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Phagocytosis of Ab-coated pathogens is mediated through FcγRs, which activate intracellular signaling pathways to drive actin cytoskeletal rearrangements. Abl and Arg define a family of nonreceptor tyrosine kinases that regulate actin-dependent processes in a variety of cell types, including those important in the adaptive immune response. Using pharmacological inhibition as well as dominant negative and knockout approaches, we demonstrate a role for the Abl family kinases in phagocytosis by macrophages and define a mechanism whereby Abl kinases regulate this process. Bone marrow-derived macrophages from mice lacking Abl and Arg kinases exhibit inefficient phagocytosis of sheep erythrocytes and zymosan particles. Treatment with the Abl kinase inhibitors imatinib and GNF-2 or overexpression of kinase-inactive forms of the Abl family kinases also impairs particle internalization in murine macrophages, indicating Abl kinase activity is required for efficient phagocytosis. Further, Arg kinase is present at the phagocytic cup, and Abl family kinases are activated by FcγR engagement. The regulation of phagocytosis by Abl family kinases is mediated in part by the spleen tyrosine kinase (Syk). Loss of Abl and Arg expression or treatment with Abl inhibitors reduced Syk phosphorylation in response to FcγR ligation. The link between Abl family kinases and Syk may be direct, as purified Arg kinase phosphorylates Syk in vitro. Further, overexpression of membrane-targeted Syk in cells treated with Abl kinase inhibitors partially rescues the impairment in phagocytosis. Together, these findings reveal that Abl family kinases control the efficiency of phagocytosis in part through the regulation of Syk function. The Journal of Immunology, 2012, 189: 000–000.
This unique actin binding ability may serve directly to couple tyrosine phosphorylation signaling events to the cytoskeleton. In this regard, Ab1 family kinases have been implicated in the regulation of cellular morphogenesis, adhesion, polarity, migration, and invasion (20–24).

Ab1 and Arg are ubiquitously expressed: Ab1 is expressed in most tissues at similar levels, whereas Arg is highly expressed in the CNS and in the thymus (25). Aberrant Ab1 and Arg fusion proteins with constitutive kinase activity have been identified as oncogenic in human lymphoid and myeloid leukemias (17). Notably, mice null for the abl1 gene are characterized by high perinatal mortality, splenic and thymic atrophy, and an increased susceptibility to infection (26). Subsequent studies have shown that the Ab1 bone marrow kinases are activated downstream of the TCR and BCR and contribute to Ag receptor signaling (27–31). Ab1 kinases are required for TCR-induced proliferation and modulate the phosphorylation of ZAP70, LAT, and Shc (27, 32). Compared to the well-documented role for Ab1 kinases in lymphocyte function, relatively little is known about the role of the Ab1 family kinases in myeloid cells. Recent reports have demonstrated that Ab1 kinase activity is required for neutrophil adhesion and spreading through β2 integrin (33) and that Ab1 kinases mediate myeloid cell migration and invasion (34, 35).

In this study, we sought to examine whether Ab1 family kinases play a role in the regulation of FcyR-mediated phagocytosis in macrophages. The inhibition or genetic deletion of Ab1 family kinases in macrophages results in decreased phagocytosis of IgG-opsonized particles. Furthermore, Ab1 kinases are activated upon FcyR engagement, are found at the phagocytic cup, and regulate the efficiency of cup formation. Mechanistically, we show that Ab1 regulates the efficiency of phagocytosis in part by promoting the phosphorylation of Syk.

Materials and Methods

Reagents

STI571 (imatinib, Gleevec) was a kind gift from Novartis. GNF-2 and SU6656 were purchased from Sigma. Piceatannol was purchased from Millipore. Sheep RBCs (sRBCs) were purchased from MP Biomedicals. Zymosan and zymosan opsonization reagents were purchased from Invitrogen.

Mice

The abflox/+ mice were generated as previously described (25) and were crossed into the ablflox/flox background to generate ablflox/flox mice. These mice were subsequently crossed with the LysM-Cre transgenic mice (The Jackson Laboratory) to generate conditional loss of Ab1 kinases in the myeloid cell lineage. Genotypes were confirmed by PCR. Mice were backcrossed to C57BL/6 at least four times during their generation. Mice were housed under specific pathogen-free conditions in the Duke University Cancer Center Isolation Facility. All studies using mice have followed the protocols reviewed and approved by the Duke Institutional Animal Care and Use Committee.

Thiglycollate-induced peritonitis

Where indicated, isolation of macrophages was performed after i.p. injection of 1 ml 3% thioglycollate solution (Sigma). After 72 h, mice were sacrificed, and the peritoneal cavity was lavaged with 5 ml HBSS to collect cells. Cells

Primary macrophages were derived from the bone marrow or isolated from the peritoneum. In all experiments using primary cells, Ab1/Arg null macrophages refer to cells derived from abflox/+; arg−/−; LysMCre+ mice, and control macrophages refer to cells derived from littermates or sex- and age-matched abflox/lox; arg+/+; LysMCre− or abflox/lox; arg+/+; LysMCre− mice. Bone marrow–derived macrophages (BMDMs) were prepared by flushing the bone marrow from the femurs and tibias of mice with a 25-gauge needle. Bone marrow cells were then collected; centrifuged, resuspended in growth media, and plated. Resident peritoneal macrophages and macrophages elicited by thioglycollate challenge (peritoneal-elicited macrophages [PEMs]) were prepared by lavaging the peritoneum of mice with 5 ml HBSS. Both peritoneal macrophages and BMDMs were cultured in 20% L-cell conditioned medium that also contained 10% heat-inactivated FBS (Invitrogen) and 1% penicillin/streptomycin in α-MEM (Invitrogen). BMDMs were used after culturing in media for 5–7 d. Peritoneal macrophage cultures were used within 1–3 d after isolation. L-cell conditioned medium was prepared by harvesting and filtering the supernatant of L929 cells (obtained from the American Type Culture Collection) grown in α-MEM (Invitrogen) supplemented with 10% FBS. RAW264.7 mouse macrophages (American Type Culture Collection) and HEK293T cells (American Type Culture Collection) were cultured in 10% FBS DMEM.

Cloning

MigR1/myr-FLAG-Syk was generated by subcloning the myr-FLAG-Syk cDNA from the pWZL vector (Addgene) into the Xhol and EcoRI sites of the MigR1 vector.

Transfection and viral transduction

PK1-vector, PK1-ArgKR, MigR1-vector, MigR1/AblPP, MigR1-AblKR, pcDNA-vector, pcDNA-myc-Syk, and PX1-Arg-YP constructs were described previously (24, 27, 36). Constructs were expressed in HEK293T cells using calcium phosphate-mediated transfection. Cells were used for experimental procedures 48 h after transfection. Retroviruses were prepared by co-transfecting HEK293T cells with PK1, PK1, or MigR1 constructs along with CMV-VSVG and gag/pol packaging constructs. After 48 h, cell retroviral supernatants were collected, filtered, and used to transduce RAW264.7 cells. RAW264.7 cells were incubated with the retroviral medium in presence of polybrene (8 μg/ml) for 48–72 h. Cells were then sorted for GFP+ expression (for MigR1 or PK1 constructs) or cultured a minimum of 3 d in puromycin (2 μg/ml for PK1 constructs) and used accordingly.

RAW264.7 cells were transiently transfected with MigR1/myr-FLAG-Syk using Fugene 6 (Roche) according to the manufacturer’s instructions.

Flow cytometry

Macrophages in single-cell suspensions were washed in FACS buffer (PBS containing 2% heat-inactivated FBS, 0.05% sodium azide). For surface staining, ~1 × 106 cells were first incubated 5 min with rat anti-mouse CD16/CD32 (mouse Fc block; BD Pharmingen) followed by staining with FITC–CD11b and PE–F4/80 or isotype controls (eBioscience) on ice for 30 min. For staining of FcyRs, PE–CD16/CD32 (eBioscience) was used. Cells were washed twice, collected on a FACScan flow cytometer, and analyzed using FlowJo (Tree Star).

RT-PCR

Total RNA was isolated from BMDMs or peritoneal macrophages using RNeasy kit (Qiagen). cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and amplified by PCR using the following primers for abl: 5′-GCCCTGGCCAGAGATCCATC-3′ and 5′-TCCCTCGAGTGTCCAGCGAGG-3′ (as described in Ref. 37).

Phagocytosis assay and microscopy

sRBC (10%) solution was pelleted and washed three times with PBS. sRBCs were opsonized with rabbit anti-sheep RBC Abs diluted 1:50 (MP Biomedicals) and incubated at 37°C with shaking for 1 h. Opsonized sRBCs were then washed three times and resuspended in PBS. Cells were counted and determined so that the sRBC/macrophage ratio was between 50:1 and 100:1. For drug studies, macrophages were preincubated 2 h with STI571 (1 μM), GNF-2 (20 μM), or a vehicle (0.1% DMSO, Sigma) before initiating phagocytosis and were maintained in drug-containing media during the assay. Macrophages were chilled on ice before addition of the opsonized sRBC; sRBCs were then added, and plates containing macrophages and sRBCs were spun down for 1 min (1000 rpm). The plates were incubated on ice for 10 min, medium was then aspirated, and warm medium was added to initiate phagocytosis. Plates were incubated at 37°C for the indicated times. Reactions were stopped by placing the plates on ice and washing away uningested particles with ice-cold PBS three times, followed by hypotonic lysis of any remaining uningested RBCs with water for 1 min. Cells were washed an additional three times with PBS followed by fixation in methanol. RBCs were visualized by light microscopy. The phagocytic index was calculated by selecting 5–10 microscope fields (×400) and scoring each field for both total number of macrophages and total number of ingested targets. A minimum of 100 macrophages were scored per coverslip. The phagocytic index indicates the number of particles ingested by 100 cells. For experiments evaluating the formation of phagocytic cups, macrophages were treated with sRBCs as described.
earlier, followed by fixation with 4% paraformaldehyde (Sigma) in PBS, permeabilization with PBS containing 0.2% Triton X-100, and blocking with 3% (w/v) BSA for 1 h. Cells were stained with either Alexa Fluor 568– or Alexa Fluor 488–conjugated phallolidin (Invitrogen) diluted 1:500 in 3% BSA (w/v) PBS for 1 h. IgG-opsinized sRBCs were stained with Alexa Fluor 568–conjugated goat anti-rabbit secondary Ab (Invitrogen) at 1:500 in 3% BSA (w/v) PBS for 1 h. Hoechst (Molecular Probes) was used to stain nuclei. The same fixation and staining procedures were used for macrophages treated with zymosan. In all experiments using zymosan, the zymosan was opsonized with zymosan opsonization reagent from Invitrogen. Phase-contrast and epifluorescence images were acquired using the Zeiss Axio Imager (Carl Zeiss MicroImaging). Confocal images were obtained using the Leica SP5 confocal scanning microscope and analyzed using LAS AF (Leica).

FcγR cross-linking

Cells were serum starved for a total of 4 h before cross-linking. In studies using pharmacological inhibitors, drugs (STI571, 10 μM; GNF-2, 20 μM; SU6656, 2.5 μM; or piceatannol, 25 μM) were added 2 h prior to cross-linking in serum-free media. After 4-h serum starvation, cells were incubated 20–30 min with 10 μg/ml 2,4-DG Abs (anti-FcγRII and anti-FcγRIII; BD Biosciences) at 4°C. Secondary cross-linking mouse anti-rat IgG (30 μg/ml; Jackson Immunoresearch Laboratories) was then added to warmed media at 37°C for indicated times before plates were set on ice, washed in cold PBS, and lysed in RIPA buffer (50 mM Tris, 1% sodium deoxycholate, 150 mM sodium chloride, 1% Nonidet P-40, 0.1% SDS) with protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin) and phosphatase inhibitors (10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate).

Immunoprecipitation and kinase assay

To immunoprecipitate Syk, HEK293T cells transiently overexpressing Syk were lysed on ice for 30 min followed by centrifugation at 17,000 × g for 10 min to remove insoluble material. Lysate (0.2 mg) was incubated with 0.4 μg anti-Syk Ab (Santa Cruz) overnight with end-over-end rotation at 4°C. To immunoprecipitate the γ-chain, RAW264.7 cells were lysed after FcγR cross-linking, and 1 mg lysate was incubated with 2 μg of anti-γ-chain Ab (Millipore) overnight. Protein G-Sepharose beads (30 μl slurry; GE Healthcare) were added for 2 h with end-over-end rotation at 4°C. Beads were then pelleted by centrifugation and washed as described (38). Syk immunoprecipitates were incubated with purified full-length or C-terminal fragment Arg kinase (0.5 μg) (gift of Dr. Tony Koleske, Yale University, New Haven, CT) in the presence or absence of 1 μM ATP for 30 min at room temperature. Sample buffer was added to stop the reaction. Samples were then boiled and run on SDS-PAGE.

Immunoblotting

Cell lysates were prepared and protein concentrations were quantified using DC Protein Assay (Bio-Rad). Cell lysate was diluted in Laemmli sample buffer and proteins were separated on 8–15% SDS-polyacrylamide gels under reducing conditions. After transfer of proteins to nitrocellulose membranes, membranes were incubated with unlabeled primary Abs overnight: anti-Abl (clone 8E9; BD Biosciences) at 1:500, anti-Arg (clone 9H5; Santa Cruz) at 1:500, anti-phospho-CRKL Y207 (Cell Signaling) at 1:1000, anti-ArgKR (Supplemental Fig. 1B). Membranes were then incubated with horseradish peroxidase–conjugated secondary Ab (Jackson ImmunoResearch Laboratories) at 1:10,000 for 1 h. Membranes were washed with TBST and developed with chemiluminescence reagent (GE Healthcare).

Statistical analysis

Values correspond to the mean ± SEM. Comparisons of a single treatment to control were evaluated by Student t test. In experiments with multiple groups, differences were evaluated by one-way ANOVA followed by Tukey’s multiple comparison test. Differences in phagocytosis between genotypes or drug treatments as function of time or in the presence or absence of Syk overexpression were evaluated using two-way ANOVA followed by Bonferroni post hoc analysis using GraphPad Prism software v.5 (GraphPad Software). Western blots were quantitated using ImageJ analysis software (National Institutes of Health). For all tests, p < 0.05 was considered statistically significant.

Results

Abl kinase activity is required for phagocytic efficiency

Tyrosine kinase signaling downstream of immune recognition receptors is required for activation of T and B cells and phagocytosis in macrophages (9, 10). Previous studies have shown that Abl kinases are activated by BCR and TCR ligation and are required for Ag-induced proliferation (27, 28, 30–32). We hypothesized that Abl kinase activity might be required downstream of the FcγR in macrophages to mediate phagocytosis. RAW264.7 murine macrophages were treated with STI571 (Gleevec, imatinib), an ATP competitive inhibitor of the Abl kinases that also inhibits PDGFR, c-Kit, and c-fms (39, 40), or with a specific allosteric inhibitor of the Abl kinases, GNF-2, which has unique specificity for the C-terminal myristate binding cleft in the kinase domain of Abl and Arg and does not inhibit other kinases (41, 42). RAW264.7 cells treated with either STI571 or GNF-2 were assayed for inhibition of Abl family kinase activity by analyzing the phosphorylation of the adapter protein CRKL on Y207, an Abl-specific site (32, 43) (Fig. 1A). CRKL phosphorylation on Y207 was reduced by 50 ± 8% when treated with STI571 and 40 ± 9% with GNF-2. As shown in Fig. 1B, treatment with either Abl kinase inhibitor markedly reduced phagocytosis of IgG-opsonized sRBCs. The inhibitory effects of STI571 and GNF-2 on phagocytosis persisted over a 45-min time course in both RAW264.7 cells and primary BMDMs (Fig. 1C, 1D). After 30–45 min, phagocytosis in macrophages treated with Abl kinase inhibitors was reduced by >60% compared with vehicle-treated cells (n = 4 experiments). RAW264.7 macrophages treated with IgG-opsonized zymosan in the presence of STI571 and GNF-2 also exhibited impaired phagocytosis, similar to the phagocytosis of sRBCs (Supplemental Fig. 1A).

To support further a role for the Abl family kinases in FcγR-mediated phagocytosis, RAW264.7 macrophages were transduced with retrovirus encoding an inactive form of Arg kinase (ArgKR), which acts in a dominant negative manner to inhibit Abl family kinase signaling (22). Macrophages expressing ArgKR phagocytosed 54 ± 5% of the sRBCs compared with control macrophages (n = 7 experiments, example shown in Fig. 1E) and also exhibited a 50% reduction in phosphorylation of CRKL on Y207, indicating reduced Abl/Arg kinase activity (Fig. 1F). Similarly, phagocytosis of IgG-opsonized zymosan was decreased in macrophages expressing ArgKR (Supplemental Fig. 1B).

Impaired FcγR-mediated phagocytosis in Abl/Arg null macrophages

We next sought to examine the requirement for Abl kinases in FcγR-mediated phagocytosis using a genetic loss-of-function approach. As global deletion of both Abl and Arg is embryonic lethal (25), we used tissue-specific inactivation of Abl. To this end, abllox/lox mice (37) were crossed into the arg–/– background (25), and the abllox/lox, arg–/– mice were crossed to mice that express Cre recombinase under the control of the lysozyme M (LysM) promoter (44). LysM is expressed in myeloid cells including monocytes, macrophages, granulocytes, and a subset of dendritic cells (44). The resulting abllox/lox, arg–/–, LysMcrt+ (from here on designated Abl/Arg null) mice were viable and demonstrated deletion of abl1 in both thiglycollate-elicited PEMS and BMDMs (Supplemental Fig. 2A), which corresponded with reduction in Abl protein and activity levels (Supplemental Fig. 2B). To test whether Abl family kinases were required for macrophage differentiation, cells were stained with the myeloid marker CD11b as well as the mature macrophage marker F4/80. Culturing bone marrow cells from wild-type and Abl/Arg null mice in CSF-1-containing macrophage...
FIGURE 1. Abl family kinase activity regulates phagocytic efficiency. (A) RAW264.7 macrophages treated with the Abl inhibitors STI571 (10 μM) or GNF-2 (20 μM) were evaluated for phosphorylation of the Abl substrate CRKL on Y207 by Western blotting. Graph shows relative levels of phospho-CRKL (pCRKL), normalized to total CRKL, from four experiments. *p < 0.05 (versus vehicle-treated control). (B) RAW264.7 were pretreated with Abl kinase inhibitors as in (A) followed by treatment with IgG-opsonized sRBCs for 30 min. Micrographs show the presence of ingested sRBCs in the macrophages. Results are representative of at least four independent experiments. Scale bar, 10 μm. (C and D) RAW264.7 macrophages (C) or BMDMs (D) were pretreated with drugs as in (A) followed by incubation with sRBCs for the indicated times. The phagocytic index was calculated as the number of ingested sRBCs in 100 macrophages. Data shown are representative of two independent experiments. *p < 0.05 (versus vehicle-treated control). (E) RAW264.7 macrophages transduced with retrovirus encoding kinase-inactive Arg (ArgKR) were treated with IgG-opsonized sRBCs for 30 min, and the phagocytic index was calculated as in (C) and (D). Scale bar, 10 μm. Results are representative of seven independent experiments. *p < 0.05 (compared with cells expressing empty vector). (F) RAW264.7 macrophages transduced with kinase-inactive Arg (PK1-ArgKR) or PK1-vector were analyzed by Western blotting with indicated Abs. Relative levels of phospho-CRKL, normalized to total CRKL, from three independent experiments are shown.

differentiation media resulted in similar numbers of CD11b<sup>+</sup>F4/80<sup>+</sup> cells (Supplemental Fig. 2C). Furthermore, recruitment of CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages to the peritoneum in response to challenge with thioglycollate was not affected by deletion of Abl and Arg (Supplemental Fig. 2D). Similarly, the percentage of CD11b<sup>+</sup>F4/80<sup>+</sup> cells isolated from the bone marrow, blood, and spleen was not affected by loss of Abl and Arg (data not shown). Taken together, these data suggest that Abl and Arg are not required for macrophage differentiation in vitro or in vivo.

To examine whether Abl kinases were required for FcγR-mediated phagocytosis, BMDMs from control and Abl/Arg null animals were challenged with IgG-opsonized sRBCs. Similar to the results obtained with Abl kinase inhibitors, the Abl/Arg null macrophages phagocytosed fewer sRBCs compared with controls (Fig. 2A). Analysis of multiple control and Abl/Arg null cells over a 60-min time course revealed that Abl/Arg null macrophages achieved a maximal reduction in the phagocytic index of 31 ± 6% after 15- to 30-min incubation with sRBCs (n = 13 experiments; ±SEM). At later time points, the Abl/Arg null cells were able to ingest similar numbers of particles as control cells (Fig. 2A, 2B). This delay in phagocytosis was also observed in Abl/Arg null macrophages isolated from the peritoneum (Fig. 2C). Abl/Arg null peritoneal macrophages exhibited a 41 ± 14% reduction in the phagocytic index compared with control macrophages after a 10- to 40-min incubation with sRBCs (n = 5 experiments; ±SEM). The Abl/Arg null macrophages also exhibited reduced phagocytosis of IgG-opsonized zymosan, particularly within the first 15 min of stimulation with zymosan particles (mean of 40 ± 9% reduction in phagocytic index compared with controls, n = 11 experiments; ±SEM) (Supplemental Fig. 3). The reduction in phagocytosis in Abl/Arg null macrophages was not due to reduced surface expression of the FcγR, as these cells expressed equivalent surface levels of CD16/CD32 compared with control macrophages (Fig. 2D). Furthermore, deletion of either Abl or Arg kinase alone was insufficient to reduce phagocytosis of sRBCs, suggesting that these kinases function redundantly in this context (Supplemental Fig. 4).

We find that pharmacological inhibition of the Abl family kinases with STI571 and GNF-2 results in a more profound and persistent impairment in phagocytosis than that induced by genetic ablation of Abl and Arg. This suggests that compensatory mechanisms may be occurring in cells genetically deleted for Abl kinases. Moreover, imatinib has inhibitory activity against c-fms, PDGFR, and c-Kit (39, 40), so it is possible that other imatinib-sensitive targets may contribute to phagocytosis. However, our finding that both pharmacological inhibition and genetic ablation of Abl and Arg result in deficits in phagocytosis suggests that these kinases are required for efficient phagocytosis.

Arg kinase is found at the phagocytic cup

Phagocytosis requires the formation of an actin-rich phagocytic cup during which contraction of the actin ring promotes membrane invagination and internalization (45). The finding that Arg kinases regulate the efficiency of phagocytosis led us to examine whether Abl family kinases were present at the phagocytic cup. RAW264.7 macrophages were transduced with YFP-tagged Arg kinase and stimulated with IgG-opsonized zymosan for a short time period to capture phagocytic cup formation. Indeed, intense regions of YFP<sup>+</sup> fluorescence were found in the membrane of cells undergoing phagocytosis, adjacent to the bound zymosan particles (Fig. 3). Further, staining for F-actin with phalloidin to mark active phagocytic cups revealed colocalization of Arg with F-actin (Fig. 3, merge). In some cases, Arg kinase was found in the membrane adjacent to zymosan particles that lacked phalloidin staining, suggesting Arg may also localize to the nascent phagosome. In contrast, in cells that were not bound to zymosan, Arg<sup>+</sup>YFP was diffusely localized at the plasma membrane and in the cytosol.
The localization of ArgYFP with the F-actin–rich cup suggests that Arg may modulate actin dynamics during phagocytosis.

Abl kinase activity regulates phagocytic cup formation
Tyrosine kinases have been implicated in regulating actin cytoskeletal rearrangements during phagocytosis. Src family kinases, for instance, have a critical role in promoting actin polymerization at the phagocytic cup after FcγR engagement (12). In contrast, Syk is required for phagocytic cup closure (14). To evaluate the role of Abl kinases during phagocytosis, RAW264.7 macrophages were pretreated with Abl kinase inhibitors followed by stimulation with IgG-opsonized sRBCs. Vehicle-treated cells exhibited robust phagocytic cup formation, but treatment with STI571 or GNF-2 resulted in markedly decreased numbers of phagocytic cups per cell, although no defects in cup closure as revealed by actin staining were observed (Fig. 4A, 4B). Likewise, Abl/Arg null BMDMs exhibited a reduction in the number of cups formed per cell compared with control cells, whereas the binding of the particles was unaffected. Abl/Arg null cells averaged 70 ± 11% of the number of cups/cell compared with control macrophages (n = 6 experiments, example shown in Fig. 4C). The localization of Arg kinase at the phagocytic cup as well as the reduction in cup formation upon Abl kinase inhibition suggested a role for Abl/Arg kinase activity in signaling events during phagocytosis.

Abl family kinases are activated by FcγR engagement and function downstream of Src family kinases during phagocytosis
To evaluate whether Abl family kinases were involved in FcγR signaling, RAW264.7 macrophages were assayed for Abl/Arg activation after FcγR engagement. We found that CRKL became phosphorylated at the Abl-specific site upon FcγR cross-linking, and this phosphorylation was reduced in the presence of the pharmacological inhibitors STI571 and GNF-2 (Fig. 5A). In the canonical model of immunoreceptor activation, the clustering of receptors leads to their tyrosine phosphorylation by Src family kinases. To test if Abl family kinases could also function to regulate immunoreceptor phosphorylation, the γ subunit was immunoprecipitated from cells after FcγR cross-linking in the presence or absence of Abl or Src kinase inhibitors. As shown in Fig. 5B, inhibiting the Abl kinases with STI571 or GNF-2 did not affect γ subunit tyrosine phosphorylation, whereas cells treated with the Src inhibitor SU6656 had reduced γ-chain phosphorylation, consistent with previous reports that Src kinases mediate FcγR phosphorylation (12, 16, 46).
Previous studies have shown that loss of Hck, Lyn, and Fgr, the Src kinases most predominantly expressed in macrophages, results in reduced phagocytosis and impaired FcγR signaling (12, 14). Activation of Abl family kinases downstream of Ag receptors depends in part on Src kinases (27). To test if Abl functions downstream of Src kinases during phagocytosis, RAW264.7 cells were pretreated with the Src kinase inhibitor SU6656 followed by FcγR cross-linking. FcγR-induced activation of Abl kinases was reduced in the presence of the SU6656 as detected by CRKL phosphorylation (Fig. 5C). Conversely, inhibition of Abl kinases with STI571 or GNF-2 failed to suppress Src activation as assessed by evaluating the phosphorylation of focal adhesion kinase (FAK) on tyrosines 576 and 577, Src-specific phosphorylation sites (47) (Fig. 5D). Together, the data suggest that Abl kinases function downstream of Src in this pathway. Phenotypically, treatment of RAW264.7 cells with SU6656 results in marked reduction in phagocytosis, similar to that observed with STI571 and GNF-2 (Fig. 5E). Furthermore, the combined inhibition of Src and Abl family kinases with SU6656 and either STI571 or GNF-2 produced a greater reduction in phagocytosis than treatment with each compound alone (Fig. 5E). These results suggest that the activation of Abl kinases in response to FcγR engagement is dependent in part on Src kinases. Inhibition of both Src and Abl family kinases may reduce phosphorylation of both common and distinct targets of these kinases in this pathway resulting in a greater reduction in phagocytosis.

Abl kinases modulate Syk activation

A critical signaling event downstream of the FcγR is the activation of the tyrosine kinase Syk. Both loss of Syk expression or inhibition of Syk kinase activity result in impaired phagocytosis (14–16, 48). To test if Abl kinases regulate the activation and phosphorylation of Syk, RAW264.7 macrophages were treated with Abl inhibitors or the Src inhibitor SU6656 and assayed for Syk phosphorylation after FcγR cross-linking. In agreement with previous studies, inhibition of Src family kinases reduced Syk phosphorylation on Y346 (which is recognized by an Ab to human Syk Y352, the equivalent site on human Syk) and on Y519 and Y520 (which are recognized by an Ab to human Syk YY525/6, the equivalent sites on human Syk) (Fig. 6A). Notably, treatment with the Abl family kinase inhibitors also reduced Syk phosphorylation on these sites (Fig. 6A). Furthermore, Abl/Arg null macrophages also exhibited reduced phosphorylation of Syk upon FcγR cross-linking (Fig. 6B). Phosphorylation of Syk on Y346 is required for both enzymatic activity as well as coupling to downstream effector molecules (49). In addition to Y346 phosphorylation, deletion or inhibition of Abl kinases in macrophages reduced phosphorylation of Syk on Y519 and Y520, which are Syk autoprophosphorylation sites required for maximal enzymatic activity (Fig. 6A, 6B) (50). These findings suggest that Abl kinases can modulate Syk activation induced by FcγR ligation. To test if Syk signaling affects Abl kinase activity, the FcγR was cross-linked in RAW264.7 cells treated with the Syk inhibitor piceatannol. Piceatannol failed to suppress Abl activation as measured by the phosphorylation of CRKL upon FcγR cross-linking.

FIGURE 4. Abl family kinase activity is required for efficient phagocytic cup formation. (A) RAW264.7 pretreated with either STI571 (10 μM) or GNF-2 (20 μM) were incubated with rabbit-IgG opsonized sRBCs for 2 min. Cells were then fixed and stained for F-actin (green) and Hoechst to mark nuclei (blue). IgG-opsonized RBCs were stained with PE-conjugated anti-rabbit Abs. Phagocytic cups were imaged by confocal microscopy. Scale bar, 10 μm. (B) RAW264.7 cells treated with inhibitors were stimulated with IgG-opsonized sRBCs for the indicated times and processed as in (A). The number of phagocytic cups present in 100 randomly selected cells was counted. Results are representative of three independent experiments. *p < 0.05 (versus vehicle-treated cells). (C) BMDMs from control and Abl/Arg null mice were stimulated with IgG-opsonized sRBCs for 5 min and processed as in (A). The numbers of phagocytic cups in control and Abl/Arg null cells were quantified as in (B). Results from two sets of control and Abl/Arg null BMDMs are quantified. Scale bar, 10 μm. Arrowheads point to F-actin–rich cups.
linking (Fig. 6C). Thus, these findings suggest that in contrast to Src kinases, Syk function is not required for FcγR-mediated activation of Abl kinases.

To investigate further the link between Abl kinases and Syk phosphorylation, constitutively active or dominant negative forms of the Abl kinase were expressed in HEK293T cells in the presence of...
Abl kinases modulate Syk activation downstream of the FcγR. (A) RAW264.7 cells were serum starved 4 h, then treated with STI571 (10 μM), GNF-2 (20 μM), or SU6656 (2.5 μM) 2 h prior to FcγR cross-linking. For cross-linking, cells were incubated with the anti-FcγR mAb 2.4G2 followed by anti-rat IgG cross-linking for 5 min. Graphs show quantitation of phospho-Syk (pSyk) Y346 and phospho-Syk YY519/20, normalized to total Syk, from three and four independent experiments, respectively. *p < 0.05 (compared with FcγR-stimulated control macrophages). (B) Control and Abl/Arg null BMDMs were serum starved 4 h, then cross-linked and processed as in (A). Graphs show quantitation of phospho-Syk Y346 and phospho-Syk YY519/20 levels, normalized to total Syk, from seven and three experiments, respectively. *p < 0.05 (compared with FcγR-stimulated control macrophages). (C) RAW264.7 cells were treated with drugs as in (A) or with piceatannol (25 μM) as indicated. After FcγR cross-linking, cells were assayed for phospho-CRKL (pCRKL) levels by Western blotting. Graph shows quantitation of phospho-CRKL levels, normalized to total CRKL, from two independent experiments. (D) 293T cells were transiently transfected with myc-tagged Syk and constitutively active Abl (AblPP) or kinase-defective Abl (AblKR) and analyzed after 48 h for phospho-Syk Y346 levels. Phospho-Syk levels were normalized to total Syk and expressed as fold change over vector. Graph shows quantitation from three independent experiments. *p < 0.05 (compared with cells expressing empty vector). (E) Syk was immunoprecipitated from 293T cells transfected with MigR1-myr-FLAG-Syk, then incubated with purified full-length Arg kinase (FL) or a C-terminal Arg fragment (CT), in the presence or absence of ATP. Phosphorylation of Syk at Y346 in the presence of full-length Arg and ATP is shown. Phospho-Syk levels were normalized to total Syk, and the fold increase in phospho-Syk after incubation with purified Arg was quantitated from three independent experiments. *p < 0.05 (versus no Arg or ArgCT).

Expression of constitutively active Abl (AblPP) increased Syk Y346 phosphorylation, whereas the phosphorylation was unaffected in cells expressing the kinase-inactive form of Abl (AblKR) (Fig. 6D). Furthermore, purified full-length Arg protein promoted the phosphorylation of FLAG-tagged Syk on Y346 in vitro (Fig. 6E). Syk Y346 phosphorylation was not detected in the absence of full-length Arg or in the presence of the C-terminal fragment of Arg, which lacks the kinase domain (Fig. 6E). Full-length Arg was unable to promote the phosphorylation of Syk on YY519/20 (data not shown), indicating that Y346 is the primary target of Abl kinases. Thus, Abl family kinases promote Syk phosphorylation in vitro and in cells.

It has been shown that the binding of Syk to phosphorylated ITAMs of the Fc receptor subunits is sufficient to induce conformational changes in Syk that lead to Syk autophosphorylation and activation (51). Syk activation is more efficient, however, when also phosphorylated in trans by other kinases (52, 53). If Abl kinases regulate phagocytosis by modulating Syk activation, we hypothesized that overexpression of a plasma membrane–targeted form of Syk may enhance phagocytosis even in the absence of Abl.
kinase signaling by shifting the equilibrium toward a more active pool of Syk. To evaluate this possibility, we used RAW264.7 macrophages overexpressing a myristoylated form of Syk in the absence and presence of the Abl/Arg-selective inhibitor GNF-2 (Fig. 7C). Overexpression of this membrane-associated Syk partially rescued the defective phagocytosis in macrophages treated with GNF-2 (Fig. 7A, 7B). These data support a model whereby Abl kinases become activated in response to FcγR ligation and modulate Syk kinase activity to promote phagocytosis (Fig. 7D).

Discussion
In this study, we show that Abl family kinases regulate the efficiency of FcγR-mediated phagocytosis and uncover a novel functional link between Abl and Syk kinases downstream of the FcγRs in macrophages. These findings suggest that Abl kinases may regulate pathogen clearance through phagocytosis. Previous reports have shown a requirement for Abl kinases in mediating the entry and dissemination of many pathogens including Shigella flexneri, Chlamydia trachomatis, HIV, and polyomavirus (54–59). Although these reports and recent findings on the role for the Abl kinases in Leishmania phagocytosis (60) support a role for Abl family kinases in pathogen uptake, the signaling pathways that are regulated by Abl kinases for efficient phagocytosis had remained elusive. Syk is an essential regulator of phagocytosis, as loss of Syk results in failure of macrophages to ingest pathogens (13, 14). In the classical paradigm, clustering of FcγRs activates membrane-associated Src kinases, which phosphorylate cytoplasmic ITAMs on the γ adapter protein (61). Phosphorylated ITAMs then serve as docking sites for the tandem SH2 domains of Syk. Upon binding the phosphorylated ITAMs, Syk undergoes conformational changes that lead to its activation. Src kinases have been shown to phosphorylate several tyrosines on Syk that potentiate its activity (52, 53). Our data show Abl kinases modulate the phosphorylation of Y346 on Syk both in vitro and in cells. Phosphorylation of Syk on Y346 disrupts Syk autoinhibitory intramolecular interactions between the interdomain B sequences and the kinase domain of Syk; moreover, binding of SH2-containing proteins to phosphorylated Y346 on Syk has been proposed to help stabilize the active conformation of the kinase (49, 62). Reduced phosphorylation of Syk on Y346 upon inhibition or loss of Abl family kinases may explain, in part, why phagocytosis in the Abl/Arg null macrophages is impaired. Previous studies have shown that Abl family kinases regulate the phosphorylation of the Syk family member ZAP70 on Y319 after TCR engagement and L-selectin ligation (27, 32, 63). Tyrosine 319 of ZAP70 is analogous to tyrosine 346 of Syk, and our finding that purified Arg kinase can directly phosphorylate this site in vitro suggests that an Abl kinase–Syk/ZAP70 signaling module may operate under diverse cell surface receptors to regulate cytoskeletal responses in hematopoietic cells.

Activation of Abl kinases downstream of growth factor, Ag, and chemokine receptors is mediated in part through Src kinases (23, 24, 27, 32, 38). We found that this paradigm also exists in during FcγR signaling in macrophages. Previous studies have shown that genetic deletion of Src family kinases in macrophages attenuates signaling from the FcγR, including Syk phosphorylation and activation (12). Our data show that pharmacological inhibition of Src impaired many FcγR proximal signaling events including decreasing the activation of Abl and Syk kinases. Notably, simultaneous inhibition of Abl and Src further reduces phagocytosis, suggesting these kinases may have unique targets or differentially regulate common targets through phosphorylating them on distinct sites. In this regard, a recent report indicated that Abl and Src form a complex in myeloid cells that regulates myeloid cell migration by modulating the activation of Rac and Cdc42 (34). The mechanism by which Abl and Src function to control activation of Rho GTPases during phagocytosis warrants further investigation.

Although Abl and Arg promote maximal Syk activation during phagocytosis, they are likely to have Syk-independent roles. Inactivation of Abl kinases reduces the formation of phagocytic cups in addition to reducing Syk phosphorylation. Notably, whereas Syk null macrophages form phagocytic cups normally, the cups fail to close (15, 48). This suggests that the Abl family kinases might regulate actin rearrangements at the phagocytic cup through mechanisms that may involve other Abl targets such as CRKII and WASP. Previous studies have shown that Abl kinases regulate Shigella flexneri invasion through phosphorylation of the adapter protein CRKII and the N-WASP actin nucleation promoting factor (54–56). Notably, both CRKII and WASP are recruited to the phagocytic cup during FcγR phagocytosis (64, 65). Phosphorylation of WASP on
Y291 is required for phagocytic cup formation (66), and Abl has been shown to phosphorylate this site in vitro (67). Future studies should address whether the Abl-mediated phosphorylation of CRKII or WASP is required for regulating F-actin organization at the phagocytic cup.

Previous studies have shown that Abl kinases modulate adaptive immune function (29, 32). The Abl single knockout mouse exhibits multiple abnormalities, among the most prominent of which are immune defects including splenic and thymic atrophy, and increased susceptibility to infection (26). Our current data suggest that in addition to lymphocyte defects, loss or inhibition of both Abl and Arg impairs myeloid cell function. Notably, the Abl kinase inhibitor imatinib has been reported to have anti-inflammatory effects in a variety of autoimmune disease models (68–71) and inhibits the functional capacity of human monocytes in vitro (72). The effects of imatinib on monocytes have previously been attributed to inhibition of the c-fms tyrosine kinase (72). Imatinib has multiple kinase and non-kinase targets (73), and our data using new and selective allosteric Abl kinase inhibitors and Abl/Arg knockout macrophages suggest that one of the relevant targets of imatinib in myeloid cells is the Abl kinase family. Our findings have potential implications for the treatment of autoimmune diseases with deregulated FcγR signaling (74). Future studies should determine whether the second-generation allosteric Abl inhibitors, which are selective for the Abl kinases, are useful for the treatment of inflammatory disorders.

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Disclosures

The authors have no financial conflicts of interest.

References
