Cross-protective effect of a novel multi-antigen-chimeric vaccine against *Streptococcus* and *Staphylococcus aureus* infection in mice

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Staphylococcal and streptococcal species are the most common pathogens that cause bovine mastitis. Induction of a broad-spectrum protective immunity against staphylococci and streptococci by combining multiple antigens into a single vaccine is highlighted. To develop a universal vaccine candidate, a GapC1-tIsdB-TRAP (GIT) construct was generated. The GIT contained the truncated GapC from *Streptococcus dysgalactiae*, and truncated IsdB and full-length TRAP from *Staphylococcus aureus*. The humoral and cellular immune responses elicited by GIT were evaluated in mice. Antibody levels against GIT displayed a consistent tendency with antibody levels against GapC, IsdB and TRAP. The level of IFN-γ was higher in the GIT group than in the IsdB group (P<0.05), and the level of IL-4 was higher in the GIT group than in the GapC or TRAP groups (P<0.05). The GIT group showed an improved protection against *Streptococcus* in comparison with GapC group. A significant difference in *S. aureus* challenge test was detected between the GIT group and the IsdB or TRAP groups (P<0.05) in per cent survival of mice, and a synergistic immunoprotection against *S. aureus* or *S. dysgalactiae* was produced in the GIT group. These results suggested that the GIT would be a promising common vaccine candidate against *S. aureus* and *Streptococcus*.

INTRODUCTION

*Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus uberis* (*S. uberis*) and *Staphylococcus aureus* (*S. aureus*) are the most common mastitis-causing pathogens in dairy cattle in many parts of the world (Kerro-Dego et al., 2012; McDougall et al., 2007). Antibiotics are most often used to combat mastitis pathogens. With increasing resistant mastitis pathogens and the potential of antibiotic residue in milk, it is necessary to prudently use antibiotics in the dairy industry. Development of an effective broad-spectrum vaccine preventing polymicrobial mastitis would be extremely desirable, leading to increased food safety and improved human health. Immunization with a combination vaccine incorporating multiple protective antigens has been proposed as a novel strategy to prevent microbial infection (Bagnoli et al., 2012; DeDent et al., 2012). Some examples have been demonstrated that antigen combinations, in particular antigen fusion, was more effective than each protein alone or an admixture of proteins in inducing protective immunity (Stranger-Jones et al., 2006; Yu et al., 2013; Zhang et al., 2002). Thus, a fusion of multiple immunogenic proteins might generate an additive protection and increase the clinical utility of the single antigen.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an essential housekeeping enzyme in the survival of bacteria, has been found to be associated with virulence and adherence in *Streptococcus* and *S. aureus* (Kerro-Dego et al., 2012; Madureira et al., 2011; Fu et al., 2013). It has been found that only one protein having GAPDH activity existed in streptococci, named GapC (Fu et al., 2013). *S. aureus* had two GAPDH proteins, GapB and GapC, and GapC was present on the surface of all *S. aureus* strains (Goji et al., 2004). Some descriptions were performed on the protective potential of GAPDH due to its multiple functions and conservation. It was reported that neonatal mice born from mothers immunized with rGapC were protected against infection with *S. agalactiae* strains (Madureira et al., 2011). GapC from *S. dysgalactiae* was a

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**Abbreviations:** GIT, GapC1-tlsdB-TRAP.
potential protective antigen against *S. dysgalactiae* bovine mastitis (Bolton *et al.*, 2004). Also, recombinant *S. uberis* GapC could confer protection against challenge with heterologous *S. uberis* strains (Fontaine *et al.*, 2002). In recent years, the cross-immunity potential of GAPDHs in different pathogenic bacteria was exposed to be attractive. *E. tarda* GAPDH had effective protection against *Vibrio* infection (Liu *et al.*, 2007). GAPDH was further demonstrated to be a broad-spectrum vaccine candidate against multiple microbial infections in aquaculture (Li *et al.*, 2012).

To increase the immune response to the GAPDH moiety, potential use of GAPDH as a component of chimeric vaccine was evaluated. A GapC/B chimera composed of two *S. aureus* proteins with homology to GAPDH, GapB and GapC, elicited strong humoral and cellular responses in mice without losing their GAPDH activity (Perez-Casal *et al.*, 2006). Similarly, vaccination with a Gap-I chimera composed of *Mycoplasma bovis* GAPDH and the host-defence peptide indolicidin resulted in a strong humoral immune response and a weak cell-mediated immune response, in spite of failure to protect feedlot cattle from challenge with *Mycoplasma bovis* (Prysiak *et al.*, 2013). This was consistent with previous findings where *Mycoplasma suis* GAPDH failed to be protective, but also induced a strong humoral and cellular immunity against *Mycoplasma suis* (Hoelzle *et al.*, 2009).

Although the role of GAPDH as a protective antigen was mixed, it was still a reliable vaccine candidate moiety. To date, no experiments about the potential of GapC cross-protection against *Streptococcus* and *S. aureus* have been investigated. We believed that the addition of a previously reported iron-regulated surface determinant B (N126-P361)* target of RNAIII-activating protein (tI dB-TRAP) from *S. aureus* (Yu *et al.*, 2013) could increase the potential cross-protective role of GapC against *Streptococcus* and *S. aureus*. In this work, we constructed a multi-antigen chimeric vaccine GIT (GapC1-tIsdB-TRAP) on GapC. The immunogenicity of GIT was demonstrated by Western blot and ELISA, and its protective efficacy against *Streptococcus* and *S. aureus* was evaluated in a lethal sepsis murine model.

**METHODS**

**Bacterial strains and cultural conditions.** The *Streptococcus agalactiae* (*S. agalactiae*) strain LS0310, *Streptococcus dysgalactiae* (*S. dysgalactiae*) strain LS0312, *Streptococcus uberis* (*S. uberis*) strain SD0306 and *Staphylococcus aureus* (*S. aureus*) strain HJI/855/23-1 were isolated from local dairy cows with mastitis, and were verified by Gram’s strain, morphology and biochemical characterizations in our laboratory. These strains were used for infection of mice in this study, and were grown in brain heart infusion (BD Difco) or tryptic soy broth (BD Difco) at 37 °C overnight. The bacteria were harvested by centrifugation and washed with sterile PBS. The washed bacteria were diluted with PBS to appropriate cell concentrations as determined at OD550 nm. *Escherichia coli* strain Match 1-T1 Phage Resistant was used for cloning purposes. Recombinant proteins were expressed in BLRBL21(DE3)pLysS (Novagen). All *E. coli* strains were grown in Luria–Bertani (LB) agar supplemented with appropriate selection at 37 °C in an incubator with 5% CO2, and broth cultures were incubated in a shaking incubator.

**Chimeric gene cloning.** The source of TRAP, IsdB and GapC and production of recombinant GapC1-tIsdB-TRAP (GIT) were as follows. The plasmids pET-32a-TRAP, harbouring TRAP from *S. aureus*, pET-32a-IsdB, harbouring IsdB from *S. aureus*, pET-32a-GapC, harbouring GapC from *S. dysgalactiae* and pET-32a-tIsdB-TRAP, harbouring fused TRAP and truncted IsdB were, previously constructed and stored in our laboratory. To select an antigenic fragment replacing a full-length GapC, the B cell epitope of *S. dysgalactiae* GapC was predicted using Proteam (IgNAR) and two online software programs (http://www.imtech.res.in/raghava/bcepred/ and http://www.iedb.org/). According to comparative analysis of the prediction results and the GenBank data, two truncated fragments of GapC was designed. One fragment of amino acid residues from M1 to A158 of GapC, containing the major B cell epitope, was named GapC1, and another fragment of amino acid residues from S131 to K133, of GapC was named GapC2 in this study. Immune protection of GapC1 and GapC2 in mice was compared with full-length GapC, and immunodominant GapC1 was demonstrated. As shown in Fig. 1, chimeric GIT on GapC2 was constructed. Firstly, GapC1 was amplified by PCR from the plasmid pET32a-GapC using the P1 and P2 primer pairs (Table 1). The PCR conditions were as follows: initial denaturation step at 94 °C for 10 min; followed by 25 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 40 s; and finished by incubation at 4 °C for 1 h. Subsequently, the purified GapC1 fragments and the synthetic oligonucleotide chain L4 were used as the PCR template. The PCR was conducted to amplify the GapC1 fused with the oligonucleotide chain L4 (GapC1-L4) by using P1 and P3 primer pairs (Table 1) under the following conditions: an initial 10 min denaturation at 98 °C; followed by 5 cycles of 50 s at 98 °C, 50 s at 50 °C and 2 min at 72 °C; and with 1 cycle of 55 °C, 50 s and 72 °C and 2 min; and with 3 cycles of 30 s at 98 °C, 50 s at 60 °C and 1 min at 72 °C; and with 25 cycles of 50 s at 98 °C, 50 s at 55 °C and 1 min at 72 °C. The following PCR was conducted by using the above PCR mixed products as PCR template. The PCR conditions were as follows: initial denaturation step at 94 °C for 10 min; followed by 25 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 40 s; and finished by incubation at 72 °C for 1 min. Secondly, the tIsdB-TRAP was amplified by PCR from the plasmid pET-32a-tIsdB-TRAP using the P5 and P6 primer pair (Table 1). The PCR conditions were as follows: initial denaturation step at 94 °C for 5 min; followed by 30 cycles of 94 °C, 50 s; 58.5 °C, 50 s; 72 °C, 80 s; and finished by incubation at 72 °C for 10 min. Thirdly, the GapC1-L4 was digested with BamHI and Sall. The tIsdB-TRAP was digested with BamHI and Sall. The digested DNA fragments, GapC1-L4 and tIsdB-TRAP, were purified with a DNA...
purification kit (Gene Tech), and were ligated with the Rapid DNA Ligation kit (Gene Tech). The PCR was carried out using the ligated mixed products as a PCR template with P1 and P6. The PCR conditions were as follows: initial denaturation step at 94 °C for 5 min; followed by 30 cycles of 94 °C, 50 s; 56 °C, 30 s; 72 °C, 2 min; and finished by incubation at 72 °C for 10 min. The purified GIT fragments were cloned into the pMD18-T vector, yielding the plasmid pMD18-GIT. The insert sequence in the plasmid was sequenced by DNA sequencing. The sequenced GIT was blasted with the public standard in GenBank. The insert sequence in the plasmid was verified by DNA sequencing.

**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’—3’)*</th>
<th>Restriction site</th>
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<tr>
<td>P1</td>
<td>GGAGAGCTCATGTTAGTATAAGTGTGAT</td>
<td>ScaI</td>
</tr>
<tr>
<td>P2</td>
<td>CTTGCCTGAGCCTGCTTCTAGCTCAGGCTAAGACGCTGAGATACTGTT</td>
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</tr>
<tr>
<td>P5</td>
<td>ACGAGATCCATACAGGACATTTAGAAGAGCGATTAGATTTATGGGATAGAT</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>CAGCTGCAATTTTATGGGATAGATTTATGGGATAGAT</td>
<td></td>
</tr>
</tbody>
</table>

*The restriction sites are underlined. To generate GIT, a 59 bp linker sequence of helix-forming bridge (LA(EAAAK)3AAA) was introduced to link GapC1 and tIsdB-TRAP.

Protein production, purification and Western blot analysis. The four plasmids, pET-32a-GIT, pET-32a-IsdB, pET-32a-TRAP and pET-32a-GapC, were transformed using electroporation into competent E. coli BL21 cells, which were then selected on ampicillin (100 μg ml⁻¹) LB agar. For expression of proteins, bacteria were grown at 37 °C and induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) for different times when OD₆₀₀ absorption reached 0.6. For protein detection and purification, bacterial cells were lysed at 4 °C with proper ultrasonication. His-tagged proteins were fractionated with Ni-NTA His-Bind Resin, according to the manufacturer’s recommendations (Novagen). Fractions containing His-tagged proteins were pooled, and endotoxin was removed by filtration through a Zeta-Plus Biofilter (CUNO). Purified proteins and induced proteins in different times were analysed by SDS-PAGE. Purified proteins were fractionated by SDSPAGE and electrotetransferred to nitrocellulose membranes. Mouse polyclonal anti-S. dysgalactiae, S. agalactiae, S. uberis and S. aureus antiserum or anti-His tag antibody was used as the primary antibody. A horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma) was used as the secondary antibody according to the manufacturer’s instructions with DAB staining.

Immunization of mice and lethal challenge. Female BALB/c mice, 6 weeks old, were purchased from the Experimental Animal Center of Changchun Institute of Biological Products. All animal experiments were performed according to the guidelines for Care and Use of Laboratory Animals (Ministry of Health, China). All the animals were housed in a special pathogen-free environment and were negative for pathogens in routine screening. Firstly, a total of 170 BALB/c mice were divided into 17 groups (10 in each group). Among them, the GIT included four groups, PBS included four groups, blank control included four groups, GapC included three groups and IsdB and TRAP each included one group. These divided groups were used for the determination of mice survival rates. Secondly, a total of 15 BALB/c mice were divided into five groups, containing three mice per group. The five groups included group GIT, GapC, IsdB, TRAP and PBS, and were used for determination of serum IgG. Meanwhile, parallel groups were conducted and used for obtaining spleen T lymphocytes. For animal immunization, mice were subcutaneously injected with 200 μl of the emulsion containing 100 μg proteins plus Freund’s complete adjuvant, PBS plus Freund’s complete adjuvant or PBS alone as controls. All groups of mice were primed at 7 weeks of age, and all groups were boosted with Freund’s incomplete adjuvant and protein at 10 weeks. For animal infection, challenge was carried out on week 12 by intraperitoneal injection with a 0.5 ml exponential-phase bacterial culture per mouse. The cell numbers of S. agalactiae strain LS0310, S. dysgalactiae strain LS0312, S. uberis strain SD0306 and S. aureus strain HJ/855/23-1 each was 6 × 10⁸, 2 × 10⁸, 1.6 × 10⁷ or 3 × 10⁷ c.f.u., respectively. The survival rates of mice were monitored for 7 days after infection.

IgG assay by ELISA. ELISA was performed to measure the IgG titres in individual mouse serum samples. Endotoxin was removed from all serum samples using the ProteoSpin Endotoxin Removal kit (Norgen Biotec Corporation) before ELISA. Briefly, 0.5 μg ml⁻¹ of purified IsdB, TRAP or GapC were coated on 96-well microtitre plates (Falcon, Fisher Scientific) overnight at 4 °C. Free binding sites were blocked with 5% skimmed milk in PBST. Individual mouse sera from three mice per group were analysed in triplicate in twofold dilutions, starting with a 100-fold dilution. Horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1/2000 in PBST was added. After 1 h of incubation, antigen-specific antibodies were detected by TMB substrate as described by the manufacturer (Bios). To stop the reaction, 100 μl of 4N sulfuric acid was added, and the optical density (OD) was measured at 450 nm. The negative sera from mice with PBS were used to determine the cut-off value according to the optimization of the ELISA procedure. The cut-off value was calculated as a mean + 3SD of the OD₄₅₀ value demonstrated by the negative serum. The serum sample was regarded as positive if the OD₄₅₀ value was higher than the cut-off value, or it was regarded as negative. Data represented the end point dilution titres.

Cytokine assays by ELISPOT. ELISPOT assay was finished using commercial kits (mouse IFN-γ and IL-4 ELISPOT sets, Dakewe Biotech) according to the manufacturer’s instructions. Briefly, the precoated ELISPOT plates were activated with serum-free CZCulture. Then, freshly prepared mice splenocytes (5 × 10⁵ cells per well) were added into the plates immediately after the addition of 10 μg ml⁻¹ GIT, IsdB, TRAP and GapC, or 10 ng ml⁻¹ of PMA in serum-free CZCulture, respectively. After incubation and washing, the plates were incubated with 2 μg ml⁻¹ biotinylated detection antibody against mouse IFN-γ or IL-4 for 1 h. ELISPOT development was performed by 1 h incubation with avidin-HRP complex, followed by incubating with the peroxidase substrate AEC for 30 min. ELISPOT spots were quantified using an immunonospy analyser (Cellular Technology).
Statistics. Values were expressed as mean±SD. Differences between groups were analysed by one-way analysis of variance (ANOVA). P<0.05 was considered as significant.

RESULTS

Protective evaluation of immunodominant fragment of GapC

The immune protection of two truncated GapC fragments, GapC1 and GapC2, was performed to establish the immunodominant fragment of GapC in mice. The constructive GapC1 and GapC2 plasmids were verified by sequencing, and recombinant proteins were analysed by SDS-PAGE and tested with polyclonal antibodies by Western blot (data not shown). Mice were immunized with purified GapC, GapC1 or GapC2, followed by challenge with 1 LD100 bacteria. The results (Fig. 2) showed that GapC1 displayed a greater protection than GapC2. Survival rates of GapC1, compared with full-length GapC, were similar following S. dysgalactiae challenge or better following S. agalactiae and S. uberis challenge. This suggested that GapC1 could replace full-length GapC in immunogenicity.

Confirmation of chimeric protein and immunogenicity analysis

To generate a recombinant GIT plasmid, the DNA fragments for the GapC1 and tIsdB-TRAP were amplified from pET32a-GapC and pET32a-tIsdB-TRAP, and further cloned into the pET32a (+) to generate a recombinant plasmid expressing the fusion protein GIT. Characterization of the plasmid indicated that it contained a 1770 bp insert (Fig. 3a). To maintain the native conformation of the chimeric protein, a nucleotide sequence encoding a helix-forming linker (LA(EAAAK)3AAA) was introduced between the GapC1 fragment and the tlsdB-TRAP fusion in this study. Following induction by IPTG and purification, an 86 kDa fusion protein was obtained (Fig. 3b). Meanwhile, an 86 kDa purified fusion protein was probed by anti-S. dysgalactiae, anti-S. agalactiae, anti-S. uberis or anti-S. aureus serum, or by an anti-His tag antibody, suggesting that the fusion protein retained GapC, IsdB and TRAP immunoreactivity (Fig. 3c–e). The successful generation of chimeric protein provided material for further vaccination and challenge.

Effects of GIT on humoral immune responses

To evaluate if the GIT was eliciting humoral immune responses, the IgG level in the sera was determined each week, from primary immunization to 35 days postimmunization. As shown as Fig. 4, following immunization with GIT, GapC, IsdB or TRAP, all mice all showed significant specific antibody levels in the sera, as compared to PBS (P<0.01). Compared with the antibodies against GapC, IsdB and TRAP, the antibody levels against GIT were not found to be significantly different. Interestingly, antibody levels in the GIT group showed a slight increase before the peak and a slow decline after the peak compared to those in GapC or IsdB groups. However, antibody levels against
GIT showed a slight decrease compared with antibody levels against TRAP before the peak. The GIT group, on the whole, showed a consistent antibody tendency with GapC, IsdB and TRAP groups. In contrast, GIT could elicit an improved humoral immune response in mice.

**GIT enhanced the T-cell immune responses**

The function of GIT on cellular immune response was further determined in mice. Two weeks after the last vaccination, the frequency of antigen-specific IL-4 and IFN-γ splenic T cells were determined by ELISPOT assay (Fig. 5). Significant numbers of IFN-γ secreting cells were detected after stimulation with GIT, compared to IsdB ($P<0.05$). Significant levels of IL-4 were detected in the GIT group compared to TRAP ($P<0.05$). These results were consistent with our previous report (Yu et al., 2013). Significant difference was also found in the frequency of antigen-specific IL-4 between the GIT group and the GapC group ($P<0.05$). However, there was no significant difference among the three single protein groups in IFN-γ and IL-4 levels. This suggested that vaccination with GIT induced an enhanced level of Th1 and Th2 response in mice.

**Immuno-protection against Streptococcus and S. aureus lethal infection**

To investigate the efficacy of GIT against *S. aureus* and *Streptococcus* infection *in vivo*, a clinical *S. aureus* isolate and three clinical *Streptococcus* isolates were used for challenge, tested by the murine sepsis model. The bacteria were serially diluted with PBS, and cell numbers of the lethal dose were determined by spread plate. As shown in Fig. 6, the survival rate of mice immunized with GIT reached a protection of 80%, 70% and 70% following *Streptococcus* challenge. This indicated that GIT improved
data revealed that GIT could provide cross-protection in mice, and would be a potential vaccine candidate against S. aureus and Streptococcus infection.

**DISCUSSION**

For the practice of polymicrobial infections in diary industry, a broad-spectrum vaccine would be preferred. Particularly, a common mastitis vaccine to prevent representative S. agalactiae, S. dysgalactiae, S. uberis and S. aureus is urgently needed. Chimeric vaccines not only can reduce the cost of antigen production and simplify immunization procedure, but also offer greater potential protection than the individual proteins. We focused our attention on pathogenesis-associated proteins from mastitis bacteria, and the protective potential of a multi-antigenic chimera. The objective of the present study was to investigate the protective efficacy of the multi-antigenic chimera GIT against Streptococcus and S. aureus.

Though GAPDH did not appear to be a suitable vaccine target against some pathogens, such as Mycoplasma bovis (Prysliak et al., 2013), Mycoplasma suis (Hoelzle et al., 2009) and Candida albicans (Gil et al., 2006), the attractive protective potential of GAPDH in many reports cannot be ignored. It has been found that GapC identity was 65.3% conserved amongst S. aureus, S. iberis, S. agalactiae and S. dysgalactiae by BLAST (data not shown), and 99.5% conserved amongst S. iberis, S. agalactiae and S. dysgalactiae (van der Merwe et al., 2011). Mouse antisera against S. dysgalactiae GapC could cross-react with GapC from S. aureus, S. iberis or S. agalactiae, while mouse antisera against S. aureus GapC could not do so in our lab (data not shown). Despite our finding that immunization with proteins sharing a close relationship was not a guarantee of cross-protection, along with everything mentioned here, S. dysgalactiae GapC was still picked in this study as an attractive vaccine moiety. Fontaine et al. showed a lack of protection against S. iberis challenge in dairy cows immunized with S. dysgalactiae GapC (Fontaine et al., 2002), but then it was demonstrated in Leigh’s letter to the editor that this was a report of an uncontrolled experiment (Leigh, 2002). The cross-protection of the truncated S. dysgalactiae GapC in our experiments also suggested the opposite to Fontaine’s results. On the other hand, S. aureus IsdB, TRAP and fusion tIsdB-TRAP were regarded as promising potential vaccine targets in previous reports (Joshi et al., 2012; Leitner et al., 2011; Yu et al., 2013). These known information allowed us to construct a chimeric GIT composed of the immunodominant fragment of GapC from S. dysgalactiae and fusion tIsdB-TRAP from S. aureus. We found that GIT could provide cross-immunoprotection against S. dysgalactiae, S. agalactiae and S. iberis in mice and this was in contrast to Fontaine’s finding again (Fontaine et al., 2002). This might be due to a different GapC construct and animal model being used in the experimental design. In particular, GIT could produce a synergistic immunoprotection against S. dysgalactiae or S. aureus in mice, which is similar to the work of Zhang et al.
It may be the spatial structure of the hybrid protein or the protective epitopes that gave rise to the synergistic immunoprotection of GIT.

Imune responses were evaluated to assess the protection conferred by GIT. In the constructive process of GIT, a helix-forming bridge LA(EAAAK)₃AAA and a flexible

(2007). Fig. 5. Specific cytokine production by splenocytes of mice immunized with the GIT chimera as compared to the proteins GapC, IsdB and TRAP. The IFN-γ (a) or IL-4 (b) ELISPOT assay was conducted 2 weeks after the last immunization. The results are given as IFN-γ SFC 10⁻⁶ cells or IL-4 SFC 10⁻⁶ cells, and the data represent the mean ± SD of three mice per group. The asterisks indicate P-values (*, P<0.05).

Fig. 6. Evaluation of the GIT vaccine protection in mice. Per cent survival after infection with 6×10⁹ c.f.u. *S. dysgalactiae* (A), 2×10⁹ c.f.u. *S. agalactiae* (B), 1.6×10⁹ c.f.u. *S. uberis* (C) and 3×10¹⁰ c.f.u. *S. aureus* (D). BALB/c mice were vaccinated or nonvaccinated, and then challenged i.p. with the lethal doses of bacteria for per cent survival, respectively. Survival of mice was monitored for 7 days (*, P<0.05, compared with IsdB or TRAP).
bridge SGSGSGSG, designed by referring the previous report (Zhao et al., 2008), were used to maintain the original conformation of GapC1, tlsdB and TRAP. Each component of the fusion GapC1-tlsdB-TRAP retained its original immunogenicity (Fig. 3c, d). No significant differences in IgG levels were found between the GIT group and single protein group as to eliciting humoral immune responses. IFN-γ is a critical cytokine that is involved in macrophage bactericidal activity (Matsuzawa et al., 2014), and IL-4 is considered an anti-inflammatory molecule (Bessoles et al., 2008). IL4 and IFN-γ favour development of Th2 and Th1 lymphocytes, respectively (Boskabady et al., 2013). The mixed Th1/Th2 responses can be assessed by IFN-γ and IL-4 ELISPOT assay. GIT was better than tlsdB at inducing IFN-γ, and better than TRAP at inducing IL-4, which is consistent with our previous report (Yu et al., 2013). Also, a significant difference was displayed between GIT and GapC at inducing IL-4. In both humoral and cellular responses, the tandem GapC1-tlsdB-TRAP induced stronger responses than did the single proteins. An interaction among GapC1, tlsdB and TRAP in GIT seems to occur. A component of GIT perhaps performs an adjuvant effect or mutual promotion of each antigenic determinant enfoldment probably occurs. In a word, GIT caused mixed Th1/Th2 immunological responses.

In present work, we continued our approach of fusing multiple antigens as a potential target for developing a mastitis vaccine, and produced the vaccine candidate GIT. It seems to be more important that fusion should contain multiple antigens that work by multiple immunological mechanisms. Use of 351R from marine fish Megalocytivirus and GAPDH from Edwardsiella tarda in a fused protein provided protective efficacy against fish Megalocytivirus infection (Shimmoto et al., 2010). Thus, we believe that our strategy will increase the possibility of combating bovine mastitis. In clinical study in the future, immunoprotection of GIT will be tested in cows. In order to promote a balanced Th1/Th2 immune response (Mutwiri et al., 2011), a suitable adjuvant such as the host-defence peptide indolicidin (Bowdish et al., 2005) and synthetic oligonucleotides known as CpG (Krieg, 2000) will be employed. In general, the present work gave useful evidence for developing a common vaccine against Streptococcus and S. aureus.

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