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Analysis of the resistence of B cell antigen receptor signalling to the inhibition of Src-family kinases

Analýza resistence signalizace přes B buněčný receptor k inhibici kináz z rodiny Src

Diploma Thesis

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Prohlášení:

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Abstract

Signalling through antigen specific receptors BCR and TCR is crucial for the development and the function of T cells and B cells. Although much is known about their signalling pathways a number of observations still remain to be clarified. In my thesis, I focused on the roles of Src-family kinases (SFKs) in the initiation of BCR- and TCR-mediated signalling. Several studies have suggested that in contrast to TCR signalling, BCR signal transduction could be initiated independently of SFKs or with only a minimal activity of these kinases. We used genetic approach to study the differences between TCR and BCR signalling apparatuses combined with inhibition of SFKs by pharmacological approach. Using this experimental set up, we show that the differences in the roles of SFKs and in the activities of SFKs needed for the initiation of BCR and TCR signalling are likely based on different composition or architecture of BCR and TCR. We further show that the SFK activity required for the initiation of TCR signalling is lower if ZAP-70 kinase is substituted with Syk kinase, which most likely reflects the different molecular mechanisms of Syk and ZAP-70 kinase activation.

Key words: Src-family kinases, BCR receptor, TCR receptor, PP2, B cells, T cells, BCR signalling, TCR signalling.

Abstrakt

Signalizace přes antigenně specifické receptory BCR a TCR je zásadní pro vývoj a funkci B a T buněk. Ačkoli je o signálních drahách těchto receptorů již mnoho známo, řada pozorování stále ještě nebyla objasněna. V rámci své magisterské práce jsem se zaměřil na roli kináz z rodiny Src (SFK) během iniciace signalizace přes receptory TCR a BCR. Některé práce ukazují, že signalizace přes receptor BCR může být, narozdíl od signalizace TCR, spuštěna nezávisle na kinázách z rodiny Src nebo pouze s minimální aktivitou těchto kináz. My jsme použili genetický přístup ke studiu rozdílů mezi signálními aparáty BCR a TCR spolu s inhibicí SFK pomocí farmakologického přístupu. Pomocí těchto experimentálních postupů se nám podařilo ukázat, že rozdílná role SFK během iniciace signalizace přes BCR a TCR je pravděpodobně zapříčiněna rozdíly ve struktuře či ve složení receptorových komplexů BCR a TCR. Zároveň jsme zjistili, že úroveň aktivity SFK potřebné k iniciaci signalizace TCR je nižší, pokud je kináza ZAP-70 vyměněna za kinázu Syk. To je pravděpodobně způsobeno rozdílným mechanismem aktivace těchto kináz.

Klíčová slova: kinázy z rodiny Src, receptor BCR, receptor TCR, PP2, B buňky, T buňky, signalizace BCR, signalizace TCR.

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List of abbreviations

APC	Allophycocyanin
BCR	B cell receptor
Blk	B-lymphocyte kinase
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
CD	Cluster of Differentiation
cDNA	Complementary deoxyribonucleic acid
Csk	C-terminal Src kinase
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
ECL1	Enhanced chemiluminescence 1
ECL2	Enhanced chemiluminescence 2
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
Fgr	Feline Gardner-Rasheed sarcoma virus
FITC	Fluorescein isothiocyanate
Fyn	Fgr/Yes related novel protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRB2	Growth factor receptor bound protein 2
Hck	Hematopoietic cell kinase

Igα	Immunoglobulin alpha chain
Igβ	Immunoglobulin beta-chain
IP3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine based activation motif
ITK	Interleukin 2 inducible T-cell kinase
LAT	Linker of activated T cells
LB	Lysogeny broth
Lck	Lymphocyte specific protein tyrosine kinase
Lime	Lck-interacting transmembrane adapter 1
LNGFR	Low affinity nerve growth factor receptor
Lyn	Lck/Yes novel tyrosine kinase
Map-kinase	Mitogen activated protein-kinase
MHC	Major histocompatibility complex
MSCV	Murine stem cell virus
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
Pag	Phosphoprotein associated with glycosphingolipid-enriched microdomains
PE	Phycoerythrin
PIP2	Phosphatidylinositol 4,5-bisphosphate
pITAM	Phosho-immunoreceptor tyrosine-based activation motif
PKC	Protein-kinase C
PLC	Phospholipase C
PVDF	Polyvinylidene difluoride
RasGRP	Ras guanyl releasing protein 1
RPMI	Roswell Park Memorial Institute medium
Scimp	SLP65/SLP76, Csk-interacting membrane protein

SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SFK	Src-family kinases
SH2	Src-homology domain 2
SH3	Src-homology domain 3
SHIP	Src homology 2 domain containing inositol-5-phosphatase 1
SHP1	Src homology region 2 domain containing phosphatase 1
SHP2	Src homology region 2 domain containing phosphatase 2
SLP65	SH2 domain containing leukocyte protein of 65 kDa
SLP76	SH2 domain containing leukocyte protein of 76 kDa
SP	Single positive
Src	Sarcoma
Syk	Spleen tyrosine kinase
TAE	Tris-acetate-EDTA
TCR	T cell receptor
Temed	Tetramethylethylenediamine
Thy1.1.	Thymocyte antigen 1.1.
Yes	Yamaguchi Y73 virus sarcoma oncogene
Yrk	Yes-related kinase
ZAP-70	Zeta chain associated protein kinase of 70kDa
IC50	Inhibitory constant 50

1 Introduction

Signalling via antigen specific receptors BCR and TCR is initiated by Src-family kinases (SFK). Although the prevailing view is that SFKs play the major role in the initiation of this signalling, several studies have suggested that in case of BCR the requirement for SFKs is much lower and this receptor is able to initiate the signalling in almost complete absence of their activity (Stepanek et al., 2013, Mukherjee et al., 2013). These observations have elicited many new questions about the initiation of BCR signalling. Moreover, explanation of this phenomenon could help us to explain several phenotypes of mice deficient in proteins involved in BCR signalling pathway.

The documented differences in the requirements for SFK activity in the initiation of BCR and TCR signalling could be potentially explained by the ability of B cell kinase Syk to phosphorylate BCR and thus initiate the signalling independently of SFKs (Wossning and Reth, 2004, Mukherjee et al., 2013). However, the data showing that Syk is able to phosphorylate BCR signalling chains are coming from non-haematopoietic cells or *in vitro* experiments and therefore they require further validation in haematopoietic cells. Additional possibilities include different composition of the antigen receptors and signalling complexes assembled after their activation which could potentially facilitate the resistance of BCR signalling to SFK inhibition.

To test these hypotheses, I modified BCR and TCR signalling apparatuses in several B cell and T cell lines. I transduced T cells with Syk kinase and overexpressed Src-family kinase in B cells. I also analysed CD19 negative B cells and expressed chimeric protein containing TCR signalling chain in B cells. In these cells, I tested the resistance of TCR and BCR signalling to the inhibition of SFK. I obtained a number of interesting results which support or disregard some of the possibilities mentioned above and which shed new light on the differences between the initiation of BCR and TCR signalling. Even though they did not result a clear-cut explanation they brought us substantially closer to solving the problem of differential requirements of TCR and BCR for SFK activity.

2 Review of the relevant literature

2.1 T cell function and development

T cells are lymphocytes characterized by the expression of T cell antigen receptor (TCR). TCR is made of surface receptor associated with CD3 complex composed of dimers of CD3 molecules and TCRζ chain. The surface receptor is a heterodimer of two variable chains α and β or γ and δ (Wucherpfennig et al., 2010). In mice and humans the majority of T cells have TCR composed of αβ chains (Paul and Lal, 2016) and thus I will further focus on αβ T cells. CD3 and TCRζ molecules are dimeric transmembrane adaptor proteins which have in the cytoplasmic domains signal transduction motifs named Immunoreceptor tyrosine-based activation motifs (ITAM) (Dai et al., 2012). TCR recognizes antigenic peptides presented on major histocompatibility complex (MHC) molecules on the cell surface. It is supported by CD4 and CD8 co-receptors recognizing other regions on MHC molecules. CD4 co-receptors bind MHC class II molecules presenting mainly peptides derived from extracellular proteins and CD8 co-receptors recognize MHC class I molecules presenting mainly peptides derived from intracellular proteins. To cover sufficiently large spectra of different peptides originating from immense variety of pathogens, there is a huge pool of T cells with different specificities toward the antigens. To avoid the self-reactivity and thus potential autoimmunity the function and specificity of T cells is tightly regulated during the development (Wucherpfennig et al., 2010).

T cell development starts in the bone marrow where lymphoid progenitors are generated. These cells then migrate to the thymus where they go through the series of well-defined developmental stages, eventually giving rise to mature T cells. The nomenclature of these stages is based on the expression CD4 and/or CD8 co-receptors. Developing T cells are going through $CD4^{-}CD8^{-}$ double negative stage (DN) to $CD4^{+}CD8^{+}$ double positive stage (DP), in the end becoming either $CD4^{+}$ or $CD8^{+}$ single positive (SP) mature T cells. In mice, the DN stage can be further subdivided into DN1-DN4 stages based on the expression of CD25 and CD44 surface markers (Germain, 2002). The development is tightly controlled by several check points. In DN3 stage cells undergo rearrangement of TCR beta chain which is than expressed together with invariant surrogate alpha chain and molecules of the CD3 complex in form of pre-TCR (Shah and Zuniga-Pflucker, 2014). Signalling through pre-TCR is crucial for further progression through the development. In the mice lacking critical components of the TCR signalling pathway, T cells are unable to proceed to the next step of the development

and die by apoptosis. This is for example the case for mice with combined deficiency of Src-family kinases (SFK) Lck and Fyn, or combined deficiency of Syk and ZAP-70 or for mice lacking the transmembrane adaptor protein LAT (described in more detail below) (Zhang et al., 1999, Cheng et al., 1997, van Oers et al., 1996b). In the DP stage pre-TCRα chain is replaced with rearranged TCRα chain. DP cells are than selected for their ability to recognize MHC I or MHC II glycoproteins in complex with peptide in a process of positive selection. Subsequently, autoreactive clones are deleted via negative selection based on high TCR affinity for self-peptides (Germain, 2002). Depending on the type of peptide-MHC glycoprotein complex they recognize, the surviving T cells are becoming either single-positive for CD4 (MHC class II.) or CD8 (MHC class I.). There is a relatively narrow window between the affinity thresholds for negative and positive selection. Such a window is maintained by a number of proteins which tune the signal coming from the receptor into the cells. Defects in these proteins than result in higher numbers of autoreactive T cells in the periphery or in defects in positive selection (Germain, 2002).

Single-positive cells then leave the thymus and circulate mainly between the blood and various lymphoid organs and tissues. Upon TCR stimulation with an antigen they become activated which is accompanied with changes in transcriptional program and it leads to their further proliferation and differentiation into different effector and memory cells. Effector T cells are relatively short lived cells actively participating in immune response (Tough and Sprent, 1995). In contrast, memory T cells could live for many years (Sallusto et al., 2004). They are not directly participating in the immune response but they could give rise to a new population of effector T cells in case the organism encounters the same pathogen for the next time (Sallusto et al., 2004). Broadly speaking, the function of effector CD8+ T cells is to eradicate infected or malformed cells by sensing non-self or damaged peptides on MHC class I molecules. Function of CD4 positive effector cells is mainly instructive. They regulate antibody responses directly by cell-cell contact with B cells as well as indirectly by production of cytokines. They also regulate activity of other leukocytes. They could be further divided into a number of different subgroups based on their function, life span and expression of different surface or intracellular markers.

2.2 TCR signalling and its regulation

TCR stimulation with an antigen leads to phosphorylation of ITAMs by SFKs. Phosphorylated ITAMs (pITAMs) serve as docking sites for SH2 domain containing proteins. One of the most important ITAM interacting proteins is protein tyrosine kinase ZAP-70. ZAP-70 is in basal condition in closed conformation in the cytoplasm (Klammt et al., 2015). Transition between the closed and open conformation is induced by binding of tandem SH2 domains of ZAP-70 to pITAMs (Klammt et al., 2015) . The open conformation is even more favoured when ZAP-70 is phosphorylated by SFKs in interdomain B located between the tandem SH2 domains and the kinase domain (Klammt et al., 2015) . Open conformation does not directly influence the kinase activity but it rather prolongs dwelling time of ZAP-70 on CD3 molecules and thus increases the probability that it will be phosphorylated in the activation loop by SFKs or by trans-autophosphorylation and hence activated (Klammt et al., 2015). Activated ZAP-70 then phosphorylates transmembrane adaptor protein LAT. PLCγ1 binds to the phosphorylated LAT and is subsequently phosphorylated and therefore activated by ZAP-70 and SFKs, as well as by ITK kinase which binds to LAT indirectly via soluble adaptor protein SPL-76 (Gresset et al., 2010). PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 binding opens calcium channels on the endoplasmic reticulum (ER). Calcium release from ER is in turn followed by even stronger influx of calcium from extracellular stores. Calcium in the cytoplasm binds to Calcineurin and thus induces NFAT signalling pathway but it also binds to many other proteins and consequently facilitate and induce many signalling pathways. DAG is bound by PKC and/or by RasGRP1. PKC than induces NFκB signalling pathway and RasGRP1 starts Map-kinase signalling pathway (Iwig et al., 2013, Smith-Garvin et al., 2009).

Although TCR and BCR signalling have many signalling branches which are leading to activation of a number of transcription factors that could be potentially probed, we have chosen the calcium response as hallmark of T cells response, because it's a signalling event which appears just step after the generation of all the other important second messengers for all the other branches of the signalling (Quintana et al., 2005) and because it can be measured with a very good time resolution.

Since T cells must proceed through both positive and negative selection the signalling pathways defining the selection thresholds have to be tightly regulated. One of the best known regulator of TCR signalling involved in these selection processes is Themis. Themis has no

catalytic activity but it's rather a scaffold protein which constitutively binds SHP1 phosphatase. Upon TCR stimulation Themis-SHP1 complex is binding indirectly to LAT via soluble adaptor protein Grb2 (Paster et al., 2015). It was shown that Themis deficient mice have block in development in DP stage where positive and negative selection take place (Fu et al., 2009, Lesourne et al., 2009). Themis deficient thymocytes show higher calcium response upon stimulation with low affinity peptides and therefore enhanced negative selection in thymus (Fu et al., 2013). In contrast to these results, mice lacking SHP1 phosphatase have normal T cell development and thus the precise role SHP1 in the function of Themis and in the regulation TCR signalling is not clear (Johnson et al., 2013). It could be potentially explained by the ability of SHP2 to compensate for SHP1 deficiency but such potential explanation lacks any experimental proof. Recruitment of SHP1 or SHP2 to plasma membrane is not restricted to Themis. Several transmembrane proteins have in the intracellular domains tyrosine phosphorylation motifs known as Immunoreceptor tyrosine based inhibitory motives (ITIMs). ITIMs are phosphorylated mostly by SFKs and serve as docking sites for phosphates such as SHP1, SHP2 or SHIP1 (Billadeau and Leibson, 2002). SHIP1 phosphatase has rather mild effect on T cell function and development (Srivastava et al., 2013), but it is crucial in B cell development and function (Pani et al., 1995). Therefore, SHIP1 is discussed in more details below.

TCR as well as BCR signalling are also regulated at the level of SFKs. But I will focus on this type of regulation later on in chapter 2.5.

2.3 B cell function and development

B cells are lymphocytes expressing antigen specific B cell receptor (BCR). BCR is a protein complex made of membrane immunoglobulin as a surface receptor and a dimer of ITAM-containing transmembrane adaptor proteins Igα and Igβ (Kurosaki et al., 2010). BCR in contrast to TCR recognizes soluble antigens. Upon antigen recognition, BCR is internalized in complex with an antigen. The complex is disassembled in the endosomes, receptors are recycled back to the cell surface or degraded and antigens are further processed (Kurosaki et al., 2010). They are cleaved into small peptides and presented to CD4+ T cells on MHC class II molecules. CD4+ helper T cell that had been previously activated with the same antigen can recognize these peptide-MHCII complexes on the B cell surface (Parker, 1993). During this cell-cell contact, B cell obtains signals necessary for the differentiation into antibody

producing plasma cell or memory B cell. Since B cell function is largely dependent on T cells which have already been selected in the thymus, the selection does not have to be as stringent as it is in case of T cells and certain level of autoreactivity is tolerated (Cambier et al., 2007). However, under some circumstances B cells are able to produce antibodies independently of T cells (Vos et al., 2000). Moreover, autoreactive B cells may cross-react with epitopes on pathogenic microorganisms and other foreign particles and get activated during regular immune response and therefore the B cell development still has to be carefully regulated and negative as well as positive selection are taking place.

B cell development starts from the lymphoid progenitors in the bone marrow. The development goes through pro-B cells stage to pre-B cell stage where the expression of the pre-B cell receptor takes place. Pre-BCR is made of rearranged heavy chain and two accessory proteins VpreB and Vλ5 which are substituting for non-rearranged light chain. Signalling through pre-BCR is crucial for the initiation of the light chain rearrangement and for survival of pre-B cells. (Geier and Schlissel, 2006). The necessity of pre-BCR signalling in the B cell development was shown on mice lacking some of the critical components of pre-BCR signalling machinery such as Igβ, Syk kinase or on mice with combined deficiency of three most important SFKs in B cells Lyn, Blk and Fyn. These mice have severe block in the B cell development at the transition from pro- to pre-B cell stage (Saijo et al., 2003, Turner et al., 1995, Gong and Nussenzweig, 1996). Pre-B cells undergo light chain rearmament and the cells which are able to express BCR turn into immature B cells. Autoreactive clones of immature B cells either undergo receptor editing to change the receptor specificity or they are deleted in a process of negative selection (Cambier et al., 2007). In comparison to T cells, the process of negative selection is not that stringent and some autoreactive clones are leaving the bone marrow together with other immature B cells. However, autoreactive B cells on the periphery are missing costimulatory signals for instance these from T cells and often progress to the state of functional unresponsiveness known as anergy (Cambier et al., 2007, Yarkoni et al., 2010). Cells that survive negative selection are leaving bone marrow as transitional B cells. Transitional B cells migrate through the blood stream to the red pulp of the spleen and then to the lymphoid follicles, where they differentiate to mature B cells (Pillai and Cariappa, 2009, Cambier et al., 2007). Two different subsets can be generated during this process. Marginal zone B cells, which can produce relatively low affinity antibody independently on T cells, and follicular B cells, which require T cells to produce antibodies and the antibodies are of comparatively higher affinity (Pillai and Cariappa, 2009).

BCR signalling is crucial not only during progression trough the development but it is also indispensable for the survival of mature B cell. It was found that inducible deletion of Igα leads to apoptosis of mature B cells. Such a serious phenotype is explained by tonic signalling of BCR which is constantly supporting the cell with pro-survival signals (Kraus et al., 2004).

2.4 BCR signalling and regulation

Similar to the TCR signalling, BCR signal transduction is initiated by phosphorylation of ITAM sequences by SFKs followed by the recruitment of Syk. Syk kinase is activated by pITAM binding and by phosphorylation in the interdomain B which both lead to the transition from the autoinhibited to the active conformation. Syk is also phosphorylated and therefore activated by SFKs and/or via trans-autophosphorylation in the activation loop of the kinase domain (Gradler et al., 2013). Activated Syk phosphorylates soluble adaptor protein SLP65 which in turn brings to the close proximity PLCγ2 and Btk kinase allowing for PLCγ2 activation by Btk (Baba et al., 2001). The catalytic activity of PLCγ2 is in the basal state inhibited by C-terminal SH2 domain which prevents the access of the substrate to the active site of the enzyme. Btk-mediated phosphorylation of PLCγ2 leads to conformational change which unmasks the active site and facilitates the enzymatic activity (Kim et al., 2004, Bunney et al., 2012). Activated PLCγ2 generates DAG and IP3 and thus activates signalling pathways in a similar fashion as does PLCy1 downstream of TCR.

As a counterbalance to ITAM phosphorylation SFKs also phosphorylate ITIM motifs present in the cytoplasmic domains of inhibitory molecules such as CD22, CD72 or CD32 (Muta et al., 1994, Adachi et al., 2000). It leads to the recruitment of negative regulators of the signalling, including phosphatases SHP1, SHP2 or SHIP1 (Billadeau and Leibson, 2002). Mutations in the genes coding SHP1 and SHIP1 phosphatases have strong impact on the function and development of B cells. Conditional Deletion of SHP1 in B cells leads to the development of autoimmunity characterized by production of DNA specific autoantibodies (Pao et al., 2007). The phenotype of the mice could be explained by the ability of SHP1 phosphatase to dephosphorylate proteins involved in BCR signalling such as Syk, Igα, Igβ or SLP65 (Pani et al., 1995, Adachi et al., 2001). Similar phenotype as in B cells specific SHP1 deficient mice is present in mice with B cell specific conditional deletion of SHIP1. These mice also suffer from autoimmune disease caused by DNA specific autoantibodies (Pani et al., 1995). SHIP1 dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to

phosphatidylinositol (3,4)-bisphosphate. PIP3 is one of the key second messengers. PIP3 is generated during TCR and BCR signalling by PI3 kinase. PIP3 is bound by PH domains of proteins that participate in the signalling, for instance Btk and PLCγ1/2 (Falasca et al., 1998). It facilitates the recruitment of these proteins to the membrane and increases the stability of the signalling complexes. Although the phenotype of SHP1 and SHIP1 deficiency in B cells is similar, the cause of the disease seems to be different. SHIP1 maintains the unresponsiveness of anergic B cells by dephosphorylation of PIP3. Once the SHIP1 is deleted the autoreactive B cells escape the anergy induction and become a danger (Pani et al., 1995).

Taken together, BCR signalling is in many aspects very similar to TCR signalling and it activates similar signalling pathways. On the other hand, it uses slightly different (though related) proteins which give the BCR signalling several unique features which I will further describe below.

2.5 SFK structure and regulation

Src family of protein tyrosine kinases consists of nine members Hck, Fgr, Lck, Blk, Lyn, Fyn, Yes, Yrk and Src. Although the domain organization as well as the primary structure (except the short N-terminal unique domain) is highly conserved among the family members, the expression pattern of each family member is different. The main SFK in B cells is Lyn but in T cells, the most important SFK is Lck (Okada, 2012).

SFKs are composed of N-terminal unique domain, followed by SH3 domain, SH2 domain, linker and kinase domain with the C terminal tail. The attachment to the plasma membrane is mediated by myrisotylation and palmitoylation of the N termini with the exception of Blk and Src which are only myrisotylated (Okada, 2012). Activity of SFKs is regulated by phosphorylation of two important tyrosine residues, activatory tyrosine located in the activation loop of the kinase domain and the inhibitory tyrosine positioned in the C-terminal tail. Phosphorylated inhibitory tyrosine is bound by the SH2 domain of the same protein. The interaction between the C-terminal tyrosine and the SH2 domain alters the conformation of the kinase domain and inhibits the kinase activity. The closed conformation is further enforced by the interaction between the SH3 domain and SH2-kinase domain linker (Okada, 2012). However, the strength of the intramolecular interactions is rather mild and could be disrupted by binding of the SH2 domain to tyrosine-phosphorylated proteins such as Igα (Pleiman et al., 1994).

The activatory tyrosine is phosphorylated by trans-autophosphorylation and the inhibitory tyrosine is phosphorylated by protein tyrosine kinase Csk. The domain organization of Csk is very similar to SFKs but in contrast to SFKs it lacks the N terminal part containing membrane localization signals and both inhibitory and activatory tyrosines. Therefore, the activity of Csk is controlled via a different mechanism. Csk kinase is activated and recruited to the plasma membrane by interaction between the SH2 domain of Csk and specific phosphotyrosine containing motifs (Okada, 2012). These motifs are present in several transmembrane adaptor proteins like PAG, LIME or SCIMP. However, mice deficient in these adaptor proteins display almost no apparent defects (Gregoire et al., 2007, Dobenecker et al., 2005, Xu et al., 2005)(Kralova et al., unpublished results). In contrast, Csk deficiency has a strong impact on the development and the function of multiple cell types resulting in embryonic lethality. Within the lymphocyte populations Csk ablation in T cells leads to TCR independent thymic development as a consequence of deregulated signalling by SFKs (Schmedt et al., 1998). Unfortunately, the role of Csk in the regulation of BCR signalling has never been studied in mice. The only evidence about the role of Csk in BCR signalling is from Csk deficient chicken B cell line DT40. These B cells have hyper-activated Lyn kinase confirming the function of Csk as a negative regulator of SFKs also in B cells (Hata et al., 1994, Okada, 2012). The reason for the differences between the phenotypes of mice deficient in the expression of Csk and those lacking Csk binding adaptor proteins is at present unclear. It could be possibly explained by compensation by other Csk interacting proteins and/or by redundancy between these adaptors.

In leukocytes, the inhibitory as well as the activatory tyrosines of SFKs are dephosphorylated predominantly by two phosphatases CD45 and CD148. Both phosphates are transmembrane proteins. Studies of mice and cell lines deficient in the expression of CD45 and/or CD148 clearly demonstrated positive regulatory function of these proteins resulting from their ability to dephosphorylate inhibitory phosphotyrosines in SFK C-terminal tails. However under specific experimental setups their involvement in the dephosphorylation of activation loop phosphotyrosines could also be distinguished (Hermiston et al., 2009). The development of CD45 deficient T cells is arrested in the DP stage as a consequence of reduced activity of SFKs (Mee et al., 1999). In contrast, the effect of CD45 deficiency on B cell development is less severe and more complex. CD45 deficient mice have increased numbers of marginal zone B cells but reduced numbers of follicular B cells and a mild block in the development in transitional B cell stage (Hermiston et al., 2005). The different impact

on B cell and T cell development is mainly explained by the redundancy between CD148 and CD45 in B cells. CD148 deficient mice have similar phenotype in B cell development as CD45 deficient mice. Moreover, CD45 and CD148 double deficient mice have block in early B cell development similar to T cells (Zhu et al., 2008). However, different role of CD45 in B cell and T cell development could be also partially explained by different necessity or different roles of SFKs in BCR and TCR signalling (discussed in detail below).

2.6 Different roles of SFKs in the initiation of BCR and TCR signalling

The first evidence for the requirement of SFKs in the initiation of TCR signalling is coming from experiments with Lck deficient human T cell line Jcam1, where the TCR stimulation is uncoupled from calcium response (Goldsmith and Weiss, 1987, Straus and Weiss, 1992). Later on, these in vitro data were confirmed using Lck knock out mice. The mice have block in DN3 stage of T cell development and thymocytes fail to phosphorylate ITAMs (van Oers et al., 1996a). However, this block is incomplete and fraction of T cells overcomes the DN3 checkpoint. This observation has been explained by the ability of another Src-family member Fyn to partially compensate for the loss of Lck, since the development of Lck/Fyn double-deficient thymocytes is completely blocked in DN3 stage (Groves et al., 1996).

In contrast to TCR signalling, the role of SFKs in the initiation on BCR signalling seems less clear. Lyn deficient mice develop autoimmunity characterized by production of autoantibodies, splenomegaly and enlarged lymph nodes. Moreover BCR-induced ITAM phosphorylation as well as calcium response is not abolished but it is only delayed (Hibbs et al., 1995, Nishizumi et al., 1995, Chan et al., 1997, Chan et al., 1998). These observations brought mainly two new important questions about the role of SFKs in BCR signalling. How Lyn negatively regulates BCR signalling and which kinases are responsible for ITAM phosphorylation? The negative role of Lyn in BCR signalling has been largely explained by the observation, that Lyn deficient B cells have hypophosphorylated ITIMs and thus they have defects in the recruitment of phosphates which balance the activation status of the cells and protect the organism from autoimmunity (Chan et al., 1998, Nishizumi et al., 1998). However, the question about the role of other kinases in BCR ITAM phosphorylation has not been completely answered. Mice with triple deficiency in Blk, Lyn and Fyn kinases display complete block in early B development and B cells in these mice are unable to pass from Pro- to Pre- B cell stage. The phenotype confirmed the indispensability of SFKs in B cell

development and pre-BCR signalling, but it did not solve the question posed above, because some proximal events of BCR signalling, including phosphorylation of ITAMs, PLCy2 and Syk kinase were still present (Saijo et al., 2003). These data suggested that yet another kinase (or kinases) is able to phosphorylate ITAM sequences and preserves at least some aspects of BCR signalling.

Different approach to study the roles of SFK in BCR and TCR signalling is based on the use of SFK specific inhibitor PP2. Treatment of T cells with PP2 completely inhibits antigen receptor signalling but treatment of B cells with PP2 only delays the signalling. The delay between BCR cross-linking and calcium response or ITAM phosphorylation is further prolonged with increasing concentration of PP2 (Fig. 1) (Stepanek et al., 2013, Mukherjee et al., 2013). Moreover, similar effect is caused by overexpression of a construct containing Csk kinase, which is targeted to the plasma membrane (Stepanek et al., 2013). The evidence that both pharmacological and genetic approaches have the same effect on the signalling argues against the possibility of experimental artefact connected with usage of the organic compound as inhibitor.

Resistance of BCR signalling to the inhibition of SFKs or to the loss of the most important SFK members has at least two possible explanations. The first is based on the possibility that Syk is able to phosphorylate ITAMs independently of SFKs. The second suggests that BCR signalling has different requirements than TCR with respect to the level of SFK activity necessary for the initiation of the signalling. Thus it is possible that even a very small residual activity of SFKs is sufficient to initiate the BCR signalling. This residual activity could be the consequence of incomplete inhibition of SFKs by PP2 or, in case of triple knock out mice, it could be the result of the compensation by additional SFK members present in B cells.

Several independent studies have shown that Syk is able to phosphorylate ITAMs in vitro and in non-hematopoietic overexpression systems. Syk in contrast to ZAP-70 was shown to be able to phosphorylate ITAMs on CD3ζ, when it was overexpressed together with chimeric proteins CD8-CD3ζ in HEK cells (Mukherjee et al., 2013). Another study used system with more physiological expression levels. They used insect cell line S2, where they partially reconstituted BCR signalling. In this system, Syk was able to phosphorylate only the membrane-distal tyrosine and the membrane-proximal tyrosine in ITAMs of Igα was not affected by Syk (Wossning and Reth, 2004). It has also been shown that when BCR is stimulated with antibodies or with high affinity antigen the calcium influx is strong and only delayed by PP2 treatment, but when BCR is stimulated with low affinity antigen PP2

treatment almost completely inhibits the signalling (Stepanek et al., 2013). Thus the extent of the resistance of BCR signalling apparatus to the inhibition of SFKs appears proportionate to the strength of the stimulation. Therefore, it is possible that upon strong stimulation of BCR, extensive aggregation of BCR enables Syk to activate itself by trans-autophosphorylation. Syk in turn may phosphorylate ITAMs and initiate the signalling independently of SFKs. Nevertheless, the ability of Syk to phosphorylate ITAMs has never been shown in hematopoietic cells. Moreover, in splenic B cells inhibition of Syk did not affect the phosphorylation of ITAMs even in PP2-treated cells. However, the ITAM phosphorylation was measured by phospho-specific antibody, which recognizes only the membrane-proximal tyrosine (Stepanek et al., 2013). Thus the possibility that Syk is able to phosphorylate ITAMs still has to be taken into account.

In this thesis, I am describing our new data characterizing the mechanism of the resistance of BCR-mediated signalling to the inhibition of SFKs and the differences in the roles of SFKs in BCR and TCR signalling. I also try to address the possibility that Syk is able to phosphorylate ITAMs and therefore induce the signalling independently of SFKs.

3 Results

3.1 SFK inhibition results in delayed antigen receptor-mediated calcium response in B cells and in its complete abolition in T cells

As a mentioned above, it has been reported before that inhibition of SFKs by PP2 leads to dose-proportional delay in BCR signalling, while even low doses of this inhibitor completely inhibit TCR signalling. Since this is a critical observation which forms the basis for all the subsequent work presented in this thesis we first wanted to verify these data and extend the concentration range of PP2 till the concentrations when the inhibitor is becoming non-specific. For these purposes, we measured the calcium response of T cell line Jurkat and B cell line Ramos in the presence of increasing doses of PP2. We loaded the cells with calcium sensitive fluorescent dye Fluo-4 and treated the cells with PP2 five minutes before the measurement or left the cells untreated. Cells were stimulated with anti BCR or anti TCR antibody. The resistance of BCR signalling to the inhibition of SFK was surprisingly high. BCR cross-linking on Ramos cells treated with 100 µM concentration of PP2 was still able to elicit calcium signalling (Fig. 1A). Although the curve representing the calcium response is missing the strong initial peak, the later sustained phase of the calcium response is not influenced by PP2 treatment. In contrast, treatment of T cells with PP2 didn't induce the delay in TCR signalling and the signalling was inhibited with very low doses of this inhibitor (Fig 1B)

3.2 Syk is not able to initiate TCR signalling independently of SFKs

In order to test if Syk is able to phosphorylate ITAMs and thus initiate the signalling independently of SFKs we reconstituted Syk/ZAP-70 deficient human T cell line P116 with Syk or ZAP-70 coding constructs via retroviral transduction. P116 cells transduced with empty vector were used as a control. To monitor the expression level of these constructs in live cells by flow cytometry, we used bicistronic vector where the inserted cDNA is connected by internal ribosome entry site with reporter gene Thy1.1. In this set up we could sort and gate different populations based on the expression of reporter gene and thus also indirectly, based on the expression level of Syk and ZAP-70 proteins coded by these vectors.

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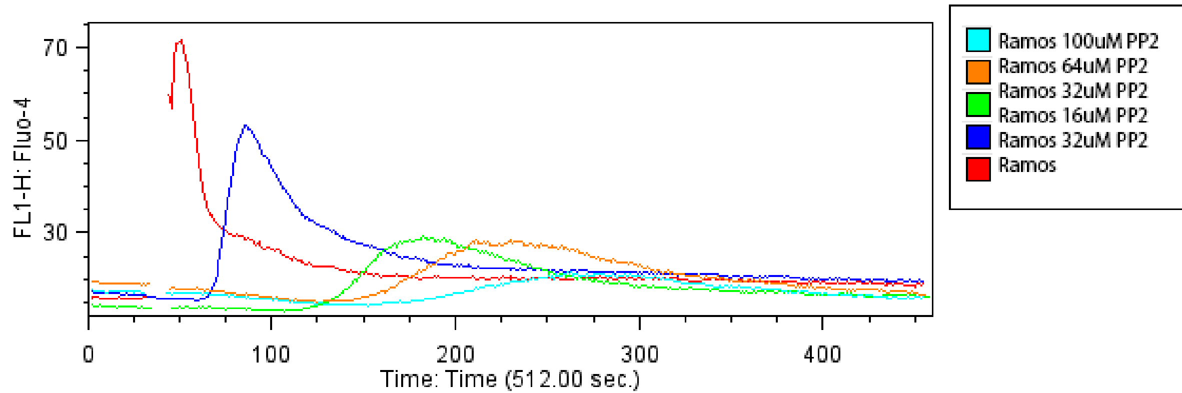

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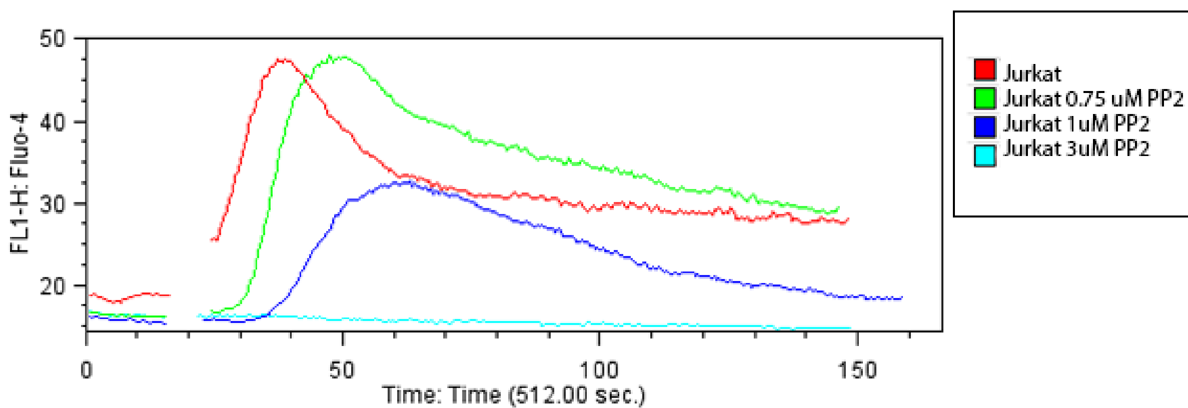
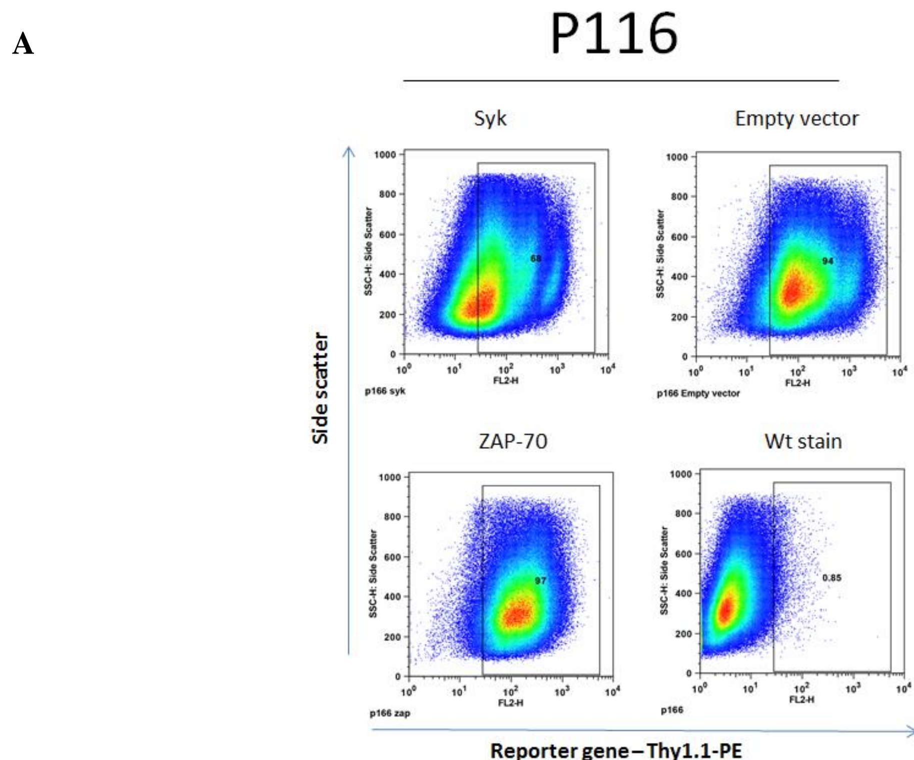


3.2.1 Figure 1.

Resistance of BCR signalling to the inhibition of SFKs by PP2. Human B cell line Ramos and human T cell line Jurkat were loaded with calcium sensitive fluorescent dye Fluo-4. Cells were incubated at 37 ^{o}C in the presence of different concentrations of PP2 for five minutes before the measurement on flow cytometer. The curve of calcium response represents median of Fluo-4 fluorescence intensity. **(A)** Calcium response of Ramos cells in the presence of 16μM, 32μM, 64μM and 100μM concentration of PP2. Ramos cells were stimulated with 10 μg/ml of anti BCR antibody (Goat anti Human IgM $F(ab')_2$) 30 s after the beginning of the measurement. **(B)** Calcium response of Jurkat cells in the presence of 0,75μM, 1μM, 3μM concentration of PP2. Jurkat cells were stimulated with anti TCR antibody (C305) 30 s after the beginning of the measurement.

We sorted the transduced cells (Fig. 2A) on cell sorter and stained them with calcium sensitive day Fluo-4 and anti Thy1.1. Cells were treated with PP2 or left untreated and stimulated with anti TCR antibody 30 s after the beginning of the measurement. The calcium response was measured on flow cytometer (Fig. 2B). The expression level of ZAP-70 in the whole population of P116 Zap cells was comparable to endogenous expression level in human T cell line Jurkat. However, the expression level of Syk in P116 Syk cells was slightly lower compared to human B cell line Ramos (Fig 2C) and it had to be compensated for by the gating strategy based on the expression of reporter gene Thy1.1. (Fig. 2A). When compared to P116 ZAP-70 cells, P116 Syk cells were more resistant to the inhibition of SFK but they were also inhibited with increasing concentration of PP2 and no delay in signalling was observed (Fig. 2B). These results show that Syk kinase is not able to initiate the signalling independently of SFKs on the T cell background where inhibition of SFK completely inhibits the calcium response.



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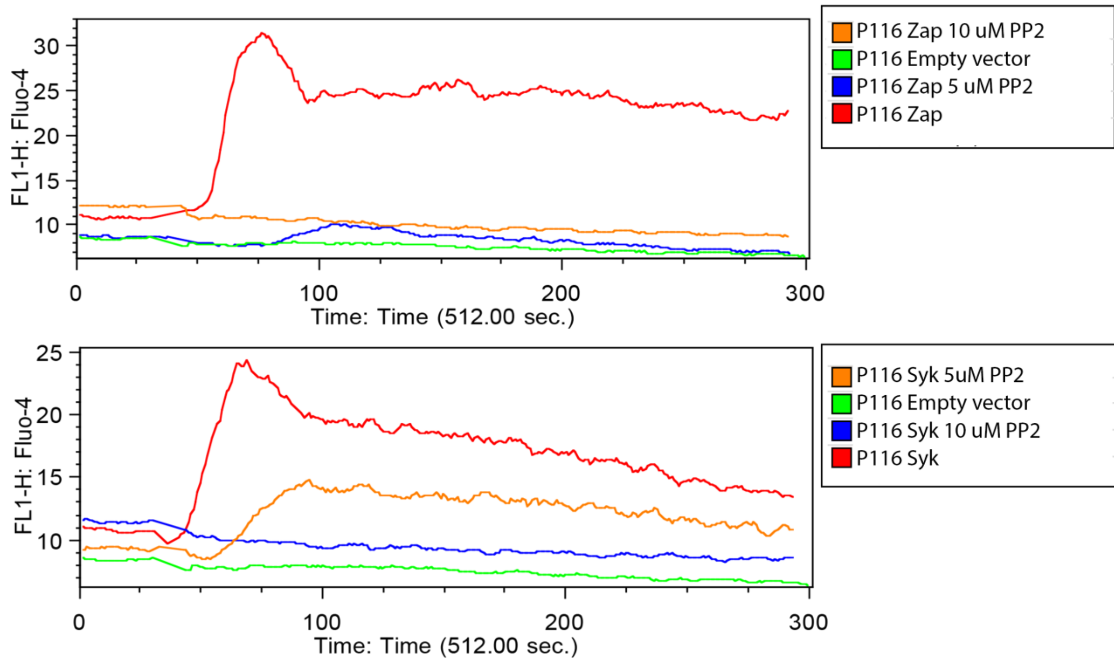

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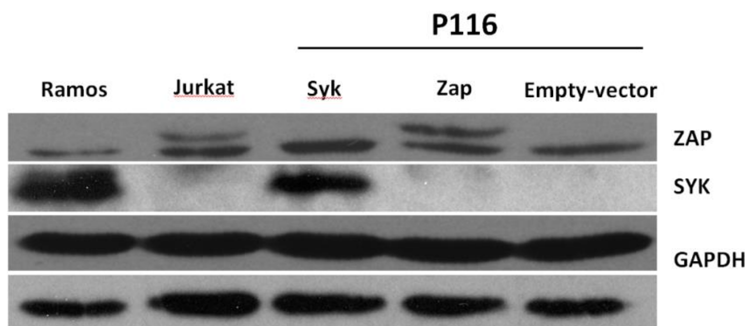

3.2.2 Figure 2.

Syk is not able to initiate the signalling independently of SFKs in T cells. **(A)** Gating strategy for the measurement in panel B. Cells were gated based on the expression of reporter gene Thy1.1. **(B)** P116 Syk, P116 ZAP-70 and P116 empty vector cells were loaded with Fluo-4 and stained with PE-conjugated anti Thy1.1 antibody and incubated without PP2 or with 5μM or 10μM PP2 at 37 °C for five minutes before the measurement. Cells were stimulated with anti TCR antibody (c305) 30 s after the beginning of the measurement.

Calcium response was measured on flow cytometer and the curve represents median of Fluo-4 fluorescence intensity. Representative experiment is showing calcium response of gated cells. **(C)** Western blot analysis of Syk and ZAP-70 protein level in P116 Syk and P116 ZAP-70 cells and comparison with endogenous expression level of Syk and ZAP-70 in Ramos and Jurkat. Cells were lysed directly in SDS-PAGE sample buffer and further processed as described in methods. Blots were stained with anti Syk (SYK-01), anti ZAP-70 (ZAP-70 ZAP-03), and anti GAPDH antibody (GAPDH-71.1). Experiments shown in figure A and B were repeated 3 times.

3.3 Overexpression of Blk does not influence the delay in BCR signalling

Since Syk failed to induce the signalling independently of SFKs, we decided to test the possibility that the resistance of BCR signalling to the inhibition of SFKs is a consequence of incomplete inhibition of SFKs. We speculated that if it were true, SFK overexpression should then shorten the delay between the receptor cross-linking and the calcium response. Moreover, IC50 value for inhibition of B cell specific Src family kinase Blk by PP2 is much higher than for other SFKs expressed in B and T cells (ref). Thus it is possible that the resistance is further influenced by the IC50 for different SFK members. To test the hypothesis, we overexpressed Blk kinase in Ramos using retroviral transduction and subsequent sorting based on the expression of reporter gene LNGFR (similarly to the reporter gene Thy1.1. as described above). The delay of the calcium response was not affected by the overexpression of Blk. Moreover, the amplitude of the response in Ramos Blk cells was slightly lower than that in Ramos cells transduced with empty vector (Fig. 3A). From these data we concluded that the resistance is not a simple result of varying sensitivity of different SFKs to PP2. To exclude the possibility that our Blk construct was non-functional we tested the ability of our Blk to phosphorylate Igα and we also tested whether overexpressed Blk is associated with plasma membrane. To test the kinase activity of Blk coded by our construct we transfected non-haematopoietic Phoenix cells with this construct and/or with Igα. Using western blot, we analysed the tyrosine-phosphorylation of the whole cell lysates. We clearly detected two phosphoproteins with molecular weight corresponding to Igα (CD79a) and Blk only in cells co-transfected with both constructs, which indicates that Blk coded by our plasmid is functional and able to phosphorylate Igα (Fig. 3B).

Membrane localisation was examined in Ramos-Blk transductants using the method for isolation of membrane and cytoplasmic fraction via differential centrifugation followed by western blot analysis. As a control of correct separation, we used GAPDH staining, since GAPDH was expected to be present exclusively in the cytoplasmic fraction. As expected, Blk kinase was detectable only in the membrane fraction, while GAPDH was present only in the cytoplasmic fraction (Fig. 3C). These data confirm that Blk coded by our plasmid was properly targeted to the plasma membrane.

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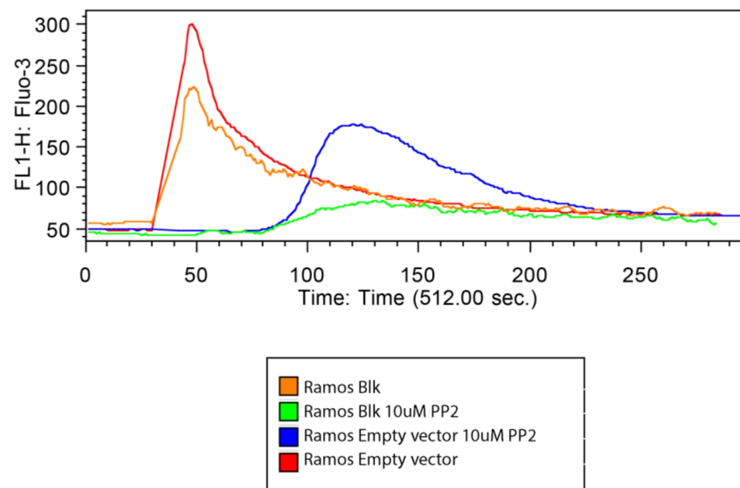

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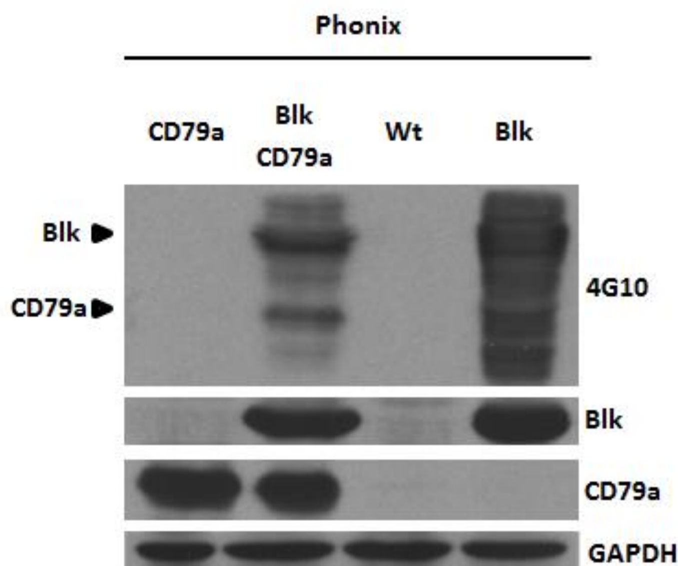

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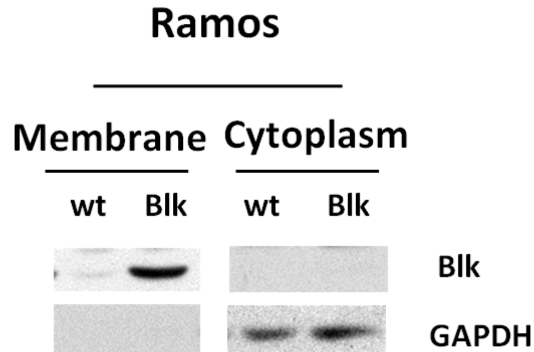

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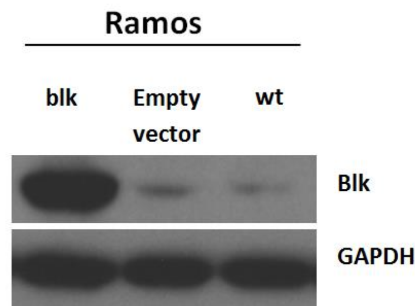

3.3.1 Figure 3.

Overexpression of SFK Blk does not influence the delay in BCR signalling. **(A)** Flow cytometry analysis of calcium response in Ramos cells overexpressing Blk kinase. Ramos Blk and Ramos empty vector cells were loaded with Fluo-3 and incubated 5 min with or without 10μM PP2 at 37 ^{0}C followed by stimulation with 10 μg/ml anti BCR antibody. Representative plot is showing calcium response of Ramos Blk and Ramos empty vector cells. The curve of calcium response represents median of Fluo-3 fluorescence intensity **(B)** Western blot analyses of Blk protein level in Ramos Blk, Ramos empty vector and Ramos. Membranes were stained with anti Blk antibody (Polyclonal) and anti GAPDH antibody (GAPDH-71.1). **(C)** Cotransfection of Blk kinase and Igα in Phoenix cell line. Phoenix cells were transfected with empty vector, Blk kinase and Igα or cotransfected with Blk and Igα. Cells were lysed in SDS-PAGE sample buffer and processed as described in Methods. Membranes were stained with anti Blk antibody (Polyclonal), anti Igα antibody (D1X5C), anti GAPDH antibody

(GAPDH-71.1) and with anti phosphotyrosine antibody (4G10). **(D)** Analyses of the membrane and cellular distribution of Blk kinase in Ramos and Ramos Blk. Membrane fraction and cellular fraction were prepared as described in Methods. Western blots were stained with anti Blk antibody (Polyclonal) and anti GAPDH antibody (GAPDH-71.1). Experiments in panel (A) were repeated 3 times and in panels (D) and (C) two times.

3.4 CD19 deficiency does not affect the delay in BCR-mediated calcium response caused by SFK inhibition

Since, we failed to alter the delay between the BCR stimulation and the onset of the calcium response by overexpression of Blk, we tried to identify other candidate proteins involved in the regulation of BCR signalling, which could influence the kinetics of the calcium response and therefore could give us an insight into the mechanism of the resistance. We selected CD19 as a potential candidate, because B cells from CD19 and Lyn double deficient mice displayed prolonged time interval between the receptor triggering and the calcium response when compared to B cells from Lyn deficient mice (Hasegawa et al., 2001). To test if CD19 deficiency is able to affect the difference in calcium response delay caused by PP2 treatment, we used CD19-deficient variant of mouse B cell line A20 (Fujimoto et al., 2002). The cells were further sorted based on CD19 expression to obtain homogenous population with no or minimal expression of CD19. To avoid potential clonal bias we reconstituted the CD19 expression in CD19-negative cells by retroviral transduction and subsequent cell sorting (Fig. 4A, 4B). We were not able to detect any differences in the calcium response delay between the CD19-negative and CD19-positive A20 cells (Fig 4C, D).

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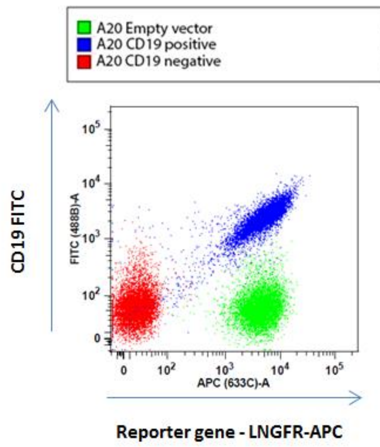

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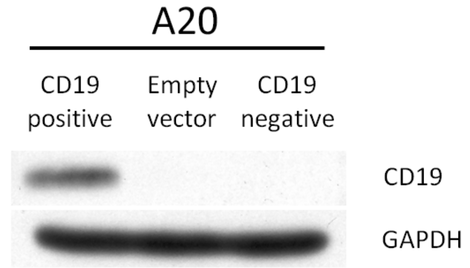

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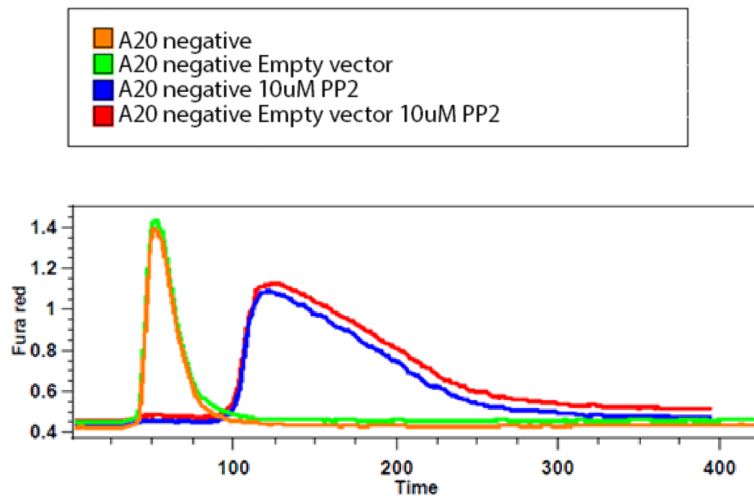

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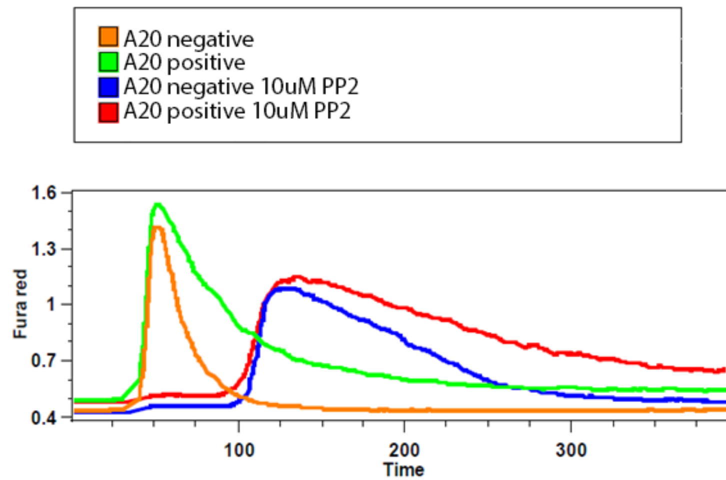

3.4.1 Figure 4.

CD19 does not alter the delay in BCR signalling after the inhibition of SFKs. **(A)** Surface expression of CD19 in CD19-negative, CD19-positive and empty vector A20 cells. Cells were stained as usually with FITC-conjugated anti-CD19 antibody and expression was measured on flow cytometer. **(B)** Western blot analyses of CD19 protein level in CD19-positive, CD19-negative and empty vector A20 cells. Cells were lysed in SDS-PAGE sample buffer and and analysed by Western blotting. Membranes were stained with anti CD19 antibody (polyclonal) and anti GAPDH antibody (GAPDH-71.1).**(C)** The calcium signalling delay is not influenced by experimental procedure connected with cell sorting and retroviral transduction. CD19 negative cells and CD19 negative cells transduced with empty vector were mixed in ratio 1:1 and stained with fluorescent sensitive dye Fura Red and with APC conjugated anti LNGFR antibody. Before the measurement, the cells were pre-warmed in 37 ^{o}C for 5 minutes with or without the presence of 10μM concentration of PP2. Cells were stimulated with 10 μg/ml anti BCR antibody (Gout anti mouse IgM F(ab')$_2$) 30 s after the beginning of the measurement. The transduced and non-transduced cells were gated based on LNGFR reporter gene expression. The curve of calcium response represents median of fluorescence intensity of Fura Red calculated as described in methods section 6.4. **(D)** CD19-negative and CD19-positive cells were mixed in 1:1 ratio and further processed and analysed as in panel (C). The experiments in figure C and D were repeated three times.

3.5 Signalling through chimeric protein CD16-CD3ζ in B cells is inhibited by PP2

The basic scheme of BCR and TCR signalling is very similar. In my opinion, the biggest difference between these signalling pathways seems to be the ability of BCR to recruit the signal transduction complex containing PLCγ. As a result BCR receptor signalling transduction complex can be assembled on Igα/β dimer, while TCR signalling transduction complex is separated from TCR and is assembled on LAT adaptor protein. Thus, it is possible that the different features and architecture of Igα/β and CD3 play an important role in the differential resistance of the BCR and TCR signalling apparatuses to the inhibition of SFKs. To test if the signalling subunits of BCR or TCR can play a role in PP2 resistance, we constructed chimeric protein composed of extracellular domain of CD16 fused with full length protein TCRζ. Therefore, we could stimulate T cells or B cells transduced with CD16-TCRζ with anti CD16 antibody without stimulation of BCR or TCR. To test if the chimeric

protein CD16-TCRζ is able to induce the signalling in a similar way as TCR, we transduced Jurkat cells with the chimeric protein. We sorted the positive cells based on the expression of reporter gene LNGFR (Fig 5A). Stimulation of CD16-TCRζ-transduced Jurkat cells with anti CD16 antibody resulted in slightly stronger calcium response than stimulation with anti TCR antibody. However, the signalling was equally well inhibited by PP2 (Fig 5B). Then we transduced Ramos cells with CD16-TCRζ and sorted the positive cells based on the reporter gene expression and surface expression of CD16-TCRζ. To avoid experimental artefact, we tested the specifity of anti CD16 antibody on Ramos cells transduced with empty vector. Anti CD16 antibody did not stimulate Ramos cells (Fig. 5C) Stimulation of CD16-TCRζ-transduced Ramos cells with anti CD16 antibody resulted in different kind of calcium response than calcium response initiated by BCR stimulation (Fig 5D). The response is slightly delayed without the initial strong peak of calcium influx. On the other hand, the plateau level during the later phases of signalling is higher when compared to the sustained calcium response elicited by BCR cross-linking. Interestingly, the signalling trough CD16-TCRζ was completely inhibited by 10μM PP2, while BCR-triggered response in the same cells was delayed but otherwise resistant to the inhibition by 10 μM PP2. These data indicate that the composition of BCR has a crucial role in the ability BCR signalling apparatus to overcome the inhibition of SFKs.

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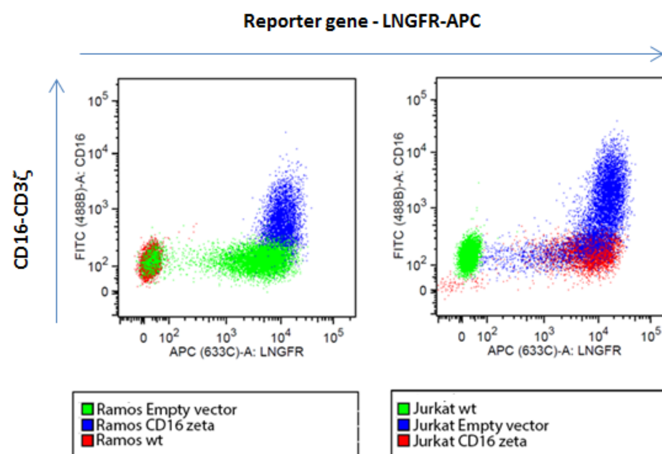

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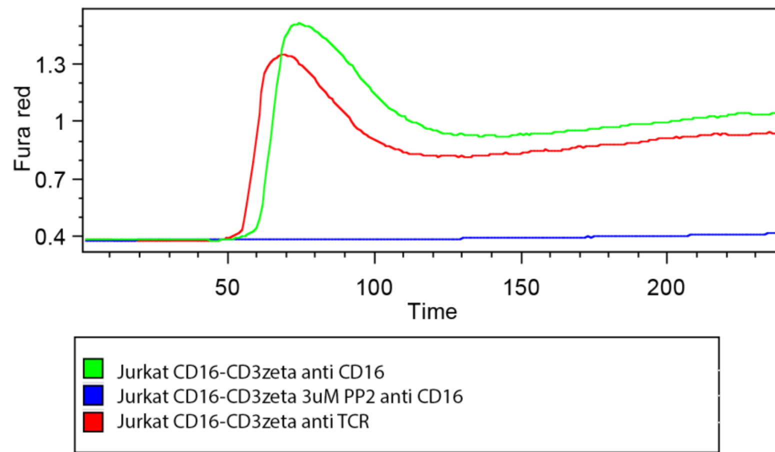

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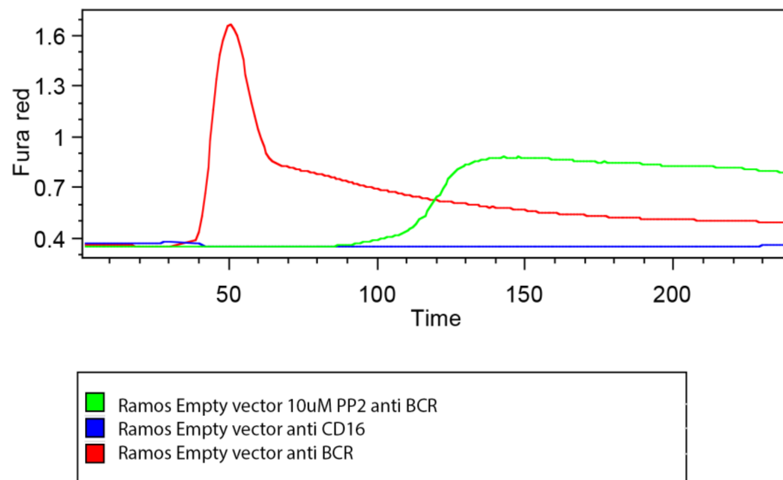

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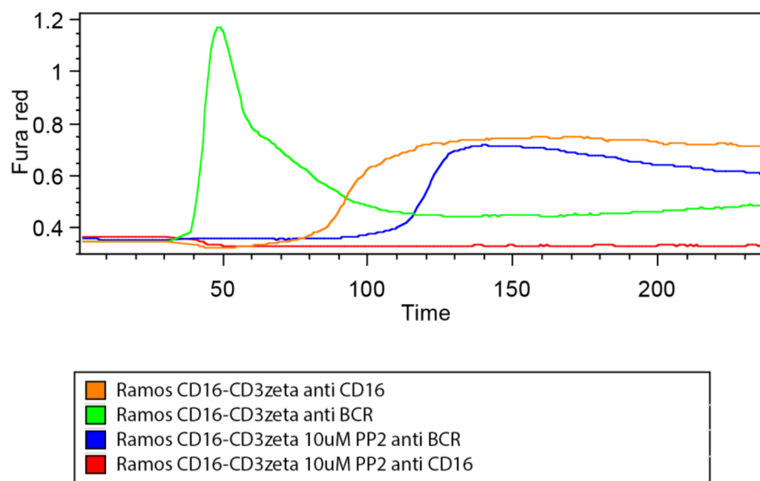

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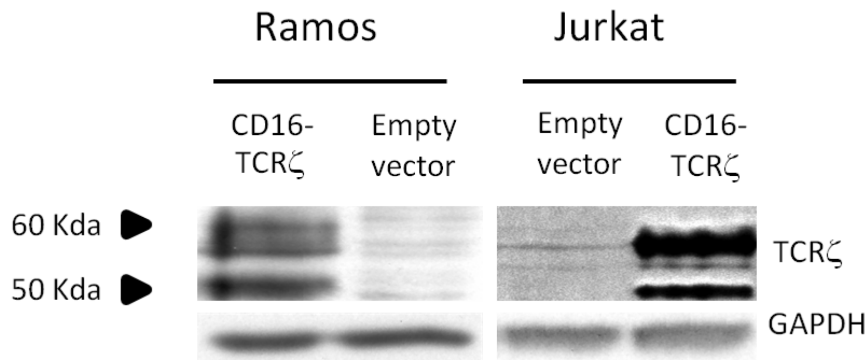

3.5.1 Figure 5 .

Signalling through chimeric protein CD16-TCRζ in B cells is inhibited by PP2. **(A)** Flow cytometry analyses of surface expression of CD16-TCRζ in Ramos CD16-TCRζ, Ramos empty vector, Ramos, Jurkat CD16-TCRζ, Jurkat empty vector and Jurkat cells. Cells were stained with FITC conjugated anti CD16 and APC conjugated anti LNGFR antibodies. **(B)** Flow cytometry analyses of calcium response in CD16-TCRζ-transduced Jurkat cells. Cells were loaded with Fura red. Before the measurement, cells were prewarmed in 37 ^{0}C for 5 minutes with or without the presence of 3μM concentration of PP2 Cells were stimulated with anti CD16 (MEM168) and anti TCR antibody (C305) 30 s after the beginning of the measurement. Calcium response represents median of Fura red fluorescence intensity calculated as described in methods section 6.4. **(C)** Stimulation of Ramos empty vector cells with anti CD16 antibody doesn't induce the signalling. Sample preparation, measurement and results analyses were made similarly as in panel (A) but Ramos empty vector cells were stimulated with anti BCR antibody or anti CD16 (MEM-168) antibody and concentration of PP2 was 10μM. **(D)** Signalling through chimeric protein CD16-TCRζ in Ramos B cells is inhibited by PP2. Sample preparation, measurement, result analyses and antibodies were the same as in panel (C). **(E)** Western blot analyses of CD16-TCRζ protein level in Ramos CD16-TCRζ, Ramos empty vector, Jurkat CD16-TCRζ and Jurkat empty vector cells. Cells were lysed in SDS-PAGE sample buffer and processed as described in Methods. Membranes were stained with anti TCRζ antibody (6B10.2) and anti GAPDH antibody (GAPDH-71.1).

4 Discussion

In my theses, I have analysed the differences in the requirements for SFKs in BCR and TCR signalling. The main goal was to explain the previous observations showing substantial differences in the sensitivity of TCR and BCR-mediated signalling to the pharmacological as well as genetic inhibition of Src-family kinases, where BCR-mediated signalling showed remarkable resistance to the inhibition of SFK activity (Stepanek et al., 2013, Mukherjee et al., 2013). One of the possible explanations was based on the ability of Syk kinase to initiate the signalling independently of SFKs (Wossning and Reth, 2004, Mukherjee et al., 2013). To test this possibility, we reconstituted Syk/ZAP-70 deficient P116 Jurkat-derived T cell line with full length Syk or ZAP-70. Although, the P116 Syk cells were more resistant to the inhibition of SFK than P116 ZAP-70, their TCR signalling was still inhibited by a relatively low dose of PP2 and almost no delay in its time course was observed. These data demonstrated that although Syk was shown to be able to phosphorylate TCRζ ITAMs *in vitro* (Wossning and Reth, 2004, Mukherjee et al., 2013), it is not capable of phosphorylating these motifs in the absence of SFK activity *in vivo*. The higher resistance of P116-Syk cells compared to P116 ZAP-70 cells to the inhibition of SFKs could be explained by the different mechanism of activation of Syk and ZAP-70 (discussed in detail in chapters 2.2. and 2.4.).

Since Syk kinase failed to induce SFK-independent signalling in P116 cells, we tried to look at the PP2 resistance from different angle. We speculated, that if the length of the lag between the BCR cross-linking and the onset of the calcium response is proportionate to the amount of the PP2 inhibitor, it could be the result of incomplete inhibition of SFKs. This seemed to be a plausible explanation, especially because one of the Src-family members, Blk, which is expressed in B cells, but not in T cells, is more resistant to PP2 inhibition than both major T cell SFKs Lck and Fyn SFKs. Thus we expected that if we overexpress Blk in Ramos B cell line the delay should be shorter. For this propose, we overexpressed Blk kinase in these cells but we didn't detect any differences in the delay between Ramos Blk and Ramos empty vector cells. Since Blk shows the highest resistance to PP2 among the Src-family mambers we concluded that the resistance of BCR signalling to the inhibition of SFK is not a simple result of differential sensitivity of B cell and T cell SFKs to PP2 and that the PP2 mediated inhibition is independent of SFK expression level. We also tried to overexpress Lyn kinase in Ramos cells but the expression level of Lyn was most likely tightly regulated in B cells and

we could not reach any significant expression increase above the endogenous level (data not shown).

The data from the previous experiments indicate that the resistance of BCR signalling to the inhibition of SFKs is not a simple result of reduced PP2 sensitivity of B cell SFKs or an ability of Syk to initiate the signalling in SFK independent manner and that this phenomenon is likely more complex. To obtain further insight into the mechanism of the resistance, we tried to modulate the delay by altering the expression of CD19. We chose CD19 as a candidate protein, because B cells from CD19 and Lyn double deficient mice showed longer delay in the onset of calcium signalling when compared to B cells from Lyn single-deficient mice (Hasegawa et al., 2001). To test the hypothesis that CD19 is responsible for the observed resistance of B cells to SFK inhibition we wanted to generate CD19-negative Ramos cells by repeated depletion of CD19-positive cells. However the expression of CD19 in Ramos cell line is homogenous and very stable and we were not able to detect any negative cells (data not shown). In contrast to Ramos, mouse B cell line A20 has non-homogenous expression of CD19 and their CD19-deficient variant has been generated before (Fujimoto et al., 2002. These cells were kindly provided to us by Thomas F. Tedder (Fujimoto et al., 2002). We further sorted these to cells obtain more homogenous population of cells with low or no expression of CD19. As a positive control, we reconstituted these cells with CD19 coded by a retroviral construct. Consistently with the published literature we saw stronger calcium response in CD19 positive cells as compared to CD19 negative cells. (Fujimoto et al., 2002). However, we didn't detect any change in the lag of calcium response after treatment with PP2 between these cell lines, demonstrating that CD19 is not involved in the regulation of PP2 resistance in B cells.

We further speculated that the composition and unique features of BCR and TCR could determine the sensitivity of calcium signalling to the inhibition of SFKs. It was shown that in basal conditions Lyn and Fyn kinases bind Igα and that the interaction is even stronger after ITAM phosphorylation (Clark et al., 1994, Pleiman et al., 1994). Thus it is possible that the co-localisation between the Igα (as a substrate) and the Lyn and Fyn kinases could facilitate ITAM phosphorylation and Lyn and Fyn activation by trans-autophosphorylation. Moreover, signal transduction complex which is assembled on BCR contains PLCγ (Engels et al., 2001) while TCR signal transduction complex containing PLCγ is assembled separately on transmembrane adaptor protein LAT (Smith-Garvin et al., 2009). Consequently, initiation of TCR signalling could be viewed as two-step process but the initiation of BCR signalling is

more straightforward. The first step in the initiation of TCR signalling is activation of ZAP-70 kinase on CD3 molecules and the second step is LAT phosphorylation and assembly of signal transduction complex containing PLCγ. In contrast, BCR recruits at least part of the PLCγ containing complexes organized by adaptor protein BLNK directly to a non-ITAM tyrosine in Igα (Engels et al., 2001). Moreover, BCR signal transduction complex is already partially assembled in steady state before BCR stimulation by antigen (Monroe, 2006). It is a consequence of tonic signalling, which is crucial for B cell survival (Kraus et al., 2004). Existence of preassembled signal transduction complex on BCR together with the ability of ITAM tyrosines to relieve SFKs from autoinhibited conformation could facilitate the initiation of the signalling even when activity of SFKs is almost completely inhibited by PP2. Moreover, since BCR signal transduction complex contains PLCγ it is possible that even if this complex is not fully active it could generate sufficient amount of IP3 to overcome the threshold for induction of calcium response.

To test if the unique features of TCR and BCR signalling are responsible for the differential sensitivity of these receptors to the inhibition of SFKs we constructed chimeric protein composed of extracellular domain of CD16 fused with full-length TCRζ. The CD16 extracellular domain allowed us to stimulate the transduced cells with anti CD16 antibodies without concurrent stimulation of TCR or BCR. This way we were able to test if the PP2 resistance is influenced by the unique properties of the antigen receptor itself. Stimulation of Jurkat cells transduced with CD16-TCRζ with anti CD16 antibody led to a similar response as TCR stimulation which confirms that the chimeric protein has similar ability to induce calcium signalling as TCR. Interestingly, the curve of calcium response in Ramos transduced with the same CD16-TCRζ construct was different from the curve of calcium response observed in Jurkat. The signalling was resulting in strong sustained calcium influx, but without the initial peak. The short delay in the signalling through chimeric protein CD16-TCRζ could be the consequence of different specificities between the SFK members expressed in B and T cells. This hypothesis is based on the observations that neither Lyn nor Blk were able to reconstitute the signalling in Lck-deficient Jurkat cell line Jcam1.6 (data not shown). The signalling through chimeric protein CD16-TCRζ in Ramos was inhibited by 10μM concentration of PP2 which suggest that differences in the composition or architecture of TCR and BCR are the most likely reasons for the differential sensitivity of these receptors to SFK inhibition.

5 Conclusions

- Protein tyrosine kinase Syk is not able to initiate TCR signalling independently of SFKs. On the other hand, and in contrast to ZAP-70, it endows TCR signalling in P116 Jurkat cell with certain level of resistance to SFK inhibition. This ability could partially explain the resistance of BCR signalling to the inhibition of SFKs.
- Overexpression of Blk does not increase the resistance of BCR signalling machinery to the inhibition by PP2, suggesting that this resistance is not much influenced by lower sensitivity of Blk to PP2 and the PP2-mediated inhibition is independent of the expression levels of SFKs.
- CD19 doesn't influence the resistance of BCR signalling to the inhibition of SFKs.
- Signalling initiated by chimeric protein CD16-TCRζ is inhibited by low dose of PP2 even when it is expressed in B cells. This indicates that the resistance of BCR signalling to the inhibition of SFKs is dependent on the unique composition of BCR rather than on differences in downstream signalling machineries.

6 Methods

6.1 Western blotting

5×10^6 cells were pelleted by centrifugation (5 min $400 \times g$) and washed by PBS. Next, cells were resuspended in 150 µl of PBS and then 150 µl of SDS-PAGE sample buffer was added. Samples were sonicated for 15 s and boiled for 2 min. SDS-PAGE was performed in Bio-Rad Mini-PROTEAN Tetra electrophoresis unit using SDS-PAGE running buffer. Separated proteins were transferred to PVDF membranes Hoeffer TE70X semidry transfer unit in blotting buffer. Before use PVDF membranes were activated by sequential washing in methanol and blotting buffer. After the transfer, membranes were blocked with 5% bovine serum albumin (BSA) in wash buffer. Next, membranes were stained overnight with antibodies diluted in wash buffer containing 1% BSA and 0,01% sodium azide. Then they were washed twice for 15 min with wash buffer, and stained 40 min with secondary antibody diluted 1:10 000 in wash buffer and washed twice for 15 min with wash buffer. ECL1 and ECL2 were mixed in ratio 1:1 and 2ml the solution was added on each blot. Membranes were exposed on KODAK medical X-ray film and developed.

6.2 Cell Culture

Ramos, Jurkat, A20 and P116 cell lines were cultivated in complete RPMI media. Phoenix cells were cultivated in complete DMEM media. All cells were cultured at 37 °C in 5% CO_2-containing atmosphere.

6.3 Surface Marker Analysis by flow cytometry

5×10^4 cells were pelleted by centrifugation, medium was discarded and the pellets were resuspended in 50 µl PBS with 1 µl antibody. Cells were stained on ice for 15 min and then washed twice with 200 µl of PBS. Before the measurement, the cells were resuspended in 100 µl PBS. Cells were measured on BD LSRII flow cytometer. Results were analysed using FlowJo 10.0.8R1 software.. Lasers and filters were used as indicated in Table 1.

6.4 Calcium response measurement by flow cytometry

10^7 cells were pelleted by centrifugation and resuspended in 1 ml of complete RPMI medium with 4 µg Fura Red or 2 µg Fluo-4 or Fluo-3. Cells were incubated for 30 min in 37 °C and then wash with 10 ml of complete RPMI media. Next the cells were resuspended in 1,6 ml of the media and the cell suspensions were stored on ice. In case the cells were also

stained by antibodies, they were washed and resuspended in 200 μl of complete RPMI media with 4 μl of antibody and then washed with 10 ml of the medium, resulting pellets were resuspended in 1,6 ml of the media and the cell suspensions were stored on ice. Before the measurement 500 ml of the cell suspension were prewarmed in 37 ^{0}C for 5 min with or without PP2. Cells were stimulated with the antibodies 30 s after the beginning of the measurement. Cells stained with Fluo-4 or Fluo-3 were measured on BD FACSCalibur flow cytometer and cells stained with Fura red were measured on BD LSRII. Lasers and Filters were used as indicated in Table 1. Results were analysed on software Flowjo 9.3.3. Fura red was excited by two lasers and the calcium response was calculated as florescent intensity obtained by the excitation with 406nm laser divided by florescent intensity obtained by the excitation with 488nm laser.

6.4.1 Table 2. Specifications of FACS analysers.

FACSCalibur	Laser wavelength (nm)	Fluorescent dye	Emission Filter
	488	Fluo-4	530/30
	488	Fluo-3	530/30
	488	PE	585/40
LSRII	406	Fura red - calcium bound	675/55
	488	Fura red - calcium free	675/40
	488	FITC	525/50
	644	APC	780/60

6.5 Cell sorting

Cells were stained as described in section 6.3., but the staining volume and amount of antibodies varied with the number of the cells. Cells were sorted on BD Influx cell sorter by operator Zdeněk Cimburek.

6.6 Transfection and/or virus production

Virus packaging cell line Phoenix-AMPHO (Phoenix) was cultivated in 10 cm dish in complete DMEM media till 80% confluency. Medium was discarded and cells were resuspended in 40 ml of DMEM media with 5% FBS but without antibiotics. Cells were plated in 6-well plate 2 ml per well and left grow overnight till 40% confluency. For every well 4 µg of DNA were diluted into 500 µl of Optimem media with 10 µl of Lipofectamine 2000 and incubated for 30 min in room temperature. Meanwhile, 500 µl of media were removed from each well. Then 500 µl of the DNA-Lipofectamine solution was added to each well and incubated overnight. Next day, the media was replaced with 1 ml of complete DMEM or in case of virus production with the complete RPMI media. Cells were harvested for western blot analysis or the virus containing supernatant was collected.

6.7 Cell transduction

Virus containing supernatants were centrifuged for 1 min at 900g to remove contaminating Phoenix cells. 5×10^5 cells to be infected were pelleted by centrifugation, the pellet was resuspended with 1 ml of the virus containing supernatant and 1ul of Lipofectamine 2000 was added. Cells were plated in 12-well plate and centrifuged for 90 min at 30 ^{0}C. The next day, cells were transferred to the cultivation bottle and cultivated as usually.

6.8 Isolation of membrane and cytoplasmic fractions

10^7 cells were pelleted by centrifugation and washed with PBS. Next, the cells were resuspended in 1ml of ice-cold hypotonic buffer and incubated for 15 min on ice. Cells were disintegrated by passing the solution thirty times through 30-gauge needle. Nuclei and intact cells were removed by centrifugation 400 × g for 5 min in 2 ^{0}C. The resulting supernatant was centrifuged for 10 min at 20000g in 2 ^{0}C to separate the membranes (pellet) and the cytoplasmic fraction (supernatant). Supernatant containing cytoplasmic fraction was further concentrated on centrifugal filter devices Amicon till the final volume reached 200 µl. Pellets were resuspended in 200 µl of PBS. Subsequently, 200 µl of SDS-PAGE sample buffer was added to both fractions and the samples were analysed by Western blotting.

6.9 Preparation of cDNA constructs

cDNA coding for individual proteins was amplified by Q5 High-Fidelity DNA Polymerase in PCR reaction from human white blood cell cDNA or in case of CD19 from mouse white

blood cell cDNA. PCR reactions were set up according protocols provided by the manufacturer (New England Biolabs). PCR products were purified by horizontal electrophoreses on 1% agarose gel in TAE buffer. Desired fragments were cut out from the gels and the DNA was purified by Zymoclean Gel DNA Recovery Kit. cDNA inserts obtained by PCR and MSCV plasmids were digested with restriction enzymes based on the primer design (Table 2.). The double digest reactions were prepared according the protocols provided by the manufacturer (Thermo Fisher Scientific). Samples were digested in 37 ^{0}C for 2 hours. Digested DNA was separated from unwanted cleavage products by horizontal electrophoreses on 1% agarose gel, cut out from the gels and purified by Zymoclean Gel DNA Recovery Kit. Plasmids were mixed with inserts in molar ration 1:5 and 250 ng of the mixture was added to the ligation reaction. Plasmids and inserts were ligated by T4 ligase in reactions which were prepared according the protocols provided by the manufacturer (Thermo Fisher Scientific) with the exception that the reactions were incubated in 16 ^{0}C overnight. 5 μl of the reaction mixture was added to freshly thawed competent cells and left on ice for 20 min. Cells were heat-shocked on 45 s in 42 ^{0}C and moved back on ice for 5 min. Subsequently, 1ml of LB media was added, cells were shacked in 37 ^{0}C for 1 h and plated on ampicillin-containing LB agar plates. The next day, 100 ml of LB media with ampicillin (0,1 g/l) were inoculated by random colonies. The bacteria cultures were cultivated for 12 h in 37 °C. Grown cultures were harvested and plasmids were isolated by JETSTAR Plasmid 2.0 Midiprep Kit. DNA was sequenced by SEQme Company with sequencing primers pre-pBABE or in case of longer inserts also with other internal primers (Table 2.). CD16-TCRζ construct was prepared by ligation of digested CD16 and TCRζ fragments prepared by PCR. The ligase reaction was carried out as described above but instead of 250 ng DNA, the reaction contained 1μg of each insert. The ligated construct CD16-TCRζ was separated from non-ligated CD16 and TCRζ cDNA by horizontal electrophoresis. Than the cDNA coding CD16-TCRζ chimeric protein was processed as described above.

6.9.1 Table 2. PCR primers and primers for sequencing

Syk Fwd.	atactcgaggccaccatggccagcagcggcatggctg	XhoI
Syk Rev	atagaattcttagttcaccacgtcatagtagtaattgc	EcoRI
ZAP-70 Fwd.	ataagatctgccaccatgccagaccccgcggcg	Bglll
ZAP-70 Rev	atagaattctcaggcacaggcagcctcag	EcoRI
TCRζ Fwd.	ataacgcgtcagagctttggcctgctggatc	Mlul
TCRζ Rev	ctatagaattcttagcgagggggcagggcct	EcoRI
CD16 Fwd.	ataggatccgccaccatgggtggaggggctggggaaag	BamHI
CD16 Rev	ataacgcgtttggtacccaggtggaaagaatgatgag	MluI
Blk Fwd.	ataagatctgccaccatggggctggtaagtagcaaaaag c	Bglll
Blk Rev	atactcgagctagggctgcagctcgtactg	XhoI
CD19 Fwd.	atactcgaggccaccatgccatctcctctccctgtctc	XhoI
CD19 Rev	gttaacgagtcacgtggttccccaagtcc	HpaI
CD19 seq	gccacagctttagatgaagg	Primer for sequencing
Pre-pBABE	cttgaacctcctcgttcg	Primer for sequencing
Syk seq	ccggcaagagagtactgt	Primer for sequencing
ZAP-70 seq	aacgtccccagacaaacc	Primer for sequencing

7 Material

7.1 Antibodies, cell lines, plasmids and other reagents

Antibodies		
Anti-Hu CD16 FITC	MEM-154	Exbio, Prague, Czech Republic,
Thy1.1. PE	HIS51	eBioscience - Thermo Fisher Scientific, Waltham, USA
CD271(LNGFR)-APC, human	ME20.4-1.H4	Miltenyi Biotech, Bergisch Gladbach, Germany
AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgM, Fc5μ fragment specific	Polyclonal	Jackson ImmunoResearch, West Baltimore, USA
AffiniPure F(ab')$_2$ Fragment Goat Anti-mouse IgM, μ Chain Specific	Polyclonal	Jackson ImmunoResearch, West Baltimore, USA
Anti-Hu CD16, isotype IgM	MEM-168	in-house
Anti-T-Cell Receptor	C305	in-house
Anti ZAP-70	ZAP-70 70/03	in-house
Anti Blk	Polyclonal	Santa Cruz Biotechnology, Dallas, USA
Anti Syk	Syk 01	in-house
Anti TCRζ	6B10.2	Santa Cruz Biotechnology, Dallas, USA
GAPDH	GAPDH-71.1	Sigma-Aldrich, St. Louis, USA
Peroxidase AffiniPure Goat Anti-Mouse IgG, Light Chain specific for Western blotting	Polyclonal	Jackson ImmunoResearch, West Baltimore, USA

Peroxidase IgG Fraction Monoclonal Mouse Anti-Rabbit IgG, Light Chain Specific	Polyclonal	Jackson ImmunoResearch, West Baltimore, USA
Ig alfa	D1X5C	Cell Signalling Technology, Danvers, USA
Anti phoshotyrosines	4G10	in-house
Anti CD19	Polyclonal	Cell Signalling Technology, Danvers, USA

Chemicals, kits, plasmids and other reagents	
Fura red	Invitrogen - Thermo Fisher Scientific, Waltham, USA
Fluo-4	Invitrogen - Thermo Fisher Scientific, Waltham, USA
Lipofectamine	Invitrogen - Thermo Fisher Scientific, Waltham, USA
Agarose	Sigma-Aldrich, St. Louis, USA
Zymoclean Gel DNA Recovery Kit	Zymo research, Irvine, USA
JETSTAR Plasmid 2.0 Midiprep Kit	Genprice, San Jose, USA
Fetal bovine serum (FBS)	Gibco - Thermo Fisher Scientific, Waltham, USA
Primers	Sigma-Aldrich, St. Louis, USA
Restriction enzymes	Thermo Fisher Scientific, Waltham, USA
Q5 High-Fidelity DNA Polymerase	New England Biolabs, Ipswich, USA
T4 DNA Ligase	Thermo Fisher Scientific, Waltham, USA
GeneRuler 1Kb	Thermo Fisher Scientific, Waltham, USA
DMSO	Sigma-Aldrich, St. Louis, USA
Opti-MEM serum reduce medium	Invitrogen - Thermo Fisher Scientific, Waltham, USA
MSCV plasmids	Modified by Laboratory of Leukocyte Signalling, Prague, Czech Republic, originally from Addgene, Cambridge, England
Human cDNA from white blood cells	Leukocyte Signalling, Prague, Czech Republic
Mouse cDNA from white blood cells	Leukocyte Signalling, Prague, Czech Republic
Fluo-3	Molecular probes, Oregon, USA
BSA	Sigma-Aldrich, St. Louis, USA
ECL1 and ECL2	Sigma-Aldrich, St. Louis, USA

Cell lines and Bacterial strain	
Ramos B cell line	Cell line deposition in laboratory of leukocyte signalling, IMG, Prague, Czech Republic
Jurkat T cell line	
P116 T cell line	
A20 B cell line	Obtained from Thomas F. Tedder, Durham, USA, department of Immunology
TOP 10 competent cells	Invitrogen - Thermo Fisher Scientific, Waltham, USA

7.2 Solutions

TAE Buffer		
Tris(hydroxymethyl)aminomethan	4,84 g/l	Serva, Heidelberg, Germany
Acetic acid	1,142 ml/l	Lachner, Neratovice, Czech Republic
Ethylenediaminetetraacetic acid	0,29 g/l	Sigma-Aldrich, St. Louis, USA

LB media		
LB Miller Broth	20 g/l	Sigma-Aldrich, St. Louis, USA

LB agar plates		
LB Miller Broth	20 g/l	Sigma-Aldrich, St. Louis, USA
Agar	15 g/l	
Ampicillin	0,1 g/l	

PBS		
NaCl	8,g/l	Sigma-Aldrich, St. Louis, USA
KCL	0,2 g/l	
KH2PO4	0,2 g/l	
Na2HPO4	1,15 g/l	

DMEM media		
NaCl	6,4 g/l	Sigma-Aldrich, St. Louis, USA
KCL	0,4 g/l	
CaCl2 * 2H2O	0,265 g/l	
MgSO4 * 7H2O	0,2 g/l	
Fe(NO3)3 * 9H2O	0,0001 g/l	
NaH2PO4	0,1528 g/l	
l arginine HCl	0,0534 g/l	
l cyst 2HCl	0,0626 g/l	
Glycine	0,03 g/l	
l histidine HCl H2O	0,042 g/l	
l isoleucine	0,105 g/l	
l leucine	0,105 g/l	
l lysine HCL	0,146 g/l	
l methionine	0,03 g/l	
l phenylalanine	0,066 g/l	
l threonine	0,09 g/l	
l serine	0,042 g/l	
l tryptophan	0,016 g/l	
l tyrosine 2Na 2H2O	0,1038 g/l	
I valine	0,094 g/l	

folic acid	0,004 g/l	
D-Ca pantothenat	0,004 g/l	
Choline chloride	0,004 g/l	
Myo-Inositol	0,0072 g/l	
Niacin amide	0,004 g/l	
Pyridoxal HCl	0,004 g/l	
Riboflavin	0,0004 g/l	
Thiamine	0,004 g/l	
Glucose	4,5 g/l	
Phenol red	0,16 g/l	

RPMI media		
NaCl	6 g/l	Sigma-Aldrich, St. Louis, USA
KCL	0,4 g/l	
Ca(NO3)2 * 4H2O	0,1 g/l	
MgSO4 * 7H2O	0,1 g/l	
L asparagine	0,05 g/l	
Na2HPO4	0,8 g/l	
l arginine	0,2 g/l	
L asparagine acid	0,02 g/l	
l cyst 2HCl	0,0653 g/l	
l glutamic acid	0,02 g/l	
Glycine	0,01 g/l	
l histidine	0,015 g/l	
Histidine l proline	0,02 g/l	
l isoleucine	0,05 g/l	
l leucine	0,05 g/l	

l lysine HCL	0,04 g/l	
l methionine	0,015 g/l	
l phenylalanine	0,015 g/l	
L proline	0,02 g/l	
l threonine	0,02 g/l	
l serine	0,03 g/l	
l tryptophan	0,005 g/l	
l tyrosine	0,02 g/l	
I valine	0,02 g/l	
Folic acid	0,001 g/l	
D biotin	0,0002 g/l	
D-Ca pantothenat	0,00025 g/l	
Choline chloride	0,003 g/l	
Myo-Inositol	0,035 g/l	
Niacin amide	0,001 g/l	
p-aminobenzoic acid	0,001 g/l	
Pyridoxine HCl	0,001 g/l	
Riboflavin	0,0002 g/l	
Thiamine	0,001 g/l	
Vitamin B12	0,000005 g/l	
Glucose	2 g/l	
Phenol red	0,005 g/l	
Glutathione	0,001 g/l	
NaHCO3	2 g/l	

Wash Buffer	
PBS	Prepared as described above
Tween20	Sigma-Aldrich, St. Louis, USA

Blotting buffer	Volume 1 l	
Methanol	200 ml	Penta, Prague, Czech Republic
Tris(hydroxymethyl)aminomethan	1,22	Serva, Heidelberg, Germany
Glycine	5,76	Sigma-Aldrich, St. Louis, USA

Complete media		
RPMI or DMEM media	900L	Prepared as described above
Penicilin	60 mg/ml	Biotika, Cambridge, UK
Gentamicin	40 mg/ml	Sandoz, Princeton, USA
Streptomycin	100 mg/ml	Sigma-Aldrich, St. Louis, USA
FBS	100 ml	Gibco - Thermo Fisher Scientific, Waltham, USA

1,5 M Tris(hydroxymethyl)aminomethane pH 6,8 , 0,4% SDS	Volume 260 ml	
Tris(hydroxymethyl)aminomethane	15,1 g	Serva, Heidelberg, Germany
10% SDS	10 ml	Merck Millipore, Darmstadt, Germany

1,5 M Tris(hydroxymethyl)aminomethane pH 8,8 , 0,4% SDS	Volume 520 ml	
Tris(hydroxymethyl)aminomethane	90,8 g	Serva, Heidelberg, Germany
10% SDS	20 ml	Merck Millipore, Darmstadt, Germany

Stacking gel 5ml		
30% Acrylamide	0,6 ml	
0,5 M Tris(hydroxymethyl)aminomethane pH 6,8 , 0,4% SDS	1,25 ml	Prepared as described above
Temed	5 µl	Sigma-Aldrich, St. Louis, USA
20% ammonium persulfate	12 µl	Sigma-Aldrich, St. Louis, USA
H2O	3,15 ml	

Stacking gel 5 ml, 10% acrylamide		
30% acrylamide	1,667 ml	
1,5 M Tris(hydroxymethyl)aminomethane pH 8,8 , 0,4% SDS	1,25 ml	Prepared as described above
Temed	2,5 µl	Sigma-Aldrich, St. Louis, USA
20% ammonium persulfate	25 µl	Sigma-Aldrich, St. Louis, USA
H2O	2,083 ml	

SDS-PAGERunning buffer		
Tris(hydroxymethyl)aminomethane	3,025 g/l	Serva, Heidelberg, Germany
Glycine	14,4 g/l	Sigma-Aldrich, St. Louis, USA
SDS	1 g/l	Merck Millipore, Darmstadt, Germany

SDS-PAGE sample Buffer	Volume 10 ml	
0,5 M Tris(hydroxymethyl)aminomethane pH 6,8 , 0,4% SDS	2,56 ml	Prepared as described above
Glycerol	2 ml	Sigma-Aldrich, St. Louis, USA
10% SDS	2 ml	Merck Millipore, Darmstadt, Germany
10% Dithiothreitol	0,5 ml	Sigma-Aldrich, St. Louis, USA

Hypotonic buffer		
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid pH 7,55	10 mM	Amresco, solon, USA
KCl	42 mM	Hemapol, Prague, Czechoslovakia
MgCl2	5 mM	Merck Millipore, Darmstadt, Germany
Protease Inhibitor Cocktail Set III	Diluted 1000x	Sigma-Aldrich, St. Louis, USA

7.3 Laboratory equipment, cell culture plastic and others

Pipetman classic	Gilson, Middleton, USA
Eppendorf tubes 1,5 and 2 ml	Eppendorf, Hamburg, Germany
PCR tubes	Axygen, Corning, USA
Eppendorf centrifuge 5417R	Eppendorf, Hamburg, Germany
Eppendorf centrifuge 5810R	Eppendorf, Hamburg, Germany
TE70X Semi-Dry Transfer Unit	AA hoefer, Holliston, USA
PowerPac Basic Power Supply	Bio-Rad, Hercules, USA
Electrophoresis BlueMarine 100	Serva, Heidelberg, Germany
FlowBox Herasafe	Thermo Fisher Scientific, Waltham, USA
Developer Fomei optimax	Fomei, Hradec Kralove, Czech Republic
Cell culture plastic, bottles, plates, tubes, pipets etc.	TPP, Trasadingen, Switzerland
BD LSRII	New Jersey, USA
BD FACSCalibur	New Jersey, USA
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	BioRad, Hercules, USA
T100 Thermal Cycler	Bio-Rad, Hercules, USA
BD influx	New Jersey, USA
Amicon Ultra-4 10K centrifugal Filter devices	Merck Millipore, Darmstadt, Germany
PVDF membrane Immobilon-P	Merck Millipore, Darmstadt, Germany

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