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Bacterial Flagellin Is an Effective Adjuvant for CD4+ T Cells In Vivo

Stephen J. McSorley,2*† Benjamin D. Ehst,* Yimin Yu,‡ and Andrew T. Gewirtz‡

Flagellin is secreted by many enteric bacteria and, upon reaching the basolateral membrane of the intestinal epithelium, activates Toll-like receptor 5-mediated innate immune signaling pathways. We hypothesized that any flagellin that gets beyond the epithelium might also regulate cells of the adaptive immune system. Here we demonstrate that the clonal expansion of naive DO11.10 CD4 T cells in response to OVA peptide (323–339) was enhanced 3- to 10-fold in the presence of purified bacterial flagellin in vivo. OVA-specific CD4 T cells were also shown to have undergone more cell division in vivo if flagellin was coadministered with OVA. Flagellin administration increased the expression of B7-1 on splenic dendritic cells, and coinjection of CTLA4-Ig, which is known to block B7 function in vivo, completely ablated the adjuvant effect on CD4 T cells. Therefore, a conserved bacterial protein produced by many intestinal microbes can modulate CD4 T cell activation in vivo. Such an adjuvant effect for flagellin has important implications for vaccine development and the generation of CD4 T cell responses to enteric bacteria. The Journal of Immunology, 2002, 169: 3914 –3919.

The recognition of conserved microbial features by the innate immune system can regulate the induction of adaptive immune responses (1, 2). For example, dendritic cells (DC)3 respond to some microbial products by secreting pro-inflammatory cytokines and increasing the surface expression of co-stimulatory molecules and peptide/MHC complex (3–5). These activated DC have the wherewithal to cause naive CD4 T cell proliferation and effector cytokine production upon recognition of cognate Ag by the T cell (6, 7). Thus, a number of microbial products are thought to function as effective adjuvants due to effects on DC, which, in turn, can influence T cell activation.

Recent studies have demonstrated that microbe-induced DC maturation/activation can be initiated by ligation of cell surface receptors that detect soluble products of microbial metabolism, allowing the host to rapidly identify common classes of infectious agents (8). Specifically, the Toll-like receptors (TLRs) are a recently described family of molecules capable of sensing bacterial cell wall components, such as LPS (9, 10), lipoteichoic acids (11), and peptidoglycan (12, 13), as well as other microbial products, such as dsRNA (14) and CpG DNA (15). Although a number of cell types are thought to express some TLRs, immature and activated DC have been shown to express a wide variety (16, 17) and are also found in close physical contact with naive T cells in vivo (18). Therefore, DC are ideally suited to recognize microbial products and present foreign Ag to naive CD4 T cells.

Bacterial flagellin has long been studied as a useful model Ag (19, 20) and was recently found to be a target of CD4 T cells during murine Salmonella typhimurium infection (21–23). In addition to being a target of the adaptive immune system, bacterial flagellin can directly activate innate immune responses in monocytes (24–26) and epithelial cells (27, 28). Specifically, exposure to flagellin in vitro induces these cells to activate NF-κB and secrete inflammatory cytokines (27, 28). This immunostimulatory capacity was recently shown to be mediated by the mammalian surface receptor TLR-5 (29, 30) that is expressed by monocytes (16), immature DC (17), and epithelial cells (29).

The innate ability to induce an inflammatory response by TLR ligands also correlates with the capacity of these products to function as effective adjuvants. For example, LPS induces an inflammatory response in the host via TLR-4 and also increases the clonal expansion of CD4 T cells in vivo (9, 10, 31, 32). Additionally, CpG DNA induces an inflammatory response via TLR-9 and can function as an adjuvant in vivo (33–35). We therefore reasoned that bacterial flagellin might function in a similar manner. Here, we demonstrate that flagellin is an effective adjuvant for CD4 T cells responding to OVA in vivo. Since flagellin is ubiquitously expressed in the gut and can be transported across gut epithelia by some pathogens (27), it may also contribute to the activation of CD4 T cells in the intestine.

Materials and Methods

Flagellin preparation

Flagellin was purified from S. typhimurium (SL3201)-conditioned medium by anion/cation exchange chromatography, as previously described (27) with one additional step added. To remove potential remaining trace levels of LPS, the purified protein was incubated with polymyxin B agarose beads (1%, v/v; Sigma, St. Louis, MO) as previously described (36). SDS-PAGE analysis revealed no contaminating proteins accompanying the expected 49-50-kDa previously described flagellin doublet. To prepare recombinant flagellin from HeLa cells, the entire FliC-coding region was prepared by PCR from S. typhimurium (SL3201) genomic DNA using the following PCR primers: GATTTACCGGCACAAGTCTAATACAA and TCTA GATTACCGCAGTAAAGAGAGGACG. This PCR product was digested with EcoRI and XbaI and inserted into pcDNA4/HisMax (Invitrogen, San

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‡ Abbreviations used in this paper: DC, dendritic cell; RAG, recombinase-activating gene; TLR, Toll-like receptor; PMN, polymorphonuclear neutrophil.

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IL-8 induction

IL-8 secretion from polarized T84 cells was assessed as previously described (27). HUVEC were plated on passage 3 in 24-well tissue culture plates and stimulated with LPS or flagellin for 6 h, after which supernatants were collected and assayed for IL-8 by ELISA. Human polymorphonuclear neutrophil (PMN) were isolated from peripheral blood of healthy donors by dextran sedimentation and density gradient centrifugation, followed by hypotonic lysis. Immediately following isolation, PMN were plated in HBSS at 10^6/ml and stimulated with Pam3Cys (a gift from M. Fenton, Boston University, Boston, MA) or flagellin for 3 h, at which time supernatants were isolated and assayed for IL-8 by ELISA. The Limulus assay kit was purchased from Cape Cod Associates (Falmouth, MA), and tests were performed according to the manufacturer’s instructions. Buffers for the Limulus assay were reconstituted using the same double-deionized (via U.S. Filter, Bradley, IL and Millipore Systems) water used throughout these studies, which, when tested by Cape Cod Associates, was found to have an endotoxin concentration of <0.005 ng/ml.

Mice and adoptive transfer

DO11.10 and DO11.10 recombinase-activating gene (RAG)-deficient TCR transgenic mice (37) were bred in a pathogen-free facility according to National Institutes of Health guidelines and screened as previously described (38). Female BALB/c (H-2b)-mice were purchased from the National Cancer Institute (Frederick, MD) and used at 8–16 wk of age. BALB/c recipient mice were adoptively transferred with 2.5 × 10^7 cells as previously described (38).

Immunization

Mice were immunized i.v. with 100 µg OVA peptide 323–339 in the presence and the absence of flagellin (10 µg) or LPS (25 µg). In some experiments aliquots of flagellin or PBS were incubated with proteinase K (100 µg/ml; Roche, Indianapolis, IN) at 37°C for 2–4 h, followed by 1 h at 70°C to denature the enzyme. Proteinase K-treated samples were mixed with OVA peptide after denaturation and immediately before i.v. injection. For in vitro restimulation, splenocytes were harvested from mice 9 days after immunization and plated in duplicate in 96-well flat-bottom plates (Costar, Corning, NY) at a final concentration of 1 × 10^6 cells/well. Cultures were incubated for 48 h in the presence or the absence of OVA peptide (323–339) and analyzed for the presence of IFN-γ and IL-4. The presence of cytokines in culture medium was measured by sandwich ELISA based on noncompeting pairs of anti-IFN-γ or anti-IL-4 mAb (BD PharMingen, San Diego, CA) according to a standard protocol, and amounts were calculated based on a standard curve generated by recombinant mouse IFN-γ or IL-4 (BD Pharamingen). For in vivo blocking experiments, CTLA-4-Ig was prepared as previously described (32), and mice were injected i.p. with 280 µg 4 h before Ag injection.

Isolation of APC for DC analysis

APC from spleens were isolated as previously described (40). Briefly, organs were subjected to mild digestion with collagenase D (Roche) at 37°C for 25 min. Low density cells were recovered by centrifugation on a 35% BSA gradient (Sigma) and then directly stained on ice.

Flow cytometry

Cell suspensions were prepared from the spleen of immunized and control mice and incubated on ice with CyChrome-conjugated anti-CD4 (BD PharMingen) and biotinylated KJ1-26 mAb (41), followed by streptavidin-PE (Caltag, South San Francisco, CA) as previously described (38).

Results

Before considering flagellin’s ability to act as an adjuvant in vivo, we sought to define the purity of our flagellin in vitro. Several lines of evidence indicate that purified bacterial flagellin can activate pro-inflammatory gene expression independently of LPS (27, 28). These include the fact that the epithelial cells used in these studies exhibited no detectable response to a broad range of LPS concentrations and that flagellin’s pro-inflammatory activity in the same cells was ablated by prior treatment with proteinase K. However, it was still possible that LPS or another bacterial product could be bound to flagellin and contribute to the pro-inflammatory activity of flagellin itself. This idea was tested experimentally by purifying flagellin from eukaryotic cells (HeLa) transfected with a plasmid encoding Salmonella flagellin (Fig. 1). This flagellin, from a eukaryotic source, induced epithelial cell secretion of IL-8 with equivalent or slightly increased potency compared with flagellin purified from S. typhimurium or Escherichia coli (Fig. 1). Therefore, the primary protein sequence of flagellin, in the absence of other bacterial products, is sufficient to induce a pro-inflammatory response in vitro. The low yield of flagellin from transfected HeLa cells precluded it from being used as an adjuvant in vivo. However, we used HeLa-produced flagellin to help quantitate potentially contaminating bacterial products in flagellin isolated from bacteria.

First, we sought to quantitate levels of LPS, since this product is known to act as an adjuvant in vivo (31, 32). The most widely used test to quantify LPS is the Limulus test, which measures the ability to activate an innate immune proteolytic cascade in crab amebocyte lysates. However, synthetic lipopeptide has also been shown to have activity in this assay (42), suggesting that crab cell lysates respond to bacterial products other than LPS. Consistent with this idea, we observed that flagellin, whether purified from bacteria or HeLa cells, had detectable activity in the Limulus assay (~1/50th that of LPS mass/volume; data not shown). As this assay does not appear able to adequately discriminate between LPS and flagellin, LPS contamination was also measured by an alternative method. HUVEC cells secreted IL-8 in response to as little as 50 pg/ml E. coli LPS, and treatment with proteinase K had no effect on its ability to induce this response (data not shown). Bacterial flagellin induced modest amounts of IL-8 secretion from HUVEC.
cells, but bacterial flagellin pretreated with proteinase K did not induce detectable IL-8 secretion at any concentration tested, the highest being 50 μg/ml (data not shown).

We next used a similar strategy to quantify the amount of lipopeptide present in our purified flagellin. Human PMN produced detectable levels of IL-8 in response to as little as 50 ng/ml of the synthetic TLR2 agonist Pam3Cys, but no response to any tested concentration of flagellin (up to 50 μg/ml), indicating that concentrations of TLR2 ligands copurified with flagellin were not significant. From these data we estimate that the 10 μg flagellin used in our in vivo studies contains <10 ng of both LPS and lipoprotein contaminants. These results combined with the failure of bacterial flagellin to activate NF-κB in HeLa cells expressing all known TLRs except TLR5 (29) strongly suggest that the in vivo bioactivity of flagellin is the result of a response to flagellin itself rather than any contaminant.

The ability of flagellin to function as an adjuvant in vivo was tested by immunizing BALB/c mice with OVA peptide (323–339) in the presence or the absence of flagellin. Splenocytes from mice immunized with OVA peptide plus flagellin produced IFN-γ upon in vitro restimulation with peptide, while splenocytes from mice immunized with OVA peptide alone or OVA peptide plus proteinase K-treated flagellin did not secrete detectable IFN-γ (Fig. 2). To examine this adjuvant effect in more detail, we tracked the in vivo response to OVA using a well-characterized adoptive transfer system (38). A trace population of OVA-specific CD4 T cells was detected in the spleen of BALB/c mice following adoptive transfer (Fig. 3A), and clonal expansion of these cells was observed, 3 days after i.v. injection of OVA peptide (Fig. 3B). Coinjection of Salmonella flagellin with OVA peptide markedly increased the clonal expansion of OVA-specific T cells compared with that of OVA peptide alone (Fig. 3C). This adjuvant effect usually accounted for a 3- to 10-fold increase in the absolute number of splenic DO11.10 cells in different experiments (data not shown). Pretreatment of flagellin with proteinase K completely ablated the flagellin-mediated enhancement of clonal expansion (Fig. 3D), consistent with the fact that proteinaceous material, including flagellin, is digested by this treatment. Proteinase K treatment itself did not affect T cell expansion, as mock (PBS) samples treated with proteinase K did not affect the response of DO11.10 T cells to OVA peptide (data not shown).

To characterize the adjuvant function of flagellin more closely we examined the kinetics of the CD4 T cell response to OVA in the presence or the absence of flagellin over a period of 15 days postimmunization. Injection of OVA peptide alone caused a transient increase in the percentage and absolute number of DO11.10 T cells, peaking on day 2 (Fig. 4). After day 2, the percentage and absolute number of DO11.10 T cells declined and eventually fell to levels below transfer only by day 15 as previously described (38). Coinjection of Salmonella flagellin increased the percentage of DO11.10 T cells found in the spleen as early as 2 days after immunization, although there was a more profound effect observed on DO11.10 T cells on day 3, the peak of clonal expansion (Fig. 4). Although the DO11.10 population also contracted between days 3 and 5 in mice coadministered flagellin, the percentage and absolute number of cells remained higher than that found in mice administered peptide alone (Fig. 4). Furthermore, the absolute number and percentage of DO11.10 T cells remained higher 15
days postimmunization in mice that had been coinjected with bacterial flagellin (141,300 ± 88,620 total KJ/spleen; 0.193 ± 0.085% KJ/spleen), compared with peptide alone (19,800 ± 11,677 total KJ/spleen; 0.027 ± 0.012% KJ/spleen).

The increased number of DO11.10 T cells found in the spleen suggested that DO11.10 cells might have proliferated more rapidly in the presence of bacterial flagellin, especially as this difference was observed very early in the response. Alternatively, it was possible that the adjuvant effect of flagellin could be explained by an inhibitory effect on the rate of cell death among responding DO11.10 T cells (43). To distinguish between these possibilities, we stained DO11.10 T cells with CFSE before adoptive transfer and examined the loss of this dye following immunization. DO11.10 T cells clearly divided in response to OVA peptide alone, and there was considerable loss of fluorescence intensity on days 2–5 compared with that in cells from transfer-only mice (Fig. 5). However, DO11.10 T cells from the spleens of mice immunized with OVA peptide plus flagellin had undergone at least one more cell division on every day analyzed (Fig. 5), indicating an increased rate of cell division in vivo.

Adjuvants are thought to influence CD4 T cell activation at least in part via the induction of B7 molecules on DC in vivo (7, 44). In agreement with this, in vivo administration of CTLA-4-Ig, that specifically blocks B7 function can reduce or totally ablate the adjuvant effect of LPS on CD4 T cells responding to OVA (32). We therefore examined the expression of B7-1 and B7-2 on splenic DC after injection of flagellin. One day after i.v. injection of flagellin, a small increase in B7-1 expression was noted on splenic CD11c-positive cells (Fig. 6A), while no significant increase in B7-2 was observed at this time (data not shown). This up-regulation of B7-1 by flagellin injection was also inhibited by pretreatment of flagellin with proteinase K (Fig. 6B), indicating that flagellin and not another bacterial contaminant was responsible for this effect. However, it was not clear from this ex vivo staining that this level of B7 induction by flagellin was sufficient to account for an adjuvant effect on OVA-specific CD4 T cells. Therefore, we directly examined the contribution of B7 to clonal expansion in the presence of flagellin by in vivo blocking with CTLA-4-Ig. Indeed, administration of CTLA-4-Ig before immunization with OVA peptide plus flagellin removed the adjuvant effect on responding DO11.10 T cells (Fig. 6C). The magnitude of this blocking effect was similar to the effect of CTLA-4-Ig on the adjuvant effect of LPS (Fig. 6C).

FIGURE 5. Flagellin increases cell division in responding DO11.10 CD4 T cells. BALB/c mice were adoptively transferred with 2.5 × 10^6 CFSE-labeled DO11.10 TCR transgenic CD4 T cells and immunized 1 day later. Groups of mice were either unimmunized (Transfer Only) or immunized i.v. with PBS (A and B, E), 10 μg Salmonella flagellin (A, dotted line), or 10 μg proteinase K-treated flagellin (B). APC from spleens were isolated, and B7-1 staining was analyzed on DC after gating on CD11c-positive cells. C, BALB/c mice were adoptively transferred with 2.5 × 10^6 RAG-deficient DO11.10 TCR transgenic CD4 T cells and immunized 1 day later. %KJ and total KJ refer to OVA-specific cells stained with the KJ1-26 Ab. Groups of mice were either unimmunized (Transfer Only) or immunized i.v. with 100 μg OVA323–339 (OVA) alone, 100 μg OVA323–339 plus 25 μg LPS (OVA/LPS), or 100 μg OVA323–339 plus 10 μg Salmonella flagellin (OVA/flagellin). Some groups of mice were injected i.p. with CTLA-4-Ig 4 h before immunization (+CTLA-4-Ig). The mean absolute number of DO11.10 CD4 T cells in the spleen ± SD is shown for each group 3 days postimmunization.

FIGURE 6. Flagellin increases B7-1 expression on splenic DC and enhances clonal expansion of DO11.10 CD4 T cells through B7. BALB/c mice were injected i.v. with PBS (A and B, E), 10 μg Salmonella flagellin (A, dotted line), or 10 μg Salmonella flagellin (B). APC from spleens after injection of flagellin (C). APC from spleens were isolated, and B7-1 staining was analyzed on DC after gating on CD11c-positive cells. C, BALB/c mice were adoptively transferred with 2.5 × 10^6 RAG-deficient DO11.10 TCR transgenic CD4 T cells and immunized 1 day later. %KJ and total KJ refer to OVA-specific cells stained with the KJ1-26 Ab. Groups of mice were either unimmunized (Transfer Only) or immunized i.v. with 100 μg OVA323–339 (OVA) alone, 100 μg OVA323–339 plus 25 μg LPS (OVA/LPS), or 100 μg OVA323–339 plus 10 μg Salmonella flagellin (OVA/flagellin). Some groups of mice were injected i.p. with CTLA-4-Ig 4 h before immunization (+CTLA-4-Ig). The mean absolute number of DO11.10 CD4 T cells in the spleen ± SD is shown for each group 3 days postimmunization.
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