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Salmonella Flagellin Induces Bystander Activation of Splenic Dendritic Cells and Hinders Bacterial Replication In Vivo

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Bacterial flagellin is a target of innate and adaptive immune responses during Salmonella infection. Intravenous injection of Salmonella flagellin into C57BL/6 mice induced rapid IL-6 production and increased expression of activation markers by splenic dendritic cells. CD11b+ and CD8α+ dendritic cells each increased expression of CD86 and CD40 in response to flagellin stimulation, although CD11b+ dendritic cells were more sensitive than the other subsets. In addition, flagellin caused the rapid redistribution of dendritic cells from the red pulp and marginal zone of the spleen into the T cell area of the white pulp. Purified splenic dendritic cells did not respond directly to flagellin, indicating that flagellin-mediated activation of splenic dendritic cells occurs via bystander activation. IL-6 production, increased expression of activation markers, and dendritic cell redistribution in the spleen were dependent on MyD88 expression by bone marrow-derived cells. Avoiding this innate immune response to flagellin is important for bacterial survival, because Salmonella-overexpressing recombinant flagellin was highly attenuated in vivo. These data indicate that flagellin-mediated activation of dendritic cells is rapid, mediated by bystander activation, and highly deleterious to bacterial survival. The Journal of Immunology, 2007, 179: 6169 – 6175.

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cells following i.v. injection remains unclear. Furthermore, irrespective of the mode of activation, it is also unclear whether certain splenic dendritic cell subsets are more responsive to flagellin activation than others. This issue is particularly important given a recent report demonstrating differential Ag processing and presentation by different dendritic cell subsets to CD4 and CD8 T cells (37).

Expression of activation markers and inflammatory cytokine production are not the only hallmarks of splenic dendritic cell activation. Previous studies have documented the rapid migration of dendritic cells into the T cell area of the white pulp following injection of microbial adjuvants such as LPS or soluble tachyzoite Ag (38, 39), microbial ligands for TLR4 and TLR11 (11, 40). Presumably, such rapid reorganization of splenic dendritic cells increases the likelihood of APCs encountering pathogen-specific T cells and therefore initiating an adaptive response to blood-borne infection. Whether flagellin induces the migration of splenic dendritic cells has not previously been examined.

*Salmonella* express flagellin when replicating in vitro, but reduce expression of this protein during intracellular growth in the mammalian host (41, 42). Such coordinated flagellin regulation has led to the idea that induction of innate inflammation by flagellin expression might be deleterious to *Salmonella* survival in vivo, although this has not been tested directly. *Salmonella* lacking flagellin expression were initially reported to have reduced virulence (43, 44), but this finding was later refuted (45, 46). The most recent data indicate that nonflagellated bacteria have reduced virulence in cell culture experiments, but are virulent, or only have slightly reduced virulence in vivo (47, 48). However, the effect of *Salmonella* flagellin overexpression has not been examined.

In this study, we examined splenic dendritic cell activation in response to flagellin administration. We demonstrate that injection of purified flagellin induces rapid production of IL-6, increased expression of activation markers, and dendritic cell redistribution in the spleen. CD11b⁺ dendritic cells were more sensitive to the effect of flagellin injection than CD8α⁺ or plasmacytoid dendritic cells. The effects of flagellin on splenic dendritic cells were not due to direct activation, but instead are mediated by flagellin activation of another bone marrow-derived cell and required MyD88 expression. Furthermore, we demonstrate that overexpression of flagellin in *Salmonella* severely limits bacterial growth in vivo. These data therefore demonstrate the importance of bacterial flagellin regulation for avoiding host innate immune activation in vivo.

Materials and Methods

Mouse strains

C57BL/6 mice were purchased from National Cancer Institute and were used at 8–16 wk of age. MyD88-deficient mice were a gift from L. Francois (University of Connecticut Health Center, Farmington, CT). All mice were housed in specific pathogen-free conditions and cared for in accordance with Research Animal Resources practices at the University of Minnesota and University of Connecticut Health Center.

Bacterial strains

Flagellar phase-fixed *Salmonella enterica* serovar typhimurium BC116 (49) was a gift from B. Cookson (University of Washington, Seattle, WA). *S. typhimurium* X7400 has a galE deletion and cannot incorporate galactose into the O-Ag or outer core. It is thus a type of deep rough LPS-deficient mutant and was provided by R. Curtiss (Arizona State University, Tempe, AZ). *Escherichia coli* strain F18 was a gift from A. Gewirtz (Emory University, Atlanta, GA). BRD509 AroA⁻ D⁻ (50) was provided by D. Xu (University of Glasgow, Glasgow, U.K.). BRD509-Flag was constructed by inserting the *Salmonella* flagellin (FlIC) gene into plasmid pTrc, electroporating into *Salmonella*, and selecting with 100 µg/ml ampicillin (Sigma-Aldrich). BRD509-ErRFP was constructed in our laboratory by introducing a previously described plasmid construct into BRD509 (51).

Flagellin purification

Flagellin was purified from *S. typhimurium* (BC116 or X7400) or *E. coli* using a modified acid-shock protocol (52). Bacteria were grown overnight at 37°C without shaking, washed, and resuspended in PBS/HCl (pH 2) for 30 min at room temperature. Supernatants were collected and flagellin was harvested by ultracentrifugation and ammonium sulfate precipitation. Silver-stained SDS-PAGE gels and antiflagellin Western blots were used to confirm the purity and identity of flagellin preparations. Residual LPS was removed by serial passage through detoxigel columns (Pierce Biotechnology). To confirm LPS removal, an aliquot from each batch of flagellin was digested with protease K, and 30–50 µg of undigested and digested flagellin was injected i.v. into C57BL/6 mice. The expression of CD80 and CD86 was determined on splenic dendritic cells 6 h later, and only batches that failed to induce any dendritic cell activation after digestion were used in this study. This in vivo assay was found to be more sensitive and reliable than the in vitro Limulus assay, as previously reported (31).

Production of bacterial flagellin in S2 cells

Flagellin was cloned from *E. coli* by PCR and inserted into plasmid pM7/BipV5, and *Drosophila* S2 cells were cotransfected with a hygromycin selection plasmid using calcium phosphate. Transfected S2 cells were selected using 200 µg/ml hygromycin B in complete Schneider’s *Drosophila* medium, and individual colonies were picked and expanded for several weeks. Individual lines were grown without serum, and flagellin expression was induced by adding 500 µM copper sulfate. Western blots using Abs against the V5 epitope and flagellin were used to confirm flagellin production in the supernatant. Flagellin was purified from insect cell supernatants using the 6-HIS tag and Ni columns before being concentrated and stored at −80°C.

Flagellin injections and *Salmonella* infection

Mice were injected with different doses of purified *Salmonella* flagellin, purified *E. coli* flagellin, insect cell flagellin, or ultrapure LPS (Alexis). At various times later, mice were sacrificed to obtain serum and spleens for dendritic cell isolation or histology. For infection, *S. typhimurium* AroA⁺ D⁻ (BRD509) or BRD509-Flag was grown overnight in LB broth without shaking and diluted in PBS following estimation of bacterial concentration using a spectrophotometer. Mice were infected i.v. in the lateral tail vein with 5 × 10^6 bacteria and monitored for signs of infection. In all experiments, the actual bacterial dose administered was confirmed by plating serial dilutions onto MacConkey agar plates. To determine bacterial colonization in vivo, spleens from infected mice were homogenized in PBS and serial dilutions were plated onto MacConkey agar plates. After overnight incubation at 37°C, bacterial plates were counted and bacterial burdens were calculated for each individual organ.

Splenic dendritic cell isolation

Spleens were harvested from mice and sequentially incubated with collagenase D (37°C for 20 min) and EDTA to liberate dendritic cells. CD11c⁺ cells were harvested by positive selection using magnetic anti-CD11c microbeads and multiple passes through selection columns (Miltenyi Biotec). Cells eluted from the columns were typically 85–95% pure CD11c⁺ cells. In some experiments, these dendritic cells were washed and placed in culture with various concentrations of bacterial flagellins overnight, and supernatants and cells were collected 24 and 48 h later for analysis. In other experiments, dendritic cells were immediately surface stained and examined by flow cytometry.

Cytokine measurement

IL-6, IL-1α, TNF-α, and IL-12p70 were measured in serum and supernatants by sandwich ELISA following the manufacturer’s recommendations.

Flow cytometric analysis

Purified dendritic cells or spleen cells were incubated for 20–45 min at 4°C in Fc block (spent culture supernatant from the 24G2 hybridoma, 2% rat serum, 2% mouse serum, and 0.01% sodium azide) in the presence of primary Abs: FITC-, PE-, CyChrome-, PE-Cy5-, or allophycocyanin-conjugated Abs specific for CD4, CD8α, CD11b, CD11c, CD40, CD80, CD86, and isotype control Abs were purchased from eBioscience and BD Biosciences. After staining, cells were fixed and analyzed by flow cytometry using a FACS Canto (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).
Bacterial flagellin induces serum IL-6 and requires bone marrow cell expression of MyD88. C57BL/6 mice were injected i.v. with 5 μg of purified Salmonella flagellin (A) or recombinant E. coli flagellin (B) produced in insect cells. At various time points later, mice were sacrificed and serum was collected. C. Wt, Myd88-deficient bone marrow chimeras and C57BL/6 mice were injected i.v. with 5 μg of purified Salmonella flagellin, and serum was collected 1 h later. IL-6 in serum samples was determined by sandwich ELISA. Each data point shows mean ± SD for three mice per group, and each graph is representative of two separate experiments.

Bone marrow chimeras

Femurs and tibias were harvested from wild-type (Wt) or MyD88-deficient C57BL/6 mice, bone marrow was recovered, and a single-cell suspension was generated. RBC were lysed, and the remaining cells were suspended in HBSS supplemented with HEPES, L-glutamine, Pen/Strep, and gentamicin. Mature T cells were removed by incubation with anti-CD90 ascites fluid (T24), followed by Low-Tox-M rabbit complement (Cedarlane Laboratories) for 45 min at 37°C. Recipient C57BL/6 mice were irradiated (1000 rad) and bone marrow cells were transferred by i.v. injection (2 × 10^6 to 5 × 10^6/mouse). Mice were rested for 8 wk before use in experimental protocols.

Immunohistology

Whole spleens were harvested and embedded in OCT (Sakura Finetek) before snap freezing in liquid nitrogen. Sections (10 μm) were cut onto slides, dehydrated in acetone, and stored at −80°C. Slides were thawed and incubated with 3% H_2O_2, Fc block, and avidin/biotin block (Vector Laboratories), before staining with Abs specific for CD11c and B220. Abs were detected using Vectastain ABC Elite (Vector Laboratories) and developed with diaminobenzidine or Vector SG (Vector Laboratories). Tissues were counterstained with 5% methyl green and mounted before images were collected using standard bright-field microscopy.

Results

Injection of mice with Salmonella flagellin induces the production of inflammatory cytokines (13, 23, 25, 26). We injected purified S. typhimurium flagellin into C57BL/6 mice and collected serum at various time points. Intravenous flagellin induced production of serum IL-6 and low levels of TNF-α within 1 h, although this disappeared from circulation by 4 h postinjection (Fig. 1A and data not shown). Serum IL-1α or IL-12 p70 was not detected at any time point following flagellin injection (data not shown). As with any biologically active microbial product, it was possible that bacterial contamination contributed to this effect. We therefore produced E. coli flagellin in S2 insect cells and compared the activity of this eukaryotic product with purified Salmonella flagellin. Flagellin produced in insect cells induced similar levels of serum IL-6, and with identical kinetics, compared with purified flagellin (Fig. 1B). Although bone marrow-derived cells are thought to be important mediators of innate responses, stromal cells can also respond to TLR ligands (53). We therefore examined whether signaling through MyD88 by bone marrow-derived cells was required for flagellin-induced IL-6. Wt bone marrow chimeras produced IL-6 in response to flagellin injection (Fig. 1C). In contrast, flagellin-induced serum IL-6 production was ablated in MyD88-deficient bone marrow chimeras (Fig. 1C). Therefore, bone marrow-derived cells respond to bacterial flagellin in a MyD88-dependent manner, and rapidly produce serum IL-6.

Next, we examined the activation of splenic dendritic cells following injection of Salmonella flagellin. Flagellin induced a substantial increase in the surface expression of CD86, and a smaller
increase in CD80 and CD40, on CD11b⁺ dendritic cells (Fig. 2, Flagellin). This activity was lost if flagellin was pretreated with proteinase K (Fig. 2, PK-Flagellin). Flagellin had only a modest effect on CD8α⁺ dendritic cells, causing a small increase in the expression of CD40, CD80, and CD86 (Fig. 2, Flagellin). In contrast, Salmonella LPS induced substantial increases in CD80, CD86, and CD40 expression on both CD11b⁺ and CD8α⁺ dendritic cells (Fig. 2, LPS). This difference in the activation of CD11b⁺ and CD8α⁺ dendritic cell subsets by flagellin was detected at several different doses of flagellin. Examination of various doses of flagellin demonstrated consistent activation of CD11b⁺ dendritic cells, but significantly reduced activity on CD8α⁺ dendritic cells, across a 100-fold range (Fig. 3). In contrast, LPS had similar effects on both subsets (Fig. 3). Flagellin also caused a small dose-dependent increase in the expression of CD40 and CD86 by plasmacytoid dendritic cells (data not shown).

Thus, plasmacytoid dendritic cells also respond to flagellin injection by increasing expression of surface activation markers, although the magnitude of this response was considerably lower than CD11b⁺ dendritic cells. Together these data demonstrate that bacterial flagellin can activate splenic dendritic cells in vivo, but has a more pronounced effect on CD11b⁺ dendritic cells.

Next, we examined the importance of MyD88 expression by bone marrow-derived cells for dendritic cell responsiveness to flagellin. CD11b⁺ and CD8α⁺ dendritic cells in Wt > Wt bone marrow chimeras increased expression of CD80, CD86, and CD40 in response to flagellin injection (Fig. 4). However, this response was reduced in MyD88-deficient > Wt bone marrow chimeras, although increased expression of CD80, CD86, and CD40 on dendritic cells was still observed (Fig. 4). These data suggest that flagellin responsiveness by stromal cells may contribute to splenic dendritic cell activation in response to flagellin.

It has been reported that murine splenic dendritic cells respond directly to bacterial flagellin, producing inflammatory cytokines and increasing expression of CD80 and CD86 (32). In contrast, other reports claim that murine splenic dendritic cells do not respond directly to bacterial flagellin (35, 36). We purified splenic CD11c⁺ dendritic cells and examined the response to purified Salmonella and E. coli flagellin in vitro. As previously reported (36), CD11c⁺ splenic dendritic cells did not increase surface expression of CD80, CD86, or CD40, and did not secrete IL-6, TNF-α, or IL-12 p70 in response to either Salmonella or E. coli flagellin, despite the fact that these preparations were clearly biologically active when injected in vivo (data not shown). These data suggest that dendritic cells are activated by flagellin in a bystander fashion rather than by direct activation.

We also examined the distribution of dendritic cells in the spleen of mice injected with flagellin. In naive C57BL/6 mice, CD11c⁺ dendritic cells were found scattered throughout the red pulp, the T cell area of the white pulp, and the marginal zone (Fig. 5A). Six hours after flagellin injection, CD11c⁺ dendritic cells were found exclusively within the T cell area of the white pulp and were strikingly absent from the red pulp or marginal zone (Fig. 5B). However, in MyD88-deficient bone marrow chimeras, CD11c⁺ dendritic cells did not undergo significant redistribution in response to flagellin injection (Fig. 5, C and D). Thus, injection of bacterial flagellin induces the redistribution of splenic dendritic cells to the T cell area of the white pulp in a MyD88-dependent manner.

Although Salmonella express flagella in culture, they markedly decrease expression of flagellin during intracellular growth (41, 42). Our data demonstrate that flagellin is an effective activator of
dendritic cells, suggesting that flagellin regulation by bacteria may be orchestrated to avoid innate immune activation. If this is correct, flagellin overexpression should be detrimental to Salmonella survival in vivo, but this has not been examined directly. We over-

expressed Salmonella flagellin in BRD509, a flagellated, attenuated strain of S. typhimurium, effectively removing the ability of bacteria to modulate flagellin expression in vivo. Despite the fact that both these strains grow well in vitro, BRD509-Flag were absent from the spleen 3 or 10 days after infection, whereas increasing numbers of BRD509 were detected over this period (Fig. 6).

Furthermore, the splenomegaly that normally accompanies Salmonella infection was significantly reduced in mice infected with BRD509-Flag. The number of splenocytes in mice infected with BRD509-Flag and the proportions of various cell populations were similar to uninfected mice (Fig. 7). In contrast, mice infected with BRD509 displayed a considerable increase in spleen cells, an increase in the proportion of CD11b+ and Gr1+ cells, and a corresponding decrease in the proportion of B and T lymphocytes (Fig.

**FIGURE 5.** Bacterial flagellin injection induces the redistribution of splenic dendritic cells. (A, B) C57BL/6 mice or (C, D) MyD88-deficient bone marrow chimeras were (B, D) injected i.v. with 5 μg of purified Salmonella flagellin, and 6 h later spleens were harvested and tissue sections were stained to detect CD11c (brown) and B220 (blue). Images are representative of several other sections from multiple mice and experiments.

**FIGURE 6.** Salmonella-overexpressing flagellin is highly attenuated in vivo. A, C57BL/6 mice were infected i.v. with 5 × 10^8 BRD509 or BRD509-Flag, and spleens were harvested 3 and 10 days later. Bacterial colonization was determined by plating serial dilutions of spleen aliquots on McConkey agar and counting Salmonella colonies the following day. Data are representative of two individual experiments. B, C57BL/6 mice were infected i.v. with 5 × 10^8 BRD509-Flag or BRD509-EaeRFP, and spleens were harvested 1, 2, and 12 days later. Bacterial colonization and in vivo plasmid stability were determined by plating serial dilutions of spleen aliquots on McConkey agar with or without ampicillin, and counting Salmonella colonies the following day.

**FIGURE 7.** Mice infected with Salmonella-overexpressing flagellin do not develop splenomegaly. C57BL/6 mice were infected i.v. with 5 × 10^8 BRD509 or BRD509-Flag; spleens were harvested 3 and 10 days later; and total cells were counted (A) or stained (B) using Abs specific for lymphocyte and macrophage populations. Data in B are from the day 10 time point and are representative of two individual experiments and four to five mice per group.
A finding consistent with differential expression of TLR5 by these regulation down-regulate the expression of flagellin during intracellular infection (7, 56, 57), this may indicate an adaptation of the innate immunity response to flagellin highly deleterious to the growth of Salmonella in vivo, and suggests an explanation for bacterial modulation of flagellin expression in vivo.

Disclosures
The authors have no financial conflict of interest.

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