

## RESEARCH ARTICLE

# Chimeric flagellin expressed by *Salmonella typhimurium* induces an ESAT-6-specific Th1-type immune response and CTL effects following intranasal immunization

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The flagellin component FliC of *Salmonella typhimurium* is capable of activating the innate immune system *via* specific interactions with TLR5 and can also act as a carrier of foreign antigen to elicit antigen-specific immune responses. Thus, we constructed an attenuated *Salmonella* strain SL5928(fliC/esat) expressing chimeric flagellin that contained the ESAT-6 antigen coding sequence of *Mycobacterium tuberculosis* inserted into the highly variable region of the *Salmonella* flagellin coding gene *fliC*<sup>i</sup>. The chimeric flagellin functioned normally, as demonstrated using a flagella swarming assay and electron microscopy. To analyze the effects of chimeric flagellin, the cell-mediated immune response and cytotoxic T lymphocyte (CTL) effects specific for ESAT-6 antigen were tested after intranasal immunization of mice with flagellated *Salmonella* SL5928(fliC/esat). The results showed that SL5928(fliC/esat) intranasal immunization can strongly elicit an ESAT-6-specific T helper (Th) 1-type immune response in mucosal lymphoid tissues, such as nasopharynx-associated lymph nodes, lung and Peyer's patches, and a Th1/Th2 response was elicited in spleen and mesenteric lymph nodes. Furthermore, intranasal immunization of SL5928(fliC/esat) produced efficient CTL effects, as demonstrated using a 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) assay. Thus, our study revealed that *Salmonella* flagellin acts as a carrier for foreign antigen and triggers strong Th1 and CTL responses during intranasal immunization. Chimeric flagellin is potentially an effective strategy for the development of novel vaccines against tuberculosis in humans and animals.

*Cellular & Molecular Immunology* (2011) 8, 496–501; doi:10.1038/cmi.2011.24; published online 15 August 2011

**Keywords:** cellular immune response; ESAT-6; flagellin; intranasal immunization; *Mycobacterium tuberculosis*

## INTRODUCTION

Tuberculosis (TB) remains a major health problem for both humans and animals worldwide. Although the Bacille Calmette–Guérin (BCG) vaccine is widely administered to prevent this disease, its efficacy remains unpredictable.<sup>1,2</sup> Therefore, there is an urgent need to develop more effective vaccines and vaccination strategies against TB. Cell-mediated immunity is thought to be essential for protection against TB,<sup>3</sup> with critical involvement of CD4<sup>+</sup> Th1 lymphocytes.<sup>4</sup> Furthermore, there is evidence that CD8<sup>+</sup> T cells,<sup>5,6</sup> as well as unconventional T cells, contribute to optimal protection.<sup>4,7</sup>

*Mycobacterium tuberculosis* (MTB) infection often occurs through the upper respiratory tract. Intranasal (i.n.) immunization with either live BCG or killed-BCG formulated in an adjuvant or recombinant adenovirus-based vaccines has been shown to provide protection against MTB.<sup>8</sup> In addition, i.n. immunization of mice with an adenoviral-based vaccine expressing Ag85A<sup>9</sup> or microparticles encapsulated with ESAT-6 elicited antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells capable of interferon (IFN)- $\gamma$  production and cytotoxic activity in the airways, lungs and mediastinal lymph nodes.<sup>10</sup> Furthermore, a previous study indicated that recombinant flagellin containing foreign antigens elicited protective immune responses in the absence of additional

adjuvant.<sup>11</sup> Effects of flagellin on CD4<sup>+</sup> T-cell proliferative and cytokine responses have also been reported.<sup>12</sup> *Salmonella* flagellins are flexible adjuvants and induce adaptive immune responses when administered by different routes or in vaccine formulations.<sup>13</sup> Therefore, the aim of the present study was to assess whether efficient cell-mediated immune responses and specific CTL effects could be stimulated by i.n. administration of flagellated *Salmonella* containing the ESAT-6 antigen coding sequences of MTB inserted in the highly variable region of *Salmonella* flagellin gene *fliC*<sup>i</sup>.

## MATERIALS AND METHODS

### Bacterial strains and plasmid

*Escherichia coli* strains DH5 $\alpha$  and BL21 (DE3) and *Salmonella typhimurium* (*Stm*) (LT2) were from our laboratory stocks. *Salmonella dublin* SL1438 (1,9,12:-:-) and SL5928 (1,9,12:-:-; Amp<sup>-</sup>, Tet<sup>+</sup>, *fliC*<sup>i</sup>::Tn10) were kindly provided by Dr Bruce AD Stocker (Stanford University, School of Medicine, Stanford, CA, USA). Phage P22 Ht *int* and *Stm* LB5000 (1,4,12:-:-; r<sup>-</sup>m<sup>-</sup>flaA66, Amp<sup>-</sup>) were kindly provided by Dr AD Harshey (University of Texas, Austin, TX, USA). pYUB412 plasmid was provided by Dr. Claude Leclerc (Pasteur Institute, Paris).

## Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the Comparative Medical Center of Yangzhou University (Yangzhou, China) and housed at the Animal Biosafety facilities. Animal experiments were conducted in strict accordance with guidelines provided by the Animal Care and Ethics Committee of Yangzhou University.

## Construction and cloning of chimeric flagellin gene *fliC/esat*

For cloning of the ESAT-6 encoding sequence, the *esxA* gene was amplified from the pYUB412 plasmid using *esat-1* (5'-AAT AGT ACT ATG ACA GAG CAG CAG TGG-3') and *esat-2* (5'-C GTA ATA TTT TGC GA A CAT CCC AGT GAC-3') primers. PCR products were purified and used in the following experiments. The *fliC<sup>i</sup>* gene encoding the phase I flagellin was amplified by PCR from *Stm* (LT2) genomic DNA using *fliC-1* (5'-ATA GAA TTC AAG GAA AAG ATC ATG GCA CAA-3') and *fliC-2* (5'-TCA AAG CTT AAC GCA GTA AAG AGA G-3') primers. The *fliC<sup>i</sup>* gene product was purified and amplified using an overlap PCR and further used to clone the chimeric flagellin gene *fliC/esat*. The coding sequence corresponding to the N-terminal domain of flagellin was amplified using *fliC-1* and F-E-2 (5'-CCA CTG CTG CTC TGT CAT AGT ACT ATT GTC TAA A-3') primers, and the C-terminal domain of flagellin was amplified using F-E-1 (5'-GTC ACT GGG ATG TTC GCA AAA TAT TAC GCC AAA-3') and *fliC-2* primers. The two PCR products were *fliC-N* and *fliC-C*, respectively. The *fliC/esat-1* fragments were cloned by overlap PCR using *fliC-1* and *esat-2* primers to amplify the *fliC-N* and ESAT-6 purified PCR products. The *fliC/esat-2* fragments were cloned by overlap PCR using *esat-1* and *fliC-2* primers to amplify the *fliC-C* and ESAT-6 purified PCR products. A chimeric fragment of *fliC/esat* was amplified from *fliC/esat-1* and *fliC/esat-2* purified PCR products by overlap PCR using *fliC-1* and *fliC-2* primers. The chimeric fragment carried the incomplete *fliC* coding sequence, in which the coding region for amino-acid residues 205–232 of flagellin had been replaced with the entire ESAT-6 coding sequence (Figure 1). The chimeric *fliC/esat* gene PCR products were purified, digested with *EcoRI* and *HindIII* and ligated into a pET30(a) expression vector to form the pET-*fliC/esat* plasmid.

## P22 phage transduction

The pET-*fliC/esat* plasmid was electrotransformed into *Stm* LB5000. The recombinant bacteria, LB5000 (pET-*fliC/esat*) (donor strain), were grown in LB broth overnight. Ten milliliters of P22 broth were added to 200  $\mu$ l of the LB culture and incubated at 37 °C on a shaker for 10 h. The donor strain/phage mixture was centrifuged for 5 min (8000 g) to pellet the cell debris, and the supernatant was collected. Several drops of CHCl<sub>3</sub> were added to the supernatant before being vortexed and stored at 4 °C. The recipient strain *Salmonella dublin* SL5928 was grown overnight in 1-ml LB. Twenty microliters of the phage lysate was added to 200  $\mu$ l of the overnight SL5928 culture and

incubated at 37 °C on a shaker for 1 h. Finally, the mixture was spread onto LB agar containing 50  $\mu$ g/ml of kanamycin.

## Swarming assays

Non-motile *S. dublin* SL5928, flagellated SL5928(*fliC/esat*) and SL5928(*fliC*) colonies were punctured using a needle and placed into 0.4% semisolid medium tubes (100 ml: tryptone 10 g, NaCl 5 g, agar 0.4 g) that were incubated at 37 °C for 6–8 h.

## Electron microscopy

Negative staining of bacteria for electron microscopy was performed with 2% phosphotungstic acid as described by Hayat and Miller.<sup>14</sup>

## Western blot assay

Flagellin of SL5928(*fliC*) and chimeric flagellin of SL5928(*fliC/esat*) were extracted by acid treatment followed by ammonium sulfate precipitation.<sup>15</sup> Under these experimental conditions, the extracted fraction was represented mainly by polymeric flagellin. The purified flagellin extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis<sup>16</sup> using a murine anti-ESAT-6 monoclonal antibody (mAb) HYB 076–08 (Abcam, Cambridge, Massachusetts, USA).

## Immunization of mice

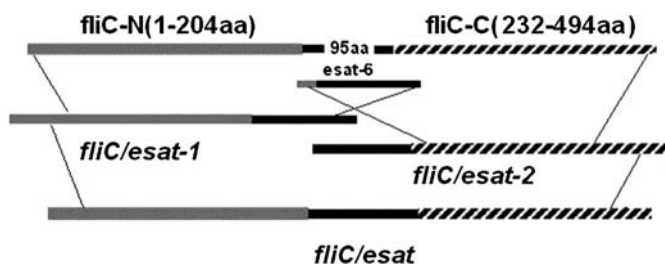
Flagellated *Salmonella* SL5928(*fliC/esat*) or non-flagellated *Salmonella* SL5928 were harvested at an optical density of 0.8. Each bacterial pellet was then resuspended in phosphate-buffered saline (PBS) with a concentration of  $5 \times 10^8$  colony-forming units/ml. The immunization of C57BL/6 mice (6–8 weeks old) was performed as described previously.<sup>17</sup> Briefly, the mice were anesthetized by intraperitoneal injection with 0.1 ml of 1% pentobarbital sodium and immunized i.n. with  $1 \times 10^7$  colony-forming units SL5928(*fliC/esat*) and SL5928 on days 0 and 18, respectively. The negative control group was immunized i.n. with PBS.

## Cell isolation

Cells were prepared from the spleens, lungs, Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and nasopharynx-associated lymph nodes (NALTs) of immunized mice. Single cells were obtained from each of these tissues by pressing the organs through a stainless steel mesh and centrifuging at 200 g for 10 min at 4 °C. Lung and spleen cell suspensions were prepared through a murine Ez-sep (Dakewei Biotech Company, Shenzhen, China) density–gradient centrifugation (800 g for 25 min at 4 °C). Total cell yields were calculated using a hemocytometer and 0.4% trypan blue to assess the viability of cells kept in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) medium with 10% fetal bovine serum (Hyclone, Logan, UT, USA).

## ELISPOT assay

The ELISPOT technique was performed as described previously.<sup>17</sup> Briefly, spleen, lung, PP, MLN and NALT cells were incubated with 10  $\mu$ g/ml of ESAT-6 peptide (1–20 aa) in a 96-well plate coated with rat anti-mouse IFN- $\gamma$  and IL-4 mAbs (BD Biosciences, San Diego, CA, USA) or ConA (5  $\mu$ g/ml; Sigma-Aldrich, St Louis, MO, USA) as a positive control for 36 h. Cytokine production was detected using biotin-labeled rat anti-mouse IFN- $\gamma$  and IL-4 mAb (BD Biosciences) and probed with alkaline phosphatase-conjugated streptavidin (BD Biosciences). The enzyme reaction was developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma-Aldrich),



**Figure 1** Diagram showing construction of the chimeric *fliC/esat* fragment.

and the results were expressed as the number of immunospots per  $10^6$  cells.

### CTL assay *in vivo*

The 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) assay used in this study demonstrated ESAT-6-specific cytotoxicity T lymphocyte effector function *in vivo* by flow cytometry. *In vivo* cytotoxic activity initiated by SL5928(fliC/esat) was established using a murine model. The donor cells were prepared from the spleen of naïve mice, washed three times with PBS and divided into two populations. One population (as target cells), CFSE<sup>high</sup> cells, was pulsed with 10  $\mu$ M ESAT-6-specific peptide (1–20 aa), incubated at 37 °C for about 45 min and then labeled with a high concentration of the fluorescent dyes CFSE (5.0  $\mu$ M). The second control population, dyestuff CFSE<sup>low</sup> cells, was left without peptide and labeled with a low concentration of CFSE (0.25  $\mu$ M). The CFSE<sup>low</sup> and CFSE<sup>high</sup> cells were mixed together (a total of  $2 \times 10^7$  cells) at a 2:1 ratio from each population and injected into each immunized mouse (7 days after the second immunization) *via* the tail vein. Splenocytes were isolated from the spleens of sacrificed mice 15 h later and analyzed by flow cytometry. The following formulas were used:

$$\text{Ratio} = \frac{\text{percentage CFSE}^{\text{low}}}{\text{percentage CFSE}^{\text{high}}}$$

$$\text{Percentage specific lysis} = 1 - (\text{ratio unprimed} / \text{ratio primed}) \times 100\%$$

### Statistical methods

Data obtained were tested by SPSS 13.0. Differences between means were assessed for statistical significance using Student's *t*-test. A value of  $P < 0.05$  was considered to be significant.

## RESULTS

### Construction and identification of the chimeric flagellin specifying ESAT-6 antigen

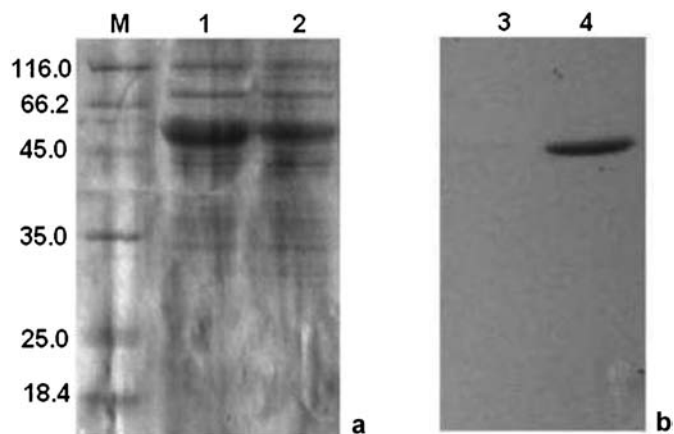
Specific primers were designed based on the *Stm* flagellin *fliC<sup>i</sup>* gene sequence to amplify the *fliC<sup>i</sup>* gene. Similarly, the ESAT-6 coding sequence was also amplified by PCR using specific primers. Overlap PCR was further used to synthesize the chimeric flagellin gene, and the ESAT-6 coding fragment was inserted into the unique hypervariable region of *fliC*. The recombinant plasmid carrying the chimeric flagellin gene was sequenced and named pET-fliC/esat. *Salmonella* SL5928(fliC/esat) were transduced with the chimeric flagellin, and the inserted ESAT-6 antigen was identified by western blot analysis. Western blot analysis using the anti-ESAT-6 mAb HYB 076–08 confirmed that the 52-kDa band corresponded to the chimeric fliC/esat protein (Figure 2).

### Swarming and electron microscopy of transduced cells

Swarming was observed within 6–8 h. Flagellated SL5928(fliC/esat) produced smooth swarming when compared with the SL5928 control (Figure 3). Electron microscopy revealed that flagellated SL5928(fliC/esat) expressed flagella similar to the wide-type control strain SL1438 (Figure 4).

### Cytokine production following *i.n.* immunization with flagellated *Salmonella* SL5928(fliC/esat)

To measure the cellular immune responses elicited by *i.n.* administration of SL5928(fliC/esat), cytokine production of IFN- $\gamma$  and IL-4 levels were quantified using an ELISPOT assay. The mean numbers of IFN- $\gamma$ -producing cells (spots/ $10^6$  cells) in the lung ( $216.67 \pm 55.08$ ), PPs ( $52.5 \pm 10.02$ ) and NALT ( $65.0 \pm 5.0$ ) were significantly increased ( $P < 0.01$ ) in SL5928(fliC/esat)-immunized mice compared to the

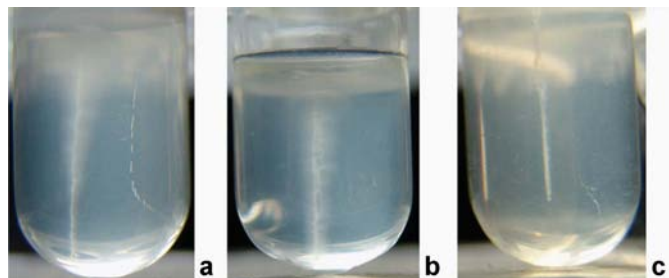


**Figure 2** Western blot analysis. Flagellin of SL5928(fliC) and chimeric flagellin of SL5928(fliC/esat) were extracted by acid treatment followed by ammonium sulfate precipitation. (a) The crude extract of fliC protein and chimeric flagellin fliC/esat were subjected to SDS-PAGE (M, standard molecular weight markers in kDa). Lane 1, crude extract of flagellin of SL5928(fliC); lane 2, crude extract of chimeric flagellin of SL5928(fliC/esat). (b) The crude extract of fliC protein and chimeric flagellin fliC/esat were transferred onto nitrocellulose and treated with anti-ESAT-6 antibodies (mAb HYB 076–08). A prominent 52 kDa band was observed (lane 4). Lane 3, crude extract of flagellin of SL5928(fliC); lane 4, crude extract of chimeric flagellin of SL5928(fliC/esat). mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

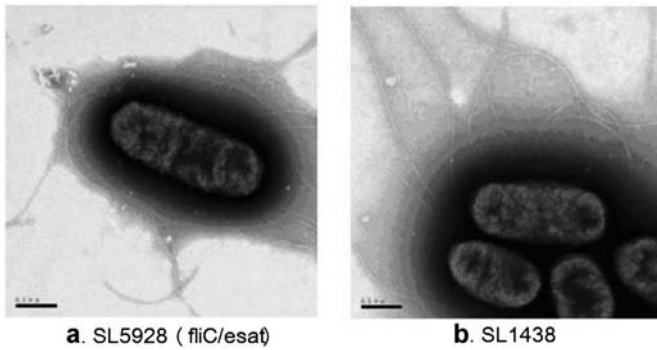
SL5928-immunized control group (Figure 5). The difference between the SL5928(fliC/esat)-immunized group and PBS control was also significant ( $P < 0.01$ ). However, the numbers of IL-4-producing cells from lungs, PPs and NALTs were significantly lower compared to those of IFN- $\gamma$ -producing cells, independent of treatment group. The numbers of ESAT-6-specific IFN- $\gamma$ -producing and IL-4-producing cells from spleen and MLNs were higher in the SL5928(fliC/esat)-immunized mice than in the SL5928-immunized group and the control group (Figure 5). These results suggest that mice receiving SL5928(fliC/esat) developed cellular immune responses against the ESAT-6 protein.

### CTL effects *in vivo*

In this study, we provide evidence that ESAT-6-specific memory CD8<sup>+</sup> T cells exert substantial Ag-specific cytotoxicity *in vivo*. We used CFSE to evaluate ESAT-specific cytotoxic T lymphocyte effector function *in vivo* by flow cytometry. As target cells, spleen cells from uninfected mice were pulsed with ESAT-6 peptide or left untreated. Each group was then labeled with different levels of CFSE and co-administered to animals that had been previously immunized with



**Figure 3** Observed motility phenotypes for the transduced cells: (a) SL5928(fliC); (b) SL5928(fliC/esat); (c) SL5928.



**Figure 4** Electron microscopy of transduced cells: (a) SL5928(fliC/esat); (b) SL1438.

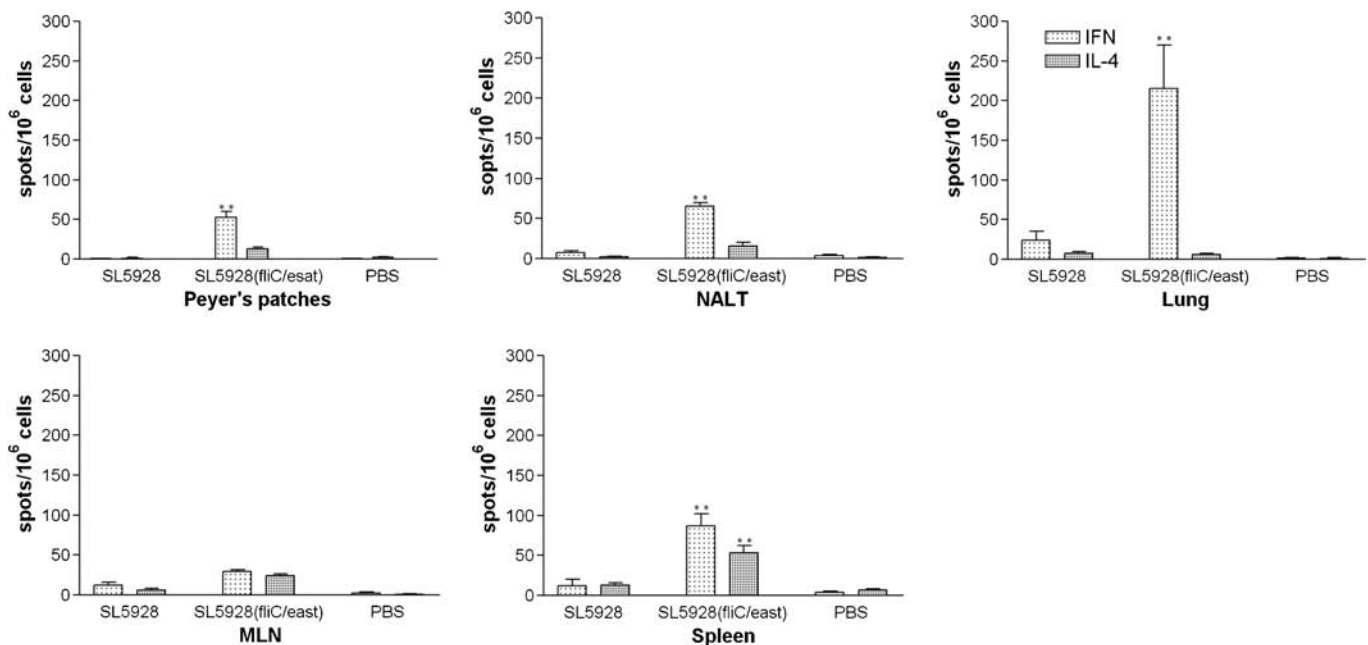
SL5928(fliC/esat) or SL5928(fliC). The frequency of the pulsed donor cell population of CD8<sup>+</sup> T cells in the spleens of recipient mice was compared with that of the donor cell population that had not been pulsed with ESAT-6 peptide. At 15 h post-transfer into mice infected by SL5928(fliC/esat) or SL5928(fliC), >75% of ESAT-6 peptide-pulsed donor cells were cleared from the spleen (Figure 6). In spleens of mice infected by SL5928(fliC), the frequencies of ESAT-6 peptide-pulsed and untreated donor cells were equal to the initial ratio (Figure 6). The marked efficiency in eliminating ESAT-6 peptide-pulsed target cells demonstrated the CTL activity of memory CD8<sup>+</sup> T cells in SL5928(fliC/esat) immunized mice.

## DISCUSSION

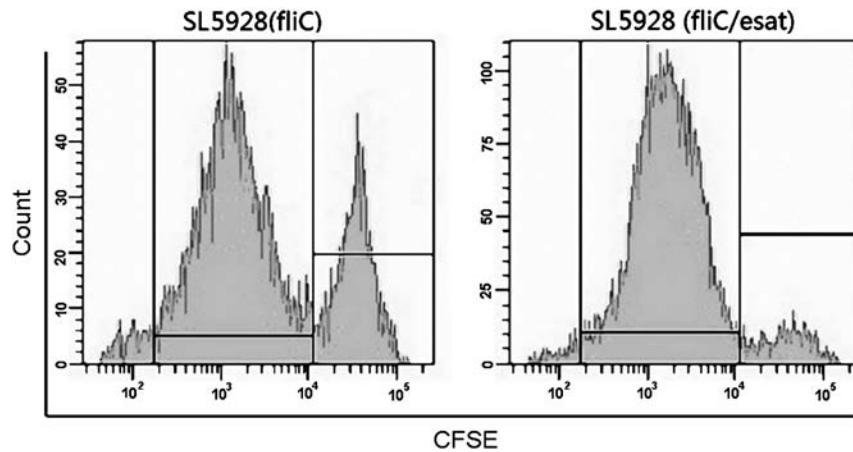
Recently, over 170 TB vaccine candidates have been tested in animal TB models<sup>18</sup> using a variety of strategies, including DNA vaccines, subunits, recombinant BCGs and live attenuated vaccines. However,

inconsistent results have been reported from these experimental models. Because protective immunity against TB requires the elicitation of cell-mediated immunity, it is necessary for candidate vaccines to elicit an effective cellular immune response or a CTL response. ESAT-6, which was originally isolated from a low-molecular-mass fraction of an MTB culture filtrate, is encoded by the RD1 region of MTB. ESAT-6 is a potent T-cell antigen and a diagnostic target for MTB and *Mycobacterium bovis* infection.

Bacterial flagellins have been studied for decades due to the importance of these proteins in bacterial motility and their adjuvant characteristics in vaccine studies. Flagellin is a highly conserved bacterial protein that elicits TLR-5-dependent inflammatory responses in both animal and plants.<sup>19</sup> Previous studies have investigated the ability of flagellin to elicit antigen-specific T-cell responses when delivered as live attenuated vaccines.<sup>20,21</sup> In addition, *Salmonella* flagellin has previously been used as an adjuvant and carrier for a synthetic peptide and to potentiate antibody production against a cholera toxin epitope.<sup>22,23</sup> Chimeric flagellin products are functional unless the 5' and 3' coding frames, which encode the regions important for secretion and polymerization of flagellin, are changed. In the *Salmonella fliC* gene, the 5' and 3' conserved regions are represented by 519 bp (encoding the N-terminal 173 amino-acid residues) and 270 bp (C-terminal 90 residues) sequences, respectively, separated by a highly variable region.<sup>24</sup> We therefore generated a flagellated *Salmonella* in which the coding region for amino-acid residues 205–232 of the flagellin was replaced with the entire coding sequence for ESAT-6 of MTB. This 'hypervariable' sequence region varies greatly in size and composition for different strains and species. However, our study demonstrated that flagellin function did not change after the foreign antigen was inserted. Furthermore, ESAT-6 expression was verified by western blotting using a specific anti-ESAT-6 monoclonal antibody HYB 076–08 (data not shown).



**Figure 5** Cytokine secretion of flagellated *Salmonella* SL5928(fliC/esat) through intranasal immunization. C57BL/6 mice were immunized intranasally with SL5928(fliC/esat), SL5928(fliC) or PBS. IFN- $\gamma$ -producing and IL-4-producing cells in the lung, PPs, NALTs, spleen and MLNs were evaluated by ELISPOT assay with ESAT-6 peptide as the stimulus. Results were expressed as the mean number of spots per 10<sup>6</sup> cells (six mice per group). \*\* $P$ <0.01 for the SL5928(fliC)-immunized and PBS-immunized mice using a Student's  $t$ -test. IFN, interferon; MLN, mesenteric lymph node; NALT, nasopharynx-associated lymph node; PBS, phosphate-buffered saline; PP, Peyer's patch.



**Figure 6** Characterization of ESAT-6-specific CTL activity *in vivo* in immunized SL5928(fliC/esat) mice. The histogram shows CFSE<sup>low</sup> untreated and CFSE<sup>high</sup> ESAT-6 peptide-pulsed target cells in the spleens of SL5928(fliC) and SL5928(fliC/esat) mice infected 15 h post-transfer, respectively. Results were representative of three experiments, each with five mice per group. CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte.

Characterization of the cytokine response following SL5928(fliC/esat) or SL5928 immunization was carried out by ELISPOT and identified lung, PP, NALT, spleen and MLN cells that produced IFN- $\gamma$  and IL-4 in response to ESAT-6 peptide stimulation. These experiments demonstrated that in mucosal-associated lymphoid tissues, such as lungs, PPs and NALTs, an anti-ESAT-6 response was Th1-associated based on the significant elevation of the Th1-associated cytokine IFN- $\gamma$ . In contrast, cells harvested from spleen and MLN produced a mixed Th1/Th2-type response based on the elevated levels of both IFN- $\gamma$  and IL-4. Our study therefore demonstrated that a Th1-associated immune response following *i.n.* vaccination with flagellated *Salmonella* SL5928(fliC/esat) was observed in the lungs, PPs and NALTs. Previous studies have also demonstrated that flagellated *Salmonella* is an effective delivery system. One study reported that the product of the gene *Rv2108* of MTB expressed in flagellin also produced the strongest Th1-like immune response.<sup>25</sup> Manocha *et al.* further demonstrated that intranasal immunization of mice with Fla-91—a recombinant flagellin containing the influenza B-cell epitope HA91-108—led to partial protection of mice from viral challenge.<sup>26</sup> High-level T-cell responses and increased IL-2 or IFN- $\gamma$  cytokine production from PPs have also been detected following *i.n.* immunization.<sup>27</sup> However, the mechanisms producing such specific immune responses in PPs remain unclear.

The induction of a Th1-like immune response to flagellin was demonstrated by *i.n.* immunization. Various routes of immunization have been investigated, including intramuscular (*i.m.*), *i.n.* and subcutaneous vaccination, as well as numerous methods of antigen delivery, such as co-encapsulation in poly-lactic acid microspheres,<sup>28,29</sup> expression by *Salmonella*<sup>30–32</sup> and DNA vaccination.<sup>33,34</sup> However, our experiments suggest that *i.n.* immunization may be the most useful method for preventing infectious respiratory diseases.

In addition, CTL responses based on CFSE *in vivo* were tested using flow cytometry to demonstrate whether chimeric flagellated *Salmonella* can induce ESAT-6-specific CTL effects. The results showed that memory T cells in the spleen of recipient mice immunized with SL5928(fliC/esat) play a substantial part in clearing the ESAT-6 peptide-pulsed target cells. In other words, ESAT-6-specific memory CD8<sup>+</sup> T cells exert substantial Ag-specific cytotoxicity *in vivo*. Similarly, Braga *et al.* also demonstrated that recombinant *S. dublin* flagella expressing the target CS (280–288) (the 9-mer (SYVPSAEQI)

synthetic H2(d)-restricted CD8<sup>+</sup> T cell-specific epitope) peptide fused at the central hypervariable domain were able to prime cytotoxic CD8<sup>+</sup> T cells.<sup>13</sup> Therefore, flagellated *Salmonella* may also offer protection by inducing efficient CTL responses.

Our study using flagellated *Salmonella* administered *i.n.* has demonstrated that flagella represent a microbial surface display system that can function as a delivery system for foreign antigen *in vivo*. Live, attenuated and flagellated *Salmonella* may therefore be a cost-effective prophylactic measure against mucosal disease.

#### ACKNOWLEDGEMENTS

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program), the National ST Major Project (no. 2008 ZX10003-010), the National Department Public Benefit Research Foundation (200903027), the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT) and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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