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Mgr. Aleš Drobek

Regulation of leukocyte signal transduction by adapter proteins with special focus on Csk anchoring proteins

Regulace leukocytární signalizace adaptorovými proteiny se speciálním zaměřením na Csk vazebné proteiny

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Školitel:

Mgr. Tomáš Brdička, PhD

Laboratoř leukocytární signalizace

Ústav molekulární genetiky, AV ČR

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Prohlášení

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1 Content

2 Abstract

Signaling through leukocyte receptors is an integral part of immune system homeostasis and it is crucial for immune response. Signal initiation and propagation has to be properly spatiotemporally controlled not to trigger pathological immune activation. Important part in the initiation and regulation of signaling is played by adaptor proteins. These adaptors can be involved in positive or negative control, or both, depending on what effector enzymes they bind. In presented work we studied the involvement of two adaptor proteins PSTPIP2 and LST1/A in leukocyte signaling. Both adaptors interact with several inhibitory enzymes which implicates these proteins in negative regulation of signaling. We extended current understanding of why the deficiency in the expression of one of these adaptors, PSTPIP2, results in the initiation and progression of autoinflammatory disorder chronic multifocal osteomyelitis disease in mice by describing its interactions with Csk kinase and SHIP1 phosphatase. In particular we have shown that PSTPIP2 deficiency leads to increased activity of signaling pathways and increased processing of IL-1β in neutrophils in response to a variety of stimuli and that binding to SHIP1 contributes to PSTPIP2-mediated suppression of inflammation. Our biochemical analysis of the second of these adaptor proteins, LST1/A, revealed that it binds inhibitory phosphatases SHP-1 and SHP-2 which mediate its inhibitory role in myeloid cell signaling. Specific receptor regulated by LST1/A is yet to be identified. Finally, we also studied in more general terms functional outcome of Csk kinase targeting to the plasma membrane in lymphoid cells. This strategy together with pharmacological inhibition of Src family kinases helped us contribute to a long standing question of the relative involvement of Src-family kinase and Syk in the initiation of BCR signaling where our data support non-redundant role of Src family kinases in BCR signaling initiation which cannot be compensated by Syk kinase.

3 Abstrakt

Signalizace přes receptory leukocytů je nedílnou součástí homeostáze imunitního systému a naprosto kritická pro imunitní odpověď. Iniciace signálu a jeho šíření musí být správně kontrolováno aby se aktivace imunitního systému nezvrhla v patologickou. Důležitou úlohu v zahájení signalizace a její regulaci hrají adaptorové proteiny. Tyto adaptory se podílejí na pozitivní nebo negativní kontrole, případně mají duální funkci. Jejich funkce je určena aktivitou enzymů, které na sebe váží. V předkládané práci jsme se zabývali dvěma adaptorovými proteiny, PSTPIP2 a LST1/A, a jejich funkcí v signalizaci leukocytů. Oba adaptory na sebe váží několik enzymů s inhibiční funkcí což poukazuje na jejich úlohu v negativní regulaci signalizace. Rozšířili jsme současné znalosti o vzniku a vývoji zánětlivého onemocnění označovaného jako chronická multifokální osteomyelitida, které se rozvíjí u myší v důsledku deficience v expresi adaptoru PSTPIP2, tím, že jsme popsali interakce tohoto adaptoru s kinázou Csk a fosfatázou SHIP1. Konkrétně jsme ukázali, že absence PSTPIP2 vede ke zvýšené aktivitě signálních drah a zvýšenému štěpení IL-1β v neutrofilech v odpovědi na celou řadu stimulací, a že vazba SHIP1 přispívá k potlačování zánětu zprostředkovanému adaptorem PSTPIP2. Biochemická analýza druhého adaptorového proteinu, LST1/A, odhalila jako interakční partnery fosfatázy SHP-1 a SHP-2, které určují inhibiční úlohu tohoto adaptoru v signalizaci myeloidních buněk. Specifický receptor, který by byl LST1/A adaptorem regulován ještě nebyl objeven. Na závěr jsme se zabývali v obecnější rovině efektem membránově cílené exprese kinázy Csk v lymfoidních buňkách. Tato strategie společně s použitím farmakologických inhibitorů kináz rodiny Src nám pomohla přispět k odpovědi na dlouho přetrvávající otázku relativní důležitosti kináz rodiny Src a kinázy Syk v iniciaci B buněčné receptorové signalizace, kde naše data podporují model nezastupitelné úlohy kináz rodiny Src v iniciaci B receptorové signalizace, která nemůže být kompenzována aktivitou kinázy Syk.

4 Introduction

4.1 Cell signaling

Signal transduction is fundamental process for essentially all cellular functions. It can be generally defined as highly coordinated transfer of signals from extracellular environment into intracellular compartments. Inside the cells the signal spreads via various signaling pathways eventually leading to functional changes within the cells. The most common means of intracellular signal propagation and regulation is signal-dependent formation of protein complexes or signaling through already preassembled multi-protein complexes and/or generation of small molecule second messengers. Regulation of such signaling cascades is often mediated via altering the subcellular localization of the components of the signaling pathways or their regulators (1).

Signal relay between cellular locations often requires dynamic protein interactions and formation of multiprotein complexes. Protein interactions within these complexes are mostly dependent on modular domains with binding function. Some of these domains recognize posttranslational modifications (PTM) such as tyrosine phosphorylation which serve to temporarily create docking sites for proteins containing these domains. A prototypical domain where the interaction is based on phosphorylation is the Src homology 2 (SH2) domain. It binds to phosphorylated tyrosine residue within a specific peptide motif. It was originally described as a functionally important conserved sequence in p130gag-fps oncoprotein, as well as in c- and v-Src and several other tyrosine kinases (2-5). Since then the number of proteins containing this domain with varying sequence specificity has grown to over 100 (6). Occurrence of SH2 domain spans various types of molecules including kinases, phosphatases and adaptor proteins (7, 8). Adaptor proteins often represent central components in protein complexes formed during the signal transduction. Adaptor proteins are either transmembrane or cytosolic and are defined as proteins lacking enzymatic activity with multiple modular binding domains and other binding sites serving to facilitate assembly of large signaling complexes (7). In addition to the SH2 domains the adaptors can also contain other domains such as one or more SH3 domains (e.g. Grb2, SLP-76) which bind to proline-rich sequences, and various short binding motifs creating multiple binding sites for effector molecules (9-11). Pleckstrin homology (PH) domain is a different example of interaction domain. PH domains predominantly interact with phosphatidylinositol-derived lipids and thus target proteins bearing these domains to various membrane compartments, such as plasma membrane,

endosomes and other membranous structures (12). Targeting to the plasma membrane and subsequent activation of the recruited protein is often the result of the activity of phosphoinositide 3-kinases (PI3K) which phosphorylate some of these lipids and which will be discussed in more detail later. Another mechanism taking part in creating interactions and signal transduction is activity of GTPases and their activators guanine nucleotide exchange factors (GEFs). For example, activity of Rac1 or Rac2 contributes to NAPDH oxidase complex assembly and its full activity during phagocytosis (13). Oligomerization is another mechanism employed in signal transduction observed for example in NOD-like and Toll-like receptors (14).

List of domains mediating protein interactions is much longer and can continue with phosphotyrosine binding domain (PTB), WW domain, 14-3-3 proteins and many others. All above mentioned examples contribute to balanced receptor signaling initiation, signal transmission and its regulation.

4.2 Proximal receptor signaling

The most important way for cells of the immune system to sense pathogens or their presence in the organism is through the vast array of receptors on the plasma membrane. Proper signaling through these receptors, essential to mount appropriate immune response, is often dependent on dynamic changes in protein tyrosine phosphorylation triggered by these receptors inside the cell. Although tyrosine phosphorylation sites represent very small fraction in the proteome (15) and protein tyrosine phosphorylation most likely does not exceed 2% of total phosphorylation (16), it is critical for signaling by a large number of receptors. Generation of signal from the engaged tyrosine phosphorylation-dependent receptor is mediated either by protein tyrosine kinase domain (RTKs) within the receptor sequence or by associated non-receptor protein tyrosine kinases (PTKs). In the immune system the most important groups of non-receptor PTKs include kinases from Src, Syk and Tec families involved in mostly positive proximal signaling in leukocytes (17). Their activity is opposed by protein tyrosine phosphatases such as SHP-1, SHP-2 or lipid phosphatase SHIP1 as well as by kinase Csk which negatively regulates Src-family kinases (SFKs) and others.

4.3 ITAM/ITIM

A typical substrate of SFKs is a sequence motif known as immunoreceptor tyrosine-based activation motif (ITAM) consisting of consensus sequence YxxI/Lx(6–12)YxxI/L (18).

It is a defining feature of the family of receptors known as immunoreceptors. These receptors are present in both innate and adaptive arms of the immune system. Examples include T-cell and B-cell antigen receptors (TCR, BCR) as well as signaling molecules coupled to ITAM containing adapters FcRγ and DAP12 such as Fc receptors and a number others (19).

Immunoreceptor signaling begins with ligand recognition and receptor clustering. This results in a cascade of signaling events starting with activation of Src-family kinases (SFK) which phosphorylate tyrosine residues in the ITAM motifs. Full phosphorylation of both tyrosine residues in the ITAM creates docking sites for tandem SH2 domains of Syk family kinases (Syk and ZAP-70) which are rapidly recruited and activated. Syk family kinases in turn phosphorylate number of downstream substrates propagating signal further from the plasma membrane to the cytoplasm and the nucleus.

Whereas ZAP-70 can be found essentially only in T cells where it plays the main role in TCR signaling and in NK cells (20), Syk has broader expression spanning B cells as well as neutrophils, monocytes and other myeloid cells. In line with its expression profile, Syk is indispensable for signal propagation from BCR, Fc receptors, integrins and several innate immunity receptors (21-24). Downstream substrates of Syk family kinases include among others various adaptor proteins such as linker for activation of T cells (LAT), B cell linker protein (BLNK) and SH2-domain-containing leukocyte protein of 76 kDa (SLP76) (10, 25, 26). All of them serve as scaffolds for the recruitment of activated signaling proteins. The most prominent components of different pathways controlled by these adaptors include various isoforms of phospholipase C gamma, protein kinase C, phosphoinositide 3-kinase, and MAPK/ERK pathway. Their activity eventually leads to translocation of transcription factors into the nucleus, as well as changes in the activity of proteins regulating cellular metabolism, migration and other aspects of cell behavior. General outputs of cell activation include proliferation, differentiation, alterations in cell migration, survival, production of cytokines and/or other effector molecules, phagocytosis and initiation of other effector functions.

Negative regulation balancing activation signal is equally important. A typical example is the signaling inhibition mediated by tyrosine-based inhibitory motif (ITIM) found in a number of transmembrane proteins. It is represented by a consensus sequence I/V/L/SxYxxL/V which is evolutionarily highly conserved (27). ITIM motif was first described in the low affinity IgG receptor FcγRIIB (28). Similar to ITAM, tyrosine residue of

the inhibitory motif is phosphorylated by SFKs. This leads to creation of a docking site which binds SH2 domain of protein tyrosine phosphatases SHP-1 and SHP-2 or phosphoinositide phosphatases SHIP1 or SHIP2. These phosphatases then down-modulate signaling by dephosphorylating ITAM motifs or downstream substrates phosphorylated during the course of signal transduction. List of the receptors and adaptors containing ITIM motifs has grown since its discovery (28) including Ig superfamily molecules (PIR-B, Sirp-1α), Siglec molecules and some C-type lectin receptors (27, 29) and many others (27, 29, 30). During the engagement of the activation pathway the inhibitory receptors are also phosphorylated at the same time. The resulting cellular response thus reflects the balance between these two signals. In low affinity binding or in the absence of binding of the ligand to the activating ITAM-bearing receptors, the inhibitory signals are mostly dominant, blocking cellular response. After high affinity receptor engagement or high number of weak activation stimuli, the signal overcomes inhibition and triggers specific response (31). This balance between activating and inhibitory signaling is characteristic feature of NK cell activation. However, these principles in various ways also regulate signaling through many other leukocyte receptors.

Inhibitory pathways based on ITIM motifs are not the only ones known to control cell activation. Potentially activating, but weak signals or monovalent ligands lead to weak phosphorylation (monophosphorylation) of ITAMs with inhibitory properties (32). This inhibitory behavior of partially phosphorylated ITAM initially observed in T cells was not connected to molecular mechanism at that time. Later on it was described in more detail for ITAM in FcRγ chain where partial ITAM phosphorylation was associated with prevalent recruitment of SHP-1 instead of Syk kinase (33). Further evidence for inhibitory function of ITAM came from study of DAP12 deficient mice which have enhanced inflammatory response to multiple stimuli (34). Mutational analysis of ITAM motifs in both adaptors showed that presence of the same tyrosine residues is necessary for activation as well as inhibition. Inhibitory mechanisms mediated by ITIM or inhibitory ITAM (designated as ITAMi) each have slightly different physiologic function. While ITIM represents immediate regulatory step tied with activation, ITAMi rather constitutes a continuous control mechanism contributing to the immune homeostasis (35, 36). In any case activity of SFKs plays crucial role in both of these mechanisms.

4.4 Src family kinases

Family of Src kinases represents relatively large group of nonreceptor tyrosine kinases. In humans it comprises 8 members (Src, Lck, Lyn, Fyn, Fgr, Hck, Blk and Yes) of common structure and similar molecular weight. Each molecule has mostly unique N-terminal part also termedSH4 which is followed by SH3 and SH2 domain. C-terminal part is composed of kinase domain (SH1) and regulatory tail (37). The unique N-terminal part contains acylation sites for myristoylation and palmitoylation, which is required for the localization to the membranes (38). Several kinases like Fyn, Lyn and Hck are expressed as multiple isoforms due to alternative splicing. Different function for each isoform has not been proven, although variances in subcellular localization have been proposed (39). Crystal structures of some of these kinases revealed interesting mechanism of regulation (40). When C-terminal tyrosine in the regulatory tail (Tyr527 in Src) is phosphorylated it binds SH2 domain in *cis* interaction. This inhibitory interaction is further consolidated by binding of SH3 domain to the linker between SH2 and kinase domain. Such condense and packed conformation renders kinase inactive. On the other hand, when inhibitory tyrosine is dephosphorylated, molecule can get into open position, tyrosine in the activation loop is accessible for autophosphorylation (Tyr416 in Src) and the kinase is then fully active (40). Regulation of these phosphorylations and thus SFK activity is dependent on the interplay of several phosphatases and C-terminal Src kinase (Csk).

4.4.1 SFK regulation by Csk

Csk is broadly expressed nonreceptor tyrosine kinase which functions as a highly specific negative regulator of SFKs. Its main function is to phosphorylate inhibitory tyrosine in their C-terminal tails. The importance of Csk is demonstrated by embryonic lethality resulting from Csk deficiency in mice (41). Crucial role of Csk in the immune system was demonstrated using granulocyte specific Csk deletion in conditional knock-out mice. These mice developed multifocal inflammation of skin and lung with strong neutrophil infiltration. Neutrophils were hyperresponsive, hyperadhesive and degranulated even in the absence of stimulation (42). Another striking phenotype of Csk deficiency was observed in different mouse model where Csk-deficient T cells could mature even in the absence of functional TCR (43).

Molecule of Csk has modular organization similar to SFKs where SH3 and SH2 domains are followed by the kinase domain in the C-terminal part. And yet, there are

significant differences with functional consequences for Csk regulation. Csk does not possess N-terminal acylation sites, thus making it a cytoplasmic protein which can bind the membrane only indirectly. Another difference is that it lacks autophosphorylation site in the activation loop as well as the C-terminal tyrosine for negative regulation (44). This sets different mechanism for the regulation of Csk when compared to SFKs. Both, SH2 and SH3 domains of Csk are positioned on the top of the kinase domain. Binding of these domains (predominantly SH2) to other proteins causes conformational change leading to enhanced kinase activity and SFKs recognition. Since Csk cannot directly bind to the plasma membrane, these interactions, especially through SH2, are crucial for its activation and bringing Csk to its substrates, SFKs (45).

Adaptor proteins are among the molecules that play a role in the translocation of Csk to the plasma membrane. There are a number of proteins, transmembrane and cytoplasmic, described to bind Csk. Among them phosphoprotein associated with glycosphingolipid-enriched membrane (PAG) (46), Lck-interacting molecule (LIME) (47), SLP Adaptor And CSK Interacting Membrane Protein (SCIMP) (48), paxillin (49) and Dok family of adaptors (50) are thought to play a role in Csk regulation. However, perhaps because of their mutual redundancy or redundancy between these proteins and other so far unknown Csk binding adaptors, no striking defects in Csk-mediated SFK regulation were observed as a result of their deficiency *in vivo* (51, 52). Phenotypes observed in paxillin and Dok family deficiencient animals are usually explained by the failure to recruit other inhibitory or modulatory enzymes (53, 54).

Inhibitory effect of Csk might be further strengthened by its association with phosphatases from the PEST family. All three members of PEST family (PTP-PEST/PTPN12, PEP/PTPN22 (Lyp in human) and HCSF/PTPN18) can interact with Csk (55). Typical example of such cooperative interaction is Lyp-Csk complex (56) where up to 50% of both endogenous proteins can interact in steady state. The resulting cooperative phosphorylation of inhibitory tyrosine and dephosphorylation of activation tyrosine can potently inhibit SFK activity. Although recently this view was challenged by the data showing that in humans the mutation disrupting this interaction has opposite effect than expected (57, 58). In addition, these phosphatases seem to be able to dephosphorylate inhibitory tyrosine as well (59).

4.4.2 SFK regulation by CD45 and CD148 phosphatases

CD45 is the most studied phosphatase in leukocytes. It mediates dephosphorylation of the inhibitory tyrosine of SFKs and thus represents a positive regulator of SFK activity (60). The importance of CD45 was established by analysis of CD45-deficient mice. In these mice strong defects in T cell signaling and development accompanied by enhanced phosphorylation of SFK C-terminal tyrosine (especially in Lck) were observed (61) However, later on a negative regulation mediated by dephosphorylation of activation tyrosine of SFKs by CD45 has also been described (62, 63). Such dual function enables CD45 to set intrinsic negative feedback and restrict SFK activity within its range. Profound effect of CD45 deficiency observed in T cells is mostly missing in B cells (64) where it is compensated by CD148 (65). The latter phosphatase is in contrast to CD45 also expressed in nonhematopoietic tissue. CD148 deficiency leads to increased phosphorylation of Lyn Tyr507 and slightly reduced activity of BCR in B cells and Fc receptors in macrophages. However these defects are much more pronounced in double-deficient mice lacking both CD148 and CD45 demonstrating the high level of redundancy between CD45 and CD148 in these cell types (65)

4.5 PI3K signaling and its regulation

PI3K pathway is activated upon many various stimulations and at least in part through SFK activity. For example, Lyn activation leads to PI3K activation in BCR signaling through CD19 (66) or to regulation of TLR signaling in macrophages by Lyn/PI3K axis (67).

The PI3Ks are group of highly evolutionarily conserved enzymes. Their specific role is to phosphorylate inositol ring of phosphatidylinositol-derived lipids in 3' position. There are a number of PI3K isoforms which can be divided into three classes with Class I being the most numerous and the most important in immune cell signaling since it can be activated in response to plethora of signals like growth factors, antigens and inflammatory stimuli. Class I PI3Ks specifically phosphorylate phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-trisphosphate (68) also abbreviated as PIP_2 and PIP_3, respectively. When PI3K pathway is activated, PIP_3 is produced at the plasma membrane and/or in early endocytic structures in very orderly and localized fashion. Elevated levels of PIP_3 can then serve as a platform for the recruitment and activation of numerous proteins through their conserved PH domains (12). A complex signaling network is initiated from this platform as tens of different types of effector molecules with PH domains can be recruited in response to PIP_3 generation (69).

The most prominent protein activated by this way is protein serine/threonine kinase Akt, also known as protein kinase B (PKB). After the recruitment to the membrane, Akt can be phosphorylated at T308 in the activation loop by phosphoinositide-dependent kinase-1 (PDK1) recruited to the membrane by similar means (70). This leads to partial activation of Akt. Full activation is achieved by phosphorylation at S473 which is mediated by mTOR complex 2 (mTORC2) (71). These phosphorylations are often used as a read-out of PI3K activity (72). Fully activated Akt mediates activation or inhibition of variety downstream effectors resulting in survival, proliferation, angiogenesis, apoptosis etc. depending on the type and physiological context of the initial signal.

The activation of pathway with such power to influence many aspects of cell physiology has to be subject to a tight control and regulation. Actions of PI3Ks are reversed or modulated by dephosphorylation of PIP_3. Phosphatase and tensin homolog (PTEN) is one of the enzymes dephosphorylating PIP_3 and the only phosphatase known to convert it back to phosphatidylinositol 4,5-bisphosphate (73, 74). PTEN thus functions as pure negative regulator of PI3Ks as it simply reverses the reaction catalyzed by these kinases. Importantly, PTEN is frequently mutated or completely lost in various types of cancer (75, 76). Another phosphatase involved in the regulation of PI3K pathway is SH2-domain–containing inositol 5‘-phosphatase 1 (SHIP1). This hematopoietic system restricted phosphatase together with more broadly expressed SHIP2, dephosphorylate 5‘ position of the inositol ring of PIP_3, generating phosphatidylinositol 3,4-bisphosphate (77). SHIP1 was originally believed to be a negative regulator of PI3K signaling. However, its product can still be recognized by several PH domains (12). Such modulation of PI3K signaling towards effectors with PH domains able to bind PI(3,4)P2 thus cannot be viewed as strictly negative. Nevertheless, deficiency in SHIP1 in mice is associated with severe and diverse phenotype characterized by strong myeloid infiltration to multiple organs, increased proliferation of myeloid progenitors, splenomegaly, osteoporosis and shortened lifespan (78, 79). These data suggest that *in vivo* the negative regulatory function of this enzyme prevails. Deleterious impact of the deregulation of PI3K signaling *in vivo* stresses the importance of its precise control.

PI3K pathway is not activated alone as it is always triggered alongside other signaling pathways to interact with and to complement these pathways to generate specific cellular response. Influences of other pathways, as well as cellular outcome are not constant and vary, based on the cell type involved and specific receptor or receptors activated. This complexity is often increased by participation of multiple PI3K isoforms that contribute to the generation of

PIP_3 pool in response to a single signal (80-82). Important examples of PI3K involvement in complex processes can be Fc-receptor mediated phagocytosis (83, 84) or BCR signaling (85, 86)

4.6 Fc receptor signaling

Fc receptors (FcRs) are part of an executive branch of antibody mediated response as they connect specificity of the adaptive immune response to the potent effector function of innate immune system (87). It is important for such powerful tool to be strictly regulated in order to prevent response against healthy tissue. Inappropriate immune response through FcRs can contribute to autoimmune diseases such as arthritis or systemic lupus erythematosus (SLE) (88). Among different immunoglobulin isotypes (IgA, IgE, IgG and IgM) and their respective receptors, IgG immunoglobulins and their specific Fcγ receptors are the most relevant for host defense against invading pathogens. There are several classes of FcγR described in rodents and their orthologues with some differences have been discovered in other mammals. FcγRI is of highest affinity capable of binding monomeric IgG. The rest are low affinity receptors binding Fc part of IgG with the dissociation constant in the micromolar range, thus capable to interact only with IgG opsonized antigen in the form of immune complexes (ICs) (89). From all FcγRs only FcγRIIB is strictly inhibitory. Since inhibitory FcγRIIB is co-expressed with activating FcγRs, ICs will bind and trigger both activating and inhibitory signals. Resulting signal, if it exceeds threshold for cell activation, will determine the strength of the response such as phagocytosis, cell degranulation or antibody-dependent cellular cytotoxicity (ADCC) (27). The balance between activating and inhibitory signals can be altered by changes in the expression levels of particular types of FcγRs. Expression of activating FcγRs can be profoundly increased after proinflammatory stimuli (LPS) or cytokines (IFN-γ) (89, 90) whereas Th2 cytokines such IL-4 or IL-10 function as down-regulators of activating FcγR expression while increasing level of FcγRIIB (91).

Phagocytosis represents essential function of professional phagocytes, such as neutrophils and macrophages. Signaling after FcR engagement necessary for phagocytosis follows classical proximal cascade already described earlier. It starts with ITAM phosphorylation by SFKs followed by Syk recruitment and activation. It is important to mention that while Syk is indispensable for FcR signal propagation (92, 93), there is a high level of redundancy among the members of Src family. Once Syk is activated it triggers activation of downstream proteins. Syk-activated phospholipase C gamma (PLCγ) localizes to

phagocytic cup (94) and uses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) as a substrate to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 diffuses into the cytosol and signals to release Ca^{2+} from intracellular stores. These second messengers then recruit and activate a number of downstream molecules including PKC isoforms and other enzymes. PLCγ as a hub for generation of second messengers (DAG, IP_3) thus represents important component of FcγR signal transduction (95).

Calcium signaling has long been considered important for phagocytosis since all FcγR can trigger it (96) although Ca^{2+}-independent phagocytosis in macrophages has also been reported (97). Interestingly, calcium increase can be caused by Ca^{2+} channels in forming phagosome rather than by release from intracelular stores alone (98). Irrespective of its source, localized calcium increase around newly formed phagosome is important for its maturation as it triggers actin depolymerization (99). PKCs are a large group of serine/threonine kinases which together have broad mode of action. Specific PKC isoform participation in phagocytosis depends on particular FcγR involved. One of the important downstream targets of PKCs appears to be ERK MAP kinase. Its activity is indispensable for successful phagocytosis in professional phagocytes (100, 101) as inhibition of this pathway leads to complete block of phagocytosis. FcR activated ERK is also important for macrophage proliferation in response to FcR engagement (102). Other fundamental downstream effectors activated, at least in part, by Syk are small GTPases from Rho family which are important for actin dynamics and formation of phagocytic cup. Syk phosphorylates Vav which is guanine nucleotide exchange factor (GEF) necessary for activation of downstream GTPases such as Rac and Cdc42 (103). Adaptor proteins also play important role in complicated signaling during phagocytosis. SLP-76 adaptor originally described in TCR signal propagation was shown to be important in integrin signaling and calcium flux and reactive oxygen intermediates generation during phagocytosis (104). Additional pathway triggered by FcR engagement is PI3K pathway. Its importance and regulation was described in the previous section. In FcR signaling it is important mainly for its involvement in actin reorganization during phagocytosis of larger particles or phagosome maturation (84) where particular PI3K isoforms are differentially required.

Signaling network triggered by FcR does not terminate after phagosome formation. Part of the signaling molecules initiate further signaling pathways involved in the late stages of phagosome maturation and phagolysosome formation.

Functional outcome of FcR engagement, beside clearance of antibody-opsonized pathogens and immunocoplexes from the body is also oxidative burst through production of reactive oxygen species (105). Fc receptor triggering is also one of the means how to stimulate secretion of cytokines. Even short lived neutrophils are able to *de novo* synthetize and secrete cytokines in response to FcR activation, namely IL-1β, IL-8 or IL-6 (106, 107).

Other important and often overlooked ligands of FcR are pentraxins. The group of pentraxins comprises of pentameric proteins such as C-reactive protein, serum amyloid P, and others. They are expressed together with other acute phase proteins during infection and are involved in innate pattern recognition of microbial pathogens and cellular debris (108, 109). Since specific antibody response after infection takes days, pentraxins represent evolutionary old innate mechanism which complements antibody-mediated opsonization.

4.7 B cell receptor signaling

B cells together with T cells represent central components of adaptive immunity. Besides their main role in antibody production, they also function as antigen presenting cells to T cells. Additionally, they are source of regulatory cytokines and chemokines. B cell development and its entire function are mainly influenced by signaling through their B cell receptor. BCR signaling can be divided in two modes, tonic and antigen dependent signaling (110, 111). Tonic signaling occurs in the absence of ligand and it is necessary for survival of naïve mature B cells. Antigen triggered signal, when accompanied by appropriate co-stimulatory signals, leads to proliferation, differentiation and eventually antibody secretion.

BCR is composed of membrane bound immunoglobulin associated with transmembrane proteins Igα and Igβ (CD79a and CD79b). Both of these proteins have ITAM motif in their cytoplasmic tail. Signaling after antigen engagement thus follows classical immunoreceptor signaling described earlier. After ITAM phosphorylation by SFKs, Lyn kinase in particular (112), Syk is recruited and activated. Interestingly, there is a discrepancy of opinion about absolute requirement of SFK for BCR signaling initiation and there have been several reports demonstrating Syk ability to phosphorylate ITAMs under specific experimental setups (113, 114). As in the case of FcRs, Syk is indispensable for BCR signaling. Further signal propagation is ensured by recruitment of adaptor protein BLNK partly through its SH2 domain to non-ITAM phosphotyrosine in Igα (115) as well as through other types of interactions with other proteins (11). These interactions and BLNK recruitment to the plasma membrane are still not fully understood. BLNK then serves as an important

scaffold for other enzymes such as Bruton's tyrosine kinase (Btk), Vav and PLCγ (116) which bind through multiple mechanisms where they individually contribute in forming signalosome (117). Downstream of Syk signal branches and in many features resembles FcR signaling cascade, such as the generation of second messenger (IP_3, DAG), calcium flux and PKC as well as PI3K activation. Calcium signaling and PKC activation lead to nuclear translocation of nuclear factor for activated T-cells (NF-AT) and nuclear factor kappaB (NF-κB) (118) as well as interferon regulatory factor 4 (IRF4) activation which is important for plasma cell differentiation (119). MAPK pathway can be activated by PLCγ through RasGRP-Ras axis (120).

While Syk has purely positive role in BCR signal transmission, Lyn functions as positive and negative regulator at the same time. In addition to ITAMs, Lyn substrates also include ITIM motifs in the inhibitory molecules such as CD22 and FcγRIIB. In the absence of Lyn hypo-phosphorylated ITIMs fail to recruit inhibitory enzymes SHP-1 and SHIP1. This can lead to hyper-reactive BCR signaling and autoimmunity which are indeed observed in Lyn-deficient mice (121, 122). Since B cells express only inhibitory FcγR, FcγRIIB/SHIP1 complex has important role in modulating immunocomplex recognition when BCR and FcγR are co-ligated. When antibody response takes place and immunocomplexes are accumulating in the body, colligation of BCR and FcγRIIB down-modulate humoral response which could otherwise turn pathological (123, 124).

4.8 Pattern recognition receptors

The main function of the immune system is to protect body against invading pathogens. To sense their presence, innate immune cells use pattern recognition receptors (PRRs) able to recognize invariable parts of pathogens. The most studied PRRs are Toll-like receptors. They are membrane bound proteins majority of which is positioned at the plasma membrane to sense extracellular ligands. Exceptions are TLR3, TLR7, TLR8 and TLR9 which can be found in endosomes (125, 126). Each receptor has predominant specificity for particular part of bacterium, viral particle or fungus, e.g. lipopolysaccharide, lipoproteins, double-stranded RNA etc. The typical outcome of TLR stimulation is activation of NF-κB pathway and production of inflammatory cytokines or activation of interferon regulatory factors triggering the interferon response (127-129).

While Toll-like receptors mainly focus on pathogens present in the extracellular space and in the endosomes, other classes of receptors are involved in protecting the cytoplasmic

space in the cell interior. This line of defense is represented by NOD-like and RIG-1-like receptors (NLRs na RLRs). NOD-like receptors represent PRR family with over 20 members which are characterized by presence of nucleotide-binding oligomerization domain (130). The role of these proteins is to recognize mainly bacterial products and various forms of cellular stress. For example NOD1 and NOD2, the first two NLRs identified, recognize γ-D-Glu-mDAP (iE-DAP) dipeptide or muramyl dipeptide (MDP) respectively (131, 132). Current model of their activation proposes conformational changes upon ligand recognition leading to oligomerization via NOD domain. Self-oligomerization is followed by recruitment of RIP2 kinase required for NF-κB and MAPK activation (133). Depending on available ligands, NLRs can act synergistically with TLRs in NF-κB activation and inflammatory cytokine production (134).

Another function of NLRs is the activation of inflammasome (135). Inflammasome is a multi-protein complex which catalyzes conversion of inactive pro-caspase-1 into active protease caspase-1. Recruitment of pro-caspase-1 can be mediated by homotypic interaction through Caspase Recruitment Domain (CARD) present in some NLRs or through adaptor protein ASC containing CARD (136, 137). Activation of inflammasome assembly is triggered by various stimuli, including pathogen associated molecular patterns (PAMPs) such as different peptidoglycans or by danger associated molecular patterns (DAMPs) like ATP, monosodium urate and other crystalline substances (138). Active heterodimeric caspase-1 can process pro-IL-1β and IL-18 into mature, biologically active form which can be released from the cells (139). Secreted IL-1β and IL-18 are important mediators of local and systemic inflammation as they induce recruitment of additional myeloid cells to the site of inflammed tissue, production of other cytokines, namely IL-6, synthesis of acute-phase proteins and expression of inflammation associated genes (140). Aside from canonical inflammasome pathway leading to caspase-1 activation, non-canonical involvement of caspase-8 and caspase-11 have also been reported (141, 142) which can lead to certain degree of functional compensation (143).

5 Aims of the study

The aim of presented thesis was to study proximal leukocyte signaling and its regulation or modulation by adaptor proteins.

- The main project of my PhD thesis was to dissect molecular mechanisms behind autoinflammatory disease called chronic multifocal osteomyelitis (CMO) in mouse. This phenotype is caused by deficiency of prolin-serin-threonin phosphatase interacting protein 2 (PSTPIP2). The major focus was on potentially dysregulated signaling in cells lacking PSTPIP2 and on the role of Csk and SHIP1 in the PSTPIP2 function.

- First of the two side projects analyzed role of transmembrane adaptor protein LST1/A. This poorly described protein with so far unknown function in immune system was chosen from genome-wide screen focused on new transmembrane adaptors. We analyzed its expression and proposed its role in the regulation of myeloid cell signaling based on its interactions with phosphatases SHP-1 and SHP-2.

- Second side project was focused on rather complex issue of initiation proximal BCR signaling. Its goal was to study the effects of the inhibitors of SFKs, including Csk and small molecule inhibitor PP2 on BCR signaling and to help answer still rather controversial question if SFKs are absolutely required for the initiation of BCR signaling, or if Syk kinase can compensate under certain circumstances.

6 Results and discussion

6.1 PSTPIP2, cytosolic adaptor protein involved in the regulation of IL-1β processing

6.1.1 Identification of PSTPIP2 as Csk-binding protein

Majority of Src-family kinases are associated with plasma membrane. However major mechanisms of the recruitment of their main regulator Csk still remain unclear. In the past our laboratory successfully used *in silico* as well as biochemical approaches to identify new adaptors binding Csk and recruiting it to the plasma membrane, including PAG, LIME and SCIMP (46-48). However, effect of their deficiencies on SFK activity in mice was negligible (51, 52). We thus speculated that other so far unknown Csk-binding proteins may exist which compensate for the lack of these proteins in animal models (48, 144). In order to find further Csk-binding adaptors we employed in vitro biochemical approach based on large-scale immunoprecipitations of the construct containing Csk SH2 and SH3 domains. This effort resulted in the identification of a novel Csk binding protein, an adaptor protein PSTPIP2 which had already been known for its involvement in autoinflammatory disease.

6.1.2 Autoinflamatory syndromes

The discovery that mutations in PSTPIP2 adaptor result in autoinflammatory disease chronic multifocal osteomyelitis in two different mouse strains has been published almost 10 years ago (145, 146). It opened the path for better understanding of the development of this and related diseases by identifying mouse models where this problem can be experimentally approached.

Autoinflammatory disorders in general are characterized by seemingly unprovoked recurrent periods of fever and localized or systemic inflammation afflicting mainly joints, skin, mucosal surfaces or bones (147, 148). These pathological conditions differ from autoimmune diseases in that they are caused mainly by the innate immune system with little or no involvement of the adaptive immunity. Many autoinflammatory diseases are caused by mutations of single genes and thus follow classical Mendelian inheritance (149). Although clinical demonstrations and severity of symptoms differ between particular diseases, a number of these diseases have a common denominator of IL-1β overproduction (140). This is the reason why these diseases are often responsive to anti-IL-1 treatment. Examples of this type of autoinflammatory diseases include Cryopyrin-Associated Periodic Syndrome (CAPS)

caused by gain-of-function mutations in NLRP3 gene (150) associated with periodic fever, joint pain and rash. Other examples are pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome caused by mutations in inflammasome regulator PSTPIP1 predominantly affecting joints (151), or Familial Mediterranean fever caused by mutations in another inflammasome regulator Pyrin (152) demonstrated also by fever, swollen joints and serosal inflammations (153). Chronic recurrent multifocal osteomyelitis (CRMO) syndrome is characterized by inflammatory bone lesions where the precise genetic basis is unknown but IL-1β overproduction seems to be involved in disease progression (154). Synovitis, acne, pustulosis, hyperostosis and osteitis (SAPHO) syndrome is very similar in symptoms to CRMO and seems to be, at least partially, responsive to anti-IL-1 treatment (155). Of these diseases PSTPIP2 deficiency in mice most closely resembles human CRMO and SAPHO. However, human patients with PSTPIP2 mutations have not been identified yet.

Due to its similarity to CRMO, the disease of PSTPIP2 deficient mice is usually described as chronic multifocal osteomyelitis (CMO). It is characterized by nonresolving sterile inflammation foci of bones (Figure 1), cartilage and skin demonstrated mostly on external body parts such as paws, tail and ears (145). The disease-causing mutations substantially reduce or abolish PSTPIP2 expression at the protein level suggesting that PSTPIP2 is a negative regulator of pro-inflammatory signaling pathways and its absence results in the lack of control of these pathways. Recently, it was described that overproduction of IL-1β through inflammasome activated caspase-1 and caspase-8 is behind disease onset (143, 156, 157). However, the molecular mechanisms linking PSTPIP2 to the suppression of IL-1β production and CMO phenotype were still missing.

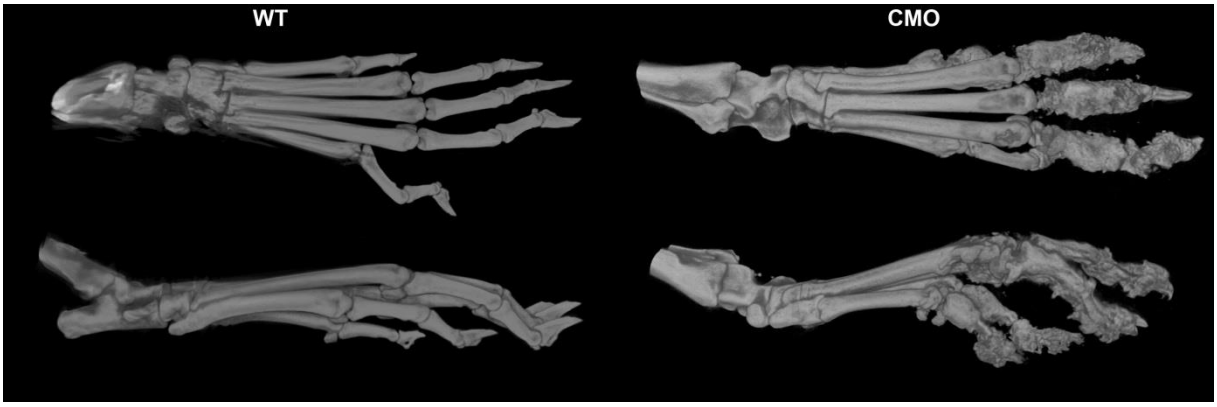

Figure 1. Visualization of microCT scan (9 μm resolution) of the right paw of WT versus CMO mice with long term non-resolving inflammation.

PSTPIP2 is composed of N-terminal F-bar domain known to bind membrane phospholipids and C-terminal tail binding PEST-family phosphatases (158) and potentially other proteins (Figure 2). It is thus likely that PSTPIP2 recruits PEST PTPs and other negative regulators of pro-inflammatory signaling interacting with its C-terminal tail to plasma membrane or other membrane structures thus facilitating their negative regulatory function. Our interest was especially caught by the observations that tyrosine residues at the C-terminus of PSTPIP2 were critical for PSTPIP2-mediated suppression of osteoclast activity (159). However, their binding partners were unknown. Our data raised the possibility that Csk could be binding these tyrosines.

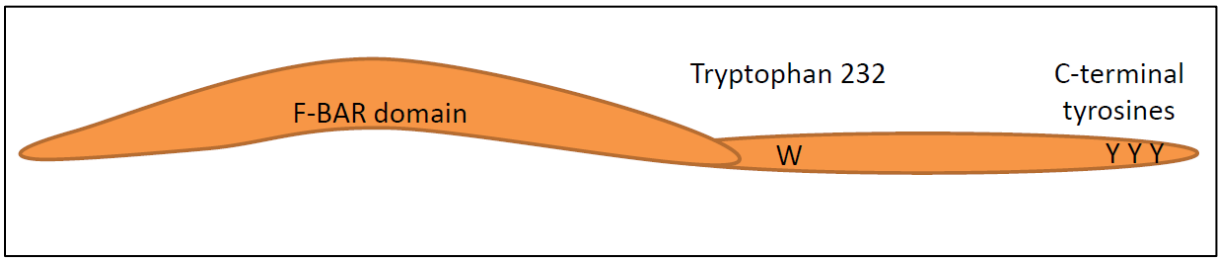

Figure 2. Schematic structure of PSTPIP2 protein. F-BAR domain mediates interaction with membranes through binding to phosphatidyl inositols. Tryptophan 232 mediates interaction with PEST family phosphatases. Phosphorylated C-terminal tyrosines are shown in this work to bind SHIP1 phosphatase.

Unfortunately we could not precisely identify the Csk binding site in PSTPIP2, because mutations of several potential binding sites disrupted PSTPIP2 stability and the protein was not expressed. Nevertheless we at least were able to say that Csk does not bind the functionally critical C-terminal tyrosines, which could be mutated without any effect on PSTPIP2 protein levels and Csk binding. In order to find the true binding partner for the C-terminal tyrosines, we performed a large-scale immunoprecipiration of WT and C-terminal tyrosine mutant of PSTPIP2. Mass spectrometry analysis of the immunoprecipitates revealed that inositol phosphatase SHIP1 specifically binds to these phosphorylated tyrosines.

6.1.3 Is macrophage activity negatively regulated by PSTPIP2?

As a next step we wanted to verify our findings by showing that pathways known to be controlled by Csk or SHIP1 are dysregulated in leukocytes from CMO mice. Available expression data at the mRNA level showed that PSTPIP2 is mostly expressed in myeloid cells such as mast cells, macrophages, granulocytes and osteoclasts. It has been described that in macrophages PSTPIP2 is phosphorylated after MCSF stimulation (160) and initially it was

believed (with some experimental support) that CMO disease is macrophage driven (145, 146, 161, 162). Because of the relatively convincing publication record pointing at macrophages as the primary cause of the disease, we decided to analyze potentially dysregulated signaling pathways in this cell type. Surprisingly, not only we were unable to find any dysregulation in any signaling pathway studied, we even failed to verify already published data describing dysregulated Erk and STAT1 signaling after MCSF treatment (162). Even additional stimuli that we newly identified as inducers of PSTPIP2 tyrosine phosphorylation, including Fc receptor or integrin crosslinking did not reveal any defects in downstream signaling responses in CMO macrophages. These negative results together with newly published data suggesting the possibility that granulocytes, rather than macrophages, may be the driving force behind CMO disease (156, 157) led us to focus more on neutrophil granulocytes.

6.1.4 PSTPIP2 regulates neutrophil activation

Neutrophils are short lived cells armed with a whole range of mechanisms how to capture, kill and destroy invading pathogens. Cytokine secretion is usually not considered as typical granulocyte weapon. However granulocytes do produce a number of cytokines such as TNF-α, IL-1β, IL-8 and MIP-1α (163) and given the large number of these cells in the blood or in inflammatory lesions, even relatively modest production of cytokine at the single cell level can have profound effects at the whole body level. After the negative experience with macrophages we decided to firstly verify published data on neutrophils. Indeed, after LPS treatment of primary neutrophils from WT and CMO mice and subsequent stimulation with inflammasome activator such as silica particles, we could readily see increased cleavage and production of mature IL-1β in CMO neutrophils. Moreover, we observed dysregulated PI3K and Erk signaling during this process. This assured us that neutrophils could really be the cell type responsible for disease initiation. Further investigation of signaling pathways revealed that other stimuli such as Fc receptor and integrin triggering also led to enhanced signaling response in CMO neutrophils. All these results indicated rather generalized hypersensitivity and over-reactiveness of CMO neutrophils.

The role of Csk binding could not be analyzed in depth, because we could not identify the Csk binding site in PSTPIP2 for the reasons mentioned above. Moreover, no differences in global level of SFK phosphorylation, whether inhibitory or in activation loop, were detected in CMO neutrophils. Reasonable explanation can be the redundancy of Csk adaptors

or regulation of a small specific pool of SFKs by PSTPIP2-bound Csk which cannot be detected by analysis of the global SFK phosphorylation in the whole cell lysates (164).

However the role of the binding of SHIP1 and PEST family phosphatases to PSTPIP2 could be tested since the binding sites were published or identified by us. Binding of SHIP1 is inducible and strictly dependent on the phosphorylation of PSTPIP2 C-terminal tyrosines while binding of PEST phosphatases is constitutive and depends on tryptophan residue W232. To analyze the effect of mutations abolishing these interactions, i.e. mutations of the three C-terminal tyrosines to phenylalanines (3YF) and mutation of tryptophane 232 to alanine (W232A), we employed conditionally immortalized primary granulocyte progenitors from CMO mice and reconstituted these with wild type or mutated versions of PSTPIP2 (165). After progenitor maturation into neutrophils, the resulting cells were challenged with LPS to stimulate pro-IL-1β synthesis and then with silica to activate inflammasome. Interestingly the neutrophils expressing either mutant form of PSTPIP2 generated higher amount of mature IL-1β when compared to cells expressing non-mutated PSTPIP2. Moreover, when we treated primary granulocytes with 3AC, a specific SHIP1 inhibitor (166), we observed increased pro-IL-1β cleavage in WT but not in CMO cells. These results indicate that both SHIP1 and PEST phosphatase binding are required for full inhibitory potential of PSTPIP2. At least some of our findings are also relevant for humans, since we observed PSTPIP2 expression, phosphorylation and binding to Csk and SHIP1 in human neutrophils too.

An intriguing recent discovery connected imbalanced intestinal microbiome composition, characterized mainly by *Prevotella* genus overgrowth, with CMO disease initiation and progression (143). This is reminiscent of other recent reports which revealed NLRs‘ role in maintaining intestinal microbiota homeostasis. Especially the function of NOD2 seems to be crucial for properly balanced microbiota composition (167-169). Moreover, inflammasome NLRs like NLRP6 and its deficiency have also been implicated in intestinal microbial changes resulting in dominance of *Prevotellaceae* species accompanied by intestinal hyperplasia and colitis prone phenotype (170). Strikingly, high fat diet reversed the microbiome changes in CMO mice shifting the balance towards microbiome composition similar to WT mice and basically eliminated disease demonstration. These data suggest that it could be possible to employ dietary modulation in human auoinflammatory conditions to moderate disease burden (143, 171).

It is not known how PSTPIP2 deficiency could predispose intestinal environment to microbiome changes. As we showed in our work, neutrophils from CMO mice are hypersensitive to a variety of stimuli. With changed intestinal homeostasis it is possible that CMO neutrophils sense this microbial disbalance and through lowered activation threshold can get easily primed. Primed hypersensitive neutrophils can then cause unprovoked sterile inflammatory response in different parts of the body. By showing increased generation of mature IL-1β by neutrophils expressing PSTPIP2 mutants unable to bind SHIP1 or PEST phosphatases we described part of the mechanism which likely drives disease initiation and/or progression at the molecular level.

6.2 Role of transmembrane adaptor LST1 in myeloid signaling

LST1 represents typical transmembrane adaptor protein (TRAP). It consists of short extracellular part followed by a transmembrane domain and cytoplasmic part which contains protein-protein interaction motifs. Adaptor itself has no enzymatic activity. Discovery of LST1 as a new potential TRAP was based on *in silico* search in human genome database.

Expression profiles from different publications showed inconsistent results describing either strong expression in immune cells or ubiquitous presence (172, 173). It was further complicated by multiple splicing variants detected (174). However, only one isoform (LST1/A) from this complex mRNA profile is conserved between mouse and human and is expressed on protein level in both organisms. We have resolved conflicting data regarding expression when we showed at mRNA level as well as at protein level that LST1/A is expressed solely in myeloid cells, monocytes and granulocytes.

The function of adaptor proteins is based on effector molecules they bind. In case of new adaptors with unknown function, interacting proteins and thus function can be extrapolated from binding motifs in adaptor's sequence. Sequence analysis revealed two potential tyrosine-based interaction motifs. Amino acid composition of these motifs showed that sequence proximal to transmembrane part fulfills the definition of ITIM motif while distal sequence resembles ITIM-like motif. This tandem organization of ITIM and ITIM-like motif is similar to the one found in Siglec family (29) and overall suggest inhibitory function of LST1/A. While majority of the ITIM motifs are present in receptors and ITAM motifs are contained in co-receptors or adaptors, peculiarity of LST1/A is that it is the other way round. Its composition of structural elements resembles ITAM bearing adaptors such as FcRγ and DAP12. However, at the same time, it contains only ITIM motifs and no ITAMs.

Phosphotyrosine-dependent interacting partners of Siglec proteins include various inhibitory molecules, such as tyrosine phosphatases SHP-1 and SHP-2, SOCS3 or ubiquitin ligase Cbl (29). Immunoprecipitation of FLAG tagged LST1/A expressed in a THP-1 cell line revealed that it binds SHP-1 and SHP-2 when phosphorylated. Phosphorylation of LST1/A is mediated by Lyn and probably other SFKs expressed in myeloid cells, but not by Syk or Btk kinase. Mutation of tyrosines in ITIM and ITIM-like motifs abolished interactions with SHP-1 and SHP-2. To verify these interactions under more physiological conditions, we used primary human monocytes for LST1/A immunoprecipitation. Similar to the cell lines, in non-stimulated monocytes immunoprecipitated LST1/A was constitutively phosphorylated and associated with SHP-1 and SHP-2. Other possible interactions (SOCS3, Cbl, SHIP1) were not detected under any conditions suggesting specific binding for SHP-1 and SHP-2 only.

Only one study analyzed *in vitro* function of LST1/A claiming that its overexpression leads to enhanced filopodia formation (175). Although we observed localization of GFP tagged LST1/A in filopodia, their formation and abundance were similar in transfected and non-transfected cells as revealed by phalloidin staining of these actin-rich structures.

Since LST1/A is an adaptor protein with no receptor domain, there is a possibility that it interacts with specific receptor or receptors and mediates their inhibitory signals. However, we were unable to detect or identify any such receptor. To overcome this problem in functional studies, we constructed chimeric protein where extracellular part of CD25 was fused to LST1/A. When model receptors (Fc receptors in this case) were co-cross-linked with CD25-LST1/A chimera in U937 cells, we could observe complete block in calcium flux in case of Fc receptor co-crosslinking with WT, but not in ITIM-mutated chimera incapable of binding to SHP-1 and SHP-2. Cross-linking of Fc receptors alone resulted in normal calcium response regardless of the construct expressed. Similarly, total protein phosphorylation in cell lysates after Fc receptor engagement was at the same level in non-transduced, WT or ITIM mutated construct. However, when CD25 and Fc receptors were co-cross-linked, overall phosphorylation was strongly reduced in the cells expressing WT but not in ITIM-mutated chimera. Furthermore, this cross-linking led to increased phosphorylation of WT LST1/A associated with increased binding of tyrosine phosphatases.

To summarize this work, we described a new transmembrane adaptor protein LST1/A and its possible role as a negative regulator in myeloid cell signaling mediated by SHP-1 and SHP-2 binding. Open questions remain regarding the identity of receptors and particular

signaling pathways which are regulated by LST1/A *in vivo.* Another important question is what outcome can be expected from LST1/A deficiency in mice or humans which is currently being addressed with the use of LST1/A deficient mouse model.

6.3 Role of Src-family kinases in the initiation of B-cell receptor signaling

Initiation of BCR signaling is a long standing and still controversial question (176). There is an issue whether ligand binding induces clustering of BCR monomers or whether it rather leads to a spatial reorganization of pre-existing BCR oligomers, but at least bivalent engagement of the receptor seems to be necessary for effective stimulation in both cases (177). Downstream of this event stands another unresolved question of proximal BCR signaling. Receptor engagement is followed by ITAM phosphorylation in Igα and Igβ. In general, ITAMs are mainly thought to be phosphorylated by SFKs. However in case of BCR several publications showed that ITAM motifs can also be phosphorylated by Syk either in vitro or in overexpression models (113, 178). These findings are supported by data from Lyn/Fyn/Blk triple-deficient mice where the proximal part of the BCR signaling is still to some extent preserved (179). In our experiments, where we tested the effects of Csk targeting to the plasma membrane we noticed that constitutively membrane-bound Csk, as well as specific SFK inhibitor PP2 had profound negative effect on BCR signaling. However, in contrast to TCR signaling it was never able to inhibit BCR signaling completely. Instead SFK inhibition rather resulted in a substantial delay of the signaling onset with relatively mild effect on the intensity of the response. These preliminary results were rather indicative of SFK-independent initiation of BCR signaling and seemed to support the idea of SFK-independent BCR signaling with novel arguments. The previous evidence for ITAM phosphorylation by Syk was mostly based on *in vitro* data and on overexpression in non-lymphoid cells (113, 114, 178). Due to these limitations these data lacked physiologically relevant credibility. Therefore we decided to investigate this phenomenon in our system, where SFKs and Syk were expressed endogenously in B cell lines and primary B cells.

We used these cells together with various SFK and Syk specific inhibitors, including membrane targeted Csk to analyze the role of SFKs and Syk in detail. Somewhat unexpectedly the main conclusion of this work eventually was that proximal BCR signaling events observed in the cells treated with SFK inhibitors are not Syk dependent but they are rather mediated by the residual SFK activity. SFK inhibition resulted in the delay of ITAM phosphorylation, similar to the delay observed in the downstream pathways and Syk inhibitors

had no effect on this delayed ITAM phosphorylation, while at the same time abolishing the downstream signaling. Moreover, even fully activated Syk kinase was not able to sustain signaling once SFKs were inhibited. Thus the delay in the onset of signaling was probably reflecting the prolonged time that the residual activity of SFK needed to overcome the threshold for signaling initiation.

One of the most convincing arguments for the SFK-independent BCR signaling was the finding showing that Lyn deficient DT40 cells were still able to signal after BCR engagement, although with significant delay when compared to WT cells (180). DT40 is a chicken B cell line believed to express only a single member of Src-family, Lyn (180). Thus its Lyn-deficient variant was supposed to express no SFKs at all. Rather intriguingly, we found that the BCR signaling in Lyn-deficient DT40 cells could still be further delayed when the cells were treated with specific inhibitor of SFKs PP2. One of the possible explanations was that in contrast to a widespread belief, Lyn deficient DT40 cells express another member or members of SFK. Indeed, when we analyzed lysates and immunoprecipitates from WT and Lyn-deficient DT40 cells using antibody specifically recognizing phosphotyrosine in the SFK activation loop, we detected unknown SFK in Lyn-deficient cells. Accordingly, real-time quantitative PCR revealed that Fyn kinase mRNA is expressed at the level of 10-20 % when compared to Lyn expression, thus offering the explanation for the ability of Lyn-deficient DT40 to signal via their BCR and to be susceptible to SFK inhibition.

All these results support the standard model of non-redundant role of SFK and Syk and of sequential involvement of SFK and Syk in BCR signaling initiation. They also brought the arguments against SFK-independent signaling initiation by Syk.

7 Conclusions

In the presented dissertation thesis, I discussed three projects dealing with signal initiation, propagation and regulation in leukocytes. Two projects described adaptor proteins, one cytoplasmic and one transmembrane, and their role in signaling on molecular level. We described the molecular basis for PSTPIP2 function in the suppression of pro-inflammatory signaling by describing its interaction with Csk and SHIP1, well-known inhibitory enzymes. Using different methods of cell activation we showed that PSTPIP2 is rather broadly involved in the regulation of neutrophil signaling. Its deficiency thus causes overall hypersensitivity of granulocytes to different environmental stimuli accompanied by dysregulation of multiple pathways, including PI3K and ERK and IL-1β processing. These data thus describe part of the molecular mechanism how PSTPIP2 deficiency eventually leads to the onset of auto-inflammatory disease.

Thorough analysis of new transmembrane adaptor protein LST1/A revealed its potential inhibitory function in myeloid cells. Through its ITIM and ITIM-like motif it binds tyrosine phosphatases SHP-1 and SHP-2, other well-known inhibitory enzymes. Combined with LST1/A constitutive phosphorylation and thus constitutive binding of phosphatases it can contribute to steady state inhibitory signaling counterbalancing activating effect of kinases. Putative receptors and pathways regulated by LST1/A are yet to be identified.

In third project we show how SFK inhibition by Csk or small molecule inhibitor affects BCR signal initiation. We presented evidence supporting sequential and non-redundant involvement of SFKs and Syk in the initial phases of BCR signaling. Additionally, our data argue against the hypothesis that Syk can initiate BCR signaling independently of SFKs.

8 Contribution

1. **PSTPIP2, a Protein Associated with Autoinflammatory Disease, Interacts with Inhibitory Enzymes SHIP1 and Csk.**

   In the main project of my PhD study I designed, performed and analyzed majority of experiments.

2. **LST1/A is a myeloid leukocyte-specific transmembrane adaptor protein recruiting protein tyrosine phosphatases SHP-1 and SHP-2 to the plasma membrane.**

   In this side project I performed transient and stable transfections of cells followed by microscope analysis (Figure 5B and Supplemental figure S1). Furthermore, I performed kinase assay (Figure 5D) and Fc receptor cross-linking experiment (Figure 6D).

3. **Non-redundant roles of Src-family kinases and Syk in the initiation of B-cell antigen receptor signaling.**

   In this project I analyzed SFK expression in DT40 cells on protein and mRNA level (Figure 7C-D). I also evaluated effect of PP2 on activation loop tyrosine phosphorylation in B and T cells (Supplemental Figure 1B).

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10 Reprint of publications

1. Drobek A, Kralova J, Skopcova T, Kucova M, Novák P, Angelisová P, Otahal P, Alberich-Jorda M, Brdicka T. *PSTPIP2, a Protein Associated with Autoinflammatory Disease, Interacts with Inhibitory Enzymes SHIP1 and Csk.* Journal of Immunology, 2015. **195**(7): p. 3416-26.

2. Draber P, Stepanek O, Hrdinka M, Drobek A, Chmatal L, Mala L, Ormsby T, Angelisova P, Horejsi V, Brdicka T. *LST1/A is a myeloid leukocyte-specific transmembrane adaptor protein recruiting protein tyrosine phosphatases SHP-1 and SHP-2 to the plasma membrane.* The Journal of Biological Chemistry, 2012. **287**(27): p. 22812-21.

3. Stepanek O, Draber P, Drobek A, Horejsi V, Brdicka T. *Nonredundant roles of Src-family kinases and Syk in the initiation of B-cell antigen receptor signaling.* Journal of Immunology, 2013. **190**(4): p. 1807-18.

PSTPIP2, a Protein Associated with Autoinflammatory Disease, Interacts with Inhibitory Enzymes SHIP1 and Csk

Ales Drobek,* Jarmila Kralova,* Tereza Skopcova,* Marketa Kucova,* Petr Novák,† Pavla Angelisová,‡ Pavel Otahal,‡ Meritxell Alberich-Jorda,§ and Tomas Brdicka*

Mutations in the adaptor protein PSTPIP2 are the cause of the autoinflammatory disease chronic multifocal osteomyelitis in mice. This disease closely resembles the human disorder chronic recurrent multifocal osteomyelitis, characterized by sterile inflammation of the bones and often associated with inflammation in other organs, such as the skin. The most critical process in the disease's development is the enhanced production of IL-1β. This excessive IL-1β is likely produced by neutrophils. In addition, the increased activity of macrophages, osteoclasts, and megakaryocytes has also been described. However, the molecular mechanism of how PSTPIP2 deficiency results in this phenotype is poorly understood. Part of the PSTPIP2 inhibitory function is mediated by protein tyrosine phosphatases from the proline-, glutamic acid-, serine- and threonine-rich (PEST) family, which are known to interact with the central part of this protein, but other regions of PSTPIP2 not required for PEST-family phosphatase binding were also shown to be indispensable for PSTPIP2 function. In this article, we show that PSTPIP2 binds the inhibitory enzymes Csk and SHIP1. The interaction with SHIP1 is of particular importance because it binds to the critical tyrosine residues at the C terminus of PSTPIP2, which is known to be crucial for its PEST-phosphatase–independent inhibitory effects in different cellular systems. We demonstrate that in neutrophils this region is important for the PSTPIP2-mediated suppression of IL-1β processing and that SHIP1 inhibition results in the enhancement of this processing. We also describe deregulated neutrophil response to multiple activators, including silica, Ab aggregates, and LPS, which is suggestive of a rather generalized hypersensitivity of these cells to various external stimulants. ***The Journal of Immunology*****, 2015, 195: 3416–3426.**

The activity of leukocyte signaling pathways must be properly controlled to prevent excessive responses with harmful consequences. This is ensured by a number of negative regulators that limit the duration and magnitude of the signaling. One such regulatory protein is the lipid phosphatase SHIP1, which controls the activity of the PI3K pathway by dephosphorylating its key mediator, phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$]. This results in the reduced activity of some of its downstream effectors (1). A prototypical example of such an effector is Akt (also known as PKB), a serine/threonine kinase, which is involved in the regulation of cell activation, proliferation, metabolism, and survival, and which is recruited to the plasma membrane by $PI(3,4,5)P_3$ for further activation (2). The deletion of SHIP1 results in the increased activity of Akt in the mast cells (3) and in the increased translocation of the Akt PH domain to the plasma membrane in neutrophils, suggesting that in myeloid cells SHIP1 regulates Akt localization and activity in vivo (4). There is also ample evidence that SHIP1 negatively regulates MAPK pathways by various mechanisms, in some cases independently of SHIP1 enzymatic activity (5).

Another critical negative regulator of leukocyte signaling is the protein tyrosine kinase Csk. It is involved in the regulation of Src-family kinases (SFKs), a family of protein tyrosine kinases indispensable to the initiation of signal transduction via ITAM-bearing immunoreceptors, and with additional and important roles in signaling by cytokine, growth factor, and pattern recognition receptors, and many others (6). Csk phosphorylates an inhibitory tyrosine residue at the C terminus of SFKs. This phosphorylated tyrosine then interacts with an SH2 domain in the same molecule, resulting in an autoinhibited conformation (7–9). Csk itself is recruited to the plasma membrane and is activated by binding to the phosphorylated tyrosine motifs of transmembrane proteins such as PAG (10, 11), LIME (12), and SCIMP (13). An additional mechanism of SFK inhibition is dependent upon protein tyrosine phosphatases (PTPs) that dephosphorylate the activation loop tyrosine necessary for SFK catalytic activity. This residue can be dephosphorylated by multiple phosphatases, including the receptor tyrosine phosphatases CD45 and CD148 (14–16), PTPs of the Shp family (17), as well as PTP LYP (PTPN22, also known as PEP in mice). In T cells, LYP/PEP forms a complex with Csk.

*Laboratory of Leukocyte Signaling, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic; †Laboratory of Structural Biology and Cell Signaling, Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic; ‡Laboratory of Molecular Immunology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic; and §Laboratory of Hemato-oncology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic

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Address correspondence and reprint requests to Dr. Tomas Brdicka, Laboratory of Leukocyte Signaling, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic. E-mail address: tomas.brdicka@img.cas.cz

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Abbreviations used in this article: 3-AC, 3-α-aminocholestane; ACN, acetonitrile; BM, bone marrow; BMDM, BM-derived macrophage; FcR, Fc receptor; PEST-PTP, proline-, glutamic acid-, serine- and threonine-rich–family PTP; $PI(3,4,5)P_3$, phosphatidylinositol-3,4,5-trisphosphate; PTP, protein tyrosine phosphatase; SFK, Src-family kinase; WT, wild type.

Together, they simultaneously dephosphorylate the activation loop tyrosine and phosphorylate the inhibitory C-terminal tyrosine of SFKs, exerting a combined inhibitory effect (18, 19). LYP/PEP is a member of a small family of PTPs known as proline-, glutamic acid-, serine- and threonine-rich–family PTPs (PEST-PTPs), consisting of only three members: PEP/LYP, PTP-HSCF (PTPN18), and PTP-PEST (PTPN12) (reviewed in Ref. 20). All three family members bind Csk via proline-rich or tyrosine-containing motifs in their central region (18, 21, 22). In addition, a conserved C-terminal homology domain of PEST-PTPs binds two related proteins, PSTPIP1 and PSTPIP2 (23, 24). These are adaptor proteins involved in the control of inflammation, and their altered function results in the development of autoinflammatory diseases (25–27). In general, these disorders are characterized by sterile inflammation and consequential tissue damage. They are mainly the result of dysregulated activity of the innate immune system with no or limited involvement of adaptive immunity (28). The autoinflammatory disease caused by PSTPIP2 mutations is, in mice, characterized by sterile inflammatory lesions in the bones and various degrees of skin and paw inflammation (26, 27, 29). The disease closely resembles the human disorder known as chronic recurrent multifocal osteomyelitis. However, human patients with genetic alterations in the PSTPIP2 gene have not been identified yet. PSTPIP2 is a member of the F-BAR family of proteins (also known as the *pombe cdc15* homology family), characterized by the presence of N-terminal F-bar domain mediating interactions with membrane phospholipids and a C-terminal tail containing various interaction motifs (30). The F-bar domain of PSTPIP2 interacts with $PI(4,5)P_2$ (31), whereas its C-terminal tail binds PEST-PTPs via the interaction motif, which includes tryptophan 232 (24, 32). The C-terminal tail of PSTPIP2 contains several tyrosines of unknown function that are phosphorylated in macrophages after exposure to M-CSF (33). Two different mouse strains where point mutations in PSTPIP2 result in the autoinflammatory disorder have been established. As a result of these mutations, one of these strains, LUPO, displays approximately a 70% reduction in PSTPIP2 protein expression (27), whereas the other strain, CMO, shows a complete absence of PSTPIP2 (34). The disease is independent of T or B cells, and it was originally attributed to the enhanced activity of macrophages and osteoclasts (27, 32, 34). In addition, more recent work has suggested that neutrophils may also be a critical cell type in disease initiation (35, 36). Similar to several other autoinflammatory syndromes, the disease appears to be, at least in part, caused by the enhanced production of IL-1β (35, 37). The molecular mechanism of how PSTPIP2 prevents autoinflammatory disease development remains largely unknown. The effects of PSTPIP2 binding to PEST-family phosphatases have been tested recently in osteoclasts and megakaryocytes, where it had an effect on their differentiation. However, in these experiments, a profound effect was also seen after the mutation of C-terminal tyrosines, which rendered PSTPIP2 especially in the case of osteoclasts essentially nonfunctional without affecting the interaction with PEST-family phosphatases (32, 38). This suggested that the binding partners of these tyrosines are critical for PSTPIP2 function. However, their identity remained unknown.

In this article, we show that these phosphorylated tyrosine residues bind the lipid phosphatase SHIP1. In addition, we also show that PSTPIP2 interacts with Csk via a mechanism that is, at least in part, independent of PEST-PTPs. Our data also bring evidence for the involvement of these inhibitory enzymes in the PSTPIP2-mediated suppression of the inflammatory response.

Materials and Methods

Abs

Abs to the following Ags were used in this study: FLAG (M2) and GAPDH (from Sigma-Aldrich, St. Louis, MO); phospho-Erk (T202/Y204), phospho-Akt (S473, T308), and Myc (9B11) (Cell Signaling Technology, Danvers, MA); Csk (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA); CD18 (YTS 213.1) (Serotec, Kidlington, U.K.); B220-biotin (RA3-6B2), TER119-biotin, Gr-1–PE (RB6-8C5), c-Kit–biotin, CD3ε-biotin, CD11b-FITC, Ly6C-PE-Cy7 (Biolegend, San Diego, CA); B220-FITC, DX5-biotin, F4/80-biotin, F4/80-PE, and F4/80-Alexa700 (eBioscience, San Diego, CA); Thy1.2-FITC, CD271-FITC, anti-FITC, and anti-biotin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany); HRP-conjugated goat anti-mouse L chain–specific Ab, HRP-mouse anti-rabbit L chain–specific, purified mouse IgG-whole molecule, goat anti-mouse $F(ab)_2$, goat anti-rat $F(ab)_2$ (Jackson Immunoresearch, West Grove, PA), Fc Bloc (2.4G2) (BD Biosciences, San Jose, CA). The rabbit antisera against SHIP1, PEP, PTP-PEST, and PTP-HSCF were a gift from Dr. A. Veillette (Institut de Recherches Cliniques de Montreal, University of Montreal). The Ab against human SHIP1 was from Exbio (Vestec, Czech Republic).

The mouse mAb that recognizes murine and human PSTPIP2 was generated by the immunization of mice (F1 hybrids of BALB/c × B10A) with a full-length recombinant murine PSTPIP2 produced in *Escherichia coli*. Splenocytes from immunized mice were fused with Sp2/0 myeloma cells and cloned by limiting dilution. Ab production was tested by ELISA and Western blotting.

To prepare mouse or human aggregated Ig, we first purified IgG from mouse serum (Sigma-Aldrich) or human AB serum (Invitrogen, Carlsbad, CA) on protein A-Sepharose (GE Healthcare, Uppsala, Sweden), transferred them to PBS, and concentrated them to 30 mg/ml on an Amicon Ultracel–30K unit (Millipore, Merck, Darmstadt, Germany). The aggregation was induced by heating to 63°C for 30 min.

Mice

CMO mouse strain (Cg-Pstpip2cmo/J) carrying the *c*.293T→C mutation in the *Pstpip2* gene (26, 29) on the BALB/c genetic background, resulting in an L98P change in the PSTPIP2 protein, and BALB/cByJ, were obtained from The Jackson Laboratory (Bar Harbor, ME). The BALB/c and F1 hybrids of BALB/c × B10A were from the animal facility of Institute of Molecular Genetics, Academy of Sciences of the Czech Republic (Prague, Czech Republic). All the experiments in this work that were conducted on animals were approved by the Animal Care and Use Committee of the Institute of Molecular Genetics and were in agreement with local legal requirements and ethical guidelines.

Cell lines and primary cells

All the primary cells and cell lines were cultured at 37°C with 5% CO_2 in the following media supplemented with 10% FCS and antibiotics: WEHI-231 cell line (ATCC) in RPMI 1640, HEK293FT cells (Invitrogen), Phoenix Eco cells (Origene, Rockville, MD), J774.2 (ATCC) cells, and immortalized macrophage progenitors in DMEM. IMDM was used for immortalized granulocyte progenitors and primary granulocytes.

Bone marrow (BM) was isolated from CMO and BALB/cByJ or BALB/c mice sacrificed by cervical dislocation. Full BM was cultured in DMEM conditioned with 10% L929 culture supernatant containing M-CSF to differentiate BM-derived macrophages (BMDMs). Osteoclasts were differentiated in DMEM supplemented with 20 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech, Rocky Hill, NJ). Murine granulocytes were isolated from BM by negative selection using B220, F4/80, DX5, c-Kit, CD3ε, and Ter119 biotinylated Abs, and anti-biotin MicroBeads, on an AutoMACS magnetic cell sorter (Miltenyi Biotec), and the purity (>90%) was determined by flow cytometry. Human granulocytes were purified from buffy coats (purchased on a commercial basis from the Blood Bank of Thomayer Hospital, Prague, Czech Republic) by sedimentation in 2% Dextran T500 (Pharmacosmos, Holbaek, Denmark) followed by Ficoll centrifugation (Ficoll-Paque PLUS, GE Health Care) and erythrocyte lysis in an ACK buffer [150 mM NH_4Cl, 0.1 mM EDTA (disodium salt), 1 mM $KHCO_3$]. The generation of immortalized macrophage progenitors has been described previously (39); in brief, BM was centrifuged over Ficoll-Paque PLUS gradient, and mononuclear cells were directly infected with ER-Hoxb8 retrovirus by spinoculation. The cells were cultivated in a medium conditioned with 1% LUTZ culture supernatant as a source of GM-CSF and 1 μM β-estradiol (estrogen; Sigma-Aldrich). The cells could be differentiated into macrophages by the withdrawal of estrogen from the media within a week. To prepare immortalized granulocyte progenitors, we used a modified version of this protocol (39). The progenitors were first enriched by the depletion of Mac-1^+, $B220^+$, and Thy1.2^+ from mouse BM

cells and cultured in the presence of IL-3, IL-6, and SCF (supplied as culture supernatants from HEK293 cells transduced with constructs coding for respective cytokines) for 2 d. Next, they were transduced with the same ER-HoxB8 construct described earlier. The transduced cells were enriched for the GMP progenitor population by FACS (Lin^-, $Sca\text{-}1^-$, $c\text{-}Kit^+$, $Fc\gamma R^+$, $CD34^+$) and propagated in a media containing 1 μM β-estradiol and 1% SCF-containing supernatant. Granulocyte differentiation was induced by β-estradiol withdrawal and the addition of 50 ng/ml G-CSF.

DNA constructs, transfection, and transduction

The CLG construct (generated by the PCR-mediated joining of individual overlapping fragments and subcloned to MSCV-IRES-Thy1.1; Clontech, Mountain View, CA) was composed of the SH2 and SH3 domains of human Csk (aa 1–171) followed by the kinase domain from human Lck (aa 225–509) with Y505 mutated to phenylalanine and then by a Myc tag sequence and GST from *Schistosoma japonicum* lacking methionine 1 (Fig. 1A). Murine PSTPIP2 was cloned from cDNA prepared by the reverse transcription (RevertAid, Fermentas, Thermo Fisher Scientific, Waltham, MA) of RNA from WEHI-231 cells. Wild type (WT) PSTPIP2 or its various mutants generated by PCR mutagenesis were cloned into the MSCV-IRES-LNGFR vector (Clontech) with an N-terminal FLAG tag. Human PSTPIP2 was cloned from cDNA prepared from peripheral blood leukocytes. A Csk-Myc construct in a pMSCV retroviral vector and its variants with inactivating point mutations in the SH2 and SH3 domains (R107K and W47A, respectively) were prepared by PCR from the analogous Csk-Myc-OFP constructs described earlier (40). Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's protocol for the transfection of Phoenix Eco cells to produce viral particles. Retrovirus-containing supernatants were then harvested, supplemented with polybrene (10 μg/ml; Sigma-Aldrich), and added to the cells. The cells were then centrifuged at 1250 × *g* for 90 min at 30°C. Infected cells were sorted based on the expression of reporter markers (Thy1.1 or LNGFR) by a BD Influx FACS (BD Biosciences).

Cell activation, lysis, and immunoprecipitation

For Western blotting, the cells were washed and resuspended in serum-free conditioned IMDM or DMEM at a concentration of 1–4 × 10^7 cells/ml. Subsequently, the cells were stimulated as indicated at 37°C. The activation of cells was stopped by the addition of an equal volume of a 2× concentrated SDS-PAGE sample buffer containing 0.1 mM diisopropyl fluorophosphate, followed by the sonication and heating of the samples (96°C for 2 min). The samples were analyzed by SDS-PAGE followed by Western blotting. For pervanadate-induced activation, the cells were incubated with 0.1 mM pervanadate for 10 min. To inhibit SHIP1 during the activation, we treated the cells with 10 μM 3-α-aminocholestane (3AC; Echelon Biosciences, Salt Lake City, UT).

For immunoprecipitation, the cells were lysed in the lysis buffer (1% lauryl maltoside [Calbiochem, Merck], 20 mM Tris [pH 7.5], 100 mM NaCl, 5 mM iodoacetamide, 50 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM Pefabloc [Sigma-Aldrich] or 100× diluted Protease Inhibitor Cocktail set III [Calbiochem]; for neutrophil granulocyte lysis, 1 mM PMSF [Sigma-Aldrich] and 0.1 mM diisopropyl fluorophosphate [Fluka; Sigma-Aldrich] were also added) at 5 × 10^6/ml for 30 min on ice. Postnuclear supernatants were then incubated for 1 h with a primary Ab (2 μg/ml), followed by 1–2 h of incubation with Protein A/G Plus agarose beads (Santa Cruz Biotechnology) or Protein G magnetic beads (Millipore, Billerica, MA) at 4°C. After washing on chromatography columns or a magnetic stand, immunoprecipitates were eluted with a 70 μl SDS-PAGE sample buffer.

In vitro kinase assay

For the in vitro kinase assay, cells were lysed in the lysis buffer where NaF, Na_3VO_4, and EDTA were omitted. The immunoprecipitation was carried out in 96-well plates precoated with 0.1 mg/ml goat anti-mouse IgG (Sigma-Aldrich) and Myc Ab (see earlier). The immunoprecipitates were incubated with a kinase buffer (25 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1% lauryl maltoside, and 0.1 μCi $\gamma[^{32}P]$-ATP per well) for 20 min at room temperature. After washing, the immunoprecipitates were eluted with a 50 μl SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography.

Mass spectrometric analysis

Protein bands were cut from the gel, then further cut into small pieces and decolorized in a sonic bath at 60°C several times with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (ACN). After complete destaining, proteins were reduced by 50 mM TCEP in 0.1 M 4-ethylmorpholine acetate (pH 8.1) for 5 min at 80°C and alkylated using 50 mM iodoacetamide in 0.1M 4-ethylmorpholine acetate (pH 8.1) in the dark at room temperature. Next, the gel was washed with water and ACN and was partly dried using a SpeedVac concentrator (Savant, Holbrook, NY). Finally, the gel was reconstituted with a cleavage buffer containing 0.01% 2-ME, 0.05M 4-ethylmorpholine acetate (pH 8.1), 10% ACN, and sequencing-grade trypsin (10 ng/μl; Promega). Digestion was carried out overnight at 37°C, and the resulting peptides were extracted with 30% ACN/0.1% TFA and subjected to mass spectrometric analysis. Mass spectra were acquired using the positive ion mode on a MALDI-FTMS APEX-Ultra (Bruker Daltonics, Bremen, Germany) equipped with a 9.4 T superconducting magnet and a SmartBeam laser. The acquisition mass range was 700–3500 m/z, and 512k data points were collected. The instrument was externally calibrated using the PepMix II peptide standard (Bruker Daltonics, Bremen, Germany). This results in a typical mass accuracy <2 ppm. A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% ACN/0.2% TFA was used as a MALDI matrix. One microliter of the matrix solution was mixed with 1 μl of the sample on the target and the droplet was allowed to dry at ambient temperature. After the analysis, the spectra were apodized using square sine apodization with one zero fill. The interpretation of the mass spectra was done using the DataAnalysis version 3.4 and BioTools 3.2 software packages (Bruker Daltonics, Billerica, MA). The proteins were identified by peptide mass fingerprinting using the search algorithm MASCOT (Matrix Science).

Results

The initial goal of this study was the identification of novel Csk-interacting proteins. To achieve this, we designed a construct (hereafter termed CLG) consisting of the SH2 and SH3 domains of Csk and the kinase domain from the SFK Lck, followed by the Myc tag and GST (Fig. 1A). The construct was expressed in the murine B cell line WEHI-231 via retroviral infection. Because many Csk-binding proteins are substrates of SFKs, we expected that the Lck kinase domain would phosphorylate proteins bound to the Csk SH2/SH3 module of the construct, and we thus label them for relatively easy detection in the in vitro kinase assay or else after phosphorylation in vivo. This would allow for the subsequent optimization and scaling up of the procedure to identify these proteins by mass spectrometry. The Myc-GST module was intended for the tandem purification of CLG using glutathione Sepharose followed by the second round of affinity purification with the anti-Myc Ab. However, this approach was eventually abandoned because of the major technical difficulties caused, presumably, by the competition between the construct and the endogenous murine GST present in the cell lysates. Hence we eventually used this construct in only single-step Myc immunoprecipitations, without engaging its GST moiety. In this setup, we immunoprecipitated the CLG fusion protein from WEHI-231 lysates and incubated the immunoprecipitate with $\gamma[^{32}P]$-ATP. The proteins radioactively labeled by the CLG Lck kinase domain were then subjected to SDS-PAGE and autoradiography. This procedure revealed several proteins with molecular masses of 75, 51–55, 37, and 16 kDa in the immunoprecipitates (Fig. 1B). Whereas the 75-kDa band most likely represented the autophosphorylated CLG construct, the identity of the other proteins remained unknown. To identify these proteins, we performed a large-scale immunoprecipitation from WEHI-231 cell lysates (from 4 × 10^8 cells) infected with the CLG construct followed by SDS-PAGE and Coomassie blue detection. In this way, we were able to detect a protein of 37 kDa, which likely corresponded to a 37-kDa band from the in vitro kinase assay (Fig. 1C). Mass spectrometry identification revealed that this protein was PSTPIP2 (Fig. 1D, Supplemental Fig. 1). Although it was known to interact with PEST-PTPs (24, 32), interaction of PSTPIP2 with Csk has not yet been described.

Because of the unusual composition of the CLG construct, we wanted to confirm that nonaltered full-length endogenous Csk interacts with PSTPIP2. To achieve this, we infected a murine WEHI-231 cell line with a FLAG-tagged PSTPIP2 construct and

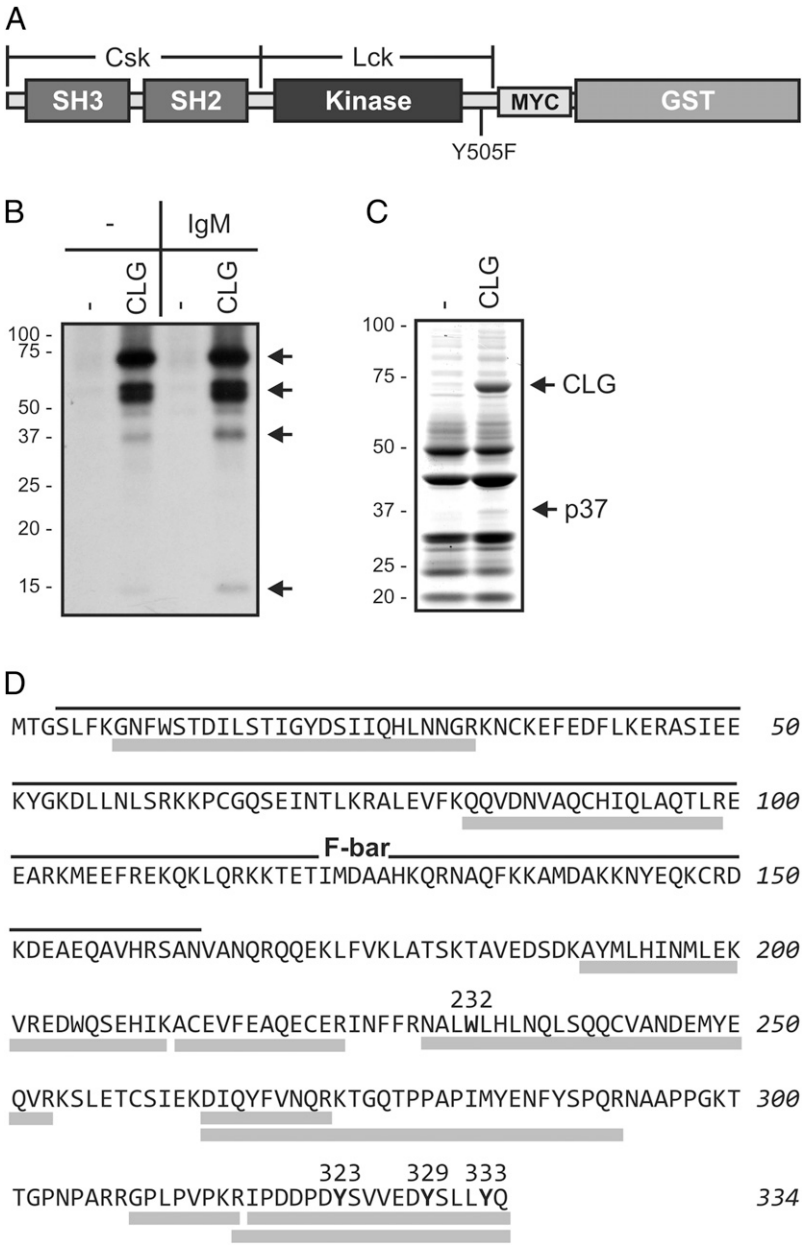

FIGURE 1. Identification of PSTPIP2 as a Csk binding protein. (**A**) Schematic representation of the CLG construct. (**B**) Anti-myc (CLG) immunoprecipitates from WEHI-231 cells transfected (or not) with the CLG construct and activated (or not) with an anti-IgM Ab were incubated with $\gamma[^{32}P]$-ATP and subjected to SDS-PAGE and autoradiography. Arrows indicate the positions of major phosphorylated species. (**C**) Large-scale anti-Myc (CLG) immunoprecipitation from lysates of WEHI-231 transfected (or not) with the CLG construct. The immunoprecipitates were separated by SDS-PAGE followed by staining with colloid Coomassie blue. Arrows show the positions of the CLG construct and p37. (**D**) Amino acid sequence of PSTPIP2. Gray bars underline the peptides identified by mass spectrometry. Functionally important sequences and residues are also labeled (F-bar domain with a black line above the sequence, important amino acid residues by numbers above the sequence).

then immunoprecipitated this protein from the lysates of infected cells. We could readily detect endogenous Csk in FLAG immunoprecipitates from PSTPIP2-FLAG–infected cells, but not from noninfected cells, demonstrating that full-length endogenous Csk interacts with PSTPIP2 (Fig. 2A). To find out which of the binding domains present in Csk is responsible for PSTPIP2 binding, we infected WEHI-231 cells with Csk constructs containing inactivating mutations in their SH2 and SH3 domains. Intriguingly, the immunoprecipitations of these mutated Csk proteins from the cell lysates revealed that the mutation of any of these domains substantially reduced or abolished the binding of PSTPIP2 to Csk, suggesting that the binding is likely cooperative, involving both the SH2 and the SH3 domains of Csk (Fig. 2B).

Because Csk is known to interact with PEST-PTPs (18, 21, 22), we wanted to find out whether the Csk–PSTPIP2 interaction is dependent on these enzymes. For this purpose, we again used WEHI-231 cells transfected with WT PSTPIP2 and its W232A mutant, which is unable to bind PEST-family phosphatases. Coprecipitation experiments revealed that the W232A mutation diminished Csk binding. However, a substantial fraction of Csk still interacted with PSTPIP2 W232A, whereas at the same time the binding of all the PEST-family phosphatases was completely abolished (Fig. 2C). These data suggested that a fraction of Csk binds to PSTPIP2 independently of PEST-PTPs, most likely via the direct interaction with PSTPIP2. In this experiment, we also demonstrated that PSTPIP2 interacts with PEP in a similar manner (requiring the presence of W232) to other PEST-family phosphatases (Fig. 2C). PEP is the last PEST-family member for which this interaction has not yet been demonstrated.

Although we identified the interaction between Csk and PSTPIP2 in the B cell line WEHI-231, macrophages were initially thought to be the critical cell type driving inflammatory disease in CMO mice (27, 34). Moreover, PSTPIP2 phosphorylation can be observed in macrophage cell line after stimulation by M-CSF (33), and the absence of PSTPIP2 resulted in an increased macrophage proliferation in response to the same stimulation (34). Thus, to study PSTPIP2 tyrosine phosphorylation and its effects on Csk binding in a more relevant cell type, we used the murine macrophage-like cell line J774. We generated constructs with phenylalanine mutations in Y323, Y329, and Y333, located in the PSTPIP2 C-terminal

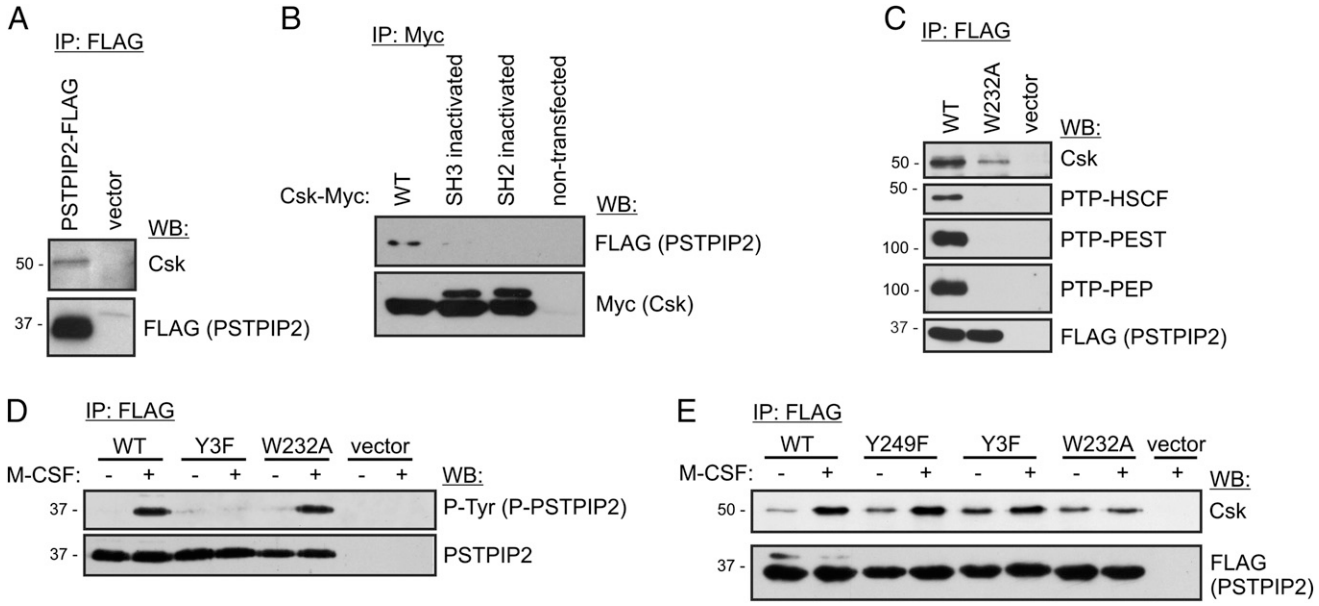

FIGURE 2. PSTPIP2 interaction with Csk. (**A**) Lysates from PSTPIP2-FLAG or vector-transduced WEHI-231 were subjected to FLAG immunoprecipitation (IP) followed by immunoblotting (WB) with indicated Abs. (**B**) Lysates from WEHI-231 transduced with PSTPIP2-FLAG and the Csk-Myc constructs with or without an inactivating mutation in the SH2 (R107K) or SH3 (W47A) domain were subjected to Myc immunoprecipitation followed by immunoblotting with FLAG and Myc Abs. (**C**) Lysates from WEHI-231 cells expressing the WT or W232A PSTPIP2-FLAG constructs were subjected to FLAG immunoprecipitation followed by immunoblotting with Abs to the indicated proteins. Empty vector-transduced cells served as a negative control. (**D**) FLAG immunoprecipitates from J774 cells transfected with indicated PSTPIP2-FLAG constructs and stimulated (or not) with M-CSF were subjected to immunoblotting with phosphotyrosine (P-Tyr) and PSTPIP2 Abs. Empty vector-transduced cells served as a negative control. (**E**) Similar immunoprecipitation as in (D) followed by immunoblotting with Csk and FLAG Abs. Only relevant parts of the blots are shown.

tail (Y3F). Y323 and Y333 have previously been reported to be responsible for the majority of PSTPIP2 tyrosine phosphorylation (24, 32), and Y329 also seemed a good candidate phosphorylation site (see *Discussion* for more details on the selection of these tyrosines). Next, we expressed these constructs, as well as the W232A construct, in J774 cells stimulated (or not) with M-CSF to induce the tyrosine phosphorylation of PSTPIP2. We observed an increase in the tyrosine phosphorylation of PSTPIP2 after M-CSF treatment, which was not affected by W232A mutation (Fig. 2D). In contrast, the mutation of all three C-terminal tyrosines (Y3F) almost completely abolished PSTPIP2 tyrosine phosphorylation (Fig. 2D), which is in agreement with previously published data (24, 32). In the next experiment, we analyzed the Csk binding to these mutants. For this purpose, we also included a mutation of Y249, which, of all PSTPIP2 tyrosines, most closely resembled the Csk-binding site (12). Similar to the previous experiment, in resting cells we observed Csk binding to WT PSTPIP2 (Fig. 2E). Moreover, upon M-CSF stimulation, Csk binding to PSTPIP2 substantially increased. However, none of the tyrosine mutations were able to reduce basal or M-CSF–induced Csk binding to PSTPIP2. In addition, when we analyzed the W232A mutant of PSTPIP2, which was unable to interact with PEST-family phosphatases, we found that in nonstimulated cells Csk binding was not altered by the W232A mutation. However, the increase in Csk binding after M-CSF stimulation was lost (Fig. 2E). We also tested a number of other mutations (Supplemental Table I), as well as their combinations with W232A, but we did not observe any additional effect on Csk binding (data not shown). Unfortunately, some of the candidate binding sites could not be analyzed because their mutations resulted in instability and degradation of the mutant protein that, as a result, could not be detected in the lysates of the transfected cells. Thus, we can conclude that, in J774 cells, PSTPIP2 constitutively binds Csk and this binding is independent of PEST-PTPs. Moreover, M-CSF stimulation results in enhanced binding, which is likely mediated by PEST-PTPs.

To better understand the function of PSTPIP2 in macrophages and to find relevant situations where the signaling pathways could be regulated by PSTPIP2, we analyzed its expression and phosphorylation in this cell type. To be able to follow the endogenous protein expression, we developed a mouse mAb specific for both human and mouse PSTPIP2. The Ab recognizes transfected (Fig. 3A) and endogenous PSTPIP2 (Fig. 3B), and works well in both Western blot analysis and immunoprecipitation experiments.

It has been previously published that PSTPIP2 expression in macrophages is increased after LPS treatment, suggesting that PSTPIP2 expression may be enhanced during inflammation. Thus, we treated BMDMs with LPS or various mostly proinflammatory cytokines and followed the PSTPIP2 expression. Interestingly, PSTPIP2 was strongly upregulated, not only by LPS but also by treatment with several proinflammatory cytokines, including IFN-γ, TNF-α, and GM-CSF, whereas treatment with IL-4 (which promotes their alternative M2 differentiation) did not change the PSTPIP2 expression (Fig. 3B). These data suggest that an increase in PSTPIP2 expression is a part of a proinflammatory differentiation program in macrophages.

To obtain the more complete information about the involvement of PSTPIP2 in the regulation of inflammatory processes in macrophages, we next tested several additional receptors involved in the macrophage inflammatory response for their ability to regulate the tyrosine phosphorylation of PSTPIP2. In this experiment, we stimulated J774 cells expressing FLAG-PSTPIP2 with M-CSF, an anti-Fc receptor (anti-FcR) Ab or an Ab against an integrin β2 subunit, as well as LPS, GM-CSF, and IFN-γ. In resting cells, we observed a low level of constitutive PSTPIP2 phosphorylation (Fig. 4A). Similar to the previously published data, M-CSF treatment resulted in increased phosphorylation. Importantly, PSTPIP2 tyrosine phosphorylation was also substantially increased after FcR

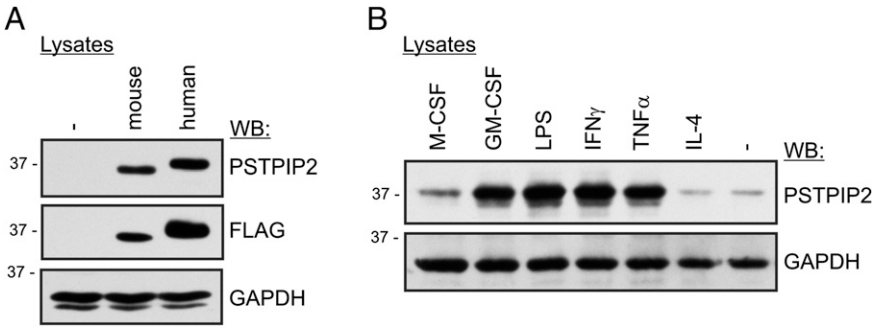

FIGURE 3. Evaluation of the PSTPIP2 Ab and regulation of PSTPIP2 expression. (**A**) HEK293 cells were transfected with FLAG-tagged mouse or human PSTPIP2. Both proteins were detected on the Western blot by the mAb to PSTPIP2. Nontransfected cells served as a negative control. (**B**) BMDMs from WT mice were treated for 24 h with LPS or indicated cytokines followed by immunoblotting with the PSTPIP2 Ab. Staining for GAPDH served as a loading control. Only relevant parts of the blots are shown.

cross-linking with more rapid kinetics (Fig. 4A). This phosphorylation peaked at 1 min and then gradually declined (Fig. 4B). It was accompanied by increased Csk binding, which was also more sustained. The binding of the PEST phosphatases PTP-PEST and PTP-HSCF did not seem to be influenced by FcR stimulation. However, the remaining family member PTP-PEP displayed a comparatively low level of binding in the resting cells, which increased substantially after FcR stimulation (Fig. 4B). Similar phosphorylation was also observed after integrin stimulation, although in this case the involvement of the FcRs could not be excluded because full-length Abs were used to cross-link the integrins in these experiments (Fig. 4A). We did not see any changes in PSTPIP2 phosphorylation after stimulation with LPS, GM-CSF, or INF-γ (data not shown).

The C-terminal tyrosines of PSTPIP2 were identified as major phosphorylation sites in PSTPIP2, and in some experiments they also appeared to be among the functionally most important residues in the PSTPIP2 protein (32, 38). In our experiments, Csk binding did not depend on the presence of these tyrosines. Therefore, we assumed that there must be an additional, unknown binding partner that interacts with these residues, and we attempted its identification. For this purpose, we used immortalized macrophage progenitors (see *Materials and Methods*). These cells are much more closely related to primary cells than tumor cell lines, and thus they are better suited for the detection of the most relevant interactions. Moreover, increased number of macrophage progenitors has been observed in CMO mice, suggesting PSTPIP2 involvement in the regulation of this population (34). Finally, these cells can be propagated in a cell culture and obtained in sufficient numbers for large-scale experiments. We infected these cells with WT PSTPIP2 and PSTPIP2 with mutated C-terminal tyrosines (Y3F) and then performed large-scale PSTPIP2 immunoprecipitations. Mass-spectrometry analysis of the proteins specifically present in the immunoprecipitates of WT, but not mutant, PSTPIP2 resulted in the identification of the lipid phosphatase SHIP1 as a novel PSTPIP2 binding partner (Fig. 5A, Supplemental Fig. 2). To confirm these data under more physiological conditions, we immunoprecipitated PSTPIP2 from the lysates of WT and CMO BMDMs stimulated with M-CSF or via FcR, and we observed a low level of constitutive SHIP1 binding that was substantially increased after both M-CSF and FcR stimulation (Fig. 5B, 5C). To verify that SHIP1 binds to the C-terminal tyrosines of PSTPIP2, we expressed WT and Y3F mutants of PSTPIP2 in CMO BMDMs and stimulated these cells with pervanadate. Indeed, we could readily observe SHIP1 binding to WT, but not to the mutant protein, confirming that functionally important C-terminal tyrosines are required for SHIP1 binding (Fig. 5D).

SHIP1 dephosphorylates the PI3K product $PI(3,4,5)P_3$, thus regulating a number of important pathways, whereas Csk inhibits SFKs, which are also expected to have a strong impact on macrophage signaling. Unexpectedly, we could not find any differences in the activity of M-CSF, LPS, or FcR-driven signaling pathways when comparing the BMDMs from WT and CMO mice (data not shown). This included the analysis of Akt and Erk phosphorylation, which are known to be regulated by SHIP as well as SFK phosphorylation at the inhibitory tyrosine (or in the activation loop) regulated by Csk. We also analyzed a number of other pathways in the macrophages (Jak-Stat, NF-κB, JNK, p38) (data not shown), but we did not see any defects. However, recently published work has suggested neutrophil granulocytes and IL-1β as probable driving forces behind inflammatory disease in CMO mice (35, 36). Thus, as a next step, we analyzed PSTPIP2 expression and function in granulocytes. Surprisingly, we found that

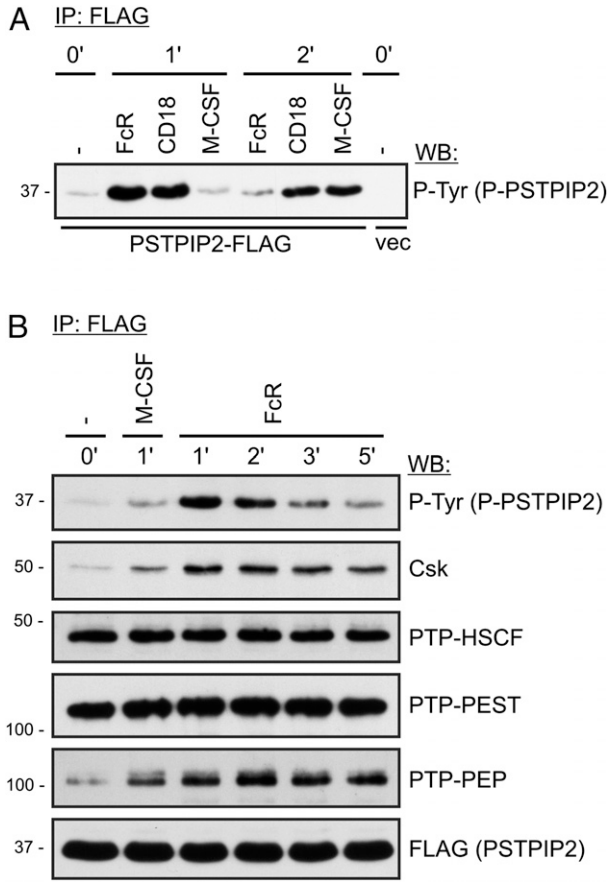

FIGURE 4. Phosphorylation and interactions of PSTPIP2. (**A**) J774 cells expressing PSTPIP2-FLAG were stimulated by M-CSF or by FcR or CD18 cross-linking for the indicated time intervals. PSTPIP2-FLAG was immunoprecipitated from the lysates of these cells and subjected to immunoblotting with the phosphotyrosine (P-Tyr) Ab. (**B**) Immunoprecipitates from M-CSF– or FcR-stimulated cells were prepared in a similar manner as in (A) and subjected to immunoblotting with the indicated Abs. Only relevant parts of the blots are shown.

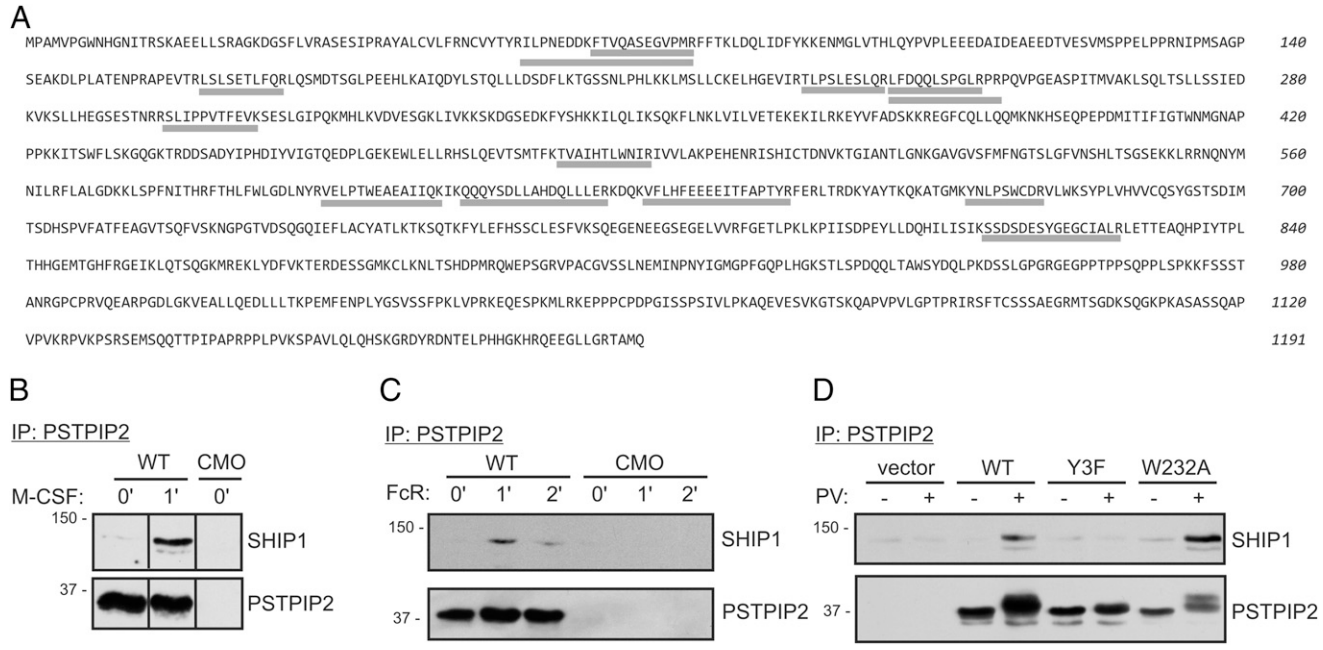

FIGURE 5. Binding of SHIP1 to PSTPIP2. (**A**) Amino acid sequence of SHIP1. Gray bars underline the peptides identified by mass spectrometry. (**B** and **C**) Lysates from WT or CMO BMDMs activated for indicated time intervals with M-CSF (B) or by FcR cross-linking (C) were subjected to PSTPIP2 immunoprecipitation followed by SHIP1 or PSTPIP2 immunoblotting. (**D**) CMO macrophages differentiated from immortalized macrophage progenitors were transduced with the indicated constructs. The cells were treated (or not) with 0.1 mM pervanadate and the lysates from these cells were subjected to PSTPIP2 immunoprecipitation followed by SHIP1 and PSTPIP2 immunoblotting. Only relevant parts of the blots are shown.

in these cells the expression of PSTPIP2 is higher than in macrophages or osteoclasts (Fig. 6A). Moreover, in contrast with our observations in the macrophages, CMO neutrophils exhibited increased FcR-triggered phosphorylation of the Erk and p38 MAPKs, as well as higher phosphorylation of Akt (Fig. 6B) and more efficient processing of IL-1β (Fig. 6C), compared with WT

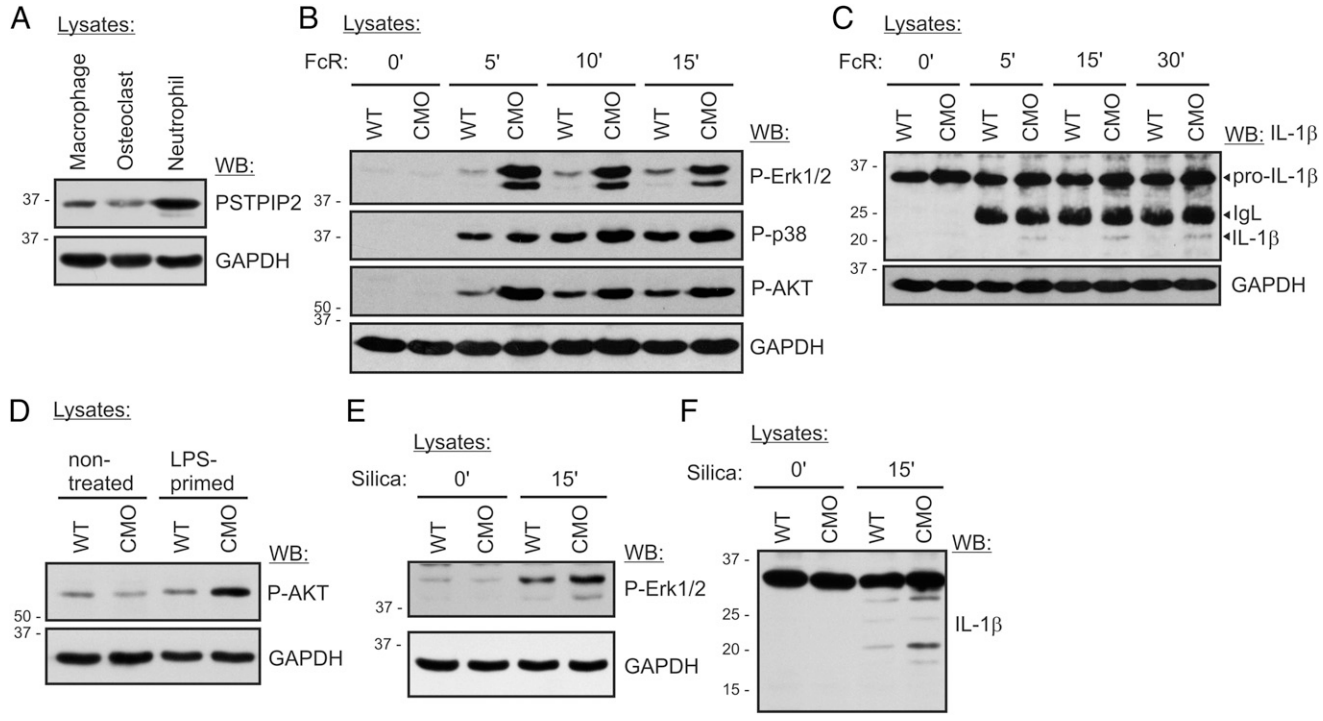

FIGURE 6. Effects of PSTPIP2 deficiency on neutrophil signaling. (**A**) Expression of the PSTPIP2 protein in the lysates of BMDMs and BM-derived osteoclasts and primary neutrophils purified from the same organ. (**B**) Erk, p38, and Akt (S473) phosphorylation in the lysates of neutrophils purified from mouse BM and treated for the indicated time intervals with mouse aggregated IgG to stimulate their FcRs. (**C**) Neutrophils were purified from mouse BM primed for 3 h with 100 ng/ml LPS and then for the indicated time intervals with aggregated mouse IgG. They were then lysed and subjected to immunoblotting with the IL-1β Ab to probe for processed IL-1β and its precursor. Note the presence of the Ig L chains from the mouse IgG used in the FcR activation. (**D**) Akt phosphorylation at S473 in the lysates of neutrophils primed for 90 min with 50 ng/ml LPS. (**E**) Erk phosphorylation in the lysates of neutrophils treated for 15 min with silica particles (50 $\mu g/cm^2$). (**F**) The same samples from (E) were probed for pro–IL-1β and its cleavage products. Only relevant parts of the blots are shown.

neutrophils. The additional analysis of signaling pathways in granulocytes isolated from CMO mice revealed that Akt phosphorylation was also increased after the relatively long (90-min) treatment of neutrophils with LPS (Fig. 6D). However, pro–IL-1β production after LPS priming did not appear to be altered in CMO neutrophils (Fig. 6C).

Finally, we also tested the effects of silica particles, a typical inflammasome activator, on CMO neutrophil signaling and IL-1β cleavage. The silica exposure in these cells resulted in the enhanced activation of MAPKs Erk1/2 (Fig. 6E), whereas no reproducible differences in Akt phosphorylation were observed in CMO mice (data not shown). To analyze IL-1β processing, we induced pro–IL-1β production in neutrophils by priming with LPS and then triggered its cleavage by subsequent incubation with silica. Although the LPS-induced pro–IL-1β production was not markedly affected in CMO neutrophils, silica exposure led to the enhanced production of mature IL-1β of 21 and 17 kDa (Fig. 6F), which are the sizes characteristic for cleavage by neutrophil serine proteases and inflammasome-dependent caspases, respectively (41). This is in agreement with the previously published data showing the enhanced secretion of IL-1β by neutrophils into the culture media under these circumstances (35, 36).

Because recent reports showed that IL-1β and inflammasome-activated caspases are critical for the disease development in CMO mice (35–37), we decided to analyze the silica-triggered IL-1β production in more detail, especially with respect to the interactions among PSTPIP2, SHIP1, and Csk described earlier. To address the question of PSTPIP2 phosphorylation under these conditions, we performed the immunoprecipitation of endogenous PSTPIP2 from unstimulated or LPS-primed and silica-stimulated BM cells. Of the cell types that express PSTPIP2 in the BM, neutrophils comprise the vast majority. Thus, we used non-separated BM cells for this experiment to increase the yield and to prevent unnecessary manipulations of these sensitive cells. Under these conditions, we observed a slight increase in PSTPIP2 phosphorylation after LPS priming followed by a much more substantial increase after silica treatment (Fig. 7A).

To study the effect of the mutations of SHIP1 binding sites in PSTPIP2 on IL-1β processing in neutrophils, we generated immortalized granulocyte progenitors by the infection of BM progenitors with the ER-HoxB8 fusion construct that, in the presence of estrogen, is maintained in the cell nucleus and blocks further differentiation. Such cells can be propagated in the cell culture and infected with retroviral constructs. The replacement of estrogen with G-CSF results in the differentiation of these progenitors into mature granulocytes. Similar to primary granulocytes, these cells are short-lived and cannot be maintained in the culture for >1 d after reaching maturity. We prepared immortalized granulocyte progenitors from CMO mice and transduced these cells with WT, Y3F, and W232A constructs of PSTPIP2. Next, we tested granulocytes differentiated from these progenitors for the processing of IL-1β. The processing of IL-1β after treatment with silica was more efficient in the cells infected with W232A and 3YF mutants when compared with WT PSTPIP2, demonstrating that despite a slightly higher expression level, the mutant proteins were unable to inhibit IL-1β cleavage as efficiently as WT PSTPIP2 (Fig. 7B). This result shows that SHIP1 and PEST phosphatase-binding sites are important for the PSTPIP2-mediated inhibition of IL-1β processing. We excluded empty-vector transfected CMO progenitors from this experiment because of their increased speed of maturation, which was not observed in PSTPIP2-infected cells (irrespective of the mutation present in PSTPIP2; data not shown). Because vector-infected cells were already progressing to apoptosis at the time when the other cell lines just reached the maturity, they could not be used as a control.

Next, to directly analyze the role of SHIP1 in IL-1β processing, we treated the WT and CMO primary granulocytes from the BM

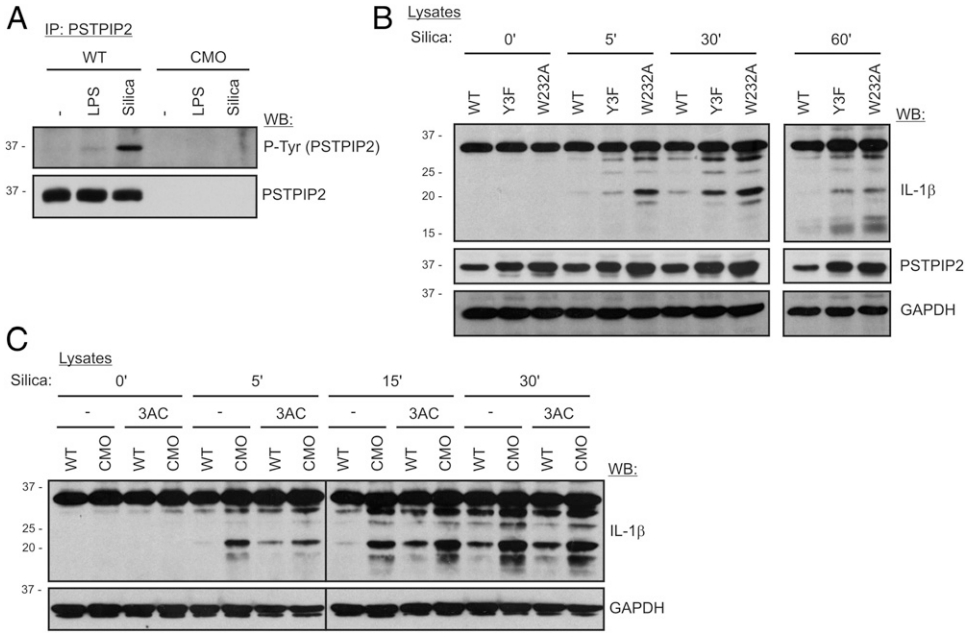

FIGURE 7. Role of SHIP1 in the PSTPIP2-mediated suppression of IL-1β processing. (**A**) BM cells were primed with 50 ng/ml LPS for 3 h followed by 15-min silica exposure (50 μg/cm^2). PSTPIP2 was immunoprecipitated from the lysates of these cells and the immunoprecipitates were subjected to immunoblotting with phosphotyrosine (P-Tyr) and PSTPIP2 Abs. Only relevant parts of the blots are shown. (**B**) Granulocytes differentiated from immortalized progenitors transduced with WT PSTPIP2 and its Y3F and W232A mutants were primed for 3 h with 100 ng/ml LPS and then stimulated by silica particles (50 μg/cm^2) for the indicated time intervals. The cells were then lysed and probed for IL-1β cleavage products. (**C**) Neutrophils purified from the mouse BM were primed with 100 ng/ml LPS for 3 h and then with silica particles (50 μg/cm^2) for the indicated time intervals in the presence or absence of the SHIP1 inhibitor 3AC (10 μM). Only relevant parts of the blots are shown.

with the specific SHIP1 inhibitor 3AC. In agreement with the proposed role of SHIP1 in PSTPIP2 function, this treatment resulted in enhanced IL-1β cleavage after exposure to silica in WT neutrophils, whereas only a very mild effect was observed in their CMO counterparts (Fig. 7C). Altogether, these data support the conclusion that interaction with SHIP1 is a part of the mechanism of how PSTPIP2 suppresses the IL-1β–mediated inflammatory response in granulocytes.

Finally, we wanted to test the expression and interactions of PSTPIP2 in primary human granulocytes. Because these cells can be obtained in relatively large numbers, they also allowed us to test the PSTPIP2 interactions. We isolated human granulocytes from fresh buffy coats and stimulated these cells with silica or human aggregated IgG (as an FcR ligand). Surprisingly, these granulocytes did not appear to interact with silica particles or to phagocytose these particles, and this treatment did not result in any PSTPIP2 tyrosine phosphorylation (data not shown). However, they vigorously responded to the FcR stimulation. This treatment resulted in the increased tyrosine phosphorylation of PSTPIP2 accompanied by the binding of Csk and SHIP1 (Fig. 8). The 100-kDa form of SHIP1 prevailing in these immunoprecipitates is likely the result of proteolytic cleavage and has been described previously (42). These results confirm that PSTPIP2 is also expressed in human granulocytes and that it participates in similar interactions as its murine counterpart.

Discussion

PSTPIP2 is known for its principal role in the development of the inflammatory disorder described as chronic multifocal osteomyelitis in mice, where its reduced expression or its complete absence is the main cause of the disease (26, 27, 34). However, the mechanism for how PSTPIP2 prevents the disease development has remained unclear. As an adaptor protein, it is likely to function via the recruitment of inhibitory enzymes and other negative regulators. The only molecules of this class previously known to bind to PSTPIP2 were the PEST-PTPs (24, 32). All three family members have been expected to bind to PSTPIP2, although the interaction has formally been proved only for PTP-HSCF and PTP-PEST (24, 32). We filled this gap by showing that PEP/LYP also interacts with PSTPIP2 in a similar manner, dependent on the residue W232 of PSTPIP2 (Fig. 2C). Mice deficient in PEP or PTP-PEST do not develop typical signs of autoinflammatory disorder (43–46), and thus it seems that the major functions of these PTPs are not related to their binding to PSTPIP2. However, if there is a redundancy among the PEST-PTPs that are associated with PSTPIP2, single family member deficiencies would probably not reveal any relevant phenotype. Moreover, additional evidence supports the importance of PEST-PTPs for the functionality of PSTPIP2. The W232A mutation in PSTPIP2, which prevented its binding to PEST-family phosphatases, diminished the ability of PSTPIP2 to inhibit the differentiation of osteoclasts and megakaryocytes (32, 38). In addition, we show in this article that this tryptophan is also important for the suppression of IL-1β processing in neutrophils. The substrate of PSTPIP2-associated PEST-PTPs remains unknown. By analogy with PSTPIP1 (47), one might speculate that their substrate would be PSTPIP2 itself, but we have not observed any increase in the phosphorylation of PSTPIP2 mutated at W232 (Fig. 2D). Thus, it seems more likely that these phosphatases dephosphorylate some of its binding partners, such as SHIP1 and Lyn or other unknown proteins in the vicinity of PSTPIP2.

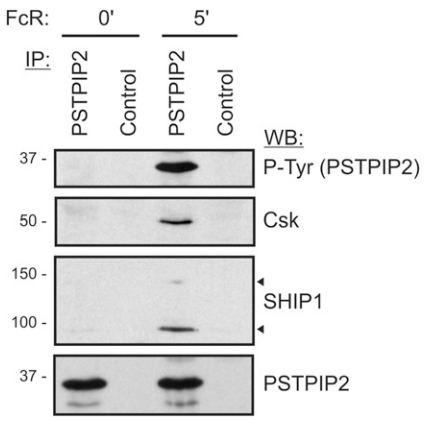

FIGURE 8. PSTPIP2 phosphorylation and interactions in human neutrophils. Human neutrophils were purified from a fresh buffy coat and activated by human aggregated IgG (300 μg/ml) for 5 min. The cells were then lysed and subjected to PSTPIP2 immunoprecipitation. The immunoprecipitates were then analyzed for the presence of the indicated molecules by immunoblotting. In the immunoprecipitations, an irrelevant isotype-matched Ab served as a negative control. Only relevant parts of the blots are shown.

Another possible role of PEST-PTPs includes the recruitment of Csk to PSTPIP2, which is further enhanced by the independent binding of Csk directly to PSTPIP2. In such a case, SFKs would be the most probable target. We did not observe any effect of PSTPIP2 deficiency on the global phosphorylation of SFKs, whether in macrophages or granulocytes. However, it is possible that PEST-PTPs together with Csk specifically inhibit the pool of SFKs associated with PSTPIP2. The interaction between PSTPIP2 and SFK Lyn was described previously in megakaryocytes (38), and we detected a similar interaction in macrophages (data not shown). The effect of Csk binding could not be fully analyzed because we were unable to identify its binding site in PSTPIP2. We suspect that the binding site is within the F-bar domain or other sequences that could not be evaluated because of the lack of mutated protein expression. Notably, the sequence integrity seems to be essential for PSTPIP2 protein stability, because other mutations, including Lupo and CMO, also result in defective protein expression (27, 34). In addition, there was a fraction of Csk that, in J774 cells, interacted with PSTPIP2 inducibly after M-CSF or FcR stimulation. The binding of the inducible Csk fraction was dependent on the interaction of PSTPIP2 with PEST-family phosphatases. The most likely candidate for bringing this additional Csk to the complex is PTP-PEP, which interacted with PSTPIP2 in an inducible manner. However, another option is that PTP-HSCF, which, unlike PTP-PEST or PTP-PEP, binds Csk via phosphorylated tyrosines in its C-terminal tail (22), is responsible for this inducible binding.

Another critical region in PSTPIP2 comprises the C-terminal tyrosines. Their mutation completely abolished the ability of PSTPIP2 to suppress the differentiation of osteoclasts and reduced its ability to inhibit megakaryocyte differentiation (32, 38). The most likely explanation was that these tyrosines recruit a negative regulatory molecule responsible for these effects. In this work, we have shown that these tyrosines are responsible for binding to the lipid phosphatase SHIP1. Indeed, Y329 and Y323 very well match the consensus binding site for the SHIP1 SH2 domain (48, 49), whereas Y333 bears a certain resemblance to the ITIM motif [hydrophobic amino acid (L) in position −2 with respect to the tyrosine], which is also known to bind the SHIP1 SH2 domain (50). All the previous work on PSTPIP2 tyrosine phosphorylation has analyzed only Y323 and Y333. It was based on the assumption that these tyrosines are analogous to the phosphorylated tyrosine residues found in the related and more thoroughly studied protein PSTPIP1 (Y344 and Y367, respectively) (24). However, our sequence analysis did not reveal any relationship between these

tyrosines. Y367 of PSTPIP1 is located inside its SH3 domain, which is not present in PSTPIP2. Moreover, it is a highly conserved residue forming a part of the SH3 domain ligand-binding pocket that is found within a number of these domains in various proteins where it is thought to regulate their interactions with respective ligands (51). The sequence surrounding Y344 of PSTPIP1 (LVYASI) is also somewhat different from Y323 of PSTPIP2 (PDYSVV), and therefore we concluded that the C-terminal tyrosines are a unique feature of PSTPIP2 not found in PSTPIP1. Notably, in the previous work, the mutation of Y323 and 333 severely reduced, but did not completely abolish, the phosphorylation of PSTPIP2, suggesting that there is an additional tyrosine phosphorylation site in PSTPIP2 (24). Y329 was shown to be phosphorylated in mast cells (52), and thus we assumed that Y329 is the most likely candidate. Therefore, we decided to mutate all three C-terminal tyrosines in PSTPIP2 at the same time for our analysis of SHIP1 binding and function. This analysis showed that these three tyrosines are required for SHIP1 binding and that the PSTPIP2 phosphorylation in their absence is negligible. Moreover, we also showed that these tyrosines are required for the maximum inhibition of IL-1β processing in neutrophils. This, together with our data demonstrating increased IL-1β cleavage in WT neutrophils in the presence of the SHIP1 inhibitor, strongly supports the idea that some of the key functions of PSTPIP2 are, at least in part, mediated by SHIP1. Additional support for this idea is also derived from the fact that the Akt and Erk pathways, which are both known to be regulated by SHIP1, are affected by PSTPIP2 absence.

When considering the role of SHIP1 in PSTPIP2 function, it is also interesting to compare the phenotypes of SHIP1-deficient mice with the phenotype caused by PSTPIP2 deficiency. Similar to CMO, SHIP1$^{-/-}$ mice develop a disease with an autoinflammatory component where inflammatory myeloid infiltrate into multiple organs and enhanced serum IL-6 levels can be observed (3, 53–55). Both strains display splenomegaly with extramedullary hematopoiesis and an increased number and proliferation of myeloid progenitor cells (3, 34, 53). In addition, they also share a phenotype of increased osteoclastogenesis and osteoclast activity, resulting in osteopenia and osteoporosis (32, 56). These features shared between SHIP1$^{-/-}$ and CMO mice suggest that they are components of a common signaling pathway. In contrast, the reduced survival of SHIP$^{-/-}$ mice because of massive myeloid cell infiltration in the lungs is not observed in CMO mice (53), and PSTPIP2 does not seem to be involved in endotoxin tolerance. This phenomenon is mainly caused by the dramatic increase in SHIP1 expression after LPS treatment (57). Despite the fact that the expression of PSTPIP2 is also increased, we did not observe any significant difference in the signaling of endotoxin-tolerized WT and CMO BMDMs, suggesting that PSTPIP2 plays no role in this process (data not shown). The more serious phenotype of SHIP1$^{-/-}$ mice is, however, not surprising, because PSTPIP2 is not the only binding partner of SHIP1, and this enzyme interacts with multiple other proteins and has an effect on other pathways.

The initial characterization of CMO and LUPO mice led to the conclusion that macrophages and osteoclasts are the major cell types responsible for the disease development (27, 32, 34). However, recent work has suggested that, in fact, granulocytes are the most important cell type triggering the disease via the increased production of IL-1β (35, 36). Indeed, we had difficulty detecting any differences in the signaling pathways between the WT and CMO macrophages. In contrast, in CMO neutrophils, we observed enhanced activity of multiple signaling pathways after LPS or silica exposure or after FcR stimulation. The CMO disease development was recently shown to require the activity of inflammasome-associated caspases (36). Indeed, we have observed increased CMO neutrophil responses to a typical inflammasome stimulator silica, which was also used in the previous studies (35, 36). Moreover, we also observed enhanced responses to FcR cross-linking, and to some extent also to TLR stimulation by LPS. This suggests that PSTPIP2 has relatively broad effects on neutrophil sensitivity to various stimuli, and that it is likely that other receptors and pathways that have not been tested yet are influenced by the absence of PSTPIP2.

Which of the receptors regulated by PSTPIP2 are responsible for disease initiation in vivo remains unclear. One possibility is that a receptor for environmental silica or other related compounds triggers the disease in living animals. Our data, as well as previous reports, show an exaggerated neutrophil response to silica treatment. However, to our knowledge, the silica receptor on granulocytes has not been identified yet, and so how silica particles initiate phagocytosis and signaling remains unclear. Fc receptors are also good candidates for triggering the disease, because they not only recognize Abs but they also bind pentraxins and pentraxin-opsonized particles (58). Pentraxins, such as C-reactive protein and serum amyloid P, are a family of acute-phase proteins involved in the recognition and opsonization of bacteria, and could well represent a link described by Lukens et al. (36) between altered bacterial microflora in the gut and inflammatory disease development in CMO mice. Moreover, FcRs use a relatively universal ITAM-dependent signaling pathway, which is also used by a number of other innate immune receptors (e.g., the C-type lectin family of pattern recognition receptors and the TREM family receptors), as well as integrins (59), and our results thus imply that signaling by these receptors will also potentially be affected. Clearly, additional research is needed to address all these possibilities, but at present our data suggest that pathways downstream of multiple receptors are affected by PSTPIP2 deficiency and, in fact, they all may contribute in concert to the CMO disease development. Our data provide evidence that SHIP1 and Csk are part of the PSTPIP2-dependent inhibitory network that regulates these pathways and prevents the development of autoinflammatory disease. Deciphering the mechanisms that govern the assembly and activity of this network will help us to understand the cause of autoinflammatory diseases, including chronic recurrent multifocal osteomyelitis and other related disorders, and will facilitate the development of successful treatments.

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Disclosures

The authors have no financial conflicts of interest.

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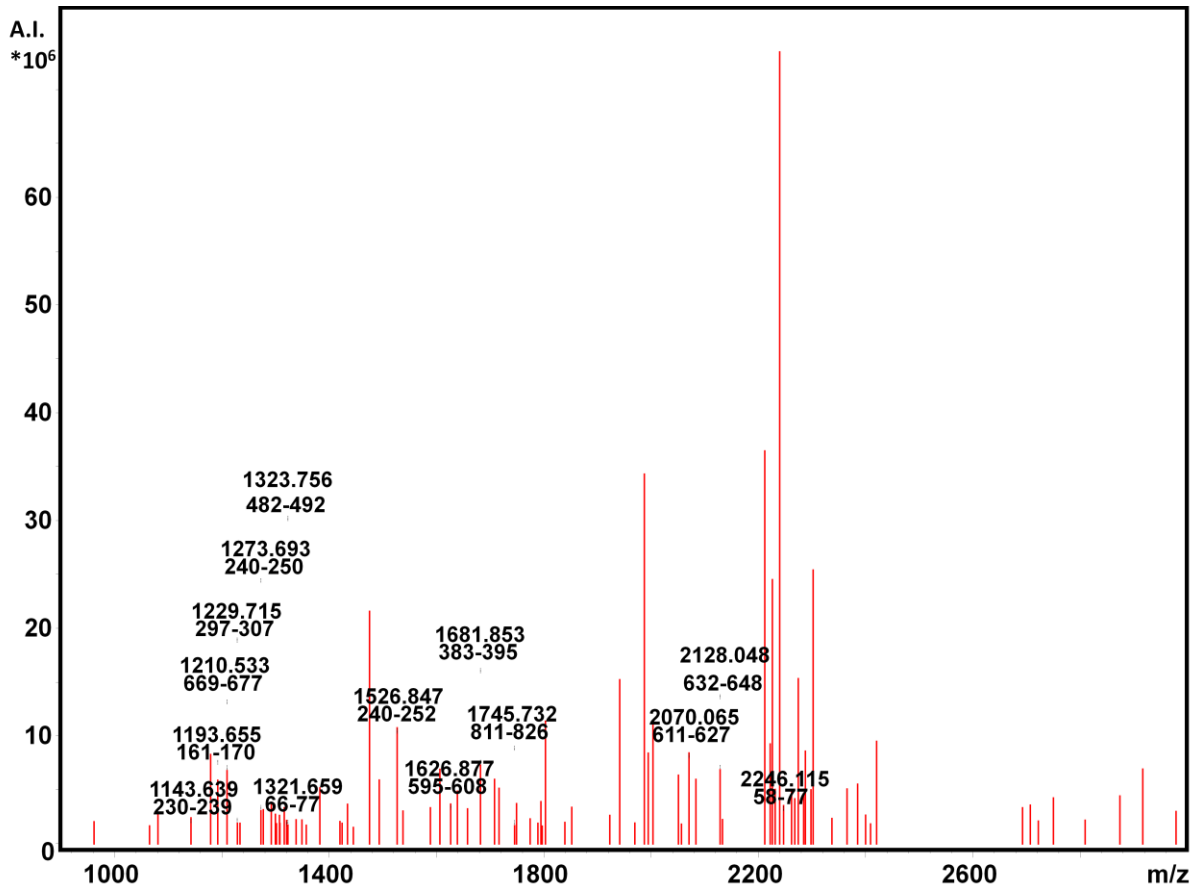
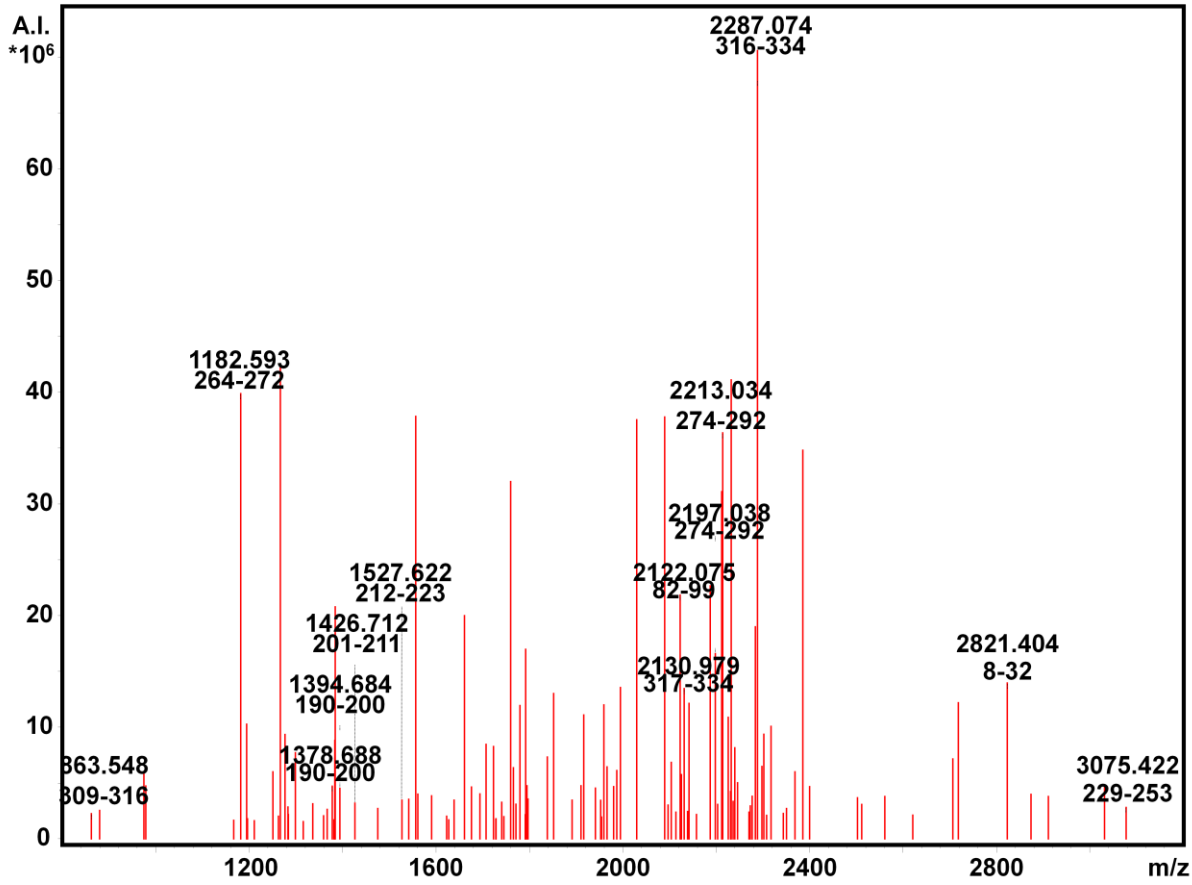
Supplemental figures and tables



Supplemental figure 1. MALDI FT-ICR MS spectrum of Proline-serine-threonine phosphatase-interacting protein 2 tryptic peptides. The position in the protein sequence and experimental mass are shown



Supplemental figure 2. MALDI FT-ICR MS spectrum of Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 tryptic peptides. The position in the protein sequence and experimental mass are shown.

Mutation	expression	Csk binding	PEST-PTPs binding	SHIP1 binding	Tyr-P
Y21F + W232A	expressed	no effect			
Y52F + W232A	expressed	no effect			
Y144F + W232A	expressed	no effect			
Y191F + W232A	expressed	no effect			
Y249F	expressed	no effect	reduced		
Y249F + W232A	expressed	no effect	abolished		
Y249,267F + W232A	expressed	no effect			
Y284F	expressed	no effect			
Y284,288F + W232A	expressed	no effect			
Y323,329,333F	expressed	no effect		abolished	abolished
W232A	expressed	reduced	abolished	slightly increased	
P278A + P279,281G	not expressed				
Deletion 278-281	not expressed				
Deletion 166-295	very low expression				
Deletion 296-334	expressed	no effect			abolished
All Y to F	not expressed				

Supplemental table 1. Effecs of PSTPIP2 mutations on PSTPIP2 expression, on PSTPIP2 binding to PEST-PTPs, Csk and SHIP1, and on PSTPIP2 tyrosine phosphorylation. Empty fields = not tested.

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LST1/A Is a Myeloid Leukocyte-specific Transmembrane Adaptor Protein Recruiting Protein Tyrosine Phosphatases SHP-1 and SHP-2 to the Plasma Membrane*[S]

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Peter Draber[1,2], **Ondrej Stepanek**[1,2], **Matous Hrdinka**[2], **Ales Drobek**[2], **Lukas Chmatal, Linda Mala, Tereza Ormsby, Pavla Angelisova, Vaclav Horejsi, and Tomas Brdicka**[3]

From the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic

Background: LST1/A was a poorly characterized protein encoded in the MHCIII locus.
Results: LST1/A is a myeloid cell-specific transmembrane adaptor associated with the tetraspanin-enriched microdomains that inhibits signaling by recruiting the protein tyrosine phosphatases SHP-1/SHP-2.
Conclusion: LST1/A is a potential negative regulator of myeloid cell signaling.
Significance: Negative regulation of signal transduction is crucial for controlling the cell response.

Transmembrane adaptor proteins are membrane-anchored proteins consisting of a short extracellular part, a transmembrane domain, and a cytoplasmic part with various protein-protein interaction motifs but lacking any enzymatic activity. They participate in the regulation of various signaling pathways by recruiting other proteins to the proximity of cellular membranes where the signaling is often initiated and propagated. In this work, we show that LST1/A, an incompletely characterized protein encoded by MHCIII locus, is a palmitoylated transmembrane adaptor protein. It is expressed specifically in leukocytes of the myeloid lineage, where it localizes to the tetraspanin-enriched microdomains. In addition, it binds SHP-1 and SHP-2 phosphatases in a phosphotyrosine-dependent manner, facilitating their recruitment to the plasma membrane. These data suggest a role for LST1/A in negative regulation of signal propagation.

The human gene *LST1* and its mouse homologue *Lst1* (also known as *B144*) are located in the MHC class III locus, which is rich in immunologically important genes (1–4). The human LST1 primary transcript has been reported to undergo extensive alternative splicing resulting in a large number of splice variants. However, only limited alternative splicing has been reported for the mouse homologue (5, 6). Translation of LST1 splice isoforms would lead to the synthesis of various soluble and membrane-bound protein products. However, it remains unclear how many LST1 splice variants really exist at the protein level. So far, the only protein product clearly detected *in vivo* is encoded by the LST1/A isoform, the only isoform conserved between human and mouse (7, 8).

Another unresolved issue is the expression pattern of LST1. Several studies reported enrichment or specific expression of LST1 in leukocytes or leukocyte-rich tissues (1, 3, 7), whereas others indicated that the expression of LST1 is relatively ubiquitous (5, 8). Moreover, it seems that production of particular isoforms could be differentially regulated, which further complicates expression analysis (5, 9).

Although the *LST1* gene has been studied extensively at the gene level and/or the mRNA level, the biological functions of the protein product(s) are largely unknown. A single published phenotypic study reported that overexpression of LST1/A in various human cell lines resulted in the formation of filopodia-like structures (7). Nevertheless, no molecular mechanism explaining how LST1 could induce the changes in cell morphology has been proposed, and thus, the physiological function of LST1/A is still unknown.

In this study, we thoroughly characterize the LST1/A isoform with the emphasis on bioinformatic analysis of its amino acid sequence, expression profile, biochemical characterization, and binding partners. Based on these data, we propose a role for LST1/A in negative regulation of cellular signaling.

EXPERIMENTAL PROCEDURES

Bioinformatics—The *in silico* search in the human genome for proteins possessing the features characteristic of known transmembrane adaptor proteins (TRAPs)[4] has been published previously (10). Sequence alignment was performed using ClustalW2 (11).

Antibodies and Reagents—Antibodies to the following antigens were used: Erk2 (rabbit), SHP-1 (rabbit), SHP-2 (rabbit) (all from Santa Cruz Biotechnology, Santa Cruz, CA); phosphotyrosine (mouse, clone 4G10, Upstate Biotechnologies); FLAG

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[S] This article contains supplemental Fig. 1.
[1] Both authors contributed equally to this work.
[2] Supported in part by the Faculty of Science, Charles University, Prague, Czech Republic.
[3] To whom correspondence should be addressed: Inst. of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic. Tel.: 420-241062467; Fax: 420-244472282; E-mail: tomas.brdicka@img.cas.cz.

[4] The abbreviations used are: TRAP, transmembrane adaptor protein; ITIM, immunoreceptor tyrosine-based inhibitory motif; Siglec, sialic acid-binding Ig-like lectin receptor; TEMs, tetraspanin-enriched microdomains; EGFP, enhanced green fluorescent protein.

tag (mouse, clone M2), GAPDH (rabbit), anti-mouse IgG-HRP (goat), anti-rabbit Ig-HRP (goat) (all from Sigma-Aldrich); CD25 (mouse IgG1, MEM-181, in-house); CD4-FITC (Beckman Coulter, Indianapolis, IN); CD8-FITC (Abd Serotec, Kidlington, UK); CD14-FITC, CD19-FITC, CD3-FITC, and CD56-FITC (all from Exbio, Vestec, Czech Republic); Thy1.1-FITC (His-51, eBioscience, San Diego, CA); mouse affinity-purified IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

The monoclonal antibody, LST1/02 (IgG1), against human LST1/A was generated by standard techniques. Briefly, F_1 (BALB/c × B10.A) hybrid mice were immunized intrasplenically with a human LST1/A peptide (amino acids 75–90) conjugated to activated mcKLH (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions. Hybridomas were prepared using Sp2/0 myeloma cells as fusion partners and selected by the standard protocol.

Cell Lines and Primary Cells—All cell lines were cultured in the indicated media supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 μg/ml gentamycin, 50 μg/ml streptomycin, and 10^4 units/ml penicillin at 37 °C in 5% CO_2. The Jurkat, Ramos, THP-1, U937 (all from American Type Culture Collection, Manassas, VA), and HeLa (kindly provided by Dr. D. Stanek, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic) cell lines were cultivated in RPMI 1640. The Hek293FT (Invitrogen), Phoenix Ampho (Origene, Rockville, MD), and K562 (UHKT cell line collection, Prague, Czech Republic) cell lines were cultivated in DMEM. HL-60 cells (UHKT cell line collection) were cultivated in Iscove's Modified Dulbecco's Medium.

Human peripheral blood mononuclear cells were prepared from buffy coats obtained from healthy donors at Thomayer University Hospital (Prague, Czech Republic) using Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation (900 × *g* for 30 min). To isolate various subpopulations, T cells, $CD4^+$ cells, $CD8^+$ cells, B cells, NK cells, and monocytes were labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD56, and anti-CD14 FITC-conjugated antibodies, respectively, followed by anti-FITC magnetic bead labeling (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were sorted using an AutoMACS magnetic cell sorter (Miltenyi Biotec), and the purity of the samples was determined by flow cytometry to be >90%. For isolation of granulocytes, a gradient composed of Histopaque-1119 (Sigma-Aldrich), overlaid by Ficoll-Paque PLUS, and freshly collected blood from healthy donors (diluted in PBS) was prepared and centrifuged (900 × *g* for 30 min). Granulocytes were collected from the interface between Ficoll-Paque PLUS and Histopaque-1119 layers, followed by erythrocyte lysis in ACK buffer (150 mM NH_4Cl, 0.1 mM EDTA, 1 mM $KHCO_3$). Where applicable, the procedures were performed after obtaining an informed consent from the donors, and in accordance with local ethical guidelines.

DNA Cloning and Transfection—The vector pcDNA3.1 containing the human LST1/A sequence was kindly provided by Dr. S. M. Weissman (Yale School of Medicine, New Haven, CT). For transient transfection experiments, the LST1/A coding sequence was cloned into the pXJ41 vector. For immunoprecipitation experiments, the construct consisting of human LST1/A followed by a FLAG coding sequence at the C terminus was cloned into MSCV-IRES-Thy1.1, a vector based on the MSCV backbone (Clontech) with the Thy1.1 reporter sequence. The tyrosine to phenylalanine mutant of LST1/A-FLAG (2YF) was prepared using PCR mutagenesis. The CD25-LST1/A-FLAG (WT and 2YF) chimeras were prepared by cloning the sequence of the extracellular part of human CD25 (coding amino acids 1–240) followed by the entire sequence of LST1/A-FLAG without the initial methionine into MSCV-IRES-Thy1.1. To prepare LST1/A-enhanced green fluorescent protein (EGFP), LST1/A sequence was cloned into pEGFP-N3 (Clontech Laboratories), and LST1/A-EGFP was subsequently ligated into MSCV-IThy1.1. All constructs were sequenced. Lyn in the pcDNA3.1 vector (kindly provided by Dr. S. Watson University of Birmingham, Birmingham, UK), Syk in pRK5 (kindly provided by Dr W. Kolanus, University of Cologne, Germany), and mouse Btk in pCDEF3 (kindly provided by Dr M. Tomlinson, University of Birmingham, Birmingham, UK), and NTAL in pFLAG-CMV (12) were used for a kinase co-transfection assay.

Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's instructions for transfection of both HEK293FT (for verification of the antibody specificity and the kinase co-transfection assay) and Phoenix Ampho cells (for production of viral particles using the MSCV-IRES-Thy1.1 vector). Retrovirus-containing supernatants were supplemented with polybrene (10 μg/ml, Sigma-Aldrich) and added to the cells. The cells were then centrifuged at 1,250 × *g* for 90 min. Infected cells were labeled with the anti-Thy1.1-FITC antibody followed by anti-FITC magnetic beads and isolated by the AutoMACS magnetic cell sorter separator.

RNA and Quantitative RT-PCR—RNA was isolated from human cell lines and sorted blood subpopulations using a mini RNA purification kit (Zymo Research). Contaminating DNA was removed using a DNA-free kit (Ambion, Austin, TX). A human normal tissue FirstChoice RNA Survey Panel and a lymph node FirstChoice Total RNA were purchased from Ambion. Human hippocampal total RNA was purchased from BioChain Institute. Reverse transcription, quantitative RT-PCR, and data analysis were performed as described previously (10). Primers specific for the human LST1/A isoform were as follows: forward, 5′-TGG AGA GGA GCT GGA CCC AGG-3′; reverse, 5′-TGG GTT GAG GAA GGT GTC TGG-3′. The unique specificity of these primers for the LST1/A isoform was confirmed by sequencing of the PCR product.

Palmitoylation Assay—Palmitoylation of LST1/A was examined using the acyl-biotinyl exchange chemistry-based method (13). Briefly, plasma membranes from 5×10^7 THP-1 cells were isolated and incubated with *N*-ethylmaleimide to block free thiols. Subsequently, palmitate residues were removed by hydroxylamine (untreated samples served as a control), and the resulting free thiol groups were labeled with biotin-HPDP (*N*-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide, Pierce, Thermo Fisher Scientific). Biotinylated proteins were then immunoprecipitated on streptavidin-agarose beads, eluted with 2× concentrated SDS-PAGE sample buffer, and analyzed by Western blotting.

Cell Activation and Inhibitor Studies—Pervanadate solution was prepared by addition of hydrogen peroxide (final concen-

tration, 0.27%) to 10 mM sodium orthovanadate, followed by 15 min of incubation at room temperature. For pervanadate-induced activation, cells (5×10^7 cells/ml) were incubated in serum-free RPMI 1640 medium with 10% pervanadate solution for 20 min. For antibody-induced activation, cells (5×10^7 cells/ml) were resuspended in serum-free RPMI 1640 medium, incubated with the indicated primary antibodies (20 μg/ml) for 30 min, washed, and activated for 2 min by goat anti-mouse antibody (10 μg/ml). For inhibitor studies, cells (10^7 cells/ml) were incubated with 2 μM Syk inhibitor IV (BAY 61-3606) or 10 μM PP2 (both from Calbiochem, Merck, Darmstadt, Germany) for 30 min at 37 °C.

Cell Lysis and Immunoprecipitation—To obtain whole cell SDS lysates, cells in serum-free RPMI1640 (5×10^7 cells/ml) were lysed by adding an equal volume of 2× concentrated SDS-PAGE sample buffer, followed by sonication.

For immunoprecipitation experiments, 5×10^7 cells were lysed in 1 ml of ice-cold lysis buffer (1 mM Pefabloc, 5 mM iodoacetamide, 1 mM sodium orthovanadate, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 20 mM Tris, pH 7.5) containing 1% dodecylmaltoside and incubated for 10 min on ice. Nuclei and debris were removed by centrifugation, and the resulting lysate was subjected to immunoprecipitation by the anti-FLAG antibody (2 μg/ml), the anti-LST1/A antibody (LST1/02, 10 μg/ml), or the isotype-matched control monoclonal antibody (anti-CD20, MEM-97, 10 μg/ml) followed by incubation with protein A/G PLUS-Sepharose (Santa Cruz Biotechnology). Immunoisolated material was eluted with 2× concentrated SDS-PAGE sample buffer containing 1% DTT and analyzed by Western blotting.

Gel Filtration and Flotation—Cells were lysed in lysis buffer containing the indicated detergent for 30 min. For flotation in a sucrose density gradient, 0.5-ml aliquots of cell lysates were mixed with 0.5 ml of 80% (w/v) sucrose in lysis buffer in an ultracentrifuge tube. Then, they were overlaid with 3.5 ml of 35% sucrose followed by 0.5 ml of lysis buffer. Samples were subjected to ultracentrifugation (200,000 × *g* for 20 h), and 0.6-ml fractions were collected from the top of the gradient. For gel filtration, lysates were first precleared by low-speed centrifugation (400 × *g* for 5 min) to remove insoluble material. 0.2-ml aliquots of the resulting supernatants were then loaded on columns containing 2 ml of Sepharose 4B pre-equilibrated in the corresponding lysis buffer. Subsequently, the lysis buffer was loaded continuously on the columns and concomitantly, 0.2-ml fractions were collected. All fractions were mixed with an equal volume of 2× concentrated SDS-PAGE sample buffer and analyzed by Western blotting.

Microscopy—HeLa cells were grown on cover slips overnight, and THP-1 cells were allowed to adhere to polylysine-coated coverslips for 15 min before processing. All samples were fixed with 4% (w/v) formaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 5 min. Blocking was performed in 2.5% BSA and 10% goat serum (Sigma-Aldrich) in PBS for 30 min. Cells were incubated with phalloidin-tetramethylrhodamine B isothiocyanate (500 ng/ml, Sigma-Aldrich) for 30 min and washed twice with PBS. The DNA dye, Hoechst 33258 (Invitrogen), was used to visualize nuclei. Images were captured with a Leica SP5 confocal microscope using the 63× objective lens (Leica Microsystems, Mannheim, Germany).

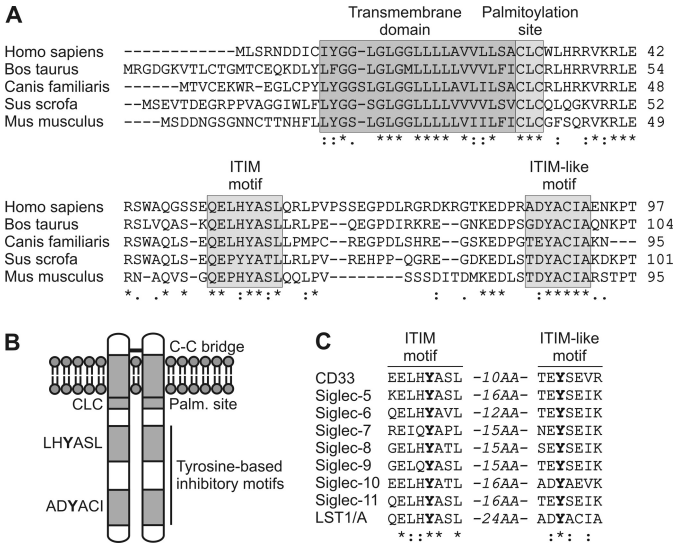

FIGURE 1. **Structure of LST1/A.** *A*, alignment of amino acid sequences of LST1/A orthologues from human (*Homo sapiens*, GenBank™ accession number: NP_995311), cattle (*Bos taurus*, XP_002702922), dog (*Canis familiaris*, XP_848911), pig (*Sus scrofa*, XP_002702922), and mouse (*Mus musculus*, O08843). The transmembrane domain, the putative palmitoylation site, ITIM, and ITIM-like motifs are highlighted. *B*, graphical depiction of human LST1/A dimer structure with a disulfide bridge. *C*, alignment of amino acid sequences of ITIM and ITIM-like region of human LST1/A with a corresponding region from members of CD33-related Siglec family. Identical (*) and more (:) or less (.) similar amino acids at particular positions in all sequences are indicated. The distance in amino acids (*AA*) separating these motifs is shown.

Flow Cytometry—Cells were stained with the indicated antibodies in PBS supplemented with 1% BSA and 10% human serum on ice for 30 min, washed, resuspended in RPMI 1640, and analyzed by a BD LSR II (BD Biosciences). For calcium measurement, cells were loaded with 5 μM Fura-Red dye (Invitrogen) in RPMI 1640 at 37 °C for 30 min. Subsequently, the cells were labeled with the indicated antibodies (50 μg/ml mouse IgG alone or with 10 μg/ml anti-CD25) on ice for 20 min. Cells were prewarmed for 5 min at 37 °C, and the relative intracellular calcium concentration was measured as a ratio of Fura-Red fluorescence intensities elicited by excitation wavelengths 405 and 488 nm. 30 s after the beginning of the measurement, Fc receptors were stimulated with goat anti-mouse antibody (10 μg/ml) and 4 min after the beginning of the measurement, 1 μg/ml ionomycin (Sigma-Aldrich) was added. Data were analyzed using FlowJo software (TreeStar).

RESULTS

LST1/A Is Transmembrane Adaptor Protein Containing ITIM and ITIM-like Motifs—LST1/A was identified as a potential TRAP during our human genome-wide *in silico* screen described previously (10). Indeed, the LST1/A protein structure exhibits key features of a TRAP (14). First, it possesses only a very short extracellular domain (including a possibly dimerizing cysteine), followed by a transmembrane domain and a comparatively larger cytoplasmic tail (7, 8). Second, the protein lacks any domain with predicted enzymatic activity. Instead, its intracellular part contains a conserved potential palmitoylation site and two putative phospho-tyrosine-based interaction

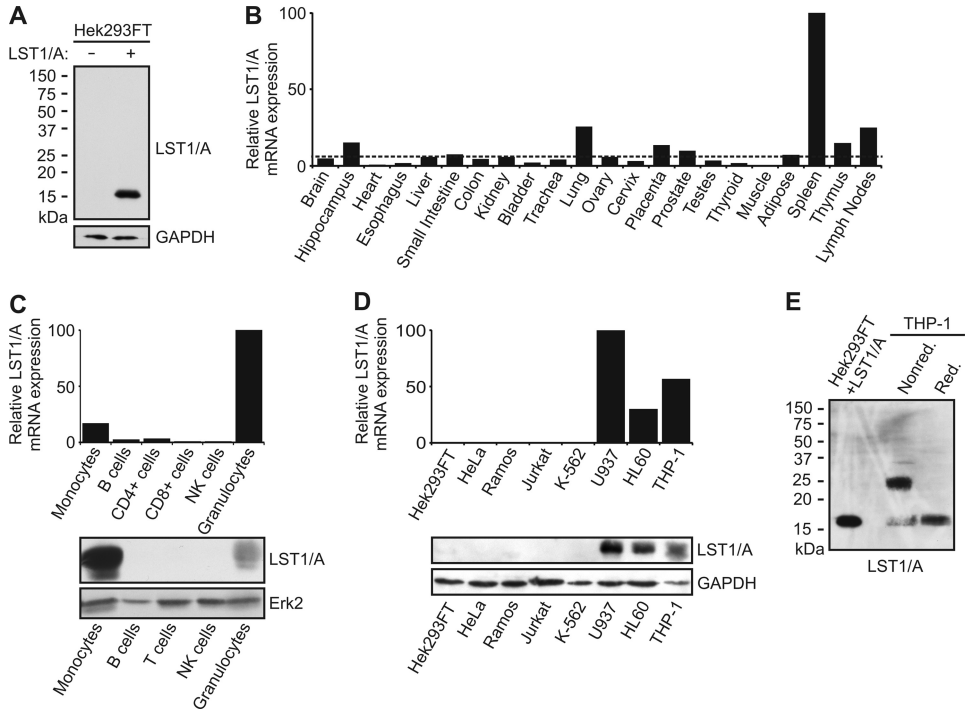

FIGURE 2. **Expression analysis of LST1/A.** *A*, Hek293FT were transiently transfected with LST1/A or an empty vector, lysed, and subjected to Western blotting using the newly developed antibody to LST1/A. Staining for GAPDH served as a loading control. *B*, expression of LST1/A mRNA in various human tissues was analyzed by quantitative RT-PCR. PCR amplifications were carried out in triplicates and mean *Ct* values were normalized to the GAPDH mRNA level. The *dashed horizontal line* indicates median from all tested tissue samples. *C*, expression of LST1/A in the isolated human peripheral leukocyte subsets was analyzed at the transcript level using quantitative RT-PCR (mean *Ct* values of triplicates were normalized to GAPDH) or at the protein level using Western blotting. Staining for Erk2 served as a loading control. *D*, expression of LST1/A in the indicated cell lines was analyzed as in *C* with GAPDH as a loading control for Western blotting. *E*, lysates of Hek293FT cells transfected with LST1/A and lysates of THP-1 cells were separated under non-reducing or reducing conditions and subjected to Western blotting with the LST1/A antibody.

motifs (Fig. 1, *A* and *B*). Orthologues of human LST1/A predicted in various mammalian species share a high level of homology in the transmembrane domain, the palmitoylation site, and both of the predicted phosphotyrosine-based interaction motifs (Fig. 1*A*).

Further examination of the human LST1/A sequence revealed that the membrane-proximal phosphotyrosine-based interaction motif constitutes a canonical immunoreceptor tyrosine-based inhibitory motif (ITIM), defined as (L/V/I)*x*Y*xx*(L/V), where *x* can be any amino acid (15), and the membrane-distal phosphotyrosine element partially fulfills the criteria for an ITIM-like motif, defined as (D/E)Y*x*E(V/I)(R/K) (Fig. 1, *A* and *B*) (16).

The tandem ITIM and ITIM-like elements strongly resemble a signaling inhibitory platform that has been found in the members of the CD33-related Siglec (sialic acid-binding Ig-like lectin receptor) family (16). The amino acid sequence of the ITIM motif is highly conserved among most CD33-related Siglecs and LST1/A. Also, substantial differences between LST1/A and CD33-related Siglecs were identified in the ITIM-like region (particularly at the +2 and +4 positions) (Fig. 1*C*). This degree of homology between LST1/A and CD33-related Siglecs suggested that LST1/A might function as an inhibitory TRAP, which could recruit some of the negative regulators of signal transduction that were described as phosphotyrosine-dependent binding partners of CD33-related Siglecs, namely SHP-1 (also known as PTPN6), SHP-2 (also known as PTPN11), SOCS3, and Cbl (16, 17).

LST1/A Is Specifically Expressed in Myeloid Cells—Conflicting results on the expression pattern of LST1 have been reported (1, 3, 5, 7, 8). Thus, we revisited this issue using a quantitative comparison of LST1/A expression levels in various cell subsets and tissues at both the mRNA and protein levels. Primers for specific RT-PCR amplification of the LST1/A isoform were designed, and a mouse monoclonal antibody against an epitope in the intracellular part of human LST1/A was developed (Fig. 2*A*). Quantitative RT-PCR performed on a panel of human tissue mRNA samples identified LST1/A mRNA in a number of lymphoid and non-lymphoid organs and tissues. By far, the highest levels of the transcript were detected in the spleen, suggesting the enrichment of LST1/A in leukocytes (Fig. 2*B*). A more detailed analysis of blood leukocyte subpopulations revealed specific expression of both LST1/A mRNA and protein in the cells of myeloid origin (monocytes and granulocytes) but not in lymphocytes (B cells, T cells, and NK cells) (Fig. 2*C*). Analogously, expression of LST1/A was detected in the cell lines derived from myeloid leukocytes (*i.e.* THP-1, U937, and HL60), but not in the cell lines of epithelial or lymphoid lineages (Fig. 2*D*). Collectively, these data indicate that LST1/A is expressed predominantly in leukocytes of the myeloid lineage.

To fully confirm that our antibody is specific for the LST1/A isoform, we compared the lysates from HEK293FT cells transfected with a vector encoding the LST1/A isoform with lysates from THP-1 cells. Upon treatment of the samples with a reducing agent DTT, the molecular weight of both transfected and endogenous LST1/A proteins were the

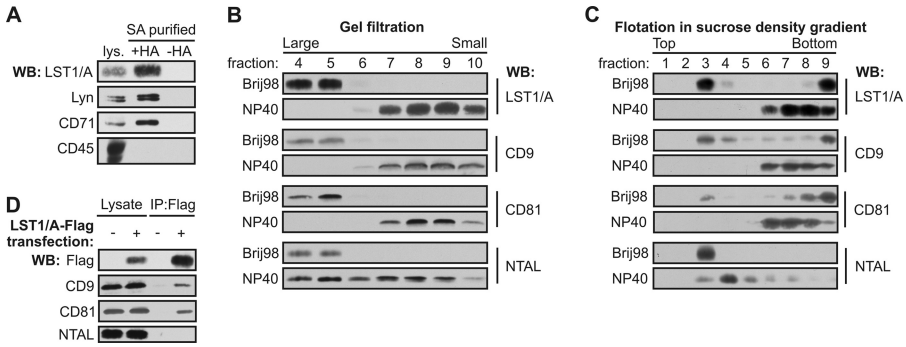

FIGURE 3. **Biochemical characterization of LST1/A.** *A*, palmitoylation of endogenous LST1/A in membranes isolated from THP-1 cells was examined using the acyl-biotinyl exchange chemistry method. Treatment of a sample with hydroxylamine (+*HA*) enabled exchange of palmitoyl residues for biotin and subsequent isolation on streptavidin (*SA*). A sample untreated with hydroxylamine (−*HA*) served as a negative control. Stainings for palmitoylated Lyn and transferrin receptor (CD71) and non-palmitoylated CD45 were used as positive and negative controls, respectively. *B* and *C*, THP-1 cells were lysed in Brij98 or Nonidet P-40 (*NP40*) and the lysates were fractionated by gel filtration on Sepharose 4B (*B*) or by flotation in the sucrose density gradient (*C*). Samples were analyzed by Western blotting using antibodies to LST1/A, tetraspanins CD9 and CD81, and the raft protein NTAL. *D*, THP-1 cells stably transfected with LST1/A-FLAG or an empty vector were solubilized in the CHAPS detergent and subjected to immunoprecipitation using the anti-FLAG antibody. Samples were stained for FLAG, CD9, CD81, and NTAL. *WB*, Western blot.

same (Fig. 2*E*). In addition, staining with our LST1/A antibody revealed a substantial shift in the apparent molecular weight of LST1/A under the non-reducing conditions, confirming the prediction that LST1/A molecules form disulfide-based covalent dimers (Fig. 2*E*).

LST1/A Resides in Tetraspanin-enriched Microdomains—Because LST1/A contains a potential palmitylation site, we examined whether endogenous LST1/A is palmitoylated, using the acyl-biotinyl exchange chemistry-based method (13). LST1/A and positive controls (Lyn and CD71) were palmitoylated, whereas CD45 (a negative control) was not (Fig. 3*A*). Palmitoylation of various transmembrane proteins could facilitate their localization to lateral membrane compartments, including membrane rafts or tetraspanin-enriched microdomains (TEMs) (18–20).

TEMs and lipid rafts can be distinguished based on their differential solubility in various detergents. Although both of these membrane compartments are resistant to mild detergents, such as Brij98 and CHAPS, TEMs, but not lipid rafts, are readily dissolved by more stringent detergents including Nonidet P-40 and Triton X-100 (12, 20–22). Similarly to tetraspanins CD9 and CD81, LST1/A was present in large complexes resistant to Brij98 and CHAPS, as detected by gel filtration on Sepharose 4B (Fig. 3*B*). LST1/A also was found in the low density fractions after sucrose density gradient centrifugation upon lysis in these mild detergents (Fig. 3*C*). However, unlike NTAL-containing lipid rafts, LST1/A-containing complexes were disrupted completely by Nonidet P-40 lysis (Fig. 3, *B* and *C*).

These biochemical characteristics indicated that LST1/A could be a component of TEMs. To further test this hypothesis, THP-1 cells stably expressing FLAG-tagged LST1/A were lysed in TEM-preserving detergent CHAPS and then subjected to immunoprecipitation by the anti-FLAG antibody. Tetraspanins CD9 and CD81, but not the raft protein NTAL, co-precipitated with LST1/A-FLAG (Fig. 3*D*), providing evidence for association of LST1/A with tetraspanins. These data demonstrate that LST1/A is a palmitoylated protein localized in TEMs.

LST1/A Localizes to Membrane Filopodia but Does Not Induce Their Formation—Thus far, only one study analyzed the potential function of LST1/A, showing that overexpression of LST1/A leads to filopodia formation in various human cell lines (7). To verify this, C-terminally EGFP-tagged LST1/A was expressed in THP-1 and HeLa cells. Confocal microscopy confirmed that LST1/A-EGFP was localized in the plasma membrane (Fig. 4*A*). To investigate whether LST1/A induces changes in cell morphology, the actin cytoskeleton was visualized by phalloidin staining. We observed clear localization of LST1/A-EGFP in actin-rich membrane protrusions. In contrast to previously published data (7), the filopodia-like structures were similarly abundant in both LST1/A-EGFP expressing and non-expressing cells as shown by the actin staining (Fig. 4*B* and supplemental Fig. 1). Therefore, the data did not confirm the role of LST1/A in the formation of filopodia or any other membrane protrusions.

LST1/A Binds SHP-1 and SHP-2 via Its ITIM and ITIM-like Element—The functions of TRAPs are largely dependent on their capacity to bind various signaling molecules. LST1/A contains two predicted phosphotyrosine-based interaction motifs that could potentially interact with SH2 domains of intracellular proteins. Due to the apparent similarity between the ITIM and ITIM-like pairs present in LST1/A and the CD33-related Siglecs, the ability of LST1/A to bind the previously described ITIM interacting SH2-containing proteins, including SHP-1, SHP-2, SOCS3, Cbl-b, and SHIP1, was tested (16, 23, 24).

For this purpose, THP-1 cells lines stably expressing C-terminally FLAG-tagged wild type (WT) LST1/A or mutant LST1/A with both tyrosines mutated to phenylalanines (2YF) were prepared. Immunoprecipitation using the anti-FLAG antibody followed by Western blotting showed that LST1/A-FLAG WT, but not the 2YF mutant, was constitutively phosphorylated in resting THP-1 cells. Moreover, only the LST1/A WT constitutively associated with protein tyrosine phosphatases, SHP-1 and SHP-2 in quiescent cells (Fig. 5*A*). Induction of strong global tyrosine phosphorylation by pervanadate treatment substantially enhanced LST1/A-FLAG tyrosine phosphorylation along with increased SHP-2 and especially SHP-1 bind-

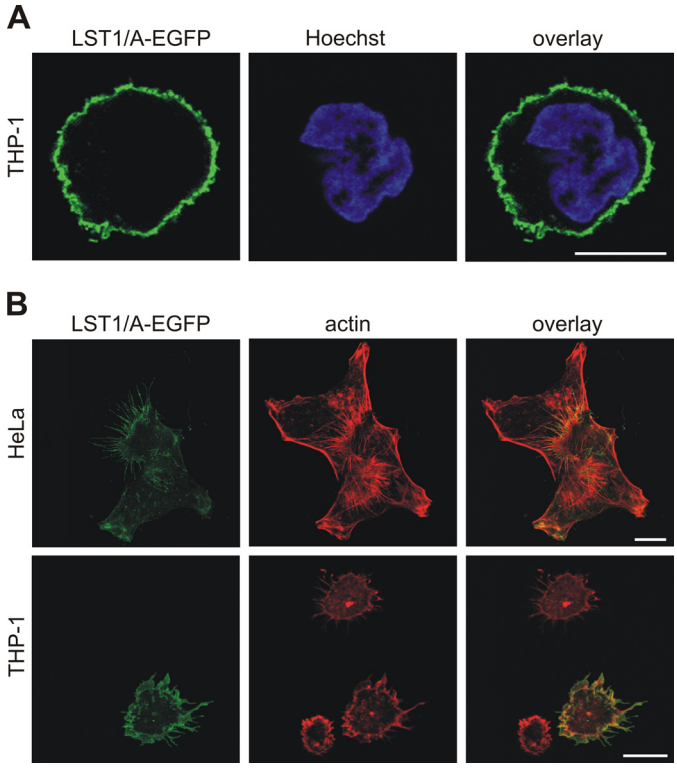

FIGURE 4. **Subcellular localization of LST1/A.** *A*, THP-1 cells were stably transfected with LST1/A-EGFP, fixed, and permeabilized, and nuclei were stained with Hoechst 33258. A representative cell with membrane localized LST1/A is shown. *B*, HeLa and THP-1 cells were transiently transfected with LST1/A-EGFP, allowed to adhere to a cover glass, fixed, and permeabilized, and the actin cytoskeleton was stained with phalloidin. Note the very similar appearance of the cells expressing and not expressing LST1/A-EGFP present in the same field. All samples were analyzed by a confocal microscope. *Scale bar*, 10 μm. For additional images, see supplemental Fig. 1.

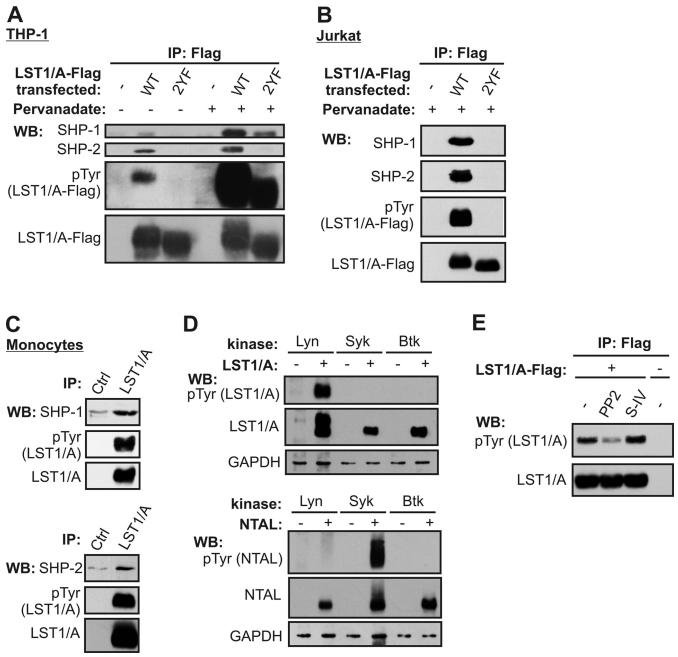

FIGURE 5. **Association of LST1/A with SHP-1 and SHP-2.** *A*, THP-1 cells and the THP-1-derived cell lines stably expressing C-terminally FLAG-tagged LST1/A wild type (WT) or LST1/A with both tyrosines mutated to phenylalanines (2YF) were treated with pervanadate or left untreated. Lysates were subjected to immunoprecipitation using the anti-FLAG antibody. The immunoprecipitated material was analyzed by Western blotting (*WB*) using antibodies to SHP-1, SHP-2, phosphotyrosine (*pTyr*), and LST1/A. *B*, Jurkat cells and Jurkat-derived cell lines stably expressing Flag-tagged LST1/A WT and 2YF mutant were treated with pervanadate, lysed, and subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with antibodies to SHP-1, SHP-2, phosphotyrosine, and LST1/A. *C*, lysates from human peripheral blood monocytes were subjected to immunoprecipitation with the anti-LST1/A antibody or with the isotype-matched control antibody followed by Western blotting using antibodies to SHP-1 (*upper panel*), SHP-2 (*lower panel*), phosphotyrosine (*pTyr*), and LST1/A. *D*, HEK293FT cells were transfected or not with a plasmid encoding LST1/A or NTAL together with a Lyn, Syk, or Btk encoding vector. After 24 h, cells were lysed, and phosphorylation of LST1/A or NTAL was analyzed by Western blotting. *E*, THP-1 cells expressing wild-type FLAG-tagged LST1/A were treated with 10 μM PP2 or 2 μM Syk inhibitor IV (*S-IV*) or left untreated. Lysates from these cells were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with phosphotyrosine and anti-FLAG (LST1/A) antibodies.

ing. The observed phosphorylated band corresponding to LST1/A in the pervanadate treated LST1/A 2YF sample could represent either phosphorylated FLAG tag (of the exogenous LST1/A-FLAG) or endogenous LST1/A co-precipitated as a result of its dimerization with the exogenous FLAG-tagged LST1/A 2YF. This possibility would also explain the detectable amount of SHP-1 co-precipitated with LST1/A 2YF after pervanadate treatment (Fig. 5*A*). Additional explanations include post-lysis phosphorylation of tyrosine 11 in the transmembrane domain. To further analyze this issue, we employed a Jurkat T cell line, which does not express any endogenous LST1/A (Fig. 2*D*). We stably transduced these cells with LST1/A WT and LST1/A 2YF constructs and followed their phosphorylation and interactions after pervanadate-mediated activation. Under these conditions, LST1/A 2YF mutant was not phosphorylated and did not interact with the SHP-1 and SHP-2 phosphatases, whereas strong tyrosine phosphorylation, as well as SHP-1 and SHP-2 binding, were readily observed for the LST1/A WT protein (Fig. 5*B*). This experiment suggested that most likely explanation for the presence of tyrosine phosphorylated band and SHP-1 and SHP-2 phosphatases in LST1/A-FLAG 2YF immunoprecipitates was the dimerization with non-mutated endogenous protein. SOCS3, Cbl-b, and SHIP1 did not co-precipitate with LST1/A WT or 2YF in THP-1 cells, suggesting that their SH2 domains are incapable of binding to the phosphorylated tyrosines of LST1/A (data not shown).

To reproduce these results in a more physiological setting, endogenous LST1/A was immunoprecipitated from primary human monocytes. Similar to the previous experiment, LST1/A was constitutively phosphorylated in the non-stimulated monocytes and co-precipitated with SHP-1 and SHP-2 (Fig. 5*C*). Taken together, these results imply that a subset of LST1/A molecules are phosphorylated at the ITIM and/or ITIM-like motifs in the resting cells and bind SHP-1 and SHP-2 in a phosphotyrosine-dependent manner. An increase in LST1/A phosphorylation leads to enhanced interaction with the protein tyrosine phosphatases.

To identify the kinase phosphorylating LST1/A, we co-expressed LST1/A in Hek293FT cells with a protein tyrosine kinase from Src (Lyn), Syk (Syk), or Tec (Btk) family. Only Lyn, but not Syk or Btk phosphorylated LST1/A in this assay (Fig. 5*D*), indicating that Lyn (and possibly other Src family kinases) are responsible for LST1/A phosphorylation. In contrast, another TRAP, NTAL, was phosphorylated mainly by Syk in this assay, which is in agreement with previously published data (12). In addition, a specific inhibitor of Src family kinases, PP2,

substantially reduced the constitutive LST1/A phosphorylation observed in THP-1 cells (Fig. 5*E*). Syk inhibitor IV, a specific inhibitor of Syk did not have any effect on LST1/A phosphorylation under these conditions (Fig. 5*E*).

LST1/A Is Able to Negatively Regulate Activating Signals Emanating from Surface Receptors—The ability of LST1/A to recruit protein tyrosine phosphatases SHP-1 and SHP-2 to the proximity of the plasma membrane suggested that LST1/A could act as a negative regulator of intracellular signaling pathways. LST1/A contains only a very short extracellular domain, and thus, it is very unlikely that it functions as a receptor. However, it is possible that LST1/A functionally associates with a so far unknown receptor(s) and that it helps to transduce their inhibitory signals.

To test the ability of LST1/A to inhibit cellular signaling pathways, we prepared a plasmid coding for a chimeric protein consisting of extracellular part of CD25 fused to LST1/A-FLAG. This chimera could be extracellularly co-cross-linked to any other surface protein such as Fc receptors, and the effect of LST1/A on downstream signaling pathways could be observed (Fig. 6*A*). First, we prepared THP-1 cells stably expressing CD25-LST1/A-FLAG WT or 2YF chimeras via retroviral transduction (Fig. 6*B*, *left panel*). Both cell lines exhibited reduced calcium response after Fc receptor stimulation, compared with non-transfected cells, probably as a consequence of the transduction procedure (Fig. 6*B*, *middle panel*). However, co-cross-linking of CD25-LST1 WT, but not the 2YF mutant, with Fc receptors further inhibited Fc receptor-mediated calcium influx, leading to almost complete loss of the response (Fig. 6*B*, *right panel*). Due to the problems with the effects of retroviral transduction on calcium flux in transduced THP-1 cells, the U937-derived cell lines stably expressing CD25-LST1/A WT or 2YF were established (Fig. 6*C*, *left panel*). In this case, the viral transduction did not affect Fc receptor signaling, but the inhibitory effects of CD25-LST1/A WT on the Fc receptor-mediated calcium influx still were observed clearly (Fig. 6*C*, *middle* and *right panels*).

Accordingly, expression of CD25-LST1/A WT or 2YF did not influence the overall tyrosine phosphorylation induced by cross-linking of Fc receptors alone (Fig. 6*D*, *left panel*). However, Fc receptor co-cross-linking with WT chimeras resulted in a substantial reduction of overall tyrosine phosphorylation in the whole cell lysates when compared with co-engagement with the 2YF chimera. (Fig. 6*D*, *right panel*). These results indicate that LST1/A might act as a negative regulator of signaling via ITIM and/or ITIM-like dependent recruitment of SHP-1 and/or SHP-2 to a specific plasma membrane compartment.

Immunoprecipitation of the chimeras showed that co-cross-linking with Fc receptors increased tyrosine phosphorylation of the CD25-LST1/A WT and enhanced SHP-1 and SHP-2 binding (Fig. 6*E*). CD25-LST1/A 2YF was not tyrosine-phosphorylated, and it co-precipitated with only a very small amount of the protein tyrosine phosphatases, probably via dimerization with endogenous LST1/A as discussed above (Fig. 6*E*).

DISCUSSION

The LST1/A protein was identified as a potential novel TRAP in our large scale *in silico* search (10). The intracellular sequence of this protein contains one canonical ITIM and an additional ITIM-like motif, which constitute a structure highly homologous to tandem phospho-tyrosine elements in the majority of CD33-related Siglecs. Interestingly, LST1/A was missed in previous genome-wide screens aimed to identify all ITIM containing transmembrane proteins, most likely because LST1/A lacks a signal peptide (15, 25). In this study, we describe the generation of a monoclonal antibody to human LST1/A and the expression and biochemical and functional analysis of LST1/A.

Our anti-LST1/A antibody was generated after immunization of mice with the peptide corresponding to amino acids 75–90 of human LST1/A. Its specificity has been verified using cells transfected with the LST1/A construct. In addition, the apparent molecular weights of the transfected LST1/A and of the endogenous LST1 protein recognized by our antibody were identical. The peptide used for the immunization was predicted to be a part of several other putative cytoplasmic LST1 isoforms identified at the mRNA level, expected to have lower molecular weight than LST1/A. However, we did not observe any additional LST1 isoforms by Western blotting when lysates and immunoprecipitates from a variety of primary cells and cell lines under both reducing and non-reducing conditions were analyzed (Fig. 2 and data not shown). Moreover, using our antibody, we detected disulfide-based LST1/A dimers. Of the putative isoforms containing the sequence of the immunogen, only LST1/A is predicted to form such dimers. Collectively, these observations provide strong evidence that the endogenous isoform of LST1 detected by our antibody is LST1/A.

Due to the inconsistencies in the previously published data on LST1 expression profile (1, 3, 5–8), we carried out a thorough expression analysis of LST1/A. It revealed that LST1/A is substantially enriched in the spleen, peripheral blood monocytes, and granulocytes, as well as the cell lines of myeloid origin, indicating that LST1/A is expressed predominantly in the leukocytes of myeloid lineage. Although this conclusion is only in partial agreement with previously published studies (1, 3, 5–8), we consider our results reliable for several reasons. 1) We analyzed LST1/A expression profile in primary tissues and blood subpopulations, as well as in cell lines both at the protein and the mRNA levels and obtained a set of mutually supporting data. 2) In contrast to previous reports (1, 3, 5–6), we concentrated on the LST1/A isoform, excluding possible interference of other splice-variants with the analysis. 3) Instead of semi-quantitative mRNA detection (5–6), we employed a quantitative RT-PCR method to monitor the mRNA level. 4) Two gene expression databases, BioGPS (26) and Gene Enrichment Profiler (27) support our data.

Thus far, the only study at least partially addressing the issue of the biological role of LST1/A suggested that LST1/A overexpression leads to the formation of filopodia, although the mechanism of this phenomenon has not been proposed (7). In agreement with this study, we observed LST1/A localization to filopodia-like structures in a number of cell types. However, when we used LST1/A-independent filopodia visualization, we failed to detect any differences in the abundance of these structures between non-transfected and LST1/A transfected cells.

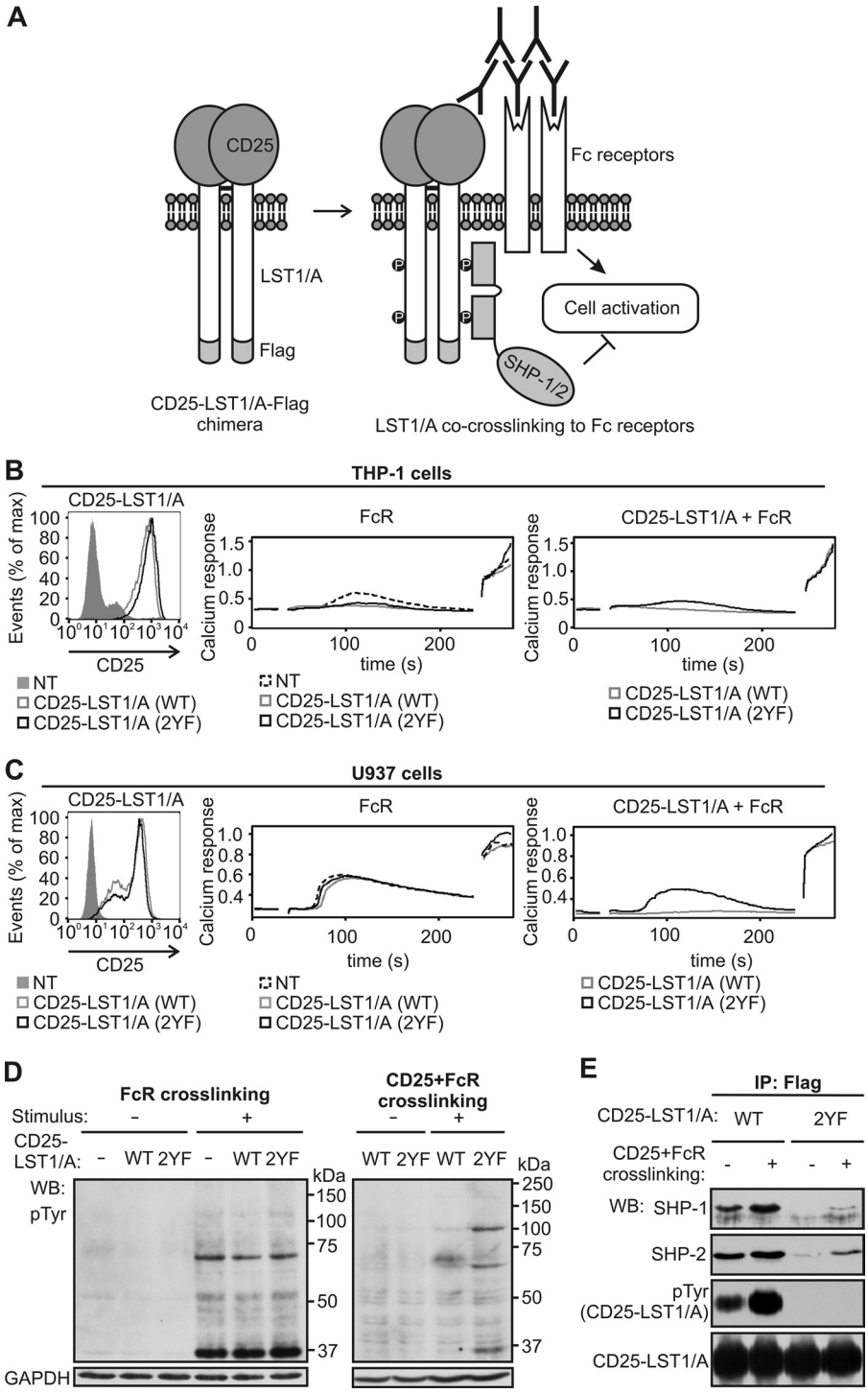

FIGURE 6. **Crosslinking of the CD25-LST1/A chimera with Fc receptors inhibits signaling.** *A*, schematic representation of the CD25-LST1/A-FLAG chimeric construct. Cross-linking of this chimera with Fc receptors is expected to inhibit Fc receptor-mediated signaling events due to the association of LST1/A with SHP-1 and/or SHP-2 phosphatases. *B* and *C*, THP-1 (*B*) or U937 (*C*) cells were left non-transfected or stably transfected with CD25-LST1/A(WT)-FLAG or with CD25-LST1/A(2YF)-FLAG, where both tyrosines in the LST1/A sequence were mutated to phenylalanines. The cells were analyzed for expression of CD25 (*left panel*) and for calcium response after cross-linking of Fc receptors alone (*middle panel*) or in combination with CD25-LST1/A (*right panel*); ionomycin was added after 4 min to measure maximum calcium response. Mean relative calcium concentration in time is shown. *D*, U937 cells expressing CD25-LST1/A(WT)-FLAG or CD25-LST1/A(2YF)-FLAG were stimulated either by cross-linking of Fc receptors (*left panel*) or by co-cross-linking of Fc receptors and CD25-LST1/A (*right panel*), solubilized, and analyzed by Western blotting using the phosphotyrosine-specific antibody (*pTyr*). Staining for GAPDH served as a loading control. *E*, U937 cells expressing the CD25-LST1/A(WT)-FLAG or CD25-LST1/A(2YF)-FLAG chimera were activated by co-cross-linking of CD25-LST1/A to Fc receptors, solubilized, and immunoprecipitated with the anti-FLAG antibody. Samples were analyzed by Western blotting using antibodies to SHP-1, SHP-2, phosphotyrosine (*pTyr*), and LST1/A.

As a starting point for the elucidation of LST1/A function, we used bioinformatic analysis of the LST1/A amino acid sequence, which revealed the presence of two evolutionarily conserved tyrosine-based motifs strongly resembling the ITIM and ITIM-like sequences in the CD33-related Siglec receptor family. Siglecs are receptors binding sialic acid residues present

in cell surface glycoproteins both in *cis* (on the surface of the same cell) and in *trans* (on another cell) (16). Members of the CD33 family are expressed mainly on various leukocyte subpopulations (17), and their function is to negatively regulate various aspects of leukocyte biology, including induction of apoptosis, inhibition of proliferation, and inhibition of immunoreceptor-induced cell activation (16) by recruiting various inhibitory molecules to their ITIM and/or ITIM-like motifs (28–30). Indeed, we observed that LST1/A bound protein tyrosine phosphatases, SHP-1 and SHP-2, in a phosphotyrosine dependent manner. Importantly, at least a fraction of LST1/A molecules was phosphorylated in resting cells (likely by Src family kinases) and constitutively associated with both protein tyrosine phosphatases. On the other hand, neither of the other binding partners of CD33-related Siglecs (SOC3, Cbl-b) nor a typical ITIM-interacting molecule (SHIP1) co-precipitated with LST1/A.

The important difference between LST1/A and Siglecs or other ITIM containing inhibitory receptors (*e.g.* FcγRIIb) is that the extracellular part of LST1/A is very short, and it is therefore highly unlikely that LST1/A would function as a cellular receptor *per se*. However, it could be functionally associated with a so far unidentified receptor(s) and could serve as an intracellular transducer of the inhibitory signal. Despite numerous attempts, we were unable to either identify a receptor, which would be associated with and/or use LST1/A as a signal transducing unit, or discover a pathway inhibited by LST1/A using myeloid cancer cell lines as a model (data not shown). One possible clue might be LST1/A localization in TEMs, where a number of important adhesion molecules and receptors (including integrins, growth factor receptors, and MHCII) are localized (18, 31, 32). It is therefore possible that LST1/A might be functionally coupled to a receptor residing in these types of microdomains.

To provide evidence that LST1/A has the ability to act as a negative regulator of signal transduction cascades downstream of surface receptors, we prepared a chimeric construct composed of the extracellular part of CD25 fused to LST1/A. As expected, we observed that cross-linking of the CD25-LST1/A chimera with Fc receptors led to the inhibition of Fc receptor-mediated calcium influx and protein tyrosine phosphorylation in the THP-1 and U937 monocytic cell lines, due to the association of LST1/A with the SHP-1 and SHP-2 phosphatases.

SHP-1 and SHP-2 are multifunctional protein tyrosine phosphatases involved in the development and differentiation of hematopoietic cell lineages as well as in the onset of inflammation (33). SHP-1 is expressed mainly in hematopoietic cells and neural tissues (33). Motheaten, viable motheaten, and *spin* mouse strains bearing loss of function mutations in the SHP-1 encoding gene (34, 35) exhibit granulocyte and macrophage hyper-responsiveness to cytokines (36) and development of severe autoinflammatory disease (35, 37). Moreover, partial SHP-1 deficiency leads to severe autoimmune disease, which develops even in the absence of B- and T- lymphocytes but is dependent on signaling via innate immune receptors, such as IL-1 and Toll-like receptors (35, 37). Most likely, cells of the myeloid lineage induce the chronic inflammation observed in SHP-1 insufficient animals. SHP-2 is expressed more ubiquitously than SHP-1 (33). In contrast to SHP-1, SHP-2 is not considered an entirely inhibitory protein tyrosine phosphatase in hematopoietic cells, as both positive and negative roles of SHP-2 in cell signaling have been described (38–41). However, in the context of Siglec mediated, and likely also LST1/A-mediated, signaling, its inhibitory function probably prevails.

We have described LST1/A as a new TRAP, which is expressed predominantly in myeloid cells and can function as a negative regulator facilitating the recruitment of SHP-1 and SHP-2 to the plasma membrane. Therefore, it is likely that some of the SHP-1 and/or SHP-2 functions in myeloid cells could be dependent on their binding to LST1/A. Our studies have clarified several unresolved issues concerning this protein and suggested that further analysis of LST1/A should focus on the negative regulation of signaling in myeloid cells. In the following studies, it will be important to determine the signaling pathways that are regulated by LST1/A *in vivo*. This should be facilitated by construction of *lst1* knock-out mice, which is currently underway.

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ADDITIONS AND CORRECTIONS

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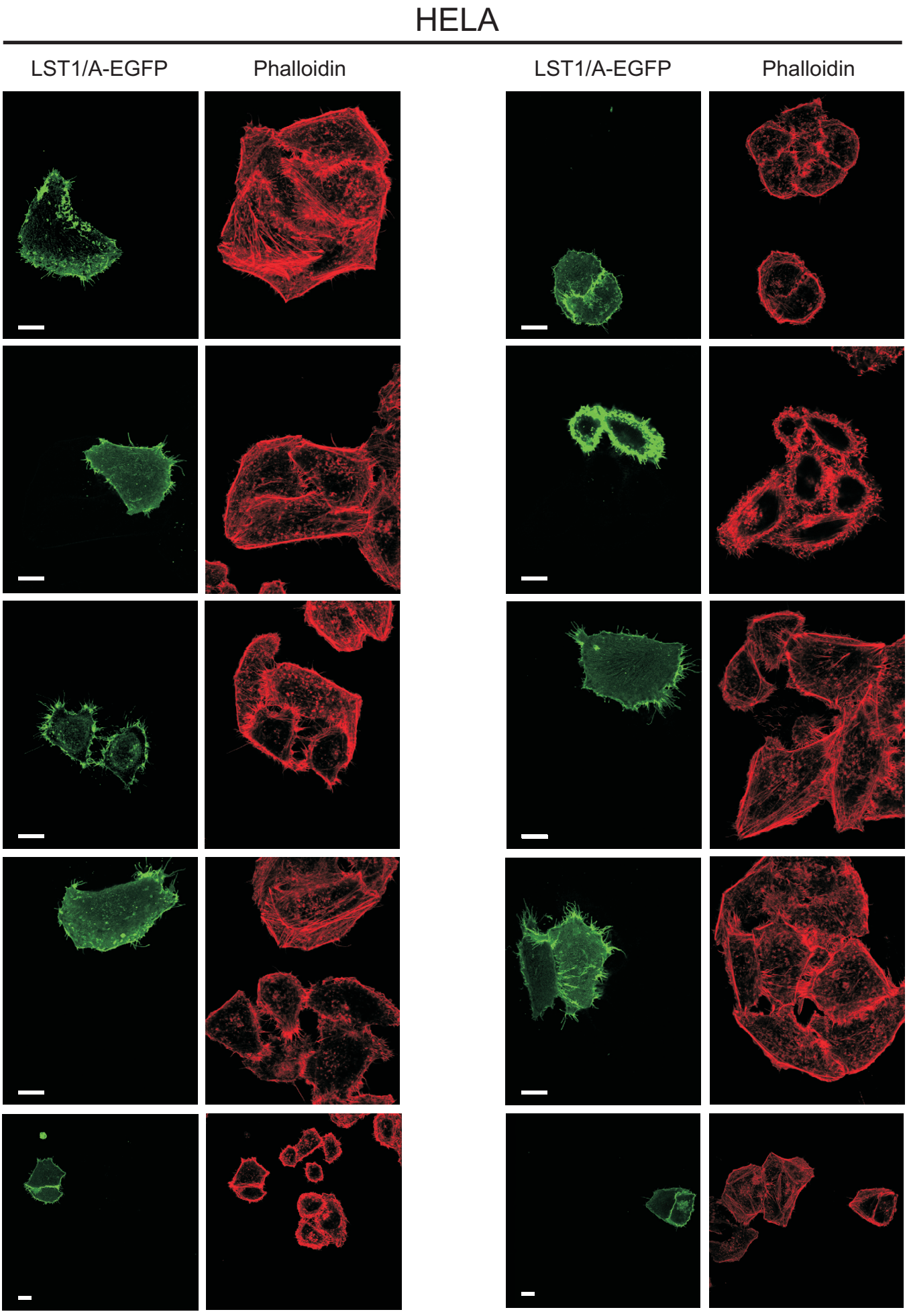
LST1/A is a myeloid leukocyte-specific transmembrane adaptor protein recruiting protein tyrosine phosphatases SHP-1 and SHP-2 to the plasma membrane.

Peter Draber, Ondrej Stepanek, Matous Hrdinka, Ales Drobek, Lukas Chmatal, Linda Mala, Tereza Ormsby, Pavla Angelisova, Vaclav Horejsi, and Tomas Brdicka

PAGE 22813:

Under "Experimental Procedures," in the section "Antibodies and Reagents," we mistakenly stated that a peptide corresponding to amino acids 75–90 of human LST1/A was used in the immunization scheme to generate LST1/A monoclonal antibodies. In fact, the correct amino acid number was 66–80. To avoid any further confusion, the peptide sequence is as follows: SSEGPDLRGRDKRGT. This does not in any way influence the data interpretation and conclusions of this article.

Supplemental figure S1



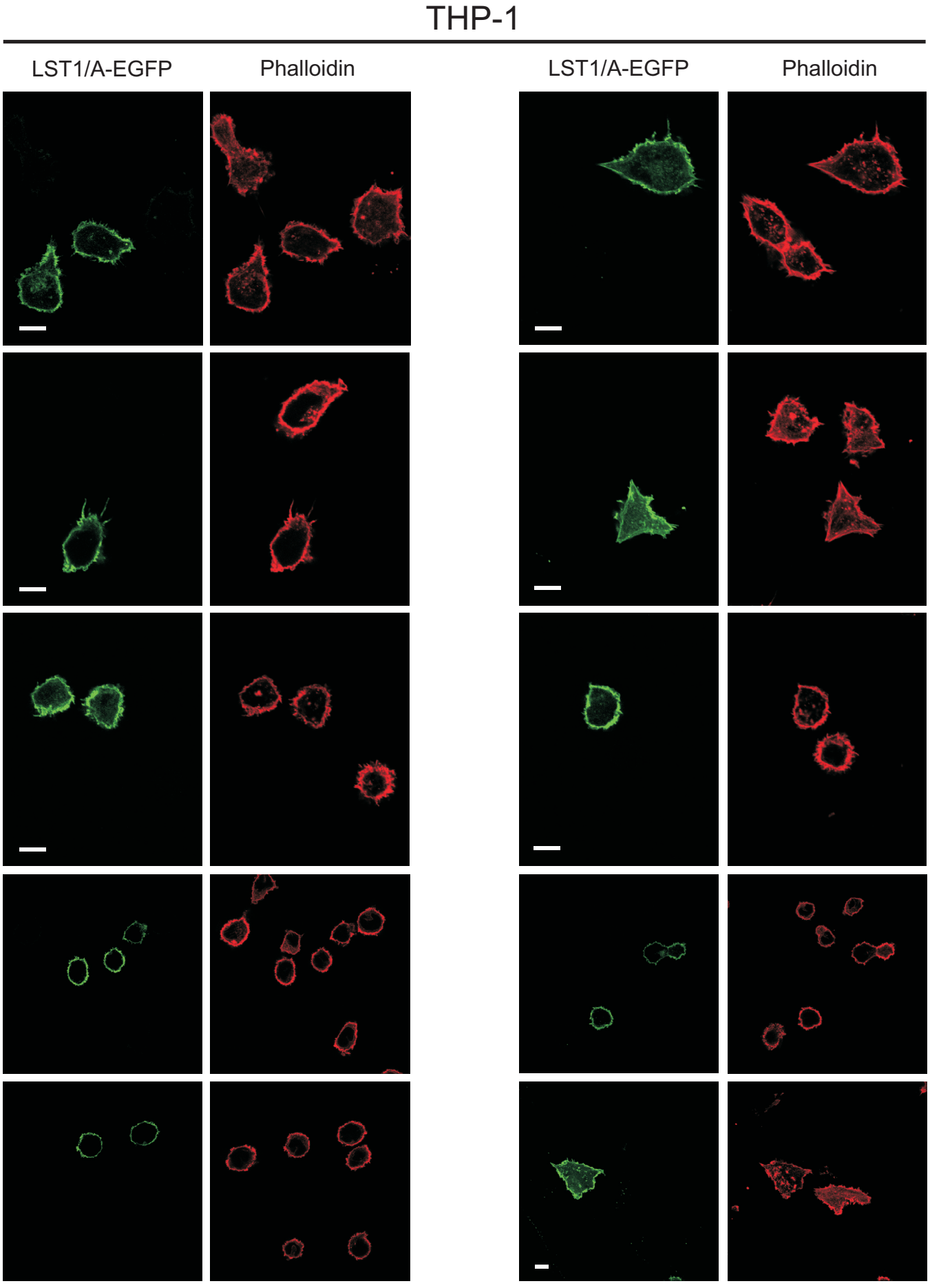


FIGURE S1. HeLa cells were transiently transfected and THP-1 stably transfected with LST1/A-EGFP, fixed, permeabilized, stained with phalloidin and analyzed by confocal microscopy. Scale bar = 10 μm.

Nonredundant Roles of Src-Family Kinases and Syk in the Initiation of B-Cell Antigen Receptor Signaling

Ondrej Stepanek, Peter Draber, Ales Drobek, Vaclav Horejsi, and Tomas Brdicka

When a BCR on a mature B cell is engaged by its ligand, the cell becomes activated, and the Ab-mediated immune response can be triggered. The initiation of BCR signaling is orchestrated by kinases of the Src and Syk families. However, the proximal BCR-induced phosphorylation remains incompletely understood. According to a model of sequential activation of kinases, Syk acts downstream of Src family kinases (SFKs). In addition, signaling independent of SFKs and initiated by Syk has been proposed. Both hypotheses lack sufficient evidence from relevant B cell models, mainly because of the redundancy of Src family members and the importance of BCR signaling for B cell development. We addressed this issue by analyzing controlled BCR triggering ex vivo on primary murine B cells and on murine and chicken B cell lines. Chemical and Csk-based genetic inhibitor treatments revealed that SFKs are required for signal initiation and Syk activation. In addition, ligand and anti-BCR Ab–induced signaling differ in their sensitivity to the inhibition of SFKs. ***The Journal of Immunology*****, 2013, 190: 1807–1818.**

The earliest events of immunoreceptor signaling include tyrosine phosphorylation catalyzed by kinases of the Src and Syk families. Src family kinases (SFKs) are cytoplasmic proteins that are associated with the plasma membrane via lipid anchors at their N termini (1, 2). At least five family members (Lyn, Blk, Fyn, Fgr, and Yes) are expressed in B cells (3). The main mechanism underlying the regulation of SFK activity depends on the phosphorylation of two key tyrosine residues. Phosphorylation of the C-terminal tyrosine (Y527 for chicken Src) is mediated by Csk and counteracted by phosphatases CD45 and CD148 in lymphocytes. This phosphorylation induces a closed conformation and inhibition of the kinase (4–7). In contrast, *trans*-autophosphorylation of the activation loop tyrosine (Y416 for chicken Src) is crucial for full kinase activity (8–10).

Syk and ZAP70 are the only members of the Syk kinase family. The recruitment of these kinases to the plasma membrane is mediated by the binding of their tandem SH2 domains to phosphorylated ITAMs in the immunoreceptor-associated chains (11–13). Activation of Syk is achieved via binding to phosphorylated ITAM and/or by phosphorylation of tyrosine residues in the interdomain B between the C-terminal SH2 domain and the kinase domain (e.g., Y346 for murine Syk) (14–16). These tyrosines seem to be phosphorylated mainly by SFKs, although autophosphorylation of these sites has also been observed under various conditions (14, 17, 18). Similar to SFKs, Syk family kinases contain (auto)phosphorylation sites in the activation loop (Y519/Y520 for murine Syk) (17). Their phosphorylation is critical for Syk-mediated signal transduction, although the exact mechanism remains a matter of debate (19–22).

Engagement of the BCR by Ag leads to the phosphorylation of ITAM in the BCR-associated Igα- and Igβ-chains (23). According to the original model of immunoreceptor signaling, this initial ITAM phosphorylation is mediated by SFKs and is a prerequisite for Syk family kinase recruitment and activation (24). In the analogous situation of TCR, this model is well supported by the existing data (25–27). In B cells, the critical role of BCR-associated ITAMs has also been demonstrated (28). However, the presumption of an essential role of SFKs in the catalysis of ITAM phosphorylation has been challenged by unexpected findings in animals deficient in several SFK members. Mice deficient in Lyn, a predominant SFK in B cells, did not display the predicted inhibition of BCR signaling. Rather, $Lyn^{-/-}$ mice exhibited enhanced B cell activation in response to BCR stimulation and developed an autoimmune syndrome, most likely because of hypophosphorylation of inhibitory receptors (29–31). The dispensability of Lyn in BCR signaling was initially explained by compensation by other SFK family members, mainly Fyn and Blk (32). Indeed, Lyn/Fyn/Blk triple-deficient mice displayed profound block in B cell development that was not observed in $Lyn^{-/-}$ mice. However, the triple-deficient mice did not exhibit any defect in anti-Igβ Ab–induced BCR-like proximal signaling at the pro-B stage (33). These data suggest that SFKs are not essential for the initiation of BCR signaling and support the hypothesis of SFK-independent phosphorylation of Igα and Igβ.

In contrast, the ability of Lyn to positively regulate BCR signaling was demonstrated in mice expressing a constitutively active Lyn mutant, which led to the hyperactivation of important signaling molecules, including Syk (34). A positive role of SFKs in proximal BCR signaling was also revealed in CD45/CD148 double-deficient mice (4) and in Lyn-deficient DT40 chicken B cell lymphoma cells (35), which both exhibited a delayed and weakened BCR response. However, neither of these models confirmed the absolute requirement for SFK activity in BCR signaling, as the signaling was far from completely blocked. In addition, the in-

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic

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Address correspondence and reprint requests to Tomas Brdicka and Ondrej Stepanek, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic. E-mail addresses: tomas.brdicka@img.cas.cz (T.B.) and ondrej.stepanek@img.cas.cz (O.S.)

The online version of this article contains supplemental material.

Abbreviations used in this article: anti-BCR, anti-BCR Ab; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetyl; NP, 4-hydroxy-3-nitrophenylacetyl; NP_5-BSA, NP conjugated to BSA at molecular ratio 5:1; NP_{25}-BSA, NP conjugated to BSA at molecular ratio 25:1; NP_{40}-Ficoll, NP conjugated to Ficoll at molecular ratio 40:1; SFK, Src family kinase; WT, wild type.

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completely inhibited BCR signaling in $Lyn^{-/-}$ DT40 cells, which are believed to express no other SFKs, was another important line of evidence supporting the existence of SFK-independent BCR signaling (32, 35, 36).

The putative SFK-independent BCR signaling is often explained by the ability of Syk to phosphorylate BCR-associated ITAMs (16, 37, 38). Indeed, Syk was reported to phosphorylate both ITAM tyrosines in Igα when coexpressed in the insect cell line S2 (15), and Syk, but not its T cell paralogue ZAP70, was reported to phosphorylate ITAMs in vitro and in COS cells (14, 39). Moreover, ectopic expression of Syk was able to rescue TCR signaling in the J.CaM1.6 T cell line, which is deficient in SFK Lck (40).

The existence of SFK-independent BCR signaling and the ability of Syk to phosphorylate BCR-associated ITAMs are broadly accepted (11, 16, 37, 38), although several important related issues have been poorly addressed so far, including the relationship between SFK-dependent and -independent signaling, SFK/Syk interplay, and the overall role of SFKs as both positive and negative regulators in the proximal steps of BCR signaling. Another important problem is that most of the evidence supporting the model of Syk-mediated ITAM phosphorylation is based on in vitro analyses and overexpression studies in cells of non–B-lymphoid origin. One of the reasons for the apparent neglect of these questions may be the lack of a suitable B cell model of SFK deficiency arising from the redundancy of multiple Src family members in B cells and their requirement for B cell development.

In this study, we used a number of novel tools including phospho-specific Abs and chemical- and protein-based inhibitors of SFKs and Syk to revisit this issue. Our results show that SFKs are indispensable for BCR signaling triggered by Ag. In contrast, we could not find any evidence for Syk-mediated ITAM phosphorylation in B cell lines and primary B cells. This brought us to the conclusion that for BCR signaling, the model of sequential activation of Src and Syk family kinases is also fully valid.

Materials and Methods

Abs and reagents

Abs to the following Ags were used: phospho-Erk (T202/Y204, amino acid numbers correspond to mouse proteins, if not indicated), phospho-Igα (Y182), phospho-Syk (Y346), phospho-Syk (Y519/520), phospho-Src family (chicken Y416), phospho-Src family (clone D49G4, chicken Y416), phospho-Lyn (Y507), phospho-Akt (S473), phospho-Src (chicken Y527), non–phospho-Src (chicken Y527), Lyn (all rabbit from Cell Signaling Technology, Danvers, MA,), phospho-SLP65 (Y84) (BD Pharmingen, BD Biosciences, San Jose, CA), GAPDH (rabbit; Sigma-Aldrich, St. Louis, MO), c-Src (rabbit, N-16; Santa Cruz Biotechnology, Santa Cruz, CA), CD3-PE (Exbio Praha, Vestec, Czech Republic), CD11b-FITC, Thy1.1-FITC (both eBiosciences, San Diego, CA), Ig κ L chain-FITC (AbD Serotec, Kidlington, U.K.), CD69-allophycocyanin, Ig λ L chain-allophycocyanin (both BioLegend, San Diego, CA), IgM-Pacific Blue (clone B76; conjugated in-house; American Type Culture Collection, Manassas, VA), B220-Dy649 (clone RA3-6B2; conjugated in-house; obtained from Dr. R. Coffman, DNAX Research Institute, Palo Alto, CA), B220-Pacific Blue (clone RA3-6B2, conjugated in-house). The following secondary Abs were used: goat anti-rabbit–Dy649 (Jackson Immuno-Research, West Grove, PA), goat anti-mouse–Alexa Fluor 488 (Invitrogen, Carlsbad, CA), goat anti-mouse–HRP IgG specific (Sigma-Aldrich), and goat anti-rabbit–HRP (Bio-Rad, Hercules, CA).

The following reagents were used for cell stimulation: anti-CD3 Ab (hamster IgG1 145-2C11; Exbio Praha), goat anti-Armenian hamster IgG, goat anti-mouse IgM $F(ab)_2$, goat anti-chicken IgY $F(ab)_2$ Abs (all from Jackson ImmunoResearch), 4-hydroxy-3-nitrophenylacetyl (NP) conjugated to BSA at molecular ratio 25:1 (NP_{25}-BSA) and 5:1 (NP_5-BSA), 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP) conjugated to BSA at molecular ratio 25:1 (NIP_{25}-BSA), and NP conjugated to Ficoll at molecular ratio 40:1 (NP_{40}-Ficoll) (all from Biosearch Technologies, Novato, CA). The following cell inhibitors were used: PP2, Syk inhibitor IV–BAY 61-3606 (both from Calbiochem, Merck, Darmstadt, Germany), and piceatannol (Sigma-Aldrich).

Cell lines and primary cells

All of the cell lines were cultured in the indicated media supplemented with 10% FBS, 2 mM glutamine, 20 μg/ml gentamicin, 50 μg/ml streptomycin, and 10^4 U/ml penicillin at 37°C in 5% CO_2. The K46-μMλ cells (obtained from Dr. J. Cambier, National Jewish Medical Research Center, Denver, CO) were cultivated in IMDM. The Phoenix Eco (Origene, Rockville, MD) cells were cultivated in DMEM. The DT40 cells (obtained from Dr. A. Weiss, University of California, San Francisco, San Francisco, CA, with kind permission from Dr. T. Kurosaki, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) were cultivated in RPMI supplemented with 1% chicken serum.

Murine spleens were collected from healthy C57BL/6j mice (Institute of Molecular Genetics Animal Facility), B1-$8i^{+/+}$ transgenic mice (41) (The Jackson Laboratory, Bar Harbor, ME), or B1-$8i^{+/-}$ heterozygotes. The B1-$8i^{+/+}$ and B1-$8i^{-/+}$ were phenotypically identical and used interchangeably. A single-cell splenocyte suspension was prepared, and erythrocyte lysis was performed in ACK buffer (150 mM NH_4Cl, 0.1 mM EDTA [disodium salt], 1 mM $KHCO_3$). Splenic B cells were isolated from the whole-spleen suspension by negative selection using anti-CD43 and anti-CD11b magnetic beads on an AutoMACS magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany), and the purity (>95%) was determined by flow cytometry. Lambda L chain–positive B cells were isolated from the whole-spleen suspension as $B220^+/Ig\lambda^+$ double-positive cells using a BD Influx (BD Biosciences). The experiments were approved by the Institutional Review Board and Animal Care and Use Committee of the Institute of Molecular Genetics.

DNA transfection and transduction

LAT-Csk-mOrange, LAT-FRB-IRES-Thy1.1, and Csk-FKBP-mOrange in the MSCV vector were described previously (42). Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's instructions for the transfection of Phoenix Eco cells to produce viral particles. Retrovirus-containing supernatants were then harvested, supplemented with Polybrene (10 μg/ml; Sigma-Aldrich), and added to the cells. The cells were then centrifuged at 1250 × *g* for 90 min. Infected K46 cells were sorted by a BD Influx FACS (BD Biosciences).

$B220^+/Ig\lambda^+$ splenocytes from B1-$8i^{+/+}$ or B1-$8i^{+/-}$ mice were cultivated for 3 d in IMDM supplemented with LPS (1 μg/ml), IL-4 (50 ng/ml), and anti-BCR Ab (anti-BCR; 5 μg/ml) before the infection. After the infection, the cells were cultivated in IMDM supplemented with LPS and IL-4 for 2 d and used in experiments.

Cell activation

For Western blotting, the cells were washed and resuspended in serum-free IMDM at a concentration of 25–50 × 10^6 cells/ml. In some experiments, the indicated inhibitors were added 5–15 min before activation. Subsequently, the cells were stimulated as indicated at 37°C. The activation of cells was stopped by addition of an equal volume of 2× concentrated SDS-PAGE sample buffer, followed by heating of the samples (94°C for 3 min) and sonication. The samples were analyzed by SDS-PAGE followed by Western blotting. For pervanadate-induced activation, the cells (5 × 10^7 cells/ml) were incubated with 1 mM pervanadate for 20 min.

For flow cytometry, splenocytes were harvested and resuspended in IMDM/10% FBS and prewarmed for 10 min at 37°C with the indicated inhibitor, if applicable. Cells were stimulated as indicated, and the activation was stopped by fixation in 2% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA).

If not indicated otherwise, we used the following doses of activators: 10 μg/ml anti-BCR Ab, 1 μg/ml NP-BSA, 1 μg/ml NP-Ficoll.

Flow cytometry

For calcium measurement, the cells were loaded with 5 μM Fura Red dye (Invitrogen) in serum-free IMDM at 37°C for 30 min. Subsequently, the cells were labeled with the indicated Abs in PBS with 1% BSA and 10% goat serum on ice for 20 min. The cells were prewarmed for 5 min at 37°C and incubated with the indicated inhibitors, if applicable, before the measurement. The relative intracellular calcium concentration was measured as a ratio of the Fura Red (43) fluorescence intensities elicited by excitation wavelengths of 405 nm (detection at 635–720 nm) and 488 nm (detection at 655–695) nm). The cells were stimulated as indicated 30 s after the measurement began. Before specific marker-dependent gating, splenic lymphocytes were gated based on their forward scatter versus side scatter properties. The median relative calcium influx is shown.

Intracellular staining was performed on cells fixed in 2% paraformaldehyde at room temperature and permeabilized in 90% methanol on ice.

The cells were stained with the indicated Abs in PBS/15% goat serum at room temperature for 45 min. Data were collected using a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA). For the calcium response analysis, FlowJo's Kinetic platform was used. The resulting curves were smoothened using the Moving Average function of the software (displayed value for each time point is the result of averaging three measured values, including this time point and its neighbors).

Quantitative RT-PCR

RNA was isolated using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). Reverse transcription was performed with RevertAid reverse transcriptase (Fermentas; Thermo Fisher Scientific) using anchored oligo (dT)20 primers. Quantitative PCR was performed using a LightCycler 480 SYBR Green I Master chemistry (Roche Applied Science, Indianapolis, IN) in triplicates with following primers (5′ to 3′): FYN-forward, TCCTCTCATACAGGGACGCT, FYN-reverse, CCAGTCTCCTTCCGAGCTGT; LYN-forward, AAATTGAAAGTTATTGAGGAGCTGG, LYN-reverse, GAGCCAGGAGTTGCCTTTCA, YES-forward, AGCAGCATTGTATGGTCGGT, YES-reverse, TTCCCGATTCACCATTCCTGG; YRK-forward, GTGCCCTACCCAGGGATGAA, YRK-reverse, CAGCACTGCACCATCACATC; HCK-forward, AGAGAAGATGCAGTTAGGCCG, HCK-reverse, TCTGTGTTCATCATGTTGCTTGC; LCK-forward, CAGCACGAGAAGGGGCTAAA, LCK-reverse, TCCGGCCGTAGGTAACAATC; SRC-forward, CACCGTCACGTCGCCTCAG, SRC-reverse, CAGTCACCTTCCGTGTTGTTGA; BLK-forward, TTGCTGGTGGCATTACCATTG, BLK-reverse, AGCTCCAGATCACGGTCACT; ACTB-forward, CCATGGATGATGATATTGCTGCG, ACTB-reverse, AACCATCACACCCTGATGTCTG. Efficiencies of the primers, determined by serial dilution method, were within the range of 92–100% and were included in the calculations of relative mRNA levels.

Statistics

For the calculation of statistical significance, a two-tailed Student *t* test (unequal variance) was used. All of the presented data are representative of at least three independent experiments.

Results

Inhibition of SFKs delays but does not abolish the BCR signaling

The primary focus of this study was the role of SFKs and Syk in the initiation of BCR signaling. Because several reports supported the concept of SFK-independent BCR signaling and/or the ability of Syk to initiate the BCR transduction cascade by phosphorylating ITAMs in Igα/Igβ, we evaluated the effects of the SFK inhibitor PP2 (44) on BCR signaling in splenic B cells and on TCR signaling in splenic T cells. We cross-linked the BCR or TCR complexes using an anti-IgM $F(ab)_2$ polyclonal Ab (anti-BCR Ab, anti-BCR) or hamster anti-CD3 Ab in combination with an anti-hamster Ab (anti-TCR), respectively. Whereas 5 μM PP2 was able to completely inhibit calcium influx after TCR stimulation, the BCR-induced calcium response was delayed but not diminished (Fig. 1A, gating strategy in Supplemental Fig. 1A). Interestingly, after 5 μM PP2 treatment, phosphorylation of the SFK activation loop tyrosine in T cells was higher than that in B cells, suggesting higher residual SFK activity in T lymphocytes (Supplemental Fig. 1B). An increase in PP2 concentration to 20 μM further delayed the calcium influx but again did not diminish the amplitude at later time points (Fig. 1B). In contrast, the inhibition of Syk using Syk inhibitor IV (45) did not delay the initiation of calcium response; rather, it reduced the amplitude during the entire monitored period in a concentration-dependent manner (Fig. 1C). Similar effects were observed after the inhibition of Syk by piceatannol (Supplemental Fig. 1C). BCR-induced phosphorylation of Igα and Syk, as well as the downstream signaling protein Erk, was delayed in the presence of PP2 in a manner similar to that of the delay in the calcium response (Fig. 1D).

The relative resistance of BCR signaling to the inhibition of SFKs and the differential effects of SFK and Syk inhibitors on BCR signaling could be explained by two hypotheses. Either the SFK inhibition was incomplete and the residual SFK activity was sufficient to initiate BCR signaling, or there is an alternative mechanism for BCR activation that is independent of SFKs. To analyze the SFK activity in PP2-treated cells, we reprobed the membranes from Fig. 1D with an Ab targeting the activation loop phosphotyrosine in the kinase domain. Although this tyrosine was substantially hypophosphorylated in the presence of PP2 in resting cells, after BCR triggering, it became markedly phosphorylated with a delay similar to that of the other examined proteins (Fig. 1E). These results suggest that the residual SFK activity may be responsible for the initiation of the observed signaling events in the presence of PP2.

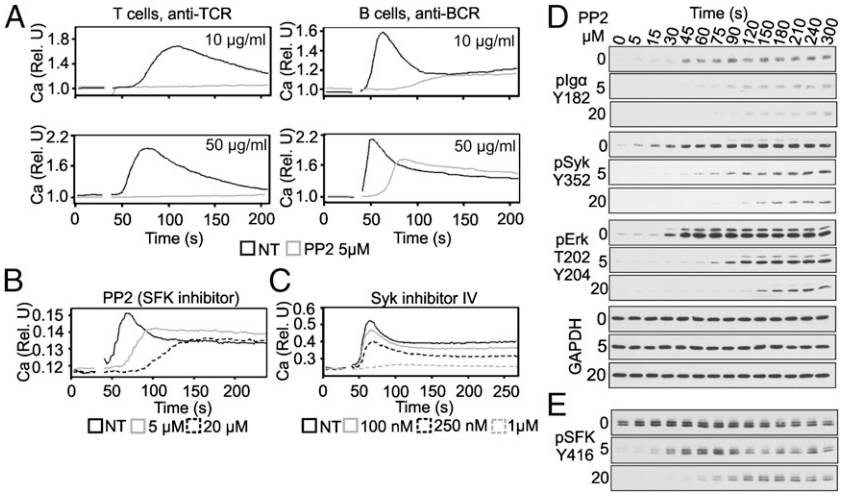

FIGURE 1. Inhibition of SFKs delays BCR signaling. (**A**) Mouse splenocytes were stained for CD19 and CD11b or for CD3 and CD11b, and treated or not with 5 μM PP2. T cells were stained with saturating concentration of anti-CD3 hamster Ab on ice before activation by the anti-hamster secondary Ab (10 or 50 μg/ml) and gated as $CD19^-/CD11b^-$ cells. B cells were activated by anti-BCR (10 or 50 μg/ml) and gated as $CD3^-/CD11b^-$ cells. The calcium influx was monitored. See Supplemental Fig. 1A for analysis of the gating strategy. (**B** and **C**) Mouse splenocytes were stained for CD3 and CD11b, activated by anti-BCR in the presence of the indicated concentration of PP2 or Syk inhibitor IV, and the calcium influx was monitored. B cells were gated as $CD3^-/CD11b^-$. (**D**) Western blot analysis of lysates from splenic B cells activated by anti-BCR in the indicated concentration of PP2. Samples were collected at the indicated time points after activation, and phosphorylation of Syk, Erk, and Igα was determined by Western blotting. (**E**) The membranes from (D) were reprobed with an Ab to SFK pY416.

Inhibition of SFKs blocks BCR signaling induced by a BCR ligand

Although BCR cross-linking via a specific Ab is widely used to mimic the BCR engagement by its ligand, such cross-linking could potentially induce effects that do not occur upon physiological BCR triggering. To analyze the effects of SFK inhibition on ligand-induced BCR signaling, we used the B cell lymphoma cell line K46-μMλ (here referred to as K46 wild type [WT]), which expresses transgenic BCR (B1-8) specific for NP (46). NP-conjugated BSA (NP_{25}-BSA) induced a rapid calcium influx, but the increase was more transient than the anti-BCR–triggered response (Fig. 2A). In agreement with our data from primary B cells, anti-BCR–induced signaling was only delayed by the inhibition of SFKs in K46 cells. Surprisingly, the NP_{25}-BSA–mediated response was substantially reduced by 5 μM PP2, and 20 μM PP2 was able to inhibit the calcium response completely (Fig. 2A), which suggests a qualitative difference between Ab- and ligand-induced BCR signal transduction. The higher sensitivity of the ligand-dependent signaling to PP2 was not the result of suboptimal concentration of NP_{25}-BSA (1 μg/ml), as even 10-fold lower concentration was sufficient to induce maximum response (Supplemental Fig. 2A).

In addition to PP2, we also used a genetically encoded protein inhibitor based on Csk, which is a kinase that very specifically phosphorylates the inhibitory tyrosine of SFKs. The Csk inhibitor consisted of an extracellular and transmembrane domain of the lipid raft protein LAT fused to constitutively active Csk (with mutations in the SH2 and SH3 domains to prevent binding to cellular proteins). This construct was C-terminally tagged with mOrange fluorescent protein (Fig. 2B) to permit the tracking of construct-expressing cells. The LAT-Csk construct was previously shown to be a potent inhibitor of TCR signaling (42). We also used a tunable variant of this inhibitor comprising the LAT transmembrane domain fused to an FRB domain and Csk-mOrange fused to an FKBP domain (Fig. 2B). The recruitment of constitutively active Csk to the plasma membrane could be induced by adding a rapamycin derivative AP21967 that dimerizes the FRB and FKBP domains. Such recruitment has been shown to regulate the inhibitory effect on SFKs (42). We prepared K46-derived cell lines expressing LAT-Csk-mOrange (K46:LAT-Csk), kinase-dead LAT-Csk-mOrange with an inactivating K222R mutation in the Csk kinase domain (K46:LAT-Csk-KD), or both components of the inducibly dimerizing LAT-Csk inhibitor (K46:dimLAT-Csk).

The expression of LAT-Csk, but not kinase-dead LAT-Csk, resulted in dramatic hyperphosphorylation of Lyn at the inhibitory tyrosine (Y507) and hypophosphorylation of SFKs at the activation tyrosine (Y416), which indicates that LAT-Csk is a potent SFK inhibitor in B cells (Fig. 2C). The expression of dimLAT-Csk induced similar effects, although to a lesser extent. The inactivation of SFKs, measured by phosphorylation of the key tyrosine residues, was enhanced by AP21967 (Fig. 2C).

We then tested the effects of LAT-Csk on the anti-BCR– and ligand-induced calcium response in K46 cells. In agreement with the PP2-based experiments, LAT-Csk completely inhibited the

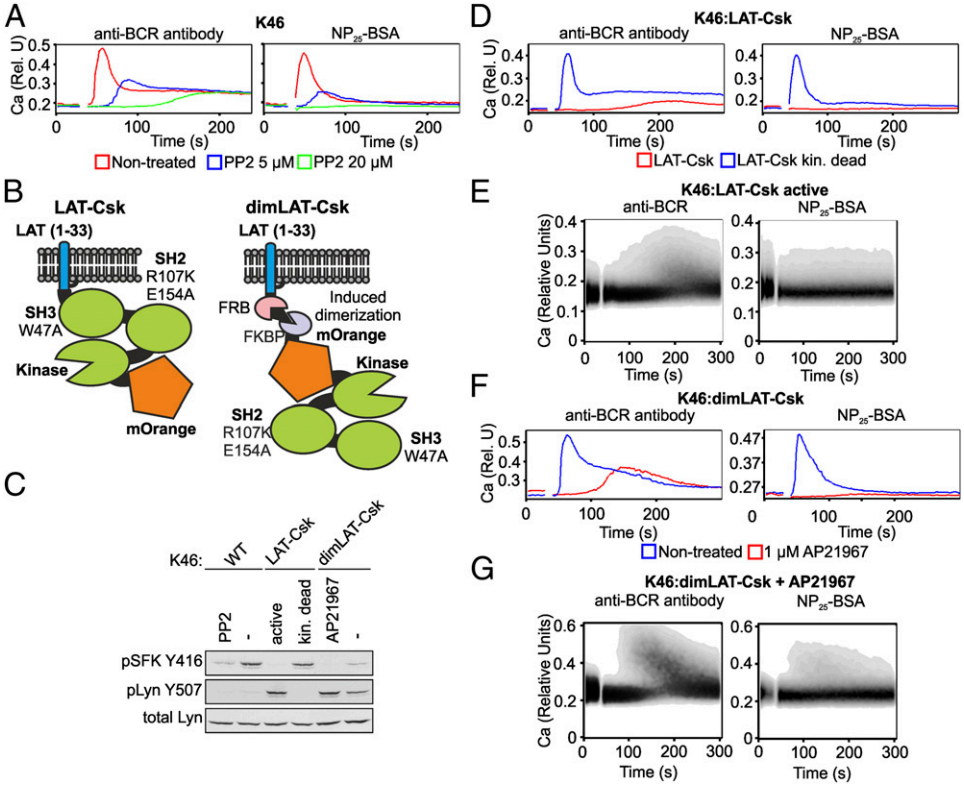

FIGURE 2. Inhibition of SFKs has different effects on Ab- and ligand-induced BCR signaling. (**A**) K46 WT cells were activated by anti-BCR or NP_{25}-BSA in the presence of the indicated concentrations of PP2, and the calcium influx was monitored. (**B**) Schematic representation of genetic Csk-based inhibitors of SFKs. (**C**) K46 cells treated or not with 5 μM PP2 for 5 min, K46:LAT-Csk and K46:LAT-Csk-KD cells, and K46:dimLAT-Csk cells treated or not with 1 μM AP21697 for 5 min were lysed and examined for phosphorylation of the activation tyrosine of SFKs and the inhibitory tyrosine of Lyn. Staining for total Lyn served as a loading control. (**D** and **E**) K46:LAT-Csk or K46:LAT-Csk-KD were activated by anti-BCR or NP_{25}-BSA, and the calcium influx was monitored. Only $mOrange^+$ cells were gated. Density plots for cells expressing active LAT-Csk are shown (E). (**F** and **G**) K46:dimLAT-Csk cells were activated by anti-BCR or NP_{25}-BSA after incubation for 5 min with or without 1 μM AP21697. The calcium influx was monitored. Only $mOrange^+$ cells were gated. Density plots for cells treated with the AP21697 are shown (G).

NP_{25}-BSA–induced calcium influx, but the cells were still able to respond to anti-BCR, although with a substantial delay (Fig. 2D, 2E). The difference in the sensitivity to SFK inhibition between the ligand-triggered and anti-BCR–triggered BCR calcium responses was reproduced in K46:dimLAT-Csk cells after the addition of the AP21967 dimerizer. Again, the anti-BCR–mediated calcium response was delayed, whereas the ligand-triggered signaling was almost completely abolished (Fig. 2F, 2G). The dimerizer did not affect the calcium signaling in K46 WT cells (data not shown). LAT-Csk also inhibited the transcriptional BCR response, as revealed by impaired CD69 upregulation after stimulation with both anti-BCR and NP_{25}-BSA (Supplemental Fig. 2B).

The experiments with NP_{25}-BSA indicated that ligand-induced BCR signaling is blocked, but anti-BCR–triggered signaling is only delayed upon the inhibition of SFKs. It was possible that NP_{25}-BSA was a weaker agonist than anti-BCR (i.e., because of low affinity of its interaction with BCR, low receptor occupancy, or limited flexibility of the protein carrier). To address this issue, we tested B1-8 agonists with lower NP:BSA ratio (NP_5-BSA), higher affinity to the BCR (NIP-BSA) (47), or attached to a more flexible carrier (NP_{40}-Ficoll). Neither the decrease in NP:BSA ratio nor the higher affinity ligand had any effect on the response in nontreated or PP2-treated cells (Supplemental Fig. 2C). In contrast, a potent T cell–independent ligand NP_{40}-Ficoll induced stronger response than NP-BSA and anti-BCR in K46 cells (Fig. 3A). However, NP_{40}-Ficoll–induced calcium signaling was totally inhibited in K46:LAT-Csk cells (Fig. 3B). PP2 did not block NP_{40}-Ficoll–triggered calcium flux completely, but again, it had a significantly stronger impact on NP_{40}-Ficoll than on anti-BCR–mediated signaling (Fig. 3C).

The BCR-induced phosphorylation of SFKs, Syk, Igα, Erk, and Akt was comparable in anti-BCR, NP_{25}-BSA, and NP_{40}-Ficoll–treated cells at 1 min after activation in K46:LAT-Csk-KD cells (Fig. 3D). The NP_{25}-BSA–induced phosphorylation appeared to be more transient for Erk, Igα, and Syk, but not Akt and SFKs upon stimulation for 5 min. LAT-Csk delayed the phosphorylation of Syk, Igα, Erk, and Akt in anti-BCR–activated cells, but almost completely abolished the phosphorylation of these proteins in NP_{25}-BSA– and NP_{40}-Ficoll–treated cells (Fig. 3D). These results support the hypothesis that anti-BCR–mediated signaling is partially resistant to the inhibition of SFKs, whereas higher SFK activity is required for ligand-induced signaling. The anti-BCR induced higher level of the activation loop phosphorylation than the ligands in K46:LAT-Csk-KD cells even at 1 min after activation, when the phosphorylation of other examined proteins was comparable (Fig. 3D). The stronger activation of SFKs by the anti-BCR could explain the partial resistance to SFK inhibition.

Interestingly, in contrast with PP2-treated primary B cells (Fig. 1E), we did not observe anti-BCR–induced phosphorylation of the activation site of SFKs in K46:LAT-Csk cells (Fig. 3D), which could be explained by the likely induction of the closed conformation of SFKs upon the inhibition by LAT-Csk. This conformation stabilizes the activation loop in the inactive position where the activation loop tyrosine might be relatively difficult to access (48). PP2-inhibited Lyn is characterized by a very low level of Y507 phosphorylation (Fig. 2C); thus, the majority of this kinase is likely in the open active-like conformation even when interacting with PP2 (49, 50). The activation loop phosphorylation site is thus more accessible and is possibly phosphorylated (in *trans*), even in the PP2-bound kinase domain.

Differences between anti-BCR– and ligand-induced signaling in primary cells

To verify our results from the K46 cells, we used primary splenocytes from the B1-8i knock-in mouse strain, which expresses the

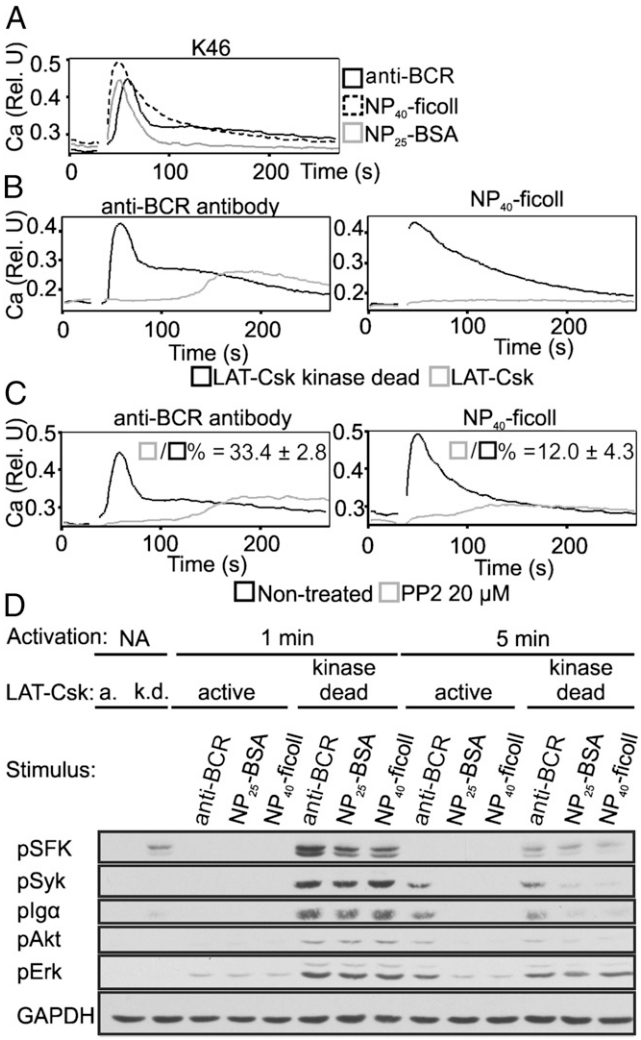

FIGURE 3. Strong BCR ligand NP_{40}-Ficoll is dependent on SFK activity. (**A**) K46 cells were activated via anti-BCR, NP_{25}-BSA, or NP_{40}-Ficoll, and the calcium influx was monitored. (**B** and **C**) K46:LAT-Csk and K46:LAT-Csk-KD (B) or K46 WT cells treated or not with 20 μM PP2 (C) were activated by anti-BCR or NP_{40}-Ficoll, and the calcium influx was monitored. Values in the boxes represent the maximum calcium response in PP2-treated cells displayed as a percentage of maximum response of nontreated cells (mean ± SEM, $n = 3$, $p = 0.034$). (**D**) K46:LAT-Csk and K46:LAT-Csk-KD cells were activated by anti-BCR, NP_{25}-BSA, or NP_{40}-Ficoll for 1 or 5 min. The phosphorylation of SFKs (Y416), Syk (Y346), Igα (Y182), Akt (S473), and Erk (T202/Y204) was analyzed by Western blotting.

B1-8 transgenic IgH (41). When paired with an Igλ L chain, the B1-8 H chain produces an NP-specific BCR (51). Because few B cells express the λ L chain, we had to sort or gate them (Supplemental Fig. 3A, 3B).

We treated the splenocytes with 5 or 20 μM PP2 and monitored the calcium influx in the $Ig\kappa^-$ B cells after activation by anti-BCR, NP_{25}-BSA, or NP_{40}-Ficoll. In the nontreated samples, the highest response was observed after activation by NP_{40}-Ficoll (Fig. 4A). The 5 μM PP2 substantially inhibited NP_{25}-BSA–mediated calcium signaling but had only a minor effect on the NP_{40}-Ficoll– and anti-BCR–mediated response. In contrast, the treatment with 20 μM PP2 resulted in significantly stronger inhibition of the response mediated by NP_{40}-Ficoll relative to the response mediated by anti-BCR (Fig. 4A).

Similar effects of PP2 were observed at the level of Igα and Erk phosphorylation. We measured the percentage of cells activating Erk upon treatment with the BCR agonists. The Erk response was

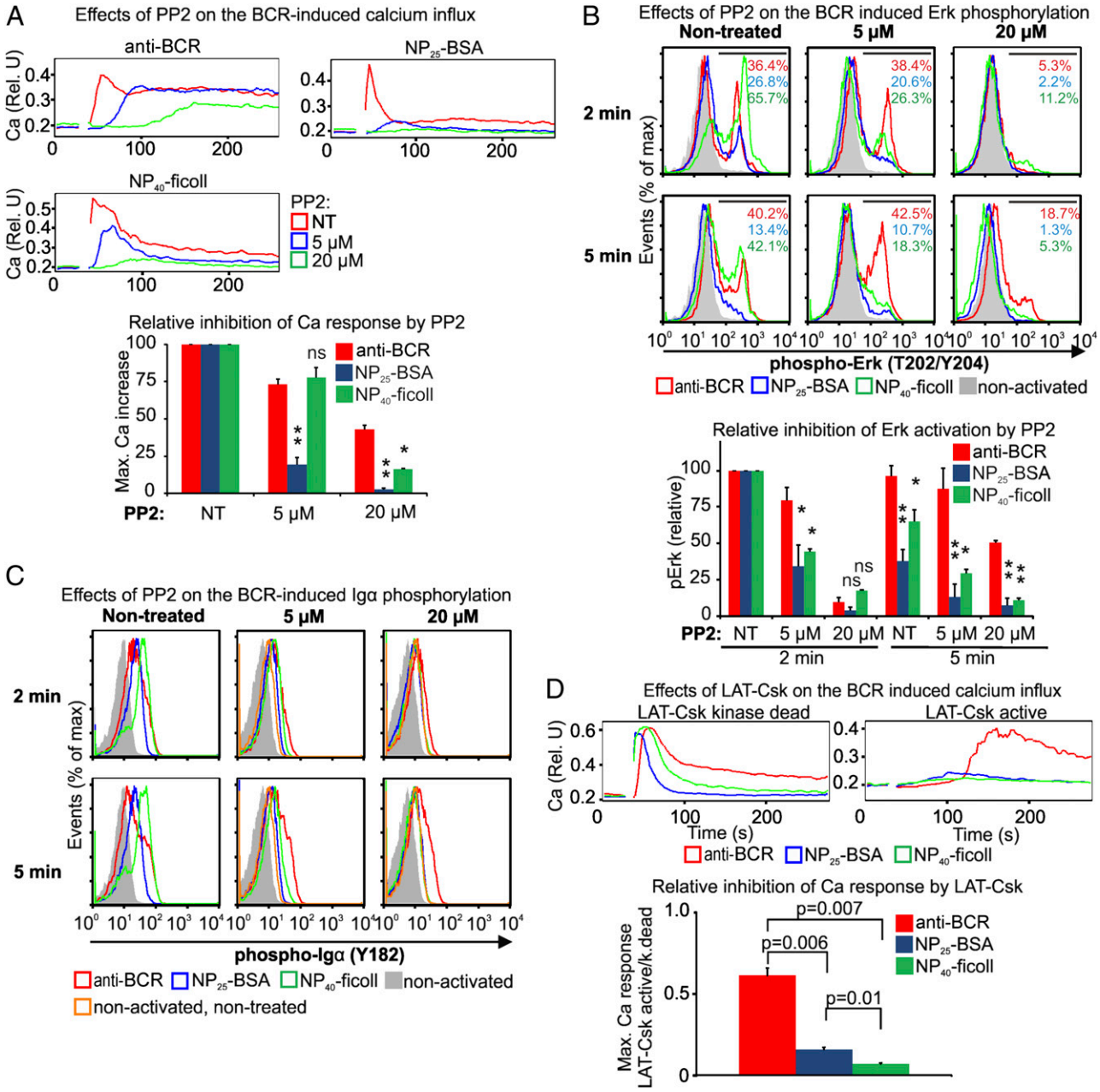

FIGURE 4. Requirement of SFKs for ligand-induced BCR signaling in primary B cells. (**A**) Splenocytes isolated from B1-8i transgenic mouse were activated by anti-BCR, NP_{25}-BSA, or NP_{40}-Ficoll in the presence of the indicated concentrations of PP2. The calcium influx in $Ig\kappa^-/B220^+$ cells was monitored. The maximum response was calculated as maximum median relative calcium concentration in the particular sample. Mean ± SEM, $n = 4$, $^*p < 0.05$, $^{**}p < 0.01$, n.s., not significant (between the indicated ligand treatment and the respective anti-BCR treatment). (**B**) Splenocytes isolated from B1-8i transgenic mouse were activated by anti-BCR, NP_{25}-BSA, or NP_{40}-Ficoll with the indicated concentrations of PP2. The phosphorylation of Erk was analyzed in $Ig\kappa^-/IgM^+$ cells. The relative pErk response was calculated as a percentage of $pErk^+$ cells for each sample. Mean ± SEM, $n = 4$, $^*p < 0.05$, $^{**}p < 0.01$ (between the indicated ligand treatment and the respective anti-BCR treatment). (**C**) Splenocytes from B1-8i transgenic mouse were activated as in (B) and stained with anti-pIgα. (**D**) $B220^+/Ig\lambda^+$ splenocytes were transduced with LAT-Csk or LAT-Csk kinase-dead. The cells were activated as indicated, and the calcium influx was monitored. The ratio of the maximal median relative calcium concentration between the LAT-Csk and LAT-Csk kinase-dead samples was calculated. Mean ± SEM, $n = 3$.

strongly inhibited by 5 μM PP2 after NP_{40}-Ficoll and NP_{25}-BSA stimulation but much less affected upon activation by anti-BCR, whereas 20 μM PP2 almost completely abolished the ligand-induced Erk phosphorylation and partially inhibited the phosphorylation of Erk mediated by anti-BCR (Fig. 4B). Although NP_{40}-Ficoll was the best inducer of Igα phosphorylation in non-treated cells, the BCR-induced phosphorylation of Igα was more efficiently inhibited after NP_{40}-Ficoll and NP_{25}-BSA stimulation than after anti-BCR stimulation (Fig. 4C).

We also transduced B cell blasts derived from $Ig\lambda^+$ splenocytes isolated from B1-8i transgenic mice with a retroviral vector encoding active or kinase-dead variants of LAT-Csk. The active LAT-Csk severely inhibited the NP_{40}-Ficoll– and NP_{25}-BSA–induced calcium influx, but only delayed and partially inhibited the anti-BCR–induced calcium response (Fig. 4D). The inhibitory effects of LAT-Csk on BCR-induced calcium signaling were concentration dependent, but unable reach the plateau at the expression levels of LAT-Csk we could achieve, indicating that these expression levels

were not sufficient to fully inhibit SFKs in LAT-Csk$^+$ cells (Supplemental Fig. 3C).

Syk does not phosphorylate Igα

Inhibition of SFKs by chemical and genetic inhibitors efficiently blocked BCR signaling induced by BCR ligands. In contrast, anti-BCR–induced signaling exhibited a partial resistance to the inhibition of SFKs. There are two possible explanations for these effects: 1) anti-BCR triggers an SFK-independent signaling pathway, or 2) the residual SFK activity is sufficient and required to initiate the downstream signaling pathways.

Because SFK-independent BCR signaling has been linked to the ability of Syk to compensate for SFK activity (37, 38), we analyzed the ability of Syk to phosphorylate ITAMs in B cells. The inhibition of Syk in K46:LAT-Csk or K46:LAT-Csk-KD cells decreased anti-BCR–induced phosphorylation of the catalytic loop tyrosines of Syk (Y519/520) and proteins downstream of Syk (SLP65, Erk, and Akt; Fig. 5A). In contrast, the phosphorylation of Igα and the interdomain B tyrosine of Syk (Y346) was not affected by the inhibition of Syk in both cell lines (Fig. 5A). Treatment of K46:LAT-Csk cells with PP2 further delayed the phosphorylation of Igα, Syk, and Erk (Fig. 5B), which shows that LAT-Csk was unable to inhibit the SFKs completely. The combination of genetic and chemical inhibitors did not block the anti-BCR–induced response entirely, probably because of the partial inhibition of Csk kinase activity in LAT-Csk chimera by PP2 (52) (Supplemental Fig. 4). We performed similar experiments in primary splenic B cells stimulated with anti-BCR after being treated with PP2, Syk inhibitor IV, PP2+Syk inhibitor IV, or left untreated. Inhibition of Syk diminished the anti-BCR–induced phosphorylation of Syk at tyrosines Y519/520 and the phosphorylation of proteins downstream of Syk, but did not affect the phosphorylation of Igα and Syk at tyrosine Y346, regardless of the inhibition of SFKs (Fig. 5C). Treatment with Syk inhibitor IV did not influence the timing of the anti-BCR–induced phosphorylation of Igα and Syk at Y346 (Fig. 5D).

Collectively, these results show that Syk activity does not play a role in the anti-BCR–induced phosphorylation of Igα (at least at the ITAM tyrosine Y182) in SFK-deficient and -sufficient B cells. Moreover, the data indicate that whereas the catalytic loop tyrosine of Syk is, at least partially, an autocatalytic site, the interdomain B tyrosine 346 is a substrate for SFKs. We conclude that

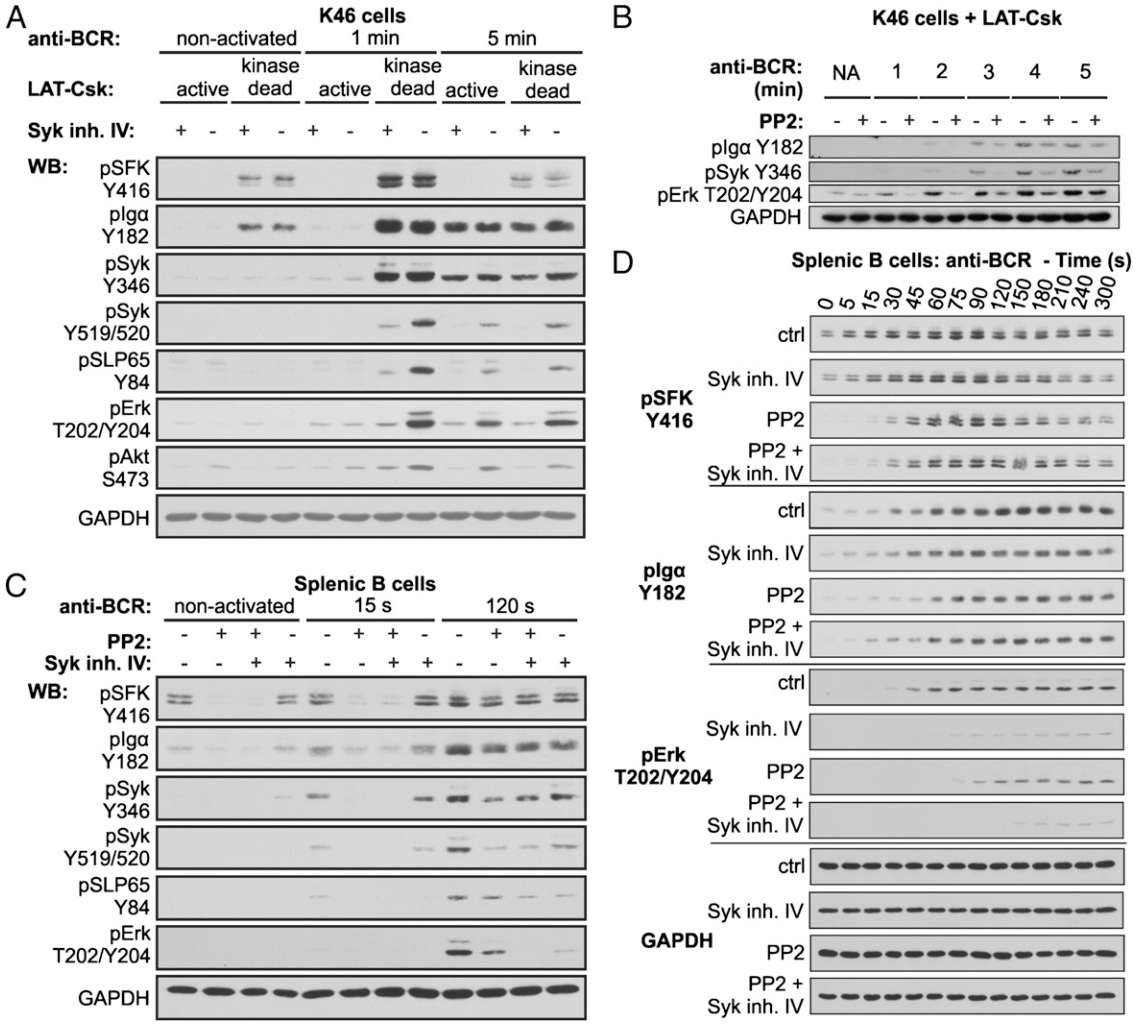

FIGURE 5. BCR-induced phosphorylation of Igα is independent of Syk activity. (**A**) K46:LAT-Csk and K46:LAT-Csk-KD cells were activated by anti-BCR in the presence or absence of 2.5 μM Syk inhibitor IV. Phosphorylation of the indicated proteins 1 and 5 min after the activation was analyzed by Western blotting. Staining of GAPDH served as a loading control. (**B**) K46:LAT-Csk cells were treated or not with 20 μM PP2 and activated by anti-BCR. Phosphorylation of Erk, Igα, and Syk at the indicated time points was analyzed by Western blotting. (**C** and **D**) Splenic B cells were treated or not with 1 μM Syk inhibitor IV and/or 5 μM PP2, and activated by anti-BCR. Phosphorylation of the indicated proteins was analyzed by Western blotting.

the initiation of anti-BCR–induced BCR signaling in our models with significant SFK inhibition still depends on the residual activity of SFKs.

SFK activity is important for sustained BCR signaling

Although the inhibition of Syk did not reveal any role in Igα phosphorylation, we tested the possibility that Syk activity is sufficient to sustain ongoing signaling upon the acute inhibition of SFKs. The inhibition of SFKs by 5 μM PP2 after BCR triggering resulted in rapid inhibition of the calcium signaling and dephosphorylation of the activation tyrosine of the SFKs, interdomain B tyrosine of Syk, and Igα (Fig. 6A, 6B). After the immediate decrease, the signaling partially recovered, especially with regard to calcium flux and Syk interdomain B tyrosine phosphorylation, probably as a result of the incomplete inhibition of SFKs as revealed by the phosphorylation of the activation loop tyrosine (Fig. 6A, 6B). The acute inhibition of anti-BCR–induced calcium signaling was also achieved by adding a dimerizer to K46:dimLAT-Csk cells (Fig. 6C). These experiments showed that SFKs are involved in not only the initiation but also the maintenance of BCR signaling.

BCR signaling in DT40 cells is dependent on SFK activity

Lyn was the only SFK detected in the chicken B cell lymphoma cell line DT40 at the level of mRNA (35). Lyn$^{-/-}$ DT40 cells were characterized by delayed, but not completely inhibited, anti-BCR–induced signaling, which suggests the existence of an SFK-independent BCR signaling pathway (35). Because our data indicate the requirement for SFKs in the initiation of BCR signaling, we tested whether BCR signaling in Lyn$^{-/-}$ DT40 cells is truly SFK independent. Treatment of DT40 WT cells with PP2 delayed calcium signaling, as expected (Fig. 7A). Importantly, PP2 also delayed calcium signaling in Lyn$^{-/-}$ DT40 cells in a concentration-dependent manner (Fig. 7A). Inhibition of the SFKs also delayed Akt and Erk phosphorylation in WT and Lyn$^{-/-}$ DT40 cells (Fig. 7B). Staining for the phosphorylated activation loop tyrosine in SFKs revealed that DT40 Lyn$^{-/-}$ cells express at least one SFK that is activated after BCR engagement (Fig. 7B).

To investigate whether DT40 Lyn$^{-/-}$ cells express another Src family member, we used a rabbit mAb that recognizes the activation loop phosphotyrosine of multiple SFKs (pSFK Y416). We used this Ab to immunoprecipitate SFKs from nontreated and pervanadate-treated WT and Lyn$^{-/-}$ DT40 cells. Cell lysates and immunoprecipitated material were stained with mAbs and polyclonal Abs to the SFK activation loop phosphotyrosine, an Ab to the phosphorylated C-terminal tyrosine of Src, an Ab to the nonphosphorylated C-terminal tyrosine of Src, and an Ab to Lyn (Fig. 7C). Although the phospho-specific Abs exhibited some level of off-target reactivity, especially after the pervanadate treatment, the additional bands not corresponding to the m.w. of the SFKs were substantially reduced after the immunoprecipitation. In samples from WT and Lyn$^{-/-}$ DT40 cells, two different Abs to pSFK Y416 stained a protein corresponding to an SFK as confirmed by its m.w. The signal was much stronger in DT40 WT cells, apparently because of the expression of Lyn. In addition, Abs to the C-terminal pY527 or nonphosphorylated Y527 (non-pY527) of Src stained a band with the same m.w. in lysates and pSFK Y416 immunoprecipitates from both cell lines. Whereas the pervanadate treatment enhanced the signal detected by the pY527-specific Ab, it reduced the signal from the non-pY527 Ab in the DT40 WT and Lyn$^{-/-}$ lysates.

To identify the non-Lyn SFK(s) in DT40 cells, we performed quantitative RT-PCR analysis using RNA isolated from DT40 WT and Lyn$^{-/-}$ cells and primers specific for LYN, LCK, FYN, BLK, SRC, HCK, YES, and YRK genes. Although the Lyn encoding transcript was the most abundant, we could detect mRNAs of other family members, among which Fyn mRNA was the most prominent (~20% of Lyn mRNA levels; Fig. 7D). Together, these results imply that DT40 Lyn$^{-/-}$ cells express SFK Fyn that is activated after BCR cross-linking and promotes BCR signaling in these cells.

Discussion

A widely accepted model of BCR signaling proposes that BCR engagement leads to the activation of SFKs, mainly Lyn, that

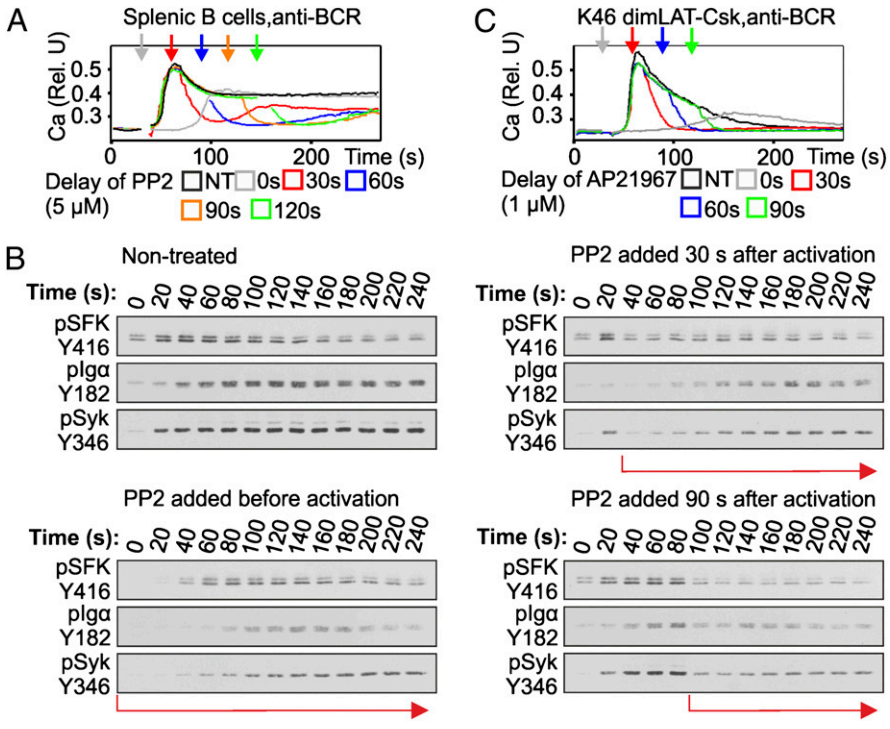

FIGURE 6. Anti-BCR–induced signaling is rapidly inhibited by acute SFK inhibition. (**A**) Splenocytes were stained for CD3 and the anti-BCR–induced calcium influx was monitored. The cells were nontreated, pretreated with 5 μM PP2, or treated with 5 μM PP2 at the indicated time points after the stimulation. B cells were gated as CD3$^-$ splenocytes. (**B**) Splenic B cells were activated by anti-BCR. A total of 5 μM PP2 was added before the stimulation or 30 or 90 s after the activation, or the cells were left nontreated. Phosphorylation of SFKs, Igα, and Syk during the onset of the response was analyzed by Western blotting. Red arrows indicate the time of inhibition of SFKs by PP2. (**C**) K46 cells expressing both components of dimLAT-Csk were activated by anti-BCR, and the calcium influx was measured. The dimerizer AP21967 (1 μM) was added before the activation or at the indicated time points after activation. Only mOrange$^+$ cells were gated.

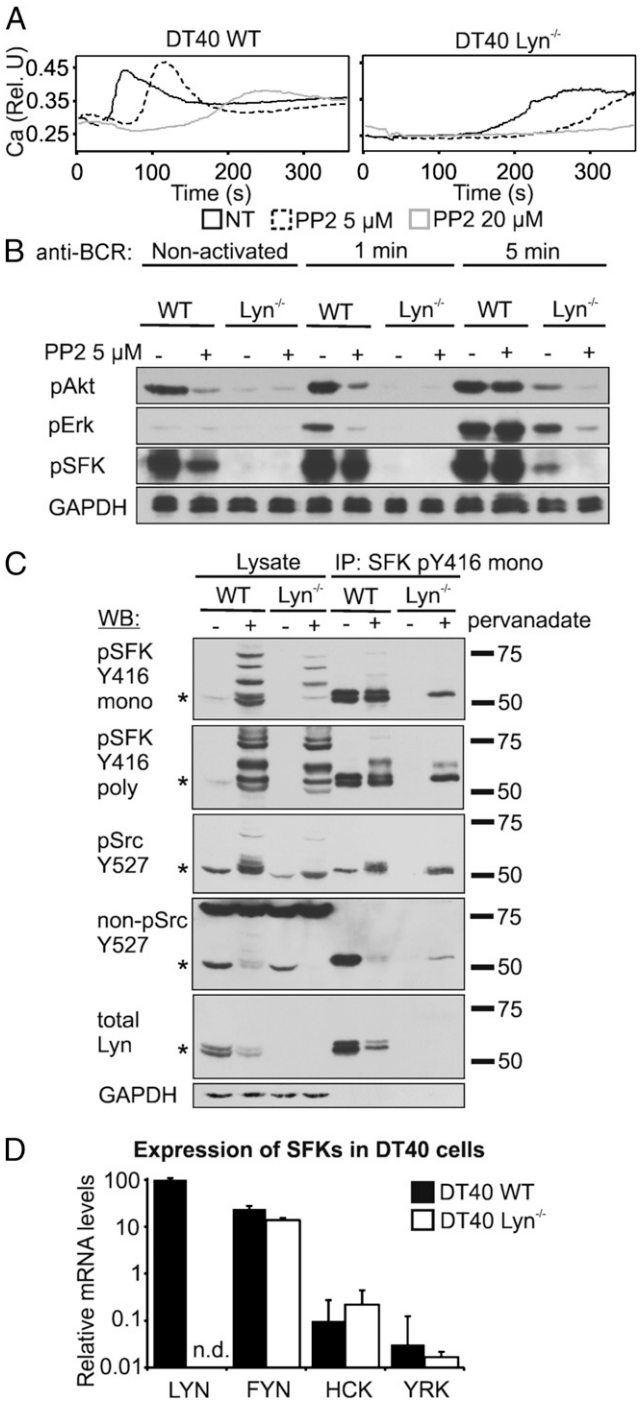

FIGURE 7. BCR signaling of Lyn-deficient DT40 cells is dependent on residual SFK activity. (**A**) WT and Lyn$^{-/-}$ DT40 cells were stimulated with the anti-BCR in the presence of 0, 5, or 20 μM PP2. The calcium influx was monitored. (**B**) WT and Lyn$^{-/-}$ cells were stimulated with the anti-BCR in the presence of 0 or 5 μM PP2. The phosphorylation of Akt (S473), Erk (T193/Y195), and the activation loop tyrosine of the SFKs (Y416) were analyzed by Western blotting. (**C**) DT40 WT and Lyn$^{-/-}$ cells were treated with pervanadate or not and subjected to immunoprecipitation with the anti-pSFK (Y416) mAb. Lysates and immunoprecipitated material were stained with Abs to anti-SFK pY416 (monoclonal and polyclonal), Src pY527, and Src non-pY527. Asterisks indicate bands with m.w. corresponding to SFKs. (**D**) Levels of mRNAs encoding Lyn, Fyn, Hck, Yrk, Yes, Src, Lck, and Blk in DT40 WT and Lyn$^{-/-}$ cells were quantified using quantitative RT-PCR. ACTB served as a reference gene. mRNAs of Blk, Src, Yes, and Lck were below the detection limit. Mean ± SEM, $n = 3$. n.d., Not detected.

phosphorylate BCR-associated Igα and Igβ to recruit and activate Syk kinase (24). Subsequently, Syk relays the signal by phosphorylating several downstream signaling proteins (16). However, the general applicability of this model is still under dispute. Whereas the activity of SFKs enhanced BCR signaling in some studies (4, 34, 35, 53, 54), other studies failed to identify a role for SFKs in the proximal steps of BCR-like signaling (33) or even showed a negative regulation of BCR signaling via the Src family member Lyn (30, 31). The main concerns about the model are based on the observation of augmented BCR signaling in Lyn-deficient primary B cells and potential SFK-independent BCR signaling attributed to the ability of Syk to phosphorylate Igα/Igβ (11, 14–16, 37–40, 55). However, the interplay between the two BCR signaling pathways (canonical and SFK independent), as well the overall role of SFKs (possessing both activation and inhibitory activity) (32) in BCR signaling remain to be elucidated. In this study, we examined the requirement for SFKs in BCR signaling and the role of SFKs and Syk in the initiation of BCR signal transduction.

Physiologically relevant evidence supporting or contradicting the model of sequential activation of kinases in BCR signaling has been largely missing, partly because of technical problems resulting from the indispensability of SFKs in B cell development (33) and from the redundancy of multiple SFK members expressed in B cells (32, 33). BCR signal transduction plays a critical role in B cell development. Mice with genetic defects in BCR signaling components including SFKs and Syk develop abnormal peripheral B cell compartments or lack peripheral B cells entirely (56, 57). In addition, some of these proteins are involved in other signaling pathways downstream of various cellular receptors (16, 58), which could interfere with the in vivo analysis of particular proteins in the context of BCR-mediated signaling. To prevent these two main problems, we performed all experiments ex vivo, where short-term inhibition of SFKs and/or Syk, as well as specific BCR stimulation of B cells, could be performed. To address the redundancy among SFKs in B cells, we used chemical or genetic inhibitors of the whole Src family.

Using a transgenic NP-specific BCR, we showed that specific ligand-induced BCR signaling requires SFK activity in K46 B cell lymphoma cells and in primary B cells. Surprisingly, the inhibition of SFKs substantially delayed the initiation of signaling but only partially reduced the intensity of the anti-BCR–induced response. A similar delay in BCR signaling was caused by the absence of Lyn in the DT40 cell line and, to a lesser extent, in primary B cells (30, 35). We analyzed in detail whether residual SFK activity or SFK-independent Syk activation was responsible for the initiation of BCR signaling under SFK deficiency. Combined treatment of the cells with LAT-Csk and PP2, as well as simultaneous inhibition of Syk and SFKs, showed that the proximal events of anti-BCR–induced signaling are not Syk dependent but are likely to be driven by the residual SFK activity. Indeed, SFKs became activated by BCR cross-linking, even in PP2-treated cells, with a delay similar to that observed with other signaling events. Thus, the small amount of noninhibited SFK molecules seemed to be sufficient to counterbalance the barrier of intracellular phosphatase activity and trigger sufficient BCR phosphorylation to promote downstream signaling. The most plausible explanation is the existence of a positive feedback loop at the level of SFKs, possibly involving the cooperation between SFKs and Igα/Igβ (59) and/or SFKs and CD19 (60, 61). The existence of such a B cell–specific SFK activation feedback mechanism (absent in TCR signaling) (62) could explain the higher sensitivity of TCR signaling to the inhibition of SFKs.

Our results failed to show SFK-independent Syk activation and phosphorylation of the ITAM in Igα by Syk. Syk activity did not

contribute to the phosphorylation of Igα under SFK-sufficient and -deficient conditions, and already activated Syk was unable to sustain ongoing signaling after an acute inhibition of SFKs. These observations challenge the proposed existence of the Syk-mediated, SFK-independent pathway and Syk/ITAM autoactivation loop in BCR signaling (14–16, 38–40, 63). Most previous experiments indicating the ability of Syk to phosphorylate ITAMs and autoactivate itself were performed using cell-free or non-B cell models. However, this activity of Syk may be relatively weak and insufficient to counterbalance the inhibitory activity of tyrosine phosphatases in B cells. Another point of consideration is that we measured the phosphorylation of only the first ITAM tyrosine in Igα (Y182). This tyrosine is critical for the initiation of BCR signaling, and multiple lines of evidence indicate that SFKs have high specificity for this tyrosine (15, 64–66). These observations combined with our data thus support the conclusion that the phosphorylation of the proximal ITAM tyrosine by SFK is a critical step in the initiation of BCR signal transduction that is completely independent of Syk. The identity of the kinase responsible for the phosphorylation of the distal ITAM tyrosine is less clear; thus, there is still a possibility that Syk is more substantially involved.

Our experiments also clarified a somewhat controversial issue concerning the identity of the kinases phosphorylating Syk (17, 18, 37, 63). Although the activation loop tyrosines of Syk (Y519/520) behaved as mainly autocatalytic sites, the interdomain B tyrosine (Y346) was predominantly phosphorylated by SFKs. Thus, both mechanisms of Syk activation, that is, binding to phosphorylated ITAM and phosphorylation of the interdomain B tyrosines (20), seem to be strictly dependent on the activity of SFKs in B cells. The observed independence of Syk phosphorylation at Y346 from the catalytic activity of Syk also suggests that Syk recruitment to the proximity of the SFKs at the BCR is not regulated by Syk catalytic activity. The finding that Syk phosphorylated ITAMs more efficiently than ZAP70 in some assays (14, 39) can be explained by the higher intrinsic catalytic activity of Syk (67) and/or higher affinity of its SH2 domains for phosphorylated ITAMs (68), not necessarily by the specificity of Syk for the ITAMs.

To our knowledge, the only direct evidence documenting SFK-independent BCR signaling in a B cell lineage was obtained using the DT40 chicken B cell lymphoma cell line. DT40 cells were believed to express only a single SFK, Lyn (32, 35, 36, 53). Thus, the delayed, but not completely inhibited, signaling in $Lyn^{-/-}$ DT40 cells could have been interpreted as SFK-independent BCR signaling (35, 38). We showed that $Lyn^{-/-}$ DT40 cells express another Src family member, Fyn, at the level only 5- to 10-fold lower than Lyn in DT40 WT cells. Low amounts of transcripts of two additional Src family members, Hck and Yrk, were also detected in DT40 cells. The non-Lyn SFKs are activated after BCR engagement and positively regulate Ab-induced BCR signaling in DT40 $Lyn^{-/-}$ cells.

Anti-BCR–induced signaling is a widely used model for BCR triggering. To our knowledge, no substantial differences between anti-BCR– and ligand-induced BCR signaling have been reported thus far. However, our data suggest that the anti-BCR cross-links BCRs in a specific way to cause enhanced SFK activation that is pronounced upon the inhibition of SFKs but apparent even in SFK-sufficient cells. However, we cannot exclude the possibility that anti-BCR mimics a specific class of ligands. For instance, B1-8 BCR has a relatively moderate affinity for NP (K_d = 2 μM) (47). Although NIP, which has 20-fold higher affinity to B1-8 than NP (47), behaved similarly in our assays, we cannot exclude the possibility that a BCR-ligand pair with even higher affinity could induce a qualitatively different response. One of the analyzed downstream signaling events was Erk phosphorylation. Triggering of the BCR resulted in bimodal Erk response where inhibition of SFKs by PP2 reduced the percentage of BCR-induced $pErk^+$ (high) cells rather than the pErk signal intensity. This type of response, where the percentage of $pErk^+$ cells corresponded to the strength of the signal, has been observed previously and has been variably explained either by switchlike behavior of the proteins involved in Erk activation or as a result of cell-to-cell heterogeneity in the expression of Erk pathway components combined with negative feedback incorporated into the pathway (see Refs. 69, 70 for more detailed discussion). Interestingly, BCR response induced by a flexible polysaccharide Ag carrier (Ficoll) was not only stronger and more sustained than NP-BSA-induced signaling, but it was also partially resistant to a low concentration of SFK inhibitor. These data might suggest that flexibility in the aggregation of BCRs by an agonist could be an important determinant of the quality of the induced signaling. In the future, it would be interesting to address this issue using a set of additional BCR-ligand pairs. Of note, different requirements for SFK activity were reported for different methods of FcεRI triggering in mast cells (71, 72). The high-intensity stimulation of FcεRI required much less SFK activity than low-intensity stimulation, but both mechanisms of mast cell activation were SFK dependent, as shown by the PP2 treatment of Lyn-deficient mast cells (71).

We believe that our results contribute to the understanding of the molecular mechanisms underlying BCR signaling, as well as the B cell–specific effects of pharmacological Src and Syk inhibitors used, tested, or considered for the treatment of various malignant and immune diseases (73–75).

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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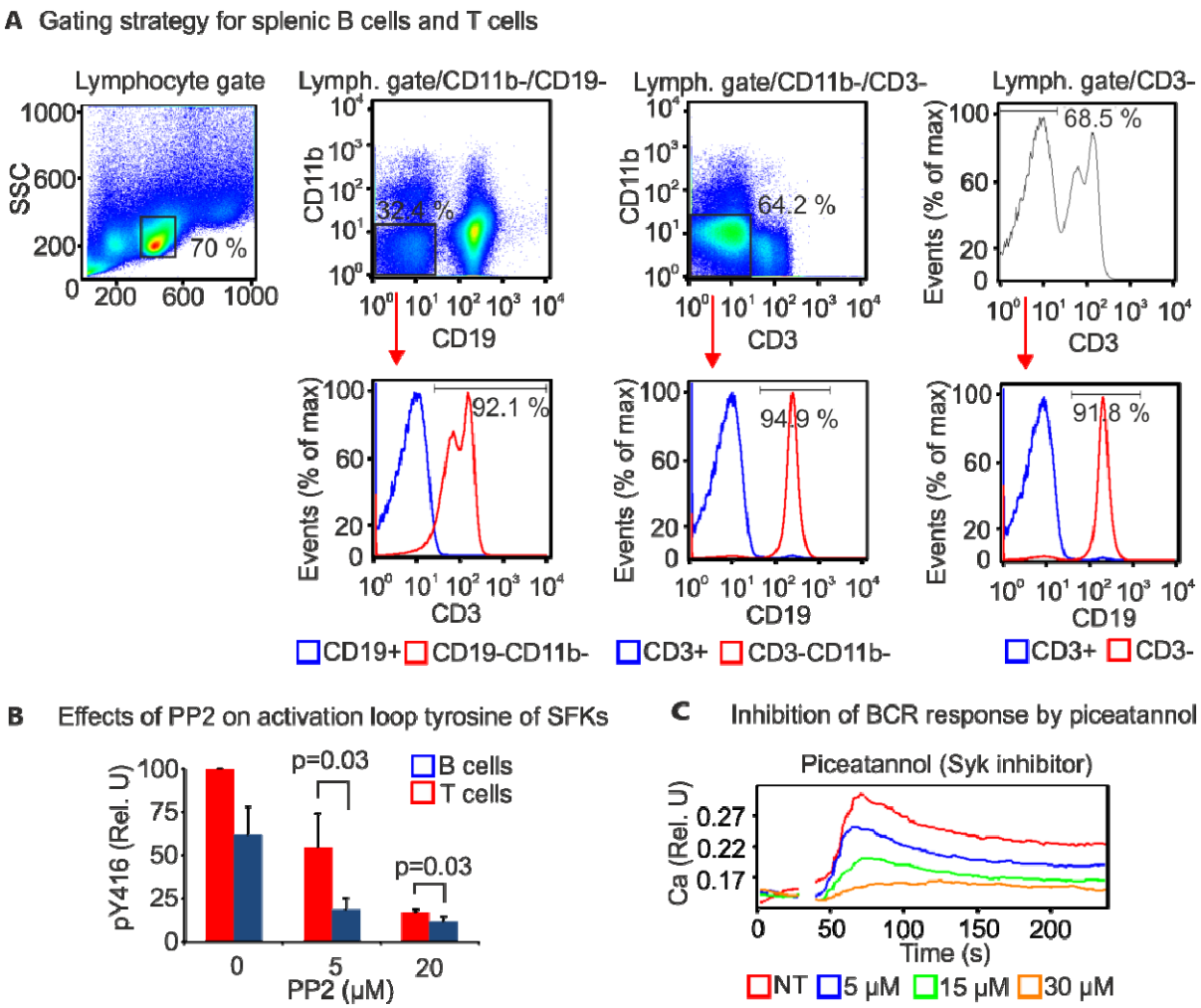
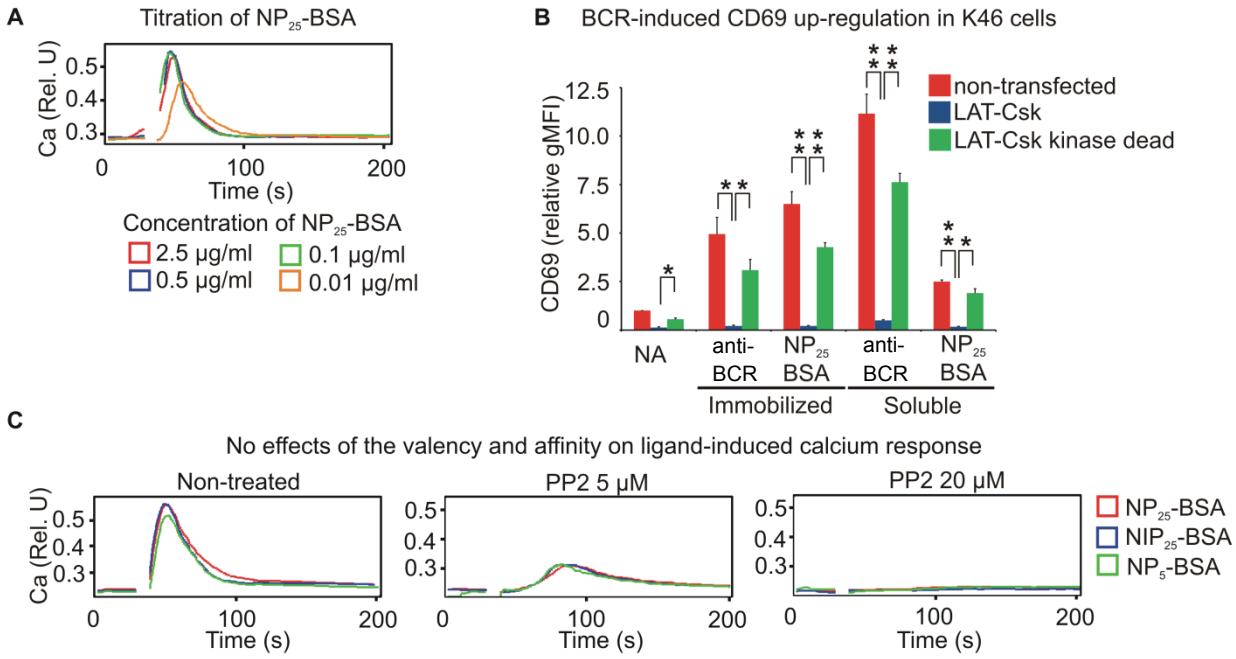
SUPPLEMENTAL FIGURES



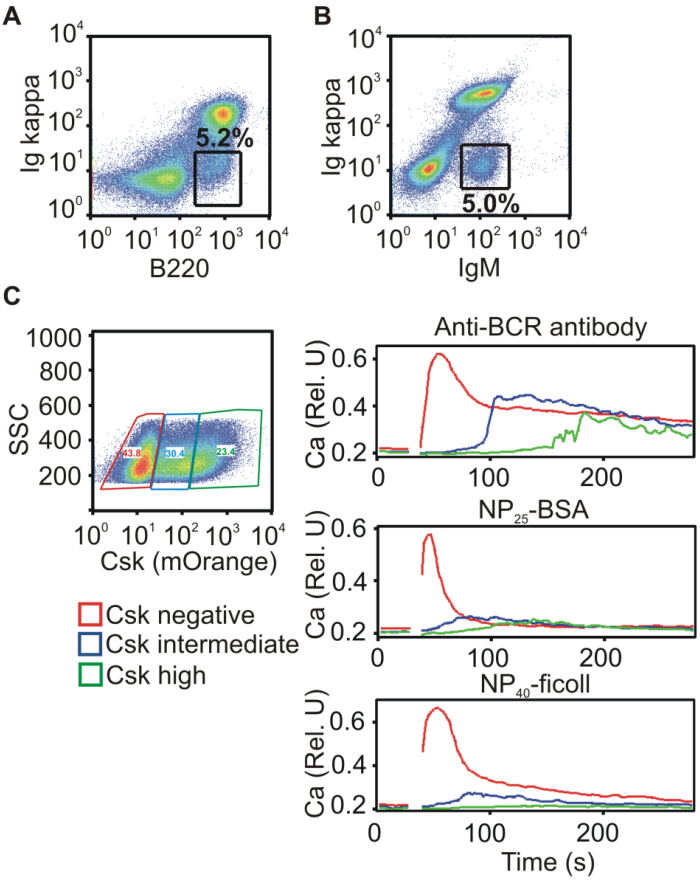
Supplemental Figure 1. Supplemental information for antibody-induced responses in splenic B and T cells. (A) Gating strategy to select splenic T cells and B cells by negative staining. The figure shows percentage of T cells ($CD3^+$) in the lymphocyte/$CD19^-CD11b^-$ gate (for Fig. 1A) and B cells ($CD19^+$) in the lymphocyte/$CD3^-CD11b^-$ gate/ (for Fig. 1A-C, and Supplemental Fig. 1C) or in the lymphocyte /$CD3^-$ gate (for Fig. 6A). (B) Splenic T cells ($CD11b^-/CD19^-$) and B cells ($CD11b^-/CD43^-$) were negatively selected by magnetic beads. The cells were incubated with indicated concentrations of PP2 for 15 min and the

phosphorylation of the activation loop tyrosine of SFKs was determined by Western blotting using pan-SFK pY416 antibody and goat anti-rabbit-IRDye 800CW (LI-COR Biosciences, Lincoln, USA). The signal was quantified using an Odyssey Infrared Imaging System (LI-COR) and normalized to GAPDH. Finally, normalized signal of pY416 antibody in PP2-non-treated T cells was set as 100 in each experiment. Mean ± SD, n=4. (C) Mouse splenocytes were stained for CD3 and CD11b, activated by anti-BCR in the indicated concentration of piceatannol, and the calcium influx was monitored. B cells were gated as $CD3^-/CD11b^-$.

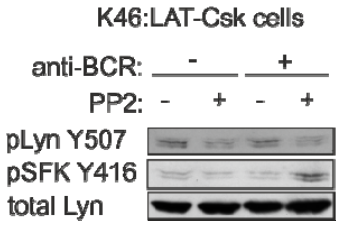


Supplemental Figure 2. Supplemental information for agonist comparison in K46 cells.

(A) Calcium response induced by indicated concentrations of NP_{25}-BSA in K46 cells was monitored. Median calcium response is shown. (B) K46 WT, K46:LAT-Csk, and K46:LAT-Csk-KD cells were stimulated with soluble or plastic-bound agonist, or left non-activated. After 13-15 h, expression of CD69 was analyzed by flow cytometry. Relative geometric mean of fluorescence is shown. The value of non-activated K46 WT cells was arbitrarily set as 1. Mean ± SEM, n=3, * $p<0.05$, ** $p<0.01$. (C) Calcium response induced by NP_{25}-BSA, NIP_{25}-BSA, and NP_5-BSA (1 µg/ml) in K46 cells treated with 0, 5, or 20 µM PP2 was monitored. Median calcium response is shown.



Supplemental Figure 3. Supplemental information for agonist comparison in primary B1-8 B cells. (A) Gating of $B220^{+}/Ig\kappa^{-}$ splenocytes analyzed in Figure 4A. (B) Gating of $IgM^{+}/Ig\kappa^{-}$ splenocytes analyzed in Figure 4B. (C) The experiment shown in Figure 4D was re-analyzed using gating of B cells expressing active LAT-Csk into three subpopulations (Csk-mOrange negative, intermediate, and high). Median calcium influx is shown for each population. Because LAT-Csk inhibited calcium response in a dose dependent manner, we hypothesize that the level of LAT-Csk expressed in primary B cells was unable to inhibit SFKs completely. This is consistent with our hypothesis that residual SFK activity is responsible for the delayed anti-BCR-induced response.



Supplemental Figure 4. PP2 interferes with the LAT-Csk-mediated inhibition of SFKs.

K46:LAT-Csk cells were treated or not with 20μM PP2 and stimulated with anti-BCR or left non-stimulated. Phoshorylation of SFK (Y416) and Lyn (Y507) was analyzed by Western blotting. PP2 treatment reduced the Csk- and LAT-Csk-mediated phosphorylation of the inhibitory tyrosine (Y507) of Lyn. Moreover, in LAT-Csk expressing cells the PP2 treatment allowed for the anti-BCR-induced auto-phosphorylation of the activating SFK tyrosine (Y416) (lane 4) that could not be observed in PP2 non-treated K46:LAT-Csk cells (lane 3). Thus, while PP2 treatment in the absence of LAT-Csk resulted in the hypophosphorylation of the SFK activation tyrosine (Fig. 1E, Fig. 2C), it had the opposite effect in LAT-Csk expressing cells. These data indicate that PP2 interferes with the LAT-Csk mediated inhibition of SFKs, probably via direct inhibition of Csk activity.