of 2B4 is reduced by inhibitory action at an ets-element in the promoter of its gene (165, 166). These negative feedback mechanisms are likely to limit excessive 2B4-mediated NK cell activation.

NTB-A-mediated signaling

Signaling through the receptor NTB-A is less well examined (fig. 3). Similar to 2B4 the engagement of NTB-A leads to receptor phosphorylation and association of SAP and EAT-2 (84, 109, 152). A study using different inhibitors of signaling pathways could show that NTB-A signaling is strongly dependent on actin reorganization, Src-family kinases and PLC-γ (152). In the same study it has been shown that EAT-2 associates with the membrane proximal ITSM, whereas SAP binds to the C-terminal ITSM (fig. 3A). A mutant receptor that could bind only SAP was unable to trigger NK cell cytotoxicity, while its counterpart that could bind only EAT-2 triggered a cytotoxic response. Furthermore NK cells with a shRNA-mediated SAP knockdown were reported to show a normal cytotoxic, but an impaired IFN-γ response after NTB-A stimulation (152). These results suggested that SAP mediates the signal for cytokine production, whereas EAT-2 transduces the signal leading to a cytotoxic response. However, these findings do not match the observation that NTB-A-mediated cytotoxicity is impaired in XLP NK cells and IFN-γ production is not (84). This difference could be due to alterations of NK cell development in the absence of SAP.

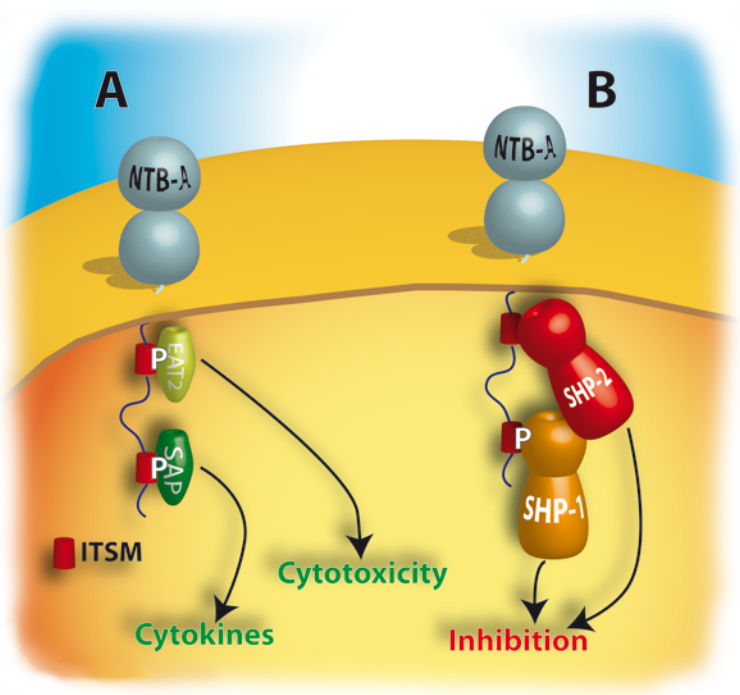

Figure 3: The model of NTB-A signaling
A: SAP and EAT-2 initiate different activating signaling pathways. **B:** A possible mechanism for inhibitory NTB-A signaling in the absence of SAP, e.g. in XLP patients. See text for details. Picture by courtesy of Claus *et al.* (39).

Similar to 2B4, NTB-A has also been reported to interact with the phosphatases SHP-1 and 2 (fig. 3B). While SHP-1 was found in complex with NTB-A regardless of its phosphorylation state, SHP-2 associated after pervanadate treatment (84). 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.

CRACC-mediated signaling

CRACC is phosphorylated after ligation and recruits the adapter EAT-2, which promotes CRACC phosphorylation through a Src-family kinase (114). There are contradicting results concerning the ability of human CRACC to recruit SAP (112, 114, 167). However, SAP is dispensable for CRACC signaling, as NK cells from XLP patients show no reduction in CRACC-mediated cytotoxicity (112). Although CRACC association with 3BP2 has not been observed (160), the CRACC signal causes phosphorylation of PLC-γ1 and 2, Akt and c-Cbl. The phosphorylation of Vav-1 and SHIP is increased to a lesser extent (114). Like 2B4 and NTB-A, CRACC has been shown to have the ability to recruit mediators of negative signaling. SHP-1 and 2, SHIP and Csk have been reported to bind to phosphorylated peptides of one CRACC ITSM (115).

CRACC signaling in mice seems to be similar to human CRACC. In mice CRACC phosphorylation has been shown to be independent of SAP, EAT-2 and ERT expression, and no association of SAP has been found (115). Like human XLP NK cells, the NK cells from SAP KO mice show no reduction in CRACC-mediated cytotoxicity (115). The importance of EAT-2 for CRACC signaling has been demonstrated by the finding that EAT-2 KO NK cells display no CRACC-mediated cytotoxicity, while SAP and ERT were dispensable. Interestingly, the absence of EAT-2 turned CRACC into an inhibitory receptor (115). This suggests that the function of CRACC can be switched from activating to inhibitory as a regulatory mechanism depending on the expression level of its pivotal adapter molecule. This is similar to the inhibitory function of 2B4 during NK cell development (95).

2 Aims of the Thesis

SLAM-related receptors are expressed on a variety of different cells of the innate and the adaptive immune system. Their general function can be described as fine-tuning of immune responses. 2B4, NTB-A and CRACC are expressed on NK cells and to some extent on T cells. The general aim of this thesis was to further elucidate the signaling mechanisms of 2B4 and NTB-A in human NK cells and to investigate the function of CRACC on T cells.

First we wanted to define amino acid residues in the extracellular part of NTB-A that are important for the homophilic interaction of two NTB-A molecules. To this end we generated NTB-A mutants and tested their ability to trigger NK cell cytotoxicity.

The second aim was to answer the unresolved questions about the early events in 2B4 and NTB-A signaling. For this purpose we wanted to investigate the early signaling events in NK cell lines with RNA interference-mediated SAP knockdown in comparison to cells with normal SAP expression. To extend our research to primary cells we also planned to establish a method for the knockdown of protein expression in primary human NK cells.

Third, CRACC is an activating receptor on NK cells, but its function on T cells has not been investigated yet. As other SRR have been shown to have co-stimulatory properties, we investigated whether this was also true for CRACC. CRACC is not expressed on the whole T cell population, we therefore also wanted to further characterize the CRACC-positive subset.

3 Materials and methods

3.1 Materials

3.1.1 Mouse monoclonal antibodies

Name	Conjugate	Source, Reference
Control IgG, MOPC21	-, PE	Sigma, Taufkirchen, Germany
anti-2B4, C1.7	-	Immunotech, Marseille, France
anti-CCR7	PE-Cy7	BD Biosciences, San Jose, CA, USA
anti-CD3, OKT3	-	ATCC, Manassas, VA, USA
anti-CD3, SK7	PE-Cy7	BD Biosciences
anti-CD4	FITC	Becton Dickinson Immunocytometry Systems, San Jose, CA, USA
anti-CD4, SK3	PerCP	BD Biosciences
anti-CD8	PerCP	BioLegend, San Diego, CA, USA
anti-CD25, SA3	FITC	BD Biosciences
anti-CD28	-	BD Pharmingen, Heidelberg, Germany
anti CD28	FITC	BD Biosciences
anti-CD45RA	APC	BD Biosciences
anti-CD69	PE	BioLegend
anti-CRACC, CS1-4	-	Stark *et al.*, 2005 (168)
anti-CRACC, 162.1	- ,PE	BioLegend
anti-Interferon-γ	FITC	BioLegend
anti-IL-2	PE	BD Biosciences
anti-NKG2D, 149810	PE	R&D Systems, Minneapolis, USA
anti-NKp30, p30-15	-	Byrd *et al.*, 2007(169)
anti-NTB-A, MAB 1908	-	R&D Systems
anti-NTB-A, NT-7	-, PE	Flaig *et al.*, 2004 (71), BioLegend
anti-phospho-tyrosine, 4G10	biotin	Upstate cell signaling solutions, Charlottesville, VA, USA
anti-SAP, SAP 23.1.5	-	Eissmann *et al.*, 2005 (82)

3.1.2 Rabbit polyclonal antibodies

Name	Source, Reference
anti-2B4	Watzl, *et al.*, 2000, (146)
anti-Actin	Sigma
anti-EAT-2	Pineda Antibody Service, Berlin, Germany, (152)
anti-Fyn	Cell Signaling Technologies, Danvers, USA
anti-NTB-A	Pineda Antibody Service, (152)

3.1.3 Secondary antibodies

Name	Conjugate	Source, Reference
goat anti-mouse IgG	HRPO	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
goat anti-mouse IgG	PE	Jackson ImmunoResearch Laboratories
goat anti-mouse IgG	-	Dianova, Hamburg, Germany
goat anti-rabbit IgG	HRPO	Santa Cruz Biotechnology, Heidelberg, Germany

3.1.4 Bacteria

E. coli strain	used for	Source
TOP10	amplification of plasmids	Invitrogen, Carlsbad, CA, USA
Stbl3	amplification of plasmids prone to homologous recombination	Invitrogen

3.1.5 Buffers

DNA-sample buffer (6 x):	0.25 % (w/v)	Bromphenol Blue
	0.25 % (w/v)	Xylene Cyanol FF
	30 % (v/v)	glycerol in H_2O

TAE (10 x), Invitrogen

Triton X-100-lysis buffer:	150 mM	NaCl
	20 mM	Tris-HCl, pH 7.4
	10 % (v/v)	glycerol
	0.5 % (v/v)	Triton X-100
	2 mM	EDTA
	10 mM	NaF
	1 mM	PMSF
	1 mM	Na-orthovanadate (for studies on protein phosphorylation)
Reducing sample buffer (5 x):	10 % (w/v)	SDS
	50 % (v/v)	glycerol
	25 % (v/v)	2-mercaptoethanol
	0.1 % (w/v)	Bromphenol Blue
	0.3125 mM	Tris-HCl, pH 6.8
MOPS buffer (20 x), Invitrogen		
Western blot transfer buffer:	24 mM	Tris
	129 mM	glycin
	20 % (v/v)	methanol
PBS (pH 7.4):	137 mM	NaCl
	8.1 mM	Na_2HPO_4
	2.7 mM	KCl
	1.5 mM	KH_2PO_4
PBST:	1 x	PBS
	0.05 % (v/v)	Tween 20
PBST/NaCl:	1 x	PBS
	0.05 % (v/v)	Tween 20
	0.5 M	NaCl

Blocking Buffer for western blot:	1 x	PBST
	5 % (w/v)	nonfat dry milk powder, Saliter, Obergünzburg
HBS 2x (pH 7.0)	54.6 mM	HEPES
	274 mM	NaCl
	3 mM	Na_2HPO_4
FACS-buffer (low protein)	1 x	PBS
	2 % (v/v)	fetal calf serum, PromoCell
FACS-buffer (high protein)	1 x	PBS
	5 % (v/v)	fetal calf serum
	0.5 % (w/v)	bovine serum albumin (BSA)

3.1.6 Cells (eukaryotic)

All culture media, fetal calf serum (FCS), non-essential amino acids and sodium pyruvate were purchased from Gibco (Invitrogen, Carlsbad, CA); donor horse serum was from Biochrom (Berlin, Germany), human serum from PromoCell (Heidelberg, Germany), PHA-P from Sigma and purified human IL-2 from Hemagen Diagnostics (Columbia, USA). If not indicated otherwise all cells were grown with 10 % (v/v) FCS and 1 % (v/v) penicillin/streptomycin (Gibco, Invitrogen).

Cell type	Origin	Culture Medium
721.221	EBV-transformed, human B cell line	IMDM
BA/F3	murine pre-B cell line	RPMI, 50 µM 2-mercaptoethanol
HEK 293T	human embryonic kidney cell line	DMEM
JY	EBV-transformed, human B cell lymphoblastoid cell line	RPMI, 50 µM 2-mercaptoethanol
NK92 C1	human NK cell line from malignant non-Hodgkin lymphoma	Alpha MEM, 12.5 % (v/v) FCS, 12.5 % (v/v) donor horse serum, 2-mercaptoethanol

P815	murine mastocytoma cell line	IMDM
Phoenix ampho	human embryonic kidney cell line, packaging cell line for amphotropic retrovirus	DMEM
primary human NK cells	isolated from peripheral blood mononuclear cells (PBMC) by negative selection	IMDM, 10 % (v/v) human serum, 10 % (v/v) non-essential amino acids, 10 % (v/v) sodium pyruvate, 100 IU/ml IL-2
primary human T cells	isolated from PBMC by negative selection	RPMI
YTS	Human leukemic NK-like cell line	IMDM, 12.5 % (v/v) FCS, 2-mercaptoethanol

3.1.7 Enzymes

Name	Use	Source
alkaline phosphatase, calf intestine (CIP)	dephosphorylation of DNA fragments	New England Biolabs, Frankfurt, Germany
Deep Vent DNA polymerase	mutagenesis PCR	New England Biolabs
restriction endonucleases	cutting of DNA	New England Biolabs
Taq DNA polymerase	PCR	New England Biolabs
T4 DNA ligase	DNA ligation	New England Biolabs

All enzymes were used in buffers provided by the manufacturer.

3.1.8 Kits

DNA Fragment Purification	Gel Extraction Kit, Qiagen, Hilden, Germany
Isolation of human NK cells	NK cell negative isolation kit, Invitrogen (formerly Dynal, Oslo, Norway)
Isolation of human T cells	Pan T cell isolation kit II, $CD4^+$ T cell isolation Kit II, $CD8^+$ T cell isolation kit, Miltenyi Biotech, Bergisch Gladbach, Germany
Plasmid DNA purification	Plasmid MiniPrep, MidiPrep or MaxiPrep Kit, Qiagen
Transfection of human NK cells	Human macrophage nucleofector kit, Lonza, Basel, Switzerland
Transfection of human T cells	Human T cell nucleofector kit, Lonza

3.1.9 Oligonucleotides

Primers for sequencing and DNA amplification

Name	Sequence (5' – 3')
H1	TCGCTATGTGTTCTGGGAAA
M13R	GGAAACAGCTATGACCAT
NTB-A 850R	CACTGAAACATACTCTAG
pSM2 Mlu f	ACGCGTGCTGTTGACAGTGAG
pSM2 Cla r	ATCGATTCCGAGGCAGTAGGC
Sp6	TTTAGGTGACACTATAG
T7	TAATACGACTCACTATAGGG

Primers for mutagenesis

Name	Sequence (5' – 3')
NTBA E26A upper	GAGTTTCCTGCAGGAGCGAAGGTCAACTTCATC
NTBA E26A lower	GATGAAGTTGACCTTCGCTCCTGCAGGAAACTC
NTBA K27A upper	GTTTCCTGCAGGAGAGGCGGTCAACTTCATCACTTG
NTBA K27A lower	CAAGTGATGAAGTTGACCGCCTCTCCTGCAGGAAAC
NTBA E37A upper	CTTGGCTTTTCAARGCAACATCTCTTGCCTTC
NTBA E37A lower	GAAGGCAAGAGATGTTGCATTGAAAAGCCAAG
NTBA E47A upper	CTTCATAGTACCCCATGCAACCAAAAGTCCAGAAAT
NTBA E47A lower	GATTTCTGGACTTTTGGTTGCATGGGGTACTATGAA
NTBA K49A upper	GTACCCCATGAAACCGCAAGTCCAGAAATCCAC
NTBA K49A lower	GTGGATTTCTGGACTTGCGGTTTCATGGGGTAC
NTBA E52A upper	AAACCAAAAGTCCAGCAATCCACGTGACTAATC
NTBA E52A lower	GATTAGTCACGTGGATTGCTGGACTTTTGGTTT
NTBA K62A upper	CTAATCCGAAACAGGGAGCGCGACTGAACTTCACC
NTBA K62A lower	GGTGAAGTTCAGTCGCGCTCCCTGTTTCGGATTAG
NTBA K92A upper	GCCCAGATATCCACAGCGACCTCTGCAAAGCTG
NTBA K92A lower	CAFGCTTTGCAGAGGTCCGTGTGGATATCTGGGC
NTB-A H54A upper	CAAAAGTCCAGAAATCGCCGTGACTAATCC
NTB-A H54A lower	GTTTCGGATTAGTCACGGCGATTTCTGGAC
NTB-A Q88A upper	GGCTCTTACAGAGCCGCGATATCCACAAAGAC

Primers for mutagenesis, continued

Name	Sequence (5' – 3')
NTB-A Q88A lower	GAGGTCTTTGTGGATATCGCGGCTCTGTAAGAG
NTB-A S90A upper	CTTACAGAGCCCAGATAGCCACAAAGACCTCTG
NTB-A S90A lower	CTTTGCAGAGGTCTTTGTGGCTATCTGGGCTCT
NTB-A L34E upper	CAACTTCATCACTTGGGAGTTCAATGAAACATCTC
NTB-A L34E lower	GATGTTTCATTGAACTCCCAAGTGATGAAGTTGACC
NTB-A L34K upper	CAACTTCATCACTTGGAAGTTCAATGAAACATCTC
NTB-A L34K lower	GATGTTTCATTGAACTTCCAAGTGATGAAGTTGACC
NTB-A T32E upper	GAAGGTCAACTTCATCGAGTGGCTTTTCAATGAAAC
NTB-A T32E lower	GATGTTTCATTGAAAAGCCACTCGATGAAGTTGACC
NTB-A T32K upper	GAAGGTCAACTTCATCAAGTGGCTTTTCAATGAAAC
NTB-A T32K lower	GATGTTTCATTGAAAAGCCACTTGATGAAGTTGACC

3.1.10 Plasmids

Name	Use	Source, Generation
pBABE NTB-A wt and mutants	stable expression of NTB-A in cell lines	Insertion via Xho I-Not I
pCR2.1 NTB-A wt	template for mutagenesis PCR	TOPO-TA cloning
pCR2.1-TOPO	cloning of PCR products	Invitrogen
phCMV-GALVenv	envelope plasmid for virus production	a gift from K. Weber, University Hospital Hamburg-Eppendorf
pLVTHM shSAP/shCD4	lentiviral vectors for stable shRNA delivery in primary cells	Addgene, Cambridge, MA, USA, Insertion via Mlu I-Cla I
pMD2.G	envelope plasmid for virus production	Addgene, Cambridge, MA, USA
pMOW NTB-A wt and mutants	stable expression of NTB-A in cell lines	Insertion via Xho I-Not I
pSHAG-MAGIC2_shSAP/shCD4	stable knockdown of SAP or CD4 in cell lines	Biocat, Heidelberg, Germany

psPAX2	packaging plasmid for viral particles	Addgene, Cambridge, MA, USA
pWPXL siNKG2D	stable knockdown of NKG2D in primary cells	a gift from C. Kalberer, University Hospital Basel, Switzerland

3.1.11 Reagents

Agarose	Gibco, Paisley, Scotland
Ampicillin	Roth, Karlsruhe, Germany
Brefeldin A	Fluka, Buchs, Switzerland
BSA	Serva, Heidelberg, Germany
BigDye Terminator v1.1 cycle	Applied Biosystems, Foster City, CA, USA
Chromium-51, as sodium chromate	Hartmann Analytik, Braunschweig
Desoxyribonucleotide trisphosphate mix	Invitrogen
DNA ladder (100 bp and 1 kb)	Invitrogen
Ethidiumbromide	Roth, Karlsruhe, Germany
recombinant human IL-2	NIH cytokine repository
recombinant human IL-15	R&D Systems, Minneapolis, USA
Kanamycin	Roth
LB broth	Invitrogen
Lipofectamine	Invitrogen
LSM solution	PAA, Pasching, Germany
Phaseolus vulgaris hemagglutinine	Sigma
Polybrene	Sigma
Polyvinylidene difluoride membrane	Millipore, Billerica, USA
Precision Plus Protein Standard	BioRad, Hercules, CA, USA
Protein G agarose	Invitrogen
Puromycin	Sigma
RetroNectin	TAKARA, Otsu, Japan
Saponin	Sigma
Streptavidin-HRPO	Jackson ImmunoResearch Laboratories
SuperSignal West Pico and Dura	Thermo

TrypLE Express	Invitrogen
Vybrant CFDA SE Cell tracer Kit	Molecular Probes, Leiden, The Netherlands
X-ray films	Perbio/Pierce, Rockford, IL, USA

3.1.12 siRNA

Name	Source
control siRNA (Non-targeting siRNA #1)	Thermo scientific, (Dharmacon)
siCRACC (ON-TARGETplus SMARTpool, SLAMF7)	Thermo scientific
siSAP (Hs_SH2D1A_3) 3'-AlexaFluor647	Qiagen
siEAT-2 (Hs_SH2D1B_1) 3'-AlexaFluor647	Qiagen

3.2 Patients

The blood samples used in this study were from patients presenting with unstable angina pectoris at University Hospital Heidelberg. Patients gave their written informed consent under a protocol approved by the institutional review board in accordance with the declaration of Helsinki.

3.3 Methods

3.3.1 Molecular biology

Agarose gel-electrophoresis

The DNA solution was mixed with DNA sample buffer before loading onto the gel. 1 % or 2 % gels were used depending on the size of the fragments (TAE, agarose, 0.00001 % Ethidiumbromide). The correct size of the DNA fragment was controlled using a DNA ladder.

DNA sequencing

Sequencing reactions were set up with the ABI Big Dye sequencing mix v1.1 according to manufacturer's instructions. The following primers were used: T7 and M13R for pCR2.1, NTB-A850R for the middle part of NTB-A constructs and H1 and Sp6 for pLVTHM. After sequencing the DNA was precipitated with ethanol, solubilized in water and analyzed on an ABI Prism 310 Genetic Analyzer.

Enzymatic cutting of DNA

Between 1 and 2 µg of DNA were incubated with 2 U of the respective restriction endonucleases for at least 1h at 37°C. Conditions of the reaction were set according to the manufacturer's instructions. The DNA fragments were separated by agarose gel electrophoresis.

Extraction of DNA fragments from agarose gels

Agarose slices containing DNA fragments were excised from the gel and DNA was extracted with the Qiagen gel extraction kit following the manufacturer's instructions.

Isolation of plasmid DNA

Bacteria were grown in 1 x LB medium with the appropriate antibiotic at 37°C over night. After harvesting the bacteria by centrifugation at 6000 x g for 5 min or 10 min, depending on culture size, DNA was isolated using Mini, Midi or MaxiPrep DNA isolation kit from Qiagen according to the manufacturer's instructions.

Ligation of DNA fragments

Insert and vector DNA were mixed at ratios ranging from 10 to 1 to 3 to 1 in ligation buffer (New England Biolabs). The mixture was incubated with 2 U T4 DNA ligase for 1 h at room temperature and used for the transformation of competent bacteria.

mRNA expression analysis

About 1 x 10^6 cells were lysed in MagNA Pure LC lysis buffer (Roche, Mannheim, Germany), frozen at -70° C and quantitative RT-PCR was performed by our cooperation partners, the group of Thomas Giese, Institute for Immunology, Heidelberg, using SEARCH LC primers.

Polymerase chain reaction (PCR)

PCR was used to amplify the shRNA-coding DNA fragments from pSHAG-MAGIC2-vectors or to insert point mutations into DNA. The conditions were fit to the respective needs. Amplified DNA fragments were cloned into pCR2.1-TOPO following the manufacturer's instructions. After mutagenesis PCR template DNA was removed using the endonuclease Dpn I and PCR products were directly used for transformation of bacteria. All mutations were confirmed by DNA sequencing.

Transformation of bacteria

Chemically competent bacterial strains were used. The transformation was carried out according to the manufacturer's instructions. Transformed bacteria were grown on LB-Agar plates at 37 °C for overnight under selection with the appropriate antibiotic.

3.3.2 Cell biology

Cell culture

All cells were grown at 37°C and 5 % CO_2 in a humidified incubator under sterile conditions. Cell lines were split on a regular basis every two to three days. Cell culture flasks were exchanged every two weeks. Cells were frozen in FCS containing 10 % DMSO at -75°C and stored in liquid nitrogen. Cell lines were thawed on a regular basis. FCS, donor horse serum and human serum were heat inactivated by incubation at 56°C for 30 min prior to use.

Cell stimulation

Cell mixing

7 to 10 x 10^7 cells of each type were resuspended at 1 x 10^5 cells/µl in IMDM, supplemented with 10 % FCS and pre-chilled on ice. Equal numbers of effector and target cells were mixed, centrifuged for 1 min at 400 x g, 4°C and incubated on ice for 10 min. Samples were then stimulated at 37°C for the appropriate time. Samples were pelleted by centrifugation at 400 x g for 5 min and 4°C, supernatant was aspirated and cells were lysed. For the time point 0 min effector and target cells were kept on ice separately and mixed immediately before lysis.

Plate bound antibodies

96-well flat bottom plates were coated overnight by incubation with goat anti-mouse IgG at a concentration of 7 µg/ml in PBS, using 50 µl per well. Plates were washed twice with PBS containing 0.5 % BSA (w/v) and then incubated for 1 h at 37°C with the appropriate antibodies for stimulation diluted in PBS containing 0.5 % BSA, again using 50 µl per well. Plates were washed again and 2 x 10^5 T cells in 50 µl medium were added to each well. T cell contact to the antibody-coated surface was intensified by centrifugation for 2 min at 400 x g, and cells were kept under normal culture conditions for 6 h, 48 h or 72 h, depending on the experimental readout.

PHA-P-stimulation

Peripheral blood T cells were stimulated by adding PHA-P to the culture medium at a concentration of 2 µg/ml. After 18 h the cells were washed twice with culture medium and then cultured in medium containing 100 IU/ml IL-2. The IL-2-containing medium was renewed every two days.

Cell lysis

Pelleted cells were resuspended in ice-cold Triton X-100 lysis buffer supplemented with 1 mM PMSF and if necessary 1 mM sodium orthovanadate and incubated on ice for 20 min. Lysates were clarified by centrifugation for 15 min at 20000 x g and 4°C.

Chromium release assays

Cell lines were grown to mid log phase, IL-2 activated primary NK cells were used at 3-4 weeks age, PHA-P-activated T cells were used 7 to 10 days after stimulation and were deprived of IL-2 for 24 h before the assays. The assay medium was IMDM, supplemented with 10 % FCS (v/v) and 1 % (v/v) Penicillin/Streptomycin in all assays. For assays with primary NK cells IL-2 was added to a final concentration of 100 IU/ml.

5 x 10^5 target cells were labeled in 100 µl medium with 100 µCi ^{51}Cr (3.7 MBq) for 1 h at 37°C. Cells were washed twice in medium and resuspended at a concentration of 5 x 10^4 cells/ml in medium. Effector and labeled target cells were mixed in 96-well V-bottom plates with a final volume of 200 µl per well. 5000 target cells/well were used in all assays. When different effector/target ratios (E/T) were used, the effector cells were plated in serial dilutions before the labeled target cells were added.

Maximum release was determined by incubation of target cells in 1 % Triton X-100. For spontaneous release, targets were incubated without effector cells in medium alone. All samples were done in triplicates. Plates were incubated for 4 h or 16 h at 37°C, 5 % CO_2. Supernatant was harvested and ^{51}Cr release was measured in a gamma counter. Percent specific release was calculated as ((experimental release – spontaneous release) / (maximum release – spontaneous release)) x 100.

In redirected lysis assays of NK cells against the target cell line P815 the antibodies were added to the effector cells to a final concentration of 0.5 µg/ml before the target cells were added. In redirected lysis assays with T cells as effector cells the anti-CD3 antibody was used in a serial threefold dilution starting at a final assay concentration of 1 ng/ml, while the final concentration of co-stimulatory antibodies was 0.5 µg/ml.

Flow-cytometry

Surface staining

Surface staining of cells was performed in 96-well V-bottom plates. About 2 x 10^5 cells were resuspended in 50 µl FACS-buffer containing 10 µg/ml of the respective primary antibody and incubated on ice for 20 min. Antibodies directly conjugated to a fluorophore were used at appropriate dilutions and cells were incubated in the dark to protect the fluorophore. After washing with FACS-buffer cells stained with unlabeled antibodies were resuspended in 50 µl PE-conjugated goat-anti-mouse IgG secondary antibody diluted 1:200 in FACS-buffer. Cells were incubated on ice for 20 min in the dark, washed again and resuspended in FACS-buffer. If necessary, cells were fixed in FACS-buffer containing 2 % formaldehyde.

For fluorescence-activated cell sorting 1 to 3 x 10^6 cells were stained and all steps were carried out under sterile conditions.

Intracellular staining

All steps for intracellular staining of cells were carried out at room temperature. Cells were fixed for 5 min in 4 % paraformaldehyde solution and permeabilized by incubation in high protein FACS-buffer containing 0.5 % (w/v) saponin for 5 min. Fixed and permeabilized cells were resuspended in 50 µl FACS-buffer with saponin containing the fluorophore-conjugated antibodies in appropriate dilutions and incubated in the dark for 20 min. To wash the cells, 150 µl of FACS-buffer with saponin were added and the cells were incubated for 5 min, before they were pelleted and resuspended in FACS-buffer.

CFDA-staining

Cells were harvested by centrifugation for 7 min at 400 x g and residual medium was carefully removed. PBS containing CFDA at a concentration of 0.5 µmol/ml was used to resuspend the cells to a density of 4 x 10^6 cells/ml. After incubation for 30 min at 37°C, 5 % CO_2 the labeling reaction was stopped by adding an excess of culture medium.

Staining of blood samples

50 µl blood were incubated with antibodies in appropriate dilutions for 20 min at 4°C in the dark. Samples were fixed and erythrocytes were lysed by addition of 2 ml BD FACS lysing solution (BD Biosciences). After incubation for 10 min at

room temperature in the dark cells were pelleted by centrifugation and resuspended in PBS.

Flow-cytometric analysis

Samples were analyzed on a BD FACScan, BD FACSCalibur or BD LSR 2 and results were evaluated using the FlowJo Software from Tree Star or FACS DiVa from BD.

Isolation of lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats or whole blood using density centrifugation over LSM solution.

Polyclonal NK cells were purified from PBMC by negative selection using a NK cell negative isolation kit, according to the manufacturer's instructions. NK cells were between 90 % and 99 % $NKp46^+$, $CD3^-$ and $CD56^+$, as confirmed by flow cytometry. After isolation the cells were resuspended in culture medium containing 1 µg/ml PHA-P and 5 ng/ml recombinant human IL-15, mixed with irradiated JY cells (5×10^5 cells/ml) and plated in 96-well round bottom plates at densities ranging from 1×10^6 to 2×10^6 cells/ml. Growing cell cultures were expanded 1:1 with culture medium.

Peripheral blood T cells were purified from PBMC with negative selection kits either for total, CD8-positive or CD4-positive T cells. T cells were then resuspended in culture medium at a density of 3×10^6 cells/ml and cultured in appropriate culture flasks.

Transfection of primary lymphocytes

Primary lymphocytes were transfected using the nucleofection technology (Lonza). 5×10^6 T cells per sample were transfected with 1 or 2 pmol of siRNA, according to the manufacturer's instructions. After nucleofection cells were taken up in AIM V medium (Gibco) supplemented with 10 % FCS.

Primary NK cells were transfected after one week of culture. 2 to 3×10^6 cells per sample were transfected with 1 or 2 pmol of siRNA using the Nucleofector solution for human macrophages and the nucleofection program X-01, following the manufacturer's instructions at all other steps.

Viral transduction

Retroviral transduction of cell lines

Retroviral gene transfer was done using the packaging cell line Phoenix ampho. At day one Phoenix cells were plated in a small tissue culture flask at a density of 1×10^6 cells in 4 ml medium and grown for 24 h. Cells were transfected with the respective plasmid using Lipofectamin according to the manufacturer's instructions. After transfection cells were grown for 12 h to 18 h. The medium was exchanged for the appropriate medium for the cells that were to be transduced and Pheonix cells were kept in culture for 24 h. Supernatant containing the viral particles was harvested and cleared by centrifugation. 0.5×10^6 cells to be transduced were resuspended in the supernatant supplemented with 5 µg/ml polybrene. Transduction was carried out as spinfection by centrifugation for 1.5 h at 1350 x g and 30°C. Spinfection was done in 12 well plates and cells were afterwards grown over night. The medium was exchanged the next day by pelleting cells and resuspending them in fresh medium. After culturing infected cells for one day puromycin was added at concentrations between 0.5 and 2 µg/ml to select for transduced cells. Transduced cells were expanded and if necessary further enriched by FACS.

Lentiviral transduction of NK cells

Viral vectors were produced in HEK 293T cells transfected with transfer vector, packaging plasmid and envelope plasmid at a ratio of 7.5 / 3.75 / 1 by calcium phosphate precipitation. To do so DNA was diluted in sterile water and 2 M calcium chloride solution was added to a concentration of 244 mmol/l. An equal volume of 2x HBS was added to the DNA solution and mixed by bubbling vigorously. The mixture was added dropwise to the HEK 293T cells. 12 h later the medium was exchanged and cells were grown for 24 h. Then the medium was collected, cleared from cells by centrifugation and filtered through 0.45 µm pore size filters.

Various transduction protocols have been tested. The virus was concentrated by ultra-centrifugation and used at MOI ranging from 10 – 20 for spinfection or the collected medium was used to concentrate the virus on RetroNectin-coated plates following the manufacturer's instructions. The transduction efficiency was measured by flow-cytometry.

3.3.3 Protein biochemistry

Homology modeling

The three dimensional structure of NTB-A was modeled using the web-based PHYRE software (http://www.sbg.bio.ic.ac.uk/~phyre/**)** (170).

Immunoprecipitation

Cell lysates from cell mix experiments were precleared by incubation with 20 µl of a 50 % slurry of recombinant protein G agarose in PBS for 30 min at 4°C. During this and all following incubations the samples were gently agitated. Protein G agarose was removed by centrifugation (1 min at 3500 x g and 4°C) and lysates were consecutively incubated with 2 µg of control antibody and 2 µg of specific antibody, each coupled to 20 µl of a 50 % slurry of recombinant protein G agarose in PBS. Incubations were for 1 h at 4°C. These steps were repeated, when more than one precipitation was done from the same lysate. Samples were washed three times with ice-cold lysis buffer and residual buffer was removed using a Hamilton syringe. Samples were frozen at -20°C until they were analyzed by SDS-PAGE and western blot.

SDS-polyacrylamid gel-electrophoresis (SDS-PAGE)

After adding reducing sample buffer, samples were boiled for 5 min at 95°C, cooled on ice and centrifuged for 1 min at 20000 x g. Samples to a maximal volume of 25 µl and 5 µl of Precision Plus Protein Standard (BioRad) were loaded on 10 % or 12 % NuPage gels (Invitrogen) and separated for 1 h 15 min at 150 V in 1 x MOPS buffer.

Western blot

After SDS-PAGE proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) for 1.5 h at 200 mA in western blot transfer buffer. PVDF membranes were activated with methanol and rinsed with transfer buffer prior to use. After western blotting, membranes were incubated for 1 h at room temperature in blocking buffer and washed for at least 3 times in PBST. Membranes were incubated with the primary antibody in PBST containing 5 % (w/v) BSA for 1 h at room temperature or overnight at 4°C. The membrane was washed at least three times with PBST/NaCl and incubated with the appropriate horseradish-peroxidase (HRPO)-conjugated secondary antibody or HRPO-conjugated streptavidin for 1 h at room temperature. Secondary antibodies were diluted 1:5 000-1:40 000 in blocking buffer. After incubation with the secondary antibody, the membrane was extensively washed

with PBST and developed using either SuperSignal West Pico or Dura and X-Ray films.

3.3.4 Statistical analysis

Statistical analysis was performed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

4 Results

4.1 The molecular basis for the homophilic NTB-A interaction

4.1.1 Mutational analysis of the homophilic NTB-A interaction

At the beginning of this work the molecular basis for the homophilic interaction of two NTB-A molecules was unknown. To find amino acid residues that contribute to the binding of two NTB-A molecules to each other, a possible structure of the extracellular part of NTB-A was predicted using homology modeling. Based on the model structure eight charged amino acid residues were chosen for mutational analysis: glutamate-26 (E26), lysine-27 (K27), glutamate-37 (E37), glutamate-47 (E47), lysine-49 (K49), glutamate-52 (E52), lysine-62 (K62) and lysine-92 (K92). The residues E26, K27, E47, K49, E52 and K92 are located on the side of the IgV-domain that is most distant to the plasma membrane. They form an alternating pattern of positive and negative charges that seemed to be a likely candidate for an interface based on electrostatic forces. We hypothesized that the four positive charges of the lysine residues could be located opposite to the negative charges of the glutamate residues of the second NTB-A in the homodimer and vice versa. The residues E37 and K62 are located at the other side of the IgV-domain and were chosen to cover a greater part of the IgV-domain. The crystal structure of the homodimer of human NTB-A published during the course of this work confirmed the predicted position of these residues (fig. 4A).

Site directed mutagenesis was performed to replace the selected amino acids with alanine. The mutated receptors were then stably expressed in the cell line BA/F3, a murine B cell line that lacks ligands for activating human NK cell receptors and is therefore a poorly lysed target cell line. Expression of the wild type form of human NTB-A makes the cells susceptible to recognition and lysis by human NK cells. Mutations that diminish capacity of binding to the wild type receptor are supposed to result in a reduced lysis of cells. To compare the lysis of BA/F3 cells induced by NTB-A-mutants to lysis induced by the wild type receptor, ^{51}Cr-release assays with IL-2-activated primary human NK cells were performed (fig. 4B).

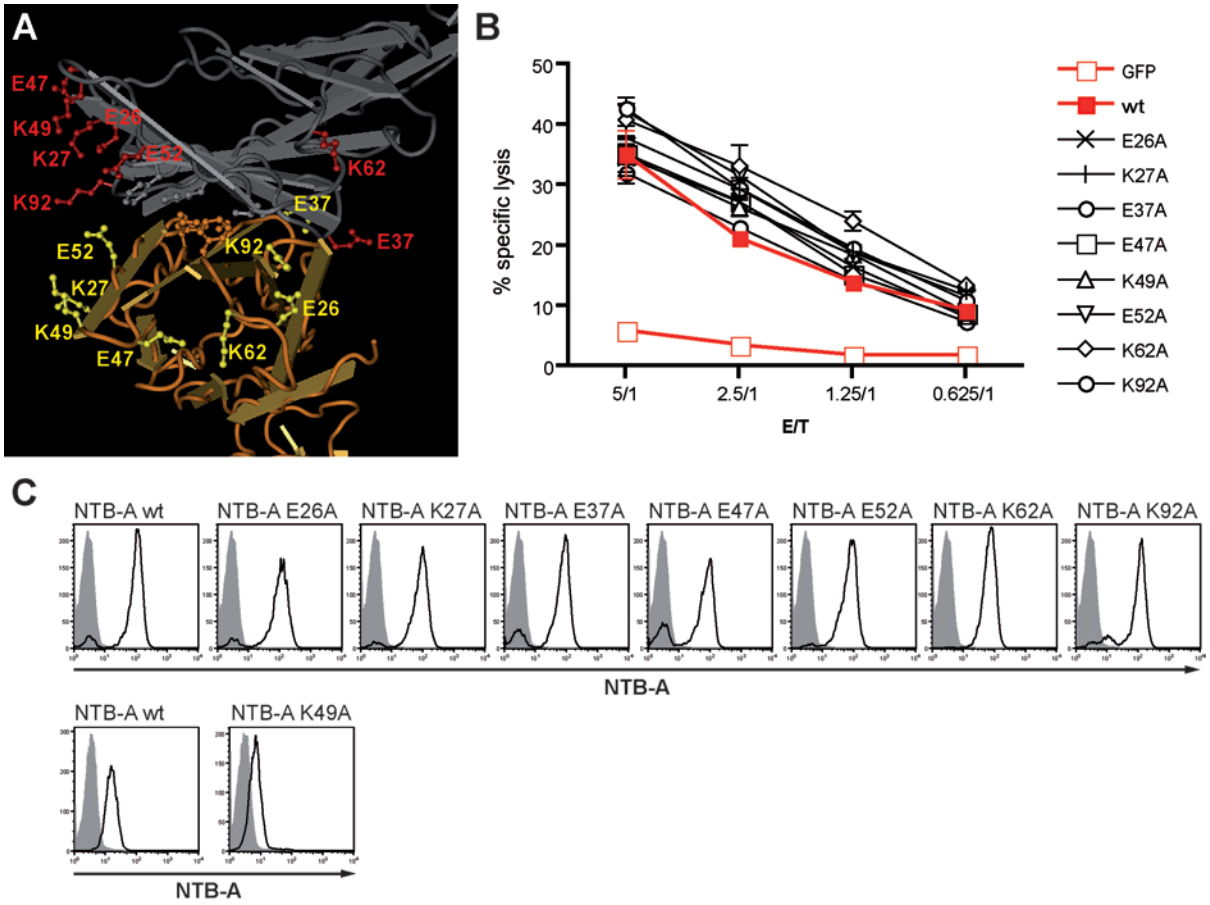

Figure 4: The charged residues at the edge of the homophilic interaction site do not contribute to the binding of two NTB-A receptors.
A: The structure of the NTB-A-homodimer with a selection of amino acid side chains. The highlighted amino acid residues were mutated to alanine to test their relevance for the interaction. **B**: BA/F3 cells stably transfected with wild type NTB-A or one of the mutated receptors were used as target cells in a 4 h ^{51}Cr-release assay with IL-2-activated primary NK cells at different effector to target (E/T) ratios. GFP-transfected BA/F3 cells were used as negative control. The specific lysis is plotted as mean of triplicates ± SD. **C:** Similar expression levels of the transfected receptors were confirmed by flow-cytometry. The mutation of lysine 49 to alanine (K49A) disrupts the binding of the antibody NT-7. Therefore the clone MAB 1908 was used (lower row). The gray histograms represent staining with an unspecific control antibody. One representative experiment of three is shown.

None of the eight mutations resulted in a reduced lysis of the respective BA/F3 cells. Similar expression levels of the wild type and the NTB-A mutants were confirmed by flow-cytometry (fig. 4C). The mutant NTB-A K49A could not be stained with the anti-NTB-A antibody NT-7. But expression of the mutant receptor could be detected using a different antibody clone (3C, lower row). This led to the conclusion that the epitope recognized by antibody clone NT-7 is located around lysine-49.

Because no difference between the lysis of wild type and the NTB-A mutants was detectable, we concluded that none of the eight amino acids contributes strongly to a functional NTB-A-dimerization. At that time-point the crystal structure of the human NTB-A-homodimer was published by Cao *et al.* (171). The structure showed that all

selected residues except E37 were indeed located outside the homophilic interface (fig. 4A).

Based on the structure of the crystallized NTB-A-homodimer Cao *et al.* defined ten amino acid residues (F30, L34, E37, S39, F42, H54, T56, R86, Q88 and S90) on each IgV-domain, which form the interface between interacting NTB-A molecules. They tested their hypothesis with a series of mutagenesis studies, substituting alanine for each of the ten amino acids. Ectodomains of NTB-A containing the single amino acid mutations were then recombinantly expressed in bacteria, refolded, purified and assessed for their dimerization in gel filtration analysis, apart from the F42A mutant that could not be refolded. With the exception of the mutation of serine-39 all mutants showed a decreased ability to form dimers in solution (171).

As the mutation of E37 showed no decrease in our functional experiments, we wanted to confirm the contribution of other residues of these ten to functional interaction of NTB-A molecules. We chose the three residues, histidine-54 (H54), glutamine-88 (Q88) and serine-90 (S90) (fig. 5A) whose mutation to alanine had a strong effect on dimerization in the gel filtration experiments reported by Cao *et al.* (171). Single mutants of the three residues to alanine were generated and H54 and S90 were both exchanged in a double mutant. The mutant NTB-A receptors were stably expressed in BA/F3 cells. Susceptibility of these cells to NTB-A-triggered cytotoxicity was tested in ^{51}Cr-release assays with primary IL-2-activated NK cells (fig. 5B). While the lysis of NTB-A Q88A-expressing cells was similar to lysis of cells expressing the wild type receptor, the H54A and S90A mutants displayed a diminished lysis. The mutation S90A showed a stronger effect on the interaction with wild type NTB-A than H54A. The double mutation H54A S90A did not reduce the lysis to a level lower than the S90A single mutation, which is only slightly above the lysis of BA/F3 cells expressing GFP instead of any activating ligand. Similar expression levels of NTB-A on the BA/F3 cells were confirmed by flow-cytometry (fig. 5C). Molecular modeling based on the crystal structure and energy calculations performed by David Nutt, our cooperation partner in bioinformatics, confirmed that our mutations did not disrupt the overall domain structure.

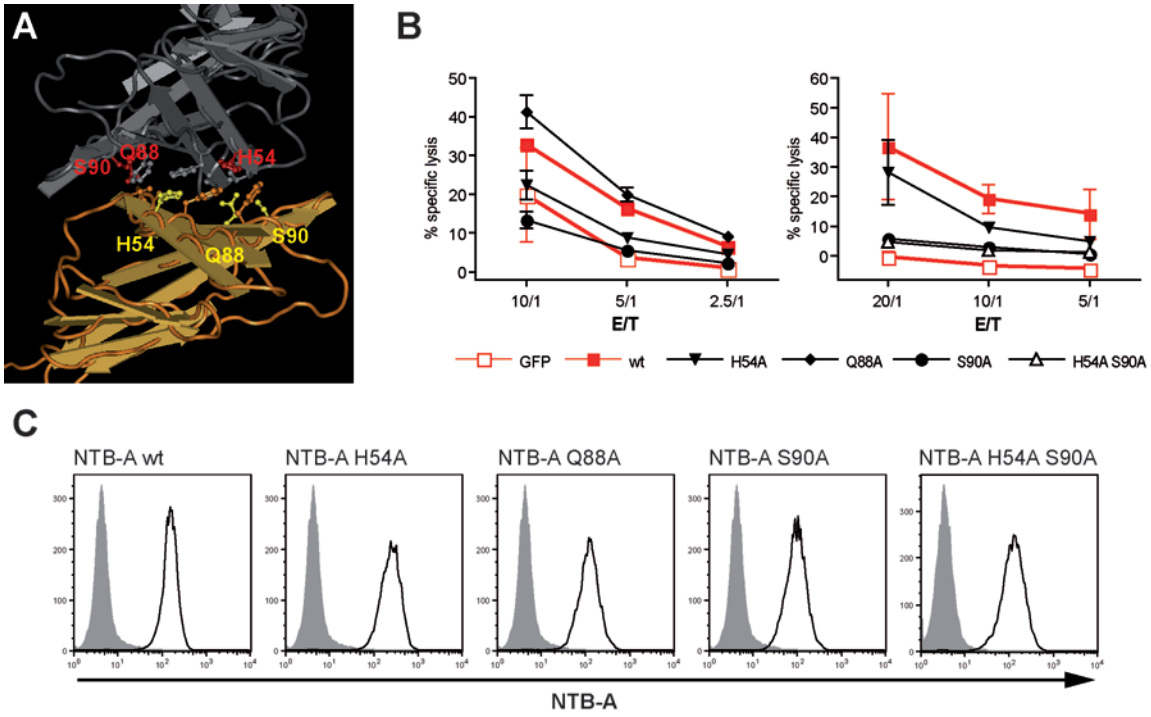

Figure 5: Mutations of the residues histidine-54, glutamine-88 and serine-90 have different impact on NTB-A function
A: The amino acid residues histidine-54 (H54), glutamine-88 (Q88) and serine-90 (S90) are highlighted in the structure of the NTB-A-homodimer. To test their functional relevance for the homophilic interaction of NTB-A each of the three was mutated to alanine in a single mutation and H54 and S90 in one double mutant. **B**: BA/F3 cells stably transfected with wild type NTB-A or one of the mutated receptors were used as target cells in 4 h ^{51}Cr-release assays with IL-2-activated primary NK cells at different E/T ratios. GFP-transfected BA/F3 cells were used as negative control. In the left diagram the effects of the three single mutants are compared, in the right diagram the double mutant is compared to the respective single mutants. The specific lysis is plotted as mean of triplicates ± SD. Two representatives of five experiments with NK cells from nine donors are shown. **C:** Similar expression levels of the transfected receptors were confirmed by flow-cytometry. The gray histograms represent staining with an unspecific control antibody.

From these results we conclude a grading in the contributions of the amino acid residues at the interface to the stability of the NTB-A-homodimer. Q88 and E37 have only a small influence on the receptor interaction, as their mutation did not lead to functional consequences. The hydrophobic interactions of H54 have a stronger relevance for dimerization, but the most important of all residues analyzed was S90, whose mutation almost totally abrogated NTB-A function.

4.1.2 Introducing complementary mutations to create a heterophilic pair of NTB-A mutants

The homophilic interaction of NTB-A makes it difficult to investigate the early events in the signaling processes after NTB-A engagement. Because there is continual engagement of NTB-A between neighboring cells in cultures of NTB-A-expressing cells, it is impossible to obtain cells expressing NTB-A in a completely unstimulated

state. To overcome this impediment we attempted to generate NTB-A mutants with complementary mutations that would render the receptors self-incompatible, but enable them to bind to each other, turning the homophilic NTB-A into a pair of heterophilic mutants.

Based on energy calculations and molecular modeling on the crystal structure of the NTB-A-homodimer our cooperation partner in bioinformatics predicted that the two amino acid residues threonine-32 and leucine-34 could be used for that purpose. These residues are located opposite each other in the homophilic interface (fig. 6A). Replacing one of the residues with lysine or glutamate would introduce a charge, but leave the overall structure of the IgV-domain intact.

Figure 6B illustrates the concept taking the mutation T32K as example. The positively charged lysine was supposed to reduce the binding to wild type receptor (fig. 6B, left panel) and to prevent dimerization with another NTB-A T32K molecule due to repulsive electrostatic forces (fig. 6B, middle panel). In the complementary mutant L34E a negatively charged glutamate residue is placed opposite the lysine and could promote receptor binding through attractive electrostatic forces (fig. 6B, right panel). We would expect NK cells expressing NTB-A T32K to lyse target cells expressing NTB-A L34E and spare target cells expressing NTB-A T32K. Lysis of target cells expressing wild type NTB-A would be reduced.

To test this concept we generated four NTB-A mutants T32E, T32K, L34E and L34K and expressed them stably in the cell line BA/F3. To obtain NK cell lines expressing only the mutant receptors, we expressed the wild type receptor or the mutants stably in the NTB-A-deficient NK cell line NKL^{4-} that has been derived from the cell line NKL by repeated fluorescence-activated cell sorting (152). The expression levels of NTB-A on all cell lines were analyzed by flow-cytometry (fig. 6C). As expected, only expression of the wild type receptor enabled the NKL cells to lyse wild type NTB-A-expressing BA/F3 cells. NKL^{4-} cells expressing the mutant receptors showed equally low cytotoxicity against BA/F3 cells expressing GFP or wild type NTB-A (fig. 6D). However, the complementary mutants did not rescue the interaction in the expected way. Target cells expressing one NTB-A mutant were poorly lysed regardless whether the NKL^{4-} expressed the complementary mutant or the same mutant (fig. 6E).

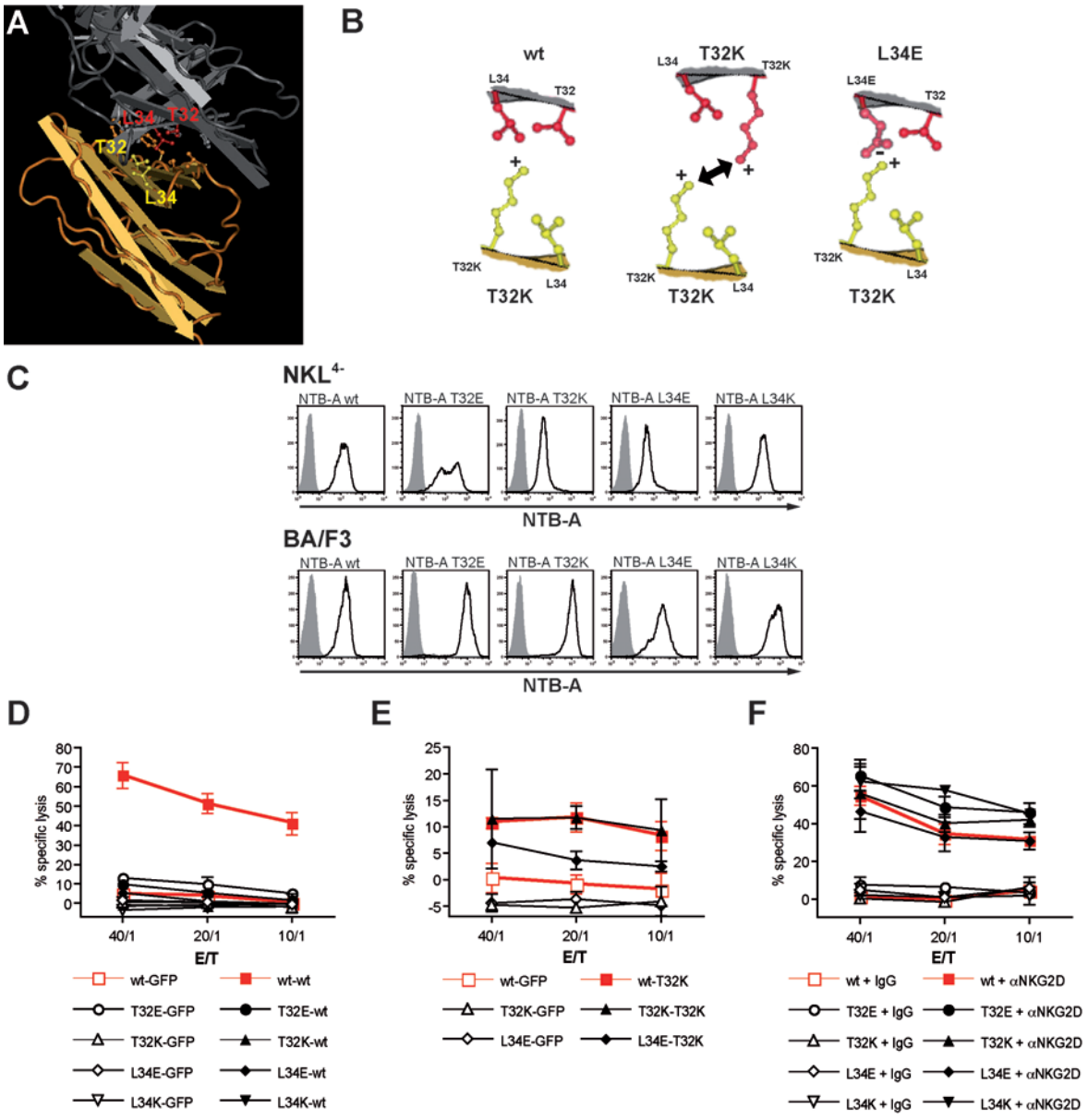

Figure 6: Introducing complementary mutations into NTB-A to create a heterophilic receptor pair

A: The positions of the two amino acid residues threonine-32 and leucine-34 in the NTB-A-NTB-A interface. By mutating the opposing amino acids to charged residues lysine and glutamate it was attempted to create a heterophilic pair of NTB-A mutants. **B:** An example to illustrate the expected effects of one mutation on the interaction with wild type NTB-A, a receptor with the identical mutation and the complementary mutant. **C:** All four possible mutations were generated and stably transfected into the NTB-A-deficient cell line NKL^{4-} and BA/F3 cells. Similar expression levels were determined by flow-cytometry. The gray histograms represent staining with an unspecific control antibody. **D:** Cytotoxicity of the transfected NKL^{4-} against BA/F3 cells expressing wild type NTB-A. **E:** Cytotoxicity of transfected NKL^{4-} against BA/F3 cells expressing the identical and complementary mutants. Cytotoxicity was tested in a 16 h ^{51}Cr-release assay at different E/T ratios **F:** To confirm the cytotoxic potential of the transfected NKL^{4-} cells, a 16 h redirected lysis assay against P815 cells was performed in the presence of control IgG or anti-NKG2D (αNKG2D) antibodies. Depicted are means ±SD of triplicates. The experiments shown are representatives of two, in case of E including all mutants and their complementary counterpart.

To exclude the possibility that the NKL^{4-} cells lost their cytotoxic potential during the retroviral transduction and the following selection process, we measured NKG2D-mediated lysis in a redirected lysis assay (fig. 6F). The NKL^{4-} cells expressing the wild type or the different mutants of NTB-A were still able to lyse target cells, but only at a low level. All ^{51}Cr-release assays had to be conducted for 16 h, because no considerable lysis could be observed after the usual 4 h incubation.

The attempt to create a heterophilic pair of NTB-A mutants by replacing the residues T32 and L34 with charged residues was unsuccessful, because these mutations only prevent the homophilic interaction, but obviously do not fit into a heterophilic interface.

4.2 Early events in SLAM-related receptor signaling

4.2.1 Association of SAP with 2B4 is dispensable for receptor phosphorylation

Receptor phosphorylation is one of the first events in SLAM-related receptor signaling. For SLAM it has been shown that SAP can bind to the unphosphorylated receptor and mediate receptor phosphorylation by recruitment of the Src-kinase FynT (150). Unphosphorylated 2B4 and NTB-A do not bind SAP (82, 152). Therefore phosphorylation of both receptors is thought to be a signaling event preceding the association of SAP. On the contrary SAP KO mice show no phosphorylation of 2B4 after engagement, although the expression of Src-kinases is not disturbed (102).

To investigate the function of SAP in these early signaling events, we made use of NK92 cells with a stably down-regulated SAP expression mediated by retroviral shRNA delivery (NK92 shSAP). These cells show a defect in 2B4-mediated cytotoxicity compared to control cells expressing an shRNA against CD4 (NK92 shCD4) while lysis mediated by the natural cytotoxicity receptor NKp30 was unaltered (fig. 7A). To test whether the signal is already impaired at the level of receptor phosphorylation, the two knockdown cell lines were stimulated by incubation with the cell line 721.221, an EBV-transformed B cell line which expresses CD48, the ligand for 2B4. Unstimulated and stimulated cells were lysed and 2B4 was immunoprecipitated from the lysates. Immunoprecipitates were then analyzed by western blotting (fig. 7B).

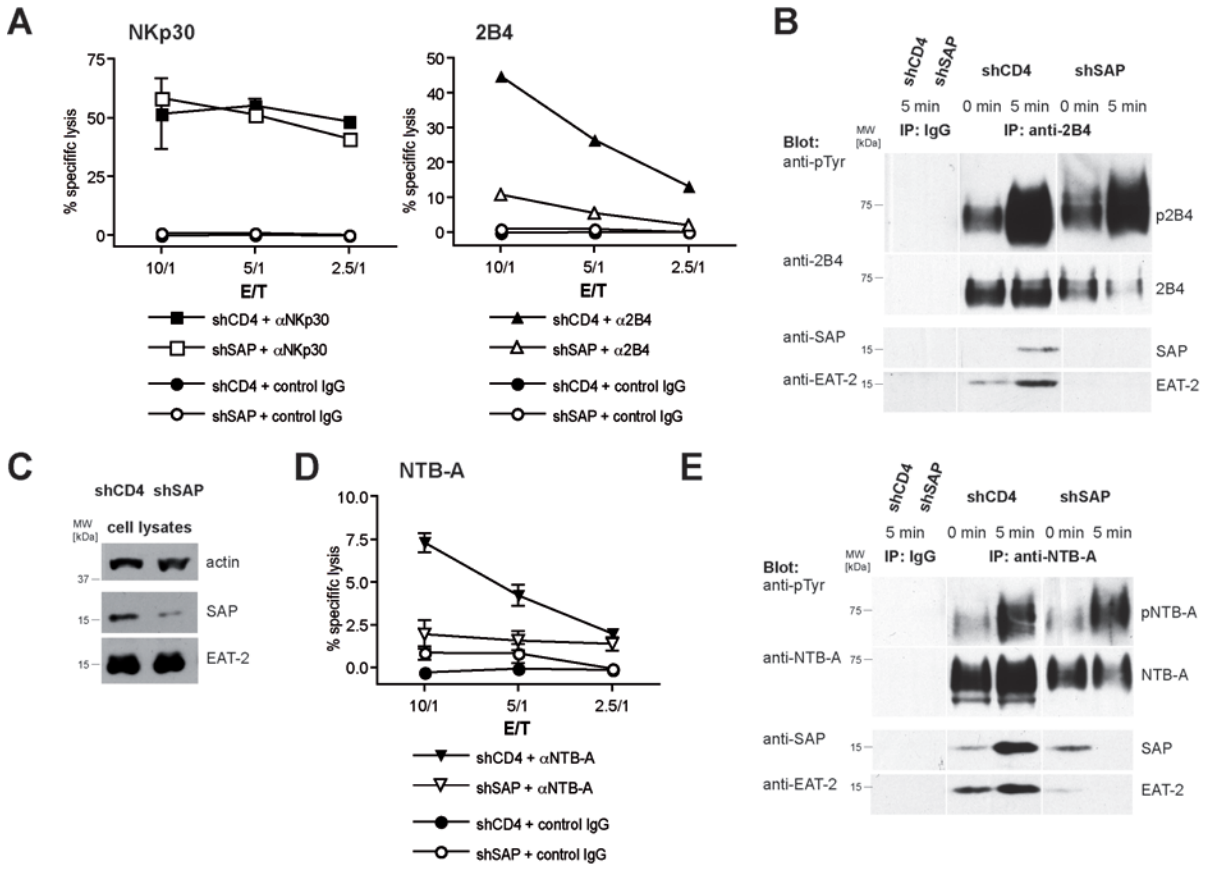

Figure 7: The effect of SAP-knockdown in NK92 cells on signaling of 2B4 and NTB-A
To study the impact of a SAP-knockdown on NK cell signaling, NK92-C1 cells stably expressing a small hairpin RNA against SAP (shSAP) or CD4 (shCD4) mRNA (as negative control) were analyzed. **A:** The two knockdown cell lines were used as effector cells in a redirected ^{51}Cr-release assay against P815 cells in the presence of IgG control antibody or antibodies against the receptors NKp30 and 2B4 at different E/T ratios. Data is shown as mean ± SD of triplicates. The results are from one representative experiment out of four. **B:** For analysis of receptor signaling equal numbers of the two cell lines were mixed with 721.221 cells and lysed at the indicated time-points. After a control immunoprecipitation with an unspecific antibody (IP: IgG) 2B4 was immunoprecipitated from the lysates. The immunoprecipitates were analyzed by western blotting. Membranes were probed with anti-phospho-tyrosine antibodies to detect receptor phosphorylation and re-probed with antibodies against 2B4 (upper panels). Antibodies against SAP and EAT-2 were used to detect co-precipitated molecules on the same membrane (lower panels). **C:** Whole cell lysates were blotted and probed with antibodies to confirm the reduced SAP levels in the NK92 shSAP cells. **D:** The two knockdown cell lines were used as effector cells in a redirected ^{51}Cr-release assay against P815 cells in the presence of IgG control antibody or antibody against NTB-A at different E/T ratios. Data is shown as mean ± SD of triplicates. The results are from one representative experiment out of four. **E:** After a control immunoprecipitation with an unspecific antibody (IP: IgG) NTB-A was immunoprecipitated from the same lysates as in B. Immunoprecipitates were analyzed by western blotting. Membranes were probed with anti-phospho-tyrosine antibodies to detect receptor phosphorylation and re-probed with antibodies against NTB-A (upper panels). Co-precipitation of SAP and EAT-2 was detected as in B (lower panels). The blots shown are representatives of at least three cell mix experiments.

The membranes were probed with anti-phospho-tyrosine antibody to detect ITSM-phosphorylation and re-probed with anti-2B4 antibody. Phosphorylation of 2B4 was increased after stimulation in control and SAP-knockdown cells (6B, upper panel). Because the extent of 2B4-phosphorylation was not diminished in shSAP cells, we conclude that phosphorylation of 2B4 is independent of SAP association. The core

function of SAP-mediated FynT recruitment is not the enhancement of ITSM phosphorylation.

4.2.2 EAT-2 recruitment to 2B4 is dependent on the presence of SAP

In the same experiments the association of the adapter molecules SAP and EAT-2 with the immunoprecipitated receptor was analyzed (6B, lower panels). In the control cells SAP was absent from the low-level phosphorylated receptor in unstimulated cells and was recruited to the activated, highly phosphorylated receptor. In the cells with reduced SAP expression level no association of SAP with 2B4 could be detected, although the SAP-knockdown was not complete, as shown by western blot analysis of whole cell lysates (fig. 7C). In contrast to SAP, receptor-bound EAT-2 was already detectable in unstimulated control cells and accumulated after stimulation. Surprisingly, EAT-2 did not bind to the receptor in the knockdown cells, neither in the unstimulated nor the stimulated state, even though its expression level remained unchanged by the SAP knockdown (fig. 7C). The 721.221 cells used to stimulate the NK92 cells in the cell mix assay do not express SAP or EAT-2 (152). Therefore the total amount of detected protein must come from the NK92 cells.

4.2.3 NTB-A-phosphorylation is also independent of SAP association

We also investigated the role of SAP in early NTB-A signaling in the NK92 shSAP cells with stably reduced SAP expression.

When testing the NTB-A-mediated cytotoxicity of the shSAP cells in comparison to the control cells, a decreased lysis was observed (fig. 7D). To analyze NTB-A phosphorylation in the absence of SAP both cell lines were stimulated by co-incubation with 721.221 cells and NTB-A was immunoprecipitated. Western blot analysis of the immunoprecipitates revealed a similar picture as for 2B4. Co-incubation with the target cells induced an increase in NTB-A phosphorylation in control cells and SAP-knockdown cells (fig. 7E, upper panel). As it has been shown that there is no background-phosphorylation of NTB-A in the 721.221 cell line (152), we can exclude that some of the phosphorylated NTB-A comes from the target cells used in the cell mix.

4.2.4 EAT-2 does not bind to NTB-A in the absence SAP

Analysis of the co-precipitated adapter molecules showed that in contrast to 2B4 the background-phosphorylation of NTB-A in unstimulated control cells was sufficient to recruit EAT-2 and SAP. The association increased with receptor phosphorylation

(fig. 7E, lower panel). In cells with reduced SAP expression we could also detect SAP and EAT-2 bound to the low-level phosphorylated receptor in unstimulated cells, but instead of accumulating at highly phosphorylated NTB-A after stimulation neither SAP, nor EAT-2 were detectable in the immunoprecipitate (fig. 7E, lower panel).
Similar to the observations made with 2B4 these results show a dependency of EAT-2-receptor association on the simultaneous binding of SAP to the receptor.

4.2.5 2B4 phosphorylation is also SAP-independent in the cell line YTS

To exclude that the observed effect is specific for the NK92 cell line, we generated a stable reduction of SAP expression in the NK-like cell line YTS by retroviral delivery of shRNA against SAP (YTS shSAP), while an shRNA against CD4 served as control (YTS shCD4). The reduction of 2B4-mediated cytotoxicity observed in the NK92 shSAP cells could be confirmed with the YTS shSAP cells (fig. 8A, left panel). NTB-A-mediated lysis by YTS shSAP was also reduced to a small extent compared to control cells (fig. 8A, right panel).
To investigate, if the early events in 2B4 signaling were affected in the same way as in the NK92 shSAP cells, YTS shSAP and YTS shCD4 cells were stimulated by co-incubation with BA/F3 cells expressing CD48. Unstimulated and stimulated cells were lysed and 2B4 was immunoprecipitated from the lysates. Receptor phosphorylation, co-precipitation and expression levels of adapter molecules were analyzed by western blotting (fig. 8B). As observed in the NK92 cells the reduction of SAP expression did not lead to a reduction of 2B4 phosphorylation. Association of SAP with the highly phosphorylated receptor after stimulation could be detected in the control cells, but was not found in shSAP cells. In contrast to the results obtained with the NK92 cells, EAT-2 could not be detected in the immunoprecipitates, possibly because of a lower expression level in the YTS cells.

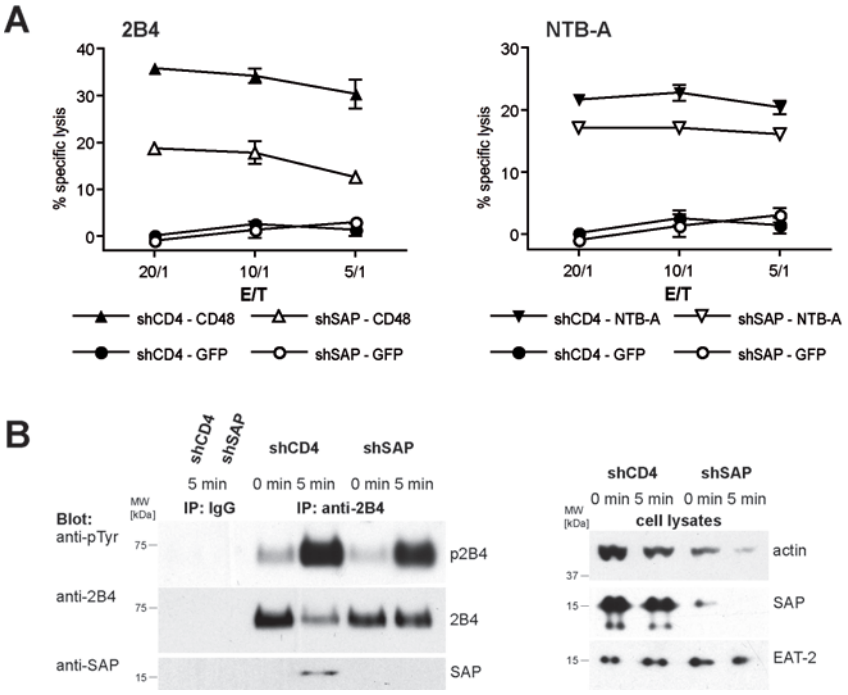

Figure 8: The effect of SAP-knockdown in YTS cells on signaling of 2B4 and NTB-A
YTS cells stably expressing a small hairpin RNA against SAP (shSAP) or CD4 (shCD4) mRNA (as negative control) were analyzed. **A:** The two knockdown cell lines were used as effector cells in a 4 h ^{51}Cr-release assay against BA/F3 cells expressing GFP, CD48 or NTB-A at different E/T ratios. Data is shown as mean ± SD of triplicates. One representative experiment out of three is shown. **B:** For analysis of receptor signaling equal numbers of the two cell lines were mixed with BA/F3 cells expressing CD48 and lysed at the indicated time-points. After a control immunoprecipitation with an unspecific antibody (IP: IgG) 2B4 was immunoprecipitated from the lysates. The immunoprecipitates of anti-2B4 antibody (left panel) and whole cell lysates (right panel) were analyzed by western blotting. Membranes were probed with anti-phospho-tyrosine antibodies to detect receptor phosphorylation and re-probed with an antibody against 2B4. Antibodies against SAP and EAT-2 were used to detect co-precipitated molecules and confirm the reduced SAP expression in shSAP cells. Re-probing with anti-actin antibody served as loading control for the lysates. EAT-2 could not be detected in the immunoprecipitates. The blots shown are representatives of at least three cell mix experiments.

4.2.6 Establishing a method for knockdown of protein expression in primary NK cells

The results obtained with the two cell lines show a dependency of 2B4 and NTB-A signaling on the presence of SAP. A similar defect in 2B4 and NTB-A-mediated cytotoxicity has been observed in NK cell from XLP patients lacking functional SAP (84, 98, 136-138). On the contrary, it has been reported that NTB-A-mediated cytotoxicity was still intact in NK cell lines, when SAP but not EAT-2 recruitment was abrogated by ITSM mutation (152). This led to the conclusion that the NTB-A signal that triggers a cytotoxic response is dependent on EAT-2, and the defect observed in XLP patients may be due to disturbances in NK cell development in the absence of functional SAP. As we observed that EAT-2 recruitment to SRR was dependent on SAP, our results supported the notion that SAP is the crucial adapter molecule for

both 2B4 and NTB-A. Therefore we wanted to confirm these findings in primary NK cells.

To knockdown SAP in primary NK cells we wanted to use a lentiviral vector expressing the same shRNA used for the RNA-interference in the cell lines. We cloned the shRNA sequences into the vector pLVTHM, a vector that contains a GFP gene as marker for transduced cells. As a positive control for transduction the vector pWPXL siNKG2D was used, a vector for RNA interference against NKG2D that also contains GFP as reporter gene (172). Primary IL-2-activated NK cells were transduced and GFP expression was detected by flow-cytometry. GFP-positive cells could be detected after transduction with each vector and the effect of RNA interference with NKG2D expression could be detected by staining the pWPXL siNKG2D-transduced cells for NKG2D (fig. 9A). But the transduction efficiency reached with either vector was low, ranging from 4 to 5 %, reaching a maximum of 10 % in one experiment with pLVTHM shSAP (fig. 9A). This efficiency is far too small to obtain sufficient cell numbers for functional assays.

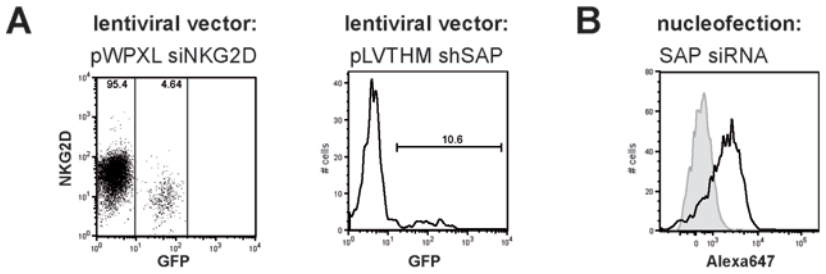

Figure 9: Lentiviral transduction of primary NK cells compared to nucleofection
A: IL-2-activated primary NK cells were transduced with the lentiviral vectors pWPXL siNKG2D or pLVTHM shSAP. Both vectors contain GFP as marker gene for transduced cells and code for small hairpin RNA against the receptor NKG2D or SAP, respectively. 12 or 15 days after transduction, respectively, the percentage of transduced cells was determined by flow-cytometry. The down-regulation of NKG2D expression on cells transduced with pWPXL siNKG2D was confirmed by staining with an anti-NKG2D antibody. The numbers give the percentage of cells in the depicted gates. The plots show the results of the most successful experiments. **B:** IL-2-activated primary NK cells were transfected with Alexa647-labeled siRNA against SAP or unlabeled control siRNA (gray histogram) using nucleofection. 20 h later the cells were washed twice and analyzed by flow-cytometry.

Thus, we decided to test, whether a transient transfection with siRNA was a more suitable tool. Using nucleofection technology we transfected primary IL-2 activated NK cells with fluorescence-labeled siRNA against SAP and analyzed the transfection efficiency 20 h later by flow-cytometry (fig. 9B). The greatest part of the NK cells was positive for fluorescence of the labeled siRNA. Therefore we chose to reduce SAP or EAT-2 expression in primary NK cells by transient transfection with siRNAs.

4.2.7 SAP is the relevant adapter molecule for 2B4 and NTB-A-triggered cytotoxicity

To confirm the results obtained with the cell lines and to elucidate the role of EAT-2, primary IL-2-activated NK cells were transfected with siRNA against SAP, EAT-2 or both. 48 h after transfection expression levels of the two adapter molecules were analyzed by western blotting of cell lysates (fig. 10A). The expression of SAP and EAT-2 was strongly reduced in cells transfected with the respective siRNA. To exclude unspecific side effects of the siRNA on the investigated signaling events equal expression of FynT was confirmed in the lysates (fig. 10A) and expression levels of 2B4 and NTB-A were confirmed by flow-cytometry (fig. 10B).

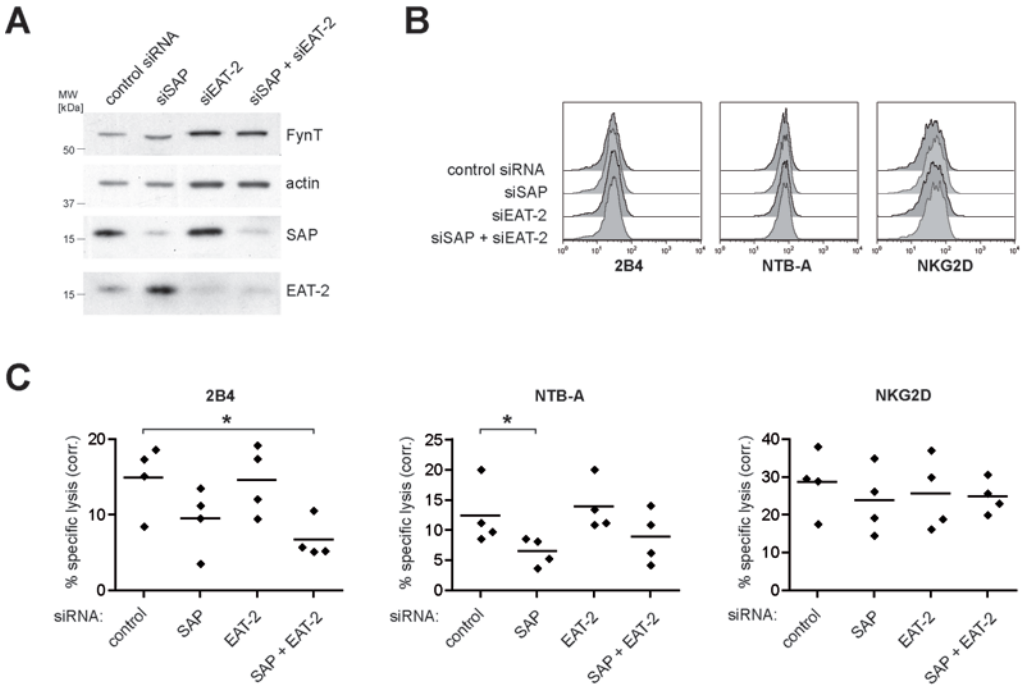

Figure 10: 2B4 and NTB-A-mediated cytotoxicity are impaired after SAP-knockdown, but not after EAT-2-knockdown in primary NK cells
IL-2-activated primary NK cells were transfected with control siRNA, siRNA against SAP, against EAT-2 or both. **A:** The knockdown was confirmed 48 h later by western blotting of whole cell lysates. **B:** Equal expression levels of 2B4, NTB-A and NKG2D 48 h after transfection were confirmed by flow-cytometry. **C:** The functional consequence of the decreased expression of the adapter molecules was tested in a 4 h ^{51}Cr-release assay against P815 cells in the presence of control antibody, or antibodies against 2B4, NTB-A or NKG2D. Results shown are from four independent experiments. Plotted is the specific lysis at an E/T ratio of 5/1 corrected by subtracting the lysis observed in the presence of control antibody for each experiment. The bars represent the mean value. Statistical significance of the reduced lysis compared to control siRNA-transfected cells was calculated using one-way ANOVA and Dunnett's post test (* indicates $p < 0.05$).

To assess the influence of SAP and EAT-2 on the signaling of 2B4 and NTB-A in primary NK cells, cytotoxicity of transfected cells against the target cell line P815 was measured in the presence of antibodies against each of the two receptors, or NKG2D as a positive control (fig. 10C).

The specific lysis was lower for 2B4 and NTB-A, when SAP expression was reduced. In contrast, a decreased expression level of EAT-2 did not result in a reduction of lysis. The lysis obtained with the double knockdown cells was diminished to a similar level as observed with the SAP knockdown cells. NKG2D-mediated lysis was not affected by the down-regulation of either adapter molecule. These effects of the knockdown were observed in all experiments performed with NK cells from different donors. However, the reduction of 2B4-mediated lysis was only statistically significant for the double knockdown and NTB-A-mediated lysis was only significantly reduced in the SAP single knockdown.

The analysis of receptor phosphorylation in primary cells was not possible, because the numbers of transfected NK cells were not sufficient.

The results obtained with primary NK cells confirm that SAP and not EAT-2 is the main mediator of signal transduction leading to cytotoxic responses after 2B4 or NTB-A engagement.

4.3 Functions of NTB-A and CRACC in T cells

Co-stimulation of T cells through NTB-A and CRACC induces activation and proliferation

For the receptors SLAM, CD84, 2B4 and NTB-A it has been shown that their engagement can enhance proliferation of T cells stimulated via their T cell receptor (73, 104, 109, 173-175). To test whether the receptor CRACC, which is expressed on a subset of T cells (fig. 11), has also co-stimulatory potential on these cells, peripheral blood T cells were stimulated with different combinations of plate-bound antibodies.

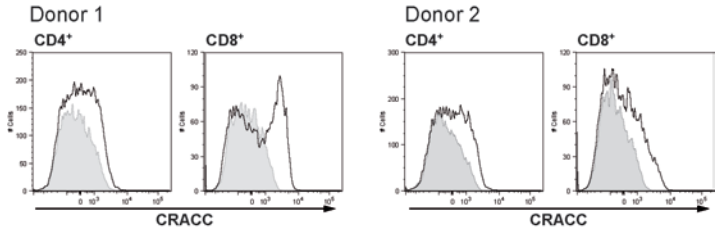

Figure 11: CRACC is expressed on a subset of T cells
Freshly isolated peripheral blood T cells were stained for CD4, CD8 and CRACC and analyzed by flow-cytometry. Results for two donors are shown to represent variability of the CRACC-positive subsets. The gray histograms represent staining with an unspecific control antibody.

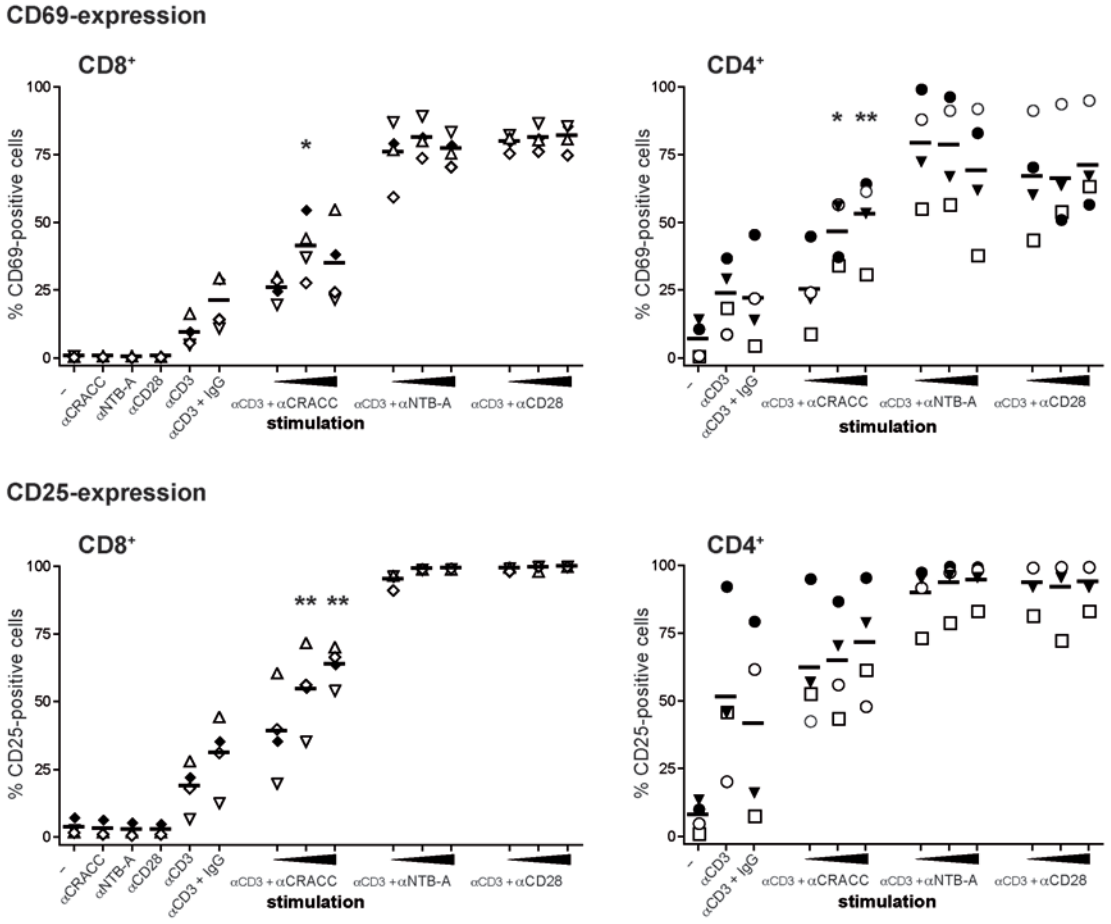

Figure 12: Co-stimulation of T cells through CRACC and NTB-A induces expression of activation markers
Peripheral blood T cells were stimulated with plate-bound antibodies. The plates were pre-coated with goat-anti-mouse-IgG antibody. Anti-CD3 antibody (αCD3) was used at a sub-stimulatory concentration of 0.01 µg/ml, alone or in combination with control antibody (IgG) at 10 µg/ml or co-stimulatory antibodies against CRACC (αCRACC), NTB-A (αNTB-A) and CD28 (αCD28) in concentrations of 0.1 µg/ml, 1 µg/ml and 10 µg/ml. αCRACC, αNTB-A and αCD28 were also tested without αCD3 at a concentration of 10 µg/ml. After 48 h the cells were harvested and stained for CD69 (upper panels), CD25 (lower panels) and CD8 (left panels) or CD4 expression (right panels). The symbols represent different donors; the bars show the mean of all four donors. The mean values obtained for the co-stimulation with CRACC were compared to value for αCD3 + IgG stimulation using one-way ANOVA and Dunnett's post test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$.

T cells used for the investigation of proliferation were labeled with CFDA prior to stimulation. The antibody against the T cell receptor component CD3 was used at a concentration too low to induce proliferation by itself and was mixed with an unspecific control antibody or antibodies against CRACC, NTB-A or CD28 at three different concentrations. These antibodies were also tested without anti-CD3 antibody.

After 48 h of stimulation the unlabeled T cells were harvested, stained for the early activation marker CD69 or CD25 (the IL-2-receptor α-chain) and CD8 or CD4 and analyzed by flow-cytometry (fig. 12). Without simultaneous stimulation of the T cell

receptor neither of the antibodies showed stimulatory capacity. Co-stimulation with anti-CRACC antibody led to a small increase of CD69 expression on CD8-positive cells and a more prominent increase on CD4-positive cells compared to the expression on cells stimulated with anti-CD3 and control antibody. CD25 was also induced through CRACC co-stimulation on both T cell subsets. The increase was more distinct than the effect on CD69 expression, although no statistical significance could be determined for the CD4-positive subset. NTB-A was as effective as the classical co-stimulatory receptor CD28 on both subsets. Nearly all CD8-positive T cells became positive for CD69 and CD25 after co-stimulation with anti-NTB-A already at the lowest concentration of 0.1 µg/ml.

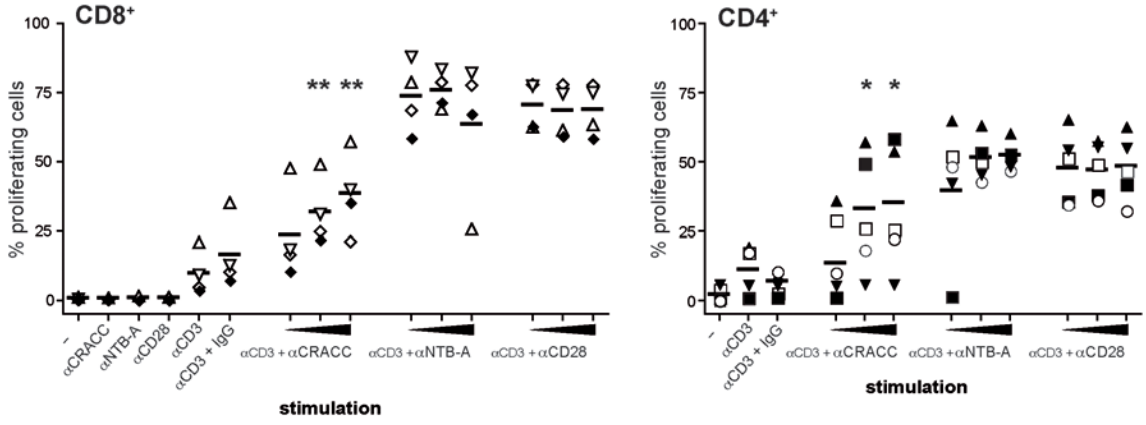

Figure 13: Co-stimulation of T cells through CRACC and NTB-A induces proliferation
Peripheral blood T cells were labeled with CFDA and stimulated with plate-bound antibodies as described for fig. 12. After 72 h the cells were harvested, stained for CD8 (left panel) or CD4 expression (right panel) and analyzed by flow-cytometry. The percentage of proliferating cells was determined based on CFDA-dilution. The symbols representing different donors correspond to the symbols used in fig. 12; the bars show the mean of all four donors. The mean values obtained for the co-stimulation with CRACC were compared to value for αCD3 + IgG stimulation using one-way ANOVA and Dunnett's post test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$.

After 72 h of stimulation the proliferation of T cells from the CD8- or the CD4-positive subset was analyzed by flow-cytometry (fig. 13). None of the antibodies induced proliferation in the absence of T cell receptor stimulation. Co-stimulation with anti-CRACC antibody induced proliferation in a dose-dependent manner, matching the observations on activation marker expression. The effect of the lowest concentration (0.1 µg/ml) was not statistically significant compared to the proliferation induced by anti-CD3 in combination with unspecific control antibody. At higher concentrations (1 or 10 µg/ml) the percentage of proliferating cells was significantly increased, both in the CD8 and CD4-positive subset, even though the response to co-stimulation of

CRACC showed a high variability between donors in the CD4-positive subset. Similar to the induction of activation markers, the proliferation induced by co-stimulation through NTB-A or CD28 was equally high. The percentage of proliferating cells reached its maximum already at the lowest concentrations.

These results show that CRACC and NTB-A have a co-stimulatory potential to activate T cells and elicit a proliferative response. The effect of co-stimulation through CRACC is possibly less prominent, because CRACC expression is restricted to a smaller subset of T cells.

4.3.1 Co-stimulation through NTB-A and CRACC induces cytokine production

To study the effect of co-stimulation through NTB-A and CRACC on the production of cytokines, peripheral blood T cells were stimulated with plate-bound antibody against CD3 at sub-optimal concentration in combination with an unspecific control antibody or antibodies against CRACC, NTB-A or CD28 for 6 h. Afterwards cells were lysed and mRNA levels of the cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, IL-17, IL-18, TNF-α, TGF-β, and IFN-γ were determined in relation to GAPDH-mRNA by quantitative RT-PCR. Where detectable, the expression pattern of cytokine mRNA showed no qualitative, but only quantitative differences between the three co-stimulators. IL-2, TNF-α and IFN-γ-mRNAs were strongly expressed after co-stimulation through CD28 (fig. 14A). NTB-A-mediated co-stimulation also increased IL-2 and IFN-γ-mRNA levels, but to a far lesser extent; and co-stimulation with CRACC led only to a very slight, but significant elevation of IFN-γ-mRNA.

To investigate cytokine production at the level of protein expression, peripheral blood T cells were stimulated for 48 h with plate-bound antibodies as described for the analysis of activation markers or proliferation. Brefeldin A was added to the cultures 4 h before analysis to inhibit exocytosis of cytokines. Expression of IL-2, IFN-γ, TNF-α and CD4 or CD8 were analyzed by intracellular staining and flow-cytometry.

IL-2 production was detected in CD4-positive cells co-stimulated with anti-CRACC and anti-NTB-A antibodies, although the variability between cells from different donors was high (fig. 14B). Similar to the results obtained for proliferation, the percentage of positive cells after CRACC-mediated co-stimulation was lower compared to that after co-stimulation of NTB-A. The observed levels of IL-2-positive cells were similar after NTB-A- and CD28-mediated co-stimulation. The induction of IFN-γ production in CD8-positive cells was only marginal after CRACC co-stimulation.

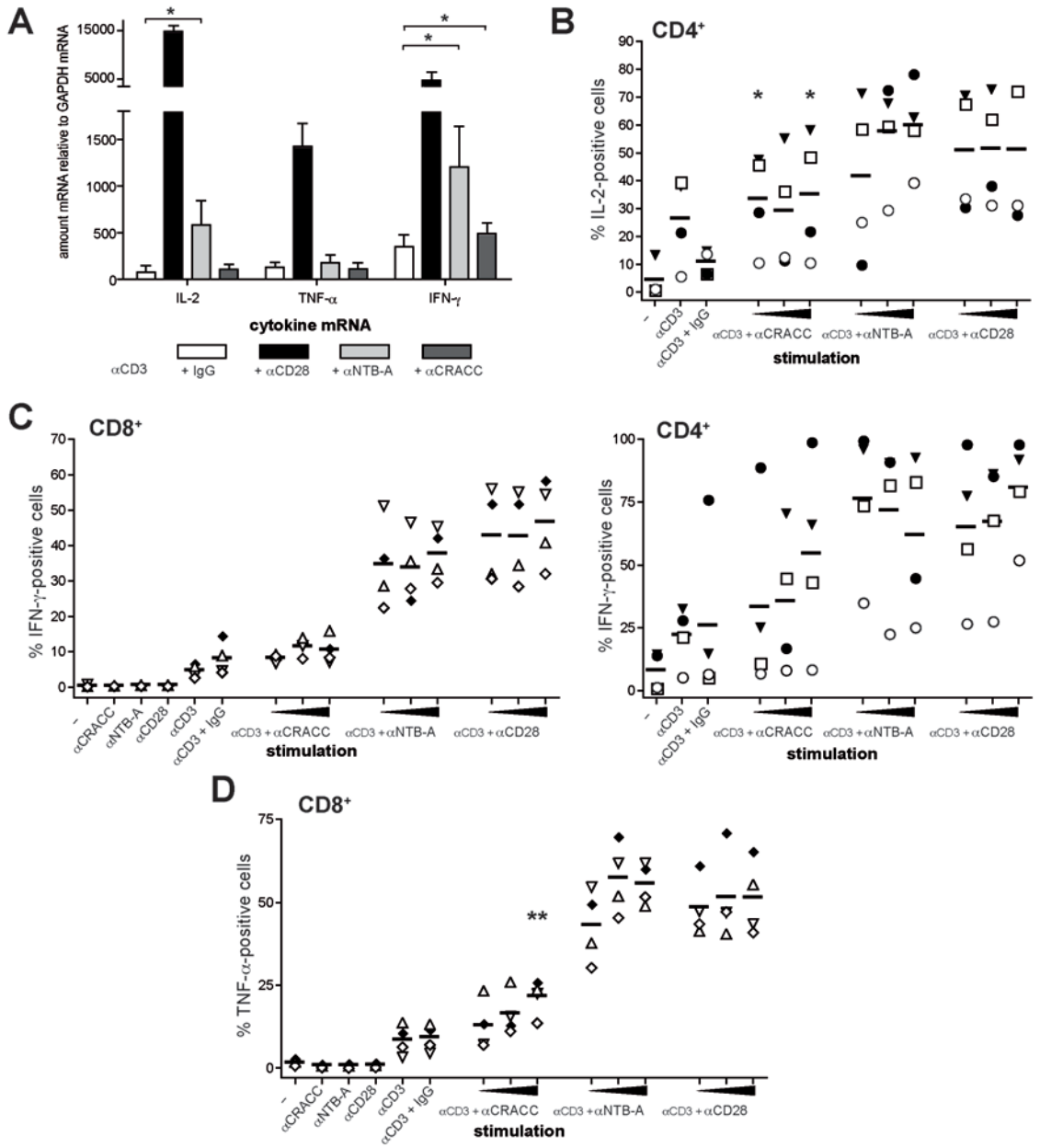

Figure 14: Co-stimulation of T cells through CRACC and NTB-A induces cytokine production
A: Peripheral blood T cells were stimulated with plate-bound antibodies. The plates were coated as described for fig. 12. Control antibody (IgG) or co-stimulatory antibodies against CRACC (αCRACC), NTB-A (αNTB-A) and CD28 (αCD28) were used at concentrations of 10 µg/ml. After 6 h the cells were harvested and mRNA expression of IL-2, TNF-α and IFN-γ relative to GAPDH-mRNA was determined by quantitative RT-PCR. The graph shows the mean of three experiments ± SD. The values obtained from the cells co-stimulated with αCRACC or αNTB-A were compared to the control cells with paired t-test. * The increased levels of IL-2-mRNA and IFN-γ-mRNA after NTB-A-co-stimulation ($p = 0.041$, each) and the increased level of IFN-γ-mRNA after CRACC-co-stimulation ($p = 0.032$) were considered statistically significant. **B – D:** The cytokine production was also analyzed at the level of protein expression. Peripheral blood T cells were stimulated as described for fig. 12. Brefeldin A was added to the cultures for the last 4 h. Cells were harvested, fixed and permeabilized. After staining with the respective antibodies cells were analyzed by flow-cytometry. **B:** IL-2 expression in CD4-positive cells. **C:** IFN-γ expression in CD8-positive (left panel) and CD4-positive cells. **D:** TNF-α expression in CD8-positive cells. The symbols representing different donors correspond to the symbols used in fig. 12 and 12; the bars show the mean of all four donors. The mean values obtained for the co-stimulation with CRACC were compared to value for αCD3 + IgG stimulation using one-way ANOVA and Dunnett's post test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$.

The effect of CRACC co-stimulation was more visible in CD4-positive cells, although without statistical significance (fig. 14C). NTB-A-mediated co-stimulation resulted in IFN-γ expression in both T cell subsets. TNF-α production of CD8-positive cells could also be detected after NTB-A co-stimulation and to a small extent after CRACC co-stimulation, but only the value obtained with the highest anti-CRACC antibody concentration was statistically significant (fig. 14D).

These results show that co-stimulation through NTB-A or CRACC is also able to induce production of the cytokines IL-2, TNF-α and IFN-γ in CD4 and CD8-positive T cells. Again, the effect of co-stimulation through CRACC is limited by the size of the subset expressing the receptor.

4.3.2 Co-stimulation with the anti-CRACC antibody is specific

To exclude the possibility that the small effects observed after co-stimulation of CRACC were due to an unspecific interaction of the anti-CRACC antibody, we investigated, whether co-stimulation with another anti-CRACC clone led to similar results. The clone CS1-4 used in previous experiments and the clone 162.1 (112) were tested at different concentrations in combination with anti-CD3 antibody in sub-optimal concentration. After 48 h the stimulated peripheral blood T cells were analyzed by flow-cytometry for CD69 and IFN-γ expression (fig. 15).

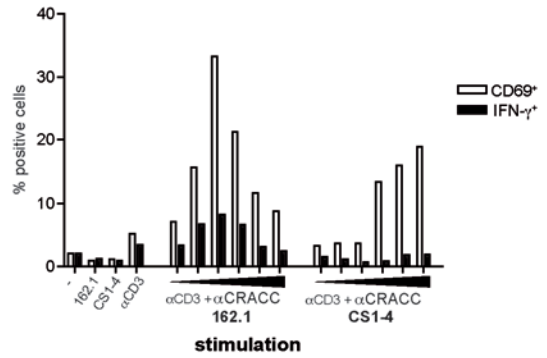

Figure 15: Comparison of the stimulation by two different anti-CRACC antibodies
Peripheral blood T cells were stimulated with plate-bound antibodies. The plates were pre-coated with goat-anti-mouse-IgG antibody. Anti-CD3 antibody (αCD3) was used at a sub-stimulatory concentration of 0.01 µg/ml, alone or in combination with increasing concentrations (0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml) of the two antibody clones against CRACC: CS1-4, the one used in previous experiments, and 162.1. After 44 h Brefeldin A was added to the cultures. 4 h later the cells were harvested, fixed and permeabilized. Flow-cytometry was performed after staining for CD69, IFN-γ and CD8.

Both antibodies could induce CD69 expression, but clone 162.1 had a stronger effect with a different dose response. At 0.1 µg/ml the percentage of positive cells had its maximum and decreased with increasing 162.1 concentrations. While co-stimulation with CS1-4 failed to induce IFN-γ production in the experiment shown in figure 15, clone 162.1 was able to induce production of IFN-γ with the same dose response observed for CD69 expression. Therefore we conclude that the co-stimulation through CRACC is a specific effect.

4.3.3 CRACC is expressed on memory T cells and activated T cells

The limited effects observed after co-stimulation of CRACC are possibly due to the small size of the T cell subset expressing the receptor. To further characterize this subset, naïve and memory T cells were screened for expression of CRACC. These subsets can be defined by the expression of CCR7 and the CD45 isoform CD45RA (23). Naïve T cells express both molecules, central memory T cells express CCR7 and CD45RO instead of RA, effector memory T cells are negative for CCR7 and CD45RA with the exception of a minor subset of effector memory T cells that re-expresses the CD45RA isoform. Freshly isolated peripheral blood T cells were analyzed by flow-cytometry for expression of these markers, CD4, CD8 and CRACC (fig. 16A). Naïve T cells neither of the CD4 nor the CD8-positive subset did express CRACC. CD8-positive cells of all memory cell types showed CRACC expression, while CD4-positive effector memory cells showed only a slight increase in CRACC staining. This is corresponding to the expression pattern on CD4 and CD8-positive T cells (fig. 11 and (112)).

Because the expression of CRACC is restricted to memory cells, we wanted to test whether activation of T cells induces CRACC expression. To this end freshly isolated peripheral blood T cells were unspecifically stimulated with PHA-P and then kept in culture with IL-2 for one week. During this time CRACC expression on CD4 and CD8-positive cells was monitored by flow-cytometry (fig. 16B). Three days after stimulation there was a strong increase in CRACC-positive cells in the CD8-positive subset and after one week all CD8-positive cells expressed CRACC. The CRACC expression on CD4-positive cells changed only marginally.

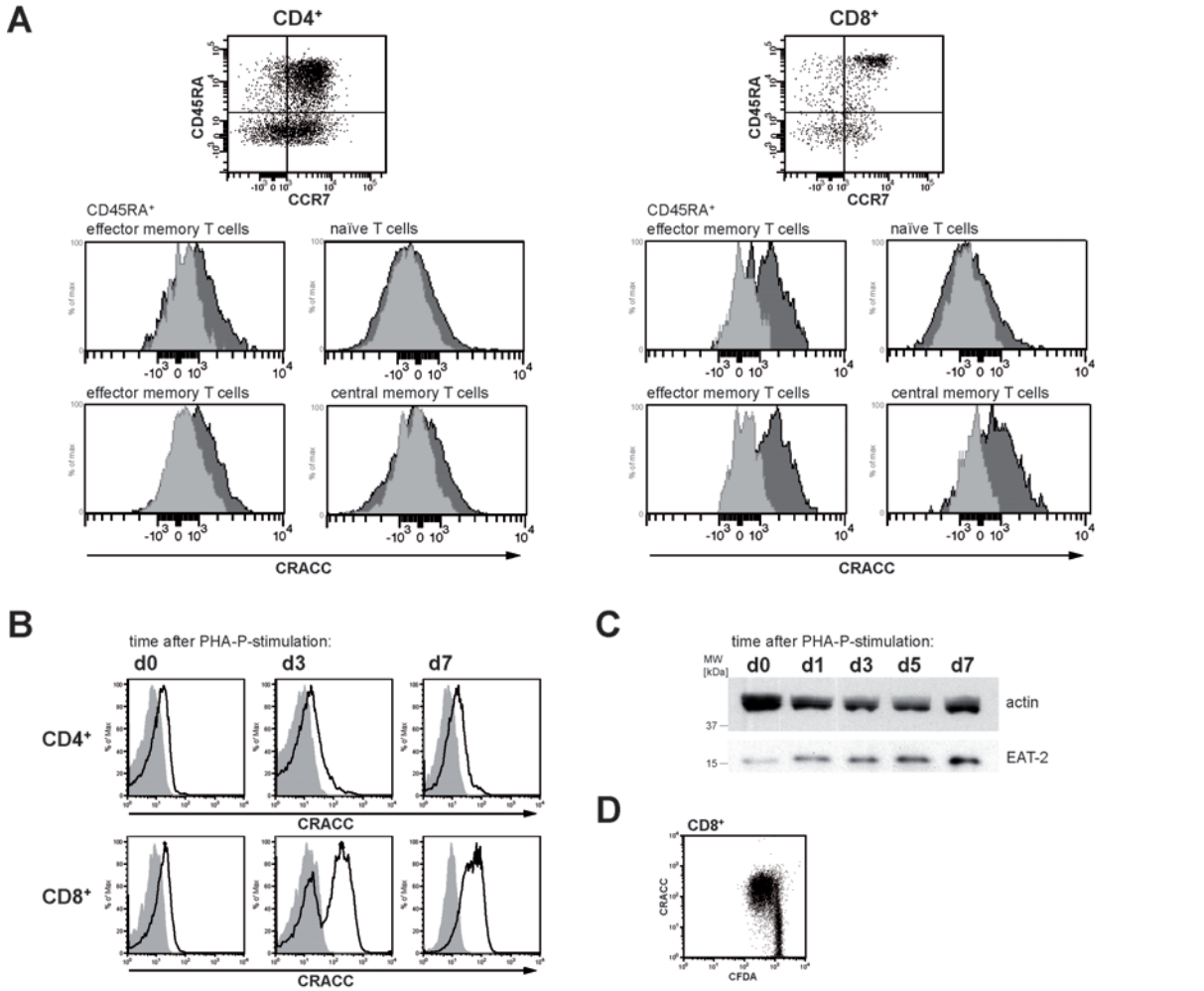

Figure 16: CRACC is expressed on memory T cells and activated T cells
A: Freshly isolated peripheral blood T cells were analyzed by flow-cytometry after staining with a control antibody or anti-CRACC antibody in combination with antibodies against CD4, CD8, CD45RA, and CCR7. According to their expression of CD45RA and CCR7 the cells were classified as naïve T cells ($CD45RA^+$, $CCR7^+$), central memory T cells ($CD45RA^-$, $CCR7^+$), and CD45-positive and negative effector T cells ($CCR7^-$). The expression of CRACC was determined on each subset among CD4-positive T cells (left panel) and CD8-positive T cells (right panel). The light gray histograms represent the staining of the control antibody of the respective subset. The results shown are representative for 8 experiments. **B:** To study the expression of CRACC on activated T cells, freshly isolated peripheral blood T cells were stimulated with 2 µg/ml PHA-P overnight, then washed and cultured in medium containing 100 IU/ml of recombinant IL-2. At the indicated time-points cells were stained for CD4, CD8 and CRACC. Gray histograms represent staining with a control antibody. **C:** 10^6 PHA-P-activated T cells were lysed at the indicated time-points to examine expression of the adapter molecule EAT-2 by western blotting of whole cell lysates. Anti-actin blot served as loading control. The blot shown is representative for five experiments. **D:** CFDA-labeled peripheral blood T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies as described for fig. 12 at a concentration of 0.01 µg/ml for both antibodies. After 72 h cells were harvested, stained for CD8 and CRACC and analyzed by flow-cytometry.

CRACC signaling is dependent on the adapter molecule EAT-2 (114) and in murine CD4-positive T cells, which do not express EAT-2, CRACC has been shown to act as inhibitory receptor (115). Therefore we studied the expression of EAT-2 in PHA-P-activated T cells by western blotting of lysates at different time-points after

stimulation (fig. 16C). Stimulation of T cells increased the expression of EAT-2 over the whole time-span investigated.

To study whether CRACC up-regulation is correlated to proliferation of T cells, CFDA-labeled cells stimulated with plate-bound antibodies against CD3 and CD28 as described for previous experiments, were analyzed for expression of CRACC on CD8-positive cells by flow-cytometry (fig. 16D). All cells that had undergone cell division expressed CRACC. In the population of cells that had not divided CRACC expression was only detected on a subset.

From these findings we conclude that CRACC is mainly expressed on activated, proliferating and memory T cells of the CD8-positive subset.

4.3.4 CRACC co-stimulation is no positive feedback mechanism to enhance proliferation

The expression of a further co-stimulatory receptor on activated and proliferating cells could function as a positive feedback mechanism. Through homophilic interaction between CRACC molecules on neighboring cells proliferation of activated T cells could be enhanced. A similar effect has been shown for the interaction of 2B4 and CD48 or SLAM on neighboring T cells (104, 174). To investigate if T cell proliferation was altered in the absence of CRACC and to further confirm the specificity of CRACC-mediated co-stimulation, freshly isolated peripheral blood T cells were transfected with control siRNA or siRNA against CRACC.

Because CRACC expression is up-regulated after stimulation and siRNA is diluted in proliferating cells, the attempt to silence CRACC could be difficult. We tested, how efficiently CRACC expression could be reduced by transient transfection with siRNA in activated cells. The efficiency of the siRNA-mediated suppression of CRACC expression was monitored by flow-cytometry after stimulating the transfected T cells with PHA-P (fig. 17A). To quantify the expression level of CRACC, the relative fluorescence index (RFI) was calculated from the mean fluorescence intensity of CRACC-staining in relation to the mean fluorescence intensity of staining with control antibody.

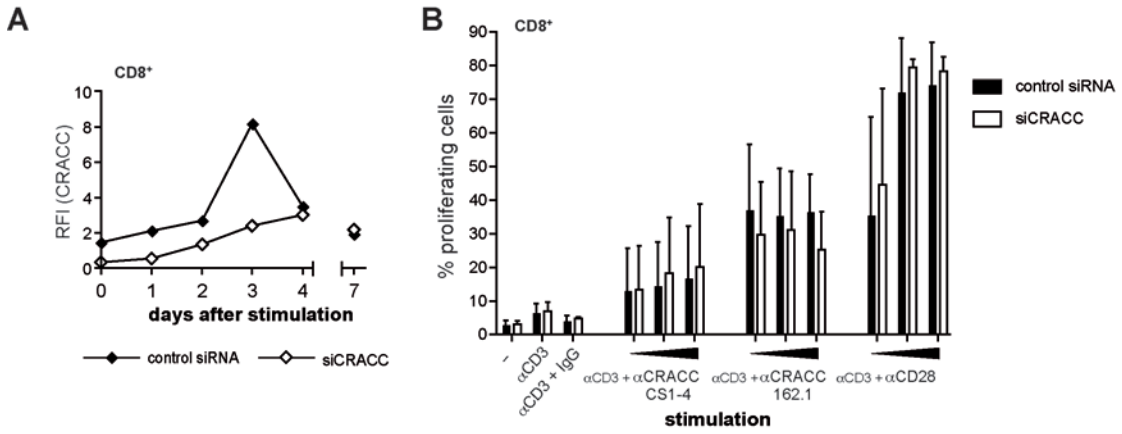

Figure 17: Knockdown of CRACC in T cells
Freshly isolated peripheral blood T cells were transfected with control siRNA or a mixture of four siRNAs against CRACC. **A:** Transfected T cells were activated with 2 µg/ml PHA-P overnight, then washed and cultured in medium containing 100 IU/ml recombinant IL-2. At the indicated time-points cells were stained for CD4, CD8 and CRACC and analyzed by flow-cytometry. The graph shows the relative fluorescence index (RFI) of CRACC-staining on CD8-positive T cells. The RFI was calculated from the mean fluorescence intensities (MFI) using the following formula: RFI(CRACC) = (MFI(CRACC) – MFI(control antibody)) / MFI(control antibody)
B: T cells transfected with the indicated siRNA were stained with CFDA and stimulated with plate-bound antibodies as described for fig. 12. The concentrations of the co-stimulatory antibodies were 0.1 µg/ml, 1 µg/ml and 10 µg/ml for anti-CRACC antibody CS1-4 and 0.01 µg/ml, 0.1 µg/ml and 1 µg/ml for anti-CRACC antibody 162.1 and anti-CD28 antibody. After 72 h cells were harvested, stained for CD8 and analyzed by flow-cytometry. The percentage of proliferating cells was determined by CFDA-dilution. The bars represent the mean values ±SD of three experiments.

The expression of CRACC was reduced in the cells transfected with anti-CRACC siRNA compared to control siRNA-treated cells for three days after stimulation. This difference was moderate during the first two days after stimulation, but became very prominent on the third day, when the expression level reached its maximum on control cells. The expression of CRACC on stimulated cells treated with siRNA against CRACC was reduced, but not completely abrogated.

As CRACC knockdown observed in PHA-P-activated cells lasted about three days, we investigated, whether the reduction of CRACC expression resulted in a distinct effect in proliferation assays. Peripheral blood T cells transfected with siRNA and labeled with CFDA were stimulated with plate-bound antibodies for 72 h similar to previous experiments. Proliferation of CD8-positive T cells was analyzed by flow-cytometry (fig. 17B). Co-stimulation with anti-CRACC antibody CS1-4 induced only very weak proliferation in T cells of the donors tested and no difference between control cells and CRACC knockdown cells was detectable. Co-stimulation with the antibody clone 162.1 was more effective. Here, the percentage of proliferating cells was slightly reduced in the CRACC knockdown cells, but the reduction was not

statistically significant. When co-stimulated via CD28, cells treated with either siRNA proliferated equally well.

This leads to the conclusion that CRACC co-stimulation between activated T cells is no positive feedback mechanism, although we cannot exclude completely that remaining CRACC is still sufficient to enhance proliferation.

4.3.5 Co-stimulation through CRACC induces proliferation in CD4 and CD8-positive cells

In the initial experiments CD4 and CD8-positive T cells were used in the mixture that is found in peripheral blood. Although the CD4-positive subset expresses CRACC to a very small extent compared to the CD8-positive T cells (fig. 16), the activation and proliferation induced through CRACC co-stimulation was similar in both subsets (figs. 11 and 12). To answer the question, whether this was due to activation of the small proportion of CRACC-positive CD4 memory cells or to a stimulatory effect of activated CD8-positive memory cells, the co-stimulation experiments were repeated with separately purified CD4 and CD8-positive cells, and a mixture of these cells.

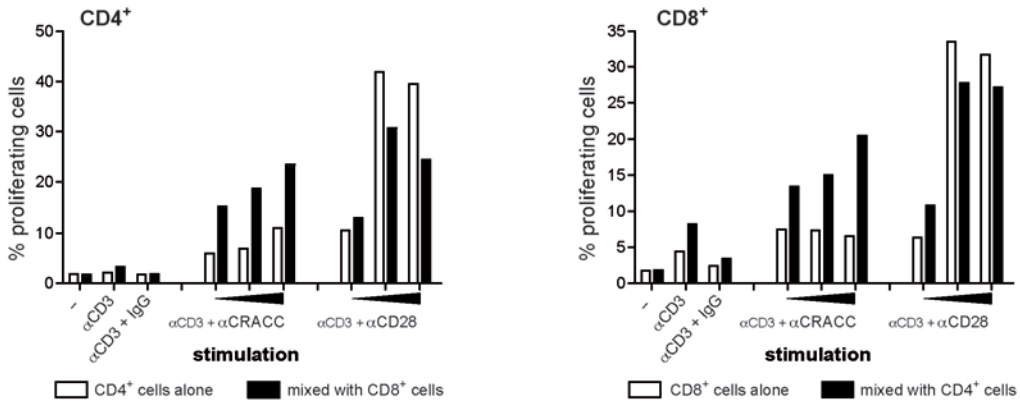

Figure 18: Separation of CD4 and CD8-positive cells reduces the effect of CRACC-mediated co-stimulation

CD4 and CD8-positive T cells were isolated separately from peripheral blood. The purity of the cells was greater than 97 % as determined by flow-cytometry. The cells were labeled with CFDA and stimulated with plate-bound antibodies as described for fig. 12. CD4 and CD8-positive cells were either stimulated alone or in a mixture consisting to 75 % of CD4-positive cells. The anti-CRACC antibody was used at concentrations of 0.1 µg/ml, 1 µg/ml and 10 µg/ml, the anti-CD28 antibody at concentrations of 0.01 µg/ml, 0.1 µg/ml and 1 µg/ml. After 72 h cells were harvested, stained for CD8 and analyzed by flow-cytometry. The percentage of proliferating cells was determined by CFDA-dilution. The graph shows representative data from one of three experiments.

After 72 h proliferation of CFDA-labeled cells was analyzed by flow-cytometry (fig. 18). CRACC co-stimulation induced proliferation of CD4-positive cells stimulated

alone, but the percentage of proliferating cells almost doubled, when CD8-positive cells were present in the culture. CD8-positive T cells showed also an increased proliferation after CRACC co-stimulation in the presence of CD4-positive cells. This led to the conclusion that CRACC co-stimulation acts on CRACC-positive cells in both T cell subsets, but is accompanied by a mutual enhancement of proliferation, possibly by secretion of cytokines or interaction of co-stimulatory receptors on neighboring cells.

4.3.6 CRACC and NTB-A do not enhance cytotoxicity

The main function of activated CD8-positive effector T cells is cytotoxicity. Because NTB-A and CRACC trigger cytotoxicity in NK cells, it could be possible that they have a similar function in cytotoxic T cells. To test this hypothesis PHA-P-activated T cells were used as effector cells in redirected lysis ^{51}Cr-release assays against the cell line P815 in the presence of antibody against CD3 in different concentrations, alone or in combination with control antibody or antibodies against NTB-A or CRACC at constant concentrations (fig. 19). CD3-mediated lysis of target cells was maximal at concentrations of anti-CD3 antibody greater than 333 pg/ml and decreased in a concentration-dependent manner. There was no change in cytotoxicity against the target cells, when the T cell receptor was stimulated together with NTB-A or CRACC.

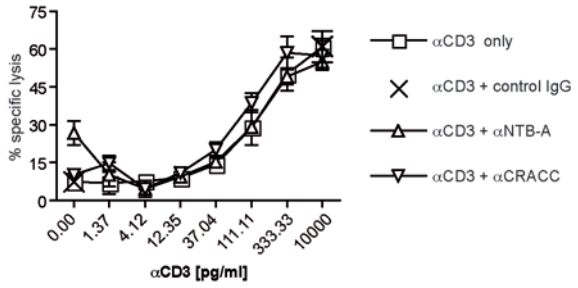

Figure 19: CRACC and NTB-A do not enhance T cell-mediated cytotoxicity
Peripheral blood T cells were stimulated with 2 µg/ml PHA-P overnight, then washed and cultured in medium containing 100 IU/ml of recombinant IL-2 for nine days. After 24 additional hours in culture without IL-2 the activated cells were used as effector cells in a 4 h redirected lysis ^{51}Cr-release assay against P815 cells in the presence of anti-CD3 antibody (αCD3) in a serial dilution, alone or in combination with a control antibody or antibodies against NTB-A (αNTB-A) or CRACC (αCRACC) at constant concentrations. The E/T ratio was 10/1. Specific lysis of target cells is plotted as mean of triplicates ± SD. One representative experiment out of five is shown.

4.3.7 CRACC is expressed on CD28-negative T cells

CRACC is expressed on antigen-presenting cells like mature dendritic cells and activated B cells (112, 113). In the presence of CD80 or CD86, the ligands for the most important co-stimulatory receptor CD28, CRACC may be dispensable or have a modulating function. But it is likely that co-stimulation through CRACC gains more importance, where CD28-mediated co-stimulation is not possible, namely in CD28-negative T cells. In humans CD28-negative cells accumulate in the T cell pool with ageing (176). This T cell population is mainly CD8-positive in healthy individuals and displays a limited T cell receptor repertoire (177). These cells are cytotoxic, but their proliferative response to antigenic stimulation or CD3-stimulation is decreased (178, 179). In the peripheral blood of patients with chronic inflammatory diseases the frequency of CD4-positive T cells lacking CD28 expression is often increased compared to healthy donors (180-182). It is assumed that these cells are involved in perpetuation and amplification of the inflammatory process (183). Due to expression of perforin and granzyme B these cells may even be able to cause direct tissue damage (184). Several receptors normally expressed on NK cells have been found on cells of the CD28-negative subset, e.g. KIR or NKG2D in rheumatoid arthritis (185, 186).

As the stimulation of these cells occurs independently of CD28, we hypothesize that co-stimulation via CRACC could play a role in chronic inflammatory diseases like it has been postulated for NKG2D co-stimulation in rheumatoid arthritis (186). To give this hypothesis a basis, we investigated if CRACC is expressed on CD4-positive CD28-negative T cells.

Blood samples of five patients suffering from unstable angina pectoris (aged between 52 and 87 years) were analyzed for expression of CD3, CD4, CD28 and CRACC by flow-cytometry. T cells were gated based upon size and granularity and CD3 expression. CRACC and CD28 expression were then studied on the CD4-positive and the CD4-negative T cells, which were considered to belong to the CD8-positive subset (fig. 20). T cells from patients 1 and 2 showed a normal expression pattern of CD28. The majority of cells in the CD4-positive subset was CD28-positive, while the CD4-negative subset of patient 2 contained a distinct population of CD28-negative cells. CRACC expression was only marginal on CD4-positive cells of these patients.

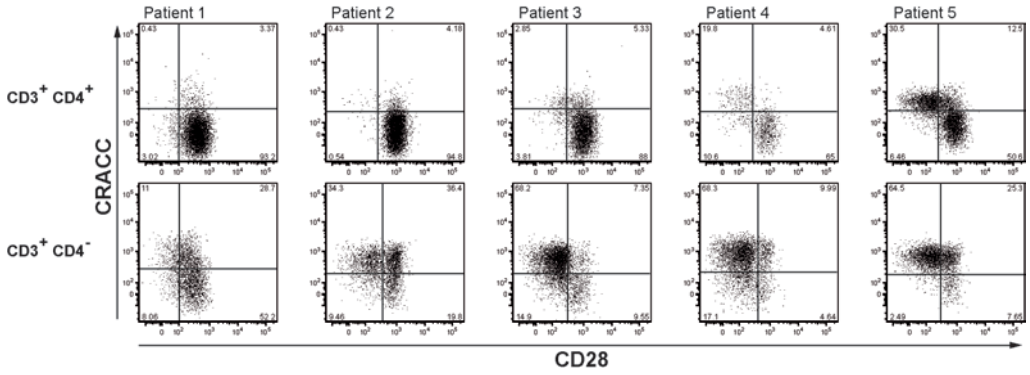

Figure 20: CRACC is expressed on CD4-positive CD28-negative T cells
Blood samples were obtained from five patients suffering from unstable angina pectoris. Samples were stained with antibodies against CD3, CD4, CD28 and CRACC and analyzed by flow-cytometry. T cells were gated based upon size and granularity and CD3 expression. CD28 and CRACC-staining are shown for CD4-positive T cells (upper row) and CD4-negative T cells, which are considered to be mainly CD8-positive cells (lower row). The numbers in the plots show the percentage of cells in the respective quadrant.

In the T cell population of patients 3 to 5 CD4-positive CD28-negative subsets were detectable. The T cells in these subsets showed distinct expression of CRACC, in contrast to the CD28-positive cells.

CD28-negative T cells were also found in the CD4-negative T cell compartments of all patients, consistent with the finding of age dependent accumulation of CD8-positive CD28-negative cells (176). These cells expressed CRACC, but CRACC-expression was not confined to CD28-negative cells in the CD4-negative population.

The expression of CRACC on the population of CD28-negative CD4-positive T cells in all patients tested supports the hypothesis that co-stimulation through CRACC could be one mechanism by which these cells are constantly activated in chronic inflammatory diseases of the vascular system.

5 Discussion

5.1 Mutational analysis of the homophilic interaction of NTB-A

The X-ray analysis of crystallized NTB-A ectodomain homodimers implied that the molecular basis for the interaction are hydrophobic contacts in the center of the molecular interface and eleven possible hydrogen bonds. Ten amino acid residues located in the IgV-domain on each molecule are involved. These residues form an interface with roughly two-fold symmetry (171). To confirm these findings Cao *et al*. performed dimerization studies with recombinantly expressed mutants of the NTB-A ectodomain. In gel filtration experiments all single mutants of these ten residues impaired the formation of dimers.

In this study the effect of several mutations on the function of NTB-A was investigated. The functional readout was cytotoxicity of wild type NTB-A-expressing NK cells induced by NTB-A mutants on the target cells. These functional assays are more suitable to estimate the contributions of single residues to the homophilic interactions in the physiological situation for some reasons. In contrast to NTB-A ectodomains expressed in bacteria as used by Cao *et al*., the NTB-A molecules expressed on eukaryotic cells are membrane-bound and glycosylated. This could have an influence on the binding properties of the receptors. Furthermore the functional assays can help to value the contributions of single residues. The strength of receptor interaction has to overcome a threshold to activate the NK cell. If a mutation has only a small effect on binding affinity, the receptor interaction may still be strong enough to trigger a response. If the mutated residue contributed strongly to receptor affinity, the mutant receptor cannot bind and does not elicit a cellular reaction.

Four of the ten residues that are involved in the interaction in the crystal structure were tested. The residue E37 was among the residues we chose for analysis, before the crystal structure was published. The other three residues, H54, Q88 and S90, were selected because their mutation had a strong effect in the gel filtration experiments reported by Cao *et al*.. In our functional assays the mutations E37A and Q88A did not impair receptor function. The mutation H54A reduced the cytotoxic NK cell response, whereas S90A almost completely abrogated NTB-A-mediated cytotoxicity (figs. 3 and 4). Single mutations to alanine of seven residues that do not

contribute to the interaction in the crystal structure did not disturb the functional interaction (fig. 4).

Comparing these results with the mutational analysis performed by Cao *et al.* one has to keep the differences in experimental setups in mind: First, Cao *et al*. investigated the affinity between two mutant receptors, whereas we studied the binding between mutant and wild type receptor. The interaction between two mutant NTB-A molecules is weaker than the one between one mutant and a wild type molecule due to the two-fold symmetry of the interface. In the first case contact is lost at two positions compared to only one point in the second case. Second, as mentioned before, the strength of the interaction has to overcome a certain threshold to trigger a cytotoxic response in the NK cell. Therefore small changes in receptor affinity are not detectable in our system. Residues that can be mutated without reducing the strength of interaction below this threshold are considered to contribute only little to the homophilic interaction. Third, because we investigated the interactions in a cellular system, our experimental setup resembles much more the circumstances, under which NTB-A interaction occurs *in vivo*. Therefore our results allow better conclusions about the importance of the four residues for the homophilic interaction under physiological conditions.

Taking these points into account we conclude that the contributions of the residues E37 and Q88 to the homophilic interaction of NTB-A are smaller than those of H54 and S90. The finding that in case of the mutation S90A removal of only one hydrogen bond results in such a strong loss of affinity shows that the specificity of the homophilic interaction is very subtle.

This may be one reason why our attempts to create a heterophilic NTB-A mutant pair failed. Because the simulation of receptor association is not feasible, the modeling for the complementary mutants was done by a more simple approach. In a model of the interacting receptors based on the crystal structure the two opposing residues were replaced with a pair of residues with opposite charge. Then the conformational changes that are likely to result from this substitution were calculated based on free energies. The results predicted that the mutations would not disrupt the overall structure of the interacting receptors. The conformational changes resulting from the different properties of the substituted residues seemed to be only small. Based on the calculations, an interaction between the complementary mutants could be possible. However, this approach cannot predict the behavior of this mutant pair in

solution, let alone whether the mutant pair would associate in a heterophilic interaction between cells.

As expected, the mutations effectively prevented the binding to wild type NTB-A or mutants of the same type. No cytotoxic response could be observed, when NK cells and target cells expressed the respective combination of receptors. However, the expression of the complementary mutants on NK cells and target cells did also not result in a cytotoxic response (fig. 6). This finding suggests that the heterophilic interaction that seemed possible in the model is not functional under the experimental circumstances.

Another critical point was that the NTB-A-deficient cells used for expression of the mutant receptors displayed a very weak cytotoxic potential (fig. 6). Under these circumstances the interaction between the complementary mutants may have been only too weak to overcome the activation threshold of these NK cells.

5.2 Early events in SLAM-related receptor signaling

5.2.1 Phosphorylation of 2B4 and NTB-A is independent of SAP

The early events after binding of 2B4 to its ligand CD48 that have been shown to be important for activating 2B4 signals are recruitment to lipid rafts (145), phosphorylation of the ITSM by Src-family kinases (145, 146), association of adapter molecule SAP (138, 149) and recruitment of the Src-kinase FynT (153, 154, 156). FynT then propagates the signal by phosphorylation of several signaling molecules, like PLC-γ or Vav-1 (146, 156). In the absence of SAP, e.g. during NK cell development or in XLP patients, 2B4 has been shown to mediate inhibitory signals. A possible explanation for this finding is that phosphatases like SHP-1 and 2 can associate with the phosphorylated receptor (82, 83). Because of their lower affinity they are displaced by SAP under normal conditions. When the amount of SAP is not sufficient to prevent the binding of phosphatases, they can counteract activating signals by dephosphorylation of signaling molecules.

For the receptor SLAM it has been proposed that FynT phosphorylates the ITSM after binding to SAP, because SAP can already associate with the unphosphorylated receptor (150). 2B4 can also be phosphorylated by FynT (82), but contradicting results have been reported regarding the role for SAP in 2B4 phosphorylation. One theory is that 2B4 is phosphorylated independently of SAP. This notion is supported by two findings. First, inhibition of tyrosine phosphatases by pervanadate treatment

could induce 2B4 phosphorylation in NK cells from XLP patients lacking functional SAP (98). Second, SAP showed no association with unphosphorylated 2B4, which excludes the possibility that SAP-mediated recruitment of FynT leads to 2B4 phosphorylation (82). On the contrary, there are reports supporting the notion that the presence of SAP is crucial for 2B4 phosphorylation. Human 2B4 expressed in HEK 293 cells was not phosphorylated unless SAP or EAT-2 were co-transfected (114). This resembles results obtained with murine 2B4. Engagement of murine 2B4 did not induce receptor phosphorylation in the absence of SAP (102, 156).

In our experiments we used human SAP knockdown NK cell lines and stimulated them by co-incubation with target cells expressing CD48, the ligand of 2B4. The knockdown of SAP was not complete, but sufficient to impair receptor function. In addition we could not detect any 2B4-bound SAP in the knockdown cells. Therefore we conclude that the reduction of SAP expression was strong enough to reveal differences between signaling in control and knockdown cells. Our results showed that the phosphorylation of 2B4 took place independently of SAP association (fig. 7). This confirms the results reported for pervanadate treatment of human NK cells, but in an experimental setup more similar to the physiological situation. As we investigated signaling in an NK cell line, we can assume that kinases involved in 2B4 phosphorylation in the physiological context were present in the system, which is not the case in non-lymphoid HEK 293 cells that have been used in the co-transfection experiments (114). This makes it likely that the reported differences for human 2B4 were caused by different experimental setups. As we used an NK cell line and stimulated 2B4 with its ligand expressed on target cells, our setup reflects the physiological situation better than the reported experiments. Therefore we conclude that in human NK cells the phosphorylation of 2B4 is mediated independent of the presence of SAP. The dependency of 2B4 phosphorylation on SAP in the murine system may reflect differences between species.

Previous reports have suggested that EAT-2 could mediate receptor phosphorylation in the absence of SAP, based on findings in transfected HEK 293 or COS-7 cells (114, 148). This possibility is excluded by our finding that EAT-2 recruitment is SAP dependent (figs. 7 and 8), which will be discussed below.

Based on the results obtained in this study we propose that the early events in 2B4 signaling happen in the following order: Engagement of 2B4 by CD48 leads to clustering of the receptor in lipid rafts. Src-family kinases that reside in the lipid rafts

phosphorylate the ITSM of 2B4 allowing SAP to bind to the receptor. In the following step the kinase FynT associates with ITSM-bound SAP and can perform its crucial function by phosphorylating molecules that activate the down-stream signaling pathways. In the absence of SAP the phosphorylated ITSM can recruit phosphatases like SHP-1 and 2, which could then inhibit signaling by dephosphorylation of signaling molecules.

In this model SAP plays no role in signaling before receptor phosphorylation. It has been reported that 2B4 must associate with lipid rafts in order to become phosphorylated (145). According to our model there should be no difference in raft recruitment of 2B4 between control and SAP knockdown cells in our experiments. It would be interesting to investigate whether this is the case. The analysis could be done by isolation of lipid rafts from stimulated cells using sucrose gradient centrifugation and comparing the amount of 2B4 in the raft fractions from the two cell lines.

The early events in NTB-A signaling have been less well described. Similar to 2B4, NTB-A is phosphorylated after engagement and SAP and EAT-2 associate with the phosphorylated receptor, but do not bind unphosphorylated NTB-A (84, 109, 152).

In this study we could show that phosphorylation of NTB-A after receptor engagement was comparable in control and SAP knockdown cells (fig. 7). This implies that phosphorylation of NTB-A is also independent of SAP. The course of signaling events seems to be the same for NTB-A and 2B4. First, the receptor is phosphorylated independently of SAP. Then the adapter molcule binds to phosphorylated ITSM and starts the signaling cascade.

5.2.2 The cytotoxic response to 2B4 and NTB-A engagement is mediated by SAP and not EAT-2

Although much is known about the role of SAP in SRR signaling, the function of EAT-2 remains unclear. Up till now, no binding partner has been identified that is recruited to phosphorylated receptors by EAT-2. In transfection experiments the over-expression of EAT-2, like SAP, has been shown to induce the phosphorylation of co-transfected receptors CD84, SLAM, Ly-9 and 2B4 (148, 187, 188). This finding led to notion that EAT-2 may also be involved in recruitment of kinases to the receptors. This put up the question whether SAP and EAT-2 mediate the same or different signaling pathways. Experiments with NK cells from XLP patients that lack functional SAP pointed to different roles for each adapter. 2B4 and NTB-A-mediated

cytotoxicity are both impaired in these NK cells despite the presence of EAT-2 (84, 98, 136-138). The fact that EAT-2 cannot compensate for the defective SAP makes it very likely that the two adapter molecules do not have interchangeable functions.

Our results are in line with the previous reports about the role of SAP for 2B4-mediated cytotoxicity in NK cells. The SAP knockdown in the cell lines NK92 and YTS and in primary IL-2-activated NK cells led to decreased cytotoxic responses upon 2B4 engagement (figs. 6, 7 and 9). This confirms the dependency of 2B4-mediated cytotoxicity on SAP association. Our finding that EAT-2 knockdown in primary cells did not impair cytotoxicity triggered by 2B4 (fig. 9) supports the notion that EAT-2 mediates signaling pathways different from those mediated by SAP.

A recent report proposed a model for the different roles of SAP and EAT-2 in signaling through NTB-A in human NK cells. Eissmann *et al*. found that SAP and EAT-2 bind to different ITSM of NTB-A (152). Mutational analysis revealed that EAT-2 binds the membrane proximal ITSM, while SAP associates with the C-terminal ITSM. When the EAT-2-binding ITSM was mutated, NTB-A-mediated cytotoxicity was abrogated, while mutation of the SAP-binding ITSM left the cytotoxic response intact. Furthermore, they reported that NTB-A-mediated cytotoxicity was unaffected by SAP knockdown in the human NK cell lines NKL and NK92. However, the production of IFN-γ after NTB-A stimulation was reduced in the SAP knockdown NK92 cells (152). This led to the model that the two adapters mediate different cellular responses to NTB-A engagement independently of each other. However, this model does not fit the observations made with NK cells from XLP patients. These cells showed impaired cytotoxicity, but normal IFN-γ production (84). Eissmann *et al*. speculated that the findings in XLP NK cells could be due to alterations of NK cell development in the absence of SAP.

The results obtained in this study contradict the model proposed by Eissmann *et al.*. We found a reduction of NTB-A-mediated cytotoxicity after SAP knockdown in the cell lines NK92 and YTS and in primary IL-2-activated NK cells (figs. 6, 7 and 9). This excludes the possibility that this finding is based on cell line specific peculiarities. At the moment we have no conclusive explanation why NTB-A-mediated cytotoxicity of the NK92 cell line was not affected by SAP knockdown in the reported experiments. We used the same knockdown vectors and tested the cells in the same experimental settings. The only obvious difference was that NTB-A-mediated cytotoxicity of our control cells was lower compared to the results reported by Eissmann *et al*. (152).

Maybe these differences are due to instability of immortalized cell lines during passaging and the selection process of transduced cells. As we could also confirm our findings in primary cells from different donors, we conclude that our results reflect the physiological situation better. Our findings also match the results reported for XLP NK cells. This strongly suggests that NTB-A-mediated cytotoxicity in human NK cells is dependent on SAP. The reduced cytotoxicity after NTB-A-engagement observed in XLP NK cells is therefore unlikely to be the result of impaired NK cell development in the absence of SAP.

Furthermore, EAT-2 knockdown in primary cells had no significant impact on cytotoxicity mediated by NTB-A (fig. 10). This finding excludes the possibility that EAT-2 functions as mediator of signaling pathways leading to cytotoxicity after NTB-A engagement. Thus we conclude that SAP mediates the main signal triggering cytotoxic responses by recruitment of FynT. The function of EAT-2 could be the initiation of yet unknown signaling pathways leading to effects not connected with the immediate cytotoxic response.

5.2.3 Association of EAT-2 with 2B4 and NTB-A is SAP dependent

The existence of two different adapter molecules, SAP and EAT-2, that can bind to phosphorylated SRR suggested that each could trigger a different signaling pathway. Our findings contradict this theory of independent signaling of SAP and EAT-2. We observed that recruitment of EAT-2 to phosphorylated 2B4 or NTB-A is impaired in cells with reduced SAP expression (fig. 7). The dependency of EAT-2 association on SAP could explain why EAT-2 does not compensate for SAP in XLP patients, although its activating function has been clearly demonstrated in SAP-independent CRACC signaling (114). Phosphorylated ITSM in CRACC only recruit EAT-2 and not SAP, which implies that EAT-2 can also recruit activating signaling molecules.

However, in over-expression experiments EAT-2 association with 2B4 has been found in both the human and the murine system when SAP was not co-transfected (114, 148). This association could be an artifact due to high expression levels of EAT-2 in the transfected cells. It is possible that EAT-2 has a lower affinity to phosphorylated 2B4 or NTB-A than to CRACC. Therefore binding of EAT-2 to 2B4 or NTB-A could only be detected, if the expression level of EAT-2 is high enough or if SAP facilitates its recruitment.

An unresolved question is, how SAP can support the association of EAT-2 with phosphorylated ITSM, as a direct interaction between the two molecules has not

been found (152). Additionally, no interaction partner that binds both molecules has been identified, besides phosphorylated SRR. Maybe the binding of SAP to one phosphorylated ITSM induces conformational changes in the cytoplasmatic tail of the receptor that make EAT-2 association easier. A further possibility is that SAP does not even have to be associated with the receptor, because the mutation of the SAP-binding ITSM of NTB-A has been shown to have no effect on EAT-2 association with the other ITSM (152).

5.2.4 An altered model of activating 2B4 and NTB-A signaling

Taking together the findings of this study we propose the following model for the early events in 2B4 and NTB-A signaling: Engagement of the receptors leads to recruitment to kinase-rich lipid rafts (which remains to be shown for NTB-A) where they become phosphorylated by Src-family kinases. The phosphorylated ITSM can be bound by SAP. ITSM-bound SAP then enables binding of the adapter molecule EAT-2 to phosphorylated 2B4 or NTB-A by means yet to be defined. The essential step is that SAP also recruits FynT through direct interaction, which activates signaling pathways by phosphorylation of downstream effector molecules.

To test this model further experiments will be necessary. It would be interesting to investigate, which signaling pathways are affected by EAT-2 knockdown in NK cells. Changes in tyrosine phosphorylation patterns after receptor stimulation could give clues about the involved molecules. This would help to identify binding partners of EAT-2, because at the moment there is no antibody available that is able to co-immunoprecipitate EAT-2-associated proteins. This could be due to the small size of EAT-2, as it might be not accessible for antibodies when it is embedded in a complex with other signaling molecules. It might be worth trying to use a tagged EAT-2-construct to get access to this signaling complex. When the signaling pathways mediated by EAT-2 are identified, it would be interesting to investigate whether these pathways are impaired in the absence of SAP. If this is the case, the dependency of EAT-2 on SAP could be confirmed.

5.3 The functions of CRACC and NTB-A in T cells

5.3.1 Co-stimulatory features of NTB-A

T cells are activated through signaling of their antigen-specific TCR. Normally, full T cell activation after engagement of the TCR is dependent on co-stimulatory signals. CD28 is regarded as the primary receptor for T cell co-stimulation. Its ligands CD80

and CD86 are expressed on antigen-presenting cells like mature dendritic cells or B cells (12). However, in the last years an increasing number of other molecules has been reported to have co-stimulatory properties. Among these are the SRR SLAM, 2B4, CD84 and NTB-A (73, 109, 173, 174, 187). In contrast, CD229 another member of this receptor family has been shown to have an inhibitory effect on T cell activation (189).

In this study our aim was to investigate whether CRACC is also a co-stimulatory receptor on T cells. Because NTB-A has already been described as a co-stimulatory SRR on human T cells, we used co-stimulation of NTB-A mainly as a second positive control besides CD28. In contrast to CRACC, NTB-A is expressed on all T cells, which allows co-stimulation of the whole T cell population via NTB-A. Thus, the observed effects were more distinct than the effects of CRACC co-stimulation.

Simultaneous stimulation of TCR and NTB-A with plate-bound antibodies has been reported to induce T cell proliferation and IFN-γ production (109). Our experiments using the same experimental approach could complete the picture of the co-stimulatory properties of NTB-A. We could show that NTB-A co-stimulation induces expression of the activation markers CD69 and the IL-2-receptor α-chain (CD25), as well as IL-2 production (figs. 12 and 14). The induction of TNF-α production in T cells (fig. 14) has not been shown before and is another feature this study adds to the known properties of NTB-A. The finding that NTB-A does not contribute to T cell-mediated cytotoxicity (fig. 19) is also new. It suggests that NTB-A co-stimulation is less important for effector functions, but plays mainly a role in the mediation of proliferative T cell responses.

The effect of NTB-A co-stimulation was similar to the effect of CD28 co-stimulation in the experiments with readout after 48 or 72 h. The enormous difference in the strength of co-stimulation was only obvious in the levels of cytokine mRNA expression after 6 h, where CD28 co-stimulation exceeded the effects of NTB-A by far (fig. 14). This implies that the kinetic of NTB-A co-stimulation is slower. Because we observed only quantitative and no qualitative differences between cytokine mRNA levels after CD28 or NTB-A expression, we conclude that NTB-A does not induce the production of a distinct cytokine pattern.

A very recent study proposed a role for NTB-A co-stimulation not in the activation and expansion of naïve cells that was investigated in our study, but in controlling the removal of activated T cells (190). Snow *et al*. could show that re-stimulation-induced

cell death, a mechanism that is involved in the contraction of the T cell pool after infection, is dependent on NTB-A and SAP. To mimic the events during the contraction phase they stimulated T cells, cultivated them for at least one week in the presence of IL-2 and then re-stimulated the cells with antibodies. They found that knockdown of SAP or NTB-A reduced the rate of apoptosis after re-stimulation. The model they propose is that NTB-A-mediated co-stimulation of activated T cells enhances the TCR-mediated signal leading to an activation level that induces apoptosis. This 'over-activation' is an interesting model how a co-stimulatory receptor could be involved in shutting down of immune responses. It would be interesting to see whether other co-stimulatory SRR have also pro-apoptotic functions on activated T cells, or if this phenomenon is specific for NTB-A.

5.3.2 CRACC like NTB-A is a co-stimulatory receptor

In this study we investigated if CRACC has also co-stimulatory properties. Simultaneous stimulation of the TCR and CRACC by plate-bound antibodies induced expression of CD69 and CD25, furthermore, production of IL-2 and proliferation (figs. 11-13). By the use of two different anti-CRACC antibody clones we excluded that the observed co-stimulation was the result of unspecific antibody interaction (fig. 15). Because the size of the CRACC-expressing T cell population was small and varied between donors (fig. 11), the observed effects were not as prominent as the effects obtained with NTB-A or CD28 co-stimulation.

Besides IL-2, the cytokine that is crucial for T cell proliferation, CRACC co-stimulation induced production of the two pro-inflammatory cytokines IFN-γ and TNF-α (fig. 13). As our analysis of cytokine mRNA showed only small changes after CRACC stimulation, it is hard to say whether CRACC induces the production of a distinct cytokine pattern. We cannot exclude that CRACC co-stimulation participates in shaping the cytokine response *in vivo*.

While SLAM and 2B4 also enhance TCR-mediated cytotoxicity (91, 191), we observed no influence of CRACC on the cytotoxic response of T cells (fig. 19). Thus, we conclude that the co-stimulatory function of CRACC mainly induces proliferation and cytokine expression.

Stimulation of cells by cross-linking surface receptors with plate-bound antibodies is a suitable method to investigate receptor function in a defined setting, but is different to the receptor-ligand interaction between cells *in vivo*. Therefore we point out that our results have not been confirmed in a more physiological setting yet. However,

antigen-presenting cells, like mature dendritic cells or activated B cells, express CRACC (112, 113). That makes it likely that co-stimulatory CRACC-CRACC interactions take place during contact of T cells to antigen-presenting cells *in vivo*. Therefore we propose that CRACC should be regarded as co-stimulator of T cell activation.

5.3.3 CRACC, a co-stimulatory receptor expressed on proliferating T cells

Our results show, that CRACC expression is induced on activated and proliferating CD8-positive T cells (fig. 16). This resembles the expression pattern that has been reported for SLAM, which is expressed on activated T cells of both CD4 and CD8-positive subsets (174, 191-193). Expression of a further co-stimulatory receptor could be a positive feedback mechanism to amplify the expansion of activated T cells during the early phase of an immune response. The co-stimulatory engagement of the homophilic receptor can take place between CRACC on the antigen-presenting cell and CRACC on the T cell or between CRACC on neighboring proliferating T cells. This feature bears also resemblance to the homophilic SLAM, which can also be induced on antigen-presenting cells (194, 195). This expression of co-stimulatory molecules on the progeny of activated T cells could also compensate for stimulation-induced down-modulation of CD28 expression. The transient loss of CD28 is thought to be a regulatory mechanism limiting the further activation of T cells (19). Delivery of secondary co-stimulatory signals through SLAM or CRACC could then fine-tune the activation of T cells by modulating strength and duration of stimulation. In our experiments T cells with a knockdown of CRACC expression showed no reduction in proliferation after CD28 co-stimulation (fig. 17). On the one hand, this could be due to the incomplete knockdown of CRACC. Maybe the remaining CRACC could still contribute sufficient co-stimulation. On the other hand, it could be possible that CRACC-mediated enhancement of proliferation gains importance at later stages of the expansion phase. This could explain, why we have observed no difference during the first three days of activation.

Recently, it has been reported that CRACC expression is also induced upon activation in murine T cells from both the CD4 and the CD8-positive subset. But in contrast to our findings in human T cells, CRACC has been shown to have an inhibitory impact on TCR-mediated T cell activation the murine system (115). This is possibly due to the lack of the adapter protein EAT-2 in murine T cells, because CRACC is an activating receptor on murine NK cells that normally express EAT-2,

but is turned into an inhibitory on NK cells of EAT-2 KO mice (114, 115). Therefore it is likely that CRACC has a function in controlling the expansion of activated T cells in mice. Whether CRACC or EAT-2 KO mice have a defect in T cell proliferation control during an infection has not been reported.

In this study we have shown that human T cells express EAT-2 (fig. 16). Based on the findings in human NK cells (114), we suggest that the co-stimulatory CRACC signaling is mediated by the recruitment of this adapter. However, it is possible that CRACC fulfills a dual function in T cell activation in humans: After stimulation CRACC and EAT-2 expression are up-regulated and enhance T cell activation. At a later stage of T cell activation a down-regulation of EAT-2 could turn the CRACC signals from activating into inhibitory signals to limit proliferation. This mechanism would function like the expression of the inhibitory receptor CTLA-4 on activated T cells (20). In both cases the ligands expressed on the antigen-presenting cell are not changed, but their effect on the T cells changes.

To prove this hypothesis the inhibitory signaling of CRACC in the absence of EAT-2 has to be shown for human T cells. A possible way to do that is to test the outcome of CRACC stimulation after knockdown of EAT-2 expression by RNA interference. Furthermore, the time course of EAT-2 expression in activated cells T cells has to be analyzed further.

One possibility we have not tested in this study is whether the induction of CRACC on proliferating T cells plays a role in the interaction between NK and T cells. CRACC engagement triggers NK cell-mediated cytotoxicity. In mice NK cells have been shown to eliminate activated CD4-positive T cells, when inhibitory receptors were blocked (196). Because activated T cells with their strong proliferative potential bear a special risk of developing lymphomas, they must be controlled tightly. Down-regulation of MHC molecules is a phenomenon observed in transformed cells (35). If loss of MHC molecules occurs on activated T cells, no inhibitory signal can counteract the CRACC-mediated NK cell cytotoxicity, and the cells can be eliminated. Therefore CRACC expression on activated T cells could also be a mechanism that facilitates the control of proliferative disorders.

5.3.4 CRACC, a co-stimulatory receptor on memory T cells

It has been a generally accepted paradigm in immunology that memory T cell responses are independent of CD28 co-stimulation. Re-activation of T cells was thought to be mediated solely through TCR signaling (197). This paradigm was

challenged by recent studies investigating the memory response to viral infection in mice. Proliferation of adoptively transferred memory CD8 T cells in response to viral infection was found to be impaired in hosts that lacked both CD80 and CD86 (198, 199). These results suggest that co-stimulatory signals are necessary for an optimal response of CD8-positive memory cells to viral re-challenge in mice.

We have found in this study that human memory T cells express the co-stimulatory receptor CRACC (fig. 16). The strongest expression was seen on CD8-positive memory cells. The expression of additional co-stimulatory receptors could lower the threshold needed for activation through TCR stimulation. This would facilitate the mounting of a strong proliferative response to antigenic re-challenge, assuming that human memory T cells are likewise dependent on co-stimulation. In this case activated T cells that differentiate into memory cells would maintain CRACC expression to facilitate re-activation.

5.3.5 A possible role for CRACC in chronic inflammatory diseases

In contrast to mice, humans and non-human primates accumulate a pool of CD8-positive CD28-negative cells with ageing (176, 200, 201). The loss of CD28 expression seems to result from repeated TCR-mediated activation and homeostatic proliferation (200, 202). The T cell receptor repertoire of these cells is limited (177). These cells are cytotoxic, and they show an impaired proliferative response to antigenic stimulation or CD3-stimulation (178, 179). Their appearance is linked to immune senescence and has also been observed in chronic viral infections, e.g. with cytomegalovirus or HIV (203, 204). CD4-positive T cells lacking CD28 expression are scarce in healthy individuals, but are often found in the peripheral blood of patients with chronic inflammatory diseases, like rheumatoid arthritis, inflammatory vascular complications or multiple sclerosis (180-182). It is assumed that these cells are involved in perpetuation and amplification of the inflammatory process (183). This notion is supported by the observation that the size of this T cell subset correlates with severity of the disease (180). As these diseases are often linked to autoimmunity, it has been speculated that this subset represents constantly stimulated autoreactive cells. In most studies, however, these cells could not be stimulated with typical autoantigens, but responded to some viral antigens or heat shock proteins (183).

CD4-positive CD28-negative T cells display a phenotype similar to cytotoxic lymphocytes: In rheumatoid arthritis several receptors normally expressed on NK

cells, like KIR or NKG2D, have been found on cells of the CD28-negative subset (185, 186). Due to expression of perforin and granzyme B these cells may even be able to cause direct tissue damage (184). Very recently, a report showed that co-stimulation of CD4-positive CD28-negative T cells from patients with rheumatoid arthritis through the 'NK receptors' NKG2D, 2B4 and DNAM-1 led to IFN-γ production and degranulation of lytic granules (205). Interestingly, none of the three receptors had co-stimulatory properties when triggered alone, but two receptors triggered simultaneously could enhance TCR-mediated responses.

In this study we have shown that CD28-negative cells of both subsets show a distinct expression of CRACC (fig. 20). In the CD4-positive subset CRACC expression was mostly confined to the CD28-negative cells. This fits to the notion that these CD4 T cells have acquired a phenotype more similar to CD8-positive effector cells with lytic granules and receptors normally found on CD8-positive cells like 2B4 and NKG2D. The expression of co-stimulatory receptors like CRACC could reduce their threshold of activation. Facilitated activation of these cells could be one of the driving forces of chronic inflammation. Therefore we conclude that CRACC-mediated co-activation of CD4-positive CD28-negative T cells is likely to play a role in chronic inflammatory diseases. Further studies will have to investigate how CRACC expression is induced in these cells, which cellular responses are triggered by CRACC co-stimulation in these cells and how these are connected to disease development and progression. It would be interesting to test, whether blocking of CRACC interactions or inhibition of CRACC signaling could dampen the exaggerated pro-inflammatory activity of the CD4-positive CD28-negative T cells. Based on these studies, new therapeutic approaches targeting CRACC may be possible.

6 References

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7 Abbreviations

APC	allophycocyanin
BSA	bovine serum albumin
CCR	chemokine receptor
CD	cluster of differentiation
CFDA	carboxyfluorescin diacetate
CRACC	CD2-like receptor activating cytotoxic cells
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
E/T	effector to target ratio
EAT-2	Ewings sarcoma virus activated transcript 2
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ERT	EAT-2 related transducer
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescin isothiocyanate
GAPDH	glycerolaldehydephosphate dehydrogenase
GFP	green fluorescent protein
HRPO	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITSM	immunoreceptor tyrosine-based switch motif
IU	international units
KIR	killer cell immunoglobulin-like receptor
KO	knockout
LSM	lymphocyte separation medium

MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger RNA
NK	natural killer
NTB-A	natural killer, T cell, B cell antigen
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PHA-P	*Phaseolus vulgaris* hemagglutinin protein
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
SAP	SLAM associated protein
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel-electrophoresis
shRNA	small hairpin RNA
siRNA	small interfering RNA
SLAM	signaling lymphocyte activation molecule
SRR	SLAM-related receptor(s)
TAE	Tris-acetate-EDTA buffer
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
wt	wild type
XLP	X-linked lymphoproliferative disease

The single letter code was used to describe amino acid residues.

8 Publications

Endt, J., P. Eissmann, S. C. Hoffmann, **S. Meinke**, T. Giese, and C. Watzl. 2007. Modulation of 2B4 (CD244) activity and regulated SAP expression in human NK cells. *Eur J Immunol* 37:193-198.

Claus, M., **S. Meinke**, R. Bhat, and C. Watzl. 2008. Regulation of NK cell activity by 2B4, NTB-A and CRACC. *Front Biosci* 13:956-965.

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