Negative Regulation of Phagocytosis in Murine Macrophages by the Src Kinase Family Member, Fgr

By Hattie D. Gresham,*‡ Benjamin M. Dale,*‡ Jeffrey W. Potter,§ Peter W. Chang,§ Charlotte M. Vines,§ Clifford A. Lowell,‖ Carl F. Lagenaur,¶ and Cheryl L. Willman§

*From the *Research Service, Albuquerque Veterans Administration Medical Center, Albuquerque, New Mexico 87108; the ‡Department of Molecular Genetics and Microbiology and the §Department of Pathology, University of New Mexico, Albuquerque, New Mexico 87131; the ‖Department of Laboratory Medicine, University of California, San Francisco, San Francisco, California 94143; and the ¶Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213*

Abstract

Ingestion of opsonized pathogens by professional phagocytes results in the generation and release of microbicidal products that are essential for normal host defense. Because these products can result in significant tissue injury, phagocytosis must be regulated to limit damage to the host while allowing for optimal clearance and destruction of opsonized pathogens. To pursue negative regulation of phagocytosis, we assessed the effect of the Src kinase family member, Fgr, on opsonin-dependent phagocytosis by mouse macrophages. We chose Fgr because it is present in high concentrations in circulating phagocytes but is not essential for Fcγ receptor–mediated ingestion by mouse macrophages. Although expression of Fgr both in a macrophage cell line and in primary macrophages significantly attenuates ingestion mediated by Fcγ receptors and CR3, it does not affect macropinocytosis or receptor-mediated endocytosis. This selective effect of Fgr is independent of its tyrosine kinase function. After Fcγ receptor cross-linking, Fgr becomes associated with the immunoreceptor tyrosine-based inhibition motif (ITIM)–containing receptor, SIRPα (a member of the signal-regulatory protein family, also known as Src homology 2 domain–containing protein tyrosine phosphatase [SHP] substrate 1 [SHPS-1], brain immunoglobulin-like molecule with tyrosine-based activation motifs [BIT], and P84) and potentiates the association of the phosphatase SHP-1 with SIRPα. This association is responsible, at least in part, for decreasing positive signaling essential for optimal phagocytosis. These data demonstrate an important negative regulatory role for this Src kinase family member and suggest that this homeostatic function must be overcome for optimal uptake and clearance of opsonized pathogens.

Key words: phagocytes • Src family kinases • protein tyrosine phosphatase

Introduction

Phagocytosis is a complex biological process of specialized cells that is essential for normal host defense against infectious pathogens and for resolution of tissue injury at sites of inflammation (1–3). We and others have hypothesized that phagocytosis is primarily a recruited function at sites of infection and inflammation because ingestion mediated by both Fcγ and complement receptors is augmented by inflammatory mediators and matrix proteins that abound at sites of tissue injury (2, 4–9). These data imply that phagocytosis is regulated. Regulatory control of phagocytosis would be of substantial benefit to the host because the production of tissue-damaging products generated during ingestion of opsonized pathogens, such as reactive oxygen and nitrogen intermediates and lysosomal enzymes, would be restricted to the site of inflammation. In this paradigm, phagocytes circulating in the vasculature would have limited phagocytic potential. However, after migration into sites of infection or inflammation, their phagocytic responses would be amplified, allowing for optimal ingestion and destruction of opsonized targets. This amplification could occur by acquiring the ability to recruit additional positive signaling molecules and/or by losing or overcoming negative signaling components that act to limit or attenuate phagocytic responses. Although considerable advances have been made in elucidating the intracellular signals essential for positive

Address correspondence to Hattie D. Gresham, Department of Molecular Genetics and Microbiology, Rm. 379, BMSB, 915 Camino de Salud, University of New Mexico, Albuquerque, NM 87131-5276. Phone: 505-272-5764; Fax: 505-272-6029; E-mail: hgresham@salud.unm.edu

signaling in phagocytosis (1–3), knowledge of the specific signal transduction components that negatively regulate phagocytosis is somewhat lacking.

Negative regulation of signal transduction cascades is necessary for homeostasis in a variety of systems. In hematopoietic and immune system cells, failure to regulate signal transduction during cell activation can result in hyperresponsive states that lead to significant pathological sequelae, such as autoimmunity and excessive inflammation (10–17). Examples include the autoimmune-mediated tissue injury and uncontrolled leukocyte activation that occur in mice with disrupted genes encoding the antiinflammatory cytokines, IL-10 (13) and TGF-β1 (14–16), and the enhanced antibody and anaphylactic responses observed in mice that lack an inhibitory FcγRIIB receptor expressed by B cells and mast cells (11, 12). These and other examples clearly demonstrate the importance of diminishing or terminating activation signals to limit tissue injury. Because the hyperresponsive states that result from failed regulation mimic several human diseases (11, 14, 17), understanding the molecular basis of negative signaling is increasing in importance.

To pursue negative regulation of phagocytosis, we looked for intracellular signaling molecules that would be expressed primarily if not exclusively in phagocytes and for which essential roles in positive signaling for phagocytosis had been eliminated. One such molecule is the Src kinase family member, Fgr. The highest concentrations of Fgr are found in mature granulocytes and monocytes, and its concentration increases with the induction of cell maturation (18–20). In addition, Fgr coimmunoprecipitates with a phagocytic receptor, FcγRII, in immune complex–stimulated neutrophils (21). However, bone marrow macrophages from mice that lack *c-fgr* by targeted gene disruption are fully able to ingest IgG-opsonized targets (22). These data suggest that Fgr is recruited to domains where phagocytic receptors cluster and where phagocytic responses are initiated but that it is not essential for ingestion to occur. Therefore, the purpose of this work is to test the hypothesis that Fgr is involved in negative regulation of phagocytosis. We show that expression of Fgr attenuates phagocytosis mediated by multiple receptors and that the suppression occurs proximal to the reorganization of the actin cytoskeleton into phagocytic cups. The molecular mechanism for this regulation involves, at least in part, the ability of Fgr to recruit increased tyrosine phosphatase activity and the phosphatase Src homology (SH)[1] 2 domain–containing protein tyrosine phosphatase (SHP)-1 to a transmembrane immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor, signal regulatory protein of the α subtype (SIRPα), after phagocytic receptor activation.

Materials and Methods

Reagents. The following reagents were obtained as indicated: S-S.1 hybridoma, which secretes murine mAb IgG2a anti–sheep erythrocyte (EIgG2a; American Type Culture Collection), tissue culture supernatant containing murine mAb IgG2b anti–sheep erythrocyte (EIgG2b; Accurate Chemical), purified rabbit polyclonal anti–sheep erythrocyte (Diamedix), purified biotin-labeled goat anti–rat $F(ab)'_2$ (Jackson ImmunoResearch Labs), purified mAb 2.4G2 rat anti–murine FcγRII/FcγRIII (PharMingen), purified mAb M1/70 rat anti–murine CD11b (PharMingen), purified mAb rat IgG1 anti–murine CD14 (PharMingen), FITC-avidin (PharMingen), purified mAb 2E6 hamster anti–murine β_2 (Endogen), FITC-labeled 70-kD dextran (Molecular Probes), rhodamine-phalloidin (Molecular Probes), and purified murine fibronectin (Calbiochem). Rat IgG1 anti–murine SIRPα (P84 antigen [23]) was purified from tissue culture supernatant by ammonium sulfate precipitation and protein G chromatography using the mAb Trap II kit (Amersham Pharmacia Biotech).

Retroviral Infections. BAC1.2F5, a subclone of a murine macrophage cell line (24) that does not express Fgr, was used for the introduction of wild-type and site-directed mutants of murine *c-fgr* by retroviral-mediated gene transfer. Murine *c-fgr* cDNA was isolated as described (25) and subsequently modified by deletion of the 5′ untranslated region to position 143 to increase translational efficiency. To generate a kinase-inactive mutant, a 21-mer oligonucleotide primer, 5′-CTT CAG CGT CCT CAC TGC CAA-3′, was used to convert Lys 279 (AAG) to Arg 279 (AGG). All mutations in *c-fgr* cDNA were confirmed by DNA sequencing (Sequenase; U.S. Biochemical). *c-fgr* constructs were cloned into the retrovial vector pLNCX (Clontech), and pCLNX-*fgr* cDNAs or a vector control were transfected into the murine packaging cell line, Psi-2 (American Type Culture Collection), as described (26). After selection in 250 μg/ml G418, subclones were screened for viral titer, clonal integration, and Fgr expression by immunoblotting. 1 ml of helper-free virus at 10^5–10^6 PFU/ml was used to infect 10^6 BAC1.2F5 cells in the presence of 4 μg/ml polybrene (Sigma Chemical Co.). After infection, clones were selected in 250 μg/ml of G418. The following clones were used in this investigation: vector control, BAC1.2F5 cells expressing a vector control; Fgr(WT), BAC1.2F5 cells expressing various levels of wild-type *c-fgr* of the p59 isoform; and Fgr(K279R), BAC1.2F5 cells expressing *c-fgr* of the p59 isoform with a K279 to R279 mutation that inactivates kinase function. Expression of Fgr protein was confirmed by Western blot analysis (see below for details). Lack of Fgr kinase activity in Fgr(K279R)-expressing cells was confirmed by an in vitro kinase assay based on autophosphorylation of Fgr immunoprecipitates. In brief, Fgr protein was immunoprecipitated with a polyclonal anti-Fgr peptide antibody (described below). The washed immunoprecipitates were incubated with [γ-^{32}P]ATP for 30 min at 30°C, the kinase reaction was terminated, and the eluted proteins were run on SDS-PAGE. Fgr(WT) cells expressed ^{32}P-labeled Fgr, whereas the Fgr(K279R) cells did not (data not shown).

To confirm that cell surface expression of phagocytic receptors was equivalent for all BAC1.2F5 clones, the cells were checked by fluorescent flow cytometry with a FACScan® cytometer (Becton Dickinson). FcγRI was assessed by direct binding of FITC-

[1]*Abbreviations used in this paper:* AI, attachment index; CSF-1, colony stimulating factor 1; ECL, enhanced chemiluminescence; EC3bi, EIgG2a, EIgG2b, and EIgGr, C3bi-, IgG2a-, IgG2b-, and rabbit IgG–opsonized erythrocyte(s); HRP, horseradish peroxidase; HSA, human serum albumin; IAP, integrin-associated protein; ITIM, immunoreceptor tyrosine-based inhibition motif; LCM, L cell–conditioned medium; MCF, mean channel of fluorescence; PDBu, phorbol dibutyrate; PECAM-1, platelet-endothelial cell adhesion molecule 1; PI, phagocytic index; PI 3-kinase, phosphatidylinositol 3-kinase; PIR-B, paired Ig-like receptor B; SH, Src homology; SHP, SH2 domain–containing protein tyrosine phosphatase; SIRPα, signal regulatory protein of the α subtype; VBS, veronal-buffered saline.

labeled purified monomeric murine IgG2a mAb S-S.1. Binding of FITC-IgG2a was not blocked by mAb 2.4G2. FcγRII/FcγRIII and CD11b were detected by indirect immunofluorescence using biotinylated rat mAb 2.4G2 and M1/70, respectively, and FITC-avidin. BAC1.2F5 clones (10^6) were incubated with 1 μg of antibody for 90 min at 0°C, washed, incubated with 0.1 μg of FITC-avidin where appropriate, washed, and fixed in 1% paraformaldehyde in PBS. Levels of expression for these phagocytic receptors were equivalent for all BAC1 clones. These cells were maintained in DMEM containing 15% FCS, 15–25% conditioned media from L929 cells as a source of CSF-1, 100 U/ml penicillin, 100 μg/ml streptomycin, 4,500 mg/l glutamine, and 200 μg/ml G418. Tissue culture reagents were purchased from GIBCO BRL. All cell lines were routinely checked for *Mycoplasma* contamination by PCR (Boehringer Mannheim).

Isolation of Primary Phagocytes. Bone marrow macrophages, resident peritoneal macrophages, and bone marrow neutrophils (PMNs) were isolated from wild-type C57BL/6 mice, from $Fgr^{-/-}$ mice in the C57BL/6 background (22), from *motheaten viable* (me^v/me^v) mice obtained from The Jackson Laboratory, and FcR knockout mice (γ chain knockout crossed with an FcRIIB knockout) in C57BL/6 × 129 mice obtained from Taconic Farms. Mice heterozygous for the targeted deletion of *fgr* were bred, and the homozygous null offspring were detected by PCR of tail vein DNA as described (22). All mice were housed under sterile pathogen-free conditions. Phagocytes isolated from homozygous null mice generated by targeting either exon 2 or 4 gave equivalent results. Resident peritoneal macrophages were obtained by lavage of the peritoneum with 5 ml buffer and were plated onto fibronectin-coated Lab-Tek chambers. Bone marrow leukocytes were flushed from the femurs. Bone marrow PMNs were isolated by NIM-2 isolation media (Cardinal Associates) as described (27). Bone marrow macrophages were cultured as described (28) with the following modifications: the nonadherent precursor cells were cultured in tissue culture flasks, instead of bacteriologic petri dishes, in the presence of 15% L cell–conditioned medium (LCM) for 3–5 d. The level of Fgr was significantly reduced in cells cultured for >5 d and in lower concentrations of LCM (2–5%).

Adherent BAC1.2F5 cells and bone marrow macrophages were released from tissue culture flasks by incubation with 5 mM EDTA in PBS for 15 min at 37°C. All phagocytes were washed twice in buffer and resuspended in HBSS containing 10 mM Hepes, 0.5 mM Ca^{2+}, 2.5 mM Mg^{2+}, 50 μg/ml gentamicin, and 1% sterile, endotoxin-free human serum albumin (HBSS with 1% HSA) at 2 × 10^6/ml. For some experiments, analyses were performed in complete tissue culture medium containing 15% LCM with qualitatively similar results. Reactions with cells in suspension were performed in 12 × 75 mm polypropylene tubes (Falcon Labware), and reactions with adherent cells were performed on either polystyrene 24-well plates (Falcon Labware) or on 8-chamber Lab-Tek glass slides (Nunc). To facilitate equivalent cell adhesion for all of the transfected cells, adherent assays were performed on plates or slides that had been coated overnight at 4°C with 2 μg/ml murine fibronectin in PBS.

Opsonization. Erythrocytes (BioWhittaker) were opsonized with polyclonal rabbit anti–sheep erythrocyte (EIgGR) as described previously (5, 6). For EIgG2a and EIgG2b (27), erythrocytes were washed in veronal-buffered saline (VBS), and 200 μl (10^9/ml) was incubated with the indicated volume of the respective hybridoma tissue culture supernatant or purified antibody for 30 min at 37°C. For EC3bi, erythrocytes were washed and suspended in dextrose-buffered VBS containing 170 mM dextrose, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (at 10^9/ml) and incubated with an equal volume of a subagglutinating dilution of rabbit IgM anti-erythrocyte (6) for 30 min at 37°C. 200 μl of EIgM was incubated with an equivalent volume of either a 1:5 or 1:10 dilution of C5-deficient mouse serum (Jackson ImmunoResearch Labs) for 60 min at 37°C. Opsonized erythrocytes were then washed and resuspended in the respective buffer at 5 × 10^8/ml.

Attachment and Phagocytosis Assays. To assess binding of the opsonized erythrocytes, washed macrophages (2 × 10^5/ml) were centrifuged at 100 *g* with either 7.5 μl of EIgG2a or EIgG2b, or 25 μl of EC3bi in 100 μl of buffer for 1 min to initiate contact. The reaction mixtures were incubated at 4°C for 30 min for EIgG2a and EIgG2b, and at 37°C for 1 h for EC3bi. Attachment was assessed by light microscopy, and the attachment index (AI) was quantitated as the number of erythrocytes bound per 100 macrophages. Additionally, attachment of EIgG2a and EIgG2b at 37°C to macrophages that had been preincubated with 10 μM cytochalasin B (Sigma Chemical Co.) to inhibit internalization gave equivalent results. For optimal EC3bi attachment, most experiments were performed in the presence of 1.0 mM Mn^{2+}. To prove that EC3bi attachment was mediated by CR3, macrophages were incubated with 1 μg of mAb 2E6 hamster anti–murine β_2 for 30 min at 0°C before the addition of EC3bi. mAb 2E6 blocked all EC3bi attachment.

Phagocytosis was assessed with macrophages either in suspension or adherent to fibronectin-coated plates. For the suspension assay, washed macrophages (2 × 10^5) were centrifuged with 7.5 μl of EIgG2a, EIgG2b, or EIgGR, or 25 μl of EC3bi at 100 *g* for 1 min. The mixtures were gently resuspended in 100 μl buffer. For EC3bi, 30 ng/ml of phorbol dibutyrate (PDBu) and 1 mM $MnCl_2$ were added to stimulate ingestion. PMN phagocytosis of EIgG2b was assessed in the presence of buffer or various concentrations of FMLP as described (27). After incubation for 30 min at 37°C, uningested erythrocytes were removed by hypotonic lysis. Phagocytosis was assessed by phase microscopy and quantitated as a phagocytic index (PI), the number of opsonized erythrocytes ingested per 100 macrophages. Additionally, the percentage of phagocytosis was tabulated by counting the number of cells ingesting at least 1 opsonized erythrocyte per 100 macrophages. Phagocytosis was also assessed using adherent cells. Washed macrophages (10^5/ml) were added to fibronectin-coated 24-well plates. After incubation at 37°C for 2 h, the cells were washed and 100 μl of EIgGR was added. After 30 min at 4°C, the nonadherent EIgGR were washed away and ingestion was initiated by incubation at 37°C for 30 min. Uningested erythrocytes were removed by hypotonic lysis. The cells were fixed with 2.5% glutaraldehyde in PBS for 10 min at room temperature. Phagocytosis was assessed by photomicroscopy.

Macropinocytosis Assay. Macropinocytosis was defined by the uptake of FITC-labeled 70-kD dextran as described by Araki et al. (29). Vector control and Fgr(WT)-expressing BAC1.2F5 cells were cultured for 16 h in the absence of LCM to remove the influence of CSF-1. Washed cells were resuspended at 10^7/ml in HBSS with 1% HSA. A 100-μl aliquot of this suspension was pulsed with FITC-labeled 70-kD dextran (5 mg/ml) for 1 h at 37°C in the absence or presence of 6 ng of murine recombinant CSF-1 (R & D Systems), washed three times, and chased for 1 h at 37°C in HBSS without HSA. In some experiments, the macrophages were preincubated with 10 nM wortmannin to inhibit phosphatidylinositol 3-kinase (PI 3-kinase) before incubation with FITC-dextran (29). The cells were washed three times at 4°C and placed on ice until fluorescence was assessed by flow cytometry. Autofluorescence of cells that were not incubated with

the FITC-dextran was subtracted to obtain specific fluorescence. The data are reported as the mean channel of fluorescence (MCF). The mean channel for autofluorescence was <2 for all experiments.

Receptor-mediated Endocytosis. Receptor-mediated endocytosis of FcγRII/FcγRIII was assessed by an adaptation of the method of Lamaze et al. (30). Receptor cross-linking and internalization were initiated by incubating 1.0×10^6 vector control and Fgr(WT)-expressing BAC1.2F5 cells at 0°C with 1 μg of rat mAb 2.4G2 in 50 μl of serum-free culture medium containing 20 mM Hepes and 0.2% BSA. After 1 h, the cells were washed and incubated with 1 μg biotinylated $F(ab)'_2$ goat anti–rat IgG. After washing, separate aliquots of the cells were incubated either at 0°C to measure the total amount of cross-linked receptors or at 37°C to initiate receptor internalization. After the indicated times (15 min, 30 min, or 1 h), all of the samples were washed and incubated with 0.1 μg FITC-avidin at 0°C to measure surface fluorescence. The samples were held at 0°C until examined by flow cytometry. The loss of surface staining on the 37°C-incubated samples was attributed to the internalization of the cross-linked receptors. The percentage of endocytosis was calculated by the following equation: % endocytosis = [(FL1 at 0°C autofluorescence) − (FL1 at 37°C autofluorescence)/(FL1 at 0°C autofluorescence)] × 100. The data are represented as the mean ± SEM, $n = 3$.

Detection of F-actin Distribution by Fluorescence Microscopy. The extent of F-actin content in phagocytic cups surrounding the opsonized E was measured by the method of Greenberg et al. (31, 32). Vector control and Fgr(WT) BAC1.2F5 cells adherent for glass coverslips were incubated with EIgGR in HBSS with 1% HSA at 4°C. After 1 h, the cells were washed three times and warmed buffer was added to initiate ingestion. After 8 min at 37°C, the cells were fixed with 1% paraformaldehyde in PBS for 30 min at 37°C. After washing, the cells were permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature, washed, and incubated with a 1:50 dilution of rhodamine-phalloidin for 2 h at 4°C. Slides were mounted with Fluoromount G (Southern Biotechnology) and examined by fluorescence photomicroscopy. The extent of actin cup formation by macrophages that had bound at least 4 EIgGR was assessed by quantitating the number of actin cups formed by 100 rosetted macrophages and the percentage of cells that had at least 1 actin cup. No actin cups were detected at 5 min after the initiation of phagocytosis.

Immunoprecipitations and Western Blotting. To assess expression of Fgr by Western blotting, a rabbit polyclonal antibody to a peptide corresponding to an NH_2-terminal sequence Phe 23–Ser 43 of murine Fgr was generated. The Ser 43 residue in the synthetic peptide was replaced by Cys to facilitate coupling of the protein to KLH. Rabbits were immunized with 200 μg peptide-coupled KLH in complete Freund's adjuvant. Affinity-purified antibodies were prepared from high titer sera on immobilized peptide columns (Sulfolink Coupling Gel; Pierce Chemical Co.). The affinity-purified antibody does not react with either murine Hck or murine Lyn. Washed and resuspended macrophages were lysed in 1% NP-40, 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM Na pyrophosphate, 2 mM Na_3VO_4, 1 mM PMSF, and 2 μg/ml aprotinin (lysis buffer). Cell lysates standardized for protein content (Bio-Rad Labs) were precleared with rabbit IgG bound to protein-Sepharose 4B (Amersham Pharmacia Biotech) before SDS-PAGE on 10% acrylamide gels. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore Corp.) and probed with the affinity-purified anti–NH_2-terminal peptide at 2 μg/ml in 10% nonfat milk in PBS. Rabbit antibody was detected by 0.05 μg/ml horseradish peroxidase (HRP)-conjugated goat polyclonal anti–rabbit IgG (Vector Labs), and the membrane was developed with enhanced chemiluminescence (ECL; Pierce Chemical Co.) and exposed to BioMax MR film (Eastman Kodak Co.).

For immunoprecipitation of SIRPα after FcγR cross-linking, vector control and Fgr(K279R) kinase-inactive BAC1.2F5 cells were cultured in the absence of LCM for 24 h. The cells were detached, washed, and resuspended in HBSS with 1.0 mM $CaCl_2$ and 1.0 mM $MgCl_2$. To aggregate FcγRs, 6×10^6 cells were incubated with 25 μg/ml of rat mAb 2.4G2 at 4°C for 30 min. Equivalent data were obtained when IgG-opsonized ghosts were used to aggregate FcγRs. After washing, the cells were resuspended to 50 μl and incubated with 25 μg/ml of goat IgG anti–rat IgG (Sigma Chemical Co.) at 37°C for 0 s, 30 s, 2 min, and 5 min. The cells were pelleted and lysed with 1% Triton X-100 in lysis buffer for 15 min at 0°C. The lysates were clarified by centrifugation, normalized for protein levels, and precleared by incubation with protein G–agarose for 1 h. SIRPα was immunoprecipitated by addition of purified rat IgG1 mAb P84 (23) bound to protein G overnight at 4°C. Beads were washed with lysis buffer containing 1 mM Na_3VO_4 and 1 mM PMSF and eluted by boiling 5 min in 2× Laemmli SDS buffer. The samples were run on SDS-PAGE, and the resolved proteins were transferred to polyvinylidene difluoride membrane. The membranes were blocked with 2% BSA, 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20, and then probed individually with the following antibodies: 0.125 μg/ml anti-PY RC20-HRP (Transduction Labs), 2 μg/ml rabbit anti-Fgr NH_2 peptide, 1 μg/ml rabbit IgG anti–SHP-1 (Upstate Biotechnology), or 1 μg/ml of rabbit IgG anti-SIRPα (Alexis). The RC20-HRP–probed membranes were developed directly by ECL, whereas the rabbit antibody–probed membranes were incubated with HRP-conjugated goat anti–rabbit IgG before ECL and exposure to film. Controls included cell lysates incubated with protein G–Sepharose alone and immunoprecipitation with a rat mAb against murine CD29.

Tyrosine Phosphatase Activity Associated with SIRPα. To assess functional tyrosine phosphatase activity associated with SIRPα after FcR activation, we incubated SIRPα immunoprecipitated from vector control and Fgr(K279R)-expressing BAC1.2F5 cells as described above with a phosphotyrosine peptide (RRLIEDAEpYAARG) overnight at 37°C. Liberated phosphate was detected by malachite green using a Tyrosine Phosphatase Assay Kit 1 (Upstate Biotechnology), following the manufacturer's instructions. Liberated phosphate was measured in picomoles from a standard curve. To assess specific phosphatase activity, the phosphate levels detected with protein G–agarose beads incubated with cell lysates in the absence of anti-P84 were subtracted from the levels detected with anti-P84. This amount was <10% of the total phosphate detected. The data are representative of two experiments, each performed in duplicate.

Results

Fgr Negatively Regulates Phagocytosis Mediated by Multiple Ligand–Receptor Pairings Both in a Macrophage Cell Line and in Primary Macrophages. To test our hypothesis that Fgr mediates negative regulation of phagocytosis, we used BAC1.2F5 cells, which exhibit many of the functions of mature macrophages (24), and which we had determined in preliminary experiments did not express Fgr protein. The cells were retrovirally infected with either a vector

control or wild-type murine *c-fgr* and assessed for the ability to bind and ingest EIgG2a, EIgG2b, and EC3bi. Both the vector control and Fgr(WT)-expressing cells had comparable levels of Src, Hck, Lyn, and Syk and expressed equivalent amounts of cell surface receptors, including FcγRI, FcγRII/FcγRIII, and CR3 (data not shown). Ingestion of both EIgG2a and EIgG2b was significantly suppressed in Fgr(WT)-expressing cells at all levels of opsonization (Fig. 1, A, C, and D). Moreover, ingestion was suppressed for both the total number of opsonized targets ingested (Fig. 1, A and C) and the percentage of cells ingesting (Fig. 1, B and D). However, FcR-mediated phagocytosis in the Fgr(WT)-expressing cells did increase with increasing concentrations of opsonin (Fig. 1, A and C), indicating that the cells were capable of mounting a phagocytic response, but that it was attenuated relative to the vector control cells. At levels of opsonization where 80–90% of the vector control cells ingested, only 40–45% of the Fgr(WT) cells were capable of internalizing the bound targets (Fig. 1, B and D). The same results were obtained with BAC1.2F5 cells adherent on fibronectin-coated plates and ingesting rabbit IgG–opsonized erythrocytes (data not shown). These data indicate that Fgr suppresses phagocytosis by FcγRs on murine macrophages and that the suppression cannot be overcome by increasing the concentration of ligand.

Because signal transduction pathways could vary for phagocytosis mediated by different classes of receptors (2, 3, 6, 33–35), the ability of Fgr to suppress ingestion could be limited to a single class of receptor, i.e., Fcγ. Therefore, we assessed the effect of Fgr expression on CR3 (CD11b/CD18)–mediated ingestion of EC3bi. Unlike FcγRs, CR3 is not constitutively able to ingest C3bi-opsonized targets, and additional stimuli, like phorbol esters, are required for efficient internalization (2, 4, 6, 33). Even in the presence of 30 ng/ml of PDBu, Fgr(WT)-expressing macrophages ingested significantly fewer EC3bi than did vector control cells (Fig. 1 E). In addition, the percentage of cells ingesting EC3bi was suppressed in Fgr(WT) cells (Fig. 1 F). Increasing the concentration of PDBu could not overcome the suppression of ingestion (data not shown). No phagocytosis of EC3bi was observed with either cell type in the absence of PDBu (data not shown). These data indicate that Fgr is able to suppress phagocytosis mediated by multiple phagocytic receptors.

One trivial explanation for our results could be that expression of Fgr alters the optimal attachment of the opsonized target to the phagocyte. Therefore, we quantitated attachment for EIgG2a, EIgG2b, and EC3bi to vector control and Fgr(WT)-expressing cells to rule out this possibility. Equivalent AIs were obtained for EIgG2a (AI = 386 ± 25 and 389 ± 16, $n = 3$, for vector control and Fgr[WT], respectively) and EIgG2b (AI = 426 ± 20.4 and 422 ± 18, $n = 3$, for vector control and Fgr[WT], respectively) whether attachment was assessed at 4°C or 37°C with cytochalasin B–treated macrophages. In addition, AIs for EC3bi binding at 37°C in the absence of PDBu but in the presence of Mn^{2+} were equivalent for the two cell types (AI = 140 ± 9 and 154 ± 9, $n = 3$, for vector control and Fgr[WT], respectively). These data indicate that Fgr suppresses FcγR- and CR3-mediated ingestion, but not ligand binding. Taken together, all of the above data suggest that Fgr suppresses ingestion mediated by multiple receptors at a point subsequent to receptor ligation.

Because overexpression of Fgr could be artificially sup-

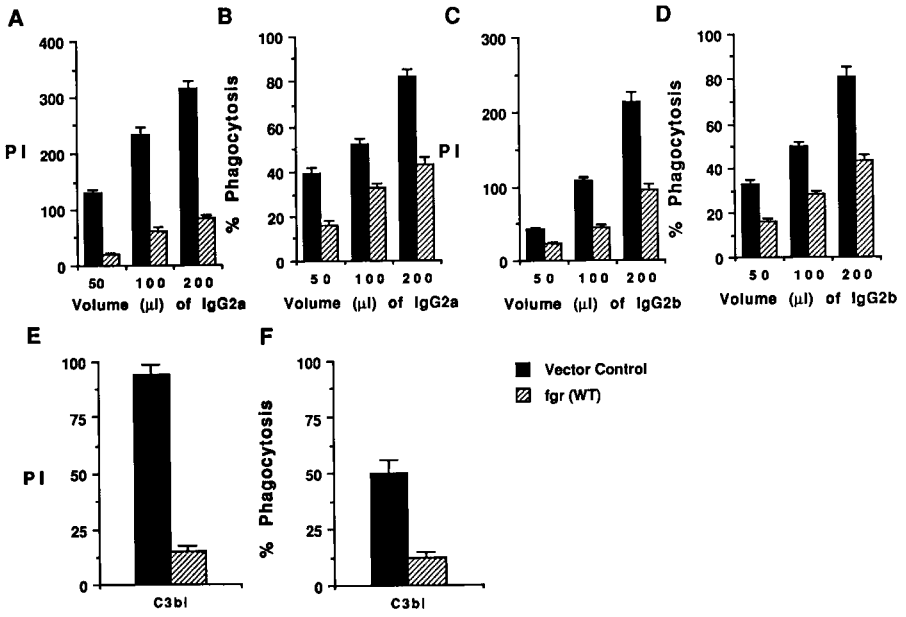

Figure 1. (A–D) Effect of increasing concentrations of opsonin, IgG2a (A and B) and IgG2b (C and D), on FcR-mediated phagocytosis by vector control (black bars) and Fgr(WT)-expressing (hatched bars) macrophages. Macrophages (2×10^5) and the appropriately opsonized erythrocytes were incubated in suspension in 100 μl of culture medium. After 30 min at 37°C, uningested erythrocytes were lysed, and phagocytosis was assessed by light microscopy. PI, the number of opsonized erythrocytes ingested by 100 phagocytes (A and C). % Phagocytosis, the number of cells ingesting per 100 phagocytes (B and D). The data are depicted as the mean ± SEM, $n = 3$. These data indicate that increasing the concentration of opsonin, either IgG2a or IgG2b, cannot overcome the suppression of phagocytosis mediated by Fgr. (E and F) Effect of Fgr(WT) (hatched bars) expression compared with a vector control (black bars) on ingestion of EC3bi by macrophages (E and F). Macrophages (2×10^5) and 25 μl of EC3bi were incubated with 30 ng/ml of PDBu in 100 μl of HBSS containing 1% HSA and 1 mM $MnCl_2$ to stimulate ingestion. After 30 min at 37°C, the uningested erythrocytes were lysed, and the PI (E) and percentage of phagocytosis (F) were assessed by light microscopy. The data are depicted as the mean ± SEM, $n = 3$. These data indicate that Fgr suppresses CR3-mediated ingestion in addition to FcγR-mediated ingestion.

pressing phagocytosis, we assessed ingestion in Fgr(WT) BAC1.2F5 cells expressing varying levels of Fgr protein and in primary phagocytes from wild-type and mice deficient in Fgr expression by targeted gene deletion (22). Three Fgr(WT)-infected clones that expressed from 1^+ to 4^+ Fgr protein compared with the Fgr-negative vector control (Fig. 2 A) were assessed for their ability to ingest EIgG2a, EIgG2b, and EC3bi. As shown in Fig. 2 B, increasing concentrations of Fgr protein resulted in significantly decreased phagocytic responses for all three types of opsonized erythrocytes. Moreover, resident peritoneal macrophages and bone marrow macrophages from $Fgr^{-/-}$ mice ingested significantly greater numbers of EIgG2a and EC3bi compared with macrophages from wild-type mice (Fig. 3, A and B). However, ingestion of EIgG2b was not as dramatically affected by Fgr expression as it was in the BAC1.2F5 cells (Fig. 3 B). The greatest difference was observed in resident peritoneal macrophages that expressed much higher levels of Fgr protein than did cultured bone marrow macrophages (data not shown). We also observed that the time, method, and concentration of LCM used for culture of the bone marrow macrophages affected Fgr expression, consistent with previous work (25). In contrast to macrophages, PMNs from $Fgr^{-/-}$ mice ingested significantly fewer EIgG2b even with FMLP stimulation to obtain optimal levels of ingestion (27). The fact that Fgr is essential for optimal FcR-mediated phagocytosis in PMNs had not been observed previously (22) and suggests that the role of Fgr in regulating PMN phagocytic responses is more complex. The data depicted in Figs. 2 and 3 indicate that expression of Fgr either in a macrophage cell line or in primary macrophages negatively regulates the magnitude of the phagocytic response.

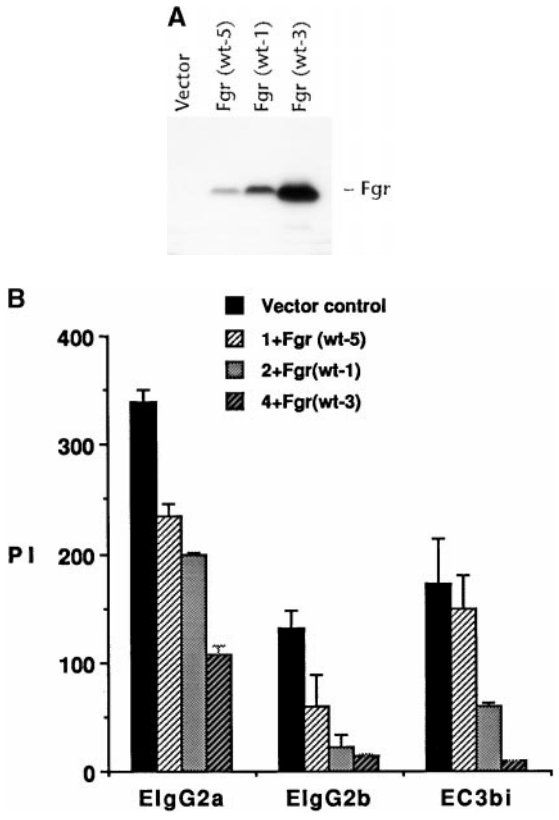

Figure 2. (A) Variable Fgr expression in three different BAC1.2F5 clones as assessed by Western blotting. (B) Effect of variable levels of Fgr(WT) expression (graded 1^+ to 4^+ by Western blotting, hatched and dotted bars) compared with a vector control (black bars) on ingestion of EIgG2a, EIgG2b, and EC3bi by BAC1.2F5 macrophages. The data are depicted as the mean ± SEM, $n = 3$. These data indicate that Fgr suppresses phagocytosis in a dose-dependent manner.

Fgr Does Not Affect Macropinocytosis, Another Membrane Fusion Event That Requires PI 3-Kinase. To assess whether Fgr expression affects other functions of macrophages that have signaling elements in common with phagocytosis, we examined the effect of Fgr expression on macropinocytosis, another mechanism by which uptake of particles as large as bacteria can occur (36, 37). Formation of macropinosomes requires PI 3-kinase and actin polymerization (29, 36), two signaling elements required by both FcγR- and CR3-mediated ingestion (29, 31, 33). We assessed macropinocytosis by the uptake of FITC-labeled 70-kD dextran (29) into macropinosomes measured both by fluorescence microscopy and flow cytometry to quantitate the amount of cell-associated fluorescence. Vector control and Fgr(WT)-expressing BAC1.2F5 cells took up the labeled dextran equivalently, and large macropinosomes were visualized in both cell types (data not shown). In addition, the level of cell-associated fluorescence was quantitatively identical as assessed by flow cytometry (MCF = 120 ± 19 vs. 104 ± 16, $n = 3$, for the vector control and Fgr[WT] cells, respectively). Moreover, inclusion of CSF-1 to stimulate macropinocytosis (29) increased the cell-associated fluorescence equivalently in both cell lines (MCF = 233 ± 53 vs. 216 ± 41; data not shown). To prove that the mechanism of uptake was similar in both the absence and presence of Fgr, we assessed the effect of 10 nM wortmannin, a PI 3-kinase inhibitor, on macropinocytic uptake. The histograms of both the vector control and the Fgr(WT)-expressing cells were shifted significantly to the left by the inclusion of wortmannin, indicating an essential role for PI 3-kinase in macropinocytic uptake by both cell lines (data not shown). These data indicate that not all mechanisms for uptake by macrophages are negatively affected by Fgr expression.

Fgr Negatively Regulates FcγR-mediated Ingestion at a Point Distal to Receptor Clustering and Aggregation, but Proximal to the Reorganization of F-actin into Phagocytic Cups. To delineate the point in phagocytosis where Fgr exerts its effect, we examined both an early (receptor cross-linking) and a later (phagocytic cup formation) process that are essential for FcγR-mediated ingestion. Opsonin-dependent phagocytosis is initiated by engagement of specific receptors with ligands on the target surface, which induces receptor aggregation and migration into membrane domains where cytoplasmic signaling molecules are activated (1–3, 37). In fact, cross-linking of FcγRII/FcγRIII with a rat mAb against these receptors and a polyclonal anti–rat antibody is sufficient to activate downstream signaling molecules that are essential for phagocytosis mediated by these receptors and to mediate receptor internalization via endocytic mecha-

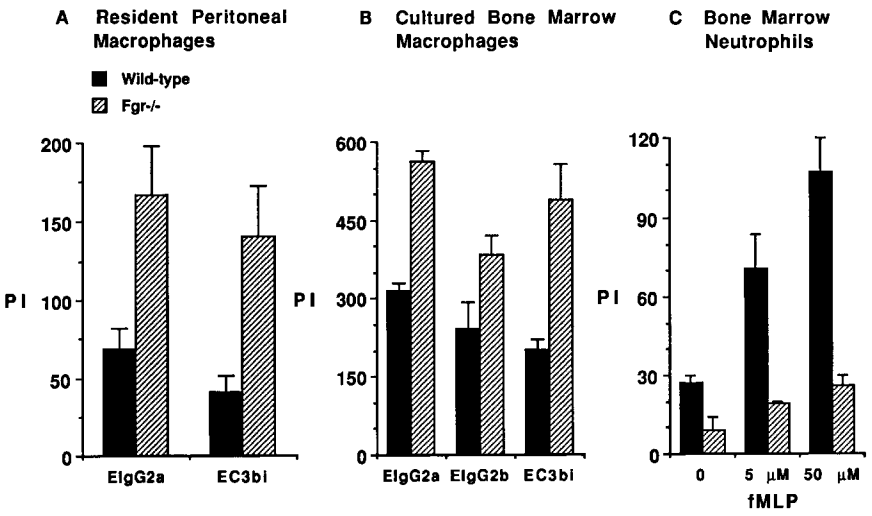

Figure 3. Comparison of phagocytic ability of resident peritoneal macrophages (A), cultured bone marrow macrophages (B), and bone marrow neutrophils (C) from $Fgr^{+/+}$ (Wild-type, black bars) and $Fgr^{-/-}$ mice (hatched bars). Mice heterozygous for deletion of *c-fgr* were bred, and homozygous null and positive offspring were detected by PCR. Resident peritoneal macrophages were obtained by lavage, bone marrow macrophages were cultured for 5 d in LCM, and bone marrow neutrophils were isolated by density gradient centrifugation. PI, the number of opsonized erythrocytes ingested by 100 phagocytes. The data are depicted as the mean ± SEM, $n = 3$–4. These data indicate that macrophages, but not neutrophils, from $Fgr^{-/-}$ mice have enhanced phagocytic function.

nisms (1, 34–38). As an example, the small GTPase, Rho, is required for phagocytosis, and the earliest step it regulates is Fcγ receptor clustering (38). Because the primary intracellular location for Src family kinases is the plasma membrane (39), Fgr could interfere with phagocytosis by blocking receptor aggregation and subsequent endocytosis. To examine this, we assessed the effect of Fgr expression on the endocytosis of FcγRII/FcγRIII induced by antibody-mediated aggregation as a measure of receptor clustering and aggregation. The percentage of internalized FcγRII/FcγRIII was equivalent for both vector control and Fgr(WT)-expressing cells at all three time points examined up to 1 h (from 36.2 ± 4.6% at 15 min to 69 ± 1.6% at 1 h for the vector control cells, compared with 34.4 ± 9.2% at 15 min and 62 ± 5.2% at 1 h for the Fgr[WT] cells; $n = 3$). Because FcγR clustering and aggregation is required for endocytosis and endocytosis is not affected by Fgr expression, these data indicate that this early event is not affected by Fgr expression. Because this early event was not involved, we wanted to determine if the kinetics of ingestion was affected by Fgr expression or whether it simply blocked ingestion after a certain number of targets were internalized. As shown in Fig. 4, Fgr expression suppressed internalization as early as 10 min, suggesting that it was attenuating the phagocytic response throughout the time course of the assay. These data indicate that Fgr suppresses ingestion at some point distal to FcγR clustering and activation and that this suppression occurs early in the internalization process.

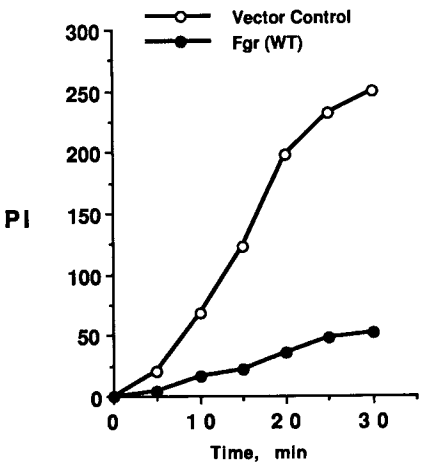

Figure 4. Effect of Fgr expression on the kinetics of ingestion of EIgGR. Vector control (○) and Fgr(WT)-expressing (●) BAC1.2F5 macrophages were allowed to attach EIgGR at 4°C for 2 h. The reaction was warmed to 37°C, and ingestion was assessed at various times as described in the legend to Fig. 1. These data indicate that Fgr expression suppresses phagocytosis at every time point during the reaction.

After FcγR cross-linking and initiation of intracellular signaling, within 5–10 min, pseudopodia containing F-actin extend over the surface of the target and create actin-rich phagocytic cups (1, 31–33). To assess the effect of Fgr on actin cup formation, adherent vector control and Fgr (WT)-expressing cells were allowed to attach EIgGR at 4°C for 2 h. After washing away the unbound EIgGR, phagocytosis was initiated by incubation at 37°C for 8 min. The distribution of F-actin relative to the bound EIgGR was visualized by rhodamine-phalloidin staining and fluorescence microscopy. At this time point, before a significant level of ingestion had occurred (Fig. 4), a majority of the vector control cells had F-actin distributed along the surface of the attached target-forming fluorescent phagocytic cups that extended from the surface of the cells (Fig. 5, A and B, arrows). In contrast, very few of the Fgr(WT)-expressing cells had demonstrable phagocytic cups even though they had bound equivalent numbers of the opsonized targets (Fig. 5, C and D, arrows). In addition, the F-actin subjacent to the attached targets did not extend as far from the surface of the cell, suggesting a blunting of pseudopod extension. To quantitate these data, we assessed the number of actin cups formed per 100 cells that had attached 4 or more EIgGR as well as the percentage of rosetting cells that had formed at least 1 actin cup. As shown in Fig. 5 E, Fgr(WT) macrophages had significantly fewer actin cups formed per 100 cells, and only 30% of these cells

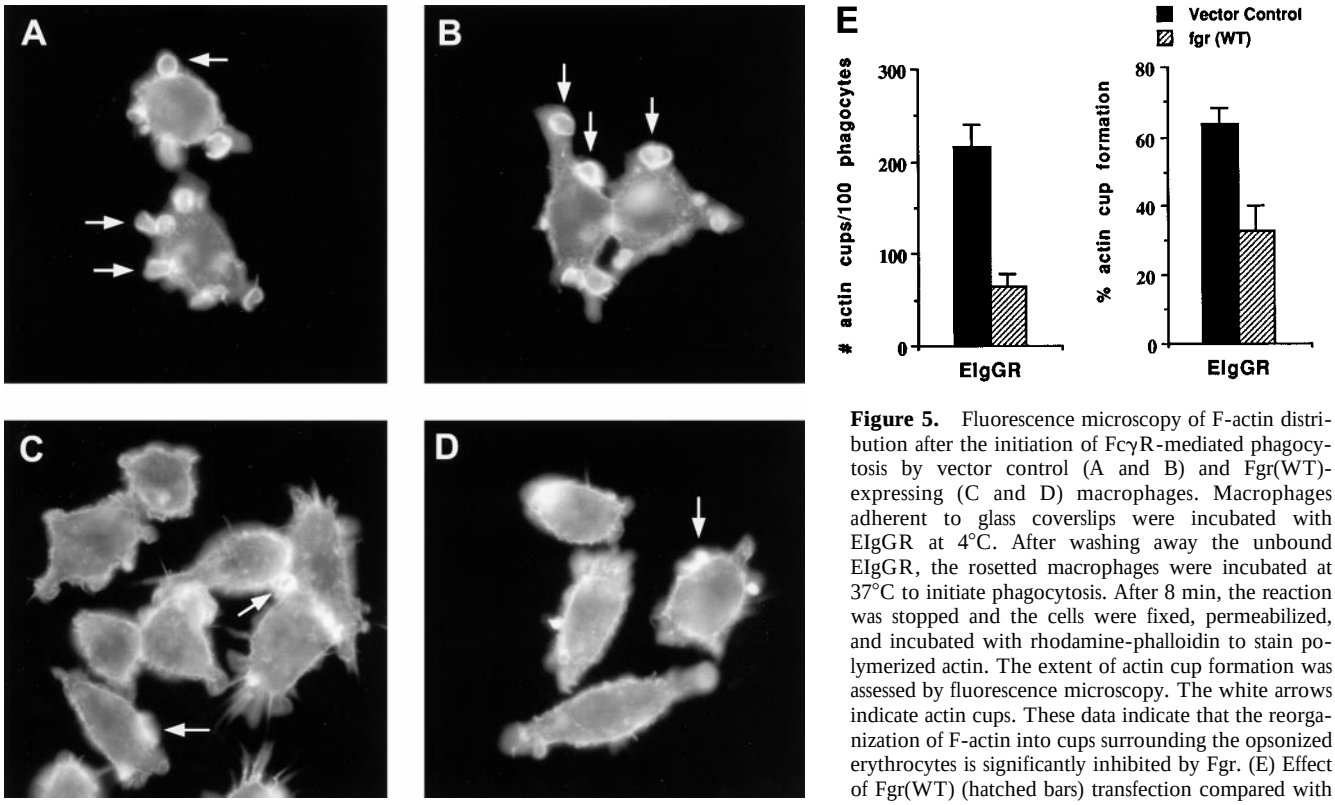

Figure 5. Fluorescence microscopy of F-actin distribution after the initiation of FcγR-mediated phagocytosis by vector control (A and B) and Fgr(WT)-expressing (C and D) macrophages. Macrophages adherent to glass coverslips were incubated with EIgGR at 4°C. After washing away the unbound EIgGR, the rosetted macrophages were incubated at 37°C to initiate phagocytosis. After 8 min, the reaction was stopped and the cells were fixed, permeabilized, and incubated with rhodamine-phalloidin to stain polymerized actin. The extent of actin cup formation was assessed by fluorescence microscopy. The white arrows indicate actin cups. These data indicate that the reorganization of F-actin into cups surrounding the opsonized erythrocytes is significantly inhibited by Fgr. (E) Effect of Fgr(WT) (hatched bars) transfection compared with a vector control (black bars) on the distribution of F-actin into cups after the initiation of FcγR-mediated phagocytosis. The extent of actin cup formation by macrophages that had bound at least 4 EIgGR was assessed by quantitating the number of actin cups formed by 100 rosetted macrophages (left) and the percentage of cells that formed at least 1 actin cup (right). The data are represented as the mean ± SEM, $n = 3$. These data indicate that Fgr suppresses both the percentage and the total number of actin cups formed during FcγR-mediated phagocytosis.

formed at least 1 actin cup, compared with 60–70% of the vector control cells. The number of actin cups correlated well with the PIs observed in the phagocytosis assays (Fig. 1). We also assessed actin cup formation with EIgG2b and obtained equivalent affects with Fgr expression (data not shown). These data indicate that Fgr negatively regulates FcγR-mediated phagocytosis at a point before the redistribution of F-actin into phagocytic cups surrounding the attached targets.

Fgr That Lacks Tyrosine Kinase Function Is Sufficient to Suppress Ingestion Mediated by Multiple Phagocytic Receptors. The members of the Src tyrosine kinase family have a homologous domain structure, and the sequence of the COOH-terminal kinase catalytic domain is highly conserved (39). Within this domain, a critical lysine residue (K279 in murine Fgr) is essential for ATP binding and phosphorylation of targeted tyrosine residues (40). Therefore, we assessed the effect of this lysine residue on the suppression of phagocytosis mediated by Fgr(WT). BAC1.2F5 cells expressing a mutant Fgr in which the lysine at position 279 was changed to an arginine (K279R) were compared with vector control and Fgr(WT)-expressing cells for phagocytic responses. Surprisingly, Fgr(K279R) was equally able to suppress ingestion mediated by both Fcγ and CR3 receptors, as was the wild-type form of murine Fgr (Fig. 6). Therefore, these data indicate that Fgr negatively regulates phagocytosis by a mechanism that does not involve its ability to phosphorylate any other protein.

Fgr Associates with a Complex of the ITIM-containing Receptor SIRPα and the Phosphatase SHP-1 after FcγR Aggregation. Positive signaling for FcγR-mediated phagocytosis involves multiple kinases, including tyrosine, lipid, and serine-threonine kinases (1–3). To address the signaling mechanism by which Fgr could be suppressing phagocytosis, we began by first assessing tyrosine phosphorylation patterns in vector control and Fgr(K279R)-expressing cells after FcγR aggregation. The use of the kinase-inactive cells (K279R), which have suppressed phagocytic responses (Fig. 6), allowed us to examine tyrosine phosphorylation independently of the additional phosphorylation mediated by Fgr. In the vector control cells, tyrosine phosphorylation of multiple proteins was increased within 0.5–5 min after FcγR aggregation (Fig. 7 A). In contrast, tyrosine phosphorylation in the Fgr(K279R) cells was significantly reduced, especially for proteins in the 95–120- and 50–65-kD range as soon as 30 s after FcR activation. Tyrosine-phosphorylated Fgr is prominent in the 56-kD range. This altered pattern of tyrosine phosphorylation after FcR aggregation in the Fgr(K279R) cells suggested that the suppression of phagocytosis could be mediated, at least in part, by the recruitment of a tyrosine phosphatase to FcγR signaling domains.

Several inhibitory pathways involving phosphatase recruitment can be triggered by the activation of ITIM-containing receptors (41). In addition to the ITIM-containing FcγRIIB

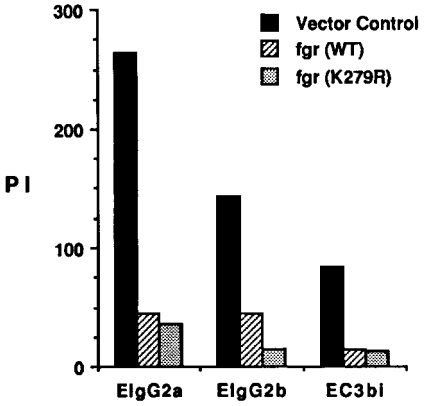

Figure 6. Effect of vector control (black bars), Fgr(WT) (hatched bars), and kinase-inactive Fgr(K279R) (dotted bars) on phagocytosis of EIgG2a, EIgG2b, and EC3bi. The experiments were performed as described in the legend to Fig. 1. The data are representative of at least three experiments. These data indicate that Fgr, which lacks tyrosine kinase activity, is sufficient for suppression of phagocytosis mediated by multiple ligand–receptor pairings.

receptor, mouse macrophages express other members of the inhibitory receptor family, including paired Ig-like receptor B (PIR-B), SIRPα, and platelet-endothelial cell adhesion molecule 1 (PECAM-1) (42–44). Because Fgr suppresses ingestion of both IgG- and C3bi-opsonized targets and because FcγRIIB is thought to inhibit only IgG-mediated responses (12), we speculated that other ITIM-containing receptors would be important for this negative regulation. Recently, both PIR-B and SIRPα were identified as major substrates and binding partners for the phosphatase SHP-1 in both BAC1.2F5 cells and bone marrow macrophages (42, 43). Because macrophages from mice with inactivating mutations in SHP-1 (*motheaten* and *motheaten viable*) are profoundly hyperresponsive, we hypothesized that SHP-1 would be a major regulator of phagocytic responses and that this regulation might be mediated through PIR-B and/or SIRPα. Because preliminary investigations revealed minimal expression of PIR-B relative to SIRPα on the vector control BAC1.2F5 cells, as suggested by others (43), we assessed whether Fgr would coassociate with SIRPα after FcγR aggregation and whether targeting SIRPα could affect phagocytic responses. To immunoprecipitate SIRPα, we used a rat mAb to an antigen first identified on neurons, P84, and subsequently shown to be identical to SHP substrate 1 (SHPS-1), BIT, and SIRPα (45). Vector control and Fgr(K279R) cells were stimulated via FcγR aggregation for various times, and the cell lysates were immunoprecipitated for SIRPα. The immunoprecipitates were subjected to immunoblotting to assess SIRPα protein expression, phosphorylated tyrosine content, Fgr association, and SHP-1 association. As shown in Fig. 7 B, the vector control and Fgr(K279R) cells expressed equivalent levels of SIRPα that was constitutively tyrosine phosphorylated. After FcγR aggregation, the level of tyrosine phosphorylation declined, and this reduction was much more pronounced in the Fgr-negative cells. After 30 s, Fgr coassociated with SIRPα in the Fgr(K279R) cells, and this association was pronounced at 5 min. Minimal Fgr levels were detected in cell lysates in-

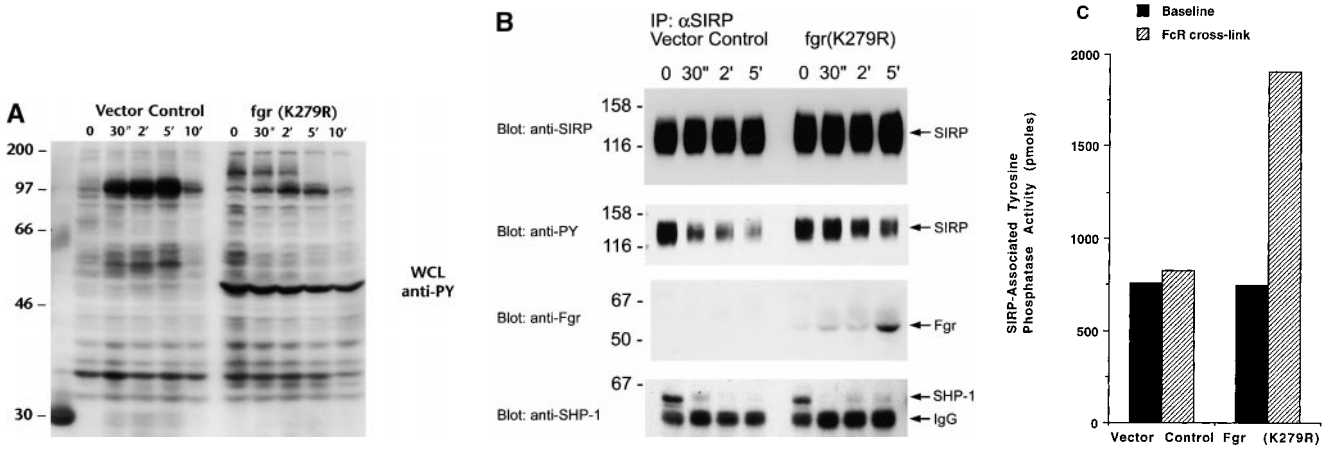

Figure 7. (A) Effect of kinase-inactive Fgr expression on the tyrosine phosphorylation pattern in whole-cell lysates after FcγR aggregation. Vector control and Fgr(K279R) BAC1.2F5 cells were incubated with rat mAb 2.4G2 anti-FcγRII/FcγRIII on ice for 1 h. After washing, the cells were incubated with intact goat anti–rat at 37°C for the indicated times. The cells were lysed and subjected to SDS-PAGE and Western blotting with anti-PY. Expression of kinase-inactive Fgr significantly suppressed tyrosine phosphorylation of several proteins as soon as 30 s after FcγR activation. (B) Coimmunoprecipitation of Fgr and SHP-1 with SIRPα after aggregation of FcγRs. Vector control and Fgr(K279R)-expressing BAC1.2F5 cells were incubated with rat mAb 2.4G2 on ice for 1 h. After washing, the cells were incubated with goat anti–rat at 37°C for the indicated times. The cells were lysed, and SIRPα was immunoprecipitated with rat mAb P84. After electrophoresis, the samples were subjected to Western blotting with either rabbit anti-SIRPα, anti-PY, rabbit anti-Fgr, or rabbit anti–SHP-1. These data indicate that after FcγR activation, Fgr associates with the tyrosine-phosphorylated 130-kD form of SIRPα and that this complex potentiates the association of SHP-1 with SIRPα. (C) Effect of Fgr expression on tyrosine phosphatase activity associated with SIRPα before and after FcγR aggregation. SIRPα immunoprecipitates from vector control and Fgr(K279R)-expressing BAC1.2F5 cells both before (black bars) and after (hatched bars) FcγR aggregation (as in A and B) were assessed for tyrosine phosphatase activity by incubation with a PY-containing peptide and measurement of liberated phosphate by malachite green absorption. The data are the mean of two experiments performed in duplicate. These data indicate that after FcγR aggregation, two- to threefold more tyrosine phosphatase activity is associated with SIRPα in the Fgr-expressing cells.

cubated with protein G alone or immunoprecipitated with rat anti-CD29 (data not shown). In addition, anti–PECAM-1 mAb did not coimmunoprecipitate with Fgr (data not shown). As reported by others (42, 43), SHP-1 was constitutively associated with SIRPα in both cells. However, in the absence of Fgr, the level of coassociated SHP-1 declined significantly after stimulation and was almost absent at 5 min. In the presence of Fgr, there was an initial decrease in coassociated SHP-1 after FcγR stimulation, with a subsequent increase at 2 and 5 min. These data suggest that Fgr coassociates with SIRPα after FcγR aggregation, and at the time of its maximal association (5 min), the tyrosine phosphorylation level of SIRPα is higher and there is a greater amount of coassociated SHP-1 compared with this time point in the absence of Fgr. To address whether this correlated with functional tyrosine phosphatase activity, we assessed the ability of the SIRPα immunoprecipitates to release phosphate from a pY-containing peptide. As shown in Fig. 7 C, after FcγR aggregation and activation, more tyrosine phosphatase activity was associated with SIRPα in the Fgr(K279R)-expressing compared with the vector control BAC1.2F5 macrophages.

To obtain evidence that SIRPα and SHP-1 could be implicated in regulating phagocytic responses, we assessed the effect of the P84 mAb against SIRPα on phagocytosis of EC3bi and EIgG2a by wild-type and $Fgr^{-/-}$ bone marrow macrophages (Fig. 8 A) and by vector control and Fgr(K279R)-expressing BAC1.2F5 cells (Fig. 8 B). Incubation of Fgr-expressing primary macrophages or BAC1.2F5 cells with mAb P84 anti-SIRPα significantly enhanced phagocytosis of both EC3bi and EIgG2a. An isotype control antibody that recognizes an antigen expressed by both cell types, CD14, had no effect. In contrast, incubation of $Fgr^{-/-}$ macrophages with anti-SIRPα had no effect on their phagocytic responses. Antibody against PECAM-1, another ITIM-containing receptor on these cells, also had no effect on ingestion by either cell type (data not shown). To assess whether the absence of the ITIM-containing receptor, FcγRIIB, could modulate this, we examined the effect of mAb anti-P84 on ingestion of EC3bi by macrophages that lack all three FcγRs, including FcγRIIB. Ingestion in the presence of the isotype control mAb was equivalent for both the wild-type and FcγR knockout macrophages (PI = 80 ± 4 vs. 76 ± 2, $n = 3$, respectively; data not shown). In addition, treatment with mAb P84 increased ingestion equivalently for both cell types (PI = 187 ± 14 vs. 176 ± 8, $n = 3$, respectively). These data indicate that the absence of the inhibitory FcγRIIB does not enhance phagocytosis of EC3bi and that anti-SIRPα is able to enhance EC3bi ingestion even in the absence of FcγRIIB. These data indicate that mAb P84 anti-SIRPα can reverse the negative regulatory effect of Fgr on FcγR- and CR3-mediated phagocytosis in a manner independent of the inhibitory FcγRIIB receptor.

To examine the role of SHP-1, we assessed ingestion of EIgG2a, EIgG2b, and EC3bi by cultured bone marrow macrophages from *motheaten viable* mice compared with control mice. As shown in Fig. 8 C, ingestion of both EIgG2a and EC3bi was markedly enhanced by me^v/me^v macrophages. As with the $Fgr^{-/-}$ macrophages (Fig. 4), ingestion of EIgG2b was not significantly augmented. To examine whether SIRPα modulates ingestion by me^v/me^v macrophages, we assessed the effect of mAb P84 on the ingestion of EIgGR by these cells. While mAb P84 significantly enhanced ingestion by wild-type macrophages (PI = 80 ± 7 control mAb vs. 247 ± 40 mAb P84, $n = 3$), it had no effect on ingestion by the me^v/me^v macrophages (PI = 267 ± 35 control mAb vs. 270 ± 34 mAb P84; data not shown). Because PIs in these cells can be >400 (Fig. 8 C), these data cannot be explained by a failure to enhance an already optimal level of ingestion. These data indicate that macrophages that contain a functionally inactive SHP-1 have enhanced phagocytic responses equivalent to those observed in $Fgr^{-/-}$ macrophages and that anti-SIRPα cannot enhance their phagocytic responses any further.

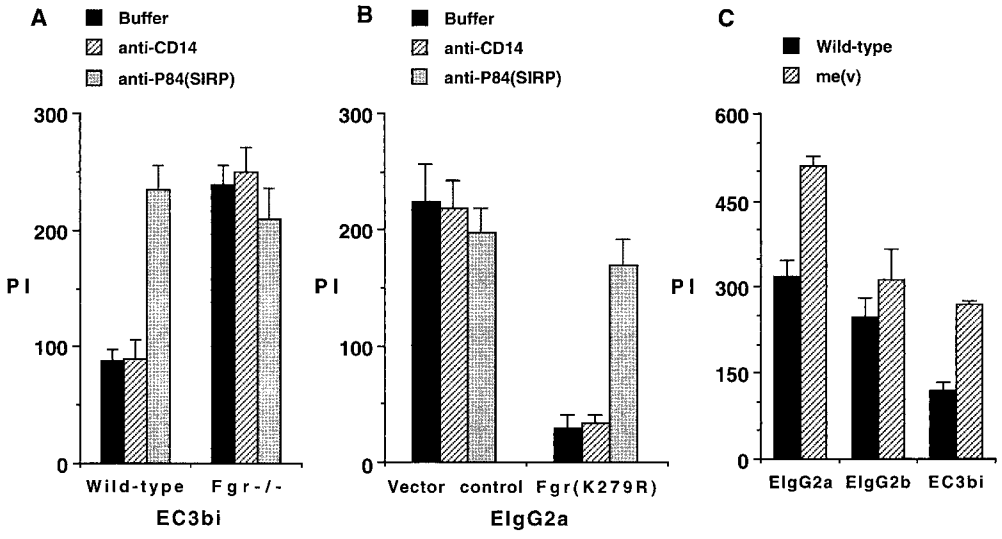

Figure 8. Effect of mAb P84 anti-SIRPα on phagocytosis by $Fgr^{+/+}$ and $Fgr^{-/-}$ bone marrow cultured macrophages (A) and vector control and Fgr(K279R)-expressing BAC1.2F5 cells (B). Cells were incubated with rat IgG1 anti-CD14 or rat IgG1 P84 for 15 min at room temperature. Ingestion of either EIgG2a or EC3bi was assessed as described. These data indicate that anti-P84 (dotted bars) enhances phagocytosis by Fgr-expressing macrophages compared with the isotype control (hatched bars). (C) Comparison of phagocytosis of EIgG2a, EIgG2b, and EC3bi by bone marrow macrophages from wild-type (black bars) and *motheaten viable* [me(v)] mice (hatched bars). These data indicate that mice with a nonfunctional form of SHP-1 have enhanced phagocytic ability.

Discussion

Phagocytes play an essential role in homeostasis by mediating the internalization and destruction of microbial pathogens. Two distinct molecular mechanisms for pathogen uptake have been proposed: one is the "zipper" mechanism that depends on repeated receptor–ligand interactions to drive pseudopod advance over the particle surface, and the other is a "trigger" mechanism that depends on the formation of membrane ruffles that fold back onto the surface of the cell, creating macropinosomes containing extracellular fluid and associated particles (37). Swanson and Baer (37) have observed that the two mechanisms differ in the magnitude of the pseudopodia produced relative to the size of the particle: zippering produces a small pseudopod and triggering a large one. These authors hypothesize that this difference arises by spatially controlled production of both excitatory and inhibitory signals in the ligand–receptor driven model of zippering (37). We contend that limitation or spatial control of the magnitude of the signal is beneficial to the host. Because ligation of FcγRs can initiate signals that generate reactive oxidants and release of lysosomal enzymes, spatial control would limit their production to the forming phagolysosome. A prediction of this hypothesis would be that negative signaling molecules could be found in zippering but not triggering mechanisms. Our present work indicates that the Src kinase family member, Fgr, is a negative signaling molecule for the zippering mechanism, phagocytosis, but not for the triggering mechanism, macropinocytosis. Moreover, the data presented in our paper are consistent with a model of negative regulation whereby Fgr raises the activation threshold necessary to generate a given magnitude of response. In this model, increasing the number of ligated and cross-linked receptors on a particle of sufficient size to trigger a phagocytic response (46) enhances the final readout of the pathway (i.e., ingestion) but cannot entirely overcome the negative signaling component contributed by the inhibitor molecule.

These data demonstrate that Fgr, like the Src family kinase member Lyn (17, 47, 48), plays an important role in negative regulation of hematopoietic cell activation. Because Lyn negatively regulates activation of the B cell antigen receptor and the high affinity receptor for IgE via phorphorylation of ITIM-containing receptors that recruit the lipid phosphatase SHIP (47, 48), we hypothesized that Fgr might work by a similar mechanism. Although we have obtained data implicating an ITIM-containing receptor expressed by macrophages, SIRPα, and a phosphatase, SHP-1 (Figs. 7 and 8), Fgr is clearly not responsible for phosphorylation of the ITIM motif in SIRPα (Fig. 7 B), and a kinase-inactive mutant is fully sufficient for the negative regulation of phagocytosis (Fig. 6). These data suggest that another kinase must be responsible for phosphorylating SIRPα and for regulating docking of SHP-1 in the plasma membrane. Because Lyn actively phosphorylates ITIMs in other receptors, it is intriguing to speculate that Lyn is responsible for phosphorylating SIRPα. If true, then the ability of Fgr to negatively regulate phagocytosis would be dependent on the function of Lyn. In this paradigm, Fgr may be functioning as an adaptor, through its SH2 and SH3 domains, to facilitate the association of SHP-1 with tyrosine-phosphorylated SIRPα (Fig. 9 B). Our data suggest a model whereby Fgr, through its SH2 domain, could protect SIRPα from dephosphorylation while allowing the recruitment and docking of SHP-1. Although we have focused the current studies on the regulation of tyrosine phosphorylation by Fgr (Fig. 7), other signaling elements, such as lipid and serine-threonine phosphatases, could also be involved. Further study will be required to determine if all of Fgr's ability to attenuate phagocytosis can be explained by recruitment of SHP-1.

These data, along with those of Hunter et al. (49) and Clynes et al. (12), which assessed the ability of inhibitory FcγRIIB to negatively regulate ingestion by activating FcγRs, are the first to define signal transduction elements that negatively regulate phagocytosis. However, there are some significant differences between our study and theirs. In their studies, the IgG-opsonized particle binds both the inhibitory receptor and the activating receptor, and the observed inhibition is dependent on specific interactions in the cytoplasmic regions of these two receptors (49). Thus, human FcγRIIB suppresses ingestion mediated by human FcγRIIA but not ingestion mediated by human FcγRIII (49), whereas in our work Fgr suppressed ingestion by multiple receptors including CR3, a non-IgG binding receptor. In fact, ingestion of EIgG2b, which is recognized by both inhibitory FcγRIIB and activating FcγRIII in murine macrophages, was not enhanced significantly in the absence of Fgr (Fig. 4) or by the lack of functional SHP-1 (Fig. 8). Moreover, the absence of FcγRIIB did not enhance ingestion of EC3bi, whereas anti-SIRPα did enhance ingestion. These data suggest that the SIRPα–Fgr–SHP-1 pathway we describe here is primarily involved in regulating other ligand–receptor pairings and is independent of FcγRIIB-mediated suppression. This fact does bring up an interesting question that relates to whether engagement of SIRPα is ligand dependent. Unlike FcγRIIB, ligands for most ITIM-containing receptors (41), including members of the SIRP family (45), have not been identified. In this regard, integrin-associated protein (IAP, CD47) was identified recently as a ligand for SIRPα expressed by neurons (P84 neutral adhesion molecule [50]). Because IAP is expressed by phagocytes and regulates matrix protein–augmented phagocytosis (51), it is intriguing to speculate that phagocytic receptors associate with molecules like SIRPα and IAP in membrane domains, and thus IAP may function as a coreceptor for SIRPα after phagocytic receptor activation.

Two other studies have examined the effect of Fgr on FcR-mediated phagocytosis by monocytes or macrophages, and both indicated that it had no effect (22, 52). There are several reasons that may explain the discrepancy between our results and theirs. First, both studies presumed that Fgr would have a positive signaling role in phagocytosis in these cells and assumed that ingestion would be in-

creased in its presence. This is reasonable because Fgr is present in high concentrations in mature phagocytes (18–20, 25, 53) and immunoprecipitates with phagocytic receptors after cell stimulation (21). Therefore, the phagocytosis assays were done with one level of opsonin, which could have represented such a high concentration that suppression of ingestion would have been difficult to observe (22, 52). In contrast, we examined phagocytosis over a wide dose–response range to demonstrate differences in the level of ingestion. In addition, the ligand on the opsonized particle affects the magnitude of the Fgr effect. For example, we found the greatest affect in wild-type cells with IgG2a- and C3bi-opsonized particles. Therefore, studies using polyclonal rabbit IgG–opsonized erythrocytes, which could target multiple receptors, might not observe negative regulation. Moreover, the work of Faulkner et al. (52) was performed with the human monocytic cell line, U937, which was transfected with human *Fgr*. U937 cells express FGR endogenously (52), and therefore, ingestion may have been suppressed constitutively such that increasing the level of FGR by transfection would not have an effect. In addition, we have found that the concentration of SIRPα expressed by phagocytic cell lines can vary (Gresham, H.D., unpublished observations), and SIRPα is required to observe the negative effects of Fgr.

Our data indicate that the role of Fgr in regulating phagocytic responses is completely different in neutrophils than it is in macrophages (Fig. 3). Although this result was unexpected, it is consistent with other data that implicate a positive signaling role for Fgr in neutrophils (53–55). Fgr exists in intracellular granules in resting neutrophils and translocates to the plasma membrane with cell activation (53). Moreover, it coimmunoprecipitates with the phagocytic receptors FcγRIIA (54) and CR3 (55) after neutrophil activation. If this positive signaling role for Fgr were mediated through its kinase function, a negative signaling component could still be contributed independently of its kinase activity through recruitment of SHP-1. Therefore, we believe that Fgr could have both positive and negative roles in regulating neutrophil phagocytic responses. Even in macrophages where a negative role for Fgr was clearly demonstrated, the magnitude of the response varied. We observed that the source of the macrophage and cell culture conditions had a significant effect on Fgr expression. These data confirm in vivo observations that indicate that the level of Fgr present in monocytic cells varies as they mature into tissue-fixed macrophages (56). In fact, some macrophage populations, such as Kupffer cells, alveolar macrophages, and Peyer's patch macrophages, do not express detectable levels of Fgr (56). Intriguingly, these cells are some of the most phagocytic cells in the reticuloendothelial system. Alternatively, exposure of macrophage populations to agents like LPS or CSF-1, which could occur at sites of inflammation or infection, can induce the expression of Fgr (25, 56). Therefore, the contribution of Fgr to the balance of positive and negative signaling in phagocytosis will depend on the phagocyte, its maturation level, its activation state, and the ligand-opsonized target.

These data significantly extend the knowledge of Fgr biology in macrophages. Although the targeted deletion of Fgr in combination with other Src kinases such as Hck and Lyn has revealed significant information about the roles of Src kinases in neutrophil and macrophage function (22, 57–59), very little has been ascertained about Fgr's unique role in hematopoietic cells. Therefore, our data demonstrating an important kinase-independent role for Fgr in negative regulation of phagocytosis are significant. While our data do not address the possible kinase-dependent functions of this Src kinase family member, it is intriguing to speculate that Fgr, like Lyn (17, 47, 48), could have both positive and negative roles in regulating hematopoietic cell function.

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References

1. Greenberg, S., and S.C. Silverstein. 1993. Phagocytosis. *In* Fundamental Immunology. 3rd ed. W.E. Paul, editor. Raven Press Ltd., New York, NY. 941–964.
2. Brown, E.J. 1995. Phagocytosis. *Bioessays*. 17:109–117.
3. Aderem, A., and D. Underhill. 1999. Mechanisms of phagocytosis. *Annu. Rev. Immunol.* 17:593–623.
4. Wright, S.D., and S.C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3bi receptor–mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149–1164.
5. Gresham, H., A. Zheleznyak, J. Mormol, and E. Brown. 1990. Studies on the molecular mechanisms of human neutrophil Fc receptor-mediated phagocytosis. *J. Biol. Chem.* 265:7819–7826.
6. Gresham, H., I. Graham, D. Anderson, and E. Brown. 1991. Leukocyte adhesion–deficient neutrophils fail to amplify phagocytic function in response to stimulation: evidence for CD11b/CD18-dependent and -independent mechanisms of phagocytosis. *J. Clin. Invest.* 88:588–597.
7. Gresham, H., J. Goodwin, P. Allen, D. Anderson, and E. Brown. 1989. A novel member of the integrin receptor family mediates Arg-Gly-Asp–stimulated neutrophil phagocytosis. *J. Cell Biol.* 108:1935–1943.
8. Gresham, H., I. Graham, G. Griffin, J.-C. Hsieh, L.-J. Dong, A. Chung, and R. Senior. 1996. Domain-specific interactions between entactin and neutrophil integrins. *J. Biol. Chem.* 271:30587–30594.
9. Pricop, L., J. Gokhale, P. Redecha, S. Ng, and J. Salmon. 1999. Reactive oxygen intermediates enhance Fcγ receptor

signaling and amplify phagocytic capacity. *J. Immunol.* 162: 7041–7048.
10. Parijs, L.V., and A.K. Abbas. 1998. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science*. 280:243–248.
11. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J.V. Ravetch. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature*. 379:346–349.
12. Clynes, R., J. Maizes, R. Guinamard, M. Ono, T. Takai, and J. Ravetch. 1999. Modulation of immune complex–induced inflammation in vivo by the coordinate expression of activation and inhibiting Fc receptors. *J. Exp. Med.* 189:179–185.
13. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin 10-deficient mice develop chronic enterocolitis. *Cell*. 75:263–274.
14. Dang, H., A.G. Geiser, J.J. Letterio, T. Nakabayashi, L. Kong, G. Fernandes, and N. Talal. 1995. SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation in TGFβ knockout mice. *J. Immunol.* 155:3205–3212.
15. Shull, M.M., I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, Z.M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, et al. 1992. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature*. 359:693–699.
16. Kulkarni, A.B., C. Huh, D. Becker, A. Geiser, M. Lyght, K.C. Flanders, A.B. Roberts, M.B. Sporn, J.M. Ward, and S. Karlsson. 1993. Transforming growth factor-β1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA*. 90:770–774.
17. Cornall, R., J. Cyster, M. Hibbs, A. Dunn, K. Otipoby, E. Clark, and C. Goodnow. 1997. Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity*. 8:497–508.
18. Notario, V., J.S. Gutkind, M. Imaizumi, S. Katamine, and K.C. Robbins. 1989. Expression of the *fgr* protooncogene product as a function of myelomonocytic cell maturation. *J. Cell Biol.* 109:3129–3136.
19. Willman, C., C. Stewart, T. Longacre, D. Head, R. Habbersett, S. Ziegler, and R. Perlmutter. 1991. Expression of the *c-fgr* and *hck* protein tyrosine kinases in acute myeloid leukemic blasts is associated with early commitment and differentiation events in the monocytic and granulocytic lineages. *Blood*. 77:726–734.
20. Willman, C., C. Stewart, J. Griffith, S. Stewart, and T. Tomasi. 1987. Differential expression and regulation of the c-src and c-fgr protooncogenes in myelomonocytic cells. *Proc. Natl. Acad. Sci. USA*. 84:4480–4484.
21. Hamada, F., M. Aoki, T. Akiyama, and K. Toyoshima. 1993. Association of immunoglobulin G Fc receptor II with Src-like protein-tyrosine kinase Fgr in neutrophils. 1993. *Proc. Natl. Acad. Sci. USA*. 90:6305–6309.
22. Lowell, C., P. Soriano, and H. Varmus. 1994. Functional overlap in the *src* gene family: inactivation of *hck* and *fgr* impairs natural immunity. *Genes Dev*. 8:387–398.
23. Chuang, W., and C.F. Lagenaur. 1990. Central nervous system antigen P84 can serve as a substrate for neutrite outgrowth. *Dev. Biol*. 137:219–232.
24. Morgan, C., J. Pollard, and E.R. Stanley. 1987. Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.2F5. *J. Cell. Physiol*. 130:420–427.
25. Yi, T.-L., and C.L. Willman. 1989. Cloning of the murine c-fgr proto-oncogene cDNA and induction of c-fgr expression by proliferation and activation factors in normal bone marrow-derived monocytic cells. *Oncogene*. 4:1081–1087.
26. Mann, R., R.C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell*. 33:153–159.
27. Caver, T., F. O'Sullivan, L. Gold, and H. Gresham. 1996. Intracellular demonstration of active TGFβ1 in B cells and plasma cells of autoimmune mice: IgG-bound TGFβ1 suppresses neutrophil function and host defense against *Staphylococcus aureus* infection. *J. Clin. Invest*. 98:2496–2506.
28. Roach, T., S. Slater, M. Koval, L. White, E. McFarland, M. Okumura, M. Thomas, and E. Brown. 1997. CD45 regulates Src family member kinase activity associated with macrophage integrin-mediated adhesion. *Curr. Biol*. 7:408–417.
29. Araki, N., M. Johnson, and J.A. Swanson. 1996. A role for phosphoinoside 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol*. 135: 1249–1260.
30. Lamaze, C., L.M. Fujimoto, H. Yin, and S. Schmid. 1997. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem.* 272:20332–20335.
31. Greenberg, S., J.E. Khoury, F. Di Virgilio, E. Kaplan, and S. Silverstein. 1991. Ca^{2+}-independent F-actin assembly and disassembly during Fc receptor–mediated phagocytosis in mouse macrophages. *J. Exp. Med*. 113:757–767.
32. Greenberg, S., K. Burridge, and S. Silverstein. 1990. Colocalization of F-actin and talin during Fc receptor–mediated phagocytosis in mouse macrophages. *J. Exp. Med*. 172:1853–1856.
33. Allen, L.-A., and A. Aderem. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med*. 184:627–637.
34. Crowley, M., P. Costello, C. Fitzer-Attas, M. Turner, F. Meng, C. Lowell, V. Tybulewicz, and A. DeFranco. 1997. A critical role for syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages. *J. Exp. Med*. 186:1027–1039.
35. Kiefer, F., J. Brumell, N. Al-Alawi, S. Latour, A. Cheng, A. Veillette, S. Grinstein, and T. Pawson. 1998. The syk protein tyrosine kinase is essential for Fcγ receptor signaling in macrophages and neutrophils. *Mol. Cell. Biol*. 18:4209–4220.
36. Swanson, J.A., and C. Watts. 1995. Macropinocytosis. *Trends Cell Biol*. 5:424–428.
37. Swanson, J.A., and S. Baer. 1995. Phagocytosis by zippers and triggers. *Trends Cell Biol*. 5:89–93.
38. Hackam, D., O. Rotstein, A. Schreiber, W.-J. Zhang, and S. Grinstein. 1997. Rho is required for the initiation of calcium signaling and phagocytosis by Fcγ receptors in macrophages. *J. Exp. Med*. 186:955–966.
39. Bolen, J., R. Rowley, C. Spana, and A. Tsygankov. 1992. The src family of tyrosine protein kinases in hemopoietic signal transduction. *FASEB J.* 6:3403–3409.
40. Xu, Y., J. Potter, and C. Willman. 1996. The function of src family tyrosine kinases in hematopoietic cells. *Leuk. Res.* 20: 229–234.
41. Bolland, S., and J. Ravetch. 1999. Inhibitory pathways triggered by ITIM-containing receptors. *Adv. Immunol*. 72: 149–177.
42. Berg, K., K. Carlberg, L. Rohrschneider, K. Siminovitch, and E.R. Stanley. 1998. The major SHP-1-binding, tyrosine-phosphorylated protein in macrophages is a member of the KIR/LIR family and an SHP-1 substrate. *Oncogene*.

17:2535–2541.
43. Timms, J., K. Carlberg, H. Gu, H. Chen, S. Kamatkar, M. Nadler, L. Rohrschneider, and B. Neel. 1998. Identification of major binding proteins and substrates for the SH2-containing protein tyrosine phosphatase SHP-1 in macrophages. *Mol. Cell. Biol.* 18:3838–3850.
44. Newman, P.J. 1999. Switched at birth: a new family for PECAM-1. *J. Clin. Invest.* 103:5–9.
45. Kharitonenkov, A., Z. Chen, I. Sures, H. Wang, J. Schilling, and A. Ullrich. 1997. A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature.* 386:181–186.
46. Koval, M., K. Preiter, C. Adles, P. Stahl, and T. Steinberg. 1998. Size of the IgG-opsonized particles determines macrophage response during internalization. *Exp. Cell Res.* 242:265–273.
47. Nishizumi, H., K. Horikawa, I. Mlinaric-Rascan, and T. Yamamoto. 1998. A double-edged kinase Lyn: a positive and negative regulator for antigen receptor–mediated signals. *J. Exp. Med* 187:1343–1348.
48. Chan, V., F. Meng, P. Soriano, A. DeFranco, and C.A. Lowell. 1997. Characterization of the B lymphocyte populations in lyn-deficient mice and the role of lyn in signal initiation and down-regulation. *Immunity.* 7:69–81.
49. Hunter, S., Z. Indik, M.-K. Kim, M.D. Cauley, J.-G. Park, and A.D. Schreiber. 1998. Inhibition of Fcγ receptor-mediated phagocytosis by a nonphagocytic Fcγ receptor. *Blood.* 91:1762–1768.
50. Jiang, P., C. Lagenaur, and V. Narayanan. 1999. Integrin-associated protein is a ligand for the P84 neural adhesion molecule. *J. Biol. Chem.* 274:559–562.
51. Brown, E.J., L. Hooper, and H. Gresham. 1990. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J. Cell Biol.* 111:2785–2794.
52. Faulkner, L., M. Patel, P. Brickell, and D. Katz. 1997. The role of the Fgr tyrosine kinase in the control of the adhesive properties of U937 monoblastoid cells and their derivatives. *Immunology.* 92:519–526.
53. Gutkind, J., and K. Robbins. 1989. Translocation of the Fgr protein-tyrosine kinase as a consequence of neutrophil activation. *Proc. Natl. Acad. Sci. USA.* 86:8783–8787.
54. Ibarrola, I., P. Vossbeld, C. Homburg, M. Thelen, D. Roos, and A. Verhoeven. 1997. Influence of tyrosine phosphorylation on protein interaction with FcγRIIA. *Biochem. Biophys. Acta.* 1357:348–358.
55. Berton, G., L. Fumagalli, C. Laudanna, and C. Sorio. 1994. β_2 integrin–dependent protein tyrosine phosphorylation and activation of the Fgr protein tyrosine kinase in human neutrophils. *J. Cell Biol.* 126:1111–1121.
56. Hatakeyama, S., K. Iwabuchi, M. Ato, C. Iwabuchi, K. Kajino, K. Takami, M. Katoh, K. Ogasawara, R. Good, and K. Onoe. 1996. Fgr expression restricted to subpopulation of monocyte/macrophage lineage in resting conditions is induced in various hematopoietic cells after activation or transformation. *Microbiol. Immunol.* 40:223–231.
57. Lowell, C., and G. Berton. 1998. Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the src-family kinases hck and fgr. *Proc. Natl. Acad. Sci. USA.* 95:7580–7584.
58. Lowell, C., and G. Berton. 1999. Integrin signal transduction in myeloid leukocytes. *J. Leukoc. Biol.* 65:313–320.
59. Meng, F., and C.A. Lowell. 1998. A beta 1 integrin signalling pathway involving src-family kinases, cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:4391–4403.