The Innate Immune Response to *Salmonella enterica* Serovar Typhimurium by Macrophages Is Dependent on TREM2-DAP12[∇]

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Macrophage recognition of *Salmonella enterica* serovar Typhimurium leads to a cascade of signaling events, including the activation of Src family and Syk kinases and the production of reactive oxygen species (ROS), which are critical for host innate defense during early stages of bacterial infection. ROS production depends on the NADPH oxidase, but little is known about the innate immune receptors and proximal adapters that regulate *Salmonella*-induced ROS. Herein, we demonstrate that serovar Typhimurium induces ROS through a pathway that requires both triggering receptor expressed on myeloid cells 2 (TREM2) and DAP12. This pathway is highly analogous to the pathways utilized by Fc receptors and integrins to regulate ROS production. Oral infection of mice with serovar Typhimurium demonstrates that the DAP12-dependent pathway regulates cecal colonization during early stages of *Salmonella* infection. Thus, DAP12 is an important regulator of *Salmonella*-induced ROS production in macrophages, and TREM2 is essential for linking DAP12 to the innate response to serovar Typhimurium.

Regulation of reactive oxygen species (ROS) production is an important innate immune defense against microbial pathogens. In the early stages of *Salmonella enterica* serovar Typhimurium infection, ROS generation by NADPH oxidase is crucial for control of bacterial growth, and in vitro studies show that ROS constitute an important bactericidal effector mechanism for macrophages infected with serovar Typhimurium (38). Host defense in later stages of *Salmonella* infection involves reactive nitrogen species, inflammatory cytokines (especially tumor necrosis factor alpha and gamma interferon), and an adaptive T-cell response (18).

ROS production is mediated by the recruitment of cytosolic proteins to the phagosomal membrane to generate a functional NADPH oxidase (1). Several receptors, including Fc receptors (FcRs) and integrins, can trigger ROS production in response to microbial pathogens (3, 23). For each of these receptors, an immunoreceptor tyrosine-based activation motif (ITAM) is critical for initiation of downstream events. FcRs contain an ITAM in their cytoplasmic tail or require the ITAM-bearing adapter Fc gamma chain (Fcγ) for surface expression, phagocytosis, and ROS production (30, 36). Integrins trigger Syk phosphorylation and neutrophil ROS production through the ITAM-containing adapters DAP12 and Fcγ (22). Activation of an ITAM-associated receptor leads to phosphorylation by Src family kinases (SFK) of the tyrosine residues within the ITAM consensus sequence. Syk family kinases are consequently recruited and activated, inducing signaling through multiple downstream pathways, including phosphatidylinositol (PI)

3-kinase and protein kinase C (PKC) activation (30, 35). In neutrophils, ROS generation has also been demonstrated to involve the adapter protein Vav, PI 3-kinase, Rho family GTPases, phospholipase C, PKC, and the actin cytoskeleton (5, 9, 10, 29).

Less is known about innate immune receptors that regulate ROS production. These include dectin-1, which recognizes 1,3-β-glucans in the cell wall of yeasts (4). Dectin-1 contains a noncanonical ITAM motif in its cytoplasmic tail, and cross-linking of dectin-1 leads to Syk phosphorylation independent of DAP12 and Fcγ (24, 36). This results in activation of the NADPH oxidase and ROS production (36). In neutrophils, carcinoembryonic antigen-related cellular adhesion molecule 3 has been identified as an ITAM-containing innate immune receptor for *Neisseria gonorrhoeae* that regulates *Neisseria*-induced ROS via a Syk-dependent mechanism (28).

Toll-like receptors (TLRs) have also been implicated in ROS production, and signaling through the TLR adapter MyD88 is required for NADPH oxidase assembly in response to gram-negative bacteria (16, 25, 26). The roles of specific TLRs in NADPH oxidase assembly and ROS generation in response to serovar Typhimurium are cell type dependent. For example, TLR4 is required for NADPH oxidase activation in human neutrophils infected with serovar Typhimurium (37), but loss of TLR4 does not affect the mouse peritoneal macrophage ROS response to serovar Typhimurium (16). Similarly, we find that the TLR4 ligand lipopolysaccharide (LPS) does not induce detectable levels of ROS in murine bone marrow-derived macrophages (data not shown). The amount of ROS generated through activation of TLR pathways is considerably less than levels that are commonly associated with antimicrobial activities and may be more relevant to potential signal transduction pathways (20).

Many pathogenic bacteria can trigger ROS production by macrophages in the absence of complement or antibodies, but

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the macrophage receptors and signaling pathways that regulate this process are unknown. Potential receptors include the integrin family of proteins. The $\beta 2$ integrin chain CD18 has been shown to bind LPS and β -glucans (17, 39), making it a potential receptor. Triggering receptor expressed on myeloid cells 2 (TREM2) has also been shown to bind several bacteria and fungi (8), and cross-linking of TREM2 on macrophage cell lines results in NO release (7). Specific innate immune receptors and proximal signaling pathways that are required for the generation of ROS in response to serovar Typhimurium have not been described. In this study, we characterize the regulation of *Salmonella*-induced ROS production by murine macrophages, demonstrating a requirement for both TREM2 and its adapter protein DAP12.

MATERIALS AND METHODS

Reagents. Chemical inhibitors for SFK (pyrrolopyrimidine 2 [PP2]), Syk kinase (piceatannol and Syk inhibitor), PI 3-kinase (wortmannin), PKC (bis-indolylmaleimide [BIM]), p38 mitogen-activated protein kinase (MAPK) (SB21290), MEK1 (PD8059), Jun N-terminal protein kinase (JNK) (JNK inhibitor 1), actin polymerization (cytochalasin D), and the proteasome (MG-132 and lactacystin) were purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO). All other reagents were purchased from Invitrogen or Sigma, unless otherwise noted.

Mice. Mice were maintained in the animal facilities of the San Francisco Veterans Affairs Medical Center and the University of Washington. All experiments were performed in accordance with AAALC guidelines and were approved by the Veterans Affairs Animal Care Committee and the University of Washington IACUC. C57BL/6 mice were purchased from Simonsen (Gilroy, CA). Homozygous DAP12-deficient mice on a C57BL/6 background were previously described (14). Femurs from the Fc γ -deficient mice on a C57BL/6 background (31) and the DAP12/Fc γ double knockouts on a C57BL/6 background (22) were provided by Charles Alpers (University of Washington) and Lewis Lanier (University of San Francisco), respectively.

Bacteria and cell lines. The bacterial strain BC840 (a green fluorescent protein [GFP]-expressing derivative of *Salmonella enterica* serovar Typhimurium SL1344) was a gift from Brad Cookson (University of Washington, Seattle, WA). The RAW 264.7 murine macrophage cell line was from ATCC. TREM2 high- and low-level-expressing derivatives of RAW 264.7 and RAW 264.7 lines expressing short hairpin RNA interference (RNAi) for TREM2 or the control construct have been described previously (14).

Luminol assays. Bone marrow-derived macrophages were grown on petri dishes in RPMI 1640 with 10% fetal bovine serum (FBS; HyClone), penicillin, streptomycin, 5 mM L-glutamine, and 50 ng/ml human macrophage colony-stimulating factor (Peprotech), and the medium was refreshed on day 4. On day 7, the adherent bone marrow macrophages were lifted from the plates with phosphate-buffered saline (PBS) containing 2 mM EDTA. The cells were resuspended in antibiotic-free RPMI 1640 with 10% FBS, 5 mM L-glutamine, 50 ng/ml human macrophage colony-stimulating factor, and 50 ng/ml murine gamma interferon, plated at 10^5 cells per well in 100- μ l volumes in white plastic 96-well tissue culture plates (Corning), and incubated overnight. The next day, the medium was removed, 50 μ l prewarmed, antibiotic-free RPMI 1640 with 10% FBS and 100 μ M luminol was added to each well, and cells were incubated for 30 to 60 min at 37°C. For inhibitor studies, the inhibitors were added to the wells at the indicated concentrations 15 min prior to addition of stimuli. DMSO alone was added to control wells. Indicated stimuli were added to wells in 50 μ l of antibiotic-free medium, and the luminescence was recorded over a 120-min interval with a plate reader (Veritas).

Salmonella infection. Age- and gender-matched 10- to 12-week-old C57BL/6 and DAP12-deficient mice were orally infected with serovar Typhimurium, following published techniques (2). Briefly, mice were pretreated with streptomycin starting 3 days prior to infection to sterilize the intestinal tract. A calculated inoculum of 10^5 log-phase *Salmonella enterica* serovar Typhimurium BC840 cells in 100 μ l was administered orogastrically via a gavage needle. The inoculum was confirmed by plating on selective MacConkey agar overnight at 37°C. To determine the count of viable *Salmonella* bacteria in the tissues, the mice were euthanized on day 3 of infection. The mesenteric lymph node chain and portions of the spleen, liver, and cecum were weighed and homogenized in 0.5 ml PBS-0.025% Triton X-100 (Polytron homogenizer). Serial dilutions of homogenates

were made and plated on MacConkey agar overnight at 37°C to determine numbers of viable organisms. Numbers of CFU were then calculated per gram of tissue.

TREM2 fusion protein binding studies. TREM2 fusion protein binding to stationary *Salmonella* strain BC840 or *E. coli* labeled with Alexa Fluor Bodipy (Molecular Probes) was determined by incubating 10^6 bacteria with 1 μ g of recombinant TREM2B/Fc or TREM1/Fc (R&D Systems) at 4°C for 1 h in staining buffer (PBS-1% FBS). Bacteria were washed with staining buffer and then incubated with allophycocyanin-conjugated goat anti-human immunoglobulin G (IgG) F(ab')₂ (Jackson ImmunoResearch) at 4°C for 30 min. After two washes in staining buffer, binding of recombinant TREM proteins to Bodipy- or GFP-conjugated bacteria was detected by using a FACSCalibur flow cytometer (BD Biosciences).

Bacterial binding to TREM2 transfectants. *Salmonella* strain BC840 was cultured overnight at 37°C in LB with 50 μ g/ml streptomycin. Bodipy-conjugated *E. coli* was purchased from Molecular Probes. The BWZ.36 (BWZ) mouse T-cell lymphoma line was kindly provided by N. Shastri (University of California, Berkeley, CA). BWZ cells expressing TREM2 were previously described and shown to bind *E. coli* in a TREM2-dependent fashion (8). BWZ or BWZ/TREM2A cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 25 μ M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were washed with staining buffer, and 10^6 cells were incubated with bacteria at a ratio of 300 bacteria per cell at 4°C for 1 h. Cells were washed twice with staining buffer, and the number of bound GFP *Salmonella* or Bodipy *E. coli* bacteria was determined by flow cytometry.

BWZ reporter assay. The BWZ line contains a *lacZ* reporter construct under the control of four copies of the NEAT promoter element (27). A stable TREM2- and DAP12-dependent *lacZ* reporter line (TD4) was derived from BWZ by retroviral transduction of DAP12 and TREM2B constructs. Viral supernatant from cells transfected with the previously described pMXpie retroviral vectors containing full-length, FLAG-tagged TREM2B and DAP12 was used to transduce BWZ cells according to published methods (14). Cells were selected in 2 μ g/ml puromycin (Sigma), and stable, puromycin-resistant subclones were screened by flow cytometry for the expression of TREM2 and DAP12 by using specific monoclonal antibodies. TD4 cells were seeded in triplicate into 96-well plates at 5×10^4 cells/well in RPMI supplemented with 1% FBS and 10 ng/ml phorbol myristate acetate (PMA). For TREM2 cross-linking, 10^6 TD4 cells were incubated with 1 μ g of the previously described anti-TREM2 monoclonal antibody 78 for 15 min at room temperature prior to seeding (14). Goat anti-mouse IgG Fab-specific F(ab')₂ (Sigma) at 10 μ g/ 10^6 cells was added to the anti-TREM2-coated TD4 wells. Cells were incubated at 37°C for 15 min with ionomycin (250 nM), dextran sulfate (0.05 μ g/ml), or *Salmonella* strain BC840 at the bacterium/cell ratio indicated, followed by the addition of gentamicin for a final concentration of 50 μ g/ml. Plates were incubated for an additional 16 h at 37°C in a 5% humidified CO₂ atmosphere and washed once with PBS. *lacZ* activity was then determined by incubating the cells with 150 μ M chlorophenol red- β -D-galactopyranoside in PBS supplemented with 100 mM 2-mercaptoethanol, 9 mM MgCl₂, and 0.125% Nonidet P-40. After 4 h, absorbance was measured at 595 nm, with correction for background absorbance at 650 nm. Values were normalized by subtracting the absorbance values of wells treated with PMA alone.

Statistical analysis. Each experiment was performed at least three times, and representative results are shown. Values shown in Fig. 3A are medians and quartiles, and statistical significance was determined by the Mann-Whitney test. Values in other figures are means and standard errors of the means.

RESULTS

Pharmacologic inhibition of *Salmonella*-induced ROS. We first used a panel of specific inhibitors to define the signaling pathways controlling *Salmonella*-induced ROS production in bone marrow-derived macrophages, comparing the effects of the inhibitors to effects on the well-characterized zymosan-induced ROS response. Inhibitors of Syk kinase (piceatannol), PI 3-kinase (wortmannin), PKC (BIM), SFK (PP2), and actin polymerization (cytochalasin) substantially inhibited ROS production in response to either serovar Typhimurium or zymosan (Fig. 1 and 2B). Inhibition of MAPK pathways demonstrated only modest inhibition of ROS production (Fig. 2A), with the most potent effect seen with the MEK1 inhibitor PD98059,

FIG. 1. *Salmonella*- and zymosan-induced ROS generation by macrophages is dependent on the activities of SFK, Syk kinase, PKC, and PI 3-kinase. Bone marrow-derived macrophages were treated with a panel of specific inhibitors for 15 min, followed by exposure to *Salmonella* strain SL1344 (left; squares) or zymosan (right; triangles). ROS production over time was detected by measuring luminol as described in Materials and Methods. *Salmonella*- or zymosan-induced ROS production by macrophages pretreated with the indicated specific inhibitor at a low dose (white squares/triangles) or a high dose (gray squares/triangles) is compared to that for control (DMSO-treated) macrophages (black squares/triangles). Black circles indicate baseline ROS production by unstimulated macrophages. The effects of specific inhibitors of SFK (PP2), Syk kinase (piceatannol), PI 3-kinase (wortmannin), and PKC (BIM) on ROS generation by macrophages are shown. Syk inhibitor decreased ROS production similarly to piceatannol (data not shown).

FIG. 2. Inhibition of the MAPK MEK1 modestly reduces ROS production, but actin polymerization is required. Experiments were performed as described for Fig. 1. Number of luminol units is graphed on the y axis and time in minutes on the x axis. ROS production by control (DMSO-treated) macrophages stimulated by *Salmonella* strain SL1344 or zymosan (black squares/triangles) and by unstimulated macrophages (black circles) is shown in comparison to the level for each inhibitor. (A) Inhibition of MEK1 (PD98059; 10 μ M or 25 μ M) reduces ROS production in response to both serovar Typhimurium and zymosan, while inhibition of p38 MAPK (SB21290; 10 μ M or 25 μ M) or JNK (JNK inhibitor 1; 5 μ M or 12.5 μ M) has little effect on ROS generation. (B) Effects of inhibition of actin polymerization (cytochalasin D) and of the proteasome (MG-132) on ROS production. Similar results were obtained for the proteasome inhibitor lactacystin (data not shown).

which reduced the peak response by approximately 50%. Proteasome inhibitors had minimal effect (Fig. 1B), suggesting that proteasome-dependent functions, such as the activation of NF- κ B, contribute little to ROS production. There was no

measurable loss of cell viability following treatment with the highest concentrations of PP2, BIM, Syk inhibitor, PD98059, JNK inhibitor 1, and cytochalasin D, as determined by trypan blue exclusion after 2 hours of treatment. Modest decreases in

cell viability were seen for treatment with the highest concentrations of piceatannol (98%), SB21290 (95%), MG-132 (94%), lactacystin (94%), wortmannin (90%), and DMSO (86%). Overall, the inhibitors had very similar effects on *Salmonella*- and zymosan-induced ROS, suggesting that serovar Typhimurium induces the ROS response through a signaling pathway similar to that activated by zymosan. Zymosan activates ROS through dectin-1, which contains an atypical but functional ITAM (36). ITAM signaling activates Syk kinase, PI 3-kinase, SFK, PKC, and actin polymerization, consistent with our findings with inhibitors for these pathways (21, 33). The importance of these signaling intermediates suggests that serovar Typhimurium, like zymosan, induces ROS generation through an ITAM-mediated pathway in macrophages.

DAP12 controls *Salmonella*-induced ROS. Known ITAM-containing molecules in macrophages include the conventional ITAM adapters Fc γ and DAP12 as well as dectin-1, which contains an unconventional ITAM motif that can nonetheless interact with Syk (24, 36). Inhibition of dectin-1 by laminarin (Fig. 3A) had no effect on *Salmonella*-induced ROS, suggesting that dectin-1 does not regulate the macrophage ROS response to serovar Typhimurium. As anticipated, laminarin treatment substantially inhibited zymosan-induced ROS (Fig. 3A). To determine whether conventional ITAM-bearing adapters contributed to *Salmonella*-induced ROS production, we tested bone marrow-derived macrophages from wild-type (WT), Fc γ ^{-/-}, DAP12^{-/-}, and Fc γ /DAP12 double-deficient mice. Only bone marrow macrophages derived from mice deficient in DAP12 demonstrated substantially reduced (>90%) ROS production after stimulation with serovar Typhimurium (Fig. 3B). In contrast, ROS response to PMA treatment did not differ significantly (Fig. 3C). Thus, DAP12 is a critical ITAM adapter for the oxidative burst in macrophages exposed to serovar Typhimurium.

DAP12-deficient mice are more susceptible to *Salmonella* infection. Based on the above-mentioned findings, we predicted that DAP12 contributes to the innate immune response to serovar Typhimurium. To test this hypothesis, we orally infected streptomycin-treated WT and DAP12-deficient mice with serovar Typhimurium. On day 3 postinfection, bacterial burden was significantly higher in the ceca of DAP12-deficient mice than in those of WT mice (Fig. 4A). This was confirmed with immunofluorescence studies that demonstrated increased bacteria colonizing the ceca of DAP12-deficient mice relative to those in WT mice (Fig. 4B). Histological examination at this time point also demonstrated increased inflammation in the ceca of DAP12-deficient mice (Fig. 4C). A trend toward increased colonization of the spleen, liver, and mesenteric lymph nodes in the DAP12-deficient mice was noted, but these results did not meet statistical significance (Fig. 4A). The differences between WT and DAP12-deficient mice were most pronounced in these early events, and no difference in overall survival was noted (data not shown).

TREM2 contributes to *Salmonella*-induced ROS. DAP12 is an adapter protein that links several receptors to Syk in a variety of innate immune cells. In myeloid cells, DAP12 is known to associate with TREM1, TREM2, TREM3, signal-regulatory protein β 1, and myeloid DAP12-associating lectin 1 (15, 33). In earlier work, we demonstrated that several types of bacteria can bind and activate the TREM2 receptor (8). Thus,

FIG. 3. ITAM signaling through DAP12 but not dectin-1 in macrophages is required for *Salmonella*-induced generation of ROS. Experiments were performed as described in the legend for Fig. 1. Number of luminol units is graphed on the y axis and time in minutes on the x axis. (A) Blockade of the dectin-1 receptor with laminarin does not block *Salmonella*-induced ROS (left), but it significantly inhibits zymosan-induced ROS (right). (B) Macrophages derived from DAP12-deficient or DAP12/Fc γ double-deficient mice fail to generate ROS in response to *Salmonella* strain SL1344, in contrast to Fc γ -deficient macrophages, in which the response resembles that of macrophages derived from WT C57BL/6 mice. KO, knockout. (C) Levels of PMA-induced production of ROS are similar for all genotypes.

TREM2 was an attractive candidate for the DAP12-associated receptor in the *Salmonella*-induced ROS pathway. To determine whether or not TREM2 contributes to *Salmonella*-induced ROS, we tested natural variants of the murine macrophage RAW 264.7 cell line that were isolated for low and high expression of TREM2 (14). Cells expressing low levels of TREM2 demonstrated decreased *Salmonella*-induced ROS compared to the line expressing high levels of TREM2, while response to zymosan was unaffected (Fig. 5A and B). Similar results were obtained using RNAi to knock down TREM2 expression in RAW 264.7 cells; TREM2 RNAi substantially reduced *Salmonella*-induced ROS without affecting the response to zymosan (Fig. 5C). In contrast, the control RNAi

FIG. 4. DAP12 deficiency results in higher cecal organism burden and inflammation at early time points after orogastric *Salmonella* infection, without a survival difference. (A) C57BL/6 (filled circles) and DAP12^{-/-} (open circles) mice ($n = 6$) were infected with 0.8×10^3 CFU *Salmonella* strain BC840. The number of viable bacteria per gram of tissue was determined at 3 days postinfection. The median number of cecal CFU/g was significantly higher in DAP12-deficient mice ($P = 0.015$, Mann-Whitney U test). Data are representative of three independent experiments. Error bars represent interquartile ranges. KO, knockout. (B) Representative photomicrographs of frozen cecal sections from *Salmonella*-infected mice show a higher burden of organisms in DAP12-deficient than in C57BL/6 animals ($\times 400$ magnification). *Salmonella* organisms are detected by green fluorescence of the GFP-expressing strain SL1344. (C) Hematoxylin-and-eosin-stained cecal sections of DAP12-deficient mice show thickened bowel walls, edematous crypts, and increased inflammatory infiltrate compared with those of C57BL/6 mice (magnifications, $\times 200$ [top] and $\times 400$ [bottom]).

demonstrated intact ROS responses to both serovar Typhimurium and zymosan (Fig. 5D). Thus, the DAP12-associated receptor TREM2 contributes to *Salmonella*-induced ROS.

TREM2 does not directly interact with serovar Typhimurium. TREM2 has been shown to bind to *E. coli* as well as to several other species of bacteria through an interaction with polyanionic ligands (8). We therefore hypothesized that TREM2 may similarly act as a receptor for polyanionic ligands on *Salmonella* bacteria. However, in contrast to what was

FIG. 5. The DAP12-associated receptor TREM2 is required for *Salmonella*-induced ROS. The murine macrophage cell line RAW 264.7 was treated with buffer alone (black diamonds), zymosan at 50 ng/ml (black triangles), or *Salmonella* strain SL1344 at 50 (black squares) or 15 (white squares) bacteria/cell, and ROS production was measured by using a luminol assay. Number of luminol units is graphed on the y axis and time in minutes on the x axis. (A) ROS production by standard RAW 264.7 cell line. (B) *Salmonella*-induced but not zymosan-induced ROS are greatly diminished in a subclone of the RAW 264.7 line expressing low levels of TREM2. (C) Suppression of TREM2 in RAW 264.7 cells by RNAi reduces *Salmonella*-induced ROS production in comparison to levels for control transfected cells, as shown in panel D. (D) ROS production by RAW 264.7 expressing control nonfunctional RNAi vectors.

FIG. 6. Serovar Typhimurium does not bind TREM2. (A) Soluble TREM2B/Fc does not bind to serovar Typhimurium, in contrast to *E. coli*. Bacteria were stained with mouse TREM2B/Fc (black histogram) or TREM1/Fc (gray histogram), followed by allophycocyanin-conjugated goat anti-human IgG secondary antibody, or with secondary antibody alone (gray filled histogram) and analyzed by flow cytometry. Similar results were obtained using a TREM2A-Ig fusion protein (data not shown). (B) Serovar Typhimurium does not bind TREM2A-expressing BWZ cells. Stationary-phase *Salmonella* strain BC840 or Bodipy *E. coli* bacteria were incubated with 10⁶ BWZ or BWZ-TREM2A cells at a ratio of 300 bacteria/cell, washed, and analyzed for Bodipy (*E. coli*) or GFP (*Salmonella*) fluorescence by flow cytometry. (C) Serovar Typhimurium does not significantly activate the TREM2- and DAP12-dependent LacZ reporter line TD4. Stationary-phase *Salmonella* strain BC840 at the indicated ratio of bacteria to reporter cells, ionomycin (Iono), cross-linked anti-TREM2 (α -TREM2) antibody, or dextran sulfate was added to triplicate wells of TD4. Cells were incubated at 37°C for 15 min, followed by the addition of gentamicin. LacZ induction was determined 24 h later. No signal was seen in BWZ cells lacking TREM2 and DAP12 (data not shown).

found for *E. coli*, soluble TREM2-Fc fusion protein failed to bind serovar Typhimurium (Fig. 6A). Further, unlike *E. coli*, serovar Typhimurium did not bind to BWZ cells expressing high levels of surface TREM2 (Fig. 6B), and serovar Typhi-

murium was unable to activate a TREM2/DAP12-dependent LacZ reporter system in BWZ cells (Fig. 6C). Thus, although TREM2 is required for the generation of ROS by macrophages in response to serovar Typhimurium, this requirement does not appear to involve direct recognition of serovar Typhimurium by TREM2.

DISCUSSION

Our studies demonstrate that efficient *Salmonella*-induced ROS production by macrophages requires both DAP12 and TREM2. ROS are critical for defense against *Salmonella* infection, especially at early time points postinfection (18), and hosts lacking NADPH oxidase have increased bacterial colonization and more-rapid death after *Salmonella* infection (19). During oral infection of naïve mice, mucosal surfaces may rely in particular on DAP12-dependent innate responses to *Salmonella*. Our in vivo studies suggest that the DAP12-dependent pathway retards *Salmonella* growth at mucosal surfaces. We did not, however, detect significant differences in overall survival between DAP12-deficient mice and control mice. It is possible that there is redundancy in signaling adapter requirements for the ROS response to serovar Typhimurium in other cell types, such as neutrophils, causing the phenotype of the DAP12-deficient mice to be much milder than that of mice globally deficient in NADPH oxidase activity. In neutrophils, for example, Fc γ and DAP12 are both capable of mediating integrin-induced ROS (22).

It is also possible that the loss of DAP12 may enhance other defense mechanisms that compensate for the loss of ROS. DAP12 has been shown to inhibit TLR and FcR responses in macrophages and dendritic cells (6, 11), and it is possible that DAP12 may have similar effects on TLR or other responses in vivo. In a colitis model of *Salmonella* infection, mucosal inflammation has been shown to require MyD88-dependent signaling in hematopoietic cells (13). Thus, increased TLR responses in DAP12-deficient mice could contribute to the increase in mucosal inflammation seen in response to serovar Typhimurium and may partially offset defective ROS production by macrophages. The effect of DAP12 deficiency on the host response to other infections is complex, with some studies showing DAP12 deficiency to be protective, as in LPS-induced endotoxemia and *Listeria monocytogenes* infection (12, 34), and others showing increased host susceptibility to D-galactosamine-potentiated endotoxemia in the absence of DAP12 (12). Additionally, DAP12-independent pathways may effectively control systemic infection such that there is no difference in survival of DAP12-deficient mice. During the systemic phase of *Salmonella* infection, multiple host defense mechanisms are utilized, including complement activation, cytokine and chemokine production, and production of antigen-specific T cells and antibodies (18). The later phases may be able to compensate for defective ROS responses in macrophages, even if they are unable to compensate for a global loss of ROS response.

The ROS response to a variety of stimuli appears to depend on ITAM signaling pathways leading to Syk activation (22, 28, 36). With the exceptions of dectin-1 and carcinoembryonic antigen-related cellular adhesion molecule 3, the upstream innate immune receptors that trigger an ROS response to specific pathogens are unknown. TREM2 does not appear to

FIG. 7. DAP12 and TREM2 initiate a signaling cascade essential for *Salmonella*-induced ROS generation. PI3K, PI 3-kinase.

be a direct receptor for serovar Typhimurium, although we cannot rule out the possibility that TREM2 can recognize serovar Typhimurium in the context of a coreceptor or in the presence of a specific host protein, similar to the requirement for LPS binding protein for TLR4 recognition of LPS. We hypothesize that TREM2 plays an intermediary role, connecting a yet-to-be-identified receptor for serovar Typhimurium to DAP12 and its signaling cascade (Fig. 7). Such a role for TREM2 has been demonstrated for the response of plexin-A1 to its ligand Sema6D on dendritic cells. TREM2 associates with plexin-A1, and loss of either TREM2 or DAP12 reduces the dendritic cell response to Sema6D (32). Similarly, CD18 does not associate directly with either DAP12 or Fcγ, yet they are required for integrin-mediated ROS in neutrophils, suggesting that a DAP12/Fcγ-associated receptor may act as an intermediary (22). Alternatively, TREM2 may interact with ligands on the macrophages themselves in a manner that promotes the generation of ROS but that we could not detect in our assays. In all, Syk activation via ITAM-containing or DAP12/Fcγ-associated receptors may be a common and essential pathway for the ROS response to a variety of pathogens.

In conclusion, our studies demonstrate that TREM2 and DAP12 are important in the innate immune response to serovar Typhimurium; they are critical for efficient ROS production by macrophages and for control of cecal colonization during oral infection in mice.

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