

# Bacterial Flagellin Stimulates Toll-Like Receptor 5–Dependent Defense against Vancomycin-Resistant *Enterococcus* Infection

Melissa A. Kinnebrew,<sup>1</sup> Carles Ubeda,<sup>1</sup> Lauren A. Zenewicz,<sup>2</sup> Nichole Smith,<sup>1</sup> Richard A. Flavell,<sup>2,3</sup> and Eric G. Pamer<sup>1</sup>

<sup>1</sup>Infectious Diseases Service, Department of Medicine, Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York;

<sup>2</sup>Department of Immunobiology, Yale School of Medicine, New Haven, Connecticut; <sup>3</sup>Howard Hughes Medical Institute, Chevy Chase, Maryland

**Treatment of vancomycin-resistant *Enterococcus* (VRE) infections is limited by the paucity of effective antibiotics. Administration of broad-spectrum antibiotics promotes VRE colonization by down-regulating homeostatic innate immune defenses. Intestinal epithelial cells and Paneth cells express antimicrobial factors on direct or indirect stimulation of the Toll-like receptor (TLR)–myeloid differentiation factor 88–mediated pathway by microbe-derived molecules. Here, we demonstrate that the TLR5 agonist flagellin restores antibiotic-impaired innate immune defenses and restricts colonization with VRE. Flagellin stimulates the expression of RegIII $\gamma$ , a secreted C-type lectin that kills gram-positive bacteria, including VRE. Systemic administration of flagellin induces RegIII $\gamma$  expression in intestinal epithelial cells and Paneth cells along the entire length of the small intestine. Induction of RegIII $\gamma$  requires TLR5 expression in hematopoietic cells and is dependent on interleukin 22 expression. Systemic administration of flagellin to antibiotic-treated mice dramatically reduces VRE colonization. By enhancing mucosal resistance to multidrug-resistant organisms, flagellin administration may provide a clinically useful approach to prevent infections in patients treated with broad-spectrum antibiotics.**

The increase of vancomycin-resistant *Enterococcus* (VRE) infections has increased at an alarming rate in the last 2 decades [1, 2]. Infection with VRE is associated with increased mortality, health care costs, and hospital stays and thus places significant burdens on the health care system [3]. Exposure to certain antibiotics leads to an increased risk of VRE colonization

and infection [4–7]. Although colonization resistance resulting from the depletion of commensal bacteria during antibiotic treatment has been cited as the reason for this association, additional mechanisms have also been implicated. Recent studies demonstrate that the intestinal microbial flora plays a critical role in regulating mucosal innate immunity [8–12]. Antimicrobial proteins (AMPs) produced by the intestinal epithelium are effector molecules that provide a first line of defense against invading commensal and pathogenic organisms. Intestinal epithelial cells and Paneth cells directly respond to bacterial products via Toll-like receptor (TLR)–MyD88-mediated pathways by secreting AMPs to kill bacteria [9, 13–15]. In addition, cells of the adaptive and innate immune system produce cytokines, such as interleukin 22 (IL-22), that regulate the expression of AMPs by intestinal epithelial cells [16].

The antimicrobial protein RegIII $\gamma$  is a secreted C-type lectin that has bactericidal activity against gram-positive bacteria and that is induced by intestinal commensal bacteria [13]. We have previously demonstrated that RegIII $\gamma$  kills VRE and *Listeria monocytogenes* in

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Reprints or correspondence: Dr Eric G. Pamer, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, Box 9, New York, NY 10065 (pamere@mskcc.org).

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the lumen of the small intestine [17, 18]. Neutralization of RegIII $\gamma$  activity with polyclonal antiserum specific to RegIII $\gamma$  increases VRE survival in the murine intestine. Antibiotic-mediated depletion of commensal bacteria dramatically reduces expression of RegIII $\gamma$ , leading to increased susceptibility to VRE; however, the delivery of recombinant RegIII $\gamma$  to the intestinal lumen reestablishes VRE killing [18]. Furthermore, the loss of commensal flora can be compensated by oral administration of lipopolysaccharide (LPS), which restores RegIII $\gamma$  expression and thus promotes VRE clearance [18]. These studies establish the important role played by RegIII $\gamma$  in limiting the early stages of VRE colonization.

A major concern with therapeutic use of TLR agonists—in particular LPS—is the potential to initiate inflammation and sepsis. Unlike LPS, bacterial flagellin (the ligand for TLR5) induces little tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\alpha$ , and RANTES (regulated on activation, normal T cell expressed and secreted) expression and does not lead to acute lung injury and sepsis on administration of moderate doses [19, 20]. Moreover, systemically administered flagellin stimulates the intestinal innate immune system and protects against the destruction of the intestinal epithelium by radiation, chemicals, and invasive pathogens [19, 21]. Unlike most other TLR ligands, flagellin is a protein and, consequently, would likely be degraded upon oral ingestion. Thus, previous studies have administered flagellin systemically. Furthermore, TLR5 expression is limited to a subset of dendritic cells in the lamina propria [22] and to the basolateral surface of epithelial cells; therefore, it is not accessible to flagellin in the intestinal lumen [23].

Here, we demonstrate that TLR5 activation restores the innate immune deficits that follow antibiotic-mediated depletion of commensal bacteria. Flagellin potently induces RegIII $\gamma$  expression in intestinal epithelial cells and Paneth cells along the length of the small intestine. Activation of TLR5-expressing hematopoietic cells and expression of IL-22 are required for flagellin-mediated RegIII $\gamma$  induction. We show that systemically administered flagellin protects antibiotic-treated mice against VRE colonization. These results suggest that flagellin administration may play a therapeutic role in the prevention of antibiotic-associated intestinal infections.

## METHODS

**Mice and bacteria.** C57BL/6 mice (6–8 weeks old) and interleukin 10 receptor  $\beta$  (IL-10R $\beta$ )-deficient breeding pairs were purchased from Jackson Laboratories. TLR5-deficient mice and IL-22-deficient mice were provided by R. Flavell (Yale University, New Haven, Connecticut) [24]. Mice were maintained in a specific pathogen-free barrier facility at Memorial Sloan-Kettering Cancer Center Research Animal Resource Center. Experiments followed approved institutional guidelines. Age- and sex-matched controls were used for all experiments. An Amer-

ican Type Culture Collection (ATCC) isolate of vancomycin-resistant *Enterococcus faecium* (stock 700221) was used for oral infections.

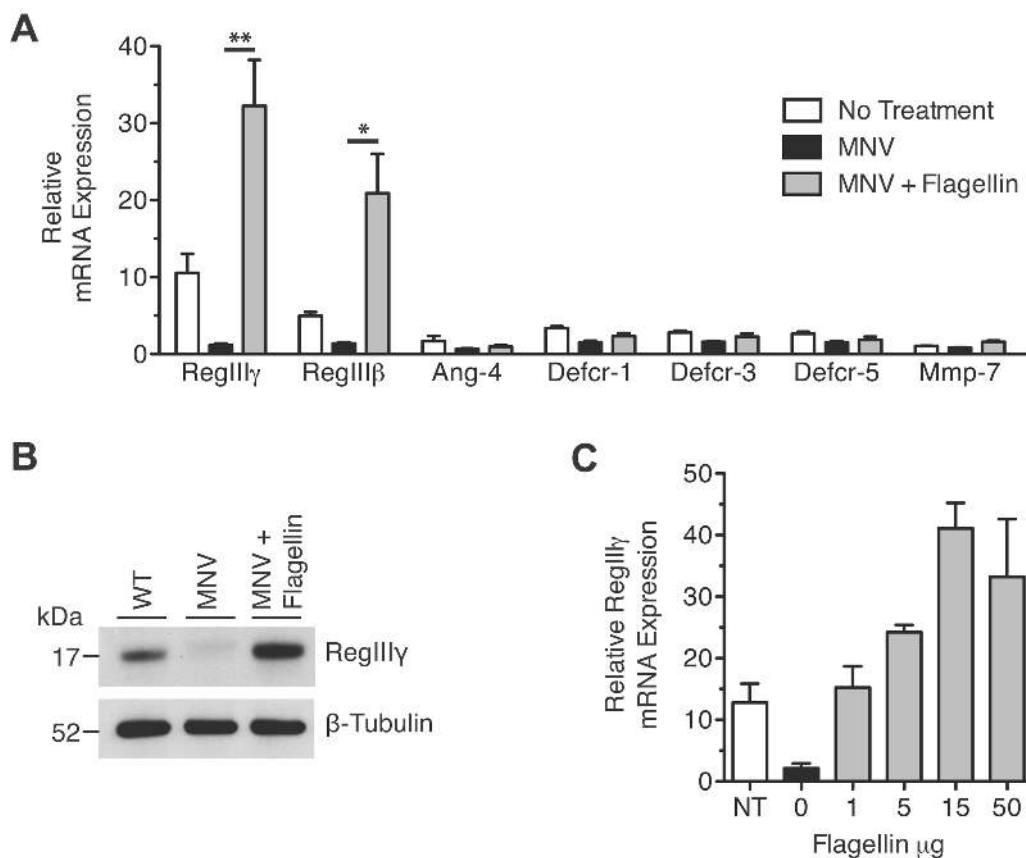
**Flagellin.** For most experiments, commercially available flagellin (InVivoGen), derived from *Salmonella typhimurium*, was used. For the dose-response experiment, flagellin was also purified from *S. typhimurium* ATCC strain 15277, using a procedure described elsewhere [25]. Contaminating LPS was removed from the flagellin preparation by serial passage through Detoxi-Gel AffinityPak columns (Thermo Scientific). The concentration of LPS was determined to be  $<2$  pg/ $\mu$ g of flagellin.

**Treatment with antibiotics and flagellin.** Drinking water was supplemented with 1 g/L vancomycin (Sigma), 0.5 g/L neomycin sulfate (Sigma), and 1 g/L metronidazole (Baxter) and provided to mice for 7 days. Unless noted otherwise, flagellin was administered by intraperitoneal injection of 15  $\mu$ g for 3 days starting on day 5 of antibiotic treatment. Mice were killed 24 h after the last flagellin injection.

**Sample collection.** For the duodenum, a 2-cm segment was excised 1 cm distal to the pylorus. For the jejunum, a 2-cm segment was collected from the exact middle of the small intestine measured longitudinally. For the ileum, a 2-cm segment located 1 cm proximal to the ileocecal valve was excised. The luminal content was flushed with phosphate-buffered saline (PBS) before tissue processing.

**Real-time polymerase chain reaction and Western blot analysis.** Freshly isolated tissue was homogenized in Trizol reagent (Invitrogen). The manufacturer's protocol for total RNA and protein extraction using Trizol was followed. DNase-treated RNA was reverse-transcribed using Oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with gene-specific Quantitect primer assays (Qiagen) and the DyNAmo SYBR Green qPCR kit (Finnzymes). Signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) transcript levels, and gene expression relative to controls treated with a combination of metronidazole, neomycin, and vancomycin (MNV) was quantitated by  $\Delta\Delta C_t$  analysis unless otherwise noted.

The extracted protein was reconstituted in a buffer of 8 mol/L urea, 1% sodium dodecyl sulfate (SDS), 0.15 mol/L Tris-HCl at pH 7.5. Identical amounts of protein were loaded on a 4%–12% SDS-polyacrylamide gel electrophoresis gel (Nupage Bis-Tris gel; Invitrogen) and transferred to a nitrocellulose membrane. RegIII $\gamma$  and the loading control protein were detected by rabbit polyclonal RegIII $\gamma$ -specific antiserum and mouse anti- $\beta$ -tubulin antibodies (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated anti-rabbit (GE Healthcare) and anti-mouse (Santa Cruz Biotechnology) antibodies. Immunostaining was revealed by chemiluminescence (GE Healthcare).



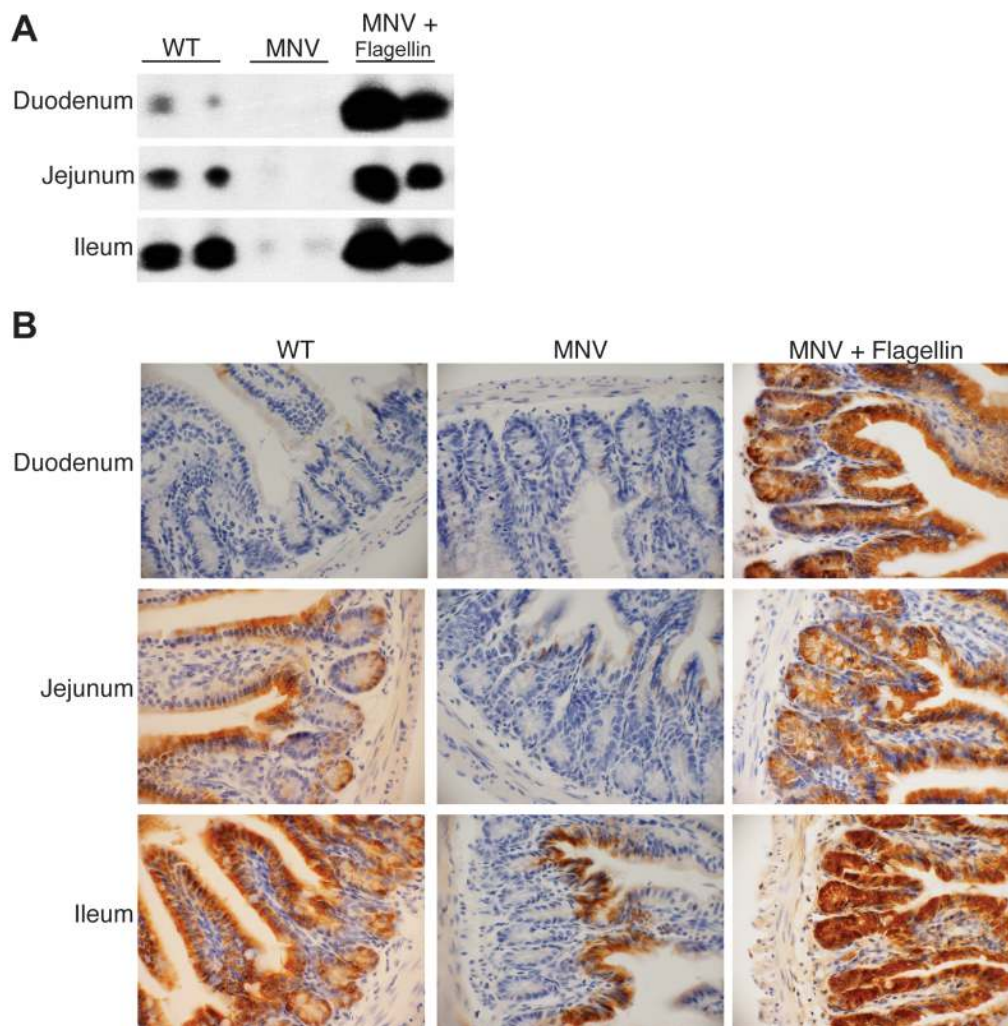
**Figure 1.** Induction of RegIII $\gamma$  and RegIII $\beta$  by flagellin in antibiotic-treated mice. *A*, Relative messenger RNA (mRNA) expression. Mice were treated with metronidazole, neomycin, and vancomycin (MNV) for 7 days. Starting on day 5 of antibiotic treatment, mice received 15  $\mu$ g of flagellin per day via intraperitoneal injection for 3 days. Mice were killed 24 h after the last flagellin injection. Transcriptional expression of RegIII $\gamma$ , RegIII $\beta$ , angiogenin-4 (Ang-4), defensin-related cryptdin 1 (Defcr-1), defensin-related cryptdin 3 (Defcr-3), defensin-related cryptdin 5 (Defcr-5), and matrilysin 7 (MMP-7) was measured by quantitative real-time polymerase chain reaction (PCR). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and determined relative to MNV-treated mice. Values are representative of at least 2 experiments and are expressed as means  $\pm$  standard errors of the mean ( $n = 4-9$ ). \* $P < .05$  and \*\* $P < .01$  by 1-way analysis of variance with the Bonferroni correction. *B*, Western blot results. Protein extracts from the distal ileum were analyzed by Western blotting with RegIII $\gamma$ -specific antiserum and anti- $\beta$ -tubulin as a loading control. Shown are representative samples from 2 experiments ( $n = 6$  for each group). *C*, Dose dependency. On day 7 of MNV treatment, mice received 0, 1, 5, 15, or 50  $\mu$ g of flagellin intraperitoneally. RegIII $\gamma$  mRNA levels were measured 24 h later by quantitative real-time PCR and were normalized to GAPDH ( $n = 5$  for each group). NT, not treated.

**Immunohistochemical analysis.** Freshly isolated tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5  $\mu$ m thickness were deparaffinized and immunohistochemically stained for RegIII $\gamma$ , using the EnVision + System-HRP kit (DakoCytomation). Polyclonal RegIII $\gamma$ -specific antiserum (1:1000) was diluted in PBS with 1% bovine serum albumin for staining. Sections were counterstained with hematoxylin.

**Generation of bone marrow chimeric mice.** Wild-type (CD45.1) or TLR5-deficient (CD45.2) recipient mice were lethally irradiated with 950 rad. Irradiated mice were injected via the tail vein with  $5 \times 10^6$  bone marrow cells derived from wild-type or TLR5-deficient donor mice. Chimerism was assessed in spleen and intestinal lamina propria 7 weeks after engraftment by flow cytometry analysis, at which time the mice were used for experiments. Lamina propria leukocytes were har-

vested according to a protocol described elsewhere [26]. Percent reconstitution was assessed by surface staining with allophycocyanin-labeled CD45.1-specific antibody and peridinin chlorophyll protein/cyanine 5.5-labeled CD45.2-specific antibody; flow cytometry analysis was performed using a BD LSR II cytometer (BD Biosciences). Mice exhibited at least 90% reconstitution with donor-derived bone marrow cells in the intestinal lamina propria (data not shown).

**VRE infection.** Mice were infected with  $1 \times 10^{10}$  colony-forming units of VRE via oral gavage on day 7 of antibiotic treatment. Twenty-four hours later, mice were killed. The distal half of the small intestine was flushed with 10 mL of PBS to collect the luminal content for plating. The intestinal wall was homogenized in PBS containing 0.1% Triton X-100 for plating. Samples were plated in serial dilutions on Enterococcosel agar



**Figure 2.** Change in the regional expression of RegIII $\gamma$  in the small intestine after flagellin administration. Mice were treated with metronidazole, neomycin, and vancomycin (MNV) for 7 days, and on day 5 flagellin was given intraperitoneally at 15  $\mu$ g per day for 3 days. Tissues from the duodenum, jejunum, and ileum were collected for Western blot analysis and immunohistochemical analysis. *A*, Protein extracts as analyzed by Western blotting using RegIII $\gamma$ -specific antiserum. Each lane is for a representative mouse from the indicated group. *B*, Immunohistochemical analysis. RegIII $\gamma$  was detected in paraffin-embedded tissue using immunohistochemistry with polyclonal RegIII $\gamma$ -specific antiserum. Positive cells were stained brown (original magnification,  $\times 400$ ). The data are representative of 2 independent experiments ( $n = 6$ ). WT, wild type.

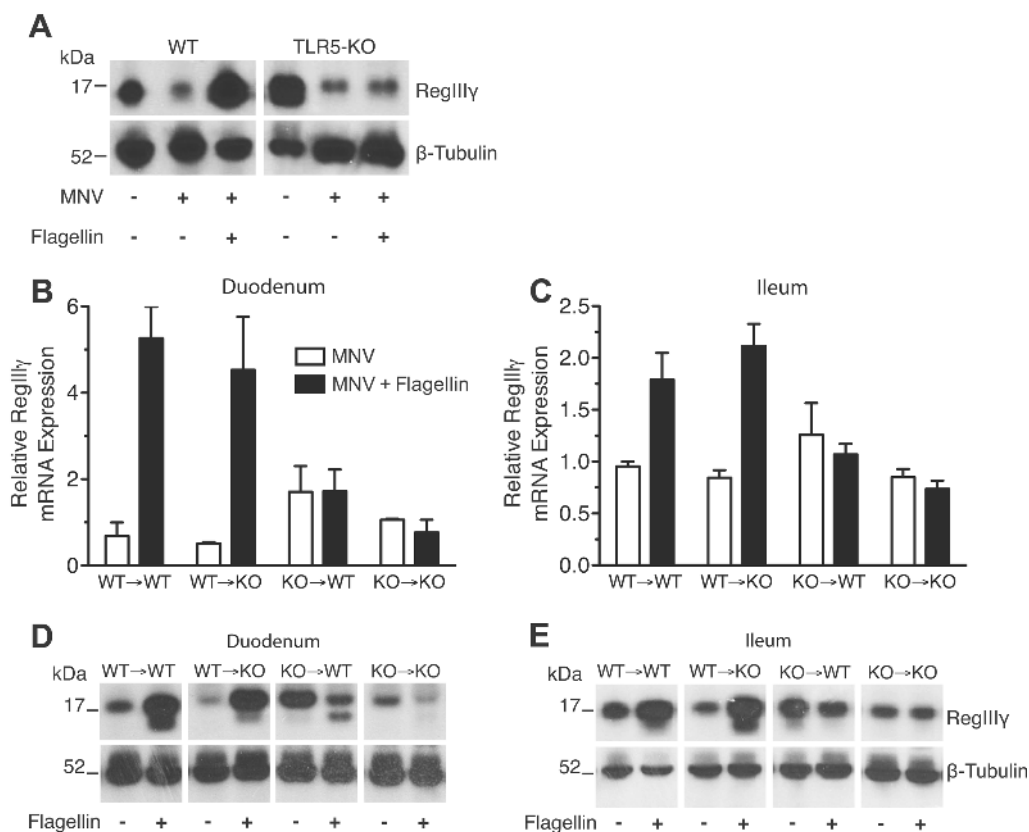
(Difco) plates supplemented with 8  $\mu$ g/mL vancomycin. Colonies surrounded by a brownish-black zone were counted.

**Statistical analysis.** Prism software (version 5a; GraphPad Software) was used to perform the statistical analysis. *P* values  $\leq .05$  were considered significant.

## RESULTS

**Induction of RegIII $\gamma$  expression by flagellin in vivo.** To determine whether flagellin can boost intestinal innate immune defense after broad-spectrum antibiotic administration, we measured mRNA levels of AMPs, previously shown to be regulated by commensal bacteria, in the distal small intestine of antibiotic-treated mice [13]. Mice were given MNV in drinking

water. MNV-treated mice were given intraperitoneal injections of flagellin derived from *S. typhimurium*, after which mRNA transcripts encoding AMPs were quantified in the small intestine. Quantitative PCR analysis showed a 30-fold induction of RegIII $\gamma$  mRNA transcripts in response to flagellin administration (Figure 1A). Western blot analysis also revealed substantial increases in RegIII $\gamma$  protein expression (Figure 1B). Similarly, mRNA levels for RegIII $\beta$ , a RegIII family member that shares  $\sim 70\%$  homology with RegIII $\gamma$ , was also strongly up-regulated by flagellin (Figure 1A). Antibiotics or flagellin did not induce significant differences in the expression of other AMPs, including defensin-related cryptidins, angiogenin-4, and matri-lysin 7 (Figure 1A). Because RegIII $\gamma$  directly mediates resis-



**Figure 3.** Requirement of Toll-like receptor 5 (TLR5)-mediated signaling in hematopoietic cells for flagellin-induced RegIII $\gamma$  expression. Mice were treated with metronidazole, neomycin, and vancomycin (MNV) for 7 days. After 5 days of MNV treatment, mice received 3 doses of 15  $\mu$ g of flagellin per day via intraperitoneal injection. *A*, Western blot analysis of protein extracts from the distal ileum, used to assess differences between wild-type (WT) and knockout (KO) mice with RegIII $\gamma$ -specific antiserum. Each lane is for a representative mouse from the indicated group. The data are representative of 2 independent experiments ( $n = 5$ ). *B–E*, Experiments in reconstituted mice. Bone marrow chimeric mice were treated with MNV and flagellin as described above  $\sim$ 7 weeks after lethal irradiation and bone marrow transfer. RegIII $\gamma$  messenger RNA (mRNA) levels were measured in the duodenum (*B*) and ileum (*C*) by quantitative real-time polymerase chain reaction, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, and expressed as means  $\pm$  standard errors of the mean. RegIII $\gamma$  protein levels in the duodenum (*D*) and ileum (*E*) were assessed by Western blot analysis using RegIII $\gamma$ -specific antiserum. Each lane of the Western blots is for a representative mouse from the indicated group. Data are representative of 2 independent experiments ( $n = 2–3$ ).

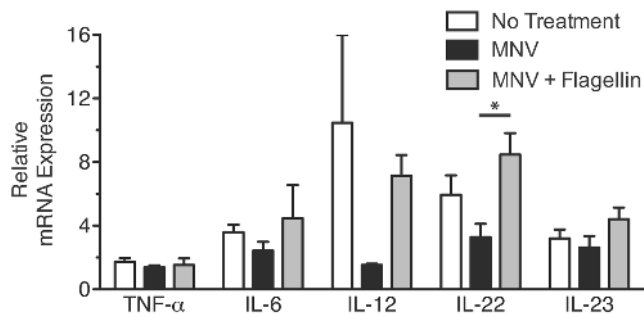
tance to VRE infection, we focused on further characterization of the RegIII $\gamma$  response.

To determine whether flagellin-induced RegIII $\gamma$  expression is dose-dependent, we administered single doses of varying amounts of flagellin to MNV-treated mice. RegIII $\gamma$  mRNA levels were significantly increased at doses as low as 1  $\mu$ g per mouse ( $P = .004$ ), compared with those in MNV-treated mice receiving no flagellin. Induction of RegIII $\gamma$  by flagellin plateaued at a dose of 15  $\mu$ g per mouse (Figure 1C).

**Change in the regional expression pattern of RegIII $\gamma$  in the small intestine after administration of flagellin.** In the small intestine, the pattern of RegIII $\gamma$  expression, along with other AMPs, reflects the density of microbial colonization, which increases in the cephalocaudal direction [13]. Under normal circumstances, RegIII $\gamma$  expression increases along this axis with the highest expression in the distal ileum [13]. Because flagellin

rapidly enters the bloodstream when administered via intraperitoneal injection [27], we asked whether the systemic administration of flagellin to antibiotic-treated mice would alter the expression pattern for RegIII $\gamma$  in the small intestine. Western blot analysis of protein extracts from the duodenum, jejunum, and ileum of MNV-treated mice showed that flagellin strongly up-regulated RegIII $\gamma$  protein levels throughout the length of the small intestine (Figure 2A).

Immunohistochemical analysis of RegIII $\gamma$  protein expression revealed staining of epithelial cells and Paneth cells of the jejunum and ileum of wild-type mice; however, RegIII $\gamma$  was not detected in the duodenum (Figure 2B). In MNV-treated mice, RegIII $\gamma$  was absent from the Paneth cells throughout the length of the small intestine. Epithelial cells at the base of the villi in the ileum, but not within the intestinal crypts, continued to stain for RegIII $\gamma$ . Systemic administration of flagellin to MNV-



**Figure 4.** Induction of cytokines by flagellin. Mice were treated with metronidazole, neomycin, and vancomycin (MNV) for 7 days. Three doses of flagellin (15  $\mu$ g per day) were administered intraperitoneally to mice beginning on day 5 of MNV treatment. Messenger RNA (mRNA) was extracted from the distal ileum to assess cytokine induction after flagellin treatment by quantitative real-time PCR. Interleukin 12 (IL-12) refers to IL-12p35, and interleukin 23 (IL-23) refers to IL23p19. Data were pooled from 2 independent experiments with a total of 4 mice per group. Levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and are expressed as means  $\pm$  standard errors of the mean. \* $P < .05$ , by 1-way analysis of variance with the Bonferroni correction.

treated mice induced RegIII $\gamma$  expression in epithelial cells and Paneth cells throughout the small intestine. Flagellin administration did not induce detectable inflammatory cell recruitment to the lamina propria of the small intestine, suggesting that RegIII $\gamma$  expression does not result from a general inflammatory response.

**Requirement of TLR5-expressing hematopoietic cells for induction of RegIII $\gamma$  by flagellin.** Because the flagellin used in these experiments was purified from *S. typhimurium*, we needed to ensure that the response to flagellin is mediated by TLR5 and not by contaminating bacterial products. To address this possibility, we measured RegIII $\gamma$  protein levels in TLR5-deficient mice and wild-type control mice receiving MNV supplemented with flagellin injections. Whereas wild-type controls treated with both MNV and flagellin showed an increase in RegIII $\gamma$  protein levels compared with wild-type mice treated only with MNV, MNV-treated TLR5-deficient mice did not show an increase in RegIII $\gamma$  expression after flagellin administration (Figure 3A). RegIII $\gamma$  protein levels in TLR5-deficient mice were comparable to those in wild-type controls and were similarly down-regulated on antibiotic treatment.

To determine whether intestinal epithelial cells are directly or indirectly activated by flagellin, we generated bone marrow chimeric mice by transferring bone marrow from TLR5-deficient or wild-type mice into lethally irradiated wild-type or TLR5-deficient recipient mice. After mice were treated with MNV and flagellin as described above, Western blot analysis and quantitative PCR were performed on tissue harvested from the duodenum and ileum. Flagellin induced RegIII $\gamma$  expression in TLR5-deficient mice reconstituted with bone marrow from

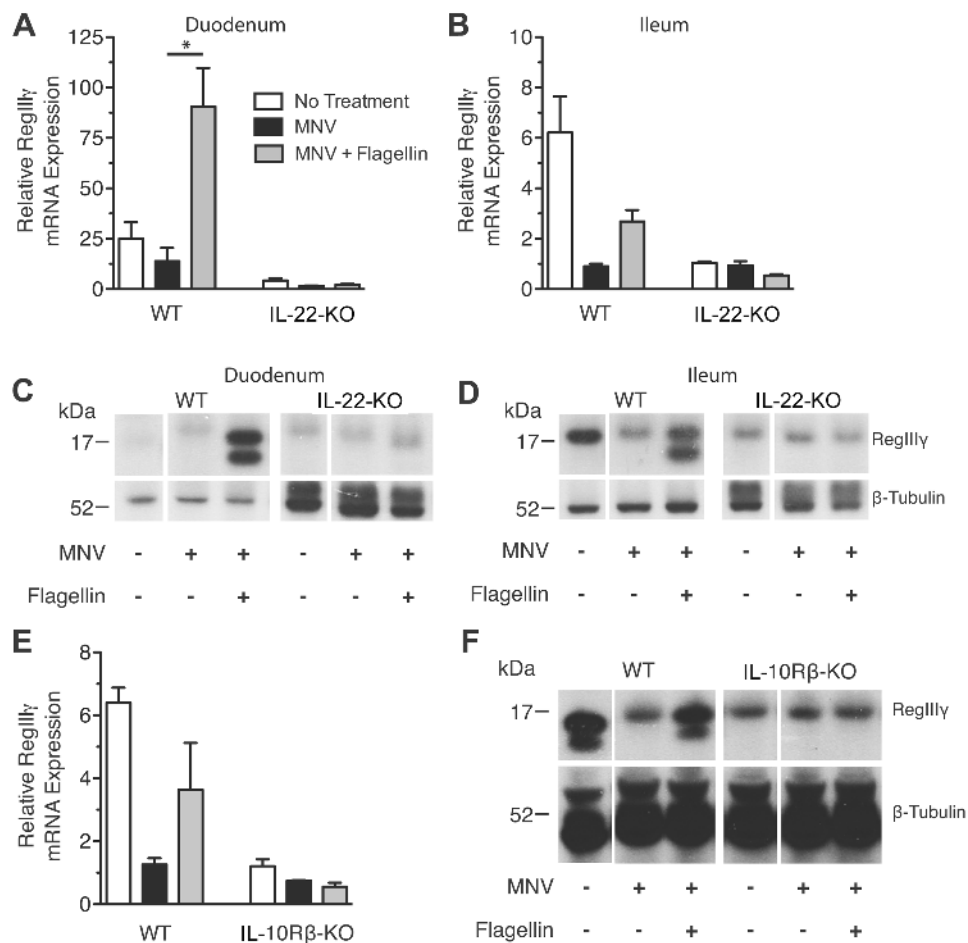
wild-type mice (Figure 3B–3E). These mice expressed TLR5 exclusively in hematopoietic cells, including lymphoid- and myeloid-derived cells. However, RegIII $\gamma$  was not induced by flagellin in wild-type mice reconstituted with TLR5-deficient bone marrow in which TLR5 was expressed predominately in non-hematopoietic tissues, including epithelial and stromal cells. These results show that flagellin-mediated RegIII $\gamma$  up-regulation requires TLR5 activation in cells of hematopoietic lineage.

**Requirement of IL-22 in flagellin-mediated RegIII $\gamma$  induction.**

Because flagellin-mediated RegIII $\gamma$  induction does not involve direct stimulation of TLR5-expressing epithelial cells, we reasoned that a cytokine produced by flagellin-activated hematopoietic cells transmits signals to epithelial cells to induce RegIII $\gamma$ . Therefore, in the distal ileum we measured levels of TNF- $\alpha$ , interleukin 6, interleukin 12, and interleukin 23, which have been shown to be induced by flagellin *in vivo* and are known to have direct and indirect effects on epithelial cells [19–21, 28]. We also determined whether flagellin induces IL-22, a cytokine that stimulates RegIII $\gamma$  and RegIII $\beta$  up-regulation during intestinal infection with the murine pathogen *Citrobacter rodentium* [29]. Analysis of mRNA transcripts for these cytokines in the small intestine revealed that flagellin modestly induced expression of IL-22 (Figure 4). In addition, IL-12 transcript levels followed a similar trend, but the level of induction did not reach statistical significance.

IL-22 is an IL-10–related cytokine that is produced by innate and adaptive immune cells and that targets epithelial cells of the digestive, respiratory, urinary, and integumentary systems, where the heterodimeric IL-22 receptor is highly expressed [30]. IL-22 has been shown to directly activate innate immune defenses and promote the proliferation and survival of epithelial cells [24]. To determine whether IL-22 plays a role in flagellin-mediated RegIII $\gamma$  induction, we compared RegIII $\gamma$  expression in MNV-treated wild-type and IL-22-deficient mice after flagellin administration. Untreated IL-22-deficient mice expressed low RegIII $\gamma$  protein and mRNA transcript levels in the distal ileum compared with wild-type mice, suggesting that IL-22 contributes to homeostatic expression of RegIII $\gamma$  (Figure 5B and 5D). Flagellin did not induce RegIII $\gamma$  expression in duodenum or ileum of IL-22-deficient mice, indicating that TLR5 stimulation of RegIII $\gamma$  expression is dependent on IL-22 (Figure 5A–5D). The administration of recombinant IL-22 to MNV-treated mice also induced RegIII $\gamma$  expression in epithelial cells throughout the small intestine in the same pattern as flagellin administration (Figure 6A–6C).

To confirm the requirement of IL-22 signaling for RegIII $\gamma$  induction, we measured RegIII $\gamma$  levels in mice lacking the IL-10R $\beta$  chain of the heterodimeric IL-22R. These mice are deficient in both IL-10 and IL-22 signaling. RegIII $\gamma$  protein and mRNA transcript levels were low in the ileum of IL-10R $\beta$ -deficient mice



**Figure 5.** Requirement of interleukin 22 (IL-22) for flagellin-mediated RegIII $\gamma$  expression. Wild-type (WT) and interleukin 22 (IL-22)-knockout [KO] or interleukin 10 receptor  $\beta$  (IL-10R $\beta$ )-deficient (IL-10R $\beta$ -KO) mice were administered metronidazole, neomycin, and vancomycin (MNV) for 7 days. Mice received 15  $\mu$ g of flagellin intraperitoneally on days 6 and 7 of MNV treatment. Tissue from the duodenum (A and C) and ileum (B and D–F) were collected for messenger RNA (mRNA) and protein extraction. A, B, and E, Results of quantitative polymerase chain reaction (PCR) was used to evaluate RegIII $\gamma$  mRNA transcript expression. Levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and expressed as means  $\pm$  standard errors of the mean. \* $P$  < .05, by 1-way analysis of variance with the Bonferroni correction. C, D, and F, Results of Western blot analysis with RegIII $\gamma$ -specific antiserum, used to assess RegIII $\gamma$  protein levels. Each lane is for a representative sample from a mouse from the indicated group. In panels A–D, data were pooled from 2 independent experiments ( $n = 8$ –10).

and were not affected by antibiotic or flagellin administration compared with wild-type controls (Figure 5E and 5F).

**Reduction in VRE colonization after administration of flagellin.** To determine whether flagellin-induced RegIII $\gamma$  expression in the small intestine restores resistance to VRE colonization in mice treated with antibiotics, mice receiving MNV were given flagellin intraperitoneally and inoculated with VRE via oral gavage. Higher levels of VRE were recovered from the wall and lumen of the distal small intestine of MNV-treated mice compared with untreated mice (Figure 7A and 7B). However, administration of flagellin to MNV-treated mice significantly reduced VRE survival in the intestinal lumen and wall ( $P < .001$ ), compared with that in mice treated only with MNV. These results demonstrate that administering flagellin to an-

tibiotic-treated mice before VRE infection can reduce VRE colonization to levels observed in mice not treated with antibiotics.

## DISCUSSION

Recent studies have revealed that antibiotic treatment compromises the innate immune system of the intestinal mucosa by depleting commensal microbes that normally stimulate epithelial cells to produce homeostatic levels of AMPs [9, 18]. Here, we asked whether the TLR5 ligand flagellin could restore innate immune deficits caused by treatment with broad-spectrum antibiotics. We show that flagellin administration induces RegIII $\gamma$  in epithelial cells along the entire length of the small intestine of antibiotic-treated mice. The extensive up-regulation

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases*.

**Figure 6.** Induction of RegIII $\gamma$  in the small intestine after administration of recombinant mouse interleukin 22 (rIL-22).

of RegIII $\gamma$  was likely caused by systemic stimulation of TLR5 and secretion of IL-22. In contrast, RegIII $\gamma$  expression is limited to the ileum under steady-state conditions because of the higher density of bacteria in the distal small intestine compared with more proximal regions [9, 13]. Our results also show that TLR5-expressing hematopoietic cells mediate flagellin-induced RegIII $\gamma$  expression. In accordance, we found that IL-22 is also required for the response, suggesting that flagellin-stimulated hematopoietic cells produce IL-22, which signals intestinal epithelial cells to express RegIII $\gamma$ . Consistent with previous studies demonstrating that oral LPS induces RegIII $\gamma$  in the small intestine and enhances resistance to VRE infection, we demonstrate that systemically administered flagellin reduces susceptibility to VRE colonization in antibiotic-treated mice.

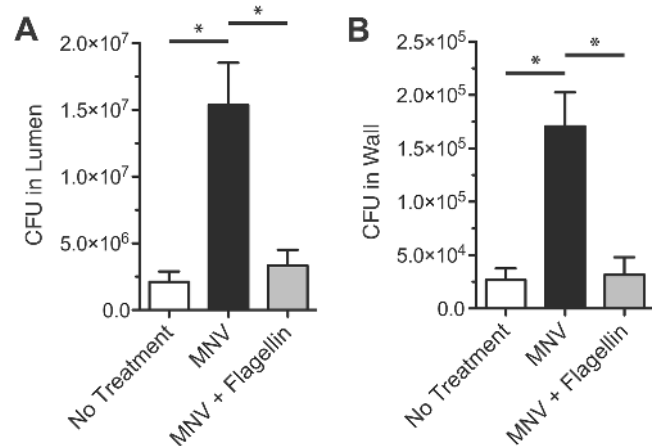
Although we have previously demonstrated that antibody-mediated blockade of RegIII $\gamma$  significantly diminishes *in vivo* killing of both VRE and *Listeria monocytogenes* [17, 18], RegIII $\gamma$  may not solely mediate flagellin's protective effects. RegIII $\beta$  is also up-regulated in response to flagellin administration. Although RegIII $\beta$  has no reported bactericidal activity, it binds to peptidoglycan and may contribute to flagellin-mediated protection against VRE [9]. Flagellin induces several cytokines that may promote VRE clearance. In addition, MyD88-mediated signals in the gut promote the repair and maintenance of the mucosal barrier [11, 19]. Together, these responses may also contribute to flagellin-mediated resistance to VRE colonization.

Both hematopoietic and nonhematopoietic cells of the intestine respond to flagellin via the TLR5-MyD88-mediated pathway [23, 31]. In the case of hematopoietic cells, the high level of TLR5 expression in the small intestine has been attributed to a subset of CD11c<sup>hi</sup>CD11b<sup>hi</sup> lamina propria dendritic cells that are important for immunoglobulin A production and type 17 T helper (Th17) cell development [22, 32]. Given that RegIII $\gamma$  expression under normal conditions depends on direct detection of commensal bacteria by intestinal epithelial cells via the TLR-MyD88-mediated pathway [9, 17], we were surprised to find that TLR5 activation of hematopoietic cells is required for flagellin-mediated RegIII $\gamma$  expression. Our results using systemic flagellin administration support an alternative pathway of RegIII $\gamma$  induction. IL-22-mediated RegIII $\gamma$  expression induced by the presence of TLR ligands within sub-epithelial tissues, as opposed to stimulation from the luminal side of the epithelium, may play a critical role in alerting epithelial cells to the loss of mucosal integrity or the presence of systemic infection. Whereas RegIII $\gamma$  expression regulated by

apical TLR stimulation is tightly controlled [18], IL-22 expression may be more indiscriminate with respect to stimulation by a variety of microbe-derived molecules once the epithelial barrier has been breached [29, 33]. Additional work is needed to identify the hematopoietic cell subsets responsible for flagellin-induced RegIII $\gamma$  expression. The candidates for the source of IL-22 include Th17 cells,  $\gamma\delta$  T cells, natural killer cells, and lymphoid tissue-inducer cells [34–38].

The protective effects of flagellin against numerous challenges, including lethal irradiation, chemical damage, and infectious agents, have been well described [19, 21]; however, the mechanism of TLR5-mediated protection has remained undefined. Prophylactic systemic administration of a TLR5 agonist results in dramatic survival after lethal irradiation [21]. High-dose ionizing radiation causes massive cell loss in the hematopoietic system and intestinal mucosa, which leads to invasion by commensal bacteria and fatal septicemia [39, 40]. NF  $\kappa$ B activation protects against lethal irradiation by initiating anti-apoptotic pathways in radiosensitive tissue [41]. The radioprotective effects of flagellin are thought to be mediated by this mechanism [21]; however, on the basis of our study, it is also possible that RegIII $\gamma$  expression induced by TLR5 activation bolsters the impaired mucosal barrier by directly killing invading bacteria.

The therapeutic use of TLR ligands has been approached



**Figure 7.** Reduction in vancomycin-resistant *Enterococcus* (VRE) colonization after administration of flagellin in mice treated with broad-spectrum antibiotics. Mice were treated with metronidazole, neomycin, and vancomycin (MNV) for 7 days. Starting on day 5 of antibiotic treatment, mice received 15  $\mu$ g of flagellin per day via intraperitoneal injection for 3 days. On day 7 of antibiotic treatment, mice were inoculated with  $1 \times 10^{10}$  colony-forming units (CFUs) of VRE by oral gavage. The luminal content (A) and intestinal wall (B) of the distal half of the small intestine were collected for bacterial counts 24 h after inoculation. Data were compiled from 3 independent experiments and expressed as means  $\pm$  standard errors of the mean ( $n = 12$ –14). \* $P < .001$ , by 1-way analysis of variance with the Bonferroni correction.



cautiously because of the potential to stimulate undesired inflammatory responses. Most efforts to manipulate the immune system have targeted the adaptive arm, with a focus on enhancing long-term immunity. The innate immune system, however, can exert a rapid and broad defense against invading organisms and, if properly timed, might be exploited as an approach to ameliorate several clinical problems. For example, TLR ligands have been used as adjuvants in a variety of vaccines, including 2 hepatitis B virus vaccines that use TLR4 agonists to induce a robust memory response [42]. One challenge, however, to the therapeutic use of flagellin is that repeated administration induces antibodies that eventually block TLR5 activation [43]. Therefore, targeting TLR5 might require the development of agonists that do not stimulate neutralizing antibody responses.

Moderate stimulation of TLR5 has not been shown to induce severe sepsis; however, this does not exclude the possibility that flagellin administration may induce inflammation. In controlled trials, flagellin has been administered to humans with few adverse effects [44–46]. In spite of this, it is possible that certain subsets of patients will experience adverse reactions to flagellin treatment. Flagellin-mediated IL-22 induction may exacerbate skin plaques in psoriasis patients [47]. Also, stimulation of TLR5 is suspected to play a role in the pathogenesis of inflammatory bowel disease [48, 49]. Thus, the therapeutic use of flagellin will require extensive clinical study with a particular focus on potential complications resulting from accentuated inflammatory responses.

The commensal flora of the gut plays a critical role in the development and maintenance of a healthy intestinal mucosa. Treatment with broad-spectrum antibiotics greatly diminishes the intestinal microbial flora, leading to increased susceptibility to a variety of bacterial infections, including with VRE and *Clostridium difficile* [4, 7, 50]. The depletion of commensal bacteria results in diminished innate immune defenses, most notably RegIII $\gamma$ , due to reduced activation of TLRs [9, 17, 18]. Our experiments provide additional evidence for the critical role played by TLRs in RegIII $\gamma$  expression and for the ability of TLR activation to reestablish AMP expression that antibiotics have impaired. Our results suggest that flagellin may have therapeutic potential and may prevent intestinal invasion with resistant microbes in patients treated with broad-spectrum antibiotics. RegIII $\gamma$  induction along the small intestine may provide an approach to restrict potentially pathogenic bacteria in the intestinal lumen, thereby limiting colonization and dissemination, both within and between individuals.

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