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Bacterial Flagellin Is an Effective Adjuvant for CD4$^+$ T Cells In Vivo[1]

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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 coinjected 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 costimulatory 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: GAATTCATGGCACAAGTCATTAATACAA and TCTAGATTAACGCAGTAAAGAGAGGACG. This PCR product was digested with *Eco*RI and *Xba* and inserted into pcDNA4/HisMax (Invitrogen, San

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

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Diego, CA) using corresponding vector sites. Following sequencing to verify the integrity of the plasmid, HeLa cells were transiently transfected using Lipofectamine (Invitrogen), and cell lysate was collected 24 h later. Recombinant flagellin was purified according to the protocol of the Probond Purification System (Invitrogen). Briefly, lysate was cleared by centrifugation, added to the Probond column, and rocked for 30 min. After washing the column, His-tagged flagellin was eluted using 350 mM imidazole, concentrated using Centricon concentrators (Millipore, Bedford, MA), dialyzed overnight in sterile PBS using a 10,000 m.w. Slide-A-Lyzer (Pierce, Rockford, IL), and stored at −80°C until use. As the yield of flagellin was too low for detection by protein assays, it was quantified by Western blot/densitometry with a mAb to flagellin (Igen, Gaithersburg, MD), using flagellin purified from bacteria as a standard.

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 placed in HBSS at 10^6/ml and stimulated with Pam_3Cys (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-2^d) 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 2.5×10^6 CFSE (Molecular Probes, Eugene, OR)-labeled (39) DO11.10 TCR or DO11.10 RAG-deficient transgenic T cells i.v. 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 (Corning, Coring, 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 PharMingen). 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). For DC analysis, cells were incubated on ice with CyChrome-conjugated anti-CD8α, FITC-conjugated anti-CD11c, and PE-conjugated anti-CD80 and anti-CD86 (BD PharMingen). A FACScan flow cytometer (BD Biosciences, Mountain View, CA) was used to collect 25,000 events after creating an appropriate live gate. FACS data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

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 (FliC). 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 quantitate 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 affect on its ability to induce this response (data not shown). Bacterial flagellin induced modest amounts of IL-8 secretion from HUVEC

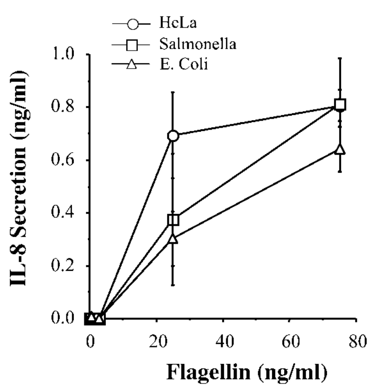

FIGURE 1. The pro-inflammatory effect of flagellin does not require associated bacterial products or bacteria-specific post-translational modification. Flagellin was purified from *Salmonella*, *E. coli* expressing *Salmonella* flagellin, or FliC-transfected HeLa cells, as described in *Materials and Methods*, and added at indicated concentration basolaterally to polarized T84 model epithelia. IL-8 was assayed in culture supernatants 5 h later by ELISA. Data show the mean ± SEM of three parallel experiments.

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 quantitate 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 Pam_3Cys, 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. 3*A*), and clonal expansion of these cells was observed, 3 days after i.v. injection of OVA peptide (Fig. 3*B*). 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. 3*C*). 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. 3*D*), 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 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

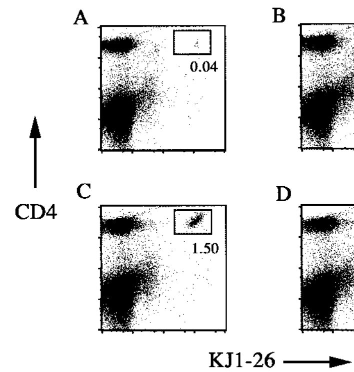

FIGURE 3. Flagellin increases the clonal expansion of naive DO11.10 CD4 T cells in vivo. BALB/c mice were adoptively transferred with 2.5 × 10^6 RAG-deficient DO11.10 TCR transgenic CD4 T cells and immunized 1 day later. KJ-126 staining was used to identify OVA-specific T cells. Groups of mice were either unimmunized (*A*) or immunized i.v. with 100 μg $OVA_{323-339}$ (*B*), 100 μg OVA($_{323-339}$) plus 10 μg *Salmonella* flagellin (*C*), or 100 μg $OVA_{323-339}$ plus 10 μg proteinase K-treated *Salmonella* flagellin (*D*). The percentage of DO11.10 CD4 T cells in the spleen 3 days later is indicated below the relevant gate. Data are representative of three individual experiments.

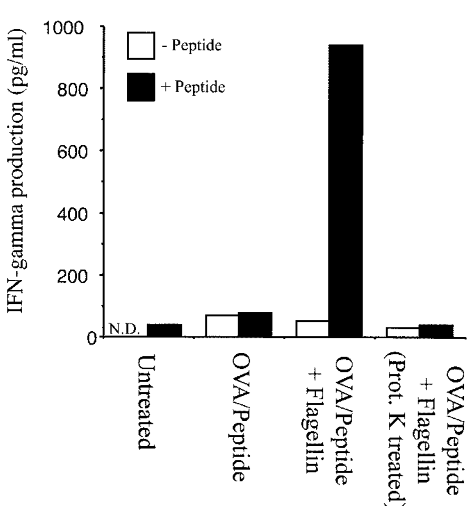

FIGURE 2. Flagellin increases IFN-γ secretion from OVA-specific CD4 T cells. BALB/c splenocytes from mice that were untreated or had received OVA peptide alone, OVA peptide plus flagellin, or OVA peptide plus proteinase K-treated flagellin 9 days previously were restimulated in vitro with OVA peptide in 96-well plates. After a 48-h culture period, supernatants were analyzed for the presence of IFN-γ by sandwich ELISA.

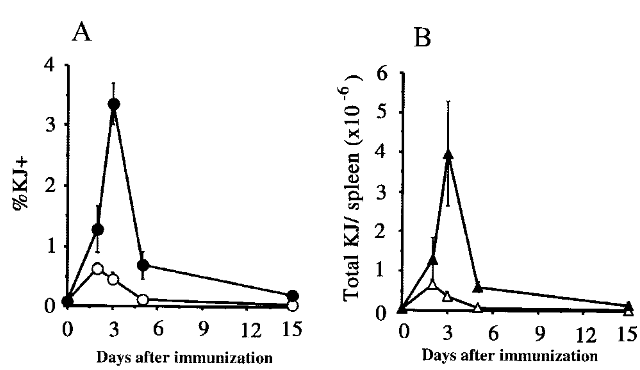

FIGURE 4. Kinetics of flagellin adjuvant effect. Groups of transferred mice were immunized i.v. with 100 μg $OVA_{323-339}$ (○) or 100 μg $OVA_{323-339}$ plus 10 μg *Salmonella* flagellin (●). %KJ and total KJ refer to OVA-specific cells stained with the KJ1-26 Ab. Data show the mean ± SD percentage and absolute number of DO11.10 T cells in the spleens of three mice per group at various times after immunization and are representative of two individual experiments.

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, as 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. 6*A*), 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. 6*B*), 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. 6*C*). The magnitude of this blocking effect was similar to the effect of CTLA-4-Ig on the adjuvant effect of LPS (Fig. 6*C*).

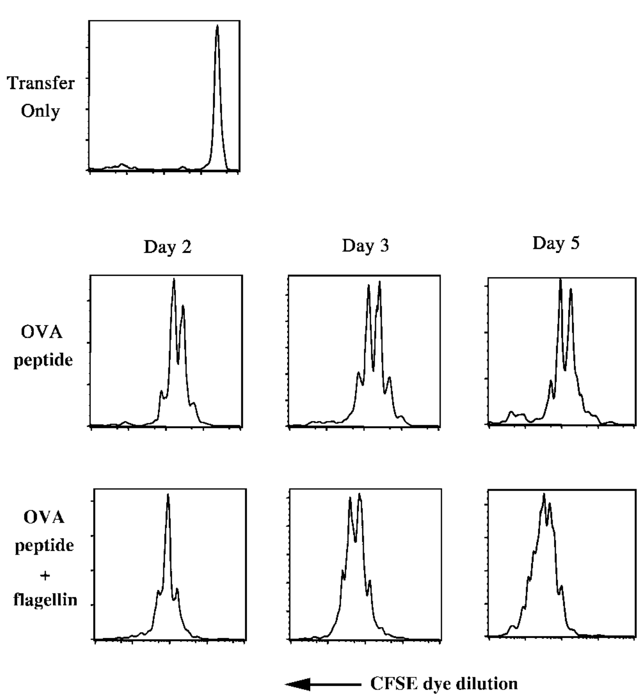

FIGURE 5. Flagellin increases cell division in responding DO11.10 CD4 T cells. BALB/c mice were adoptively transferred with 2.5×10^6 CFSE-labeled DO11.10 TCR transgenic CD4 T cells and immunized 1 day later. Groups of mice were either unimmunized (Transfer Only) or immunized i.v. with 100 μg $OVA_{323–339}$ (OVA peptide) or 100 μg $OVA_{323–339}$ plus 10 μg *Salmonella* flagellin (OVA peptide + flagellin). CFSE dye dilution is shown after gating on DO11.10 CD4 T cells in the spleen at days 2, 3, and 5 postimmunization. Data are representative of two individual experiments.

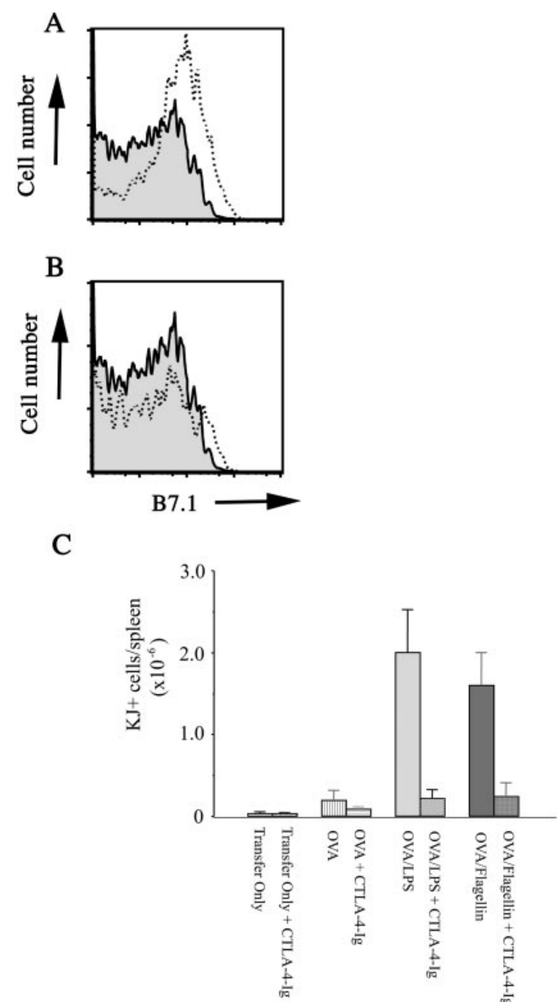

FIGURE 6. Flagellin increases B7-1 expression on splenic DC and enhances clonal expansion of DO11.10 CD4 T cells through B7. BALB/c mice were injected i.v. with PBS (*A* and *B*, ▨), 10 μg *Salmonella* flagellin (*A*, dotted line), or 10 μg proteinase K-treated flagellin (*B*). APC from spleens were 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 $OVA_{323–339}$, (OVA) alone, 100 μg $OVA_{323–339}$ plus 25 μg LPS (OVA/LPS), or 100 μg $OVA_{323–339}$ 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.

Discussion

Our data demonstrate that a microbial protein, bacterial flagellin, can function as an adjuvant for CD4 T cells in vivo when administered i.v. with Ag. We have not examined the adjuvant effect of flagellin on CD4 T cells when given by other routes. An examination of the role of flagellin on T cell responses to orally administered Ag may be particularly informative. Flagellin enhanced the clonal expansion of naive CD4 T cells in vivo and induced recall production of IFN-γ in vitro. The presence of flagellin during CD4 T cell priming resulted in increased Ag-specific T cell numbers at the peak of clonal expansion and after clonal contraction had occurred. Such an effect is likely to be advantageous for the host immune response to a flagellated pathogen, since this would result in a larger pool of pathogen-specific T cells. Although our results have not examined the immune response to enteric bacteria, it is likely that flagellin expression in the intestine could modulate the immune response to mucosal Ags.

It is likely that flagellin increases clonal expansion of T cells by influencing cell division and not cell death, since CFSE dye dilution experiments revealed more cell division by DO11.10 T cells in the presence of flagellin. However, we cannot rule out an additional effect of flagellin on the rate of T cell death in vivo. We think it likely that flagellin influences the rate of CD4 T cell division by increasing the expression of B7 molecules on DC in vivo. The enhanced clonal expansion of OVA-specific CD4 T cells in vivo correlated with the capacity of flagellin to induce B7-1 expression on DC in vivo and was also blocked by CTLA-4-Ig treatment. Although our blocking experiments do not specifically target the expression of B7 molecules by DC, it is likely that the block occurs during T cell interactions with this cell subset in vivo.

Although a number of TLR agonists function as adjuvants, flagellin might be a particularly attractive candidate for the development of synthetic adjuvants. In contrast to all other defined TLR agonists, flagellin is a protein, allowing it to be encoded within current nucleic acid based vaccine vectors (e.g., plasmid-carrying liposomes and viruses). In support of this idea, we demonstrate that recombinant flagellin made in eukaryotic cells (as opposed to bacteria) is equipotent at inducing TLR5-mediated responses compared with the bacterial product (Fig. 1). Recent studies suggest that the TLR5-activating region of flagellin is comprised of amino acids from various portions of this 47-kDa molecule (45) and will serve as important groundwork for developing such a strategy.

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