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# Protein-tyrosine Phosphatase Shp2 Positively Regulates Macrophage Oxidative Burst\*

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**Background:** Innate immune cell oxidative burst is needed to combat pathogens.

**Results:** Loss of Shp2 phosphatase reduces, whereas increased Shp2 phosphatase function enhances, ROS production.

**Conclusion:** The Shp2 phosphatase domain is specifically required for optimal oxidative burst in macrophages.

**Significance:** Humans bearing aberrancies of Shp2 phosphatase or of Shp2-containing signaling pathways may be prone to impaired or excessive ROS production.

Macrophages are vital to innate immunity and express pattern recognition receptors and integrins for the rapid detection of invading pathogens. Stimulation of Dectin-1 and complement receptor 3 (CR3) activates Erk- and Akt-dependent production of reactive oxygen species (ROS). Shp2, a protein-tyrosine phosphatase encoded by *Ptpn11*, promotes activation of Ras-Erk and PI3K-Akt and is crucial for hematopoietic cell function; however, no studies have examined Shp2 function in particulate-stimulated ROS production. Maximal Dectin-1-stimulated ROS production corresponded kinetically to maximal Shp2 and Erk phosphorylation. Bone marrow-derived macrophages (BMMs) from mice with a conditionally deleted allele of *Ptpn11* (Shp2<sup>fllox/fllox</sup>;Mx1Cre+) produced significantly lower ROS levels compared with control BMMs. Although YFP-tagged phosphatase dead Shp2-C463A was strongly recruited to the early phagosome, its expression inhibited Dectin-1- and CR3-stimulated phospho-Erk and ROS levels, placing Shp2 phosphatase function and Erk activation upstream of ROS production. Further, BMMs expressing gain of function Shp2-D61Y or Shp2-E76K and peritoneal exudate macrophages from Shp2D61Y/+;Mx1Cre+ mice produced significantly elevated levels of Dectin-1- and CR3-stimulated ROS, which was reduced by pharmacologic inhibition of Erk. SIRP $\alpha$  (signal regulatory protein  $\alpha$ ) is a myeloid inhibitory immunoreceptor that requires tyrosine phosphorylation to exert its inhibitory effect. YFP-Shp2C463A-expressing cells have elevated phospho-SIRP $\alpha$  levels and an increased Shp2-SIRP $\alpha$  interaction compared with

YFP-WT Shp2-expressing cells. Collectively, these findings indicate that Shp2 phosphatase function positively regulates Dectin-1- and CR3-stimulated ROS production in macrophages by dephosphorylating and thus mitigating the inhibitory function of SIRP $\alpha$  and by promoting Erk activation.

Macrophages are phagocytic cells that function as the body's global first line defense against invading pathogens. Upon interaction with pathogen-producing stimuli, macrophages internalize large particulate microorganisms and generate microbicidal reactive oxygen species (ROS)<sup>3</sup> produced by activated NADPH oxidase (1). NADPH oxidase is composed of membrane-integrated gp91<sup>phox</sup> and p22<sup>phox</sup>, as well as the four cytosolic components p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, and Rac2 (2, 3). Individuals with chronic granulomatous disease have inherited germ line mutations within variable components of the NADPH oxidase complex and suffer from recurrent, life-threatening bacterial and fungal infections, highlighting the imperative nature of competent ROS production by the innate immunity (4, 5).

Shp2, a protein-tyrosine phosphatase encoded by the *PTPN11* gene, promotes activation of Ras-Erk signaling and plays an essential role in hematopoietic cell development (6, 7). Genetic disruption of murine *Ptpn11* within hematopoietic lineages leads to rapid loss of blood cell production of all lineages (8, 9). In humans, gain of function *PTPN11* mutations are commonly found in children with Noonan syndrome and juvenile myelomonocytic leukemia (10, 11). Although no *PTPN11* mutations have been found to be associated with clinical immune deficiency, Shp2 is a critical signaling component of leptin receptor-dependent protection against the parasitic

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<sup>3</sup> The abbreviations used are: ROS, reactive oxygen species; BMM, bone marrow-derived macrophage; GOF, gain of function; PEM, peritoneal exudate macrophage; SOZ, serum opsonized zymosan; IMDM, Iscove's modified Dulbecco's medium; DPI, diphenyleneiodonium chloride; IP, immunoprecipitation.

pathogen *Entamoeba histolytica* (12), and children bearing germ line loss of function *LEPR* mutations are susceptible to respiratory infections (13). Further, previous studies found that Shp2 regulates the phosphorylation of transcription factors HoxA10 and ICSBP, leading to transcriptional repression of the NADPH oxidase components gp91<sup>phox</sup> and p67<sup>phox</sup> and preventing myeloid terminal differentiation (14, 15); however, no studies have examined the function of Shp2 phosphatase in ROS production in terminally differentiated macrophages or neutrophils, which may reveal a novel role for Shp2 in innate immunity and ROS production.

Macrophages are capable of detecting and responding to pathogen-derived molecules such as fungal glucans and lipopolysaccharides, because they express cell surface pattern recognition receptors such as C-type lectins. Dectin-1 is a C-type lectin expressed on macrophages that responds to  $\beta$ -glucan-containing particles derived from fungal cell walls and stimulates Src- and Syk-dependent signaling (16). Dectin-1 stimulation results in activation of the Ras-Erk pathway, production of microbicidal ROS, and induction of expression of the inflammatory cytokines TNF $\alpha$  and IL6. In humans, loss of function mutations in *DECTIN-1* confer a state of increased susceptibility to mucocutaneous *Candida albicans* and invasive aspergillosis (17, 18).

Based on the known high expression of Shp2 in macrophages and its well defined role as a positive regulator of the Ras-Erk pathway, we hypothesized that Shp2 promotes normal innate immunity by positively up-regulating particulate-stimulated NADPH oxidase activation and abrupt production of ROS, known as oxidative burst. To address this hypothesis, we examined the correlation of Shp2 activation to peak ROS production in zymosan-stimulated peritoneal exudate macrophages (PEMs) and examined the putative placement of Shp2 in the Dectin-1-stimulated pathway employing genetic studies and pharmacologic studies using the Syk inhibitor R406 and the Erk inhibitor SCH772984. Genetic disruption of *Ptpn11* resulted in reduced macrophage ROS production in response to both zymosan (Dectin-1 stimulation) and serum opsonized zymosan (SOZ, complement receptor 3 stimulation), indicating a positive function of Shp2 in oxidative burst. Structure-function studies using various Shp2 loss of function and gain of function constructs indicated that the phosphatase function of Shp2 is specifically required for positive regulation of particulate-stimulated oxidative burst. Mechanistic studies demonstrated that Shp2 exerts its positive effect on ROS generation by dephosphorylating the myeloid inhibitory immunoreceptor, SIRP $\alpha$  (signal regulatory protein  $\alpha$ ), and by promoting Erk activation.

## EXPERIMENTAL PROCEDURES

**Reagents**—Chemicals were purchased from Sigma-Aldrich unless otherwise stated. PBS, pH 7.2, penicillin/streptomycin, neomycin, IMDM, and DMEM were from Invitrogen; FCS was from HyClone Laboratory (Logan, UT). The ECL detection kit came from Pierce. Rabbit polyclonal antibody against p40<sup>phox</sup> and mouse monoclonal antibody T-Syk were from Upstate Biotechnology (Lake Placid, NY), rabbit polyclonal antibody against p47<sup>phox</sup> was from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal antibody against p67<sup>phox</sup> was from

BD Biosciences. Rabbit polyclonal and mouse monoclonal antibodies against Shp2 were from Santa Cruz Biotechnology (catalog no. SC-280) and BD Biosciences (catalog no. 610621), respectively. Anti-SIRP $\alpha$  polyclonal antibody was from Abcam (catalog no. 53721), and mouse dectin-1/CLE7A antibody (clone 218838) was from R&D Systems, Inc. (Minneapolis, MN). Anti-phospho-SIRP $\alpha$  was generated as previously described (19). Anti-gp91<sup>phox</sup> mAb 54.1 and anti-p22<sup>phox</sup> mAb NS2 were kindly provided by A. J. Jesaitis (Montana State University) (20). All the other antibodies, including anti-phospho-antibodies, were obtained from Cell Signaling Technology (Beverly, MA) unless otherwise stated. Zymosan A from *Saccharomyces cerevisiae* was from Sigma (Z-4250). The FITC-labeled zymosan particles were from Invitrogen. Phycoerythrin-Mac-1 and allophycocyanin-F4/80 were from BD Pharmingen.

**Animal Husbandry**—Mice bearing a conditional gain of function (GOF) *Ptpn11* allele (*LSL-Shp2*<sup>D61Y/+</sup>) or a conditional floxed *Ptpn11* allele (*Shp2*<sup>lox/flox</sup>) have been described (9, 21, 22) and were crossed with mice bearing the Mx1Cre transgene to generate experimental (Shp2D61Y;Mx1Cre+ or Shp2<sup>fl/fl</sup>;Mx1Cre+) and negative control (Shp2D61Y;Mx1Cre− or Shp2<sup>fl/fl</sup>;Mx1Cre−) animals. All animals received three intraperitoneal injections with 300  $\mu$ g of poly(I·C) (GE Healthcare) to induce *Ptpn11* recombination. All mice were maintained under specific pathogen-free conditions at the Indiana University Laboratory Animal Research Center (Indianapolis, IN), and this study was approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

**Plasmid Construction**—The cDNAs encoding WT Shp2 or Shp2-R32K and Shp2-C463A mutants were cloned into EcoRI and KpnI sites of pEYFP-N1 (Clontech) to generate YFP-tagged Shp2 constructs, and the constructs were confirmed by sequencing. The YFP-tagged Shp2 cDNAs were then ligated into pMSCV (Clontech) for use in generation of retroviral supernatants. Preparation and characterization of the pMIEG3-WT Shp2, Shp2-D61Y, and Shp2-E76K was described previously (23).

**Retroviral Transduction of Bone Marrow and Macrophage Differentiation**—Retroviral transduction of murine bone marrow low density mononuclear cells (C57Bl/6 background) with MSCV-WT Shp2-YFP, Shp2-R32K-YFP, or Shp2-C463A-YFP or with MIEG3-WT Shp2, Shp2-D61Y, or Shp2-E76K was performed as described (23, 24). Transduced cells were sorted by fluorescence-activated cell sorting (FACSCalibur; BD Biosciences) prior to macrophage differentiation, as described (23). Activity, immunostaining, and live images were analyzed 6–7 days after macrophage differentiation.

**PEM Preparation**—Eight weeks after poly(I·C) treatment, Shp2D61Y;Mx1Cre+ and negative control Shp2D61Y;Mx1Cre− animals were injected with 1 ml of 3% thioglycollate to induce peritoneal inflammation. 72 h later, peritoneal cells were harvested by lavage with PBS, and macrophages were isolated by culturing cells at  $1 \times 10^6$  cells/ml in IMDM with 20% heat-inactivated serum and 2% penicillin-streptomycin in a tissue culture dish for 2 h and then removing nonadherent cells (25). After overnight incubation in IMDM with 20% heat-inactivated serum and 2% penicillin-streptomycin, PEMs were used to perform functional and biochemical analyses.

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**Preparation of Particulate Stimuli**—Zymosan and SOZ A particles (Sigma; Z-4250) were prepared as previously described (26–28). A synchronized phagocytosis assay was used for zymosan- or SOZ-induced NADPH oxidase activity assays (29, 30). Briefly, macrophages in 200  $\mu$ l of PBSG (PBS plus 0.9 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 20 mM dextrose) were incubated on ice for 5 min in 50  $\mu$ M luminol and 20 units/ml HRP, and then 25  $\mu$ l of cold zymosan or SOZ (final concentration, 400  $\mu$ g/ml) were added. Cells and particles were spun at 1200 rpm for 5 min at 4 °C and then immediately placed at 37 °C in the luminometer.

**ROS Detected by Chemiluminescence in Intact Cells**—ROS production during synchronized phagocytosis of SOZ or zymosan stimulation was measured in the presence of 20  $\mu$ M luminol with 20 units/ml HRP (24, 30, 31). An Lmax microplate luminometer (Molecular Devices, Sunnyvale, CA) was used to record luminescence as previously described (24, 26). For some experiments, macrophages were preincubated at 37 °C with Syk inhibitor, R406, NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI), or Erk inhibitor for 30 min.

**Phagocytosis Assays**—Synchronized phagocytosis was performed as previously described (24). Briefly, 2  $\times$  10<sup>6</sup> macrophages were washed with PBS and resuspended in 3 ml of PBSG, added to 6-well plate, and kept on ice for 5 min, followed by adding 300  $\mu$ l of cold SOZ or zymosan (4.4  $\mu$ g/ $\mu$ l in PBSG) for a final concentration of 400  $\mu$ g/ml. Plates were immediately centrifuged at 1200 rpm for 5 min at 4 °C and then transferred to 37 °C for indicated time. For biochemical studies, cells were washed in 3  $\times$  PBS, and 60  $\mu$ l of lysis buffer was added to each well. For the phagocytic assays, the macrophages were plated at 0.5  $\times$  10<sup>6</sup> cells/well in 12-well plates in macrophage differentiation medium incubated at 37 °C for 24 h. After washing, FITC-labeled zymosan particles (100  $\mu$ g/ml) (Molecular Probes, Invitrogen) were added to the cells, and cells were incubated at 37 °C for indicated time. The cells were then put on ice and washed thoroughly to remove unbound particles with PBS. The macrophages were detached and analyzed by flow cytometry immediately.

**Western Blots**—Macrophages were lysed in lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 100 mM NaF, 10 mM sodium pryophosphate, 1 mM PMSF, 1  $\times$  protease inhibitor mixture set I (Calbiochem), 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.1 mM ZnCl<sub>2</sub> (32, 33). 10  $\mu$ g of protein lysate was subjected to SDS-PAGE and immunoblotting using ECL detection (24, 26). YFP expression was also analyzed by flow cytometry (FACSCalibur; BD Biosciences) (24, 26).

**Immunoprecipitation (IP)**—100  $\mu$ g of cell lysates were adjusted to a total volume of 1 ml in IP incubation buffer (50 mM Hepes, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.1% BSA, 50 mM NaF, 1 mM PMSF, 1  $\times$  protease inhibitor mixture set I (Calbiochem), 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.1 mM ZnCl<sub>2</sub>). 2  $\mu$ g of anti-Shp2 polyclonal antibody (SC-280; Santa Cruz Biotechnology) was added to the cell lysates and incubated at 4 °C for 1 h, followed by addition of 25  $\mu$ l of protein A/G Plus-agarose (Santa Cruz Biotechnology) and incubated for 2–3 h at 4 °C. The beads was washed three times in buffer (50 mM Hepes, pH 7.4, 1% Triton X-100, 120 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 1  $\times$  protease inhibitor mixture set I (Calbiochem), 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.1 mM ZnCl<sub>2</sub>), resuspended in 25  $\mu$ l of 2  $\times$

SDS-PAGE loading buffer, and boiled for 5 min, and supernatant was subjected to SDS-PAGE and immunoblotting with anti-phospho-SIRP $\alpha$  or anti-Shp2.

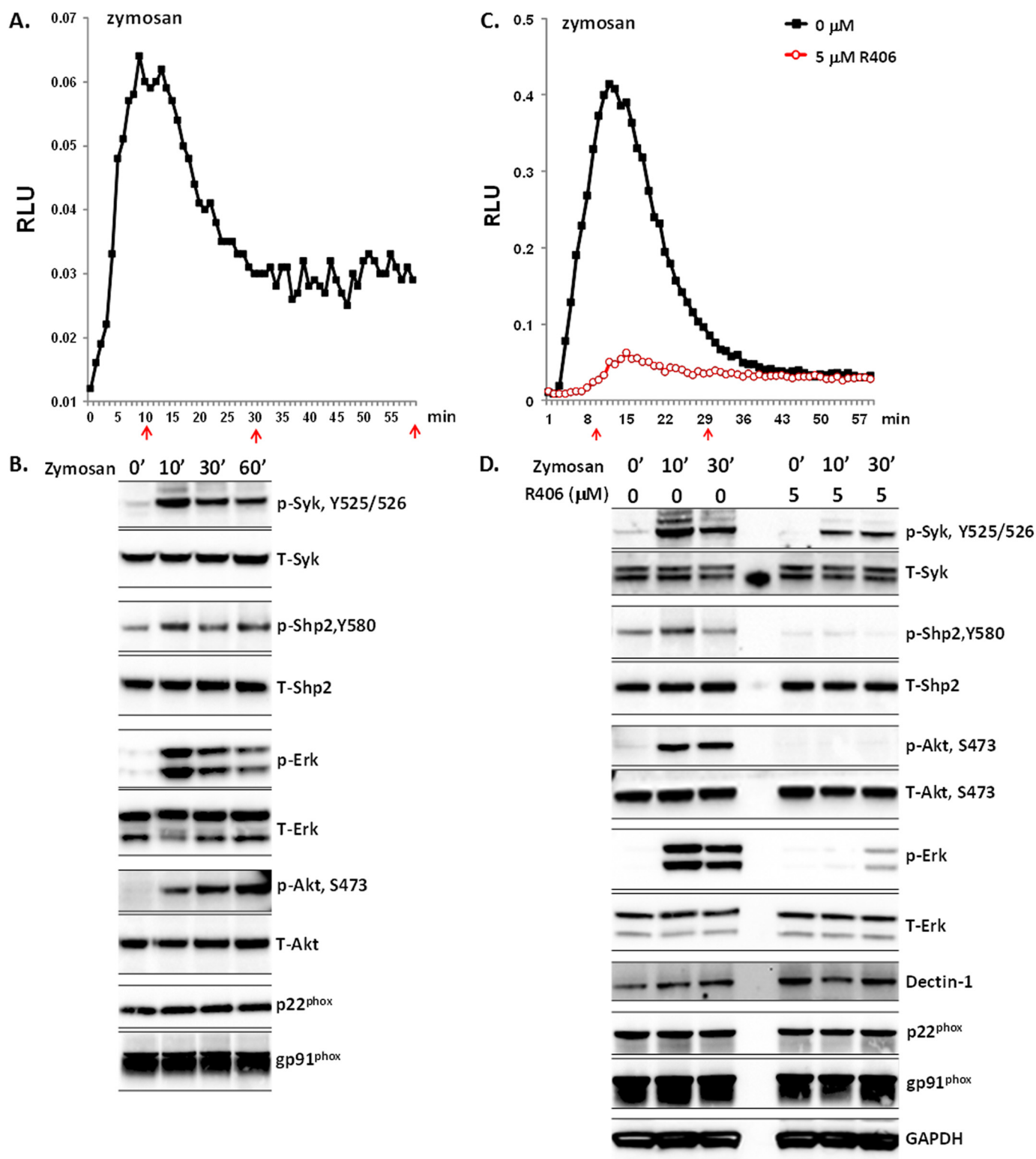
**Immunofluorescence Microscopy**—Immunostaining for Shp2 was performed after synchronized phagocytosis. Briefly, 1  $\times$  10<sup>6</sup> PEMs or BMMs in 2 ml of PBSG were added to coverslip-bottomed dishes (MatTek Cultureware, Ashland, MA) and incubated for 5 min on ice prior to adding 300  $\mu$ l of zymosan or SOZ (final concentration, 400  $\mu$ g/ml). The cells and particles were spun down at 1200 rpm for 5 min at 4 °C and then incubated at 37 °C for 10 min for PEMs or 30 min for BMMs. Phagocytosis was terminated by placing the cells on ice, which were then washed with cold PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS, blocked with 10% goat serum plus 2% BSA in PBS, and immunostained with anti-Shp2 followed by Alexa Fluor 568 goat anti-rabbit IgG. For co-staining, cells were immunostained with anti-SIRP $\alpha$  polyclonal Ab (catalog no. 53721; Abcam) and anti-Shp2 mAb (catalog no. 610621; BD Biosciences) followed by Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG1. Cells were imaged on a spinning disk (CSU10) confocal system mounted on a Nikon TE-2000U inverted microscope with an Ixon air-cooled EMCCD camera (Andor Technology, South Windsor, CT) and a Nikon Plan Apo 100 $\times$  1.4 N.A. objective. Images shown are representative of at least three independent experiments.

**Live Cell Imaging by Confocal Videomicroscopy**—SOZ-induced phagocytosis in WT mouse BMMs expressing YFP-tagged WT Shp2, Shp2-C463A, or Shp2-R32K was filmed using a spinning disk (CSU10) confocal system mounted on a Nikon TE-2000U inverted microscope with an Ixon air-cooled EMCCD camera (Andor Technology, South Windsor, CT) and a Nikon Plan Apo 100 $\times$  1.4 N.A. objective as described previously. All images were analyzed with Metamorph software (Universal Imaging, Downingtown, PA). Each type of experiment was performed on at least three independent occasions. Live images were collected in a single confocal plane (1  $\mu$ m) with 514-nm excitation and 0.3 s of exposure with a time lapse of 10 s.

**Statistical Analysis**—Groups were compared using unpaired, two-tailed, Student's *t* test.

## RESULTS

**Shp2 Functions Downstream of Syk in Dectin-1 Signaling**—Previous work has demonstrated that Dectin-1 stimulation with  $\beta$ -glucan-containing particles leads to Ras-Erk pathway activation and ROS production in a Syk-dependent manner (16). Given that the protein-tyrosine phosphatase Shp2 positively regulates Ras-Erk pathway signaling and is known to be crucial for normal hematopoietic cell development and function (6, 7), we hypothesized that Shp2 functions in the Dectin-1 signaling pathway to promote particulate-stimulated ROS production in macrophages. To address this hypothesis, we used PEMs to examine the kinetics of Shp2 phosphorylation at tyrosine 580 (indicating that Shp2 is in its open, active conformation) (34) in response to zymosan and correlated Shp2 phosphorylation with peak ROS production and activation of the known Dectin-1-responsive signaling molecules, Syk, Erk, and Akt. Zymosan exposure induced maximal ROS production at



**FIGURE 1. Zymosan-induced ROS production and the activation of Dectin-1-stimulated signaling molecules in WT mouse PEMs.** *A*, ROS production in  $0.01 \times 10^6$  PEMs upon zymosan ( $400 \mu\text{g/ml}$ ) stimulation. *B*, immunoblot demonstrating phosphorylation of Syk, Shp2, Erk, and Akt at different time points during zymosan-triggered activation in mouse PEMs. *C*, ROS production in  $0.05 \times 10^6$  PEMs upon zymosan ( $400 \mu\text{g/ml}$ ) stimulation in the absence or presence of the Syk inhibitor R406 ( $5 \mu\text{M}$ ). *D*, immunoblot demonstrating phosphorylation of Syk, Shp2, Erk, and Akt at different time points during zymosan-triggered activation in mouse PEMs in the absence or presence of Syk inhibitor R406 ( $5 \mu\text{M}$ ).  $10 \mu\text{g}$  of cell lysate was used in immunoblotting. Assays were performed in triplicate. *RLU*, relative light units.

10 min poststimulation, and this time point corresponded to maximal activation of Syk, Shp2, and Erk (Fig. 1, *A* and *B*). As expected, Akt was also activated by zymosan stimulation; how-

ever, peak Akt activation was at 1 h poststimulation and after the peak ROS production, suggesting that Akt serves to support sustained ROS production rather than promoting immediate

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and maximal ROS production. Upon treatment with the Syk inhibitor, R406, ROS production was strongly suppressed, which correlated kinetically with reduced Syk, Shp2, Erk, and Akt activation (Fig. 1, C and D). Importantly, R406 treatment did not suppress expression of p22<sup>phox</sup> and gp91<sup>phox</sup> (Fig. 1D), suggesting that reduced ROS is not due to altered expression of NADPH oxidase components but is instead due to reduced NADPH oxidase function.

**Genetic Disruption of Shp2 Results in Reduced Zymosan- and SOZ-stimulated ROS Production**—We next used a mouse model bearing a conditionally floxed allele of *Ptpn11* (Shp2<sup>fl/fl</sup>; Mx1Cre+) (9). Shp2<sup>fl/fl</sup>;Mx1Cre+ and negative control Shp2<sup>fl/fl</sup>; Mx1Cre− mice were treated with 300 μg of poly(I-C) every other day for three doses. 4–6 weeks following poly(I-C) treatment, animals were euthanized followed by isolation of bone marrow low density mononuclear cells, which were cultured in M-CSF to generate BMMs. Based on Mac1 and F4/80 staining, cultured BMMs from the Shp2<sup>fl/fl</sup>;Mx1Cre+ and Shp2<sup>fl/fl</sup>; Mx1Cre− mice demonstrated a similar level of terminal differentiation (Fig. 2A). However, following stimulation with zymosan (Dectin-1 stimulation) or with SOZ (complement receptor 3 stimulation), the Shp2<sup>fl/fl</sup>;Mx1Cre+ BMMs demonstrated a reduced oxidative burst compared with the Shp2<sup>fl/fl</sup>;Mx1Cre− BMMs (Fig. 2, B–D). Because Shp2 had previously been found to regulate FcγR-induced phagocytosis in macrophages (35), we next examined whether the reduced ROS production was due to a global reduction in phagocytosis; however, the phagocytic index was similar for the Shp2<sup>fllox/fllox</sup>;Mx1Cre+ and Shp2<sup>fllox/fllox</sup>;Mx1Cre− BMMs (Fig. 2E). Moreover, we found that expression of various components of the NADPH oxidase complex (p22<sup>phox</sup>, gp91<sup>phox</sup>, p40<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup>) were expressed at similar levels in the Shp2<sup>fl/fl</sup>;Mx1Cre+ and Shp2<sup>fl/fl</sup>; Mx1Cre− BMMs (Fig. 2F), indicating that the reduced ROS seen in the Shp2<sup>fllox/fllox</sup>;Mx1Cre+ BMMs is not due to reduced expression of *phox* proteins.

When examining Dectin-1 signaling, both total Shp2 and phospho-Shp2 levels were reduced in the Shp2<sup>fl/fl</sup>;Mx1Cre+ BMMs, and as anticipated Erk activation was reduced in a correlative fashion (Fig. 2G). Reduced Shp2 expression did not have an effect on overall phospho-Akt levels, suggesting that the Shp2-regulated Erk activation is more relevant to abrupt and maximal ROS production. Consistent with the pharmacologic data using R406, reduced Shp2 expression and activation did not cause reduced activation of Syk. Collectively, these functional and biochemical studies suggest that Shp2 functions downstream of Syk in the Dectin-1-stimulated signaling pathway and positively up-regulates Dectin-1-stimulated ROS levels.

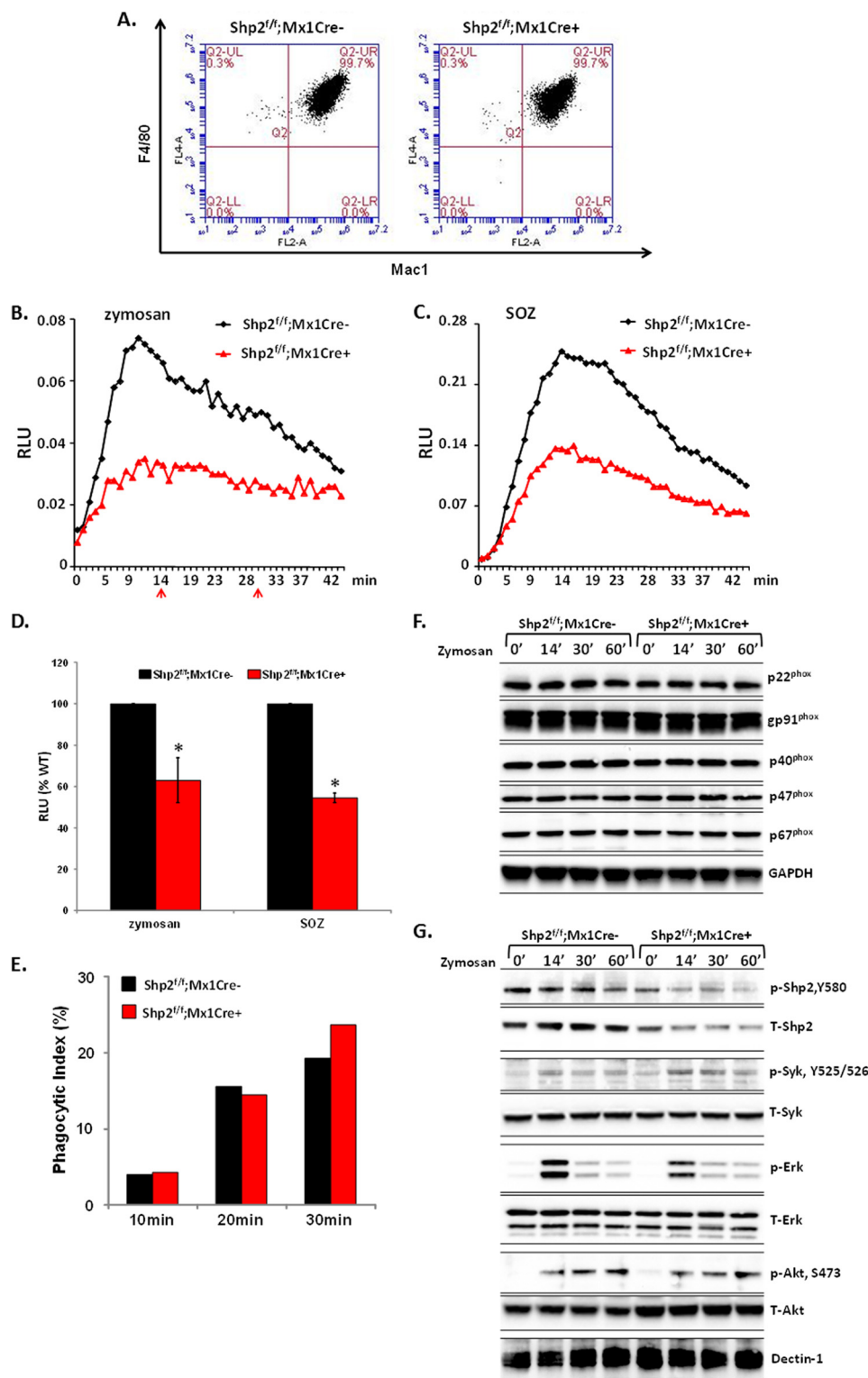
**Shp2 Is Recruited to the Phagosome and Requires Tyrosine Phosphatase Function to Promote ROS Production**—Given the positive role of Shp2 on zymosan- and SOZ-stimulated ROS production, we next imaged SOZ-stimulated BMMs to determine whether Shp2 is recruited to the phagosome membrane, the location of the NADPH oxidase complex. Consistent with the positive functional findings, we found that Shp2 strongly co-localizes with F-actin on the phagosomal cup and early phagosomes (Fig. 3A).

To define the biochemical role of Shp2 in ROS production, we generated YFP-tagged Shp2 constructs bearing mutation of

the N-SH2 (R32K) or phosphatase (C463A) domains (Fig. 3B). These constructs were retrovirally introduced into murine bone marrow low density mononuclear cells followed by sorting to enrich for YFP+ cells and generation of BMMs. Sorted, differentiated macrophages expressed similar levels of each of the Shp2 constructs based on YFP expression (Fig. 3C) and immunoblot analysis (Fig. 3D), expressed similar levels of the NADPH oxidase components, p67<sup>phox</sup>, p22<sup>phox</sup>, and gp91<sup>phox</sup> (Fig. 3D), and differentiated similarly based on Mac1 and F4/80 expression (Fig. 3E). When subjected to zymosan or SOZ stimulation, mutation of either the N-SH2 domain or the phosphatase domain resulted in reduced ROS production (Fig. 3F). Given the functional effect of the N-SH2 domain and phosphatase dead mutants, we next compared the subcellular phagosomal membrane localization of WT Shp2 to the point mutants using time lapse confocal videomicroscopy. The N-SH2 domain mutant was not recruited to the phagosome; however, the phosphatase dead mutant was strongly recruited to the phagosome, even more intensely than WT Shp2 (Fig. 3G). These findings suggest that the phosphatase function of Shp2 specifically is needed for the positive regulation of NADPH oxidase.

**GOF Shp2 Mutants Enhance Zymosan- and SOZ-stimulated ROS Production**—Based on the finding that expression of phosphatase dead Shp2-C463A resulted in reduced ROS production, we reasoned that macrophages expressing juvenile myelomonocytic leukemia-associated GOF Shp2 mutants (10, 23), characterized to have increased phosphatase activity (10, 36), would produce elevated zymosan- and SOZ-stimulated ROS levels. To examine this hypothesis, we retrovirally introduced WT Shp2, Shp2-D61Y, or Shp2-E76K into murine bone marrow low density mononuclear cells followed by *in vitro* differentiation to BMMs. We demonstrated increased total Shp2 expression (compared with vector-transduced cells; Fig. 4A); however, the NADPH oxidase protein components were similar in all cells (Fig. 4A). As anticipated, BMMs retrovirally expressing GOF Shp2-D61Y or GOF Shp2-E76K produced significantly elevated levels of ROS in response to zymosan and SOZ compared with BMMs retrovirally expressing WT Shp2 (Fig. 4, B and C). Additionally, Shp2-D61Y was strongly recruited to the SOZ-stimulated phagosomal cup and early phagosome, similar to that seen with WT Shp2 (Fig. 4D).

To utilize a more physiologic model, we examined ROS production using PEMs from Shp2D61Y;Mx1Cre+ mice, in which Shp2D61Y is under the endogenous control of the *Ptpn11* promoter (21). PEMs collected from the Shp2D61Y;Mx1Cre− and Shp2D61Y;Mx1Cre+ mice demonstrated a similar level of terminal differentiation based on F4/80 and Mac1 staining (Fig. 5A) and expressed similar levels of the NADPH oxidase components, p40<sup>phox</sup>, p22<sup>phox</sup>, and gp91<sup>phox</sup> (Fig. 5E). Consistent with the retrovirally transduced cells, PEMs from Shp2D61Y; Mx1Cre+ mice produced substantially higher levels of zymosan- and SOZ-stimulated ROS compared with PEMs from Shp2D61Y;Mx1Cre− mice (Fig. 5, B–D). Using Shp2D61Y; Mx1Cre+ PEMs, we interrogated the Dectin-1-stimulated signaling pathway to further clarify the contribution of Syk, Shp2, Erk, and Akt activation to the elevated ROS production in response to zymosan. As predicted, we found increased phospho-Shp2 and enhanced Erk activation in the GOF Shp2-ex-

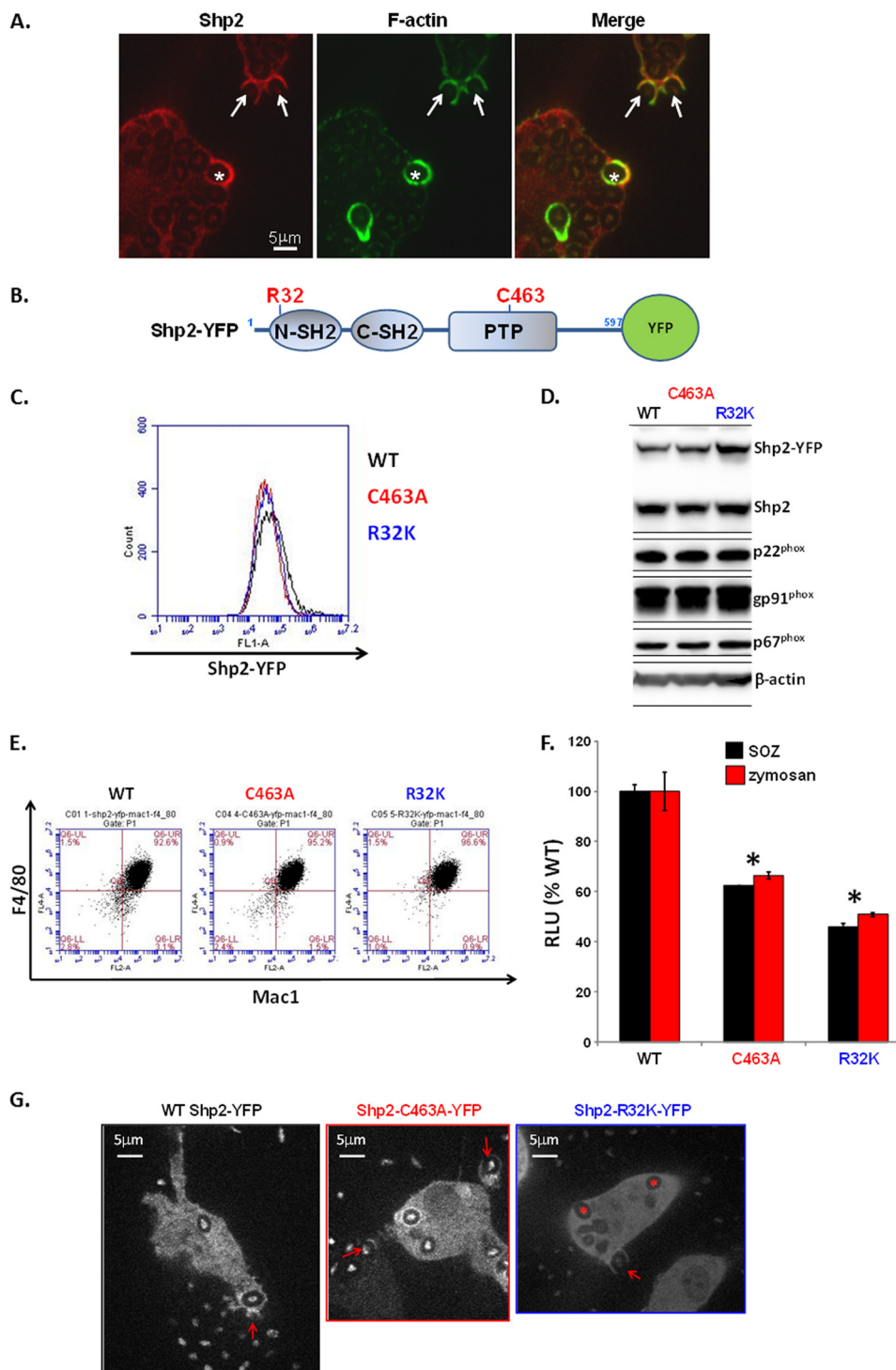


**FIGURE 2. Zymosan- or SOZ-induced ROS production and the activation of Dectin-1-stimulated signaling molecules in *Shp2<sup>fl/fl</sup>;Mx1Cre-* and *Shp2<sup>fl/fl</sup>;Mx1Cre+* mouse BMMs.** *A*, flow cytometry demonstrating the expression level of macrophage membrane markers Mac1 and F4/80 in *Shp2<sup>fl/fl</sup>;Mx1Cre-* and *Shp2<sup>fl/fl</sup>;Mx1Cre+* BMMs. *B* and *C*, ROS production in  $0.05 \times 10^6$  *Shp2<sup>fl/fl</sup>;Mx1Cre-* or *Shp2<sup>fl/fl</sup>;Mx1Cre+* BMMs upon zymosan (*B*) or SOZ (*C*) stimulation. *D*, ROS production compiled from three independent experiments ( $n = 3$ ). \*,  $p < 0.01$  for *Shp2<sup>fl/fl</sup>;Mx1Cre+* compared with *Shp2<sup>fl/fl</sup>;Mx1Cre-* in response to zymosan or SOZ. *E*, phagocytic index. 10,000 cells were analyzed by flow cytometry, and the phagocytic index for each cell type was calculated as described under "Experimental Procedures." *F*, representative immunoblot showing NADPH oxidase *phox* protein expression. *G*, representative immunoblot demonstrating phosphorylation of Shp2, Syk, Erk, and Akt at different time points during zymosan-triggered activation in *Shp2<sup>fl/fl</sup>;Mx1Cre-* and *Shp2<sup>fl/fl</sup>;Mx1Cre+* BMMs. RLU, relative light units.

pressing cells compared with control cells (Fig. 5E). Akt activation was not substantially elevated in the *Shp2D61Y*-expressing cells, suggesting that Shp2 regulation of Erk activation is more

relevant for zymosan-induced maximal ROS production. Interestingly, Erk activation was sustained longer in these cells compared with that observed using WT PEMs (Fig. 1) or the bone

## Functional Contribution of Shp2 to Oxidative Burst



**FIGURE 3. Expression of Shp2 mutants (Shp2-C463A and Shp2-R32K) leads to reduced zymosan- and SOZ-stimulated ROS production.** *A*, localization of endogenous Shp2 upon SOZ stimulation and staining with anti-Shp2 (Santa Cruz, SC-280) and Alexa Fluor 555 goat anti-rabbit IgG in WT BMMs. Alexa Fluor 488 phalloidin was used to stain F-actin, which specifies early phagosomes. *Arrows* and *asterisks* denote Shp2 accumulation on the phagosome cups and early phagosomes, respectively. Representative images from three independent experiments. *B*, structural motifs of YFP-tagged Shp2 constructs. *C*, flow cytometry indicating the expression level of YFP-tagged WT Shp2 (black), Shp2-C463A (red), or Shp2-R32K (blue). *D*, immunoblot demonstrating expression of YFP-tagged Shp2 and endogenous Shp2 in transduced BMMs expressing YFP-tagged WT Shp2, Shp2-C463A, or Shp2-R32K, as well as expression of NADPH oxidase components, gp91<sup>phox</sup>, p22<sup>phox</sup>, and p67<sup>phox</sup>. *E*, flow cytometry demonstrating the expression level of macrophage membrane markers Mac1 and F4/80 in the populations of BMMs expressing YFP-tagged WT Shp2, Shp2-C463A, or Shp2-R32K. *F*, ROS production in BMMs expressing YFP-tagged WT Shp2, Shp2-C463A, or Shp2-R32K compiled from three independent experiments ( $n = 3$ ). \*,  $p < 0.01$  for Shp2-C463A versus WT or Shp2-R32K versus WT in response to SOZ or zymosan. *G*, distribution of YFP-tagged WT Shp2, Shp2-C463A, or Shp2-R32K during SOZ-induced phagocytosis in BMMs. *Arrows* indicate the cup of phagosomes, and *asterisks* indicate the internalized phagosomes. *Bars*, 5  $\mu\text{m}$ . *RLU*, relative light units.



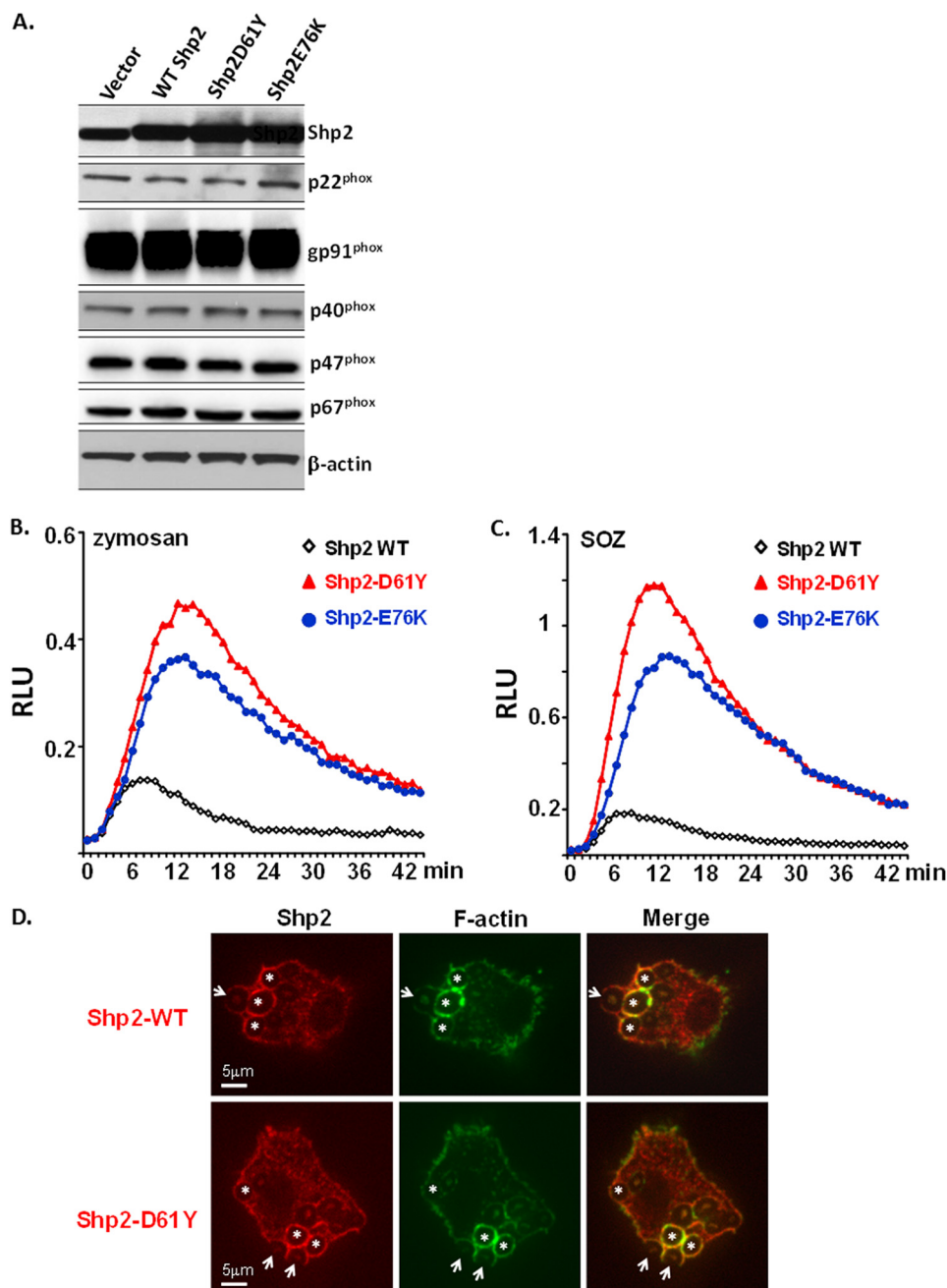


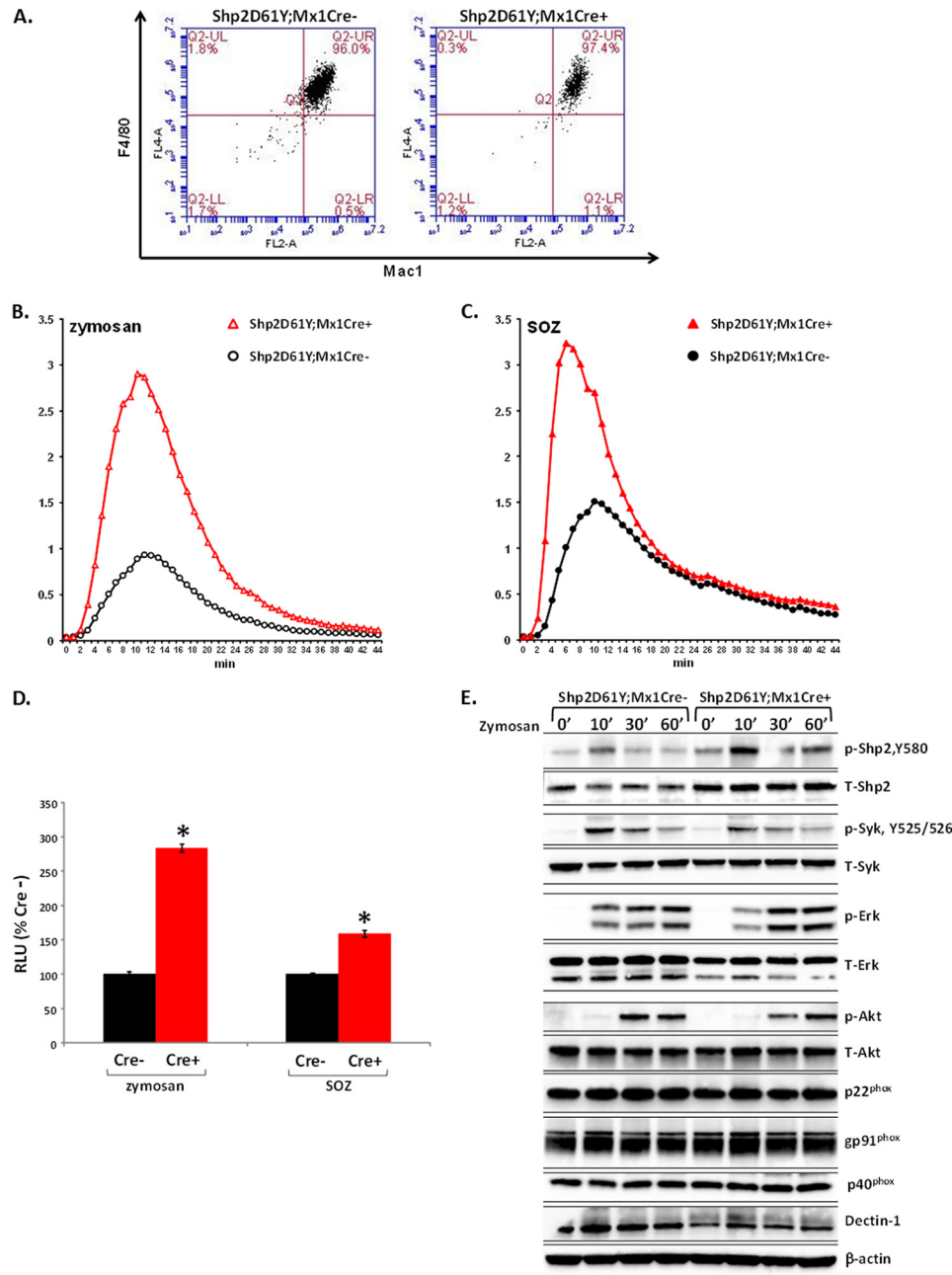
FIGURE 4. ROS generation in Shp2D61Y- and Shp2E76K-expressing BMMs. *A*, immunoblot demonstrating Shp2 and *phox* protein expression in WT BMMs retrovirally expressing empty vector (MIEG3), WT Shp2, or GOF mutants Shp2-D61Y and Shp2-E76K. *B* and *C*, ROS production in  $0.1 \times 10^6$  BMMs expressing WT Shp2 or GOF mutants Shp2-D61Y and Shp2-E76K upon zymosan (*B*) or SOZ (*C*) stimulation. *D*, localization of WT Shp2 and Shp2-D61Y upon SOZ stimulation and immunofluorescent staining with anti-Shp2 (SC-280; Santa Cruz) and Alexa Fluor 555 goat anti-rabbit IgG in WT BMMs. Alexa Fluor 488 phalloidin (Life Technologies) was used to stain F-actin. Arrows and asterisks denote Shp2 accumulation on the phagosome cups and early phagosomes, respectively. Bars, 5  $\mu$ m. RLU, relative light units.

marrow-derived macrophages from Shp2<sup>f/f</sup>;Mx1Cre<sup>-</sup> mice (Fig. 2). This difference in Erk inactivation kinetics might be due to a strain difference of the Shp2D61Y;Mx1Cre animals, which are on a mixed C57Bl/6-Sv129 background rather than a pure C57Bl/6 background. Notwithstanding, these findings are consistent with the need for Erk to be activated for maximal ROS production; however, they suggest that Erk inactivation is not imperative for the return of ROS to baseline levels. Consistent with previous results using R406 (Fig. 1) and Shp2<sup>f/f</sup>;Mx1Cre<sup>+</sup> BMMs (Fig. 2), Syk activation was not altered in the presence of

GOF Shp2D61Y (Fig. 5E), again suggesting that Shp2 functions downstream of Syk in the Dectin-1 signaling pathway. Together, these findings support a model placing Shp2 downstream of Syk in Dectin-1 signaling and indicate that Shp2 phosphatase function positively regulates NADPH oxidase activity via promoting Erk activation.

*Erk Activation Promotes ROS Production in Response to Zymosan Stimulation*—Several previous studies have found that ROS *per se* functions as a signaling molecule to promote Erk activation (37). These studies were performed in nonphagocytic cells, and

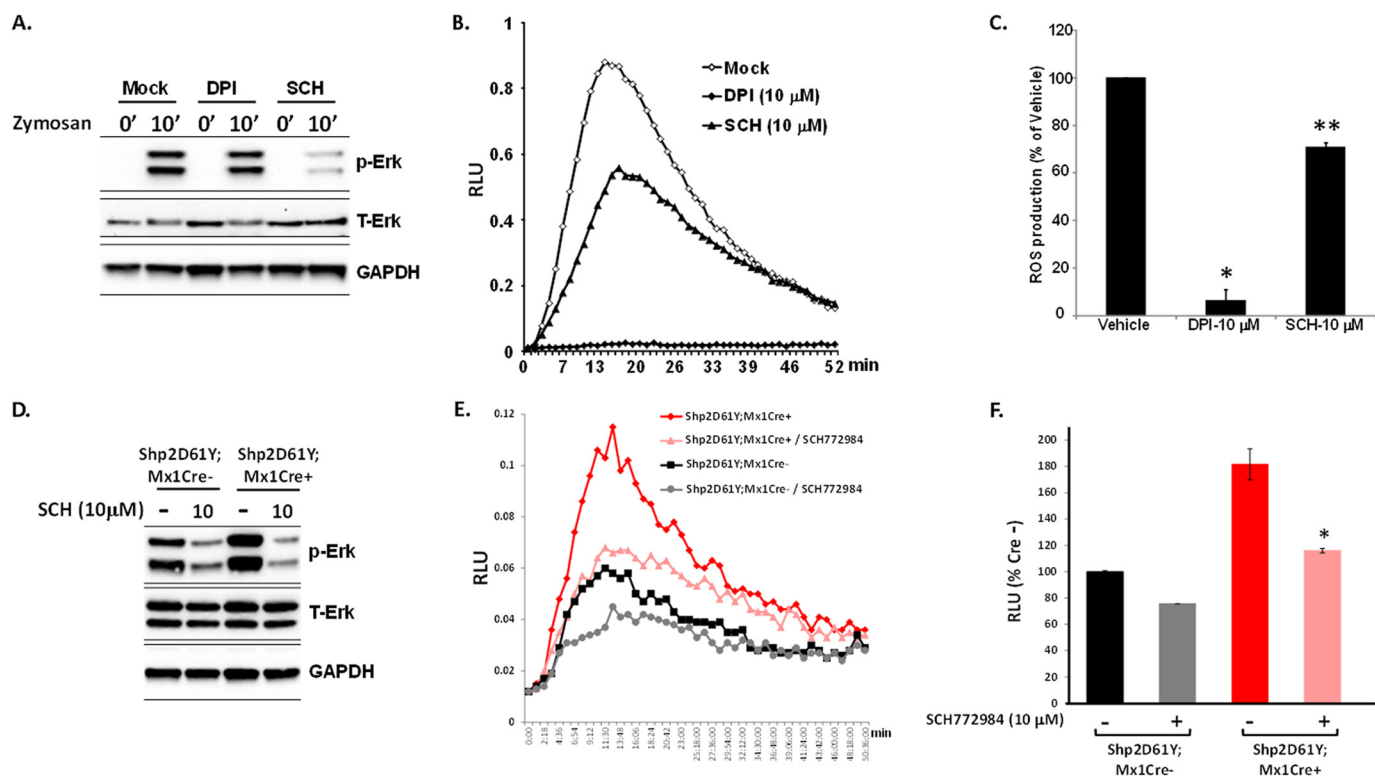
## Functional Contribution of *Shp2* to Oxidative Burst



**FIGURE 5. ROS production and the activation of Dectin-1-stimulated signaling molecules in *Shp2D61Y;Mx1Cre*<sup>-</sup> and *Shp2D61Y;Mx1Cre*<sup>+</sup> mouse PEMs upon zymosan or SOZ stimulation.** *A*, flow cytometry demonstrating the expression level of macrophage membrane markers Mac1 and F4/80 in *Shp2D61Y;Mx1Cre*<sup>-</sup> and *Shp2D61Y;Mx1Cre*<sup>+</sup> PEMs. *B* and *C*, ROS production in *Shp2D61Y;Mx1Cre*<sup>-</sup> and *Shp2D61Y;Mx1Cre*<sup>+</sup> PEMs upon zymosan (*B*) and SOZ (*C*) activation. *D*, ROS production compiled from three independent experiments ( $n = 3$ ). \*,  $p < 0.01$  for *Shp2D61Y;Mx1Cre*<sup>+</sup> versus *Shp2D61Y;Mx1Cre*<sup>-</sup> in response to zymosan or SOZ. *E*, representative immunoblot demonstrating protein level of phosphorylated and total Shp2, Syk, Erk, and Akt, as well as *phox* proteins at various time points during zymosan-triggered activation in *Shp2D61Y;Mx1Cre*<sup>-</sup> and *Shp2D61Y;Mx1Cre*<sup>+</sup> PEMs. RLU, relative light units.

typically the cells were cultured in supraphysiologic concentrations of H<sub>2</sub>O<sub>2</sub>. On the other hand, other studies demonstrate that Erk promotes NADPH oxidase activation and ROS production (38–40). To determine whether Erk activation is upstream or downstream of ROS in zymosan-stimulated macrophages, we first examined zymosan-stimulated Erk activation in the presence of the conventional NADPH oxidase inhibitor, DPI. Although incubation with DPI essentially obliterated zymosan-stimulated ROS, Erk activation was unchanged (Fig. 6, A–C). In concordance with these findings, bone marrow-derived macrophages from X-linked chronic granuloma-

tous disease mice (gp91<sup>phox</sup><sup>-/-</sup>) (41), which are devoid of ROS production, also demonstrated no change in zymosan-stimulated phospho-Erk levels (data not shown). We next treated cells with the Erk inhibitor SCH772984 and found a significant reduction of ROS that correlated with reduced Erk activation (Fig. 6, A–C), suggesting that Erk functions upstream of zymosan-stimulated oxidative burst. To further define the linear arrangement of Shp2, Erk, and ROS production, we examined the effect of Erk inhibition on ROS production in *Shp2D61Y*-expressing macrophages. Hyperactivated Erk in *Shp2D61Y*-expressing macrophages was reduced by treatment with



**FIGURE 6. Erk inhibition reduces zymosan-stimulated ROS production.** *A*, representative immunoblot demonstrating p-Erk levels during zymosan-triggered activation in the absence and presence of NADPH oxidase inhibitor DPI or Erk inhibitor SCH1772984. *B*, ROS production in  $0.1 \times 10^6$  WT PEMs in the presence of DPI or SCH1772984. *C*, ROS production compiled from three independent experiments ( $n = 3$ ).  $^* p < 0.0001$  comparing DPI-treated to vehicle-treated;  $^{**} p < 0.01$  for SCH1772984-treated versus vehicle-treated. *D*, representative immunoblot demonstrating p-Erk levels in Shp2D61Y;Mx1Cre<sup>-</sup> and Shp2D61Y;Mx1Cre<sup>+</sup> bone marrow-derived macrophages in the absence or presence of Erk inhibitor SCH1772984. *E*, ROS production in  $0.03 \times 10^6$  Shp2D61Y;Mx1Cre<sup>-</sup> and Shp2D61Y;Mx1Cre<sup>+</sup> bone marrow-derived macrophages in the absence or presence of SCH1772984. *F*, ROS production compiled from three independent experiments ( $n = 3$ ).  $^* p < 0.01$  for SCH1772984-treated versus vehicle-treated in Shp2D61Y;Mx1Cre<sup>+</sup> cells. RLU, relative light units; SCH, SCH1772984.

SCH772984, similar to that observed with WT Shp2-expressing macrophages (Fig. 6*D*). In a corresponding manner, inhibition with SCH772984 reduced the enhanced ROS levels in the zymosan-stimulated Shp2D61Y-expressing macrophages to near WT levels (Fig. 6, *E* and *F*). These findings support the hypothesis that Erk is downstream of Shp2 and upstream of NADPH oxidase and ROS production in zymosan-stimulated macrophages.

*Shp2 and Phospho-SIRP $\alpha$  Co-localize on Phagosomes and Interact Biochemically in Dectin-1-stimulated Cells*—Given the positive role of Shp2 phosphatase function in promoting zymosan- and SOZ-stimulated ROS, we investigated putative Shp2 phosphatase substrates that might regulate Erk and NADPH oxidase activation. SIRP $\alpha$  (signal regulatory protein  $\alpha$ ) is a myeloid inhibitory immunoreceptor highly expressed on macrophages, requires tyrosine phosphorylation to exert its inhibitory effect, and has been shown to be a physiological substrate of Shp2 phosphatase (42, 43). Previous studies indicate that dephosphorylation of SIRP $\alpha$  leads to increased NF $\kappa$ B-stimulated expression of inflammatory cytokines and NADPH oxidase protein components such as gp91<sup>phox</sup> (19, 44). However, because we see a rapid effect of the various Shp2 mutants on ROS production independent of the time needed for NF $\kappa$ B-stimulated gene expression and protein synthesis, we anticipated that zymosan- or SOZ-stimulated SIRP $\alpha$  dephosphorylation would be correlated with NADPH oxidase activation and ROS production.

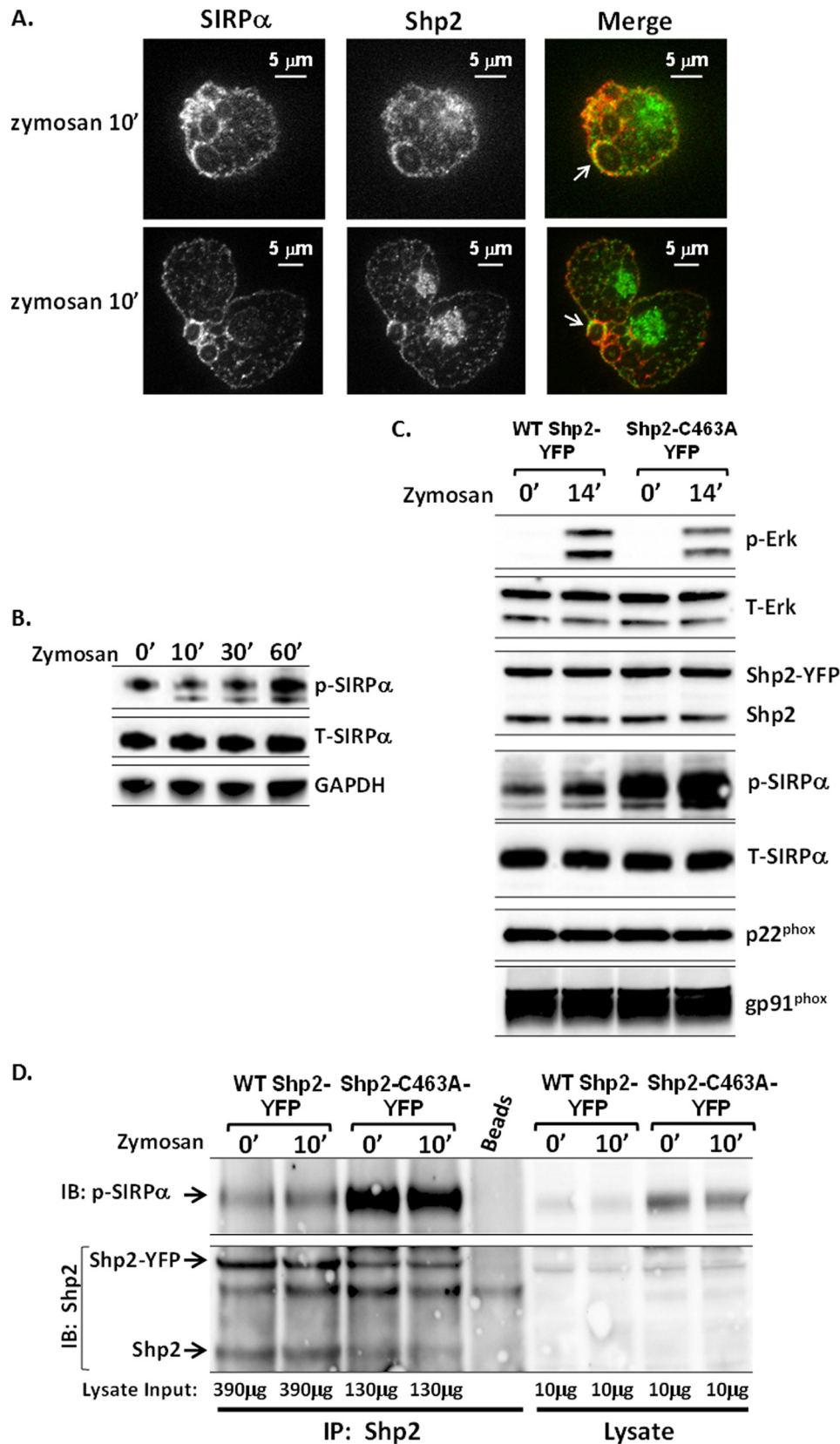
To address this hypothesis, we first examined the subcellular localization of SIRP $\alpha$  in zymosan-stimulated PEMs and found that SIRP $\alpha$  is strongly associated with the phagosome membrane at 10 min poststimulation, which correlates kinetically with zymosan-stimulated Shp2 activation and ROS production (data not shown). We next conducted co-localization studies between Shp2 and SIRP $\alpha$  on the phagosome membrane at peak ROS production and found that indeed Shp2 and SIRP $\alpha$  co-localize on phagosome membranes (Fig. 7*A*). However, the actual percentage of phagosomes showing co-localization of Shp2 and SIRP $\alpha$  at peak ROS production was only  $\sim 10$ –15% of phagosomes. Given the relatively low prevalence of Shp2-SIRP $\alpha$  co-localization at the time of peak ROS production, as well as the previous observation that phosphatase dead Shp2-C463A remained more strongly associated with the phagosome membrane compared with WT Shp2 (Fig. 3*G*), we hypothesized dephosphorylation of SIRP $\alpha$  and disruption, rather than maintenance, of the Shp2-SIRP $\alpha$  interaction is needed for maximal activation of NADPH oxidase.

To examine this idea further, we examined zymosan-stimulated phospho-SIRP $\alpha$  levels and correlated phospho-SIRP $\alpha$  with ROS production. As predicted, the phospho-SIRP $\alpha$  level was lowest at the time of maximal ROS production (10 min after zymosan stimulation; Fig. 7*B*) and at the time of maximal Shp2 phosphorylation (Fig. 1*B*). If SIRP $\alpha$  is a substrate of Shp2 in response to phagocytic stimuli, we predicted that cells

## Functional Contribution of Shp2 to Oxidative Burst

expressing phosphatase dead Shp2-C463A would demonstrate elevated phospho-SIRP $\alpha$  levels in response to Dectin-1 stimulation. Accordingly, we found substantially higher levels of

phospho-SIRP $\alpha$  at baseline and in response to zymosan stimulation in the Shp2-C462A-expressing BMMs compared with the WT Shp2-expressing cells (Fig. 7C). Importantly, the ele-



vated levels of phospho-SIRP $\alpha$  correlated to reduced levels of phospho-Erk (Fig. 7C), suggesting that Shp2 dephosphorylation of SIRP $\alpha$  constrains the SIRP $\alpha$  inhibitory function to permit normal Erk activation.

These findings support a model whereby Shp2 interacts with phosphorylated SIRP $\alpha$  and in which Shp2, upon SIRP $\alpha$  dephosphorylation, is released and available to augment activation of the Ras-Erk pathway, promote association of the NADPH oxidase protein complex, and enhance production of ROS. To address this hypothesis, we performed co-IP assays to examine the interaction between phospho-SIRP $\alpha$  and WT Shp2 compared with Shp2-C463A. Although we did observe an interaction between WT Shp2 and phospho-SIRP $\alpha$  (Fig. 7D), this interaction was in fact difficult to detect and required the use of ~3-fold more protein lysate for the co-IP compared with the amount of lysate used to detect the very strong interaction between Shp2-C463A and phospho-SIRP $\alpha$  (Fig. 7D). The modest biochemical interaction observed between WT Shp2 and phospho-SIRP $\alpha$  is consistent with the relatively low number of phagosomes demonstrating WT Shp2-SIRP $\alpha$  co-localization upon zymosan stimulation (Fig. 7A) and supports the notion that Shp2 dephosphorylation of SIRP $\alpha$  and disruption of the Shp2-SIRP $\alpha$  interaction is needed for optimal activation of Erk and NADPH oxidase.

## DISCUSSION

Innate immune cell production of microbicidal ROS in response to foreign stimuli is vital for clearance of invading pathogens and immunocompetence. However, ROS production needs to be regulated in a temporal and spatial manner to minimize nonspecific damage to healthy tissues. Within this manuscript, we have demonstrated that the protein-tyrosine phosphatase, Shp2, is a novel player in the Dectin-1-stimulated signaling pathway and functions downstream of Syk and upstream of Erk to promote NADPH oxidase activation and oxidative burst. Further, we found that Shp2 activation and dephosphorylation of the inhibitory immunoreceptor, SIRP $\alpha$ , correlates kinetically with peak ROS production in Dectin-1-stimulated macrophages.

Shp2 is highly expressed in hematopoietic cells and has previously been shown to promote IL-6 production by promoting activation of NF $\kappa$ B (45). Additionally, Shp2 has been shown to be an important component of the leptin receptor signaling pathway in protecting against the parasite *E. histolytica*, and a component of this protection is due to STAT3-regulated IL-6 production (12). However, the role of Shp2 in oxidative burst has never been described. We first found that cells bearing genetic disruption of *Ptpn11* produce lower levels of zymosan- and SOZ-stimulated ROS. Using a series of Shp2 point mutants,

we found that both the N-SH2 domain and the phosphatase domain of Shp2 are needed for zymosan- and SOZ-stimulated ROS. Importantly, videomicroscopy demonstrated that Shp2-R32K (N-SH2 domain mutant) failed to be recruited to the phagosome, whereas phosphatase dead Shp2-C463A was strongly recruited to phagosomes and was retained more intensely than WT Shp2 (Fig. 3G). These findings indicate that the N-SH2 domain is needed for Shp2 recruitment to the phagosome; however, once recruited, the phosphatase function of Shp2 is needed to promote ROS production. Supporting this idea, GOF Shp2 mutants Shp2-D61Y and Shp2-E76K increased both zymosan- and SOZ-stimulated ROS (Figs. 4 and 5).

Given the apparent positive functional role of Shp2 phosphatase on ROS production and the kinetic correlation of Erk activation with maximal ROS production (Fig. 1, A and B), we reasoned that Shp2 exerts its positive effect on NADPH oxidase activation via positive regulation of Erk, because Shp2 has been shown to positively up-regulate Erk activation in response to multiple stimuli (34, 46, 47). This notion is consistent with previous studies that have demonstrated positive regulation of Erk activation on NADPH oxidase (38–40). We found that the Erk inhibitor, SCH772984, reduced ROS production in both WT cells and in cells expressing GOF Shp2D61Y (Fig. 6), placing Erk downstream of Shp2 and upstream ROS production.

We next interrogated potential Shp2 substrates that may be involved in the regulation of particulate-stimulated Erk activation and ROS production. SIRP $\alpha$ , also known as SHPS-1/BIT/CD172a, was first identified as a phosphorylated glycoprotein that interacted with Shp2 in response to insulin stimulation, and expression of catalytically inactive Shp2 resulted in increased tyrosine phosphorylation of SIRP $\alpha$  (48). SIRP $\alpha$  contains extracellular immunoglobulin-like domains and four intracellular tyrosine residues followed by amino acid sequence XX(L/V/I), indicating sites of tyrosine phosphorylation (43). Several studies indicate that SIRP $\alpha$  may be a physiological substrate for Shp2 (42, 43). Early functional studies in fibroblasts indicated that overexpression of SIRP $\alpha$  inhibited receptor tyrosine kinase- and cytokine receptor-stimulated cell proliferation and that the intracellular tyrosine residues were vital for the SIRP $\alpha$  inhibitory function (42). Given the high expression level of SIRP $\alpha$  in macrophages, investigators found that SIRP $\alpha$  negatively modulates inflammatory cytokine production via down-regulation of NF $\kappa$ B-regulated expression (19, 49). Additionally, one study found that SIRP $\alpha$  phosphorylation functioned to inhibit soluble stimulation (phorbol 12-myristate 13-acetate)-induced plasma membrane ROS by down-regulating expression of the NADPH oxidase component, gp91<sup>phox</sup> (44).

**FIGURE 7. SIRP $\alpha$  phosphorylation and interaction with Shp2 in zymosan-stimulated WT Shp2- and Shp2-C463A-expressing BMMs.** A, co-localization of SIRP $\alpha$  and Shp2 in WT mouse PEMs 10 min after zymosan-induced phagocytosis and immunofluorescent staining with anti-SIRP $\alpha$  (catalog no. 53721; Abcam) and anti-Shp2 (BD Biosciences; catalog no. 610621) and Alexa Fluor 568 goat anti-rabbit IgG and goat 488 anti-mouse IgG1, respectively. B, immunoblot demonstrating reduced levels of phospho-SIRP $\alpha$  upon zymosan stimulation of WT PEMs. C, phosphorylation of SIRP $\alpha$  and Erk in BMMs retrovirally transduced with YFP-WT Shp2 or YFP-Shp2-C463A. Total Shp2, SIRP $\alpha$ , Erk, p22<sup>phox</sup>, and gp91<sup>phox</sup> were also examined using antibodies described under "Experimental Procedures." D, binding of WT Shp2 and Shp2-C463A mutant to tyrosine-phosphorylated SIRP $\alpha$  in macrophages before and after zymosan stimulation. Cell lysates from macrophages expressing YFP-WT Shp2 or YFP-Shp2-C463A were subjected to IP anti-Shp2 antibody (SC-280; Santa Cruz Biotechnology) followed by immunoblotting (IB) with anti-phospho-SIRP $\alpha$  or anti-Shp2 (catalog no. 610621; BD Biosciences). 390  $\mu$ g of protein lysis from macrophages expressing YFP-WT Shp2 and 130  $\mu$ g of protein lysis from macrophages expressing YFP-Shp2-C463A were used for IP. 10  $\mu$ g of protein lysis was used to do immunoblot analysis. The data are representative of three separate experiments.

## Functional Contribution of Shp2 to Oxidative Burst

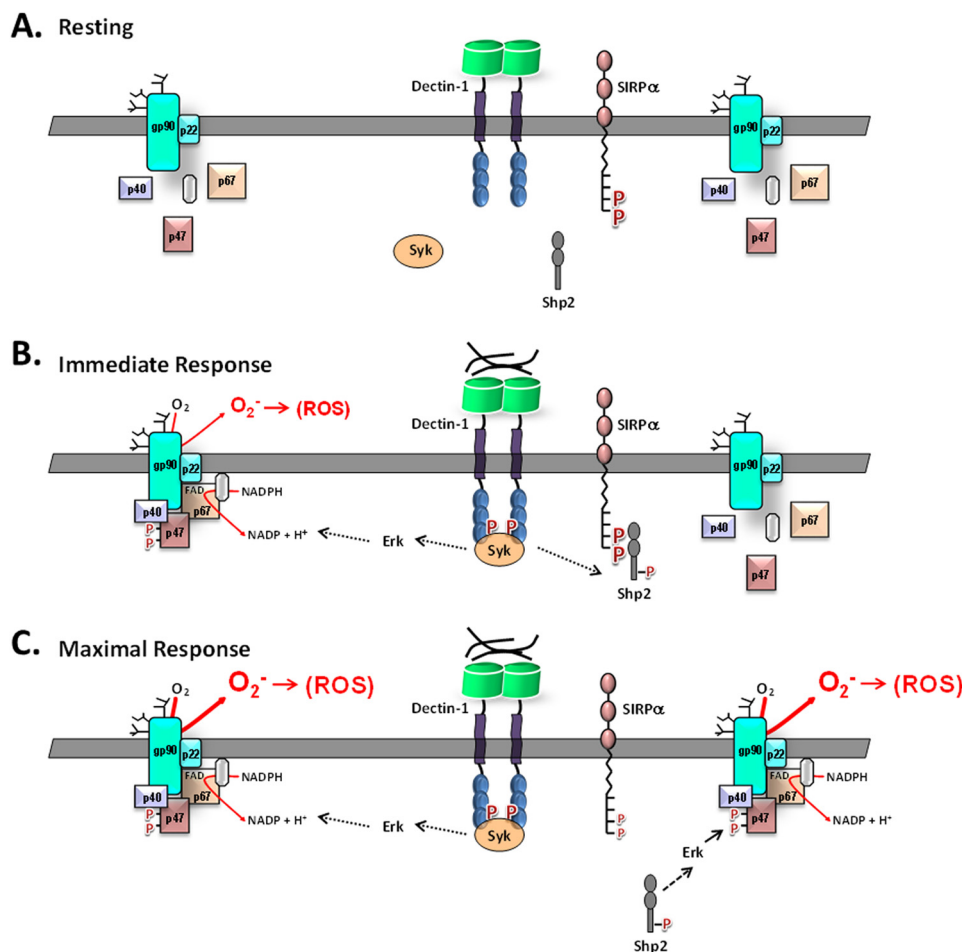


FIGURE 8. **Schematic diagram indicating proposed mechanism of Shp2 and SIRP $\alpha$  interaction in response to Dectin-1 stimulation.** A, cells at resting state. B, immediate Dectin-1-stimulated ROS production in a Syk-dependent, Shp2-independent manner. C, following Shp2 dephosphorylation of SIRP $\alpha$ , maximal Dectin-1-stimulated ROS is produced in both a Syk-dependent and Shp2-dependent manner.

To date, however, no previous studies have identified SIRP $\alpha$  as an important regulatory molecule in particulate-stimulated oxidative burst. We first defined that SIRP $\alpha$  is indeed present on early phagosomal membranes in response to zymosan stimulation with similar kinetics to Shp2 phagosomal recruitment. In fluorescent microscopy studies, however, although we found that Shp2 and SIRP $\alpha$  co-localize on the early phagosome (Fig. 7A), we were surprised to find that the co-localization was modest and seen in only  $\sim 10$ – $15\%$  of phagosomes at the time corresponding to maximal ROS production (10 min poststimulation). These findings suggest that SIRP $\alpha$  phosphorylation recruits Shp2 to the phagosome membrane and that the Shp2-SIRP $\alpha$  interaction delays the full oxidative burst by sequestering Shp2 from the Ras-Erk pathway, thus deferring maximal Erk activation and ROS production. The increased association of phosphatase-dead Shp2-C463A with the phagosome (Fig. 3G) and the increased biochemical interaction between Shp2-C463A and SIRP $\alpha$  (Fig. 7D) in conjunction with reduced ROS production support this model.

Although our data support a positive role for Shp2 phosphatase in particulate-stimulated ROS production by dephosphorylating and thus repressing the inhibitory role of SIRP $\alpha$ , it is important to put these findings into context with previous studies in this signaling pathway. First, studies from the Eklund lab

demonstrated that Shp2 functions to dephosphorylate the transcription factors ICSBP and HoxA10, resulting in reduced transcription and expression of the NADPH oxidase protein components gp91<sup>phox</sup> and p67<sup>phox</sup> (14, 15). These studies very nicely provide a rational mechanism of how GOF Shp2 mutants may inhibit expression of genes needed for myeloid cell terminal differentiation and thus promote leukemogenesis.

Additionally, van Beek *et al.* (44) demonstrated that ectopic expression of SIRP $\alpha$  in PLB-985 cells functioned in an inhibitory manner by repressing gp91<sup>phox</sup> expression during granulocytic or monocytic differentiation, leading to reduced soluble stimulation (phorbol 12-myristate 13-acetate)-induced ROS. Notably, these previous studies were performed in undifferentiated or differentiating myeloid cells, whereas our studies were performed in terminally differentiated macrophages. Importantly, in terminally differentiated macrophages, we did not find a difference in expression of the various NADPH oxidase components in cells lacking Shp2 (Fig. 2), expressing loss of function Shp2 mutants (Fig. 3), or gain of function Shp2 mutants (Figs. 4 and 5). Contrasting our studies to this previous work highlights an interesting concept that in undifferentiated myeloid cells, Shp2 and SIRP $\alpha$  may be more important for regulating transcription factor function and myeloid cell differentiation (50, 51), whereas in terminally differentiated cells, Shp2 and SIRP $\alpha$  are more impor-

tant for regulating phosphorylation and subcellular localization, respectively, of cytoplasmic signaling proteins.

Further, whereas many studies focus on the inhibitory role of SIRP $\alpha$  in myeloid cell immune function, others have found that SIRP $\alpha$  can also play a positive role in immune cell function (52). Importantly, this positive regulatory role of SIRP $\alpha$  was found in the context of Shp1-expressing hematopoietic cells, whereas many of the original studies defining a negative regulatory role of SIRP $\alpha$  were performed in fibroblasts which express only Shp2 (52). Because both Shp1 and Shp2 are expressed in hematopoietic cells and have both been found to interact with SIRP $\alpha$  and because Shp1 is conventionally thought to play a negative regulatory role and Shp2 is thought to play a positively regulatory role in hematopoietic cell function, these considerations bring into relief the speculative idea that Shp1 may function to dephosphorylate and inhibit the positive regulatory role of SIRP $\alpha$  (leading to a net negative signal), whereas Shp2 may function to dephosphorylate and inhibit the negative regulatory role of SIRP $\alpha$  (leading to a net positive signal). Although beyond the point of the current study, it may be of interest to compare the effect of phosphatase dead Shp1 with that of phosphatase dead Shp2 in particulate-stimulated ROS production in terminally differentiated macrophages or neutrophils.

Based on our findings within the current study, we have clarified a model that places Shp2 activation downstream of Dectin-1-stimulated Syk and upstream of Erk and ROS production (Fig. 8). Previous work defined that upon stimulation, Dectin-1 is phosphorylated (likely by members of the Src family of kinases) resulting in Syk recruitment, Ras-Erk pathway activation (16), and immediate production of ROS (Fig. 8, A and B). Findings within this study demonstrate that Dectin-1 stimulation leads to activation of Shp2 in a Syk-dependent manner and that phospho-Shp2 is recruited to phosphorylated SIRP $\alpha$  (Fig. 8B). Once Shp2 dephosphorylates SIRP $\alpha$ , Shp2 is available for additional positive regulation of the Ras-Erk pathway, leading to maximal ROS production (Fig. 8C). A potential means of Erk activation of NADPH oxidase function is phosphorylation of p47<sup>phox</sup>, which has been found previously in neutrophils (40, 53). It is possible that the SIRP $\alpha$  interaction with Shp2 defers maximal ROS production for the purpose of mitigating or preventing unwarranted ROS release to normal tissues. These findings also have application for improved understanding of the childhood leukemia, juvenile myelomonocytic leukemia, because aberrantly elevated ROS production from activating *PTPN11*-expressing innate immune cells may account for the common difficulty in clinically differentiating juvenile myelomonocytic leukemia from microbial and viral infections (54–56). Collectively, our findings demonstrate that Shp2 positively regulates oxidative burst at least in part by promoting Erk activation and that the Shp2-SIRP $\alpha$  interaction may fine tune the optimal timing and location for ROS production.

*Acknowledgments*—We thank Dr. Jesaitis for antibodies 54.1 against gp91<sup>phox</sup> and NS2 against p22<sup>phox</sup>. We also thank the Indiana University Simon Cancer Center Flow Cytometry Core and Indiana Center for Biological Microscopy. We gratefully acknowledge the administrative assistance of Marilyn L. Wales.

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