In vitro and in vivo macrophage function can occur independently of SLP-76


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Abstract

Expression of SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76), a hematopoietic cell-specific adapter protein, is required to couple Syk family tyrosine kinase activation to downstream mediators such as phospholipase C (PLC)-γ following TCR, platelet collagen receptor and mast cell FcεR stimulation. In addition to T cells, mast cells and platelets, SLP-76 is expressed in monocytes and macrophages. To determine the role of SLP-76 in FCγR-stimulated signaling pathways in macrophages, we examined cultured bone marrow-derived macrophages (BMM) from SLP-76−/− and wild-type mice. In this study, we show that FCγR cross-linking rapidly induces tyrosine phosphorylation of SLP-76 in wild-type BMM. Surprisingly, however, BMM from SLP-76−/− mice activate ERK2 and phosphorylate PLC-γ2 following FCγR ligation. Furthermore, SLP-76−/− BMM display normal FCγR-dependent phagocytic function and reactive oxygen intermediate production. SLP-76−/− and SLP-76+/c9059/c9059 BMM secrete comparable levels of IL-12 in response to lipopolysaccharide and IFN-γ. To examine macrophage function in vivo, SLP-76−/− mice were challenged i.v. with Listeria monocytogenes. SLP-76−/− mice survive and efficiently contain the acute phase of infection similar to wild-type mice but exhibit a stable chronic infection attributed to the lack of mature T cells. These data show that, although SLP-76 is required to couple Syk family PTK activity to downstream mediators and effector functions in FCγR-induced pathways in some cell types, activation of FCγR-dependent pathways occurs independently of SLP-76 in BMM.

Introduction

Adapter proteins are molecules that lack intrinsic catalytic activity but regulate intracellular signaling pathways through the binding and recruitment of signaling molecules into multimeric complexes. Recently, critical roles for several adapter proteins have been established in a number of signaling pathways (1–3). One such adapter molecule is the SH2-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76). SLP-76 was initially characterized in T lymphocytes as a protein consisting of an N-terminal acidic domain, a central proline-rich domain and a C-terminal SH2 domain (4). The acidic domain contains tyrosine residues that become phosphorylated by the Syk family protein tyrosine kinase (PTK), ZAP-70, upon TCR engagement (5,6). Tyrosine phosphorylation of SLP-76 is required to bind to the Rac/Rho guanine nucleotide exchange factor, Vav (7,8), and the adapter protein, Nck (9). The proline-rich domain constitutively binds to the hematopoietic-specific adapter protein, Gads (which is structurally homologous to Grb2) (10–13), and the C-terminal SH2 domain binds another adapter protein, SLAP-130/FYB (14,15).

Several recent observations have promoted our understanding of SLP-76 function in TCR-stimulated signaling pathways. Studies performed using a SLP-76-deficient Jurkat T cell line revealed that SLP-76 is required for TCR-induced IL-2 promoter activity. In this mutant cell line, TCR ligation stimulates ZAP-70 phosphorylation, but not phospholipase C (PLC)-γ1 phosphorylation, calcium influx or extracellular signal-regulated kinase (ERK) activation (16). These data...
suggest that SLP-76 functions at a point downstream of ZAP-70 activation but upstream of PLC-\(\gamma\)1 and ERK activation. In addition, SLP-76-deficient mice lack mature T cells due to a complete block in thymocyte development at an early stage (17,18). Development through this stage requires signals mediated by the pre-TCR, which has been shown to also employ many of the same signaling components utilized by the mature TCR (19,20).

SLP-76 is also expressed in other hematopoietic cell types, including platelets, NK cells, mast cells and monocytes/macrophages (4,21–24). In platelets, engagement of the collagen receptor leads to the activation of Syk (25). Platelets from Syk-deficient mice display a defect in aggregation and granule release in response to collagen stimulation (26). Interestingly, platelets from SLP-76\(^{-/-}\) mice demonstrate a similar functional defect in response to collagen, despite their ability to activate Syk (27), suggesting that SLP-76 functions to translate PTK activation to distal signaling events in both platelets and T cells.

Another family of receptors that elicits signals analogous to those of the TCR are the Fc\(\gamma\) receptors (Fc\(\gamma\)R). The Fc\(\gamma\)R belong to the Ig gene superfamily and contain a homologous extracellular domain that recognizes the Fc portion of IgG molecules. There are three classes of Fc\(\gamma\)R: Fc\(\gamma\)RI and Fc\(\gamma\)RII form a multimeric complex with \(\gamma\) chain dimers in monocytes and macrophages, while Fc\(\gamma\)RII exists as a monomer (28). Similar to the TCR and collagen receptor complexes, both the \(\gamma\) chains and the intracytoplasmic domain of Fc\(\gamma\)RII bear an immunoreceptor tyrosine-based activation motif (ITAM) that becomes phosphorylated upon receptor ligation and subsequently associates with the SH2 domains of Syk (29). Cross-linking Fc\(\gamma\)R on monocytes and macrophages by IgG-opsonized particles allows for the efficient engulfment and clearance of the bound particles, including invading pathogens or infected cells. These engulfed particles can then be processed and presented to T cells in the context of MHC class II molecules on their cell surface. In addition, Fc\(\gamma\)R-mediated signals stimulate the production of microbicidal reactive oxygen intermediates (ROI) and the expression of inflammatory cytokines by macrophages, which can then regulate other components of the host immune response (28). A requirement for Syk in Fc\(\gamma\)R-mediated functions was shown in Syk-deficient mice in which bone marrow- or fetal liver-derived macrophages were unable to internalize surface-bound IgG-opsonized sheep erythrocytes (30,31).

Consistent with a role for SLP-76 in transducing signals via ITAM-bearing receptors in T cells and platelets, recent studies examining mast cells derived from SLP-76-deficient mice revealed a marked defect in signaling via the high-affinity receptor for IgE (32). Surprisingly, however, we have found that NK cells from SLP-76-deficient mice appear to function normally, even when stimulated via antibody-dependent cellular cytotoxicity which requires engagement of Fc\(\gamma\)R (33). Therefore, in this study we asked whether Fc\(\gamma\)R could function in bone marrow-derived macrophages (BMM) from SLP-76-deficient mice. Our results demonstrate that SLP-76 is expressed in murine macrophages and is rapidly tyrosine phosphorylated in wild-type BMM upon stimulation with IgG-opsonized sheep red blood cells. However, SLP-76 is not required to activate ERK2 or phosphorylate PLC-\(\gamma\)2 in response to Fc\(\gamma\)R stimulation. Consistent with these biochemical findings, SLP-76-deficient BMM are able to bind and internalize IgG-coated sheep red blood cells comparable to wild-type BMM. Production of ROI in response to various stimuli, including IgG-coated beads, is similar between SLP-76\(^{-/-}\) and SLP-76\(^{+/+}\) BMM. In addition, IL-12 secretion by BMM in response to lipopolysaccharide (LPS) and IFN-\(\gamma\) does not require SLP-76. Finally, SLP-76-deficient mice challenged i.v. with sublethal doses of Listeria monocytogenes survive and resist fulminant infection similar to wild-type mice, albeit with a persistent chronic infection attributed to the lack of T cells in these mice. Together, these results suggest that although SLP-76 is required to couple Syk family PTK to some receptor-induced pathways, it is not required to link Syk to downstream mediators in response to Fc\(\gamma\)R stimulation in BMM.

Methods

Mice

Generation of the SLP-76\(^{-/-}\) and SLP-76\(^{+/+}\) mouse strains was described previously (17). Mice were raised at the University of Iowa Animal Care Facility under pathogen-free conditions and used in accordance with the National Institutes of Health guidelines.

Culture of BMM

Murine BMM were cultured according to a commonly used protocol described previously (34). Briefly, bone marrow cells from femurs and tibias were seeded onto plastic Petri dishes, containing RPMI 1640 supplemented with 15% L929 cell-conditioned supernatant (LCM), 10% FCS, 100 U/ml penicillin, 1000 U/ml streptomycin and 20 mM l-glutamine, and cultured at 37°C, 5% CO\(_2\). After 3–4 days, non-adherent cells were transferred to Petri dishes, containing fresh media. After an additional 3–4 days, non-adherent cells were discarded and the remaining monolayer of cells was used for subsequent experiments after a total of 7–10 days of culture. Adherent cells obtained through this protocol were 99% macrophages as determined by morphology and surface expression of Mac-1.

Preparation of stimuli

Antibody-coated sheep erythrocytes (AE) were prepared by washing sheep erythrocytes (Elmira Biologicals, Iowa City, IA) 3 times in PBS. Washed erythrocytes were either ready for use (as uncoated erythrocyte controls) or incubated with subagglutinating concentrations of rabbit anti-sheep red blood cell antibodies (ICN, Costa Mesa, CA) at 37°C for 30 min then washed twice in PBS before use. Antibody-coated beads used in ROI experiments were prepared by incubating polystyrene beads in 2.5% glutaraldehyde overnight, followed by several washes. Beads were then incubated with 0.1% BSA in PBS for 6 h, followed by incubation in 0.5 M ethanolamine overnight. BSA-coated beads were opsonized with rabbit anti-BSA IgG antibody for 6 h and then washed before use. Zymosan A particles (Sigma, St Louis, MO) were washed 3 times in PBS before use. Complement-opsonized zymosan (COZ) was prepared by incubating
washed zymosan A particles with freshly isolated human serum at 37°C for 30 min followed by two washes in PBS.

**Immunoprecipitations**

For each condition, 7–8×10^6 BMM were plated onto a 60 mm tissue culture-treated dish and allowed to adhere overnight at 37°C, 5% CO₂ in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and l-glutamine. Adherent cells were then washed twice in HBSS and then rested in HBSS for 30 min at 37°C, 5% CO₂ prior to stimulation. HBSS (for unstimulated conditions) or the indicated particulate stimulus was added and allowed to precipitate onto the cellular monolayer by centrifugation at 450 g for 2 min at 4°C. Dishes were then transferred to a 37°C, 5% CO₂ incubator for the indicated stimulation times. For pervanadate (PV) stimulations, a solution containing 0.1 mM Na₃VO₄/3 mM H₂O₂ was added to the dishes for 2 min at room temperature. Cell lysates were prepared by scraping and collecting cells in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.5 and 1 mM EDTA), containing protease inhibitors (50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin A and 1 mM PMSF) and phosphatase inhibitors (400 nM sodium vanadate, 10 mM NaF and 10 mM sodium pyrophosphate). Anti-murine SLP-76 sheep polyclonal antisera (sheep antisera raised against murine SLP-76 amino acids 136–235) or purified anti-PLC-γ2 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were conjugated to GammaBind (Pharmacia Biotech, Uppsala, Sweden) beads for 2 h at 4°C. Lysates were subjected to immunoprecipitation with the conjugated beads for 2 h at 4°C. Precipitates were washed 4 times in high-salt NP-40 lysis buffer (500 mM NaCl) and twice in NP-40 lysis buffer. Remaining protein complexes were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with either anti-phosphotyrosine mAb diacetate (H₂DCFDA) (Molecular Probes, Eugene, OR) at (Pharmacia Biotech, Uppsala, Sweden) beads for 2 h at 4°C and allowed to bind to the macrophages on ice for 20 min followed by centrifugation at 450 g for 1 s at 4°C. Phagocytosis was initiated by discarding cold media, adding fresh warm media and incubation at 37°C, 5% CO₂ for the indicated times. For cells stimulated with either unopsonized or IgG-opsonized erythrocytes, unopsonized erythrocytes remaining after the stimulation were lysed in ACK hypotonic solution (1.55 M Na₃Cl, 0.1 M potassium bicarbonate and 1 mM EDTA) for 30 s at room temperature, washed 3 times in PBS, fixed in Permeafix (Ortho Diagnostic, Raritan, NJ) and then mounted onto glass slides. For cells stimulated with zymosan or COZ, cells were treated as above with the omission of the hypotonic lysis. Ingestion was visualized under phase-contrast microscopy (×400) and macrophages were scored for the number of ingested particles per 100 macrophages for each condition by a blinded reader.

**Measurement of reactive oxygen species production**

BMM were plated into a 96-well flat-bottom plate (50,000 BMM/well) and allowed to adhere overnight at 37°C, 5% CO₂. Cells were then washed twice in HBSS (with calcium) and incubated in either HBSS or 20 μM dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes, Eugene, OR) at room temperature for 30 min. After incubation, the plate was chilled on ice for 10 min prior to addition of the stimuli. Stimuli were allowed to bind to the macrophage cells on the monolayer by centrifugation (450 g) for 3 min at 4°C followed by immediate transfer to a 37°C spectrophotometric plate reader (Fluostar). Analysis was performed by using an excitation wavelength of 480 nm and measuring fluorescence emission at 540 nm. Data were quantified by measuring the change in fluorescence per 5×10⁶ BMM over time.

**IL-12 ELISA**

BMM (8×10⁶ BMM/well) were incubated at 37°C, 5% CO₂ for 24 h in media alone or media containing 1 μg/ml LPS or LPS plus 1000 U/ml IFN-γ (R & D Systems, Minneapolis, MN) in 96-well plates. Culture supernatants from each condition were assessed for IL-12 by ELISA using plates coated with anti-murine IL-12 antibody (p40/p70; PharMingen, San Diego, CA). Serial dilutions of purified recombinant mouse IL-12 (R & D Systems) were used for a reference standard. Plates were incubated overnight at 4°C, followed by several washes in PBS/0.05% Tween (T-PBS) and incubation at room temperature for 4 h with 0.5 μg/ml biotin-conjugated rat anti-IL-12 (PharMingen) in T-PBS/1% BSA. Wells were then washed extensively and incubated with horseradish peroxidase-conjugated streptavidin (Zymed, San Francisco, CA) at room temperature for 1 h, followed by several washes.

**In vitro ERK2 activity assay**

For each condition, 6×10⁶ BMM were plated onto a 60 mm tissue culture-treated dish and incubated overnight as described above. Cells were then washed and rested in HBSS at 37°C, 5% CO₂ for 30 min prior to stimulation. Cells were stimulated with either the particulate stimuli as described above or 50 ng/ml phorbol myristate acetate (PMA; Sigma, St Louis, MO) for 5 min at room temperature or 1 μg/ml LPS (0127:B8; Sigma, St Louis, MO) for 20 min at 37°C. Cell lysates were prepared by scraping cells in Triton X-100 lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.6 and 150 mM NaCl), containing 1 mM PMSF, 1 mM aprotinin, 1 mM sodium vanadate, 50 mM NaF and 0.5 mM EGTA. Lysates were subjected to immunoprecipitation with anti-ERK2 antibodies (Santa Cruz Biotechnology) for 2 h at 4°C. Immune complexes were washed extensively and resuspended in kinase buffer (20 mM Tris, pH 7.5, 13 mM MgCl₂ and 1.5 mM EGTA), containing 1 mg/ml myelin basic protein (MBP) (Sigma), 20 μM ATP and [γ-³²P]ATP (8 μCi/ml) for 15 min at room temperature. Proteins were subjected to SDS–PAGE, followed by Coomassie blue staining to ensure equal amounts of MBP substrate per sample and autoradiography to visualize phosphorylated MBP.
Levels of IL-12 were detected using the TMB peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MA) and measurement of absorbance at 490–650 nm.

**In vivo Listeria experiments**

Growth, maintenance and infection of mice with virulent *L. monocytogenes* strain 10403s were performed as described (35). To assess levels of *L. monocytogenes* infection at day 4 or day 12, mice were euthanized followed by harvesting of spleens and livers. Liver sections were weighed and tissues were then homogenized in 0.2% NP-40 lysis buffer. Samples were plated directly or following dilution on tryptic soy agar plates containing 50 µg/ml streptomycin. Plates were incubated overnight at 37°C and then colonies were counted. Results are reported as c.f.u. per spleen or gram of liver. Statistical significance was determined by Student’s t-test.

**Results**

**FcγR stimulation induces tyrosine phosphorylation of SLP-76 in wild-type BMM**

In macrophages, PTK activation has been shown to be required for FcγR-dependent signaling events and functions, including the actin-dependent phagocytosis of IgG-opsonized particles (36,37). SLP-76 becomes tyrosine phosphorylated by Syk family PTK upon TCR ligation in T cells (6,38) and collagen receptor ligation in platelets (27). In a previous report, our laboratory has demonstrated SLP-76 expression in primary murine macrophages (24). We therefore addressed whether SLP-76 is tyrosine phosphorylated upon FcγR stimulation in macrophages, using cultured BMM derived from C57BL/6 mice. In this study, IgG-opsonized sheep erythrocytes (AE) were used to engage macrophage FcγR. As a control, BMM were also stimulated with various particles known to be engulfed through distinct receptors.

As shown in Fig. 1, while zymosan particles stimulate only a modest increase in SLP-76 phosphorylation at both 5 and 15 min of stimulation (Fig. 1, lanes 2 and 3), AE induce a significant increase in SLP-76 tyrosine phosphorylation within 5 min of stimulation (Fig. 1, lanes 6 and 7). Kinetic studies of AE-induced SLP-76 phosphorylation revealed maximal phosphorylation within 2 min of stimulation (data not shown). This phosphorylation is not due to an effect induced by sheep red blood cells themselves, since BMM stimulated with unopsonized erythrocytes (Fig. 1, lanes 9 and 10) show no phosphorylation of SLP-76 above that of unstimulated BMM. Stimulation with COZ particles (Fig. 1, lanes 4 and 5) also induces significant SLP-76 phosphorylation but only after longer stimulation times. From these data, we conclude that SLP-76 is expressed in murine BMM and is rapidly tyrosine phosphorylated upon FcγR stimulation. In addition, while zymosan does not induce significant SLP-76 phosphorylation, engagement of complement receptors by COZ does induce SLP-76 phosphorylation, albeit with slower kinetics than that seen with FcγR stimulation.

**PLC-γ2 is inducibly phosphorylated and ERK2 is activated in response to FcγR stimulation in SLP-76−/− BMM**

A previous report from our laboratory established that SLP-76 mice have normal numbers of macrophages in splenocyte populations, despite the absence of peripheral T cells (17). Furthermore, we found no significant difference between the ability of bone marrow-derived progenitors from SLP-76+/− and SLP-76−/− mice to proliferate and differentiate in vitro. We found that equivalent numbers of bone marrow-derived cells from SLP-76+/− and SLP-76−/− mice give rise to comparable numbers of adherent cells that are morphologically similar after 5 days of growth in culture with 15% LCM (data not shown). From these observations, we conclude that SLP-76 is not required for macrophage development either in vivo or in vitro.

We then sought to determine whether SLP-76 is required to promote specific signaling events in macrophages. It has been demonstrated that SLP-76 is critical for mediating TCR- and collagen receptor-induced PLC-γ phosphorylation in T cells and platelets respectively (16,27). Tyrosine phosphorylation of PLC-γ is required for activation of its enzymatic activity, leading to inositol 1,4,5-trisphosphate and diacylglycerol production (39). In macrophages, PLC-γ2 is stimulated upon FcγR cross-linking, presumably due to activation by Syk (40). To determine if SLP-76 is required for PLC-γ2 activation in macrophages, we examined FcγR-induced PLC-γ2 tyrosine phosphorylation in SLP-76+/− and SLP-76−/− BMM (Fig. 2). BMM from SLP-76+/− and SLP-76−/− mice were either left unstimulated (Fig. 2, lanes 1 and 6), or stimulated with AE (Fig. 2, lanes 2, 3, 7 and 8), COZ (Fig. 2, lanes 4 and 9) or the non-specific phosphatase inhibitor, PV (Fig. 2, lanes 5 and 10). In both SLP-76+/− and SLP-76−/− BMM, COZ modestly stimulates PLC-γ2 phosphorylation by ~2-fold when compared to corresponding unstimulated controls. Within 5 min of stimulation with AE, PLC-γ2 phosphorylation is significantly induced, and is sustained for at least 10 min in both SLP-76+/− and SLP-76−/− BMM, indicating that SLP-76 is not required for activation of PLC-γ2 in response to FcγR stimulation.
the host immune response is to ingest particles, including To test if BMM could ingest IgG-coated particles in the cytosis requires PTK-dependent signaling events and fails to occur in the absence of SLP-76 (41). Phagocytosis of yeast particles by macrophages was internalized AE, using phase-contrast microscopy. In Fig. Phagocytosis of IgG-coated sheep red blood cells by BMM dependent cytoskeletal changes. FcγR-stimulated phagocytosis requires PTK-dependent signaling events and fails to occur in BMM from Syk-deficient mice (30,31,36,37). To test if BMM could ingest IgG-coated particles in the absence of SLP-76, we stimulated SLP-76+/− BMM with IgG-coated sheep erythrocytes for various times at 37°C. Macrophages were then fixed and scored for internalized AE, using phase-contrast microscopy. In Fig. 4(A), we show that BMM from SLP-76+/− mice are able to ingest AE at a rate comparable to that of SLP-76−/− BMM. In addition, SLP-76−/− BMM are able to phagocytose a similar number of COZ and zymosan particles as SLP-76−/− BMM by 15 min at 37°C (Fig. 4B). These data show that SLP-76 is not required for FcγR-mediated phagocytosis of IgG-coated sheep erythrocytes, providing further evidence that FcγR-induced signaling events required for phagocytosis proceed independently of SLP-76.
SLP-76 in FcγR-dependent signaling in macrophages

Fig. 5. ROI production in response to various stimuli by SLP-76+/− and SLP-76+/+ BMM. SLP-76+/− (black bars) and SLP-76+/+ (white bars) BMM were preloaded with the oxidant-sensitive fluorescent dye dichlorohydrofluorescein diacetate and then either left unstimulated (Unstim), or stimulated with zymosan (Zym), COZ or antibody-opsonized polystyrene beads (AB) at 37°C in a spectrofluorometric plate reader. Fluorescence emission at 540 nm wavelength was measured over time. Each condition within an experiment was performed in triplicate. Bars represent the mean rate of ROI production expressed as the change in fluorescence/min/5 x 10⁴ BMM (±SEM). Data depicted is one representative experiment of three independent studies.

Fig. 4. Phagocytosis of AE by SLP-76+/− and SLP-76+/+ BMM. (A) SLP-76+/− (squares) and SLP-76+/+ (diamonds) BMM were allowed to adhere to glass coverslips overnight (~5000 BMM/cover slip) followed by addition of either AE or uncoated sheep erythrocytes at 4°C for 20 min. Phagocytosis was initiated by replacing cold media with warm media and allowing stimulation to occur at 37°C for the indicated times. Cells stimulated with uncoated erythrocytes were incubated at 37°C for 15 min. Following stimulation, uningested sheep erythrocytes were lysed in hypotonic solution, cells were fixed and the number of ingested erythrocytes per 100 macrophages was assessed by visualization under phase-contrast microscopy (×400). Data represent the mean values (±SEM) of three independent experiments. In all experiments, none of the uncoated erythrocytes were ingested by the BMM. (B) SLP-76+/− (black bars) and SLP-76+/+ (white bars) BMM were incubated with either COZ or zymosan (Zym) particles at 4°C for 20 min. Phagocytosis was initiated as described above, followed by stimulation at 37°C for 15 min. Coverslips were then washed several times in PBS and cells were fixed. Phagocytosis was quantified, using phase-contrast optics as described above. Bars represent the mean number of particles per 100 macrophages (±SEM) from two independent studies.

ROI production and IL-12 secretion by BMM occur in the absence of SLP-76

In addition to phagocytosis, macrophages help to clear pathogenic organisms by producing ROIs, which confer important microbicidal activity against engulfed pathogens. To test whether SLP-76 plays a required role in ROI production, we stimulated SLP-76+/− and SLP-76+/+ BMM with zymosan, COZ or antibody-coated beads, then measured ROI production over time using the intracellular oxidant-sensitive dye, dichlorohydrofluorescein diacetate. In Fig. 5, we show that the rate of ROI production by SLP-76+/− BMM is similar to that of SLP-76+/+ BMM in response to each stimulus examined.

Macrophages also react to pathogens by secreting cytokines that regulate other cellular components of the host's immune response. For example, LPS derived from Gram-negative bacteria and IFN-γ have been shown to act in synergy to stimulate IL-12 secretion by macrophages (45). IL-12 can then stimulate other cells of the immune system, including NK cells (46,47). To examine the role of SLP-76 in mediating IL-12 secretion, we treated SLP-76+/−, SLP-76+/+ and SLP-76+/+ BMM with LPS and IFN-γ. As shown in Fig. 6, stimulation with LPS alone induces only modest IL-12 secretion in SLP-76+/−, SLP-76+/+ and SLP-76+/+ BMM. However, stimulation with both LPS and IFN-γ induces a significant increase in IL-12 production above that of LPS alone, and at a level that was comparable between all three sets of BMM. These

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**Table 1. SLP-76−/− mice survive in vivo challenge with *L. monocytogenes***

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Death</th>
<th>Frequency</th>
<th>Infection level in surviving mice at day 12</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>Days after infection</td>
<td>Log_{10} c.f.u./spleen (± SD)</td>
</tr>
<tr>
<td>SLP-76+/+</td>
<td>3</td>
<td>0/3</td>
<td>−</td>
</tr>
<tr>
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<td>9</td>
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<td>3/3</td>
<td>4,4,4</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Below the limit of detection.
<sup>b</sup>ND, not determined.

Survival and levels of chronic infection after injecting mice with a sublethal dose (1.6–2.0 × 10<sup>5</sup>) of virulent *L. monocytogenes* 10403s. Survival of mice was monitored over 12 days after injection. Survival results are reported as number of deaths per number of mice injected within each group (frequency). For each death that occurred over the 12 days, the number of days before the subject expired is reported. At day 12, all surviving mice were euthanized, spleens and livers harvested, and tissues homogenized. Dilutions of the homogenates were plated onto tryptic soy agar plates. After 24 h of incubation at 37°C, colonies were counted and results reported as Log<sub>10</sub> c.f.u. per spleen or g of liver (± SD). For both SLP-76+/+ and SLP-76−/− mice, numbers were below the limit of detection for this assay.

Experimental data demonstrate that SLP-76 is not required for the production of reactive oxygen species or IL-12 in BMM.

**SLP-76-deficient mice survive and efficiently control acute infection by *L. monocytogenes* in vivo**

The above data provide *in vitro* evidence, suggesting that macrophages from SLP-76−/− mice are capable of responding to pathogenic challenges *in vivo*. To test this notion, we challenged SLP-76−/−, SLP-76+/+ and SLP-76−/− mice with low doses of *L. monocytogenes*. In wild-type mice, this dose of *L. monocytogenes* does not result in fulminant lethal infection largely due to functions of the innate immune response, including those provided by macrophages and NK cells (48). However, if innate immunity is sufficiently compromised, overwhelming infection in the spleen and liver ensues during the acute stage and death occurs within days. This has been demonstrated in TNF receptor- and IFN-γ-deficient mice, which succumb to infection by this dose of *L. monocytogenes* within the first week of infection (35,49). To determine if SLP-76−/− mice have an appropriate innate immune response to control the acute phase of infection against *L. monocytogenes*, SLP-76+/+, SLP-76−/−, SLP-76+/− and TNF−/− mice were injected i.v. with 1000–2000 *L. monocytogenes*, and acute survival was determined (Table 1). In our experiments, all three TNF−/− mice died within 4 days of infection. As expected, none of the SLP-76+/+ and SLP-76+/− mice succumbed to infection during the course of the experiment. To assess chronic infection, spleens and livers from the surviving mice were scored for c.f.u. of *L. monocytogenes* on day 12 following injection. Spleens and livers from the SLP-76+/+ and SLP-76+/− mice showed c.f.u. that were below the limit of detection for this assay, suggesting that the infection had been successfully cleared. In the SLP-76−/− mice, only one of the five infected mice died (at 10 days after injection). However, unlike wild-type or SLP-76+/− mice, spleens and livers from SLP-76−/− mice contained a significant number of Listeria at 12 days, indicating chronic infection, presumably due to the complete lack of mature T cells in SLP-76−/− mice.

While SLP-76−/− mice appear to mount an innate immune response sufficient to avoid acute lethality by *L. monocytogenes*, it remains possible that differences in response to the bacteria during the early stage of infection (days 2–4) exist. In other words, it is possible that SLP-76−/− mice exhibit a defect in their innate immune response, which is not sufficient to result in fulminant infection but results in a decreased ability to efficiently contain bacterial titers as well as wild-type mice. To address this possibility, SLP-76+/+ and SLP-76−/− mice were injected i.v. with the same dose of *L. monocytogenes* as in the experiment shown in Table 1. Four days after injection, spleens and livers of mice were scored for *L. monocytogenes* c.f.u. All of the SLP-76+/+ and SLP-76−/− mice survived up to the fourth day post-infection when spleens and livers were

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**Fig. 6.** IL-12 secretion in response to LPS and IFN-γ. BMM from SLP-76+/+ (black bars), SLP-76−/− (gray bars) and SLP-76+/− (white bars) were either left unstimulated or stimulated with LPS or LPS plus IFN-γ together for 24 h. Each condition was performed in triplicate. Concentrations of IL-12 (µg/ml) present in culture supernatants were assessed by ELISA. The experiment depicted reflects one representative experiment of three independent studies.
PTK, ZAP-70, becomes transphosphorylated by Src family SLP-76 and BLNK/SLP-65, thereby providing recruitment sites for the tandem SH2 FcγRIIITAM associated with FcγRII.

Vav in B cells (54). BLNK/SLP-65 was originally identified in cellular models of receptor-induced activation. Similar to the substrate of Syk tyrosine kinase activity following BCR signaling, FcγRIIITAM activate Syk tyrosine kinase to generate microbicidal ROI (28). Several comparisons have been drawn between FcγRIIITAM and ZAP-70 show a discrete defect in thymocyte development at an early stage that requires signals mediated from an immature TCR (pre-TCR) (56). A similar defect is found in SLP-76-deficient Jurkat T cells (17,18), suggesting that SLP-76 and Syk PTK function in the same pathway during thymocyte development. In addition, SLP-76-deficient Jurkat T cells demonstrate a defect in TCR-induced PLC-γ1 phosphorylation, calcium influx and ERK activation, although ZAP-70 activation remains intact (16). These data support the model that SLP-76 acts as a critical mediator downstream of Syk activation in both pre-TCR- and TCR-induced signaling pathways leading to thymocyte development or T cell activation. Similar to the defects observed in T cells, platelets and mast cells from SLP-76−/− mice fail to signal normally via ITAM-bearing receptors, suggesting that SLP-76 can serve a function in other hematopoietic cell types similar to its role in T cells. In contrast, however, signaling via ITAM-bearing receptors on NK cells seems to be intact in SLP-76−/− mice (33). In this report, we examined the function of SLP-76 in macrophages and found that SLP-76 does not appear to be required for macrophage development in vivo nor for macrophage proliferation and differentiation in culture with media containing LCM as a source of macrophage colony stimulating factor (M-CSF).

In addition to not being required for macrophage development, FcγR-mediated PLC-γ2 phosphorylation and ERK activation occur independently of SLP-76 in BMM. One potential explanation for this finding is that there is another factor in macrophages that functionally compensates for the lack of SLP-76. B cells, which do not express SLP-76, contain an homologous adapter protein, B cell linker protein (BLNK/SLP-65) (57). Similar to SLP-76 in TCR signaling, BLNK/SLP-65 is a substrate of Syk tyrosine kinase activity following BCR ligation and has been shown to associate with PLC-γ, Grb2 and Vav in B cells (54). BLNK/SLP-65 was originally identified as a B cell-specific molecule; however, we have recently detected BLNK/SLP-65 mRNA expression in BMM (data not shown). This finding suggests that some functional redundancy may exist within FcγR-mediated signaling events in macrophages. This question can now be addressed with the advent of mice deficient in BLNK/SLP-65 (58,59). However, if SLP-76 and BLNK/SLP-65 play wholly overlapping roles in macrophage FcγR-mediated pathways, a defect in macrophage function may only be observed after generating mice deficient in both SLP-76 and BLNK/SLP-65.

**Fig. 7.** SLP-76−/− mice demonstrate appropriate innate immunity to efficiently contain bacterial titers during the acute stage of infection. SLP-76−/− and SLP-76+/− mice were injected with virulent L. monocytogenes as above. Four days after injection, all mice were euthanized, and spleens and livers were harvested. The c.f.u. of L. monocytogenes were determined as in the previous experiment. Results are reported as log_{10} c.f.u. per spleen or liver as indicated. Bars represent mean determinations (±SD) for seven SLP-76−/− (shaded bars) or six SLP-76+/− (white bars) mice pooled from two independent experiments. Student's t-test indicates no significant difference between the number of c.f.u. obtained from SLP-76−/− and SLP-76+/− spleens or livers.

Recovered from the mice. The c.f.u. obtained from both spleens (Fig. 7) and livers of SLP-76−/− and SLP-76+/− mice were comparable, suggesting that innate immune responses against L. monocytogenes occurs efficiently in the absence of SLP-76.

**Discussion**

Macrophages express FcγR, which mediate binding to IgG-opsonized particles. Cross-linking of these FcγR activates various intracellular signaling pathways, leading to an array of effector functions, including actin-dependent engulfment and clearance of bound particles, production of cytokines, and generation of microbicidal ROI (28). Several comparisons have been drawn between FcγR signaling events and other cellular models of receptor-induced activation. Similar to the BCR and TCR complexes, FcγR complexes are associated with intracytoplasmic ITAM-containing domains (50). Interestingly, the ITAM-bearing chain contained within FcγRII and FcγRIII is also associated with a platelet collagen receptor (51). Upon stimulation of collagen receptors on platelets or Fcγ-R on macrophages, chain phosphorylation is induced (51,52). Similar to ITAM contained within antigen receptors, ITAM associated with FcγR and the collagen receptor are tyrosine phosphorylated following receptor stimulation, thereby providing recruitment sites for the tandem SH2 domains of Syk family PTK (51,53). In T cells, the Syk family PTK, ZAP-70, becomes transphosphorylated by Src family PTK upon association with the activated TCR complex, leading to up-regulation of its enzymatic activity (54). ZAP-70 then phosphorylates several downstream substrates, including the linker for activated T cells (LAT), PLC-γ1 and SLP-76 (6,55). Here, we show that SLP-76 is also rapidly tyrosine phosphorylated following FcγR stimulation in macrophages. Although we can only presume that SLP-76 phosphorylation in macrophages is dependent upon Syk, this event coincides with the time course of FcγR-induced Syk activation and tyrosine phosphorylation events reported by others (30,31,52).

Since SLP-76 is a substrate for Syk family PTK in T cells, one model for SLP-76 function holds that SLP-76 serves to couple Syk PTK activity to one or more downstream effectors. This hypothesis is supported by genetic studies performed in mice and the Jurkat T cell line. Mice deficient in both Syk and ZAP-70 show a discrete defect in thymocyte development which may only be observed after generating mice deficient in both SLP-76 and BLNK/SLP-65.
It is also conceivable that neither SLP-76 nor BLNK/SLP-65 are required to couple Syk to downstream signaling molecules. This notion is supported by studies in which co-expression of FcγR and Syk in COS cells support the ability of the transfected cells to phagocytose IgG-coated particles in an actin-dependent manner (60,61), indicating that expression of hematopoietic-specific proteins, other than FcγR and Syk, is not absolutely required for FcγR-induced phagocytosis.

It should be noted that since our experiments utilized a stimulus that presumably engages all three classes of FcγR, it is possible that we were not able to detect a signaling defect specific to one or two of the FcγR classes. There is evidence to suggest that signaling requirements initiated by different classes of FcγR are not identical to one another (60). In addition, we have not examined macrophages derived from other tissues such as the peritoneum, which show distinct phenotypic differences from BMM derived in culture in the presence of M-CSF. However, although we cannot generalize our findings to all monocytes and macrophages derived from other tissues, the ability of SLP-76/−/− mice to control the acute phase of infection by L. monocytogenes suggests that components of the innate immune response, which include macrophages, do not require SLP-76 to prevent lethal fulminant infection by this bacterium. At the same time, this observation does not exclude the possibility that other FcγR-mediated functions by macrophages are defective in the absence of SLP-76, since control of Listeria infections is not likely to be solely dependent upon FcγR-mediated functions. In addition, other cellular components of the innate immune response such as NK cells play important roles in containing the acute phase of infection by Listeria (62). We have recently shown that several functions of SLP-76-deficient NK cells, including antibody-dependent cellular cytotoxicity and cytokine secretion, are normal (33) and these may be sufficient to overcome subtle defects in macrophage function. The observation that SLP-76/−/− mice display significant Listeria c.f.u. in their spleens and livers at day 12 indicates chronic infection. This is likely due to the complete absence of T cells, and thus adaptive immunity, in these mice. A similar response to Listeria infection has been demonstrated in other T cell-deficient mice (63,64). However, we cannot exclude the possibility that a defect in macrophage function may lend some contribution to the chronic infection.

Although our results do not show a requirement for SLP-76 in the functions examined here, there is a potential that SLP-76 plays an important role in other receptor-induced pathways in monocytes/macrophages. For example, cellular adhesion to various substrates requires cytoskeletal rearrangements mediated by specific integrins. Some of these integrin receptors have been shown to activate Src and Syk family PTK activities in macrophages (65,66), and may require a subset of molecules that is distinct from those required for FcγR-mediated phagocytosis. Interestingly, tyrosine phosphorylation of SLP-76 in T cells mediates its association with the Rho GTPase guanine nucleotide exchange factor, Vav (7,8). In addition, it recently has been shown that SLP-76 phosphorylation also induces its association with the adapter protein, Nck (9). Nck has been shown to interact with the p21-activated protein kinases (Paks) and Wiskott–Aldrich Syndrome Protein (WASP) (67–69). Both the Paks and WASP are regulated by Rac and Cdc42, and are implicated in mediating actin polymerization (70–73). The observation of a multimolecular complex in T cells involving SLP-76, Vav and Nck raises the possibility that SLP-76 may function to couple Rho GTPase activation to downstream effector proteins that can then regulate cytoskeletal rearrangements. Future studies will address the role of SLP-76 in specific integrin-dependent events as well as its requirement in other monocyte/macrophage functions such as chemotaxis.

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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AE</td>
<td>antibody-coated sheep erythrocytes</td>
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<tr>
<td>COZ</td>
<td>complement-opsonized zymosan</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>LCM</td>
<td>L929 cell-conditioned supernatant</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
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<tr>
<td>paks</td>
<td>p21-activated protein kinases</td>
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<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
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<td>PTK</td>
<td>protein tyrosine kinase</td>
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<td>ROI</td>
<td>reactive oxygen intermediates</td>
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<td>PV</td>
<td>pervanadate</td>
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<tr>
<td>WASP</td>
<td>Wiskott–Aldrich syndrome protein</td>
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References


