

Recognition of the flagellin–TLR5 pathway offers novel opportunities for the experimental therapy of various forms of shock, acute respiratory distress syndrome, inflammation and infection.

The Flagellin—TLR5 Axis: Therapeutic Opportunities

by Lucas Liaudet,
Amitabha Deb, Pál Pacher,
Jon G. Mabley,
Kanneganti G.K. Murthy,
Andrew L. Salzman and
Csaba Szabó

Bacteria synthesize large sized surface structures known as flagella through the ordered polymerization of protein subunits. This process results in a planar or tubular regular structure that has evolved to accomplish specific functions. These functions are required for their survival/spread in a particular environment through chemotaxis, motility and nutrition. Flagella are the most complex tubular structures known in bacteria. Flagella are composed of a helical rigid filament, a torsion adapter or hook and a proton-fueled rotator known as the basal body. Pili and fimbriae are similar helical structures but are less complicated. They also function to help in attachment to specific surfaces. Each flagellum consists of approximately 20,000

Summary

Motile bacteria synthesize large-sized surface structures known as flagella through the ordered polymerization of protein subunits. Flagellin, a protein of 40–60 kDa, is the principal constituent of the flagellum; each flagellum consists of approximately 20,000 flagellin molecules. An alignment of the amino acid sequences from different Gram-negative species shows a high degree of similarity in the amino and carboxy terminal domains. In contrast, the central hypervariable regions of these proteins are quite divergent. Recent work reveals that—in addition to playing a role in bacterial adhesion—monomeric flagellin, a protein component of flagellated bacteria, can also act as a soluble immunostimulatory and proinflammatory factor, activating the immune/inflammatory axis via the Toll-like receptor 5–nuclear factor- κ B axis. Monocytes and macrophages, as well as intestinal and pulmonary epithelial cells, respond to monomeric flagellin at low concentrations. Administration of flagellin at doses comparable to or lower than that of bacterial lipopolysaccharide (endotoxin) can induce prominent local and systemic immune/inflammatory responses *in vivo*. Recognition of the flagellin–TLR5 pathway offers novel opportunities for the experimental therapy of various forms of shock, sepsis, acute respiratory distress syndrome, bacterial inflammation and infection. © Prous Science 2002. All rights reserved.

flagellin molecules. The protein flagellin (coded by the gene *fliC*) is the major structural component of bacterial flagella. The filament is mainly composed of the proteins *fliC* or *fliB* that is dependent on phase variation. Bacterial flagellins have molecular masses of approximately 40–60 kDa. An alignment of the amino acid sequences from different Gram-negative species shows a high degree of similarity in the amino and carboxy terminal domains. In contrast, the cen-

tral hypervariable regions of these proteins are quite divergent. Differences in length within the hypervariable domains account for most of the variation in molecular mass among different species.^{1–3} Recently, the crystal structure of the flagellin molecule has revealed a structure resembling an aircraft with two wings and a body. The α -helices of N-terminal and C-terminal chains interact and form a rod-shaped structure. This hydrophobic rod-shaped domain forms the central

axis, while the hypervariable region forms the outer surface.⁴

The assembly of flagella in pathogens like *Salmonella* is similar to the secretion of virulence factors, and requires a complex regulation of export machinery across both inner and outer bacterial cell membranes. It is not surprising that certain components of the secretion system used in flagellar biosynthesis are structurally and functionally homologous to the components of the type III secretion system used in the export of virulence factors in *Salmonella* and *Shigella* spp. The striking similarities between the two secretory systems also indicate that flagellar export machinery might be an additional mechanism for secretion of virulence factors. Flagella are composed of nearly 40 proteins. Only two of the nearly 40 proteins in flagella assembly are dependent on the general secretory pathway. The external components are all secreted through a specific pathway similar to the type III systems identified in some pathogens.⁵

Role of flagellin in bacterial adhesion

Although flagellum is primarily recognized as being the locomotive organelle of motile bacteria, it has also been reported to serve as an adhesive structure.⁶ There is no evidence, however, that flagella alone are involved in attachment. Some studies have shown that flagellin (the monomeric functional unit of flagella) is more important in invasion than bacterial adherence to intestinal cells. The flagellin gene (*fliC*) has been disrupted in *Salmonella enterica* serotype *enteritidis*. The mutation does not affect expression of downstream genes like *fliU*.⁷ Infection experiments with differentiated Caco-2 cells revealed that the mutant was about 50-fold less invasive than the wild-type strain, while the bacterial adherence was unaffected.⁷ It has also been observed that *fliC*, *fliD* and crude flagella are responsible for adherence *in vitro* to mouse cecal mucus. The tissue association in the mouse cecum of a nonflagellated strain was approximately

10-fold lower than that of a flagellated strain belonging to the same serogroup, confirming the role of flagella in adherence.⁸

Similar conclusions were drawn from studies investigating the behavior of bacteria on biofilms. Communities of microorganisms, which adhere to various surfaces, form biofilms.⁶ These bacterial biofilms have advantages in both growth and survival compared with their planktonic forms. Although several factors are thought to affect surface attachment, including extracellular material, nutrients and temperature, recent studies showed flagellation and motility facilitate attachment in both biotic and abiotic surfaces.⁶ Thus, flagella can contribute to the establishment of the bacteria to human cells, although the role of individual components of the flagellar apparatus is yet to be evaluated in the context of the whole attachment process.

Flagellin as a bacterial modulin

Multiple components of Gram-negative and Gram-positive bacteria possess the ability to stimulate the release of proinflammatory cytokines from monocytes and macrophages. These components are collectively called modulins. These include lipoteichoic acid and peptidoglycan from Gram-positive organisms, lipoarabinomannan from mycobacteria, lipoprotein from mycoplasma and porins as well as lipopolysaccharides (LPSs) from Gram-negative organisms.⁹⁻¹³

In general, the innate immune system recognizes pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents but not on the host. Toll-like receptors are now known to recognize the PAMPs. Most of the ligands that are recognized are derived from pathogens, suggesting that the Toll-like receptors (TLRs) are critical to sensing invading microorganisms. Pathogen recognition by TLRs provokes rapid activation of innate immunity by inducing produc-

tion of proinflammatory cytokines and up-regulation of co-stimulatory molecules. Activated innate immunity subsequently leads to adaptive immunity. Distinct TLRs can exert distinct, but overlapping, sets of biological effects.⁹⁻¹³

Recent investigations have revealed an important role for flagellin in the induction of host proinflammatory responses (as opposed to its role in the attachment process). Flagellin, at low concentrations, activates macrophages, monocytes and intestinal and pulmonary epithelial cells *in vitro*, and induces the release of a host of proinflammatory mediators *in vitro* and *in vivo*.¹⁴⁻²² The inflammatory mediators that are produced *in vivo* in response to flagellin include tumor necrosis factor- α (TNF- α), macrophage inflammatory protein-1 α (MIP-1 α), interleukin-6 (IL-6), IL-12p40 and IL-10, as well as nitric oxide, produced by the inducible isoform of nitric oxide synthase (iNOS).³ It appears that the central downstream pathway that mediates flagellin's proinflammatory and immunostimulatory effects involves the activation of TLR5 (see below in detail). In addition, it has recently been observed that *Pseudomonas aeruginosa* flagellin elicits host cell responses through binding to a glycolipid receptor, asialoGM1 (ASGM1) in the host cell membrane. ASGM1 lacks transmembrane and cytoplasmic domains. Flagellin ligation to ASGM1 promotes ATP release from host cells, followed by autocrine activation of a nucleotide receptor. This process results in activation of phospholipase C, Ca²⁺ mobilization and other downstream signaling cascades.²³ The potential relationship between TLR5 and ASGM1 has not yet been explored.

The flagellin-TLR-5 receptor-NF- κ B axis

Both *Drosophila* and mammalian Toll receptors are transmembrane proteins with a large extracellular domain that contains multiple leucine-rich repeats. The receptors also contain a cytoplasmic domain homologous to

that of the IL-1 receptor and therefore referred to as a TIR domain, for Toll/IL-1 receptor homology domain. The similarities between members of the Toll family and members of the IL-1 receptor are not restricted to their structure. Both families induce activation of NF- κ B and share many components of the NF- κ B signaling pathway.^{11–13} While TLR2 is recognized as the receptor for Gram-positive bacterial components, and TLR4 is the receptor for bacterial LPS, until recently, the ligand for TLR5 remained elusive. In a line of recent investigations, the ligand for the TLR5 receptor was recently identified as flagellin.²² It has been established that the mammalian TLR5 recognizes bacterial flagellin from both Gram-positive and Gram-negative bacteria. CHO cells expressing human TLR5 and a luciferase-linked reporter have been exploited to screen for PAMPs recognized by the receptor. TLR5 did not respond to any of the PAMPs known to stimulate TLR pathways, such as LPS, lipopeptide, yeast cell wall or peptidoglycan, except flagellin. Flagellin binds to TLR5, and TLR5–flagellin interaction culminates in the activation of NF- κ B, which is required for the transcriptional induction of many proinflammatory cytokines. Another line of confirmatory evidence that flagellin is the cognate ligand of TLR5 came from the fact that deletion of flagellin genes from *Salmonella typhimurium* has been found to abrogate TLR5-stimulating activity.¹⁹ *S. typhimurium* possesses two genes for flagellin: *fljB* and *fliC*. Culture supernatants of *fljB-fliC*+ contained TLR-5 stimulating activity, whereas culture supernatants from the same strain lacking both flagellins (*fljB-fliC*-) expressed no TLR5-stimulating activity. The lack of both flagellin genes had no effect on TLR2-stimulating activity. These results indicate that flagellin is the principal TLR5-stimulating activity present in *S. typhimurium* culture supernatant. It has also been observed that recombinant expression of the *Listeria monocytogenes* flagellin gene (*flaA*) induces

TLR5 activation, in contrast to the nontransformed strain.¹⁹

As already mentioned above, we reported in 2001 that flagellin induces the production of a host of inflammatory mediators *in vitro* and *in vivo*²¹ in a fashion that is independent of the activation of the TNR4 receptor.²¹ In a parallel, independent investigation, it was also found that intraperitoneal injection of flagellin induces the systemic production of IL-6.²² Moreover, mice deficient in the adaptor protein MyD88, which is required for TLR signaling, were found completely unresponsive to flagellin.²² It was concluded, therefore, that—similar to TLR2 and TLR9—MyD88 is an essential signal transducer of TLR5 for signaling. (It is noteworthy that mechanisms are also known of TLR signaling in an MyD88-independent fashion, e.g., TLR4 can induce costimulatory molecule up-regulation in an MyD88-independent way.)²²

An unusual aspect of TLR5 is that it recognizes flagellin, a protein rather than a PAMP. Flagellin also does not undergo posttranslational modification that will distinguish it from host cellular proteins. However, the amino- and carboxy-termini of flagellin are extremely conserved, presumably because they form a hydrophobic core of the flagella and have significant structural constraint on variability.^{1–3}

There are both common and unique aspects that mediate TLR family responses. TLRs have been shown to participate in the recognition of pathogens by the innate immune system, but it is not clear how a restricted family of receptors has the capacity to recognize the wide spectrum of TLR stimuli known to exist. It has been reported that two members of the TLR family, TLR2 and TLR6, coordinate together to activate macrophages by Gram-positive bacteria and zymosan, whereas TLR2 recognizes bacterial lipopeptide, without TLR6. The cytoplasmic domain of TLR2 can form functional pairs with TLR6 and TLR1, and this interaction leads to cytokine

production. Thus, the cytoplasmic tails of TLRs are not functionally equivalent, with certain TLRs requiring assembly into heteromeric complexes, whereas others are active as homomeric complexes.²⁴ From the TLR5 studies, it is apparent that TLR5 does not require any cooperation from other TLRs for production of cytokines in response to flagellin.²² However it is still an open question whether PAMPs other than flagellin are also recognized by TLR5 and if there is an interaction among TLR5 and other TLRs.

An important feature shared by many bacterial modulins including flagellin is that they signal monocytes and macrophages via intracellular signal transduction factors associated with the IL-1 receptor/Toll-like receptor family. Proteins in the IL-1R/TLR family are grouped based on conserved sequences in their cytoplasmic domains that are required for signal transduction.^{9,11–13} Upon activation of the IL-1R and TLRs, several common events are triggered, including recruitment and activation of the IL-1R associated kinase (IRAK) via the adaptor protein MyD88, formation of a complex between TNF receptor-associated factor 6 (TRAF6) and activation of downstream cascades leading to activation of NF- κ B and other factors required for transcription of proinflammatory cytokine genes. The common pathway of activation of NF- κ B via IRAK-TRAF6- transforming growth factor β -activated kinase 1 (TAK1)-IKK is also followed for TLR5 like the other Toll receptors (Fig. 1). However infection of pathogenic bacteria also leads to activation of NF- κ B in an MKK3/6-p38 mitogen-activated protein kinase-dependent pathway leading to activation of Elk-1 apart from this conventional pathway. The pathogen-induced activation of NF- κ B inducing kinase NIK-IKK α/β -IkB α pathway and MKK3/6-p38 kinase pathway may bifurcate at TAK1 (Fig. 1).^{11–13} It is not yet known if purified flagellin activates the p38 pathway. Thus, interaction of bacteria through TLR5 leads to activation of NF- κ B and Elk-1, which

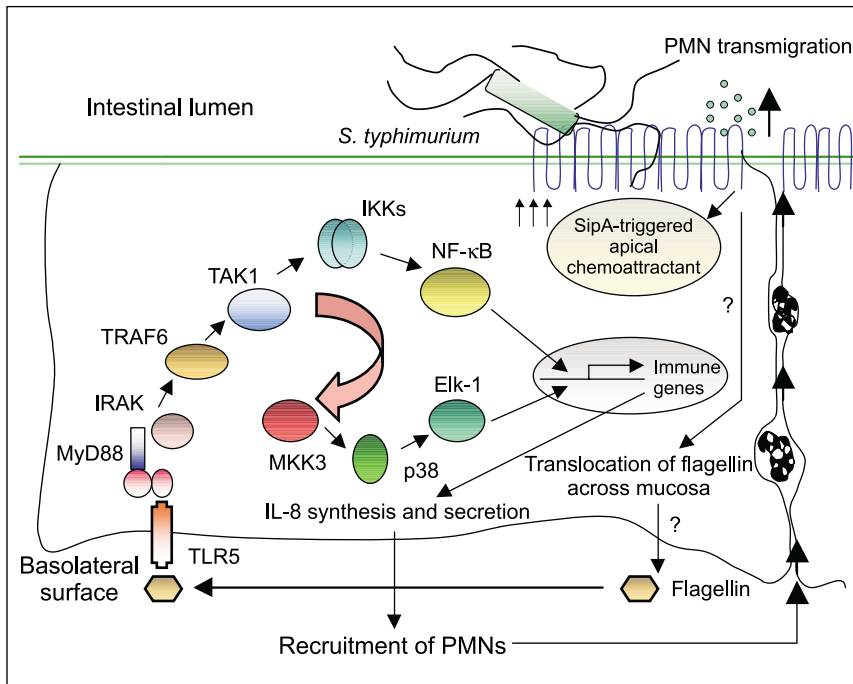


Fig. 1. A diagrammatic representation of some of the putative cellular signal transduction mechanisms of the action of flagellin in intestinal epithelial cells. The translocation of flagellin in the intestinal cells is poorly understood. The consensus is that flagellin binds TLR5 to activate IKK-dependent and -independent pathways to activate nuclear factor- κ B and p38 kinase required for induction of inflammatory genes that include interleukin-8 (IL-8). IL-8 mediates a number of important processes that includes PMN (polymorphonuclear cell) transmigration.

and TLRs, it appears that different TLRs may share a number of common signaling features such as IRAK and MyD88. However they may also exhibit substantial differences in co-factor requirements that result in relatively unique signaling mechanisms.²⁵

The focus of flagellin-TLR5 research is now focused more in understanding the mechanism of flagellin transport and how it is recognized by receptors like TLR5 and in the details of the molecular events at the receptor after flagellin-TLR5 binding. In a number of reports, it has been demonstrated that flagellin that contacts basolateral epithelial surface is proinflammatory, but apical flagellin has no effect. Nevertheless, nitric oxide production, along with a potent activation of NF- κ B, can also be observed in cultures of intestinal epithelial cells in response to flagellin in nonpolarized cell systems *in vitro* (Fig. 2).²¹ Use of different truncated versions of flagellin have been used to determine the regions involved in

is known to drive cell transcription of immune response genes in the nucleus.

Recently, it has been observed that Gram-negative flagellin induces self-tolerance, which is associated with a block of release of IRAK from TLR5. Prior exposure to flagellin results in a subsequent state of flagellin tolerance in human monocytes, THP-1 cells, Jurkat cells and cos cells. The tolerance pathway does not need protein synthesis. Flagellin did not tolerize monocytes and THP-1 cells to LPS. However, LPS treatment of monocytes and THP-1 cells resulted in a state of flagellin cross-tolerance. The same study showed that release is essential for IRAK activity, since the TLR5-associated IRAK lacks kinase activity. LPS-induced cross-tolerance to flagellin is also associated with a block in IRAK release from TLR5. Although the detailed mechanism is yet to be unfolded, on the basis of all these results obtained with the IL-1 receptor

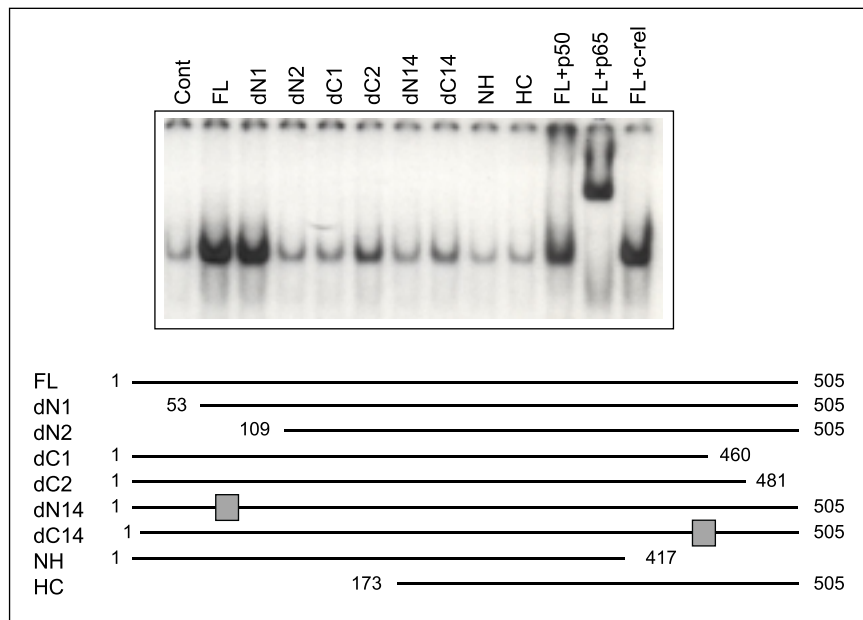


Fig. 2. Delineation of flagellin regions relevant for the activation of nuclear factor- κ B (NF- κ B) in nonpolarized epithelial cells. DLD-1 cells were treated with purified flagellin (recombinant²¹). Different truncated forms of *Salmonella muenchen* flagellin were tested for NF- κ B activation in nuclear cell extracts, and 5 μ g of the nuclear extract was analyzed in electrophoretic mobility shift assay. dN14 and dC14 are deletion mutants in which deletions are replaced by homologous amino acid sequences unrelated to flagellin. The results show that these regions are critical for activation of NF- κ B. Supershifts with p50, p65 and c-rel antibodies were used to identify the specific binding of NF- κ B subunits.

activation of NF- κ B, as shown in Figure 2.

The flagellar locus *fliE* controls the secretion of flagellin. A transposon insertional mutant of *S. typhimurium* in *fliE* locus does not affect their adherence or invasion in intestinal epithelial cells. However, such mutants are unable to induce IL-8 secretion in host cells.²⁶ Unlike the wild-type counterpart, the *fliE* mutant failed to secrete flagellin and lacked any surface assembly of flagella. The mutants did not activate the I κ B α /NF- κ B signaling pathway or induce the coordinated trans-epithelial migration of isolated human neutrophils. A trans-complementation of the *fliE*-deficient mutants with a wild-type *fliE* containing plasmid restored all the defects. This observation shows that *fliE* is an upstream regulator of flagellin secretion and its associated signaling cascades further downstream. It is also possible that secretion of flagellin as a virulence factor and regulation of flagellin as a component of flagellar assembly process are differentially regulated. It is likely that one process is sensed by the interaction of bacteria with human cells, whereas the flagellar assembly process is independently regulated at the requirement of its own survival through motility. Future studies should utilize TLR5-deficient cells and animals in order to confirm all these conclusions about TLR5 being the critical signal transducer for flagellin.

Recently, *fliE* has been reported to be an upstream regulator of flagellin and thus found to control flagellin-mediated signaling processes. Disruption of a flagellar basal body protein *fliE* in *Salmonella* was shown to abrogate flagellin production and failed to induce a proinflammatory response in epithelial cells.²⁶ It is likely that the regulation of flagellin production and assembly, as it relates to the flagellar attachment process, is differentially regulated than the secretion process of flagellin. Although much of the molecular mechanism of the intracellular transport process for flagellin remains to be characterized, it is clear that fla-

gellin plays an important role as a virulence factor by interacting with the cells externally and internally, and contributes significantly to many disease conditions in pathogen infected individuals (see below).

Conserved flagellin domains involved in host cell activation

Because flagella are a feature of many strains of bacteria, it is not surprising that there is a significant homology in flagellin amino acid sequence among distant bacterial species. Sequence alignment of flagellin protein from a variety of bacterial species shows a high degree of sequence conservation in the N-terminal (160 amino acids) and C-terminal (85 amino acids) domains. The central hypervariable region varies in size and sequence among different bacterial species and accounts for most of the variation in molecular mass (35 to 60 kDa).

Although in earlier studies in human monocytes, McDermott and colleagues proposed that the central hypervariable region is essential in inducing the production of TNF- α ,¹⁶ recent studies in epithelial cells point toward the importance of the N- and C-terminal domains. Eaves-Pyles and colleagues used r6-histidine (6HIS)-tagged fusion constructs from the *Salmonella dublin* (SD) *fliC* flagellin gene. A full-length r6HIS SD flagellin (6HIS flag) potently induced NF- κ B activation in Caco-2BBE cells and in fact was as potent as native-purified SD flagellin. Fusion proteins representing the D3, amino or carboxyl regions alone did not induce proinflammatory mediators. The results with a recombinant protein containing the amino D1 and D2 and carboxyl D1 and D2 separated by an *Escherichia coli* hinge (ND1-2/ECH/CD2) indicated that D1 and D2 were bioactive when coupled to an ECH element to allow protein folding. This chimera, but not the hinge alone, induced NF- κ B activation, and nitric oxide and IL-8 production in intestinal epithelial cell lines. The potent proinflammatory activity of flagellin, therefore, was

identified to reside in the highly conserved N and C D1 and D2 regions.²⁷ Close examination of the N-terminus region identified another highly conserved domain of 22 amino acids in length (amino acid 31-52), designated as the RINSA domain. It should be possible that the antibodies raised against N-terminus part of *Salmonella* or *E. coli* flagellin recognize flagellins from *Pseudomonas*, *Serratia* or *Proteus* and so forth. To test this hypothesis we raised monoclonal and polyclonal antibodies to the RINSA and the entire N-terminus region of *Salmonella muenchen* flagellin. As shown in Figure 3, *Salmonella* flagellin antibodies reacted with recombinant flagellin from *E. coli*, *Serratia* and *Proteus*.

Distribution of TLR5

Considering the function of TLRs, it is not surprising that high expression can be found at sites of host-microbe interaction. TLRs were initially found to be expressed in all lymphoid tissue but were most highly expressed in peripheral blood leukocytes. Expression of TLR mRNA has been found in monocytes, B cells, T cells and dendritic cells.¹⁶⁻¹⁸ Responsiveness to PAMPs (rather than the level of expression alone), however, should remain a critical factor in confirming the expression of TLR family members. In some cases like TLR4, some other molecules also determine responsiveness (such as RP104). B cells weakly express TLR4 relative to macrophages, yet they possess robust LPS responsiveness because they express RP105, another TLR family member.²⁸ The expression of TLRs on cells of the monocyte/macrophage lineage is consistent with the role of TLRs in modulation of inflammatory responses via cytokine release and expression of co-stimulatory molecules. Therefore, TLR expression at peripheral sites as well as in immune cells that migrate them enables TLRs to serve the innate immune system at sites of pathogen invasion.

TLR5 is specifically expressed in monocytes, immature dendritic cells

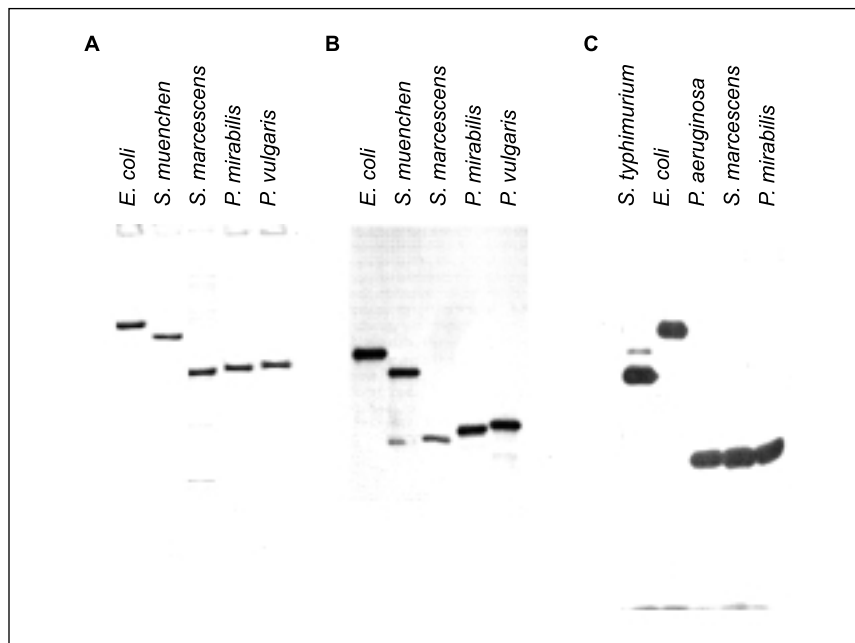


Fig. 3. Evidence for antibodies recognizing flagellins produced by various bacterial species. **A.** Purification of recombinant flagellin from different organisms. **B** and **C.** Antibodies have been raised against the N-terminus and the RINSA region of flagellin, and they successfully recognize flagellin in the bacterial extracts from different microorganisms, as evidenced by immunoblot analysis.

and epithelial cells.^{29,30} A particularly high TLR5 expression was found in the lung tissue, an observation that may have relevance for the potent flagellin-induced inflammatory responses in the same organ (see below).

Intestinal epithelial cells regulate the expression of TLR5 to the basolateral side of the cell (see above), presumably to respond to invasive pathogens, such as *Salmonella*, while ignoring noninvasive, commensal *E. coli*. Regulation of TLR gene expression is of vital importance to the understanding of the diversity of TLR responses, as TLRs share many signaling components and responsiveness, which mostly requires expression of a particular TLR. On the basis of the expression pattern, TLR can be classified as ubiquitous (TLR1), restricted (TLR2, TLR4, TLR5) or specific (TLR3). It is likely that expression and regulation of distinct but overlapping ligand-recognition patterns may underlie the existence of a large, seemingly redundant TLR family.

Flagellin and epithelial cell activation

Whereas immune cells, most notably monocytes/macrophages, represent the pivotal effectors of the innate immune system,^{31–33} it is noteworthy that epithelial cells also possess the ability to generate innate immune responses upon recognition of certain microbial products. In a manner similar to immune cells, epithelial cells utilize NF- κ B as a rapidly inducible transcriptional activator in response to immune and proinflammatory signals.³⁴ Such ability is particularly important for epithelial cells in the gastrointestinal tract, which form a highly specialized barrier separating two distinct environments, thus maintaining the delicate balance between the gut lumen and the underlying tissue. On the one hand, intestinal epithelial cells must remain “tolerant” to the large amount of bacteria that make up the gut microflora. These same cells, however, must be able to rapidly detect invading pathogenic microbes in order to generate an appropriate immune response. In the past few

years, the novel concept has emerged that flagellin from motile enteric microorganisms is a central bacterial mediator eliciting immune activation of intestinal epithelial cells.

In the late 1990’s, three independent groups, working on the interactions between enteropathogenic Gram-negative bacteria and intestinal epithelial cells (or enterocytes), published reports suggesting that a secreted bacterial factor might elicit inflammatory activation in these cells.^{18,34–36} In our laboratory, we demonstrated that different strains of *Salmonella* could activate NF- κ B in the human intestinal epithelial cell line Caco-2 cells independently from bacterial invasion of the enterocytes,³⁵ an observation later confirmed by Gewirtz and colleagues.³⁴ These results implied that bacterial adhesion, rather than invasion, or the release of a secreted factor, was sufficient to induce NF- κ B activation in intestinal epithelial cells. These data were completed by the results of Steiner et al., who demonstrated that a pathogenic strain of *E. Coli* induced the secretion of the chemokine IL-8 by Caco-2 cells.¹⁸ IL-8 is a potent chemoattractant cytokine that induces the local recruitment of activated polymorphonuclear cells at sites of inflammation. The release of IL-8 by Caco-2 cells was observed as little as 3 hours after exposure to either live bacteria or cell-free culture filtrates. In the same study, it was shown that the IL-8-releasing activity was heat stable, sensitive to protease degradation and was not inhibited by polymyxin B (which binds to and neutralizes the action of LPS).³⁶

Following these initial studies, the same groups of investigators worked on identifying the putative bacterial factor responsible for the previously described epithelial activation. Steiner’s group reported that purified flagella from various *E. Coli* isolates were able to induce IL-8 secretion in Caco-2 cells and that aflagellic mutants of *E. Coli* were unable to induce IL-8 secretion.¹⁸ Chromatographic purification of the IL-8–

releasing activity revealed a single protein, identified as flagellin. Purified flagellin appeared extraordinarily potent as an IL-8 releasing factor, with a half maximal activity at 10^{-10} M.¹⁸ At the same time, our group published an extensive report on the fundamental role of flagellin as an inducer of proinflammatory cascades in two different intestinal epithelial cell lines, namely Caco-2 cells and DLD-1 cells.²¹ Using first a strain of *S. dublin*, we found that the addition of sterile-filtered medium (after an overnight growth of bacteria to 10^{12} colony forming units/ml) to Caco-2 and DLD-1 cells induced I κ B α degradation and NF- κ B nuclear translocation, as well as the induced expression of iNOS and the up-regulation of nitric oxide production. Purification and protein isolation disclosed a single 55 kDa band as the nitric oxide-inducing and NF- κ B-activating activity of the filtered medium. Micro-sequence analysis of this 55 kDa band followed by a Genbank database search produced an exact match with published sequences of *Salmonella* flagellin.²¹

The role of flagellin was further substantiated by a series of distinct observations published in our report. First, sterile-filtered medium from aflagellin mutants of *Salmonella* species were unable to induce NF- κ B activation and nitric oxide production in Caco-2 and DLD-1 cells. Second, purified *Salmonella* flagellin induced nitric oxide production with an extremely high potency, attested by a threshold inducing concentration of 100 pg/ml and an EC₅₀ of 2.5 ng/ml. Finally, we demonstrated that rabbit antiserum raised against the N-terminus fragment of recombinant *S. muenchen* flagellin completely abolished the nitric oxide-inducing activity of a broad range of recombinant flagellins derived from a series of Gram-negative motile bacterial species (*E. coli*, *S. muenchen*, *Serratia marcescens*, *Proteus mirabilis* and *Proteus vulgaris*). These effects were clearly independent from any possible contamination with LPS, given that they were not influenced by the addition of

the LPS-binding agent polymyxin B, and also that pure LPS at a concentration of 1 μ g/ml (i.e., several orders of magnitude greater than the used concentrations of flagellin) did not induce NF- κ B activation in our epithelial cell lines.²¹ The activation of intestinal epithelial cells by flagellin in the *in vitro* experiments was also confirmed in additional studies performed *in vivo*. Mice were challenged with an intraperitoneal injection of purified *S. dublin* flagellin (10 μ g/mouse). Twenty-four hours later, the animals were sacrificed, and samples from the distal ileum were harvested and processed for the immunohistochemical detection of iNOS. A massive expression of iNOS was detected in the epithelial lining of the gut mucosa following flagellin administration, contrasting with the absence of iNOS immunoreactivity in control mice.²¹

After the report by Steiner and our own studies, additional data were published on the role of flagellin in the inflammatory activation of intestinal epithelial cells. Ogushi and colleagues demonstrated that flagellin from *S. enteritidis* strongly activated the expression of β -defensin-2, an antimicrobial peptide playing an important role in host defense at mucosal surfaces, by Caco-2 cells.³⁷ β -Defensin activation was preceded by the nuclear translocation of NF- κ B, confirming our previous demonstration that flagellin has the ability to activate this transcription factor in mammalian cells.²¹ In a second report, the same group showed that NF- κ B activation by flagellin was related to an increase in intracellular calcium, suggesting a tight coupling between a putative flagellin cell surface receptor with intracellular pathways of calcium signaling.³⁸ Further confirming the proinflammatory activity of flagellin, it was recently shown that stimulation of Caco-2 cells with *Salmonella* flagellin triggers the expression and secretion of the CCL20 chemokine.³⁹ The CCL20 chemokine is the unique attractant of immature dendritic cells, which are bone marrow-derived antigen-presenting cells with the ability to

induce primary immune responses. Thus, the recruitment of dendritic cells into the epithelium is a prerequisite to initiate a specific, adaptive, immune response. These data therefore suggest that interaction between flagellin and intestinal epithelial cells is instrumental to initiate adaptive immune responses in the gut.³⁹

Two recent reports provided new insights into the signal transduction pathways underlying this newly discovered interaction between bacterial flagellin and mammalian epithelial cells. In a first study published in 2001, Gewirtz and colleagues found that activation of IL-8 secretion by a model, polarized, intestinal epithelium stimulated by *S. typhimurium* required the transepithelial migration of a "proinflammatory factor."²⁰ The translocation of this factor from the apical to the basolateral surface was indeed necessary to elicit IL-8 secretion. Purification of this proinflammatory factor revealed that it was of bacterial origin and was in fact flagellin. Although the mechanism underlying flagellin translocation remained unknown, this study strongly suggested that a flagellin receptor was present at the basolateral, but not apical, side of the intestinal epithelium.²⁰ This observation makes considerable sense, given that normal intestinal mucosa is continuously exposed to commensal bacteria and their products, notably flagellin. The polarization of flagellin sensing at the basolateral membrane then prevents any inflammatory activation under normal conditions. In contrast, this polarization would allow a rapid sensing of the presence of pathogenic flagellated organisms following any breach in the intestinal mucosa. In turn, this might favor the development of an immediate immune response against the invading pathogen. In a second set of experiments, the same group of researchers worked to identify this putative flagellin receptor at the basolateral membrane.¹⁹ Following the previous identification that flagellin is the unique ligand of TLR5²² (see above), these authors demonstrated

that intestinal cells express TLR5 exclusively at their basolateral surface, and that cell surface expression of TLR5 conferred NF- κ B-dependent gene expression in response to flagellin.¹⁹

Overall, the data described above consistently define a novel concept in the interactions between bacteria and mammalian epithelial cells. The step-wise observations that flagellin induces proinflammatory gene expression in enterocytes through the activation of the transcription factor NF- κ B, and that this response depends on the basolateral expression of TLR5, opens exciting therapeutic perspectives for a number of human diseases. Indeed, this newly discovered innate immune mechanism is relevant not only for gastrointestinal diseases induced by invasive pathogens,³⁷ but may also be instrumental in the development and perpetuation of inflammatory bowel diseases. In this scheme, ligation of basolateral TLR5 by flagellin secreted from commensal organisms might occur in any state of epithelial barrier dysfunction (Fig. 1). In turn, this would play an important role in inducing or exacerbating the intestinal inflammation characterizing these chronic inflammatory disorders. Pharmacological interference with the flagellin/TLR5 signaling pathway may therefore represent a novel therapeutic strategy for both clinical enteric infections with invasive Gram-negative pathogens, as well as possibly for the treatment of inflammatory bowel disease.

Flagellin as a pathogenetic factor in the pathogenesis of systemic inflammation and shock

Sepsis and septic shock are heterogeneous clinical syndromes complicating the course of serious infections. Sepsis is defined as a systemic inflammatory response syndrome caused by an infection.⁴⁰ The definition of systemic inflammatory response syndrome relies on the presence of several criteria, including tachycardia, tachypnea, fever (or hypothermia) and

leukocytosis (or leukopenia). According to the currently accepted terminology, special subcategories of sepsis are severe sepsis (or the sepsis syndrome), with dysfunction in one or more organ systems, and septic shock, with hypotension not responsive to intravenous fluid loading. Most forms of sepsis have a poor prognosis, with a mortality rate ranging from 30% to 50%.⁴¹ Septic shock is the most common cause of mortality in surgical intensive care units and the thirteenth most common cause of death in the United States. The prevalence of these pathologies is continuously increasing because of the extended longevity of patients with chronic illnesses, the increased occurrence of immunosuppression and the more frequent use of invasive procedures.⁴¹

Over the past decade, our pathophysiological understanding of sepsis has substantially improved. It is now well recognized that the innate immune system plays a major role in host defense against infection. Recognition of invasive microbial pathogens is mediated by pattern recognition receptors on the surface of immune cells that recognize pathogen-associated molecular motifs. In Gram-negative sepsis—which represents the most frequent form of clinical sepsis—it is generally considered that the LPS component of the outer bacterial membrane represents a major trigger of innate immune responses. LPS-binding protein, CD14 and the TLR4 receptor are key molecules for the recognition of LPS by cells of the myelomonocytic lineage. In turn, immune cells activated by microbial pathogens release numerous effector molecules, which orchestrate the innate and adaptive host defenses. Such molecules include cytokines, proteases, oxidants and free radicals, adhesion molecules, lipid metabolites and nitric oxide. Uncontrolled immune activation, resulting in overstimulation of this physiological host response ultimately leads to the development of overwhelming inflammation, cardiovascular collapse and multiple organ damage that characterize

the clinical syndrome of septic shock.^{42,43}

As mentioned previously, LPS has long been considered as the main component of Gram-negative bacteria triggering inflammation and shock. However, several important differences distinguish bacterial and LPS-induced shock, notably regarding the kinetics and distribution of proinflammatory gene expression. The existence of these differences supports the concept that bacterial components distinct from LPS are additional triggers of systemic inflammation and shock during the course of severe Gram-negative infections. Several lines of evidence accumulated over the last 3 years now strongly suggest that flagellin is another major proinflammatory mediator released from Gram-negative pathogens. In addition to its primary role as a structural component of the bacterial flagella, flagellin may also act as an exotoxin, being released from motile Gram-negative bacteria *in vitro* (see above) and being detectable in significant amounts as a free circulating protein in the blood of septic rats²¹ as well as in septic patients.^{44,45} As mentioned earlier, various lines of independent investigations have indicated that flagellin can induce the expression of proinflammatory mediators by monocytes and epithelial cells *in vitro*, resulting from the activation of NF- κ B.¹⁴⁻²²

In view of these data obtained *in vitro*, we have conducted a series of experiments *in vivo* to specifically address the potential role of flagellin as a mediator of systemic inflammation and shock.²¹ We first demonstrated that mice injected intraperitoneally with 10 μ g of purified *S. dublin* flagellin showed a systemic release of a host of proinflammatory cytokines, including TNF- α , IL-6, IL-12 and the chemokine MIP-1 α . In addition, we found that flagellin administration induced the up-regulated production of the potent vasodilator nitric oxide, which is currently considered as a major mediator of sepsis-induced hypotension.⁴⁶ Using immunohisto-

chemical techniques, we found in particular that flagellin induced a massive expression of iNOS in intestinal epithelial cells,²¹ indicating that flagellin not only acts on immune effector cells such as monocytes/macrophages, but also on epithelial cells, as detailed in the previous section of this review. Of note, the release of cytokines and nitric oxide were already observed when minute doses of flagellin were administered (1 µg/mouse), indicating that this bacterial component is a very potent activator of innate immune responses *in vivo*.²¹

We then evaluated the hemodynamic behavior of anesthetized mice challenged with intravenous recombinant *S. muenchen* flagellin.²¹ We found that flagellin induced a progressive hypotension, culminating into death after approximately 4 hours. This hypotension was associated with a severe impairment of vascular responsiveness to vasoconstricting agonists in *ex vivo* aortic rings, a typical feature of septic shock. Interestingly, the hypotension and the vascular hypocontractility elicited by flagellin were noted in both endotoxin-insensitive (Balb/c) mice and endotoxin-resistant (C3H/HeJ) mice. The latter animals typically do not develop LPS-induced shock, because of a point mutation in the gene encoding TLR4, responsible for LPS responsiveness.¹¹⁻¹³ These observations indicated, first, that the effects of flagellin could not be related to any contamination with LPS and, second, that these effects were mediated by a receptor distinct from TLR4.²¹ Confirmation and extension of our working hypothesis was given by Hayashi and colleagues, who reported a few months after our initial publication that flagellin mediates innate immune response through activation of the TLR5 receptor.²²

We completed our investigations by evaluating the influence of flagellin on the development of oxidant stress (an important mechanism of organ failure during septic shock), tissue

neutrophil accumulation (a hallmark of activated inflammatory processes) and organ damage.⁴⁷ In these experiments, we also compared the effects of flagellin with those of LPS to determine possible differences in the response to these two toxins derived from Gram-negative bacteria. Conscious mice were challenged either with 100 µg of recombinant *S. muenchen* flagellin or an equal dose of *S. enteritidis* LPS. We found that flagellin induced a widespread oxidative stress, evidenced by an increase in malondialdehyde and a decrease in reduced glutathione in most organs, which was particularly severe in the liver, the heart and the lungs, and was significantly greater than the effect of LPS. In addition, flagellin induced an early recruitment of inflammatory cells into the lungs as well as biological signs of liver injury. In contrast, LPS induced neutrophil accumulation in the gut and produced renal injury.⁴⁷ It is relevant that the particularly severe alterations noted in the liver and lung following flagellin administration were highly consistent with a previous study reporting a predominant distribution of TLR5, the flagellin receptor, in these two organs.²⁸

Overall, these different sets of data support the concepts that: 1) flagellin is a potent trigger of inflammatory processes *in vivo*, culminating into cardiovascular failure and multiple organ dysfunction; and 2) distinct responses are triggered by flagellin and LPS, which is in agreement with the distinct cell receptors (TLR5, respectively TLR4) involved in the recognition of these two components released from Gram-negative pathogens. Obviously, the findings presented herein open new therapeutic perspectives for the treatment of clinical septic shock. One can envision that pharmacological interventions able to interfere with the flagellin/TLR5 signaling pathways might represent novel strategies to down-regulate the overwhelming inflammation and cardiovascular collapse complicating severe Gram-negative infections.

Role of flagellin in pulmonary inflammation and acute respiratory distress syndrome

Acute lung inflammation culminating in acute respiratory distress syndrome is a major complication of Gram-negative bacterial sepsis.⁴⁸ This syndrome is characterized by massive alveolar collapse, reduced lung compliance and a severe impairment of gas exchanges leading to refractory hypoxemia. Patients suffering from acute respiratory distress syndrome require prolonged periods of mechanical ventilation, and the mortality of this syndrome remains higher than 40%.⁴⁸ The mechanisms linking a distant focus of infection to remote lung inflammation remain elusive. The commonly held view is that bacterial toxins released into the circulation activate interconnected inflammatory cascades in the lung, ultimately leading to diffuse alveolar damage.⁵³ Bacterial LPS has so far been held responsible for triggering this chain of events.^{49,50} Whether additional bacterial toxins play a role in the development of acute pulmonary inflammation during Gram-negative sepsis remains an unresolved issue. Given the potent proinflammatory activity of flagellin and its ability to induce the recruitment of neutrophils into the lung (see above),⁴⁷ we tested the hypothesis that this toxin might represent a previously unrecognized mediator of acute lung inflammation in Gram-negative sepsis. In fact, this hypothesis was supported by an earlier report by Feldman and colleagues,⁵¹ who showed that the pulmonary inflammation elicited in mice by the intratracheal instillation of *P. aeruginosa* was markedly reduced when an aflagellic mutant of the organisms were injected. These authors also found that purified flagellin, directly instilled into the lungs, induced a significant pulmonary inflammation.

We performed *in vitro* and *in vivo* experiments to specifically address the role of flagellin on lung inflammation.^{45,52} Cultured human alveolar epithelial cells (A549) were exposed

to various concentrations of recombinant *S. muenchen* flagellin. After 24 hours, the secretion of IL-8 by these cells was assayed in the culture medium. (As mentioned above, IL-8 is a potent chemoattractant cytokine that induces the local recruitment of activated polymorphonuclear cells at sites of inflammation.) We also measured by fluorescence cell sorting analysis the expression of the intercellular adhesion molecule ICAM-1, an important protein involved in cell-cell interactions, by A549 cells. We found not only that flagellin was able to induce IL-8 secretion and ICAM-1 expression, but also that these effects of flagellin were extraordinarily potent. Indeed, half-maximal stimulation of IL-8 synthesis was obtained at a concentration of 2×10^{-14} M (1 pg/ml), and maximal effect at 2×10^{-10} M. In addition, the threshold concentration of flagellin inducing ICAM-1 expression was 2×10^{-13} M (10 pg/ml). In contrast, in similar conditions, we could not observe any effect of LPS (at concentrations up to 1 μ g/ml) on IL-8 production and ICAM-1 expression.^{45,52}

In vivo experiments were conducted in conscious mice receiving various intravenous doses of flagellin. The animals were sacrificed at selected time points and bronchoalveolar lavage fluid was obtained for the measurement of various inflammatory parameters. At doses as low as 1 μ g/mouse, flagellin induced a severe form of acute lung inflammation starting after 1 hour and reaching a peak after 4 hours, indicated by a massive release of TNF- α , IL-1 β and the chemokines MIP-1 α and MIP-2. These effects were associated with the recruitment of leukocytes—most notably polymorphonuclear granulocytes—into the alveolar spaces and by the development of significant lung hyperpermeability to proteins, which are normally restricted to the intravascular space.^{45,52} Additional dose-response experiments indicated that flagellin retained its proinflammatory potential even when administered at doses as low as 10 ng/mouse. Finally,

we noted that flagellin induced the nuclear translocation of the transcription factor NF- κ B in lung extracts obtained 1 hour after flagellin injection,⁴⁵ thereby reproducing previous *in vitro* findings. The high potency of flagellin noted in these experiments suggests that this toxin might represent an important mediator of lung inflammation during clinical Gram-negative sepsis. In this respect, we have obtained preliminary data showing that free flagellin circulates in the plasma of septic patients at concentrations that would be sufficient to induce significant lung inflammation. Indeed, a positive correlation between the levels of circulating flagellin and the severity of lung inflammation could be demonstrated in these patients.^{44,45}

Our findings that flagellin is a potent inducer of pulmonary inflammatory processes *in vivo* suggested that its neutralization might exert beneficial effects in conditions of Gram-negative bacteria-mediated pulmonary inflammation. In order to directly test this possibility, we have tested the effect of immunization against *S. dublin* flagellin in a severe pneumonia model induced by *S. marcescens*. Immunized animals exhibited significant improvements over nonimmunized ones, when challenged with live *S. marcescens* administered directly into the trachea. As illustrated in Figure 4, immunized mice had a significant improvement in survival and also disclosed a marked reduction in the proinflammatory and oxidant response triggered by the bacteria. This effect was unrelated to altered bacterial clearance or neutrophil trafficking, indicating that the results were more likely related to a down-regulation of the proinflammatory effects of released flagellin than to a suppression of bacterial invasion (Fig. 4).

Thus, a host of data emerge regarding the role of flagellin as a powerful trigger of inflammation, shock and acute respiratory distress syndrome during Gram-negative bacteria infections. Our results, coupled with the findings that immunization against fla-

gellin offers significant protection against inflammatory processes induced by flagellated organisms, might offer new antiinflammatory and antishock strategies for the future in severe forms of clinical Gram-negative infections.

Strategies to counteract flagellin-mediated immune/inflammatory responses

Active immunization may be a valid strategy to counteract flagellin-induced inflammatory responses. Recent studies have demonstrated that a significant fraction of *Salmonella*-specific CD4+ cells respond to the flagellar protein *fljC* *in vivo* during vaccination and that responses to flagellin are sufficient to protect mice from lethal infection.⁵³ These studies also mapped stimulating domain of flagellin to a 14-amino acid region within a constant region of the C-terminus. Similarly, mice vaccinated with *Campylobacter* or *Salmonella* flagellin elicited a protective immune response against bacterial infection (Fig. 4).⁵⁴

Passive immunization may be an alternative approach to counteract flagellin-induced inflammatory responses. We have recently tested whether anti-flagellin polyclonal antibodies would neutralize the production of nitric oxide induced by conditioned medium or recombinant flagellin. As a positive control, lapine antiserum raised against the N-terminus of *S. muenchen* flagellin abolished the nitric oxide-inducing activity of recombinant *S. muenchen* flagellin in interferon gamma primed Caco-2BBE cells.²¹ The antiserum also abolished the nitric oxide-inducing activity of recombinant flagellins or conditioned medium derived from a series of Gram-negative motile bacterial species (*E. coli*, *S. muenchen*, *S. marcescens*, *P. mirabilis* and *P. vulgaris*).²¹ The latter findings imply that flagellin may represent a crucial factor in the induction of nitric oxide production by conditioned medium obtained from flagellated Gram-negative bacilli. The above data

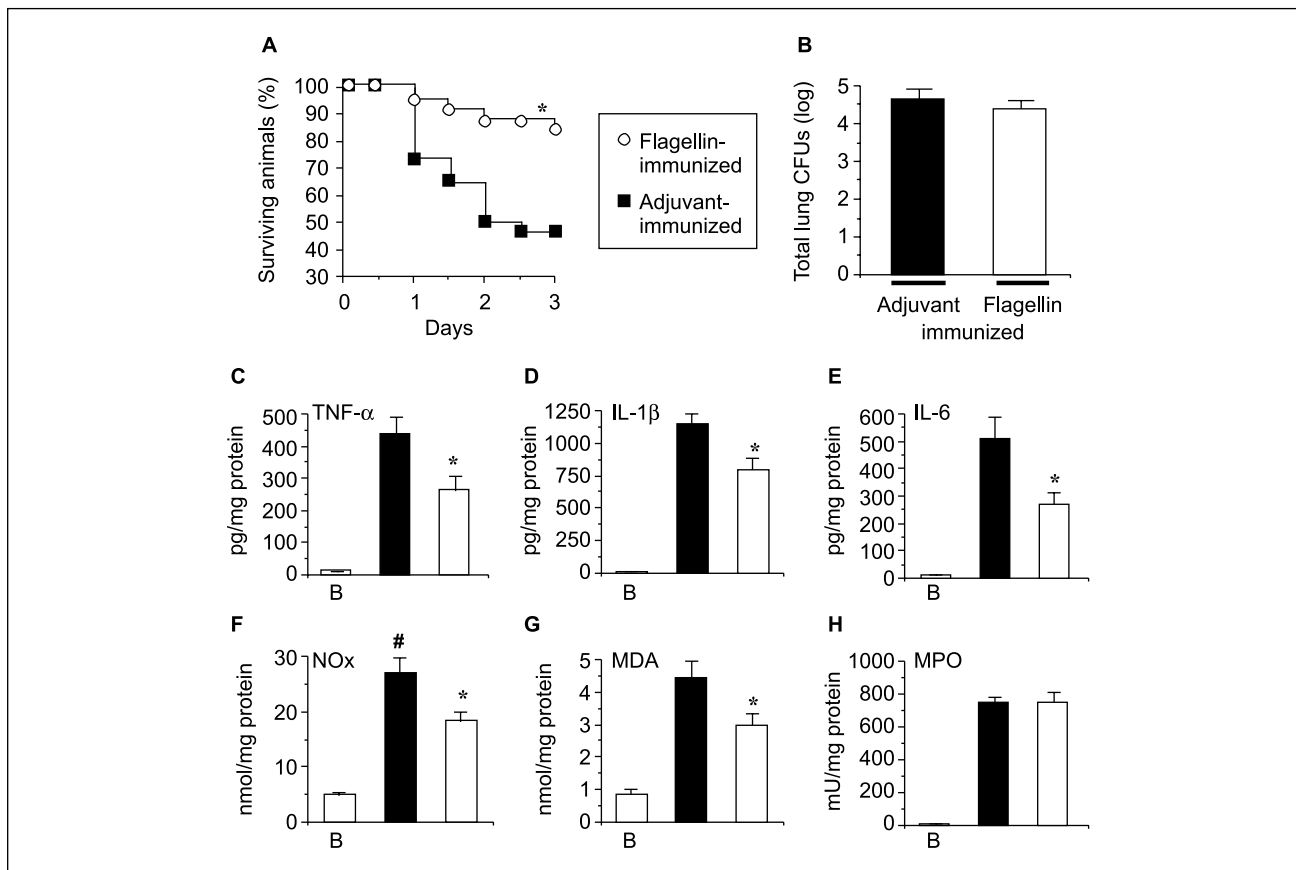


Fig. 4. Protection in a severe murine pneumonia model by active immunization against flagellin. Male Balb/C mice were immunized with 50 μ g of recombinant *Salmonella dublin* flagellin²¹ in complete Freund's adjuvant i.p. Two additional injections were given after 2 and 4 weeks with incomplete Freund's adjuvant. Control mice received Freund's adjuvant only. At 12 weeks, *Serratia marcescens* suspension (4×10^6 bacteria/ml) was instilled intratracheally. The survival of mice immunized with flagellin ($n = 58$) was improved when compared with that of mice receiving adjuvant alone ($n = 42$). **A.** Bacterial load (determined by plating serial 10-fold dilutions of lung homogenates onto Trypticase soy agar) was comparable in the flagellin-immunized and control groups of mice. **B.** In a second set of studies, eight mice immunized with flagellin and eight adjuvant-treated mice were killed 24 hours after the instillation of 2×10^5 bacteria/mouse. Age-matched mice ($n = 8$), not exposed to bacteria, were used as baseline control. **C.** Lungs were excised and cytokine, chemokine and nitrite/nitrate (NOx) levels, as well as myeloperoxidase (an indicator of neutrophil accumulation) and malondialdehyde levels were measured in the homogenates. Immunization against flagellin reduced tumor necrosis factor- α (TNF- α); **(C)**, interleukin-1 β (IL-1 β); **(D)**, IL-6 **(E)**, NOx **(F)** levels in pneumonia and attenuated pulmonary malondialdehyde **(G)**, but not myeloperoxidase (MPO; **H**) levels.

provide an example for the neutralization by polyclonal antibodies of the inflammatory response-inducing ability of flagellin. It is conceivable that similar neutralization may also be achievable by monoclonal antibodies. It remains to be tested whether such antibodies are able to modify the severity of various forms of inflammation, shock and acute respiratory distress syndrome in animal models of disease and, ultimately, in humans.

Other approaches may also be conceivable to counteract flagellin-mediated inflammatory responses. For instance (at least in theory), one could

interfere with the binding of flagellin to the TNFR5 receptor by developing competitive or noncompetitive peptide or small molecule inhibitors or antagonists. So far, no such approach has been made public in the literature. An additional approach may target downstream pathways triggered by TLR5 receptor activation. A distinct feature of this latter approach may be its broad nature: as noted above, signal transduction pathways such as NF- κ B are shared between many proinflammatory ligand/receptor systems, including the TLR4 and TLR2 systems, and many others.

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Lucas Liaudet, Amitabha Deb, Pál Pacher, Jon G. Mabley, Kanneganti G.K. Murthy and Andrew L. Salzman are researchers and Csaba Szabó M.D., Ph.D., is CSO and Director of Research and Development at Inotek Pharmaceuticals Corporation, 100 Cummings Center, Suite #419E, Beverly, MA 01915, U.S.A. Dr. Szabó is also Professor of Surgery at the University of Medicine and Dentistry of New Jersey, Newark, U.S.A. E-mail: szabocsaba@aol.com