Responses to the soluble flagellar protein FliC are Th2, while those to FliC on *Salmonella* are Th1

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Features of the Th1 or Th2 phenotype start to develop during CD4 T cell priming. This study of the response to the bacterial flagellar protein FliC shows that either Th1 or Th2 responses can be induced in mice depending upon how FliC is presented. This is shown by assessing the cytokine mRNA and class of FliC-specific plasma cells induced *in situ*. Soluble recombinant (r)FliC and polymerized FliC are strongly Th2 polarizing, inducing IL-4, NIP45 and c-Maf mRNA as well as ε and γ1 switch transcripts and switching to IgG1. CD28-requirement for this switching shows its T cell dependence. rFliC was unable to induce markers of Th1 activity including IL-12, T-bet and IFN-γ. Conversely, when FliC is presented in its native context surface-bound on live, flagellated *Salmonella,* switching is predominantly to IgG2a (IgG2c in C57BL/6 mice), reflecting Th1 activity. The development of divergent FliC-specific polarization to either Th1 or Th2 indicates that the context in which this antigen is encountered rather than its intrinsic immunostimulatory properties determines the direction of Th polarization.

Key words: T helper differentiation / Flagellin / *Salmonella* / Plasma cells

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1 Introduction

The flagellae of *Salmonella enterica* serovar Typhimurium *(S. typhimurium)* are surface-exposed. They are involved in bacterial adhesion and locomotion and can be longer than the bacterium to which they are attached. The major constituent of each flagellum is a repeating unit of flagellin and a single *Salmonella* bacterium can have up to ten flagella each with 30,000 or more flagellin molecules [1]. There are two flagellin genes in *S. typhimurium*, *fliC* and *fljB.* An individual bacterium can express one but not both proteins at the same time. The flagellar proteins, FliC or FljB, are important inducers of immune responses against flagellated bacteria that can stimulate monocytes and DC to produce cytokines, chemokines and nitric oxide [2–7]. Flagellin is also a major target of the CD4 T cell response to *S. typhimurium* [8]. A conserved region of flagellin can induce human and mouse DC maturation through the innate recognition molecule TLR5 [2, 3, 9–11].

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Abbreviations: ***S. typhimurium***: *Salmonella enterica* serovar Typhimurium ***Salmonella***: *S. typhimurium* strain SL3261 **CGG:** Chicken gamma globulin **NP:** (4-Hydroxy-3-nitrophenyl) acetyl **TLR:** Toll-like receptor ***B.***: *Bordetella* **d:** Day(s)

The way innate signals activate DC maturation to APC via recognition of conserved pathogen-associated molecular patterns can critically influence the severity of disease resulting from infection. The activators of such innate signals include the different TLR [12–14], which mainly signal through the adaptor molecule MyD88 [12]. Absence of MyD88 deviates responses to Th2 indicating a generic TLR capacity to drive Th1 responses [15, 16], although reports describing TLR stimulation resulting in Th2 induction have been published [17–19].

After encountering Ag, DC can migrate to the T zone of secondary lymphoid tissue where they present processed Ag to naive CD4 T cells [20]. Successful cognate interaction primes T cells giving them Th characteristics that may be polarized to show Th1 or Th2 activity. This polarization can be detected *in vivo* within 72 h of immunization [21, 22]. During priming CD4 T cells become able to produce type-specific cytokines and, through cognate interaction with Ag-activated B cells, induce Ab responses [21–23]. Th2 responses can be induced by Ag such as alum-precipitated OVA and chicken gamma globulin (CGG). Primary responses to soluble proteins generally require additional innate stimuli from molecules such as LPS [24], but soluble rFliC by itself induces immune responses, possibly through additional signals induced by its interaction with TLR5 [2, 25]. Markers of Th2 activity include IL-4, induction of

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the transcription factor – c-Maf [26] and NF-AT interacting protein – Nip45 [27] as well as T cell-mediated B cell switching to IgG1 and IgE. Th1-inducing Ag include intracellular bacteria like *S. typhimurium* and Th1 markers include IFN-γ, T-bet and T-dependent B cell switching to IgG2a [28, 29]. The γ2c replaces the γ2a heavy chain gene in Th1 responses in C57BL/6 mice, the main strain used in this study [30].

Primed CD4 T cells induce B cells, through cognate interaction, to grow either as plasmablasts in extra-follicular foci, or in B cell follicles where they form germinal centers. While T cells initiate extra-follicular plasmablast development they have no continued influence over plasmablast growth and differentiation [31]. Switching in B cells can be induced by Th cells both at the time of primary interaction in the T zone as well as during centrocyte selection in germinal centers [32–34].

Recently, it has been shown that purified soluble flagellin can induce anti-FliC IgG1 and stimulate the maturation of TLR5-expressing splenic and bone marrow-derived DC via a MyD88-mediated pathway [2]. This study clearly demonstrates that flagellin can drive a Th2-polarized response to co-administered Ag. In the current study we independently corroborate and extend these findings using recombinant soluble rFliC and soluble polymeric flagellin purified from the attenuated *aroA*$^{-}$ strain of *S. typhimurium*, SL3261 (referred to as *Salmonella*). We show that the polarization in the FliC-specific plasma cell response to FliC presented in its native context bound to live *Salmonella* is quite different. Presented in this way the anti-FliC antibody induced is predominantly Th1-reflecting IgG2c. Thus the switching pattern to FliC, and consequently the direction of the Th response and its protective potential against infection depends on how the Ag is initially encountered.

2 Results

2.1 Purification of rFliC and flagellin

Protein purity of rFliC was confirmed by SDS-PAGE. Contaminating proteins detectable after elution from the Ni-NTA column were not observed after affinity chromatography (Fig. 1A). Western blotting using an anti-6x histidine mAb was used as a secondary method to show that the cloned *FliC* gene was expressed in frame (Fig. 1B). Further confirmation of protein identity was achieved by trypsin digest and analysis of the fragments by electrospray tandem mass spectrometry (data not shown). Flagellin purified to homogeneity from cells after acid-treatment is shown in Fig. 1C. No detectable

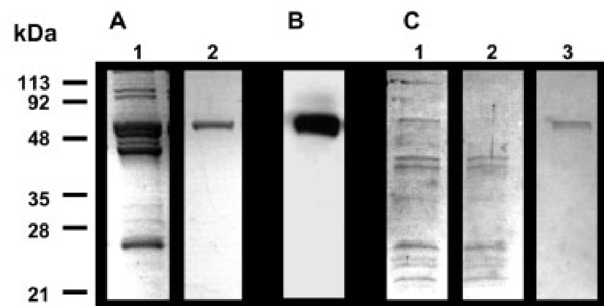

Fig. 1. Purification of rFliC and flagellin. (A) Elution of rFliC after histidine-tag purification showed the presence of contaminating proteins (lane 1). These were removed by affinity chromatography using a FliC-specific mAb (lane 2). (B) Western blot showing a single band is detected by a poly-histidine-specific mAb. (C) Flagellin was purified by acid-treatment of whole organisms (lane 1). Following affinity chromatography the flagellin was removed from other proteins (lane 2) and eluted as homogeneous flagellin (lane 3).

contaminating proteins were carried over after affinity purification. The amount of contaminating LPS administered after endotoxin removal was $<$0.1 endotoxin unit per mouse. rFliC prepared in this manner, unlike LPS, selectively induced phenotypic markers of activation on murine splenic DC but not B cells (data not shown).

2.2 Soluble FliC induces a strong Th2 response equivalent to the response to alum-precipitated protein

The Th2 and Th1 gene expression profiles, respectively induced by soluble rFliC, alum-precipitated rFliC or live *Salmonella* are shown in Fig. 2 and 3. These were assessed in the draining popliteal LN 7 days after s.c. immunization in the foot and compared to those in non-immunized mice. Background levels of transcription of these genes in popliteal LN of SPF mice are generally low or non-detectable (Fig. 2 and 3). A strong Th2 response to soluble rFliC is indicated by the induction of IL-4, Nip-45 and c-Maf mRNA and γ1 and ε germ-line transcripts. All of these genes were strongly induced 7 days after immunization with soluble rFliC or alum-precipitated rFliC; *p*-values for soluble rFliC or alum-precipitated rFliC compared with non-immunized mice, or mice immunized with *Salmonella* were all $<$0.005. Indeed c-Maf and Nip45 transcript-levels following immunization with *Salmonella* were lower than in non-immunized mice ($p<0.005$) (Fig. 2).

Th1 activity was assessed from mRNA levels for IL-12, IFN-γ, the global Th1 transcription regulator T-bet [35] and germ-line γ2c IgH region transcripts. Fig. 3 shows

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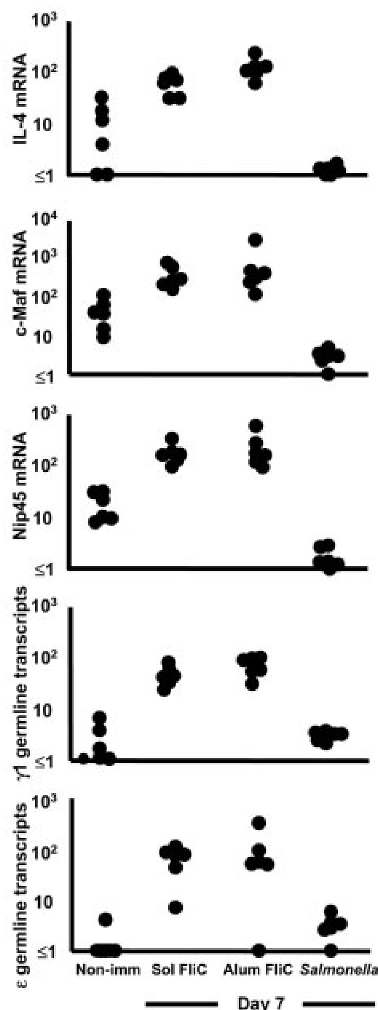

Fig. 2. Immunization with soluble rFliC selectively induces a Th2 activity that is not evoked by *Salmonella*. Mice were immunized in the footpad with soluble rFliC, alum-precipitated rFliC or live *Salmonella* and the draining popliteal LN were isolated 7 days later. Responses were compared between non-immunized (Non-imm) mice and groups of mice immunized with soluble (sol) rFliC, alum-precipitated (alum) FliC or *Salmonella*. Each point shows data from a single LN draining the site of s.c. immunization or sham immunization. Data are representative of at least two repeats.

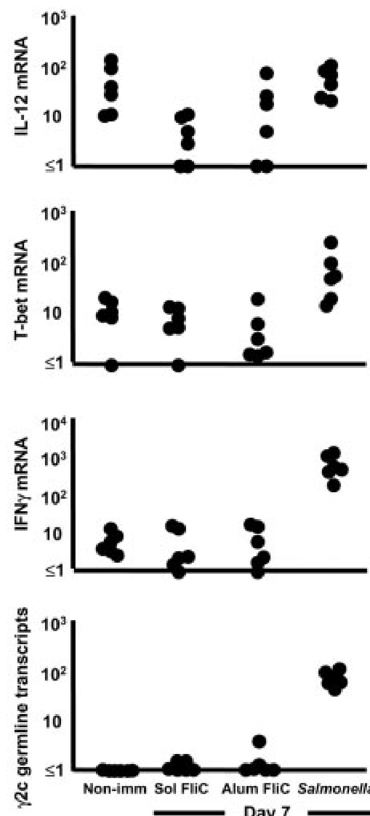

Fig. 3. Immunization with *Salmonella* induces Th1 activity that is not evoked by soluble rFliC. Mice were immunized in the footpad with soluble (sol) rFliC, alum-precipitated (alum) rFliC or live *Salmonella* and the draining popliteal LN isolated 7 days later. Responses were compared between these groups of immunized mice and non-immunized mice (Non-imm). Each point is the result from a single LN draining the site of s.c. immunization or sham immunization and data are representative of at least two experiments.

strong induction of the Th1 markers IFN-γ, T-bet and γ2c germ-line transcripts by *Salmonella* (p-values <0.005, <0.01 and <0.005, respectively compared with non-immunized controls). In contrast rFliC induced no significant differences in the levels of these transcripts. Background levels of IL-12 mRNA in non-immunized mice were high and remained high after immunization with *Salmonella,* while IL-12 levels fell significantly in response to soluble rFliC ($p<0.005$). Thus the response induced by soluble rFliC shows an unambiguous Th2 profile 7 days after immunization and bore no similarity to the Th1 response induced by *Salmonella*.

2.3 Dependence of the FliC-specific antibody response on T cell activation via CD28

It has been reported that flagellin can act as a T-independent Ag (reviewed in [36]). To test this in the current experimental model responses to soluble rFliC in wild-type mice and CD28-deficient mice were compared. CD28 is critical for the induction of T-dependent antibody responses [37]. FliC-specific plasma cell induction required T cell activation via CD28 (Fig. 4, left hand panel), since the numbers of FliC-specific plasma cells induced by rFliC were over 80-fold lower in CD28-

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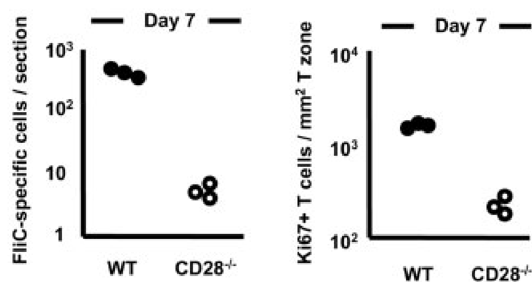

Fig. 4. CD28-deficient mice are unable to induce FliC-specific plasma cells in response to soluble rFliC. Mice were immunized i.p. and the spleen isolated 7 days later. FliC-specific plasma cells were identified by immunohistology. Staining for CD3 expression and Ki67 was used to identify T cells in the T zone that had progressed to G1 or further in the cell cycle. Data are representative of at least two experiments.

deficient mice. Also, no FliC-specific serum IgG1 or IgG2a was induced in the CD28-deficient mice (data not shown). T cell proliferation in the T zone 7 days after immunization was nearly 8-fold lower in CD28-deficient mice (Fig. 4, right hand panel). Thus soluble rFliC induction of switched FliC-specific plasma cells is T dependent.

2.4 Switched plasma cells induced with soluble rFliC are predominantly IgG1

To confirm the FliC-specificity of these T cell responses we studied switching in FliC-specific plasma cells during responses to FliC presented in different contexts (Fig. 5). Fourteen days after immunization with rFliC alone IgG1 dominated the specific response and IgG2c was undetectable (Fig. 5A). Conversely the anti-FliC serum Ab induced by *Salmonella* was mainly IgM with some switching to IgG2c (Fig. 5A). After co-administration of rFliC and *Salmonella* a mixed Th1 and Th2 switching pattern was induced synchronously (Fig. 5A). These serological findings are reflected in the numbers of switched FliC-specific plasma cells in the spleen, which clearly show the Th1 anti-FliC switching pattern induced by *Salmonella* (Fig. 5B and C).

To test if FliC polymerization affected the switching pattern mice were immunized with flagellae isolated from *Salmonella*. Fig. 5D indicates that the responses to flagellae and rFliC gave comparable Th2 switching to IgG1. Thus polymerization of FliC is not responsible for the anti-FliC Th1 response induced by live *Salmonella*. Live *Salmonella* plus isolated flagellae again gave bi-directional switching to IgG1 and IgG2c.

2.5 Secondary Ab responses to *Salmonella* in rFliC-primed mice retain the Th polarization induced in the primary response

The stability of switching induced by priming with rFliC was assessed by challenging mice with *Salmonella* or rFliC 35 days later. There is a clear significant secondary IgG1 response in mice primed and boosted with rFliC (Fig. 6A and B; $p<0.05$) indicating the Th2 pattern of switching is retained in the secondary response. During the 4 days after boosting rFliC-primed mice with rFliC the number of FliC-specific plasma cells in the spleen rose approximately 100-fold. When rFliC primed mice were challenged with live *Salmonella* there was a clear secondary extrafollicular response yielding FliC-specific plasma cells (Fig. 6B) even though at this early stage the Ab titers had not risen significantly (Fig. 6A). During the 4 days after boosting rFliC-primed mice with *Salmonella* the number of FliC-specific extrafollicular plasma cells had increased 5-fold. Switching patterns in these plasma cells reflect a Th2 dominance of the CD4 T cell help, for among these FliC-specific plasma cells medians of some 40% had switched to IgG1 and 10% had switched to IgG2a. Thus rFliC induced memory T cells do not appear to be re-educated to display Th1 switching patterns after challenge with live *Salmonella.* The possibility that IgG1 switched memory B cells masked such re-education was considered. This seems unlikely as between 41 and 52% of the FliC-specific plasma cells in the recall extra-follicular response had not switched from IgM (Fig. 6B, right hand panel). Consequently it appears that FliC-specific B cells with the potential to switch were recruited into this recall response and very few switched to IgG2c. Switching in memory B cells during secondary extra-follicular Ab responses is possible as has been noted previously [23]. Thus the isotype of antibody that is induced in the primary response persists during recall responses. The immunizing dose of live *Salmonella* was the maximum that could be given without producing substantial symptoms. This threshold precluded testing whether higher doses of *Salmonella* might influence the direction of FliC-specific switching.

2.6 rFliC induces severe adverse reactions in *Salmonella*-primed mice

It was not possible to study the stability of the Th1 response to FliC induced by *Salmonella* as rFliC reproducibly induced a severe adverse reaction in mice primed with *Salmonella.* Batch variation of rFliC used for challenge was not responsible, as rFliC given at the same time to non-primed mice or mice primed with rFliC caused no adverse reaction. OVA given to mice primed with *Salmonella* expressing OVA also caused no adverse reaction.

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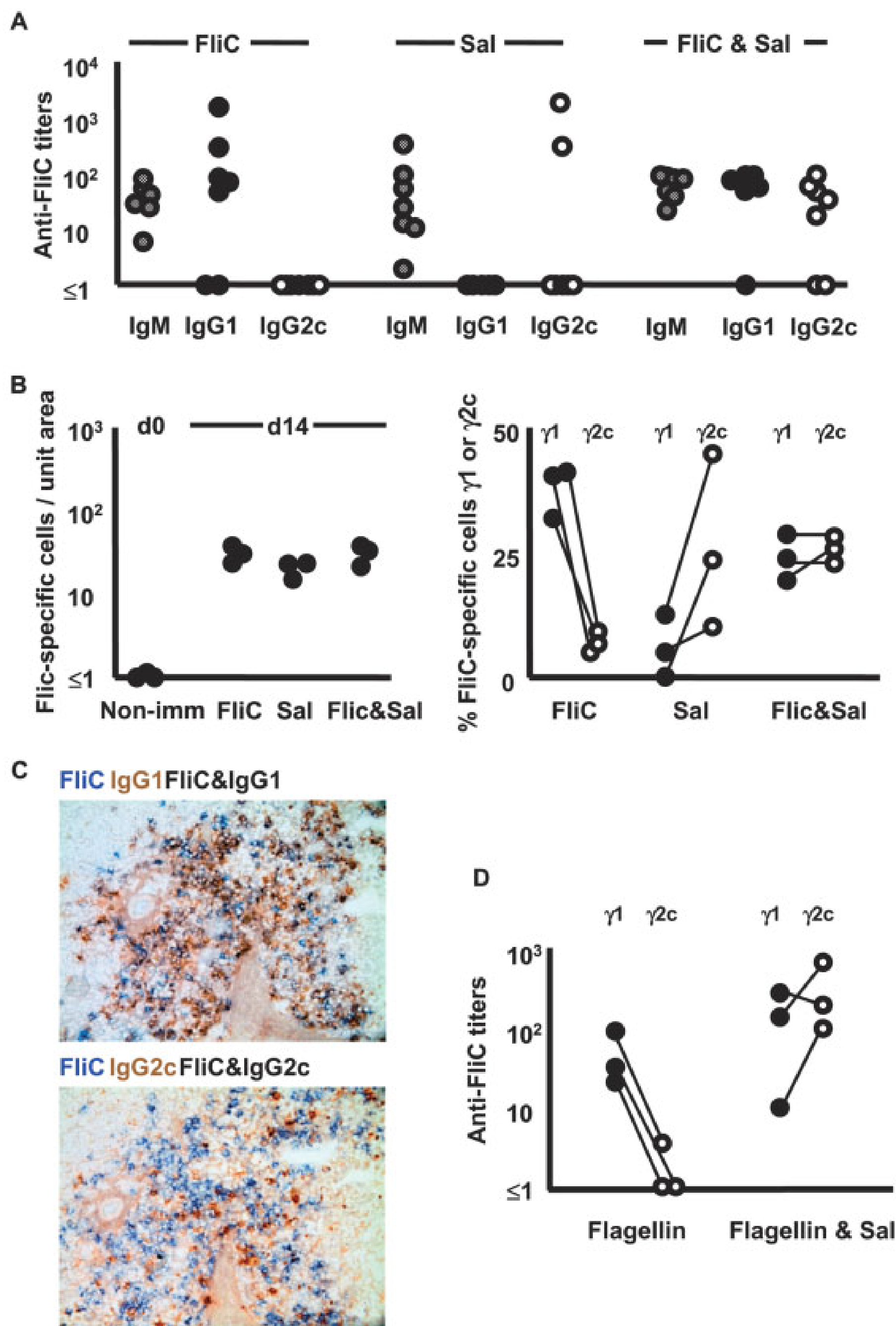

Fig. 5. Ig class switching, shown by serum Ab titers (A) and splenic plasma cell numbers (B) 14 days after immunization. Lines link the responses from individual mice. The responses show the continued Th2 polarization in the anti-soluble rFliC response shown in Fig. 2. Mice were immunized i.p. with rFliC, or *Salmonella* (Sal), or rFliC with Sal. γ1 and γ2c indicate the proportion of FliC-specific plasmacytoid cells respectively producing IgG1 or IgG2c. (C) Serial sections of mice immunized with rFliC stained for FliC-specific cells and IgG1 (top panel) or IgG2c (lower panel) included to illustrate the technique. In both panels FliC-specific plasma cells are blue, and in the upper panel, brown cells are IgG1-producing and black cells contain FliC-specific IgG1. In the lower panel, brown cells are IgG2c producing and black cells are IgG2c-producing FliC-specific plasma cells. (D) shows a Th2 switching pattern induced by purified flagellae (Flagellin) and a mixed response by co-immunization with *Salmonella*. Bars link the subclass-specific antibody titers from individual mice. Non-immunized mice had $\leq$2 FliC-specific plasma cell per unit area and Ab titers $<$1 (data not shown). Data are representative of at least two repeats.

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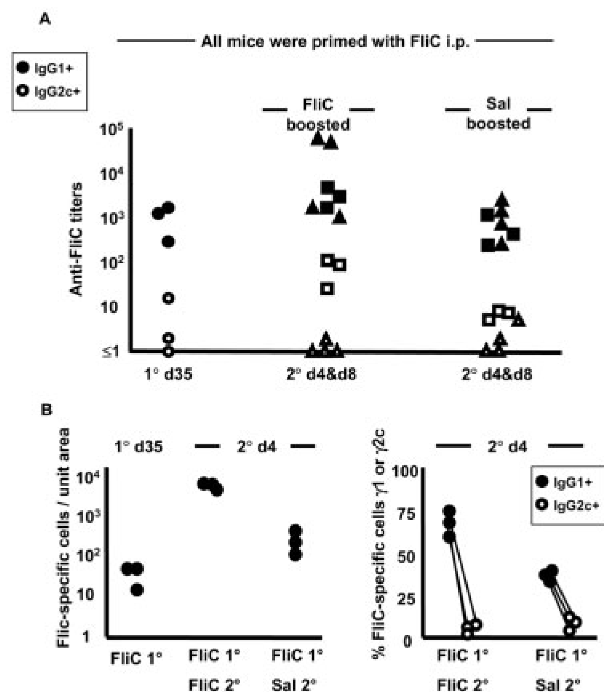

Fig. 6. Stability of Th polarization after secondary immunization. (A) Antibody titers of mice 35 days after priming i.p. with rFliC and of similarly primed mice 4 days (squares) or 8 days (triangles) after challenging with either rFliC or *Salmonella*. (B) Left panel: numbers of FliC-specific plasma cells in the red pulp 35 days after immunization and 4 days after secondary challenge. Right panel: the proportion of FliC specific plasmacytoid cells producing IgG1 or IgG2c. Lines link data from individual mice. Data are representative of at least three experiments.

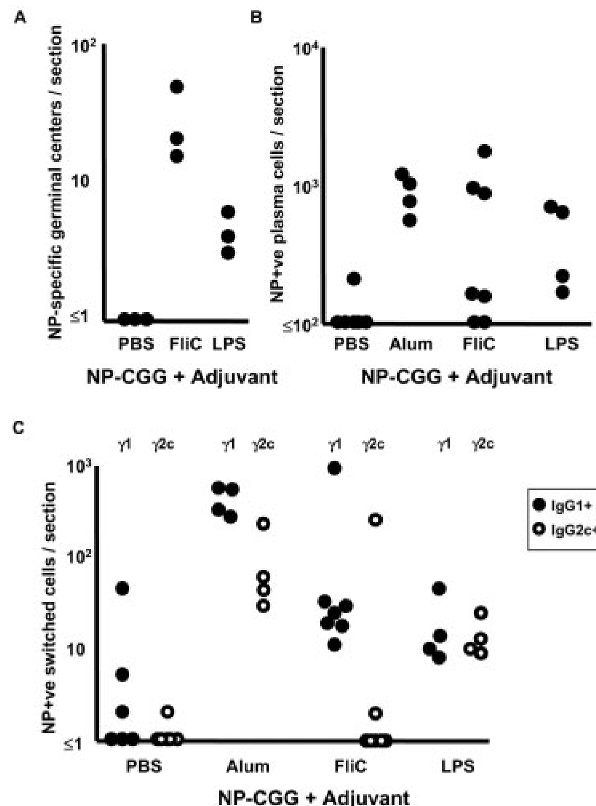

Fig. 7. rFliC can act as an adjuvant to promote immune responses to soluble protein Ag. Mice were immunized i.p. with soluble NP-CGG with or without rFliC or LPS or alum-precipitated NP-CGG and the responses assessed 7 days later. (A) Total numbers of NP-specific germinal centers identified by immunohistological staining to identify NP-specific cells. These single-positive cells were distinguished from follicular DC which were identified by the presence of both NP-binding and IgG2c-containing immune complex. (B) Total numbers of NP-specific extra-follicular plasmacytoid cells per section. (C) Numbers of IgG1 switched (closed circles) and IgG2c switched (open circles) NP-specific plasma cells per section. Non-immunized mice had ≤1 NP- or CGG-specific plasma cell per unit area. Data are representative of at least two experiments.

2.7 rFliC acts as an adjuvant that selectively drives Th2 responses to protein Ag

The i.p. response to alum-precipitated NP-CGG was compared with that to soluble NP-CGG given i.p. alone or with rFliC or LPS. Soluble NP-CGG alone gave a weak response (Fig. 7A–C, left hand columns). rFliC induced a striking increase in numbers of NP-specific germinal centers (Fig. 7A). The addition of any of the three adjuvants resulted in significant increases in numbers of NP-specific plasma cells induced (Fig. 7B; $p<0.02$). Similar numbers of NP-specific IgG1 and IgG2c plasma cells were induced with added LPS (Fig. 7C). In contrast, alum-precipitated NP-CGG or NP-CGG with rFliC induced significantly more switching to IgG1 than IgG2c (Fig. 7C; $p<0.02$ and <0.05, respectively). The absence of endotoxin in the NP-CGG and rFliC preparations (<0.1 endotoxin units per immunizing dose) excludes contaminating LPS influencing the adjuvant effect of rFliC.

3 Discussion

This report shows that the flagellar protein rFliC acts as an adjuvant and polarizes the immune response against itself and co-administered Ag towards Th2. This offers a major advantage to using rFliC over model Ag such as haptenated-OVA or CGG, which require exogenous adjuvants such as alum or CFA to induce strong polarized antibody responses. Adjuvants can contribute to the direction of polarization, for instance this is typically to Th2 with alum [38]. The direction of the immune response against FliC was influenced not by the intrinsic properties

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of FliC, but by the context in which it was encountered. McSorley and colleagues [8] showed that in C57BL/6 mice immunized with *Salmonella* the majority of *Salmonella*-specific cells secreting IFN-γ were FliC specific. Selective switching to IgG2c in this response confirms that the Th response to FliC as surface-attached flagellae on *Salmonellae* is Th1.

The Th2 polarized FliC-specific response to soluble rFliC or flagellin resembles the response seen to most alum-precipitated or aggregated proteins. A recent *in vitro* study showed that DC matured with flagellin induced a Th1 profile in allogeneic T cells. LPS also behaved in a similar manner whereas schistosome egg Ag polarized to a Th2 response [39]. The *in vivo* relevance of this observation that flagellin matures DC that prime T cells to adopt Th1 activity is questioned both by our study and the recently published report from Didierlaurent and colleagues [2] that soluble FliC primes CD4 T cells to show Th2 activity. The study by Didierlaurent showed that highly purified monomeric FliC isolated from *Salmonella* acts as a Th2 adjuvant to polarize responses to OVA in normal mice and in an adoptive transfer OVA-specific model. Their purified protein was able to up-regulate maturation markers on splenic and bone marrow-derived DC in a MyD88-dependent manner. DC were shown to be transcribing TLR5 actively and the involvement of other TLR in the maturation of these APC in response to flagellin was excluded. Co-immunization with OVA resulted in upregulation of Th2-associated cytokines that were detected after *in vitro* re-stimulation with OVA. Furthermore mice produced IgG1 specific to OVA and FliC. Additionally OVA and flagellin given in combination to splenic DC can markedly up-regulate CD80 [25]. Thus murine DC can respond to flagellin. This result is in contrast with a previous report [3]. The reason for the discordance between these two groups is not clear. Irrespective of the differences noted by these groups it is clear that highly purified flagellin can have an adjuvant effect in mice ([2, 25] and the current study). What is a more moot point is the mechanism by which it acts as an adjuvant.

Why does this powerful Th2 adjuvant lose this property and evoke a Th1-directed switching to IgG2c when it is attached to *Salmonella?* Linkage is crucial, for soluble rFliC given with *Salmonella* still induces switching to IgG1 in FliC-specific B cells. This double switching pattern to IgG1 and IgG2c when soluble rFliC and *Salmonella* are given together may reflect relatively independent Th1 and Th2 responses proceeding together that have not been induced by the cytokine environment of the T zone where the naive CD4 T cells are being primed. Evidence for parallel synchronously evolution of Th1 and Th2 in the same LN has been seen previously in the response to NP-CGG and killed *B. pertussis.* In this the Th2 response to NP-CGG and the Th1 response to *B. pertussis* proceed independently in the same LN although the *B. pertussis* acted as an adjuvant for the response to the hapten-protein conjugate [21]. These findings pointed to the possibility of cytokine-independent polarization of CD4 T cells during priming, which has subsequently been confirmed, particularly in relation to IL-4 and IL-13 by several groups [40–43, 22]. An alternative explanation for bi-directional switching when both rFliC and *Salmonella* are given together that Ag nonspecific switching is provoking IFN-α production [44], seems unlikely in this situation. IFN-α, possibly acting through BAFF [45] induces switching to all IgG subclasses. The absence of IgG1 anti-FliC production following immunization with *Salmonella* suggests that these switching pathways were not major players in the immunizing conditions tested in the present study. In addition, little or no FliC-specific IgG3 was detectable on day 14 after primary immunization with rFliC and live *Salmonella* (data not shown).

Boosting *Salmonella*-primed mice with rFliC led to rapid morbidity. This identifies a potential danger for the use of flagellin as an *in vivo* immunogen or adjuvant in humans. The nature of this severe adverse reaction requires further investigation. It is possible that the reaction reflects a response to persisting organisms within the host. *S. typhimurium* is known to persist in the gall bladder and may be present for extended periods in the reticulo-endothelial system [46].

In conclusion, soluble rFliC is a powerful immunogen that acts as its own adjuvant. It can also act as an adjuvant for soluble proteins that normally do not evoke responses unless they are alum precipitated, or are given with other conventional adjuvants. The capacity of FliC to evoke an almost pure Th1 or Th2 switching pattern dependent upon the context of encounter suggests that factors additional to the MyD88 signaling pathways are at play in TLR assisted CD4 T cell priming. The ability to identify FliC-specific plasma cells in combination with the use of recently described flagellin-specific transgenic mice [47] will provide powerful tools in developing our understanding of the development and expansion of the Ag-specific immune response.

4 Materials and methods

4.1 Mice, bacteria, and reagents

Matched groups of mice were used at 8–14 weeks. C57BL/6 and BALB/c mice were obtained from HO Harlan OLAC Ltd (Bicester, UK). CD28-deficient mice [37] were provided by Dr. Graham Anderson, University of Birmingham. *Salmonella*

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enterica serovar Typhimurium strain SL3261 (*Salmonella*) is an attenuated *aroA*⁻ strain [48]. Bacteria were cultured in Luria Bertani broth. For immunizations, mid-log phase bacteria were used. Reproducibility was confirmed by culturing bacteria. Cloning was performed in *Escherichia coli* TOP10F cells (Invitrogen; Paisley, UK) and protein expression in *E. coli* BL21 (Novagene, Nottingham, UK). *S. typhimurium* LPS (Quadratech, Epson, UK) and restriction enzymes (Roche, Lewes, UK) were obtained commercially; (4-hydroxy-3-nitrophenyl) acetyl (NP)-CGG was synthesized as described [22].

4.2 Generation of rFliC

FliC was amplified from *Salmonella* to contain a 5′ *Nde*I and 3′ *Xho*I site without a stop codon. The product was cloned into pCR2.1TOPO (Invitrogen), excised via *Nde*I and *Xho*I restriction and ligated into pET22b$^+$ (Novagene), allowing the addition of a poly-histidine tail. rFliC was isolated from mid-log phase cultures of rFliC-expressing *E. coli* by cell disruption in 8 M urea (pH 8.0) [4] and Ni-NTA chromatography (Qiagen, Crawley, UK). rFliC was eluted with 8 M urea pH 6.5 and dialyzed before purification to homogeneity by affinity chromatography using an anti-FliC mAb (kindly supplied by Drs. Martin Woodward and Roberto La Ragione, VLA, Weybridge, UK). Remaining minimal amounts of LPS were removed using a Detoxi-Gel column (Perbio) before filter sterilization. LPS concentrations were determined using the QCL-1000® Chromogenic LAL Test Kit (Cambrex, Wokingham, UK).

4.3 Flagellin purification

Whole flagella were isolated from *Salmonella* as described [49] by harvesting bacteria from overnight cultures and resuspending in a minimal volume adjusted to pH 2.0 using 2 M HCl. The solution was mixed for 1 h before centrifugation at 48,000×g for 1 h. The supernatant was recovered and the pH increased to 7 using 4 M NaOH. Flagellin was then further purified by affinity chromatography and LPS depleted as before.

4.4 Immunizations

Mice were immunized i.p. or s.c. at the following doses: rFliC, 20 μg in PBS; flagellin, 20 μg; *Salmonella,* 10^5 live organisms; 50 μg soluble NP-CGG. LPS co-administered with rFliC was given at the maximum safely tolerated dose of 1 μg. When LPS was given with NP-CGG the safely tolerated dose of 50 μg LPS was used. Alum-precipitation of rFliC and NP-CGG was as described [50].

4.5 Immunohistology

The acetone-fixed frozen spleen sections (5 μm) were stained to detect CD3, Ki67, IgG1 and IgG2c as described in [23, 43], except the mAb used to detect IgG2c was R19–15 from BD PharMingen. FliC-binding cells were detected using biotinylated rFliC and StreptABComplex-alkaline phosphatase (Dako). NP-binding cells were detected with NP conjugated to sheep Ig, then bound by biotinylated rabbit anti-goat antibodies (Dako) and StreptABComplex-alkaline phosphatase (Dako). NP-specific germinal centers were identified as NP-staining follicular areas. Specific Ig containing cells were confirmed as plasmacytoid cells by staining for CD138.

4.6 Reverse transcription of mRNA and its relative quantitation by PCR

RNA from LN was extracted using the RNeasy kit (Qiagen, Crawley, UK), eluted in water containing 1 mg of oligo-dT_{12-18} (Amersham Pharmacia Biotech, High Chalfont, UK) and denatured at 70°C for 10 min. RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Paisley, UK) at 42°C for 60 min. Relative quantitation of specific cDNA species to β-actin message was carried out in a multiplex PCR on the ABI 7700 (Applied Biosystems, Warrington, UK) as described [22]. Probes for cytokines and switch transcripts were labeled with FAM (Applied Biosystems), while β-actin probes were labeled with VIC (Applied Biosystems). Sequences for β-actin, γ1 germ-line transcripts, γ2c germ-line transcripts, ε germ-line transcripts, IL-4, IL-12, IFN-γ and T-bet are published [22, 43]. Sequences for c-Maf: forward: ACC TCG GTC TTG CAC TTT GC; reverse: TGC CTG GCT CTT ATG GTT ACT ATT ATT; probe: CTC CGG ATC CTT CGC GTG CGT. Sequences for Nip45: forward: TTG CAT TTT CAG CCT TGG GTA T; reverse: TGC CAA GAA GTG TGT ACC AGA CA; probe: CTC TCT GCC TGC CAG GAC TCT AGC CA. Reactions contained Universal PCR Master Mix (Applied Biosystems), β-actin primers and probe, test gene primers and probe, and cDNA template. Standard TaqMan PCR reaction conditions were used with 45 cycles. Relative quantification of signal per cell was achieved by setting thresholds within the logarithmic phase of the PCR for β-actin and the test gene and determining the cycle number at which the threshold was reached (C_T). The C_T for the target gene was subtracted from the C_T for β-actin. The relative amount was calculated as $2^{\Delta CT}$.

4.7 Ag-specific ELISA

ELISA were performed as described [22] with the test Ag, FliC, coated on plates at 5 μg/ml.

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4.8 SDS-PAGE and Western blotting

Proteins were separated on 12% (v/v) acrylamide gels and detected using the Gelcode blue stain (Perbio). For Western blotting, proteins were transferred onto nitrocellulose; and histidine-tagged proteins were identified using an anti-poly-histidine mAb (Roche).

4.9 Statistics

Two tailed statistical analysis uses the Mann-Whitney test.

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