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# NK cell regulation

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# 1 Abstract

Here we employ a combination of synthetic biology in Schneider cells and verification of these results with pharmacological inhibitors in primary human immune cells to investigate immune cell regulation. Taking advantage of the high transient co-transfection rate in Schneider cells, we were able to create a minimal signaling pathway containing an ITAM-motif, SYK kinase and its target SLP-76. The co-expression of all ITAM-containing adapters investigated resulted in strong activation of SYK kinase as observed by SLP-76 phosphorylation. Mutation experiments showed that this was dependent on both functional tyrosines within the ITAM motif as well as the SH2 domains of SYK kinase. Additionally, we observed a requirement for co-expression of Src kinases for ZAP70 mediated phosphorylation of SLP-76, whereas SYK was activated just by the presence of ITAMs without the need for Src kinases. Inhibitor experiments further underlined this requirement. Src-dependent SYK/ZAP70 activation and subsequent phosphorylation of SLP-76 could be controlled by co-expression of Csk or CD45, providing a molecular basis for this pathway *in vivo*.

In primary NK cells, we could demonstrate that SLP-76 is phosphorylated after cross-linking of ITAM-associated receptors as predicted by the synthetic approach. NK cells express both SYK kinases SYK and ZAP70, whereas B cells only express SYK and T cells only express ZAP70. To further investigate the differences in the activation of SYK kinases, we stimulated all 3 cell types in the presence of Src or SYK kinase inhibitors. We could show that ZAP70 activation in T cells could be effectively inhibited by Src kinase inhibitors while NK cells and B cells were only partially inhibited at the same concentration thus validating our reconstructed pathway in Schneider cells.

## 1.1 Zusammenfassung (abstract in german)

Zur Untersuchung der Regulation von NK-Zellen wurden in der vorliegenden Arbeit Signalkaskaden in Schneiderzellen nachgebaut. Dafür wurden bekannte Komponenten von Signalkaskaden aus NK-Zellen transient in Schneiderzellen co-transfiziert und die Phosphorylierung von kritischen Signalmolekülen untersucht. Mit Hilfe dieses Ansatzes konnten wir in Schneiderzellen eine Signalkaskade mit 3 essentiellen Komponenten nachbilden. Ein Adaptermolekül das ein ITAM-Motiv enthält war ausreichend SYK zu aktivieren und somit SLP-76 zu phosphorylieren. Durch Mutationsexperimente konnten wir zeigen, dass diese Aktivierung abhängig von Tyrosinen im ITAM-Motiv und den SH-2 Domänen von SYK ist. Die SYK-Kinase ZAP70 hingegen benötigte die Co-Expression einer Src-Kinase um aktiviert zu werden. Mit Hilfe von Inhibitorexperimenten konnte diese Abhängigkeit weiter demonstriert werden. Die Aktivierung von Src-Kinasen konnte in diesem Zusammenhang durch Co-Expression von Csk und CD45 reziprok reguliert werden.

In primären NK Zellen konnten wie zeigen, dass SLP-76 nach Kreuzvernetzung von ITAM-assoziierten Rezeptoren - wie von unserem synthetischen Ansatz vorhergesagt – phosphoryliert wird. NK Zellen exprimieren die SYK Kinasen SYK und ZAP70, B Zellen nur SYK und T Zellen nur ZAP70. Um die Unterschiede der Aktivierung durch Src-Kinasen weiter zu untersuchen wurden alle 3 Zelltypen in Anwesenheit von Src oder SYK Inhibitoren stimuliert. So konnten wir zeigen, dass die Aktivierung von ZAP70 durch Inhibition von Src-Kinasen in T Zellen unterdrückt wird, während NK und B Zellen nur teilweise inhibiert werden.

# 2 Introduction

## 2.1 NK cells

In the mid-1970s, a novel immune cell type was described based on its ability to lyse allogeneic tumor cells without the need for prior sensitization. The term "natural cytotoxicity" was introduced to describe this feature and the cells mediating this effect were named natural killer (NK) cells [1-5]. In the last 40 years, much progress has been made in the understanding of the function and regulation of NK cells. We now know that NK cells contribute to effective innate immune responses and provide the first important line of defense against parasites, viruses and cancer [6-9]. NK cells derive from the common lymphocyte progenitor, but they are independent of a functional thymus and - unlike the antigen receptors of B and T cells - rely on germ-line-encoded surface receptors that do not undergo somatic recombination. While NK cells can be found among blood lymphocytes, NK cells reside in organs [10] and can play a role in organ homeostasis as well [11].

The immune system is classically grouped into innate and adaptive immune system. The first and rapid response upon infection is delivered by the innate immune system composed of macrophages, dendritic cells, mast cells, basophils, eosinophils, neutrophils and NK cells. T and B cells mediate the adaptive immune response, which is characterized by slow initiation and the generation of memory. Adaptive immune cells rely on the expression of recombination activation genes – which NK cells do not express – to generate an infinite number of B and T cell receptors enabling specificity in the adaptive response after clonal expansion. In contrast, NK cells mediate their effector functions rapidly without the requirement for further development. However, recent reports indicate memory-like features of NK cells which will be discussed later.

Human NK cells are typically identified as lymphocytes that express CD56 and lack expression of CD3. Expression of NKp46 is also mostly restricted to NK cells and therefore frequently used as a marker as well. In human blood, NK cells represent 5-15% of the total peripheral blood lymphocytes (PBL) and are grouped into $CD56^{dim}$ and $CD56^{bright}$ cells with $CD56^{dim}$ NK cells accounting for up to 90% of all NK cells in peripheral blood and spleen [12, 13]. In contrast, equal numbers of $CD56^{dim}$ and $CD56^{bright}$ NK cells are found in the liver and $CD56^{bright}$ NK cells are abundant in other organs as well [11].

### 2.1.1 NK cell effector functions as part of the immune response

High cytotoxic NK cell activity was shown to be associated with a 40% lower cancer risk compared to NK cells from people with low cytotoxic NK cell activity [14]. Human NK cell deficiency is rare and immune-functions are severely compromised in the absence of NK cells. Reports have shown increased susceptibility to Herpesvirus and Papillomavirus infections [15]. Because of their ability to eliminate transformed cells, NK cells are increasingly used for immunotherapy where NK cells are expanded and activated *ex vivo* [16-18]. Recent studies have also focused on the expression of chimeric antigen receptors (CAR) in NK and T cells to improve immunotherapy [19, 20]. Despite their limited ability to infiltrate tumor tissue, NK cells have several advantages over T cells expressing CARs, like spontaneous cytotoxic activity and a different less inflammatory set of cytokines [21].

NK cells not only control infections as part of the classical immune response but also protect against transformed and potentially malignant cells. Tumors develop out of uncontrollably proliferating cells, which are usually the result of mutations. These mutations can occur spontaneously upon exposure to certain chemicals or radiation. Given the sheer number of cells in the human body the occurrence of transformed cells is unavoidable. Therefore, many mechanisms – among them the constant surveillance of NK cells - exist to ensure that these cells are recognized and eliminated either by inducing apoptosis or direct killing. The ability of NK cells to spontaneously recognize and kill transformed cells is therefore an important line of defense against cancer. Briefly, NK cells take part in the defense against infections, the regulation of immune responses as well as the surveillance of stressed or transformed cells. NK cells exert their immune functions by cellular cytotoxicity, the secretion of cytokines and chemokines as well as contact with other immune cells [8].

For many NK cell effector functions direct cell contact with its target cell is required. This interaction between target cell and effector cell has been termed immunological synapse (IS). Formation of a lytic immune synapse provides additional regulatory steps by bringing NK cell receptors and their ligands in physical proximity and is critical for directed secretion of effector molecules [22-24]. The IS enables the rearrangement of receptors, ligands, adhesion molecules, signaling components and effector molecules on and in the NK cell as well as on the target cell to enhance signaling between effector and target cell as well as provide a direction for the secretion of effector molecules. Briefly, the formation of the synapse starts upon contact with the target cell and rearrangement of the actin cytoskeleton. Next, the microtubule-organizing centre (MTOC) of the NK cell and the secretory lysosomes are polarized towards the IS. Thereafter secretory lysosomes dock with the plasma membrane at the IS, before fusing in the final stage with the plasma membrane and releasing their cytotoxic

contents. Cytotoxic proteins contained within secretory lysosomes are released towards the target cell during this process. The major cytotoxic proteins released by NK cells are perforin and granzymes [25]. Perforin creates pores in the membrane of the target cell to facilitate the entry of granzymes into the target cell. Granzymes are serine proteases that cleave a variety of targets, like caspases, that induce target cell death through apoptosis. As cytotoxic granules fuse with the membrane and undergo exoxcoytosis, the lyososomal protein CD107a is incorporated into the NK cell membrane. Hence, NK cell degranulation can be measured by incorporation of CD107a in the NK cell membrane [26]. CD107a, also known as Lysosomal-associated membrane protein 1 (LAMP-1), is found primarily in lysosomal membranes and has been reported to protect NK cells during degranulation from self-reactivity [27]. NK cells can also use Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), both belonging to the TNF family, to induce target cell death by apoptosis. How cytotoxic cells detect target cell death and know when to release their target remains still unclear, but there is evidence of serial killing by NK cells. Hence, it is likely that a mechanism exists to ensure the release of NK cells only upon successful elimination of the target cell.

A recent study indicated that mechanical force exerted by cytotoxic T lymphocytes (CTL) via the immunological synapse contributed to perforin pore formation and that artificially increased target cell tension augmented pore formation and killing [28]. While NK cells share many features of the IS with CTLs, differences in kinetics and mechanism have been reported [29], therefore similar experiments need to be conducted with NK cells to examine the influence of mechanical force.

The main cytokines released by NK cells are the type 1 cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). NK cell derived IFN-γ provides protection against viruses in humans [30] and was shown to control viral replication in vitro [31, 32]. In addition, IFN-γ can activate other cell types such as DCs, macrophages, T cells and promote upregulation of MHC-1 molecules on other cells [33]. NK cell derived IFN-γ has also been reported to improve the outcome of liver fibrosis contributing to organ homeostasis [34]. The accumulation of activated TNF-α producing NKp44+ NK cells in the human liver has been reported to correlate with liver fibrosis and viral load [35]. Both IFN-γ and TNF-α have been reported to aid NK cell cytotoxicity by NF-Kb dependent upregulation of ICAM-1 expression on target cells, thereby assisting conjugate formation between NK and target cells [36].

NK cells might also regulate other cell types as well as other NK cells by cytotoxicity. Activated T cells can be eliminated by NK cells to prevent overactivation [37, 38]. It has been reported that activated mouse NK cells can be killed by other NK cells after membrane

transfer of the NKG2D ligand Rae-1 from tumor cells [39]. Murine NK cells deficient for the IFN-a receptor have also been shown to express increased levels of NKG2D ligands and are therefore susceptible to killing by other NK cells [40].

### 2.1.2 NK cell education

One important step for the understanding of NK cell regulation was the realization that NK cells preferentially kill cells with low or no major histocompatibility complex (MHC) class I expression that led to the formulation of the "missing-self hypothesis" [41]. The identification of MHC class I specific inhibitory receptors supported this concept. These receptors signal through an immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail and are phosphorylated upon binding to MHC class I presumably by Src kinases. They can recruit and activate phosphatases such as SHP-1/2 and SHIP to the receptor clusters, interfering with activating signaling pathways by dephosphorylation. Early studies implied that NK cells express at least one inhibitory receptor specific for self MHC-1, but subsequent studies found subpopulations without inhibitory receptors for self MHC-1 in mice and humans [42, 43]. In addition, NK cells in MHC-1 deficient hosts are not autoreactive. Thus, another mechanism has to exist to prevent NK cells lacking inhibitory receptor expression from autoreactivity. NK cells lacking inhibitory receptor expression were found to be hyporesponsive when triggered. This adaptation of the reactivity of NK cells depending on the inhibitory receptor ligand matches is generally referred to as NK cell education and assures the self-tolerance of NK cells. Several theories have been proposed to explain this process and the evidence so far provides support for different theories. In the "licensing" model [44], signaling by inhibitory receptors allows NK cells to gain functional competence so that only NK cells with functional inhibitory receptors become mature. In the "disarming" model [45], chronic stimulation by activating receptors renders NK cells hyporesponsive or anergic. Inhibitory signals could counteract this constitutive activation resulting in functional NK cells. As self-specific inhibitory receptors don't seem to function as an all or nothing signal, the "rheostat model" was proposed [46]. In this theory, stronger inhibitory signals would increase the functional responsiveness of NK cells.

Given the available data today, all proposed theories can explain certain aspects of NK cell education and are still discussed in the NK cell community [47, 48].

### 2.1.3 NK cell maturation

NK cells are – like B and T cells - derived from common lymphocyte progenitors (CLP) and NK cells are thought to develop primarily in the bone marrow. Both cytokines and transcription factors drive and control NK cell development. Interleukin 15 (IL-15) is critical for the development of NK cells and required constantly during their lifespan [49, 50]. The transcription factor PU.1 is important for early NK cell development and Id2 and Tox are required for later stages [51] whereas Eomes and T-bet regulate the final stages of NK cell development [52].

According to the expression of surface molecules, NK cell populations have been grouped into different maturation stages. There is a developmental relationship between the different subpopulations, suggesting a differentiation of mature NK cells starting from $CD56^{bright}$ via $CD56^{dim}$, $CD57^{-}$, $CD62L^{+}$, $CD94/NKG2A^{+}$ to the more differentiated $CD56^{dim}$, $CD57^{+}$, $CD62L^{-}$, $CD94/NKG2A^{-}$ NK cells. While $CD56^{bright}$ cells are not very cytotoxic, they are especially good at producing IFNγ after stimulation with pro-inflammatory cytokines, such as interleukin IL-12 and IL-18. This activity is gradually lost during the differentiation towards the more cytotoxic $CD56^{dim}$, $CD57^{+}$ NK cells. In contrast, these most differentiated NK cells can produce more IFNγ when triggered via activating surface receptors and this IFNγ competence has recently been linked to the epigenetic remodeling of the IFNG promoter [53]. In addition to the subpopulations defined by these maturation markers, NK cells can be grouped by the expression of other surface molecules as well. Indeed, one study with just 37 parameters [54] found over 30.000 NK cell subpopulations. In addition, NK cell diversity increases with age and after viral infections, so this heterogeneity might be beneficial [55].

### 2.1.4 NK cell memory

Recent studies have shown that NK cells can also acquire memory or memory-like functions, thereby challenging the classical distinction between innate and adaptive. NK cells that exhibit a more potent secondary response were first described in a mouse model of delayed-type hypersensitivity (DTH) using hapten or viral antigens [56, 57]. Recombination-activating gene (RAG)-deficient mice, lacking T and B cells, were sensitized with a hapten or a viral antigen and showed an NK cell-specific DTH response when challenged later with the same antigen. This antigen-specific type of NK cell memory is confined to CXCR6-positive liver NK cells [58]. In another form of antigen-specific NK cell memory, the receptor responsible for the effect is known. Cytomegalovirus (CMV) infections in mice have been shown to induce a rapid and clonal-like expansion of a NK cell subset expressing Ly49H, which recognizes the CMV-encoded protein m157 [59]. These NK cell memory subsets show

enhanced immune responses upon secondary challenge with CMV. The activating receptor DNAX accessory molecule-1 (CD226) cooperates with Ly49H for the expansion of these memory NK cells by signaling through Fyn and protein kinase Cη (PKCη) [60].

CMV infection is also associated with the generation of memory NK cells in humans, where the expansion and long-term persistence of $NKG2C^+$ NK cells can be observed [61, 62]. However, NKG2C does not seem to be involved in the direct recognition of CMV [63]. Interestingly, similar expansions of $NKG2C^+$ NK cells have been observed during and after other virus infections, such as hantavirus, HIV and hepatitis B [64], but they were always restricted to human CMV-seropositive individuals.

*In vitro* exposure of NK cells to a combination of IL-12, IL-15 and IL-18 generates memory-like cells that show enhanced effector functions [65, 66]. *In vivo*, inflammation or other immune responses could result in the exposure of NK cells to these cytokines. Dendritic cells are the main producers of IL-12 and IL-18, thereby regulating NK cells [67]. However, in the current situation, it is very difficult to judge how much memory NK cells can contribute to immune responses against secondary infections with the same pathogen.

### 2.1.5 Relation to other ILCs

In recent years, other immune cells with comparable cytokine-induced responses have been identified and therefore NK cells have been reclassified as innate lymphoid cells (ILC). Parallel to T cell classifications, three major groups of ILCs have been defined on the basis of similarity in their production of signature cytokines, developmental requirements, and phenotypic markers. RNA analysis in tonsils revealed four distinct populations corresponding to ILC1 cells, ILC2 cells, ILC3 cells and NK cells. [68] All ILC subsets are $CD45^+$ lineage negative lymphocytes, meaning that they lack markers for the common lineages including T cells, B cells and cells of a myeloid origin. ILCs depend on IL-7 for their development and survival and concordantly express the receptor for IL-7 (CD127). Together with ILC1s, NK cells constitute group 1 ILCs, which are characterized by their capacity to produce interferon-γ and their functional dependence on the transcription factor T-bet, similarly to how type 1 helper T ($T_H1$) cells are defined.

## 2.2 NK cell receptors and signaling

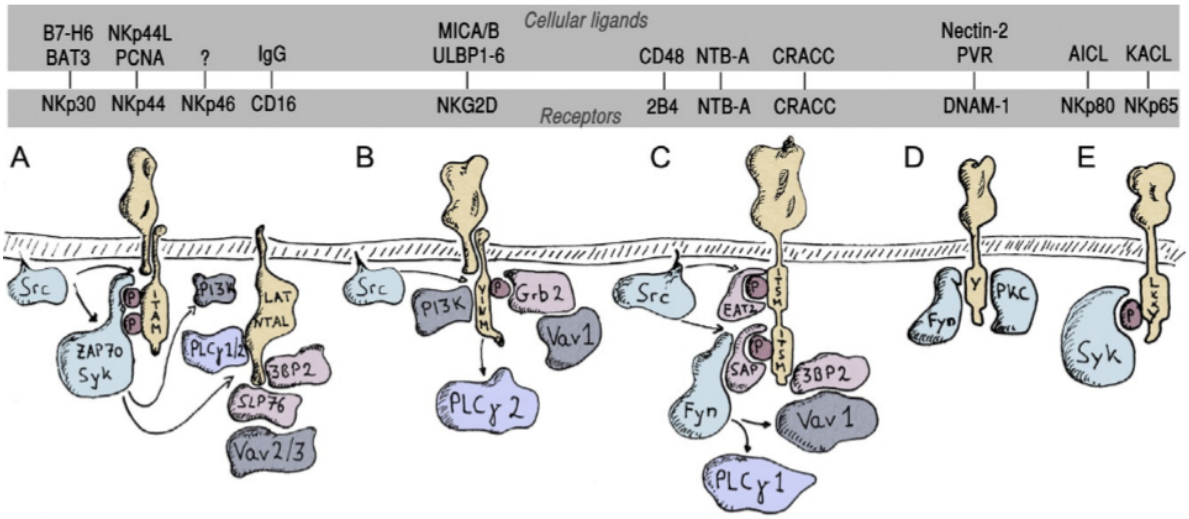


**Figure 1. Major activating NK cell receptors, their known ligands and downstream signaling.** With permission from Watzl: How to trigger a killer [9]

NK cell activity is tightly regulated by an interplay between activating and inhibitory cell surface receptors. With the exception of the Fc receptor CD16, most activating NK cell receptors recognize ligands expressed by infected or transformed cells (Fig. 1). Inhibitory receptors recognize MHC class 1 molecules. Synergy between activating receptors is necessary to overcome inhibitory signals. One additional regulatory mechanism that has been described recently is the organization of receptors and signaling components as microclusters on the cell membrane. Recent reports indicate these microclusters might play a role in NK cell regulation as well. It has been shown that receptor clustering at the lytic synapse is important for the generation of robust signaling in NK cells [69]. Altering the membrane organization of activating receptors may be important to how NK cell self-tolerance is achieved [70] and phosphorylation of the inhibitory KIR2DL1 receptor occurs in signaling microclusters at the synapse between an NK cell and its target [71]. The major activating and inhibitory NK cell receptors, their known ligands and downstream signaling are summarized in this chapter.

### 2.2.1 ITAM-associated receptors

One important receptor-associated signaling motif is the immunoreceptor-tyrosine based activation motif (ITAM). ITAMs were described 30 years ago on the basis of their sequence homology [72] and consist of paired YxxL/I motifs separated by a defined interval (YxxL/I-X6-8-YXXL/I). The main activating receptors of T cells and B cells, TCR and BCR, associate with partner chains that contain ITAM motifs in their cytoplasmatic tail and therefore signaling through ITAMs has been extensively studied in these cell types [73].

In human NK cells, activating killer cell Ig-like receptors (KIR-S), CD94/NKG2C-E, and NKp44 associate with DAP12, while NKp46, NKp30, and CD16 pair with CD3ζ and FcεRγ, through which they trigger NK cell functions.

Canonically, upon receptor engagement Src-family kinases (SFK) phosphorylate the two tyrosines in the ITAM and these in turn form a binding site for the Src-homology domain 2 (SH2) domains of the SYK family kinases SYK and ZAP70. Both ZAP70 and SYK possess tandem SH2 domains that mediate their specific and selective interaction with doubly phosphorylated ITAMs. The combined action of SFK and SYK family kinases is sufficient for the full activation of downstream signaling pathways and for eliciting effector responses. However, the existence of non-canonical ITAM signaling without the need for SFK has been reported in B cells.

Recruitment of these tyrosine kinases results in phosphorylation of transmembrane adapter molecules such as LAT and NTAL as well as phosphorylation of cytosolic adapter molecules like SLP-76 and 3BP2. These adapters provide binding sites to many downstream signaling molecules and facilitate the assembly and phosphorylation of signaling complexes. These include PI3K, PLCγ and Vav2,3.

**CD16**

In contrast to other activating NK cell receptors, CD16 is a Fc receptor and therefore recognizes the Fc part of an antibody. This makes NK cells a versatile tool against a wide array of pathogens and malignant cells despite their limited and germ-line coded repertoire of activating receptors. In a process called antibody-dependent cellular cytotoxicity (ADCC), NK cells recognize target cells whose membrane-surface antigens have been bound by specific antibodies. As many therapies increasingly make us of monoclonal antibodies or IVIg, these affect NK cells via engagement of CD16 [74]. CD16 is the only activating NK cell receptor that can trigger resting NK cells when stimulated in isolation. Recently, a subpopulation of human NK cells has been described that is deficient for the FcεRγ signaling adaptor. NK cells lacking FcεRγ display poor cytotoxicity but significantly enhanced IFNγ production upon CD16 stimulation [75]. FcεRγ$^{-}$ NK cells have a CD56$^{dim}$ phenotype and the existence of this subset is also associated with prior human CMV infection [76]. However, these NK cells also demonstrate enhanced responses against other viruses [77]. How the selective expression of adapter chains regulates NK cell signaling remains to be investigated.

**Natural cytotoxicity receptors (NCRs)**

Classically defined as activating NK cell receptors mediating NK cell cytotoxicity and production of inflammatory cytokines, the NCR family consists of the three known members NKp30, NKp44 and NKp46. NCR's are expressed on NK cells and subsets of other ILCs, some T cell and NKT cell subsets, and can recognize a variety of ligands derived from viruses, parasites, bacteria and transformed or malignant cells [78]. NKp46 and NKp30 have been reported to bind to several pathogen-derived ligands. For NKp30, two cellular ligands have been identified, BAG6 and B7-H6. NKp44 also binds to several pathogen-derived ligands and to the cellular ligands PCNA and NKp44L. Some NCR ligands remain to be identified. All NCRs rely on adapter chains to activate signaling pathways after receptor engagement and – like CD16 - their signaling might be partially regulated by different expression of these adapter chains. NKp44 associates with DAP12, while NKp46 and NKp30 pair with CD3ζ and FcεRγ.

Despite association with the same adapter chains as CD16, only CD16 is known to trigger primary non-activated NK cells when stimulated alone. One explanation for that could be a difference in the interaction between CD16 or the NCRs and the adapter chains [79]. A key molecule where activating signals converge could be SLP-76 [80], which will be discussed later.

### 2.2.2 NKG2D

NKG2D is an activating receptor that recognizes cellular ligands on transformed or infected cells. These ligands include MICA, MICB and ULBP1-6, which are usually not expressed in healthy adult tissue at significant levels that activate NK cells [81]. Expression of these proteins can be induced by hyperproliferation, transformation or infection by pathogens [82]. In humans, NKG2D is associated with DAP10. DAP10 contains a YINM tyrosine-based signaling motif. Upon receptor engagement, Src kinases phosphorylate DAP10 which can recruit PI3K or Vav1 through the adapter GRB2. Structural analysis has shown that one NKG2D homodimer associates with four DAP-10 chains [83], therefore both pathways could be triggered simultaneously by one receptor homodimer.

### 2.2.3 SLAM-related receptors

Another group of NK cell receptors is part of the signaling lymphocyte activation molecule (SLAM) receptor family. 2B4, NTB-A and CRACC are members of this group expressed on human NK cells. These receptors contain immunoreceptor tyrosine-based switch motifs

(ITSM) in their cytoplasmatic tail. 2B4 binds to CD48 [84] while NTB-A and CRACC are their own ligands and interact in a homophilic manner. CD48 is expressed on all human hematopoietic cells. Upon receptor engagement, Src family kinases phosphorylate the ITSM motifs leading to the recruitment of small SH2-domain-containing molecules like SAP and EAT2. SAP recruits the Src kinase Fyn [85, 86], which can initiate further downstream signaling. The motif of these receptors is named a "switch" motif because in the absence of SAP they undergo a "switch-of-function" and mediate inhibitory signals [87].

### 2.2.4 DNAM-1 and NKp80

In addition to the previously mentioned groups of activating NK cell receptors there are several receptors that also contain tyrosine-based activation motifs. The most prominent receptors are DNAM-1 and NKp80.

Known ligands for DNAM-1 are Nectin-2 (CD112) and PVR (CD155). Both ligands have been reported to be upregulated on tumor cells [88, 89]. DNAM-1 has tyrosine-based signaling motifs in its cytoplasmic tail and can recruit the Src-family kinase Fyn and PKC [90, 91]. DNAM-1 deficiency in mice has been reported to affect the expansion and generation of memory NK cells [60].

NKp80, also known as killer cell lectin-like receptor subfamily F, member 1 (KLRF1), is a type II transmembrane C-type lectin-like receptor [92]. NKp80 signals through a hemiITAM motif in its cytoplasmatic tail [93, 94]. NKp80 induces NK-cell mediated cytotoxicity and cytokine production through interaction with its ligand AICL. Expression of NKp80 correlates with NK cell maturation in secondary lymphoid tissue and has been proposed as a marker of NK cell maturation in tissue [95].

### 2.2.5 Inhibitory receptors

Human NK cells recognize self MHC-1 molecules by killer cell immunoglobulin-like receptors (KIR). These receptors transmit inhibitory signals via an immunoreceptor tyrosine-based inhibitory motif (ITIM). Upon engagement by MHC-1, the ITIM motif is phosphorylated presumably by Src kinases followed by recruitment of phosphatases like SHP-1, SHP-2 and SHIP [96]. Recruitment of phosphatases interferes with the signaling by activating receptors. KIRs have evolved from the Ig-superfamily and consist of type 1 transmembrane glycoproteins with two or three Ig-like domains and possess either a short or long cytoplasmic tail [97, 98]. In humans, the KIR receptors KIR2DL2/2DL3, KIR2DL1, and KIR3DL1 are specific for the HLA-C1, HLA-C2, and HLA-Bw4 ligands, respectively [99].

CD94–NKG2A heterodimers belong to the C-type lectin family of receptors. They recognize the non-classical MHC molecule HLA-E [100] and signal through ITIMs as well. HLA-E binds peptides derived from the leader sequence of classical MHC-1 molecules [101], therefore providing NK cells with a mechanism to indirectly monitor the expression of MHC-1 on target cells.

### 2.2.6 Common downstream signaling and intersection points

Many activating signals share common signaling components. All activating receptors or their adapters are phosphorylated by Src family kinases. No kinase of this family is essential for NK cell functions, but pharmacological inhibition of all Src-family kinases blocks ITAM and non-ITAM signaling and therefore NK cell effector functions. NK cells express a large number of Src family kinases, including Fyn, Lck, Lyn, Hck, c-Fgr, Src and c-Yes [102-104]. SFK can be reciprocally regulated by Csk and CD45. CD45 specifically dephosphorylates the C-terminal inhibitory tyrosine of SFK and has been reported to be a critical positive regulator of Lck and Fyn in T cells [105]. Csk is thought to be the kinase that phosphorylates the C-terminal inhibitory tyrosine of SFK, driving SFK towards an inactive state. While deficiencies in ZAP70 or SYK will severely impair T cell and B cell functions [106, 107], respectively, NK cell maturation and cytotoxic activity are not significantly impaired even when ZAP70 and SYK are both absent as not all receptors rely on SYK family kinase signaling. But ITAM-coupled receptors have been reported to be functionally impaired in the absence of SYK family kinases [108].

Two downstream molecules of particular interest for this study are VAV1 and SLP-76 as they are phosphorylated upon engagement of several activating NK cell receptors and might function as intersection point of activating and inhibitory signals.

**SLP-76**

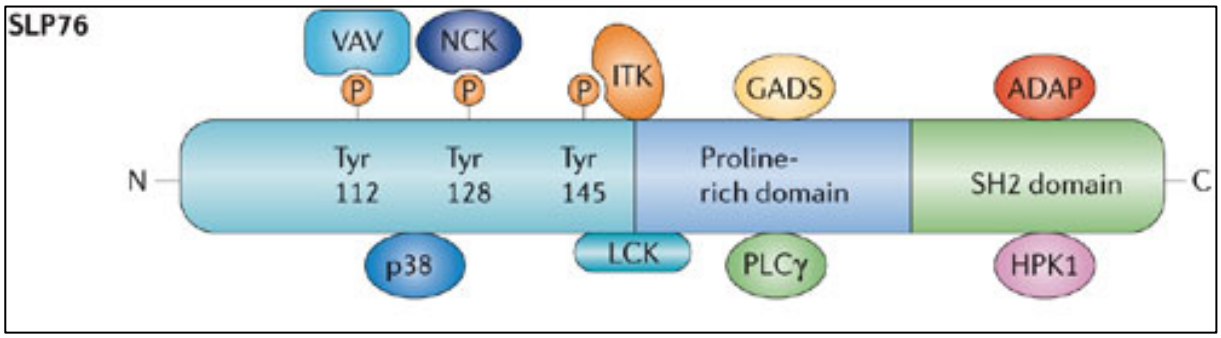


**Figure 2. Structure and interaction partners of SLP-76.** SLP-76 contains inducibly phosphorylated tyrosines in the amino (N) terminus, a central proline-rich domain and a carboxy (C)-terminal SH2 domain. These domains mediate binding to several other signal-cascade intermediates as shown. ADAP, adhesion- and degranulation-promoting adaptor protein; BTK, Bruton's tyrosine kinase; GADS, GRB2-related adaptor protein; GRB2, growth-factor-receptor-bound protein 2; ITK, interleukin-2-inducible T-cell kinase; NCK, non-catalytic region of tyrosine kinase; PLCγ, phospholipase Cγ. With permission from Koretzky et al., Nat Rev Imm 2006 [109].

SLP-76 was first described in T cells and plays an important role in TCR signaling but SLP-76 is highly expressed in spleen, thymus and other peripheral blood leukocytes as well. While one earlier study found SLP-76 to be dispensable for NK cell effector functions [110] these findings were contradicted in later studies taking advantage of inducible SLP-76 KO mice [111] and SLP-76 has been shown to be important for NK cell development, cytotoxicity and IFN-γ production [112, 113]. Signaling through SLP-76 by activating receptors is critical for NK cell education [114].

Phosphorylation of SLP-76 at two tyrosines, Tyr113 and Tyr128, has been proposed as intersection point of activating signals by different receptors [80]. It was shown that stimulation of the 2B4 receptor resulted primarily in phosphorylation of Tyr113 whereas stimulation via NKG2D or DNAM-1 induced phosphorylation of Tyr128. Thus, these phosphorylation sites could provide a molecular basis for the synergy of activating NK cell receptors. In support of this hypothesis, engagement of CD16 induces the phosphorylation of both Tyr113 and Tyr 128 and CD16 alone is sufficient to trigger the activation of resting NK cells. SLP-76 functions as an adapter and is known to interact with many other key signaling molecules like Vav, p38, Lck, PLCγ to form signaling complexes upon stimulation (Fig. 2) [109]. SLP-76 is also critical for T cell activation by the TCR [115] and a homologue expressed in B cells termed SLP-65 (encoded by BLNK) important for signaling by the BCR [116].

Furthermore, SLP-76 has been reported to play an essential role in the formation of microclusters surrounding the TCR upon activation [117, 118]. SLP-76 functions as a scaffolding protein that can interact with many other proteins. Previously described

interactions partners include, but are not limited to Vav1, p38, Itk, Nck, Lck, ADAP, GADS and PLCγ [109, 119].

**Vav1**

Vav1 is a member of the Dbl family of guanine nucleotide exchange factors (GEF) for the Rho family of GTP binding proteins. Vav1 contributes to the activation of Rho-family GTPases like Rac1 and Cdc42 that are important regulators of actin reorganization.

While NKG2D selectively signals via Vav1 [120], ITAM-based receptors rely more on Vav2 or Vav3 for their function [121]. NK cells only deficient for Vav1 show an impairment in NK cell cytotoxicity [122] but when all three Vav isoforms are absent NK cell functions are severely compromised [121]. Vav1 has been described as an intersection point to integrate activating and inhibitory signals through regulation of Vav1 phosphorylation levels by Src family kinases and the phosphatase SHP-1 [123].

## 2.3 Schneider cells (S2)

In synthetic biology, scientists rebuild functional minimal signaling systems which can then be easily manipulated to enhance mechanistic understanding. Schneider cells, isolated from *Drosophila melanogaster* embryos in 1972 [124], provide a useful platform for this approach. Historically, S2 cells have often been used for large-scale preparative protein expression [125-127]. Moreover, elements of the early T cell receptor (TCR) signaling pathway also have been expressed in S2 cells in order to investigate the exact specificity of antibodies directed against phosphorylation sites in the ITAMs of TCR-CD3 subunits after their phosphorylation by the Src family kinases Lck and/or Fyn [128]. They easily take up large amounts of DNA enhancing co-transfection while maintaining a reasonably high transfection efficiency (about 30%). The evolutionary distant environment should prevent interference of *Drosophila* proteins with the heterologously expressed mammalian signaling system. In previous studies, the S2 system was used to investigate signaling of the BCR [129-132] and a synthetic approach applied to elucidate TCR and ITAM signaling [133]. Other studies employed S2 cells to investigate human transcription factors [134, 135]. For similar reasons, Schneider cells were utilized by the Long lab as target cells where only the transfected ligands were recognized by NK cells without interference by other mammalian molecules [80, 136].

For transient co-transfection experiments in Drosophila Schneider cells, the expression vector pRmHa-3 containing an inducible metallothionein promoter [137] was used. Thus, expression of the transiently transfected vectors could be induced by non-toxic concentrations of copper sulphate. By only transiently transfecting the cells with a sufficient efficiency, there is no need for a long selection process to further enrich transfected cells.

# 3 Objective

The aim of this study is to use a synthetic approach to predict NK cell signaling and verify these results in primary human NK cells. Pitfalls of conventional approaches to signaling studies that are conducted with inhibitors or by knockdown are often unspecific side-effects. Redundancy of many proteins like Src family kinases provide challenges to signaling studies as well. Furthermore, signaling processes are very complex and often overlapping *in vivo* and important components of an investigated pathway could easily be missed. Thus, rebuilding a functional pathway by a synthetic approach could help our understanding of essential components of NK cell signaling. A useful tool for this approach that has been previously described in the literature are the evolutionary distant S2 cells that can co-express many transiently transfected proteins at once. Together with conventional signaling studies in primary cells to validate results obtained in the artificial S2 system, this could be a powerful approach to investigate NK cell signaling.

Thus, we aimed to recreate a functional minimal NK cell signaling pathways utilizing S2 cells. This pathway should be inducible by cross-linking or engagement with natural ligands. Additionally, we wanted to examine possible intersection points for inhibitory and activating signals by recreating respective pathways in S2 cells. Obtained results and postulated pathways should be validated in primary NK cells by use of inhibitors.

# 4 Results

## 4.1 Transient Co-transfection in Schneider cells

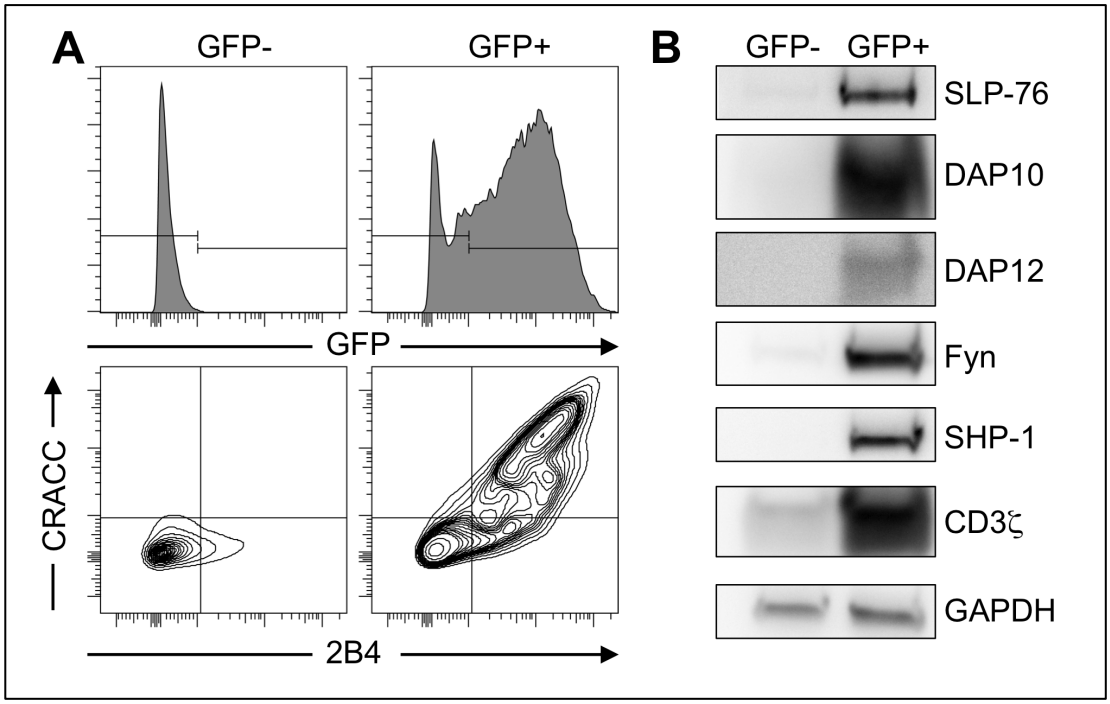


**Figure 3. High co-transfection rate of multiple genes in transiently transfected Schneider cells. (A)** Schneider S2-cells were co-transfected with plasmids coding for 2B4, CRACC, DAP10, DAP12, CD3ζ (CD247), SLP-76, SYK and GFP. Expression was induced by $CuSO_4$. After 36-48 hours GFP- and GFP+ cells were enriched by cell sorting and analyzed by FACS for expression of GFP, 2B4 and CRACC. Results are representative of three independent experiments. **(B)** Western blot analysis of co-expressed proteins in the total cellular lysate of transiently transfected S2 cells from (A) enriched for GFP- and GFP+ by cell sorting. GAPDH was used as a loading control. Results are representative of five independent experiments.

To test for the high co-transfection efficiency in S2 cells, S2 cells were transiently transfected with plasmids encoding for GFP, SLP-76, SYK, Fyn, DAP10, DAP12, SHP-1 and for the receptors 2B4 and CRACC. This resulted in about 30% GFP positive cells, which were then sorted by FACS as GFP+ and GFP-. Analysis of protein expression by Western Blot and FACS clearly indicated that only GFP+ cells expressed high levels of all the transfected proteins while there were almost undetectable levels of the transfected proteins in GFP- cells (Fig. 3). FACS analysis of 2B4 and CRACC confirmed surface expression of both receptors and revealed that most cells co-expressed both receptors. This demonstrates that transfected S2 cells take up all available plasmids and subsequently co-express many heterologous proteins transiently with a reasonably high efficiency. Furthermore, using GFP as a marker, we were able to enrich the co-transfected cells by sorting. Thus, the S2 cell system provides a suitable platform for the setup of an artificial signaling system that is easy to manipulate as already demonstrated by others [129-132].

## 4.2 Limitations of the Schneider cell system

### 4.2.1 Src-kinases are constitutively active in the S2-system

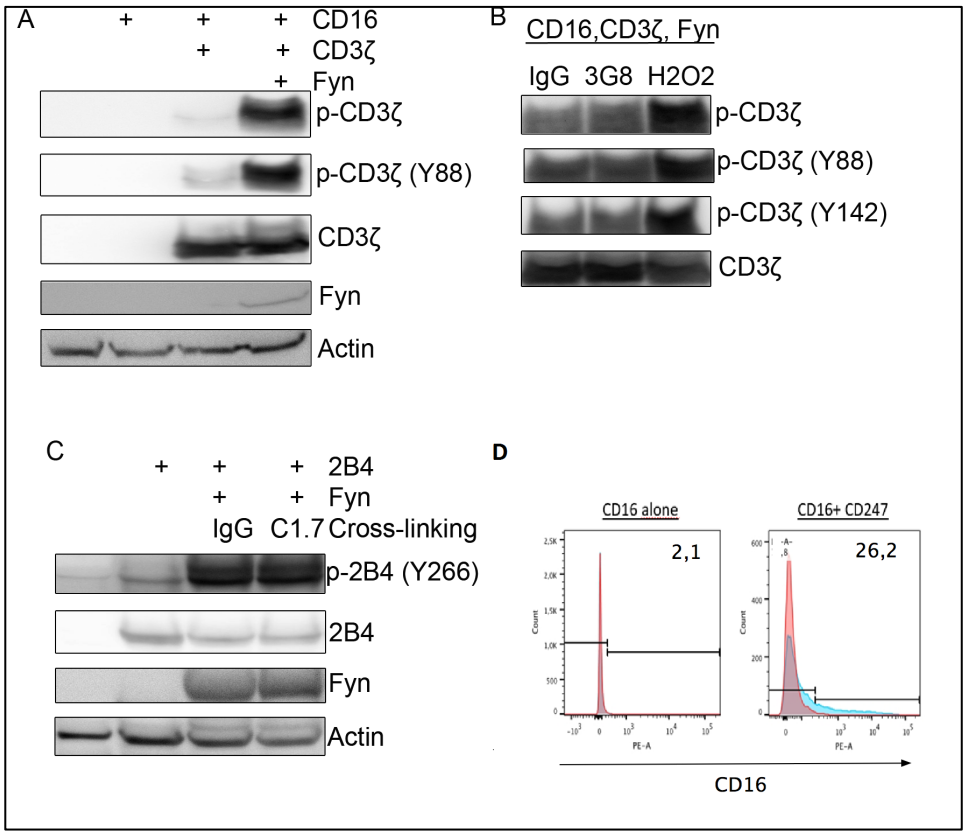


**Figure 4. Src kinases are constitutively active in the S2 system**. (A) S2 cells were co-transfected with combinations of CD16, CD3ζ and Fyn and analyzed by Western Blot as described before. (B) S2 cells were co-transfected as in (A) and incubated with isotype control (IgG) or CD16 specific antibodies (3G8) on ice before cross-linking for 2' at 28°C with goat-anti-mouse IgG. Treatment with $H_2O_2$/Pervanadate was used to induce maximal phosphorylation. Phosphorylation of CD3ζ was analyzed by Western Blot. (C) S2 cells were co-transfected with 2B4 alone or 2B4 and Fyn and treated as in (B). (D) S2 cells were co-transfected with CD16 alone or CD16 and CD3ζ (CD247). Membrane expression of CD16 was analyzed by FACS (isotype control in red, specific CD16 staining in blue).

To test the capabilities of the S2 system to reconstruct a minimal signaling pathway, we started with the co-expression of CD16 and its associated adapter chain CD3ζ or 2B4 together with the Src kinase Fyn, respectively (Fig 4). We observed substantial constitutive phosphorylation of the CD3ζ chain and the 2B4 cytoplasmatic tails when Fyn was co-expressed. While CD3ζ phosphorylation by Fyn was independent of CD16 expression, membrane expression of CD16 was only detected when co-expressed with CD3ζ. As reported by others [138], CD3ζ is necessary for CD16 membrane expression similar to human immune cells.

Ideally, the minimal signaling system would be inducible by cross-linking the expressed receptors with specific antibodies to investigate the interaction of different receptor signaling pathways. Therefore, we cross-linked CD16 co-expressed with CD3ζ and Fyn or 2B4 co-

expressed with Fyn. Cross-linking of the receptors did not result in increased phosphorylation of their respective signaling motifs.

### 4.2.2 DPI-experiments did not result in an inducible system

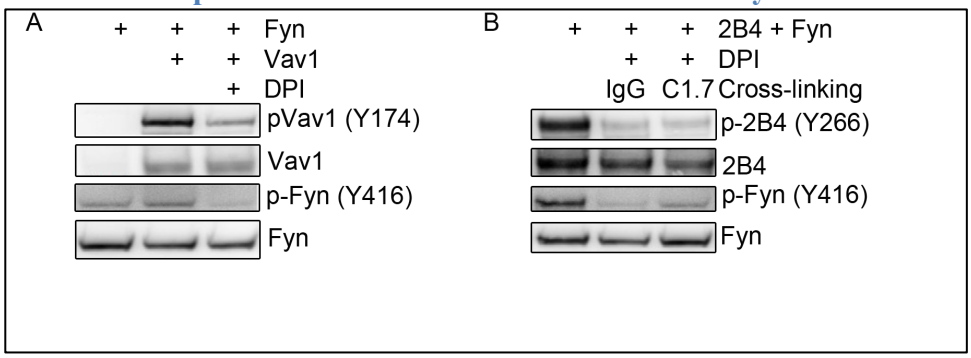


**Figure 5. Pre-treatment with DPI reduced autophosphorylation.** (A) S2 cells were transiently co-transfected with the indicated proteins as described before and pretreated with DPI for one hour before harvesting. (B) S2 cells were co-transfected as before with the indicated proteins. Then the cells were cross-linked as described before with specific 2B4 antibody (C1.7) or isotype control (IgG).

The activity of Src kinases is regulated by phosphorylation and dephosphorylation of activating and inhibitory tyrosines. Like other studies before, we observed constitutive phosphorylation and activation of Fyn [129]. One reason for that could be the missing activity of inhibitory phosphatases. Protein tyrosine phosphatases (PTP) have been reported to be regulated by the redox equilibrium inside the cells. $H_2O_2$ production leads to the oxidation of a reactive cysteine in the catalytic center of the PTP and therefore reversible inhibition of its enzymatic activity. Additionally, high $H_2O_2$ levels have been reported to directly activate SFK [139]. Rolli et al reported elevated $H_2O_2$ production in S2 cells in line with their phagocytic activity [129]. $H_2O_2$ production can be inhibited by diphenylene iodonium (DPI), an inhibitor of flavoproteins like the NADPH oxidase. Therefore, we pretreated the cells with DPI before harvesting as described by others. Another well described substrate of Fyn is Vav1. We observed Vav1 phosphorylation upon co-expression with Fyn in S2 cells. (Fig. 5A). DPI pretreatment of cells did reduce the constitutive phosphorylation of Fyn itself and Fyn-mediated Vav phosphorylation upon co-expression in S2 cells (Fig. 5A).

Pretreatment with DPI did show decreased phosphorylation of 2B4 by Fyn upon co-expression, but did not show an increase in 2B4 phosphorylation in combination with cross-linking of the 2B4 receptor by a specific antibody compared to isotype control (Fig. 5B).

Thus, we could decrease the constitutive activity of Fyn by treatment with DPI, but could not induce its activation by cross-linking of co-expressed receptors like 2B4. This is in line with previous studies that were also not able to recreate an inducible pathway in S2 cells [129].

### 4.2.3 Trying to reconstruct a 2B4 minimal signaling pathway

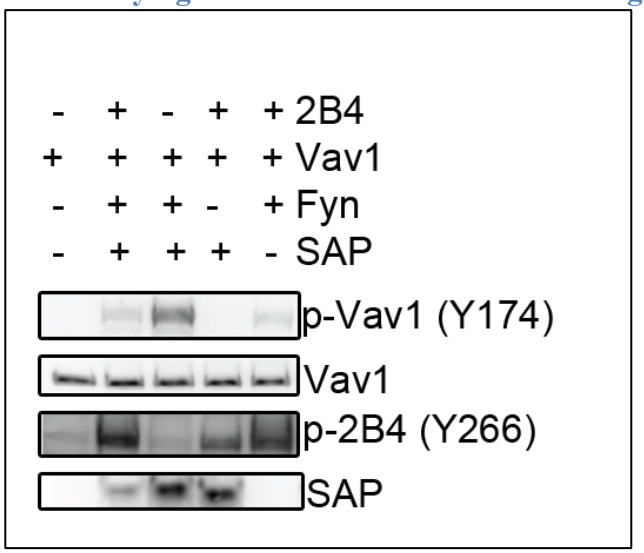


**Figure 6. Co-expression of SAP does not affect 2B4 or Vav1 phosphorylation.** S2 cells were co-transfected as described before with the indicated proteins and analyzed by Western Blot.

As we are trying to recreate a minimal signaling pathway it could be the case that essential signaling molecules are missing to induce a significant increase in phosphorylation after receptor cross-linking. Therefore we co-expressed several NK cell signaling adapters that have been reported to play important roles in early signaling of the NK cell receptors 2B4 and CD16, respectively. For 2B4 this includes the adapter protein SAP, which binds and recruits the Src kinase Fyn to the cytoplasmatic ITSM motifs of 2B4. In patients suffering from X-linked lymphoproliferative disease (XLP1), SAP is nonfunctional, not only abolishing the activating function of 2B4, but rendering this receptor inhibitory [87]. Another important adapter that has been reported to recruit Vav1 to 2B4 upon receptor activation is 3BP2. Co-expression of 2B4, Fyn and Vav1 together with SAP did not result in increased phosphorylation of 2B4 or Vav1 (Fig. 6) in various combinations. The absence of 2B4 even increased Fyn-dependent Vav1 phosphorylation, presumably because it's the only substrate for Fyn when 2B4 is not co-expressed. Additional co-expression of 3BP2 and/or LAT in various combinations or exchanging SAP for EAT2 did not affect phosphorylation levels of 2B4 or Vav1 either (data not shown). Pre-treatment with DPI or cross-linking did not significantly affect phosphorylation levels either (data not shown). Thus, our attempts to reconstruct a functional minimal signaling pathway dependent on co-expression or even activation of the 2B4 receptor were not successful. SFK phosphorylated every co-expressed substrate and their activity was not affected or regulated by co-expression with other components of the 2B4 pathway in S2 cells.

### 4.2.4 Expression of phosphatases

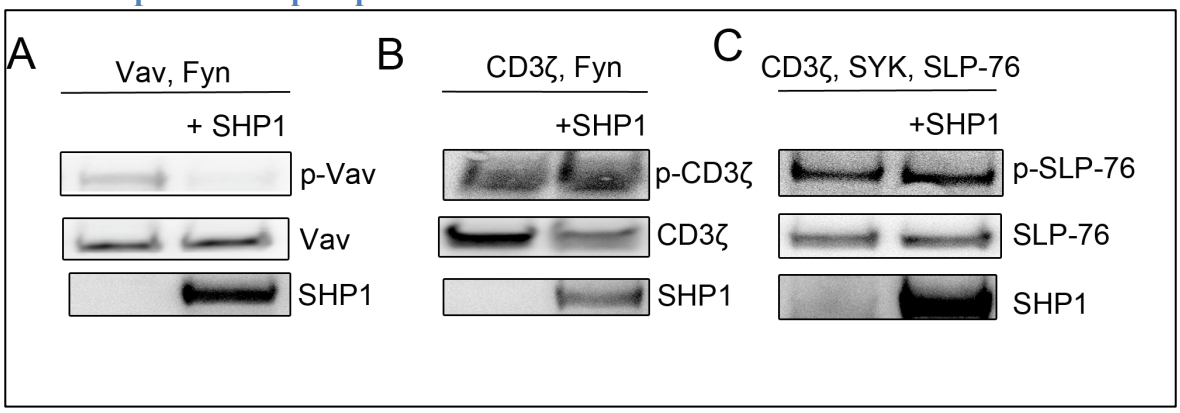


**Figure 7. Co-expression of SHP-1 leads to dephosphorylation of Vav1, but not CD3ζ or SLP-76.** (A) Vav1 and Fyn were co-expressed in S2 cells with or without SHP-1 and Vav1 phosphorylation was detected by WB. (B) CD3ζ and Fyn were co-expressed in S2 cells with or without SHP-1 and CD3ζ phosphorylation was observed by WB. (C). CD3ζ, SYK and SLP-76 were co-expressed in S2 cells with or without SHP-1 and SLP-76 phosphorylation was detected by WB.

Phosphatases play a prominent role in NK cell signaling by dephosphorylation of activating signaling molecules thereby interfering with activating signals. In a previous study, co-expression of the phosphatase SHP-1 in S2 cells could successfully counteract phosphorylation by SYK and Src kinases [129]. To investigate if co-expression of SHP-1 in S2 cells could lead to dephosphorylation of other co-expressed signaling components in S2 cells, we co-expressed SHP-1 in different combinations of the previously observed combinations inducing constitutive phosphorylation. Thus, we co-expressed SHP-1 with Vav1 and Fyn (Fig. 7A). This decreased the observed phosphorylation of Vav1 on the activating tyrosine Y174 significantly. In contrast, the phosphorylation of CD3ζ by Fyn was not affected by co-expression of SHP-1 (Fig. 7B). SYK-mediated and ITAM-dependent phosphorylation of SLP-76 was also not affected by co-expression of SHP-1 (Fig. 7C).

Phosphatases are recruited by inhibitory receptors containing ITIM motifs, therefore it might be possible that the presence of an ITIM motif could enhance SHP-1 phosphatase activity. Co-expression of KIR2DL1, an inhibitory NK cell receptor containing ITIM motifs in its cytoplasmatic tail, did not enhance SHP-1 activity in different combinations or when cross-linked by specific antibodies (data not shown). Therefore, SHP-1 demonstrated substrate specificity as well as constitutive activity upon co-expression in S2 cells.

## 4.3 ITAM – SYK – SLP-76

### 4.3.1 ITAM-expression results in SYK activation and SLP-76 phosphorylation

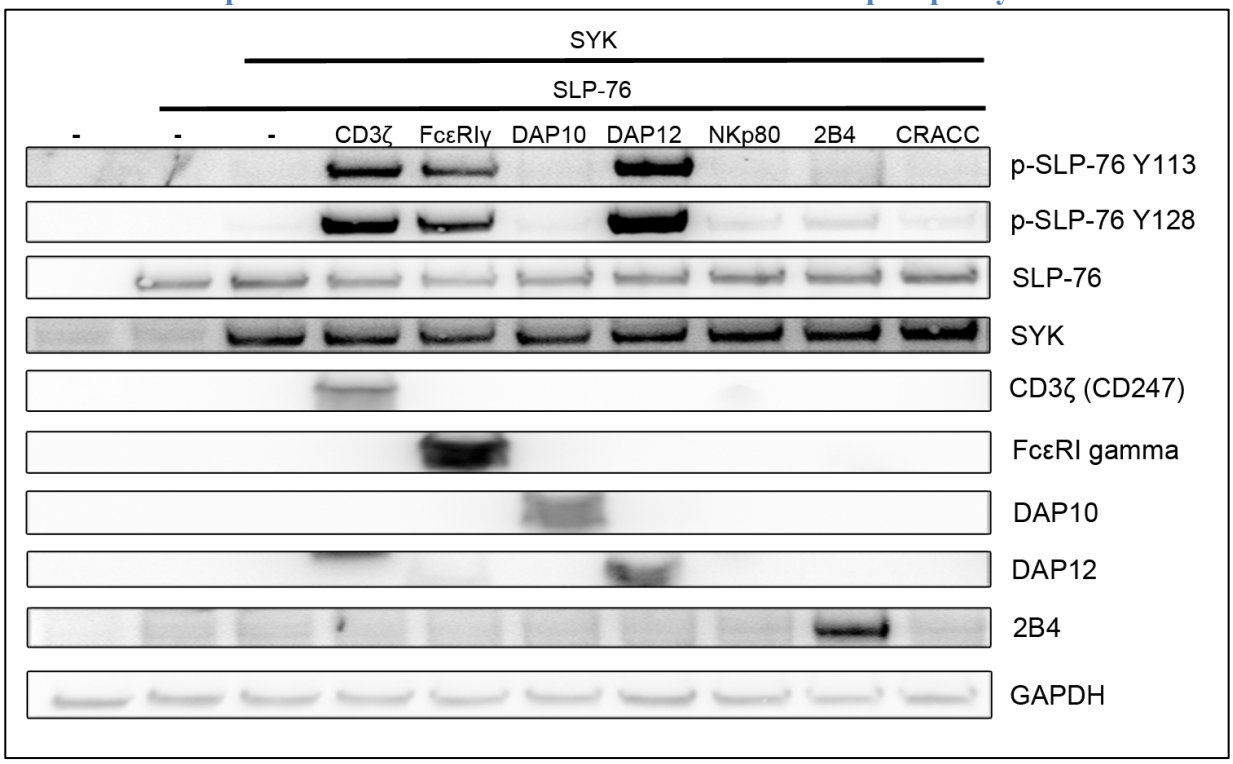


**Figure 8. SLP-76 phosphorylation in the presence of SYK and ITAM-containing receptors**. S2-cells were co-transfected with the indicated proteins. Equal numbers of FACS-enriched GFP+ cells were analyzed by Western Blot for SLP-76 phosphorylation and expression of the transfected proteins. Expression of NKp80 and CRACC was verified by FACS analysis (Supplementary data). Results are representative of at least 5 independent experiments.

We then used the S2 cell system to rebuild an ITAM-based signaling pathway resulting in SLP-76 phosphorylation. Expression of SLP-76 alone in S2 cells did not result in its phosphorylation, demonstrating that *Drosophila* kinases do not significantly phosphorylate human SLP-76 (Fig. 8). Co-expression of SYK also did not result in SLP-76 phosphorylation. However, co-expression of SLP-76 and SYK together with ITAM bearing signaling adapters such as CD3ζ, FcεRγ or DAP12 induced phosphorylation of SLP-76 at tyrosines Y113 and Y128. Therefore we could identify a non-canonical pathway for ITAM-mediated SYK activation that does not rely on SFK. This pathway is very similar to a previously described pathway utilizing transiently co-transfected S2 cells. In this earlier study, the more B cell specific ITAM-containing Ig-$\alpha$ was co-expressed with SYK and the B cell adapter SLP-65, a homologue of SLP-76. Co-expression of all three molecules in S2 cells resulted in SLP-65 phosphorylation. We could replicate these results with more NK cell specific signaling components. Moreover, we compared the ability of other non-ITAM receptors to activate SYK without the co-expression of SFK. Co-expression of 2B4 and CRACC, which both carry ITSM motifs in their cytoplasmatic tail, with SLP-76 and SYK did not lead to SLP-76 phosphorylation. Co-expression of NKp80, which carries a hemiITAM motif, with SLP-76

and SYK also did not induce SLP-76 phosphorylation. Thus, only ITAM-containing adapters were able to enhance SYK activity and result in SLP-76 phosphorylation. Taken together, we could confirm and expand the earlier report of an SFK-independent ITAM-SYK-SLP-76/65 pathway in S2 cells with signaling components expressed by NK cells.

### 4.3.2 ITAM-activation of other kinases

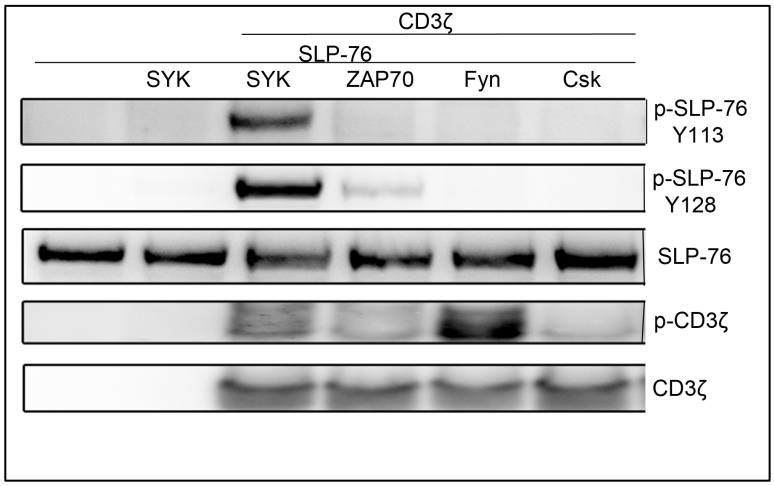


**Figure 9. Ability of other kinases to phosphorylate SLP-76 in S2 cells when CD3ζ is co-expressed.** S2-cells were co-transfected with the indicated proteins. Equal numbers of FACS-enriched GFP+ cells were analyzed by Western Blot. Representative of three independent experiments.

Next we tested the ability of other kinases to phosphorylate SLP-76 when co-transfected with an ITAM-containing protein. Therefore, we co-transfected S2 cells with CD3ζ and SLP-76 and the kinases SYK, ZAP70, Fyn and Csk. SYK induced the strongest phosphorylation of SLP-76. While the SYK-family kinase ZAP70 could phosphorylate SLP-76 to a lesser extent, no phosphorylation was detectable when the Src-family kinases Fyn or the kinase Csk were co-transfected (Fig 9). In addition, we analyzed the phosphorylation status of CD3ζ. Here we observed that Fyn induced by far the highest level of CD3ζ phosphorylation, while SYK and ZAP70 phosphorylated CD3ζ at lower levels. SYK phosphorylated CD3ζ more than ZAP70. Csk did not induce any detectable CD3ζ phosphorylation.

SLP-76 is a known substrate of SYK and ZAP70. In the S2 system, both SYK kinases demonstrated the ability to phosphorylate SLP-76 upon co-expression with CD3 ζ. However, SYK is significantly more activated than ZAP70 in this setting.

### 4.3.3 Mutations of SYK and CD3ζ

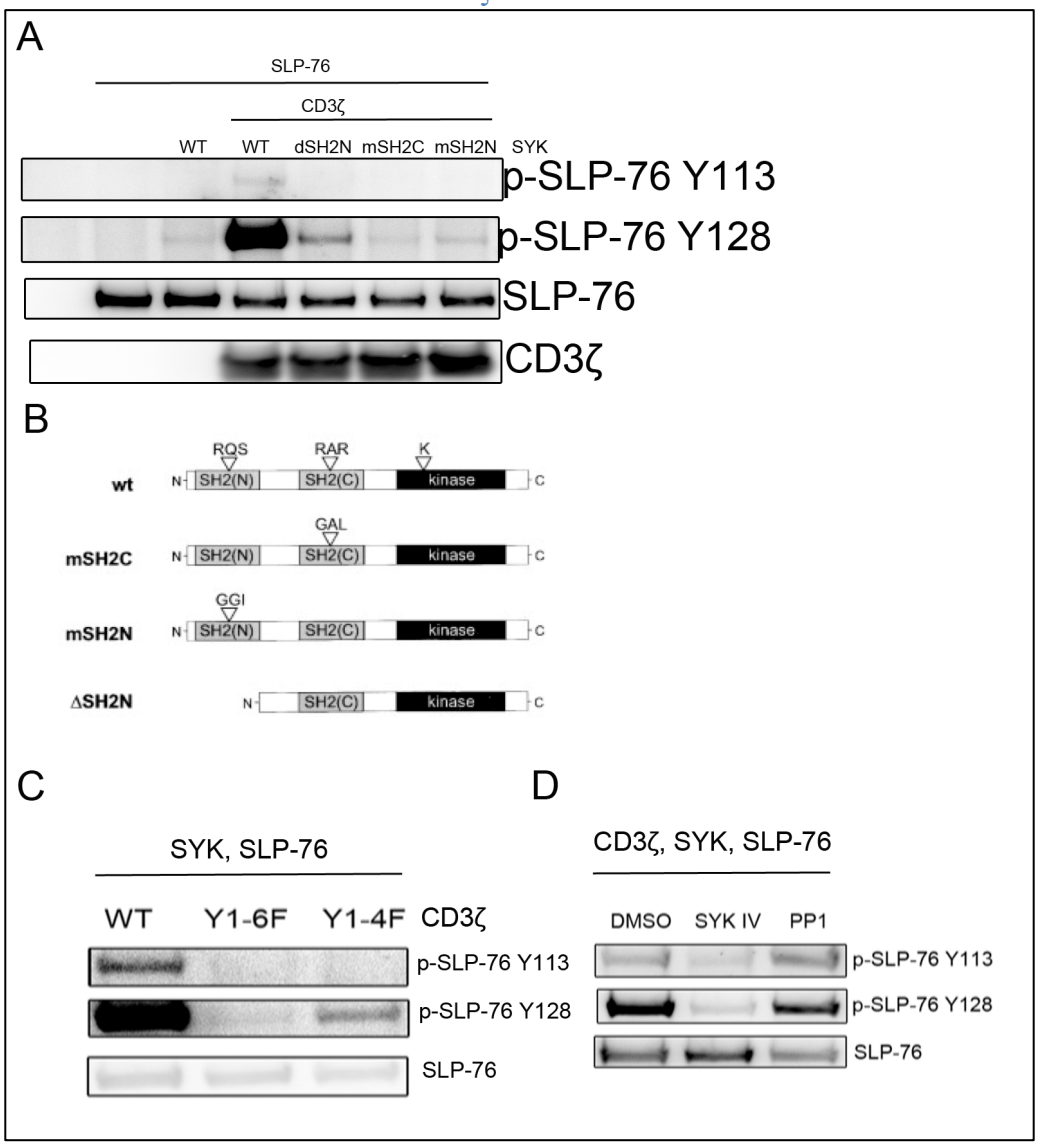


**Figure 10. Structure and investigated mutations of SYK kinase.** S2-cells were co-transfected as described before with GFP, SLP-76 and mutations or WT of CD3ζ and SYK. (A) Analysis of SLP-76 phosphorylation upon co-expression of GFP, SLP-76, CD3ζ and either wildtype (wt) SYK or the indicated SYK SH2-domain mutants. All Western Blots are representative of at least three independent experiments. (B) SYK mutations utilized in (A). With permission from Rolli et al. Mol Cell 2002 [129]. (C) Analysis of SLP-76 phosphorylation upon co-expression of GFP, SLP-76, SYK and either wildtype (wt) CD3ζ or the indicated CD3ζ ITAM-mutants. (D) SLP-76 phosphorylation was analyzed in GFP+ cells upon treatment with the SYK-family kinase inhibitor SYKIV, the Src-family kinase inhibitor PP1 or DMSO as a carrier control.

To further investigate the conditions necessary for SYK activation and subsequent phosphorylation of SLP-76, we made use of the easy manipulation of the S2-system. Canonically, the SH2 domains of SYK can bind to phosphorylated ITAM motifs.

Using CD3ζ mutants we observed no SLP-76 phosphorylation when all tyrosines of the ITAMs were replaced by phenylalanine, whereas the presence of a single intact ITAM was sufficient to result in some SLP-76 phosphorylation (Fig. 10C). Similarly, using SYK mutants we found that SLP-76 phosphorylation was dependent on both SH2 domains, as we did not detect any phosphorylation when the N-terminal SH2 domain was deleted or the N-terminal or the C-terminal SH2 domain was inactivated by a point mutation (Fig. 10A, B). These data

demonstrate that the minimal signaling pathway in S2 cells still relies on the canonical interaction of the SYK SH2 domains with the (phosphorylated) tyrosines of the ITAM despite being independent of Src-family kinases.

To further explore the involvement of SYK and Src-family kinases we used pharmacological inhibitors during the co-expression of CD3ζ, SYK and SLP-76. Syk Inhibitor IV (SYKIV), (or BAY 61-3606 or CAS 732938-37-8), is a cell-permeable, ATP-competitive, reversible, and highly selective inhibitor of SYK tyrosine kinase activity. SYKIV does not affect Src kinase activity according to *in vitro* assays conducted by the manufacturer. Its specificity targeting SYK kinase activity over ZAP70 has been demonstrated before [140]. Blocking SYK kinase activity by the inhibitor SYKIV in the S2 cells completely blocked the SLP-76 phosphorylation (Fig. 10D), demonstrating that the kinase activity of co-transfected SYK is essential for this minimal signaling pathway. The Src Inhibitor PP1 (or CAS 172889-26-8) controls the biological activity of Src family kinases. This small molecule inhibitor primarily inhibits SFK while affecting the activity of other kinases only at much higher concentrations according to the manufacturers *in vitro* assays. Blocking Src-family kinases with the inhibitor PP1 in this experiment did not impact SLP-76 phosphorylation, additionally excluding the possibility that *Drosophila* Src-related kinases may be involved.

### 4.3.4 SYK vs. ZAP70 activation

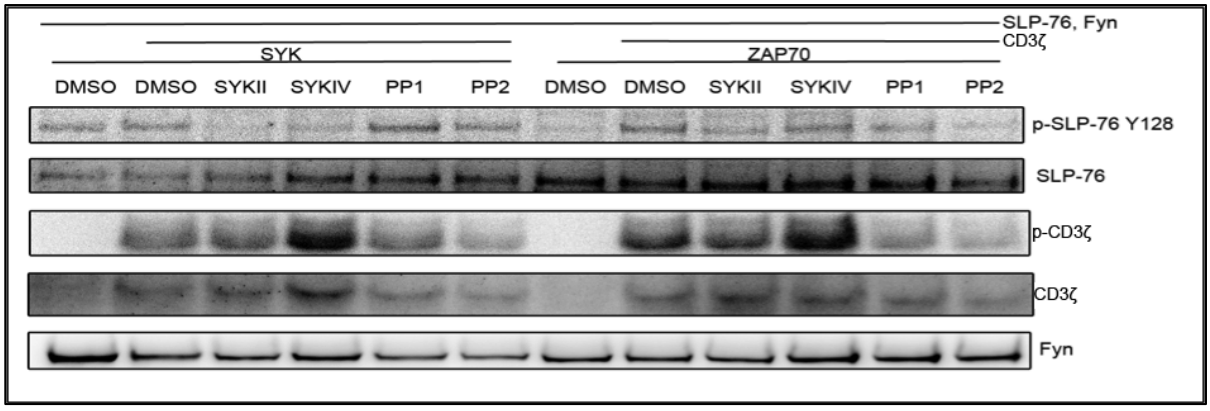


**Figure 11. ZAP70 but not SYK is dependent on Src kinases for ITAM-mediated activation.** SLP-76, Fyn and CD3ζ were co-expressed with either SYK or ZAP70 in S2 cells as described before. Cells were treated with the indicated inhibitors or DMSO control for one hour before harvesting. Phosphorylation and expression of proteins was observed by WB.

The canonical ITAM pathway is initiated by Src family kinases that phosphorylate the ITAM motif, therefore providing binding sites for the SH2 domains of SYK and ZAP70. This activates SYK and leads to phosphorylation of its targets. To test if increased ITAM

phosphorylation also leads to increased activation of SYK and therefore increased phosphorylation of SLP-76 in S2 cells, we co-expressed CD3ζ, SYK or ZAP70 and SLP-76 with the Src family kinase Fyn (Fig. 11). Co-expression of Fyn clearly enhanced phosphorylation of CD3ζ in both setups. Of note, co-expression of Fyn did result in SYK activation without the need for co-expression of CD3ζ. However, co-expression of Fyn seemed to enhance SLP-76 phosphorylation more in the presence of ZAP70 than in the presence of SYK. To investigate this difference in activation by Fyn, we treated the S2 cells with specific inhibitors as before. Additionally to SYKIV, we used the SYK kinase inhibitor SYKII. SYKII is a cell-permeable pyrimidine-carboxamide compound that acts as a potent, selective, reversible, and ATP-competitive inhibitor of SYK while affecting ZAP70 and other kinases only at much higher concentrations (manufacturers datasheet). In addition to PP1, we used the SFK inhibitor PP2 as well. PP2 is a potent, reversible, ATP-competitive, and selective inhibitor of the Src family of protein tyrosine kinases that does not significantly affect the activity of ZAP70 and other non-Src family kinases (manufacturers datasheet). Further confirming this regulation of ZAP70 by Fyn, pretreatment with SYK inhibitors SYKII and SYKIV decreased SLP-76 phosphorylation more when SYK was co-expressed, whereas Src kinase inhibitors PP1 and PP2 affected SLP-76 phosphorylation more when ZAP70 was co-expressed instead of SYK (Fig. 11).

Therefore, this experiment demonstrates that both SYK and ZAP70 can be activated by co-expression of Fyn. However, the activation of ZAP70 seemed to be more dependent of kinase activity of SFK than SYK.

### 4.3.5 Co-expression of Vav1 enhances SLP-76 phosphorylation

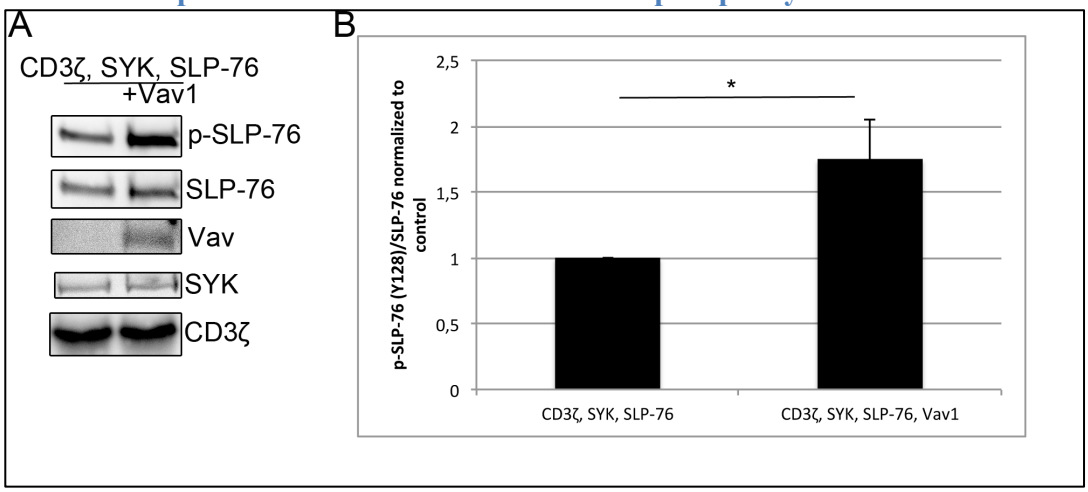


**Figure 12. Co-expression of Vav1 enhances SLP-76 phosphorylation.** S2 cells were transiently co-transfected as described before with CD3ζ, SYK and SLP-76 or CD3ζ, SYK, SLP-76 and Vav1. (A) SLP-76 phosporylation and protein expression was analyzed by WB. (B) SLP-76 phosphorylation was quantified using Image J software (Version 1.47v). The integrated density of each band was measured using the gel analysis function of ImageJ and normalized to SLP-76 using ImageJ. N=3. Statistical analysis was performed using unpaired t-test.

A well known interaction partner of SLP-76 is Vav1 [109], which also plays a central role in NK cell signaling [123]. We observed enhanced phosphorylation of SLP-76 when the previously described minimal signaling pathway of CD3ζ, SYK and SLP-76 was co-expressed together with Vav1 (Fig. 12). Presumably, binding of Vav1 protects SLP-76 against dephosphorylation by phosphatases. Vav1 itself was also phosphorylated in this setting, but this phosphorylation was independent of CD3ζ expression (data not shown).

### 4.3.6 CD45 and Csk control ZAP70 via Fyn

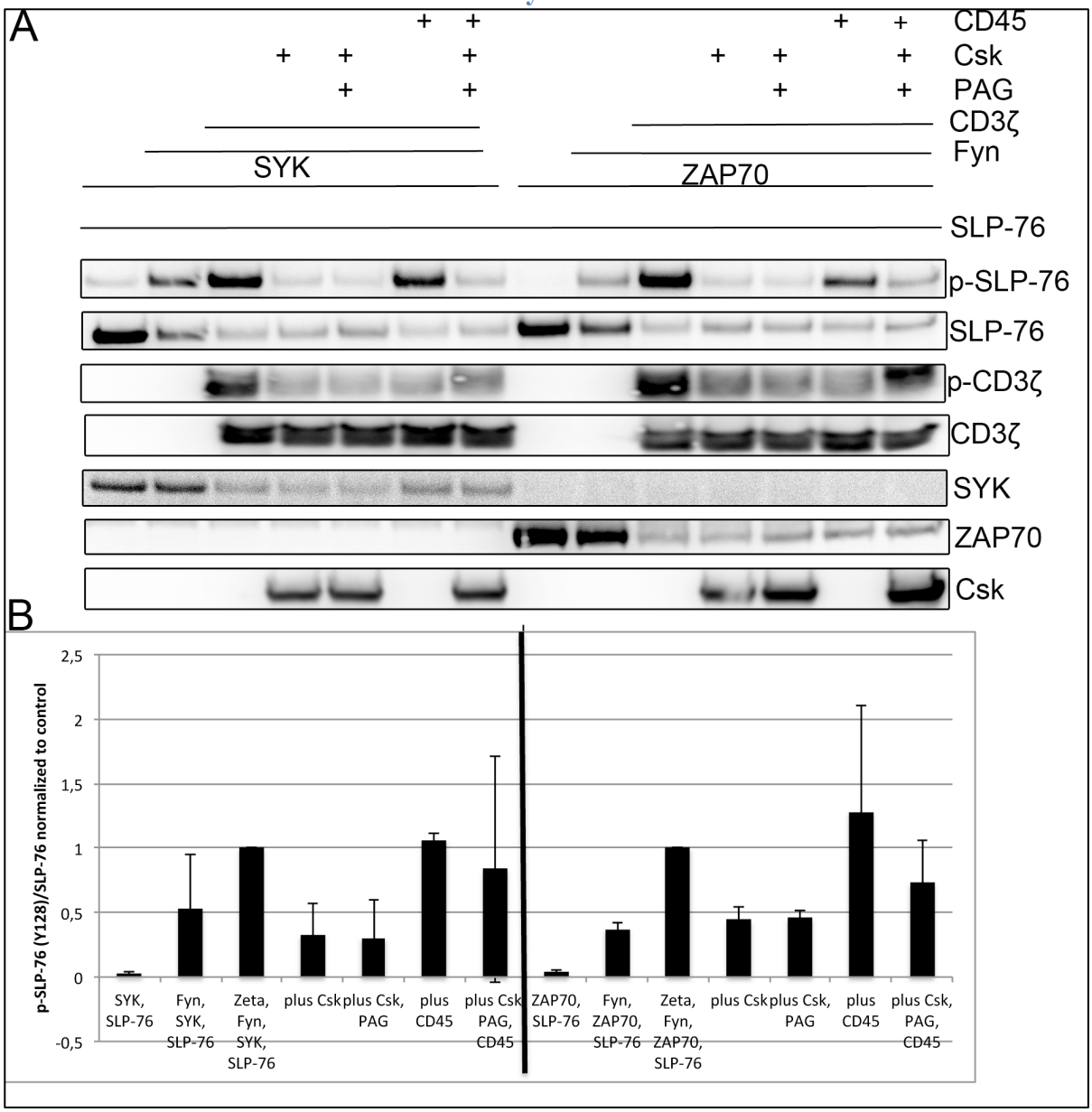


**Figure 13. Csk and CD45 can control SYK and ZAP70 activation via Fyn. (A)** S2 cells were co-transfected with the indicated proteins and analyzed for SLP-76 phosphorylation and protein expression by WB as before. (B) Quantification of SLP-76 phosphorylation normalized to SLP-76 expression and lane 3 with CD3ζ, Fyn, SYK and SLP-76 (left) or lane 10 with CD3ζ, Fyn, ZAP70 and SLP-76 (right). Expression of CD45 was verified by FACS and expression of PAG was not detectable. (Supplementary results). N=2 for left side (SYK), N=3 for right side (ZAP70)

Src kinases can be controlled by Csk and CD45 [141]. Csk phosphorylates inhibitory tyrosines on SFK, thus inhibiting kinase activity. Csk is recruited to the membrane by the adapter protein PAG [141]. Csk, via its SH2 domain, constitutively associates with tyrosine-phosphorylated PAG, a lipid raft targeted transmembrane adapter protein [142, 143]. PAG expression should therefore also affect SFK function [144]. Upon TCR stimulation, PAG is transiently dephosphorylated, thereby releasing Csk from the plasma membrane [145, 146]. CD45 dephosphorylates inhibitory tyrosines and might be necessary for full SFK activation. In a similar setup expressing CD3ζ, Lck and ZAP70 in HEK cells, a combination of Csk, PAG and CD45 was shown to control Lck and ZAP70 activity [141]. Therefore, we tried co-expressing Csk, PAG and CD45 with either CD3ζ, Fyn, SYK and SLP-76 or CD3ζ, Fyn, ZAP70 and SLP-76 to investigate if this affects SLP-76 phosphorylation (Fig. 13). Co-expression of Csk decreased SLP-76 phosphorylation for both SYK and ZAP70. However, co-expression of PAG did not further decrease the observed SLP-76 phosphorylation. Co-expression of CD45 did not affect SYK-mediated SLP-76 phosphorylation when activated by co-expression of CD3ζ and Fyn, but increased ZAP70-mediated SLP-76 phosphorylation in the same setup. Additionally, co-expression of CD45 seemed to counteract the inhibition of both SYK and ZAP70 activation by Csk.

Despite our attempts to normalize protein expression between samples when a different number of plasmids was co-transfected by including empty pD-vector, we observed stronger expression of SLP-76, SYK and ZAP70 when co-expressed with fewer proteins in this experiment (Fig. 13A). The differences in expression of SLP-76 were taken into account by normalization of p-SLP-76 to SLP-76 for the quantification (Fig. 13B). Thus, the reduced phosphorylation of SLP-76 upon co-expression with Csk for both SYK and ZAP70 could be attributed to their reduced expression. However, additional expression of CD45 together with Csk and PAG in the sample containing the most co-transfected proteins in this experiment showed increased phosphorylation of SLP-76. Therefore at least an activating role of CD45 is likely despite the reduced expression levels of SYK and ZAP70.

This experiment is only representative of 2 independent experiments; therefore no statistical analysis was possible.

## 4.4 Primary and activated NK cells

### 4.4.1 ITAM-receptor cross-linking leads to SYK and Src dependent SLP-76 phosphorylation in NK cells

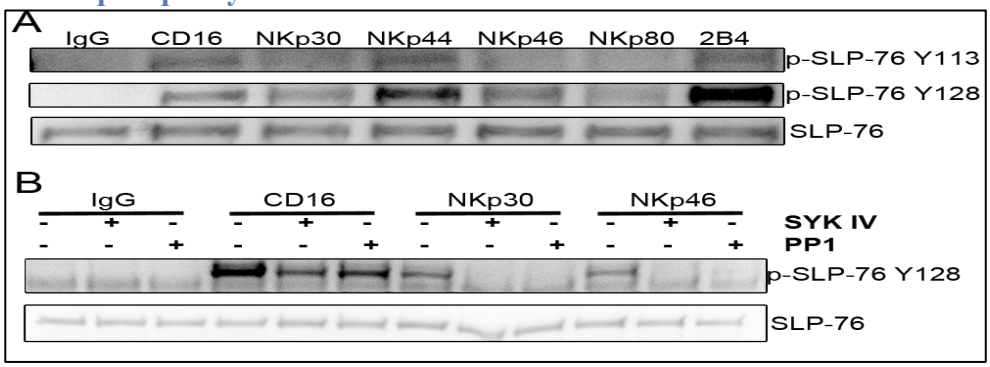


**Figure 14. SLP-76 is phosphorylated in primary and activated NK cells after cross-linking of ITAM-associated receptors.** (A) IL-2 activated expanded human NK cells were pre-incubated with the mAbs specific to the indicated receptors or an isotype control for 30' on ice, followed by cross-linking with secondary goat F(ab')2 anti-mouse IgG at 37°C for 2 min. Cell lysates were analyzed by Western Blot for SLP-76 phosphorylation. (B) Freshly isolated, resting human NK cells were pre-incubated with the indicated inhibitors or DMSO control for 30 min at 37°C followed by incubation with mAbs specific to the indicated receptors or an isotype control for 30 min on ice, followed by cross-linking with secondary goat F(ab')2 anti-mouse IgG at 37°C for 2 min. Cell lysates were analyzed by Western Blotting for SLP-76 phosphorylation. All Western Blots are representative of at least three independent experiments and donors.

Our previous results would predict SLP-76 phosphorylation in a SYK-dependent manner after engagement of ITAM-coupled receptors in NK cells.

To test for the induction of SLP-76 phosphorylation in human NK cells we first used IL-2 activated, cultured human NK cells and stimulated different activating receptors by antibody-mediated cross-linking. We observed phosphorylation of SLP-76 at both tyrosines (Y113 and Y128) after stimulating CD16, NKp30, NKp44 or NKp46, all of which are associated with ITAM-bearing adapter chains (Fig. 14A). Stimulation of NKp80, which signals via a hemi-ITAM, did not result in detectable SLP-76 phosphorylation, whereas the ITSM-containing 2B4 receptor could induce strong SLP-76 phosphorylation at both tyrosines in activated NK cells. Next, we used pharmacological inhibitors to test which signaling pathways are involved in the SLP-76 phosphorylation. In freshly isolated, resting human NK cells, triggering of CD16, NKp30 and NKp46 also resulted in SLP-76 phosphorylation (Fig. 14B). This was blocked by inhibiting SYK or Src-family kinases, suggesting that in these cells the canonical ITAM-based signaling pathway is operational.

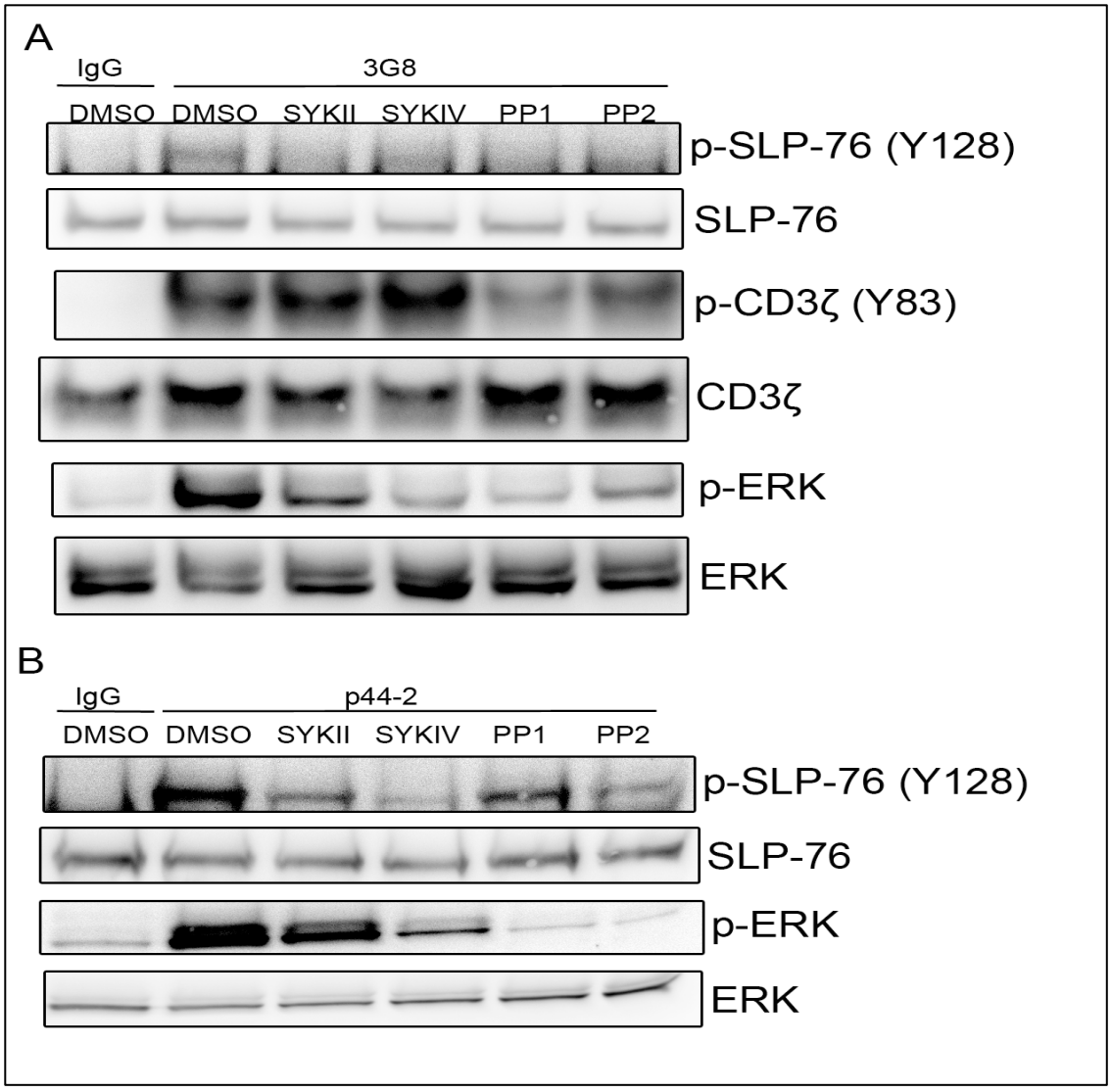


**Figure 15. Stimulation of CD16 and NKp44 in activated NK cells.** IL-2 activated and expanded NK cells were pre-incubated with mAbs specific to either (A) CD16 or (B) NKp44 or an isotype control for 30' on ice, followed by cross-linking with secondary goat F(ab')2 anti-mouse IgG at 37°C for 2 min. Cell lysates were analyzed by Western Blot for SLP-76 and ERK phosphorylation.

To further confirm the results obtained in primary rested NK cells and investigate possible differences in signaling, we stimulated IL-2 activated, cultured human NK cells via CD16 (Fig. 15A, Fig. 16) or NKp44 (Fig. 15B, Fig. 17) with cross-linking by specific antibodies. The presence of inhibitors specific for SYK kinases, SYKII and SYKIV, decreased the phosphorylation of SLP-76 upon receptor cross-linking by both receptors significantly. Inhibitors specific for Src family kinases, PP1 and PP2, also decreased SLP-76 phosphorylation levels upon receptor engagement of CD16 or NKp44.

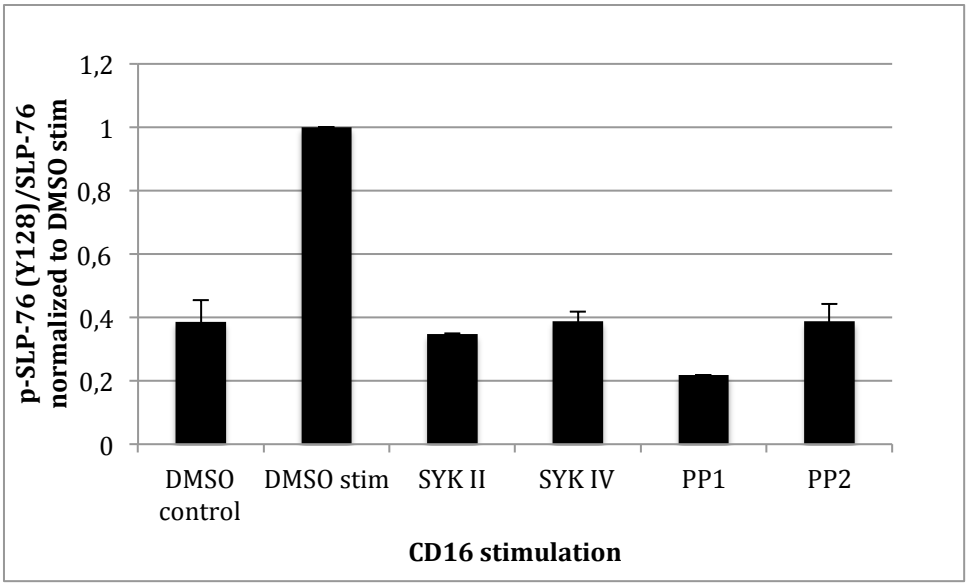


**Figure 16.Quantification of CD16 stimulation from Fig. 15A.** The protein bands were quantified using Image J software (Version 1.47v). The integrated density of each band was measured using the gel analysis function of ImageJ and normalized to SLP-76 and to the DMSO-treated stimulated control. N=2.

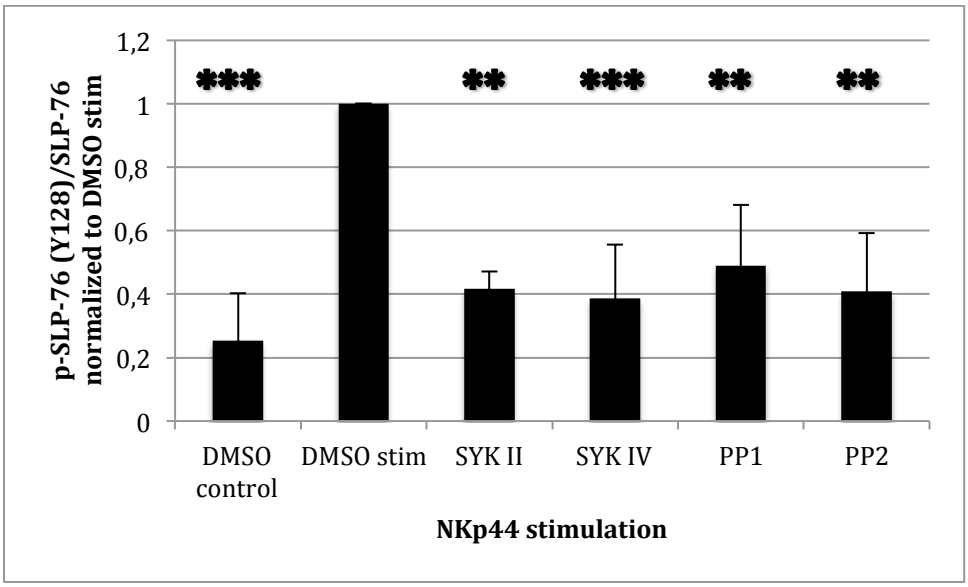


**Figure 17. Quantification of NKp44 stimulation from Fig. 15B.** The protein bands were quantified using Image J software (Version 1.47v). The integrated density of each band was measured using the gel analysis function of ImageJ and normalized to SLP-76 and to the DMSO-treated stimulated control. N=3. Statistical analysis was performed by one-way ANOVA and stars indicate significant differences to DMSO-treated stimulated control.

### 4.4.2 Establishing Phosflow

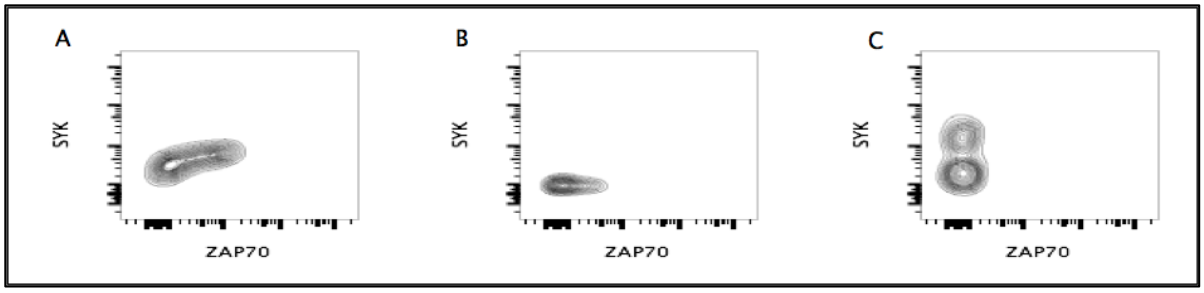


**Figure 18. Differential expression of SYK kinases in human immune cells.** Human PBMCs were analyzed by intracellular staining for expression of SYK and ZAP70. PBMCs were additionally stained for expression of CD3, CD19 and CD56 to identify NK, T and B cells. Expression of SYK and ZAP70 in (A) NK cells (CD3-CD56+), (B) T cells (CD3+CD56-) and (C) B cells (CD3-CD19+). Gating on immune subpopulations as depicted in supplementary results.

The S2 results indicated that ZAP70 activation relies more on SFK than SYK activation (Fig. 11). While NK cells are known to express both SYK and ZAP70, T cells express only ZAP70 and B cells express only SYK. Thus, it would be a nice validation to stimulate these three cell types in the presence of SFK inhibitors and investigate possible differences in SLP-76 or SLP-65 phosphorylation. SLP-65 is the homologue of SLP-76 expressed in B cells [109]. Using intracellular flow cytometry with phospho-specific antibodies (Phosflow), it should be possible to analyze all three cell types in the same sample of freshly isolated, rested human PBMCs without the need for further isolation or separation.

First we examined the expression of SYK and ZAP70 by intracellular flow cytometry in NK cells, T cells and B cells (Fig. 18). As described in the literature [147] we found that NK cells are mostly double positive for both SYK and ZAP70, whereas T cells or B cells express only ZAP70 or SYK, respectively. Thus, comparing the sensitivity of these three cell types to Src and SYK kinase inhibitors upon ITAM-dependent stimulation might provide insights into differential regulation of SYK and ZAP70 by Src family kinases.

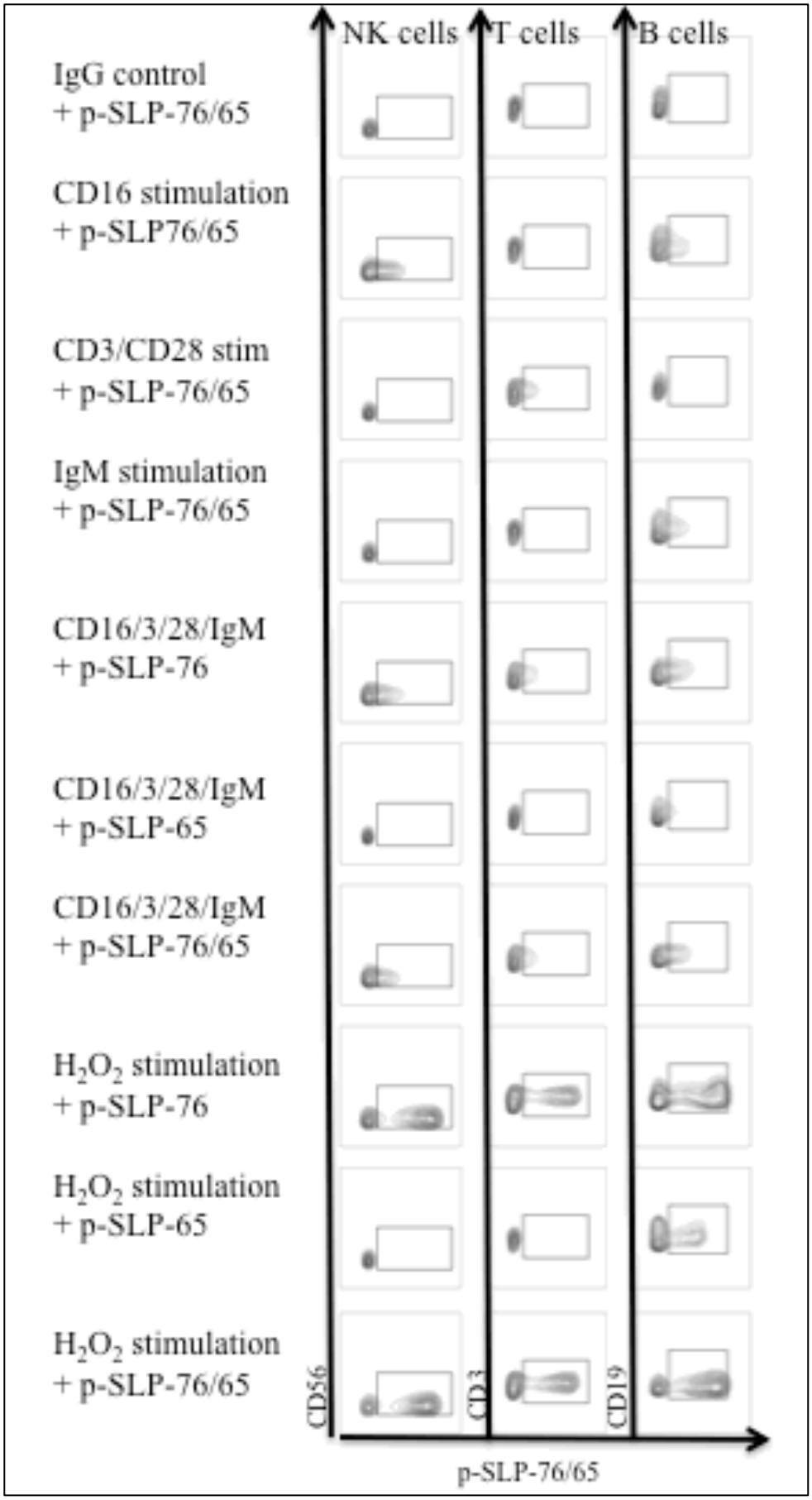


**Figure 19. Preliminary flow cytometry results to establish Phosflow.** Primary, rested human PBMCs were incubated on ice for 30 min with antibodies specific for CD3 (UCHT1), CD28, CD16 (3G8) or isotype control (MOPC-21) as indicated.

Cells were washed and subsequently incubated with secondary goat-amti-mouse and/or IgM as indicated for 15' on ice. To induce cross-linking, the cells were incubated for 2' at 37°C and immediately fixed. To induce maximal phosphorylation, cells were treated with $H_2O_2$/pervanadate for 2' at 37°C- Staining of surface markers and intracellular staining for SLP-76 and/or SLP-65 phosphorylation was performed according to the manufacturers protocol. Lymphocytes were identified by FSC/SSC, NK cells as CD3-CD56, T cells as CD3+CD56- and B cells as CD3-CD19+. For percentage of positive cells see Table 1.

Table 1: Percentage of p-SLP-76/65 positive cells

| IgG | CD16 | CD3/CD28 | IgM | $H_2O_2$ | SLP-76 Y128 | SLP-65 Y84 | NK cells | T cells | B cells |
|---|---|---|---|---|---|---|---|---|---|
| ■ | | | | | ■ | ■ | 0,29 | 0,19 | 2,3 |
| | ■ | | | | ■ | ■ | 40,6 | 0,41 | 19,6 |
| | | ■ | | | ■ | ■ | 0,16 | 17,9 | 2,05 |
| | | | ■ | | ■ | ■ | 1,77 | 0,72 | 19,5 |
| | | ■ | ■ | | ■ | | 32,3 | 16 | 29 |
| | | ■ | ■ | | | ■ | 0,28 | 0 | 8,63 |
| | | ■ | ■ | | ■ | ■ | 28 | 16,2 | 30,3 |
| | | | | ■ | ■ | | 74,2 | 46,2 | 63,6 |
| | | | | ■ | | ■ | 0,44 | 0,09 | 31,2 |
| | | | | ■ | ■ | ■ | 78,6 | 50,2 | 77,7 |

To establish intracellular flow cytometry with phospho-specific antibodies in primary human PBMCs, we first stimulated the populations of interest alone or in different combinations. Additionally, the cells were treated with $H_2O_2$/pervanadate as a positive control (Fig. 19 and Table 1). Furthermore, we used antibodies specific for p-SLP-76 (Y128) or p-SLP65 (Y84) alone or in combination to test their specificity. Ideally, it would be possible to stimulate NK, B and T cells in the same sample to investigate the effects of Src and SYK inhibitors on the different cell types. Therefore we tested the specificity of the stimulation as well as the SLP-76 and SLP-65 phospho-specific antibodies in a preliminary experiment. Stimulation of the CD16 receptor by cross-linking led to a significant increase in p-SLP-65/76 positive cells both in the NK cell and the B cells population. Cross-linking of CD3 and CD28 only increased the percentage of p-SLP-65/76 positive T cells confirming a specific stimulation. Stimulation of B cells by IgM only increased the percentage of p-SLP-65/76 B cells. To test the specificity of p-SLP-76 and p-SLP-65, both conjugated to the same fluorophore AF647, we stimulated PBMCs by cross-linking of CD16, CD3 and CD28 and stained with p-SLP-65 and p-SLP-76 alone or in combination as before. We observed an increase of cells positive for p-SLP-76 in all three populations without p-SLP-65, indicating either unspecific binding or the expression and phosphorylation of SLP-76 in B cells. Of note, recent studies reported the expression of SLP-76 in some B cell subpopulations associated with leukemia [148]. Using only p-SLP-65 while stimulating all three cell types in the same sample increased the percentage of p-SLP-65 positive B cells. To further confirm these results, we stimulated the

cells with $H_2O_2$ /pervanadate to induce maximal possible phosphorylation. All three cell-types showed increased binding to p-SLP-76 alone upon $H_2O_2$ /pervanadate stimulation but only B cells showed binding to p-SLP-65 alone. Using both p-SLP-76 and p-SLP-65 antibodies further increased the percentage of positive B cells compared to p-SLP-76 or p-SLP-65 alone while not significantly increasing the percentage of positive NK cells or T cells compared to SLP-76 alone. To summarize, B cells and T cells can be stimulated specifically by cross-linking CD3/CD28 and IgM and this stimulation simultaneously analyzed by Phosflow, respectively. Given the unspecific binding of p-SLP-76 to B cells and their stimulation by CD16, it was not possible to stimulate all three cell types in the same sample. Thus, we stimulated NK cells and T cells in one sample and B cells in a separate sample while still using PBMCs from the same donor and using the same inhibitor stocks on the same day to gain comparable results.

### 4.4.3 Phosflow: PBMC stimulation with inhibitors

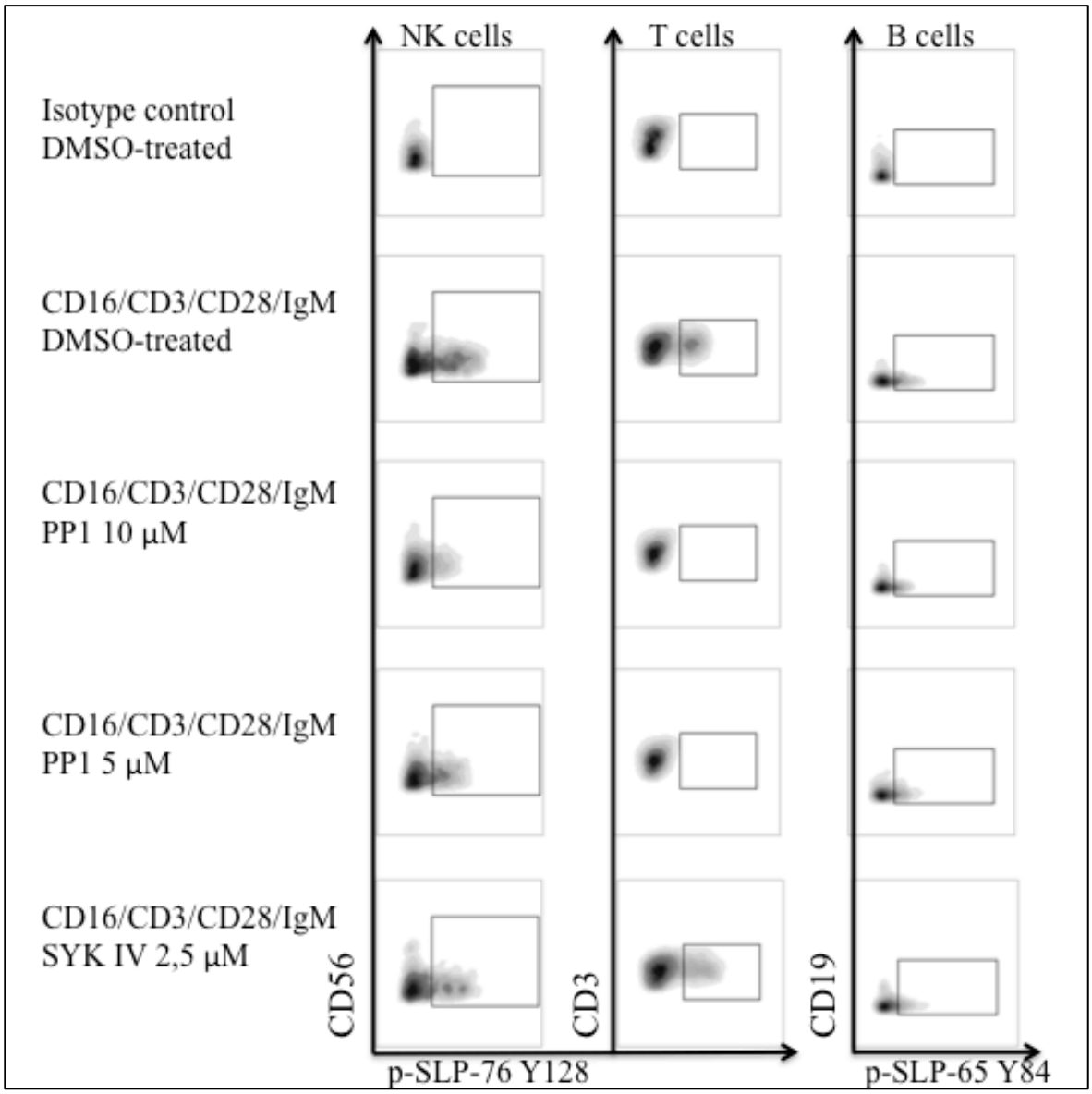


**Figure 20. Inhibitor treatment revealed differential requirements for SFK in NK, T and B cells**. Primary, rested human PBMCs were stimulated with antibodies specific for CD3 (UCHT1), CD16 (3G8) or isotype control (MOPC-21) by cross-linking with secondary goat-anti mouse for 2' at 37°C in the presence of inhibitors or DMSO as before. Representative FACS-analysis of PBMCs stimulated with antibodies inducing NK, T or B cell activation via ITAM-coupled receptors or isotype control.

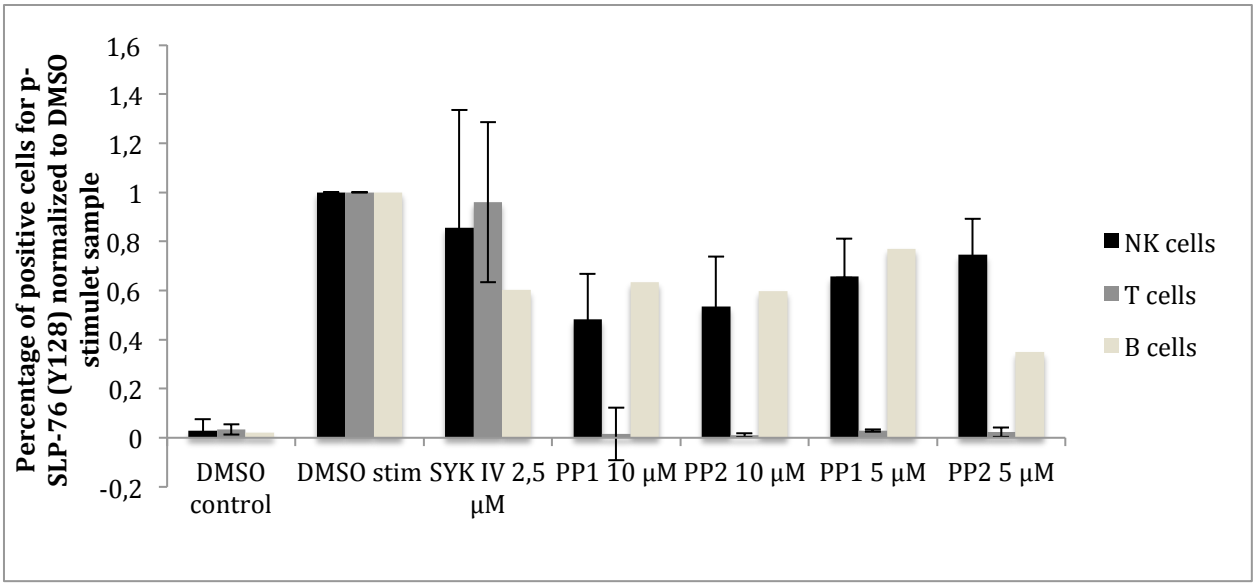


**Figure 21. Quantification of Phosflow results from Fig. 18. N=3 for NK and T cells, N=1 for B cells.**

Next, we investigated the phosphorylation of SLP-76 and SLP-65 in NK and T or B cells in the presence of inhibitors specific for Src family and SYK kinases. Taking advantage of Phosflow, we could detect the phosphorylation of SLP-76 in stimulated NK and T cells in the same sample thereby directly comparing the effects of inhibitors. B cells had to be stimulated and analyzed in a separate sample given the preliminary results indicating unspecific stimulation of B cells by CD16 antibody as well as unspecific binding of the p-SLP-76 antibody. However, the PBMCs for B cell stimulation were prepared next to the NK/T cell stimulation using the same cell and inhibitor concentrations.

Treatment with an inhibitor for SYK kinase but not ZAP70, SYKIV, decreased p-SLP-65 positive B cells while hardly affecting the percentage of p-SLP-76 positive NK or B cells compared to the DMSO-treated stimulated control. Treatment with Src family kinase inhibitors PP1 and PP2 completely abolished the phosphorylation of SLP-76 in T cells at both 10 µM and 5 µM. However, the percentage of p-SLP-76 positive NK cells was reduced but not completely inhibited. At the lower 5 µM concentration, NK cell phosphorylation of SLP-76 upon stimulation was partly restored while still completely inhibited in T cells. B cells seemed even less affected by inhibition of Src family kinases than NK cells. For B cells, the data is only representative of only one donor thus excluding these results from statistical analysis and making further validation necessary.

## 4.5 Supplementary Results

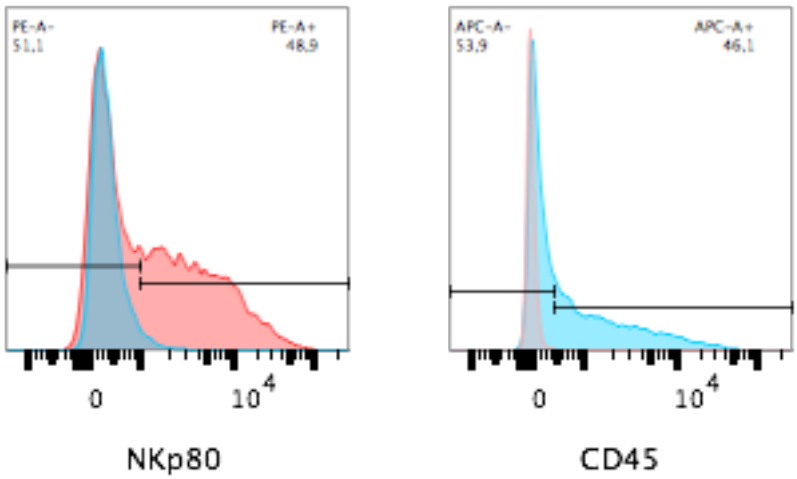


**Figure 22. Supplementary results 1: Expression of NKp80 and CD45. Untransfected stained control in red, examples for co-transfected S2 cells stained for NKp80 or CD45 in blue.**

The expression of NKp80 and CD45 could not be verified by WB. Therefore, we analyzed NKp80 and CD45 expression by FACS. Shown are representative FACS results for expression of NKp80 and CD45 in co-transfected S2 cells.

These results confirmed the expression and membrane localization of NKp80 and CD45 in S2 cells.

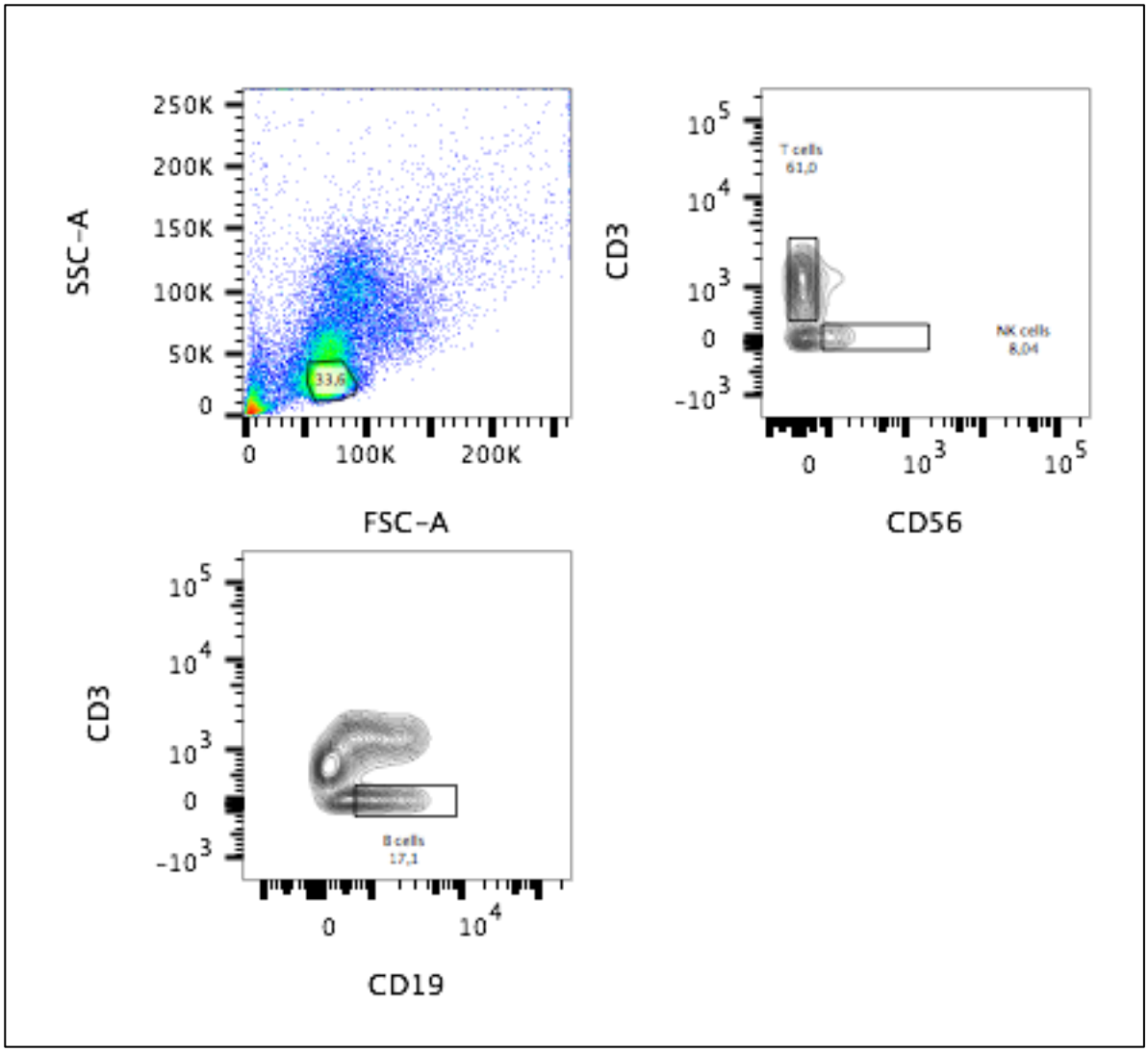


**Figure 23. Gating strategy for Phosflow.** Lymphocytes were identified by FSC/SSC according to size and granularity. T cells were CD3+CD56-, NK cells were CD3-CD56+ and B cells CD3-CD19+. Here, B cells were stained after permeabilization leading to unspecific binding of CD19 in CD3+ cells.

# 5 Discussion

**The S2 system**

**The S2 system as a tool for synthetic biology**

As demonstrated in the first experiment (Fig. 3), S2 cells can be transiently co-transfected with up to 10 different plasmids at once as shown by others [129]. Here we sorted the cells by co-expression of GFP and could therefore show directly that GFP positive cells express the other transfected proteins as well. Co-transfection of more than 3 proteins in S2 cells has only been verified indirectly by co-expression of SHP-1 or for only 2 proteins by flow cytometry [129]. We could directly demonstrate for the first time that S2 cells can be efficiently and transiently co-transfected with up to 10 different plasmids in one sample. This was further confirmed by FACS analysis of transfected receptors 2B4 and CRACC in the same experiment as most cells were double-positive for both. Integration of heterologously expressed adapters like CD3ζ into the cell membrane was thereby confirmed as well as reported by others before. Similar to human immune cells [138], CD16 did not incorporate into the membrane in S2 cells without the co-expression of CD3ζ or FcεR1γ. Taken together, these experiments showed that the S2 system could be used to rebuild and manipulate human signaling pathways by co-expression of heterologously expressed proteins. The evolutionary distance between insect cells and human cells should prevent interactions of intrinsic *Drosophila* proteins with the heterologously expressed mammalian proteins. However, it should be noted that despite their evolutionary distance to humans, S2 cells express many signaling components similar to human cells including homologues of ITAM receptors [149, 150] and Vav1 [151]. Despite our best efforts to control for the activity of *Drosophila* proteins by treatment with inhibitors or $H_2O_2$/pervanadate, we cannot exclude that the possibility that *Drosophila* adapter proteins, phosphatases or kinases take part in our reconstructed pathways. Differences in post-translational modifications between human and insect cells might also affect interactions [152].

**SFK are constitutively active when expressed in S2 cells**

Furthermore, the S2 system proved to be of limited use when investigating pathways involving SFK. As demonstrated by others before and confirmed by our results, SFK are constitutively active when expressed in S2 cells. As this constitutive activation of Src family kinases could be a consequence of elevated $H_2O_2$ levels in S2 cells as suggested by others, we pretreated the S2 cells with the inhibitor DPI before harvesting to reduce $H_2O_2$ production.

Indeed, we observed less autophosphorylation as well as phosphorylation of co-expressed Fyn-targets 2B4 and Vav1 (Fig. 4, 5). However, cross-linking of 2B4 or CD16 did not lead to an increase in 2B4 or CD3ζ phosphorylation even when pretreated with DPI, respectively. While we could reduce autoactivation of Fyn by treatment with an inhibitor reducing $H_2O_2$ levels, this might also interfere with the activation by cross-linking.

As we could be missing essential signaling components to induce significant phosphorylation of 2B4 or Vav1 upon receptor cross-linking, we co-expressed more suspected components. We tried to recreate a 2B4 or CD16 minimal signaling pathway by co-expressing suspected components. For 2B4, we co-expressed the adapter protein SAP, that has been described as critical for activating signaling by 2B4 (Fig. 6). Co-expression of SAP did not influence the observed phosphorylation of 2B4. Additionally, we tried co-expression of other downstream molecules like EAT2, 3BP2, SLP-76, Vav1 and LAT. Co-expression of these proteins together with 2B4 and Fyn did not affect the constitutive phosphorylation of 2B4. Vav1 was phosphorylated every time when Fyn was co-expressed, independently of the co-expression of other proteins. The same could be observed for 2B4 phosphorylation (data not shown). Similar attempts involving CD16 were unsuccessful as well (data not shown). Thus, it was not possible to recreate an inducible minimal signaling pathway involving SFK.

Additionally, we wanted to make this pathway inducible by cross-linking or engagement by its natural ligands. This would provide an additional level of validation for a potential minimal signaling pathway. Experiments co-expressing the 2B4 receptor with several combinations of downstream signaling molecules co-expressed in S2 cells did not result in enhanced phosphorylation when cross-linked by specific 2B4 antibodies or co-expression of the 2B4 ligand CD48 (data not shown).

**The phosphatase SHP-1 is active and specific when expressed in S2 cells**

Phosphatases play a key role in NK cell signaling, not only interfering with signaling by activating receptors but also to attenuate signaling after activation. We tried co-expressing the phosphatase SHP-1 with 2B4, Fyn and Vav1 and observed reduced phosphorylation of Vav1 when SHP-1 was co-expressed (Fig. 7). This indicated that SHP-1 can directly dephosphorylate Vav1 as reported by others [123, 153]. Like Src family kinases, SHP-1 seemed to be constitutively active when expressed in S2 cells. Co-expression of the inhibitory KIR2DL1 receptor containing ITIM motifs in its cytoplasmatic tail did not further enhance SHP-1 activity (data not shown). In contrast to an earlier study where SHP-1 co-expression in

S2 cells did reduce SYK-mediated SLP-65 phosphorylation [129], we did not observe a reduction in SYK-mediated SLP-76 phosphorylation.

To summarize, these results demonstrate that SHP-1 is active when co-expressed in S2 cells and can counteract Fyn-mediated Vav1 phosphorylation. Thus, SHP-1 demonstrated substrate specificity when expressed in S2 cells as it did not dephosphorylate 2B4 or CD3ζ. Therefore, the S2 system can be used to investigate potential targets of SHP-1. In contrast to our findings, SLP-76 has been reported as a substrate of SHP-1 in T cells and NK cells [154]. Additionally, SYK kinases are under control of inhibitory receptors in NK cells, likely mediated by SHP-1 [155, 156]. Moreover, SHP-1 was constitutively active like SFK when expressed in S2 cells. Nonetheless, SHP-1 co-expression did not inhibit SYK activation or dephosphorylated SLP-76 in our experiments. As we could not enhance SHP-1 activity by co-expression of an ITIM motif (data not shown), it was impossible to recreate an inhibitory pathway in S2 cells.

**Limitations of the S2 system**

Taken together, our findings revealed serious limitations in the usefulness of S2 cells to recreate a heterologously expressed signaling pathway. Given the constitutive activation of key signaling proteins like SFK, it was not possible to reconstruct a functional minimal signaling pathway relying solely on SFK for activation. Any given pathway would need to be controlled by selective expression of its components to control for autoactivation. As we established a plasmid-database of well over 50 signaling components relevant for NK cell signaling, it was mathematically impossible to test all combinations. Taking educated guesses, we tested many combinations of NK cell signaling components co-expressed in S2 cells to recreate a functional minimal signaling pathway, but many combinations remained untested and could be used in future experiments. Given the limitations of the S2 system, results have to be carefully controlled. Furthermore, we could not induce or enhance activation via external stimuli.

However, we were able to identify a minimal signaling pathway using SYK kinases for activation, similar to studies before [129]. Our findings were carefully controlled for their dependency on the presence of an activating ITAM motif by selective co-expression and inhibitor experiments.

## Predictions from the S2 system

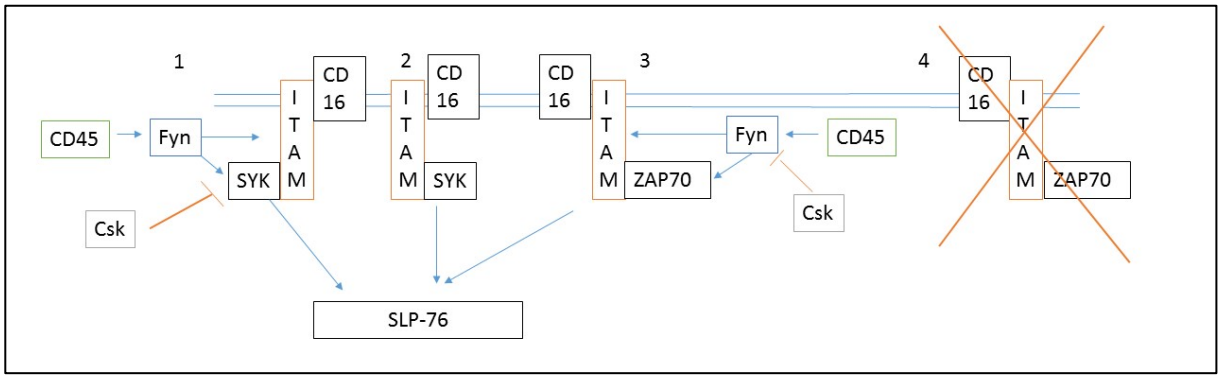


**Figure 24. Proposed signaling pathways. (1) & (3) Canonical SYK/ZAP70 ITAM pathway. Fyn phosphorylates ITAM motifs and activates SYK/ZAP70. Activated SYK/ZAP70 phosphorylates SLP-76. (2) & (4) Non-canonical SYK-based ITAM pathway. SYK phosphorylates the ITAM motifs and is activated. Subsequently, SLP-76 is phosphorylated. ZAP70 alone cannot activate itself or phosphorylate ITAMs in this model.**

We could identify a minimal signaling pathway involving SYK kinases and their activation by ITAMs. Taken together, we were able to indentify three pathways dependent on ITAM co-expression resulting in SLP-76 phosphorylation while we could exclude one possibility (Fig. 25). Additionally to the well described canonical ITAM-SFK-SYK/ZAP70-SLP-76 pathway, we could identify a non-canonical pathway for SYK, but not ZAP70. In this pathway, SYK kinase is activated by a positive feedback loop that can amplify the signal without the need for SFK. Further experiments revealed that only SYK, but not ZAP70 can take part in this non-canonical pathway. Additionally, we could confirm the canonical activation of SYK and ZAP70 by SFK in S2 cells. In this setting, SFK could be negatively regulated by co-expression of Csk or positively regulated by co-expression of CD45. As SFK activate SYK and ZAP70 in the canonical pathway, SLP-76 phosphorylation could be indirectly regulated by their co-expression in this setting. In summary, our results predict the existence of canonical ITAM pathways relying on SFK for SYK and ZAP70 activation. Furthermore, we could construct a non-canonical pathway for SYK with NK cell specific signaling molecules. The observed pathways will now be discussed in detail.

### ITAM – SYK – SLP-76

In our synthetic biology approach we found SLP-76 phosphorylation upon co-expression of an ITAM-containing adapter, SYK and SLP-76 in S2 cells (Fig. 8). Similar to previous studies [129], the initiation of this signaling pathway did not require engagement of a surface receptor and was likely induced by the overexpression of the ITAM-containing signaling chain in the S2 cells. We co-expressed and cross-linked CD16 together with CD3ζ, SYK and SLP-76 but observed no increase in SLP-76 phosphorylation upon cross-linking (data not

shown). While we only saw SLP-76 phosphorylation upon co-expression of ITAM-containing receptors in the S2-cells (Fig. 8), this does not exclude the phosphorylation of SLP-76 upon triggering of non-ITAM activating receptors as other adapters and kinases might build a different signaling pathway. In previous studies SLP-76 phosphorylation was partially induced by cross-linking of non-ITAM receptors such as NKG2D, 2B4 and DNAM-1 ([80] and Fig. 4A). This indicates that these pathways might function independently of SYK kinase. Indeed, NKG2D-DAP10 seems to rely on Src-family kinases [157] and triggers human NK cell cytotoxicity via a SYK-independent pathway [158, 159]. The same pathway has been described in T cells as well [160]. In contrast, triggering of ITAM-based receptors such as CD16 in NK cells induces the phosphorylation and activation of SYK and ZAP70 [161, 162] and these kinases are essential for the function of the receptors [163].

Co-expression of DAP12 together with SYK and SLP-76 induced the strongest activation of SYK and subsequent phosphorylation of its target SLP-76. However, we did not quantify this finding. In chimeric antigen receptor (CAR) construction studies, DAP12 was shown to induce higher cytotoxicity than CD3ζ in this setting [164]. A possible explanation for this finding could be the ability of DAP12 ITAMS to activate SYK better than CD3ζ ITAMs as observed in the present study.

**A non-canonical pathway**

In our S2-cell system SYK-mediated SLP-76 phosphorylation did not require the activity of Src-family kinases. This differs from the canonical ITAM-based signaling pathway, where Src-family kinases are required for ITAM phosphorylation thereby providing a binding site for SYK activation. There have been previous reports for non-canonical ITAM signaling. In platelets, phosphorylation of SLP-76 was shown to be essential for ITAM receptor signaling [165] and SYK kinase was shown to be more important than Src-kinases for this signaling pathway [166]. Additionally, a similar minimal signaling pathway involving just SYK and an ITAM receptor has previously been shown to result in phosphorylation of SLP-65, a SLP-76 related molecule in B cells [129].

**Structural requirement for SH2 domains of SYK and ITAM domains of CD3Zeta**

Our mutagenesis data show that the minimal signaling pathway in S2-cells still relied on the presence of tyrosines in the ITAM receptor and on a functional SH2 domain in SYK (Fig. 10). This indicates that this non-canonical signaling pathway is still dependent on the canonical interaction of the SYK SH2 domains with the (phosphorylated) tyrosines of the ITAM despite

being independent of Src-family kinases. Therefore, SYK may be sufficient for ITAM-phosphorylation under certain conditions. In contrast to earlier studies in B cell activation, we found that both the C terminal and the N terminal SH2 domain of SYK are critical for activation by ITAMs, whereas others identified the C terminal SH2 domain of SYK as the dominant regulatory site [130].

**Kinase specificity**

In line with a previous study [129] we detected CD3ζ phosphorylation in the presence of SYK and lower phosphorylation in the presence of ZAP70 (Fig. 9), supporting this hypothesis. There have been several studies demonstrating that SYK may be more important for the function of ITAM-based receptors in NK cells than ZAP70 [155, 167, 168]. While testing the ability of other kinases involved in NK cell signaling to phosphorylate SLP-76 when co-expressed with CD3ζ, in addition to SYK only ZAP70 demonstrated a weak ability to phosphorylate SLP-76 in S2 cells whereas Fyn, Lck and Csk did not phosphorylate SLP-76 in this setting. Of note, the Src kinase Fyn did phosphorylate CD3ζ significantly more than both SYK kinases SYK and ZAP70. These findings are in line with experiments by other groups that observed SYK mediated phosphorylation of SLP-76 in transfected Sf9 cells and to lesser extent by ZAP70, but not by the Src kinase Lck [169].

**Enhanced by Vav1**

A known interaction partner of SLP-76 is Vav1. While trying to assemble a functional ITAM-based pathway with signaling components downstream of SLP-76, we observed that SLP-76 phosphorylation was significantly enhanced in the previously described CD3ζ, SYK, SLP-76 minimal signaling pathway (Fig. 12). Vav1 is a well described interaction partner of phosphorylated SLP-76 [109, 170]. How Vav1 enhances SLP-76 phosphorylation in the S2 system remained unclear. It could be that binding to Vav1 protects phosphorylated SLP-76 against dephosphorylation by S2 phosphatases. Vav1 could also contribute to SYK activation or facilitate the interaction between SLP-76 and SYK. Interactions between Vav1 and SYK have been reported before [171]. Vav1 has also been reported to enhance SLP-76 microclusters in T cells [172].

**SFK and SYK/ZAP70**

In the S2 system, we found that SYK can also be activated by co-expression of Fyn but this activation was further enhanced by co-expression of CD3ζ (Fig. 11). Co-expression of ZAP70

with SLP-76 alone or with CD3ζ did not result in significant SLP-76 phosphorylation. SLP-76 was phosphorylated by ZAP70 when Fyn was co-expressed with SYK and this was further enhanced by co-expression of CD3ζ. Taken together, these results show that activation of ZAP70 seemed to be more dependent on Src family kinases than SYK, suggesting that ZAP70 is more involved in the canonical ITAM signaling pathway. This may be correlated with the fact that we saw weaker CD3ζ phosphorylation induced by ZAP70 compared to SYK so that additional phosphorylation by Fyn could enhance this signaling pathway (Fig. 9). The dominance of SYK over ZAP70 may therefore also be influenced by the fact that SYK can engage a non-canonical ITAM-based signaling pathway independently of Src-family kinases. SYK has been described as less dependent on SFKs for its catalytic activation, as it is able to trans-autophosphorylate and activate itself, although its activation is visibly enhanced by SFK [173]. In contrast, ZAP70 is highly dependent on SFK for its activation [174]. While SYK and ZAP70 are very similar in structure, each consisting of two SH2 and a kinase domain, ZAP70 has less than 50 percent homology with SYK in amino acid sequence [175]. Other studies have shown that ITAM binding by ZAP70 relieves an auto-inhibitory conformation of ZAP70 and that phosphorylation of specific tyrosine residues within the interdomain separating the C-terminal SH2 domain and the kinase domain (interdomain B) by SFK are important for regulating the activity of ZAP-70 [106, 176].

To further test this difference in regulation by Src family kinases, we treated the S2 cells with inhibitors specific for SYK but not ZAP70 or Src family kinases. Indeed, treatment with SYK kinase inhibitors SYKII and SYKIV decreased SLP-76 phosphorylation when expressed in the CD3ζ, Fyn, SYK, SLP-76 pathway whereas Src family kinases inhibitors PP1 and PP2 did not. In contrast, treatment with Src family kinase inhibitors PP1 and PP2 decreased SLP-76 phosphorylation when expressed in the CD3ζ, Fyn, ZAP70, SLP-76 pathway. In a similar approach, COS cells were transiently co-transfected with different combinations of chimeric CD8 receptor, SYK or ZAP70 and Lyn [177]. Comparable to the observations in this study, SYK was activated when co-expressed with an ITAM motif and able to phosphorylate the ITAM, but ZAP70 relied an activation by the Src kinase Lyn. SYK could also be activated by co-expression with Lyn alone.

Given that SYK can also be activated by Fyn in the S2 system, this could explain why we could not detect any SLP-76 phosphorylation upon SFK-inhibition in NK cells by Western Blot as the canonical pathway seems to induce stronger phosphorylation of SLP-76 than the non-canonical SFK-independent pathway.

**Findings apply to other cell types as well**

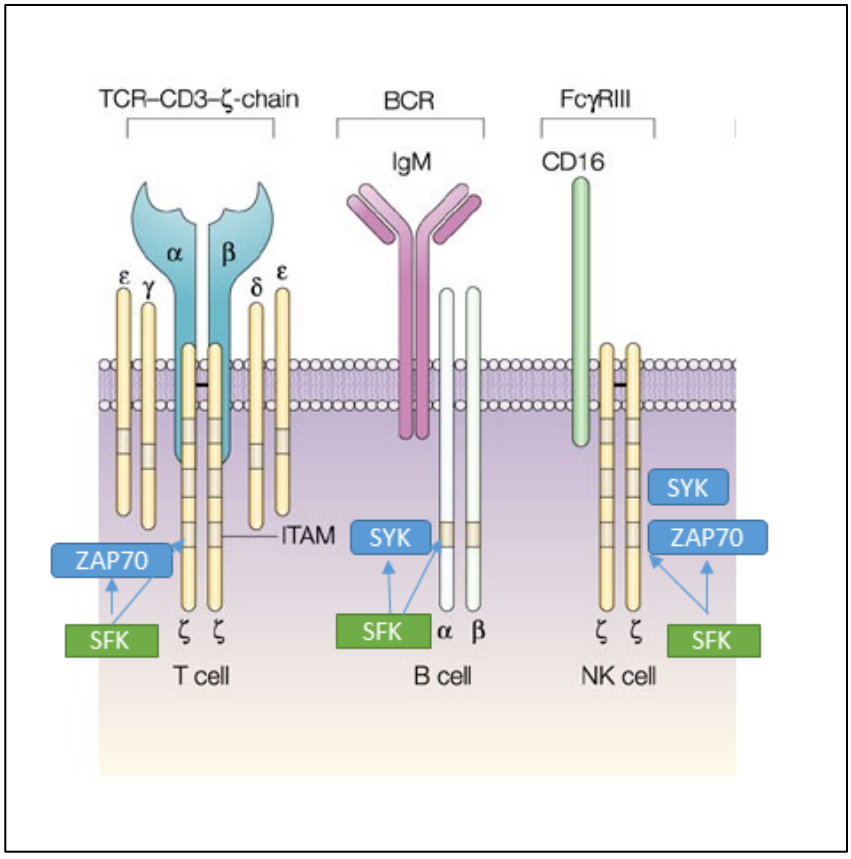


**Figure 25 Major ITAM-associated receptors and their canonical downstream signaling.** The T cell receptor (TCR), which uses the CD3–ζ-chain complex for signalling; the B-cell receptor (BCR), which interacts with the signal-transducing subunits Igα (also known as CD79a) and Igβ (also known as CD79b); the low-affinity Fc receptor for IgG (FcgammaRIII; also known as CD16), expressed by natural killer (NK) cells and associates with dimers of either the ζ-chain or the structurally closely related gamma-chain (the IgE Fc receptor gamma-subunit). Canonically, the respective ITAMs are phosphorylated by SFK, which is followed by recruitment and activation of SYK family kinases to the phosphorylated ITAMs. T cells express the SYK family kinase ZAP70, whereas B cells express the SYK family kinase SYK. NK cells express both SYK and ZAP70. Edited and with permission from [178]. (SFK = Src family kinases)

Our recreated signaling pathways did not rely on the expression of a specific receptor for their activation. Therefore, our findings should apply to all cell types that express these signaling molecules as well. For example, T cells express the CD3ζ chain as part of the TCR as well as ZAP70 and SLP-76 (Fig. 26).

**What does this mean for NK cells?**

Stimulating primary NK cells via activating receptors associated with ITAM-motifs resulted in SLP-76 phosphorylation in primary (Fig. 14) and activated (Fig. 15, 16, 17) NK cells as predicted by the minimal signaling pathway reconstructed in S2 cells. SLP-76 phosphorylation after CD16 stimulation has been reported before [80], but to our best knowledge this is the first report of SLP-76 phosphorylation after cross-linking of NCRs.

SLP-76 phosphorylation could be effectively inhibited by the specific SYK inhibitors SYKII and SYKIV, demonstrating that SYK plays a critical role in SLP-76 phosphorylation in NK cells (Fig. 14, 17, 18). Moreover, SFK inhibitors PP1 and PP2 also inhibited SLP-76 phosphorylation as observed by Western Blot (Fig. 14, 15). This is in line with previous findings that observed inhibition of SLP-76 phosphorylation in primary NK cells after cross-linking of CD16 by PP1 and SYKIV as well [80]. These results would indicate that the canonical pathway relying on SFK kinases for activation of SYK is functional in NK cells. However, results obtained by FACS analysis revealed differential requirements for SFK comparing SYK/ZAP70 expressing NK cells and ZAP70 expressing T cells that will be discussed later. Given the greater sensitivity of flow cytometric analysis compared to Western Blot, these results showed that SLP-76 phosphorylation in NK cells is less reliant on SFK than in T cells. This could be attributed to the expression of SYK in NK cells thus validating the non-canonical ITAM-SYK-SLP-76 pathway observed in S2 cells (Fig. 8).

**How could this affect NK cell receptor synergy?**

It is also interesting to speculate what these results might mean for synergistic signaling by activating NK cell receptors as described before. We observed phosphorylation of both tyrosines Y113 and Y128 after cross-linking of ITAM-associated receptors CD16, NKp30 and NKp46 in primary human NK cells (Fig. 14). To our best knowledge, this is the first time that SLP-76 phosphorylation was investigated after cross-linking of NKp30 and NKp46 in primary human NK cells. Both NCRs are not able to trigger NK cell effector functions alone on primary rested cells. Resting and IL-2-activated NK cells differ in their requirement for co-activation [179] and it was suggested that synergistic SLP-76 phosphorylation is one reason why co-engagement of different activating receptors can stimulate resting NK cells [80]. This could also explain why in cytokine stimulated NK cells engagement of a single activating receptor results in phosphorylation of both tyrosines in SLP-76, resulting in sufficient NK cell activation. This indicates that SLP-76 cannot be the only intersection point of activating signals and NCR signaling therefore probably converges by another mechanism or molecule. Additionally, we observed phosphorylation of tyrosine Y113 and Y128 in activated NK cells when triggered via the 2B4 receptor. Only tyrosine 113 is phosphorylated when rested cells are triggered via 2B4 [80]. However, in activated NK cells engagement of 2B4 alone can trigger NK cell effector functions. Thus, this switch in SLP-76 phosphorylation of 2B4 between primary rested and activated NK cells could provide a molecular basis for the

different requirements of synergistic signals. Respective pathways in primary and activated NK cells leading to SLP-76 phosphorylation by 2B4 and other non-ITAM receptors remain to be investigated. Cytokine stimulated NK cells can also show memory-like responses [17]. Epigenetic regulation was shown to be one reason for the enhanced function of memory-like NK cells [53]. However, a cytokine-induced switch in ITAM-based signaling from a canonical to a Src-independent, minimal SYK-dependent pathway may additionally modify the reactivity of these cells

Cross-linking of NKp80 in activated NK cells did not result in detectable SLP-76 phosphorylation. NKp80 signals through an atypical ITAM motif termed "hemiITAM" in its cytoplasmatic tail that has been reported to have impaired capability to recruit SYK. This could provide an explanation for the observation that NKp80 does not activate SYK in the S2 system (Fig. 8) or phosphorylates SLP-76 upon cross-linking in activated NK cells. However, we and others observed SLP-76 phosphorylation induced by cross-linking of 2B4, that signals through ITSM motifs that do not recruit SYK either.

**Comparing NK, T and B cell signaling in primary cells**

As knockdown by siRNA in primary NK cells is hard to achieve, we compared the sensitivity of NK cells to SYK and SFK inhibitors with other immune cells only expressing SYK or ZAP70 by flow cytometry. A key advantage of this approach is the simultaneous stimulation and analysis of NK cells and T cells in the same sample. This enhances comparability as both cell types were stimulated in the presence of the exact same inhibitor concentrations and antibodies for flow-cytometric analysis. Furthermore, PBMCs are more representative of the *in vivo* situation as possible interactions with other mononuclear blood cells are still possible. B cells could not be analyzed in the same sample because of unspecific binding of the p-SLP-76 antibody.

In contrast to our findings by Western blot, flow cytometric analysis by intracellular staining did reveal differences in SLP-76 phosphorylation of primary NK cells after CD16 stimulation and inhibitor treatment (Fig, 21, 22). As flow cytometry can be conducted with 10-15 times fewer cells than Western Blotting, it could be that we did not load enough cells on our gels to see low level differences in SLP-76 phosphorylation after inhibitor treatment. Another possible explanation for this difference could be the lower threshold of antibodies binding to a given antigen for detection by flow cytometry. Therefore, we might detect lower levels of phosphorylation by flow cytometry than by Western Blot. In an earlier study, PP1 inhibited SLP-76 phosphorylation by CD16 cross-linking in primary NK cells significantly more than

SYKIV [80]. This difference was observed by Western Blot. However, we detected differences by flow cytometry for NK cell inhibition as well as between NK cells and T cells (and one B cell donor).

The same concentration of SYKIV that seemed to block SLP-76 phosphorylation in isolated primary or expanded activated NK cells after CD16 cross-linking did not block SLP-76 phosphorylation in NK cells when cross-linked via CD16 in PBMCs when analyzed by flow cytometry. This could be attributed to a lower detection threshold of phosphorylated SLP-76 by flow-cytometry and would likely be mediated by the ITAM/SFK/ZAP70 pathway. In line with this theory, B cells are more and T cells less affected by the same SYKIV concentration upon stimulation, although both not statistically significant. In line with these results, a previous study compared the effects of SYKIV treatment on B cell and T cell calcium flux upon activation and found that this inhibitor only reduced calcium flux in B cells [140]. A potential other explanation could be unspecific binding of the p-SLP-76 antibody to other unaffected phosphorylated proteins which we observed regularly by Western Blot. It could also be the case that NK cells are less resistant to the SYKIV inhibitor when isolated than as PBMCs.

Moreover confirming the differential regulation by SFK of SYK and ZAP70, inhibition of SFK revealed significant differences in SLP-76 phosphorylation levels between NK cells and T cells.

Given the possible pathways predicted by the S2 system, these differences in sensitivity to SYK and SFK inhibitors could be explained by expression of SYK and ZAP70. In the S2 system, SYK phosphorylates SLP-76 by a SFK-independent pathway consisting of an ITAM, SYK and SLP-76. Additionally, SYK could be activated and subsequently phosphorylated SLP-76 by the canonical pathway involving SFK. ZAP70 could only phosphorylate SLP-76 by the canonical ITAM-SFK-ZAP70-SLP-76 pathway. Therefore, our findings postulate three ITAM-based minimal signaling pathways that result in SLP-76 phosphorylation.

NK cells express both SYK and ZAP70, thereby inhibition of SYK still leaves the ZAP70 pathway to phosphorylate SLP-76. Inhibition of SFK by PP1 and PP2 abolishes the ZAP70 pathway relying on activation by SFK, leaving only the SFK-independent SYK pathway to phosphorylate SLP-76. Thus, these findings seem to confirm the functionality of the non-canonical SFK independent ITAM-SYK-SLP-76 pathway in primary NK cells. This is further validated by the observation that SLP-76 phosphorylation was completely abrogated in T cells stimulated in the same sample. However, this could also be explained by differences in resistance or uptake of the inhibitors.

While our Phosflow results for B cells are only representative of one donor, they seem to confirm that B cells are more resistant to inhibition of SFK than T cells or NK cells. In this single experiment with B cells, B cell sensitivity seemed highest to inhibition by SYKIV and least affected by inhibition of SFK compared to both T and NK cells. This would further confirm that ZAP70 expressing cells rely more on SFK than SYK expressing cells. In line with these findings, a recent study found that PP1 could block calcium flux in T cells but not in B cells upon receptor cross-linking [140].

If BCR signaling in B cells can be induced by a Src-independent pathway only by SYK is still debated. Some studies suggest that both pathways are possible [129], others report that only the canonical pathway involving Src family kinases is functional in B cells [180-182]. A recent study described differences in dependency on Src family kinase for SYK activation when the BCR was stimulated by antibody or ligands [183]. While antibody-induced stimulation was only delayed but not abolished when Src kinase activity was inhibited by PP2 or a constitutively active LAT-Csk construct, ligand-induced BCR stimulation was completely abrogated when Src family kinases were inhibited [183]. Therefore, differential regulation of SYK by SFK could calibrate the threshold for BCR receptor activation. Further supporting this proposed mechanism to fine-tune the BCR response, ligation by monovalent or multivalent ligands seems to be differentially regulated by an interplay of SYK and SFK in B cells as well [184]. It is interesting to speculate if a similar mechanism applies to NK cells. As NK cells additionally express the SYK kinase ZAP70, functional differences could be regulated by expression of SYK and ZAP70. Both SYK and ZAP70 function as adapter proteins as well [166, 185], therefore differences in interaction partners could enhance respective specific signaling pathways initiated by SYK or ZAP70. Loss of SYK expression has been associated with memory-like NK cells after HCMV infection [186]. Lck, Fyn and Zap70 deficient murine NK cells still have natural cytotoxicity mediated by SYK [155].

In another study, the absence of CD45 affected ZAP70 but not SYK signaling in murine NK cells [167]. In murine studies, SYK was indispensable for DAP12 signaling but ZAP70 not necessary [168]. Many other cell types have been reported to be affected by differential expression of SYK and ZAP70 that can affect cell functions. In osteoclasts, SYK is critical for cytoskeleton organization. Osteoclasts deficient for SYK could be rescued by expression of SYK, but not ZAP70 as evidenced by cytoskeleton organization and SLP-76 phosphorylation. Expression of ZAP70 even inhibited SYK-mediated phosphorylation of SYK, Vav3 and SLP-76 [187]. In platelets, the SLP-76 tyrosines are important for ITAM-

signaling [165] and SYK seems to be more important than Src for activation and SLP-76 phosphorylation [166].

**Control by Csk, CD45**

The kinase Csk and the phosphatase CD45 reciprocally regulate phosphorylation of the inhibitory tyrosine of the Src family kinases Lck and Fyn [105, 188, 189]. CD45 can desphosphorylate the negative regulatory tyrosine of Src family kinases [105] while it is phosphorylated by Csk. The adapter protein PAG is critical for membrane recruitment of Csk to receptor complexes [141]. In a similar approach utilizing HEK-cells, co-expression of CD45, Csk and PAG with CD3ζ, ZAP70 and Lck controlled CD3ζ and activating Lck/ZAP70 phosphorylation [141]. Therefore, we co-expressed CD3ζ, Fyn, SYK or ZAP70, SLP-76 with combinations of CD45, Csk and PAG. We found that co-expression of Csk inhibited SLP-76 phosphorylation mediated by both SYK and ZAP70 in S2 cells, presumably by inhibiting SFK (Fig. 13). As SYK was co-expressed with Fyn in this experiment, inhibition of Fyn by Csk was sufficient to block SYK activation. While the non-canonical pathway should still be functional, Fyn-mediated activation of SYK could induce a stronger phosphorylation. Co-expression of PAG, reported by others as necessary for membrane recruitment of Csk [141], did not further reduce SLP-76 phosphorylation levels. However, expression of PAG could not be verified as there was no commercial antibody available. Co-expression of CD45 counteracted Csk and restored SLP-76 phosphorylation and presumably enhanced it when co-expressed in the ZAP70 pathway. These results showed that SFK can be regulated reciprocally by Csk and CD45, directly affecting SFK-mediated activation of SYK and ZAP70 when co-expressed with ITAMs. Our results could provide the molecular basis for previous obervations by others as discussed. The non-canonical ITAM-SYK-SLP-76 pathway without co-expression of Fyn was not affected by CD45 or Csk co-expression in S2 cells (data not shown).

Studies in T-cell lines and in the CD45-deficient mouse underline the importance of CD45 for SFK activity. CD45-deficient T-cell lines contain Lck and Fyn molecules that are hyperphosphorylated at their C-terminal negative regulatory tyrosines and demonstrate a significant reduction in TCR-stimulated phosphotyrosine induction [190, 191]. CD45-deficient mice have few peripheral T cells since TCR signaling during thymic development is greatly impaired [192, 193]. However, B cell development was not impaired in these studies, further supporting our model if differential regulation of SYK and ZAP70 expressing cell types.

In CD45 deficient murine NK cells, CD45 independent ITAM signaling is mediated by SYK, but not by ZAP70 [167]. A possible molecular basis for this report could be the non-canonical SYK-pathway described in this study. In the absence of CD45, SFK cannot be activated as the inhibitory tyrosine is not dephosphorylated, thus only the non-canonical SYK activation without SFK remains active. Signaling pathways in CD45-deficient NK cells are dysregulated and lead to differentially regulated cytotoxicity and cytokine production [194]. Expression of different CD45 isoforms [195] might also affect NK cell effector functions via regulation of SFK and subsequently SYK family kinases.

**Concluding remarks**

The initial findings regarding the ITAM-SYK-SLP-76 pathway are very similar to a study conducted in S2 cells as well but using more B cell specific ITAMs and SLP-65 [129]. Nevertheless, we could confirm some of these findings with NK cell specific signaling components while also revealing additional differences in the regulation of SYK and ZAP70 by SFK. We could successfully reconstruct a functional non-canonical minimal signaling pathway in S2 cells. Additionally, we could demonstrate that activation of ZAP70 relies on SFK as in the canonical ITAM pathway. SYK could also be activated by the canonical pathway. Furthermore, activation of ZAP70 could be reciprocally regulated via Fyn by Csk and CD45. We could confirm the existence of all three postulated pathways in primary immune cells taking advantage of the differential expression of SYK and ZAP70 in NK, B and T cells. SYK expressing NK and B cells were more resistant to inhibition by SFK inhibitors than ZAP70-only expressing T cells, providing evidence for the non-canonical ITAM-SYK-SLP-76 pathway. While we could not validate the regulation of the canonical pathway by Csk and CD45 in primary immune cells, our findings could provide the molecular basis for observations by others [167] in the absence of CD45. Taken together, our data support the existence of a non-canonical ITAM-SYK-SLP-76/SLP-65 pathway while also confirming the canonical ITAM-SFK-SYK/ZAP70-SLP-76/SLP-65 pathway and its reciprocal regulation by Csk and CD45. However, given the possibility that primary immune cells were stimulated by cross-linking with specific antibodies, we cannot exclude that the non-canonical pathway is an artifact induced by the artificial and strong stimulus.

# 6 Outlook

Given the observed limitations of the S2 system for signaling studies, the obtained results should be taken with care. While we could elucidate differences in regulation of SYK and ZAP70 in S2 cells and validate these results by treatment of primary immune cells with inhibitors, further validation by other methods like selective siRNA knockdown of either SYK or ZAP70 should be conducted. These experiments could provide further insight into how SYK and ZAP70 affect NK cell effector functions, which have not been investigated in this study.

Additionally, the results obtained with primary cells were induced by cross-linking. As cross-linking by antibodies is an artificially strong stimulus, this could induce signaling pathways that do not exist *in vivo*. Our observed non-canonical ITAM-SYK-SLP-76 pathway could be such an artifact. Therefore, further studies with natural ligands have to be performed to test the validity of this pathway *in vivo*.

Richard Feynman:

“We are at the very beginning of time for the human race. It is not unreasonable that we grapple with problems. But there are tens of thousands of years in the future. Our responsibility is to do what we can, learn what we can, improve the solutions, and pass them on.” [196]

# 7 Methods

## 7.1 Cell culture

### 7.1.1 NK cell isolation and culture

Human blood samples from healthy donors were drawn for research purposes at IfADo. All donors gave written consent. All media and supplements were from Thermo Fisher Scientific, unless stated otherwise. Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll density gradient centrifugation (PAN-Biotech, Germany). Human NK cells were purified from PBMCs using the Dynabeads Untouched Human NK Cell kit (Thermo Fisher Scientific) according to manufacturers instructions. For NK cell activation and expansion, purified NK cells were cultured in 96-well round-bottom plates (Nunc) with irradiated K562-mbIL15-41BBL (kind gift from Dario Campana) in IMDM Glutamax supplemented with 10% FCS and 1% Penicillin / Streptomycin, IL-2 (100 U/ml, NIH Cytokine Repository) and IL-15 (5 ng/ml, Pan Biotech). IL-21 (100 ng/ml, Miltenyi Biotec) was added at the first day. NK cells were between 90% and 99% $CD3^-$, $CD56^+$ and $NKp46^+$ as assessed by flow cytometry.

### 7.1.2 Schneider cell culture and transient transfection

Schneider S2-cells (Invitrogen) were grown in Schneider's *Drosophila* medium (Serva) supplemented with 10% FCS at 26°C without $CO_2$. Cells were passaged every 2 to 3 days to maintain exponential growth. S2 cells were transfected using Lipofectamine 2000 (Life Technologies, Inc.). For transfections, the S2 cells were seeded in a 6-well plate (Sarstedt) at $1 \times 10^6$ per well in 3 ml medium and incubated over night. Supercoiled plasmids (0.3 µg of each plasmid were adjusted with pRmHa-3 to equal DNA concentrations per well) were complexed with lipid (6 µl Lipofectamine 2000 Reagent) in 0.5 ml OptiMEM Glutamax medium (Life Technologies, Inc.). The complex was incubated at room temperature for 30 min and then added drop-wise to the cells. After 24-48 h $CuSO_4$ (0.5 mM) was added to the culture for 36-48 h to induce the expression of proteins. For inhibitor experiments, SYKIV inhibitor (Calbiochem, 2.5 µM), PP1 (Biomol, 10 or 5 µM) or DMSO solvent control were added to the cells 1 h prior to harvesting. $CuSO_4$-induced GFP+ cells were enriched by cell sorting (BD Jazz). Purity of GFP positive cells was greater than 80% after enrichment. Receptor expression was analyzed by FACS using the following antibodies: 2B4-APC (C1.7), CRACC-PE (162.1), NKp80-PE (5D12) all from Biolegend. All samples were measured on a LSR Fortessa and data were analyzed using the FlowJo software (TreeStar, Inc.). To inhibit

$H_2O_2$ production, S2 cells were incubated for 1 hr with DPI (Diphenyleneiodonium chloride) solved in DMSO (final concentration: 16.7 µg/ml or 53 µM).

## 7.2 Molecular biology and biochemisty

### 7.2.1 SDS-PAGE and Western Blotting (Wet blot)

Equal cell numbers of enriched GFP+ S2-cells or NK cells were lysed on ice in lysis buffer (0,5% Triton X, 2 mM EDTA, 10 mM NaF, 20 mM Tris-HCl, 150 mM NaCl, 10% (v/v) glycerol, pH 7,3) supplemented with proteinase and phosphatase inhibitor cocktails (Roche). Cell lysates were cleared by centrifugation and the supernatants mixed with reducing SDS sample buffer (5x concentrated buffer was added to the sample in a 1:5 ratio). The proteins were denatured by boiling the lysates for 5 min at 95°C. The proteins were separated by discontinuous SDS-PAGE [197]. The acrylamide concentration was chosen depending on the size of the proteins of interest. A higher percentage of acrylamide leads to a better separation of proteins of lower molecular weight, whereas lower percentages are optimal for the separation of proteins with higher molecular mass. Usually a acrylamide concentration of 10% or 12% was used.

A PVDF membrane (ImmobilonTM-FL, Millipore) was activated for 1 min in 100% MetOH. Whatman paper was also incubated for 1 min in the transfer buffer. A stack was built up with a sponge with three Whatman paper each on top and bottom, PVDF membrane and SDS gel in between. The transfer was performed in a transblot tank (BioRad) in transfer buffer. Since the PVDF membrane surface binds any protein, free binding sites were blocked by incubation of the membrane for 1 h at RT with 5% milk powder in PBS-T. Afterwards, the membrane was washed three times for 5 min with PBST and incubated for 1 h at RT or 4°C overnight with the indicated primary antibodies. After washing to remove unspecifically bound antibodies, bound primary antibody was detected by a horseradish peroxidase (HRP) labeled secondary antibody. HRP oxidizes its substrate luminol to an instable compound that emits light while decaying. The chemoluminescence was detected using a Fusion Fx7 (Filber).

*Table 2: 10% SDS gels*

| Stacking gel | | Separating gel (10%) | |
|---|---|---|---|
| $H_2O$ | 4.45 ml | $H_20$ | 3.4 ml |
| Upper buffer 4X | 1.9 ml | Lower buffer 4X | 2 ml |
| Acrylamide | 1.2 ml | acrylamide | 2.7 ml |
| 10% APS | 75 µL | 10% APS | 50 µL |
| TEMED | 7.5 µL | TEMED | 7.5 µL |

*Table 3: 12% SDS gels*

| Stacking gel | | Separating gel (12%) | |
|---|---|---|---|
| $H_2O$ | 4.45 ml | $H_20$ | 2.8 ml |
| Upper buffer 4X | 1.9 ml | Lower buffer 4X | 2 ml |
| Acrylamide | 1.2 ml | acrylamide | 3.2 ml |
| 10% APS | 75 µL | 10% APS | 75 µL |
| TEMED | 7.5 µL | TEMED | 7.5 µL |

Upper buffer: 0.5 M Tris, 0.4%SDS in dH2O, pH 6.8
Lower buffer: 1.5 M Tris, 0.4% SDS in $dH_2O$, pH 8.8

**NK cell stimulation**

For inhibitor experiments, NK cells were preincubated with the indicated inhibitors or solvent control for 30' at 37°C and subsequent steps conducted in the presence of inhibitors. For antibody-mediated crosslinking, rested or activated NK cells were preincubated with the following antibodies at 10 µg/ml for 30 min on ice: NKp80 (5D12), NKp46 (9E2), CD16 (3G8) from Biolegend. NKp30 (p30-15), NKp44 (p44-8) were produced in our lab. After washing with medium, NK cells were stimulated by crosslinking with goat anti-mouse $F(ab')_2$ Ab (20 µg/ml, Dianova) at 37°C for 2 min, washed in ice-cold PBS, lysed and analyzed by Western Blotting as described above.

### 7.2.2 Cloning

For transient co-transfection experiments in *Drosophila* Schneider cells, the expression vector pRmHa-3 (Fig. 26) containing an inducible metallothionein promoter [137] was used. Some vectors were obtained from BIOSS.

The cDNAs of human NK cell signaling components were inserted into pRmHa-3 (Fig. 27) and verified by sequencing (GATC Biotech).

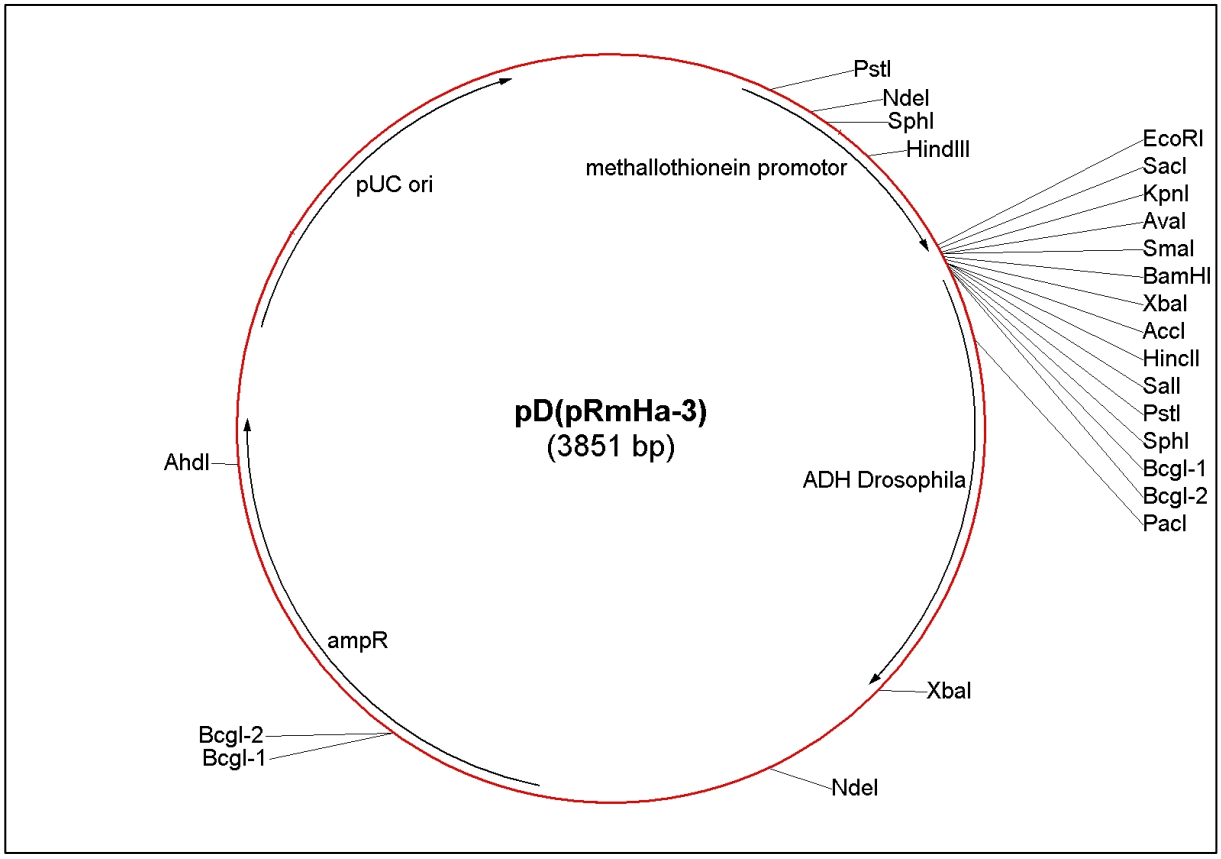


**Figure 26. Map of pRmHA-3**

List of plasmids cloned in our lab:

| Plasmid | Insert | Cloning sites | Cloned by |
|---|---|---|---|
| pD-DAP10 | NM_014266 | | Mina |
| pD-DAP12 | NM_003332.3 | SalI/NotI | Mina |
| pD-NKp80 | NM_016523 | SalI/NotI | Mina |
| pD-GFP | | | Simone |
| pD-SAP | NM_002351 (SAP) | SalI/NotI | Frank |
| pD-EAT2 | NM_053282 (EAT2) | SalI/NotI | Frank |
| pD-LCK | NM_005356 | SalI/NotI | Mina |
| pD-SLP76 | NM_005565 | SalI/NotI | Mina |
| pD-VAV1 | NM_005428.3 | SalI/NotI | Mina |
| pD-SHIP1 | NM_001017915.2 | KpnI/BamHI | Mina |
| pD-CD16 | NM_001127592.1 | EcoRI/NotI | Mina |
| pD-NKp30 | | SalI/NotI | Frank |
| pD-NK46 | | SalI/NotI | Frank |

| | | | |
|---|---|---|---|
| pD-NKG2D | | | Christian |
| pD-2B4 | NM_016382.3 | SalI/NotI | Mina |
| pD-CRACC | NM_021181 | SalI/NotI | Mina |
| pD-KIR2DL1 | NM_014218 | SalI/NotI | Mina |
| pD-FceR1G | NM_004106 | | Mina |
| pD-LAT | | | Mina |
| pD-GRB2 | NM_002086.4 | SalI/NotI | Mina |
| pD-PAG1-FLAG | | EcoRI | Mina |
| pD-3BP2 | NM_003023.4 | SalI/NotI | Mina |
| pD-CrkL | NM_005207.3 | SalI/NotI | Mina |
| pD-ITK | NM_005546.3 | SalI/NotI | Mina |
| pD-BTK | NM_000061.2 | SalI/NotI | Mina |
| pD-CSK | NM_004383 | SalI/NotI | Mina |
| pD-PLCG1 | NM_002660 (silent mutation C-> A) | SalI/NotI | Mina |
| pD-CD48 | NM_001778.3 | EcoRI/SalI | Mina |
| pD-p85 | NM_181523.2 | SalI/NotI | Mina |
| pD-p110 | NM_005026.3 | SalI/BamHI | Mina |
| pD-CD45RABC | | | Mina |
| pD-CD45RO | | | Mina |

### 7.2.3 Flow cytometric analysis (FACS)

For flow cytometric analysis, S2 cells were washed with FACS buffer and resuspended in 100µl FACS-buffer. After staining for 20' on ice, cells were washed and analyzed by flow cytometry. The FACS analysis was performed using a BD LSR Fortessa flow cytometer (BD Biosciences). Cell sorting was performed on a BD Jazz (BD Biosciences). The data analysis was done with FlowJo v. 6.1.1 analysis software (Tree Star Inc.).

**Phosflow stimulation and staining**

For detection of phosphorylated intracellular proteins by FACS, BD Phosflow was applied according to the manufacturers instructions.

Freshly isolated PBMCs were preincubated on ice with primary antibodies against CD16 (3G8), CD3-PerCP (UCHT1) and/or CD28 for 30'. After washing, the PBMCs were incubated with secondary goat anti mouse IgG. For BCR stimulation, cells were preincubated with IgM. Cells were incubated at all times in the presence of inhibitors at the indicated concentrations or DMSO vehicle control. To induce stimulation, the cells were incubated for 2' at 37°C and immediately fixed with BD Cytofix buffer for another 10' at 37°C. Subsequently, the cells were washed and permeabilized by adding BD Phosflow Perm Buffer II for 30' on ice. Cells were then washed twice and stained with the following antibodies: p-SLP-76 Y128 AF647, p-SLP-65 AF647, CD56 BV421, CD19 AF700 (all from BD), CD3 PerCP (Biolegend). Cells were washed and analyzed on a BD LSRFortessa.

# 8 Material

## 8.1 Buffers and solutions

| | |
|---|---|
| Blocking buffer | |
| ECL solution 1 | 250 mM luminol (Sigma-Aldrich), 90 mM p-coumaric acid (Sigma-Aldrich) in 0.1 M Tris-HCl pH 8.5 |
| ECL solution 2 | 0.1% $H_2O_2$ in 0.1 M Tris-HCl pH 8.5 |
| FACS buffer | PBS + 2% FCS |
| FACS washing buffer | PBS + 2% FCS |
| FCS | Gibco |
| Lysis buffer | 0.5% Triton X, 2 mM EDTA, 10 mM NaF, 20 mM Tris-HCl, 150 mM NaCl, 10% (v/v) glycerol, pH 7.3 |
| PBS | Gibco |
| Phosflow fixation buffer | BD Phosflow Fixation buffer |
| Phowflow permeabilization buffer | BD Perm 2 |
| Primary antibody solution | 5% BSA, 0,1% $NaN_3$ in PBST |
| SDS-PAGE running buffer | MOPS buffer (Invitrogen) |
| SDS-PAGE sample buffer | 300 mM Tris, 46% glycerol, 10% SDS, β-mercaptoethanol, bromophenol blue in dH2O |
| Secondary antibody solution | PBST |
| Transfer buffer | |
| WB washing buffer | PBS + Tween 20 |

## 8.2 General equipment

| | |
|---|---|
| Autoclave | Systec DX-150 |
| CASY cell counter | Modell TT, Roche Diagnostics |
| Cell culture 6 well plates | Sarstedt |
| Centrifuges | Heraeus Fresco 21 (Thermo Scientific)<br>Heraeus Megafuge 40R (Thermo Scientific) |
| Falcon tubes | Sarstedt |
| Filtration systems, sterile | Stericup, Milipore Corporation, Bedford, MA, USA |
| Flow cytometers | BD LSR Fortessa<br>BD FACSJazz |
| Incubators | HERAcell 240i, Heraeus Instruments GmbH, Fellbach Germany |
| pH-Meter | PB-11 (Sartorius) |
| Pipetboy | Accu Jet Pro (Brand) |
| Pipettes | Gilson |
| Power supply for SDS-PAGE/WB | Power Pac (Biorad) |
| SDS-PAGE and Tank-Blot equipment | Biorad |
| Sterile hood | SAFE 2020; Thermo Scientific |
| Thermomixer | Thermomixer comfort (Eppendorf) |
| Vortex | IKA; Bioblock Scientific |
| Western Blot detection | Vilber Fusion Fx 7 |

| | |
|---|---|
| Purified water | Milli Q Direct 8 (Millipore) |

## 8.3 Chemicals

| | |
|---|---|
| Dimethyl sulfoxide (DMSO) | J.T. Baker / Sigma |
| Acrylamid | Roth |
| Ammonium persulfate | Roth |
| Bovine serum Albumin (BSA) fraction V | Roth |
| Calciumchlorid-dihydrate (S2 supplement) | Merck |
| EDTA | |
| Ethanol | Roth |
| Methanol | Roth |
| Milk powder | Saliter |
| PageRuler™ Prestained Protein Ladder, 10 to 180 kDa | Thermo Fisher Scientific #26616 |
| Phosphatase inhibitor cocktail | Roche |
| Protease inhibitor cocktail | Roche |
| Sodiumhydrogencarbonate | Grüssig |
| ß-mercaptoethanol | |
| TEMED | Sigma |
| Tris | Roth |
| Tween 20 | Roth |

## 8.4 Disposables

| | |
|---|---|
| Eppendorf tubes | Sarstedt |
| FACS tubes | BD |
| Gloves | Latex gloves from Sempermed |
| Pipettes 5/10/25/50 ml | Sarstedt |
| PVDF membrane | Immobilon, Millipore |
| Tissue culture flasks | Sarstedt |
| Watman paper | Sigma |

## 8.5 Cell culture medium and supplements

| | |
|---|---|
| S2 medium | SERVA |
| Fetal calf serum (FCS) | Gibco |
| HEPES | Gibco |
| PBS | Dulbecco's PBS (Gibco) |
| RPMI 1640 | Gibco |
| Penicillin-Streptomycin | Gibco |
| L-Glutamine | Gibco |
| Lipofectamine 2000/3000 | Life Technologies |
| IL-2 | NIH Cytokine Repository |
| IL-15 | Pan Biotech |
| Il-21 | Milteny Biotec |
| OptiMEM | Life Technologies |

### 8.5.1 Inhibitors

| | |
|---|---|
| SYKII | Cayman Chemical Company |
| SYKIV | Cayman Chemical Company |
| PP1 | Cayman Chemical Company |
| PP2 | Cayman Chemical Company |
| DPI (Diphenyleneiodonium chloride) | Sigma |

## 8.6 DNA

The following plasmids were obtained from the BIOSS Centre for Biological Signalling Studies in Freiburg and verified in our lab by sequencing:

pD-Syk, pD-Zap70, pD-Fyn, pD-SHP-1, pD-TCRZeta, pD-ZetaY1-4F, pD-ZetaY1-6F

## 8.7 Antibodies

### 8.7.1 Primary antibodies for FACS analysis and Phosflow

For Phosflow:

p-SLP-76 Y128 AF647, p-SLP-65 AF647, CD56 BV421, CD19 AF700 (all from BD), CD3 PerCP (Biolegend).

For expression analysis: 2B4-APC (C1.7), CRACC-PE (162.1), NKp80-PE (5D12), CD45-PE all from Biolegend.

### 8.7.2 Primary antibodies for Western Blot

Antibodies: pY113-SLP-76 (J80-373), pY128-SLP-76 (J141-668.36.58) (BD Biosciences); isotype control mouse IgG1 (MOPC-21) (Sigma); SLP-76 (#4958), ZAP70 (D1C10E) (Cell signaling); CD3-Z, Syk (SYK-01) (Biolegend); Fyn, DAP10, DAP12, FceRg, 2B4, SHP-1, GAPDH (GA1R) (Thermo Scientific); Csk (C14520 Transduction Laboratories).

### 8.7.3 Secondary antibodies

**Detection**

HRP Goat anti-mouse IgG (Biolegend # 405306) Goat Polyclonal Ig

HRP Donkey anti-rabbit IgG (Biolegend # 406401) Donkey polyclonal Ig

HRP Goat anti-rat IgG (Biolegend # 405405) Goat Polyclonal Ig

**Cross-linking**

Goat IgG anti-Mouse IgG (H+L)-unconjugated. (Dianova #115-005-003 Jackson ImmunoResearch)

## 8.8 Cells

| | |
|---|---|
| Primary rested PBMCs | Isolated from blood donations in our lab |
| Cultivated, expanded NK cells | Cultivated from isolated NK cells as described |
| Schneider (S2) cells | Purchased from Invitrogen |
| Feeder cells for NK cell culture | Gift from Dario Campana |

# 9 Abbreviations

| | |
|---|---|
| 3BP2 | SH3 domain-binding protein 2 (aka SH3BP2) |
| Ab | Antibody |
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| BCR | B cell receptor |
| CLP | common lymphocyte progenitor |
| Csk | C-terminal Src kinase |
| CTL | Cytotoxic lymphocytes |
| DAP10 | DNAX-activation protein 10 (aka HCST) |
| DAP12 | DNAX-activation protein 12 (aka TYROBP) |
| DMSO | Dimethyl Sulfoxide |
| EAT2 | EWS/FLI1-activated transcript 2 (aka SH2D1B) |
| FACS | Fluorescence-activated cell sorting |
| FCS/FBS | Fetal bovine serum |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| Grb2 | Growth factor receptor-bound protein 2 |
| HRP | horseradish peroxidase |
| FN-γ | Interferon gamma |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| ILC | Innate lymphoid cells |
| IS | Immunological synapse |
| ITAM | Immunotyrosine-based activation motif |
| ITIM | Immunotyrosine-based inhibitory motif |
| ITSM | immunoreceptor tyrosine-based switch motif |
| IVIg | Intravenous immunoglobulin |
| kDa | Kilo-Dalton |
| KIR | Killer-cell immunoglobulin-like receptors |
| LAT | Linker for activation of T-cells family member 1 |
| Lck | Lymphocyte cell-specific protein-tyrosine kinase |
| MHC | Major histocompatibility complex |
| NCR | Natural cytotoxicity receptor |
| NCR | Natural cytotoxicty receptors |

| | |
|---|---|
| NK cell | Natural killer cell |
| PI3K | Phosphoinositide 3-kinase |
| PLCy | Phospholipase Cy |
| RT | Room temperature |
| S2 cells | Schneider cells |
| SAP | SLAM-associated protein (aka SH2D1A) |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SFK | Src family kinases |
| SH2 | Src homology 2 |
| SH3 | Src homology 3 |
| SHIP1 | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 |
| SHP-1 | Src homology region 2 domain-containing phosphatase-1 |
| SLAM | signaling lymphocytic activation molecule |
| SLP-76 | SH2 containing leukocyte protein of 76 kDa (also LCP2) |
| SRR | SLAM-related receptors |
| SYK | Spleen tyrosine kinase |
| TCR | T cell receptor |
| TNF-α | Tumor necrosis factor alpha |
| uL | Mikroliter |
| uM | Mikromolar |
| WB | Western Blot |
| ZAP70 | 70 kDa ζ-chain associated protein |

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