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Flagellin Treatment Protects against Chemicals, Bacteria, Viruses, and Radiation[1]

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Sudden exposure of human populations to chemicals, pathogens, or radiation has the potential to result in substantial morbidity. A potential means of rapidly protecting such populations might be to activate innate host defense pathways, which can provide broad protection against a variety of insults. However, innate immune activators can, by themselves, result in severe inflammatory pathology, which in large part is driven by hemopoietic-derived cytokines such as TNF-α. We reasoned that, because it preferentially activates epithelial cells, the TLR5 agonist flagellin might not induce severe inflammatory pathology and yet be an ideal agent to provide such non-specific protection, particularly at the mucosal surfaces that serve as a front line of host defense. In accordance, we observed that systemic treatment of mice with purified flagellin did not induce the serologic, histopathologic, and clinical hallmarks of inflammation that are induced by LPS but yet protected mice against chemicals, pathogens, and ionizing radiation. Flagellin-elicited radioprotection required TLR5, the TLR signaling adaptor MyD88, and was effective if given between 2 h before to 4 h after exposure to irradiation. Flagellin-elicited radioprotection was, in part, mediated via effects on cells in bone marrow but yet rescued mortality without a pronounced rescue of radiation-induced anemia or leukopenia. Thus, systemic administration of flagellin may be a relatively safe means of providing temporary non-specific protection against a variety of challenges. ***The Journal of Immunology,*** **2008, 180: 8280–8285.**

Humanity remains vulnerable to large outbreaks of disease caused by radiation, toxic chemicals, viruses, or antibiotic-resistant bacteria. A potential means of rapidly protecting at-risk populations from such outbreaks might be to use agents that activate endogenous pathways of host defense, particularly ones that rapidly induce cytoprotective and/or antimicrobial gene expression. To date, the limited efforts in this area have largely focused on using the TLR4 agonist LPS/endotoxin as a potential means to protect against both irradiation and infectious disease (1). However, LPS administration can be quite dangerous, inducing rapid sepsis at high doses and causing severe lung inflammation in mice at doses as low as 1 mg/kg body weight (2). Furthermore, while LPS potently activates immune cells, it is a poor stimulator of epithelial cells (3), which are often the first cells to interact with most pathogens and chemicals, and thus may not provide optimum protection against these types of challenges, which prominently affect mucosal surfaces. In contrast, the TLR5 agonist flagellin, the molecular subunit of bacterial flagellin, is a potent activator of innate immune signaling pathways in epithelial cells (4) but has generally observed to be a poor activator of hemopoietic cells, such as macrophages and dendritic cells (DC)[3] (5). Although flagellin-treated epithelial cells up-regulate select cytokine expression, cytoprotective genes, and other host-defense factors (6, 7), they have not generally been observed to make significant amounts of the "master" proinflammatory cytokines, such as TNF-α (8), that mediate much of "adverse effects" associated with LPS. Thus, we reasoned that systemic treatment with flagellin might be relatively safe and yet provide broad non-specific protection against a variety of challenges. Herein, we describe that systemic administration of flagellin was indeed safe and protected mice against chemical, bacterial, viral, and radiation challenge.

Materials and Methods

Reagents

Flagellin was purified from *Salmonella typhimurium* (SL3201, *fljB*$^-$) and purity verified as previously described (9). *Escherichia coli* LPS O111:B6 was purchased from Sigma-Aldrich. Like many commercial preparations of LPS, it likely contains contaminating TLR2 agonists (10). D-galactosamine was purchased from Sigma-Aldrich. Dextran sodium sulfate (DSS) was purchased from MP Biomedicals.

Mice

All experiments used 12-wk-old mice that had been purchased from The Jackson Laboratory, except for mice lacking MyD88 and TLR5, which were generated/maintained as previously described (9). All mice were on the C57BL/6 background except for rotavirus experiments, which used 8-wk-old BALB/c mice (C57BL/6 are resistant to rotaviral infection). In *Salmonella* and radiation experiments, any mice that lost >25% of body weight or appeared moribund were euthanized.

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[3] Abbreviations used in this paper: DC, Dendritic cell; FER, flagellin-elicited radioprotection; DSS, dextran sodium sulfate; CBC, complete blood-cell count; BMC, bone marrow cells.

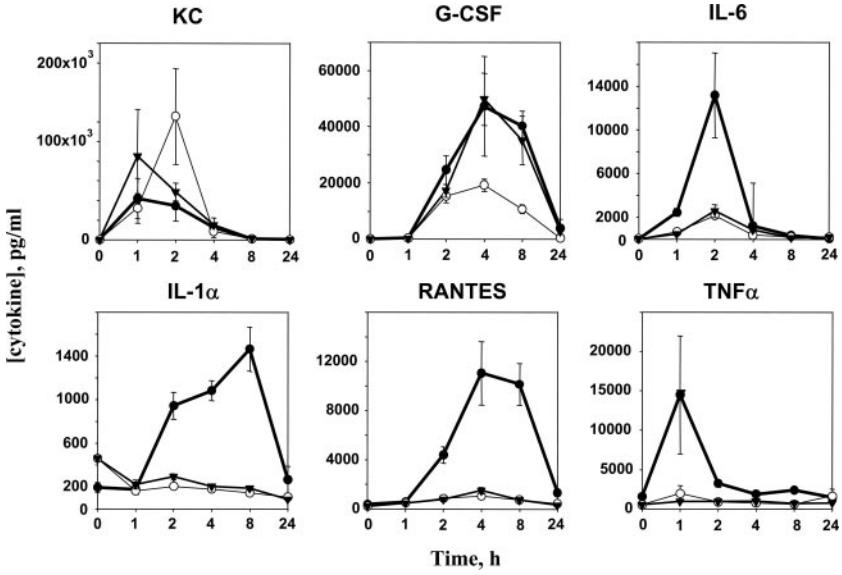

FIGURE 1. Systemic flagellin and LPS induces distinct cytokine profiles. Groups of C57BL/6 mice ($n = 5$) were i.p. injected with LPS (10 μg, filled circles) or flagellin (10 or 50 μg, open circles or triangles, respectively). Sera was isolated at indicated times and cytokine levels measured by bead-based multiplex assay.

Assessment of bioactivity of LPS and flagellin

Mice were i.p. injected with indicated concentration of flagellin or LPS or vehicle (PBS) in a fixed volume of 0.1 ml. Serum was isolated at indicated times via retro-orbital plexus. Cytokines were measured via multiplex bead-based assay on a Luminex 100 (Bioplex Manager 4.0). Body weight and blood glucose using Accu-Chek strips were also measured. In D-galactosamine sensitized sepsis model mice were administered 20 mg of D-galactosamine 15 min before administration of indicated amounts of flagellin or LPS and mortality was monitored for a period of 24 h.

LPS-induced lung injury

Animals were sacrificed 24 h after administration of flagellin or LPS i.p. and lungs harvested for histological analysis and determination of wet:dry ratio. To harvest for histology, the trachea was cannulated and the lungs were fixed by inflation with 4% paraformaldehyde. Following overnight fixation, tissue was embedded in paraffin, sectioned, and stained. For wet:dry analysis, lungs were weighed, desiccated under vacuum overnight at 45°C, and weighed again. The wet lung mass was divided by the dry lung mass to give the wet:dry ratio.

DSS-induced colitis

Mice were administered vehicle (PBS) or 20 μg of flagellin i.p. 2 h before placing them on drinking water containing 2.5% DSS for 7 days, at which time mice were euthanized. Colitis was assessed as previously described (11).

Salmonella *infection*

Mice were administered vehicle (PBS) or 50 μg of flagellin i.p. and, 2 h after, orally colonized with 1×10^6 CFU of *S. typhimurium* (SL3201) that were grown as previously described (12) and monitored for mortality.

Rotavirus infection

Mice were administered vehicle (PBS) or 50 μg of flagellin i.p. and, 2 h after, given orally 0.1 ml of 1.33% sodium bicarbonate and colonized with 1×10^8 PFU Rhesus rotavirus (provided by M. Estes, Baylor University, Houston, TX) and relative level of fecal rotaviral Ags measured by ELISA as previously described (13).

Whole body γ-radiation

Mice were exposed to whole body γ-radiation of 8 Gy using γ Cell 40 ^{137}Cs irradiator at a dose rate of 75 rads/min, except MyD88KO that were given 6 Gy of radiation. Flagellin (50 μg) (or indicated concentration) was administered i.p. 2 h (or indicated time) preceding (or following) irradiation and body weights and mortality was monitored. LPS (10 μg) was administered 10 h before the radiation.

Blood analysis

Mice were bled retro-orbitally in BD Microtainer EDTA tubes (BD Biosciences) and analyzed by HESKA CBC-Diff, Veterinary Hematology System.

Bone-marrow transfers

Donor (C57BL6) mice received 50 μg of flagellin i.p. After 24 h, bone marrow cells (BMC) were isolated from femur in DMEM supplemented with 10% FBS. Recipient mice (C57BL6) mice were given whole body γ-radiation (8.5 Gy). After 3 h, these recipient mice received erythrocyte free BMC (1×10^5 cells/mouse) retro-orbitally and monitored for mortality.

Results

Flagellin elicits a non-pathologic profile of cytokine induction and lacks LPS' tendency to cause adverse events

A major impediment to the potential therapeutic and/or prophylactic use of innate immune activators is their potential to induce dangerous levels of proinflammatory cytokines and, consequently, result in inflammation, multiorgan damage, or even septic shock. This is best documented for LPS, which, when systemically administered, induces the expression of a panel of proinflammatory cytokines referred to as a "cytokine storm" (11). A substantial portion of LPS-induced cytokine secretion is thought to come from macrophages, which are, in vitro, exquisitely sensitive to LPS but are unresponsive to bacterial flagellin (5). In stark contrast, epithelial cells respond robustly to picomolar concentrations of flagellin and are generally unresponsive to LPS (14). Thus, we reasoned that systemic administration of flagellin might not mimic the "cytokine storm" induced by LPS and, consequently, might be less likely to result in acute injury. Indeed, we observed that, compared with LPS, an equal or 5-fold greater amount of systemically administered flagellin by mass (an equimolar dose) induced very little TNF-α, IL-1α, and RANTES and induced only a modest level of IL-6 (Fig. 1). In contrast, compared with LPS, flagellin induced similar levels of G-CSF and induced markedly greater levels of the human IL-8 homologue KC. Thus, flagellin is not necessarily a weaker agonist than LPS, but rather induces a distinct response that might have less potential to cause injury.

Next, we examined whether such systemic treatment with flagellin shared or lacked LPS' potential for toxicity and/or inflammatory pathology. First, we examined whether flagellin treatment induced the acute lung injury induced by LPS. In accordance with our previous work, mice administered *E. coli* LPS via i.p. injection showed marked histopathologic inflammation and pulmonary fluid accumulation within 24 h (2). In contrast, lungs of flagellin-treated mice did not look different from control lungs (Fig. 2, *A* and *B*). Such lack of lung pathology was observed at

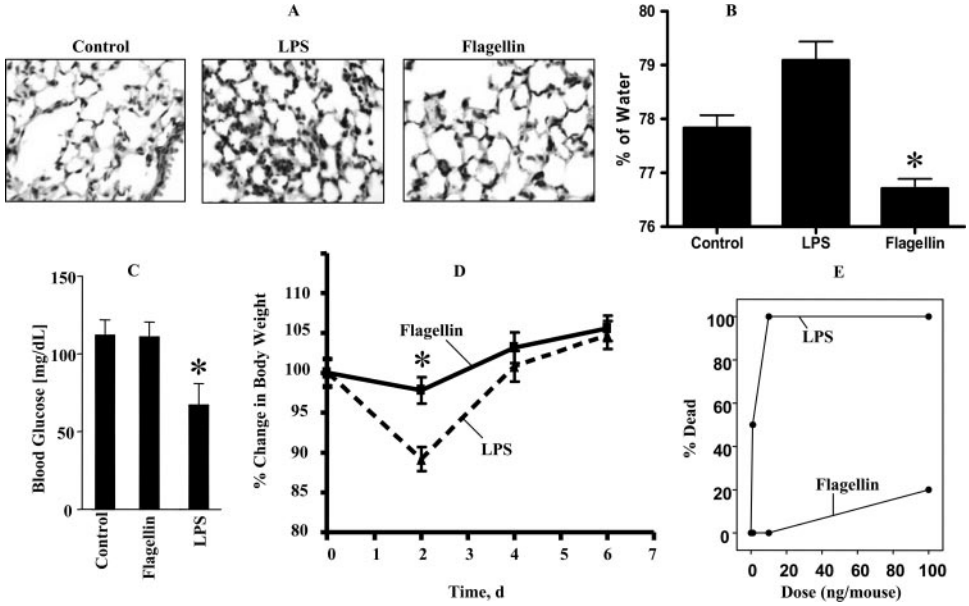

FIGURE 2. Flagellin lacks LPS' ability to induce acute lung injury, weight loss, and septic shock. *A* and *B*, Groups of C57BL/6 mice ($n = 5$) were i.p. injected with 20 μg of LPS or 50 μg of flagellin. Mice were euthanized 24 h later. Lung histopathology (*A*) and water content were assayed (*B*) at 24 h. *C*, Blood glucose was measured at 24 h. *D*, Body weight was followed for 6 days. *E*, Mice were injected with 20 mg D-galactosamine and 15 min later injected with indicated dose of LPS or flagellin. Survival was followed for 48 h.

flagellin dose 25 μg/mouse and following treating mice 10 times with flagellin every 48 h (data not shown). Moreover, flagellin-treated mice lacked the acute hypoglycemia and weight loss that occurs in mice treated with LPS (Fig. 2, *C* and *D*). Lastly, we compared the ability of LPS and flagellin to induce death in a well-characterized model of sepsis in which mice are sensitized by administration of D-galactosamine (15). In stark contrast to LPS, flagellin had only modest ability to induce death in this sepsis model (Fig. 2*E*). Together, these results indicate that, compared with LPS, flagellin lacks high potential to induce severe adverse events.

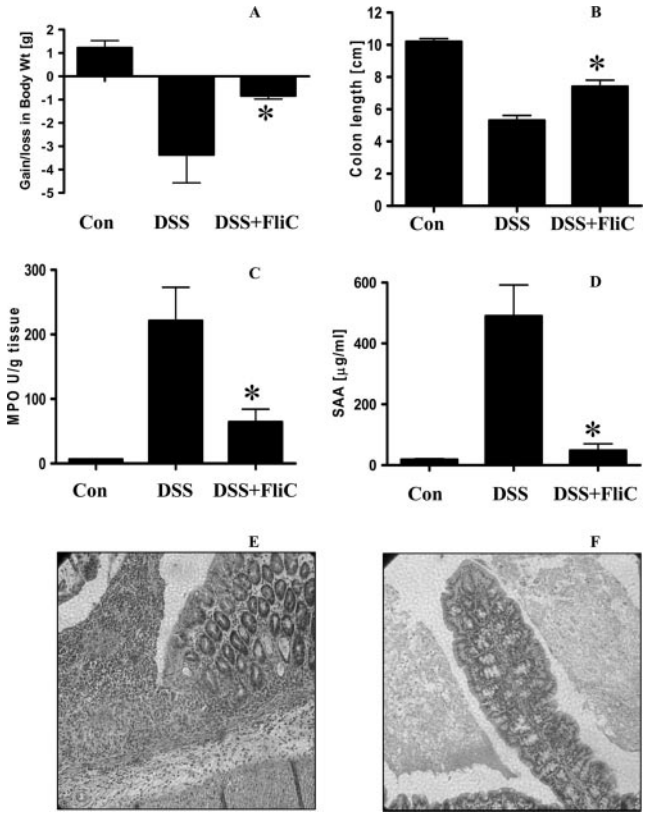

FIGURE 3. Flagellin treatment protects mice against the chemical DSS. Groups of C57BL/6 mice ($n = 5$) were i.p. injected with vehicle (PBS) or 50 μg flagellin and then placed on drinking water containing 2.5% DSS for 7 days. *A*, Change in body weight over the 7-day DSS treatment. *B*, Colon length. *C*, Level of colonic myeloperoxidase, which is commonly used to quantitated PMN infiltration. *D*, Concentration of serum amyloid A (SAA), which serves a general marker of inflammation. *E* and *F*, Representative H&E stained sections of colon from mice receiving DSS only (*E*) or DSS preceded by flagellin treatment (*F*).

Flagellin protects the gut against orally ingested chemicals and pathogens

Flagellin treatment is a potent activator of host defense/cytoprotective gene expression in intestinal epithelial cells (6, 7). Consequently, we hypothesized that flagellin treatment might protect mice against ingestion of the chemical DSS, which induces severe acute colitis underlied by early destruction of intestinal epithelium (16). The primary clinical features of this model are loss of body weight, shortening of the colon, and robust inflammation that can be observed by histopathology or quantitated by measuring levels of the neutrophil product myeloperoxidase. Levels of the general inflammatory marker serum amyloid A correlate with disease severity in this model (17). These changes induced by DSS were all greatly abrogated by a single treatment of flagellin administered

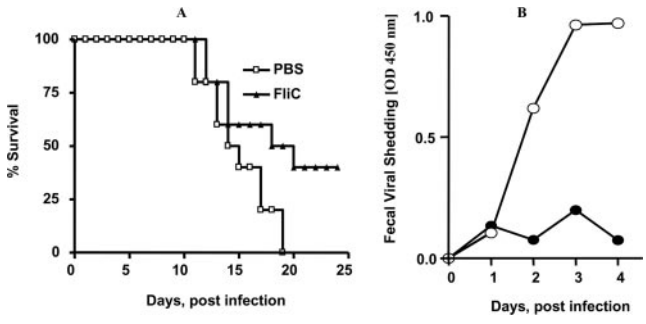

FIGURE 4. Flagellin protects mice against orally administered pathogens. *A*, Mice were i.p. injected with vehicle (PBS) or 50 μg flagellin and then administered 10^6 CFU of *Salmonella typhimurium* (SL3201) by oral gavage. Survival was followed for 25 days. *B*, Mice were given 1–10^8 PFU Rhesus rotavirus. Levels of fecal viral Ags were measured by ELISA.

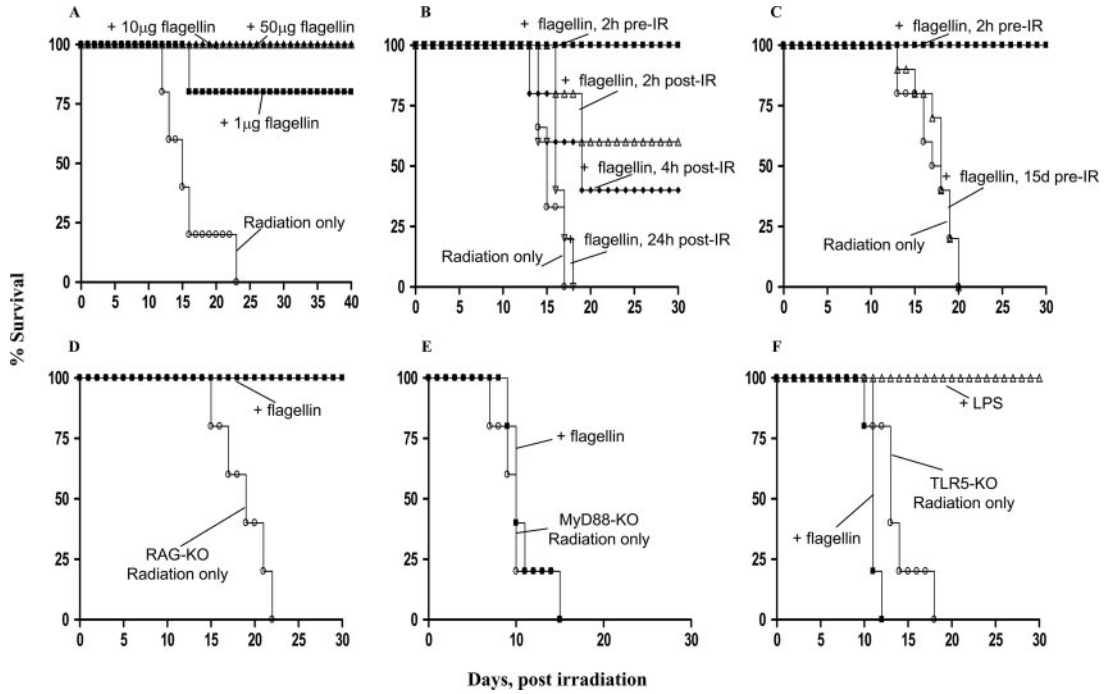

FIGURE 5. Flagellin protects mice against radiation by a TLR5/MyD88-mediated mechanism. Groups of mice ($n = 5$, C57BL/6 or indicated strain) were i.p. injected with flagellin as indicated before or preceding being exposed to 8 Gy γ-radiation. Survival was followed for 30-0 days. *A*, Mice were pretreated with indicated dose of flagellin 2 h before irradiation. *B*, Mice were given 50 μg flagellin 2 h preceding or indicated time following exposure to radiation. *C*, Mice were given 50 μg flagellin 2 h or 15 days preceding exposure to radiation. *D*, RAG1-KO mice were given 50 μg flagellin 2 h preceding exposure to radiation. *E*, MyD88KO mice were given 50 μg flagellin 2 h preceding exposure to radiation. *F*, TLR5KO mice were given 20 μg LPS or 50 μg flagellin 2 h preceding exposure to radiation.

upon initial exposure to DSS indicating that flagellin can protect the gut from this chemical (Fig. 3).

Next, we examined whether flagellin treatment might protect mice against orally administered pathogens. Mice were challenged with a lethal dose of *S. typhimurium* that resulted in 100% mortality within 20 days postinfection. Administration of flagellin 2 h before mice were infected delayed and ultimately reduced mortality by 40% (Fig. 4*A*). Next, mice were challenged with rotavirus. Rotavirus infection in adult mice does not result in clinical indicators of disease but serves as a well defined model of intestinal viral infection in which infectivity/replication can be quantitated by measuring levels of viral Ags in feces (18). Pretreatment of mice with flagellin markedly reduced the degree of rotavirus infection (Fig. 4*B*). Thus, prophylactic treatment with flagellin can reduce the potential of an exposure to a pathogen to result in severe infection.

Flagellin protects mice against irradiation by a mechanism requiring TLR5 and MyD88

Another potential hazard to human populations might be exposure to ionizing radiation, which results in damage to many organ systems including rapidly dividing mucosal surfaces and BMC. LPS has been shown to protect the gut against radiation (19), although its effect on mortality has not been well characterized possibly due to concerns of detrimental events associated with LPS administration. Thus, we next examined whether flagellin might have the ability to protect mice from a lethal dose of γ-irradiation. Untreated irradiated mice all died 2–3 wk postirradiation. Systemic

Table I. *Effects of flagellin on CBC*[a]

	White Blood Cell (10^3/mm^3)	Lymf (10^3/mm^3)	Gran (10^3/mm^3)	Mono (10^3/mm^3)	Hemotocrit (%)	RBC (10^6/mm^3)	Hemoglobin (g/dL)	Platelets (10^3/mm^3)
FliC only	CBC performed 24 h after FliC injection							
Con	8.6	6.9	1.2	0.5	46.23	10.4	15.6	557
SE	0.72	0.55	0.12	0.12	0.94	0.18	0.2	9.1
FliC	4.9	3.5	0.97	0.47	43.3	9.8	14.7	538.3
SE	0.1	0.12	0.12	0.033	0.43	0.1	0.12	72.1
Rad/FliC	CBC performed 7 days after irradiation and FliC injection							
Con	7.3	5.7	1.1	0.5	43.1	10.1	14.7	573.8
SE	0.68	0.45	0.28	0.071	0.43	0.14	0.22	168
Rad only	0.13	0	0	0	32.1	7.9	11.2	225.3
SE	0.033	0	0	0	0.93	0.32	0.45	68
Rad/FliC	0.075	0	0	0	34.4	8.4	11.9	134.3
SE	0.025	0	0	0	0.76	0.27	0.25	10.5

[a] Groups of mice ($n = 3$, C57BL/6) were given an i.p. injection containing 50 μg of flagellin or vehicle (PBS) (*top*). Another group of mice was given 50 μg flagellin i.p. or PBS vehicle 2 h prior to receiving 8.0 Gy rads (*bottom*). CBC was measured 24 h postinjection or 7 days postirradiation, respectively.

administration of 1 μg of flagellin, given 2 h before irradiation, protected 75% of mice against this challenge, whereas doses of 10 or 50 μg uniformly protected against this lethal challenge (Fig. 5*A*). Flagellin-treated irradiated mice were euthanized at 40 days, at which time they appeared healthy and had intestines with normal histopathologic appearance. Although optimal protection required flagellin be administered prophylactically, a significant degree of protection was observed if flagellin was administered up to 4 h following irradiation, whereas there was no benefit of administering flagellin 24 h postirradiation (Fig. 5*B*).

We next turned our focus to trying to understand the mechanism by which flagellin treatment can protect mice from these challenges. We focused on FER largely because it provided a highly reproducible simple readout (i.e., survival). First, we considered the possibility that FER might involve adaptive immunity. The rationale for this notion was that flagellin elicits a robust adaptive immune response that can broadly recognize a variety of bacterial flagellins (20) and, thus, it seemed plausible that flagellin might be providing broad protection against opportunistic pathogens that might prey upon an irradiated host. First, we examined whether FER would be evident if mice were irradiated 2 wk following flagellin treatment, at which time adaptive immune response to flagellin would be robust while innate responses would be likely diminished. FER was not evident in this case (Fig. 5*C*), suggesting adaptive immunity to flagellin was not sufficient to mediate FER. Moreover, RAG1-KO mice, who lack T and B lymphocytes, could be fully protected against radiation by flagellin treatment (Fig. 5*D*), indicating that adaptive immunity is not necessary for FER. Next, we examined whether required the best-characterized pathway of innate immune recognition of flagellin, namely MyD88-mediated TLR5-signaling (21). We observed that mice lacking MyD88 or TLR5 could not be protected against radiation by flagellin (Fig. 5, *E* and *F*). As a control experiment, we verified that mice lacking TLR5 could still be protected by the TLR4 agonist LPS. Thus, FER is mediated by TLR5-mediated innate immune signaling.

We next considered that flagellin's ability to elicit cytokine production might underlie FER. Indeed, one of the cytokines induced by flagellin, namely G-CSF, is itself a radioprotectant particularly in the setting of radiation therapy for cancer (22). G-CSF is thought to act largely on BMC, in particular, boosting their ability to proliferate, which results in reducing radiation-induced leucopenia. Thus, we measured the effects of flagellin on complete blood-cell counts (CBC) alone and in response to radiation (Table I). By itself, i.p. injection of flagellin modestly reduced circulating levels of neutrophils and lymphocytes, suggesting such cells may be an ultimate, direct or indirect, target of flagellin possibly via mobilizing these cells to peripheral tissues. One week following exposure to radiation, irradiated mice had a significant degree of anemia and severe leukopenia, which was not affected by flagellin pretreatment. Thus, flagellin did not protect against radiation-induced anemia or leukopenia (Table I). Next, to examine whether FER might be mediated by an effect on BMC, we examined whether bone marrow from mice given flagellin might have an increased ability to rescue mice from lethal radiation. Specifically, bone marrow was isolated from unirradiated mice injected with flagellin or vehicle (PBS) and then 1×10^5 cells transferred to irradiated mice. Transfer of this relatively small number of cells isolated from control mice did not reduce radiation-induced mortality. In contrast, transferring BMC from mice treated with flagellin partially rescued radiation-induced mortality (Fig. 6), but again did not affect CBC 7 days postirradiation (data not shown). Together these results suggest that FER is not mediated by simple boost of blood cell production/survival but yet BMC may be one key ultimate target of flagellin-induced innate immune response that mediates FER.

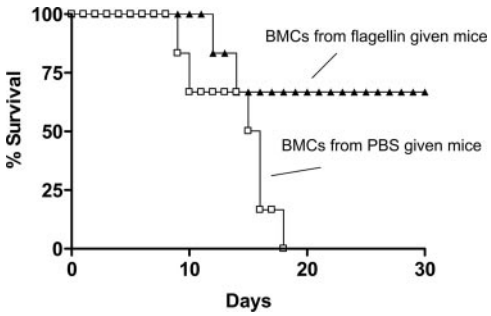

FIGURE 6. Bone marrow from flagellin-treated mice has increased ability to protect against radiation. Groups of mice ($n = 3$, C57BL/6) were i.p. injected with 50 μg flagellin or vehicle (PBS). Then, 24 h later, BMC were isolated and injected retro-orbitally (1×10^5 cells/mouse) into mice, which had just completed an exposure of 3 h to 8.5 Gy γ-radiation. Survival was followed for 30-0 days.

Discussion

Manipulation of the adaptive immune system has long been a central focus of medicine. Approaches to activate adaptive immunity in a targeted manner underlie vaccination while strategies to nonspecifically suppress adaptive immunity have permitted life-saving organ transplantation. In contrast, while much effort has been directed toward developing approaches to inhibit innate immunity, particularly to treat inflammation, strategies to activate innate immunity to treat or prevent disease have not been substantially explored. The primary risk of this strategy is the potential to cause severe inflammatory pathology although, when facing a lethal challenge, this risk may be justified. Moreover, not all innate immune activators are equivalent. In particular, as shown herein, flagellin lacks high potential to cause the severe detrimental affects that have been observed in response to administration of LPS. The primary advantage of a medical strategy to activate innate immunity is that such approaches can potentially provide quite broad protection against a variety of challenges. Indeed, it is the innate immune system that efficiently handles the vast majority of every day challenges that all eukaryotes encounter. In accordance with this notion, we observed herein that activating TLR5-mediated innate immunity with purified flagellin protected mice against a broad and diverse group of challenges including chemical, microbial, and radiological hazards.

The mechanisms by which flagellin treatment protected mice from such challenges are not yet well defined but, based on studies examining flagellin-induced gene expression and the cell types that mediate the flagellin-induced response, some reasonable speculation can be made, particularly for FER, possibly extending to the other diseases models we examined. Firstly, considering that epithelial cells derived from various tissues including the gut, lung, and kidney respond robustly to flagellin (4, 23, 24) while most populations of murine macrophages and DC are hyporesponsive to purified soluble flagellin (5, 25) suggests a major role for the former in mediating the effects of systemically administered flagellin. In accordance, we have recently shown that the majority of the flagellin-induced elevation in serum cytokines is mediated by MyD88 signaling in non-hemopoietic cells (26). Production of cytokines by epithelial cells may play a role in FER. Specifically, we envisage that flagellin-induced cytokines, such as G-CSF, might boost production of innate immune cells that can protect mice from opportunistic infections that develop from irradiation, due to leukopenia and loss of intestinal mucosal barrier—referred to as "gastrointestinal syndrome." That bone marrow isolated from flagellin-treated mice had increased ability to protect against radiation supports this possibility. Additionally/alternatively, epithelial production of the neutrophil chemoattractant KC might increase

recruitment of these cells to the mucosal surfaces, thus better protecting these sites against potential infection. Thus, while flagellin treatment did not rescue radiation-induced leukopenia in blood, it is possible that it resulted in greater numbers of innate immune cells at mucosal surfaces or, somehow, boosted the quality of these cells. We are currently designing approaches to test this possibility.

Another potential mechanism that might underlie FER is that flagellin's actions on epithelial cells render them better able to withstand challenge. Indeed, in addition to severe leukopenia, apoptosis of intestinal epithelial cells is thought to be one of the primary events underlying radiation sickness during radiotherapy in cancer patients. Although flagellin induces both pro- and anti-apoptotic gene expression, its induction of anti-apoptotic gene expression generally prevails in vitro and in vivo, making flagellin a potent cytoprotectant of intestinal epithelial cells (7, 12). Additionally, flagellin induces expression of genes with direct antibacterial activity and activates heat-shock protein expression, which better equip these cells to survive a bacterial challenge (27) that may result from irradiation. Understanding the mechanisms by which flagellin exerts its protective effects, which will likely have different correlates of protection against different challenges, remains a key challenge in this area.

Once the mechanism is better understood, it may ultimately be possible to develop specific paradigms to use flagellin treatment to protect human populations against select dangers. In the case of microbial challenges, we would envision that activation of innate immunity might be advantageous when the exposure to the infecting agent was not anticipated and/or when vaccines are not available. Activation of innate immunity in such a scenario need not clear the pathogen but simply slow it down to allow time for adaptive immunity to become activated. In the case of exposure to chemicals or radiation that might occur, for example, in the terrible event of an intentional radiological attack, we envision that rapid administration of flagellin might substantially attenuate the ensuing mortality. Moreover, in light of its limited propensity to induce the adverse events associated with LPS administration, it might be safe and reasonable to administer flagellin when exposure to a challenge was thought to be imminent, albeit not assured. Given that human DC do not share the hyporesponsiveness to flagellin exhibited by murine DC (5), its possible adverse events might be more severe in humans, but previous studies in which *Salmonella* flagellin were administered to human populations to study Abs did not report such events (28–31), suggesting an acceptable dosing regimen may be achievable. Thus, we propose that strategies to activate innate immunity with relatively safe innate immune activators, such as flagellin, should be considered for development for potential specialized use in certain types of public health crises.

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Disclosures

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