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Salmonella flagellin is a potent carrier–adjuvant for peptide conjugate to induce peptide-specific antibody response in mice

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ABSTRACT

As an agonist to innate immune system, *Salmonella* flagellin has been proven to be a potent adjuvant either admixed or genetically fused with antigens and applied to a variety of vaccines against infectious diseases. However, relatively little is known about its carrier–adjuvant effect for conjugate vaccine. Conjugation is an effective approach often used to make haptens such as some peptides and polysaccharides immunogenic and in some cases used to make poor immunogens more immunogenic. In the current study, *Salmonella* flagellin was tested for its carrier–adjuvant effect in a peptide conjugation. The recombinant *Salmonella* flagellin (rFliC) purified from *Escherichia coli* was firstly modified by maleimide groups, then coupled with a synthetic peptide (EXP153:CDNNLVSGP) that is a B-cell epitope derived from *Plasmodium falciparum* exported protein-1 to generate the conjugate of EXP153–rFliC. Bioactivity assay showed that both chemical modification and conjugation did not apparently impair the TLR5-ligand activity of rFliC. EXP153–rFliC was used to immunize BALB/c mice via subcutaneous route, and the sera obtained from immunized mice were examined by ELISA and IFA. While no detectable antibody responses were induced by the peptide admixed with rFliC, the robust peptide-specific antibody responses were observed in mice immunized with the peptide conjugated to rFliC in the absence of any additional adjuvant. The immune sera induced by the conjugate recognized the native protein of malaria parasite. The data obtained from this study demonstrate the carrier–adjuvant activity of *Salmonella* flagellin in peptide conjugate immunization and indicate its promising application for conjugate vaccine research and development.

1. Introduction

Toll-like receptors (TLRs) belong to a family of pattern recognition receptors that recognize highly conserved structures known as pathogen-associated molecular patterns or danger/damage associated molecular patterns and play a key role in innate immune responses [1,2]. When a host immune system is exposed to pathogens, antigens are captured by antigen presenting cells (APCs) and then processed and presented via the major histocompatibility complex to T cells through T cell receptors. Meanwhile, co-stimulatory molecules are up-regulated and pro-inflammatory cytokines and chemokines are produced due to the stimulation of APCs by pathogen-associated molecular patterns through TLRs. With these two signals, naive T cells are activated and adaptive immune responses are initiated [3]. Therefore, TLR ligands are often employed as adjuvant by scientists in a broad range of vaccine research and development [4,5].

Bacterial flagellin, a monomer subunit that polymerizes to form filaments of bacterial flagella, is a ligand of TLR5 when it occurs extracellularly [6]. *Salmonella* flagellin has been demonstrated to be a potent adjuvant for immune responses and tested in a variety of vaccines against bacteria [7–9], viruses [10,11] and parasites

Abbreviations: FliC, phase 1 flagellin of *Salmonella enterica* serovar Typhimurium; rFliC, recombinant FliC expressed in *Escherichia coli*; rFliC-M, maleimide modified rFliC treated with Sulfo-EMCS; EXP153, synthetic malaria peptide derived from *Plasmodium falciparum* exported protein-1; Sulfo-EMCS, *N*-[ε-maleimidocaproyloxy] sulfosuccinimide ester.

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[12,13] either admixed or genetically fused with antigens. Very importantly, previous experiments showed that *Salmonella* flagellin did not promote IgE mediated allergic response in animals, and its adjuvant activity was not impaired by pre-existing anti-flagellin antibodies [8]. Several influenza vaccines with their antigens fused to *Salmonella* flagellin have been recently trialed and appeared to be immunogenic and well tolerated in humans [14,15]. However, little knows about *Salmonella* flagellin served as carrier protein for conjugate vaccine except one research which investigated the immune effect of a *Salmonella* double-valent antigen constructed by conjugation of *Salmonella* core O-polysaccharide to its flagellin [16,17].

Peptide-based vaccines are widely tested because of their relatively easier preparation with modern techniques [18] and capability to target epitope-specific immune responses. However, most peptides are low- or even non-immunogenic. It is critical to enhance their immunogenicities to induce robust peptide-specific immune responses. Conjugation of peptide to an appropriate carrier is an effective approach that is often used to resolve this problem for the development of peptide-based vaccines.

Plasmodium falciparum exported protein-1 (PfEXP-1) is a 23 kDa protein that is expressed at erythrocytic and pre-erythrocytic stages [19]. PfEXP-1 can be secreted by parasites and found in parasitophorous vacuole membrane as integral protein and in vesicles within cytoplasm of infected erythrocytes [20,21]. Naturally acquired antibodies obtained from different endemic regions responded to this protein [22,23]. An eight amino acid fragment (PfEXP-$1_{153\text{-}160}$, DNNLVSGP) of PfEXP-1 forms a B-cell epitope recognized by the inhibitory monoclonal antibody 8E7/55 [24]. The animal immune sera raised by the PfEXP-$1_{153\text{-}160}$ peptide conjugated to diphtheria toxoid presented the capacity to significantly inhibit the in vitro growth of malaria parasites [25].

In this study, the value of *Salmonella* flagellin as carrier–adjuvant was evaluated in a peptide conjugation with PfEXP-$1_{153\text{-}160}$ as model peptide. The recombinant phase 1 flagellin of *S.* Typhimurium purified from *Escherichia coli* was modified by maleimide groups and then coupled with the synthetic PfEXP-$1_{153\text{-}160}$ peptide named EXP153. The resultant conjugate was further characterized and used to immunize mice. The antibody responses raised against EXP153 were analyzed.

2. Materials and methods

2.1. Expression and purification of rFliC

The gene of phase 1 flagellin of *S. enterica* serovar Typhimurium was PCR amplified from the genomic DNA of SL7207 strain with flic-F (5′-GCCCATGGCACAAGTCATTAATACAAAC-3′) and flic-R (5′-GCCTCGAGACGCAGTAAAGAGAGGACG-3′) as primers. PCR products were digested with *Nco* I and *Xho* I and ligated into the vector of pET28a(+) (Novagen, EMD Millipore) digested with the same enzymes to construct the recombinant plasmid pET28a(+)-fliC-6His. Successful construction was confirmed by restriction enzyme analysis and sequencing. The FliC gene sequence of SL7207 strain was identical to the reported sequence of LT2 strain deposited in GenBank (GenBank accession: STM1959).

The recombinant plasmid of pET28a(+)-fliC-6His was transformed into *E. coli* BL21(DE3) (Novagen, EMD Millipore). After induction with isopropyl β-D-1-thiogalactopyranoside (Promega), bacterial cells were harvested and disrupted by sonication. Sonicated cell supernatant was applied to a Ni-Sepharose column (GE Healthcare) to purify histidine-tagged *Salmonella* flagellin (~53 kDa) in accordance with the manufacturer's instruction. After desalting with a PD-10 column (GE Healthcare), the recombinant protein was treated with endotoxin removal resins (Pierce, ThermoFisher Scientific). The purified protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot under non-reducing condition.

2.2. Chemical modification of rFliC

The chemical linker of Sulfo-EMCS (Pierce, ThermoFisher Scientific) was used to add maleimide groups onto rFliC following the procedure described previously [26]. The protein was treated by different concentrations of Sulfo-EMCS at 0.5, 1, 2 and 4 mM, respectively. The modified rFliC (rFliC-M) was examined by SDS-PAGE under non-reducing condition, and the content of maleimide group added on rFliC-M (moles of maleimide per mole of rFliC) was determined by indirect Ellman's reaction [27].

2.3. Preparation of EXP153–rFliC conjugate

The peptide EXP153 (CDNNLVSGP) was synthesized by Beijing SBS Genetech (China). The sequence of DNNLVSGP is derived from PfEXP-1 (GenBank accession: AF246997). A cysteine at the N-terminus of the peptide was added to facilitate conjugation to maleimide modified proteins [28]. The lyophilized peptide was dissolved in the buffer of PBS-E (1× PBS, 5 mM EDTA, pH 7.2), and the content of free sulfhydryl in the peptide solution (moles of free thiol per mole of peptide) was calibrated by Ellman's reaction [27].

To make EXP153–rFliC conjugate, EXP153 was added in excess to react with the rFliC-M that was treated by 2 mM of Sulfo-EMCS. After 1 h incubation at room temperature, the remaining free sulfhydryl was measured by Ellman's reaction to determine the consumption of the peptide in the reaction, by which the conjugation ratio of EXP153 to rFliC (moles of EXP153 per mole of rFliC) within the conjugate was calculated. The unconjugated peptides in the reaction mixture were then removed by filtration through a 10 kDa MWCO spin filter (EMD Millipore). The resultant conjugate was examined by SDS-PAGE under non-reducing condition.

2.4. Bioactivity assay of rFliC samples

Two cell lines of HEK-293 and HEK 293-mTLR5 (InvivoGen) were used to assess TLR5-ligand activities of the rFliC samples (rFliC, rFliC-M and EXP153–rFliC) following the procedure as described [12]. Native *Salmonella* flagellin (nFliC) (InvivoGen) and bovine serum albumin (BSA) (Sigma–Aldrich) were used in the assay as positive and negative control, respectively. A commercial enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech) was used to measure the level of the human interleukin 8 (hIL-8) secreted in the cell culture supernatant. The hIL-8 level of each sample was obtained by comparing its absorbance after subtracting the background value to the standard curve made with known concentrations of hIL-8.

2.5. Animals and immunization

Two independent animal studies were carried out in the laboratory animal facility of Second Military Medical University and in compliance with the university's policy of animal care and use. BALB/c female mice aged 6–8 weeks were purchased from Shanghai SLAC Laboratory Animal (China) and randomly assigned into five experimental groups of five mice in the first study or three groups of six in the second.

In the first mouse study, five vaccine formulations were prepared: (1) rFliC/Freund: rFliC formulated with complete or incomplete Freund's adjuvant (Sigma–Aldrich) for the first and second immunization, respectively, (2) EXP153+ rFliC: EXP153 and rFliC mixture in saline, (3) EXP153–rFliC: conjugate in saline, (4) EXP153–rFliC/Alum: conjugate formulated with alum adjuvant

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