Gram-negative Flagellin-induced Self-tolerance Is Associated with a Block in Interleukin-1 Receptor-associated Kinase Release from Toll-like Receptor 5*

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Flagellin from a number of Gram-negative bacteria induces cytokine and nitric oxide production by inflammatory cell types. In view of the evidence that flagellin responsiveness is subject to modulation, we explored the possibilities that a prior exposure to flagellin might result in a state of reduced flagellin responsiveness or tolerance and that lipopolysaccharide (LPS) and flagellin may induce a state of cross-tolerance to each other. Our results demonstrate that a prior exposure to flagellin results in a subsequent state of flagellin tolerance in human monocytes, THP1 cells, Jurkat cells, and COS-1 cells. Tolerance occurs within 2 h after addition of flagellin and does not require protein synthesis. Flagellin did not induce tolerance to LPS in monocytes and THP1 cells; however, LPS treatment of monocytes and THP1 cells resulted in a state of flagellin cross-tolerance. Flagellin-induced self-tolerance is not the result of a decrease in the steady-state level of toll-like receptor 5 (TLR5) or interleukin-1 receptor associated kinase (IRAK), but it is associated with a block in the release of IRAK from the TLR5 complex in flagellin-tolerant cells. Release is essential for IRAK activity because the TLR5-associated IRAK lacks kinase activity. LPS-induced cross-tolerance to flagellin is also associated with a block in IRAK release from TLR5. These results provide evidence for a novel mechanism of TLR signaling control.

Evidence from a large number of studies demonstrates the importance of cytokine production in the protective response against Gram-negative pathogens. We (1, 2) and others (3) reported that flagella from Gram-negative bacteria such as Salmonella enteritidis and Pseudomonas aeruginosa induce cytokine production (e.g. TNF-α and IL-1) by human monocytes. Genetic evidence pointed to a role for flagellin, the major structural protein of the flagellum (1). Subsequently, we demonstrated that purified recombinant flagellin is an extraordinarily potent inducer of cytokine and nitric oxide production by monocytes (4, 5). Half-maximal stimulation of monocytes and THP1 cells was achieved with picomolar concentrations of flagellin. The proinflammatory action of flagellin has been confirmed by other investigators using Caco-2 cells and model epithelial systems (6–12).

Flagellin, like lipopolysaccharide (LPS) (13–16), signals via a toll-like receptor (TLR/IL-1 receptor-associated kinase (IRAK)-dependent pathway (5, 8, 17). LPS signals via TLR4, whereas flagellin utilizes TLR5 (8, 17). The activation of IRAK by flagellin is a relatively rapid process; maximal activation of IRAK activity in human and murine monocytes occurs within 5–10 min after the addition of flagellin to the cells (5).

In vitro studies with cultured monocytes and neutrophils as well as analysis of these cell types from patients with Gram-negative sepsis have established that prior exposure to LPS induces a transient state of cellular tolerance to subsequent stimulation by LPS (see e.g. Refs. 18–20). LPS tolerance is present within hours after an initial exposure to LPS (21–24). LPS tolerance may have evolved as a mechanism to limit the mediator storm that is induced by LPS and which is responsible for the pathologic events associated with LPS-induced septic shock. Because of the potential importance of LPS tolerance in the host response to Gram-negative infection, the underlying mechanism(s) that govern the induced state of LPS tolerance continues to be a subject of intense investigation. Although the mechanism for this effect has not been established definitively, evidence has been obtained and arguments made in favor of a role for a labile repressor (22), secretory leukocyte protease inhibitor (25), the down-regulation of TLR4 expression (26) and decreased IRAK expression (27). Investigators have also analyzed the potential for LPS, lipoarabinomannan (from mycobacteria) and lipopeptides (from mycoplasma) to induce a state of cross-tolerance to each other. The available evidence indicates that LPS can induce tolerance to several unrelated inducers including lipoarabinomannan (28) mycoplasma lipopeptides (29) and IL-1 (20), all of which utilize TLR-dependent signaling pathways.

In view of the evidence that flagellin responsiveness is subject to modulation (2) and is TLR5/IRAK-dependent (5, 8, 17), we explored the possibilities that a prior exposure to flagellin would result in a state of reduced flagellin responsiveness and that LPS and flagellin may induce a state of cross-tolerance to each other.

EXPERIMENTAL PROCEDURES

Cells and Reagents—THP1 cells were obtained from the American Type Tissue Culture Collection. Jurkat cells (a human T cell line) constitutively expressing the SV40 large T antigen were kindly provided by Dr. Amnon Altman, La Jolla Institute for Allergy and Immunology, San Diego. These cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum and 20 μg/ml gentamicin. COS-1...
Flagellin Tolerance

Monocytes or THP1 cells (2 × 10⁶) were incubated overnight in the absence (0) or presence of 10⁻⁷ m flagellin (F) or 100 ng/ml LPS (L). The cells were then washed and incubated in the absence or presence of flagellin or LPS for 4 h. The culture medium was then harvested, centrifuged to remove cells, and assayed for TNF-α content. The values represent the mean ± S.D. of triplicate samples.

| Incubation conditions (primary/secondary) | Peripheral blood monocytes | THP1 cells |
|------------------------------------------|---------------------------|-----------|
| 0/0                                      | 19,500 ± 1,500            | 15,900 ± 800 |
| 0/L                                      | 1,890 ± 40                | 5,500 ± 300 |
| O/F                                      | 212 ± 30                  | 200 ± 100 |
| L/L                                      | 97 ± 7                    | 12,100 ± 200 |
| F/P                                      | 15,300 ± 60               | 200 ± 100 |
| F/F                                      | 0                         | 0         |

TABLE I

Induction of tolerance by flagellin and LPS in human monocytes and THP1 cells

*RESULTS*

*Exposure of Cells to Flagellin Induces a Subsequent State of Flagellin Tolerance—* We began by determining whether a primary incubation of human monocytes or THP1 cells with flagellin would result in a subsequent state of flagellin tolerance. In addition, we evaluated the potential ability of flagellin and LPS to induce tolerance to each other. Human monocytes and THP1 cells were incubated with or without 10⁻⁷ m flagellin or 100 ng/ml LPS for ~18 h, washed, and then incubated for an additional 4 h in the presence or absence of flagellin or LPS. Culture supernatants were collected and assayed for TNF-α content. As shown in Table I, unstimulated human monocytes and THP1 cells (0/0) did not produce detectable levels of TNF-α.

As expected, both types of cell generated high levels of TNF-α in response to a single exposure to flagellin or LPS (0/F and 0/L, respectively). As expected, a primary exposure of cells to LPS resulted in a marked reduction in subsequent responsiveness to LPS (L/L). In addition, a primary exposure to LPS also dramatically reduced the response to flagellin (L/F). This result differs from our earlier observation that LPS-tolerant human monocytes retain some responsiveness to intact flagella (2). We believe that the difference in magnitude of the post-LPS response to purified flagellin versus intact flagella may be caused by the strength of signal provided by intact flagella as opposed to purified flagellin because intact flagella may possess the ability to cross-link a far greater number of receptors than flagellin. Like LPS, flagellin also induced a state of subsequent self-tolerance (F/P) in human monocytes and THP1 cells. However, flagellin did not markedly reduce the response to LPS (F/L). These findings raised the interesting possibility that flagellin and LPS may modulate TLR signaling potential by different mechanisms.

*IRAK Kinase Activity and Protein Levels during Flagellin Tolerance*—In view of these initial results with flagellin and the observation that LPS tolerance may be linked, at least in part, to a decrease in the level and activity of the IRAK (27), we analyzed the effect of a primary exposure to flagellin or LPS on the subsequent activation of IRAK in THP1 cells. THP1 cells were incubated overnight with or without flagellin or LPS, washed, and then incubated in the presence or absence of flagellin for 10 min or LPS for 30 min. As expected, flagellin (Fig. 1A, second lane, 0/F) (5) and LPS (third lane, 0/L) (27)
induced IRAK activation as measured by phosphorylation of myelin basic protein. Quantitative analysis of the kinase results are shown in Fig. 2 (black bars). In line with the TNF-α results presented in Table I, a prior exposure to flagellin almost completely inhibited the activation of IRAK (F/F) but had no effect on the response to LPS (F/L). As expected, a prior exposure to LPS blocked the subsequent response to flagellin (L/F).

In confirmation of the results of Li et al. (27), a prior exposure to LPS resulted in a marked decrease in the level of the IRAK protein (Fig. 1B, seventh lane, and Fig. 2, gray bars). Note that the lower prominent band in Fig. 1B is the nonphosphorylated form of IRAK, whereas the upper band is the phosphorylated form of the protein. In contrast to the results with LPS, a prior exposure to flagellin (fourth lane, F/F) did not result in a decrease in IRAK protein levels. However, the combination of overnight exposure to flagellin and a 15-min incubation with LPS did result in a decrease in nonphosphorylated IRAK (sixth lane, F/L). However, there was clearly sufficient IRAK in the cells to obtain a level of active IRAK which was similar to that observed with cells receiving only a single exposure to flagellin or LPS (Fig. 1A, second and third lanes). Because the block in flagellin-induced IRAK activation occurs without any evident change in IRAK protein expression, it seemed likely that the level of IRAK protein is not a contributing factor in the induction of flagellin tolerance. In addition, we concluded from these experiments that LPS and flagellin tolerance may be mediated, at least in part, by distinct mechanisms.

Flagellin Tolerance Is Not Restricted to Monocytes—To determine whether flagellin tolerance is restricted to cells of the monocytic lineage, we determined whether this condition could be induced in Jurkat cells, a human T cell line. Using an NF-κB-Luc reporter construct, we established that Jurkat cells are highly responsive to flagellin, with 50% of the maximal response being achieved with $7 \times 10^{-12}$ μg flagellin (data not shown). As with monocytic cells (5), flagellin induced a marked increase in IRAK enzyme activity in Jurkat cells (Fig. 1C, 0/F). However, overnight incubation of these cells with flagellin completely blocked the subsequent ability of to induce IRAK activation (fourth lane, F/F). In addition to Jurkat cells, we have found that flagellin tolerance can be induced in COS-1 and HEK293 cells (data not shown). Thus the ability of flagellin to induce a subsequent state of nonresponsiveness to itself is not restricted to cells of the monocytic lineage but rather appears to be a general property of TLR5-positive, flagellin-responsive cells.

Time Course for the Onset of Flagellin Tolerance—To determine the minimal time required for the onset of flagellin tolerance, we stimulated Jurkat cells with flagellin for 4, 12, 18, and 24 h prior to a second 15-min exposure to flagellin. After the second incubation, the level of IRAK activity was assessed. Control cells were incubated with flagellin for a single 15-min period before the measurement of IRAK activity. As shown in Fig. 3A, flagellin tolerance was maximal within 4 h after an initial exposure to flagellin and was sustained over the ensuing 24 h. In view of the relatively rapid onset of flagellin tolerance, we next determined whether protein synthesis was required. Jurkat cells were incubated in the presence or absence of 10 μg/ml cycloheximide for 15 min before a subsequent 2- or 4-h incubation in the presence of flagellin. The cells were then washed and incubated with flagellin for 15 min before the measurement of IRAK activity. Control cells were incubated with or without flagellin for a single 15-min period. As presented in Fig. 3B, cycloheximide treatment did not block the onset of flagellin tolerance. Furthermore, these results also demonstrate that flagellin tolerance occurs as early as 2 h after an initial exposure to the protein.

Lack of an Effect of High Level IRAK Expression on Flagellin Tolerance—Although the results presented in Fig. 1 are consistent with the notion that flagellin tolerance is not caused by a decline in IRAK expression, we tested this hypothesis by transiently transfecting Jurkat cells with the NF-κB-Luc reporter construct and an IRAK cDNA under the transcriptional control of a constitutively active cytomegalovirus promoter. Because the Jurkat cells express the SV40 large T antigen and the IRAK expression vector contains an SV40 origin of replication, we expected that the transfected cells would constitutively express very high levels of IRAK. If flagellin tolerance is not linked to the level of IRAK, then flagellin should induce self-tolerance even though the cells express a relatively high level of IRAK protein. A representative experiment is presented in Table II. Because flagellin has a relatively rapid effect on the expression of luciferase, there was a significant level of residual luciferase activity in cells incubated overnight in the presence of flagellin (F/0). However, a second incubation with flagellin did not result in an increase in luciferase activity (F/F). This was the case whether or not the cells were transfected with the IRAK-FLAG expression plasmid. The elevated level of NF-κB-dependent luciferase expression in unstimulated cells transfected with an IRAK expression vector has been observed by other investigators (30–33). Using Western blots, we determined that flagellin had no effect on the level of IRAK protein expression in the transfected cells (data not shown). Taken together, the results presented in Figs. 1 and 2 and Table II establish that flagellin tolerance does not involve an effect on IRAK expression.

Flagellin Tolerance and TLR5 Expression—In addition to a decrease in IRAK expression, LPS tolerance has also been associated with the down-regulation of TLR4 expression (26). Because flagellin tolerance might simply be the result of the inhibition of TLR5 expression, we analyzed the steady-state level of TLR5 in control and flagellin-tolerant cells using flow cytometry. Jurkat cells were incubated overnight in the presence or absence of $10^{-9}$ μg flagellin and then analyzed for surface expression of TLR5. The samples were incubated se-
Fig. 2. Quantitative analysis of IRAK activity and protein level during tolerance in THP1 cells. The data in this figure were derived from the quantification of the results in Fig. 1 using the Alpha Innotec Imaging System. F, flagellin; N, no addition.

A.

1° Flagellin: 2° Flagellin: MBP

B.

1° Flagellin: Cycloheximide: 2° Flagellin: MBP

Fig. 3. Time course for the induction of flagellin tolerance in Jurkat cells. A, Jurkat cells were incubated with flagellin for 4, 12, 18, or 24 h before a second 15-min incubation with flagellin. Control cells were incubated with or without flagellin for 15 min. All of the cultures were harvested after the second flagellin incubation and analyzed for IRAK activity. B, Jurkat cells were incubated in the presence or absence of 10 μg/ml cycloheximide for 15 min before the addition of flagellin for an additional 2 or 4 h. Control cells were incubated with or without flagellin for 15 min. All of the cultures were harvested after the second flagellin incubation and analyzed for IRAK activity.

TABLE II
Effect of constitutive IRAK expression on the induction of flagellin tolerance in Jurkat cells

Jurkat cells (5 × 10⁶) were transfected with pNF-xB-Luc and pRL-TK. One set of cells was also transfected with IRAK. The cells were incubated overnight in the absence (0) or presence of 10⁻³ m flagellin (F). The cells were then washed and incubated in the presence or absence of flagellin for 4 h. The cells were harvested and assayed for inducible luciferase as well as constitutive Renilla luciferase activity. The values have been normalized using the Renilla luciferase activity in each sample. The percent tolerance was calculated using the following formula: Percent tolerance = 100% B[(F/F) – 0/0] – [(F/0 – 0/0)/(F/F – 0/0)] × 100.

| IRAK transfection | Incubation conditions (primary/secondary) | Luciferase activity | Tolerance |
|-------------------|-----------------------------------------|---------------------|------------|
| No                | 0/0                                     | 40                  |            |
|                   | 0/F                                     | 3,150               |            |
|                   | F/0                                     | 1,205               |            |
|                   | F/F                                     | 1,250               | 99         |
| Yes               | 0/0                                     | 1,045               |            |
|                   | 0/F                                     | 6,365               |            |
|                   | F/0                                     | 3,485               |            |
|                   | F/F                                     | 3,805               | 94         |

sequentially with an affinity-purified goat antibody raised against a peptide within the extracellular domain of TLR5 and a fluorescein isothiocyanate-labeled rabbit anti-goat IgG and then analyzed using a FACS Calibur flow cytometer (Fig. 4). As is evident from the flow cytometry data, overnight incubation with flagellin did not result in a decrease in the steady-state level of surface TLR5. Identical results were obtained using fixed, permeabilized control and tolerant Jurkat cells and a rabbit antibody directed against a peptide in the cytoplasmic domain of TLR5 (data not shown).

TLR5/IRAK Association during Flagellin Tolerance—In view of the observations that flagellin tolerance does not involve changes in the level of TLR5 or IRAK, we evaluated the possibility that flagellin tolerance may develop because of a reduced ability of IRAK to associate with the TLR5 receptor complex. Previous work has demonstrated that the association of IRAK with the IL-1 receptor complex is dependent on stimulation of cells with IL-1 (34–36). Similarly, the association of IRAK with TLR2 appears to be dependent on cellular stimulation (37). We began by assessing TLR5-IRAK association in TLR5-positive, flagellin-responsive COS-1 cells that were transiently transfected with TLR5-FLAG and IRAK expression plasmids. The cells were transfected, rested for 24 h, and then incubated overnight in the presence or absence of flagellin. Subsequently, the cells were washed and then incubated with flagellin for 0–30 min. Cell lysates were prepared, and the TLR5-FLAG was immunoprecipitated with anti-FLAG antibody. After electrophoresis and transfer of proteins to PVDF membranes, the membranes were probed for the presence of IRAK using an anti-IRAK antibody. The blots were then stripped and reprobed for TLR5 using the anti-FLAG antibody. In all cases, the level of TLR5 was the same in each lane (data not shown). In marked contrast to the earlier studies with the IL-1 receptor or TLR2, IRAK was associated with TLR5 in unstimulated control cells (Fig. 5A). Stimulation of the cells with flagellin for 0.5–30 min did not dramatically change the level of associated IRAK. Similar results were obtained with flagellin-tolerant COS-1 cells. In all cases, the TLR5-associated IRAK migrated in the gel in a manner consistent with the phosphorylated form of the protein (IRAK-P). The migration of this band was shifted after treatment with the serine/threonine-protein phosphatase 2A, i.e., the protein phosphatase 2A-treated IRAK migrated more rapidly that IRAK-P as a broad band, the majority of which migrated with the nonphosphorylated form of the protein (data not shown).

In view of the earlier studies on IRAK association with the IL-1 receptor and TLR2, we were concerned that the association of IRAK with the TLR5 receptor complex in unstimulated cells might be the result of the overproduction of IRAK in transfected cells. Therefore, we assessed the association of
endogenous IRAK with TLR5 in cells transiently transfected with only TLR5-FLAG. As shown in Fig. 5B, endogenous IRAK was associated with TLR5 in unstimulated control and tolerant cells, and stimulation did not appear to change the level of TLR5-associated IRAK dramatically. The constitutive association of IRAK with TLR5 was also observed in untransfected THP1 cells (see Fig. 7).

The results presented in Fig. 5 raised the possibility that flagellin signaling via TLR5 might be different from the process observed with the IL-1 receptor or TLR2. Perhaps IRAK activation and signaling in response to flagellin is not dependent on IRAK release from TLR5. To evaluate this possibility, we took advantage of the observation that TLR5/IRAK complexes are very poorly immunoprecipitated with the anti-IRAK antibody.2 Apparently, the epitope recognized by the anti-IRAK antibody is not accessible when the protein is associated with the TLR5 complex. Therefore, we prepared cell lysates from unstimulated and flagellin-stimulated THP1 cells and incubated the lysates with anti-IRAK antibody (Fig. 6, left and middle lanes) or anti-TLR5 antiserum (right lane). The resultant immunoprecipitates were then assayed for kinase activity using myelin basic protein (MBP) as a substrate.

Fig. 6. TLR5-associated IRAK lacks kinase activity. THP1 cells were incubated in the presence (F) or absence (O) of flagellin for 15 min before the preparation of cell lysates. The lysates were incubated with either anti-TLR5 or anti-IRAK antibody and protein G. The immunoprecipitates were then analyzed for IRAK activity using myelin basic protein (MBP) as a substrate.

In view of the observation that flagellin stimulation did not produce a detectable change in the level of TLR5-associated IRAK (Fig. 5), we considered the possibility that the flagellin-induced release of IRAK-P may involve an exchange reaction that replaces IRAK-P with the nonphosphorylated form of IRAK. Because the TLR5-associated IRAK is always found in the phosphorylated form, the newly bound IRAK would have to be phosphorylated rapidly. This type of exchange reaction would provide a reasonable explanation for the ability of LPS to render cells cross-tolerant to flagellin (Table I; Figs. 1 and 2). By decreasing the level of free IRAK (Fig. 1B and Ref. 27), LPS would be expected to impair the release of IRAK-P severely. If this were the case, then we would expect to find IRAK-P still associated with TLR5 in LPS-tolerant cells, even though the level of free IRAK was reduced dramatically. Therefore, we made THP1 cells tolerant to flagellin or LPS and analyzed the levels of TLR5-associated IRAK (using anti-TLR5 antibodies in the immunoprecipitation reaction) and free IRAK (using anti-IRAK antibody) after a second 30-min incubation with flagellin. Control cells were incubated with flagellin for only the 30-min period. As shown in Fig. 7, IRAK-P was associated with TLR5 in control cells (first lane). Almost all of the IRAK in the anti-IRAK immunoprecipitate was nonphosphorylated (second lane). Similar results were obtained with the lysates from flagellin-tolerant cells (third and fourth lanes). However, a very different pattern was observed with lysates from LPS-tolerant cells (fifth and sixth lanes). As expected, the level of free IRAK (as assessed by its immunoprecipitation with anti-IRAK antibody) was reduced dramatically (sixth lane). However, the level of TLR5-associated IRAK was not markedly different from control or flagellin-tolerant cells (fifth lane). In conjunction with the results presented in Figs. 5 and 6, these results provide strong support for the hypothesis that flagellin self-tolerance is caused by a block in the release of IRAK-P from the TLR5 complex, thus preventing IRAK-P from expressing its kinase and signaling activities.

2 S. B. Mizel and J. A. Snipes, unpublished observations.
Western blot.

Immunoprecipitated with either anti-TLR or anti-IRAK antibodies and (L5 and 27). However, it is also possible that LPS, like flagellin, reduction in the availability of free IRAK (Fig. 6; see also Refs. of TLR5-associated IRAK-P may not occur because of a marked release of IRAK-P and thus maintains a constant level of TLR5-association may be the result of a flagellin-induced ex-
gaged. Alternatively, the relatively constant level of IRAK-P-
vate the IRAK/H18528 signaling pathway (4), it is indeed possible that IRAK-P release between unstimulated and flagellin-stimulated cells. Thus our assay system may not result to diverse microbial products.

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