Clostridium difficile Tx_A_{C314} and SLP-36kDa enhance the immune response toward a co-administered antigen

Paola Brun,¹ Melania Scarpa,¹ Alessia Grillo,¹ Giorgio Palù,¹ Carlo Mengoli,¹ Alfonso Zecconi,² Patrizia Spigaglia,³ Paola Mastrantonio³ and Ignazio Castagliuolo¹

Correspondence
Ignazio Castagliuolo
ignazio.castagliuolo@unipd.it

¹Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Padua, Italy

²Department of Animal Pathology, Hygiene and Health, University of Milan, Milan, Italy

³Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

This study evaluated the *in vivo* adjuvant activity of two peptides derived from *Clostridium difficile*: a fragment of the receptor-binding domain of toxin A (Tx_A_{C314}) and a fragment of the 36 kDa surface-layer protein (SLP-36kDa) from strain C253. Their ability to affect the magnitude, distribution and polarization of the immune response against fibronectin-binding protein A (FnbpA), a protective vaccine antigen against *Staphylococcus aureus*, was evaluated using two different routes of immunization: intranasal and subcutaneous. It was shown that (i) the route of immunization affected the magnitude of the immune response; (ii) both peptides enhanced the production of circulating anti-FnbpA IgG and IgA; (iii) following mucosal immunization Tx_A_{C314} was more effective than SLP-36kDa at inducing antibody in the gastrointestinal tract; (iv) the adjuvant influenced the Th1/Th2 balance; and (v) Tx_A_{C314} was more effective than SLP-36kDa in inducing a cell-mediated response. These studies provide insight into the ability of different *C. difficile*-derived peptides to differentially affect and polarize the activity of the immune system and on their potential use as adjuvants in newly developed vaccines.

Received 31 October 2007
Accepted 19 December 2007

INTRODUCTION

Bacterium-derived products are the most powerful immunomodulatory molecules of the mucosal-associated immune system (Fallon & Alcamì, 2006). Thus components of the bacterial cell wall, recognized through a family of surface and cytoplasmic receptors, i.e. Toll-like receptors and nucleotide-binding oligomerization domain isoforms, expressed in epithelial and immune cells modulate the development of adaptive immune responses against mucosal antigens (Cario, 2005; Nestle *et al.*, 1998). Furthermore, bacterial toxins are among the strongest antigens and a few enterotoxins such as cholera toxin (CT) secreted by *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxin (LT) are among the most powerful mucosal adjuvants identified to date (Aizpurua & Russell-Jones, 1988).

Abbreviations: CT, cholera toxin; DC, dendritic cell; FnbpA, fibronectin-binding protein A; IFN- γ , gamma interferon; IL-2, interleukin-2; i.m., intramuscularly; i.n., intranasally; LT, heat-labile enterotoxin; s.c., subcutaneously; SLP, surface-layer protein; SLP-36kDa, fragment of the 36 kDa SLP; TcdA, toxin A.

Recent studies have reported that several *Clostridium difficile* proteins are immunodominant antigens, as they efficiently stimulate *in vivo* the immune system associated with the intestinal mucosa (Mukherjee *et al.*, 2002). Thus significant levels of circulating antibodies against *C. difficile*-derived surface-layer proteins (SLPs), the predominant outer-surface component, toxin A (TcdA) and toxin B have been demonstrated in a large portion of the human population (Kyne *et al.*, 2000). Indeed, *C. difficile*-derived proteins specifically interact with a variety of immune and non-immune cells in the intestinal mucosa. Thus TcdA binds specifically to epithelial cells and lamina propria phagocytes, inducing the release of a vast array of immunomodulatory cytokines (Castagliuolo & LaMont, 1999). We have also recently reported that a non-toxic fragment of the C-terminal domain of TcdA, Tx_A_{C314}, comprising a region of the receptor-binding domain, conserved the ability to bind to intestinal epithelial cells and to stimulate immune cells (Castagliuolo *et al.*, 2004). Tx_A_{C314} exerted a significant adjuvant activity following *in vivo* co-administration with weak peptide antigens, such as keyhole limpet haemocyanin and hen egg lysozyme

(Castagliuolo *et al.*, 2004). Recently, we and others (Cerquetti *et al.*, 2002; Eveillard *et al.*, 1993) have reported that SLPs from *C. difficile* bind to intestinal epithelial cells and induce the release of regulatory cytokines in dendritic cells (DCs) (Ausiello *et al.*, 2006), suggesting that these proteins may also boost immune responses *in vivo*.

The goal of this study was to evaluate the ability of TxAc₃₁₄ and a fragment of the 36 kDa SLP (SLP-36kDa) to enhance and polarize the immune response toward a Th1- or Th2-type response. In particular, we evaluated the ability of TxAc₃₁₄ and SLP-36kDa to trigger a specific humoral, mucosal and cell-mediated response following mucosal or subcutaneous administration. As test antigen, we used a recombinant peptide comprising a surface adhesion factor, fibronectin-binding protein A (FnbpA), derived from a pathogenic *Staphylococcus aureus* strain that plays a key role in bacterial mucosal colonization (Lammers *et al.*, 1999).

METHODS

Expression and purification of recombinant TxAc₃₁₄, SLP-36kDa and FnbpA. TxAc₃₁₄, SLP-36kDa and FnbpA were expressed in *E. coli* BL21(DE3). cDNAs encoding TxAc₃₁₄ from *C. difficile* strain VPI 10463 (ATCC) and FnbpA from *S. aureus* strain 1706 (Zecconi *et al.*, 2005) were cloned in expression vector pGEX-2T as described previously (Castagliuolo *et al.*, 2004, 2006). The protein SLP-36kDa from *C. difficile* C253 has been characterized previously (Cerquetti *et al.*, 2000). In this study, the *slpA* gene region of 978 bp of p36 without the peptide signal was amplified using primers GST2 (5'-GCTGGATCCACTGGAACACAAGGT-3'), incorporating a *Bam*HI restriction site, and GST7 (5'-TGTATCATTTGCGAATTCAGTTTC-3'), incorporating an *Eco*RI restriction site. The PCR fragment was cloned into the expression vector pGEX-6P-1. The recombinant proteins were purified by affinity chromatography using a glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech), according to standard procedures. The integrity, purity and concentrations of the recombinant proteins were determined by SDS-PAGE and Bradford (Bio-Rad) protein assays. Triton X-100 and bacterial LPS were removed by sequential single-step affinity chromatography using Calbiosorb Adsorbent Resin (Calbiochem) and Endotoxin Removing Gel (Pierce), respectively (Castagliuolo *et al.*, 2004). The levels of endotoxin contamination in the recombinant protein preparations were determined to be less than 0.01 IU ml⁻¹ (*Limulus* amoebocyte assay; BioWhittaker), which corresponded to less than 0.1 pg LPS administered per mouse.

Immunization protocols. Male BALB/c mice (8–10 weeks old), purchased from Charles River Laboratories, Oderzo, Italy, were used in all experiments. Mice were housed under controlled temperature and humidity conditions and received standard pelleted chow and tap water *ad libitum*. All procedures involving animal studies were carried out following the guidelines recommended by the Institutional Animal Care and Use Committee of the University of Padova, Italy. Mice were injected either subcutaneously (s.c.) or intranasally (i.n.) with 10 µg purified peptide antigen (FnbpA) alone (negative-control mice) or with a peptide mixture consisting of equal amounts of recombinant TxAc₃₁₄ or SLP-36kDa as potential adjuvant together with FnbpA. In addition, a group of mice were injected either s.c. or i.n. with recombinant TxAc₃₁₄ or SLP-36kDa alone to assess the specificity of their effect. As a reference, 10 µg purified FnbpA mixed with Freund's adjuvant was injected intramuscularly (i.m.).

A total of 8–12 mice were used for each experimental condition. Mice were bled 1 week prior to the first immunization and then randomly allocated to an experimental group. Three doses of vaccine were given at 2-week intervals: at time 0, and after 2 and 4 weeks. After a further 3 weeks, the animals were sacrificed. For i.n. administrations, 30 µl was transferred to the nasal cavity of non-anaesthetized mice through fine tips attached to a micropipette. For s.c. administration, a total of 30 µl was injected into the interscapular region of non-anaesthetized mice using a tuberculin syringe. For i.m. immunization, purified recombinant FnbpA was administered into the right tibialis anterior muscle supplemented with an equal volume of complete Freund's adjuvant in a total volume of 30 µl. Control mice received equal volumes of sterile, endotoxin-free PBS, either i.n. or s.c.

Body fluid collection. Serum and intestinal washes were collected 3 weeks after the third immunization. Blood samples were collected in deeply anaesthetized animals by cardiac puncture. Blood was left at room temperature for 2 h, centrifuged and the serum was stored at -20 °C until used. The small intestine, from the duodenum to the ileo-caecal valve, was surgically removed and flushed with 3 ml ice-cold PBS containing a protease inhibitor mixture (50 mM EDTA, 120 µg aprotinin ml⁻¹, 150 µg tosyl-L-lysine chloromethyl ketone ml⁻¹, 20 µg pepstatin A ml⁻¹, 10 µg leupeptin ml⁻¹, 10 µg antipain ml⁻¹ and 10 µg chymostatin ml⁻¹; Roche). Washes were centrifuged (10 000 g for 10 min) to remove faecal matter and mucus. The clear supernatants were collected and stored at -20 °C.

Detection of specific antibodies by ELISA. To determine the level of specific anti-*S. aureus* FnbpA in the serum and intestinal washes, standard indirect ELISAs were performed as described previously using purified recombinant *S. aureus* FnbpA as the capture antigen. To quantify anti-FnbpA antibodies, for each plate a reference curve was determined by coating the microtitre plate wells with known amounts of purified mouse IgG and IgA (ICN). The concentration of specific anti-FnbpA antibodies was determined by subtracting the mean value obtained in non-immune animals (background) from values obtained in each experimental (immune) mouse (Castagliuolo *et al.*, 2004).

Purification of spleen mononuclear cells and assessment of cell-mediated immune responses. Spleens were removed aseptically 49 days after the primary immunization, and spleen mononuclear cells were purified and cultured as described previously (Castagliuolo *et al.*, 2004). Cells from each animal were seeded at a concentration of 10⁶ ml⁻¹ and cultured in medium alone or in the presence of purified FnbpA (10 µg ml⁻¹) in a humidified 5% CO₂ incubator at 37 °C. As a positive control, cells were exposed to phytohaemagglutinin at 5 µg ml⁻¹. To measure cytokine release, after 5 days, cultures were harvested by centrifugation and the supernatants were collected, fractionated and stored at -80 °C. The levels of interleukin-2 (IL-2) and gamma interferon (IFN-γ) were measured using commercially available ELISAs (Biosource), running each sample in duplicate. To assess lymphocyte proliferation after 96 h, [³H]thymidine [1 µCi (37 kBq) per well] was added to each well and cells were cultured for an additional 24 h before harvesting. Cells were then collected by centrifugation and washed twice with ice-cold PBS, and [³H]thymidine incorporation was determined using a β-scintillation counter. Splenocyte proliferation was expressed as the proliferation index, as described previously (Castagliuolo *et al.*, 2004).

Statistical analysis. Results were expressed as means ± SEM. Statistical analysis was performed using analysis of variance and Bonferroni's test. Statistical significance was considered for *P* < 0.05.

RESULTS AND DISCUSSION

TxA_{C314} and SLP-36kDa enhance the production of anti-FnbpA-specific serum IgG and IgA

Mucosal and subcutaneously administered vaccines are extremely appealing, as they can ideally trigger systemic and mucosal responses (Holmgren *et al.*, 2003). However, the strength of the specific immune response elicited is generally disappointing due to inadequate numbers of DCs at the site of antigen administration and the poor efficacy of the adjuvant present in the vaccine in activating DCs and priming T-lymphocyte responses. Thus strategies to augment the immunogenicity of mucosal-delivered vaccines are intended mainly to identify substances able to induce DC recruitment, activation and maturation (McKenzie *et al.*, 2004). It has recently been reported that *C. difficile*-derived SLPs are able to modulate DC activity (Ausiello *et al.*, 2006), and in preliminary *in vitro* studies, we have observed that TxA_{C314} adjuvant activity *in vivo* depends on DC activation; therefore, we assessed the ability of recombinant peptides to modulate the immune response towards a surface antigen of *S. aureus* able to confer protective immunity against tissue colonization (Mamo *et al.*, 1994). As shown in Fig. 1, following s.c. or i.n. immunization of mice with TxA_{C314} or SLP-36kDa co-administered with recombinant *S. aureus* FnbpA, we observed a significant increase in specific anti-FnbpA IgG in all of the animals immunized compared with administration of the peptide antigen alone. Indeed, TxA_{C314} and SLP-36kDa were equally effective at enhancing specific IgG production following i.n. or s.c. administration, whereas TxA_{C314} was more effective than SLP-36kDa in enhancing the production of FnbpA-specific serum IgA. In addition, in accordance with previous reports, s.c. administration was more effective than i.n. administration at triggering specific systemic antibody responses (De Magistris, 2006). Serum antibodies reacted with specific recombinant proteins as well as with native proteins obtained from lysates of reference *S. aureus* strains, as determined by ELISA and Western blotting (data not shown). In addition, s.c. or i.n. administration of TxA_{C314} or SLP-36kDa alone did not affect the level of specific anti-FnbpA IgG or IgA (data not shown).

TxA_{C314} and SLP-36kDa enhance the production of specific anti-FnbpA mucosal IgA

As specific mucosal immune responses are instrumental in protecting the host against a number of pathogens that use mucosal surfaces as a port of entry, we determined the strength of the immune response elicited at the gastrointestinal mucosal surfaces, taking into account the importance of the common mucosal response. Specific anti-FnbpA IgA was detected at low levels on the gastrointestinal mucosa of mice immunized by administration i.n. or s.c. of FnbpA alone, whereas the presence of TxA_{C314} or SLP-36kDa significantly enhanced the level of specific mucosal IgA anti-FnbpA (Fig. 2). As expected

Fig. 1. Level of systemic anti-FnbpA IgG and IgA in mice immunized i.n. or s.c. BALB/c mice (8–12 per group) were immunized i.n. or s.c. on days 0, 14 and 28 with FnbpA (10 µg) alone (open bars) or supplemented with 10 µg TxA_{C314} (filled bars) or 10 µg SLP-36kDa (shaded bars). Three weeks after the last immunization, blood was collected by cardiac puncture and serum concentrations of IgG (a) and IgA (b) reactive against FnbpA were measured by ELISA. The level of specific anti-FnbpA antibodies was determined by preparing a reference curve using mouse IgG or IgA and subtracting the background from each sample (non-immune mice). Results are shown as means ± SEM. *, $P < 0.01$ versus FnbpA alone; †, $P < 0.05$ versus FnbpA alone; ‡, $P < 0.05$ versus FnbpA plus TxA_{C314} or SLP-36kDa i.n.

(Holmgren *et al.*, 2003; De Magistris, 2006), the i.n. route was more effective than the s.c. route of administration in stimulating mucosal anti-FnbpA IgA. Furthermore, TxA_{C314} was slightly more effective than SLP-36kDa at enhancing mucosal anti-FnbpA IgA production following i.n. administration.

TxA_{C314} and SLP-36kDa induce a mixed immune response

As polarization of the immune response plays a key role in protecting the host against specific pathogens, to characterize further the adjuvant activity of TxA_{C314} and SLP-36kDa we determined the serum levels of anti FnbpA-specific IgG₁ and IgG_{2a}, following either i.n. or s.c. vaccine

Fig. 2. Faecal anti-FnbpA IgA antibodies. BALB/c mice (8–12 per group) were immunized i.n. or s.c. on days 0, 14 and 28 with FnbpA (10 µg) alone (open bars) or supplemented with 10 µg TxA_{C314} (filled bars) or 10 µg SLP-36kDa (shaded bars). Three weeks after the last immunization, animals were sacrificed, the intestine was carefully removed and the luminal content was flushed with 1 ml ice-cold PBS and centrifuged, and the level of anti-FnbpA IgA was measured by ELISA. Results are shown as means ± SEM. *, $P < 0.01$ versus FnbpA alone; †, $P < 0.05$ versus FnbpA alone; ‡, $P < 0.05$ versus FnbpA plus TxA_{C314} i.n.

administration. As shown in Fig. 3, both immunization protocols with a peptide antigen in combination with TxA_{C314} or SLP-36kDa induced a mixed Th1/Th2-type immune response, as indicated by the production of both IgG subclasses (IgG₁ and IgG_{2a}). Mice immunized with FnbpA plus TxA_{C314} or SLP-36kDa via the s.c. route showed higher FnbpA-specific IgG_{2a} levels than following vaccine administration through the i.n. route, supporting previous results indicating that the s.c. route is more effective at polarizing the immune response towards a Th1 response (Uddowla *et al.*, 2007).

TxA_{C314} is more effective at inducing antigen-specific cell-mediated responses

As shown in Fig. 4, spleen lymphocytes obtained from mice immunized s.c. or i.n. using only recombinant FnbpA exhibited a modest proliferative response and negligible release of IFN- γ and IL-2 following *in vitro* challenge with recombinant FnbpA or *S. aureus* lysate (data not shown). However, spleen cells isolated from mice immunized with FnbpA in the presence of TxA_{C314} or SLP-36kDa and challenged *in vitro* with the peptide antigen released significant amounts of IL-2 and IFN- γ and showed a significant increase in the proliferative response (Fig. 4). In addition, lymphocyte proliferation and cytokine release were significantly more pronounced in cells purified from mice immunized with FnbpA in the presence of TxA_{C314} and challenged *in vitro* with FnbpA than in cells obtained from mice immunized with SLP-36kDa and FnbpA (Fig. 4), either i.n. or s.c.

Fig. 3. Level of anti-FnbpA IgG₁ and IgG_{2a} in mice immunized i.n. or s.c. BALB/c mice (8–12 per group) were immunized i.n. or s.c. on days 0, 14 and 28 with FnbpA (10 µg) alone (open bars) or supplemented with 10 µg TxA_{C314} (filled bars) or 10 µg SLP-36kDa (shaded bars). Three weeks after the last immunization, blood was collected by cardiac puncture and serum concentrations of IgG₁ (a) and IgG_{2a} (b) reactive against FnbpA were measured by ELISA. The level of specific anti-FnbpA antibodies was determined by preparing a reference curve using mouse IgG₁ or IgG_{2a} and subtracting the background from each sample (non-immune mice). Results are shown as means ± SEM. *, $P < 0.01$ versus FnbpA alone; †, $P < 0.05$ versus FnbpA plus TxA_{C314} or SLP-36kDa i.n.

Thus co-administration of either TxA_{C314} or SLP-36kDa with a peptide antigen induced a strong humoral and cell-mediated immune response. The profile of antigen-specific IgG subclasses and of cytokine released from immune splenocytes challenged *in vitro* with *S. aureus* FnbpA is compatible with the induction of a mixed Th1/Th2-type immune response for both *C. difficile*-derived proteins. However, TxA_{C314} induced a stronger cell-mediated response and higher levels of IgG_{2a}, indicating a more pronounced Th1-type branch. This complex and less well-defined profile of the immune response is not unprecedented: following immunization, Th1- and Th2-type responses can be observed at different stages of the

Fig. 4. Induction of anti-FnbpA-specific T-cell responses by i.n. or s.c. administration of a peptide vaccine. BALB/c mice (8–12 per group) were immunized i.n. or s.c. on days 0, 14 and 28 with PBS alone, FnbpA alone or FnbpA supplemented with 10 µg TxA_{C314} or 10 µg SLP-36kDa. Three weeks after the last immunization, spleens were collected and lymphocytes were purified and seeded at a concentration of 10⁶ cells ml⁻¹ in 24-well Costar plates. (a, b) To assess antigen-specific cytokine release, cells were cultured for 5 days in medium alone (open bars) or in the presence of *S. aureus* FnbpA (10 µg ml⁻¹; filled bars). The culture medium was collected by centrifugation and assayed for IFN-γ and IL-2 by ELISA. Results are shown as means ± SEM. *, *P* < 0.01, and †, *P* < 0.05, versus cells cultured in medium alone. (c) To assess antigen-specific T-cell proliferation, [³H]thymidine (37 kBq per well) was added 18 h before harvesting to cells obtained from non-immune mice (hatched bars), FnbpA alone (open bars), or FnbpA supplemented with TxA_{C314} (filled bars) or SLP-36kDa (shaded bars). Cells were collected and incorporated radioactivity was measured. Data are expressed as the proliferation index corresponding to the ratio of counts min⁻¹ obtained in antigen-stimulated versus non-stimulated cells. Results are shown as means ± SEM. *, *P* < 0.01 versus mice injected with PBS, FnbpA alone and FnbpA plus SLP-36kDa; †, *P* < 0.05 versus FnbpA alone.

immune response and depend on specific antigen/adjuvant combinations as well as on the route of vaccine administration (Flegel *et al.*, 1991; Nakagawa *et al.*, 1996). The molecular mechanisms leading to a more prominent production of Th1- or Th2-type cytokines and different IgG isotypes are presently not completely understood; however, we can speculate that both TxA_{C314} and SLP-36kDa can activate different intracellular signal cascades, which eventually trigger the release of different cytokines from immune and non-immune cells (Ausiello *et al.*, 2006; Moser, 2001; Warny & Kelly, 1999). Cytokines present in the microenvironment at the time of priming, as well as the relative expression of co-stimulatory molecules, are key elements driving the CD4⁺ T-cell response towards a particular Th profile and therefore boost the development of an immune response or favour tolerance (Jones *et al.*, 2001; Moser, 2001). In addition, as DCs, with pivotal roles in the initiation and subsequent development of immune responses, are a heterogeneous family of cells strongly influenced by the surrounding microenvironment, it is possible that DCs positioned in the nasal mucosa and subcutaneous tissues respond differently to the same molecules (Svensson & Kaye, 2006; Svensson *et al.*, 2004).

A common feature of the most powerful mucosal adjuvants, such as CT, *E. coli* LT and plant lectins, is a carbohydrate-recognition domain able to bind simultaneously to several specific membrane receptors, inducing their clustering (Rappuoli & Pizza, 2000). Indeed, non-toxic CT and LT mutants retaining the ability to bind to mucosal epithelial cells maintain their adjuvant activity (Pizza *et al.*, 2001). Both peptides used in this study exhibit in principle the ability to bind and cluster multiple

receptor molecules on the cell membrane. Indeed, SLP monomers can auto-assemble in regular supramolecular structures, whereas TxA_{C314} comprises a region of the receptor-binding domain of TcdA that shows a lectin-like structure (Castagliuolo *et al.*, 2004; Heyman *et al.*, 2007). Therefore, it is possible that, following binding to cell membranes, these peptides can induce receptor clustering that triggers the development of specific intracellular signalling cascades, activating immune cells and enhancing the immune response.

In summary, we have shown that two immunodominant peptides derived from *C. difficile* – a recombinant non-toxic region of the C-terminal domain of TcdA and a region of the 36 kDa SLP – demonstrate robust adjuvant properties when co-administered with a relevant peptide antigen via the mucosal (i.n.) or s.c. route. In addition, the profile of antigen-specific IgG subclasses and of cytokine release from immune splenocytes challenged *in vitro* with the test antigen used in this study is compatible with a different ability to polarize the immune response.

REFERENCES

- Aizpurua, H. J. & Russell-Jones, G. J. (1988). Identification of classes of proteins that provoke an immune response upon oral feeding. *J Exp Med* **167**, 440–451.
- Ausiello, C. M., Cerquetti, M., Fedele, G., Spensieri, F., Palazzo, R., Nasso, M., Frezza, S. & Mastrantonio, P. (2006). Surface layer proteins from *Clostridium difficile* induce inflammatory and regulatory cytokines in human monocytes and dendritic cells. *Microbes Infect* **8**, 2640–2646.
- Cario, E. (2005). Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2. *Gut* **54**, 1182–1193.
- Castagliuolo, I. & LaMont, J. T. (1999). Pathophysiology, diagnosis and treatment of *Clostridium difficile* infection. *Keio J Med* **48**, 169–174.
- Castagliuolo, I., Sardina, M., Brun, P., DeRos, C., Mastrotto, C., Lovato, L. & Palù, G. (2004). *Clostridium difficile* toxin A carboxyl-terminus peptide lacking ADP-ribosyltransferase activity acts as a mucosal adjuvant. *Infect Immun* **72**, 2827–2836.
- Castagliuolo, I., Piccinini, R., Beggiao, E., Palù, G., Mengoli, C., Ditadi, F., Vicenzoni, G. & Zecconi, A. (2006). Mucosal genetic immunization against four adhesins protects against *Staphylococcus aureus*-induced mastitis in mice. *Vaccine* **24**, 4393–4402.
- Cerquetti, M., Molinari, A., Serafino, A., Sebastianelli, A., Diociaiuti, M., Petruzzelli, R., Capo, C. & Mastrantonio, P. (2000). Characterization of surface layer proteins from different *Clostridium difficile* clinical isolates. *Microb Pathog* **28**, 363–372.
- Cerquetti, M., Serafino, A., Sebastianelli, A. & Mastrantonio, P. (2002). Binding of *Clostridium difficile* to Caco-2 epithelial cell line and to extracellular matrix proteins. *FEMS Immunol Med Microbiol* **32**, 211–218.
- De Magistris, M. T. (2006). Mucosal delivery of vaccine antigens and its advantages in pediatrics. *Adv Drug Deliv Rev* **58**, 52–67.
- Eveillard, M., Fourel, V., Barc, M. C., Kerneis, S., Coconnier, M. H., Karjalainen, T., Bourlioux, P. & Servin, A. L. (1993). Identification and characterization of adhesive factors of *Clostridium difficile* involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secreting HT-29 cells in culture. *Mol Microbiol* **7**, 371–381.
- Fallon, P. G. & Alcamí, A. (2006). Pathogen-derived immunomodulatory molecules: future immunotherapeutics? *Trends Immunol* **27**, 470–476.
- Flegel, W. A., Müller, F., Daubener, W., Fischer, H. G., Hadding, U. & Northoff, H. (1991). Cytokine response by human monocytes to *Clostridium difficile* toxin A and toxin B. *Infect Immun* **59**, 3659–3666.
- Heyman, A., Levy, I., Altman, A. & Shoseyov, O. (2007). SP1 as a novel scaffold building block for self-assembly nanofabrication of submicron enzymatic structures. *Nano Lett* **7**, 1575–1579.
- Holmgren, J., Czerkinsky, C., Eriksson, K & Mharandi, A. (2003). Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine* **21** (Suppl. 2), S89–S95.
- Jones, H. P., Hodge, L. M., Fujihashi, K., Kiyono, H., McGhee, J. R. & Simecka, J. W. (2001). The pulmonary environment promotes Th2 responses after nasal-pulmonary immunization with antigen alone, but Th1 responses are induced during instances of intense immune stimulation. *J Immunol* **167**, 4518–4525.
- Kyne, L., Warny, M., Qamar, A. & Kelly, C. P. (2000). Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med* **342**, 390–397.
- Lammers, A., Nuijten, J. M. & Smith, H. E. (1999). The fibronectin binding proteins of *Staphylococcus aureus* are required for adhesion to and invasion of bovine mammary gland cells. *FEMS Microbiol Lett* **180**, 103–109.
- Mamo, W., Boden, M. & Flock, J. I. (1994). Vaccination with *Staphylococcus aureus* fibrinogen-binding proteins (FgBPs) reduces colonisation of *Staphylococcus aureus* in a mouse mastitis model. *FEMS Immunol Med Microbiol* **10**, 47–53.
- McKenzie, B. S., Brady, J. L. & Lew, A. M. (2004). Mucosal immunity: overcoming the barrier for induction of proximal responses. *Immunol Res* **30**, 35–71.
- Moser, M. (2001). Regulation of Th1/Th2 development by antigen-presenting cells *in vivo*. *Immunobiology* **204**, 551–557.
- Mukherjee, K., Karlsson, S., Burman, L. G. & Akerlund, T. (2002). Proteins released during high toxin production in *Clostridium difficile*. *Microbiology* **148**, 2245–2253.
- Nakagawa, I., Takahashi, I., Kiyono, H., McGhee, J. R. & Hamada, S. (1996). Oral immunization with the B subunit of the heat-labile enterotoxin of *Escherichia coli* induces early Th1 and late Th2 cytokine expression in Peyer's patches. *J Infect Dis* **173**, 1428–1436.
- Nestle, F. O., Aljagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. & Schadendorf, D. (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* **4**, 328.
- Pizza, M. G., Giuliani, M. M., Fontana, M. R., Monaci, E., Douce, G., Dougan, G., Mills, K. H., Rappuoli, R. & Del Giudice, G. (2001). Mucosal vaccines: nontoxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* **19**, 2534–2541.
- Rappuoli, R. & Pizza, M. G. (2000). Bacterial toxins. In *Cellular Microbiology*, pp. 193–220. Edited by P. Cossart, P. Boquet, S. Normark & R. Rappuoli. Washington, DC: American Society for Microbiology.
- Svensson, M. & Kaye, P. M. (2006). Stromal-cell regulation of dendritic-cell differentiation and function. *Trends Immunol* **27**, 580–587.
- Svensson, M., Maroof, A., Ato, M. & Kaye, P. M. (2004). Stromal cells direct local differentiation of regulatory dendritic cells. *Immunity* **21**, 805–816.
- Uddowla, S., Freytag, L. C. & Clements, J. D. (2007). Effect of adjuvants and route of immunizations on the immune response to recombinant plague antigens. *Vaccine* **25**, 7984–7993.

Warny, M. & Kelly, C. P. (1999). Monocyte cell necrosis is mediated by potassium depletion and caspase-like proteases. *Am J Physiol* **276**, C717–C724.

Zecconi, A., Binda, E., Borromeo, V. & Piccinini, R. (2005). Relationship between some *Staphylococcus aureus* pathogenic factors and growth rates or somatic cell counts. *J Dairy Res* **72**, 203–208.