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Research Article

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Cumulative expression of the two Lyn isoforms creates a threshold for macrophage signaling in the absence of receptor engagement

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Abstract

It has been unclear whether phenotypic differences between LynA- and LynB-knockout mice, each expressing only one splice form of the Src-family kinase (SFK) Lyn, are due to isoform-specific functions or dose effects from differences in overall Lyn expression. To assess the functions of LynA and LynB, we generated a genetic series in which LynA and LynB expression levels vary independently, allowing us to test whether upregulation of one Lyn isoform could compensate for loss of the other. We used macrophages from this dose series to test signaling at steady state and in response to pharmacological pan-SFK activation. We found that LynA and LynB are equally capable of phosphorylating the positive-regulatory tyrosine kinase Syk and the negative-regulatory inositol phosphatase SHIP1, to a level determined by the cumulative expression level of LynA + LynB protein at steady state. Despite the dependence of steady-state and induced phosphorylation of upstream signaling intermediates on the level of Lyn expression, Erk and Akt signaling in response to pan-SFK activation is enabled by any Lyn expression, insensitive to further upregulation of either isoform. Thus, expression of either LynA or LynB at steady state leads to balanced potential for positive-regulatory and negative-regulatory signaling, setting a maximal response in the absence of a microbial encounter.

Keywords: Lyn, macrophage, SHIP1, steady-state regulation, Syk kinase.

1. Introduction

Src-family kinases (SFKs) initiate or suppress signaling responses in many receptor pathways, including those associated with immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs).[1] Phosphorylation of dual ITAM tyrosines by activated SFKs leads to recruitment and partial activation of the tyrosine kinase Syk,[1–3] where SFK-mediated phosphorylation of the Syk SH2-kinase (interdomain-B) linker promotes its full activation via activation-loop autophosphorylation.[4] Together, the SFKs and Syk phosphorylate actin-remodeling proteins, adaptors, and second messenger-generating enzymes, which activate Erk, Akt, and other pathways, inducing an antimicrobial response.[2,3,5] Conversely, phosphorylation of ITIMs by SFKs leads to the recruitment and activating phosphorylation of phosphatases. The ITIM-binding inositol phosphatase SHIP1 is particularly important for limiting immune-cell responses, dephosphorylating the membrane-anchored second-messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and blocking further propagation of signaling.[1,6]

Among the SFKs, Lyn is generally the primary mediator of suppressive signaling.[7,8] Using knockout (KO) bone-marrow-derived macrophages (BMDMs), we have found a unique requirement for Lyn in maintaining a steady-state set point of SHIP1 phosphorylation, which limits low-level phosphorylation of Erk and Akt in resting cells.[9] Steady-state phosphorylation of SHIP1 is the key barrier between activation of SFKs/Syk and a proinflammatory response. Activation of

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SFKs in the absence of a pathogen or interacting particle induces further phosphorylation of SHIP1, with activated Lyn and the SFKs Hck and Fgr capable of performing this function. However, during engagement of the hemi-ITAM-containing receptor Dectin-1 with particulate ligands, SHIP1 is protected from further phosphorylation,[9] suggesting that ITIMs are sequestered away from activated SFKs in the phagocytic synapse. Thus, the degree of steady-state SHIP1 phosphorylation, regulated by Lyn expression, serves as a rheostat, with basal regulation, not induction of SHIP1 function, determining the ability of the cell to accumulate PIP_3 and propagate proinflammatory signaling in the absence of a microbial encounter.[9]

Lyn is expressed as two splice variants, LynA and LynB, which are produced by alternative splicing of *Lyn* RNA transcript.[8,10] The unique-region insert in LynA is subject to autophosphorylation-induced ubiquitination and degradation, conferring cell-type-specific regulation independently of LynB.[11,12] To test the contributions of LynA and LynB to immune-cell function, we used CRISPR/Cas9 gene editing to delete LynA by disrupting the unique insert ($LynA^{KO}$) or introduce a point mutation into the intra-exon LynB splice site ($LynB^{KO}$).[13] Single-isoform deletion of LynA and LynB had distinguishable effects, with loss of LynB the dominant driver of inflammation and autoimmunity.[13] Interpretation of this finding, however, was complicated by the observation that LynB is the more highly expressed isoform in most cell types.[13] Therefore, it was unclear whether to attribute phenotypic differences to isoform-specific functions or to dose effects from losing more overall Lyn function with deletion of LynB.

For this study, we took advantage of our observation that deleting one Lyn isoform leads to upregulation of the other[13] to create a genetic series with varying LynA and LynB expression. This allowed us to test the independent functions of LynA and LynB and whether upregulation of one isoform could compensate for loss of the other, uncoupling isoform specificity from dose effects. To examine the intrinsic potential of LynA and LynB to phosphorylate substrates without the spatial complexities of specific receptor engagement,[9,14] we derived macrophages from transgenic mice in which the SFK-inhibitory kinase Csk is replaced with an analog-sensitive variant (Csk^{AS}) that is selectively inhibited by a bulky derivative of the small molecule PP1. Csk^{AS} has wild-type (WT)-like SFK-inhibitory function until inhibited with 3-IB-PP1, at which point the loss of Csk inhibitory function leads to robust, receptor-independent activation of SFKs.[11,12,15–17]

Using this unique toolkit, we demonstrate that both LynA and LynB phosphorylate Syk and SHIP1 at tonically low levels in resting macrophages and inducibly during pan-SFK activation. Furthermore, cumulative steady-state expression levels of LynA and LynB determine the extent of Syk phosphorylation and early signing. To our surprise, this signaling set point is insensitive to changes in Lyn protein abundance over time after signal initiation. Lyn-dose-dependence, however, is lost further downstream, suggesting that regulation of the macrophage steady state by Lyn limits the potential magnitude of the proinflammatory response in the absence of a pathogen encounter.

2. Methods

2.1. Mice

Mice were derived on a C57BL/6 background. LynA knockouts or LynB knockouts with supraphysiological expression of the remaining isoform ("UP" genotypes) were generated by homozygous breeding of CRISPR-generated LynA-knockout mice ($LynB^{+/+}LynA^{KO}$) or LynB-knockout mice ($LynA^{+/+}LynB^{KO}$).[13] LynA or LynB knockouts with WT-like expression of the remaining isoform ("PHYS" genotypes) were generated by crossing homozygous LynA-knockout or LynB-knockout mice to neomycin (neo)-disrupted complete Lyn^{KO} mice,[18] yielding hemizygous animals ($LynB^{+/-}LynA^{KO}$ or $LynA^{+/-}LynB^{KO}$).[13] Lyn-knockout Csk^{AS} mice were generated by crossing each knockout to $Csk^{KO}Csk^{AS}$, in which WT-like Csk function is provided by transgenic Csk^{AS} until addition of the Csk^{AS}-specific inhibitor 3-IB-PP1.[13,15–17] Because Csk^{KO} is embryonic lethal,[19] $Csk^{KO}Csk^{AS}$ and $Csk^{+/-}$ parents carrying the various Lyn knockouts were bred together to generate experimental animals. Animals were genotyped from genomic DNA by polymerase chain reaction (PCR, Transnetyx, Inc., Memphis, TN) and secondarily by immunoblotting, where possible. Animal studies were performed in compliance with the University of Minnesota/American Association for Accreditation of Laboratory Animal Care and National Institutes of Health policy, under Animal Welfare Assurance number A3456-01 and Institutional Animal Care and Use Committee protocol 2506-43046A. All animals were housed in specific-pathogen-free conditions and attended by licensed veterinarians.

2.2. Preparation of BMDMs

BMDMs were prepared as previously described,[12] starting with bone marrow flushed from femora and tibiae of 10-15-wk-old male and female mice. After hypotonic lysis of erythrocytes, cells were cultured in untreated plates (Corning, Manassas, VA, USA) in Dulbecco's Modified Eagle Medium (Corning) supplemented with 10% heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, CA, USA), 0.11 mg/ml sodium pyruvate (Corning), 2 mM penicillin/streptomycin/L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 10% CMG-14-12-cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF). After 7 days of culture, BMDMs were detached from plates with 5 mM ethylenediaminetetraacetic acid and replated at 1×10^6 cells/well in untreated 6-well plates (Corning) in M-CSF-free medium supplemented with 25 U/ml IFN-γ (Peprotech, Rocky Hill, NJ, USA) for inflammatory priming overnight.

2.3. Cell stimulation

Cells expressing the Csk^{AS} transgene had growth rates and steady-state signaling comparable to WT in the absence of the Csk^{AS} inhibitor 3-IB-PP1. To induce pan-SFK activation, BMDMs were treated with 10 μM 3-IB-PP1[9,11,12] and incubated at 37°C before quenching on ice and lysing in 4% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 128 mM Tris base, pH 6.8. After scraping cells off the plate and incubating 5 min at 37°C, lysates were collected and sonicated on a Bioruptor (Diagenode Inc, Denville, NJ, USA) at 50% duty for 3 min to shear DNA. Lysates were then boiled for 15 min and stored at -20°C.

2.4. Immunoblotting

Samples were separated on 7% NuPage Tris-Acetate gels (Invitrogen, Carlsbad, CA, USA) before transfer to Immobilon-FL PVDF membrane (EMD Millipore, Burlington, MA, USA). Revert Total Protein stain (LI-COR Biosciences, Lincoln, NE, USA) was used to quantify the total protein content in each lane. After destaining, membranes were cut as appropriate and blocked 1 h with TBS Intercept Blocking Buffer

(LI-COR). Membranes were then incubated overnight at 4°C with primary antibodies, treated 1.5 h with secondary antibodies, and visualized on an Odyssey CLx near-infrared imager (LI-COR). Densitometry quantification was performed in ImageStudio (LI-COR), as described previously.[20] Antibodies were obtained from Cell Signaling Technologies (Danvers, MA, USA): rabbit anti-pSyk^{Y352} 65E4 #2717, rabbit anti-pSHIP1^{Y1020} #3941, rabbit anti-pErk1/2$^{T202/Y204}$ D13.14.4e #4370, rabbit anti-pAkt^{S473} 193H12 #4058, mouse anti-β-Actin 8H10D10 #3700; Abcam (Cambridge, UK): mouse anti-LynA + B Lyn01 #1890; Santa Cruz (Santa Cruz, CA, USA): mouse anti-Syk (4D10) #sc-1240; and LI-COR: donkey anti-mouse IgG 800CW #925-32212, donkey anti-rabbit IgG 800CW #925-32213, donkey anti-mouse IgG 680LT #925-68022, and donkey anti-goat IgG 680LT #926-32214. After densitometric analysis, blot images were rotated as necessary. Brightness, and contrast corrections were applied linearly and uniformly across each image.

2.5. Quantification of RNA and treatment with actinomycin D

BMDMs were treated with 10 μg/ml actinomycin D (ActD) (Sigma-Aldrich) for up to 4 h before lysing in TRIzol (Thermo Fisher, Waltham, MA, USA). RNA was extracted in chloroform (Sigma-Aldrich) and purified using RNeasy kits (Qiagen, Hilden, Germany). Synthesis of cDNA for quantitative reverse-transcription (qRT)-PCR was performed using qScript kits (Quantabio, Beverly, MA, USA). SYBR green was used to detect *LynA* (5′-to-3′ forward (f): ATCCA ACGTC CAATAA ACAGCA, reverse (r): ATAAG GCCAC CACAA TGTCAC), *LynB* (f: TCGAA GACTC AACCA GTTCC TGAA, r: TGAAG GACAA GTCAT CTGGG TG), *Myc* (f: TCCTG TACCT CGTCC GATTC, r: AATTC AGGGA TCTGG TCACG) or *Ppia* (Cyclophilin) (f: TGCAG GCAAA GACAC CAATG, r: GTGCT CTCCA CCTTC CGT) in 1 μg total RNA via qRT-PCR. Relative $2^{-\Delta\Delta Ct}$ values, with *Ppia* as a reference, were calculated.[21]

2.6. Graphing and statistics

Data comprise at least 3 biological replicates, performed on different experiment days, with cells derived from different individual mice. Cells from male and female mice yielded comparable results and were analyzed together. Graphing and statistical analyses were performed in Prism software, version 10.6.1 (GraphPad, Boston, MA, USA).

3. Results

3.1. Generating macrophages with varying expression of LynA and LynB mRNA and protein

To test the relative contributions of overall Lyn expression and isoform specificity during SFK activation, we took advantage of the observation that deleting LynA or LynB induces upregulation of the remaining isoform.[13] This allowed us to generate a genetic series in which expression levels of LynA and LynB could be varied independently. Homozygous single-isoform LynA or LynB knockouts were crossed with complete Lyn^{KO} for hemizygous, WT-like expression of the remaining isoform ("PHYS" genotypes) or paired with a matching homozygous parent for supraphysiological expression of the remaining isoform ("UP" genotypes) (Fig. 1a). We generated this dose series on a Csk^{AS} background (Fig. 1b), which supplies WT-like Csk function, maintaining a normal cellular resting state in the absence of the Csk^{AS} inhibitor 3-IB-PP1.[9,11,12,15–17]

After correcting for total protein staining in each lane (Figure S1a and b), relative expression levels of LynA protein and LynB protein were quantified. As expected, homozygous single-isoform knockouts (UP genotypes) upregulated the remaining isoform of Lyn (Fig. 1c), and abundances of the remaining isoforms in the PHYS genotypes were comparable to WT (Fig. 1d). Although the remaining Lyn isoform was upregulated in both UP genotypes, LynB was upregulated almost 2-fold more than LynA, leading to higher total Lyn (LynA + LynB) expression in LynB^{UP}A^{KO} BMDMs than in LynA^{UP}B^{KO} BMDMs (Fig. 1e).

Upregulation of LynA in the LynB-knockout genotypes was expected because mutating the *LynB* RNA splice site should direct all RNA splicing toward production of *LynA*.[13] However, upregulation of LynB in the LynA-knockout genotypes was not originally anticipated—*LynA* splicing should occur, but premature termination and nonsense-mediated decay would eliminate that fraction of *Lyn* mRNA. To determine the origin of Lyn upregulation, we assessed mRNA levels via isoform-specific qRT-PCR. Although both isoforms were expressed at supraphysiological levels in the UP genotypes, *LynB* mRNA was upregulated almost 2-fold more than *LynA* (Fig. 1f). As the half-lives of *LynA* and *LynB* mRNAs were comparable, as revealed by an ActD chase assay (Fig. 1g), the relative abundance of *LynB* mRNA is likely due to increased production.

3.2. Syk phosphorylation in BMDMs is regulated by the cumulative dose of Lyn at steady-state

To test the intrinsic abilities of LynA and LynB to initiate signaling in the absence of receptor engagement, we activated the SFKs pharmacologically by treating transgenic Csk^{AS} BMDMs with the small molecule 3-IB-PP1. As expected,[9] complete knockout of LynA and LynB severely impaired production of phosphorylated (p)Syk^{Y352}, an SFK substrate,[22,23] in response to 3-IB-PP1 treatment (Fig. 2a). Interestingly, expression of either LynA or LynB alone increased the induction of Syk phosphorylation in a dose-dependent manner (Fig. 2b). Although either isoform could clearly perform this function, expression of LynA in the LynB-knockout genotypes was less effective at restoring Syk phosphorylation than expression of LynB in the LynA-knockout genotypes.

Upon activation, LynA is ubiquitinated and degraded 5-fold more rapidly than LynB,[11,12] so we initially assumed that degradation during treatment might underlie the relative paucity of LynA function. We therefore quantified degradation of LynA and LynB in the knockouts (Fig. 2c) and adjusted the pSyk quantifications to remove contributions of differential abundance of total Lyn (LynA + LynB) protein at each treatment time point. To our surprise, significant differences in Syk phosphorylation remained, suggesting that Lyn degradation over time does not explain the differences in signaling between LynA-expressing genotypes and LynB-expressing genotypes (Figure S2a) or between the PHYS and UP genotypes (Fig. 2d). Together, these results suggested that activation-induced degradation is not the key underlying difference in Syk-phosphorylating activity among the various knockouts.

After confirming that there was no genotype-dependent loss of total Syk protein during the 3-IB-PP1 treatment timeframe (Figure S3a and b), we returned to our initial observation that LynB protein is more abundant than LynA in macrophages and more upregulated than LynA in the UP genotypes. We therefore tested the effects of adjusting the pSyk quantifications to remove contributions of differential steady-state expression of total Lyn. This correction more effectively eliminated differences between LynA-expressing

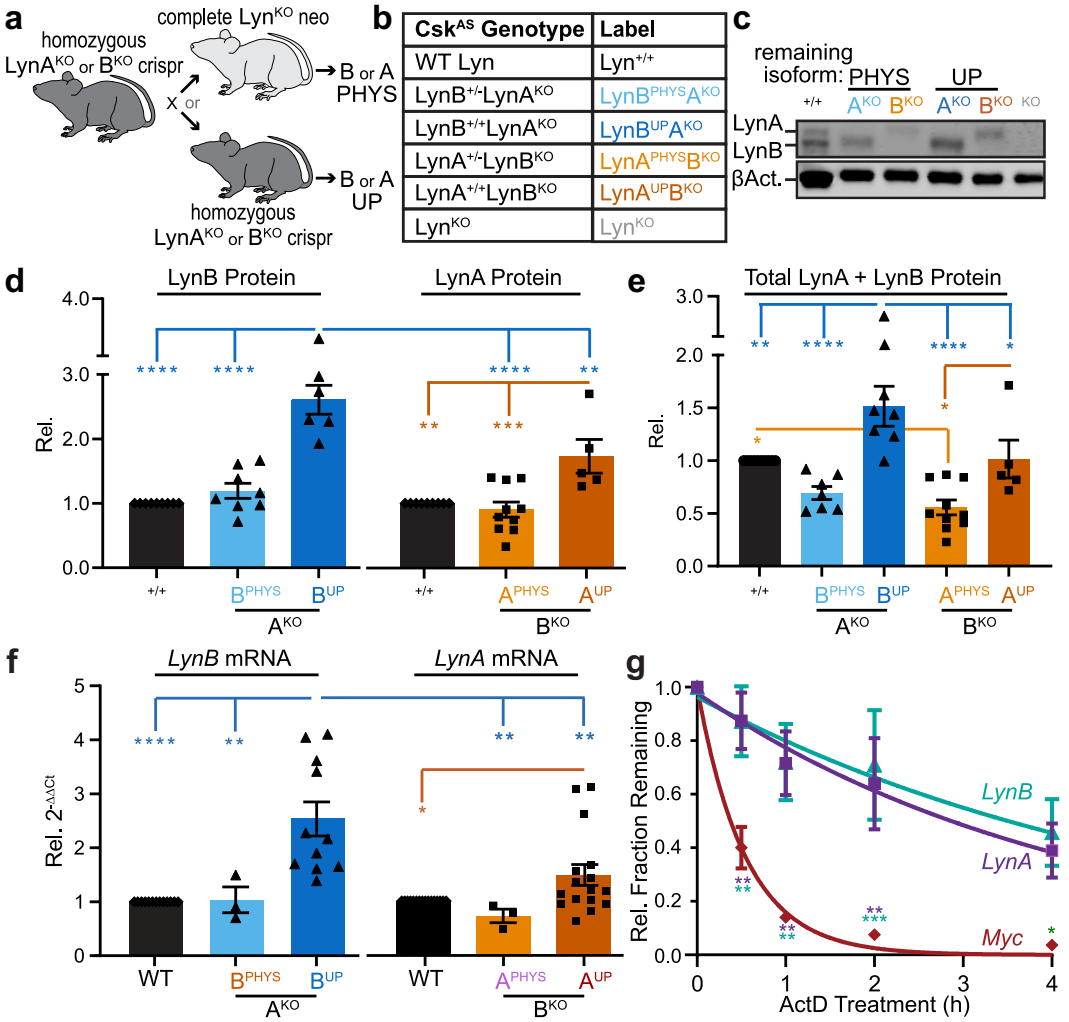


Figure 1 Titration of LynA and LynB expression in BMDMs with a Lyn-knockout genetic series. a) Homozygous single-isoform LynA or LynB knockouts (LynA^{KO} or (Lyn)B^{KO} crispr) crossed with neo-disrupted complete Lyn^{KO} (PHYS progeny) or crossed with another homozygous parent (UP progeny). b) Nomenclature for the Lyn-knockout series; homozygous (WT) expression of LynA and LynB on a Csk^{AS} background is Lyn$^{+/+}$. c) Representative immunoblots showing expression of LynA and LynB protein in BMDM lysates from the Lyn-knockout series; for clarity, the word "Lyn" is omitted from the lane headings. β-Actin (βAct.) is a visual reference for loading. Densitometric quantification, corrected for total protein staining in each lane of d) LynB protein (left), shown relative (Rel.) to LynB protein in Lyn$^{+/+}$; LynA protein (right), shown relative to LynA protein in Lyn$^{+/+}$; and e) total LynA + LynB protein in BMDM lysates, shown relative to total Lyn in Lyn$^{+/+}$. LynA and LynB proteins were undetectable in their respective knockouts. Error bars: standard error of the mean (SEM), n = 5-11 independent replicates. Significance (Sig.) was assessed using 1-way (1w)-ANOVA with Tukey's test for multiple comparisons: ****$P < 0.0001$, ***$P = 0.0001$, **$P = 0.0036$-0.0069, *$P = 0.0112$-0.0434. No significant differences (ns) were found unless indicated. f) qRT-PCR quantification of *LynB* and *LynA* mRNA, corrected for expression of *Ppia* (Cyclophilin) and shown relative to *LynA* or *LynB* in WT BMDMs. LynA and LynB mRNAs were undetectable in their respective knockouts. Error bars: SEM, with n = 11-13 for WT and UP genotypes and 3 for PHYS genotypes. Sig. assessed using 1w-ANOVA with Tukey's test: ****$P \leq 0.0001$, **$P = 0.016$– 0.0071, *$P = 0.0120$-0.0400. g) Quantification of mRNA in WT BMDMs during ActD treatment, fit with a model of single-exponential decay. Rapidly degraded *Myc* is a control for ActD efficacy. Error bars: SEM, n = 3. Sig. assessed using 2w-ANOVA with Tukey's test: ***$P = 0.0005$, **$P = 0.0014$-0.0093, *$P = 0.0217$; otherwise ns.

genotypes and LynB-expressing genotypes (Figure S2b) and between PHYS and UP genotypes (Fig. 2e). Thus, differences in Syk phosphorylation are best explained by differences in the steady-state abundance of Lyn protein, with no intrinsic functional differences between the two Lyn isoforms.

3.3. Dose-dependent effects of Lyn in signal initiation are lost downstream of Syk

We next evaluated the effect of Syk phosphorylation on downstream signal propagation. We have previously observed[9] that complete Lyn^{KO} leads to reduced phosphorylation of PLCγ2^{Y1217}, a Syk substrate,[24] in response to pan-SFK activation. Expression of either LynA or LynB was sufficient to restore pPLCγ2, but some of the dose effects were lost in LynB knockouts, which express lower levels of total Lyn (Fig. 3a and b). This suggests that the Lyn-dose-dependence of downstream protein phosphorylation becomes weaker as signaling is propagated down the pathway, indicating that a threshold level of Syk activity is sufficient to continue signal transduction.

We have also reported that complete Lyn^{KO} leads to reduced phosphorylation of Erk$^{Y202/T204}$ and increased phosphorylation of Akt^{S473} in response to pan-SFK activation.[9] Notably, these downstream effects were not sensitive to LynA or LynB upregulation in the UP genotypes, largely uncoupled from the extent of Syk and PLCγ2 phosphorylation

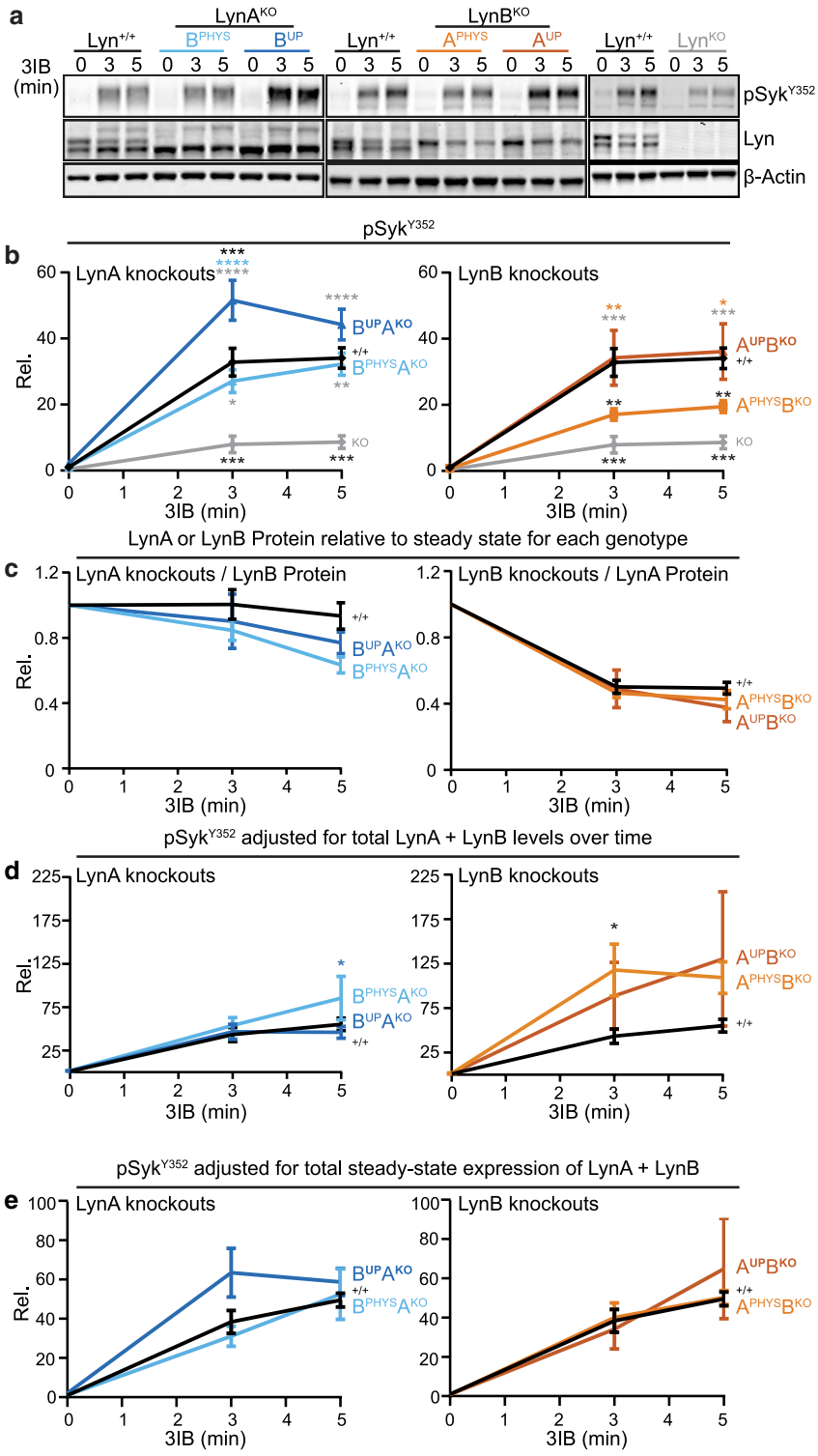


Figure 2 Steady-state expression levels of LynA and LynB determine the extent of Syk phosphorylation during pharmacological SFK activation: a) representative immunoblots showing Lyn and pSyk^{Y352} in lysates from Csk^{AS} BMDMs during treatment with 10 μM 3-IB-PP1 (3IB), a maximally SFK-activating concentration.[9] b) Quantification of pSyk^{Y352}, corrected for the total protein content in each lane and shown relative to the average signal in untreated Lyn$^{+/+}$. Error bars: SEM, n = 4-15. Sig. assessed separately for each graphed set using 2-way (2w)-ANOVA with Tukey's test: ****$P \leq 0.0001$, **$P = 0.0017$-0.0078, *$P = 0.0101$-0.0329; otherwise ns. c) Quantification of LynB protein and LynA protein during treatment with 3-IB-PP1. No significant differences were found with 2w-ANOVA with Tukey's test. d) Data from panel b, adjusted to remove effects of changing total Lyn protein levels in each genotype at each time point, n = 6-9. Sig. assessed using 2w-ANOVA with Tukey's test: *$P = 0.0205$-0.0239, otherwise ns. e) Data from panel b, adjusted to remove effects of differing steady-state expression of total Lyn protein in each genotype (ns).

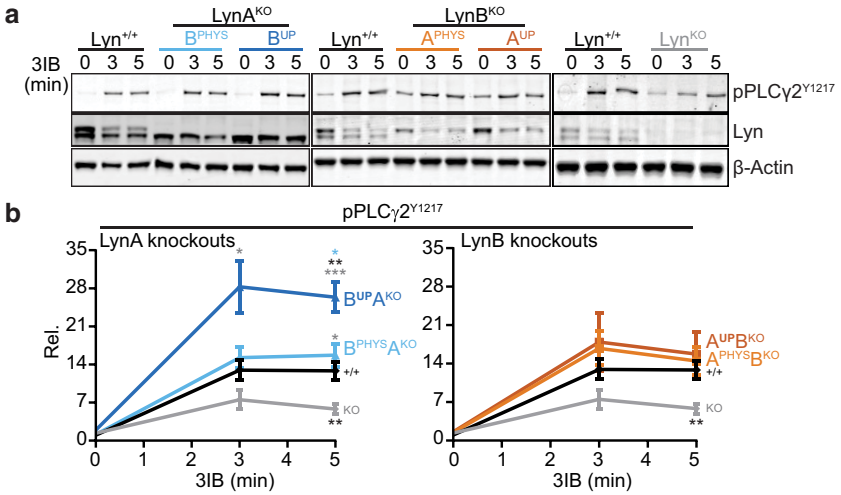


Figure 3 Dose dependence of LynB in downstream phosphorylation of PLCγ2. a) Representative immunoblots showing pPLCγ2^{Y1217} in BMDM lysates. b) Quantification of pPLCγ2^{Y1217}, corrected for the total protein content in each lane and shown relative to the average signal in untreated Lyn$^{+/+}$. Error bars: SEM, n = 4-17. Sig. assessed using 2w-ANOVA with Tukey's test: ***P = 0.0007, **P = 0.0073-0.0090, *P = 0.0158-0.0456; otherwise ns.

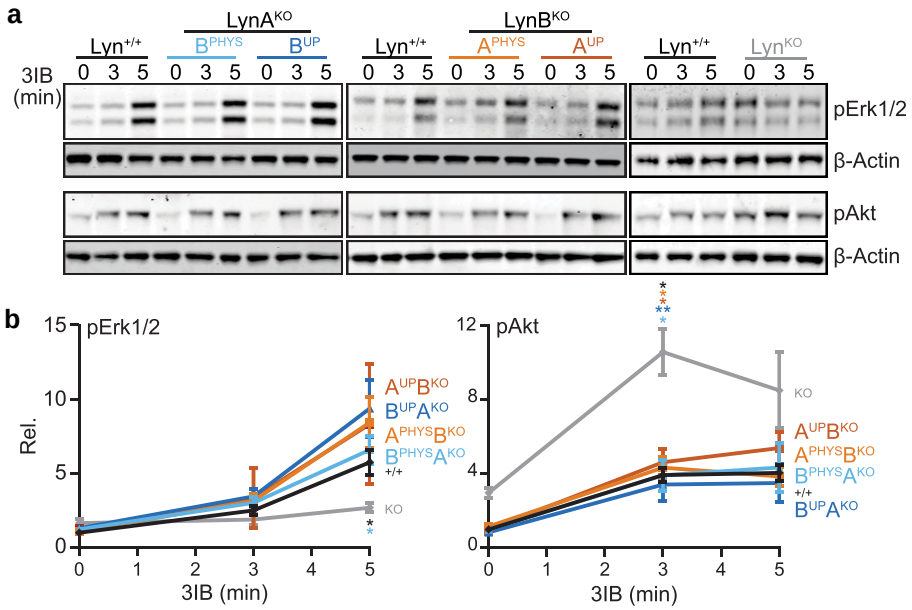


Figure 4 Beyond a threshold of LynA or LynB expression, the Erk and Akt pathways are restored to WT-like function. a) Representative immunoblots showing pErk1/2$^{Y202/T204}$ and pAkt^{S473} in BMDM lysates. b) Quantification of pErk1/2$^{Y202/T204}$ and pAkt^{S473}, corrected for the total protein content in each lane and shown relative to the average signal in untreated Lyn$^{+/+}$. Error bars: SEM, n = 4-11. Sig. assessed using 2w-ANOVA with Tukey's test: **P = 0.0087, *P = 0.0110-0.0430; otherwise ns.

upstream (Fig. 4a and b). These findings suggest that a modest level of upstream signaling activity is sufficient to promote a WT-like downstream response to pan-SFK activation.

Akt is hyperactivated in the absence of Lyn due to a failure to maintain low-level steady-state phosphorylation of SHIP1, which leads to basal accumulation of the Akt-activating second messenger PIP_3.[9] We therefore assessed phosphorylation of SHIP1^{Y1020} to test the roles of the two Lyn isoforms in maintaining this rheostatic SHIP1 function. Expression of either LynA or LynB restored WT-like levels of steady-state SHIP1 phosphorylation (Fig. 5a and b); as with Syk phosphorylation, these effects were dose dependent. In contrast, 3-IB-PP1 treatment induced robust phosphorylation of SHIP1 in a Lyn-independent manner (Fig. 5c). This finding reinforces the importance of low-level steady-state activities of Lyn and SHIP1, balanced to define the signaling potential of the cell in response to later stimulation. Our study further defines this steady-state signaling process as setting a threshold for downstream activation of the Erk and Akt pathways, with dose-dependent effects dissipating in favor of a limited-magnitude all-or-none response in the absence of a receptor-engaging microbial encounter.

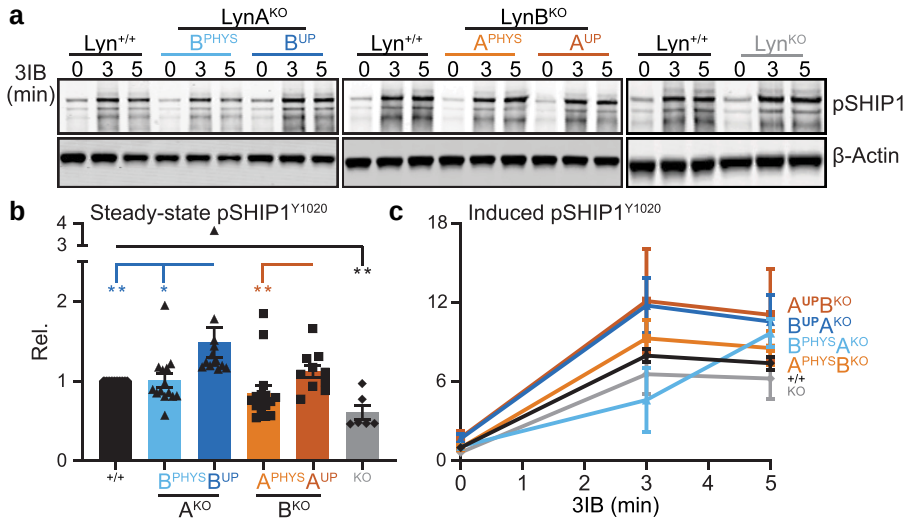


Figure 5 Steady-state but not induced phosphorylation of SHIP1 is dependent on expression of either LynA or LynB. a) Representative immunoblots showing pSHIP1^{Y1020} in BMDM lysates. b) Quantification of pSHIP1^{Y1020} in resting macrophages. Data are corrected for the total protein content in each lane and shown relative to the average signal in untreated Lyn$^{+/+}$. Error bars: SEM, with n = 4-11. For consistency with previous data analysis, significant differences between A-knockout and B-knockout genotypes were not assessed. Differences between single-isoform and $^{+/+}$ genotypes were assessed using 1w-ANOVA-Tu test: $^{**}P = 0.0090$, $^{*}P = 0.0128$-0.0390; otherwise ns. Differences between $^{+/+}$ and ^{KO} genotypes were assessed using Welch's *t* test, $^{**}P = 0.0064$. c) Quantification of pSHIP1 in 3-IB-PP1-treated BMDMs. Data are corrected for the total protein content in each lane and shown relative to the average signal in untreated Lyn$^{+/+}$. Error bars: SEM, with n = 4-11. Sig. assessed using 1w-ANOVA-Tu test (ns).

4. Discussion

To parse the relative contributions of isoform-specific roles and dose effects of LynA and LynB, we generated a genetic series of single-isoform LynA^{KO} and LynB^{KO} mice, in which the remaining isoform is expressed at a WT-like or supraphysiological level. We then probed the intrinsic abilities of LynA and LynB to initiate signaling by using a small-molecule inhibitor of their primary negative regulator (Csk) in Csk^{AS}-transgenic macrophages to induce pan-SFK activation. Although LynB has a greater role in phosphorylating Syk, it is also the more highly expressed isoform and more profoundly upregulated than LynA in homozygous single-isoform (UP-genotype) macrophages. Of note, this upregulation is mediated by increased production of *LynB* mRNA. It is tempting to speculate that expression levels of the two Lyn isoforms, exploited as tools in the current study, are regulated in a complementary fashion in WT cells to tune the cumulative abundance of Lyn, potentially providing for more stable Lyn protein levels after a burst of activation-induced LynA degradation.

Although Lyn abundance decreases during induced signaling, due to activation-induced degradation of LynA,[11,12] it is the basal expression of total LynA + LynB protein that determines the extent of Syk phosphorylation induced by pan-SFK activation. LynA and LynB are equally capable of phosphorylating ITAM-associated signaling machinery, and overexpression of either Lyn isoform can compensate for loss of the other.

The observation that steady-state expression of Lyn is the key factor in SFK-induced signaling is consistent with our earlier finding that the extent of basal phosphorylation of SHIP1, largely performed by Lyn, creates a set point for downstream signaling.[9] LynA and LynB both have dose-dependent contributions to low-level steady-state phosphorylation of SHIP1. However, further phosphorylation of SHIP1 during pan-SFK activation can occur independently of Lyn.[9] This suggests that c-Cbl/ubiquitin-mediated degradation of Lyn has a primary function in regulating the steady-state abundance of Lyn protein and determining the sensitivity of the cell to future induction of signaling. Indeed, we have found that Csk^{AS} mast cells, which express almost no c-Cbl, are more sensitive than macrophages to Erk-pathway activation in response to 3-IB-PP1 and that ectopic expression of c-Cbl in mast cells suppresses this effect.[11] Furthermore, c-Cbl^{KO} BMDMs, which have impaired activation-induced degradation of all the SFKs, especially LynA at early timepoints,[11] have increased capacity to signal through the Erk pathway in response to pan-SFK activation—we now interpret these observations as effects on steady-state levels of total Lyn protein, maintained by RNA expression, splicing, and steady-state ubiquitination and degradation of small pools of basally active Lyn.

Our previous pathway characterization showed Lyn expression to be necessary for upstream Syk and PLCγ2 phosphorylation but not PI3K phosphorylation, which promotes balanced Erk and Akt signaling during pan-SFK activation.[9,12] It is therefore striking that low levels of LynA or LynB expression are sufficient to support WT-like activation of the Erk and Akt pathways, despite dose-dependent phosphorylation of Syk and, to a lesser extent, PLCγ2. This uncoupling of downstream from upstream signal strength may be a function of basal SHIP1 phosphorylation, which counters the signal-promoting functions of Syk and PLCγ2 via a PIP_3 bottleneck.[9] This steady-state rheostat may explain the necessity of coordinating total expression of LynA and LynB: if expression of either isoform changes, for instance after activation-induced degradation, cells can upregulate either or both isoforms to maintain the steady-state pSHIP1 necessary to regulate later signal strength.

While this study focuses on the intrinsic potential of LynA and LynB to phosphorylate their substrate Syk and initiate ITAM-pathway signaling, isoform-specific functions may yet be revealed in other signaling pathways or contexts. However, LynA or LynB can promote TLR4 and TLR7 signaling in BMDMs,[25] and their identical kinase domains are not likely to have intrinsic substrate preferences. If the two Lyn isoforms are found to function similarly in the broader context of cell signaling, then phenotypic differences in the single-isoform knockout mice[13] may simply be dose effects, with

loss of LynB driving more severe disease because it is the more highly expressed isoform in relevant cell types.

Our findings support a model in which cumulative LynA + LynB expression effects a steady-state inhibitory program by phosphorylating SHIP1 and restricting macrophage activation by SFKs in the absence of a pathogenic threat. Increased inhibitory signaling in the macrophage steady-state creates a bottleneck between initiating events and productive signal propagation, protecting the cell from spurious activation and reinforcing the importance of the Lyn/pSHIP1 rheostat in macrophage regulation.

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Author contributions

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Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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