Generation of a Novel System for Studying Spleen Tyrosine Kinase Function in Macrophages and B Cells[1]

Allison L. Miller,* Chao Zhang,† Kevan M. Shokat,† and Clifford A. Lowell*[2]

Spleen tyrosine kinase (Syk) is a nonreceptor tyrosine kinase that is expressed primarily in hematopoietic cells. Because this protein has been implicated in processes such as Fc-mediated phagocytosis, BCR signaling, oxidative burst, degranulation, cytokine secretion, and integrin-mediated outside-in signaling, it is hypothesized that Syk may be a viable target in the treatment of a variety of autoimmune and inflammatory diseases. Because efforts to design a small-molecule therapeutic that specifically inhibits Syk have been largely unsuccessful, and genetic studies of Syk have been hampered by the fact that $syk^{-/-}$ mice die in utero, we have taken a chemical genetic approach to study the function of Syk. Specifically, we have created a mutant form of Syk that retains its wild-type function, but is susceptible to inhibition by enlarged derivatives of the tyrosine kinase inhibitor, PP1. We report in this study that Syk M442A S505A reconstituted wild-type function when introduced into murine $syk^{-/-}$ bone marrow-derived macrophages and $syk^{-/-}$ DT40 chicken B cells, as determined by functional and biochemical assays. Furthermore, after screening a series of PP1 derivatives, we identified one compound, namely 2,3-DMB-PP1, that specifically inhibited Syk M442A S505A, but not wild-type Syk. This system provides us with the power to characterize immune functions that are Syk specific, and furthermore, it provides us with a tool to assess how inhibition of Syk may alter an immune response and influence disease pathogenesis and/or progression. *The Journal of Immunology,* **2009, 182: 988–998.**

The human genome encodes 518 kinases that comprise ~2% of all genes (1), making them one of the largest gene families in eukaryotes. The biological importance of these kinases is highlighted by the fact that over 30% of current drug development efforts are directed at targeting kinases (2). However, because the majority of these kinases share some degree of structural similarity, it has been challenging to develop inhibitors that bind to and inhibit a specific kinase without having an effect on other members of the kinome (3, 4). To circumvent these obstacles and to generate a system with the power to interrogate the functions of a specific kinase, a chemical genetic approach was developed. This approach involves mutation of a structurally conserved bulky gatekeeper residue to alanine or glycine in the active site of the kinase. The unique space created by the gatekeeper mutation renders the mutant kinase susceptible to inhibition by an enlarged inhibitor (5), thus creating what is called an analog-sensitive kinase allele (ASKA).[3] This strategy has been used for a number of kinases, including v-Src (5), Fyn (5), Cla4p (6), Grk2 (7), Lck (8), and ZAP70 (9), among others (7). In some cases, this technology has been taken a step further, and analog-ASKA have been introduced in vivo by generating transgenic or knock-in animals (10, 11). Notably, the inhibitors appear to be fast acting and nontoxic when used in vitro and in vivo (10).

Spleen tyrosine kinase (Syk) is one of the 90 members of the tyrosine kinase family of kinases (1), and is known to be expressed in neutrophils (12), mast cells (13), macrophages (14), B cells (15), dendritic cells (16), and epithelial cells (17). Studies from our laboratory and others have identified critical roles for this tyrosine kinase in a variety of immunological events, including Fc-mediated phagocytosis (18), BCR signaling (19), oxidative burst, degranulation, cytokine secretion, and integrin-mediated outside-in signaling (20). Src-family kinases bind to and phosphorylate ITAMs. Syk is then recruited to these ITAMs, where it is activated to initiate downstream events. In vivo studies to interrogate the function of Syk have been limited by the lack of a specific inhibitor, and by the fact that $syk^{-/-}$ mice die during development due to a fusion of the vasculature with the lymphatic system (21). To date, inquiries into the function of Syk have used nonspecific inhibitors such as piceatannol, small interfering RNA to Syk, or $syk^{-/-}$ cells isolated from irradiated wild-type mice that have been injected with fetal liver preparations of $syk^{-/-}$ embryos. However, there are inherent limitations to these studies. It is for this reason that we set out to generate an ASKA of Syk that we could use to study the roles of Syk kinase in intracellular signaling pathways both in cell lines and eventually in vivo.

Crowley et al. (18) have shown that $syk^{-/-}$ bone marrow-derived macrophages have a complete defect in Fc-dependent phagocytosis. Although these macrophages can form actin cups around the opsonized particle, they are unable to internalize it. Using a colorimetric assay and a novel flow-based assay for Fc-mediated phagocytosis, we tested the ability of primary wild-type ($syk^{+/+}$) and $syk^{-/-}$ bone marrow-derived macrophages that were retrovirally reconstituted with wild-type or mutant versions of Syk, to phagocytose Ig-coated SRBC. These studies demonstrated that two ASKA mutants, Syk M442A (Syk MA) and Syk M442A, S505A (Syk MASA), not only restored the ability of $syk^{-/-}$ macrophages to perform Fc-mediated phagocytosis, but were able to do so at near wild-type levels. These findings were further confirmed using

*Department of Laboratory Medicine, and †Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143

Received for publication August 22, 2008. Accepted for publication November 12, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants R01AI68150 and R01AI65495.

[2] Address correspondence and reprint requests to Dr. Clifford A. Lowell, Department of Laboratory Medicine, University of California, 513 Parnassus Avenue, Box 0451, San Francisco, CA 94143.

[3] Abbreviations used in this paper: ASKA, analog-sensitive kinase allele; pERK, phospho-ERK; ptyr, phosphotyrosine; Syk, spleen tyrosine kinase.

calcium flux and signaling assays in $syk^{-/-}$ DT40 B cells that had been stably transfected with wild-type Syk or Syk MASA. Together these studies suggested that Syk MASA approximates the activity of wild-type Syk. We then showed that Syk MASA was susceptible to specific inhibition to the compound 2,3-DMB-PP1 in a dose-dependent fashion. In conclusion, we have generated a system that gives us the power to functionally interrogate Syk activity in a biologically and immunologically relevant system. These studies have laid the groundwork to generate a viable mouse whose cells express a form of Syk that can be specifically inhibited by a small-molecule inhibitor.

Materials and Methods

Plasmids and mutagenesis

The cDNA for Syk was subcloned from pBluescript into pMIGW, an internal ribosome entry site-GFP vector. Before subcloning, the following mutants were generated using QuikChange site-directed mutagenesis kit (Stratagene): M442A, M442G, M442A S505A, M442A S505G, M442G S505A, and M442G S505G.

Isolation and culturing of bone marrow-derived macrophages

Wild-type and $syk^{-/-}$ bone marrow was isolated from the tibia and femur bones of wild-type B6 mice, and bone marrow chimera mice were generated by using fetal liver cells from $syk^{-/-}$ embryos, respectively. Chimeras were generated in lethally irradiated mice, as previously described (22). Syk deficiency was confirmed by intracellular staining and/or Western blot. Marrow was eluted in PBS/20 mM HEPES. Clumps were removed by rigorous pipetting and passage through a 70-μm nylon mesh. Erythrocytes were lysed using ACK lysis buffer. Cells recovered were plated in T-75 flasks in MEMα without nucleosides, which was supplemented with 10% FCS, penicillin (100 U/ml)/streptomycin (100 mcg/ml), 2 mM glutamine, and 10% CMG supernatant (recovered from 293 cells expressing rM-CSF) and incubated overnight at 37°C/5% CO_2 before retroviral gene transfer.

Retrovirus production/titration

Retroviral stocks were generated by transient transfection of 293T cells with the empty pMIG-W vector (10 μg), or pMIG-W containing wild-type or mutant Syk (10 μg), and plasmids containing Moloney murine leukemia virus gag-pol (10 μg) and vesicular stomatitis virus glycoprotein envelope (15 μg) with Lipofectamine 2000 (Invitrogen). Supernatants were harvested at 48 and 72 h posttransfection, and after filtration through a 0.2-μm filter, were stored at −20°C until use. To titer the retroviral stocks, 3T3 cells were plated in six-well plates and were allowed to incubate overnight. The next day, cells were counted from two wells, and this number was averaged and considered to be the starting number of 3T3 cells. Virus was diluted in DMEM/10% FCS, penicillin (100 U/ml)/streptomycin (100 mcg/ml), and 8 μg/ml polybrene, and plated on the 3T3 monolayers. Forty-eight hours later, the 3T3 cells were trypsinized from the plates, and the percentage of cells that were GFP^+ was assessed. This percentage was multiplied by the number of starting 3T3 cells and the dilution factor of the virus to calculate the virus titer.

Retrovirus gene transfer

Nonadherent bone marrow cells were harvested from T-75 flasks 1 day postharvest. These cells were resuspended to 7.5×10^6 cells/ml in DMEM/10% FCS and penicillin (100 U/ml)/streptomycin (100 mcg/ml). In a volume of 15 ml, 7.5×10^6 cells were mixed with 10% CMG, polybrene (final concentration of 8 μg/ml) (Sigma-Aldrich), HEPES (final concentration 20 mM), and 6–12 ml of retrovirus supernatant. The 15 ml was divided equally among the wells of nontissue culture-treated six-well plates, and the plates were spun for 1 h at 2000 rpm, at 20°C. The plates were then returned to the incubator from 4 h to overnight. The medium on the cells was changed from the spinfection medium to MEMα without nucleosides, which was supplemented with 10% FCS, penicillin (100 U/ml)/streptomycin (100 mcg/ml), 2 mM glutamine, and 10% CMG supernatant. Cells were used in assays after a minimum of 6 days in culture following spinfection. Medium was changed on these cells every 3 days. A similar spinfection protocol was used for generating stable lines of $syk^{-/-}$ DT40 cells that expressed wild-type Syk or Syk MASA; however, the medium used for culturing the DT40 cells was RPMI 1640 with 10% FCS, penicillin (100 U/ml)/streptomycin (100 mcg/ml), 2 mM glutamine, and 50 μM 2-ME. Postretroviral infection, the DT40 cells were sorted twice based on GFP expression to obtain homogenous populations of Syk-expressing cells.

Phagocytosis assays

We used three methods to measure Fc-mediated phagocytosis activity. The colorimetric assay was adapted from published work by Jungi (23). Briefly, macrophages were retrovirally infected with pMIG retroviruses, were sorted on the basis of GFP expression, and were plated on 96-well tissue-culture treated plates in 250 μl of MEMα with penicillin (100 U/ml)/streptomycin (100 mcg/ml) and 0.5% BSA at a concentration of $3–4 \times 10^5$ cells/ml. The cells were allowed to incubate at 37°C for 1–4 h to allow the cells to firmly adhere. During this time, SRBC (PML Microbiologicals) were opsonized. To do this, SRBC were washed twice with $DGVB^{2+}$ (2.5 mM Veronal (barbital; Sigma-Aldrich), 75 mM NaCl, 2.5% dextrose, 0.05% gelatin, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.5) buffer), and were then resuspended in RPMI 1640 medium. Anti-SRBC Ab (Rockland) was added to the SRBC at a dilution of 1/64 (dilution determined by a hemagglutination assay). The SRBC were incubated at room temperature with the Ab for 1 h while rocking. The SRBC were then washed twice with $DGVB^{2+}$ and were resuspended at 2% in RPMI 1640. Cells were maintained out of the light at 4°C before use. Once the macrophages were firmly adherent, medium was replaced with 200 μl of fresh MEMα with penicillin (100 U/ml)/streptomycin (100 mcg/ml) and 0.5% BSA. Medium (50 μl) and unopsonized or opsonized SRBC were added to the wells of macrophages and were left to incubate for various amounts of time. The SRBC/medium was aspirated from the wells. The macrophages were washed with 250 μl of PBS; uninternalized SRBC were lysed with 200 μl of ACK; and then the macrophages were washed a second time with 250 μl of PBS. Macrophages were then lysed with 50 μl of 0.3% SDS, and 200 μl of diaminobenzidine substrate (Sigma-Aldrich) was added to the wells to measure the peroxidase activity associated with the SRBC. Color change was allowed to proceed for 1 h at room temperature, in the dark. Absorbance at 470 was measured using the Spectramax Plus plate reader. Increases in absorbance correspond to increases in phagocytosis of SRBC. In experiments in which PP1 derivatives were tested for their ability to specifically inhibit the mutant forms of Syk, inhibitors were added just before the addition of the SRBC and were present for the duration of the assay.

A similar protocol was used for the microscopic and flow-based assays for Fc-mediated phagocytosis; however, several changes were made. The microscopic assay was done identically to the colorimetric assay; however, instead of lysing the macrophages with 0.3% SDS, the macrophages were fixed with 3.7% formaldehyde, and the number of internalized SRBC per macrophage was counted in 100–200 cells. The phagocytic index represents the number of internalized SRBC per 100 macrophages. In the case of the flow-based assay, 4×10^5 cells were put into polypropylene tubes. No prior sorting was required. The remainder of the experiment was conducted like the colorimetric assay; however, at the conclusion of this assay, cells were resuspended in cold PBS with 2% FCS and 0.05% sodium azide. Cells were then run through a FACSCalibur (BD Biosciences), and side scatter of the GFP^+ and GFP^- populations was assessed. In experiments in which PP1 derivatives were tested for their ability to specifically inhibit the mutant forms of Syk, inhibitors were added 30 min before the addition of the SRBC, and were present for the duration of the assay.

Western blots

DT40 cells were resuspended in RPMI 1640 supplemented with penicillin (100 U/ml)/streptomycin (100 mcg/ml), 2 mM glutamine, and 50 μM 2-ME at 100×10^6 cells/ml. Cells were maintained at 37°C in a water bath for 15 min before stimulation. If the experiment involved an inhibitor, it or DMSO was then added and the cells were preincubated in its presence for additional 15 min. After this period, 50 μl of cells was removed and lysed in 50 μl of 2× concentrated, nonreduced SDS-PAGE sample buffer (128 mM Tris (pH 6.8), 10% glycerol, and 4% SDS) to be used as the unstimulated control. A total of 5 μg of anti-chicken IgM (Bethyl Laboratories) was then added to the cells, and after 10 min, an additional 50 μl of cells was lysed in 50 μl of 2× sample buffer. Before running these cell lysates on a gel, they were ultracentrifuged at 100,000 rpm for 30 min at 20°C. Supernatants were collected, reduced with DTT at a final concentration of 1%, and then boiled for 5 min. A total of 5–10 μl of the reduced lysate was loaded per well in a 10% NuPage bis-Tris gel. Gels were probed with 4G10 supernatant (1:20), anti-ERK2 (1:2000) (Santa Cruz Biotechnology), anti-phospho-ERK (pERK; 1:1000) (Cell Signaling Technology), or anti-Syk (N-19; 1:1000) (Santa Cruz Biotechnology).

Calcium flux

A total of $35–50 \times 10^6$ DT40 cells was resuspended in 1 ml of RPMI 1640 supplemented with 2% BSA, penicillin (100 U/ml)/streptomycin (100 mcg/ml), 20 mM HEPES, 2 mM glutamine, and 50 μM 2-ME. To the cells, 10 μl of indo-I was added. The cells were rocked for 1 h at room temperature,

	Subdomain V	Subdomain VII
c-Src	/-IYIVTEYMSKG-/	/-VCKVADFGLAR-/
Cdk2	/-LYLVFEFLHQD-/	/-SIKLADFGLAR-/
Syk-WT	/-WMLVMEMAELG-/	/-YAKISDFGLSK-/
Syk-MA	/-WMLVAEMAELG-/	/-YAKISDFGLSK-/
Syk-MG	/-WMLVGEMAELG-/	/-YAKISDFGLSK-/
Syk-MASA	/-WMLVAEMAELG-/	/-YAKIADFGLSK-/
Syk-MASG	/-WMLVAEMAELG-/	/-YAKIGDFGLSK-/
	↑ 442	↑ 505

FIGURE 1. Partial sequence alignment of c-Src and Cdk2 with wild-type and mutant forms of Syk. The gatekeeper amino acid of Syk, M442, was mutated to an alanine or a glycine. In addition, gatekeeper mutants were paired with mutations of S505 to an alanine or a glycine. The gatekeeper residue is in red, and the second-site residue is in blue. The positions of these residues in Syk are noted below.

while protected from light. The cells were then washed before being resuspended at ~25–40 × 10^6 cells/ml in the aforementioned medium and maintained on ice. A total of 300 μl of these cells was used for each sample. Before running the sample on the LSRII, the cells were warmed at 37°C for 3 min. UV emission was recorded for 30 s before anti-IgM stimulation (20 μg/ml in a volume of 30 μl) to provide a baseline. Calcium flux was recorded for a total of 4 min.

Inhibitor synthesis

The 1-NA-PP1, 2-NA-PP1, 1-NM-PP1, and 2-NM-PP1 were synthesized, as previously described (24). The synthesis of the other PP1 analogues will be described elsewhere (C. Zhang and K. M. Shokat, manuscript in preparation).

Results

Generation of an ASKA of Syk

We mutated the gatekeeper residue M442 in Syk to an alanine (M442A) or glycine (M442G) to create analog-sensitive Syk (Fig. 1). In addition, a second-site mutation, S505A, was introduced into the single-mutant M442A in an attempt to stabilize the mutant enzyme as well as to enhance inhibitor sensitivity, because it was found that residues larger than alanine at this position sometimes impede the binding of PP1 analogues (6).

Syk M442A S505A reconstitutes Syk function in bone marrow-derived macrophages

To determine whether the Syk mutants described above were able to reconstitute wild-type Syk function, we retrovirally introduced them into *syk*$^{-/-}$ bone marrow cells using the pMIG-W retrovirus, which contains an internal ribosome entry site-GFP. Once differentiated into macrophages, these cells were sorted based on GFP expression and were subsequently subjected to a colorimetric assay for Fc-mediated phagocytosis to assess Syk activity. Upon examination of our gatekeeper mutants, we observed that Syk M442A (Syk MA) reconstituted the ability of *syk*$^{-/-}$ macrophages to phagocytose opsonized SRBC to ~80% of wild-type macrophages, or *syk*$^{-/-}$ macrophages reconstituted with wild-type Syk. In contrast, mutation of M442 to a glycine appeared to markedly affect the function of Syk because this allele was only able to reconstitute ~40% of the Fc-mediated phagocytosis observed in wild-type macrophages (Fig. 2*A*). Similar assays were conducted to test the activity of

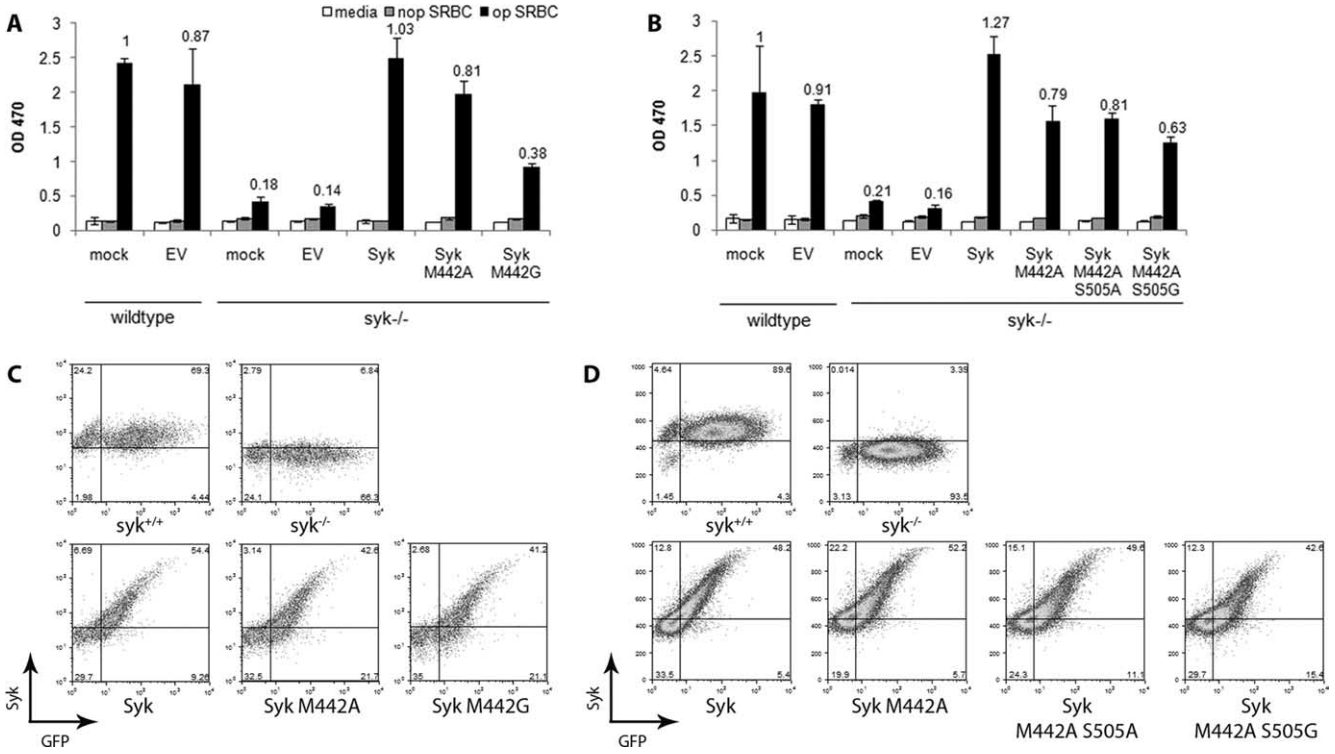


FIGURE 2. Syk M442A and Syk M442A S505A reconstituted Fc-mediated phagocytosis in *syk*$^{-/-}$ bone marrow-derived macrophages. Fc-mediated phagocytosis, as measured by the colorimetric assay, for gatekeeper mutants (*A*) and secondary mutations, paired with the gatekeeper mutant, M442A (*C*). Numbers appearing above the black bars (indicating phagocytosis of opsonized SRBC (op SRBC)) represent the relative amount of phagocytosis relative to wild-type bone marrow-derived macrophages. Macrophages infected with the pMIG-W backbone are designated EV (empty vector). Incubation of macrophages with medium (white) or nonopsonized (nop SRBC) (gray) were used as negative controls. The ability of these mutants to reconstitute phagocytosis was not a result of differences in their expression levels (*B* and *D*). These studies were conducted two to three times with two to four replicates per group. Error bars represent the SD of the mean.

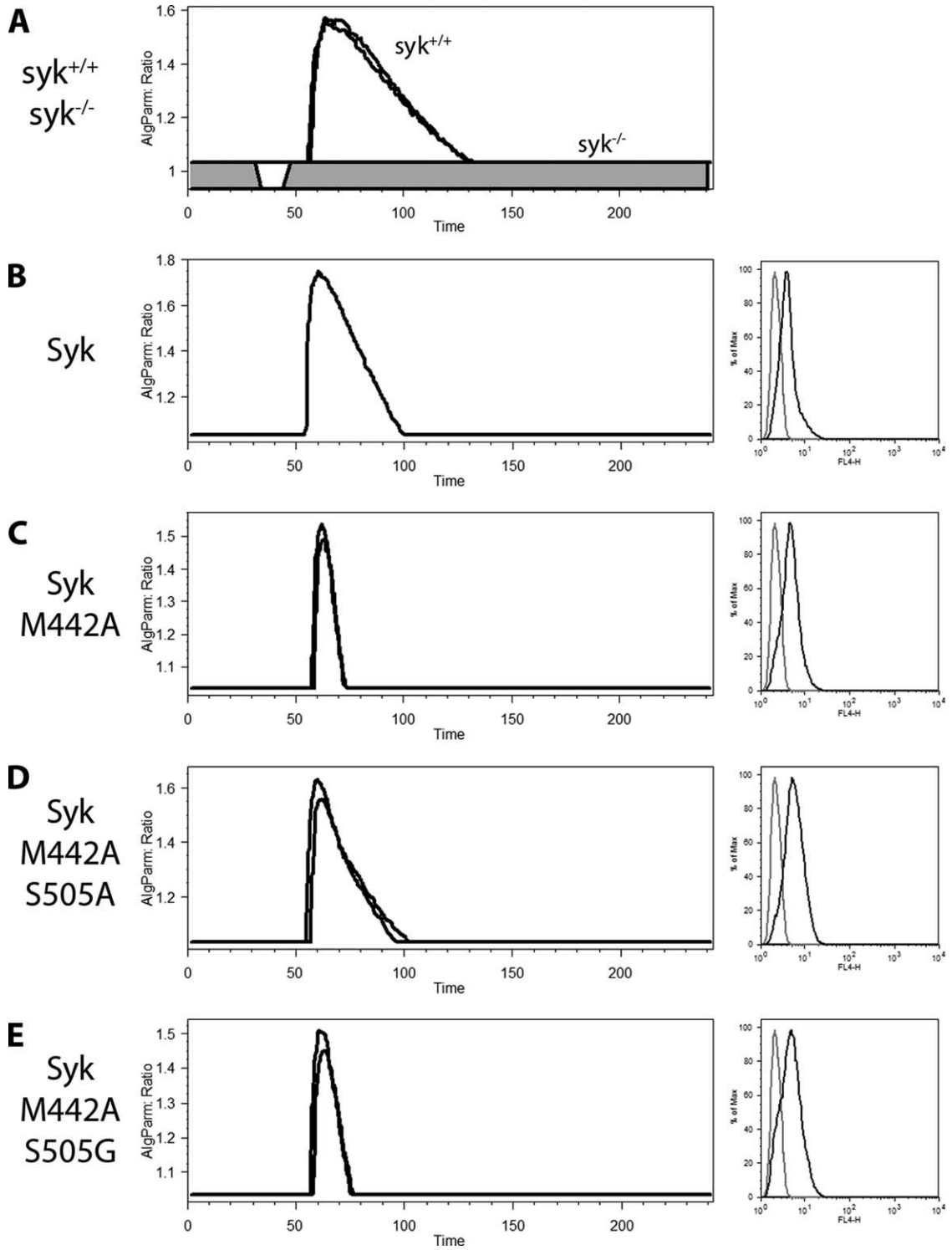


FIGURE 3. Syk M442A S505A reconstitutes the ability of $syk^{-/-}$ B cells to flux calcium following BCR ligation. Calcium flux for wild-type DT40 cells and $syk^{-/-}$ DT40 cells (*A*), as well as $syk^{-/-}$ DT40 cells reconstituted with Syk (*B*), Syk M442A (*C*), Syk M442A S505A (*D*), and Syk M442A S505G (*E*) following stimulation with anti-IgM (20 μg/ml). Levels of expression for wild-type Syk and Syk mutants are similar for each of the DT40 cell lines and appear to the *right* of the calcium flux plots. These experiments were repeated twice, with one to three replicates per sample.

the Syk alleles containing mutations in the secondary site. Syk M442A S505A (Syk MASA)-expressing macrophages, like those expressing Syk M442A, reconstituted ~80% of the Fc-mediated phagocytosis observed in wild-type macrophages (Fig. 2*C*). However, Syk M442A S505G (Syk MASG) (Fig. 2*C*) was found to be a less active form of Syk. Secondary site mutations paired with the M442G gatekeeper mutation failed to rescue the activity of this allele; they instead resulted in a further decrement in Fc-mediated phagocytosis (data not shown).

To determine whether the ability to reconstitute Fc-mediated phagocytosis was related to the expression levels of the mutant forms of Syk, we performed intracellular staining for Syk on the sorted cells that were used in the phagocytosis assays. The retrovirally transduced forms of Syk were expressed at similar levels

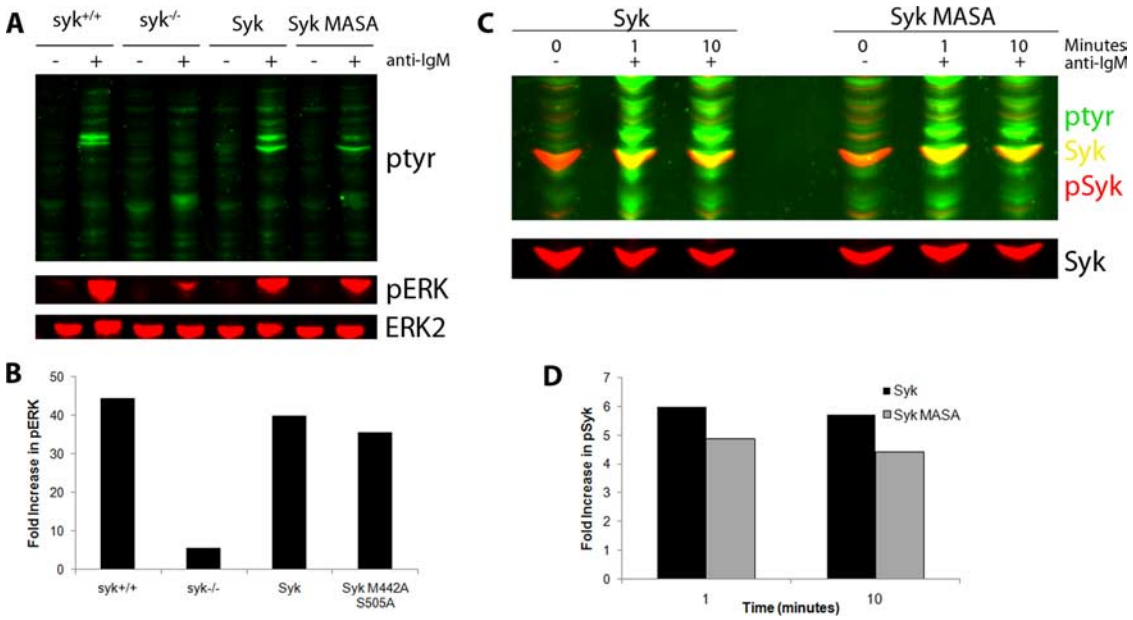


FIGURE 4. Syk M442A S505A reconstitutes phosphorylation of signaling proteins in $syk^{-/-}$ B cells. *A*, The ptyr and pERK levels following 10 min of anti-IgM stimulation (5 μg). Fold increases in pERK were normalized to ERK2 levels and are quantitated in *B*. Phospho-Syk levels at 1 and 10 min post-anti-IgM stimulation (5 μg) (*C*). Levels of phospho-Syk were assessed by probing a Western blot with anti-ptyr (green) and anti-Syk (red) and looking for areas of overlap (yellow). Fold increases in phospho-Syk were normalized to total Syk and are quantitated in *D*. These experiments were repeated three times.

(Fig. 2, *B* and *D*), suggesting that expression levels of the kinases were not responsible for the functional differences observed in the macrophages.

Syk MASA reconstitutes wild-type Syk function in DT40 B cells

To further establish which mutant Syk allele had the capability to reconstitute wild-type Syk activity, we took advantage of a line of $syk^{-/-}$ DT40 B cells that were established by Kurosaki and colleagues (25). It is known that Syk is essential in mediating BCR signaling. Therefore, we chose to introduce wild-type Syk, Syk MA, Syk MASA, and Syk MASG into $syk^{-/-}$ DT40 cells to generate stable lines that express these proteins, so that we could then use them to assess parameters of B cell signaling.

Having established these cell lines, we first chose to monitor calcium flux in $syk^{+/+}$ and $syk^{-/-}$ DT40 cells, as well as $syk^{-/-}$ cells that had been reconstituted with wild-type Syk, Syk MA, Syk MASA, or Syk MASG, following stimulation with anti-IgM. The $syk^{+/+}$ DT40 cells exhibited a substantial calcium flux after BCR ligation, whereas the $syk^{-/-}$ DT40 cells were completely impaired in their ability to flux calcium, as determined using the indo-1 calcium indicator (Fig. 3*A*). Reconstitution of $syk^{-/-}$ cells with either wild-type Syk (Fig. 3*B*) or Syk MASA (Fig. 3*D*) was sufficient to allow these cells to flux calcium similar to the magnitude and duration of wild-type cells following anti-IgM stimulation. A calcium flux was observed following anti-IgM stimulation of $syk^{-/-}$ DT40 cells expressing Syk MA (Fig. 3*C*) or Syk MASG (Fig. 3*E*); however, it was transient. Although the magnitude of these fluxes was similar to what was observed in wild-type DT40 cells, or $syk^{-/-}$ cells reconstituted with wild-type Syk, the duration was significantly less. Intracellular staining for Syk, shown to the right of each calcium flux panel, demonstrated that signaling differences between the mutants were not due to different expression levels of the kinases.

Because Syk MASA was found to be the mutant allele that most closely recapitulated the activity of wild-type Syk, we next evaluated its ability to restore B cell signaling in $syk^{-/-}$ DT40 cells. DT40 cell lines were stimulated with anti-IgM for 10 min and were then assessed for levels of phosphotyrosine (ptyr) and pERK (p44/p42). Reconstitution of $syk^{-/-}$ DT40 cells with wild-type Syk or Syk MASA resulted in the restoration of the banding pattern of phosphorylated proteins observed in wild-type DT40 cells (Fig. 4*A*). When we specifically monitored phospho-ERK post-anti-IgM stimulation, we observed a 44-fold increase in wild-type DT40 cells. DT40 cells reconstituted with wild-type Syk or Syk MASA and stimulated with anti-IgM responded similarly and up-regulated pERK levels 40- and 34-fold, respectively (Fig. 4*B*). To further establish that Syk MASA was working similarly to wild-type Syk, we assessed Syk protein levels and phosphorylation status with or without anti-IgM stimulation in our DT40 stable transfectants. As predicted from our intracellular staining (Fig. 3, *B* and *D*), Syk protein was expressed at similar levels in $syk^{-/-}$ DT40 cells that were reconstituted with wild-type Syk or Syk MASA (Fig. 4*C*). In resting cells, Syk is unphosphorylated; however, upon anti-IgM stimulation, both wild-type Syk and Syk MASA become phosphorylated, as evidenced by the yellow color observed with the overlay of the Western blots for Syk and ptyr (Fig. 4*C*). These levels are dramatically increased relative to resting cells at 1 min following anti-IgM stimulation, and remain elevated at 10 min following anti-IgM stimulation (Fig. 4*D*). Results from these biochemical experiments agree with our phagocytosis data and suggest that Syk MASA retains ~80–85% of the activity of the wild-type kinase.

The 1-NA-PP1, 2-NA-PP1, and PoP-PP1 inhibited Fc-mediated phagocytosis in wild-type bone marrow-derived macrophages

Having identified a mutant form of Syk that retained approximately the same degree of activity as wild-type Syk, we next wanted to identify an inhibitor that specifically acted on this mutant kinase. A series of 15 PP1 analogues that present various groups at C3 of the pyrazolopyrimidine ring to target the enlarged gatekeeper pocket were chosen as candidate inhibitors for the mutant Syk. Before assessing their effects on Syk MASA, we initially

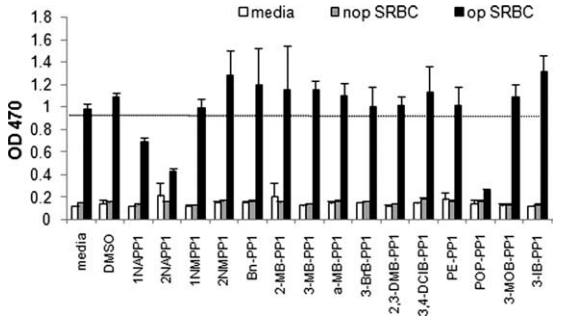


FIGURE 5. The 1NAPP1, 1NAPP1, and CZ29 inhibit Fc-mediated phagocytosis in wild-type bone marrow-derived macrophages. PP1 derivatives were used at a concentration of 6 μM and were added to macrophages just before medium (white), nonopsonized SRBC (nop SRBC) (gray), or opsonized SRBC (op SRBC) (black). The assay measured phagocytosis at a 30-min timepoint. The dotted line represents the level of phagocytosis observed in wild-type macrophages incubated with op SRBC in the presence of medium. All conditions were tested in triplicate. Error bars represent the SD of the mean.

screened these compounds for their specificity by assessing their effects on Fc-mediated phagocytosis in wild-type cells. Of the 15 compounds, 1-NA-PP1, 2-NA-PP1, and PoP-PP1 inhibited Fc-mediated phagocytosis in wild-type cells, thus suggesting that these compounds were having nonspecific effects on the pathway underlying Fc-mediated phagocytosis (Fig. 5). These compounds were discarded in the subsequent experiments in which we tested the efficacy of the remaining inhibitors on Syk MASA.

Development of a novel, flow cytometric-based assay for Fc-mediated phagocytosis

Although we used the colorimetric assay of phagocytosis as a high throughput method for screening our library of inhibitors, this method was often limited by cell numbers and cell purity following sorting. To circumvent these variables, we developed a novel, flow cytometric-based assay for Fc-mediated phagocytosis. This assay was built on the premise that as macrophages phagocytose SRBC, their side scatter increases. Thus, we can quantitate the degree of phagocytosis in an unsorted population of cells by using a flow cytometer to gate on the population of Syk-expressing, GFP^+ macrophages to assay their change in side scatter after incubation with opsonized SRBC, in the presence or absence of an inhibitor. The GFP^- cells can be used as an internal negative control. To establish that changes in side scatter are indeed indicative of the degree of phagocytosis, we incubated $syk^{+/+}$ and $syk^{-/-}$ macrophages with medium, unopsonized SRBC, or opsonized SRBC, and monitored their change in side scatter over time. Fig. 6*A* shows the side-by-side comparisons of the histograms representing side scatter for wild-type and $syk^{-/-}$ macrophages at 5, 15, 20, and 30 min after the addition of medium or unopsonized/opsonized SRBC. As expected, the side scatter does not increase with the addition of unopsonized SRBC. In contrast, following the addition of opsonized SRBC, the side scatter of the wild-type macrophages increases with time, whereas the side scatter of the Syk-deficient macrophages remains mostly unchanged.

To prove that this assay was accurately measuring phagocytosis, we ran a side scatter, colorimetric, and microscopic assay for Fc-mediated phagocytosis in parallel to compare the methods used in this study with the classical method of quantitating Fc-mediated phagocytosis. Data from the flow cytometry-based side scatter assay (represented as the change in median side scatter), the colorimetric assay (represented as the change in absorbance at 470 nm), and the microscopic assay (represented by phagocytic index) are presented in *C–E* of Fig. 6, respectively. Furthermore, in Fig. 6*F*, we show representative images of wild-type and $syk^{-/-}$ macrophages that had been incubated with Ig-opsonized SRBC for 10, 20, and 30 min. Our results show that the kinetics of phagocytosis are the same in each of the three assays. Furthermore, in all three experiments, Fc-dependent phagocytosis occurred only in wild-type cells. In subsequent experiments, we extended the time course to assay changes in side scatter as long as 1 h after addition of the RBC (data not shown). However, we consistently observed peak levels of phagocytosis at 30 min, and thus, we used this timepoint as the standard endpoint for our experiments.

The 2,3-DMB-PP1 specifically inhibits Syk MASA

Wild-type Syk and Syk MASA were retrovirally transduced into $syk^{-/-}$ bone marrow, and GFP-expressing macrophages were sorted and subsequently assayed for phagocytic capacity. We initially screened the panel of PP1 analogues using the colorimetric assay. Three compounds, namely 1-NM-PP1, 3-MB-PP1, and 2,3-DMB-PP1, displayed an ability to specifically inhibit the ASKA of Syk, but not wild-type Syk (Fig. 7*A*). These results indicate that whereas 1-NM-PP1 and 3-MB-PP1 do inhibit our Syk mutants, 2,3-DMB-PP1 is the most effective inhibitor of Syk MASA, because it reduced the level of Fc-mediated phagocytosis to the level observed in $syk^{-/-}$ macrophages. The fact that these compounds are effective even when added just before the SRBCs confirms that they have excellent cell permeability and can rapidly bind to their intracellular target. It should be noted that 2,3-DMB-PP1, although inhibiting our mutant forms of Syk, also appeared to moderately diminish phagocytosis of $syk^{-/-}$ macrophages retrovirally transduced with wild-type Syk (Fig. 7*A*). Because we did not see an effect of this compound on $syk^{+/+}$ macrophages (Fig. 5), and this result was not recapitulated in other phagocytosis experiments, we feel certain of the specificity of the 2,3-DMB-PP1 for Syk MASA.

Next, we chose to use the side scatter assay for phagocytosis to verify our results with the inhibitors from the colorimetric assay. Wild-type or $syk^{-/-}$ macrophages expressing Syk or Syk MASA were preincubated with DMSO, 1-NM-PP1, 3-MB-PP1, and 2,3-DMB-PP1 at a concentration of 5 μM for 30 min before the addition of opsonized SRBC, after which phagocytosis was determined by assessing the change in median side scatter. The incubation of Syk- or Syk MASA-expressing macrophages with opsonized SRBC and DMSO results in an increase in side scatter relative to those that were incubated with medium alone. We observed that the increase in side scatter in macrophages reconstituted with wild-type Syk was unchanged in the presence of the inhibitors. However, addition of 2,3-DMB-PP1 significantly decreased the side scatter shift of macrophages expressing only the Syk MASA mutant, but not wild-type Syk. Although both 1-NM-PP1 and 3-MB-PP1 were also able to reduce the side scatter of the Syk MASA-expressing macrophages that were incubated with opsonized SRBC, these changes were modest relative to what was observed with 2,3-DMB-PP1 (Fig. 7*B*).

In addition to confirming the efficacy of 2,3-DMB-PP1 using Fc-mediated phagocytosis as a readout, we also tested 1-NM-PP1, 3-MB-PP1, and 2,3-DMB-PP1 for their ability to specifically inhibit calcium flux in DT40 cells expressing Syk MASA, following anti-IgM stimulation. Wild-type or $syk^{-/-}$ DT40 cells, as well as $syk^{-/-}$ DT40 cells that had been reconstituted with wild-type Syk or Syk MASA were loaded with indo-1, incubated with DMSO or inhibitor (1 μM), and were subsequently stimulated with anti-IgM. Both 1-NM-PP1 and 3-MB-PP1 were able to partially inhibit calcium flux in cells expressing Syk MASA, but not in cells expressing wild-type Syk. As before,

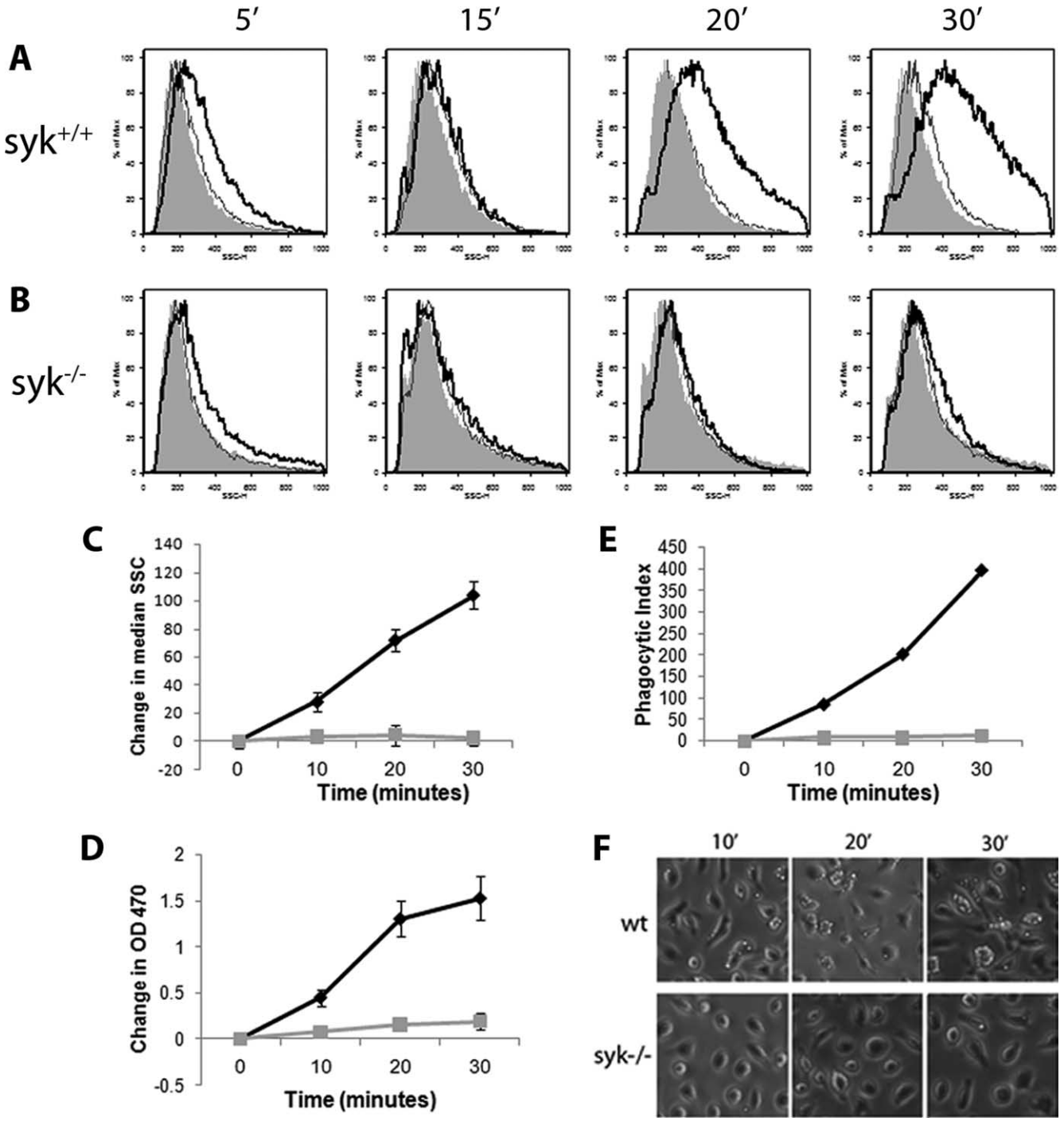


FIGURE 6. A novel FACS-based method of assessing SRBC phagocytosis based on changes in side scatter. Incubation of macrophages with opsonized SRBC (black trace), but not nonopsonized SRBC (gray face) or medium (gray fill), causes a time-dependent increase in the side scatter of wild-type (*A*), but not Syk-deficient (*B*) cells. A comparison of the side scatter (*C*), colorimetric (*D*), and microscopic (*E*) methods of quantitating Fc-mediated phagocytosis in wild-type (black) and *syk*$^{-/-}$ (gray) macrophages, along with representative images of wild-type and *syk*$^{-/-}$ macrophages that have been incubated with opsonized SRBC 10, 20, and 30 min (*F*). Samples for the side scatter and colorimetric assays were run in quadruplicate and triplicate, respectively, and are represented as the change in median side scatter or OD470 relative to macrophages incubated with medium alone. In microscopy studies, the number of phagocytosed SRBC was counted in 200 cells of each type, at each time point, and was used to calculate phagocytic index, which is the number of phagocytosed SRBC per 100 macrophages.

2,3-DMB-PP1 was found to be the most potent inhibitor, because it completely and specifically ablated calcium flux in DT40 cells reconstituted with Syk MASA (Fig. 7, *C* and *D*). It should be noted that the calcium flux curves for Syk-deficient cells expressing Syk MASA in this experiment differed from the calcium flux curves observed for DT40 cells expressing wild-type Syk. The calcium response given by *syk*$^{-/-}$ DT40 cells reconstituted with Syk MASA was variable in our studies; in some cases the calcium flux post-anti-IgM stimulation was monophasic, and in other cases it was biphasic. This may be due to Syk MASA having a less stable interaction with the BCR-signaling complex as compared with wild-type Syk, because the

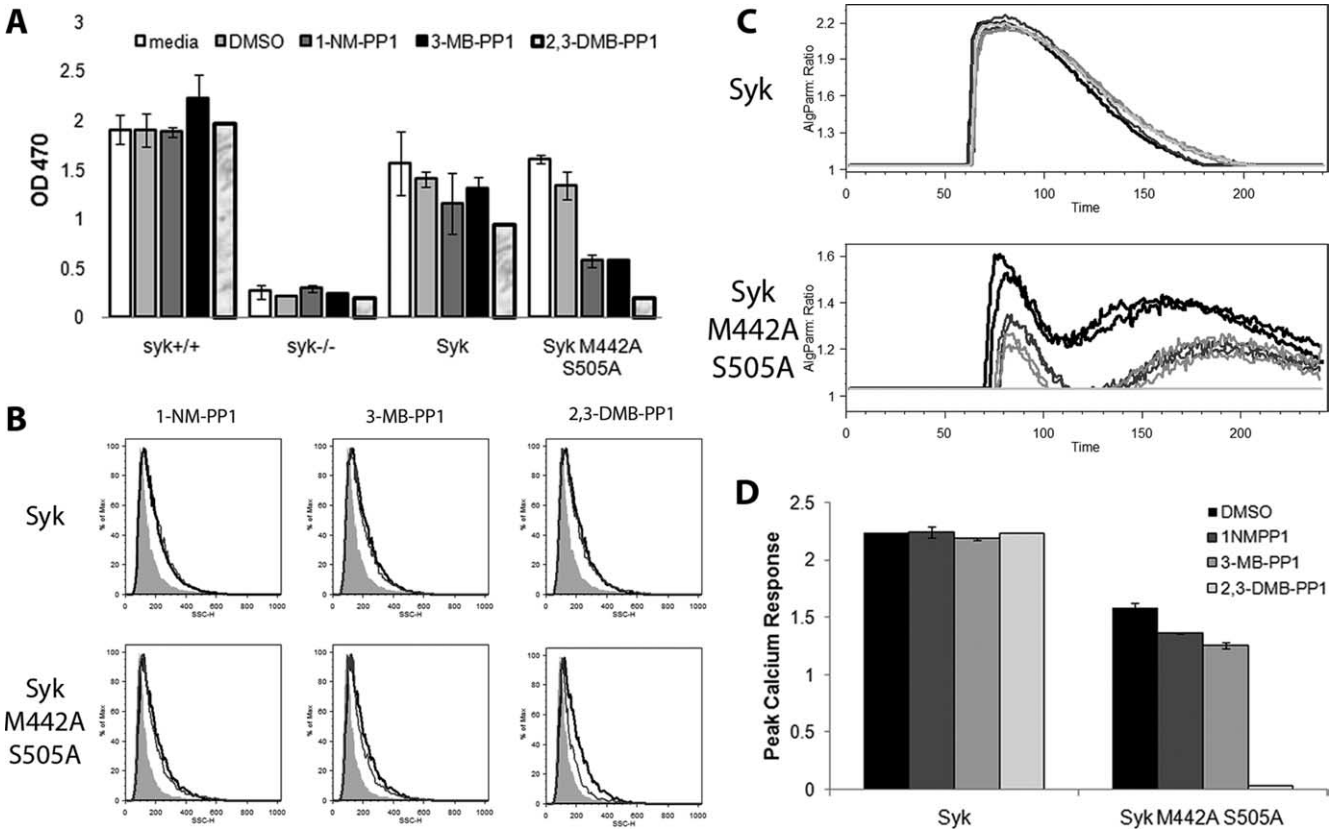


FIGURE 7. The 2,3-DMB-PP1 specifically inhibits Fc-mediated phagocytosis and calcium flux in Syk M442A S505A-expressing macrophages and B cells, respectively. Inhibition of Fc-mediated phagocytosis of opsonized SRBC in the presence of medium (white), DMSO (light gray), 1-NM-PP1 (dark gray), 3-MB-PP1 (black), and 2,3-DMB-PP1 (marble), as determined by the colorimetric assay (*A*). The 2,3-DMB-PP1 was used at a concentration of 6 μM. Experiments were repeated three times, with $n = 2$–3 per experiment. Inhibition of Fc-mediated phagocytosis of opsonized SRBC in the presence of 1-NM-PP1, 3-MB-PP1, and 2,3-DMB-PP1, as determined by the side scatter assay (*B*). Macrophages incubated with medium, opsonized SRBC and DMSO, and opsonized SRBC and inhibitor (5 μM) are shown in gray fill, gray trace, and black trace, respectively. Side scatter was assessed after 30 min. Experiments were done twice with $n = 2$ per experiment. Inhibition of calcium flux in the presence of DMSO (black), 1-NM-PP1 (dark gray), 3-MB-PP1 (medium gray), and 2,3-DMB-PP1 (light gray flat line) (*C*). Compounds were used at 1 μM. These results are quantitated in *D*. Experiments were repeated twice with $n = 2$ per experiment. Error bars represent SD of the mean.

introduced mutations may slightly alter protein-protein interactions. If true, this may explain why Syk MASA can functionally, but not always fully reconstitute Syk function in all of the parameters we tested.

Dose response and kinetic analysis of 2,3-DMB-PP1 on Syk MASA

To determine the dose at which 2,3-DMB-PP1 was most effective to specifically inhibit Syk MASA, we used the flow cytometric-based assay for Fc-mediated phagocytosis. We found that 5 μM was the minimum concentration of inhibitor required to reduce the phagocytosis observed in Syk-deficient macrophages reconstituted with Syk MASA to the levels seen in $syk^{-/-}$ cells without significantly altering phagocytosis of Syk-deficient cells reconstituted with wild-type Syk (Fig. 8*A*, *top panel*). At concentrations of 10 μM, nonspecific effects of the compound on wild-type Syk start to occur (data not shown). The doses of 2,3-DMB-PP1 used in the data shown did not alter the ability of wild-type cells to efficiently take up the opsonized SRBC, indicating that the inhibitor selectively inhibited the MASA-expressing cells (Fig. 8*A*, *top panel*). The $syk^{-/-}$ GFP-negative cells, present in the same assay as the Syk-expressing, GFP-positive cells, were also unaffected by the 2,3-DMB-PP1 (Fig. 8*A*, *bottom panel*). To further validate these results, we used microscopy to assess Fc-mediated phagocytosis in Syk-deficient macrophages that had been retrovirally reconstituted with wild-type Syk or Syk MASA, in the presence or absence of 2,3-DMB-PP1. Similar to what has been observed in our colorimetric assay, Syk MASA reconstituted ~80–85% of Fc-mediated phagocytosis as compared with macrophages expressing wild-type Syk. Furthermore, only macrophages expressing the mutant form of Syk were susceptible to chemical inhibition with 2,3-DMB-PP1 (Fig. 8*B*).

In addition to a dose-response curve, we also subjected these same populations of macrophages to DMSO or 2,3-DMB-PP1 and performed a time-course analysis. As before, we saw that Syk MASA reconstituted the ability of $syk^{-/-}$ macrophages to carry out Fc-mediated phagocytosis at levels comparable to $syk^{+/+}$ and Syk-deficient cells reconstituted with wild-type Syk. At all time points tested, 2,3-DMB-PP1 specifically inhibited Fc-mediated phagocytosis in $syk^{-/-}$ cells reconstituted with Syk MASA, as evidenced by a decrease in side scatter relative to cells not treated with the compound (Fig. 8*C*).

Whereas our colorimetric assay for phagocytosis suggested that Syk MASA reconstituted ~80% of wild-type Syk activity, results from the side scatter method for assessing phagocytosis demonstrated that our mutant functioned equivalently to $syk^{+/+}$ macrophages or $syk^{-/-}$ macrophages reconstituted with wild-type Syk. To rule out the possibility that this was a result of increased expression of Syk MASA, we performed intracellular staining for

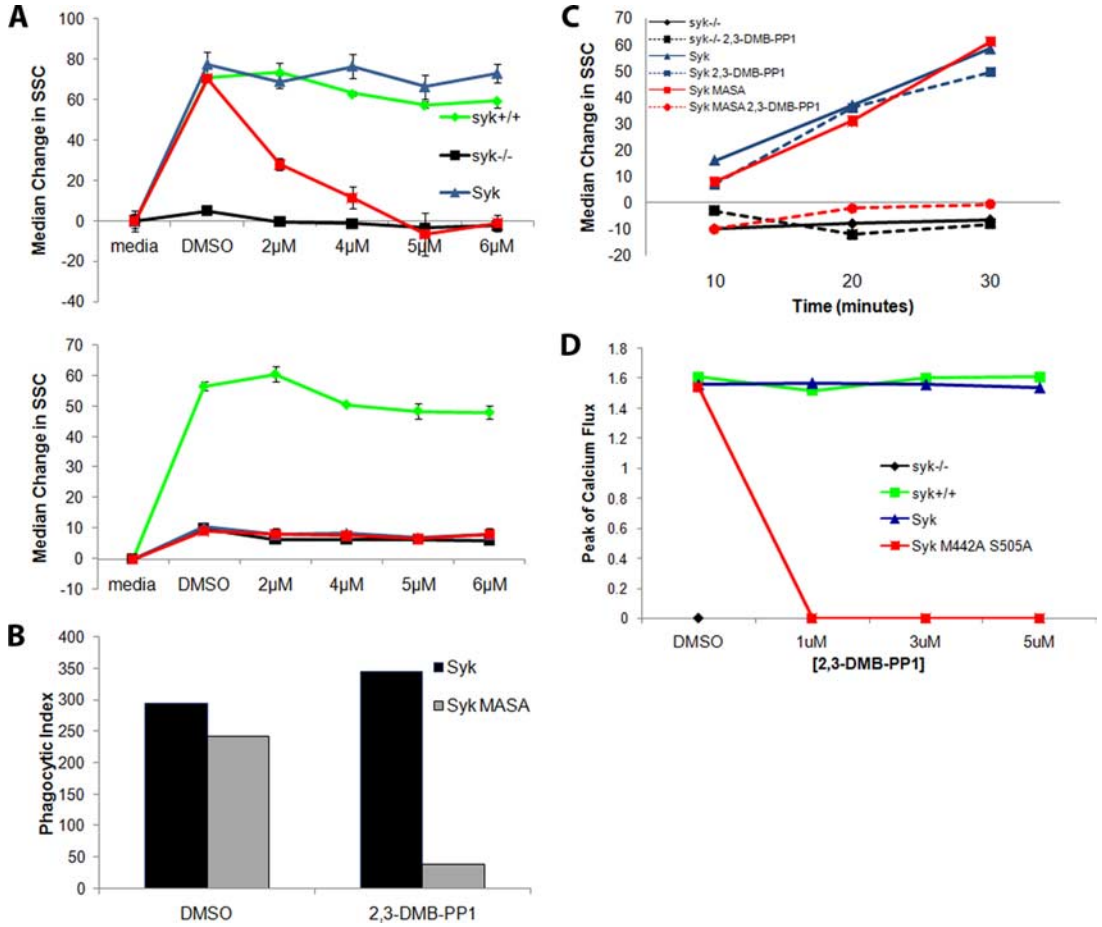


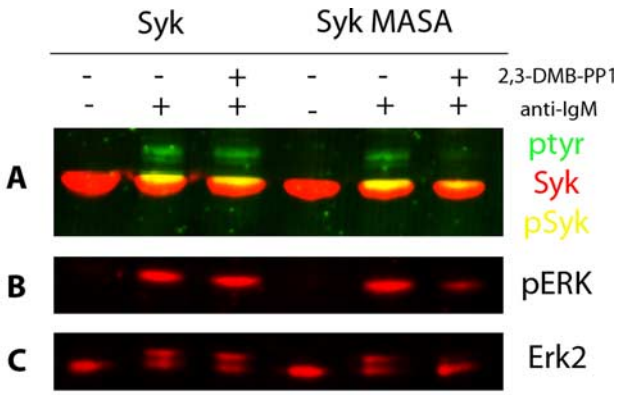
FIGURE 8. The 2,3-DMB-PP1 specifically inhibits Fc-mediated phagocytosis and calcium flux in a dose-dependent fashion. Effects of 2,3-DMB-PP1 on Fc-mediated phagocytosis in the GFP^+ (*A*, *top panel*) or GFP^- (*A*, *bottom panel*) populations of wild-type (green) and $syk^{-/-}$ (black) macrophages transduced with pMIGW-empty vector, or $syk^{-/-}$ macrophages tranduced with wild-type Syk (blue) or Syk M442A S505A (red). Fc-mediated phagocytosis in $syk^{-/-}$ macrophages that were reconstituted with wild-type Syk (blue) or Syk MASA (red) in the presence or absence of 2,3-DMB-PP1, as assessed by microscopy at 30 min postaddition of opsonized SRBC (*B*). Phagocytic index is the number of phagocytosed SRBC per 100 macrophages. Effects of 2,3-DMB-PP1 on Fc-mediated phagocytosis over time, as determined by the side scatter assay (*C*). Black, blue, and red represent $syk^{-/-}$ macrophages retrovirally transduced with pMIGW-empty vector, pMIGW-Syk, or pMIGW-Syk M442A S505A, respectively. Dotted lines represent treatment with 2,3-DMB-PP1. The 2,3-DMB-PP1 was used at a concentration of 5 μM. Error bars represent the SD of the mean. All phagocytosis experiments were conducted three times, with each condition being tested in duplicate. Effects of 2,3-DMB-PP1 on calcium flux in wild-type DT40 cells (green) or $syk^{-/-}$ DT40 cells expressing wild-type Syk (black) or Syk M442A S505A (gray) (*D*). Calcium flux experiments were done twice, with a $n = 2$ per experiment. Error bars represent SD of the mean.

Syk. We found that expression of Syk MASA was similar, although surprisingly slightly less than wild-type Syk (data not shown). Therefore, we could conclude that Syk MASA has the potential to completely restore Fc-mediated phagocytosis in $syk^{-/-}$ macrophages. Our inability to detect this previously was most likely due to slight differences in the purity of and the Syk expression in the sorted cells used in the colorimetric and microscopic assays for Fc-mediated phagocytosis.

In addition to testing our inhibitors in phagocytosis assays, we also performed a dose response to 2,3-DMB-PP1 using calcium flux as a readout. Our results indicate that calcium flux was completely abolished even at 1 μM 2,3-DMB-PP1, the lowest concentration of inhibitor that we tested (Fig. 8*D*). We failed to see an effect of 2,3-DMB-PP1 on wild-type DT40 cells or $syk^{-/-}$ DT40 cells reconstituted with wild-type Syk.

The 2,3-DMB-PP1 specifically inhibits signaling in Syk MASA-transfected DT40 cells

Having established that 2,3-DMB-PP1 is able to specifically inhibit Fc-mediated phagocytosis and calcium flux in cells expressing Syk MASA, but not wild-type Syk, we wanted to determine whether it could similarly inhibit phospho-ERK and phospho-Syk activation in DT40 cells expressing Syk MASA. DT40 cells stably expressing either wild-type Syk or Syk MASA were rested in serum-free medium, preincubated with DMSO or 5 μM 2,3-DMB-PP1, and then stimulated for 10 min with anti-IgM before cell lysis. Cell lysates were subsequently probed for ptyr, pERK, Syk, and ERK2. The 2,3-DMB-PP1 treatment of $syk^{-/-}$ DT40 cells expressing Syk MASA resulted in decreases in ptyr, pSyk, and pERK after 10 min of anti-IgM stimulation (Fig. 9, *A* and *B*). The two bands present in some of the lanes of the ERK2 blot represent the phosphorylated and unphosphorylated forms of the protein (Fig. 9*C*). When quantitated, these differences were found to be a ~2-fold decrease in phospho-Syk and a ~3-fold decrease in phospho-ERK. There did appear to be a nonspecific effect of 2,3-DMB-PP1 on $syk^{-/-}$ DT40 cells that had been reconstituted with wild-type Syk; however, it was substantially less than that observed for $syk^{-/-}$ DT40 cells reconstituted with Syk MASA. These data indicate that in addition to phagocytosis and calcium flux, 2,3-DMB-PP1 was able to selectively block the signaling responses in DT40 cells expressing the Syk MASA mutant.



pSyk	1	12.73	9.23	1	10.22	5.13
pERK	1	39.73	32.36	1	61.14	22.57

FIGURE 9. The 2,3-DMB-PP1 inhibits phosphorylation of signaling proteins in $syk^{-/-}$ B cells expressing Syk M442A S505A. Effects of 2,3-DMB-PP1 on the phosphorylation of a variety of signaling proteins, including Syk (*A*) and ERK (*B*). Levels of phospho-Syk were assessed by probing a Western blot with anti-ptyr (green) and anti-Syk (red) and looking for areas of overlap (yellow) (*A*). Fold increases in phospho-Syk were normalized to total Syk. Fold increases in pERK were normalized to ERK2 (*C*). Quantitation is displayed in the chart below the blots. These experiments were conducted three times.

Discussion

Because phosphorylation events in a cell are critical for inter- and intracellular communication, and because these signaling networks are often deregulated in disease states, much effort has gone into developing kinase inhibitors in the hopes of treating diseases such as cancer and autoimmune and inflammatory disorders (26). Unfortunately, the high structural conservation among kinases has posed a challenge in generating an inhibitor that is specific for a particular kinase (5). To circumvent this obstacle, Shokat et al. pioneered a chemical-genetic approach to be able to study the function of individual kinases in an experimental system. This method takes advantage of the fact that most kinases contain a bulky amino acid (i.e., methionine or threonine), referred to as the gatekeeper residue, which governs entrance into a hydrophobic pocket in the ATP binding site. By mutating the gatekeeper residue to a smaller residue, one could render a kinase susceptible to an engineered inhibitor that would be designed to fit in the enlarged ATP-binding pocket.

Levin et al. (27) recently applied the chemical genetic strategy to the study of a Syk-family kinase, ZAP70, because ZAP70-deficient mice are void of functional T cells. We have now extended this approach further to the other member of the Syk-family kinases, Syk kinase. Syk-deficient mice are embryonic lethal due to a fusion of the vasculature with the lymphatic system (21), which makes study of the kinase in hematopoietic cells difficult (due to the need to generate fetal liver chimeras) and studies in nonhematopoietic cells impossible. Moreover, loss of Syk could affect the development of cell populations (for example, the B cell lineage), thus making studies of some mature cell types problematic. For these reasons, we became interested in making an ASKA of Syk that would allow us to probe further into the immunological roles of Syk.

Mutation of the gatekeeper amino acid is often a functionally silent mutation; however, there are instances in which mutation of the gatekeeper can alter the structure of the enzyme and render it inactive. Under these circumstances, a second-site mutation, identified through random mutagenesis or rational sequence analysis, can be introduced to restabilize the kinase and salvage the approach (7). In addition to providing stability to the kinase, a second-site mutation can also be used to generate more space and hydrophobicity in the gatekeeper pocket of the enzyme, thus leading to more efficient inhibition of the kinase by PP1 analogues. Upon examination of the structure of Syk, we noticed that a hydrophilic residue, S505, sits in close proximity to its hydrophobic ATP-binding pocket. In comparison, Src (28) and Cdk2, two distinct kinases that both tolerated their respective gatekeeper mutations, contain an alanine at the analogous position of S505 in Syk. Thus, we predicted that mutation of residue S505 to an alanine might confer stability and/or enhance inhibition of this kinase. Our studies found that the S505A mutation accomplished both of these things. Specifically, the magnitude and duration of the calcium flux observed in Syk-deficient DT40 cells reconstituted with wild-type Syk were equaled only by those reconstituted with Syk MASA, but not Syk MA or Syk MASG. Furthermore, in data not shown, it appeared that Syk MASA was slightly more inhibited with 2,3-DMB-PP1 than was Syk MA.

To assess the activity of the Syk ASKA mutants, we used functional and biochemical assays in primary bone marrow-derived macrophages and DT40 B cells. FcγR-dependent phagocytosis in macrophages (18) and phosphorylation events (15, 29, 30) and calcium flux (25) following BCR ligation are known to require Syk activity. Results from our studies approximated the activity of Syk M442A S505A to be 80–100% of wild type. It was clear that whereas Fc-mediated phagocytosis (as assessed using the FACS-based method of FcγR-dependent phagocytosis) could be fully restored in $syk^{-/-}$ macrophages expressing Syk MASA, signaling parameters were often only partially restored to 80–85% of wild type. This may be due to the fact that different thresholds of Syk activity are required to mediate different cellular responses. Although reconstitution of Fc-mediated phagocytosis did not reveal functional differences between Syk MA and Syk MASA, calcium flux experiments did. Only the double mutant was capable of mediating a calcium flux that resembled that which was observed in wild-type DT40 cells, or $syk^{-/-}$ cells reconstituted with wild-type Syk. This suggested that the secondary mutation of S505 to an alanine was necessary and sufficient to stabilize the Syk MA mutant.

Having identified an ASKA of Syk that retained wild-type functions, we next sought to identify an inhibitor that can specifically block the function of the mutant kinase without having an effect on the wild-type form of Syk. After screening 15 compounds, 2,3-DMB-PP1 was found to be the most efficacious at accomplishing this. The 2,3-DMB-PP1 blocked both signaling reactions (Syk and ERK activation), as well as phagocytosis and calcium flux only in cells expressing the Syk MASA mutant allele.

In addition to observing differences in the ability of Syk MASA to reconstitute various Syk-dependent activities, we also observed differences in the susceptibility of these activities to inhibition with 2,3-DMB-PP1. Specifically, calcium flux was completely abolished with a 1 μM dose of 2,3-DMB-PP1, whereas a dose of 5 μM was required to fully prevent phagocytosis of opsonized SRBC. The heightened sensitivity of calcium flux to inhibition by small molecules has previously been observed (2, 27). As before, this may partially be explained by considering the threshold of signaling required for a particular cellular activity (2). If only small amounts of Syk activity are required to mediate a cellular output, then a great deal of inhibition would be required to fully block that process. Conversely, if a large amount of Syk activity is required to mediate a cellular output, then even partial inhibition may reduce the activity of the kinase below the required threshold.

Following from this hypothesis and examining the data presented in this manuscript, one would predict that Fc-mediated phagocytosis requires a low threshold of Syk activity, whereas calcium flux requires a high threshold. However, this hypothesis

does not fully explain the observation that Syk MASA fails to fully reconstitute the tyrosine phosphorylation seen in wild-type cells, and yet, it is only partially inhibited following treatment with 2,3-DMB-PP1. One hypothesis to explain these results is that the protein scaffold of Syk may play important noncatalytic roles that contribute to phosphorylation. This, plus possible redundant players to Syk, may explain why the incomplete restoration and blockade of Syk MASA signaling parameters may still allow for the restoration and blockade of functional parameters such as calcium release and phagocytosis.

Oh et al. (31) recently reported the generation of an ASKA of Syk that was created for the purpose of understanding the role of Syk in B cell function. In this study, we report the generation of a second ASKA of Syk that differs from theirs not only in terms of the inhibitor used, but also in the location of the secondary mutation. Although both studies rely on modifying Syk at secondary sites to mimic the sequence and structure of Src, a kinase that was tolerant to a gatekeeper mutation (28), distinct secondary sites were chosen to achieve this. Oh et al. (31) conducted their studies solely in DT40 chicken B cells and monitored phosphorylation events and activation of NF-κB and NF-AT. Our work not only includes assays using DT40 cells, but also includes studies with primary bone marrow-derived macrophages, in which signaling from a different Syk-dependent receptor system was examined (i.e., FcγR-mediated phagocytosis). Together, these studies have established that generating ASKA of Syk is a valid approach for studying Syk function.

Our interest in generating an ASKA of Syk extends beyond the power that it gives us to tease out the functions of Syk in vitro. We ultimately chose to develop this system for the power is gives us to examine the function of Syk in vivo. We are currently generating a knock-in mouse that will express Syk MASA in place of wild-type Syk. We predict, based on the ability of Syk MASA to restore normal BCR and FcγR signaling responses, that the Syk MASA animal will develop normally in utero, but its cells will be sensitive to inhibition with 2,3-DMB-PP1. We firmly believe that a mouse expressing Syk MASA in place of wild-type Syk will be a powerful tool for elucidating the biological roles of Syk in murine models of infectious and autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

References

1. Manning, G., D. B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam. 2002. The protein kinase complement of the human genome. *Science* 298: 1912–1934.
2. Knight, Z. A., and K. M. Shokat. 2005. Features of selective kinase inhibitors. *Chem. Biol.* 12: 621–637.
3. Sicheri, F., I. Moarefi, and J. Kuriyan. 1997. Crystal structure of the Src family tyrosine kinase Hck. *Nature* 385: 602–609.
4. Davies, S. P., H. Reddy, M. Caivano, and P. Cohen. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 351: 95–105.
5. Bishop, A. C., K. Shah, Y. Liu, L. Witucki, C. Kung, and K. M. Shokat. 1998. Design of allele-specific inhibitors to probe protein kinase signaling. *Curr. Biol.* 8: 257–266.
6. Weiss, E. L., A. C. Bishop, K. M. Shokat, and D. G. Drubin. 2000. Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. *Nat. Cell Biol.* 2: 677–685.
7. Zhang, C., D. M. Kenski, J. L. Paulson, A. Bonshtien, G. Sessa, J. V. Cross, D. J. Templeton, and K. M. Shokat. 2005. A second-site suppressor strategy for chemical genetic analysis of diverse protein kinases. *Nat. Methods* 2: 435–441.
8. Denzel, A., K. J. Hare, C. Zhang, K. Shokat, E. J. Jenkinson, G. Anderson, and A. Hayday. 2003. Cutting edge: a chemical genetic system for the analysis of kinases regulating T cell development. *J. Immunol.* 171: 519–523.
9. Levin, S. E., C. Zhang, T. A. Kadlecek, K. M. Shokat, and A. Weiss. 2008. Inhibition of Zap-70 kinase activity via an analog-sensitive allele blocks T cell receptor and CD28 superagonist signaling. *J. Biol. Chem.* 283: 15419–15430.
10. Chen, X., H. Ye, R. Kuruvilla, N. Ramanan, K. W. Scangos, C. Zhang, N. M. Johnson, P. M. England, K. M. Shokat, and D. D. Ginty. 2005. A chemical-genetic approach to studying neurotrophin signaling. *Neuron* 46: 13–21.
11. Holt, J. R., S. K. Gillespie, D. W. Provance, K. Shah, K. M. Shokat, D. P. Corey, J. A. Mercer, and P. G. Gillespie. 2002. A chemical-genetic strategy implicates myosin-1c in adaptation by hair cells. *Cell* 108: 371–381.
12. Asahi, M., T. Taniguchi, E. Hashimoto, T. Inazu, H. Maeda, and H. Yamamura. 1993. Activation of protein-tyrosine kinase p72syk with concanavalin A in polymorphonuclear neutrophils. *J. Biol. Chem.* 268: 23334–23338.
13. Benhamou, M., N. J. Ryba, H. Kihara, H. Nishikata, and R. P. Siraganian. 1993. Protein-tyrosine kinase p72syk in high affinity IgE receptor signaling: identification as a component of pp72 and association with the receptor γ chain after receptor aggregation. *J. Biol. Chem.* 268: 23318–23324.
14. Greenberg, S., P. Chang, and S. C. Silverstein. 1994. Tyrosine phosphorylation of the γ subunit of Fcγ receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. *J. Biol. Chem.* 269: 3897–3902.
15. Yamada, T., T. Taniguchi, C. Yang, S. Yasue, H. Saito, and H. Yamamura. 1993. Association with B-cell-antigen receptor with protein-tyrosine kinase p72syk and activation by engagement of membrane IgM. *Eur. J. Biochem.* 213: 455–459.
16. Fanger, N. A., D. Voigtlaender, C. Liu, S. Swink, K. Wardwell, J. Fisher, R. F. Graziano, L. C. Pfefferkorn, and P. M. Guyre. 1997. Characterization of expression, cytokine regulation, and effector function of the high affinity IgG receptor FcγRI (CD64) expressed on human blood dendritic cells. *J. Immunol.* 158: 3090–3098.
17. Yanagi, S., R. Inatome, T. Takano, and H. Yamamura. 2001. Syk expression and novel function in a wide variety of tissues. *Biochem. Biophys. Res. Commun.* 288: 495–498.
18. Crowley, M. T., P. S. Costello, C. J. Fitzer-Attas, M. Turner, F. Meng, C. Lowell, V. L. Tybulewicz, and A. L. DeFranco. 1997. A critical role for Syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages. *J. Exp. Med.* 186: 1027–1039.
19. Pleiman, C. M., D. D'Ambrosio, and J. C. Cambier. 1994. The B-cell antigen receptor complex: structure and signal transduction. *Immunol. Today* 15: 393–399.
20. Mocsai, A., C. L. Abram, Z. Jakus, Y. Hu, L. L. Lanier, and C. A. Lowell. 2006. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 7: 1326–1333.
21. Cheng, A. M., B. Rowley, W. Pao, A. Hayday, J. B. Bolen, and T. Pawson. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature* 378: 303–306.
22. Mocsai, A., M. Zhou, F. Meng, V. L. Tybulewicz, and C. A. Lowell. 2002. Syk is required for integrin signaling in neutrophils. *Immunity* 16: 547–558.
23. Jungi, T. W. 1985. A rapid and sensitive method allowing photometric determination of erythrophagocytosis by mononuclear phagocytes. *J. Immunol. Methods* 82: 141–153.
24. Bishop, A. C., C. Y. Kung, K. Shah, L. Witucki, K. M. Shokat, and Y. Liu. 1999. Generation of monospecific nanomolar tyrosine kinase inhibitors via a chemical genetic approach. *J. Am. Chem. Soc.* 121: 627–631.
25. Takata, M., H. Sabe, A. Hata, T. Inazu, Y. Homma, T. Nukada, H. Yamamura, and T. Kurosaki. 1994. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. *EMBO J.* 13: 1341–1349.
26. Rokosz, L. L., J. R. Beasley, C. D. Carroll, T. Lin, J. Zhao, K. C. Appell, and M. L. Webb. 2008. Kinase inhibitors as drugs for chronic inflammatory and immunological diseases: progress and challenges. *Exp. Opin. Ther. Targets* 12: 883–903.
27. Levin, S. E., C. Zhang, T. A. Kadlecek, K. M. Shokat, and A. Weiss. 2008. Inhibition of ZAP-70 kinase activity via an analog-sensitive allele blocks T cell receptor and CD28 superagonist signaling. *J. Biol. Chem.* 283: 15419–15430.
28. Bishop, A. C., J. A. Ubersax, D. T. Petsch, D. P. Matheos, N. S. Gray, J. Blethrow, E. Shimizu, J. Z. Tsien, P. G. Schultz, M. D. Rose, J. L. Wood, et al. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* 407: 395–401.
29. Richards, J. D., M. R. Gold, S. L. Hourihane, A. L. DeFranco, and L. Matsuuchi. 1996. Reconstitution of B cell antigen receptor-induced signaling events in a nonlymphoid cell line by expressing the Syk protein-tyrosine kinase. *J. Biol. Chem.* 271: 6458–6466.
30. Qin, S., T. Inazu, M. Takata, T. Kurosaki, Y. Homma, and H. Yamamura. 1996. Cooperation of tyrosine kinases p72syk and p53/56lyn regulates calcium mobilization in chicken B cell oxidant stress signaling. *Eur. J. Biochem.* 236: 443–449.
31. Oh, H., E. Ozkirimli, K. Shah, M. L. Harrison, and R. L. Geahlen. 2007. Generation of an analog-sensitive Syk tyrosine kinase for the study of signaling dynamics from the B cell antigen receptor. *J. Biol. Chem.* 282: 33760–33768.