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Both Radiosensitive and Hemopoietic Cells Promote Innate and Adaptive Immune Responses to Flagellin

Catherine J. Sanders,*† Daniel A. Moore, III,* Ifor R. Williams,*† and Andrew T. Gewirtz1*

The TLR5 agonist flagellin induces innate and adaptive immune responses in a MyD88-dependent manner and is under development as a vaccine adjuvant. In vitro studies indicate that, compared with other bacteria-derived adjuvants, flagellin is a very potent activator of proinflammatory gene expression and cytokine production from cells of nonhemopoietic origin. However, the role of nonhemopoietic cells in promoting flagellin-induced immune responses in vivo remains unclear. To investigate the relative contributions of the nonhemopoietic (radiosensitive) and the hemopoietic (radiosensitive) compartments, we measured both innate and adaptive immune responses of flagellin-treated MyD88 radiation bone marrow chimeras. We observed that radiosensitive and radiosensitive cells played distinct roles in the innate response to flagellin, with the radiosensitive producing the majority of the TNF-α, IL-12, and IL-6 cytokines and the radiosensitive cells most of the KC, IP-10, and MCP-1 cytokines. Direct activation of either compartment alone by flagellin initiated dendritic cell costimulatory molecule up-regulation and induced a significant humoral immune response to the protein itself as well as to coinjected OVA. However, robust humoral responses were only observed when MyD88 was present in both cell compartments. Further studies revealed that hemopoietic and nonhemopoietic expression of the cytokines TNF-α and IL-6, but not IL-1, played an important role in promoting flagellin-induced Ab responses. Thus, in vivo both radiosensitive and hemopoietic cells play key nonredundant roles in mediating innate and adaptive immune responses to flagellin. *The Journal of Immunology, 2008, 180: 7184–7192.

Bacterial flagellin, the monomeric subunit of bacterial flagella, plays a major role in the generation of both innate and adaptive immune responses upon host exposure to flagellated bacterial pathogens. Flagellin is a particularly potent activator of epithelial cells, which, when exposed to flagellin, produce proinflammatory mediators that induce immune cell trafficking and activation, including the recruitment of neutrophils and dendritic cells (DC)2 (1, 2). The epithelial cell response to flagellin is mediated by TLR5 (2). Flagellin can be considered a dominant innate immune activator of intestinal epithelial cells (IEC) in that it is necessary and sufficient to recapitulate the proinflammatory gene expression that is induced in these cells by colonization with flagellated pathogens such as Salmonella typhimurium (3, 4). Such responses of IEC to flagellin exposure is in stark contrast to that of other TLR ligands in that IEC are minimally responsive to the TLR4 ligand LPS and the TLR9 ligand CpG DNA (5, 6).

Flagellin is also a major target of adaptive immunity. Specifically, upon Salmonella infection flagellin is a dominant T cell epitope and a major target of the humoral immune response (the basis of H serotyping) (7–9). Such adaptive immune responses to Salmonella are necessary to clear primary infection and protect against future infection by this pathogen. Flagellin is also a dominant antigenic target of the elevated adaptive immune response associated with Crohn’s disease, a chronic inflammatory disease primarily affecting the gastrointestinal tract (10, 11). Specifically, Crohn’s disease patients display elevated levels of serum Abs to flagellin isolated from Escherichia coli and the Clostridia species. In mice, the transfer of flagellin-specific T cells into immunodeficient hosts results in colitis, suggesting that elevated adaptive immunity to flagellin may play a role in driving Crohn’s disease. Thus, understanding the mechanisms that mediate the adaptive immune response to flagellin is germane to understanding the generation of both protective and potentially pathogenic immune responses. Although such naturally acquired adaptive immune responses to flagellin occur in a complex context, exposure to purified flagellin is sufficient to result in strong IgG and IgM immune responses to this protein, suggesting that flagellin is not merely highly antigenic but may also promote adaptive immunity (9, 12). Indeed, coinjection of flagellin plus OVA induces OVA-specific CD4+ T cell activation and IgG production (13, 14). Consequently, flagellin has gained attention as a possible vaccine adjuvant. Flagellin expression with bacterial or viral Ag leads to innate immune functions, potent humoral immunity, and protection against infection with influenza A, Yersinia pestis, and tetanus toxin (15–17). In the case of lethal West Nile Virus, this protection is conferred by neutralizing Abs, specifically demonstrating the protective nature of flagellin-induced Abs (18).

Although TLR ligands are generally thought to induce Th1-type immunity (19), the Th1- and Th2-type immune responses induced by flagellin suggest its capacity to induce a wide variety of immune responses (14, 20, 21).

Flagellin’s ability to act as an adjuvant is thought to result, at least in large part, from its ability to activate TLR5. In accordance, mice lacking the signaling adaptor protein MyD88 (referred to as Myd88-knockout (ko) mice), which is thought to mediate all TLR5 signaling (14, 22, 23), fail to generate flagellin-specific Abs both in response to purified flagellin or upon the induction of experimental colitis (24). In general, the promotion of adaptive

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2 Abbreviations used in this paper: DC, dendritic cell; IEC, intestinal epithelial cells; IP-10, IFN-γ-induced protein of 10 kDa; KC, keratinocyte-derived chemokine; ko, knockout; WT, wild type.

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immunity by TLR ligands is thought to be due to the direct ligation of TLRs on DC (25). However, whether flagellin might promote adaptive immunity by such a mechanism is unclear, in that there have been contrasting reports regarding the ability of flagellin to directly induce activation of DC (14, 26, 27). Although several studies have observed that flagellin can induce up-regulation of costimulatory molecules on DC in vivo, it is unclear whether this occurs by direct recognition of flagellin by the DC or whether DC are indirectly activated via bystander cell populations (14, 28, 29).

In the case of the TLR ligands LPS (TLR4) and CpG (TLR9), direct activation of nonhemopoietic cell types is insufficient for DC activation (30). However, the case with flagellin and TLR5 ligation may be quite different, particularly when considering in vitro studies suggesting that flagellin preferentially activates epithelial cells (2, 3).

Due to flagellin’s remarkable ability to activate nonhemopoietic cell populations such as epithelia in vitro and the ambiguity regarding its ability to directly induce DC activation, we hypothesized that flagellin’s activation of nonhemopoietic cells in vivo influences both innate and adaptive immunity to flagellin. To investigate this hypothesis, we sought to generate mice in which only their hemopoietic and/or nonhemopoietic cells would be directly activated in response to flagellin. Because all responses to purified flagellin are MyD88 dependent (14, 22–24), we used the approach of generating bone marrow irradiation chimeras whereby endogenous bone marrow (wild type (WT) or MyD88ko) was depleted by irradiation and reconstituted with bone marrow from a donor mouse (WT or MyD88ko). Although in this approach the terms radiosensitive and radiosensitive are the precise functional classes of cells that one can study, our results herein and the characterization of this approach by others indicate that the radiosensitive cells studied by this approach are largely hemopoietic (bone marrow derived) cells while the radiosensitive class of cells contains few cells of hemopoietic origin. In this study, we observed that both hemopoietic and nonhemopoietic cells contributed to flagellin-induced acute cytokine secretion, up-regulation of costimulatory molecules on DC, and flagellin’s ability to elicit Ig to itself and OVA. Bone marrow chimeric mice generated using TNF-αko and WT or IL-6ko and WT demonstrated that either cytokine from either compartment assisted in promoting robust Ab responses to flagellin. Consequently, strategies using flagellin as a vaccine adjuvant should directly activate MyD88 signaling in both bone marrow-derived and radiosensitive cells.

Materials and Methods

Reagents/injections

All reagents were administered i.p. Native flagellin was isolated from *Salmonella typhimurium* and its purity was verified as previously described (13, 31). Unirradiated mice received 10 μg of flagellin per mouse while mice that had been subjected to irradiation (to generate bone marrow chimeras) received 40–50 μg of flagellin per mouse. The higher doses given to irradiated mice was based on preliminary observations that irradiated animals tended to exhibit reduced responses to this bacterial product. CpG ODN 1826 was given at 50 μg/mouse (TriLink Biotechnologies). OVA (grade IV; Sigma-Aldrich) was given at 50 μg/mouse. TNF-α and IL-6 (R&D Systems) “carrier-free” were administered at 1.5- and 4-μg doses, respectively. The term “carrier-free” is used by the manufacturer to indicate that additional proteins (e.g., BSA) were not added to the material.

Mice

MyD88-deficient mice, originally developed by S. Akira (Osaka University, Osaka, Japan) (32), that were backcrossed at least 10 times to C57BL/6 were a gift from M. Sherman (Emory University, Atlanta, GA). All other mice were purchased from The Jackson Laboratory, including IL-6-deficient (B6.129S2-Il6tm1Kopf/J), TNF-α-deficient (B6.129S6-Tnfαtm1Gkl/J), and IL-1R-deficient (B6.129S7-Il1r1tm1Imx/J) mice. In accordance with guidelines from The Jackson Laboratory, the following mice served as appropriate WT controls: IL-6 and IL-1R mice (C37BL/6J) and TNF-α-deficient mice (B6129S2F2/J). These mice were used between 6 and 10 wk of age. For radiation bone marrow chimera experiments, CD45.1 allele-expressing B6.129L-PpcrePep3b/B6J served as WT mice, thus allowing an assessment of the degree of chimerism achieved. These mice have been backcrossed at least 22 times onto C57BL/6J. Mouse studies were approved by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA).

Preparation and evaluation of radiation bone marrow chimeric mice

Bone marrow cells were eluted from the femora and tibiae of donor mice. Recipient mice were gamma irradiated once with 11 Gy using a 137Cs irradiator and then i.v. injected with 2 × 10^6 bone marrow cells in PBS of the appropriate donor strain. All recipients were males between 6 and 12 wk of age, but age matched within experiments. Mice were allowed to rest posttransfer between 2 and 4 mo before experimental use. For 2 wk immediately following transfer, mice received drinking water supplemented with 2 mg/ml neomycin sulfate (Mediatech). At least 8 wk after bone marrow transfer, blood samples were taken via retro-orbital or submandibular pouch bleed. The degree of bone marrow chimerism was determined.

FIGURE 1. Degree of chimerism in irradiated bone marrow (BM) transplanted mice. Mice were irradiated, given injections of bone marrow, and given 3 mo to reconstitute. A. Representative flow cytometric analysis of PBMC of a WT mouse reconstituted with MyD88ko bone marrow. Ninety-nine percent of CD45-expressing cells are of donor origin (i.e., CD45.2^+^). B. Three chimeric groups were made using MyD88ko and WT mice. Percentage of PBMC of donor origin is the average of six mice ± SD.

<table>
<thead>
<tr>
<th>Chimera</th>
<th>BM donor</th>
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<th>% PBMC of donor origin</th>
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<td>WT → WT</td>
<td>WT</td>
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<td>MyD88KO → WT</td>
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<td>98.6 +/- 0.6%</td>
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<tr>
<td>WT → MyD88KO</td>
<td>WT</td>
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by flow cytometric evaluation of PBMC isolated through centrifugation of blood samples over Histopaque-1077 (Sigma-Aldrich). The differential expression of the CD45 allele of all ko mice (CD45.2) compared with B6.SJL-PtprcaPep3b/BoyJ mice (CD45.1) was used to evaluate donor vs host hemopoietic cell composition.

**DC isolation**

Mice were injected i.p. with 50 μg of flagellin per 100 μl of PBS or 100 μl PBS alone, sacrificed after 6 h, and spleens were harvested. Spleens were digested with 1 mg/ml collagenase type IV (Worthington Biochemical) in complete DMEM plus 2% FBS for 30 min at 37°C to liberate DC. A single cell suspension was generated and cells were washed twice in PBS supplemented with 2 mM EDTA and 1% FBS and mesh filtered before staining for flow cytometry.

**Flow cytometric analysis**

To assess bone marrow chimerism, expressions of T, B, myeloid, and NK cell populations in PBMC were analyzed by flow cytometry. Percentages of cells of donor origin ranged from 95 to 100%. Flow cytometric stains were performed for 30 min at 4°C in 24G2 hybridoma culture supernatant.
ELISA analysis

Mouse sera from chimeric mice and unirradiated MyD88ko controls were evaluated for cytokines using a BioSource multiplex cytokine ELISA kit (Invitrogen). The measurements were by the "Luminex Core" facility of Baylor University (Houston, TX). Serum cytokine levels of reconstituted IL-6- and TNF-α-deficient mice were obtained using mouse IL-6 and mouse TNF-α cytokine ELISA kits (R&D Systems). Ab ELISA plates (MP Biomedicals) were coated with 2 μg of OVA or 100 ng of S. typhimurium FliC/well in 0.1 M NaHCO₃, buffer (pH 9.6) overnight at 4°C. Plates were washed in ELISA wash buffer (HBSS, 0.5% goat serum, and 0.1% Tween 20) and serum was applied in various dilutions for 1 h at 37°C. After three additional washes, HRP-conjugated sheep anti-mouse IgG (GE Healthcare) and goat anti-mouse IgG1 (Catag Laboratories) were added at 1/1000 and 1/2000, respectively, in wash buffer for 1 h at 37°C. Plates were developed using tetramethylbenzidine substrate (Kierkeaegard and Perry Laboratories-KPL), stopped using H₂SO₄, and read at 450 nm on a SpectraMax Plus plate reader (Molecular Devices). Ab titers were defined by the reciprocal of the serum dilution equivalent to three times the ELISA plate background (coated, no sample).

Statistics

Significance was determined using the Student’s t test (GraphPad Prism software). Differences were noted as significant when p < 0.05.

Results

To understand the relative roles of radioresistant (i.e., nonhemopoietic) and radiosensitive (i.e., hemopoietic) cell populations in the induction of immune responses to flagellin, we constructed radiation bone marrow chimeras made from MyD88ko mice and WT mice. Although both strains of mice were on a C57BL/6 back- ground, which has a CD45 allele referred to as CD45.2, the WT mice used had been engineered to carry the CD45.1 allele. This difference in CD45 subtype enabled an assessment of the degree of chimerism in our mice by measuring the relative percentages of CD45.1 vs CD45.2 on PBMC by flow cytometry, which indicated that ~98% of these cells were of donor origin (Fig. 1). Irradiated MyD88ko mice given MyD88ko bone marrow had a high rate of mortality and were not amenable to this approach of study. Thus, unirradiated MyD88ko mice served instead as negative control mice (i.e., expected to lack innate and adaptive immune responses to flagellin) in these studies.

Flagellin is a rapid and potent inducer of a myriad of immune cytokines in vivo (1, 28, 33). To assess which cell compartment may be responsible for production of certain acute cytokines, we injected chimeric mice with OVA alone or flagellin plus OVA i.p., bled the mice after 90 min, and evaluated the levels of serum cytokines using a multiplex cytokine ELISA. Control chimeric mice (WT→WT) mice exhibited robust inductions of keratinocyte-derived chemokine (KC or CXCL1; approximate equivalent of human IL-8), IFN-γ-induced protein of 10 kDa (IP-10 or CXCL10), and the MCP-1 monocyte/macrophage chemotactant protein (CCL2), moderate inductions of IL-6, IL-12, and IL-1α, and a modest but statistically significant induction of TNF-α in accordance with previous studies of unirradiated C57BL/6 mice (28, 33) (Fig. 2A). For unknown reasons, possibly due to damage induced by the radiation process, the overall cytokine levels (indeed, all types of immune responses measured) appeared to be lower in irradiated/reconstituted mice than that observed in unirradiated mice. For this reason, experiments involving irradiated mice used higher doses of flagellin than experiments not involving such mice. In agreement with the currently held view that all responses to soluble flagellin are MyD88 dependent, negative-control MyD88ko mice lacked induction of all measured cytokines. Mice that had MyD88 in only their hemopoietic cells (WT→MyD88ko) produced TNF-α and IL-12 levels comparable to those of WT control chimeras (WT→WT) mice, suggesting that cells of hemopoietic origin may promote Th1-like responses to flagellin. IL-6 was secreted primarily by the hemopoietic compartment, although radiosensitive cells could produce this cytokine as well. In contrast, the radiosensitive compartment (MyD88ko→WT) was the primary source of acute KC and MCP-1 and the lorne source of IP-10.

We next sought to determine whether the relative role of radiosensitive and radiosensitive cells in mediating flagellin-induced cytokine production was specific for flagellin or would extend to another TLR agonist. Thus, we generated a parallel set of WT/ MyD88 bone marrow chimeras (confirming that they had a high degree of chimerism; data not shown) and measured serum cytokines in response to a synthetic ligand for TLR9, CpG ODN, which, like flagellin, acts via a MyD88-dependent signaling pathway (34). The pattern of acute cytokine production elicited by CpG ODN differed from that induced by flagellin (e.g., much more TNF-α and IP-10, less KC, IL-12, and IL1α). More central to this study was that, in contrast to the flagellin-induced cytokine production, the majority of all cytokines elicited by CpG ODN were made by radioresistant cells (Fig. 2B). Thus, the notion that radioresistant cells play a major role in mediating systemic cytokine production in response to TLR agonists may be especially true for flagellin.

Directly activated radioresistant and hemopoietic cells promote phenotypic DC maturation

Activation of DC is a critical step in the induction of adaptive immunity. It is unclear whether in vivo induction of DC maturation by flagellin is mediated by the direct ligation of TLR5 on DC (whether murine DC express TLR5 is not clear) or whether an indirect mechanism of activation is at play. To investigate how flagellin mediates DC maturation in vivo, we treated MyD88 bone marrow chimeras with flagellin i.p. and assessed the activation of DC in the spleen. Such assays defined DC as cells that expressed high levels of CD11c and detectable levels of MHC II and assessed their activation state by measuring their surface expression of CD80 and CD86. Splenic DC from control chimeras (WT→WT) displayed increased levels of CD80 and CD86, albeit at levels somewhat lower than those observed in DC from unirradiated C57BL/6 mice treated identically (Refs. 14 and 29 and our data not shown) (Fig. 3). Splenic DC from WT→WT mice did not display detectable up-regulation of CD40 (data not shown). In accordance
with a recent report, splenic DC from MyD88ko→WT mice showed modest but detectable up-regulation of these two markers, although the level of activation observed was below that seen in mice with WT bone marrow (35). Splenic DC from WT→MyD88ko mice up-regulated CD80 and CD86 to a comparable level as that of cells from WT→WT mice. These results suggest that in vivo DC maturation can result from flagellin-induced responses of radioresistant cells, albeit only modestly, and does not absolutely require direct activation of TLR signaling in DC. However, in contrast to results from ex vivo studies in which murine DC obtained from spleen or bone marrow failed to respond to flagellin ex vivo (26, 27), the expression of MyD88 on only hematopoietic cells in vivo was sufficient to result in DC maturation in vivo.

Direct activation of either radioresistant or hematopoietic compartments by flagellin can induce humoral immunity

Flagellin is a potent inducer of adaptive immunity as reflected by CD4+ T cell activation and by protective humoral immune responses (13–18). Such responses to flagellin are thought to be dependent upon the activation of innate immunity in general and TLR5/MyD88 in particular (18, 24). In light of the above-described results indicating that MyD88 in radioresistant cells accounted for a substantial portion of flagellin-induced cytokine responses and that these cells have the ability to promote DC maturation in response to flagellin, we hypothesized that the direct activation of only this cellular compartment might be sufficient to result in a flagellin-specific Ab response. We injected radiation bone marrow chimeras with 50 μg of flagellin i.p. Serum was isolated on day 0 and day 14. Serum flagellin-specific IgG levels in MyD88ko→WT mice at day 14 were significantly higher than those in the same mice at day 0 or in MyD88ko mice 14 days after flagellin treatment (Fig. 4A). However, the flagellin-specific IgG titers in these mice were roughly 40% of those attained in WT control chimeras. Thus, although direct activation of radioresistant cells alone by flagellin can promote development of humoral immunity, it is not sufficient for a robust, WT-like Ab response.

We next investigated flagellin’s ability to act as an adjuvant when MyD88 was absent in either the radiosensitive or the radioresistant cell compartment. All three groups of WT/MyD88 bone marrow chimeras and unirradiated MyD88ko were i.p. injected with 50 μg OVA alone (OVA only) or 50 μg OVA plus 50 μg flagellin (B). Serum was isolated on day 0 or day 14. A, Flagellin-specific IgG titers (mean ± SEM; n = 3–5 mice/group). B, OVA-specific IgG and IgG1 titers; n = 3–5 mice/group ± SEM. An asterisk (*) indicates that marked difference was statistically significant (p < 0.05).

**FIGURE 4.** Ab production profile by flagellin-treated bone marrow chimeras. MyD88 BM chimeric mice and unirradiated MyD88ko were i.p. injected with 50 μg of flagellin (A) or 50 μg OVA alone (OVA only) or 50 μg OVA plus 50 μg flagellin (B). Serum was isolated on day 0 or day 14. A, Flagellin-specific IgG titers (mean ± SEM; n = 3–5 mice/group). B, OVA-specific IgG and IgG1 titers; n = 3–5 mice/group ± SEM. An asterisk (*) indicates that marked difference was statistically significant (p < 0.05).

**FIGURE 5.** Induction of Abs to flagellin in IL-6ko, IL-1Rko, and TNF-ako mice. Indicated mice (WT or cytokine-deficient) mice and corresponding WT controls were i.p.-injected with 10 μg of flagellin or an equivalent volume of HBSS. Sera isolated on day 14 were evaluated for flagellin-specific IgG; n = 3 mice/group ± SEM. An asterisk (*) indicates that marked difference was statistically significant (p < 0.05).
IL-6 and TNF-α promote humoral immunity to flagellin

In view of the general importance of cytokines in promoting the activation of adaptive immunity and our observation that MyD88 signaling in radioresistant cells is important for flagellin-induced production of cytokines and its promotion of humoral immunity, we hypothesized that flagellin-induced cytokines may be important for flagellin’s ability to induce Ab responses. Thus, we investigated whether select cytokines might play nonredundant roles in the induction of adaptive immunity to flagellin. First, we examined flagellin-specific Ab titers from individual cytokine or cytokine receptor knockout mice. We injected IL-6ko, TNF-αko, and IL-1Rko mice with flagellin or PBS and then examined flagellin-specific IgG titers 14 days later. IL-6ko and TNF-αko mice generated significantly lower titers than their respective WT controls, whereas IL-1Rko mice generated WT-level titers (p < 0.05) (Fig. 5). These data suggest that IL-6 and TNF-α may play important roles in the development of flagellin-induced adaptive immune responses.

We next investigated whether the early time points at which TNF-α and IL-6 are made can be connected to the Ab-promoting properties of these two cytokines. Acute TNF-α, measured systemically at 90 min, appears to be produced entirely by the hemopoietic compartment while acute IL-6 can be secreted by either compartment (but mainly hemopoietic cells) (Fig. 2). To determine whether acute TNF-α and acute IL-6 are responsible for the ability of these cytokines to promote flagellin-induced Abs, we coinjected specific cytokine ko mice with recombinant mouse TNF-α or IL-6 and purified flagellin and then measured flagellin-specific Ab titers from sera harvested from blood drawn 14 days later. Acute TNF-α acted as an adjuvant to promote flagellin-specific Abs in TNF-αko mice (Fig. 6B). These data suggest that because hemopoietic cells are the sole sources of acute TNF-α in response to flagellin, acute TNF-α made by this cell compartment may play an important role in promoting flagellin-induced adaptive immunity. Analogous administration of recombinant mouse IL-6 plus flagellin was unable to promote flagellin-specific IgG in IL-6ko mice, suggesting that even though flagellin is a potent inducer of IL-6, the ability of IL-6 to promote flagellin-induced adaptive immunity may be due to its...
production at a later time in the immune response (Figs. 6A and 2, respectively) (33).

To investigate the relative contributions of radioresistant and hemopoietic cell populations to either IL-6- or TNF-α-induced promotion of adaptive immunity to flagellin, we generated both sets of bone marrow chimeras using IL-6- or TNF-α-deficient mice (Fig. 7A). Chimeric mice were treated with 50 μg of flagellin and serum was harvested from blood taken on days 0 and 14. Mice that had the IL-6 gene in only their radioresistant cells (IL-6ko→WT) or only their hemopoietic cells (WT→IL-6ko) were able to generate flagellin-specific IgG titers to a similar extent as WT→WT controls and well above that of IL-6ko→IL-6ko (Fig. 7B). Next, we tested the role of hemopoietic vs radioresistant TNF-α in the promotion of flagellin-induced Abs. TNF-αko→WT and WT→TNF-αko mice were both able to develop Abs to flagellin above that of TNF-αko→TNF-αko mice, although at levels significantly below that of WT→WT mice (Fig. 7C). Thus, while either cell compartment can supply the IL-6 needed for a WT-level Ab response to flagellin, TNF-α produced by both hemopoietic and radioresistant compartments is required for a robust Ab response.

**Discussion**

Although first appreciated in immune cells, TLRs are in fact expressed by a variety of cell types and tissues in a manner enabling the host to mount appropriate immune responses according to how a pathogen may enter and traffic through the host. Such TLR expression is not only important in host defense against pathogens but also mediates immune responses that occur when bacterial products attain systemic bioavailability, which can occur in infection, vaccination, and, in the case of flagellin, appears to occur in sepsis and some intestinal disease states (10, 11, 36, 37). Thus, while the results described herein of systemically administering flagellin and/or the model Ag OVA can most easily be interpreted in terms of the context of flagellin-based vaccines/ads, they may also provide insights into the protective and potentially pathogenic immune responses that target enteric bacteria. In particular, this study focused on investigating the contributions of the often overlooked nonhemopoietic cell compartment, which in vitro studies suggest is highly responsive to flagellin (38). We examined multiple stages of the immune response in bone marrow chimeric mice given flagellin with or without the model Ag OVA to define the relative roles of hemopoietic and nonhemopoietic (i.e., radioresistant) cells. We report distinct nonoverlapping roles for these cell compartments in mediating both innate and adaptive immune responses to flagellin.

Our data indicate that nonhemopoietic cells play a significant role in the early elevation of serum cytokines in response to flagellin treatment. Specifically, direct activation of MyD88-dependent signaling in nonhemopoietic cells was required for most of the induction of KC and MCP, which promote recruitment/activation of neutrophils and monocytes, respectively. Given the central role of these leukocytic populations in mediating the clinical manifestations/tissue damage that characterize acute inflammation, it would thus seem likely that nonhemopoietic cells would likely play a part in any potential acute reactogenicity that might be associated with flagellin-based vaccines/ads. Moreover, it seems reasonable to speculate that nonhemopoietic cells might play a substantial role in driving acute inflammation in response to flagellated enteric bacteria. The observation that nonhemopoietic cells also contributed significantly to the production of these cytokines in response to the immunomodulator/TLR9-agonist CpG DNA suggests that these cells may contribute to inflammation/reactogenicity in a variety of scenarios although the relative proportion of these cytokines produced by nonhemopoietic cells was significantly greater in response to flagellin, which is consistent with the notion that flagellin is a particularly potent activator of the nonhemopoietic cell compartment. Although our methodology did not address the specific nonhemopoietic cell type/tissue responsible for these cytokines, the fact that epithelial cell lines derived from the gut, lung, and kidney are uniformly highly responsive to flagellin suggests that these cells/tissues are likely involved (33, 39, 40).

A key event in innate immune activation that bridges innate and adaptive immunity is up-regulation of the surface expression of costimulatory molecules on DC, referred to as DC maturation. The low expression of TLR5 by murine DC and their inability to respond to flagellin ex vivo led to the reasonable suggestion that flagellin-induced DC activation in vivo occurs by an indirect (i.e., bystander) mechanism of activation (27). In accordance with this possibility and a recent report by Salazar-Gonzalez et al., we observed that the expression of MyD88 in only nonhemopoietic cells resulted in a significant level of DC maturation, albeit at levels considerably less than those observed when both cell compartments expressed MyD88 (35). It seems reasonable to speculate that such indirect DC activation might be mediated by paracrine mediators produced by nonhemopoietic cells, but none of the cytokines that we observed to be produced by these cells have been observed to be able to induce DC maturation and, thus, the mechanism remains under investigation. In any case, the means by which nonhemopoietic cells promote DC maturation may not be very important in that the expression of MyD88 in only hemopoietic cells was sufficient to result in up-regulation of DC costimulatory molecules to the same level as seen in mice with MyD88 in all cells. Such flagellin-induced, MyD88-mediated DC activation might reflect direct activation of these cells by flagellin or could perhaps result from a bystander mechanism involving cytokines produced by other hemopoietic cells. Hemopoietic cell types, NK cells, and monocytes, have been reported to secrete cytokines in response to flagellin (29, 41, 42). We find that hemopoietic cells are responsible for most acute IL-12 and TNF-α, which would seem reasonable candidates to potentially mediate bystander activation of DC. Another possibility is that in vivo DC may indeed respond directly to flagellin, but whether they respond ex vivo as they do in some studies depends upon the specific experimental conditions used (14). Alternatively, perhaps DC present in the spleen basally lack responsiveness to flagellin but the activated DC observed in the spleen 6 h following systemic treatment with flagellin are an inherently different population of DC that trafficked to the spleen in response to flagellin treatment. In accordance with this possibility, Uematsu et al. recently observed that intestinal DC, but not splenic DC, exhibit a TLR5-mediated response to flagellin ex vivo (26).

In accordance with DC maturation being a key event in the induction of adaptive immunity, flagellin was able to promote Ab responses to mice that had MyD88 only in their hemopoietic cells. However, the level of response was considerably reduced compared with mice with MyD88 in both cell compartments. Moreover, the expression of MyD88 only in nonhemopoietic cells was sufficient for flagellin promotion of such Ab responses. Thus, in perhaps what might be considered the most interesting aspect of our study, our results demonstrate that the activation of nonhemopoietic cells is important for promoting humoral immunity in response to flagellin. There is some precedence to this concept in that TLR recognition events in both the hemopoietic and nonhemopoietic compartments are required for the development of effector T cells (43) in response to HSV-2. The marked reduction in cytokine...
production in mice lacking MyD88 in nonhemopoietic cells suggests a role for these mediators in promoting adaptive immunity in response to flagellin. The fact that such mice exhibited WT levels of DC maturation argues against these cytokines promoting humoral immunity via effects on DC. However, it is possible that there was a delayed induction of robust DC activation in the chimeric mice in which only nonhemopoietic cells had MyD88 and/or that assays of splenic DC at early time points are not generally reflective of the degree of DC maturation of mice given specific inoculations. Such caveats notwithstanding, it seems reasonable to speculate that a key contribution of nonhemopoietic cells to humoral immunity might be to promote cell trafficking that allows for efficient interactions between innate immune cells and lymphocytes. In support of this notion, we observed that nonhemopoietic cell MyD88 was required for robust serum elevation in chemokines for innate immune cells and absolutely required for the induction of the T cell chemokine IP-10. It also seems reasonable to speculate that the reduced induction of IL-6 and/or IL-1α production in mice lacking nonhemopoietic MyD88 might contribute to their reduced humoral immune response to flagellin/OVA. Lastly, we recognize that immune alterations brought on by nonhemopoietic cell-specific expression patterns of TLR4 and other TLRs. However, it should be noted that differences in cell-specific expression patterns of TLR4 and other TLRs may lead to differential regulation of downstream signaling pathways, which likely contribute to the observed differences in immune responses.

In conclusion, although MyD88 signaling in only hemopoietic or nonhemopoietic cells led to significant immune responses, these responses were much more robust when both cell compartments had functional MyD88 signaling. Such synergy between TLR signaling in hemopoietic and radioresistant cells likely reflects the complexity and efficiency of immune responses that have evolved to protect against pathogens and should be taken into account when designing vaccine strategies based on flagellin.

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Disclosures

The authors have no financial conflict of interest.

References

cell proliferation and activation directly and through dendritic cell-NK cell interactions. *J. Leukocyte Biol.* 78: 888–897.


CORRECTIONS


Fig. 4A was published twice, once in error in place of Fig. 5. The correct Fig. 5 is shown below. The published legend is correct, but shown again here for reference.

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Fig. 8 of the paper correctly shows amplification of an mRNA fragment for P2Y12, demonstrating that the murine dendritic cell line XS106 expresses mRNA for P2Y12. However, all references to the mRNA fragments for P2Y12, the primers utilized for RT-PCR, and the expression of P2Y12 by XS106 cells were incorrectly written as P2Y11, including in the legend of Fig. 8, the abstract, Materials and Methods, and Results. All of these should be P2Y12. The authors regret any inconveniences caused by this error.

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Table II contained an incorrect setting of the Cm1 probe column heading. Under the heading “VH probe” there should be two columns, *BamHl/NcoI* and *AvaII*. The correct Table II is shown.

In Fig. 5C, the colors indicating rhesus macaques (RMs) and sooty mangabeys (SMs) are switched. In the correct version shown below, RMs are the black lines, and SMs are the red lines.

In addition, in the legend for Fig. 5E contained minor errors. The corrected version follows:

*E.* Flow cytometric analysis of the mean proportions of total, naive, and memory CD4+PD-1+ T cells (left panel) and total, naive, memory, and effector CD8+ PD-1+ T cells from peripheral blood of SIV-negative (open bars; \( n = 19 \)) and naturally infected chronic SIV-positive SMs (filled bars; \( n = 32 \)).

**FIGURE 5.** Corrected panel C.