

# A Critical Role for Syk in Signal Transduction and Phagocytosis Mediated by Fc $\gamma$ Receptors on Macrophages

By Mary T. Crowley,\* Patrick S. Costello,<sup>§</sup> Cheryl J. Fitzer-Attas,\*  
Martin Turner,<sup>§</sup> Fanying Meng,<sup>‡</sup> Clifford Lowell,<sup>‡</sup>  
Victor L. J. Tybulewicz,<sup>§</sup> and Anthony L. DeFranco\*

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*From the G. W. Hooper Foundation, \*Department of Microbiology and Immunology, and  
‡Department of Laboratory Medicine, University of California, San Francisco, California  
94143-0552; and the §National Institute for Medical Research, London NW7 1AA, United Kingdom*

## Summary

Receptors on macrophages for the Fc region of IgG (Fc $\gamma$ R) mediate a number of responses important for host immunity. Signaling events necessary for these responses are likely initiated by the activation of Src-family and Syk-family tyrosine kinases after Fc $\gamma$ R cross-linking. Macrophages derived from Syk-deficient (Syk<sup>-</sup>) mice were defective in phagocytosis of particles bound by Fc $\gamma$ Rs, as well as in many Fc $\gamma$ R-induced signaling events, including tyrosine phosphorylation of a number of cellular substrates and activation of MAP kinases. In contrast, Syk<sup>-</sup> macrophages exhibited normal responses to another potent macrophage stimulus, lipopolysaccharide. Phagocytosis of latex beads and *Escherichia coli* bacteria was also not affected. Syk<sup>-</sup> macrophages exhibited formation of polymerized actin structures opposing particles bound to the cells by Fc $\gamma$ Rs (actin cups), but failed to proceed to internalization. Interestingly, inhibitors of phosphatidylinositol 3-kinase also blocked Fc $\gamma$ R-mediated phagocytosis at this stage. Thus, PI 3-kinase may participate in a Syk-dependent signaling pathway critical for Fc $\gamma$ R-mediated phagocytosis. Macrophages derived from mice deficient for the three members of the Src-family of kinases expressed in these cells, Hck, Fgr, and Lyn, exhibited poor Syk activation upon Fc $\gamma$ R engagement, accompanied by a delay in Fc $\gamma$ R-mediated phagocytosis. These observations demonstrate that Syk is critical for Fc $\gamma$ R-mediated phagocytosis, as well as for signal transduction in macrophages. Additionally, our findings provide evidence to support a model of sequential tyrosine kinase activation by Fc $\gamma$ R's analogous to models of signaling by the B and T cell antigen receptors.

Cross-linking of receptors for Fc regions of IgG (Fc $\gamma$ Rs)<sup>1</sup> triggers cellular events that are crucial for a variety of immune responses. These include phagocytosis, production of cytokines and chemokines, release of agents that damage microorganisms or infected cells, and changes in expression of cell surface proteins involved in cell-cell adhesion and antigen presentation (1, 2). The important roles for these receptors in antibody-mediated allergic and inflammatory responses have been demonstrated in mice made deficient for Fc $\gamma$ Rs by targeted gene disruption (3, 4). Thus, the Fc $\gamma$ Rs allow the humoral and cellular aspects

of immunity to communicate and cooperate in expanding, sustaining, and regulating immune responses.

Signaling events triggered by Fc $\gamma$ R cross-linking are believed to be largely analogous to the events induced by engagement of B cell and T cell antigen receptors. Tyrosine kinases of the Src and Syk families become activated and associate with specific recognition sequences known as immunoreceptor tyrosine-based activation motifs (ITAMs), contained within the intracellular domains of some of the Fc $\gamma$ R subunits. Targets of these activated tyrosine kinases include the Fc $\gamma$ R itself, enzymes that generate second messengers (e.g., phospholipase C- $\gamma$ 1 and phosphatidylinositol 3-kinase [PI 3-kinase]), and regulators of Ras and other Ras-like G proteins (e.g., Shc, Vav) (5).

An important function of Fc $\gamma$ Rs on macrophages and monocytes is their ability to promote phagocytosis. Ingestion of IgG-coated cells serves to remove and destroy invading

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<sup>1</sup>Abbreviations used in this paper: AA, arachidonic acid; EA, erythrocytes coated with antibody; Fc $\gamma$ R, Fc receptor for IgG; GST, glutathione S-transferase; GST-c-Jun, GST fused to the NH<sub>2</sub> terminus of c-Jun; ITAM, immunoreceptor tyrosine-based activation motif; JNK, Jun NH<sub>2</sub>-terminal kinase; LCM, L cell conditioned media; PI, phosphatidylinositol.

microorganisms or infected cells. In addition, phagocytosis provides a means for internalizing antigen for processing and presentation to T cells (6). The molecular mechanisms by which Fc $\gamma$ Rs trigger the phagocytic process are poorly understood. A role for Fc $\gamma$ R-mediated protein tyrosine phosphorylation in inducing phagocytosis is suggested by the finding that protein tyrosine kinase inhibitors block phagocytosis of IgG-coated particles (7–9). Moreover, the intracellular tyrosine kinase Syk associates with Fc $\gamma$ RII (10) and with the tyrosine phosphorylated  $\gamma$  chain of Fc $\gamma$ RI (11) and Fc $\gamma$ RIII (12), and has been implicated in Fc $\gamma$ R-mediated phagocytosis. For example, COS-1 cells transfected with human Fc $\gamma$ Rs exhibit enhanced phagocytosis upon cotransfection of human Syk (8). Similarly, cells expressing Fc $\gamma$ RIII-Syk (CD16-Syk) chimeras can phagocytose particles that cross-link the CD16 portion of the molecule (13); chimeras containing kinase-inactive Syk do not mediate internalization. How Syk promotes Fc $\gamma$ R-mediated phagocytosis is unclear, but inositol phospholipid metabolism is likely to be an important downstream signaling event since wortmannin, a potent inhibitor of PI 3-kinase, prevents Fc $\gamma$ R-mediated phagocytosis (8). To test directly the importance of Syk for Fc $\gamma$ R-induced signaling and phagocytosis, we have examined these events in cultured macrophages derived from mice genetically deficient for Syk. The role of Syk in signal transduction in response to Fc $\gamma$ R engagement and stimulation with the bacterial endotoxin LPS were also examined. The results reported here demonstrate that Syk is required for Fc $\gamma$ R-induced phagocytosis, but not for phagocytosis of latex beads or microorganisms. In addition, Syk was found to play an important role for many Fc $\gamma$ R-induced signaling events, but not for various LPS-induced signaling events or biological responses.

## Materials and Methods

**Antibodies.** The hybridomas producing the 2.4G2 monoclonal rat antibody, the MAR18.5 mouse anti-rat Ig  $\kappa$  chain monoclonal antibody, and the anti-sheep red blood cell monoclonal antibodies TIB 111, TIB 114, and TIB 109, were obtained from the American Type Culture Collection (Rockville, MD). Monoclonal antiphosphotyrosine-agarose was purchased from Sigma Chemical Co. (St. Louis, MO). The 4G10 antiphosphotyrosine antibody was prepared and used as described (14). Horseradish peroxidase-conjugated sheep anti-mouse IgG antibody was obtained from Amersham Corp. (Arlington Heights, IL). Horseradish peroxidase-conjugated sheep anti-rabbit IgG antibody was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IL). Protein G-Sepharose was obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Anti-human Vav protein antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Antibodies to p38, Erk1/2, and Jun NH<sub>2</sub>-terminal (JNK) kinases were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-Syk antibody has been described (15). Anti-Shc antisera was generated by immunizing with a glutathione S-transferase (GST) fusion protein containing amino acids 359–473 of the human Shc protein. Specific antibodies were obtained by affinity column purification of antibodies

reactive with the Shc-GST fusion protein, but not with GST alone.

**Reagents.** Wortmannin was obtained from Calbiochem Corp. (San Diego, CA). Fluorescent phalloidins were from Molecular Probes Inc. (Eugene, OR). Myelin Basic Protein was from Sigma Chemical Co. The recombinant GST fused to the COOH-terminal of Jun (GST-c-Jun) fusion protein was purified from *Escherichia coli* lysate as described (16). Purified LPS from *Salmonella minnesota* was obtained from List Biological Laboratories (Campbell, CA).

**Cells.** Mice heterozygous for a disruption of the *syk* gene (17) were bred, and the resulting embryos were dissected at day 17 of gestation to obtain the fetal liver. Genotyping of offspring was performed by Southern blotting as described previously (17). Single-cell suspensions were resuspended at 10<sup>6</sup> cells per ml in RPMI-1640 media supplemented with 10% FCS, 2 mM sodium pyruvate, 1 mM glutamine, and 50  $\mu$ M 2-mercaptoethanol, non-essential amino acids, and 10% L cell conditioned media (LCM) as a source of M-CSF and GM-CSF. The adherent monolayer cultures generated by this protocol contained primarily cells of the macrophage lineage as determined by adherent morphology, expression of CD11b, CD16, and CD32, but not Gr-1, or B or T lymphocyte markers.

Generation of the *hck*<sup>-</sup>/*fgr*<sup>-</sup>/*lyn*<sup>-</sup> strain of mice is described elsewhere (18). Macrophages from normal and *hck*<sup>-</sup>/*fgr*<sup>-</sup>/*lyn*<sup>-</sup> mice were isolated from bone marrow. Marrow from tibia and femur bones was eluted in DMEM. Clumps were removed by rigorous pipetting and passage through 70- $\mu$ m nylon mesh. Erythrocytes were lysed by adding 2 vol of 1.44% NH<sub>4</sub>Cl. The cells in these suspensions were recovered by centrifugation. Cells were cultured in media described for fetal liver cultures. Nonadherent cells were removed 1–2 d later and transferred to a fresh 150-mm plate. Nonadherent cells were removed from this secondary culture 4 d later and discarded. The adherent cultures were used 5–7 d after initial harvest from the mice, when confluency was achieved. Cultures generated via this protocol were almost purely macrophages, as indicated by the criteria listed above, and contained very few, if any, fibroblasts.

**Binding and Phagocytosis of Antibody-coated Erythrocytes (EA).** EA were prepared in calcium- and magnesium-free PBS by incubating sheep erythrocytes (Accurate Chemical and Scientific Corporation, Westbury, NY) (10<sup>9</sup>/ml) with a subagglutinating concentration of rabbit anti-sheep red blood cell antibodies (Nordic Immunology, Tilburg, Netherlands). Cultured macrophages were grown on 12-mm glass coverslips and incubated at 37°C with EA (10<sup>7</sup>/ml) for 30 min. Unbound EA was washed away, and some coverslips were fixed in 2% glutaraldehyde in PBS for 30 min, and were mounted for phase microscopy of rosettes as described below. To observe internalization, some coverslips were incubated in hypotonic buffer (1.4% NH<sub>4</sub>Cl) at room temperature for 5 min to lyse uninternalized EA before fixation. For studies on the rate of phagocytosis, EA particles were first bound to macrophages on ice for 30 min, followed by a 5-min centrifugation at 150 g. Phagocytosis was initiated by addition of warm medium, and cultures were placed in a 37°C incubator. Uninternalized EA were lysed by addition of H<sub>2</sub>O for 30 s, and cells were fixed as above.

**Fluorescence Microscopy.** To visualize actin cups, cells on coverslips were incubated in the presence or absence of cytochalasin D (1  $\mu$ M) for 15 min. Cells were incubated 2–4 min with EA, and were then fixed in 3.7% formaldehyde for 10 min. Alternatively, to achieve a high number of stable actin cups, cells were incubated on ice with EA (10<sup>7</sup>/ml) for 30 min. Unbound EA were

washed away, and slips were processed for fluorescence microscopy. Cells on 12-mm glass coverslips were fixed in 3.7% formaldehyde for 10 min, and were then permeabilized in .01% Triton X-100 in PBS for 5 min. Cells were then stained with BODIPY-phalloidin or Oregon green-phalloidin (5 U/ml in PBS) for 15 min. Cells were observed by phase contrast and fluorescence microscopy (Axiophot microscope; Carl Zeiss, Inc., Thornwood, NY). To visualize LPS-induced actin-rich structures, cells on coverslips were incubated at 37°C with or without LPS (100 ng/ml) for 1 h.

**Cell Stimulation.** Before stimulation for signaling experiments, cells were starved for 2 h in culture media lacking LCM. For Fc $\gamma$ R stimulation, adherent cultures were incubated on ice for 30 min with 2.4G2 hybridoma supernatant. Cells were then warmed to 37°C and treated with cross-linking antibody (10  $\mu$ g/ml MAR18.5) for indicated times. Unstimulated cultures were incubated on ice, and at 37°C in culture media without LCM. For LPS activation, cells were starved as above. LPS (1  $\mu$ g/ml) was then added directly to culture vessels for the indicated times. Plates of Fc $\gamma$ R- and LPS-stimulated cells were washed with cold PBS containing 1 mM sodium vanadate. Ice-cold lysis buffer consisting of 1% Triton-X-100, 50 mM Hepes buffer, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 100 mM NaF, 1 mM PMSF, and 1 mM NaVO<sub>4</sub> was added directly to the flasks. Cultures were lysed on ice for 10 min.

**Immunoprecipitations and Immunoblotting.** Anti-Shc immunoprecipitations were carried out as described previously (19). For antiphosphotyrosine immunoprecipitations, samples were adjusted to RIPA (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris buffer, pH 8.0). Immunoprecipitations were carried out for 8–10 h at 4°C. SDS-PAGE and immunoblotting were carried out as described previously (19), with the exception that to detect the electrophoretic mobility shift of p42 Erk2, soluble lysate proteins were separated on 12.5% SDS-PAGE containing an acrylamide/bis-acrylamide ratio of 120:1, and were transferred to nitrocellulose.

**Immunoprecipitation and In Vitro Protein Kinase Assays.** Cell lysates prepared as described above were precleared with 25  $\mu$ l of protein A-Sepharose, and were then incubated with 10  $\mu$ l of anti-Syk, anti-JNK, or anti-Erk2 antibody for 1 h on ice. Immune complexes were collected on 25  $\mu$ l Protein A-Sepharose, and were washed twice with lysis buffer and twice with kinase assay buffer (25 mM Hepes, pH 7.6, 20 mM MgCl<sub>2</sub>, 20 mM  $\beta$ -glycerol phosphate, 1 mM DTT, or 20 mM MOPS, pH 7.6, 20 mM MgCl<sub>2</sub>, 30 mM  $\beta$ -glycerol phosphate, 5 mM EGTA, and 1 mM DTT for JNK or Erk kinase assays, respectively). Reactions were performed at 30°C for 15 min in the presence of 10  $\mu$ Ci of  $\gamma$ -[<sup>33</sup>P]ATP using 20–25  $\mu$ g GST-c-Jun or Myelin Basic Protein as substrate for JNK or Erk2, respectively. Anti-Syk immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer (20 mM Tris HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 0.1% NP-40). The beads were incubated in 50  $\mu$ l of kinase assay buffer plus 10  $\mu$ Ci of  $\gamma$ -[<sup>33</sup>P]ATP for 10 min at room temperature. Erk1/2, JNK, and Syk kinase reactions were stopped by adding SDS-PAGE sample buffer, separated by SDS-PAGE and transferred to nitrocellulose, and were analyzed using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA).

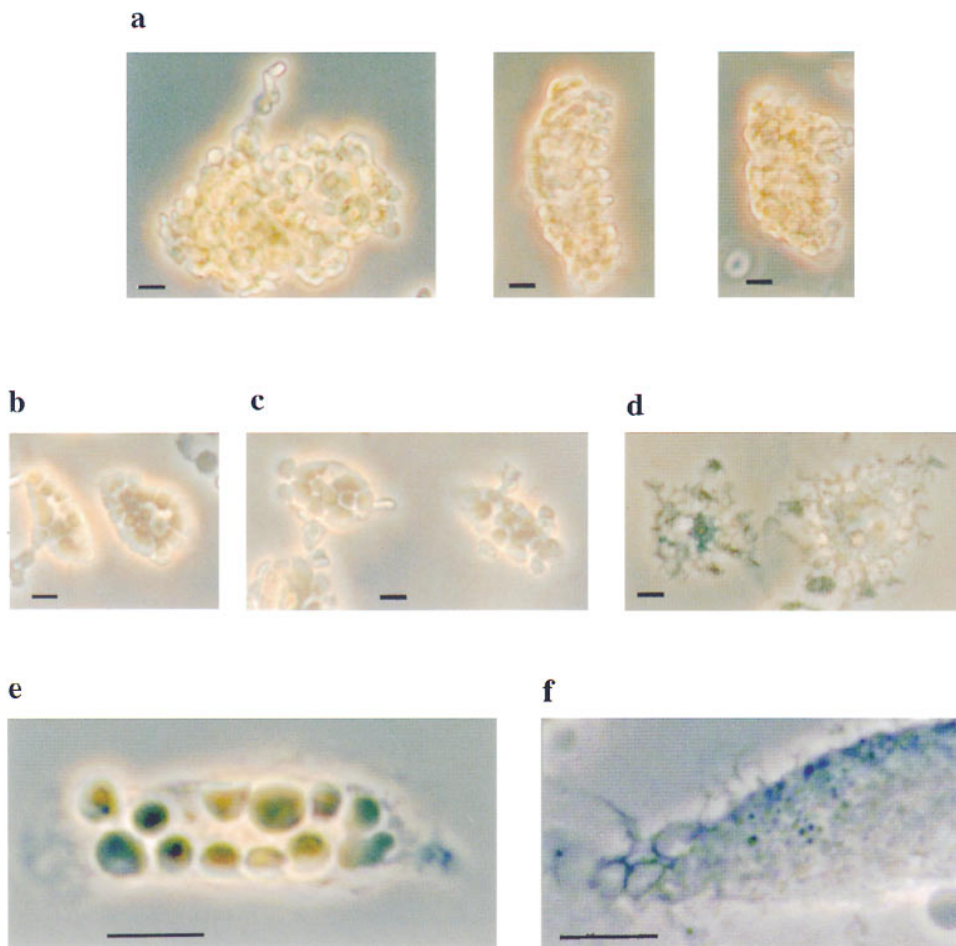
**Cytokine ELISA Assays.** Macrophages were incubated in culture media containing LCM, in 24-well plates at a density of 5  $\times$  10<sup>4</sup>/well in 1.5 ml of media. Cultures received LPS at the indicated doses, and supernatants were collected from cultures at the indicated times after initial exposure. Duplicate supernatants were collected from two separate wells at each time point. Supernatants were stored at –80° until assay. Cytokine concentrations in the

supernatants were measured using murine cytokine ELISA detection kits from Biosource International (Camarillo, CA).

## Results

**Defective Fc $\gamma$ R-Mediated Phagocytosis in SYK<sup>–</sup> Macrophages.** The engulfment of cells and particulate matter is an important function of the tissue macrophage. Among the receptors known to promote phagocytosis are the Fc $\gamma$ Rs and the complement receptor. To examine the relative importance of Syk and members of the Src-family of tyrosine kinases for Fc $\gamma$ R-mediated phagocytosis, we established primary cultures of fetal liver-derived wild-type and Syk-deficient macrophages, or of bone marrow-derived wild-type and Src-family kinase-deficient (Hck<sup>–</sup>/Fgr<sup>–</sup>/Lyn<sup>–</sup>) macrophages in CSF-1-containing medium. None of the other known Src-family tyrosine kinases were detected in the latter macrophages (18).

The capacity of these cultured fetal liver-derived or bone marrow-derived macrophages to mediate particle uptake via their Fc $\gamma$ Rs was tested (Fig. 1). Macrophages were incubated with IgG-coated sheep erythrocytes (EA) either on ice or at 37°C. Wild-type, Syk-deficient, and Hck<sup>–</sup>/Fgr<sup>–</sup>/Lyn<sup>–</sup> cells bound EA equally well under either condition. At 37°C, wild-type and Hck<sup>–</sup>/Fgr<sup>–</sup>/Lyn<sup>–</sup> cells internalized the bound EA particles, although the Src family-deficient macrophages internalized the bound particles more slowly. To examine this difference more readily, the macrophages were allowed to bind the EA particles at 0°C, and were then warmed to 37°C to permit internalization to proceed. Under these conditions, wild-type macrophages internalized the EA particles rapidly, with a substantial percentage internalized by 5 min at 37°C, and near maximal internalization by 10 min. In contrast, the Hck<sup>–</sup>/Fgr<sup>–</sup>/Lyn<sup>–</sup> macrophages had internalized very few particles by 5 min, but had internalized a substantial percentage by 10 min. At this time, there was less internalization than that seen with wild-type macrophages, but after 30 min the difference was minimal (data not shown). In contrast, Syk-deficient macrophages began engulfment, but were unable to complete phagocytosis of the bound EA particles. This effect was readily apparent when the macrophages were subjected to hypotonic conditions that lysed the erythrocytes attached to the surface of the macrophage, but not the fully engulfed EA (Fig. 1, *b* and *d*). Additionally, wild-type macrophages bound and internalized erythrocytes coated with IgG3, IgG2a, or IgG2b antibodies, whereas Syk<sup>–</sup> macrophages bound but failed to internalize these particles (data not shown). Antibody-coated erythrocytes remained bound to Fc $\gamma$ Rs on the surface of Syk<sup>–</sup> macrophages for several days in culture, indicating that the inhibition of phagocytosis was complete, and was not simply delayed. The phagocytosis observed in these experiments was Fc $\gamma$ R dependent as uncoated erythrocytes were not phagocytosed or bound by normal or Syk<sup>–</sup> macrophages. These findings demonstrate an absolute requirement for the presence of Syk to accomplish Fc $\gamma$ R-mediated phagocytosis in macrophages.

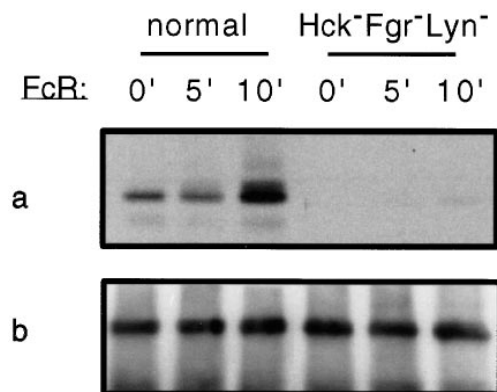


**Figure 1.** Binding and phagocytosis of antibody-coated EA by wild-type, Hck<sup>-/-</sup>Fgr<sup>-/-</sup>Lyn<sup>-/-</sup>, and Syk<sup>-/-</sup> macrophages. Adherent wild-type, Hck<sup>-/-</sup>Fgr<sup>-/-</sup>Lyn<sup>-/-</sup>, and Syk<sup>-/-</sup> macrophages (a, left to right) were incubated with EA (10<sup>7</sup> cells per ml) for 30 min at 37°C before removal of unbound EA. Uninternalized EA were lysed in b–f. All normal macrophages (b and e) and Hck<sup>-/-</sup>Fgr<sup>-/-</sup>Lyn<sup>-/-</sup> macrophages (d) contained many internalized erythrocytes. The large load of ingested material resulted in a loss of cell body extensions, and a more rounded appearance of the macrophages. None of the Syk-deficient macrophages contained any internalized erythrocytes (d and f). These macrophages had a frilled appearance due to the interlacing of actin-rich macrophage cell membrane structures around each bound erythrocyte. Bar, 10 μm.

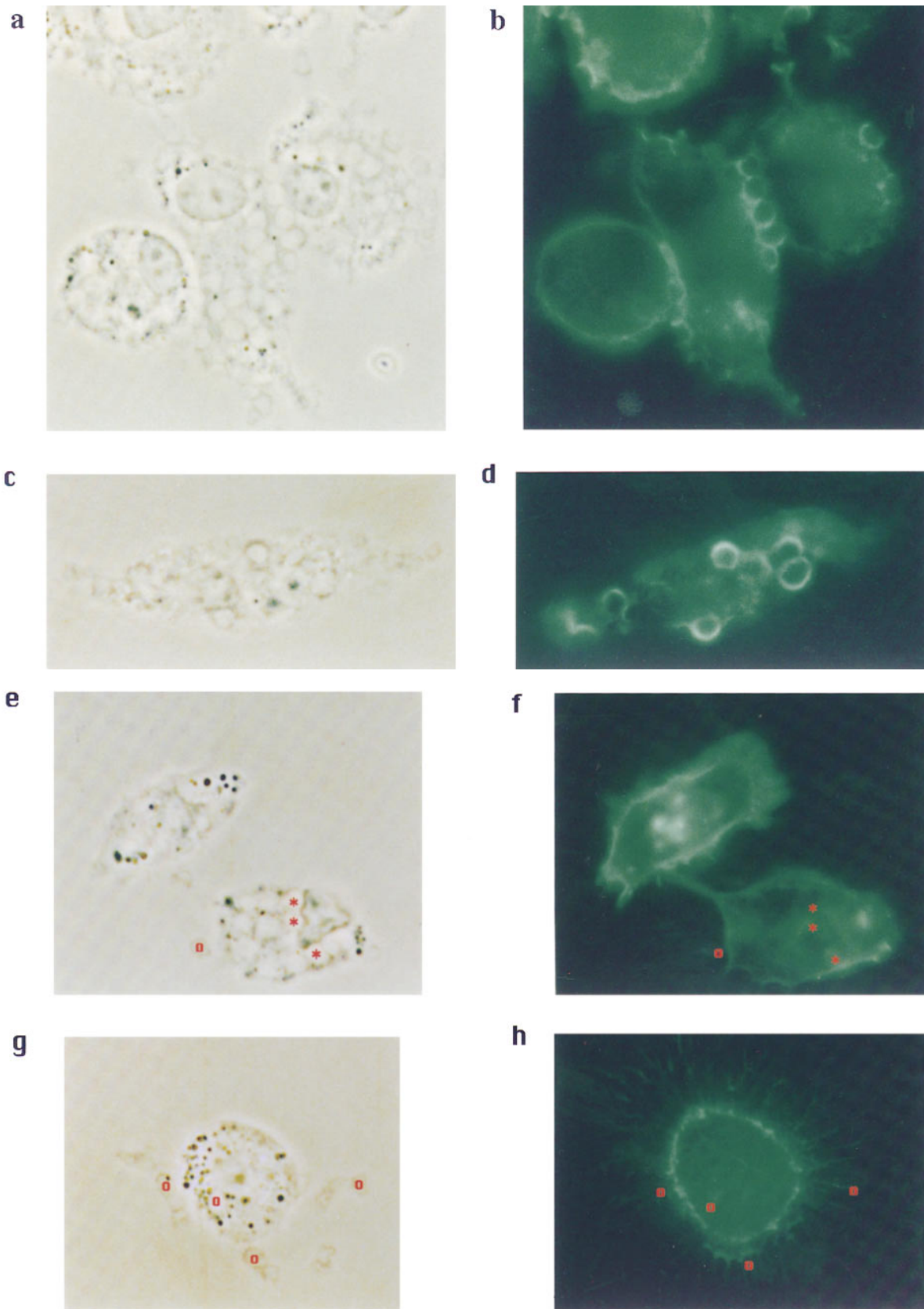
The difference in phagocytic ability of Syk<sup>-/-</sup> macrophages compared to Hck<sup>-/-</sup>/Fgr<sup>-/-</sup>/Lyn<sup>-/-</sup> macrophages was unexpected, as both of these types of kinases are thought to play an important role in signaling by the family of receptors that includes FcγRs and the antigen receptors of B and T cells (5, 20). Studies in a variety of systems have suggested that Src-family tyrosine kinases are responsible for initiating signaling of these immunoreceptors by phosphorylating the two tyrosines of the receptor ITAM, and thereby providing a mechanism for recruiting and activating the tyrosine kinase Syk (or ZAP-70 in T cells) to the engaged receptor (21–23). Indeed, as predicted by this general model, we found that Syk was only poorly activated after FcγR cross-linking in Hck<sup>-/-</sup>/Fgr<sup>-/-</sup>/Lyn<sup>-/-</sup> macrophages as compared to its activation in normal cells (Fig. 2). It should be noted, however, that there was a small amount of FcγR-induced activation of Syk in the Src family mutant cells, indicating that Syk could participate in FcγR signaling to some extent, even in the absence of these three Src-family tyrosine kinases.

The defect in FcγR-mediated phagocytosis of Syk-deficient macrophages did not represent a general defect in the phagocytic ability of these cells. There were no discernible differences in the abilities of wild-type, Syk-deficient, or Hck<sup>-/-</sup>/Fgr<sup>-/-</sup>/Lyn<sup>-/-</sup> macrophages to phagocytose latex beads

ranging in size from 0.2 to 5.5 μm, or to engulf and destroy yeast and *E. coli* (data not shown). Furthermore, after pretreatment with LPS, all three macrophage populations exhibited increased phagocytic capacity for bacteria (data



**Figure 2.** Greatly decreased FcγR-mediated Syk activation in Hck<sup>-/-</sup>/Fgr<sup>-/-</sup>/Lyn<sup>-/-</sup> macrophages. Bone marrow-derived macrophages were incubated with growth media (0') or anti-FcγR mAb 2.4G2 followed by anti-rat IgG cross-linking for the indicated times. The protein kinase activity of Syk was assessed by in vitro autophosphorylation of immunoprecipitated Syk followed by SDS-PAGE and autoradiography (a). Anti-Syk immunoblotting (b) showed that equivalent amounts of Syk protein were present in each reaction.



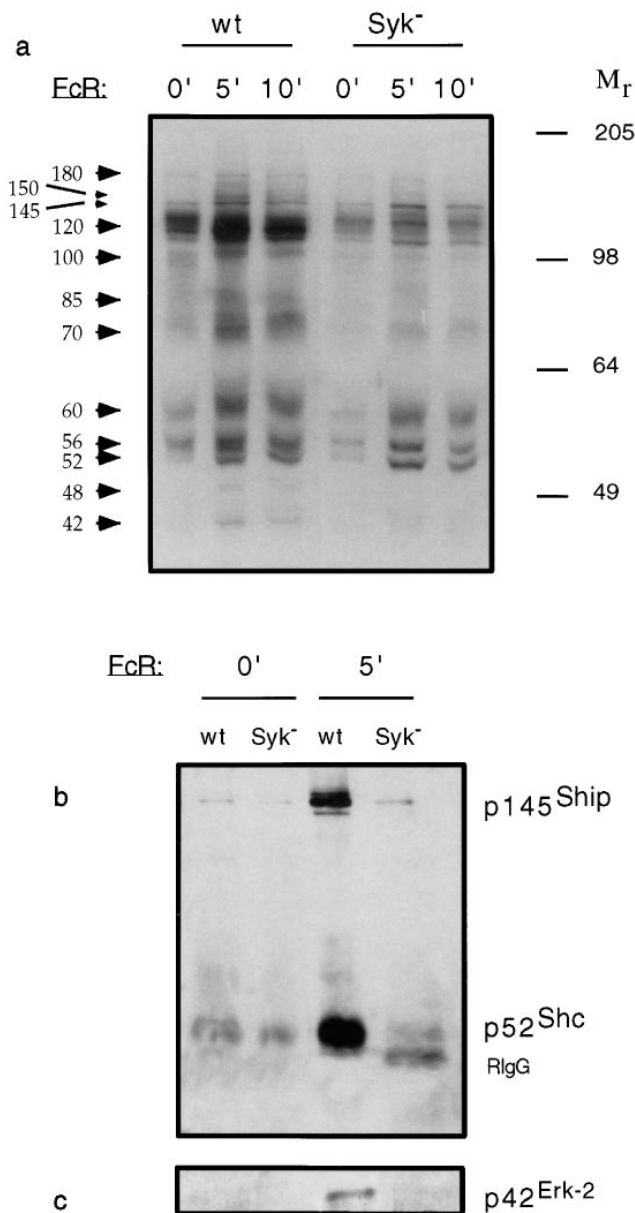
**Figure 3.** Formation of actin-rich membrane cups around bound EA in normal and Syk-deficient macrophages. Macrophages were incubated with EA on ice for 20 min. Fixed and permeabilized normal (*a* and *b*) and Syk-deficient (*c* and *d*) cells were stained with Oregon green-phalloidin to visualize F-actin. The bound erythrocytes are apparent under phase microscopy (*a* and *c*). Corresponding actin-lined cups visible by fluorescence microscopy (*b* and *d*) are evident. Alternatively, after incubation on ice, cells were washed and further incubated for 20 min at 37°C, during which time there was dissolution of actin cup structures in Syk-deficient macrophages (*g* and *h*) as well as internalization of the EA in the case of normal cells (*e* and *f*). Several internalized EA (\*) and bound, noninternalized EA (o) are indicated by symbols inside the erythrocytes.

not shown). The attachment and engulfment of microorganisms most likely represents binding of complex carbohydrates on the surface of the microbe to lectin-like glycoproteins or integrins expressed by the macrophage (24). Evidently these receptors do not require Syk for triggering phagocytosis.

To characterize further the defect in Fc $\gamma$ R-mediated phagocytosis of Syk<sup>-</sup> macrophages, we next examined the position of actin structures after EA binding. Actin is likely to play an important role in phagocytosis since it is concentrated in the macrophage cytoplasm surrounding the particle being engulfed, and since cytochalasin D treatment, which blocks actin polymerization, abrogates phagocytosis without affecting particle binding (25, 26). We used fluorescent phalloidin to visualize the polymerized actin cytoskeleton in Syk-deficient and wild-type cells. Phagocytic, actin-lined cups formed around antibody-coated erythrocytes bound by Syk-deficient cells, and were indistinguishable from those formed by wild-type cells (Fig. 3, a-d) or by Hck<sup>-</sup>/Fgr<sup>-</sup>/Lyn<sup>-</sup> cells (data not shown). Actin cup formation occurred at 4°C or at 37°C, and was blocked by cytochalasin D treatment. Syk-deficient cells were presumably unable to effect the closure of the pocket generated by the advancing membrane edges that created the phagocytic cup. How macrophages and other professional phagocytes achieve this final stage of phagocytosis is unknown. At 37°C, but not at 4°C, the actin cup structures were dynamic, and their dissolution occurred at about the same time as internalization was completed in wild-type macrophages. Interestingly, Syk<sup>-</sup> cells also exhibited dissolution of the actin cups around the bound EA, although these particles were never internalized or released.

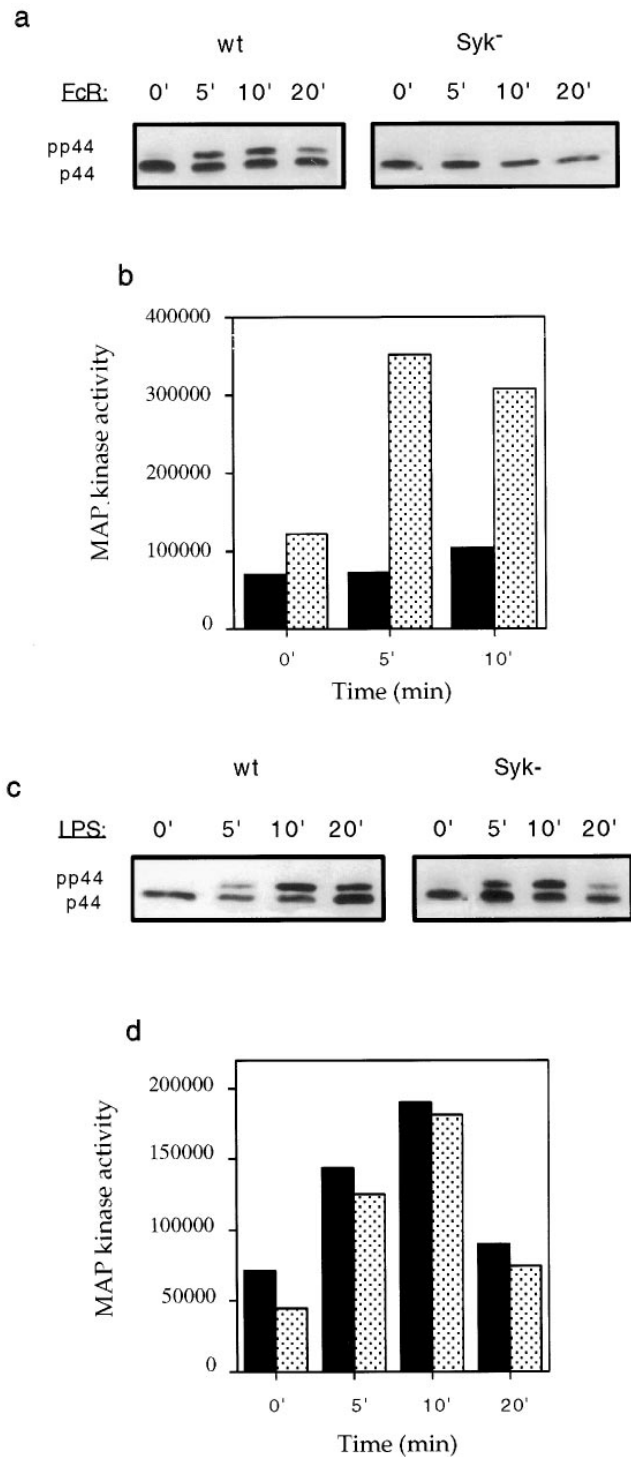
We next examined whether Syk participates in other macrophage-signaling pathways involving cytoskeletal rearrangements. Macrophages responding to LPS develop distinct morphological changes, including the development of actin-rich ruffles. As LPS stimulation of macrophages leads to tyrosine phosphorylation of Syk (19), we asked whether Syk participated in LPS-induced cytoskeletal changes. When exposed to LPS, Syk<sup>-</sup> cells developed actin-rich ruffles indistinguishable from those induced on normal cells (data not shown). Thus, LPS does not require Syk to effect these changes in the actin-based cytoskeleton.

**Impaired Signaling Via Fc $\gamma$ R In Syk<sup>-</sup> Macrophages.** To examine the mechanism behind the observed phagocytic defect of Syk<sup>-</sup> macrophages, we looked for defects in tyrosine phosphorylation of cellular proteins and defects in signal transduction pathways known to be dependent upon receptor-activated tyrosine kinases. After cross-linking of Fc $\gamma$ RII and Fc $\gamma$ RIII on the surface of Syk<sup>-</sup> and wild-type macrophages with the antibody 2.4G2, total cellular proteins were analyzed by SDS-PAGE and antiphosphotyrosine immunoblotting. We observed a number of proteins that were inducibly tyrosine phosphorylated in normal cells. The most obvious of these had apparent molecular weights of 180, 150, 145, 120, 100, 85, 70, 60, 56, 52, 48, and 42 kD, and are indicated by arrows in Fig. 4 a. Syk<sup>-</sup> macrophages were defective in most aspects of this re-



**Figure 4.** Impaired Fc $\gamma$ R-induced tyrosine phosphorylation of cellular substrates in Syk-deficient macrophages. Cells were incubated with growth media (0') or anti-Fc $\gamma$ R mAb 2.4G2 followed by anti-rat IgG cross-linking for the indicated times. Cells were lysed, and were subjected to SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies (a). The molecular mass (in kD) of markers and the positions of major phosphotyrosine-containing proteins are indicated at the right and left of a, respectively. Alternatively, cell lysates were subjected to immunoprecipitation with anti-Shc antibodies (b) or anti-Erk2 antibodies (c). Erk2, Shc, and Shc-associated proteins were resolved by SDS-PAGE, and their phosphorylation status was determined by immunoblotting with antiphosphotyrosine antibodies.

sponse. Some of the induced tyrosine phosphoproteins, for example, the 42-kD band corresponding to Erk2 (Fig. 4 c), were conspicuously absent upon Fc $\gamma$ R stimulation in Syk<sup>-</sup> cells, and many other proteins became only weakly phosphorylated after receptor cross-linking. In contrast, a few proteins, including two at 56 and 52 kD which may corre-



**Figure 5.** Impaired Fc $\gamma$ R-induced MAPK activation in Syk-deficient macrophages. Wild-type and mutant macrophages were incubated with growth media (0') or with anti-Fc $\gamma$ R mAb 2.4G2, followed by cross-linking with anti-rat IgG for the indicated times. The electrophoretic mobility shift of p44 MAP kinase was assessed by SDS-PAGE, and by immunoblotting with anti-MAP kinase antibodies (a). Alternatively, Erk1 and Erk2 activity was assessed directly by immunoprecipitation with anti-Erk2 antibodies and measurement of the ability to phosphorylate the substrate myelin basic protein in vitro (b and d). Unstimulated or Fc $\gamma$ R-stimulated wild-type (stippled bars) or Syk<sup>-</sup> (black bars) macrophages were tested (b). Values shown represent <sup>33</sup>PO<sub>4</sub> incorporation from labeled ATP into the substrate protein. Seven separate experiments using Syk-deficient cells

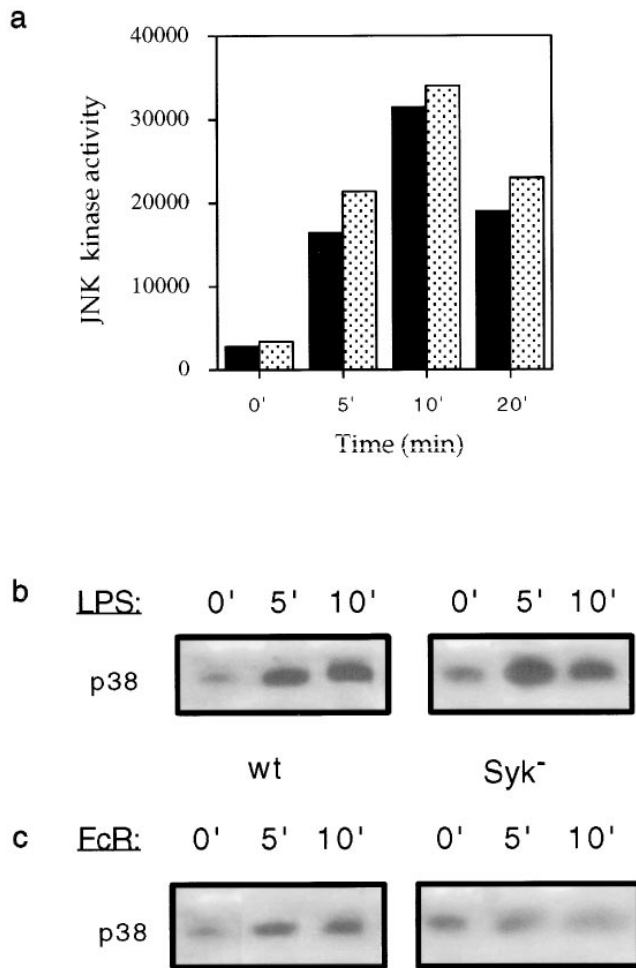
respond to members of the Src family of tyrosine kinases, did exhibit induced tyrosine phosphorylation comparable to that seen in wild-type cells.

We then examined the induced tyrosine phosphorylation of the important signaling components Shc and p145<sup>Shp</sup> (Fig. 4 b). Within 5 min of Fc $\gamma$ R cross-linking in normal cells, we observed increased tyrosine phosphorylation of Shc and p145<sup>Shp</sup> as well as the induced association of Shc with p145<sup>Shp</sup>, in agreement with previous results (19). In contrast, in Syk-deficient cells, Fc $\gamma$ R cross-linking induced only weak tyrosine phosphorylation of Shc and p145<sup>Shp</sup>. These results show that Syk plays a principal role in the rapid tyrosine phosphorylation of many cellular substrates after Fc $\gamma$ R cross-linking.

**Requirement for Syk in Fc $\gamma$ R-induced Activation of MAP Kinases.** The lack of Fc $\gamma$ R-induced tyrosine phosphorylation of p42<sup>MAPK</sup> (Erk2) in the Syk<sup>-</sup> macrophages (Fig. 4) was quite striking. MAP kinases become activated upon dual phosphorylation of threonine and tyrosine residues in a TxY motif. In the case of Erk1 and Erk2, this dual phosphorylation results in a mobility shift upon SDS-PAGE. Therefore, we examined the effect of Fc $\gamma$ R stimulation on Erk1 and Erk2 electrophoretic mobility. As expected, p42<sup>MAPK</sup> (Erk2) and p44<sup>MAPK</sup> (Erk1) exhibited a clear shift in mobility in normal macrophages stimulated through the Fc $\gamma$ Rs. This response was absent in Syk<sup>-</sup> macrophages (Fig. 5 a and data not shown). Moreover, in vitro kinase assays revealed that Fc $\gamma$ R stimulation led to a large increase in Erk2 MAP kinase activity in wild-type macrophages, but to a small or no increase in Syk<sup>-</sup> macrophages (Fig. 5 b).

These results do not reflect a generalized defect in MAPK activation in the Syk-deficient macrophages, since the ability of LPS to induce a mobility shift of p44 Erk1 and the activation of Erk2 were normal (Fig. 5, c and d). LPS stimulation of macrophages strongly activates not only the Erk1/2 MAP kinases, but also the other two known types of mammalian MAP kinases, p38 and JNK1/JNK2. Enzymatic activation of JNK occurred normally in LPS-stimulated Syk<sup>-</sup> macrophages (Fig. 6 a). Similarly, LPS-induced tyrosine phosphorylation of p38 occurred normally in Syk<sup>-</sup> macrophages (Fig. 6 b). Fc $\gamma$ R cross-linking also induced a modest increase in p38 immunoprecipitated with antiphosphotyrosine antibodies from wild-type macrophages, although this response was less robust than was the response seen with LPS (Fig. 6 c). As was seen with Erk1 and Erk2, the p38 response to Fc $\gamma$ R engagement was largely absent in Syk-deficient macrophages. We did not find JNK kinase activity increased in response to Fc $\gamma$ R cross-linking in the fetal liver-derived macrophages, or in murine macrophage cell lines such as P388D1 and RAW 264.7 (data not shown).

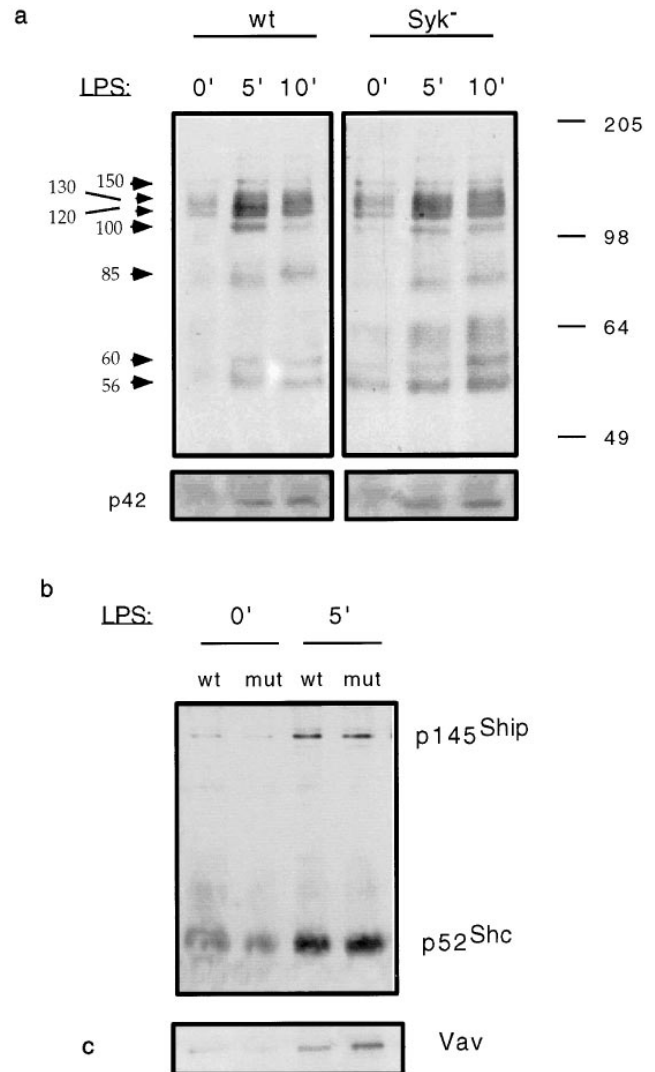
gave similar results. LPS-induced mobility shift of Erk1 (c) and enzymatic activation of Erk2 (d) was also assessed in wild-type (stippled bars), or Syk<sup>-</sup> (black bars) macrophages exposed to 1  $\mu$ g/ml LPS for the indicated times. Similar results were obtained in seven separate experiments.



**Figure 6.** Activation of JNK and tyrosine phosphorylation of p38 in Fc $\gamma$ R-stimulated and LPS-activated macrophages. Activation of JNK in wild-type (stippled bars), or Syk<sup>-/-</sup> (black bars) macrophages exposed to 1  $\mu$ g/ml LPS for the indicated times was assessed by immunoprecipitation with anti-JNK antibodies, followed by *in vitro* phosphorylation of a c-Jun-GST fusion protein substrate (a). For analysis of p38 phosphorylation, phosphotyrosine-containing proteins were immunoprecipitated from lysates of unstimulated (0'), LPS-stimulated (b), or Fc $\gamma$ R-activated (c) macrophages. Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and the region containing p38 was probed with anti-p38 antibody. Similar results were obtained in three experiments comparing Syk-deficient and normal macrophages.

As the activation of MAP kinases in response to LPS was not affected by the absence of Syk, we next examined LPS-induced tyrosine phosphorylation of cellular proteins. We did not observe any defects in LPS-induced tyrosine phosphorylation of the examined cellular substrates, including Vav, Shc, and p145<sup>Shp</sup> (Fig. 7). Thus, Syk-deficient macrophages were not defective in any of the examined signaling responses to LPS.

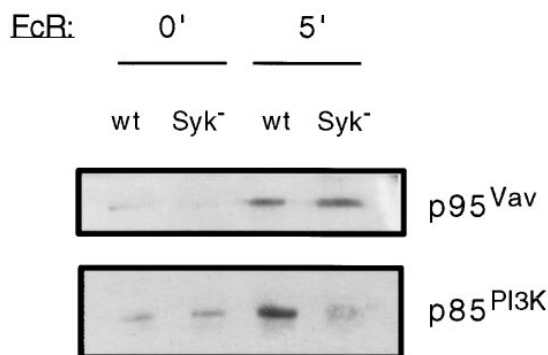
**Fc $\gamma$ R Signaling Through Vav and p85 PI 3-Kinase in Syk<sup>-/-</sup> Macrophages.** We next examined the effect of loss of Syk on Fc $\gamma$ R-induced tyrosine phosphorylation of Vav and of the p85 subunit of PI 3-kinase. In normal macrophages, p85 and Vav became immunoprecipitable with anti-



**Figure 7.** Lack of defect in LPS-induced tyrosine phosphorylation of cellular substrates in Syk<sup>-/-</sup> macrophages. Cells were incubated with media or media plus 1  $\mu$ g/ml LPS for the indicated times. Cells were lysed, and the lysate proteins were subjected to SDS-PAGE and immunoblotting with antiphosphotyrosine antibody (a). Molecular mass markers (in kD) and major phosphotyrosine-containing substrates are indicated on the right and the left of a, respectively. The Erk2 MAPK band, as confirmed by subsequent immunoblotting with anti-Erk2 antibodies (not shown), is indicated as p42. Alternatively, cell lysates were subject to immunoprecipitation with anti-Shc antibodies (b) and immunoblotting with antiphosphotyrosine antibody, or to immunoprecipitation with antiphosphotyrosine antibody and immunoblotting with anti-Vav (c) antibodies. The positions of proteins of interest are indicated.

phosphotyrosine antibodies after Fc $\gamma$ R cross-linking (Fig. 8). It is possible that the immunoprecipitated proteins were themselves phosphorylated on tyrosine, or alternatively, that they were precipitated by virtue of an association with another protein that was tyrosine phosphorylated upon cell stimulation. Relatively harsh conditions were used for the antiphosphotyrosine immunoprecipitations, so weak protein-protein interactions were likely disrupted. In Syk<sup>-/-</sup> macrophages, immunoprecipitation of Vav by antiphosphoty-



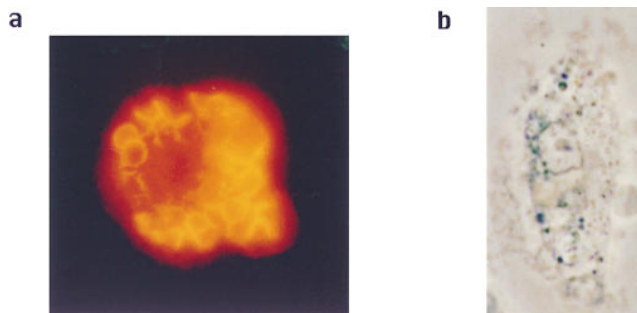


**Figure 8.** Altered FcγR-induced signaling events associated with p85 PI 3-kinase, but not with Vav in Syk<sup>-</sup> cells. Fetal liver-derived macrophage cultures were stimulated through their FcγR for 5 min. Cellular lysate proteins were immunoprecipitated with antiphosphotyrosine antibodies, and the immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotting with anti-Vav and anti-p85 PI 3-kinase antibodies as indicated.

rosine antibodies was still induced by FcγR stimulation, but immunoprecipitation of p85 was abolished (Fig. 8). Vav or its associated proteins may be substrates for members of the Src family of kinases that are associated with the FcγRs (27). The absence of p85 in antiphosphotyrosine immunoprecipitates, however, suggested that signaling reactions involving PI 3-kinase were defective in Syk-deficient macrophages.

The perturbation of PI 3-kinase signaling in Syk-deficient macrophages was particularly interesting as PI 3-kinase has been implicated in FcγR-mediated phagocytosis in the human monocyte cell line U937 (28), and in murine bone marrow-derived macrophages (29) by virtue of the blocking effect of a potent and selective inhibitor of PI 3-kinase, wortmannin. We found that wortmannin also prevented phagocytosis of EA by fetal liver-derived cultured macrophages. This inhibitor did not affect FcγR-induced polymerized actin accumulation around bound particles (Fig. 9), and thus blocked phagocytosis at a stage similar to the block seen in Syk-deficient macrophages. In contrast to its effects on FcγR-mediated phagocytosis, wortmannin did not affect macrophage phagocytosis of yeast, latex beads, or *E. coli* (data not shown).

**Cytokine Production By Syk<sup>-</sup> Macrophages.** Cross-linking of FcγR receptors on some macrophage populations can trigger the production and release of a variety of proinflammatory cytokines, bioactive lipids, and cytotoxic oxidants (30). The nature of the responses are dependent upon the tissue origin of the macrophage, since different macrophage types have distinctive properties in this regard. Unfortunately, cross-linking of FcγRs of fetal liver-derived cultured macrophages did not generate a respiratory burst in either wild-type or Syk-deficient cells. FcγR cross-linking also failed to trigger detectable production of TNFα, IL-1β, or IL-6 (data not shown). In contrast, LPS caused these macrophages to release a number of proinflammatory mediators, including TNFα, IL-1β, IL-6, and IL-12 (Fig. 10).

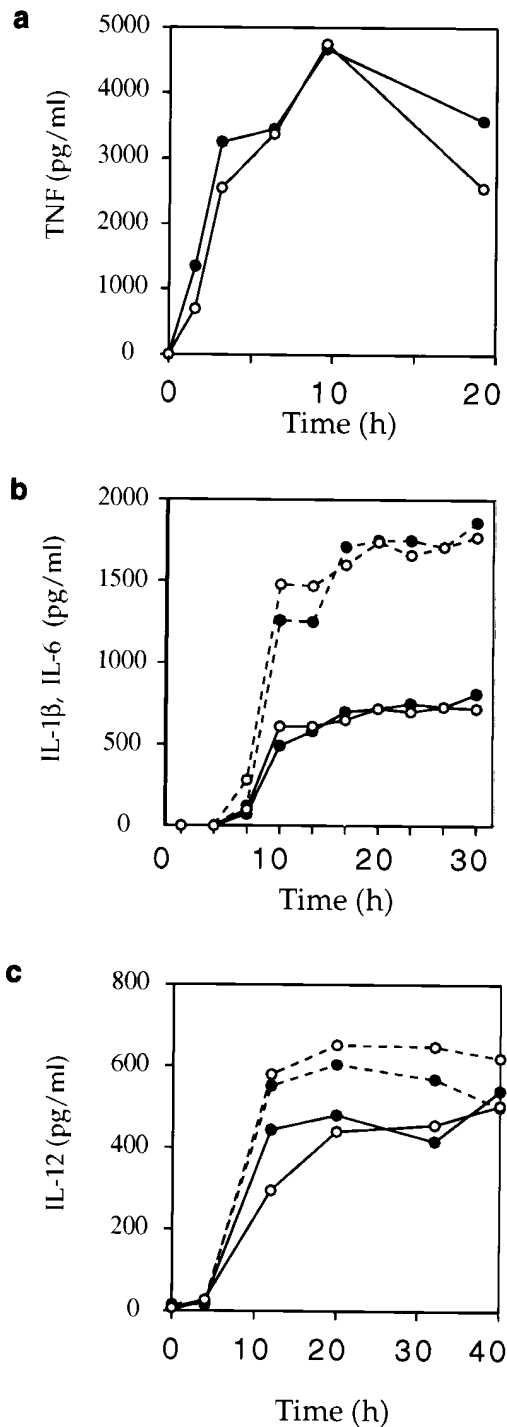


**Figure 9.** Effect of the PI 3-kinase inhibitor wortmannin on FcγR-mediated phagocytosis and actin-based phagocytic cup formation. Macrophages grown on coverslips were pretreated with wortmannin (50 nM), for 20 min, and were then incubated with EA either for 20 min on ice to obtain stable actin-lined phagocytic cups (a), or at 37°C for 40 min, after which time bound, but not internalized erythrocytes were lysed with 1.44% NH<sub>4</sub>Cl (b). Fixed and permeabilized cells were stained with BO-DIPY-phalloidin, and were examined by phase microscopy (a) or by fluorescence microscopy to visualize F-actin (b).

LPS induction of TNFα release from Syk-deficient macrophages was indistinguishable from that induced from wild-type macrophages (Fig. 10 a). TNFα production during the 4 h after cell stimulation was no different in cells that were or were not starved of the growth factors in L-Cell-conditioned media before and during such short-term assays (data not shown). Cell viability beyond 4 h could not be maintained without conditioned medium, however, so later times were not tested in its absence. In addition, LPS-induced release of IL-1β, IL-6, and IL-12 (Fig. 10, b and c) were not impaired in Syk-deficient macrophages. Thus, the signaling pathways that are involved in production of proinflammatory cytokines by macrophages responding to LPS can function independently of the Syk tyrosine kinase.

## Discussion

In prior experiments, we and others observed that stimulation of macrophages with LPS or FcγR cross-linking resulted in increased tyrosine phosphorylation and enzymatic activation of Syk (11, 19, 31). In this study, we have addressed the role of Syk in LPS- and FcγR-mediated signaling events and in downstream biological events by examining these responses in vitro in cultured macrophages from mice in which the gene for Syk had been deleted by gene targeting. In each case examined, the responses of Syk<sup>-</sup> macrophages to LPS were not different from the responses of normal macrophages. These responses included early signaling events such as activation of the Erk1/2 and JNK MAP kinases, and downstream events such as production of the cytokines TNF-α, IL-1β, IL-6, and IL-12. It is still possible that Syk participates in one or more responses to LPS that were not examined. Nonetheless, it is clear that Syk does not play a central role in mediating many of the LPS-triggered signaling events in macrophages. As tyrosine kinase inhibitors block many LPS responses of macrophages



**Figure 10.** Production and release of inflammatory cytokines in Syk-deficient and wild-type macrophages responding to LPS. Syk<sup>-/-</sup> (●) or wild-type (○) cultured macrophages were stimulated with 50 ng/ml LPS for the time indicated, and concentrations of TNF-α (a), IL-1β (b, solid line), IL-6 (b, dashed line), IL-12 (c, solid line), or IL-12 from γ-IFN (100 U/ml) primed macrophages (c, dashed line) were determined by ELISA.

(32–34), it seems likely that tyrosine kinases other than Syk play critical roles in mediating LPS responses.

In contrast to the normal LPS responses of Syk-deficient macrophages, a number of FcγR-mediated signaling events

were severely compromised, and FcγR-mediated phagocytosis was completely blocked in these mutant macrophages. FcγR-triggered tyrosine phosphorylation of an array of cellular proteins, including Shc and p145<sup>Shp</sup>, was impaired in Syk<sup>-/-</sup> macrophages. FcγR-triggered activation of Erk2 and p38 MAP kinases was likewise impaired. Since the tyrosine phosphorylation of Shc triggers its association with Grb-2 and SOS (35), which may lead to the activation of the Ras–Raf pathway, and subsequently to Erk1/2 MAP kinase activation, the defect in Erk2 activation in Syk<sup>-/-</sup> cells may be attributable to deficient Shc-related signaling events. Additionally, Syk appeared to be important for FcγR-induced signaling events involving the p85 subunit of PI 3-kinase. In wild-type macrophages, stimulation through the FcγR resulted in increased quantities of this protein and Vav in antiphosphotyrosine immunoprecipitates. In FcγR-stimulated Syk<sup>-/-</sup> cells, increased amounts of Vav, but not p85, were observed in antiphosphotyrosine immunoprecipitates. These results revealed an important role for Syk in some, but not all FcγR-induced signaling events.

Since FcγRs share subunits and ITAM signaling sequences with B and T cell antigen receptors and FcεRI, the importance of Syk for FcγR signaling observed here is consistent with studies indicating a requirement for Syk or the related tyrosine kinase in T cells, ZAP-70, for signaling by these receptors (36–40). Antigen or Fc receptor cross-linking triggers phosphorylation of receptor subunit ITAMs, probably by different members of the Src family of kinases (41), and subsequent recruitment of Syk or ZAP-70 tyrosine kinases to the phosphorylated ITAMs. It is currently thought that Syk and ZAP-70 are then primarily responsible for phosphorylation of important signaling targets. Given the similarities of the lymphocyte antigen receptors and FcγRs, it is likely that initiation of FcγR signaling similarly requires the sequential activity of these two families of tyrosine kinases. With this in mind, we examined FcγR-mediated signaling events in macrophages genetically deficient for the three Src family members ordinarily expressed in macrophages Hck, Fgr, and Lyn. We found that FcγR-mediated Syk activation was impaired in Hck<sup>-/-</sup>/Fgr<sup>-/-</sup>/Lyn<sup>-/-</sup> macrophages. This observation provides additional strong evidence for a model of sequential activation of Src family and Syk tyrosine kinases by the FcγRs.

In addition to its role in early signal transduction events, Syk was also found to be absolutely required for FcγR-mediated phagocytosis in macrophages. Syk<sup>-/-</sup> macrophages bound antibody-coated particles by their FcγRs, and initiated engulfment of the particles with actin-based cytoplasmic extensions. In the absence of Syk, however, the cells were unable to internalize fully these antibody-coated particles. Evidently, Syk was important for the FcγR-triggered cellular events necessary to extend further and fuse the leading edge of the macrophage membrane that surrounded the particle. These results are in agreement with findings demonstrating a role for Syk in FcR-mediated phagocytosis in various artificial systems. For example, coexpression of Syk in COS-1 transfectants expressing FcγRs promoted efficient FcγR-mediated phagocytosis (8), and chimeric trans-

membrane proteins containing Syk kinase domains were capable of triggering phagocytosis by COS-1 cells (13). In the latter case, the kinase activity of Syk was sufficient and necessary to trigger phagocytosis in COS cells. Interestingly, we found that Fc $\gamma$ R-mediated phagocytosis was only delayed in Hck<sup>-</sup>/Fgr<sup>-</sup>/Lyn<sup>-</sup> macrophages, despite severely diminished Syk activation. Evidently, the reduced number of activated Syk molecules clustered beneath cross-linked Fc $\gamma$ Rs in Hck<sup>-</sup>/Fgr<sup>-</sup>/Lyn<sup>-</sup> macrophages were still sufficient to allow phagocytosis to proceed with only a moderate delay.

The phagocytic defect in Syk<sup>-</sup> macrophages may reflect the lack of Syk kinase action needed to activate signaling pathways necessary for Fc $\gamma$ R-mediated phagocytosis, as suggested by experiments in Cos-1 cells (13). Alternatively, the physical association of Syk with phosphorylated receptor tails may allow it to serve an adaptor-type function at cross-linked receptors to allow recruitment of other important proteins to the site of particle binding. Chacko et al. have described such a role for Syk in Fc $\gamma$ RII signaling in platelets (42). In any case, the experiments reported here demonstrate that Syk is a crucial element in Fc $\gamma$ R-mediated phagocytosis by cells in which it is a naturally occurring and biologically important response after cross-linking of these receptors. Importantly, the phagocytic defect in Syk<sup>-</sup> macrophages was limited to phagocytosis via activated Fc $\gamma$ Rs. Phagocytosis of microorganisms and synthetic particles was unaffected by the absence of Syk. Interesting in this regard are observations indicating that there are distinct cytoskeletal structures involved in phagocytosis of particles bound to different receptors (43).

Several Fc $\gamma$ R-induced signaling events have been implicated as participating in phagocytosis. Fc $\gamma$ R cross-linking triggers phospholipase A<sub>2</sub> activation and subsequent arachidonic acid (AA) release, and it was found that the AA was a critical component in phagocytosis (44). In those studies, a block in Fc $\gamma$ R-mediated phagocytosis was overcome by providing exogenous AA in the culture media. The phagocytic defect in Syk<sup>-</sup> macrophages was not remedied by addition of exogenous AA (data not shown). It is still possible that local production of AA plays an important role in Fc $\gamma$ R-mediated phagocytosis, but that this is only one of several required Syk-dependent signaling events.

Another signal transduction protein implicated in Fc $\gamma$ R-mediated phagocytosis is PI 3-kinase (28). Fc $\gamma$ R engagement induced an increase in p85 protein immunoprecipitable by antiphosphotyrosine antibodies in normal macrophages, but not in Syk<sup>-</sup> macrophages. Moreover, we noted a striking similarity between the phagocytic defect in Syk<sup>-</sup> cells, and the stage at which phagocytosis was blocked by treatment of normal cells with the PI 3-kinase inhibitor wortmannin. In both cases, actin cups formed, but could not close off to internalize the bound particle (Figs. 3 and 9). In agreement with our results, another group has recently reported that

wortmannin blocks phagocytosis at this stage (29). Thus, it is attractive to propose that the defect in Syk<sup>-</sup> macrophages results from a failure to activate a signaling pathway involving PI 3-kinase. Syk may be required to activate and/or to properly localize PI 3-kinase activity during phagocytosis. Interesting in this regard is the observation that Fc $\gamma$ RII cross-linking on platelets induced the association of the Fc $\gamma$ RII with PI 3-kinase (42).

It should be noted that Fc $\gamma$ R signaling in Syk<sup>-</sup> macrophages also showed a defect in a second signaling component that acts on inositol-containing phospholipids, p145<sup>Ship</sup>. This molecule is an inositol phosphatase that hydrolyzes the 5-phosphate of phosphatidylinositol (3,4,5)P<sub>3</sub>, the product of PI 3-kinase, to generate phosphatidylinositol (3,4)P<sub>2</sub> (45, 46). It is not known how the activity of p145<sup>Ship</sup> is regulated, but Fc $\gamma$ R engagement leads to tyrosine phosphorylation of p145<sup>Ship</sup> and its association with both Syk and Shc (19). These signaling events were also defective in the Syk-deficient macrophages, and thus, p145<sup>Ship</sup> function is also likely to be impaired in Syk<sup>-</sup> cells stimulated via Fc $\gamma$ R cross-linking.

How PI 3-kinase and/or p145<sup>Ship</sup> may be acting in the phagocytic process is of course not known. One possibility is suggested by the connection between PI 3-kinase and small G proteins involved in cytoskeletal rearrangements. The Rho family GTPases Rho, Rac, and CDC42 play important roles in the regulation of the actin cytoskeleton to produce membrane ruffling, filopodia, and lamellipodia (47), and likely play an important role in phagocytosis. PI 3-kinase is thought to play a role in coordinating these responses. For example, activation of Rac via the PDGF receptor requires synthesis of PI 3-kinase phosphoinositide products and association of PI 3-kinase with receptor cytoplasmic domains, indicating that PI 3-kinase can act upstream of Rac (48). Similarly, inhibitors of PI 3-kinase prevent agonist-stimulated guanine nucleotide exchange on Rac (49).

The phagocytic process may be analogous to other processes that require the combined activities of Rho-family proteins. Nobes et al. concluded that stimulation of CDC42 induced the formation of filopodia (47). CDC42 also can stimulate Rac, which in turn initiates a process whereby the filopodia are filled in by advancing lamella. This process may be related to the events in Fc $\gamma$ R-mediated phagocytosis. That is, the phagocytic cup may be a scaffold of filopodia-like structures. The formation of actin cups may be followed by filling in over the cup by advancing lamellipodia. According to this hypothesis, a key issue is how the activities of Rho-family members are orchestrated to allow phagocytosis to proceed successfully. The final events in phagocytosis may be defective in Syk-deficient macrophages because signaling events, perhaps involving Rho family proteins, PI 3-kinase, and p145<sup>Ship</sup>, are not activated or localized properly in the absence of Syk.

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Address correspondence to Dr. Mary T. Crowley, Scripps Clinic and Research Foundation, 10550 N. Torrey Pines Rd., IMM 25, La Jolla, CA 92037. Phone: 619-784-2195; FAX: 619-784-9180; E-mail: mtcrow@scripps.edu

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