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Characterization of CD4$^+$ T Cell Responses During Natural Infection with *Salmonella typhimurium*[1]

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CD4$^+$ T cells are important for resistance to infection with *Salmonella typhimurium*. However, the Ag specificity of this T cell response is unknown. Here, we demonstrate that a significant fraction of *Salmonella*-specific CD4$^+$ T cells respond to the flagellar filament protein, FliC, and that this Ag has the capacity to protect naive mice from lethal *Salmonella* infection. To characterize this Ag-specific response further, we generated FliC-specific CD4$^+$ T cell clones from mice that had resolved infection with an attenuated strain of *Salmonella*. These clones were found to respond to an epitope from a constant region of FliC, enabling them to cross-react with flagellar proteins expressed by a number of distinct *Salmonella* serovars. *The Journal of Immunology*, 2000, 164: 986–993.

The causal agent of human typhoid fever is the Gram-negative bacterium, *Salmonella typhi*, which is transmitted via contaminated food and water. Despite the widespread use of antibiotics, this disease remains a significant health care problem in developing countries, where as many as 30% of typhoid patients fail to recover due to lack of treatment or a substantial delay in antibiotic administration (1). Live, attenuated vaccine strains of *Salmonella* have the potential to be a cost-effective prophylactic measure in combating this disease, yet the mechanism of protective immunity provided by such vaccination is not fully understood, and the Ags important for the induction of immunity are largely unknown.

S. typhi does not cause systemic disease in mammalian hosts other than humans. This is probably due to a requirement for specific interactions with the human cystic fibrosis transmembrane conductance regulator for penetration of the gut epithelium and subsequent visceral infection to occur (2). However, a murine model of human typhoid is provided by infection of susceptible mice with *S. typhimurium*, which causes a lethal, systemic disease. After gaining entry to the host, *Salmonella* infect macrophages and persist in an intracellular vacuole by preventing phago-lysosomal fusion (3) and delaying vacuole acidification (4).

As with other intracellular micro-organisms, such as *Leishmania* and *Mycobacteria*, there is an absolute requirement for Th1-like CD4$^+$ T cells to produce or induce macrophage-activating cytokines such as IFN-γ and TNF-α to resolve infection (5, 6). Thus, mice lacking class-II restricted CD4$^+$ T cells succumb to infection with attenuated *Salmonella*, whereas mice lacking class-I restricted T cells or $\gamma\delta$ T cells resolve infection (7, 8). Further evidence that cellular immunity is required for the resolution of a primary *Salmonella* infection is demonstrated by experiments using IFN-γR knockout mice (8) or mice treated with neutralizing Abs to IL-12 (9), neither of which are able to resolve infection with an attenuated strain. A similar requirement for cellular immunity has been observed in humans with certain genetic or acquired cellular immunodeficiencies that confer an increased susceptibility to *Salmonella* infection (10, 11).

It has long been known in the murine model that protective immunity can be conferred by immunization with attenuated bacteria (12). Thus, susceptible strains of mice that resolve infection with an attenuated strain develop specific immunity, allowing resistance to secondary challenge with a virulent strain. However, it is not known which Ags are responsible for inducing such resistance, and there have been few reports of successful immunization of susceptible mice with purified *Salmonella* proteins. The best characterized of these are the *Salmonella* porins, which have been shown to protect susceptible mice against challenge with virulent *Salmonella* (13). A number of other bacterial extracts have been shown to be effective in protecting against *Salmonella* infection, but the identity of the immunizing Ag was not determined (14, 15). In addition, limited attempts at transferring immunity with *Salmonella*-specific T cell lines have failed to identify the specificity of the response (16, 17).

In this paper we report the characterization of a CD4$^+$ T cell response induced by attenuated *Salmonella* in C57BL/6 mice. We demonstrate that a significant fraction of CD4$^+$ T cells respond to the flagellar protein, FliC, in vivo during vaccination with an attenuated strain, and that responses to FliC are sufficient to protect against infection with a virulent strain. We also identified a stimulating epitope within a constant region of FliC as well as the TCRs that recognize this epitope. Finally, we demonstrated that responses to this peptide arise in vivo only upon secondary infection with *Salmonella*.

Materials and Methods

Mice

Female C57BL/6 (H-2^b), BALB/c (H-2^d) mice were purchased from the National Cancer Institute (Frederick, MD) and used at 8–16 wk of age for immunization experiments and for preparing APC populations.

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Table I. *Salmonella strains*

Strain	Relevant Properties	Source and Reference
SL1344	Virulent, hisG, xyl, rpsL	B. Stocker
SL3261	Attenuated SL1344 ΔaroA, hisG, xyl, rpsL	B. Stocker
BC116	SL1344 hin108::Tn10dCm (fljB off)	This study
BC115	SL3261 hin108::Tn10dCm (fljB off)	This study
TH453	fliC::Tn10	M. Homma
S. cholerasuis (7001)	Serotype 6,7:c:1,5	ATCC
S. enteritidis (13076)	Serotype 1,9,12:g,m:-	ATCC
S. gaminara (8324)	Serotype 16:d:1,7	ATCC
S. paratyphi (9150)	Serotype 1,2,12:a:-	ATCC
Shigella sonnei		ATCC

Bacterial strains, infection, and Ag preparation

Mice were infected with *Salmonella* as previously described (18). Bacterial strains (Table I) were grown overnight in Luria-Bertoni medium and diluted in PBS after estimating bacterial concentration using a spectrophotometer. Immediately before oral infection by gavage, mice were given a 5% sodium bicarbonate solution to neutralize stomach acids, thus promoting infection. The effective dose of bacteria given to the mice was verified by plating out dilutions of the bacterial solution on MacConkey agar plates. In all experiments the estimated bacterial concentration differed from the actual concentration by <3-fold (data not shown). Heat-killed *S. typhimurium* (HKST)[3] (3) was prepared from an overnight culture of SL1344 that was washed twice in PBS before incubation at 65°C for 1 h. Other heat-killed *Salmonella* or *Shigella* were prepared in a similar manner, and each strain was assessed for motility in soft agar to confirm flagella expression before the preparation of heat-killed bacteria. FliC was prepared from phase-fixed bacterial strains BC115 and BC116 using both shearing and differential centrifugation as previously described (19, 20). FliC was further purified using a Sephadex G-200 column, collecting fractions that contained a single band of the relevant m.w. on Coomassie-stained SDS-PAGE gels. These FliC fractions were concentrated and pooled before the presence of FliC in these preparations was confirmed by Western blotting using an mAb specific for FliC (Biogenesis, Sandown, NH) that detected a single band coincident with the band detected by Coomassie staining. FliC was the only protein detected by silver staining (data not shown).

The IFN-γ ELISPOT

The frequency of *Salmonella*-specific and FliC-specific IFN-γ-producing T cells was determined by the ELISPOT assay (21). C57BL/6 mice were infected orally with 1×10^9 SL3261, and $CD4^+$ T cells were purified by positive selection from infected or uninfected spleens. Spleen cells were incubated on ice with FITC-conjugated anti-CD4 (PharMingen, San Diego, CA) followed by anti-FITC microbeads (Miltenyi Biotech, Auburn, CA) before selection using a magnetic cell sorter according to the manufacturer's instructions. After this procedure samples were routinely 97–99% $CD4^+$ cells, as assessed by flow cytometry after two serial passages through the columns. Purified $CD4^+$ cells (5×10^6/well) were added to 24-well tissue culture plates containing 5×10^6 irradiated syngeneic splenocytes as APC. These wells then were supplemented with medium alone, HKST (5×10^7/ml), purified FliC (100 μg/ml), or peptide (10 μM) and incubated for 48 h at 37°C (these concentrations were determined to be optimal for IFN-γ production). Live cells were recovered using Histopaque (density, 1.083; Sigma, St. Louis, MO) and added in serial dilutions to 96-well microtiter plates (Millipore, Bedford, MA) that had been coated with purified anti-IFN-γ (PharMingen). After 20 h, cells were removed, and spots were visualized using biotinylated anti-IFN-γ (PharMingen) and avidin peroxidase (Sigma) in conjunction with 3-amino-9-ethylcarbazole substrate (Sigma). Spots were counted under a dissecting microscope and then recounted by an investigator who was unaware of the experimental protocol to confirm accurate counting.

Immunization and Ab measurement

Mice were immunized twice, s.c. in the flanks with 200 μg of FliC or OVA mixed 1/1 with CFA, at 2-wk intervals. One week after the second dose, blood was obtained from the retro-orbital plexus, and serum was prepared. The Ab to FliC and OVA was measured using an ELISA method as previously described (18). Briefly, Ag was coated on 96-well microtiter plates (Costar, Cambridge, MA), and serum was added to plates in serial dilutions. After washing, bound Ab was detected using goat anti-mouse IgG1 and IgG2a HRP-conjugated Abs (Southern Biotechnology Associates, Birmingham, AL).

Generation of T cell lines and clones

T cell lines were prepared as previously described (22). Briefly, a single cell suspension of spleen cells from FliC-immunized or orally infected mice was cultured with HKST for 4 days. Live cells recovered from this culture were rested for an additional 10 days in the absence of Ag before a further round of restimulation with Ag and APC. Clones were derived from the line by limiting dilution in 96-well plates after stimulation with HKST and the addition of recombinant human IL-2 (Roche, Indianapolis, IN). Clones from 96-well plates that had <30% positive wells were expanded in 24-well plates before being assessed for reactivity to HKST. Clones were maintained by repeated cycles of stimulation with HKST followed by expansion with rIL-2 and at least 1 wk of rest before further restimulation.

Proliferation assays

T cells (1×10^4/well) were added to 96-well plates containing 5×10^5 irradiated APC and serial dilutions of HKST, purified FliC, or heat-killed *Escherichia coli* expressing pMal-FliC. After 2 days of stimulation, 1 μCi of [^{3}H]thymidine was added to each well, and incorporated thymidine was measured the following day using liquid scintillation spectrophotometry.

Construction and characterization of pMal-FliC and derivatives

The construction of these strains has been described in detail previously (23). Briefly, *fliC* from SL1344 chromosomal DNA was amplified by PCR using standard techniques and ligated into the vector pMIE1. This construct, which encodes a 90-kDa MalE-FliC fusion protein, was digested sequentially by exonuclease III and S1 nuclease to make nested deletions in *fliC* from the 3′ end. The PCR products from these constructs were examined for the extent of deletion and were ordered by size. *E. coli* DH5α-containing plasmids were grown to mid-log phase in Luria-Bertoni broth and induced for 3–4 h with 500 μM isopropyl β-D-thiogalactoside (IPTG) before being washed and killed at 65°C. We define regions of *S. typhimurium* FliC by sequence homology as described previously (24), numbering from the N-terminal alanine of the mature polypeptide translated from the corrected sequence for *fliC* (GenBank accession no. D13689): regions I (aa 1–100), II (aa 101–160), III (aa 161–179), IV (aa 180–300), V (aa 301–323), VI (324–360), VII (aa 361–421), and VIII (aa 422–494).

Flow cytometry

T cell lines, clones, or freshly isolated spleen cells (1×10^6) were incubated on ice for 15 min with blocking solution (anti-Fc receptor supernatant from 24G2; 2% rat serum, 2% mouse serum, and 0.01% sodium azide) before staining with the following Abs; PE-conjugated anti-Vβ2, -3, -4, -5.1/5.2, -6, -7, -8.1/8.2, -8.3, -10, -11, and –13; biotin-conjugated anti-Vβ9, -12, and –14; FITC-conjugated Vα2, -3.2, -8, and -11.1; NK1.1; or CD4. Streptavidin-PE was used as a detecting agent for biotin-labeled Abs. After staining, cells were washed and analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

[3] Abbreviations used in this paper: HKST, heat-killed *Salmonella typhimurium*; ELISPOT, enzyme-linked immunospot.

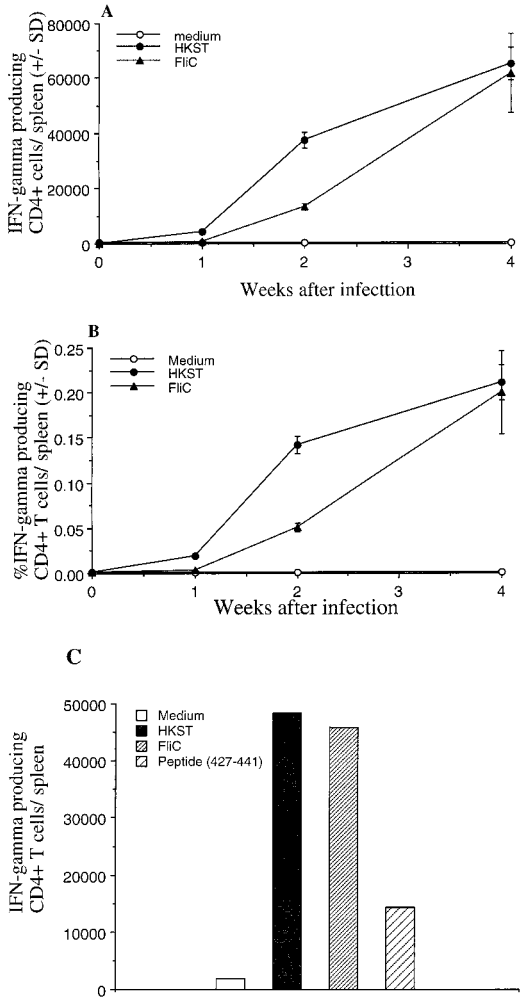


FIGURE 1. The numbers of HKST- and FliC-specific IFN-γ-producing CD4$^+$ T cells increase during infection. CD4$^+$ T cells were purified from mice orally infected with SL3261, and the frequency of IFN-γ-producing cells was determined by ELISPOT. Total numbers of IFN-γ-producing cells per spleen (*A* and *C*) or percentage of CD4$^+$ cells producing IFN-γ (*B*) after primary (*A* and *B*) or secondary (*C*) infection with SL3261 are shown. The mean ± SD of two separate ELISPOT measurements per group from two pooled, infected spleens are shown for each time point.

The TCR sequencing

Sequencing of TCR variable regions was accomplished using the method of Candiasis et al. (25). Briefly, VβDβJβ or VαJα regions were amplified by nested RT-PCR from each T cell clone using degenerate primers. These DNA fragments were gel purified and cloned into the pGem-3Z vector (Promega, Madison, WI). The inserted DNA was sequenced using M13 primers (Promega), upstream and downstream of the insert site.

Results

CD4$^+$ T cells respond to FliC during Salmonella *infection*

It is clearly established that CD4$^+$ T cells are critical for the resolution of *Salmonella* infection (7, 8). However, the Ags recognized by T cells during infection are largely unknown. As a first step in this direction, we estimated the frequency of IFN-γ producing, *Salmonella*-specific CD4$^+$ T cells during infection using an ELISPOT assay. These experiments demonstrated that *Salmonella*-specific IFN-γ producing CD4$^+$ T cells can be detected in the spleen as early as 1 wk after oral infection, although the frequency of responding cells is low at this time point (Fig. 1). *Salmonella*-specific CD4$^+$ T cells increased in number from ~4,000/spleen at 1 wk postinfection to 60,000/spleen at 4 wk (Fig. 1*A*). At 4 wk postinfection this number accounted for ~0.2% of all CD4$^+$ T cells in the spleen (Fig. 1*B*). In uninfected mice, the frequency of *Salmonella*-specific CD4$^+$ T cells was below the limit of detection in this assay (estimated at 1,000 cells/spleen, data not shown).

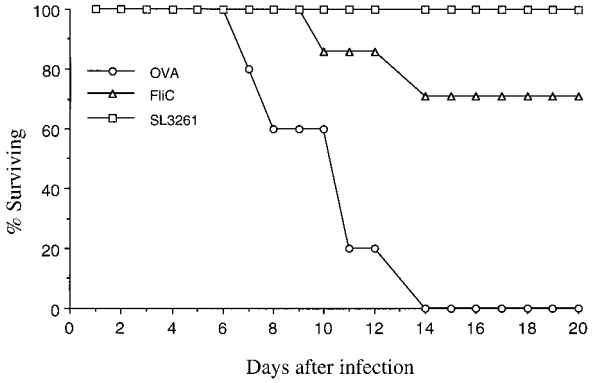


FIGURE 2. Immunization with purified FliC protects mice against oral challenge with virulent *Salmonella*. Groups of mice were immunized as described in *Materials and Methods* and challenged orally with 1×10^5 virulent *S. typhimurium*, SL1344, 1 wk following the boost. Mice were monitored daily for fatalities. Data show the percentage of death in OVA (17 total) or FliC groups (17 total) over the first 20 days. Surviving mice lived until the termination of the experiment at around 3 mo postchallenge. The OVA-immunized mice did not differ significantly from a naive group (data not shown). The SL3261 group represents mice orally immunized with 1×10^9 SL3261 at least 6 wk before challenge. Pooled results from three separate experiments are shown.

To date the only *S. typhimurium*-specific T cell clone for which the Ag has been identified was derived from C3H mice and is specific for the flagellar Ag, FliC (23). We measured the frequency of IFN-γ-producing CD4$^+$ T cells responding to FliC in *Salmonella*-infected C57BL/6 mice (Fig. 1). A large fraction of *Salmonella*-specific, CD4$^+$ T cells responded to FliC at both 2 and 4 wk postinfection, indicating that FliC is a major target for the immune response during primary infection with SL3261. However, T cells specific for unknown Ags, other than FliC, are clearly responding to *Salmonella*, especially during the first 2 wk of infection.

Similarly, after secondary challenge with SL3261, a large fraction of *Salmonella*-specific IFN-γ-producing CD4$^+$ T cells recognized FliC (Fig. 1*C*). One week after i.v. challenge with the attenuated strain ~0.15% of CD4$^+$ T cells responded to both FliC and HKST in this assay, indicating that there were ~45,000 cells/spleen with FliC specificity. Thus, FliC-specific T cells also account for a large fraction of all IFN-γ-producing, *Salmonella*-specific CD4$^+$ T cells during a secondary response.

Purified FliC can protect mice against lethal challenge

Because the magnitude of the CD4$^+$ T cell response to FliC was large in infected mice, it was possible that the response to FliC would be sufficient to protect mice against challenge with the virulent strain, SL1344. It has been reported that protection of mice against *S. typhimurium* requires the transfer of both immune T cells and immune serum (26). We therefore immunized mice with purified FliC or OVA and first confirmed that FliC-immunized mice make serum Ab responses to HKST (data not shown). Furthermore, challenge with a lethal dose of the virulent strain, SL1344, demonstrated that mice immunized with FliC were protected from infection with virulent *Salmonella*, although the degree of protection was less than that provided by the live vaccine strain

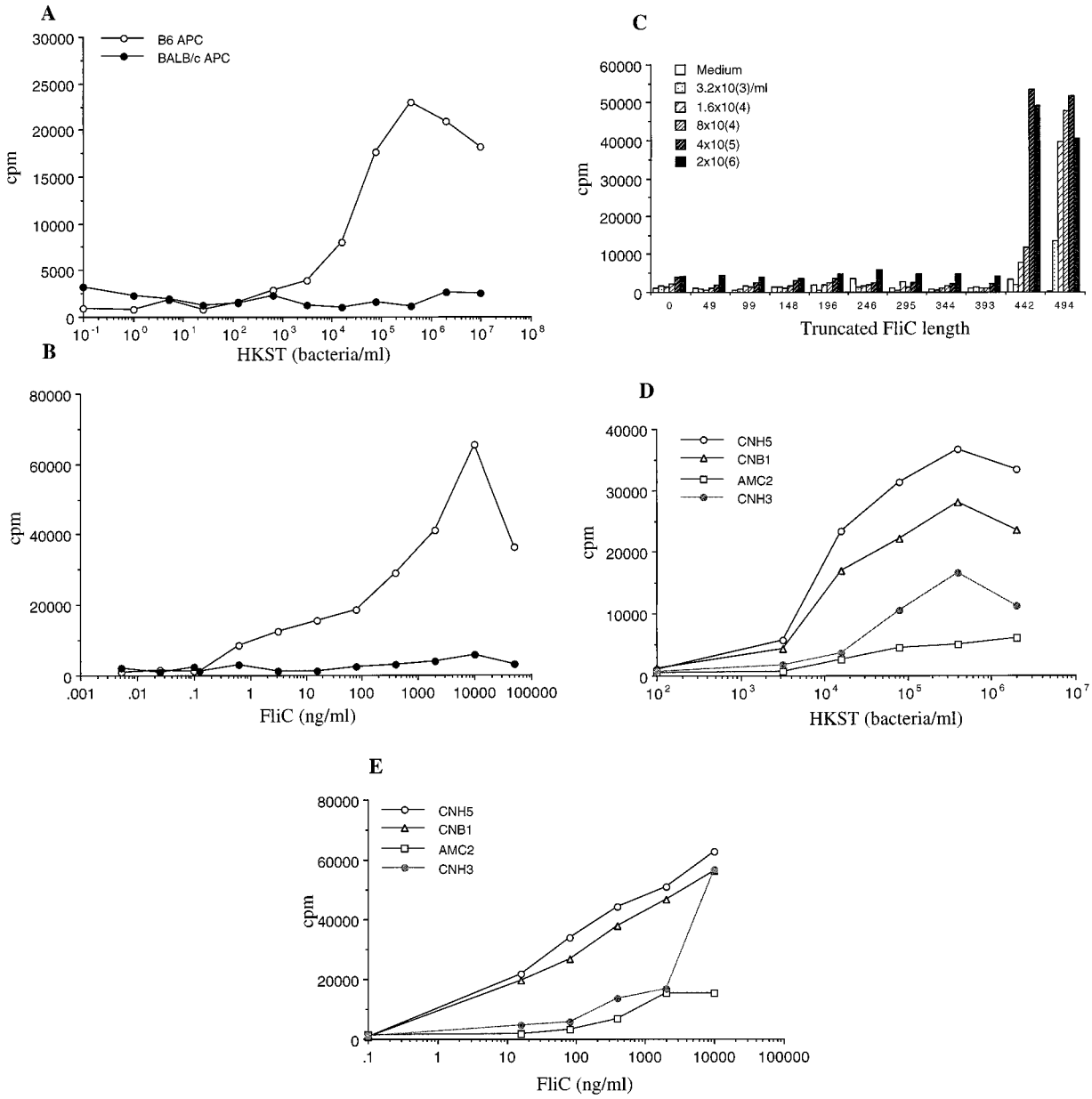


FIGURE 3. A T cell line and clones from infected mice respond to a FliC epitope. A T cell line and clones were prepared as described in *Materials and Methods* and stimulated (1×10^4/well) with HKST (*A* and *D*), FliC (*B* and *E*), or truncated FliC proteins expressed in *E. coli* (*C*) in the presence of irradiated syngeneic (B6) or allogeneic (BALB/c) APC (5×10^5/well). In *C*, the proliferation of the line in response to increasing lengths of FliC is shown. Proliferation was measured by [^{3}H]thymidine incorporation at 72 h after stimulation. Data are representative of three individual experiments.

(Fig. 2). Thus, immune responses to FliC alone can protect against *Salmonella* infection, although responses to other Ags may be required for optimal protection.

A T cell line from infected mice responds to FliC

To further analyze CD4$^+$ T cell responses to *Salmonella typhimurium* in vaccinated mice, we generated a CD4$^+$ T cell line from C57BL/6 mice that had resolved an infection with the attenuated strain, SL3261. After three rounds of stimulation with HKST in vitro, the line was responsive to HKST presented by APC expressing H-2^b but not H-2^d MHC molecules (Fig. 3*A*). As shown in Fig. 3*B*, this line was also stimulated by purified FliC presented by syngeneic spleen cells. The specificity of this FliC response was confirmed by generating an OVA-specific CD4$^+$ T cell line from OVA-immunized C57BL/6 mice that responded to OVA presented by I-A^b, but did not proliferate in response to the FliC preparation (data not shown). Flow cytometric analysis confirmed that cells in the line expressed CD4 (data not shown), and because B6 mice lack the I-E class II molecule, the FliC peptides responsible for stimulating the line must therefore be presented in the context of I-A^b. To identify the epitopes recognized by FliC-reactive CD4$^+$ T cells within this line we generated a series of truncated MalE-FliC fusion proteins expressed in *E. coli*, as previously described (23). This analysis showed that only a single region of FliC, close to the carboxyl terminus, was recognized (Fig. 3*C*).

T cell clones respond to a constant region within FliC

The line described above was cloned by limiting dilution, and all clones were tested for responsiveness to both HKST and FliC. All clones (26 in total) that responded to HKST also responded to FliC

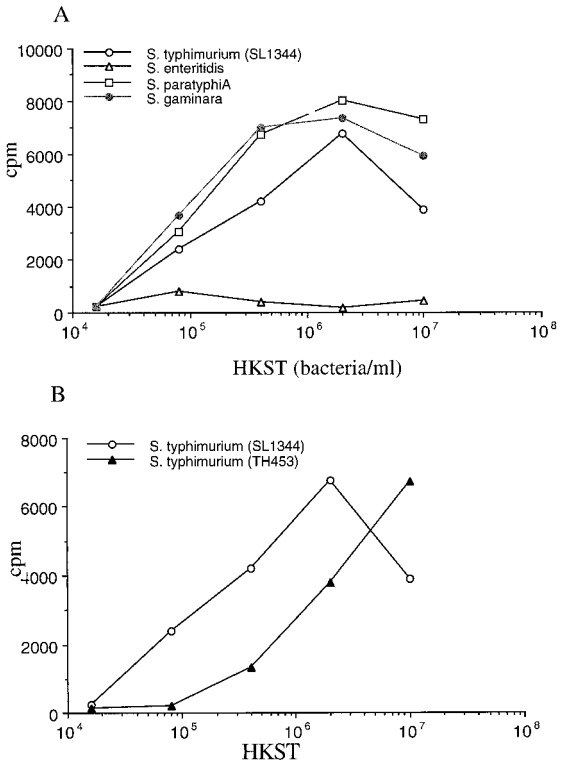


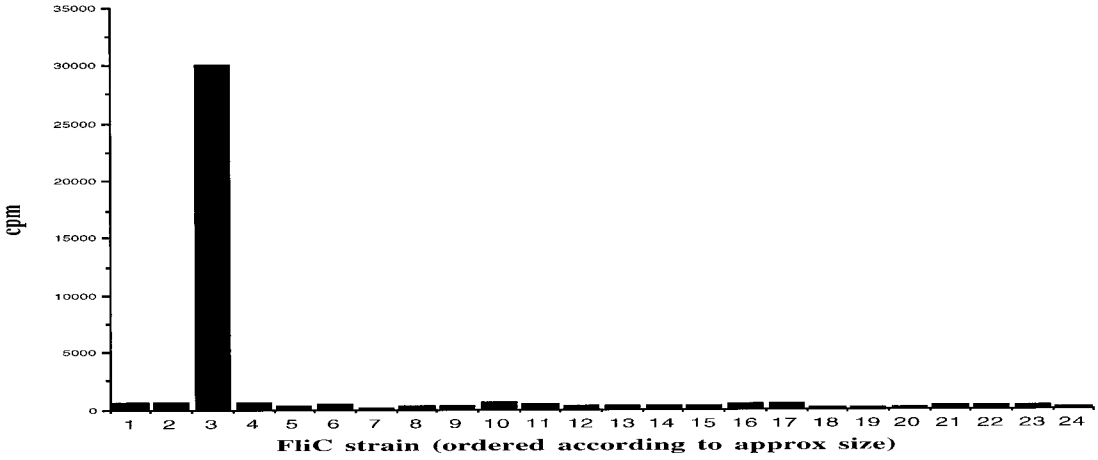
FIGURE 4. Clones respond to other *Salmonella* serovars and an FliC$^-$ strain. T cell clone CN.B1 (1×10^4/well) was restimulated with either HKST or heat-killed *S. enteritidis*, *S. paratyphi A*, or *S. gaminara* at various doses (*A*) or the FliC-nonexpressing strain TH453 (*B*) in the presence of irradiated syngeneic APC (5×10^5/well). [^{3}H]Thymidine incorporation was measured as described in *Materials and Methods*. Data are representative of two individual experiments with this particular clone.

in vitro. The responses of four representative clones are shown in Fig. 3, *D* and *E*. These clones were further tested for responsiveness to the same truncated FliC fusion proteins described above. As expected from the limited mapping of the line (Fig. 3*C*), all clones responded to the same C-terminal region of FliC (data not shown).

The FliC protein sequence has been divided into eight regions based on homology between *Salmonella* serovars (24). The initial epitope mapping indicated that the T cells recognized either region VII or VIII, both of which have relatively conserved amino acid sequences between serovars (75 and 100% homology for regions VII and VIII, respectively). Thus, it was possible that these clones might recognize FliC proteins from other distinct *Salmonella* serovars due to the sharing of a conserved epitope. Indeed, consistent with a constant region epitope, we found that these clones responded to a conserved epitope in FliC produced by *S. paratyphi*, *S. gaminara*, *S. typhimurium* (Fig. 4*A*), and *S. cholerasuis* (data not shown), but that is not present in *S. enteritidis* (Fig. 4*A*) or *Shigella sonnei* (data not shown).

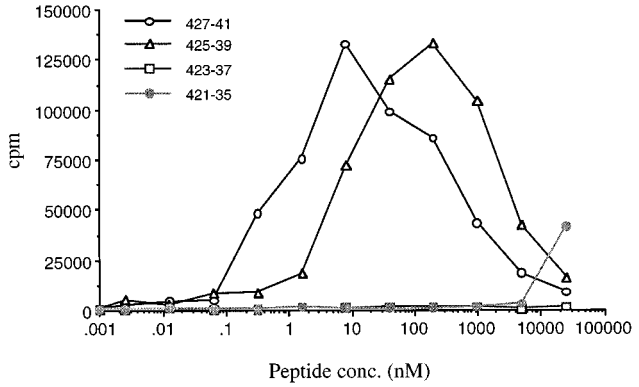
S. typhimurium has two genes encoding flagellin proteins, FliC and FljB, which are expressed alternately by phase variants, so that a bacterium never naturally expresses both gene products simultaneously. There is a significant degree of homology between these two proteins within the C-terminal portion of the amino acid sequence (27). To assess whether the epitope was contained in a region homologous to both structural genes we stimulated the clones with a phase-fixed variant of *S. typhimurium* that contains a transposon insertion in *fliC* and can therefore only produce FljB. As shown in Fig. 4*B*, the clones still responded to these bacteria in the absence of FliC, although the response was weaker than that to FliC-expressing bacteria.

Together these data indicate that this FliC epitope recognized by a CD4$^+$ T cell from naturally infected C57BL/6 mice is located in



	Carboxyterminal FliC sequeneces of relevant strains	Stimulation
WT	QVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIED	yes
1	QVDTLRSDLGAVQNRFNSAIT	no
2	QVDT	no
3	QVDTLRSDLGAVQNRFNSAITNLG	yes
4	QVD	no

FIGURE 5. Further epitope mapping and sequencing of truncated FliC products places the epitope in constant region VIII. T cell clone CN.B1 (1×10^4/well) was restimulated with truncated FliC products expressed in *E. coli* (1×10^6/well) in the presence of irradiated syngeneic APC (5×10^5/well). [^{3}H]Thymidine incorporation was measured as described in *Materials and Methods*. Data are representative of two individual experiments with this clone.



FliC peptide sequences

421-35	RSDLGAVQNRFNSAI
423-37	DLGAVQNRFNSAITN
425-39	GAVQNRFNSAITNLG
427-41	VQNRFNSAITNLGNT

FIGURE 6. Precise mapping of epitope using synthetic peptides. Clone CN.B1 (1×10^4/well) was restimulated with four overlapping synthetic peptides (spanning positions 421–441 of FliC) and irradiated syngeneic APC (5×10^5/well). Thymidine incorporation was measured as described in *Materials and Methods*. Data are representative of two individual experiments with this clone.

a region common to a number of distinct *Salmonella* serovars and is present in both phase variants.

The dominant epitope is contained within the constant C-terminus of FliC

We constructed additional truncated FliC fusion proteins that overlap the region thought to contain the stimulatory epitope recognized by our T cell clones. The genes encoding MalE-FliC truncations closest to the epitope, indicated by loss of responsiveness in the stimulation assays, were then subjected to DNA sequence analysis. All T cell clones tested responded in a similar fashion to the representative clone CN.B1 shown in Fig. 5. These data suggest that the terminal NLG at positions 437–39 are critical to maintain responsiveness. We therefore synthesized four overlapping 15-mer peptides around this region and used these to stimulate the clones. As shown in Fig. 6, both peptides 427–441 and 425–439 were able to stimulate the clones; 427–441 was more efficient. This epitope, as predicted from the extensive cross-reactivity in the proliferation experiments described above, is found within constant region VIII, which has almost 100% homology between *Salmonella* serovars and between FliC and FljB (Table II). Indeed, there is extensive homology between this region of FliC and the flagellar sequence of other bacteria, such as *Shigella sonnei* and *E. coli*, further emphasizing the conserved nature of this epitope. As all nonresponding bacteria tested to date have an N to D substitution at position 433 (Table II), this may indicate a contact residue for either MHC or TCR molecules.

FliC-specific clones are CD4$^+$, Vβ2/Vα10$^+$, and NK1.1$^-$

To assess heterogeneity among the FliC-specific clones from infected mice we stained each clone for the TCR variable region using mAbs. We initially screened with a panel of Vβ- and Vα-specific mAbs specific for 15 Vβ-chains and four Vα-chains. All clones were found to be both CD4$^+$ and Vβ2$^+$ and did not stain with any other β-chain-specific Ab or any of the four Vα Abs tested (data not shown). It was recently reported that infection of beige mice with *Salmonella* induces a population of IL-4-secreting NK1.1$^+$ T cells (28), and that a class II-dependent NK1.1$^+$ $\gamma\delta$ cell population is induced after peritoneal infection with *Salmonella* (29). Since the majority of CD4$^+$ NK1.1$^+$ T cells express a limited range of TCR Vβ-chains including Vβ2, we stained all clones with an Ab against NK1.1 to rule out the possibility that we had cloned an NK-T cell. However, none of the clones stained positively for NK1.1, while the Ab was able to detect an NK1.1$^+$ population in the spleen of uninfected B6 mice (data not shown).

As none of the anti-Vα Abs stained the clones, we had no information regarding Vα usage. Therefore, we determined the TCR usage of four representative FliC-specific clones by RT-PCR using degenerate primers to amplify the VDJ region of the β-chain or the VJ region of the rearranged α-chain (25). As shown in Table III all four clones contained in-frame transcripts for Vβ2 and Vα10 with identical junctional regions, indicating that these four clones were derived from a single FliC-specific CD4$^+$ T cell. It was therefore possible that despite cloning the T cell line after only three rounds of stimulation in vitro, the T cell line was composed mainly of a single dominant clone. We therefore generated a second T cell line from mice immunized s.c. with FliC/CFA and again made a number of T cell clones. These were assessed in a stimulation assay using truncated FliC fusion proteins as described above. In summary, all of these clones (10 of 10) responded to the FliC$_{427-441}$ epitope described above (data not shown). In addition, we sequenced the TCR α and β variable regions from two of these clones, and these data indicate a more heterogeneous TCR usage (Table III). Together these data suggested that the FliC epitope 427–441 is an epitope recognized by T cell clones derived from C57BL/6 mice regardless of initial priming conditions in the mouse.

Table II. *FliC epitope sequences in other bacteria*[a]

Bacteria	Protein	Sequence of FliC at 427–441	Response
S. typhimurium	(FliC, serotype i)	VQNRFNSAITNLGNT	Yes
S. typhimurium	(FljB)	VQNRFNSAITNLGNT	Yes
S. cholerasuis	(FliC, serotype c)	VQNRFNSAITNLGNT	Yes
S. paratyphi A	(FliC, serotype a)	VQNRFNSAITNLGNT	Yes
S. gaminara	(FliC, serotype d)	VQNRFNSAITNLGNT	Yes
S. enteritidis	(FliC, serotype g,m)	IQNRFDSAITNLGNT	No
S. sonnei	(FliC)	VQNRFDSAITNLGNT	No
E. coli	(FliC)	VQNRLDSAVTNLNNT	No

[a] Underlined amino acids indicate difference from *S. typhimurium* sequence. Response indicates >3-fold proliferation above background in stimulation assay using FliC-specific clone CN.B1.

Table III. *Junctional sequences of 6 T cell clones specific for FliC epitope 427–441*[a]

Clone	Source	α- and β-Chain Junctional Sequences
CN.B1	Infected mouse	Vα10 Jα10 **CTC TGT GCT ATA GAT** TCG ACT **GGA GGC TAT AAA** L C A I D S T G G Y K Vβ2 Dβ1 Jβ1.2 **ACC TGC AGT GCA** CTG GGG GCG CGA **AAC TCC GAC TAC ACC** T C S A L G A R N S D T T
JL.F1	Infected mouse	—[b]
KD.D3	Infected mouse	—[b]
JL.E7	Infected mouse	—[b]
SB.F10	FliC-immunized mouse	Vα3.4 JαBM2T3.1 **TGT GCT GTG AGC GC**G GAG **TAT AAC CAG GGG AAG** C A V S A E Y N Q G K Vβ12 Dβ1 Jβ1.3 **CTG TGT GCC AGC AGT** TGG GAT **TCT GGA AAT ACG CTC** L C A S S W D S G N T L
SB.A8	FliC-immunized mouse	Vα2.2 JαTT11 **TAC TTC TGT GCA GCA AGT** TGG GGG **TAT GGG AGC AGT GGC** Y F C A A S W G Y G S S G Vβ15 Dβ2 Jβ2.4 **CTC TGT GGT GCT** CGG GGA CTG GGG GGG **AGT CAA AAC ACC** L C G A R G L G G S Q N T

[a] Functional rearranged TCR junctional sequences with amino acids are shown for T cell clones from *Salmonella*-infected (CN.B1, JL.F1, KD.D3 and JL.E7) or FliC-immunized mice (SB.F10 and SB.A8). Underline bases indicate additions. Nonfunctional rearrangements on the other chromosome were also sometimes amplified from a number of clones but are not shown for clarity of figure.

[b] Sequences exactly as for CN.B1.

FliC$_{427–441}$-specific CD4$^+$ T cells are more numerous during secondary infection

To determine the proportion of FliC-specific CD4$^+$ T cells that respond to epitope 427–441 in vivo, we used the ELISPOT technique to measure the frequency of IFN-γ-producing CD4$^+$ T cells from *Salmonella*-infected mice after in vitro restimulation with peptide. Surprisingly, we were unable to detect a response to this epitope at any stage of a primary infection (data not shown). However, upon secondary challenge with SL3261, responses to this peptide were clearly detected, accounting for approximately one-third of all FliC-specific IFN-γ-producing cells (Fig. 1*C*).

Discussion

Previous attempts to vaccinate mice against *Salmonella* using porins, a group of major outer membrane proteins, have demonstrated that these Ags can stimulate a protective response to a challenge strain of limited virulence (13). We have demonstrated here that FliC can provide significant protection to naive C57BL/6 mice and that FliC is also the major target of CD4$^+$ T cells during primary and secondary infection.

FliC was initially identified as a target for CD4$^+$ T cells from experiments with *S. typhimurium*-infected C3H mice (23). There is also evidence from infection of human volunteers with attenuated *S. typhi* that FliC is probably a major target of the cellular immune response, as T cells from these infected patients proliferated in response to flagellar extracts in vitro (30). In this report we extend these studies and demonstrate with a quantitative ELISPOT assay that FliC is a major target for CD4$^+$ T cell responses during natural infection of C57BL/6 mice with attenuated *S. typhimurium*.

Why does FliC elicit such a strong response? It has been reported that despite a requirement for flagellar expression for motility, FliC is not a virulence factor, i.e., nonmotile *Salmonella* lacking FliC are just as virulent in vivo as motile FliC-expressing strains (31). Therefore, the immune system does not specifically target *Salmonella* proteins required for growth in the host. However, other factors, such as the amount and physical localization of FliC on the bacterial surface and the biochemical process of self assembly that occurs at the tip of the filament, may mean that there is an abundance of soluble FliC secreted into the surrounding environment. Thus, it may be one of the first Ags to which dendritic cells have access when they encounter *Salmonella* during the early stages of infection.

The very nature of FliC as an Ag may also encourage an immune response. Flagellar proteins from *Salmonella* have long been the subject of study by immunologists due to their immunogenic properties and the capacity for polymerization and depolymerization of the filament (32). In addition, a recent report has described the production of TNF-α from a human promonocytic cell line in response to FliC (33), suggesting that there may be an inherent adjuvant effect attributable to FliC itself. Any or all of these factors might contribute to the selection of FliC as a target for CD4$^+$ T cell responses during infection with *S. typhimurium*.

The identification of FliC as an Ag involved in the primary CD4$^+$ T cell response after infection with a vaccine strain of *Salmonella* and the capacity of this response to provide protection from a virulent organism may aid in the development of subunit and live attenuated *Salmonella* vaccines. Expression of heterologous epitopes within the FliC molecule itself has already been shown to be an effective strategy to induce immune responses to heterologous proteins (34). The factors discussed above that may be responsible for a CD4$^+$ response to FliC might similarly enhance responses to these inserted epitopes.

The conserved nature of the I-A^b binding peptide described here indicates that there may be a significant degree of cross-protective immunity between serovars after infection with *Salmonella*. Previous attempts to map T cell epitopes of *S. muenchen* in FliC-immunized BALB/c (H-2^d) mice using overlapping synthetic peptides identified major epitopes within regions I and II, the conserved amino-terminal portion of the molecule (35). The FliC epitope identified from *Salmonella*-infected C3H/HeJ mice was located in hypervariable region VI in the middle of the protein. Thus, there is no clear pattern in recognition of FliC epitopes across MHC haplotypes.

Virtually all the T cell clones that we have isolated from infected mice are specific for the 427–441 peptide. Thus, it was surprising that 427–441-specific $CD4^+$ T cells could not be detected by ELISPOT during the primary infection. It is possible that in vitro culture conditions favor clones responding to this epitope and that T cells responding to other undefined epitopes account for the majority of the natural primary response to FliC in vivo. However, T cells responding to peptide 427–441 account for a significant fraction of FliC-specific T cells during a secondary response. It may be that the presence of FliC-specific B cells during this secondary restimulation serves to change the nature of the peptides presented or the APC involved in stimulating FliC-specific $CD4^+$ T cells. This possibility is currently under investigation in our laboratory.

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