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Innate Immune Recognition of Conserved Structural Features of Bacterial Flagellin Shapes Host Antibody Responses

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Abstract

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Within this dissertation, we have determined how components of the host's innate immune system and structural features of flagellin, a pathogen associated molecular pattern (PAMP) and the major structural component of bacterial flagella, determine isotype specific antibody responses. Bacteria use flagella for chemotaxis toward energy and nutrient resources. For *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) the primary structural protein comprising the flagella is flagellin, which is encoded by the genes, *fljB* and *fliC.* The primary flagellin molecule, FliC, has been crystallized revealing that its structure consists of four domains: D0, D1, D2 and D3. D0 is recognized by Naip5 and Naip6 (Naip5/6), which initiates the assembly of the inflammasome and caspase-1 activation. D1 is recognized by Toll-like receptor 5 (TLR5), which signals through adaptor protein MyD88 to initiate MAPK signaling and NF-κB activation. Using purified flagellin from *S.* Typhimurium, we dissected the contribution of innate flagellin recognition pathways to promote antibody responses towards flagellin and co-administered ovalbumin in mice. We demonstrate IgG2c responses towards flagellin were TLR5- and inflammasome-dependent; IgG1 was the dominant isotype and partially TLR5- and inflammasome-dependent. In addition, a substantial flagellin-specific IgG1

response was induced through a TLR5-, inflammasome-, and MyD88-independent pathway. To address how flagellin triggers the TLR5-, inflammasome-, and MyD88-independent pathway, we turned our attention to the flagellin molecule's highly conserved recognition sites located on domains D0 and D1, and its hypervariable D2 and D3 (D2/D3) domains. To determine how FliC's structural features affect the TLR5-, inflammasome-, and MyD88-independent pathway, we destroyed the D0 (FliC-C) or the D1 ($FliC^{TLR5}$) recognitions sites, or deleted the D2/D3 domain ($FliC^{D0/D1}$), and performed primary and secondary immunizations with C57BL/6 mice. Results from FliC-C, $FliC^{TLR5}$, $FliC^{D0/D1}$ immunized mice indicate that primary antibody responses towards flagellin are strictly dependent on FliC's D2/D3 domain. Robust secondary antibody responses in mice require TLR5 and caspase-1, or MyD88, and FliC's D2/D3 domain. This is the first formal demonstration that flagellin's D2/D3 domain is required for induction of primary antibody responses and robust secondary antibody responses. The compilation of our results identifies a novel recognition pathway for bacterial flagellin that plays a significant role in flagellin's immunogenicity.

Flagellin also works as an adjuvant when co-administered with ovalbumin, but only promotes moderate IgG1 anti-OVA responses following secondary immunizations. In contrast to flagellin's modest adjuvancy towards co-administered antigens, we demonstrate that covalent linkage of antigens to flagellin significantly enhances the antigen's immunogenicity. By coupling antigens to flagellin, we tested several flagellin-HIV fusions and identified one construct that was capable of inducing antibodies specific for the 4E10 epitope of HIV's gp41 membrane proximal external region (MPER). These results establish a flagellin vaccine platform that may be exploited to enhance the immunogenicity of poorly immunogenic epitopes to combat debilitating diseases such as influenza, tuberculosis, malaria, and HIV.

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Dedication

I would like to dedicate this dissertation to my parents, Américo López-Rodríguez and Georgina Yglesias. It is because of their constant love, support and encouragement that I have been able to succeed at the highest level of my field and continually strive to better myself. Without my parents I would not be where I am today, and for this, I will forever be indebted to them. Therefore, I dedicate this dissertation to them, knowing I can never pay them back for the opportunities they have already given me and for the future opportunities that I would not have if not for them.

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The Pathology Department at the University of Washington has been an amazing support network for me and has not only provided me the opportunity to acquire a Ph.D., but has also given me the resources to attend numerous conferences and present my work across the country. A special thank you (and big bear hug) goes to the director of the Path Grad Program, Steve Berard. Steve's constant enthusiasm and support for the past five years has been amazing and irreplaceable.

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Introduction

Introduction to adjuvants

In today's world, vaccines are the most effective means to prevent infections, attenuate the spread of disease, and limit morbidity and mortality in humans. While most successful vaccines are a result of live, attenuated pathogens, there are many other infectious diseases in which attenuated vaccines have not yet been developed for. Moreover, there are many other pathogens, like HIV, in which an attenuated form is impractical due to its inability to confer protection and potential to become pathogenic. Therefore, vaccination strategies against pathogens such as HIV, have focused on using nonliving antigens such as inactivated viruses or recombinant antigens. While recombinant antigens are incapable of causing disease, and have limited toxicity to the host, they also have weak immunogenicity, inducing a poor immune response from the host towards the selected pathogen. For this reason, subunit vaccines require additional help to boost the host's immune response towards themselves. Thus, adding adjuvants (from *adjuvare*, to help) to a subunit vaccine helps to increase the magnitude and breadth of immune responses, enhance immunological memory, and direct appropriate immune responses towards pathogens. Unfortunately, in the U.S.A., we have a limited number of approved adjuvants and, in most instances, their mechanisms of action is unknown. Hence, it is critical to have a better understanding of adjuvants, in order to help develop safer and more effective vaccines against infectious diseases.

Bacterial flagellin is key for virulence

Motility assists bacteria during invasion and colonization of the host (1). The flagellum is the molecular machine that propels some Gram-negative and Gram-positive bacteria (Fig. 1) (2-4). Numerous pathogens are flagellated, including bacteria that infect mucosal surfaces. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), a major cause of diarrheal illness worldwide, uses flagella to travel through the mucus to reach the epithelial barrier and invade host cells (5, 6).

During an infection, hosts use pattern recognition receptors (PRR) to identify microbe associated molecular patterns (MAMPs) and alert the immune system of a pathogen invasion (7). PRRs are germline encoded and do not undergo gene rearrangement (8). Hosts are capable of recognizing multiple MAMPs encoded by *S.* Typhimurium such as LPS (TLR4), lipoproteins (TLR1, 2, 6), CpG-DNA (TLR9), and flagellin (TLR5). Flagellin is the primary protein of flagella and is encoded by *S.* Typhimurium genes *fljB* and *fliC* (3). Detection of flagellin alerts the host to a flagellated bacterium that has broken through the epithelial barrier or has entered the cell cytosol (8). There are three known PRRs that are responsible for sensing flagellin: Toll-like receptor 5 (TLR5) and nucleotide-binding and oligomerization domain-like receptors (NLRs) Naip5 and Naip6 (Naip5/6) (9-13). Activation of these innate immune receptors promotes inflammatory and immune responses, such as microbicidal activity, cytokine production and dendritic cell (DC) maturation (14-18). Flagellin uptake by DCs leads to antigen presentation and the generation of T cell-dependent antibody responses (19).

TLR5 expression and function

Until recently, the collection of data about which cell types express TLR5 was heavily dependent on mRNA and biological responses to flagellin. Gewirtz *et al*. demonstrated that TLR5

recognizes flagellin that has gained access to the basolateral surface of polarized epithelia (17, 20). Results from the Hawn group at University of Washington indicated that murine alveolar macrophages are able to respond to FliC (from *S.* Typhimurium) or FlaA (from *Legionella pneumophila*), while bone marrow derived macrophages (BMDMs) and a murine macrophage cell line (RAW 264.7) were both unable to be stimulated by purified flagellin monomers (Fig. 3) (21). On the contrary, in another report, other murine macrophage cell lines (J774A.1) are responsive to high doses of purified FliC (≥10μg/mL) (22). Recently the Miyake group from the University of Tokyo has generated a monoclonal anti-mouse TLR5 antibody that confirms the previously mentioned findings for leukocytes; epithelial cell reactivity with this monoclonal antibody (mAb) has not yet been reported (23). In addition to supporting the previous findings, the Miyake group demonstrates that neutrophils do express TLR5, but do not respond to flagellin by secretion of cytokines or chemokines. Along with extensive investigation into the agonist that stimulates TLR5, considerable research has been conducted to understand which populations of DCs recognize flagellin and what role recognition of flagellin has in the immune response. Several groups have shown that murine DCs respond to flagellin. Uetmatsu *et al*., revealed that a sub-population of lamina propria DCs (LPDC) are $TLR5^{+}$ and specialize in the recognition of flagellin (23-25). Furthermore, additional studies have observed that there are several host compartments, primarily the spleen and intestines, that have resident DCs that constitutively express TLR5 and are responsive to flagellin monomers (15, 23, 26, 27). Mucosal DCs tightly regulate the expression of TLR5. TLR5 can also be upregulated in bone marrow derived DCs (BMDC) via the vitamin A metabolite retinoic acid (RA) and can be enhanced further by stromal cell products (28). However, TLR5 expression has been shown to be downregulated with treatment of TLR ligands (28).

TLR5 requires the adaptor protein MyD88 in order to initiate downstream signaling (13, 29). TLRs (except TLR3), as well as IL-1R family members, contain a TIR (Toll-IL-1R) domain that homotypically interacts with the TIR domain of MyD88 (30). TLR5 signaling results in the secretion of a wide array of inflammatory cytokines and chemokines, both *in vivo* and *in vitro*. Human epithelial cells (Calu-3 and A549 cell lines) and DCs (differentiated from human PBMCs) respond to flagellin monomers and release IL-8 (20, 31, 32). Murine $CD11c^+$ lamina propria cells and alveolar macrophages respond to flagellin by releasing IL-6, IL-12, and TNF (21, 25). Early reports from mouse models indicated that purified flagellin monomers were able to upregulate key inflammatory markers, such as TNF, IL-12/23p40, IL-6, and Cxcl1 (homologue of human IL-8), however we have been unable to reproduce TNF secretion using similar mouse models (33). Kinnebrew *et al*. recently showed that $CD103^+$ $CD11b^+$ LPDCs respond to flagellin by secreting large amounts of IL-23 (27). These results indicate that epithelial cells, macrophages and DCs in humans and mice are responsive to flagellin in a TLR5-dependent fashion (34). Although a large body of work indicates that TLR5 recognition of flagellin monomers requires MyD88, a recent report demonstrates that TLR5 also retains MyD88-independent activity as an endocytic receptor that facilitates the presentation of processed flagellin on MHC class II molecules (35). These reports demonstrate that flagellin recognition through TLR5 in a MyD88-dependent fashion occurs on various cell types and plays a key role in innate immunity through the release of pro-inflammatory cytokines and chemokines.

Naip5 and Naip6 expression and function

Intracellular sensors, such as NLRs, are germline encoded PRRs and are another vital component of microbial surveillance. NLRs respond to MAMPs and endogenous signals (36). NLRs form a

multimeric complex, termed the "inflammasome," which leads to secreted mature forms of IL-1β and IL-18, lipid release, and cell death via pyroptosis (37-39). The inflammasome complex is capable of recognizing flagellin that leads to casapase-1 (Casp1) activation. Miao *et al*. observed that cytoplasmic flagellin activated Casp1 in a Nlrc4-dependent manner (10). Further work by Vance *et al*. showed that Naip5 and Nlrc4 are required for Casp1 activation by the amino acid C-terminal portion of *Legionella pneumophila* (*L. pneumophila*) and *S.* Typhimurium flagellins (40). Recent work, by the Shao and Vance groups, has demonstrated that flagellin recognition by the inflammasome is dependent on direct interaction of flagellin with Naip5 and Naip6 (Naip5/6), leading to oligomerization with Nlrc4, and the activation of Casp1 (12, 41). While Naip5/6 recognizes flagellin *in vitro* in transfected HEK293T cells, the role of Naip6 remains unclear since Naip5 appears to be the primary NLR for FliC recognition in BMDMs (12, 41, 42). Moreover, *in vivo* observations from our group has recently shown that Naip5$^{-/-}$ mice given intraperitoneal (i.p.) injections of flagellin were unable to upregulate IL-18, identical to Casp1$^{-/-}$ mice. Our recent observations suggest that we are unable to use IL-18 as a viable measurement for Naip6 recognition of flagellin, despite Naip6's persistence in vivo (33).

Inflammasome signaling is the result of the activation of Casp1. As described above Naip5/6 are capable of recognizing flagellin, and require oligomerization with Nlrc4 in order to recruit Casp1 and initiate processing of pro-IL-18 and pro-IL-1β, and pyroptosis (36, 37, 43). It has been well established that murine macrophages and DCs are able to recognize cytoplasmic flagellin and activate the inflammasome complex. However, it is still unclear if epithelial cells and other cell types are able to recognize intracellular flagellin (37, 44, 45).

FliC's recognition by the innate immune system is a result of conserved structural domains

The flagella that allows for *S.* Typhimurium motility is primarily composed of FliC (Fig. 2) (3). The structure of flagellin has revealed that within its boomerang-like shape, it retains four domains: D0, D1, D2, and D3 (Fig. 2) (46, 47). TLR5 recognition of flagellin is dependent on the conformation and the highly conserved amino acid residues of the D1 domain (48, 49). In contrast, *Helicobater pylori* (*H. pylori*) flagellin has evolved to evade TLR5 detection as a result of amino acid substitution at residues 89-96 (49, 50). Recently, Yoon *et al*. crystallized the zebrafish TLR5 structure interacting with *Salmonella enterica* serovar Dublin (*S*. Dublin) FliC and confirmed that the D1 domain of FliC directly interacts with TLR5 at two primary interfaces, including absolutely conserved flagellin residues Arg^{90} and Glu^{114} (51). Using functional and structural data, we have mutated conserved residues in the D1 domain of FliC, in order to ablate TLR5 recognition and signaling.

The D0 domain of FliC is recognized by Naip5/6. Numerous reports have shown that Naip5/6 recognition is dependent on the C-terminal of domain D0 (10, 11, 13, 40, 41, 52). While TLR5 recognition of domain D1 is entirely dependent on the tertiary structure of FliC (40). Full-length flagellin monomer is capable of activating the inflammasome independently of Naip5, however recognition of the C-terminus of flagellin alone is Naip5-dependent (41). The Vance group went on to demonstrate that Naip5/6 recognition of D0 is ablated when the highly conserved leucine residues 490, 492, and 493 are substituted for alanines (40). Previous groups, along with our own lab's unpublished results, have observed that the recognition of FliC by the innate immune system is responsible for flagellin's antigenic properties. Meanwhile, our lab has observed from *in vitro* and *in vivo* experiments that the addition of a 6x histidine-tag (6xHis) to

either the N- or C-terminus of flagellin significantly affects inflammasome recognition of flagellin that agrees with previously published reports (41).

Antibody responses towards flagellin

Purified flagellin monomers induce robust antibody responses towards itself. As has been discussed, flagellin is a robust agonist of the innate immune system, stimulating a select population of cells. Gewirtz and others have reported that flagellin is capable of generating antibody responses, which are dependent on both innate recognition and T cell responses (19, 22, 53-55). Purified flagellin monomers are capable of activating DCs in a TLR5-MyD88-dependent manner, as seen by the upregulation of MHC class II, CD80, and CD86 (15, 34, 56). Activation of DCs leads to T cell activation, which is required for a humoral response against monomeric flagellin (19, 34, 53). The T cell responses are likely to be influenced by the cytokines that are stimulated by flagellin. Recognition of purified flagellin monomers in mouse models leads to release of Cxcl1, IL-6, IL-23, and IL-18 (33, 44, 55). Sanders *et al*., demonstrated that antibody responses towards *S*. Typhimurium FliC are dependent upon αβ T cells and independent of γδT cells, while serum cytokine responses are independent of T cells (19, 34). In C57BL/6 (BL/6) mice flagellin induced antibody responses are heavily biased towards IgG1 rather than IgG2c isotype specific responses against flagellin monomers (34, 53). While the TLR5-MyD88 flagellin recognition pathway is essential for robust systemic cytokine secretion, neither are required for antibody responses towards purified flagellin (33, 55). However, multiple labs have observed that MyD88 is required for IgG2c and IgG1 isotype specific responses towards flagellin (34, 55). Gewirtz and colleagues observed that either TLR5 or Nlrc4 is sufficient for the induction of humoral immunity, and that these innate flagellin recognition pathways are redundant (55). However, in the absence of both TLR5 and Nlrc4 mice no longer generate

antibodies against flagellin (22, 55). Conversely, our group has recently shown that TLR5, Casp1, and MyD88 are not required for anti-flagellin IgG1 responses, providing evidence to support the idea of an undefined novel third pathway promoting antibody responses towards flagellin (33).

Flagellin as a molecular adjuvant

Flagellin is an attractive adjuvant because of its ability to stimulate the innate immune system and induce robust humoral responses (13, 18). As discussed above, flagellin is capable of being recognized by TLR5 and the inflammasome, which induces a robust pro-inflammatory response. These immunogenic characteristics have led to flagellin being investigated as an adjuvant for human vaccines. Reports have shown when flagellin is co-immunized with antigens, it induces a humoral response towards the co-immunized antigen (22, 33, 54-57). We will refer to the adjuvant activity of flagellin towards co-injected antigens as extrinsic adjuvancy. Protective immunity was observed when the F1 antigen of *Yersinia pestis* (*Y. pestis*) was co-administered with flagellin and lead to the protection of wild-type (WT) mice when challenged via the intranasal route with virulent *Y. pestis* (57). Additionally, the hypervariable domain flagellin is not required for flagellin's extrinsic adjuvancy (54, 58). Flagellin's robust extrinsic adjuvancy requires TLR5 and the inflammasome recognition (22, 33, 55). Within this dissertation we have characterized the required components of the innate immune system and the structural features of flagellin necessary to impart a humoral response towards antigens (such as ovalbumin) when co-administered with FliC (33).

Flagellin is not only an attractive candidate as a stand-alone adjuvant, but it is also a unique protein that can be re-engineered to create novel antigens. As an antigen flagellin stimulates a robust humoral response towards itself in WT mice due to its recognition by TLR5

and the inflammasome (22, 55, 56). While the highly conserved domains D0 & D1 are required for robust pro-inflammatory responses and adjuvancy, the D2 and D3 (D2/D3) hyper-variable region is thought dispensable and can be replaced without compromising immunogenicity. Flagellin is also advantageous as a fusion protein since we can take advantage of the detailed understanding concerning the regulation and structure of the flagellar apparatus. We have investigated how to direct antibody responses to epitopes of interest that replace the hypervariable D2/D3 region of flagellin. Thus, the hypothesis for my graduate work is, innate immune recognition of flagellin's highly conserved amino acid residues regulates isotype-specific antibody responses.

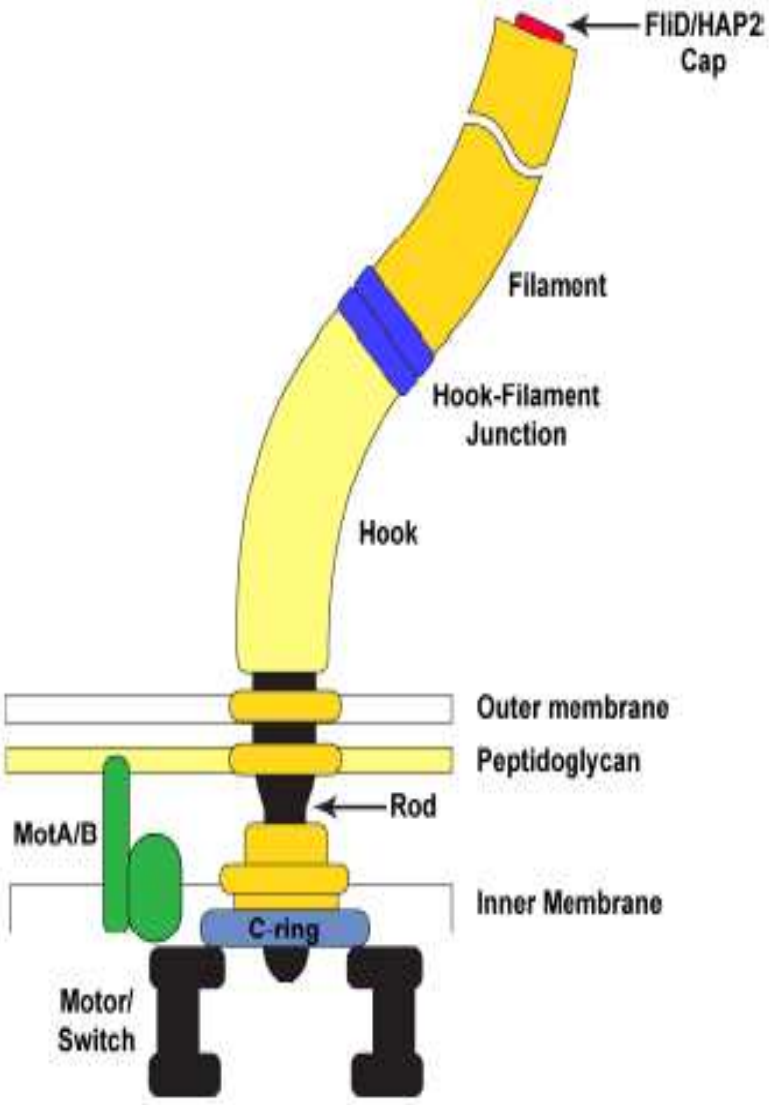

Figure 1. Structure of bacterial flagellum
Adapted from (4).

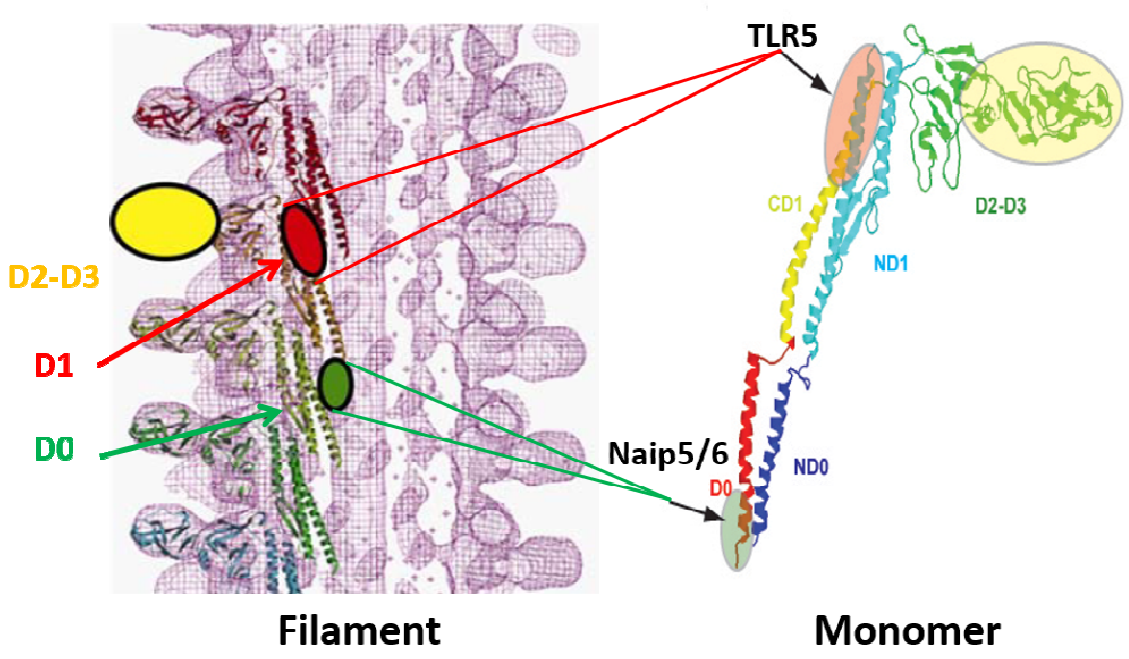

Figure 2. The flagellin filament and monomer
A density map of the flagellar filament alongside monomeric FliC with all its domains labeled. During a bacterial infection, anti-flagellin antibodies are directed to the exposed D2/D3 domain (yellow). TLR5 recognizes D1 (red) and Naip5/6 recognizes D0 (green). Adapted from (47).

Chapter II: Flagellin induces antibody responses through a TLR5- and inflammasome-independent pathway

Introduction

Innate immunity is responsible for both sounding the alarm of pathogen invasion and directing cellular and humoral immunity (59, 60). The innate immune system recognizes pathogens with germline encoded PRRs that respond to conserved pathogen associated molecular patterns (PAMPs) (8, 61, 62). Two key groups of PRRs are membrane bound TLRs and cytosolic NLRs. TLRs recognize structurally diverse PAMPs, including nucleic acids, glycolipids, lipoproteins, and proteins. The only known protein ligand for human TLRs is bacterial flagellin, which is recognized by TLR5 (17).

Flagellin is exposed on the surface of flagellated bacteria and is a major antigenic target of the immune system in a wide variety of hosts, ranging from plants and invertebrates to vertebrates (9, 46, 63). In *S.* Typhimurium flagellin is encoded by the genes *fliC* and *fljB*, with *fliC* being the primary gene (3). FliC is a potent immunogen that is capable of inducing strong immune responses to itself (intrinsic adjuvancy) and co-administered antigens (extrinsic adjuvancy) (19, 22, 44, 55-57, 64-66). The intrinsic and extrinsic adjuvancy of flagellin has been attributed to conserved structures in its D0 domain, recognized by Naip5/6, and its D1 domain, recognized by TLR5 (9-13, 17, 47, 67, 68). Studies from several groups have established that recognition of FliC by the innate immune system leads to microbicidal activity, cytokine production, and DC activation (14, 15, 18). Immunization of mice with FliC elicits robust T cell activation and T cell-dependent antibody responses (15, 19, 22, 26, 53, 55, 69-72).

Flagellin, the ligand for TLR5, has been shown to induce a T_H2 biased response (34, 53, 69), and is currently being developed as a vaccine adjuvant (73, 74). Because flagellin is a

protein, the molecule can be engineered for vaccine development to retain immunogenicity and display foreign epitopes of interest from pathogens such as: influenza (hemagglutinin and matrix proteins) *Y. pestis,* and *H. pylori* (FlaA flagellin) (57, 75-79). Compared to the co-administration of flagellin with an antigen, flagellin fusion proteins elicit enhanced humoral responses and are therefore a more alluring alternative for vaccine design (60, 73, 80-82). The greater efficacy of the flagellin-antigen fusions suggests that proximity of the antigen to the adjuvant allows for enhanced antigenicity

NLRs are cytosolic sensors that oligomerize after ligand recognition and form multi-protein complexes termed inflammasomes (36, 83). A broad range of pathogen derived and endogenous signals initiate inflammasome formation and one of its triggers, alum, has been used for decades as an adjuvant that elicits T_H2 type responses towards co-administered antigens (84, 85). The best-studied NLR, Nlrp3, is required for alum induced activation of the inflammasome (85, 86), but alum also utilizes an inflammasome-independent pathway to induce T_H2 immunity (87, 88). The Naip family of NLRs activates the inflammasome in a Nlrc4-dependent manner (11, 12). Murine Naip2 recognizes the rod proteins of some bacterial type III secretory systems, whereas murine Naip5 and Naip6 recognize flagellin (11, 12, 38). Mouse Naip1 and human NAIP recognize the needle protein of some bacterial type III secretion systems (11, 12, 38, 89). Recognition of these protein ligands by the Naip proteins induces oligomerization with Nlrc4, leading to recruitment and activation of Casp1 (90). Active Casp1 processes pro-IL-1β and pro-IL-18 into mature forms for secretion, and initiates a form of cell-mediated death termed pyroptosis (37). The Nlrc4 system has been recently found to contribute to flagellin-induced antibody production in mice, in a manner that is redundant with TLR5 (22). In the absence of

TLR5, Nlrc4 is required for flagellin's immunogenicity (22). The isotype specificity of Nlrc4 inflammasome-dependent antibody responses is unknown.

TLR5 is expressed on the surface of epithelial cells, neutrophils, monocytes and DCs (17, 23, 48). Flagellin recognition by TLR5 induces its dimerization and signaling through adaptor protein MyD88 (29, 51). Activation of DCs via TLR5 leads to the upregulation of MHC class II, CD80, and CD86, and the secretion of cytokines, such as IL-23, IL-6 and Cxcl1 (15, 19, 69). TLR5 also promotes flagellin uptake and presentation that is required for efficient T cell activation (19, 34, 72, 82). Thus, TLR5 recognition of flagellin induces multiple pathways that are beneficial properties for adjuvants. In the absence of the Nlrc4 inflammasome, TLR5 is required for flagellin's immunogenicity (22).

TLR5 and the major cytokine outputs of the Nlrc4 inflammasome, IL-1β and IL-18, require MyD88 for signaling (91). Despite this commonality antibody responses towards flagellin are maintained in MyD88-deficient mice (55). Thus, MyD88-independent pathways emanating from either TLR5, such as flagellin uptake (35, 72), or the Nlrc4 inflammasome (39) may also contribute to flagellin-specific antibody responses. Herein, we dissect the innate immune components that are responsible for flagellin's intrinsic and extrinsic adjuvant properties, as well as the production of isotype specific antibody responses to flagellin and a model co-administered antigen, ovalbumin (OVA). These studies define the innate immune components that are required to generate robust isotype specific antibody responses towards FliC or co-administered antigens, and uncover a novel TLR5, inflammasome-, and MyD88-independent flagellin recognition pathway that contributes to flagellin's immunogenicity**.**

Results

TLR5 and the Naip5 inflammasome control distinct early cytokine responses in vivo. Innate recognition of FliC leads to cytokine and chemokine production that contributes to host defense and adaptive immunity. To characterize similarities and differences in early phase of innate detection of flagellin we defined the pathways necessary for triggering flagellin-dependent cytokine responses *in vivo* using WT, TLR5$^{-/-}$, Naip5$^{-/-}$, Casp1$^{-/-}$, MyD88$^{-/-}$ and TLR5$^{-/-}$/Casp1$^{-/-}$ (DKO) mice. Mice were injected i.p. with 30 µg of FliC, isolated and purified from *S.* Typhimurium (Fig. 3), and sera were assessed at 2 and 4 hours after flagellin injection. WT mice produced IL-6, Cxcl1, IL-12/23p40, and IL-18 (Fig. 4). At the 2 hour time point serum Cxcl1, IL-6 and IL-12/23p40 were TLR5- and MyD88-dependent (Fig. 4A, C, D). Serum IL-6 and IL-12/23p40 levels were also partially dependent on Naip5 and Casp1 (Fig. 4C, D). In contrast, flagellin induction of IL-18 was TLR5- and MyD88-independent, but entirely dependent on Naip5 and Casp1 (Fig. 4B). At 4 h post-injection the role of TLR5 and the Naip5 inflammasome in flagellin induced cytokine production was more complex (Fig. 4). IL-12/23p40 and IL-18 continued to be dependent on the flagellin sensors, TLR5 and Naip5 inflammasome, respectively (Fig. 4A, B). However, IL-6 and Cxcl1 were detected at 4 h in TLR5$^{-/-}$, but not MyD88$^{-/-}$ or TLR5/Casp1 DKO mice, suggesting a delayed cytokine cascade, where flagellin induced IL-18 or other Casp1-dependent factors induce Cxcl1 and IL-6 in a MyD88-dependent manner (Fig. 4C, D). Thus, the innate immune receptors TLR5 and Naip5 function both independently and in concert to regulate early cytokine production induced by flagellin. Flagellin has also been reported to induce low levels of TNF and IL-1β in mice (22, 55, 92). In our studies, we found no significant flagellin-dependent induction of IL-1β or TNF at 2 and 4 h post-injection in any of the mice (data not shown). For TNF, it is likely that the 2 h time point missed the early and low

level induction which typically peaks around 1-1.5 h post-injection (data not shown) (92, 93). Other investigators have also had difficulty detecting IL-1β in mouse serum, suggesting that technical issues may have precluded our ability to detect the low levels reported by Bedoui and colleagues (44, 55, 92).

IgG1 isotype specific responses are MyD88-independent. Flagellin is a major antigenic target during bacterial infections and when injected as a purified protein also induces antibodies against itself and co-administered antigens. Because flagellin is being developed as a platform for recombinant vaccines, we dissected the innate immune pathways needed to generate isotype specific antibody responses against flagellin or a co-administered antigen, OVA. BL/6 and MyD88$^{-/-}$ mice were injected i.p. with 30 μg of FliC and boosted with the same dose of flagellin 3 weeks later. The immunized animals were bled prior to immunization (naïve serum) and at two weeks post primary and secondary immunizations. We tested the sera for IgG1 and IgG2c antibodies against FliC. After primary immunizations the IgG1 anti-FliC median titer was 3160 in WT mice and 316 in MyD88$^{-/-}$ mice (Fig. 5A). Following the secondary immunization, IgG1 anti-FliC median titers increased more than one hundredfold in both WT and MyD88$^{-/-}$ animals, and IgG1 titers remained significantly reduced in MyD88$^{-/-}$ compared to WT mice (Fig. 5A).

In contrast to IgG1, FliC immunized BL/6 mice generated low titers of IgG2c anti-FliC, which were best detected following the secondary immunization (Fig. 5B). The IgG2c anti-FliC response was absolutely dependent on MyD88$^{-/-}$ (Fig. 5B). Our data demonstrates that IgG2c and a portion of IgG1 anti-flagellin responses are MyD88-dependent. Our results also support a MyD88-independent pathway for IgG1 anti-flagellin antibodies in BL/6 mice.

TLR5 and the inflammasome play largely redundant roles in IgG1 anti-FliC responses. We next examined the individual components of innate flagellin recognition for their contribution to the anti-FliC antibody responses. Compared to WT mice, TLR5$^{-/-}$, Naip5$^{-/-}$, and Casp1$^{-/-}$ mice produced similar IgG1 titers towards flagellin (Fig. 6A-C). In contrast, the IgG2c anti-FliC responses were significantly reduced in TLR5$^{-/-}$, Naip5$^{-/-}$, and Casp1$^{-/-}$mice following secondary immunizations (Fig. 6D-F). Our results suggest that each individual innate recognition pathways for flagellin contributes to the generation of IgG2c anti-FliC responses. In contrast, loss of TLR5 or the individual inflammasome molecules, Naip5 or Casp1, did not affect robust IgG1 anti-FliC responses.

MyD88-independent IgG1 anti-FliC responses are also TLR5- and inflammasome-independent. To determine if TLR5 or the inflammasome contributes to MyD88-independent IgG1 anti-FliC responses, we generated *TLR5* and *Casp1* DKO mice. TLR5/Casp1 DKO mice had significantly reduced IgG1 anti-FliC titers, but maintained moderate titers as seen in MyD88$^{-/-}$ mice (Fig. 7A). As expected, the DKO animals had significantly reduced IgG2c responses towards FliC (Fig. 7B). These results strongly suggest that the MyD88-independent anti-FliC IgG1 response is mediated by a novel pathway that is distinct from the known TLR5 and inflammasome pathways for flagellin recognition.

Serum IgA anti-FliC responses are TLR5 and MyD88 dependent and Casp1-independent. We tested naïve and boost sera of BL/6 mice and knockout mice and assessed IgA anti-FliC titers following secondary immunizations of FliC **s**ince previous *in vitro* and *in vivo* studies have shown that monomeric flagellin is capable of inducing IgA responses towards itself (94, 95). IgA anti-FliC responses were detected at low levels following the secondary immunization (Fig. 8A). Consistent with recent results of Cunningham and colleagues (94), our

results with TLR5$^{-/-}$, MyD88$^{-/-}$ and DKO mice show IgA anti-FliC responses are TLR5- and MyD88-dependent (Fig. 8B, E, F). Furthermore, the IgA anti-FliC titers were detected in Naip5$^{-/-}$ and Casp1$^{-/-}$ mice, suggesting that the inflammasome is not required for IgA anti-FliC responses (Fig. 8C, D).

Flagellin's adjuvancy towards extrinsic antigens is partially dependent on TLR5 and the inflammasome. Next, we examined how flagellin regulates antibody responses towards a co-administered (extrinsic) antigen, OVA. Mice immunized with OVA alone failed to generate anti-OVA responses, whereas mice co-injected with FliC and OVA generated anti-OVA antibodies (Fig. 9A). We also tested the isotype specificity of the FliC-dependent anti-OVA antibody responses and found that in all animals tested, IgG1 was the only isotype detected against OVA; no IgG2c or IgA was generated against OVA (data not shown). The IgG1 anti-OVA responses were similar in WT, TLR5$^{-/-}$, Naip5$^{-/-}$, and Casp1$^{-/-}$ mice (Fig. 9B-D). The IgG1 anti-OVA titers were significantly reduced in MyD88$^{-/-}$ mice and approached, but did not reach, statistical significance in DKO animals ($p=0.07$) (Fig. 9E, F). Thus, the IgG1 and anti-OVA and IgG1 anti-FliC responses appear to be regulated by similar MyD88-dependent & -independent mechanisms.

Defective flagellin-induced IL-18 in A/J mice. A/J mice contain a hypofunctional *Naip5* that is associated with susceptibility to *Legionella pneumophila* (11, 12, 83, 96). We assessed the serum cytokines in response to FliC two hours post i.p. injection with 30 µg FliC. A/J and BL/6 mice produced equivalent amounts of IL-6, IL-12/23p40, and Cxcl1 (Fig. 10A-C). However, A/J mice failed to produce IL-18 (Fig. 10D). As with BL/6 mice, A/J mice did not produce detectable TNF or IL-1β (Fig. 10E, F). Thus the flagellin-induced cytokine responses in

A/J mice are consistent with intact TLR5 and impaired Naip5 recognition of flagellin due to the hypofunctional *Naip5*$^{A/J}$ allele.

Augmented IgG1 and IgG2a anti-FliC responses in A/J mice. We compared isotype specific antibody responses against FliC in A/J and BL/6 mice and their respective *MyD88* deficient strains. In contrast to BL/6 mice, A/J mice had more robust antibody responses to flagellin after primary and secondary immunization, with approximately 10-fold increased IgG1 anti-FliC titers following primary and secondary, and greater than 100-fold increased IgG2a/c anti-FliC titers following primary and secondary immunizations (Fig. 8A, B). The IgG2a responses in A/J mice were partially MyD88-dependent, whereas IgG1 anti-FliC titers were reduced in A/J MyD88$^{-/-}$ mice, trending towards significance (P=.08) (Fig. 8A, B). In contrast to BL/6 mice, which have a strong IgG1 biased antibody response against flagellin, A/J mice had a balanced antibody response towards flagellin, with equivalent IgG1 and IgG2a anti-FliC titers.

Anti-OVA responses in A/J mice are partially dependent on MyD88. A/J and A/J MyD88$^{-/-}$ mice were immunized with FliC and OVA. As with IgG2c responses in BL/6 mice, IgG2a anti-OVA antibody responses were undetectable in A/J mice (data not shown). After primary immunizations in both A/J and A/J MyD88$^{-/-}$ animals, IgG1 anti-OVA antibodies were at titers of 100 or less in all mice (Fig. 8C). In A/J mice following secondary immunizations with FliC and OVA, IgG1 anti-OVA responses were robust, with titers reaching a median of 10^5 (Fig. 8C). As for BL/6 MyD88$^{-/-}$ and A/J MyD88$^{-/-}$ mice had reduced, but detectable IgG1 anti-OVA titers compared to that of their WT counterparts (Fig. 8C).

MyD88 suppresses IgA anti-FliC responses in A/J mice. Sera from A/J and A/J MyD88$^{-/-}$ was also assessed for IgA anti-FliC responses. In contrast to BL/6 mice, A/J mice did not generate significant IgA anti-FliC following two immunizations (Fig. 9A). Conversely, the

A/J MyD88$^{-/-}$ mice produced a substantial IgA anti-FliC titer after two immunizations (Fig. 9B). These results illuminate the fine intricacies between different strains of mice, resulting in quantitative and qualitative differences in isotype specific responses generated against FliC.

Discussion

Dissecting the innate immune pathways that recognize flagellin's structural properties and promote adaptive immune responses is vital for the rational design of flagellin based vaccines. Flagellin based fusion proteins are currently being developed as vaccines for infectious diseases (73, 74) and assessed in phase I and II clinical trials (76-78). Flagellin is also being developed as a therapeutic agent to treat infectious diseases, toxic exposures and cancers (97-99). Several studies have shown that flagellin's adjuvancy is dependent on TLR5 recognition (73-75). More recently murine Naip5 and Naip6 have been demonstrated to recognize bacterial flagellin and activate the Nlrc4 inflammasome (11, 12). Additional studies have indicated that inflammasome mediated detection of flagellin also contributes to its immunogenicity (55). Vijay-Kumar et al. demonstrated that TLR5 and the Nlrc4 inflammasome play redundant roles in flagellin-induced antibody responses, with neither being necessary and either being sufficient for flagellin-induced antibody responses (22). This same group determined that MyD88 is not required for flagellin induced IgG antibody responses, suggesting that a MyD88-independent pathway emanates from either TLR5 or the Nlrc4 inflammasome, and contributes to anti-flagellin IgG production.

In our study, we dissected the role of flagellin detection by the innate immune system in generating isotype specific antibody responses to flagellin itself (intrinsic adjuvancy) and co-administered OVA (extrinsic adjuvancy). While neither deletion of TLR5 nor the Naip5 inflammasome alone were sufficient to reduce IgG1 anti-FliC antibodies, deletion of both TLR5 and Casp1 or elimination of MyD88 significantly reduced IgG1 responses towards flagellin.

However, IgG1 anti-FliC responses were still detected in MyD88 and TLR5/Casp1 DKO mice, suggesting flagellin is recognized by a third uncharacterized pathway. This conclusion is supported by our studies in A/J MyD88$^{-/-}$ mice, which are naturally impaired in Naip5 (96). The A/J MyD88$^{-/-}$ mice produced reduced, but moderate IgG1 and IgG2a anti-FliC responses. The combined data from immunizations performed in BL/6 and A/J mice support our working hypothesis that a novel third pathway contributes to antibody responses towards flagellin and that this pathway is independent of MyD88 and known innate detection pathways for flagellin recognition, TLR5 and Naip5. Uncloaking this third pathway would help understand the complexities of innate immune recognition of flagellated pathogens, and will also be critical for the rationale design of flagellin-based vaccines. It will be critical to assess whether or not this third pathway is conserved in humans, and how this pathway may influence the generation of robust cellular and humoral responses, and the establishment of long-term immunity.

Our data also demonstrates that in BL/6 mice, TLR5 and the Naip5/Nlrc4/Casp1 inflammasome work in parallel to drive IgG2c anti-FliC responses in a MyD88-dependent manner. The MyD88-dependency of the IgG2a/c antibody response is even more apparent in A/J mice, which have quantitatively greater antibody responses and markedly enhanced IgG2a response that is equivalent to the IgG1 response. The studies in A/J mice demonstrate that the IgG2a primary response has greater dependence on MyD88 than the IgG1 response, as seen in BL/6 mice. However, unlike BL/6, A/J mice also had a MyD88-independent component for the IgG2a response. This suggests that underlying genetic differences between BL/6 and A/J mice contribute to isotype specificity and overall quantity of anti-flagellin immune responses.

Although this MyD88-independent pathway did not contribute to IgG2c responses in BL/6 mice, this may be due to the low magnitude of the IgG2c responses toward flagellin

observed in BL/6 mice. Thus, it will be important to further dissect the components of the immune systems in BL/6 and A/J mice that are responsible for MyD88-independent responses against flagellin. Similarly, the structural components of bacterial flagellin that dictate MyD88-independent antibody production are uncharacterized. Understanding this third pathway of flagellin is another piece to the puzzle that can be utilized to enhance flagellin based vaccine design.

Our results from BL/6 mice demonstrate that IgA anti-FliC responses are TLR5- & MyD88-dependent, consistent with a recently published report (94). We add to this body of work by demonstrating that the inflammasome does not impact IgA anti-FliC titers and thus does not compensate for TLR5-deficiency. Our results from the BL/6 mice are congruent with the data from Cunningham and colleagues, which indicate that $CD103^{+}$ DCs, from the lamina propria, expressing TLR5 prime $Foxp3^{+}$ Tregs to induce flagellin-specific IgA in the mesenteric lymph node (94). Conversely, our data from A/J and A/J $MyD88^{-/-}$ mice indicate that IgA anti-FliC responses may be regulated differently in different strains of mice. In A/J mice, MyD88-dependent signals suppressed IgA production. The mechanism for suppression of IgA anti-FliC responses in A/J mice is currently unknown and requires further investigation.

It is well publicized that flagellin, when used as an adjuvant, imparts an IgG1 isotype specific response towards co-administered antigens, presumably due to TLR5 recognition (15, 53, 55, 57). Our data support the conclusion that TLR5 and the Naip5/Nlrc4/Casp1 inflammasome play overlapping roles for IgG1 responses towards OVA when co-administered with FliC (extrinsic adjuvancy). Because the anti-OVA IgG1 is reduced but detectable in both $MyD88^{-/-}$ and DKO mice, our data also indicates that the MyD88-independent pathway contributes to the adjuvancy of FliC towards OVA. Therefore, our immunization studies support

the existence of a third pathway for flagellin recognition that also contributes to flagellin's adjuvancy.

Interestingly, humans contain one full length *Naip* homolog, *NAIP* (100, 101). Human NAIP recognizes needle proteins from bacterial type three secretory systems (12); however, there are also reports indicating that NAIP recognizes flagellin (102, 103) and that copy number variation for the human *NAIP* gene may affect ligand detection (101, 104). Although flagellin recognition by human NAIP has been implicated with cellular assays (102), it has recently been demonstrated by multiple groups, that mouse Naip1 and human NAIP, both recognize needle proteins from type III secretions systems from several bacteria, not bacterial flagellin (11, 12, 89).

In addition, it has been reported that TLR11 can recognize flagellin in mice of the BL/6 background (105). It is possible that TLR11 contributed to antibody responses towards FliC. However, like all other TLRs, except TLR3 (106, 107), TLR11 is MyD88-dependent (108). Because FliC immunized MyD88$^{-/-}$ and TLR5/Casp1 DKO mice have similar phenotypes, TLR11 does not appear to contribute significantly to flagellin-dependent antibody responses. In any case, the translational implications of TLR11 recognition of flagellin for vaccine development in humans are of little relevance, since human TLR11 is a nonfunctional pseudogene (63).

Our data indicate that flagellin is a potent adjuvant for IgG1 and IgG2a/c isotype specific responses toward itself. In addition, our data indicates that TLR5 and MyD88 contribute to the IgG2a/c immune responses in both BL/6 and A/J mice. Our studies revealed that the IgG2a/c vs. IgG1 bias of the anti-flagellin humoral response was strongly influenced by mouse genetic background. Because BL/6 mice have a weaker and IgG1 biased anti-flagellin antibody response,

A/J mice may be a more relevant model for deciphering the immunogenic characteristics of flagellin and the host innate immune pathways needed for robust anti-flagellin immune responses. Since A/J animals generate a robust IgG1 and IgG2a anti-FliC responses in a Naip5-independent manner, studies in A/J mice may also be more relevant to human vaccine development.

In both A/J and BL/6 mice, antibody responses generated against OVA were solely IgG1, with no detectable IgG2a/c anti-OVA antibodies. It is not clear why flagellin induces distinct isotype specific responses against intrinsic (FliC) and extrinsic (OVA) antigens. It is conceivable that increased proximity of antigen to the immunogenic portions of flagellin (sites recognized by TLR5, inflammasome, and 3rd pathway) dictates a more robust IgG2a/c antibody response to intrinsic antigens. Further dissection of the cellular and molecular pathways that distinguish and differentiate responses towards intrinsic and extrinsic antigens are needed.

At present, the identity of this third pathway that promotes anti-FliC responses is unknown. The flagellin utilized in our immunizations is a monodispersed species, as expected for flagellin monomers (Fig. 3); therefore, it is unlikely that the third pathway represents a T cell-independent response to flagellin polymers (109-111). Our results from TLR5/Casp1 DKO mice indicate that the third pathway does not utilize either TLR5 or the inflammasome. Thus, the possibility that TLR5 heterodimerizes with another TLR, such as the proposed TLR4/5 heterodimers, is an unlikely explanation for the third pathway (18, 112). It is formally possible that TLR11 could function through a MyD88-independent mechanism, or that Naip5 or Naip6 could signal independently of Casp1. The existence of both of these novel pathways would provide a complex explanation for our results. Although we cannot exclude this possibility of multiple novel signaling pathways emerging from the known flagellin receptors, we favor the

most parsimonious explanation for our data, which is a third pathway for flagellin recognition that functions independently of MyD88, TLR5 and the inflammasome. Therefore, we believe the most suitable set of experiments to compliment the work presented here would be to reengineer endogenous FliC from *S.* Typhimurium with destroyed recognition sites at domains D0 and D1. Immunizations conducted with these reengineered proteins and complimented with various knockout mice, would allow us to confirm a third innate flagellin recognition pathway that promotes flagellin's robust antigenicity.

Our results demonstrate that flagellin's adjuvancy toward intrinsic and extrinsic antigens can be attributed to at least three pathways in our animal model: TLR5, the Naip5/Nlrc4/Casp1 inflammasome, and a novel MyD88-independent pathway. It is unclear what constitutes the third novel pathway for flagellin-dependent antibody responses, and what structures on flagellin are required for this activity. Additionally, it is unknown whether the third pathway is evolutionarily conserved and contributes to flagellin-induced immunity in humans. These questions beg further investigation in order to enhance our understanding of the biological properties of flagellin, and how flagellin may be used as an adjuvant, a vaccine platform, and therapeutic agent.

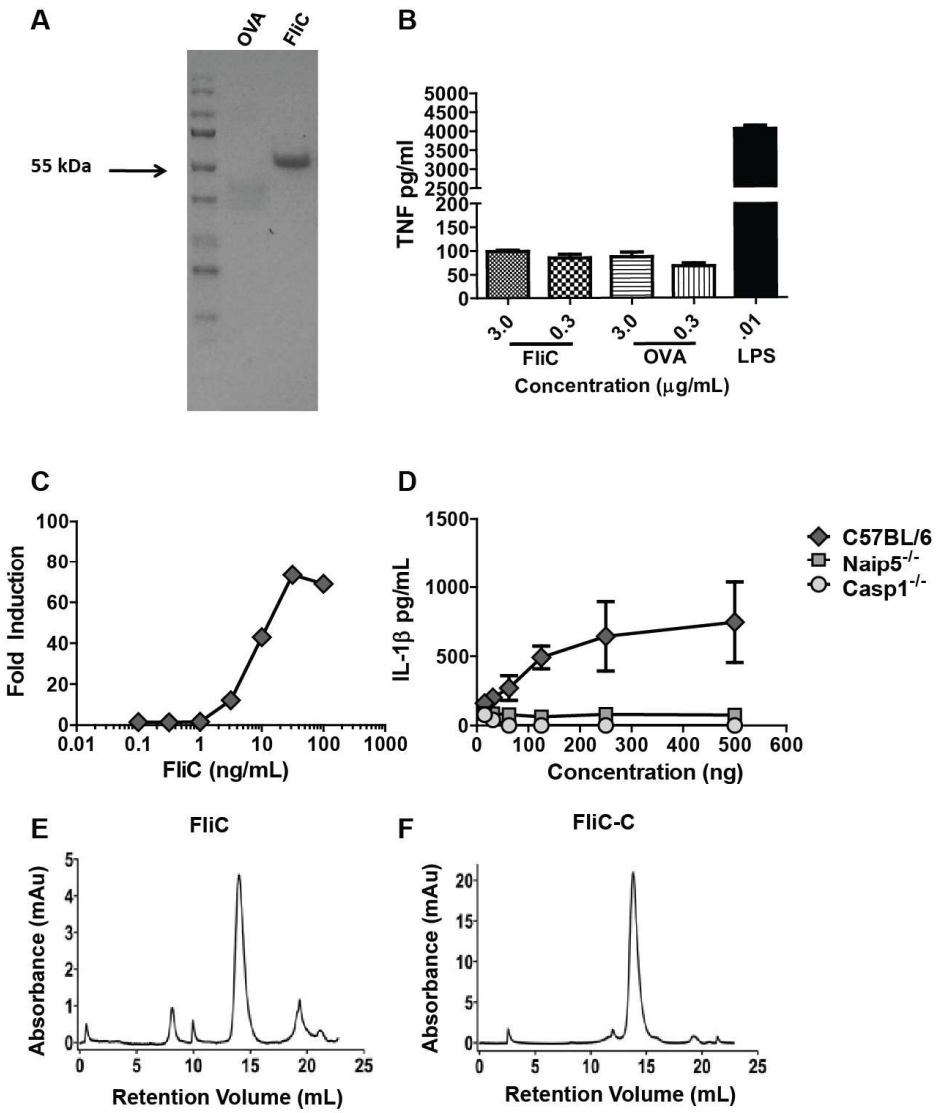

Figure 3. Highly purified flagellin is free of exogenous innate immune agonists, and is a robust agonist of TLR5 and the inflammasome. Highly purified FliC and OVA contained less than 0.1 pg endotoxin (0.001 EU) per µg of protein (data not shown), and consisted of a single protein of approximately 55 kDa (**A**). Flagellin did not activate RAW 264.7 cells, which do not express TLR5 and are unresponsive to flagellin (**B**). RAW 264.7 cells stimulated with either PBS, FliC, or OVA had less than 100 pg/mL TNF from collected supernatants, while cells given 10 ng/ml LPS produced greater than 4000 pg/mL in supernatants collected following overnight treatment (**B**). TLR5 biological activity was determined using CHO cells stably transfected with murine TLR5 and a NF-κB luciferase reporter (**C**). Flagellin induced Naip5- and caspase-1-dependent IL-1β secretion in LPS primed BMDMs with an EC_{50} of approximately 100 ng/mL (**D**). Size exclusion chromatography demonstrated that FliC purified from *S.* Typhimurium (FliC) (**E**) or recombinant FliC purified from E. coli (FliC-C) (**F**) eluted at the same volume as a monodispersed species, consistent with flagellin monomers.

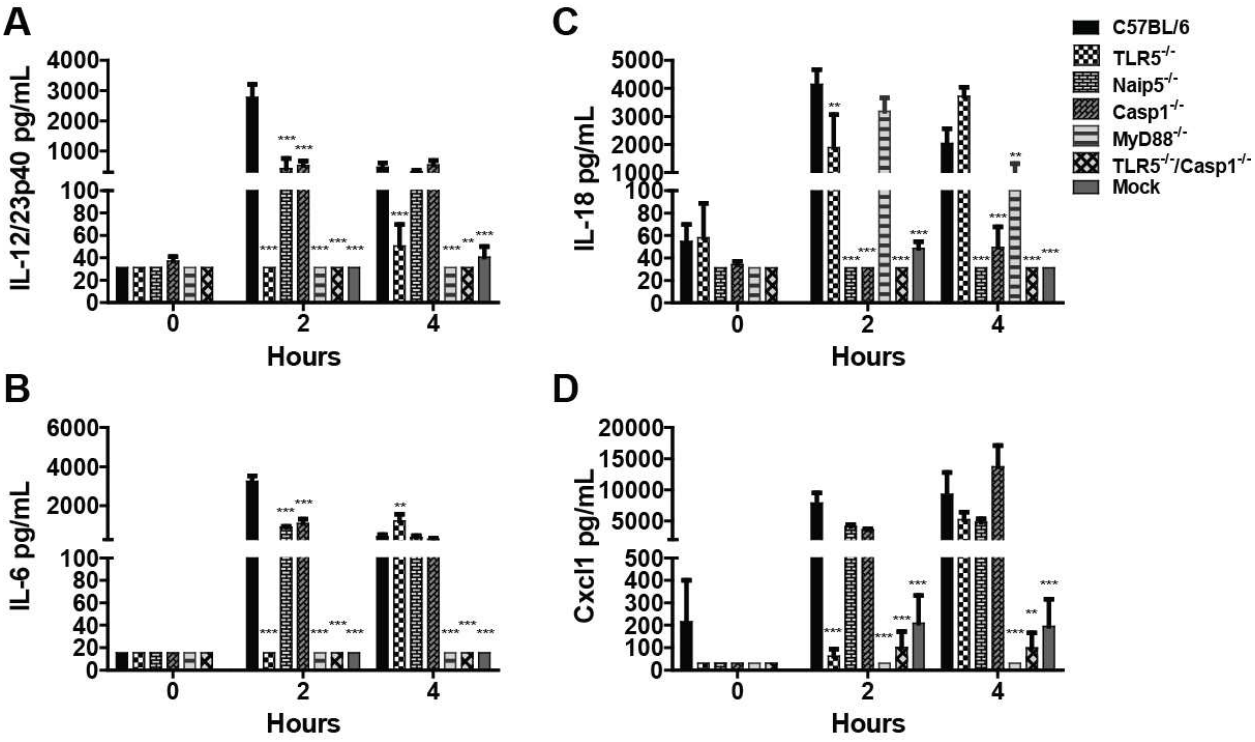

Figure 4. Flagellin induced cytokines are differentially regulated by TLR5, Naip5, Casp1, and MyD88. WT (n=7-12), TLR5$^{-/-}$ (n=4-8), Naip5$^{-/-}$ (n=3), Casp1$^{-/-}$ (n=4-11), DKO (n=3-4), MyD88$^{-/-}$ (n=3-6), and mock (n=8-10) mice were injected i.p. of FliC (30 µg) or PBS (mock). Serum was collected 2 hours after injections and cytokine levels were determined by ELISA. IL-12/23p40 **(A**), IL-18 **(B**), IL-6 **(C**) and Cxcl1 **(D**). All groups have a minimum n=3. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post-test: ** = P<0.01, *** = P<0.001.

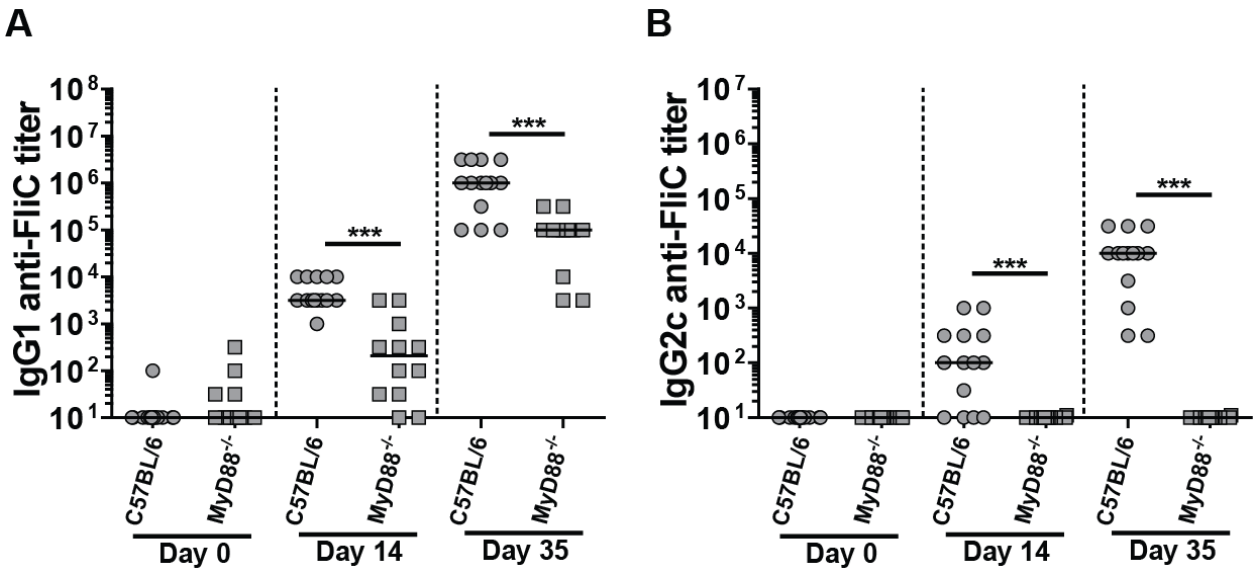

Figure 5. IgG1 anti-FliC responses are partially and IgG2c anti-FliC are entirely MyD88-dependent. WT (n=9) and MyD88$^{-/-}$ (n=7) mice were immunized with 30 µg FliC on day 1 and 21 and sera collected on days 14 and 35. Naïve, day 14, and day 35 sera were analyzed for IgG1 **(A)** and IgG2c **(B)** antibody responses against FliC by ELISA. Data is a combination of two-independent experiments with n=3-5 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: ** = $P < 0.01$.

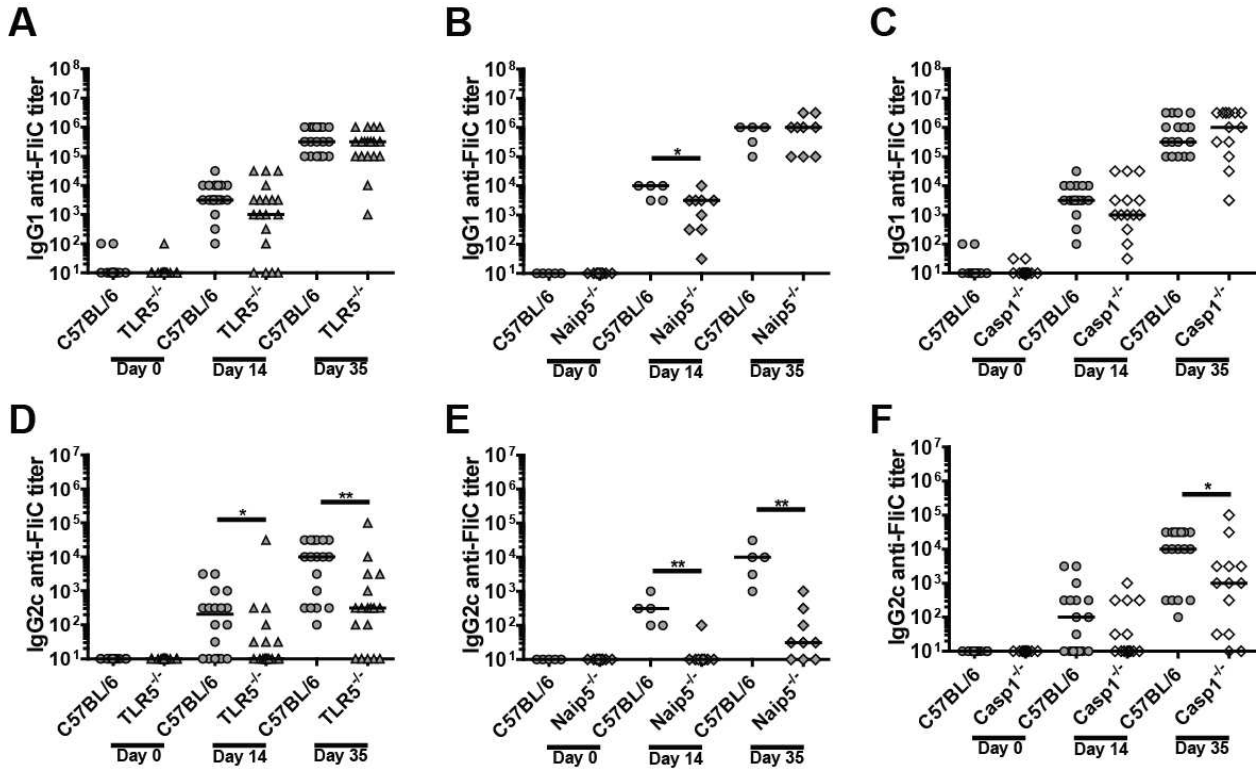

Figure 6. IgG2c anti-FliC responses are partially TLR5- and inflammasome-dependent. TLR5$^{-/-}$ (n=19) **(A, D)**, Naip5$^{-/-}$ (n=9) **(B, E)**, and Casp1$^{-/-}$ (n=14) **(C, F)** mice were immunized twice on day 1 and day 21 and sera collected on days 14 and 35. Naïve, day 14, and day 35 sera were analyzed for IgG1 (**A-C**) and IgG2c (**D-F**) isotype specific antibody responses against FliC by ELISA. Data is a combination of 2-3-independent experiments with n=3-6 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= $P < 0.05$, ** = $P < 0.01$.

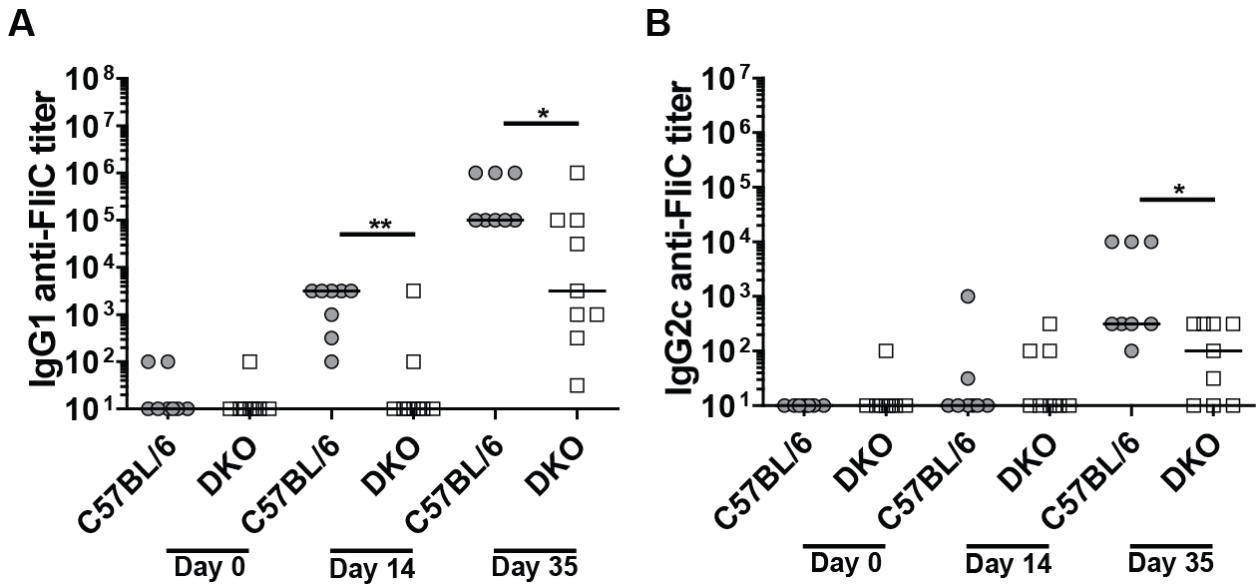

Figure 7. IgG1 anti-FliC responses are partially TLR5- & caspase-1-independent. TLR5$^{-/-}$ /Casp1$^{-/-}$, DKO (n=10) mice were immunized twice on day 1 and day 21 and sera collected on days 14 and 35. Naïve, day 14, and day 35 sera were analyzed for IgG1 (**A**) and IgG2c (**B**) isotype specific antibody responses against FliC by ELISA. Data is representative of two-independent experiments with n=4-5 per group. Statistical analyses were done using Mann-Whitney analysis of individual groups: ** = $P < 0.01$.

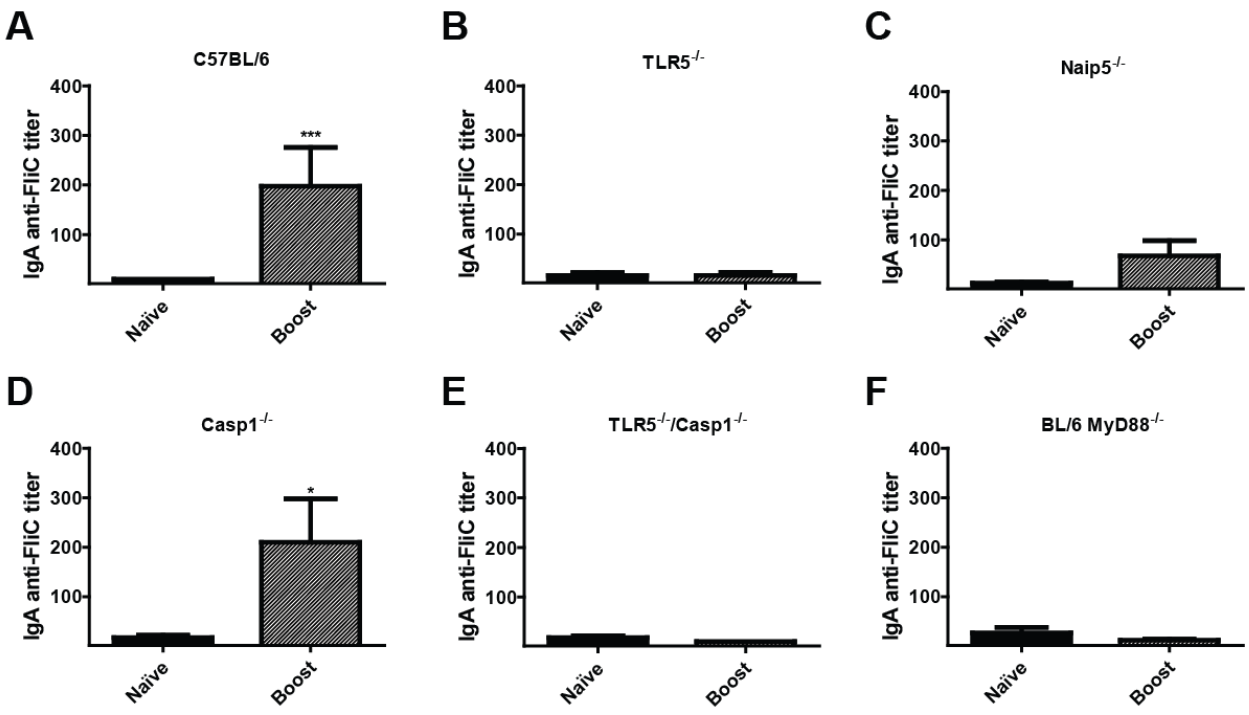

Figure 8. IgA anti-FliC responses are TR5- & MyD88-dependent. C57BL/6 (n=17) **(A)**, TLR5$^{-/-}$ (n=14) **(B)**, Naip5$^{-/-}$ (n=9) **(C)**, and Casp1$^{-/-}$ (n=11) **(D),** TLR5$^{-/-}$/Casp1$^{-/-}$, DKO (n=9) **(E)**, MyD88$^{-/-}$ (n=11) **(F)** mice were immunized on day 1 and day 21 and sera was collected on days 0 (naïve), 14 and 35. Naïve and day 35 sera (boost) were analyzed for IgA specific antibody responses against FliC by ELISA. Data is a combination of 2-3-independent experiments with n=3-6 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= $P < 0.05$, *** = $P < 0.001$.

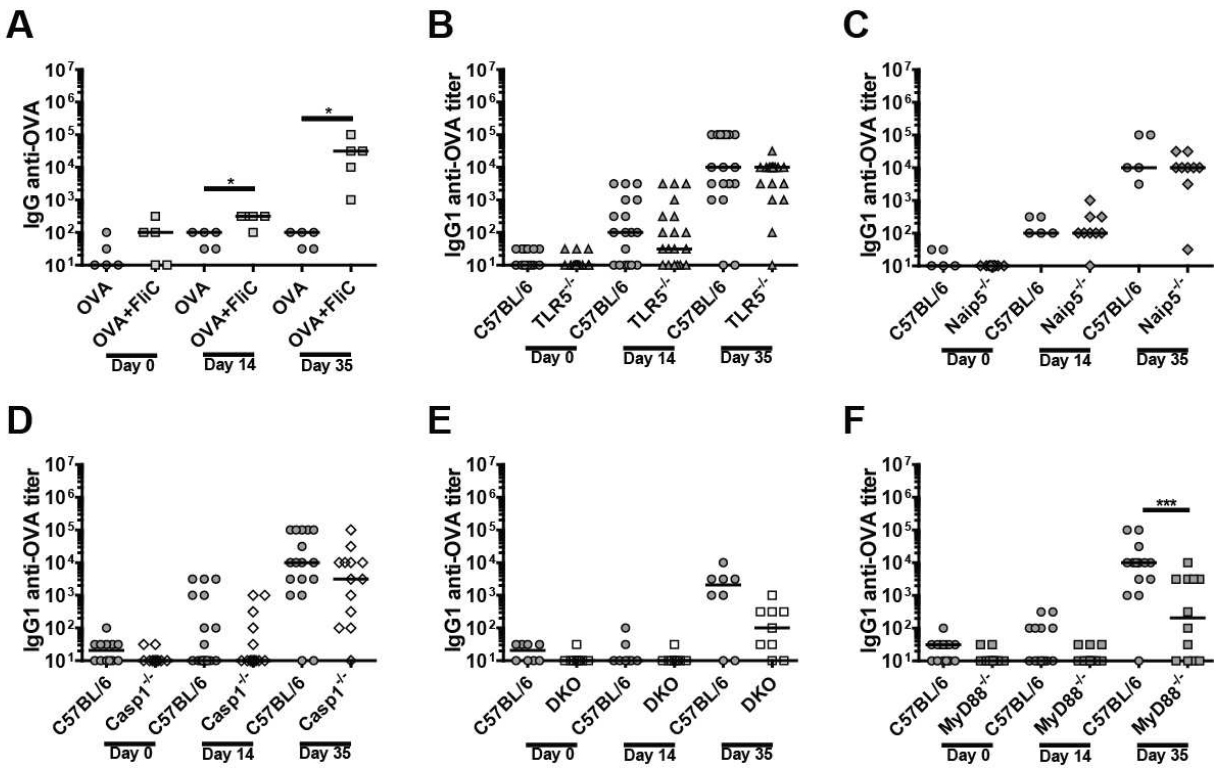

Figure 9. Flagellin induced IgG1 anti-OVA responses are partially MyD88-dependent. WT, mice were immunized twice on day 1 and day 21 with OVA alone (n=5) or OVA plus FliC (n=5), and sera collected on days 14 and 35. **A)** Naïve, day 14, and day 35 sera were analyzed for IgG1 specific antibody responses against OVA by ELISA. **B-F)** WT, TLR5$^{-/-}$ (n=19) **(B)**, Naip5$^{-/-}$ (n=9) **(C)**, Casp1$^{-/-}$ (n=14) (**D**), MyD88$^{-/-}$ (n=7) (**E**) and TLR5$^{-/-}$/Casp1$^{-/-}$, DKO (n=10) (**F**), mice were immunized twice on day 1 and day 21 with OVA plus FliC, and sera collected on days 14 and 35. Naïve, day 14, and day 35 sera were analyzed for IgG1 specific antibody responses against OVA by ELISA. **B-F)** Data is a combination of 2-3-independent experiments with n=3-6 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= $P < 0.05$, *** = $P < 0.001$.

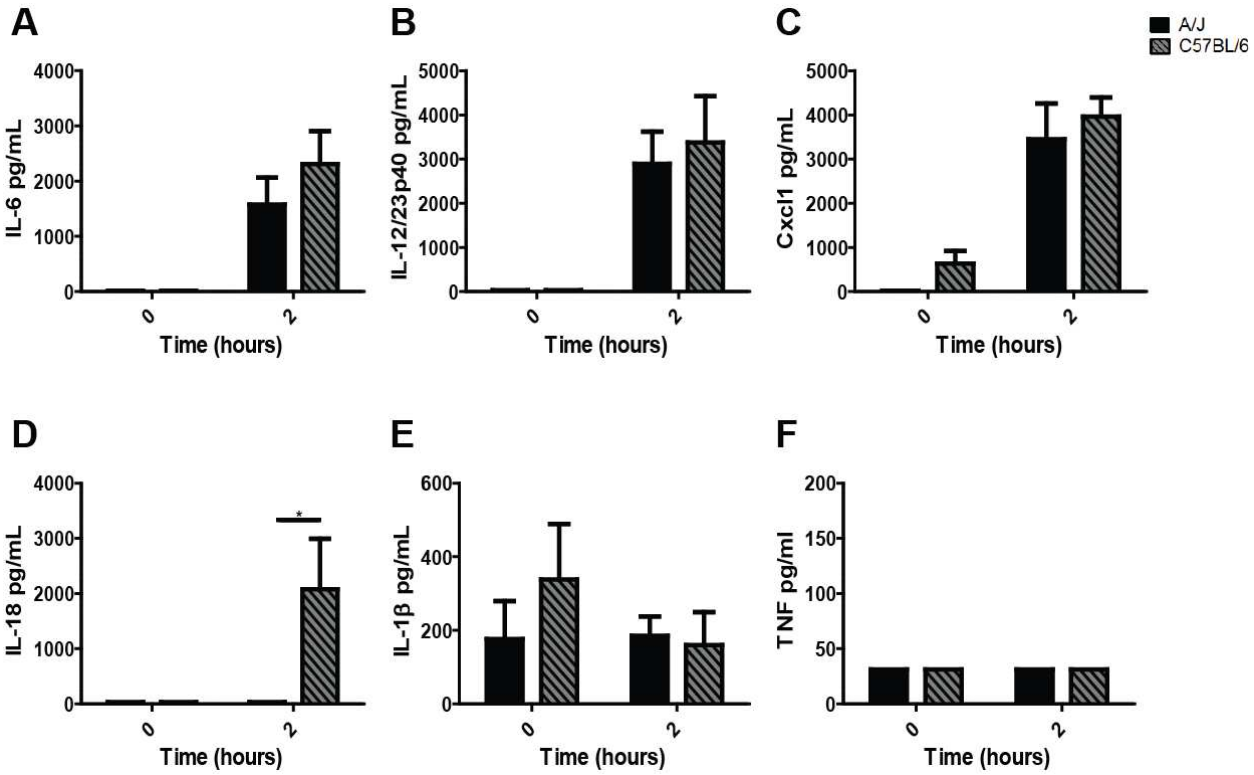

Figure 10. Defective flagellin-induced IL-18 in A/J mice. C57BL/6 (n=5) and A/J (n=5) mice were injected i.p. with FliC (30 µg). Serum was collected 2 hours after injections and cytokine levels were determined by ELISA: Cxcl1 (**A**), IL-6 **(B**), IL-12/23p40 **(C**), IL-18 **(D**), IL-1β **(E**), and TNF **(F**). Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post-test: * = $P<0.05$.

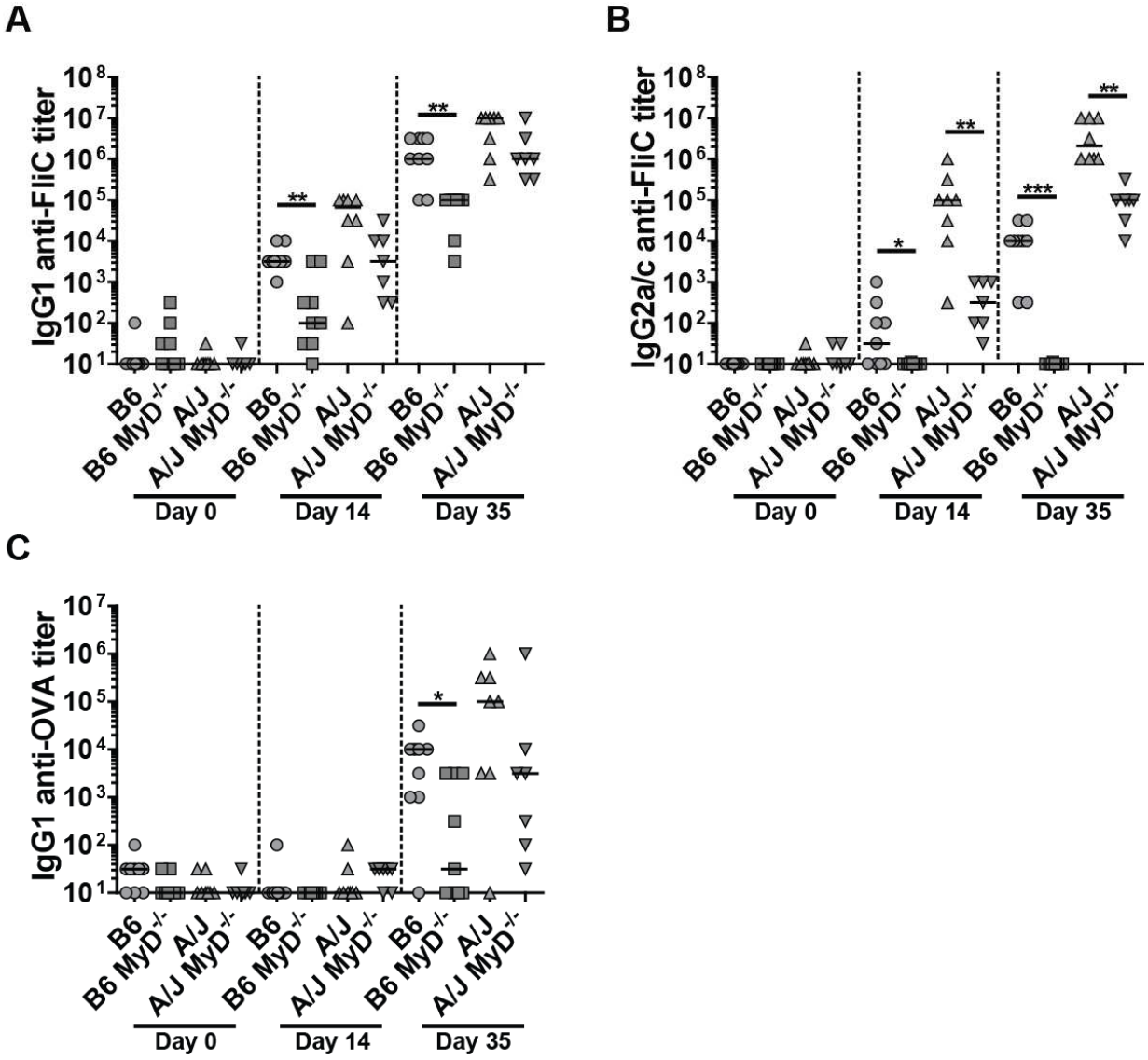

Figure 11. Augmented IgG1 and IgG2a anti-FliC responses in A/J mice are partially MyD88-dependent. C57BL/6 (n=9) and A/J mice (n=7), and their MyD88-defiecient counterparts (n=7-9) were immunized twice on day 1 and day 21 with FliC plus OVA and sera collected on days 14 and 35 were analyzed for IgG1 (**A**), and IgG2a or IgG2c (**B**) responses against FliC by ELISA. **C)** Naïve, day 14, and day 35 sera were assessed for IgG1 anti-OVA responses by ELISA. Data is a combination of two-independent experiments with n=3-5 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= $P<0.05$, ** = $P<0.01$.

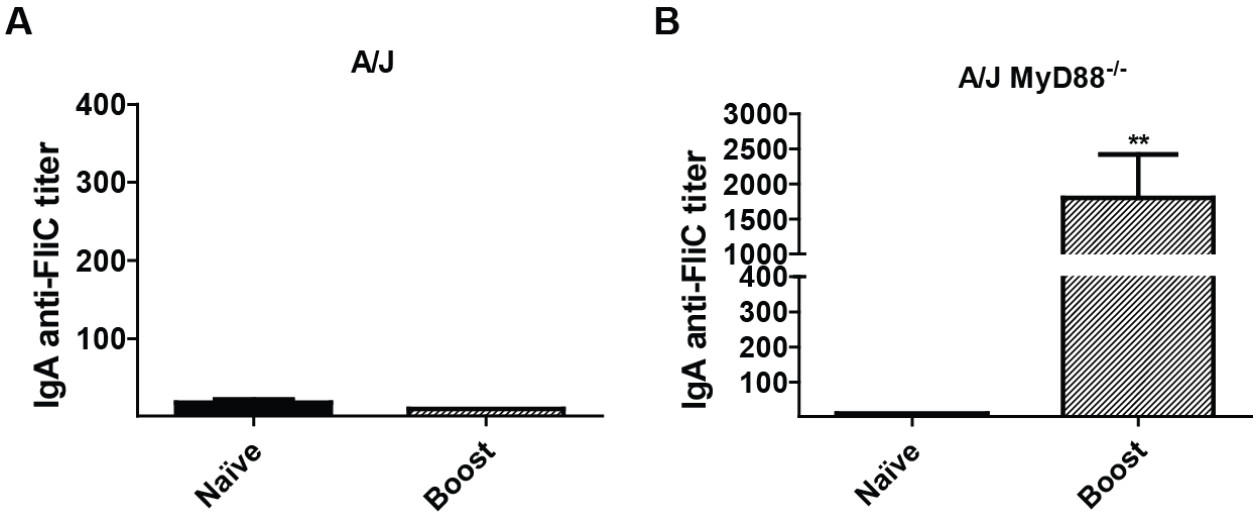

Figure 12. A/J MyD88$^{-/-}$ mice generate robust IgA anti-FliC responses. A/J mice (n=8), and their MyD88-defiecient counterparts (n=7) were immunized twice on day 1 and day 21 with FliC plus OVA and sera collected on days 0 (naïve), 14 and 35. Naïve and day 35 sera were analyzed for IgA specific antibody responses against FliC by ELISA **(A, B)**. Data is a combination of two-independent experiments with n=3-4 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: ** = P<0.01.

Chapter III: Flagellin's hypervariable D2/D3 domain, but not the TLR5 and Naip5/6 recognition sites are required for primary anti-flagellin antibody responses

Introduction

The innate immune system is armed with germline encoded PRRs that recognize a multitude of PAMPs (8, 106). The recognition of PAMPs by PRRs leads to the induction of pro-inflammatory responses, including the activation of mononuclear phagocytes that engulf, process, and present antigens initiating adaptive immune responses (8, 61, 62, 106). Two sets of PRRs that have been intensely studied over the past decade are TLRs and NLRs (36, 39, 113). TLRs have been shown to recognize various PAMPs, ranging from bacterial lipopeptides (TLR1, 2, 6) to viral nucleic acids (TLR3, 7, 8, 9), but the only human TLR to recognize a proteins is, TLR5 (17, 63).

TLR5 recognizes flagellin and is located on the cell surface of epithelial cells, neutrophils, monocytes, and DCs (9, 17, 23). TLR5 signaling is dependent on the adaptor protein MyD88 that is essential for downstream signaling via NF-κB and MAPK pathways (30, 51). TLR5 recognition of flagellin induces cytokine and chemokine production that is MyD88-dependent (19, 33, 55). Several studies support the requirement for TLR5 signaling via MyD88 to induce T cell-dependent antibody responses towards flagellin (15, 19, 26, 35). However, recent studies have also shown that TLR5 enhances anti-flagellin T cell responses independently of MyD88 and Nlrc4, and suggest that TLR5 also functions as an endocytic receptor to enhance processing and flagellin presentation (35, 72). We have shown that flagellin induces antibody responses that are independent of the known TLR5, Naip5/Nlrc4/Casp1 inflammasome, and

MyD88 signaling pathways, suggesting that flagellin activates a third novel pathway to promote antibody responses (22, 33, 55).

Another set of PRRs that play an essential role in detecting various PAMPs are NLRs (52). NLRs are a key group of cytosolic sensors that are able to detect both PAMPs and endogenous danger signals that lead to the activation of the inflammasome. One set of distinct NLRs is the Naip family. In mice, there are at least four Naips: Naip1, Naip2, Naip5, and Naip6 that recognize needle, rod, or flagellin proteins from various bacterial species (12, 38). Naip5/6 recognize cytosolic flagellin and activate the inflammasome through recruitment of Nlrc4, which triggers Casp1 (13, 37). Activation of the Naip5/6 inflammasome leads to the secretion of bioactive forms of IL-18 and IL-1β, calcium dependent secretion of eicosanoids, and cell death, pyroptosis (37). Both the IL-18R and IL-1R also require MyD88. Similar to TLR5, the inflammasome does not play a significant role in generating IgG1 anti-FliC responses following FliC immunizations (6, 33). Thus, TLR5 and the Naip5/6 inflammasome have redundant roles in the induction of IgG1 antibody responses towards FliC. Mice that are deficient in both TLR5 and inflammasome mediated recognition, have significantly reduced IgG1 and barely detectable IgG2c anti-FliC responses (33). These results suggest that TLR5 and the inflammasome work together to promote IgG1 and IgG2c anti-FliC responses and that neither are absolutely required for flagellin-induced antibody responses.

S. Typhimurium flagellin, FliC, is comprised of four domains: D0, D1, D2, and D3 (46, 47). The crystal structure and cryoelectron microscopy of FliC and the flagellar filament have revealed that the D0 (Naip5/6) and D1 (TLR5) domains, which are buried deep within the filament of polymeric flagellin, are recognized by the host's innate immune system (46, 47). The Naip5/6 recognition site of FliC has been mapped to the carboxy-terminal 35 amino acids of the

D0 domain, but is also influenced by the amino-terminal D0 domain (10, 40, 41). FliC's TLR5 recognition site has been mapped to the highly conserved amino acid region of the D1 domain, which has been confirmed in the recent crystal structure of flagellin with zebrafish TLR5 (48, 49, 51).

The D2 and the D3 domains (D2/D3) of FliC are largely exposed on the outer surface of the flagellar filament, and are the regions of the protein that are recognized by serotype specific antibodies during natural *Salmonella* infections (114-117). While most studies have implicated the TLR5 recognition site of FliC as the critical component of flagellin's adjuvancy and immunogenicity, there are also reports that suggest the hypervariable region, made up of D2/D3 domain are required for FliC's immunogenicity (58, 118-120).

Utilizing flagellin's known recognition sites, our group set out to determine how FliC's innate immune stimulatory sites in domains D0 and D1 contribute to the MyD88-independent pathway that promotes IgG1 anti-FliC responses. In this study we selectively destroyed either the Naip5/6 (D0) or TLR5 (D1) recognition sites to create two proteins, FliC-C and $FliC^{TLR5}$, which lack Naip5/6 or TLR5 innate stimulatory activity, respectively. To determine whether the D0 and D1 sites are sufficient for FliC's immunogenicity we deleted the D2/D3 domain, and also tested a natural flagellin molecule from *Listeria monocytogenes* (*L. monocytogenes*), FlaA, which retains TLR5 and Naip5/6 stimulatory activity, but does not contain a D2 or D3 region (49). Using this panel of mutant flagellin proteins and mice with targeted deficiencies, we determined the molecular requirements for flagellin induced antibody responses.

Immunizing WT, $MyD88^{-/-}$ and DKO mice, with the four different flagellin monomers demonstrated how modifications to the different innate recognition sites and domains affect FliC's intrinsic and extrinsic adjuvancy. We performed primary and boost immunizations on

WT, MyD88$^{-/-}$ and DKO mice with either WT FliC (isolated from *S.* Typhimurium), FliC-C, FliC^{TLR5}, FliC$^{D0/D1}$, or FlaA, all co-immunized with the non-immunogenic antigen, OVA. Our results show that destroying either the D0 or D1 recognition site does not eliminate Naip5/6- and TLR5-independent IgG1 anti-FliC or anti-OVA responses. Immunizations conducted with FliC$^{D0/D1}$ or rFlaA in MyD88$^{-/-}$ showed significantly reduced primary and secondary IgG1 and ablated IgG2c anti-flagellin responses. While DKO mice had completely ablated IgG1 and IgG2c primary and secondary antibody responses towards flagellin. Our results support that there is a TLR5- and inflammasome-independent pathway that requires that D2/D3 domain of flagellin. Conversely, the TLR5-, Casp1-, and MyD88-indpendent IgG1 anti-OVA were still maintained when FliC$^{D0/D1}$ or rFlaA were used as adjuvants. In summary, our data demonstrates that the third unknown pathway that promotes IgG1 towards FliC is dependent on the hypervariable region of FliC, domains D2 and D3, but MyD88-independent IgG1 anti-OVA responses do not require D2 or D3 structural features of FliC.

Results

TLR5 and inflammasome activity are independent of FliC's D2 and D3 domains.

Flagellin has been well described as a TLR5 and Naip5/6 agonist that promotes robust humoral immunity in mice. In addition, we have shown that TLR5-, Casp1-, and MyD88-independent factors also promote isotype specific antibody responses towards *S.* Typhimurium FliC. To determine which flagellin structures modulate antibody production we designed three variant FliC proteins that have destroyed either TLR5 (FliC^{TLR5}) or Naip5/6 (FliC-C) recognition sites, or deleted both the D2 and D3 domains (FliC$^{D0/D1}$) (Fig. 13). A coomassie gel of all three proteins indicate that they were expressed at the appropriate molecular weights (Fig. 14). To determine whether or not the immunogenicity of flagellin was unique to *Salmonella* and other

closely related proteins, we also purified distantly related *L. monocytogenes* flagellin, FlaA (Fig. 13). FlaA is predicted to lack most of the D2 and D3 domains of FliC and is similar in size to $FliC^{D0/D1}$ (Fig. 14). FlaA is capable of stimulating TLR5, yet shares only 38% similarity with FliC.

Following expression and purification of all four proteins, we tested TLR5 and inflammasome activity. The TLR5 stimulatory activity of FliC-C (EC_{50}=8.49 +/-6.85 ng/mL), $FliC^{D0/D1}$ (EC_{50}=0.48 +/-0.15 ng/mL) and FlaA (EC_{50}=2.78 +/-2.59 ng/mL) were comparable to FliC isolated from *S.* Typhimurium (EC_{50} = 4.23 +/-4.17 ng/mL), whereas the $FliC^{TLR5}$ variant had significantly diminished TLR5 stimulatory activity (EC_{50} = 659.63 +/-393.02 ng/mL, P<.01) (Fig. 15A). In contrast to our anticipated outcomes for inflammasome activation, $FliC^{TLR5}$, $FliC^{D0/D1}$, and FlaA yielded greatly reduced IL-1β production compared to equivalent concentrations of WT FliC (Fig. 15B). Meanwhile, the FliC-C variant, which is mutated in the ultimate residue (R495P) and has a C-terminal 6xHis-tag, was also a poor inducer of IL-1β production, even at high doses (Fig. 15B). None of the proteins induced IL-1β production in $Casp1^{-/-}$ BMDMs (Fig. 15C). Thus, *in vitro* characterization of our flagellin proteins demonstrated that the $FliC^{TLR5}$ and FliC-C mutations selectively abrogated TLR5 and Naip5/6 recognition, respectively, but also established that the addition of a 6xHis-tag to either N- or C-terminal does interfere with inflammasome activation.

Early TLR5-dependent cytokine production in response to flagellin is independent of FliC's D2 and D3 domains. Intraperitoneal injection of flagellin induces TLR5- and Naip5/6-dependent cytokine production (22, 33, 55). Since biological activity of our recombinant flagellin molecules *in vivo* may be influenced by factors that are not present *in vitro*, we tested the ability of our mutant proteins to induce cytokine production in *in vivo*. BL/6 mice were

injected i.p. with 30 µg of individual protein, and sera was then collected at two and four hours post injection and tested for cytokine production. Mice that received WT FliC, FliC-C, FliC$^{D0/D1}$, and FlaA had robust production of IL-12/23p40 at both two and four hours post injection (Fig. 16A). As our results from our *in vitro* assay would predict, mouse i.p. injections with FliC-C, FliC^{TLR5}, FliC$^{D0/D1}$, or FlaA had significantly reduced IL-18 and IL-6 when compared to mice that received WT FliC injections, at both the 2 and 4 hour time points (Fig. 15B, C). Following injections with WT FliC, FliC-C, FliC^{TLR5}, FliC$^{D0/D1}$, or FlaA mice had no detectable TNF in their sera at either time point (Fig 15D). Our results demonstrate that early TLR5-dependent cytokine production is independent of the D2/D3 domain. Moreover, cytokines that are dependent or partially-dependent on inflammasome activity are significantly reduced following i.p. injections with proteins that contain a 6xHis-tag, independently of the D2/D3 domain.

IgG1 and IgG2c anti-flagellin responses are partially dependent on FliC's conserved recognition sites and the D2/D3 domain. Flagellin's immunogenicity and adjuvancy have been largely attributed to recognition of the conserved D0 and D1 domains by innate immune receptors Naip5/6 and TLR5 (29). Although these innate immune receptors contribute to flagellin-dependent antibody responses, additional factors are also critical (33). To address the possibility of novel recognition pathways emanating from the conserved recognition sites in flagellin's D0 and D1 domains, we tested the ability of our *de novo* flagellin mutants to induce antibody responses. Mice were immunized twice with 30 µg of recombinant protein on day 0 and 21 and blood was drawn two weeks after each immunization to assess IgG1 and IgG2c titers. Primary (day 14) anti-flagellin IgG1 responses from mice immunized with FliC, FliC-C and FliC^{TLR5} did not differ significantly (Fig. 17A). Secondary (day 35) anti-flagellin IgG1 responses for FliC^{TLR5} immunized mice were significantly lower than mice immunized with WT FliC and

FliC-C, but FliC^{TLR5} mutant protein still induced strong antibody responses with a median titer of $3x10^5$, respectively (Fig. 17A). As anticipated, IgG2c isotype specific responses were both significantly reduced following primary and secondary immunizations with FliC-C and FliC^{TLR5} compared to FliC (Fig. 17B). Four out the five mice that received immunizations of FliC^{TLR5} had no detectable IgG2c specific responses following a secondary immunization, whereas FliC-C immunized mice only had a half log reduction in their median IgG2c titers following secondary immunization (Fig. 17B). Our immunization studies with FliC-C demonstrates that IgG1 anti-flagellin are independent of the Naip5/6 recognition site and IgG2c is partially dependent on inflammasome recognition. Meanwhile, mice immunized with FliC^{TLR5} demonstrate that IgG1 and IgG2c isotype specific responses are partially dependent on the D0 and D1 structural features of FliC. FliC^{TLR5} abrogated anti-flagellin responses is most likely attributable to it not only having a mutated D1 recognition site, but also an N-terminal 6xHis-tag possibly interfering with Naip5/6 recognition.

Primary anti-flagellin antibody responses are dependent on flagellin's D2/D3 domain. We next immunized WT mice with WT FliC, FliC$^{D0/D1}$, or FlaA. Primary IgG1 anti-flagellin titers from WT mice immunized with FliC$^{D0/D1}$, or FlaA were all below our limit of detection and significantly lower than mice immunized with WT FliC (Fig. 17C). After secondary immunization with FliC$^{D0/D1}$ and FlaA, anti-flagellin IgG1 antibody responses were now detectable, but significantly reduced relative to WT FliC (approximately 30- and 100-fold reduction in median titers for FliC$^{D0/D1}$ and FlaA, respectively) (Fig. 17C). Similar to anti-flagellin IgG1 primary responses, anti-flagellin IgG2c responses towards FliC$^{D0/D1}$ and FlaA were both below our limit of detection, and following secondary immunizations the anti-flagellin IgG2c titers also remained significantly lower than responses from WT FliC immunized mice

(approximately 300- and 100- fold reduction in median titers for $FliC^{D0/D1}$ and FlaA, respectively) (Fig. 17D). Thus, primary anti-flagellin IgG1 and IgG2c responses are dependent on the D2 and D3 domains, and the TLR5 and Naip5/6 stimulatory D0 and D1 domains are insufficient for flagellin to induce robust primary anti-flagellin antibody responses. The D2/D3 domain is not required for the generation of secondary anti-flagellin IgG1 and IgG2c responses, but both were significantly attenuated in $FliC^{D0/D1}$ and FlaA immunized mice, signifying that robust isotype specific anti-flagellin responses are also partially dependent on the D2/D3 domain.

MyD88-independent anti-flagellin IgG1 antibody responses do not require flagellin's TLR5 or Naip5/6 recognition sites. We recently demonstrated that anti-flagellin IgG2c responses are largely MyD88-dependent, and that a substantial proportion of the anti-flagellin IgG1 response proceeds through an undefined MyD88-independent pathway (33). To further characterize this pathway, we immunized $MyD88^{-/-}$ mice with WT FliC, FliC-C, or $FliC^{TLR5}$ to determine how conserved structural features of flagellin contribute to the production of anti-flagellin IgG1 in the absence of MyD88. In contrast to WT mice (Fig. 17), $MyD88^{-/-}$ mice immunized with FliC-C and $FliC^{TLR5}$ showed no significant difference in anti-flagellin IgG1 titers on days 14 or 35, when compared to WT FliC immunized counterparts (Fig. 18A). As expected $FliC^{TLR5}$ immunized $MyD88^{-/-}$ mice had no detectable IgG2c following primary and secondary immunizations and were not significantly different than WT FliC immunized $MyD88^{-/-}$ mice (Fig. 18B). Unexpectedly though, $MyD88^{-/-}$ mice that were immunized with FliC-C had significantly higher IgG2c responses than $FliC^{TLR5}$ immunized mice, with their median titer reaching 10^2, respectively (Fig. 18B). These results indicate that MyD88-independent anti-

flagellin IgG1 responses do not emanate from either the TLR5 or the Naip5/6 recognition sites on bacterial flagellin.

MyD88-independent anti-flagellin IgG1 primary antibody responses require flagellin's D2/D3 domain. We next immunized MyD88$^{-/-}$ mice with FliC$^{D0/D1}$ or FlaA. Similar to WT immunized mice (Fig. 17), FliC$^{D0/D1}$ or FlaA immunized MyD88$^{-/-}$ mice did not make detectable primary anti-flagellin IgG1 responses (Fig. 18C). After MyD88$^{-/-}$ mice received a secondary immunization of FliC$^{D0/D1}$ or FlaA, they had significantly reduced anti-flagellin IgG1 antibody responses compared to WT FliC immunized mice (Fig. 17C). Although, both FliC$^{D0/D1}$ and FlaA immunized mice produced detectable anti-flagellin IgG1 secondary responses, FlaA immunized mice had a subtle, but significant half-log increase in their median IgG1 titers compared to FliC$^{D0/D1}$ immunized mice (Fig. 17C). As expected, there were no significant differences in IgG2c anti-flagellin responses following primary and secondary immunizations with WT FliC, FliC$^{D0/D1}$, or FlaA in MyD88$^{-/-}$ mice (Fig. 17D). Our results from MyD88$^{-/-}$ immunizations once again demonstrate that primary anti-flagellin IgG1 responses require FliC's D2/D3 domain. Surprisingly, after secondary immunizations with FliC$^{D0/D1}$ or rFlaA, we did not detect modest anti-flagellin IgG1 responses, suggesting that secondary anti-flagellin MyD88-independent responses are partially independent of the D2/D3 domain. Since both FliC$^{D0/D1}$ and FlaA are potent activators of TLR5 it is possible that MyD88-independent pathways emanating from TLR5, contribute to modest anti-flagellin IgG1 secondary responses observed in MyD88$^{-/-}$ mice (Fig. 17C) (35, 39).

TLR5- and Casp1-independent anti-flagellin antibody responses do not require flagellin's TLR5 or Naip5/6 recognition sites. To determine whether or not TLR5 and inflammasome recognition of FliC$^{D0/D1}$ and FlaA contributed to the modest MyD88-independent

anti-flagellin IgG1 secondary responses we immunized TLT5$^{-/-}$/Casp1$^{-/-}$ mice with our panel of flagellin proteins to establish how different sites in the flagellin protein promote TLR5- and Casp1-independent antibody responses. DKO mice were immunized with FliC-C and FliC^{TLR5} as described above. After primary and secondary immunizations, WT FliC, FliC-C and FliC^{TLR5} had no significant differences in anti-flagellin IgG1 or IgG2c responses (Fig. 19A, B). Thus, the anti-flagellin IgG1 responses in TLR5/Casp1 deficient mice do not require TLR5 or Naip5/6 recognition sites (Fig. 19A, B).

TLR5- and Casp1-independent anti-flagellin antibody responses require flagellin's D2/D3 domain. We next immunized DKO with either WT FliC, FliC$^{D0/D1}$ or FlaA to determine whether elimination of the D2/D3 hypervariable region of flagellin affects TLR5- and Casp1-independent antibody responses. IgG1 titers following primary and secondary immunization were significantly attenuated following immunizations with flagellin proteins that lack flagellin's D2/D3 domain (Fig. 19C). Primary IgG1 responses were undetectable in all DKO mice that received FliC$^{D0/D1}$ or FlaA (Fig. 19C). Secondary immunizations show significantly reduced IgG1 in FliC$^{D0/D1}$ and FlaA immunized mice compared to mice immunized with WT FliC (Fig. 17C); the median anti-flagellin IgG1 titers were 1,000 fold lower for FlaA and 10,000 for FliC$^{D0/D1}$ compared to WT FliC immunized mice (Fig. 19C). Anti-flagellin IgG2c primary responses had no significant differences between WT FliC, FliC$^{D0/D1}$, or rFlaA, with a majority being below our limit of detection (Fig. 19D). Secondary immunizations with FliC$^{D0/D1}$ had no detectable anti-flagellin IgG2c responses (Fig. 19D). DKO mice immunized with FlaA had reduced anti-flagellin IgG2c responses compared to WT FliC, but did not reach statistical significance (Fig 19D). Thus, TLR5- and Casp1-independent antibody responses are dependent on flagellin's D2/D3 domain.

TLR5- and Casp1-independent IgG1 anti-OVA responses do not require the Naip5/6 or the TLR5 recognition sites. Flagellin also works as an adjuvant toward antigens that are physically linked to flagellin and induces predominantly IgG1 antibody responses against the antigen. Similar to anti-flagellin antibody responses, antibody responses directed against model antigens co-injected with flagellin are partially dependent on TLR5, Casp1, and MyD88 (33). Mice immunized with FliC-C, FliC^{TLR5}, FliC$^{D0/D1}$, or FlaA, also received 30 µgs of OVA i.p. on day 0 and 21. WT mice immunized with WT FliC, FliC-C, or FliC^{TLR5}, plus OVA showed no significant differences in their IgG1 anti-OVA titers following secondary (day 35) immunizations (Fig. 20A). Similarly, DKO mice immunized with OVA with WT FliC, FliC-C or FliC^{TLR5} used as adjuvants displayed no significant differences in their secondary anti-OVA titers (Fig. 20B). Previously, we described a TLR5-and Casp1-independent IgG1 response towards OVA when co-immunized with flagellin and here we show that this MyD88-independent pathway does not stem from either the D0 or D1 domains of FliC (33).

MyD88-independent IgG1 anti-OVA responses are independent of the D2/D3 domain. Since we have shown that the TLR5- and Casp1-independent pathway that promotes IgG anti-OVA responses does not require either the Naip5/6 or TLR5 recognition sites we immunized mice with WT FliC, FliC$^{D0/D1}$ or FlaA plus OVA to observe how the D2/D3 domains affects extrinsic adjuvancy. BL/6 mice immunized with WT FliC, FliC$^{D0/D1}$ or FlaA plus OVA have similar IgG1 anti-OVA responses on day 35 following secondary immunizations (Fig. 20C). Secondary anti-OVA IgG1 responses in DKO mice immunized with WT FliC, FliC$^{D0/D1}$, or FlaA, plus OVA show an overall decrease in median titers compared to WT mice (Fig. 20C), but no significant differences in titers were observed regardless of co-immunized flagellin (Fig. 20D). Hence, unlike TLR5- and Casp1-indepdent IgG1 anti-flagellin responses which are

dependent on FliC's D2/D3 domain, TLR5- and Casp1-independent IgG1 anti-OVA responses are independent of the D2/D3 features of FliC.

Discussion

Previously, we described how innate flagellin receptors, TLR5 and Naip5/6, work in concert to promote IgG1 and IgG2c anti-FliC responses (33). Although anti-flagellin IgG2c responses are largely dependent on TLR5, Casp1 and MyD88, the anti-flagellin and anti-OVA IgG1 responses were only partially dependent on these host molecules, revealing a TLR5-, Naip5/6-, and MyD88-independent pathway utilized by flagellin to induce antibody responses (33). In this report, we provide a more detailed understanding of this pathway by examining the structural components of bacterial flagellin that contribute to antibody responses in mice. Consistent with our previous studies in knockout mice, WT mice immunized with FliC-C or $FliC^{TLR5}$ displayed significant reductions in IgG2c (T_H1) responses, confirming that T_H1 type antibody responses against flagellin are influenced by both TLR5 and the Naip5/6 inflammasome (33). These results support our previous conclusion that flagellin recognition through TLR5 and Naip5/6 work in concert to promote robust IgG2c responses (33). As we anticipated IgG2c anti-FliC responses were MyD88-dependent, regardless of immunogen used (Fig. 18).

Our results from WT mice immunized with FliC-C or $FliC^{TLR5}$ also support our conclusion that TLR5 and Naip5/6 play redundant roles in generating anti-flagellin responses (33). Since anti-flagellin IgG1 antibodies are the dominant isotype produced in flagellin immunized BL/6 mice, these results are also consistent with the previous conclusions of Vijay-Kumar *et al*., that either TLR5 or Naip5/6 recognition of FliC is sufficient for anti-flagellin IgG responses (22, 33). No significant differences were observed in IgG1 anti-FliC titers from DKO mice immunized with either FliC, FliC-C, or $FliC^{TLR5}$, demonstrating that the third unknown

pathway that promotes IgG1 responses against FliC does not emanate from the conserved TLR5 or the Naip5/6 recognition sites (Fig. 20A, B).

Results from BL/6 mice immunized with $FliC^{D0/D1}$ or FlaA indicate that primary anti-flagellin responses are dependent on the D2/D3 domain and secondary anti-flagellin antibodies produced are TLR5- and Naip5/6-dependent (Fig. 20). We conclude that the TLR5- and Casp1-independent anti-flagellin responses do not require the highly conserved sites in flagellin's D0 and D1 domains that are recognized by Naip5/6 and TLR5, respectively. Therefore, our data demonstrates that the conserved TLR5 and Naip5/6 recognition sites on flagellin, which were previously considered essential for flagellin's immunogenicity, contribute, but are not required for anti-flagellin responses (57, 64, 65, 76-78, 118, 121).

We also assessed how deletion of the hypervariable D2 and D3 domains affect cytokine production and flagellin's immunogenicity and adjuvancy. Consistent with *in vitro* biological activity, i.p. injection of $FliC^{D0/D1}$ and FlaA induced TLR5-dependent cytokine production that was equivalent to WT FliC injected mice (Fig. 16). This is consistent with the results of Yoon *et al*., which demonstrated that recombinant flagellin protein consisting of predominantly the D0 and D1 domains of *S.* Dublin FliC was capable of inducing robust cytokine responses in mice (51). Our findings contradict the results of Nempont *et al*., who found that deletion of flagellin's hypervariable region was incapable of inducing cytokine responses in mice, but capable of activating TLR5 *in vitro* (58).

In contrast to cytokine induction our immunization studies with either $FliC^{D0/D1}$ or FlaA demonstrated that primary IgG1 and IgG2c anti-flagellin responses are entirely dependent on the D2/D3 domain. Thus, flagellin's immunogenicity is strongly influenced by the D2/D3 domain. Secondary anti-flagellin IgG1 responses were observed with $FliC^{D0/D1}$ and FlaA, and these

responses were TLR5-, Casp1-, and MyD88-dependent. These results suggest that the D2/D3 domain is critical for rapid antibody responses against flagellin, and that in the absence of the D2/D3 domain activation of the innate immune system via TLR5 and Casp1 is required for flagellin's immunogenicity. Our results are also in alignment with McDonald *et al*., Nempont *et al*., and Burdelya *et al*., who all demonstrate that *Salmonella's* flagellin intrinsic adjuvancy is partially dependent on the D2 and D3 domains of flagellin even while immunogenic D0 and D1 domains remain intact (58, 118, 119). Conversely, TLR5- and Casp1-independent IgG1 anti-OVA responses were not affected by the absence of the D2/D3 domain (Fig. 20C, D). At this time it remains unclear what flagellin-dependent pathway is mediating the TLR5- and Casp1-independent anti-OVA responses. However, our IgG1 anti-OVA responses do agree with Nempont's *et al*., results and conclusion, that show the extrinsic adjuvancy of FliC are independent of the D2/D3 structural features of flagellin (58). However, these results disagree with McDonald's *et al*., who found that a flagellin D2/D3 deletion protein co-immunized with West Nile viruses (WNV) EIII antigen (STF2Δ+EIII), was unable to generate IgG anti-EIII responses. We have also found that flagellin's ability to function as an adjuvant is dependent on the co-administered antigen, and some proteins are still poor antigens when mixed with flagellin, but become good antigens when covalently linked to flagellin. Thus the WNV EIII domain may also be a weak antigen that requires physical linkage to flagellin in order to gain sufficient immunogenicity to generate anti-EIII antibody responses (118).

Our immunization results with $FliC^{D0/D1}$ and FlaA in WT mice support previous results from multiple labs and further demonstrates that robust primary anti-flagellin antibody responses require the hypervariable D2/D3 domain. Our results also illustrates that the D2/D3 domain is not required for secondary antibody responses or for flagellin to function as an adjuvant for the

co-administered antigen, OVA. In the absence of D2/D3, secondary anti-flagellin IgG1 responses entirely TLR5- and Casp1-dependent. Thus, immunogenic properties of $FliC^{D0/D1}$ and FlaA are strictly dependent on TLR5 and Casp1.

As expected, $MyD88^{-/-}$ mice immunized with $FliC^{D0/D1}$ and FlaA were unable to generate IgG2c responses towards $FliC^{D0/D1}$ or FlaA (Fig. 18D). In contrast to DKO mice, anti-flagellin IgG1 responses were reduced but not ablated in $MyD88^{-/-}$ mice following immunizations with $FliC^{D0/D1}$ or FlaA (Fig. 18C). There are at least two possible explanations for the anti-flagellin IgG1 responses observed in $MyD88^{-/-}$ mice: 1) TLR5 may function independently of MyD88, possibly by promoting flagellin uptake and MHC II presentation (35), 2) Naip5/6 inflammasome may function independently of MyD88 by inducing other Casp1-dependent factors, such as biologically active eicosanoids (39).

Our results illustrate that in addition to the host's innate TLR5 and Naip5/6 recognition pathways, FliC's D2/D3 domain is also required for optimal anti-flagellin antibody responses, but not adjuvancy (Fig. 21). This result has important implications for design of immunogens and vaccines that utilize flagellin. Our studies indicate that manipulation of the D2/D3 domain of FliC may also affect the potency of the immunogen. These conclusions are supported by results from Nempont *et al*., and Burdelya *et al*., which also demonstrate that deletion of flagellins hypervariable D2/D3 domain significantly reduce anti-flagellin responses (58, 119). One major unresolved question is the mechanism responsible for D2/D3 domain enhancement of anti-flagellin antibody responses. Since D2/D3 is important for anti-flagellin, but not anti-OVA antibody responses, we believe that D2/D3 may enhance anti-flagellin antibodies by targeting flagellin to sites within lymphoid tissue that promote antibody responses. We think that it is less likely that D2/D3 is acting via a novel innate immune receptor and the induction of cytokines.

Understanding how FliC's D2/D3 domain enhances anti-flagellin antibody production is puzzling. Since the D2/D3 has very little homology with other flagellin molecules and varies greatly from the highly conserved D0 and D1 domains, which are recognized by Naip5/6 and TLR5. Sequence alignment of FliC with other flagellin's reveals that a small portion of the D2 domain is conserved amongst flagellated bacteria, whereas the D3 domain is unique to a very small subset of *S. enterica serovar* Typhimurium with serotype Hi (data not shown). Thus, it remains unclear what amino acid region from the D2 and D3 domains mitigates this third unknown pathway. Nempont *et al*., described three different FliC deletions Δ204-292, Δ191-352, and Δ174-400 that all appear to have reduced immunogenicity, although the most severe attenuations are apparent in Δ191-352, and Δ174-400 deletions (58). In addition to these studies, Taylor *et al*., and Carapau *et al*., and our own observations (data not shown) indicate that deletion of the D3 domain is inconsequential to IgG1 anti-FliC titers, suggesting that the D2 domain may be critical for MyD88-independent anti-flagellin IgG1 responses (58, 78, 120). Future studies to define the precise structures on D2/D3 that are required to enhance antibody production will be helpful to understand FliC's biological activity, to utilize for vaccine development.

While our findings demonstrate that, in addition to the TLR5 and Naip5/6 recognition sites, the D2/D3 domain of flagellin also promote its robust antigenicity. However, the mechanism responsible for host recognition of the D2/D3 domain and induction of potent primary antibody responses remains to be elucidated. One possible mechanism is suggested by results observed over four decades ago by Nossal, Ada and colleagues. Their studies with *Salmonella enteritidis serovar* Adelaide (*S.* Adelaide) flagellin and flagella demonstrated that flagellin is targeted to lymphoid follicles in rats (122). Furthermore, they demonstrated that the

targeting of flagella and flagellin to lymphoid follicles was influenced by microbiota and radiosensitive cells, suggesting that natural antibodies may be responsible for lymphoid follicle targeting (123).

The rationale design of effective vaccines to meet the clinical needs of combating numerous infectious diseases such as influenza, malaria, and HIV requires in depth knowledge of the molecular mechanisms of immunogens to enhance vaccine efficacy. There is ample mouse model data that support the role of flagellin's TLR5 and Naip5/6 stimulatory activity in promoting immune responses, but we provide evidence for an additional third pathway that also promotes flagellin's robust antigenicity. Herein, we demonstrate that, in fact, there is an additional feature on FliC, the D2/D3 domain that controls robust primary antibody responses independent of the known innate immune recognition pathways for bacterial flagellin. Further characterization of the D2/D3 domain pathway will provide valuable insight into novel strategies for flagellin based vaccine designs.

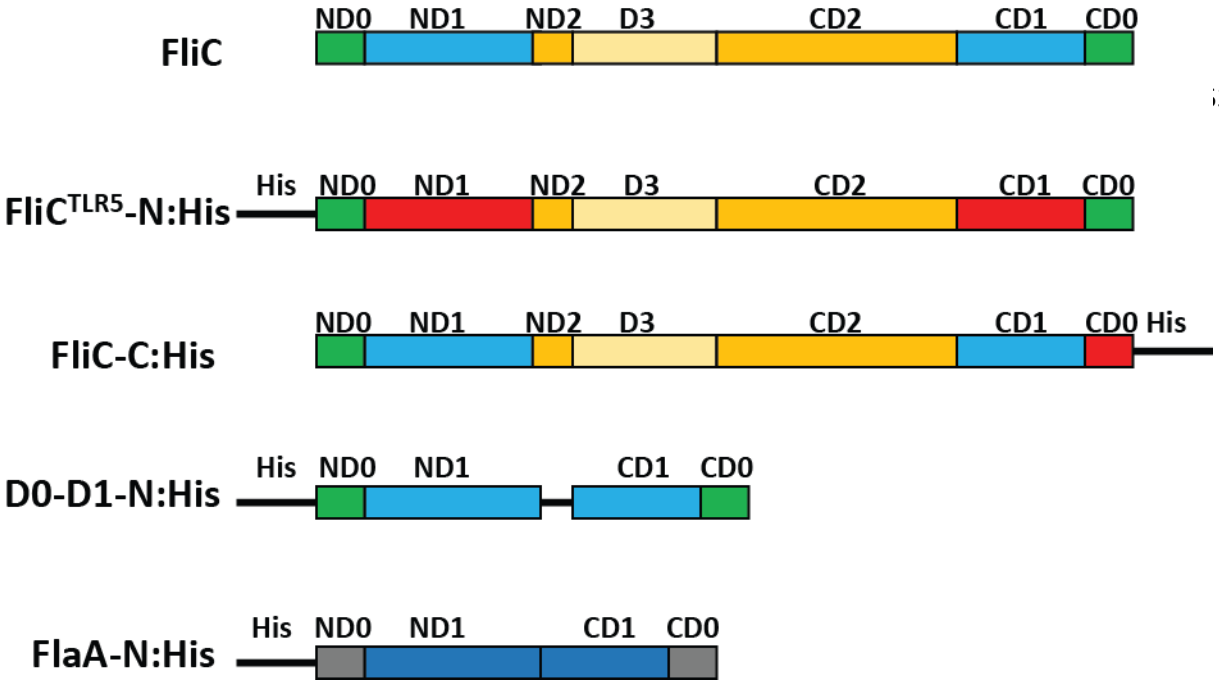

Figure 13. Diagram of flagellin proteins
Flagellin mutants with destroyed recognition sites are depicted with red domains.

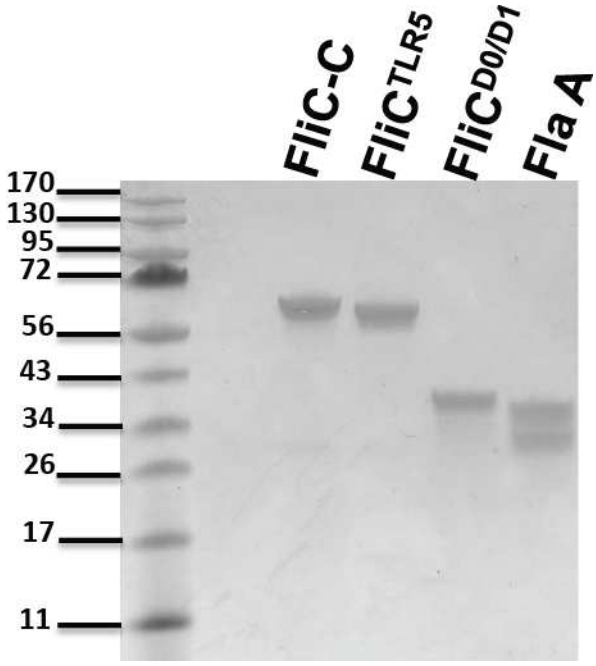

Figure 14. Purified recombinant flagellin proteins ran on SDS-PAGE confirmed by coomassie blue staining.

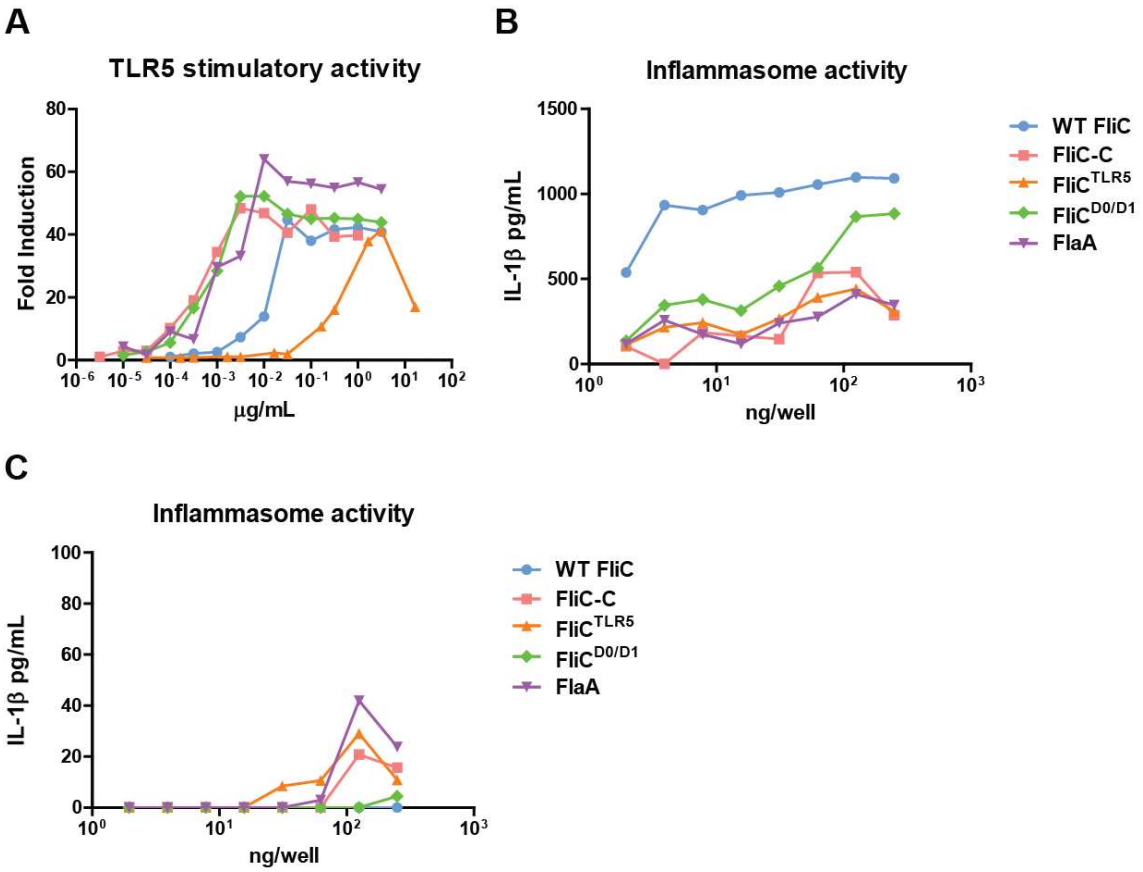

Figure 15. Recombinant flagellin proteins retain robust TLR5 activity. TLR5 and inflammasome biological activity for the different flagellin proteins were assessed by using CHO cells stably transfected with murine TLR5 and a NF-κB luciferase reporter (**A**) or induced Naip5- and Casp1-dependent IL-1β secretion in LPS primed BL/6 (**B**) or Casp1$^{-/-}$ (**C**) BMDMs.

Proteins	EC_{50} ng/mL	SD +/- ng/mL
WT FliC	4.23	4.17
FliC-C	8.49	6.85
$FliC^{TLR5}$	659.63	393.02
$FliC^{D0/D1}$	0.48	0.16
FlaA	2.78	2.59

Table I. Flagellin proteins and their corresponding TLR5 stimulatory activity EC_{50} +/-SD. Data are representative of three independent experiments.

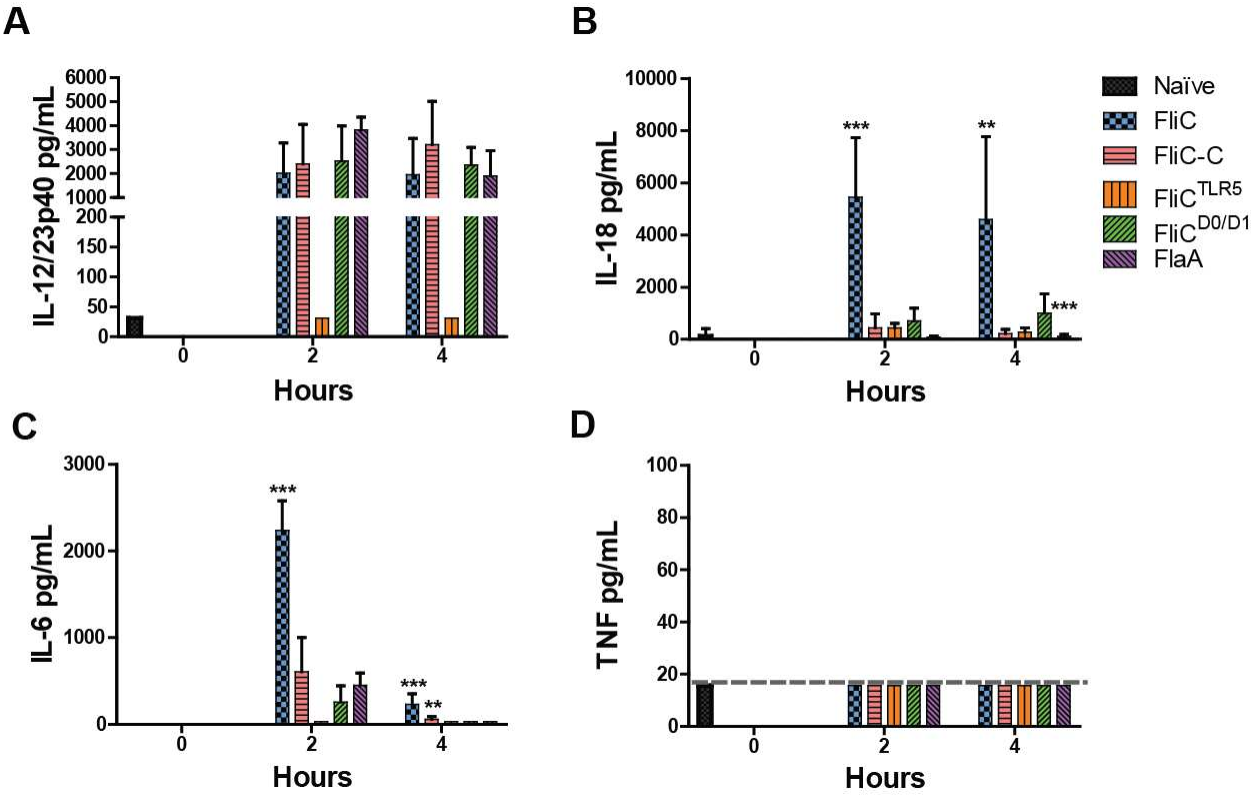

Figure 16. Inflammasome-dependent cytokines are significantly reduced following i.p. injections with recombinant flagellin proteins. WT FliC (n=5), FliC-C (n=5), FliC^{TLR5} (n=5), FliC$^{D0/D1}$ (n=5), FlaA (n=5), and naïve (n=5) mice were injected i.p. of flagellin (30 µg). Serum was collected 2 and 4 hours after injections and cytokine levels were determined by ELISA. IL-12/23p40 **(A)**, IL-18 **(B)**, IL-6 **(C)** and TNF **(D)**. All groups have an n=5. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post-test: ** = P<0.01, *** = P<0.001.

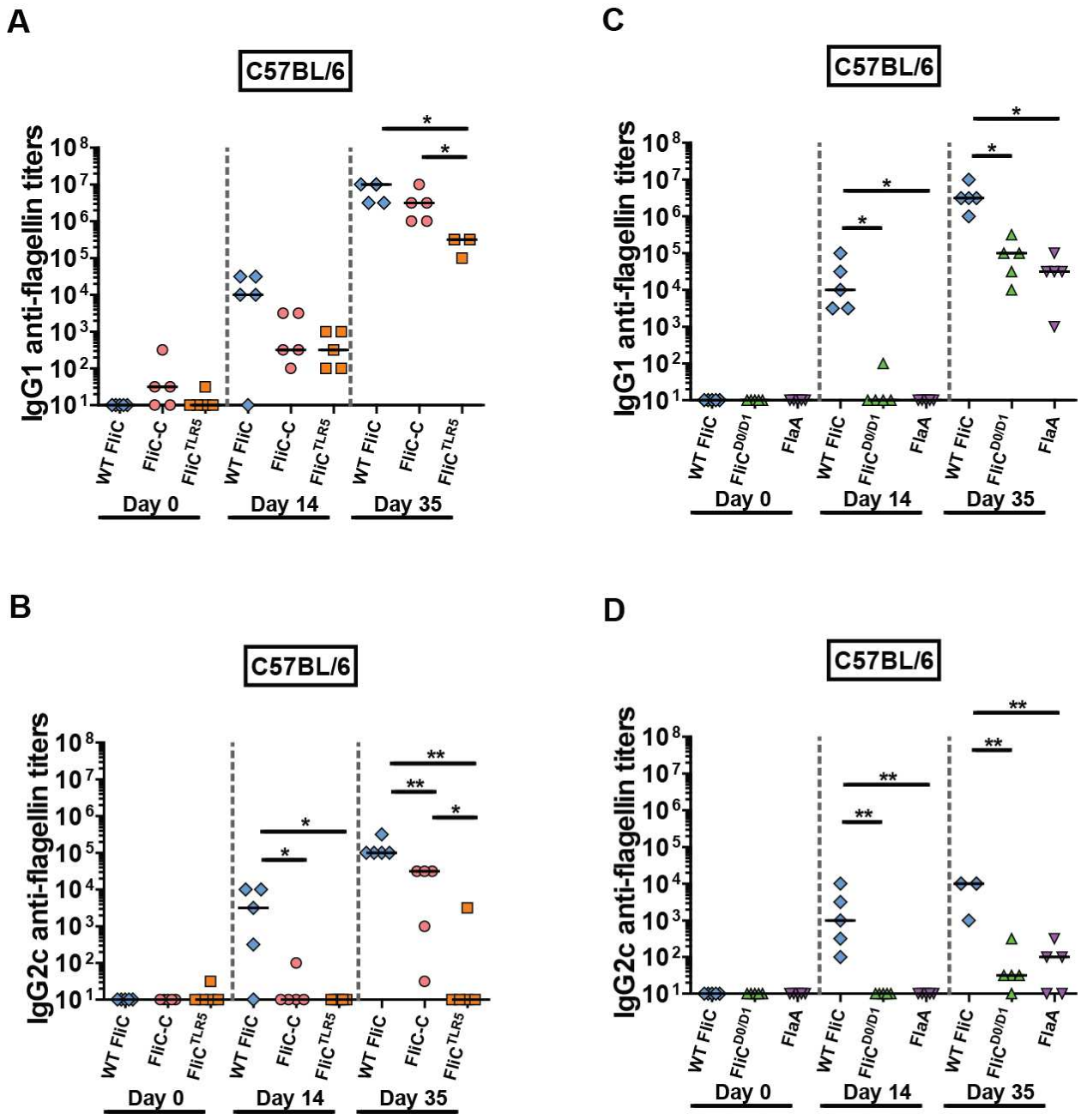

Figure 17. Primary IgG1 and IgG2c anti-flagellin responses in WT mice are D2/D3-dependent. C57BL/6 (n=5) were immunized twice on day 1 and day 21 with either WT FliC, FliC-C, $FliC^{TLR5}$, $FliC^{D0/D1}$, FlaA, plus OVA and sera collected on days 14 and 35 were analyzed for IgG1 (**A, B**), and IgG2c (**C, D**) responses against immunogen by ELISA. All groups have an n=5 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= $P<0.05$, ** = $P<0.01$.

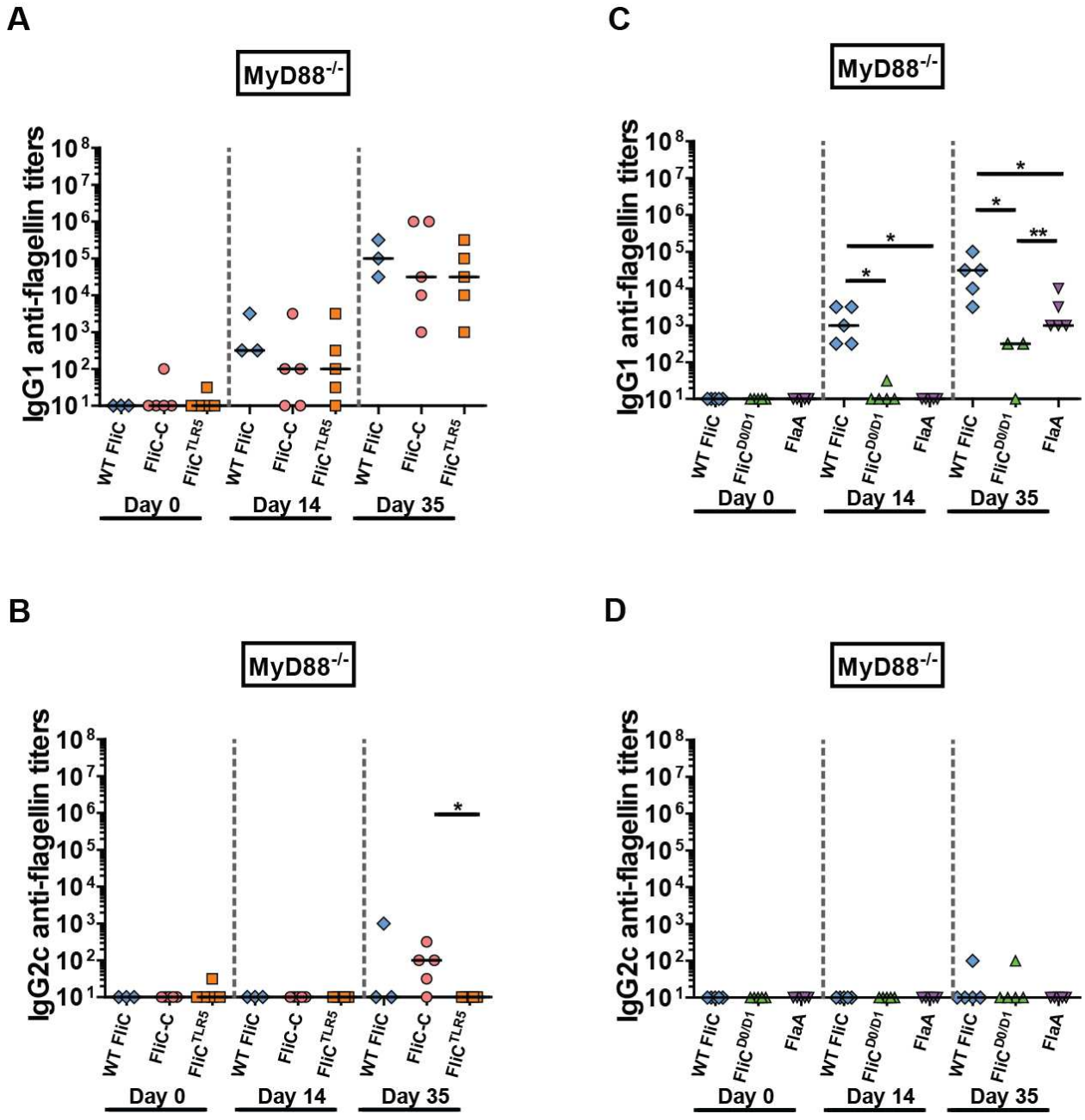

Figure 18. Secondary IgG1 anti-flagellin responses are partially D2/D3-dependent in MyD88$^{-/-}$ immunized mice. MyD88$^{-/-}$ were immunized twice on day 1 and day 21 with either WT FliC, FliC-C, FliC^{TLR5} , FliC$^{D0/D1}$, FlaA, plus OVA and sera collected on days 14 and 35 were analyzed for IgG1 (**A, B**), and IgG2c (**C, D**) responses against immunogen by ELISA. All groups have an n=3-5 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= P<0.05, ** = P<0.01.

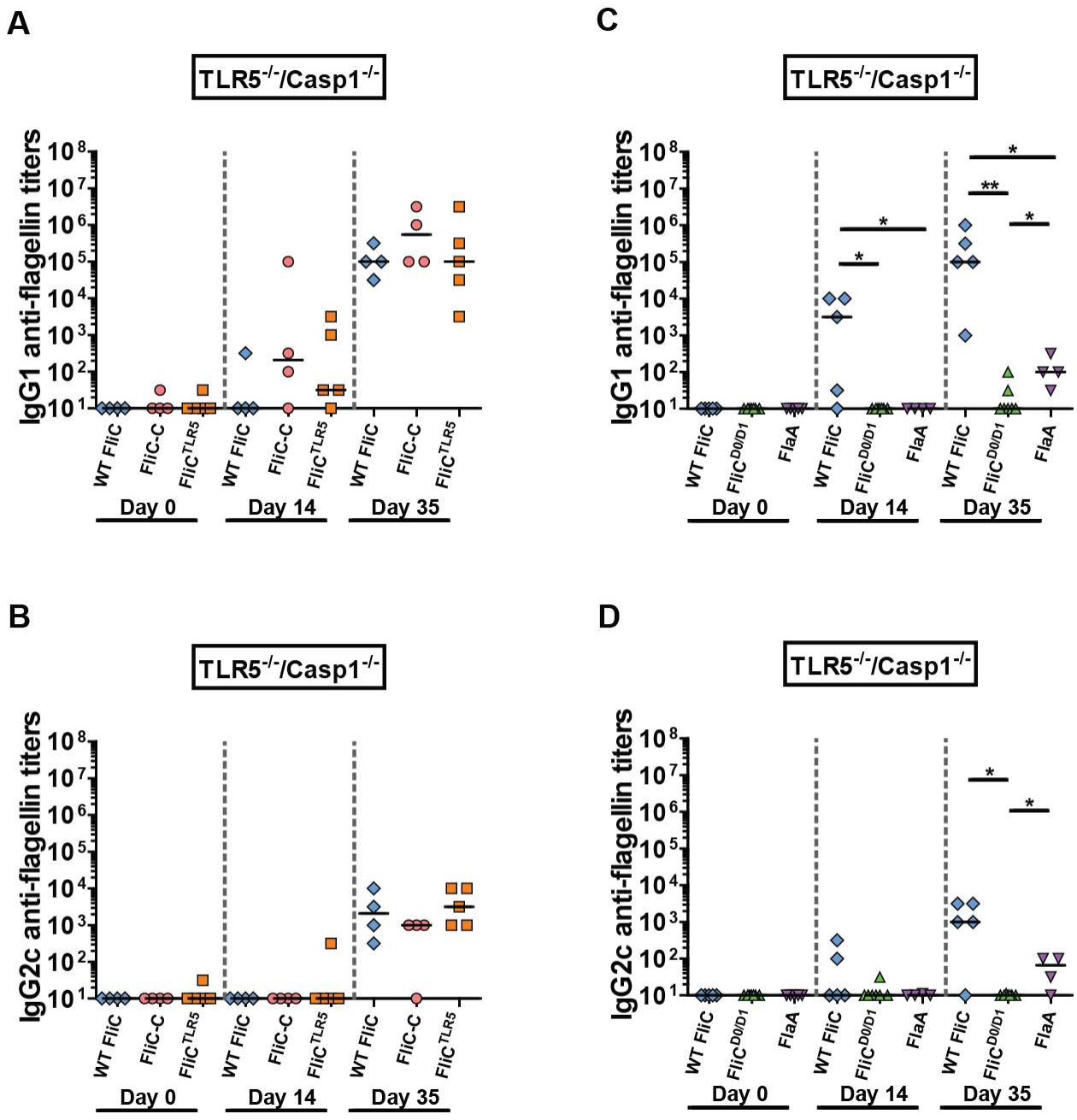

Figure 19. Primary and secondary IgG1 and IgG2c anti-flagellin responses are dependent on FliC's D2/D3 domains in the absence on TLR5 and inflammasome recognition. DKO mice were immunized twice on day 1 and day 21 with either WT FliC, FliC-C, $FliC^{TLR5}$, $FliC^{D0/D1}$, FlaA, plus OVA and sera collected on days 14 and 35 were analyzed for IgG1 (**A, B**), and IgG2c (**C, D**) responses against immunogen by ELISA. All groups have an n=4-7 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= P<0.05, ** = P<0.01.

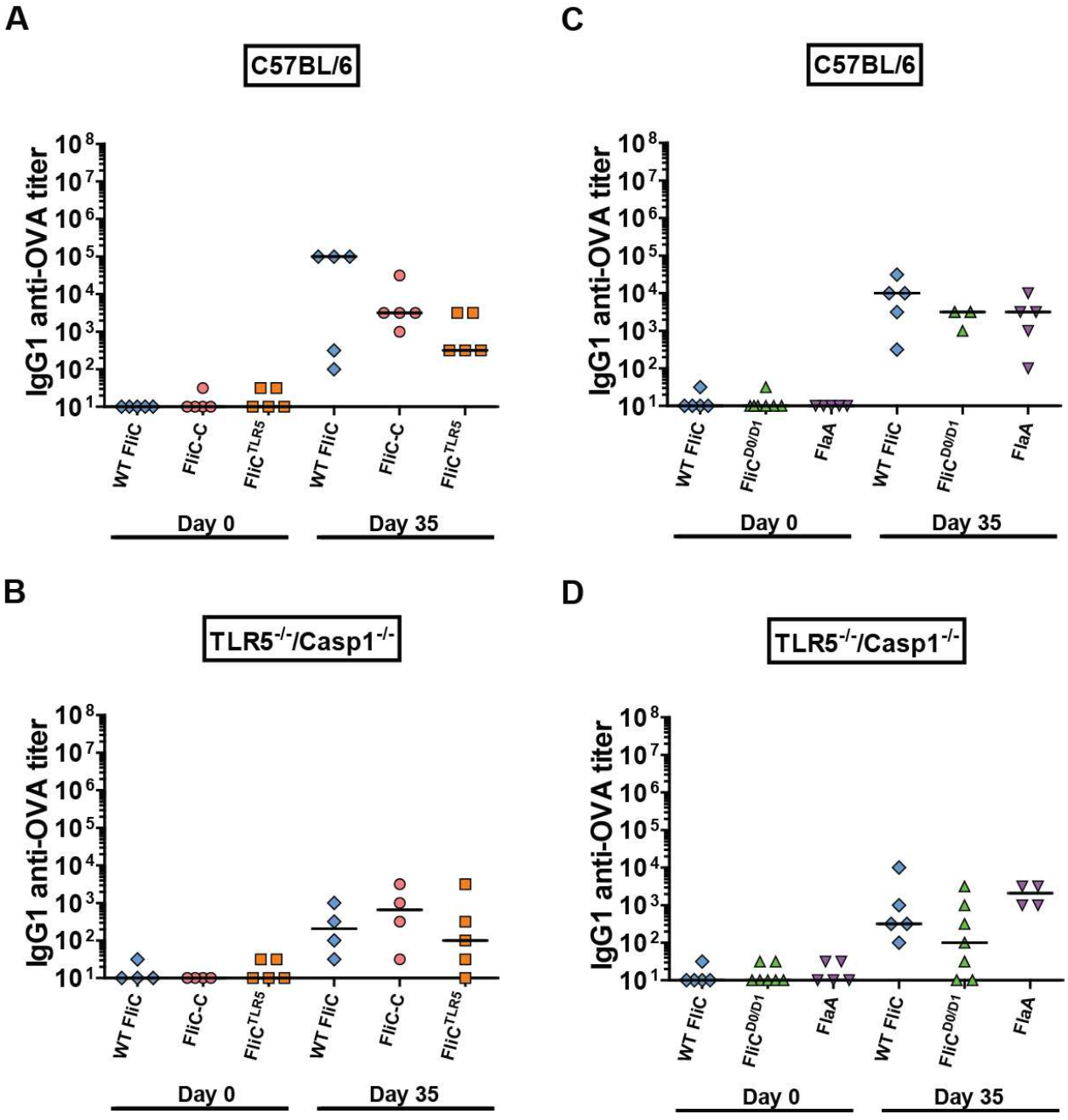

Figure 20. Flagellin induced IgG1 anti-OVA responses are independent of FliC's D2/D3 domain. WT and DKO mice were immunized twice on day 1 and day 21 with either WT FliC, FliC-C, $FliC^{TLR5}$, $FliC^{D0/D1}$, FlaA, plus OVA. Naïve, and day 35 sera were analyzed for IgG1 specific antibody responses against OVA by ELISA in WT (**A, C**) and DKO (**B, D**) mice. All groups have an n=4-7 per experiment.

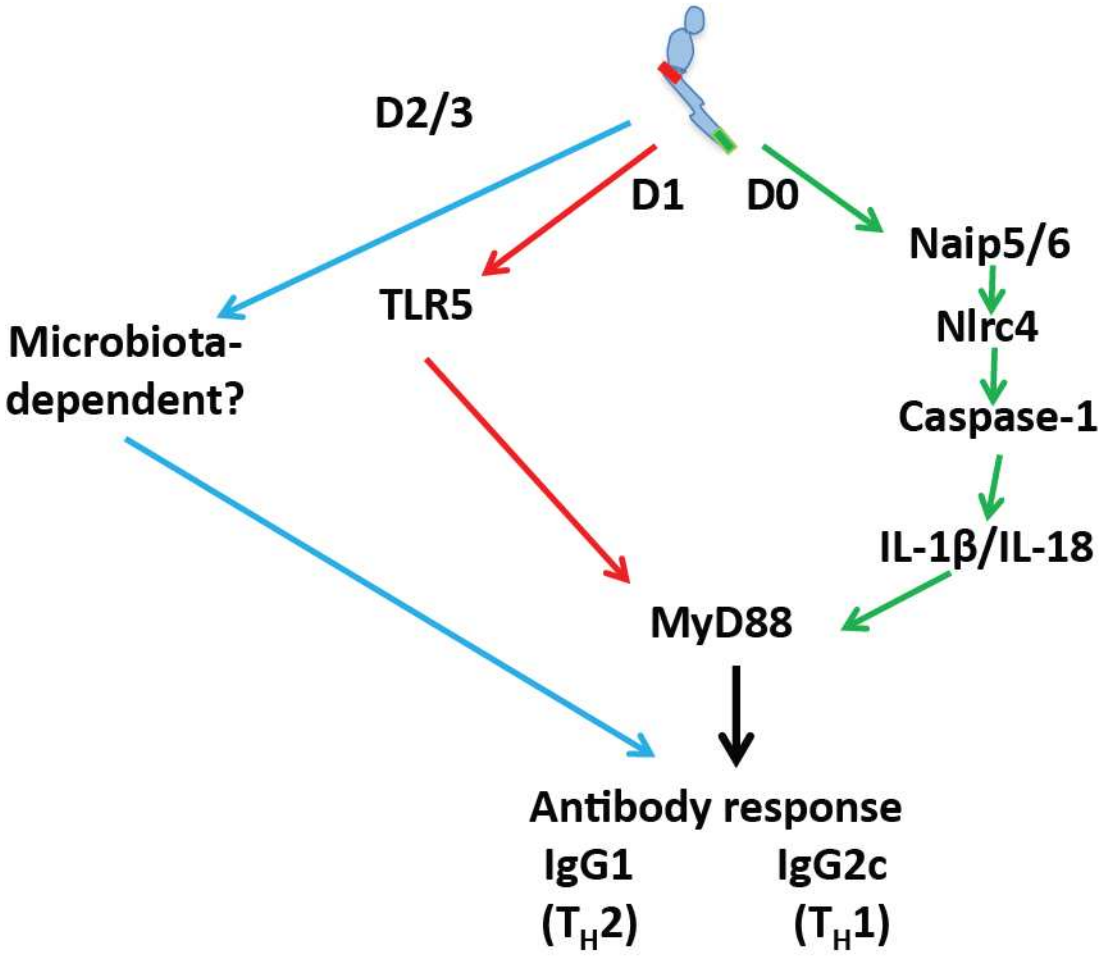

Figure 21. The three recognition pathways that promote anti-flagellin antibody responses.

Chapter IV: Generation of anti-4E10 epitope specific antibodies in mice with flagellin-HIV epitope-scaffold fusion proteins

Introduction

In 1989, Charles Janeway proposed that adjuvants (immunologists' "dirty little secret") were PAMPs, and that PAMP recognition by host germline encoded PRRs prompts long-term cellular and humoral immunity (124). Over the past decade it has indeed become evident that activation of the innate immune system is critical for the host's efficient clearance of pathogens and establishing protective immunity (80, 125). One family of PRRs that has been the major focus of adjuvant research is TLRs and their agonists. TLRs and their agonists can be classified into three broad groups based on their agonists' chemical composition: the first group, TLRs 2/1, 2/6, and 4, recognize lipid containing molecules; the second group, TLRs 3, 7, 8 and 9, respond to nucleic acid; and the third group, TLRs 5 and 11, recognize proteins. Since TLR11 is a pseudogene in humans, TLR5 has the unique role of being the only human TLR to recognize the protein bacterial flagellin (126).

Bacterial flagellin is the principle component of the flagellar apparatus enabling bacterial motility (127). Flagellin has been targeted throughout evolution by the immune systems of a broad range of multicellular organisms, including plants, invertebrates, and most animals (9, 46, 51, 63). *Salmonella's* flagellin is especially well studied at multiple levels, including x-ray and high resolution electron microscopy, which have revealed the structure of the flagellin monomer, with its "boomerang" like shape comprised of four domains: D0, D1, D2, and D3, which

polymerizes into 11 protofilaments that together form the flagellar filament (46, 47). TLR5 recognizes the highly conserved D1 domain of monomeric flagellin (46-48, 51).

Flagellin's unique attribute of being a protein adjuvant, makes it amenable to genetic manipulation and recombinant protein engineering. Hence, there has been intensive scrutiny of how the host's immune system recognizes and responds to monomeric flagellin (29). Like other TLR agonists, flagellin injections in mice induces rapid cytokine and chemokine production such as IL-23, IL-6 and Cxcl1, and upregulation of classical APC activation markers, such as MHC II, CD80, and CD86, in a TLR5-dependent manner (15, 33, 55). Immunizations of mice conducted with monomeric flagellin induces $CD4^+$ and $CD8^+$ T cell responses, as well as T cell-dependent T_H1 (IgG2a/c) and T_H2 (IgG1) type humoral responses, with a strong T_H2 (IgG1) bias observed in BL/6 mice (19, 33, 53, 55, 82, 128). These properties are currently being exploited to develop TLR5-dependent vaccines for human use (76-78, 120, 121, 129).

There are currently a limited amount of approved adjuvants in the U.S.A. and their efficacy is generally considered to be lacking (130, 131). Bacterial flagellin and flagellin fusion proteins are one promising avenue in this pursuit to generate vaccines that are more effective at generating long-term immunity with minimal side effects. Several flagellin fusion proteins have been designed including fusion with antigens from: WNV, *Y. pestis*, influenza, malaria, and HIV. In general, these immunogens retained TLR5 stimulatory activity and their immunogenicity in mice and hence, deemed TLR5-dependent (65, 118, 120, 129, 132, 133). In addition, clinical trials using flagellin-fusion proteins with influenza epitopes generated neutralizing antibodies, with minimal adverse side effects at low doses (<9 µg), suggesting that flagellin fusion proteins are a viable vaccine platform in humans (76-78).

Our group has recently demonstrated that flagellin's immunogenicity is also influenced by a novel third TLR5-independent pathway that is dependent on the D2/D3 domain of flagellin (33). However, the D3 portion of *S*. Typhimurium FliC is the most variable among flagellated bacteria, rendering D3 the most suitable region of FliC to allow for replacement with weak immunogens. Herein, we demonstrate that FliC is a poor adjuvant when co-immunized with weak antigens such as GFP and is unable to enhance GFP's immunogenicity. Conversely, when GFP replaces the D3 domain of FliC (FliC-GFP), GFP's immunogenicity is significantly enhanced. Thus, demonstrating that linking FliC's D0/D1/D2 domains to immunogenically poor antigens can enhance the host's antibody responses towards the antigen.

The 4E10 antibody is one of the first broadly neutralizing anti-HIV antibodies described (134, 135). 4E10 recognizes the highly conserved membrane proximal external region (MPER) of gp41, the transmembrane glycoprotein, with an ectodomain largely responsible for trimerization and critical for cellular invasion (136-139). Several epitope-scaffolds have been generated that display the 4E10 epitope on the surface of non-viral heterologous proteins. However, despite good binding characteristics, these epitope-scaffolds were poor inducers of anti-4E10 epitope specific antibodies in rabbits (140). Hence, we designed three FliC-HIV epitope-scaffold fusion proteins that retain FliC's D0/D1/D2 domains and have placed HIV epitope-scaffolds that display the broadly neutralizing epitopes 4E10 or 447. Immunizations with our three different FliC-HIV fusions demonstrate that flagellin fusion proteins are able to promote robust antigenicity against the immunogen and the epitope-scaffold, but only one of the fusion proteins promoted antibody specificity towards HIV's MPER 4E10 epitope. Our results demonstrate that coupling weak immunogens such as epitope-scaffolds to FliC's conserved D0/D1/D2 domains promotes robust antibody responses, however epitope specific responses

towards HIV's neutralizing epitopes requires additional empirical studies to determine the best FliC-HIV fusion vaccine that elicits robust HIV epitope specificity.

Results

FliC-GFP fusion proteins retain TLR5 stimulatory activity. We and several other groups have previously shown that FliC from *S.* Typhimurium can be used as an adjuvant when co-injected with other antigens, such as ovalbumin and the *Y. pestis* F1 antigen (33, 55, 58). However, the ability of flagellin to function as an adjuvant appears to be dependent on the nature of the co-administered antigen, since the immunogenicity of some antigens, such as WNV EIII domain, are not enhanced by co-immunization with FliC (118). GFP is a poor antigen when injected into mice alone, but fusion of GFP to flagellin's carboxy-terminus enhances GFP's immunogenicity and ability to induce $CD8^+$ and $CD4^+$ T cell responses (128). Because fusion proteins to the amino- or carboxy-terminal ends of flagellin will also impair the ability of flagellin to form polymers, we wanted to determine whether or not replacement of the FliC D3 domain with GFP is also an effective means to enhance GFP's immunogenicity. Recently the D3 domain of FliC has been successfully replaced with superfolder GFP, and this fusion protein retains GFP's fluorescence properties and reportedly polymerizes into filaments (141). The FliC-GFP fusion activates TLR5 as well as full length FliC (FliC EC_{50}=5.03 +/- 4.79 S.D. ng/mL and FliC-GFP EC_{50}=3.38 +/- 4.47 S.D. ng/mL) (Table II) (Fig. 22).

Coupling GFP to FliC significantly enhances anti-GFP responses. We immunized mice with either GFP alone, GFP+FliC, or the FliC-GFP fusion protein (141). All mice were immunized twice, 21 days apart and blood was drawn 14 days following, each immunization. First, we examined isotype specific anti-FliC responses from A/J mice that were immunized with GFP, GFP+FliC or FliC-GFP fusions. As anticipated, mice that were immunized with GFP alone

had no detectable IgG1 or IgG2a anti-FliC responses either after primary or secondary immunization (data not shown). Following primary and secondary immunizations mice immunized with FliC-GFP had significantly lower anti-FliC IgG1 responses compared with GFP+FliC immunized mice (Fig. 23A). The primary IgG2a anti-FliC responses were low and did not differ significantly between GFP+FliC and FliC-GFP mice (Fig. 23). Mice receiving secondary immunizations with FliC-GFP had significantly lower IgG2a anti-FliC titers than mice immunized with GFP+FliC (Fig. 23B).

We next tested sera for anti-GFP IgG1 and IgG2a responses following primary and secondary immunizations. Primary immunization results from mice immunized with GFP and GFP+FliC, both showed very low IgG1 anti-GFP responses (Fig. 23C). FliC-GFP immunized mice had significantly higher anti-GFP IgG1 primary and secondary responses; FliC-GFP mice achieved median anti-GFP IgG1 titers of 10^6, following secondary immunizations (Fig. 23C). Conversely, mice receiving secondary immunization of either GFP or GFP+FliC, demonstrated subtle increases in their anti-GFP IgG1 titers, which were significantly lower than FliC-GFP immunized animals (Fig. 23C). Primary immunizations with either GFP, GFP+FliC, or FliC-GFP in all mice had low anti-GFP IgG2a responses (Fig. 23D). Mice receiving secondary immunizations of FliC-GFP fusion protein generated robust IgG2a anti-FliC responses, which were significantly higher than the GFP and GFP+FliC immunized mice (Fig. 23D). Thus, physically linking a weak antigen, in this case GFP, to FliC promotes robust antibody responses against the antigen, which are significantly better than simply co-administering the antigen with a TLR5 agonist.

Induction of anti-HIV antibodies with FliC-HIV epitope-scaffold fusion proteins. Although computational design of epitope-scaffolds that are recognized by HIV broadly

neutralizing antibody has been highly successful, this success has not been successfully translated into effective immunogens that are capable of inducing broadly neutralizing antibodies against HIV (140, 142). These epitope-scaffolds were shown to have high affinity binding to monoclonal antibody (mAb) 4E10 and showed high structural mimicry of the 4E10 epitope (140). However, when used as an immunogen, they had poor immunogenicity, even in the presence of additional adjuvants (140). We hypothesized that the epitope-scaffolds are weak antigens like the WNV EII protein and GFP, and that fusion of the 4E10 epitope-scaffolds to FliC would increase their immunogenicity and probability of inducing 4E10-like broadly neutralizing antibodies. To test this hypothesis, we identified two 4E10 and one 447 epitope-scaffolds that were structurally compatible with substituting into the D3 domain of FliC. We substituted FliC amino acid residues 190-286, with epitope scaffolds T88 (4E10), T117 (4E10), or T284 (447), creating fusion proteins: F88 (4E10), F117 (4E10), and F284 (447). The fusion proteins when expressed, were shown to be at the correct predicted molecular weight (Fig. 24A). We next tested TLR5 stimulatory activity and determined that F284 (EC_{50}=3.61 +/-2.96 ng/mL) was comparable to FliC (EC_{50}=5.03 +/-4.79 ng/mL), while both F88 (EC_{50}=217 ng/mL) and F117 (EC_{50}=303 +/-364.1 ng/mL) had about 40-60-fold reductions in their TLR5 stimulatory activity (Fig. 24B). Thus, substitution of the D3 domain with the 4E10 epitope scaffolds interferes with TLR5 recognition, possibly through the formation of multimers (140).

FliC-HIV fusion proteins are potent immunogens. We immunized BL/6 mice four times with 30 µg of either F88, F117, or F284. Two weeks following each immunization, we drew blood and tested the sera for antigen-specific antibody responses. All of the FliC fusion proteins generated high titer antibodies against themselves (median titers of 10^6 – 3.16 x 10^6), which were equivalent to anti-FliC titers seen in FliC immunized mice (Fig. 25 and data not

shown). Our results show that linking HIV epitope-scaffolds to FliC promotes robust antibody responses directed against the epitope-scaffolds, which was not affected by differences in TLR5 stimulatory activity.

F117 generates strong 4E10 epitope-specific antibody responses. To determine whether mice immunized with the FliC HIV epitope-scaffold fusions generated desirable antibody responses against the HIV epitopes we tested all sera with a panel of epitope-scaffolds control proteins to determine the epitope specificity of the antibody responses. We used sera collected after the fourth immunization, since these had the highest titers. We examined the sera reactivity towards T117 (4E10), T93 (4E10), and MICA (a 6xHis-tagged non-epitope scaffold protein), as a negative control. F88 mice had low, but significantly higher anti-T117 responses than anti-MICA (Fig. 26A). However, F88 mice had anti-T93 responses that were comparable to anti-MICA responses (Fig. 26A). Mice immunized with F117 had significantly higher antibody responses against heterologous 4E10 epitope-scaffolds T88 and T93 than negative control anti-MICA responses (Fig. 26B). In contrast to both F88 and F117 immunized mice, F284 immunized mice did not exhibit any significant responses to heterologous 447 epitope-scaffolds (Fig. 26C). Thus, immunizations with FliC-HIV fusion proteins promote robust intrinsic adjuvancy to the epitope-scaffolds, but differ in their ability to induce antibody responses against the desired HIV epitopes. Out of three FliC-HIV immunogens only F117 generated HIV epitope specific antibody responses.

Discussion

Herein, we have illustrated that flagellin's adjuvancy is dramatically influenced by physically linking antigens to the flagellin molecule. Mixing flagellin with poor antigens can enhance the antigens immunogenicity, but this effect is dependent on antigenic properties of the

co-administered antigen. Weak antigens, such as GFP (Fig. 23) become highly immunogenic when coupled to flagellin. We demonstrate that flagellin's D0/D1/D2 domains are sufficient to enhance immunogenicity of weak antigens like GFP. Cuadros *et al.*, have demonstrated that flagellin-GFP fusion proteins activate APC secretion of pro-inflammatory cytokines, and induce the formation of GFP specific $CD8^+$ and $CD4^+$ T cells, suggesting that one benefit of coupling antigens to flagellin is enhanced production of antigen-specific T cell responses (128).

Because FliC fusion dramatically enhanced anti-GFP antibody responses we wanted to determine whether or not FliC could also enhance the immunogenicity of other weak antigens. Several HIV epitopes have been identified to be recognized by broadly neutralizing antibodies (bnAb) and recently, HIV epitope-scaffolds have been generated that bind these bnAbs with high affinity (140, 143-146). However, these HIV epitope-scaffolds are poor immunogens and lack the ability to induce desirable anti-HIV bnAbs. The bnAb 4E10 recognizes an epitope within the MPER of gp41 (147). The 4E10 epitope has been successfully introduced into non-viral scaffolding proteins that retain the alpha-helical structure of 4E10 and mimics HIV's gp41 structure (140). However, the 4E10 epitope-scaffolds were inherently weak antigens, even in the presence of additional adjuvants (140, 142). We hypothesized that their poor immunogenicity is one factor contributing to the inability of the epitope-scaffolds to induce desirable antibody responses.

We were able to successfully produce three FliC-HIV fusion proteins by replacing the entire D3 domain with epitope-scaffolds that displayed either HIV's 4E10 or 447 epitopes. While the 447 fusion (F284) retained good TLR5 stimulatory activity, both 4E10 fusion (F88 & F117) had reduced TLR5 stimulatory activity (Fig. 25). Despite differences in their TLR5 stimulatory activity, all fusions were capable of inducing robust antibody responses against their respective

immunogens, which is consistent with published data from our lab and others that indicate the TLR5 activation is not essential for flagellin's immunogenicity (33, 35, 55). However, the ability to induce antibody responses that were specific for the desired HIV epitope was not universal, as only F117 generated significant 4E10 specific antibody responses. In contrast, both F88 and F284 induced significant responses against their respective epitope-scaffolds, but unable to induce significant antibody responses against the 4E10 or 447 epitopes, respectively (Fig. 26). These results suggest that FliC-HIV epitope-scaffold fusion proteins are capable of inducing antibodies that recognize the broadly neutralizing 4E10 epitope in mice and that such fusions are good candidates for future testing.

A successful AIDS vaccine will need to induce potent and broadly neutralizing antibodies towards HIV's envelope glycoprotein (148-150). Antibodies that recognize HIV's gp41 4E10 epitope have been shown to have one of the widest breadths of HIV-1 neutralization (98%) (151). Unfortunately, bnAbs that recognize 4E10 have also been shown to recognize self-antigens in humans and mice (152, 153). 4E10 heavy-chain knock-in mice have shown that indeed 4E10 antibodies do react with self-antigens and also displayed significant B cell dysregulation, consistent with the elimination of autoreactive antibodies by negative selection mechanisms (152). However, here we demonstrate that F117 immunized mice were able to generate antibodies that successfully recognized the 4E10 epitope, suggesting that there could be other antibodies that are generated against the 4E10 epitope that are not self-recognizing. Hence, understanding the structural basis of 4E10 epitope recognition by antibodies generated in mice following F117 immunizations is critical in understanding if 4E10 bnAbs may be induced without self cross-reactivity.

The moderate success of the RV144 HIV vaccine trial recently completed in Thailand and the documentation of bnAbs in many HIV-infected long-term survivors has reinvigorated interest in developing vaccines that induce antibody-mediated immunity against HIV (154). Our studies with FliC-HIV fusion proteins demonstrate that flagellin enhanced immunogenicity of the HIV epitope-scaffolds and successfully lead to specific anti-4E10 antibodies for one of the fusions. Our results also indicate that enhancing immunogenicity is beneficial, but insufficient to induce the desirable HIV epitope-specific antibodies. It is unclear what factors influence the successful generation of HIV epitope-specific antibodies. At the present time it seems most likely that empiric testing of multiple flagellin epitope-scaffold fusions will be required to identify constructs with desired activity. We also focused on replacing the D3 domain in this study, in order to attempt to generate polymerizable antigens or antigens that could be synthesized and displayed by bacteria. Unfortunately, all of the fusion proteins failed to be secreted by *Salmonella* and were incapable of forming flagellar filaments *in vitro* and *in vivo* (data not shown). Fusions of antigens to the amino- and carboxy-terminus to flagellin have been successfully used for generating vaccines against influenza and other microbes (29, 65, 76, 78, 120, 132). Future studies should also investigate fusing HIV epitope-scaffolds to the amino- and the carboxy-termini, which will also increase the number of scaffolds that can be fused to flagellin since structural constraints imposed by D3 domain replacement will be lifted. The evaluation of additional HIV epitopes is also warranted as well as potential advantages of flagellin fusion to mucosal (i.e. intranasal) immunization and the generation of mucosal anti-HIV-immunity.

The successful anti-4E10 specificity generated following F117 immunization demonstrates the ability to rationally design an HIV vaccine that elicits epitope specific

antibodies against a broadly neutralizing epitope. Thus, our results have broad implications for the design of vaccines designed to elicit antibody responses against elusive conserved epitope. Namely, enhancing the immunogenicity of the epitope-scaffold via fusion to flagellin, increasing the likelihood of detecting desirable antibody responses. Our studies demonstrate that structural design of flagellin epitope-scaffold fusion proteins combined with empiric testing for induction of epitope-specific antibody responses in mice is a promising pre-clinical pipeline to select promising vaccine candidates for higher level experimental and clinical trials.

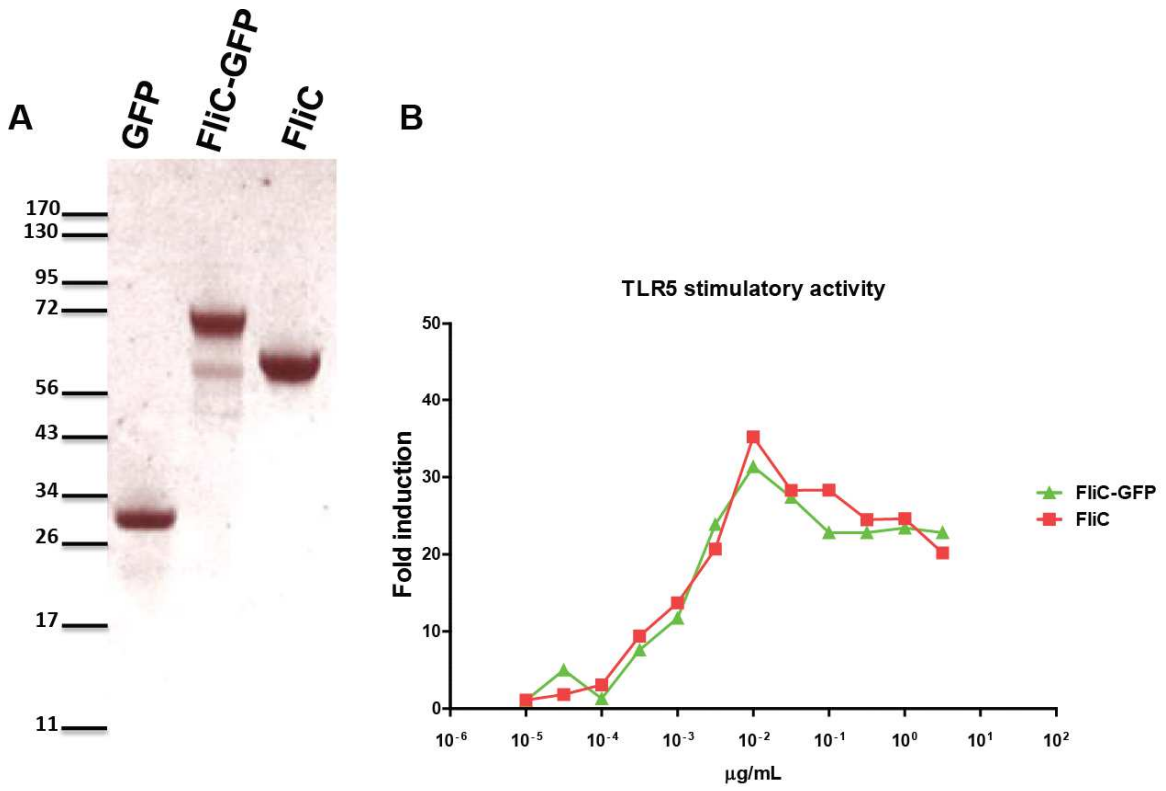

Figure 22. FliC-GFP fusion protein maintains TLR5 stimulatory activity compared to FliC. Purified recombinant GFP, FliC, and FliC-GFP proteins ran on SDS-PAGE confirmed by coomassie blue staining that all three have the appropriate molecular weight (**A**). TLR5 and biological activity for FliC and FliC-GFP were assessed by using CHO cells stably transfected with murine TLR5 and a NF-κB luciferase reporter (**B**).

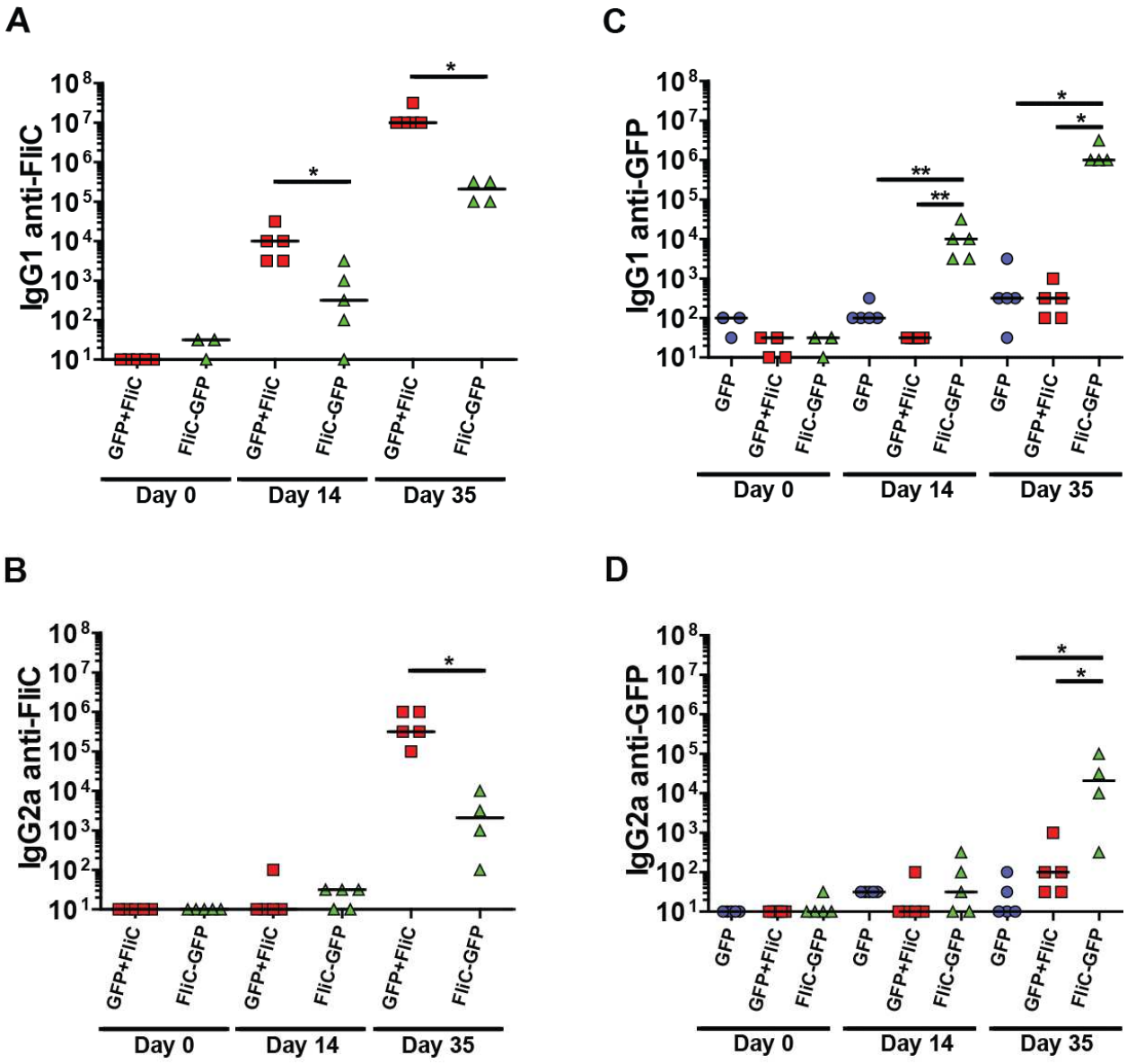

Figure 23. Covalently linking GFP to FliC significantly enhances anti-GFP IgG1 and IgG2a responses. A/J mice were immunized twice on day 1 and day 21 with either GFP (alone), GFP+FliC, or FliC-GFP and sera collected on days 14 and 35 were analyzed for IgG1 (**A, C**), and IgG2a (**B, D**) responses against immunogen by ELISA. All groups have an n=4-5 per experiment. Statistical analyses were done using Mann-Whitney analysis of individual groups: *= $P<0.05$, ** = $P<0.01$.

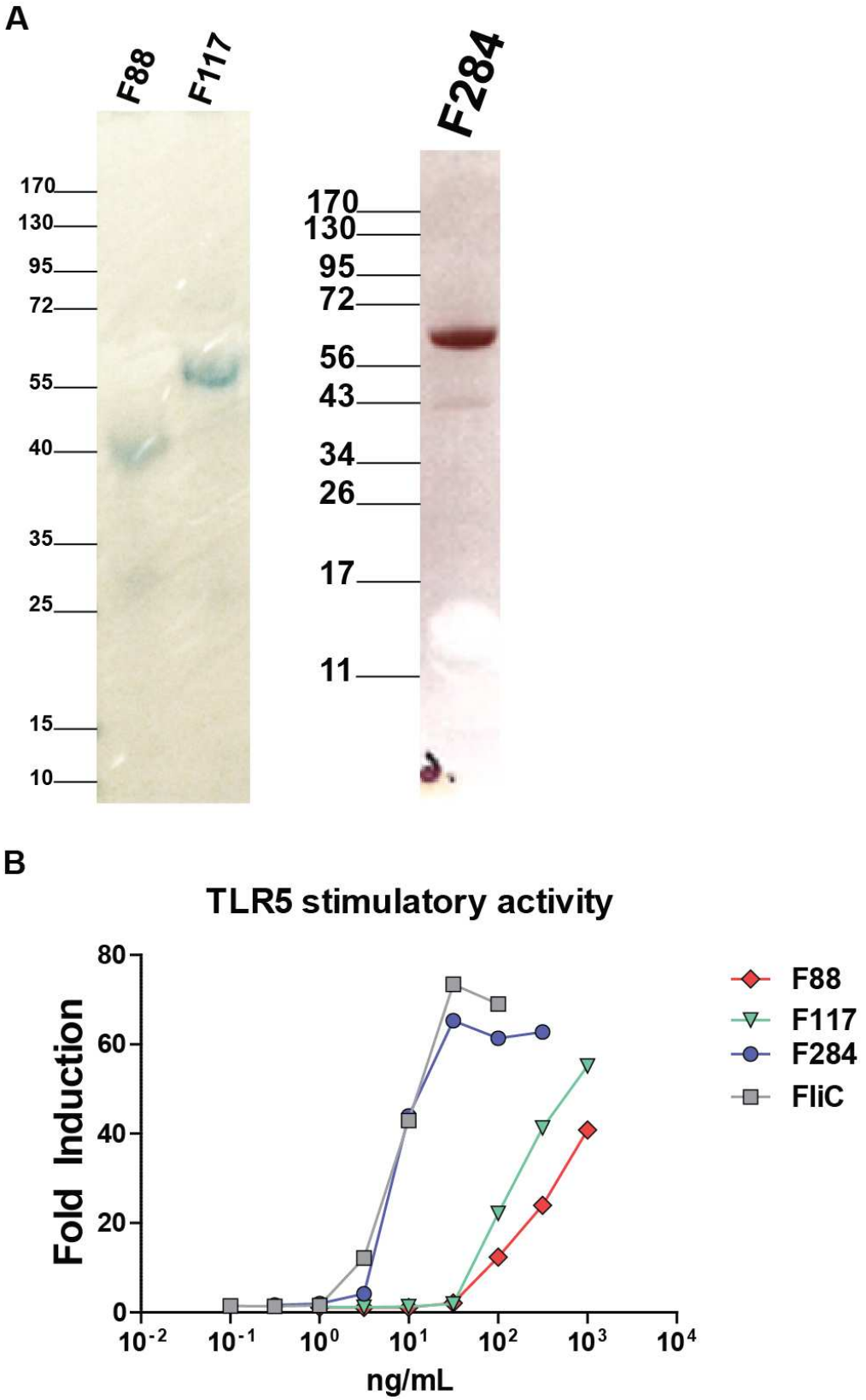

Figure 24. FliC-HIV fusion protein F284 maintains TLR5 stimulatory activity compared to

FliC. Purified recombinant F88, F117, and F284 proteins ran on SDS-PAGE confirmed by coomassie blue staining that all three have the appropriate molecular weights (**A**). TLR5 and biological activity for F88, F117, F284 and FliC were assessed by using CHO cells stably transfected with murine TLR5 and a NF-κB luciferase reporter (**B**).

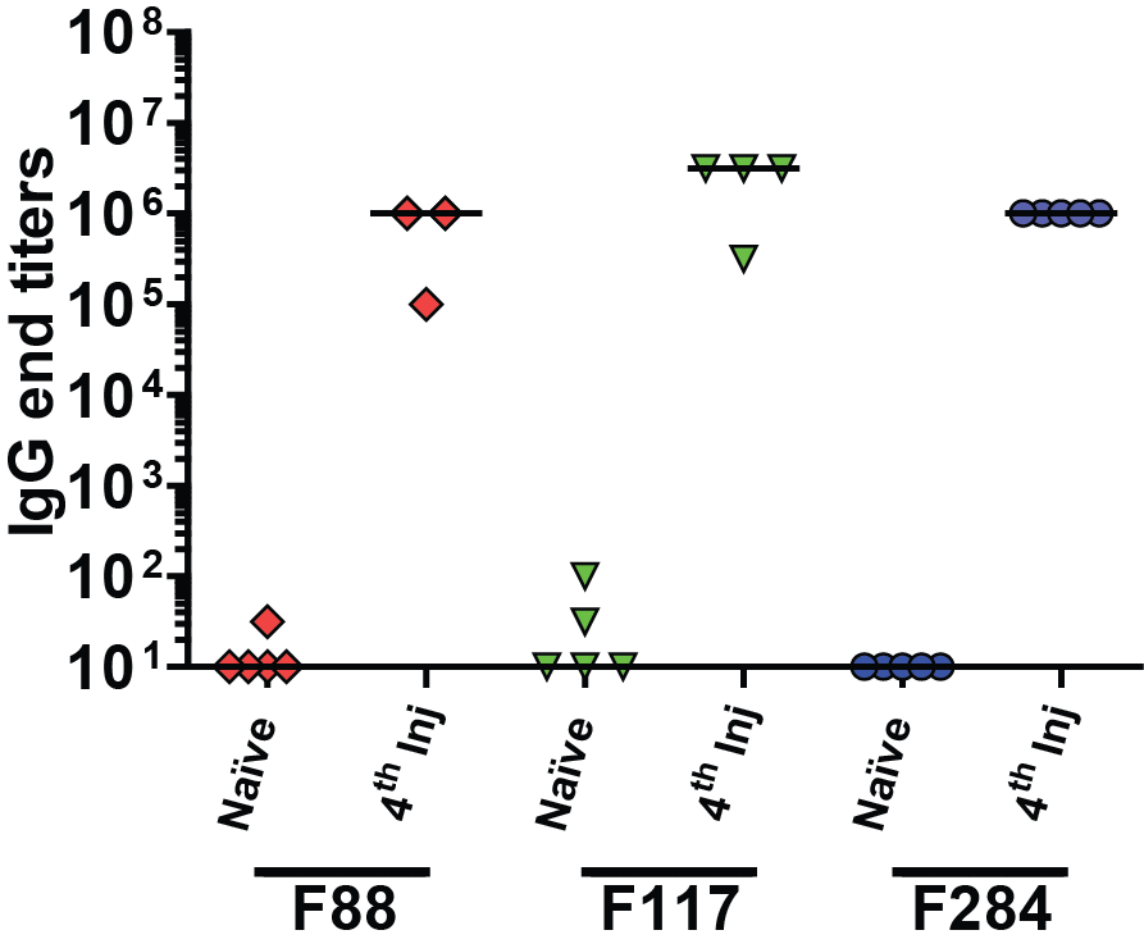

Figure 25. FliC-HIV fusion proteins retain robust immunogenicity. C57BL/6 mice were immunized four times with either F88, F117, or F284. Sera collected two weeks following the final immunization was analyzed for IgG responses against the immunogen by ELISA. All groups have an n=4-5 per experiment.

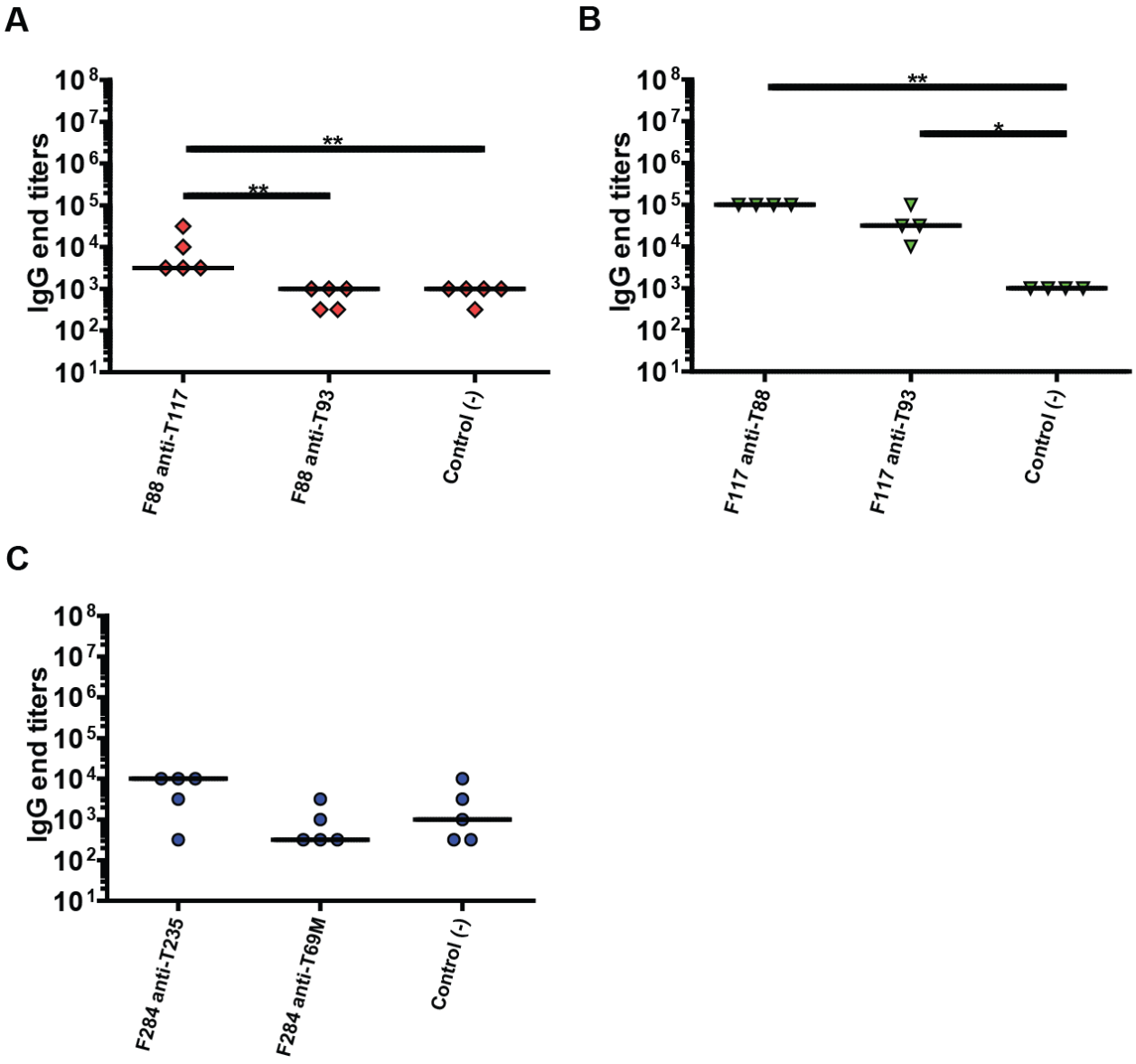

Figure 26. F117 fusion protein generates anti-4E10 specific antibody responses. C57BL/6 mice were immunized four times with either F88 (**A**), F117 (**B**), or F284 (**C**). Sera collected two weeks following the final immunization was analyzed for IgG responses against corresponding epitope-scaffolds by ELISA. All groups have an n=4-5 per experiment.

Proteins	EC_{50} ng/mL	SD +/- ng/mL
FliC	5.03	4.79
FliC-GFP	3.38	4.47
F88	217.5	**ND**
F117	303.3	364.1
F284	3.61	2.96

Table II. Flagellin fusion proteins and their corresponding EC_{50} +/-SD. ND, Not determined. Data are representative of a minimum of three independent experiments.

Chapter V: Conclusion

Herein, I have described studies that model a comprehensive examination of how the host's innate recognition of flagellin's highly conserved amino acid residues promote antibody responses towards itself and mediates its adjuvancy. Prior to our work, there was some evidence suggesting that recognition of flagellin by TLR5 and Naip5/6 did not completely explain flagellin's immunogenicity, and that additional flagellin recognition mechanisms existed and were critical for promoting flagellin-induced antibody responses (22, 35, 39, 55, 122, 123). Using mice deficient in flagellin recognition pathways, we conclusively demonstrated that a novel third pathway exists in mice, which contributes to flagellin's robust immunogenicity that is independent of TLR5 and the inflammasome (33). Furthermore, my studies provide a detailed analysis of how the different structural features of *Salmonella enterica serovar* Typhimurium flagellin mediates anti-flagellin antibody responses. Our work provides a foundation for future studies in the lab to springboard from, to address the question of, "What is the molecular basis of this third pathway?"

There are many future avenues of research that need to be conducted in order to completely understand the molecular and cellular mechanisms that are responsible for the third pathway that I have described herein. Some potential explanations as to why $FliC^{D0/D1}$ and FlaA are incapable of generating primary anti-flagellin responses are: flagellin molecules may bind to gangliosides in a D2/D3-dependent manner (155, 156), natural antibodies stemming from microbiota are directed towards the D2/D3 domain (123, 157), FliC is interacting with other cell surface markers via D2/D3, or in C57BL/6 mice there is a hole the B cell repertoire for these proteins. However, this final explanation is highly unlikely since previous studies have

demonstrated that the highly conserved D0/D1 domain is recognized by antibodies during immunization with monomeric flagellin (71).

The explanation I favor for this unknown pathway is provided by experiments conducted by Nossal, Ada, Miller, and colleagues over 4 decades ago (122, 123). They showed that flagella and flagellin isolated from *S.* Adelaide were targeted to the lymphoid follicles shortly after immunization of rats (122). Using germ free and irradiated rats, they demonstrated that lymphoid targeting of flagella was dependent on microbiota and radiosensitive cells, suggesting that microbiota-induced natural immunity are responsible for targeting flagella to lymphoid follicles and that this may be important for robust primary antibody responses that are induced by flagellin (123). We are planning immunization studies with germ-free mice and various flagellin proteins to understand if and how the microbiota influences flagellin's immunogenicity, once a germ-free animal facility has been established at the University of Washington. If successful, these studies will lead to additional studies to understand how the microbiota influences flagellin's immunogenicity and test to see if low affinity antibodies, such as IgM, may be responsible.

My thesis work provides a framework to begin to identify the precise host cellular and molecular mechanisms that comprise the third pathway. However, prior published studies may provide insight into the critical amino acid residues in flagellin's D2/D3 domain that influence primary antibody responses. Over 40 years ago, Ada and colleagues performed multiple immunizations with cyanogen bromide cleaved flagellin proteins on Wistar rats and demonstrated that a cleaved portion, "fragment A" of *Salmonella* flagellin, retained its ability to localize to lymphoid follicles (157). Our lab has contacted Gus Nossal in reference to these previous experiments, since the flagellin molecule used in those studies was from *S.* Adelaide,

but unfortunately this flagellin gene from their strain was never cloned. Currently, in the NCBI database there are two *S.* Adelaide flagellins (accession numbers AAA53490 and EHC38209). Fortunately, both proteins in the NCBI database have identical sequences and both contain exact sequence matches to "fragment A" from *S.* Adelaide (158), which will allow us to test *S.* Adelaide flagellin from either sequence.

Sequence alignments of *L*. *monocytogenes* FlaA, *S.* Typhimurium FliC, and *S.* Adelaide flagellins, indicate that the majority of the homology of all three proteins resides in the D0 and D1 domains, and a limited portion of the D2 domain (Fig 27). The aligned sequences reveal that "fragment A" of *S.* Adelaide corresponds to *S.* Typhimurium FliC residues 144-383, which consists of the carboxy terminal portion of the ND1 domain and the majority of the D2/D3. These data provide a series of breadcrumbs to follow and, more importantly, a starting point to identify the precise amino acid residues required for FliC's D2/D3-dependent immunogenicity. Future experiments in the lab will include immunizations with flagellin proteins that have additional deletions to further isolate the FliC residues that are required for TLR5- and inflammasome-independent immunogenicity. Currently, protein expression and purification of FliC's D2/D3 has commenced and immunizations will begin shortly to determine if the D2/D3 domain is sufficient to induce primary antibody responses independent of Naip5/6 and TLR5 recognition. Moreover, if the D2/D3 domain of flagellin is sufficient to promote primary and secondary responses, it will demonstrate that a functional TLR5 is not required for flagellin to work as an adjuvant. Understanding if FliC's D2/D3 domain can function independently of TLR5 is a critical component to flagellin-based adjuvant research, since approximately 10% of humans have a TLR5 mutation rendering it non-functional, and knowing if D2/D3 is sufficient

for primary and secondary responses will be critical for those individuals with TLR5 polymorphisms (31).

Early phase I and II clinical trials with flagellin fusion proteins have demonstrated that flagellin fusion proteins elicit antigen specific antibodies with minimal adverse side effects to patients (76-78, 121). My graduate studies demonstrate that flagellin is a poor adjuvant to intrinsically weak antigens (such as GFP), and that physically coupling weak antigens to FliC significantly enhances the antigens' immunogenicity (159). Using this information, we engineered FliC fusion with HIV epitope-scaffolds and have established a platform for coupling weak antigens to flagellin to promote robust antibody responses. The strategy to design flagellin fusion proteins with HIV epitope-scaffolds has been uniformly successful in generating antibody responses against the epitope-scaffolds, but inconsistent in obtaining antibody responses towards the desired HIV epitopes, which usually represent less than 10% of the surface area on the epitope-scaffold and less than 3% of the surface area of the FliC-HIV epitope-scaffold fusion. At the present time it is unknown what factors dictate a successful immunogen and empirical testing of novel immunogens will be required to select for promising vaccine candidates. Nonetheless, we believe that flagellin based fusion with epitope-scaffolds is a proven platform that can be utilized to generate robust vaccines that confer long term-immunity (129).

The flagellin-based adjuvant platform is also a transferable to other infectious diseases. As an example, Correia *et al*., demonstrates that epitope-scaffolds for respiratory syncytial virus (RSV) are capable of generating neutralizing antibodies in macaques (160). We believe that designing flagellin fusion proteins with these epitope-scaffolds will enhance the immunogenicity of the RSV epitope-scaffold proteins and increase efficacy of an RSV epitope-scaffold based vaccine.

In closing, my thesis work demonstrates that in addition to TLR5 and Naip5/6, there is a novel third pathway required for robust anti-flagellin responses and is dependent on flagellin's D2/D3 domain. Our work with flagellin fusion proteins suggest that the D2 domain is probably the crucial component although additional studies are required to confirm this idea. These three pathways function together to promote flagellin's robust T_H1 (IgG2a/c) and T_H2 (IgG1) humoral responses and our structural definition of the flagellin components that contribute to each of these pathways will help future efforts to exploit the immunogenicity of flagellin for vaccine design. My dissertation studies illustrates how the host's innate recognition of flagellin and FliC's conserved and non-conserved amino acid regions work in concert with one another to promote flagellin's intrinsic and extrinsic adjuvancy. Translational implications of flagellin-based vaccines are currently being realized, and additional studies to address the remaining fundamental questions of flagellin's immunogenicity, including the molecular nature of the third pathway and whether it is conserved in humans, will further assist efforts to utilize bacterial flagellin to design effective vaccines against worldwide debilitating diseases such as: influenza, malaria, tuberculosis, and HIV.

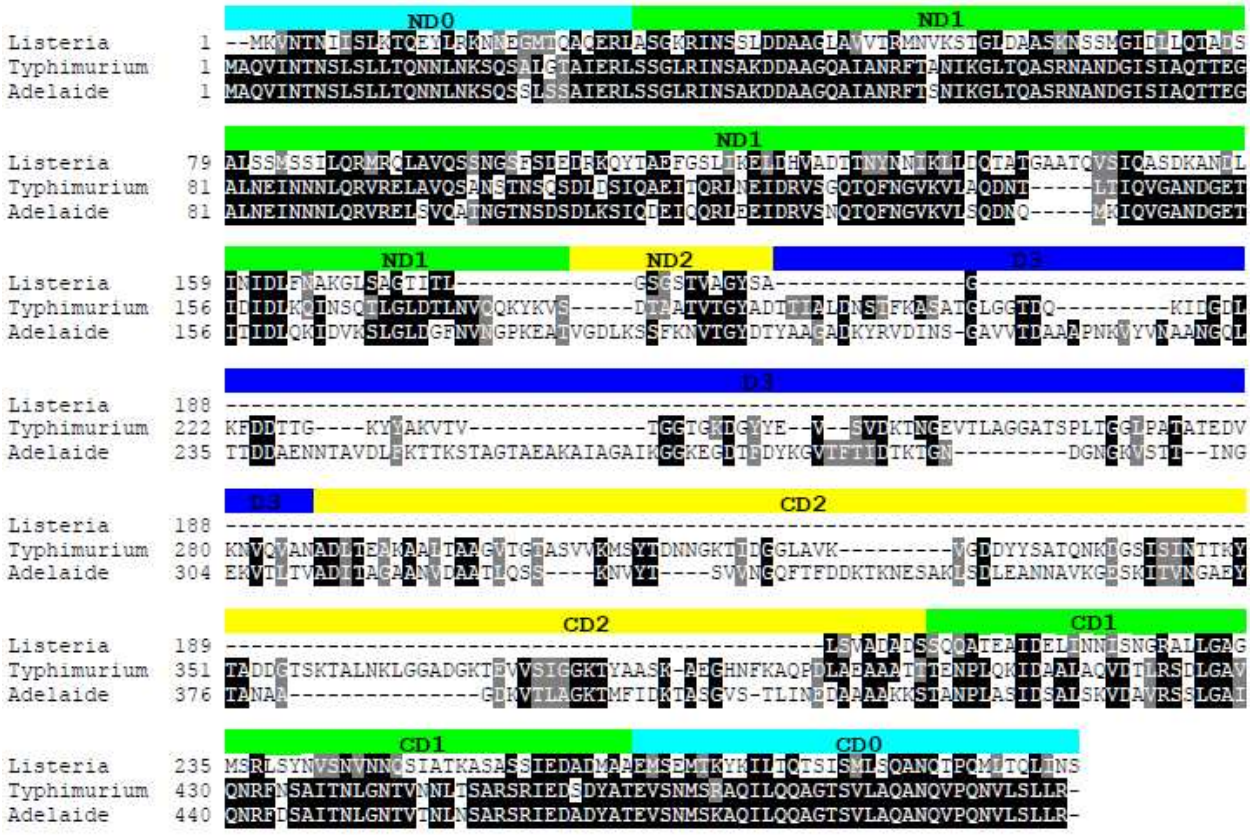

Figure 27. Sequence alignment *L. monocytogenes, S.* Typhimurium, and *S.* Adelaide flagellins demonstrates homology of all three proteins resides in the D0 and D1 domains, and a limited portion of the D2 domain. D0 and D1 domains are 1-178 and 407-495 (light blue and green) and D2 and D3 179-406 domains (yellow and dark blue).

Materials and Methods

Methods from Chapter II

Protein isolation and purification. Wild-type flagellin monomers were isolated from *S.* Typhimurium strain SL1344 (Δ*flgM*); purity was verified as previously described (49, 67). OVA was purchased from Sigma and ultrafiltered (Amicon) to reduce endotoxin. Removal of residual endotoxin from isolated flagellin monomers and OVA (Sigma-Aldrich) was performed by using polymyxin B columns (Thermo scientific). Endotoxin levels were <1 pg/μg of protein, as measured using the limulus colorimetric assay (Lonza). The purified flagellin was characterized biochemically and its biological activity for TLR5 and Naip5-dependent was determined prior to mouse studies (supplementary Figure 1).

NF-κB luciferease reporter assay. CHO K1 cells were stably transfected with mouse TLR5 cDNA cloned into the pEF6 V5/His TOPO vector (Invitrogen) or the empty vector, plus the ELAM-LUC plasmid; luciferase assays were performed as previously described (48, 49).

Protein transfection. Bone marrow derived macrophages (BMDMs) were prepared from femurs of C57BL/6 mice, Naip5-deficient mice, caspase-1-deficient mice, or A/J mice and cultured in RPMI 1640 supplemented with 10% FBS (ATLAS biologicals), 10% L-cell supernatant (CSF1 source), 2mM L-glutamine, and 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) (10, 21, 161). All assays were performed in triplicate and each experiment was repeated at least twice. BMDMs were primed with 10 ng/mL of ultrapure LPS (List Biologicals) for 3 hours to induce pro-IL-1β expression prior to protein transfection, using

Profect-P1-lipid based protein delivery reagent (Targeting Systems) as previously described (10, 52); IL-1β secretion was determined by ELISA (Duoset R&D).

Mice and immunizations. The University of Washington Institutional Animal Care and Use Committees approved all animal protocols. Mice were bred and housed in a specific pathogen-free facility at the University of Washington. A/J and C57BL/6 animals were purchased from Jackson Laboratories and bred in-house. Naip5$^{-/-}$ (40), caspase-1 (Casp1$^{-/-}$) (162), MyD88$^{-/-}$ (163) and TLR5$^{-/-}$ (25) mice were all generated on the C57BL/6 background and bred in-house. TLR5$^{-/-}$/Casp1$^{-/-}$ mice were generated and bred in our animal facility. A/J MyD88$^{-/-}$ mice were generated by back-crossing the MyD88 deletion onto the A/J background for nine generations and then crossing to generate homozygous MyD88$^{-/-}$ mice. 8-14 week old matched animals were used in all experiments. Retro-orbital bleeds were performed on all animals prior to immunization to obtain naïve serum. Mice received two sequential i.p. immunizations with 30 μg FliC and 30 μg OVA separated by 21 days. Blood was drawn two weeks following each immunization.

Cytokine analysis. Mouse sera were evaluated for cytokine responses following i.p. injections of 30 μg of FliC or PBS using commercially-sourced ELISA kits according to manufacturer's instructions (Duoset, R&D Systems). IL-18 cytokine analysis was determined by ELISA, using anti-mouse IL-18 (Clone 74; R&D Systems) as a capture antibody, and biotinylated anti-mouse IL-18 (Clone 93-10C; R&D Systems) as a detection antibody.

Antibody analysis. High binding capacity 96 well plates (COSTAR) were coated with 1 μg/mL of monomeric FliC or ovalbumin diluted in PBS (OmniPur) and allowed to incubate overnight at room temperature (RT). Plates were washed three times with PBS containing 0.05% tween-20, and blocked for 1 hour RT in PBS containing 1% BSA (Sigma). Plates were washed

and serial dilutions of serum were added to the wells and incubated for 1 hour at RT. Plates were washed again and horse radish peroxidase conjugated secondary antibodies (anti-IgG1, -IgG2a, -IgG2c–HRP) (Jackson Immunoresearch), or –IgA-HRP (Biolegend) were added and incubated for another hour at RT. Plates were developed with TMB substrate (Thermo), stopped with H_2SO_4 and absorbance was read at 450 nm (Molecular Devices). Antibody end point titer was defined by the reciprocal of the maximal serum dilution that exceeded three times the standard deviation above the mean background absorbance.

Statistical analysis. Significance was determined by one-way ANOVA with Bonferroni multiple comparison post-test or Mann-Whitney test, using Graphpad Prism 5 software. Differences were noted as significant when $P < 0.05$.

Methods from Chapter III

Creation of flagellin proteins. The FliC-C, FliC^{TLR5}, and FliC$^{D0/D1}$ gene blocks were ordered from Life technologies. All FliC gene blocks were based from *S*. Typhimurium strain SL1344 *fliC* (GenBank accession number: CBW17983) encoding amino acids 1-494. FliC-C contained substitution of amino acid R495P and the addition LVPRGSHHHHHH at the carboxyl terminus. Protein FliC^{TLR5} had amino acid substitutions at R90E, Q97A, E114R, R118E, E121R, D419R, R422D, and the addition of LVPRGSHHHHHH at the amino terminus. The FliC$^{D0/D1}$ protein encoded amino acids 1-176 and 391-495 with linker SPGISGGGGGILDSMG from 176-391 and the addition LVPRGSHHHHHH at the amino terminus. The FlaA gene block was also ordered from Life technologies. The FlaA construct was based from *L. monocytogenes* strain 10403 *flaA* (GenBank accession number: ACI49720) encoding amino acids 1-287 and the addition LVPRGSHHHHHH at the amino terminus. DNA fragments of FliC-C, FliC^{TLR5},

FliC$^{D0/D1}$, and FlaA were then cloned into pet29b (GenScript) and transformed into flagellin negative BL21 *E. coli* cells for expression (Invitrogen).

Purification of bacterial flagellin. Wild-type FliC flagellin monomers were isolated from *S.* Typhimurium strain SL1344 (Δ*flgM*); purity was verified as previously described (49, 67). OVA was purchased from Sigma and ultrafiltered (Amicon) to reduce endotoxin. Wild-type FlaA flagellin monomers were isolated from *L. monocytogenes* 10403; purity was verified as previously described. Removal of residual endotoxin from isolated flagellin monomers and OVA (Sigma-Aldrich) was performed by using polymyxin B columns (Thermo scientific). Endotoxin levels were <1 pg/μg of protein, as measured using the limulus colorimetric assay (Lonza). Single colonies of BL21 *E. coli* cells transformed with pet29b flagellin containing plasmids (FliC-C, FliC^{TLR5}, FliC$^{D0/D1}$, and FlaA) were expanded from starter cultures in Luria Broth and then induced with 1 mM IPTG when OD595 reached 0.6, plus kanamycin (100 mg/ml), incubated for 4 hr at 37º C and then overnight at 16º C. Cultures were then pelleted and stored at 20º C. Pellets were resuspended in standard buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, 0.5 mg/ml lysozyme), sonicated on ice, and clarified by centrifugation. Supernatants were tumbled with 10 ml of nickel-NTA resin (Superlow NTA, QIAGEN) for 30 min at 4 º C. The resin was then rinsed twice with 10 mM imidazole, once with standard buffer plus 20 mM imidazole and eluted with standard buffer plus 250 mM imidazole. Eluates were concentrated by ultrafiltration (Amicon Ultra, Millipore) and filtered through 0.22 mm Ultrafree-MC spin columns (Millipore). Proteins were then purified by preparative size exclusion chromatography (SEC) on Superdex 75 16/60 columns (GE Healthcare) at room temperature in 25 mM PIPES (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 0.02% w/w sodium azide (PNEA). The eluted protein is further purified by passage through a Proteospin Endotoxin Removal Maxi

Kit (Norgen) to remove any residual LPS. Endotoxin levels were <1 pg/μg of protein, as measured using the limulus fluorescent assay (Lonza). All purified flagellin was characterized biochemically and its biological activity for TLR5-dependent activity was determined prior to mouse studies.

NF-κB luciferease reporter assay. CHO K1 cells were stably transfected with mouse TLR5 cDNA cloned into the pEF6 V5/His TOPO vector (Invitrogen) or the empty vector, plus the ELAM-LUC plasmid; luciferase assays were performed as previously described (48, 49).

Protein transfection. Bone marrow derived macrophages (BMDMs) were prepared from femurs of C57BL/6 mice, Naip5-deficient mice, caspase-1-deficient mice, or A/J mice and cultured in RPMI 1640 supplemented with 10% FBS (ATLAS biologicals), 10% L-cell supernatant (CSF1 source), 2mM L-glutamine, and 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) (10, 21, 161). All assays were performed in triplicate and each experiment was repeated at least twice. BMDMs were primed with 10 ng/mL of ultrapure LPS (List Biologicals) for 3 hours to induce pro-IL-1β expression prior to protein transfection, using Profect-P1-lipid based protein delivery reagent (Targeting Systems) as previously described (10, 33, 52); IL-1β secretion was determined by ELISA (Duoset R&D).

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all animals prior to immunization to obtain naïve serum. Mice received two sequential i.p. immunizations with 30 μg flagellin and 30 μg OVA separated by 21 days. Blood was drawn two weeks following each immunization.

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Antibody analysis. High binding capacity 96 well plates (COSTAR) were coated with 1 μg/mL of monomeric flagellin or ovalbumin diluted in PBS (OmniPur) and allowed to incubate overnight at room temperature (RT). Plates were washed three times with PBS containing 0.05% tween-20, and blocked for 1 hour RT in PBS containing 1% BSA (Sigma). Plates were washed and serial dilutions of serum were added to the wells and incubated for 1 hour at RT. Plates were washed again and horse radish peroxidase conjugated secondary antibodies (anti-IgG1, -IgG2c–HRP) (Jackson Immunoresearch) were added and incubated for another hour at RT. Plates were developed with TMB substrate (Thermo), stopped with H_2SO_4 and absorbance was read at 450 nm (Molecular Devices). Antibody end point titer was defined by the reciprocal of the maximal serum dilution that exceeded three times the standard deviation above the mean background absorbance.

Statistical analysis. Significance was determined by one-way ANOVA with Bonferroni multiple comparison post-test, unpaired student's T-test, or Mann-Whitney test, using Graphpad Prism 5 software. Differences were noted as significant when $P < 0.05$.

Methods from Chapter IV

Protein expression and purification. DNA segments encoding FliC and FliC-HIV epitope-scaffold fusion constructs were synthesized with optimized codon usage and RNA structure (Codon Devices, Genscript Corp.), subcloned into pET29 (EMD Biosciences), and transformed into BL-21 *E.* coli (Invitrogen). Epitope-scaffolds T88, T117, and T284 were expressed as previously described (164). The superfolder GFP (GFP) pET29b and the FliC-GFP pET23b plasmids were generously provided by Dr. Ferenc Vodervisztand then transformed into transformed into BL-21 Star *E.* coli (Invitrogen) for expression (141). Single colonies were expanded from starter cultures in Luria Broth and then induced with 1 mM IPTG when OD595 reached 0.6, plus kanamycin (100 mg/ml), incubated for 4 hr at 37º C and then overnight at 16º C. Cultures were then pelleted and stored at 20º C. Pellets were resuspended in standard buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, 0.5 mg/ml lysozyme), sonicated on ice, and clarified by centrifugation. Supernatants were tumbled with 10 ml of nickel-NTA resin (Superlow NTA, QIAGEN) for 30 min at 4 º C. The resin was then rinsed twice with 10 mM imidazole, once with standard buffer plus 20 mM imidazole and eluted with standard buffer plus 250 mM imidazole. Eluates were concentrated by ultrafiltration (Amicon Ultra, Millipore) and filtered through 0.22 mm Ultrafree-MC spin columns (Millipore). Proteins were then purified by preparative size exclusion chromatography (SEC) on Superdex 75 16/60 columns (GE Healthcare) at room temperature in 25 mM PIPES (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 0.02% w/w sodium azide (PNEA). The eluted protein is further purified by passage through a Proteospin Endotoxin Removal Maxi Kit (Norgen) to remove any residual LPS. Endotoxin levels were <1 pg/μg of protein, as measured using the limulus fluorescent assay (Lonza). All purified

flagellin was characterized biochemically and its biological activity for TLR5-dependent activity was determined prior to mouse studies.

NF-κB luciferase reporter assay. CHO K1 cells were stably transfected with mouse TLR5 cDNA cloned into the pEF6 V5/His TOPO vector (Invitrogen) or the empty vector, plus the ELAM-LUC plasmid; luciferase assays were performed as previously described (48, 49).

Mice and immunizations. The University of Washington Institutional Animal Care and Use Committees approved all animal protocols. Mice were bred and housed in a specific pathogen-free facility at the University of Washington. A/J and C57BL/6 animals were purchased from Jackson Laboratories and bred in-house. 8-14 week old matched animals were used in all experiments. Retro-orbital bleeds were performed on all animals prior to immunization to obtain naïve serum. Mice received two sequential i.p. immunizations with 30 μg GFP alone, or 30 μg flagellin and 30 μg GFP, or 30 μg FliC-GFP, separated by 21 days. Otherwise, mice received four sequential i.p. immunizations with 30 μg flagellin, T88, T117, T284, F88, F117, or F284 separated by a minimum of 21 days. For all immunized animals blood was drawn two weeks following each immunization.

Antibody analysis. High binding capacity 96 well plates (COSTAR) were coated with 1 μg/mL of monomeric flagellin, GFP, FliC-GFP, T69M, T88, T93, T117, T235, T284, F88, F117, F284, or gp140 diluted in PBS (OmniPur) and allowed to incubate overnight at room temperature (RT). Plates were washed three times with PBS containing 0.05% tween-20, and blocked for 1 hour RT in PBS containing 1% BSA (Sigma). Plates were washed and serial dilutions of serum were added to the wells and incubated for 1 hour at RT. Plates were washed again and horse radish peroxidase conjugated secondary antibodies (anti-IgG, -IgG1, -IgG2a–HRP) (Jackson Immunoresearch), were added and incubated for another hour at RT. Plates were

developed with TMB substrate (Thermo), stopped with H_2SO_4 and absorbance was read at 450 nm (Molecular Devices). Antibody end point titer was defined by the reciprocal of the maximal serum dilution that exceeded three times the standard deviation above the mean background absorbance.

Statistical analysis. Significance was determined by Mann-Whitney test, using Graphpad Prism 5 software. Differences were noted as significant when $P < 0.05$.

Methods from Chapter V

Flagellin sequence alignments. Flagellin sequences were aligned using ClustalW2 and displayed with boxshade.

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